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Reliability of photobioreactors in life support systems: an investigation of methods to restore *Chlamydomonas reinhardtii* cultures after heat stress

by

Maria Johansson

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Approved Dissertation Committee

Prof. Dr. Matthias Ullrich
Jacobs University Bremen

Prof. Dr.-Ing Clemens Posten
Karlsruhe Institute of Technology

Prof. Dr. Alexander Lerchl
Jacobs University Bremen

Dr. Klaus Slenzka
OHB System AG/International Space University

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Life Sciences & Chemistry

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Summary

Microalgal cultures in photobioreactors are essential in biological life support systems for space flight. Such photobioreactors need to have a high level of reliability as the crew depends on the life support system. However, photobioreactor cultures are sensitive to environmental parameters outside of their tolerance range, and crew time for maintenance and repair is limited. This work aimed to increase resilience and decrease vulnerability of photobioreactor cultures by exploring methods for restarting photobioreactor cultures after heat shock, with as little use of crew time as possible.

Using the microalga *Chlamydomonas reinhardtii*, two paths for restarting a photobioreactor culture were explored. In the first path we tested the heat resistance of *C. reinhardtii* biofilms, hypothesizing that microalgal biofilms would have a higher heat tolerance than planktonic cultures. That would make it possible to use a subset of microalgal culture, grown as a biofilm inside the planktonic photobioreactor culture, to restart the planktonic culture after a heat shock event, making it essentially self-restarting.

Firstly, a suitable biofilm substrate had to be found. We used a ceramic, alumina (Al_2O_3), which was chemically inert and could be manufactured with a variety of porosities. The surface of the substrate could be functionalized through silanization to give it different physico-chemical properties. A number of combinations of porosities and surface functionalization were tested for biofilm growth and potential harm to cells. It was found that cell health and re-growth ability did not decrease for cells growing on the alumina substrates regardless of porosity or surface functionalization. Alumina with pore sizes around the size of a *C. reinhardtii* cell had the most cells attaching to them. These alumina were used for the heat stress tests.

To test heat tolerance of *C. reinhardtii* biofilm, and compare it to that of planktonic cultures, we designed, built and calibrated a heat stress test setup. It consisted of lab size photobioreactors immersed in heated water baths. We could not, however, detect any difference in heat stress tolerance between *C. reinhardtii* grown as a biofilm and *C. reinhardtii* planktonic culture.

The second path explored the suitability of a silica sol-gel, manufactured with a novel method, for encapsulation of *C. reinhardtii* cells for medium term storage. The silica sol gel manufacturing method has been designed to be less stressful to biological cells and also consists of fewer steps than other similar methods described in literature. It would therefore be more suitable than those methods when personnel time is limited. The method proved

successful, the algal cells survived and stayed healthy for several weeks. The sol gel did not have detrimental effects on cell health and it stayed stable during the run of the experiments.

This work did not result in a self-restarting mechanism for a photobioreactor. However, we have established that porous alumina with pore sizes around one cell length are appropriate as biofilm substrates. We have also designed and tested a heat test setup and mapped the heat tolerance of *C. reinhardtii* including acclimatization time. And we have confirmed that silica sol gel manufactured with a new method can be used to store microalgal cells for several weeks. The relative ease of handling of the silica sol gel manufacturing method makes it a possible alternative for storing cells in biological life support systems, to be used for starter cultures in photobioreactors.

Contents

Abbreviations

BLSS Biological life support system

CFU Colony forming units

EPS Extracellular Polymeric Substances

HSP Heat shock protein

HSR Heat stress response

LSS life support system

PBRs Photobioreactors

ROS Reactive oxygen species

RT Room temperature

TAP Tris acetate phosphate medium

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Chapter 1

Introduction

1.1 Research motivation and thesis outline

The overall goals of life support system (LSS) design for manned space flight is mission success and minimized risk of crew health problems. Each component of the system contributes to its reliability. For longer missions, in which food production is necessary, biological organisms must be included in the LSS, making it a biological life support system, a BLSS. Photobioreactors (PBRs) with microalgae are included in biological life support systems in manned space flight to provide air regeneration through the microalgae's photosynthetic machinery, which converts transforms carbon dioxide to oxygen. They are therefore key components in the BLSS, and their reliability and resilience is important to ensure the BLSS reliability. This means that not only does it need to provide enough of its products to fulfill the needs of the crew, it also needs to be maintainable and repairable so that it can continue to provide the system with its products over a long period of time.

Algal cultures are sensitive to stress, and the environment outside the BLSS would be very different from the algae's natural environments. Different combinations of extreme temperature differences, low pressure, varying gravity, and high levels of ionizing radiation would have to be handled by the BLSS design. The PBR culture can easily fail should any of the barriers against the outer environment fail, or should the BLSS fail to keep the internal system conditions, such as temperature or pH, at healthy levels. Commercial bioreactors in industry are designed to give the cultures a highly controlled environment. The bioreactor is monitored by personnel, and should it break down it will be repaired or replaced. However, personnel time in manned space flight is limited, as are energy and material resources. A faulty bioreactor in such a location can't be inexpensively replaced. To further complicate matters, storage of algae is complicated even in controlled lab environments,

as they are sensitive to cryopreservation. Even if the low survival rates of cryopreserved algae would be off-set by storing large amounts of cells, cryostorage requires freezers to be kept constantly at low temperatures, introducing yet a vulnerability to the BLSS. The sensitivity of the microorganisms in combination with the limited resources for handling the bioreactor, limitations in storage methods for algal cells, and the harsh outer environment increases the importance of having a robust BLSS design, including the PBRs.

A system's reliability can be defined as the probability at any given time that it has not failed. A LSS component can contribute to the overall system reliability, not only by its own reliability, but by its resilience - its ability to return to normal operation after a failure. A PBR of medium reliability that can be quickly and inexpensively restored might contribute more to the LSS's reliability than a reliable air-regeneration device that cannot be restored at all should it break down. Therefore a PBR of medium reliability but which is easily restorable might be preferable, if the LSS is designed to allow for occasional PBR downtime [9].

The overall purpose of the work described in this thesis was to explore methods for restoring photobioreactors after a failure of the biological culture, with minimum or no use of personnel time, the latter making the PBR essentially self-restarting. To this end two forms of immobilization of microalgae were studied, biofilm formation and gel encapsulation, in attempts to make use of naturally occurring mechanisms in cells for recovery of a cell culture after a stress event.

Section 1.2 provides background information on PBRs as well as on BLSS, and on the chosen model alga in this work, *Chlamydomonas reinhardtii*. In section 1.3 the thesis aims are presented, followed by an outline of the research strategy in section 1.4. In chapter 2 the results of the thesis work are shown with each section consisting of a manuscript done as part of the thesis work. An introduction to the manuscripts of the chapter can be found on page 29. Finally, the results are discussed in chapter 3.

1.2 Background

1.2.1 Photobioreactors

A photobioreactor, or PBR, is a device for growing microalgae in a controlled environment. Microalgae are grown for their ability to use sunlight energy to build energy rich molecules, which can be used in a range of products; from fuels such as biodiesel [25] or hydrogen gas [22], to high value products such as food supplements or pharmaceuticals [127]. They have been grown commercially for decades, usually in open raceway ponds [20]. A PBR however, is a closed system, where the microalgal culture is grown in a light-flooded vessel. A PBR culture can more easily be kept axenic than an open pond culture and culture conditions are easier to control, which increases microalgal yields. The disadvantage is that a PBR system is more expensive to build and run than an open pond system [78].

In order to grow, the microalgal culture needs sunlight, carbon dioxide, and a nutrient medium containing a nitrogen source, phosphorus, and trace elements. Algal cultures can be grown as batch cultures, or as continuous cultures. PBRs have been built and studied in many different designs. The common elements for all designs is a bioreactor vessel that lets light through to the cell culture, gas inlets and outlets for enriching the culture with CO_2, nutrient medium inlets and outlets, and a mixing mechanism, see Figure 1.1.

Since the purpose of a PBR is to make use of energy from sunlight to allow growth of cell cultures its vessel is designed to let as much light as possible through to the cell culture, while still being practical for CO_2 supply, mixing and harvesting. The vessels that in early designs were made in cylinder shapes, have evolved into tubes or thin, flat vessels [37]. Recent research has also looked at leading light through to the culture via glass sponges inside the reactor [66].

The carbon dioxide is added to the bioreactor culture using CO_2-enriched air. Several research projects have looked at using microalgae for remediation of carbon dioxide from flue gas from power plants or cement plants, which make inexpensive CO_2 sources [40]. The gas supply is often used to mix the culture as well, by designing the reactor so that the gas bubbles travel up through the length of the vessel, in a so called “bubble column”. Sometimes the shape of the vessel itself, or structures inside the vessel, have been designed to allow the gas flow to do the mixing while letting as much carbon dioxide as possible dissolve in the liquid on its way up.

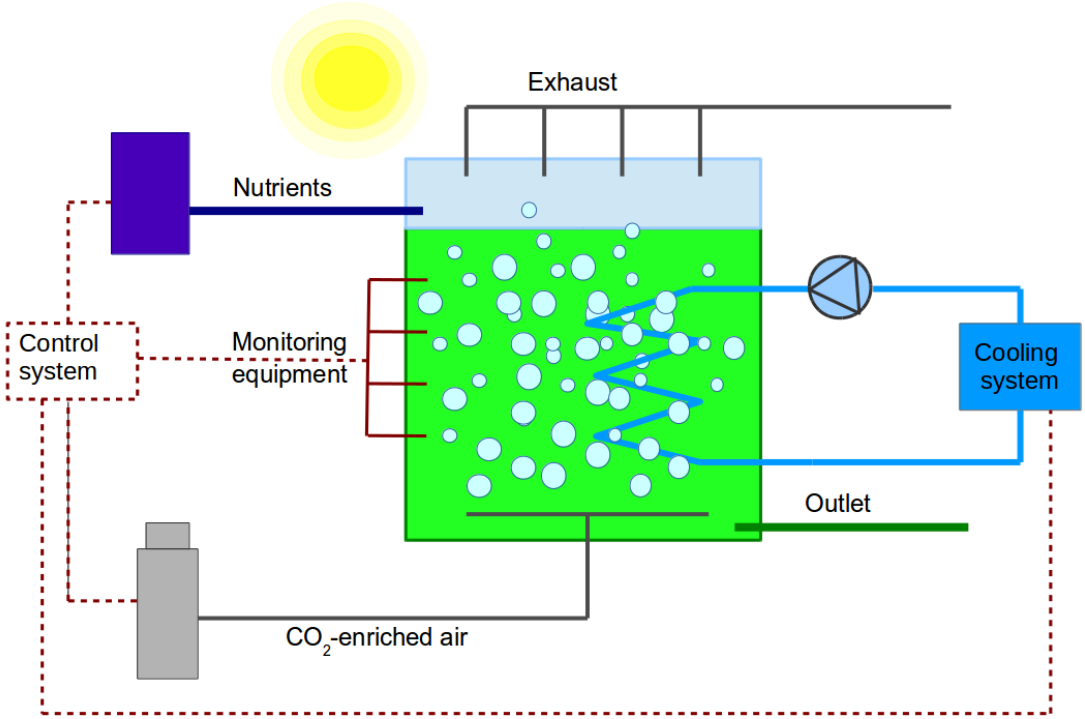

Figure 1.1: The main technical components of a photobioreactor. *The bioreactor vessel, here shown as a flat plate vessel, has inlets for air enriched with carbon dioxide and for nutrients, and outlets for air and for old culture. The air inlet also help mixing the bioreactor culture by being placed in the bottom of the bioreactor vessel so that the air bubbles up through the culture. Monitoring equipment, such as temperature and pH sensors, keep track of the culture's state and the control system adjusts culture conditions accordingly, for example by switching on or off the cooling system.*

Connected with a PBR in production is also monitoring equipment as well as control equipment for pH, temperature, and culture density. The production facility would also be monitored and managed by personnel.

If the product is oil or a high value product like for instance omega-3 fatty acids the algae biomass need to be harvested and the product extracted. The harvesting and extraction processes are energy intense, mainly because of the dewatering process. Algal hydrogen production systems, on the other hand, often use a two-phase process in which the algae are grown to a certain concentration in the first phase and then are switched to anaerobic, hydrogen producing conditions in the second phase [22]. The product, hydrogen gas, is then collected from the gas outlet.

Another version of PBRs is to use biofilm reactors. In these, the algal culture is grown as a biofilm on a technical substrate subjected to a liquid flow. A biofilm reactor offers the advantage of easier harvesting with less need for dewatering and also a more controllable culture; in the case of hydrogen production growing the cells as a biofilm makes it easier to cycle them from one culture phase to the other [53]. One study successfully tested restarting a biofilm reactor after harvesting with the cells that were left on the substrate [69].

1.2.2 Photobioreactors in life support systems

Life support in manned space flight includes all components that are needed to keep the crew fed, healthy and safe. This means providing breathable air, food and water, as well as removing waste. Life support also means keeping the crew sheltered from radiation and other environmental factors, but this aspect of LSSs is beyond the scope of this thesis.

Food, air, and water can be stored on board for shorter mission durations. For longer missions, however, these materials would need to be resupplied or recycled. Space launches are costly, so regeneration of air and water onboard keeps the mission cost down [7]. The higher the closure of the regeneration loops - in order words the higher the degree of material reuse - the less material need to be resupplied. The degree of material loop closure can be measured as the percentage of material being reused, by weight. The first priorities are to regenerate air, the most critical material for crew safety, and water, wich makes up the bulk weight of material in the LSS. This can be done by physico-chemical methods; in the International Space Station (ISS)

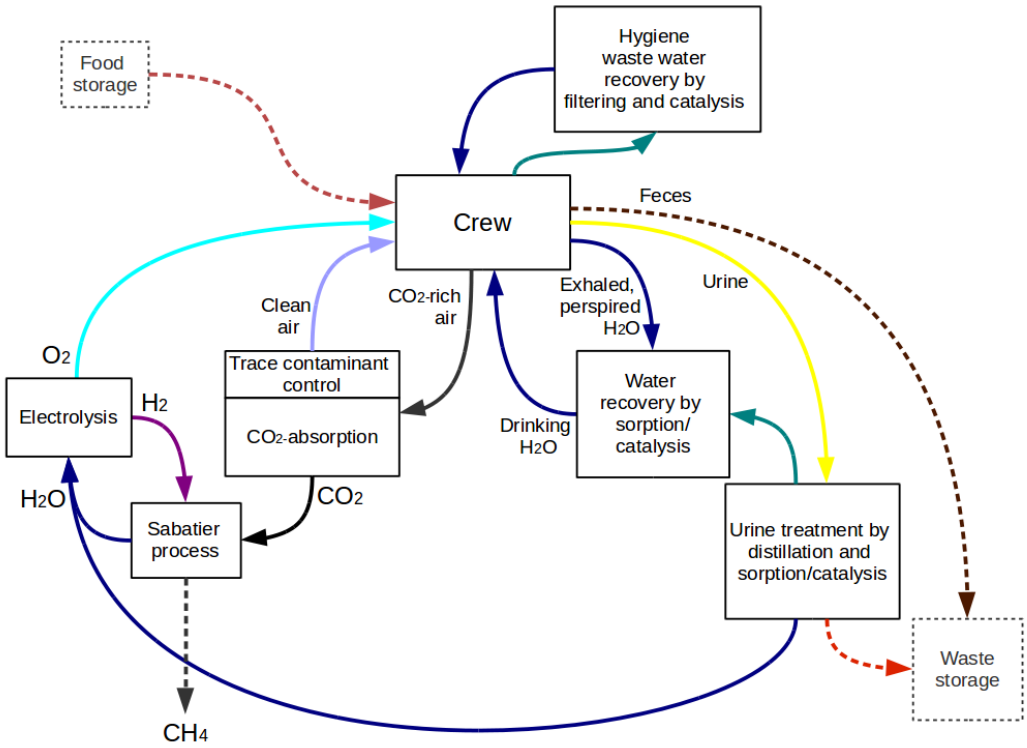

Figure 1.2: ISS life support system schematic. *The International Space Station, ISS, is supported by a number of systems employing physico-chemical mechanisms to recover water and oxygen for the space station crew. A significant percentage of the material mass in in any life support system is water, which is why recovering it is prioritized. This is done in several parallel but separate systems so that relatively clean water from exhalation and perspiration is recovered as drinking water, and water for washing is filtered and cleaned to be used again as wash water. Water in urine is used for oxygen instead. In this system there are no biological organisms to make food, therefore carbon cannot be recirculated. Oxygen is recovered from exhaled carbon dioxide and the carbon is vented off the station in the form of methane. The lack of biological organisms also means that carbon, nitrogen and other nutrients in feces and urine cannot be recovered, and goes to waste storage instead.*

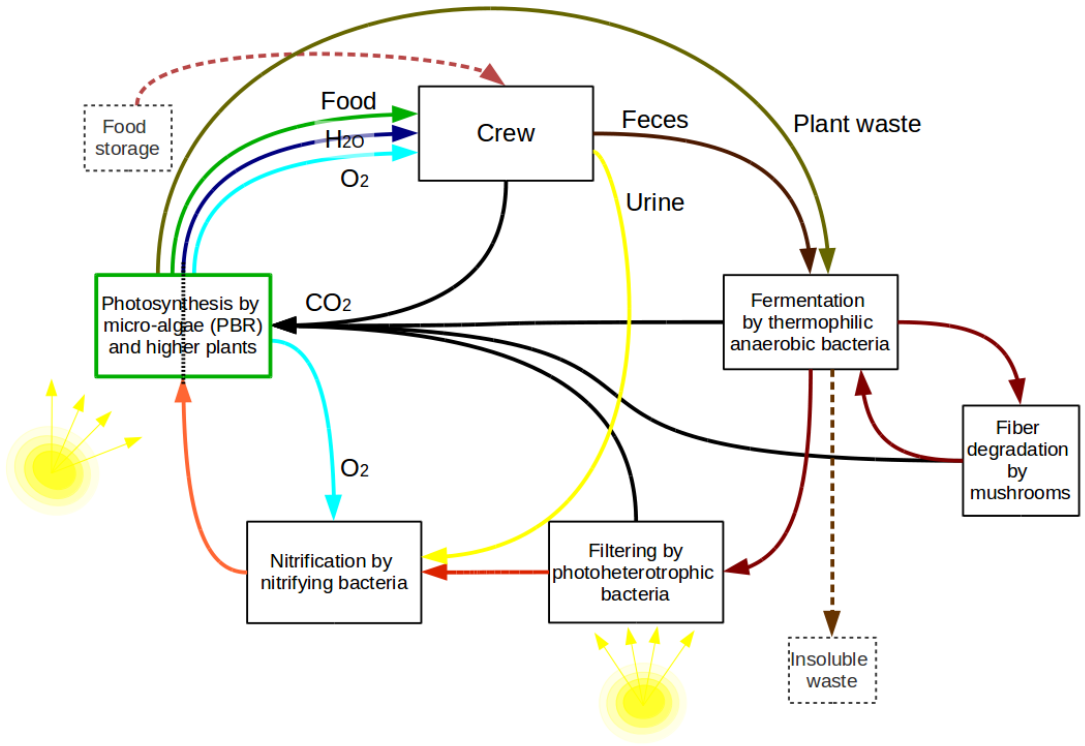

Figure 1.3: Melissa schematic. *The research system Melissa, "MicroEcological LIfe Support System Alternative", is an attempt to build a biological life support system modeled on a lake ecosystem. This system has a higher degree of material recovery than the ISS system, but unlike the ISS system it has not yet been used in flight. It has been built up in a pilot plant at the University of Barcelona in Spain. The system is built in a loop where feces are first fermented down by thermophilic bacteria to avoid propagating bacteria harmful to humans. Fiber degradation can be done by an additional mushroom compartment. Some waste is still insoluble and the loop will never be completely closed, making it necessary to still have a food store. Waste is then treated by nitrifying bacteria. Two photosynthetic compartments with microalgae and higher plants uses nutrients and CO2 from the other compartments to produce food. They also produce oxygen, and by collecting transpiration water from the higher plants the crew is also supplied with drinking water.*

air revitalization and water recovery is done through a connected set of filters, dehumidifiers, electrolyzers and Sabatier reactors [117], see illustration in Figure 1.2.

For missions that need a high degree of independence, for example missions far away from Earth, it would be important to include at least some biological components in LSSs; at longer distances resupply missions become increasingly expensive and uncertain, so a LSS that also produces food becomes desirable [7]. Even if water represent the largest amounts of material in a LSS, closing the carbon cycle by producing food from reused waste and breathing air - that is reuse the carbon from food that the crew eats - increases the safety of the mission by making it independent of food resupply. As yet, no physico-chemical method can produce food. Photosynthetic organisms however, bind carbon from carbon dioxide into complex carbon molecules using energy from sunlight. Oxygen is produced as a byproduct of photosynthesis, which from the point of view of a LSS designer means that air regeneration is a service coupled to food production. Since the photosynthetic organisms would use nutrients from waste streams coming from the crew, water regeneration and waste management are other services emerging from food production.

Research on BLSSs have been done in several different research programs, using different design philosophies. All of these must use microorganisms for cleaning of water and regeneration substances, but they can be included in designs either in mixed communities in artificial wetlands or in axenic cultures in more controllable bioreactors. American programs BioHome from NASA and the rather famous Biosphere-2, have chosen to use artificial wetlands. Several other programs, however, have used microalgae PBRs for oxygen production because of their high degree of controllability - the air regeneration output can be matched to the oxygen consumption by changing the light conditions or temperature of the reactors, and the algae can be used as animal feed or fertilizers, or in smaller amounts for food. Two programs using PBRs are the Soviet/Russian BIOS-3 system [47] with research taking place in the 1970s through to the 90s and the ongoing MELiSSA program [48] financed by the European Space Agency that was started in the mid-1980s. The BIOS-3 system used only a few biological components; waste was incinerated and the ashes used as nutrients for two photosynthetic compartments - one with higher plants for food production and one with microalgae (*Chlorella vulgaris*) in PBRs for controllable oxygen production. The whole system was hermetically sealed, and in tests lasting several months very high degrees of material loop closure were reached — 91 % of the material was reused in the experiments that included algae [47]. Some micro-nutrients were lost however,

bound as insoluble compounds in the waste incineration ash. The system will therefore still be dependent on food storage or resupply to some degree.

The MELiSSA system, illustrated in Figure 1.3, takes a different approach and attempts to regenerate most of the chemical compounds in waste using a loop of coupled bioreactors, beginning with a mixed thermophilic bioreactor culture breaking down waste, then using other bioreactor cultures for breaking down organic carbon compounds and for nitrification. Finally, energy is bound back in the system with two photosynthetic compartments - one for higher plants and one for microalgae *(Spirulina platensis*). It is a more complex system than BIOS-3, and at least theoretically reaches a higher degree of closure — up to 95 % [51].

Including biological organisms in the LSS might also make biofuel production possible. Since the early 2000s much research has been done on hydrogen production using the microalgae *Chlamydomonas reinhardtii* [124]. PBRs in the LSS could conceivably be coupled not only with air regeneration and food production, but also with hydrogen production. Hydrogen can be used in fuel cells that can drive machinery in the LSS or in other mission systems. Other research has studied the feasibility of including fuel cells in BLSSs [10].

1.2.3 Common stress factors for photobioreactor cultures

PBR cultures can be subjected to different types of stress or adverse conditions, arising both inside the culture and externally. The cell culture environment is very cramped for the cells, and stirring and gas supply to the culture leads to turbulence which can cause shear stress [110].

As the PBR culture is supplied with CO_2 and nutrients an imbalance between supply and demand might cause a non-optimal chemical environment; when CO_2 supply does not keep up with demand for example, the pH rises [25]. The CO_2 supply is also important to balance out the oxygen generated by photosynthesis. The enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco) which fixes carbon from carbon dioxide into biologically available forms of carbon can also bind oxygen. Since carbon dioxide and oxygen compete for binding spaces on Rubisco, photorespiration can occur if the partial pressure of dissolved oxygen is too high [110]. If the dissolved oxygen levels are even higher it can, in combination with light, lead to formation of radicals causing oxidative stress [25]. In order to balance the supply of CO_2 and nutrients with growth, sensors should be installed in the reactor which

allow control over pCO_2, pO_2 and pH. Placing several sensors along the axis of the reactor makes it possible to discover potential chemical gradients [94].

However, sensors and associated controlling functions can also be an indirect cause of stress. In one test, foaming occurred in the PBR, temporarily preventing an addition of nitric acid from reaching the main culture. The purpose of the nitric acid was to lower the pH, but since it did not reach the culture the pH sensors measured a high pH, and more nitric acid was added. When the foaming dissolved the combined dose of all nitric acid additions lead to a strong overshoot in pH [129].

Another important balance is that of the nutrient medium. If not all salts in the medium are used up in the same rate they are supplied by, they will accumulate. This happened in one test, where PBRs were run successfully for several months but all cultures in the end crashed due to ion accumulation [71].

One of the most common stresses of cultures in PBRs is an externally rising temperature. The downside of using solar radiation as an energy source for PBRs is that it also contains a large amount of infrared radiation. This part of the solar spectrum cannot be used by the photosynthetic organisms but is absorbed by the bioreactor culture and heats it up. In addition, the efficiency of the photosystem is limited; nearly 90 % of the photosynthetically active radiation is lost as heat and contributes to the heating of the culture. Tests with outdoor cultures have shown that they easily reach temperatures over 40 °C, if not cooled [77]. In one test, outdoor PBR cultures reached 47 °C [93].

Numerous ways of cooling the PBRs exist. Some suggest putting the cultures in water baths [94, 110], or growing the cultures in plastic bags floating in the sea [134]. Commonly used solutions are to spray water on the reactors using evaporative cooling or to have a heat exchange element with cooling water inside the PBR [25, 110]. Water spraying can be a resource intense method, if water is scarce. It is also possible to use materials that reflect part of the solar spectrum in the PBR vessel, lowering the amount of heat reaching the reactor [94], although such materials are more expensive than the plastics many up-scaled PBRs are made of.

Even with cooling methods PBR cultures, exposed to sunlight, risk overheating due to exceptional weather conditions for which the cooling system was not dimensioned, or because the cooling system or the equipment controlling it, failed.

1.2.4 Reliability and resilience of photobioreactors and systems using them

Measuring reliability of a BLSS is not a trivial task. Definitions of reliability, and other related concepts vary, but Hashimoto et al [55], describes system performance in terms of reliability, resilience, and vulnerability, where reliability is defined as the probability at any given time that the system will work satisfactorily; resilience as how quickly the system returns to a satisfactory state once a failure has occurred; and vulnerability as the likely magnitude of a failure, should it occur.

A system's efficiency, reliability, resilience and vulnerability must be measured against the objective of the system. For a space LSS the objective is stated for example as "ensuring mission success", keeping the crew safe and healthy, or keeping the crew productive. A crew that suffers bad health is of course an unproductive crew, so these objectives could all be seen as different ways of expressing the same objective. Between the objectives of high system efficiency, high reliability, high resilience, and low vulnerability exist important trade-offs. An example is a PBR using an axenic culture of a high productivity species, instead of naturally occurring consortia of species, which increases the efficiency of the PBR, but also makes it more likely that the bioreactor culture will be taken over in case of contamination by other microorganism species - it has an increased vulnerability.

These kinds of trade-offs mean that making the system as reliable as possible might go against the system objectives. Especially in a LSS it is not only important to make the system "fail-safe" (increase reliability) but it is also important to aim for "safe fails" (decreased vulnerability), to use concepts introduced by Holling [60]. An example of a LSS failing safely is one that have large buffers of oxygen, food and other critical materials. The buffers give the crew time to try to repair the system or try to return home, in case of a life support failure, before the failure affects their health or ability to work and make decisions [67]. Buffers increase a system's resilience and decreases its vulnerability.

Another method for system designers to decrease vulnerability is to add redundant components, so that a failing LSS component can be replaced by its spare. In space systems, however, each added kilogram to the system adds significant cost to the mission. For a PBR culture vulnerability could also be decreased and resilience increased if organisms are stored outside of the PBR. These backup cultures adds little extra weight, making it a cheaper form of redundancy. Both these methods need crew time to replace or repair the

bioreactors. In space missions crew time is expensive; the more time spent on maintenance and repair of the LSS, the less time will be available for the main mission objectives. Bartsev and Okhonin suggested a method for increasing reliability and resilience in BLSS which reduces both redundancy and crew time for repairs: using several bioreactors, working in parallel. Should one of them fail, the others can be boosted to cover for the failing reactor while it recovers, using the self-restoring capability of biological cells to let the bioreactor regrow "on its own" from the cells surviving the failure [9].

A PBR would only be one component (or several parallel components performing the same task) in a LSS. The bioreactor's reliability may not need to be translated directly into reliability for the whole system, if the system is designed to allow the bioreactor to fail. This non-linearity and trade-off effects such as those discussed above, are some of the factors making it difficult to calculate reliability, resilience and vulnerability for ECLS. Databases for failure exist for space systems but they rely on operational data [67], and fully realized BLSS do not exist other than as concepts and lab models as yet. Reliability calculations have to be handled with theoretical calculations instead and can be done using different mathematical principles. Even with full-scale ECLS it would be hard to calculate reliability since they have to be functional for a very long time. A test to see if a ECLS meets the criteria of mean time to failure less than 0.01 would mean running a system for 100 years, or running 100 systems for 1 year [8].

The reliability of single components, such as bioreactors, can and have been tested however. In general, bioreactors can fail both due to problems with the reactor culture itself or its reaction to external stress or due to failures of the technical equipment supporting the bioreactor. Biological organisms often have narrower tolerance ranges than technical components in terms of temperature, pH and other parameters. However, according to Bartsev et al [6] the experiences from BIOS-3 show that when a bioreactor fails, it is because of the technical components of the systems, not the biological. Strayer et al, [129], had similar experiences. As mentioned above, Bartsev et al [9] advocates for the organisms self-restoring capability — used properly that capability can be used to increase the resilience of biological components to be greater than technical components.

1.2.5 *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is taxonomically affiliated to the phylum Chlorophyta, class *Chlorophyceae*, order *Chlamydomonadales*, family *Chlamydomonadaceae* and genus *Chlamydomonas*. It is a well-studied microalga that has

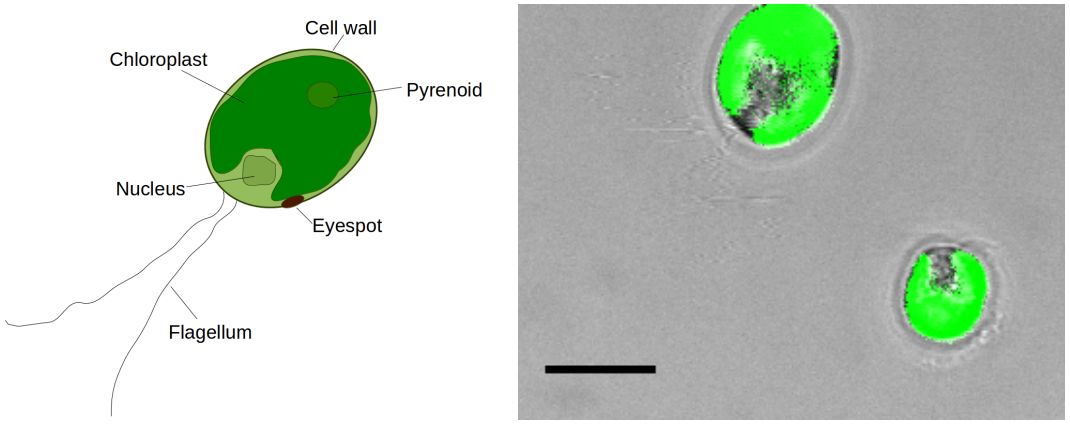

Figure 1.4: Chlamydomonas reinhardtii. *To the left a schematic of* C. reinhardtii, *showing the anterior flagella and its chloroplast. Close to the flagella is the eyespot, with which the cell can sense light intensity. The pyrenoid houses cell's the carbon-concentrating mechanism. To the right is a light microscope picture of two* C. reinhardtii *cells overlaid with a chlorophyll fluorescence picture, taken with a confocal laser scanning microscope, the scale bar is 10 µm. The picture shows the outline of the single chloroplast in each cell. Chlorophyll fluorescence is emitted in the red part of the light spectrum; the emission wavelength filter was set to 660-703 nm when this picture was taken. In this picture the chlorophyll fluorescence is shown in green to represent chlorophyll.*

been used extensively as a model organism in chloroplast studies [82] and has been suggested as a model organism for plant heat stress response [58]. Its hydrogen producing capability is being studied in applied research, where efforts to develop efficient hydrogen producing *C. reinhardtii* strains, as well as PBR technology for culturing these strains, are being made [74, 78].

Chlamydomonas cells are haploid, except the zygospore which is diploid. The nuclear genome consists of 17 chromosomes which contain 15 000 protein coding genes. The total genome size, including the chloroplastic and mitochondrial genomes, is 121 megabases [54]. The *C. reinhardtii* genome has been fully sequenced in the *Chlamydomonas* genome project [88, 109], and is available online [85].

The alga is a mixotroph, it can do photosynthesis as well as grow heterotrophically if supplied with acetate as a carbon source. When deprived of sulphur, its photosynthesis is reduced while cell respiration is constant, leading to anoxia in the cell. Under these conditions an Fe-hydrogenase is produced, which reduces protons to molecular hydrogen, resulting in hydrogen gas release from the cell. This helps the cell to maintain activity in the electron transport chains in chloroplasts and mitochondria, thus maintaining some ATP production necessary for basic cell functions [43, 87].

The cells are approximately 10 µm long when new, but during their growth stage they grow to 2.2 their original size before mitosis [84]. The cells have two anterior flagella, see depiction in Figure 1.4, allowing swimming motility where the flagella move in a "breaststroke" pattern [54]. The flagella can also be used for gliding motility on substrates [18]. A light sensitive eyespot near the base of the flagella gives the cells photo sensing capability [116]. *C. reinhardtii* cells have been shown to be phototactic; they swim down a light gradient towards dimmer light if they find themselves in light levels that would be harmful to the photosynthetic machinery of the cells, and they swim up the gradient towards stronger light if they find themselves in low light conditions [42]. It has only one large chloroplast which is cup-shaped and fits around the nucleus. The cell wall of *C. reinhardtii* contains no cellulose, it consists of hydroxyproline-rich glycoproteins [90].

It has two different reproduction cycles, asexual and sexual. In the asexual cycle the cells undergo mitosis one, two or three times while still retained within the cell wall, after which the daughter cells are released (two, four or eight daughter cells depending on the number of cell divisions). When the cells are subjected to a dark:light cycle the cells grow in size during the light period, but the mitosis and release of daughter cells takes place at the end of the dark period [13]. The longer the light period, the higher the number of cell divisions that take place.

The sexual cycle is triggered by unfavorable environmental conditions and requires the mating of two mating strains, mt+ and mt-. Zygospore are formed by the fusion of two cells of opposing mating strains. Zygospores are very resistant to adverse conditions [125], and the cell can survive in this form for a long time before undergoing meiosis and continuing the cell cycle. In most research that does not directly test the sexual cycle only a mt+ or a mt- strain is used, so that the cells only have asexual reproduction.

C. reinhardtii has been flown in space biology experiments. One study tested if more radiation tolerant species can be developed through directed evolution [16]. Its stickiness to glass during its cell cycle has also been tested in microgravity conditions [128]. Another study found that the active growth stage of *C. reinhardtii* cell cultures was extended in microgravity [44].

Little work on biofilm formation of *C. reinhardtii* has been made, although it is known that it can excrete an extracellular matrix consisting of acidic polysaccharides [90]. It has however been reported to stick to glass [128], and one study used self-immobilization of *C. reinhardtii* to glass and silica beads as a means of cycling hydrogen-producing cells between sulfur-rich and sulfur-deprived conditions [53].

1.2.6 Heat shock response and heat stress tolerance in *Chlamydomonas* and other species

When a cell is subjected to heat stress, a heat stress response, HSR is triggered. In 2015 a review of studies of HSR in *Chlamydomonas* was published [121], with a model of HSR derived both from studies on *C. reinhardtii* and other species.

Heat stress affects the fluidity of cell membranes, cell metabolism, cell division, DNA replication and DNA repair, and also causes misfolding of proteins and failure to assemble protein complexes. In addition, elevated temperatures affect the environment of the cells; solubility of both O_2 and CO_2decreases, but the availability of CO_2decreases faster than that of O_2, making CO_2 less available to the cells.

When photosynthetic cells experience heat stress, their first response is to inactivate Rubisco activase, which in turn means that Rubisco will be inactivated so that carbon fixation ceases [39]. In order to maintain membrane viscosity, cells also change the ratio of saturated to unsaturated fatty acids in the membrane by exchanging fatty acids in it with longer and more saturated fatty acids synthesized de novo [123].

Another rapid response to heat stress is the arrest of DNA replication and repair. Mammalian cells arrest cell growth and division under stress, probably to avoid DNA damage [137]. Hemme et al. [58] have shown that *C. reinhardtii* cells also arrest cell division and DNA replication under stress, although cell growth continues at a reduced pace. They also showed that when cells are shifted back from heat stress to normal conditions their pre-stress state is not reestablished, although they resume their ability to divide after 8 hours of recovery time.

Heat stress leads to misfolding or failed folding of proteins, the occurrence of which triggers expression of a group of proteins called heat shock proteins, HSPs. A metabolic shift away from bulk protein synthesis to synthesis of stress response proteins like HSPs is another rapid response to heat stress. HSPs act as chaperones to newly formed proteins and help refold damaged proteins. HSP expression has often been used as a marker for heat shock response of *C. reinhardtii* [121]. These studies show that HSP expression starts when cells are shifted from 20 ° to 39 °C – 41 °C [132], from 24 °C to 36 °C [72] or from 25 °C to 37 °C [121].

Synthetization of HSPs take time, so cell survivability increases if they are subjected to induction heat stress at a lower temperature before being subjected to higher temperatures. This allows the cells to start the process of

HSP synthetization before the stress gets too severe. The effect of induction time on survival of *C. reinhardtii* cells have been studied for example by Hema et al. [57].

The stress response is not constant over time; after about 3 hours of stress the combined effects of reduced bulk protein synthesis and accelerated synthesis of HSPs will have reduced the concentration of misfolded or unfolded proteins. This leads to inactivation of stress kinases. HSP levels after heat shock are modulated compared to pre-stress levels [58]. When heat stress goes on for longer, up to 24 hours, membrane viscosity is restored through the exchange of fatty acids and ATP and NADPH is no longer needed for fatty acid synthesis. Rubisco activase can therefore regain activity and activate Rubisco and carbon assimilation [58].

However, when Rubisco reactivates, the increased relative ratio of dissolved O_2 to dissolved CO_2in the cell environment combined with Rubisco's decreased selectivity for CO_2 at elevated temperatures, will lead to photorespiration [58, 70]. Photorespiration results in formation of H_2O_2, a reactive oxygen species, ROS. Furthermore, when fatty acid synthesis is no longer a sink for ATP and NADPH the PS electron chain becomes overreducted, leading to PS antenna uncoupling and increased Mehler reactions producing another ROS: O_2^-. The increase of ROS leads to increased ROS scavenger expression as a late response to heat stress [95, 58]. Heat stress tolerance has been tested on *Chlamydomonas* in a few studies, but no extensive mapping of heat tolerance at different temperatures and with different acclimatization times could be found in the existing literature.

1.2.7 Stress tolerance of biofilms

Cell survivability under stress depends on the state of the cell. Cells with a slower growth rate are less sensitive to stress than those that grow faster [12]. Cells in biofilm grow slower than those in liquid culture and may therefore be more resistant to stress.

Cells growing as a biofilm have been shown to be more tolerant to several types of stress than cells growing in liquid culture [27, 81]. This could have several explanations, the biofilm itself is for example a barrier to harmful compounds. It is also a barrier to dehydration as was shown for facade growing algae [56]. Natural biofilms contain many different microbial species, that can promote each others growth by forming nutrient chains. The biofilm, whether it contains many species or one, retains nutrients and extracellular

enzymes close to the cells, helping nutrient intake. When enzymes stay close to the cells producing them productive phenotypes are promoted, increasing the overall productivity of the biofilm [139]. Cells growing in a biofilm also show stronger expression of heat response genes than those in liquid culture [112], even when temperatures are not elevated, leading to cells being prepared for stress before it happens.

1.3 Aims of thesis

The overall aim of this thesis was to investigate methods to increase resilience and decrease vulnerability of PBR cultures. Two methods for restoring a PBR culture after a breakdown were investigated in lab-scale, both intended to restore a *Chlamydomonas reinhardtii* planktonic culture after heat stress. The first method acts internally in the PBR. Investigations aimed at finding out if a subset of cells grown as a biofilm inside the planktonic PBR culture would survive stress better than the planktonic culture and therefore be able to restore it after stress. When results showed no difference between heat stress survival of *C. reinhardtii* planktonic culture and biofilm culture a second method for restoring a PBR culture was investigated. The second method acts externally by restoring the PBR from cells stored outside the PBR, protected from stress, encapsulated in a silica gel.

Two research questions and three specific aims guided the research:

Question I: "Can a subset of a *C. reinhardtii* culture grown as a biofilm be used to restore the culture after it has broken down due to heat stress?"

Aim i) To explore attachment and cell viability status of *C. reinhardtii* grown as a biofilm on alumina supports with micro-scale pores, with respect to different pore sizes and surface properties of the alumina.

Aim ii) To test heat stress survival of *C. reinhardtii* when growing as a biofilm on alumina supports compared to survival as a planktonic culture.

Question II: "Is immobilization in sol-gel a possible method for storage of viable *C. reinhardtii* cells?"

Aim iii) To test long term stability and survival of *C. reinhardtii* immobilized in a sol-gel.

1.4 Research strategy

The green alga *Chlamydomonas reinhardtii* was chosen for this work because it has shown promise as a hydrogen producing organism, is known to function well in closed bioreactors, and because it is a well-studied organism which has been used as a model organism in photosynthesis research and the effect of temperature on photosynthesis. Its hydrogen producing ability could be interesting for some space applications, such as in situ fuel production. *C. reinhardtii* is described more closely in section 1.2.5.

The investigations were carried out with a focus on heat stress, as this is one of the most common stresses in PBR cultures. The PBR culture needs light as an energy source, but only a portion of the light is used for photochemistry. A significant portion of it which is absorbed by the light harvesting antenna in the photosynthetic machinery is re-emitted as heat, thus heating of the culture. Furthermore, heat stress could easily arise in a closed LSS due to failure of technical equipment. Further information on stress factors in PBRs is given in section 1.2.3, and previous work *C. reinhardtii* heat tolerance is described in section 1.2.6.

For research question I, "Can a subset of a *C. reinhardtii* culture grown as a biofilm be used to restore the culture after it has broken down due to heat stress?", a biofilm substrate had to be found. Therefore, aim i) was to test *C. reinhardtii* biofilm growth on a certain type of porous alumina supports. The alumina was chosen because it is an inert material whose surface properties can easily be modified with chemical treatments via silanization. Furthermore, the porosity of the material can be controlled. This means that many different alumina varieties could be produced, with different surface characteristics and surface structures. The alumina material is described in detail in section 1.4.1. The results of the biofilm growth experiments are reported in chapter 2.2.

Aim ii) was to compare heat stress survival of *C. reinhardtii* biofilm on alumina support to heat stress survival of planktonic culture. A heat test setup was designed and built in order to carry out such tests. The setup is described in section 1.4.2. In order to know the temperature tolerance range of *C. reinhardtii* a literature study was conducted (see section 1.2.6), but the tolerance was also tested in the lab, with the help of the work of MSc student Alaa Al-Hashimi, as described in 2.3. The result of the biofilm heat tolerance experiments can be found in 2.4.

For research question II, "Is immobilization in sol-gel a possible method for long-term storage of viable *C. reinhardtii* cells?", *C. reinhardtii* was immobilized in a sol-gel started from colloidal silica particles, using a new method in

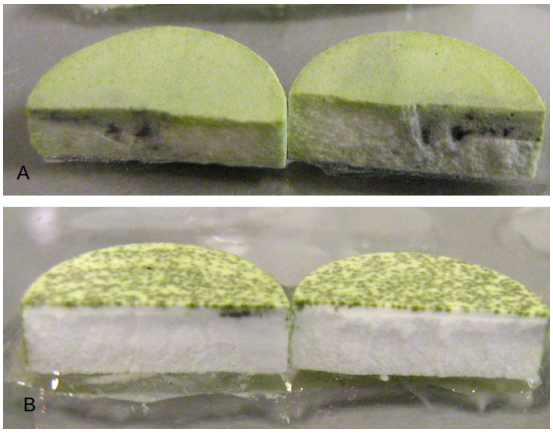

Figure 1.5: Cut coupons. *The photos show two coupons having been cut in half by a scalpel. The black areas in middle of A are residues from the scalpel cutting the coupons. Coupon A is made out of ceramic stirred at 400 rpm, and coupon B from ceramic stirred at 1100 rpm in the manufacturing process. A therefore have larger pores than B. Most of the green cell growth is concentrated on or near the surface of the coupons. In A the growth is spread a little further inside the coupon than in B. Compare to fluorescence picture of cut coupons in 2.2.*

which the technical manufacturing of the gel and the mixing of gel and biological cells are done in two separate processes. The work was part of a larger study where this immobilization method was tested on several different algal species. *C. reinhardtii* was included as a representative of a fresh water alga. The sol-gel process is described in section 1.4.3. The alumina supports used with the sol-gel were different from the alumina used in the previous tests, in that the pores were in macro-scale so that water could flow through them. The results of the immobilization study is reported in 2.5.

1.4.1 Porous alumina ceramic

Al_2O_3, or alumina, is a ceramic material that is bio-inert and insoluble in water. It is often used for medical implants [41]. It is used as an abrasive in industry due to its hardness, and in sunscreen due to its white color and chemical inertness. One test have described toxicity of alumina nano-particles to fresh-water algae, but the authors attributed the toxicity to the properties of the material and to leaching of aluminium ions, Al^{3-}[103]. Other experimenters have used alumina as a support for sensitive human cell cultures successfully [17].

The biofilm substrates used in the work described in this thesis have been manufactured from α-Al_2O_3 powder in an innovative process, in which the

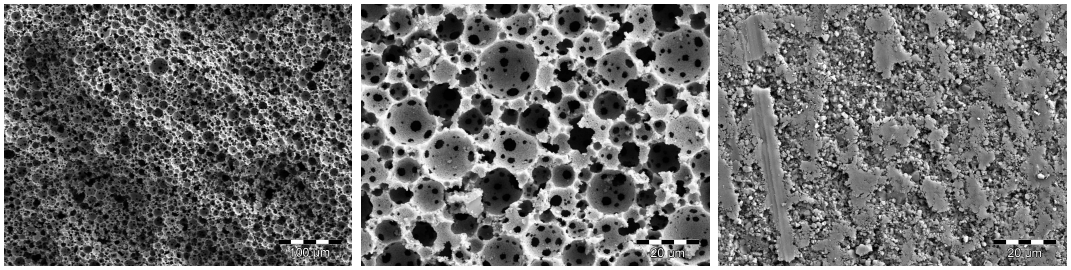

Figure 1.6: Alumina structure. *The scanning electron micrographs (SEM) show three different variants of alumina. The picture to the left shows an alumina that was stirred at 1500 rpm and has been sandblasted after manufacturing as a means of gaining extra surface. It is shown at 200x magnification and the scale-bar is 100 µm. The middle pictures shows a 1000x magnification of a ceramic stirred at 1500 rpm, the scale-bar is 20 µm. This ceramic was not sandblasted. The individual alumina kernels making up the bulk can be seen, and it is also possible to see that the windows between pores are smaller than pores themselves. The picture to the right shows a non-porous control ceramic at 1000x magnification, scale-bar 20 µm. SEM-pictures courtesy of Christian Soltmann.*

porosity of the resulting ceramic foam can be tightly controlled, the HAPES method [5]. They were manufactured and measured by Christian Soltmann, Novelpor, and followed the same procedure that was described in [73], where the same type of ceramics were used as spawning plates for clownfish. One aim of the work in the first research track was to find out whether the alumina produced with the HAPES method would have a toxic effect on *C. reinhardtii*. See examples of the alumina substrates in Figure 1.5.

The manufacturing process starts with suspension of α-Al_2O_3-powder in deionized water with an anionic polyelectrolyte dispersion agent. Decane (70 vol%) and the anionic surfactant sodium lauryl sulfate (0.33 vol%) are added before the suspension is stirred, creating a high alkane phase emulsified suspension, HAPES. The stirring rate decides the porosity of the final ceramic foam; higher stirring rates will result in smaller decane droplets which will result in smaller pores in the alumina, see Figure 1.6. The emulsified suspension is poured into a mould and is allowed to dry in room temperature under ambient pressure for 4 days followed by drying at vacuum for 1 day. The final step is sintering of the foam at 1400 °C for 2 hours.

The porosity of the ceramic foams was measured by the manufacturer using mercury intrusion porosimetry. Pore sizes ranging from 4 to 50 µm on the surface of the ceramic were produced for the work in this thesis. Each ceramic foam has pores in a range of sizes, the manufacturing process controls the average and extent of that pore size range. The *C. reinhardtii* cell interaction with the ceramic depend on the surface pore size, but also on the size of the windows between pores inside the material, as this determines how easy it is

Stirring rate/name	Pore size range on surface	Average window size between pores
Non-porous controls	—	—
1500 rpm	∼4-16 µm	∼2-3 µm
1100 rpm "S"	5-20 µm	3 µm
700 rpm "M"	10-40 µm	7 µm
400 rpm "L"	30-50 µm	16 µm

Table 1.1: Ceramic foam porosities measured through mercury intrusion. *The porosity of a ceramic foam is controlled by the stirring rate of the ceramic emulsion in the manufacturing process, the foams are presented in this table with the stirring rates that produced them (rpm = revolutions per minute). For the foams that were used in the cell attachment tests their names as used in section 2.2 are also given; S = small, M = medium, L = large. The 1500 rpm foams average pore window size has not been measured, only estimated from SEM-pictures. Listed are also the non-porous alumina controls that were used in the cell attachment tests.*

for cells and liquid to penetrate the ceramic. The average pore window sizes ranged from 2 to 16 µm. Pore sizes around the size of the *C. reinhardtii* cells (10 µm) were chosen, in order to maximize the surface area on which the cells could attach. Several pore size distributions were manufactured for the pilot tests, see table 1.1. SEM-pictures showed that samples stirred at 1500 rpm and 1100 rpm were very similar in pore size distribution and pre-studies to the cell attachment experiments showed that attachment did not differ between those two pore size distributions, hence the 1500 rpm samples were not used. The experiments did, however, use non-porous controls - ceramics with solid surfaces manufactured with the process described above, but without stirring, see Figure 1.6.

The chemical properties of the alumina surface can be modified by attaching molecules with different functional groups to it. Without surface modification the alumina is slightly hydrophobic. Several surface modifications were tested in pre-studies (see Table 1.2). These were prepared using two different modification methods — acid immersion and silanization. Immersing ceramic in acid makes molecules bind to the surface by themselves. Silanization on the other hand is a process where a molecule is attached to a metal oxide surface via a silane-bridge; the hydroxyl groups on the metal oxide surface displace alkoxy-groups on the silane. The silanizations done for this work were done using a wet-chemical method in which silane (attached to the desired functional groups) is dissolved in a mixture of water and acetone. The ceramic was incubated with this mixture for 16 hours and was then washed with pure water-acetone mixture and dried at 70 °C for one hour. In this work several different surface modifications were chosen, on the basis of a literature search. The literature shows that algae attach better to hydrophobic materials [101, 122], probably due to hydrophobic interaction, so a treatment

that would make the alumina hydrophobic, with hexadecyltrimethoxysilane, was chosen. Electrostatic interaction with the negatively charged surface of algal cells and the positively charged surface of a substrate also enhances algal attachment [27], so a treatment with aminopropyltrimethoxysilane was chosen to give the alumina surface positive charge. Other surface modifications tested and listed in Table 1.2 were designed to test other surface properties. They did not perform well or at least did not outperform the aminopropyl- and hexadecyl- treatments in pre-studies, and were not tested further.

In the sol-gel test another variety of porous alumina was used, with millimeter-scale pores that allowed water to flow through the substrates, see Figure 1.10. These substrates were not produced with the HAPES method, but in a direct foaming method described in [5]. The sol-gel is described in section 1.4.3.

Molecule	**Treatment**	**Induced surface property**
Oxalic acid 700 µmol/g	Immersion	Negative surface charge, hydrophilicity
Benzoic acid 700 µmol/g	Immersion	Negative surface charge, hydrophobicity
Benzoic acid 3.6 mmol/g	Immersion	Negative surface charge, hydrophobicity
Dicarbonic acid	Immersion	Chelating
Aminopropyltrimethoxysilane*	Silanization	Positive surface charge
Methacryloxypropyltrimethoxysilane	Silanization	Weakly hydrophobic, protein binding
Glycidoxypropyltrimethoxysilane	Silanization	Weakly hydrophobic, protein binding
Hexadecyltrimethoxysilane*	Silanization	Strongly hydrophobic
—	Sandblasting	Increased surface area

Table 1.2: Ceramic surface modification treatments that were tried in pre-tests. *The two treatments marked * were chosen for the final cell attachment and heat stress tests, together with ceramic samples without any surface treatment. Most treatments were designed to change either surface charge or hydrophobicity/-philicity or both as algal cells have been shown to attach via electrostatic as well as hydrophobic interaction. One exception is dicarbonic acid immersion which was chosen as organic acids have chelating properties, which has been connected to* C. reinhardtii *palmelloid formation in one test [65]. Protein binding treatments were chosen as they might bind protein on the algal cell wall surface. One surface treatment, sandblasting, is physical, not chemical. This was done to increase the surface area of the coupon, so that more cells could attach. Without surface treatment alumina is weakly hydrophobic.*

1.4.2 Heat stress experiment setup design and validation

The experiments that compared the heat tolerance of *C. reinhardtii* biofilm to that of its liquid culture were conducted in a heat test setup that was designed specifically for these tests by the thesis author and by professor Matthias Ullrich, and were manufactured by Bernd Schmeyers, OHB. This

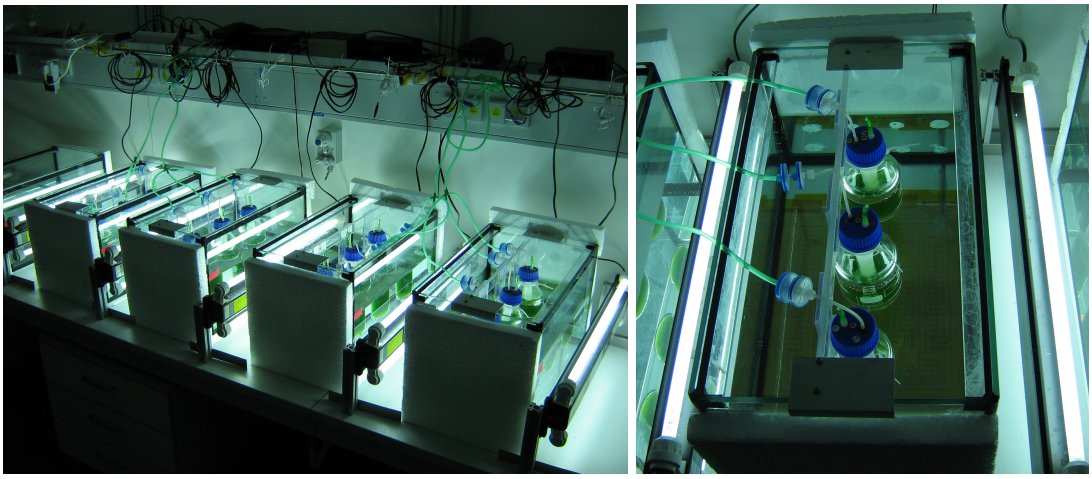

Figure 1.7: Heat stress experiment setup. *Four aquaria were used as transparent heat baths. The electric heating sheet used to control temperature of the heat bath can be seen underneath the aquarium in the picture to the right. Lights were placed between the aquaria. The heat baths were isolated with styrofoam underneath the heating sheet, on the sides and during the experiments also on the top. In these pictures the styrofoam on top has been removed to show the layout of the experiment setup.*

section describes the the test setup and the work done to validate its temperature stability.

The purpose of the setup was to enable testing of liquid cultures and biofilm cultures at four different temperatures simultaneously, keeping all other conditions equal. It consisted of four water baths, in the form of rectangular glass aquaria filled with approximately 30 l of water with temperature controlled electric heating sheets underneath them, see Figure 1.7. The water baths were insulated with styrofoam (approximately 5 cm thick) underneath the heating sheets, on both ends and on top. Fluorescent lights bulbs were placed on each side outside of the aquaria. These had a bluish spectrum (9000 K) to allow the most light to reach the algal cultures. Light with shorter wavelengths (bluish light) is absorbed more slowly by water than light with longer wavelengths (reddish light). Each water bath was illuminated by four 24 W bulbs, in a 12:12 day:night cycle all through the growth phase and heat test.

The algae were grown in small lab PBRs manufactured from 500 ml cylindrical Schott bottles, see Figure 1.8. These were designed to simulate conditions in large-scale PBRs and allowed growth and heat stress treatment of 330 ml liquid culture and three biofilm substrates in the same vessel. An air inlet was made from a hole in the cap via a tube that led the air into the bottom of the PBR. Air was pressed through a gas inlet and the liquid culture by an aquarium pump. The air mixed the culture and provided it with carbon

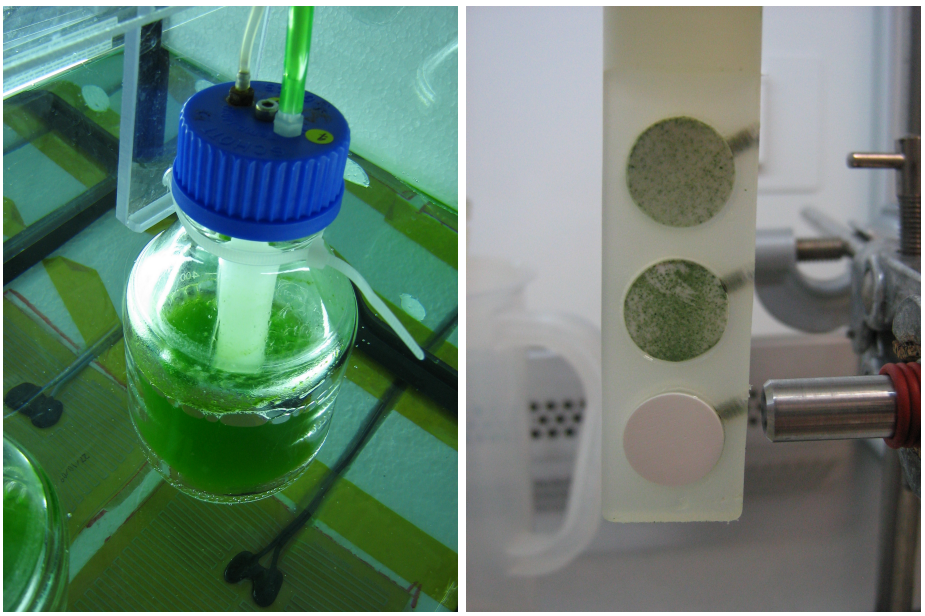

Figure 1.8: Lab bioreactor and alumina coupon holder with coupons. *The picture to the left shows one of the lab bioreactors immersed in the heat bath. It is hanging from a plexiglass support, to which it is fastened with a cable tie. The alumina coupon holder fastened to the cap can be seen sticking down in the middle of the bioreactor fluid. To the right the lower part of a coupon holder with coupons can be seen. These coupons have been immersed in bioreactor fluid during an experiment and have algal cells attached to them, in differing amounts depending on their surface treatments.*

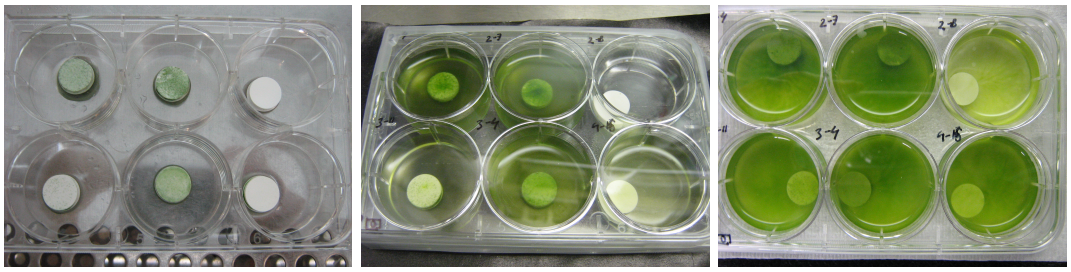

Figure 1.9: Illustration of a re-growth test. *The three pictures show the same well-plate at different points in time, and illustrates a re-growth test. In the picture to the left the coupons have just been removed from the coupon holder of a bioreactor after an experiment. They have been placed in the wells of the plate which will then be filled with TAP-medium. The middle picture shows the same well-plate three days later, after it has been incubated in a growth chamber. It is possible to see differences in cell concentration between the different wells. The picture to the right shows the well-plate seven days after the first picture was taken, most of the cell cultures in the wells have reached maturity and it is harder to see a difference between them.*

dioxide when it bubbled through the culture. No extra carbon dioxide was added to the air. A gas outlet in the cap of the bottle let gas out from the PBR. Both gas outlet and inlet had 0.2 µm filters, to avoid contamination of the culture.

Each lab PBR had a substrate holder fastened to the inside of its cap (see Figure 1.8). The holders were cylindrical rods with three circular holes, 12 mm in diameter, in which round alumina coupons could be fastened, see Figure 1.9. The substrates were thus immersed in the liquid culture, in the center of the bottle. The PBRs were hung from a support fastened at the top of the aquarium so that they were immersed almost to the cap in temperature controlled water.

The bottles were placed in the water baths, which were kept at room temperature, RT, and were kept there during the growth phase of cultures, in order to change conditions as little as possible between growth phase and stress phase. The culture vessels were removed from the water baths, temporarily, shortly before the heat stress tests, so that the water bath temperatures could be raised to the three different target temperatures. Experiments were conducted with three bottles in each heat bath and with four heat baths at different temperatures in parallel.

The temperature stability of the water baths was studied in a series of tests designed to be relevant for the already tested heat tolerance of *C. reinhardtii* (Section 2.3). Several parameters were tested; the time to heat up the liquid in the PBRs immersed in the water bath, the difference in temperature between the top and the bottom of the PBR, and temperature stability during a research relevant time interval. Between 40 °C and 50 °C the PBRs heated up to within -0.1 °C/+0.4 °C of their target temperature in 30 minutes and were able to keep the temperatures with less than 0.2 °C variation for 30 minutes after that. The temperature difference between top and bottom of the PBR was negligible.

The 30 minutes it took to heat up the PBR culture functioned as an acclimatization period for the *C.reinhardtii* cells. The acclimatization period length varied only slightly between the different temperatures. As was shown in the heat tolerance tests described in Section 2.3 acclimatization impacts the cell survival rate. The situation is most similar to the gradual heating tests. Large scale PBR cultures also experiences acclimatization periods before heat stress, due to their large volume, so acclimatization was an important feature of the heat tests.

To evaluate if the algae heat tolerance tests done in thermomixers could be applied to the PBRs in water baths several survival rate pre-tests with liquid algae cultures in the PBRs was done, with CFU and re-growth samples

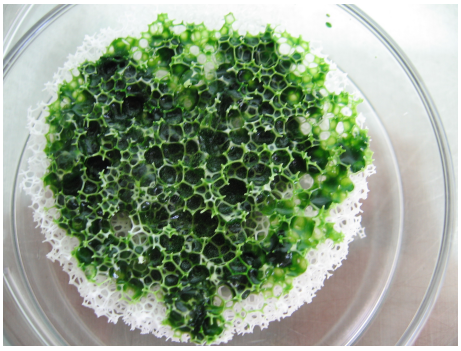

Figure 1.10: Sol-gel enveloped alumina. *Photograph of a macro-porous alumina support with silica sol-gel containing* C. reinhardtii *cells, taken shortly after gel has been added to the support. The alumina disc is approximately 8 cm in diameter. The macro-porous structure allows water to flow through the support, making it possible to use them in a flow-through bioreactor as a lab-scale waste-water treatment device.*

(see figure 1.9) taken before and after the heating period. The thermomixer tests on *C. reinhardtii* had shown differences in survival rates within 30 minutes of stress after acclimatization. The aim was to find a heat stress period that allowed detection of as small differences in heat stress response between samples as possible. The optimal heating period for experiments in the heat test setup, the time from that the PBRs at room temperature were put in the pre-heated baths was estimated to be 60 minutes; 30 minutes to heat up the flasks and 30 minutes of stress time. Repeated tests showed, however, that 50 minutes heating period in the heat test setup gave differences in cell survival for exponentially growing cultures of one order of magnitude between 40 °C, 42 °C and 44 °C samples, so this interval was ultimately used in the heat stress experiments.

1.4.3 Sol-gel encapsulation

Sol-gel encapsulation of living *C. reinhardtii* cells was studied as an alternative method of PBR restart, in which encapsulated cells are stored outside the PBR and manually inserted into it to inoculate a new culture. The sol-gel is transparent to light, and is also porous enough to let nutrients and carbon dioxide through to the cells. Thereby the cells can continue to grow inside the gel, while being protected from contamination and dehydration. The encapsulation slows down cell metabolism so that cells survive longer than they would if they were in free culture [35]. These properties of encapsulated living cells suggest that encapsulation could be a suitable method for for long-term storage of *C. reinhardtii* cells. Advantages of encapsulation over methods like

cryopreservation, especially from the point of view of life support systems, includes no required access to freezers and fast start of cultures from stored samples.

Encapsulation of biological cells and enzymes is a large research area as it offers advantages for biotechnological production: encapsulated cells shift their metabolism from cell growth to production of other metabolites, and it separates the cell culture from its metabolites.

Encapsulation has been done on bacteria, mammalian cells, algal cells and enzymes in a variety of gel materials. Materials such as alginate are inexpensive, and are easy to manufacture, but many organic gels have limited stability and will rupture eventually [142, 79]. Inorganic gels, such as silica or alumina gels are stable and have therefore been studied for use with biological cells. One promising method of encapsulation is the use of silica sol-gel. "Sol" stands for solution and the name "sol-gel" refers to the method of synthetization: a *sol*ution of metal monomeres, oligomeres and colloidal particles is *gel*ated by the creation of chemical bonds between them. However, manufacturing routes for inorganic gels were first developed for use without biological cells, which meant that the formation of by-products such as alcohols were not seen as a problem. Earlier work on encapsulation of biological cells in inorganic gels has used glycerol or other additives to protect the cells against harmful by-products from gel formation.

In this work the viability of *C. reinhardtii* cells and gel stability of a silica gel manufactured with a novel method has been tested, for the purpose of answering research question two. The method has two benefits: the first is that colloidal silica particles are used, which minimizes shrinking of the gel. This minimizes pressure on the cells and results in a porous gel structure that allows fast exchange of nutrients and gases. The second benefit is that the technical manufacturing of the gel and the biological mixing of the cells and the gel is done in two separate processes so that cells can be kept at biological conditions throughout the process and additives can be avoided. An example of algae encapsulated in sol-gel can be seen in Figure 1.10, and the results of the experiments are described in 2.5.

Results

2.1 List of manuscripts and statements of contributions

Results are represented by the following manuscripts which were prepared during the work with this PhD thesis:

***"Chlamydomonas reinhardtii* growth on alumina supports: comparing pore sizes and surface treatments"** Cell attachment experiments on alumina of various pore size distribution and with different surface treatments showed that the alumina system is suitable for *C. reinhardtii* biofilm growth and that pore sizes in the range of 30 - 50 µm with pore windows around 16 µm had the most cells attaching to them. Alumina with no surface treatment or treated with aminopropyltrimethoxysilane showed the best results. These types of alumina were therefore used in the heat stress survival on alumina work.

All authors contributed to the conception and design of the study and to the writing of the article. Maria Johansson conducted all experiments, collected and assembled all data, and drafted the article; Maria Johansson, Christian Soltmann and Matthias S. Ullrich analyzed and interpreted the data.

***"Chlamydomonas reinhardtii* heat stress survival when growing as a biofilm on porous alumina substrates"** Heat stress survivability and re-growth ability of *C. reinhardtii* cells attached to alumina as compared to liquid culture cells were tested. No difference in survivability or re-growth ability could be detected in these tests, and it was therefore decided to continue survivability tests with the sol-gel system instead.

All authors contributed to the conception and design of the study and to the writing of the article. Maria Johansson conducted all experiments, collected and assembled all data, and drafted the article; Maria Johansson, Christian Soltmann and Matthias S. Ullrich analyzed and interpreted the data.

"A new process for silica sol-gel encapsulation of algae for storage purposes" Long-term survival of *C. reinhardtii* enveloped in a silica sol-gel was tested. The results showed that *C. reinhardtii* can survive for several months in such a system.

All authors contributed to the conception and design of the study. Maria Johansson and Christian Soltmann conducted all experiments, collected and assembled all data. Maria Johansson, Christian Soltmann and Matthias S. Ullrich analyzed and interpreted the data. Maria Johansson drafted the article, and Christian Soltmann, Matthias S. Ullrich contributed to the writing of it.

Additionally, CHAPTER 2.3 describes the results of the heat tolerance tests performed in preparation of the heat stress tests described in chapter 2.4. It gives a comprehensive picture *C. reinhardtii* survivability in a wide temperature range and at various heat stress times, something which is not readily available from the existing literature.

The work was carried out as a lab rotation project by M. Sc. student Alaa al-Hashimi under the supervision of Maria Johansson. Maria Johansson designed the study. Alaa al-Hashimi contributed to the design of the study, conducted all experiments, collected and assembled all data. Maria Johansson and Alaa al-Hashimi analyzed and interpreted the data. Maria Johansson wrote the text of chapter 2.3.

2.2 *C. reinhardtii* growth on alumina supports

The following manuscript has been submitted.

Chlamydomonas reinhardtii growth on alumina supports: comparing pore sizes and surface treatments

Authors:

Maria Johansson*; Christian Soltmann; Klaus Slenzka; Matthias S. Ullrich

*corresponding author: m.johansson@jacobs-university.de

Abstract

Microalgae can be used commercially to produce various products such as vitamins, fish feed and energy carriers like hydrogen. Growing microalgae as a biofilm makes it easier to control the environment of the algal culture. Liquid culture production, on the other hand, offers high biomass yields. In this study *Chlamydomonas reinhardtii* surface attachment and growth on alumina substrates with different porosities, with or without surface treatments, have been tested. The surface treatments were silanization with either aminopropyltrimethoxysilane or hexadecyltrimethoxysilane. The purpose was to find out what porosities and surface properties of the alumina allowed the most cells to attach. The results of the study show that more *C. reinhardtii* cells attach to alumina with pore sizes around 30-50 μm and pore windows around 16 μm than to alumina with smaller pores. Silanization with hexadecyltrimethoxysilane had a negative effect on cell attachment, especially for the alumina with smaller pore sizes. The future aim of this work is to find a material on which cells attach and survive stress events in a liquid culture bioreactor, allowing them to function as an inoculum for a restored liquid culture after the stress event.

Keywords:

Alumina; Algae; Algal attachment; Biofilm; *Chlamydomonas reinhardtii*; Photobioreactors

2.2.1 Introduction

Growing microalgae as a biofilm offers advantages over planktonic cell culture in commercial algae production; several high-value products such as fatty acids, vitamins, and compounds that can be used as drugs are produced by algal cells when they are exposed to stress [124], and biofilm reactors make control of environmental conditions for the cells easier, making it possible to stress them at specific levels and at specific times and durations. Such reactors also offer technical advantages during harvesting of algal cultures to be used as biofuel [52].

Microalgae have been grown commercially for decades, mainly for food supplements, pigments, and fish feed [127]. This production has used planktonic cultures grown in open ponds. It has been proposed to also use microalgae for commercial production of lipids or hydrogen as biofuel [124, 22]. One organism that has received particular attention in this research is *Chlamydomonas reinhardtii*, because of its ease of cultivation, the vast knowledge about its biology, and its natural ability to produce hydrogen [87, 75].

C. reinhardtii is a microalga which, in addition to its photosynthetic ability, can use acetate as a carbon source. It is oval, approximately 10 μm in length, and has two anterior flagella with which it can move in liquid culture using "breaststrokes" [54]. The flagella are also used in cell-to-cell interaction, in cell-substrate interaction, to anchor the cells [18], as well as for gliding motility on solid media [54]. *C. reinhardtii*'s cell wall means that the flagellar membrane is the only domain of the cell membrane accessible from outside [18]. The cell wall consists of hydroxyproline-rich glycoproteins and the species can also produce a gelatinous extracellular matrix. In light-dark cycles the *C. reinhardtii* cell cycle is synchronized, in a 24 hour cycle cells typically undergo two or three divisions.

In order to make biofuel production economically feasible the algae have to be grown in low-cost systems [94], and without major energy inputs [26]. The production is foreseen to take place in closed systems to keep cultures axenic, but also because it is easier to control the impact of environmental factors [78]. Research effort has been spent for example on developing bioreactor designs which allow more light to be utilized without stressing the algae [37], on cost effective production systems [83], and on genetically modifying the algae to increase their metabolic productivity [75].

One research avenue has been the study of biofilm reactors [22, 91]. Biofilm reactors are already used in water treatment facilities, bioremediation, and in

some biotechnological applications [113, 96], but are less common for axenic microalgal culture. Growing the organism in a biofilm could help make the process more controllable [53], increase light penetration into the culture [66], decrease water and energy use of the bioreactor [102], and decrease harvesting costs [52, 69].

Much of the research on biofilm reactors has concentrated on finding the right substrate for cells to grow on, but factors that increase bacterial and algal cell attachment to surfaces have also been studied in ecosystem and biofouling research. The cells attach either via their cell appendages, or via their cell bodies, through several possible mechanisms. Electrostatic interaction with charged surfaces is possible since bacterial and microalgal cell surfaces often, but not always, are electronegative [27, 4]. Also, the surfaces of many micro-organisms, including microalgae, are often hydrophobic, making hydrophobic interaction another possible attachment mechanism [4, 101, 122]. Hydrophobicity is not always correlated to attachment though [64]. Functional groups on the cell surface can also form covalent bonds [27], and enable acid-base interactions [101, 45] with a substrate. These chemical forces compete with each other, with attracting forces being able to dominate over repulsive ones, and vice versa [27]. It is therefore difficult to predict whether a microalga species will attach to a specific substrate or not, even if their respective surface properties and the chemical environment around them would all be fully known.

The properties of the substrate surface are important mostly in the initial attachment phase. Differences in biofilm productivity between samples of different substrates diminish when biofilm is allowed to form for a longer time [45]. This may be due to the production of extracellular polymeric substance, EPS, which can have other chemical properties than the cell surface [15].

Another important factor for biofilm formation is surface roughness. Porosity and surface roughness increases the surface area of a given amount of substrate, so that porosity increases the number of cells attached to a surface [27, 122]. Porous substrates with pore sizes one to five times the cell size have the most cells attaching to them [131]. When pores are smaller than the cells there will be fewer contact points between the substrate and the cell surfaces and it will be harder for cells to attach, whereas when pores are approximately the same size as the cells there are more contact points than on a flat surface [31]. Surface roughness allows the biofilm to grow without being sheared off [2].

The study reported in this paper examines porous alumina as a support material for *C. reinhardtii* grown in a biofilm. A manufacturing method for alu-

mina in which porosity can be controlled was used [5]. Aluminium ions, Al^{3+}, are cytotoxic, but alumina, Al_2O_3, is chemically inert. Porous alumina ceramics have been used as a support when growing human cells [17], and alumina coated SiO_2 nanoparticles are less toxic than non-coated ones [59]. However, one study on alumina nano-particles found that they were toxic to algae [103]. This was thought to be a combined effect of the size and shape of the nanoparticles and leaching of Al^{3+} ions from them. Other studies found metal-oxide surfaces to be beneficial to attachment, because metal oxides contain hydroxide bonds in solution, which can interact with the cell surface in hydrogen bonding [27].

The purpose of this work was to test materials that can support a *C. reinhardtii* biofilm, with the future intention of using such biofilms as heat stress resistant inocula to a liquid culture, after the liquid culture has broken down. The main aim of the study has been to find out how porosity and surface treatments of alumina substrates affect the number of cells attached to it, and how the alumina affect the cells' ability to grow and produce daughter cells.

2.2.2 Materials and Methods

Algae species, media, and culture maintenance

Chlamydomonas reinhardtii wild type SAG 11-32b was used in this study. Cultures were maintained on TAP-medium agar plates [50], at 23 °C. Culture maintenance as well as experiments were done under fluorescent cool white light in a day:night cycle of 14:10 hours. Experiments were done in liquid TAP-medium at room temperature. The temperature of the liquid culture itself was checked regularly with a laser thermometer and was never above 30 °C. TAP-medium contained acetate, thus allowing the cells to grow heterotrophically as well as do photosynthesis.

Alumina ceramic

Porous alumina ceramic was manufactured as described in [5]. The pore size ranges resulted from the stirring rate in the manufacturing process. For this study three different stirring rates were used: 400 rpm, 700 rpm and 1100 rpm. The pore size ranges resulting from these stirring rates have been described in [73], here they are simply called "Large" or L, "Medium" or M, and "Small" or S, see fig. 2.1. The "Large" pore size range is approximately 30-50

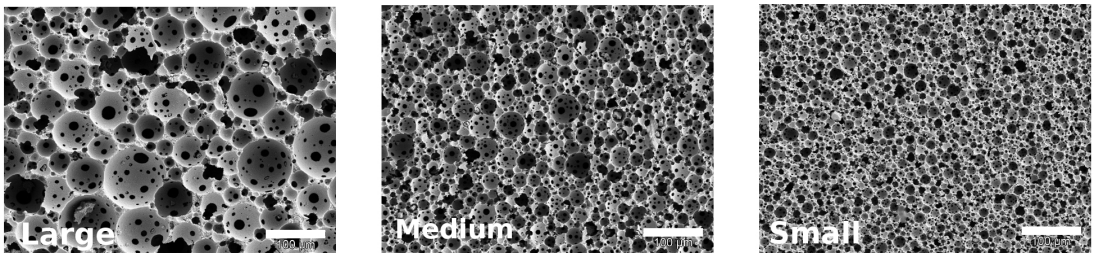

Figure 2.1: Alumina porosity. *SEM-pictures of the alumina ceramic used in these test. The pictures show the three different pore size distributions used, from left to right "Large", "Medium", and "Small". Scale bars are 100 µm.*

µm on the surface, with an average size of the windows between pores, d_{50}, of 16 µm. The "Medium" range is approximately 10-40 µm on the surface, with an average pore window size of 7 µm, and the "Small" range is approximately 5-20 µm on the surface, with an average pore window size of 3 µm. In some experiments non-porous alumina coupons were used as controls, here called "NP". The surfaces of some coupons were modified by silanization either with aminopropyltrimethoxysilane (A) or with hexadecyltrimethoxysilane (H). The amino groups change the surface charge of the alumina, possibly enabling electrostatic interaction with the *C. reinhardtii* cells. The purpose of the hexadecyl-treatment was to make the surface hydrophobic thereby enabling hydrophobic interactions between the cells and the surface. Each experiment also used coupons with no surface functionalization, showing the surface properties of the ceramic itself (C). The combination of three different pore size distributions with three different surface treatments resulted in 9 different types of coupons tested in the experiments.

CDC biofilm reactor

The alumina coupon types to be tested were manufactured in round coupons 12 mm in diameter that were fastened in polypropylene holders in a continuous flow stirred tank reactor; a CDC biofilm reactor from Biosurface Technologies Corporation, Bozeman, MT, USA. The reactor vessel was in the shape of a cylinder with a stirrer in the center creating a flow parallel to the coupon surfaces. The liquid culture volume in the reactor was approximately 350 ml. A stirring rate of 100 rpm was used, and the continuous flow rate was 0.45 ml/min. The reactor held 8 polypropylene holders, and each holder held three coupons, so that 24 coupons were tested at a time. One reactor run lasted 7 days with inoculation of the reactor from a pre-culture being done on the first day. At the last day of the run the ceramic coupons were taken out and tests were performed on them, as described below.

Re-growth curves

re-growth curves were made to estimate how fast a culture could regrow from the biofilm on an alumina coupon. Each coupon used for this test was taken out of the biofilm reactor, rinsed with TAP-medium, and put into a well on a 6-well plate, where the wells are 35 mm in diameter. 10 ml TAP-medium was poured into each well and the well-plates were put into a controlled environment growth chamber, with illumination and temperature conditions as described for the maintenance of algal cultures. Optical density, OD, in the liquid was measured in a spectrophotometer at 750 nm, once a day at the same time of day. The logarithms of the OD-values compared to OD at starting time ($\ln(OD/OD_0)$ were calculated, and a logarithmic function was fitted to these values using the SciDAVis software [11]. The function used was the Richards-function reparametrized as described in [144]. The time to reach an OD of 0.15, corresponding to $1.6x10^6$ colony forming units per ml, was calculated from the fitted function. Statistical significance was calculated using a two-tailed Student's t-test, with two samples assuming unequal variances. The values for aminopropyl samples and hexadecyl samples were each compared to the non-functionalized samples of corresponding pore sizes, using a 95% confidence interval.

PAM analysis

Chlorophyll fluorescence measurements were made using a MINI-PAM Photosynthesis Yield Analyzer from Heinz Walz GmbH, Eiffeltrich, Germany. Coupons were removed from holders after a biofilm reactor run, and rinsed with TAP-medium to remove non-attached cells. The coupons with attached cells were dark-adapted for 30 min before being measured. The MINI-PAM measures the background fluorescence of the sample, F_0, and the fluorescence after a light impulse, F, and then calculates the "yield". The yield is the same as the Genty-parameter which is a measure of the quantum yield of photosystem II linear electron transport [46]. In most nutrient-replete phytoplankton the yield is around 0.65 [38]. Stress or other factors which decrease the efficiency of photosystem II will result in a lower dark adapted yield value indicating increased efficiency of non-photochemical quenching [86]. For coupons with few cells on them the PAM measurements were not reliable, as the signal was too weak. Therefore, only coupons where the F_0-value falls within the calibration range were included in the analysis.

Confocal Laser Scanning Microscopy

Some coupons were imaged in a confocal laser scanning microscope, LSM 510 Meta; Carl Zeiss Jena GmbH, Jena, Germany. Those coupons were rinsed with TAP-medium after they were removed from the biofilm reactor, and then kept in fresh TAP-medium until they could be imaged. The time between the coupons being taken out and the imaging was up to six hours. Shortly before being imaged the coupons were rinsed with phenosafranine solution (0.5 mg phenosafranine per ml TAP-medium), which stains the contents of dead cells [140]. The images were taken with three different channels; one for chlorophyll autofluorescence (excitation wavelength 477 nm and emission wavelength filter 660-703 nm), one for phenosafranine fluorescence (excitation wavelength 514 nm and emission wavelength filter 531-617 nm), and one channel to image the alumina surface by catching light scattered from the surface (excitation wavelength 633 nm and emission wavelength filter 628-649 nm). The images were taken at several randomly chosen locations on the coupon surfaces and at several depths for each location, making "z-stacks". The same strategy was used to take images of the exposed interior face of coupons that had been cut in half.

Cells in each channel of the picture were counted using the ImageJ software [111]. LSM-pictures were converted to Image5D format using the LSMToolbox [106]. Contrast and brightness were adjusted for each channel so that all cells could be seen, then composite images of the z-stacks were made. Cell counts for each channel were made either by transforming the z-projection to a binary image and counting cells using the "Analyze particles" command, or by using the "Find maxima"-command directly on the z-projection. A cell with a phenosafranine signal was counted as dead. Dead cells show both phenosafranine fluorescence and autofluorescence, therefore the number of live cells was taken to be the cell count from the autofluorescence channel with the phenosafranine channel cell count subtracted.

2.2.3 Results and discussion

After biofilm reactor incubation, attached algae on the diverse coupons were subjected to fresh medium in order to test for re-growth. Optical densities typically reached 0.8 - 0.9 with the clearest differences between the coupon types early in the re-growth phase. Fig. 2.8 shows the time it took the cultures regrown from the biofilm reactor coupons to reach an optical density of 0.15. As can be seen, the pore size was the most important factor for re-growth time; the coupons with larger pores had shorter re-growth times.

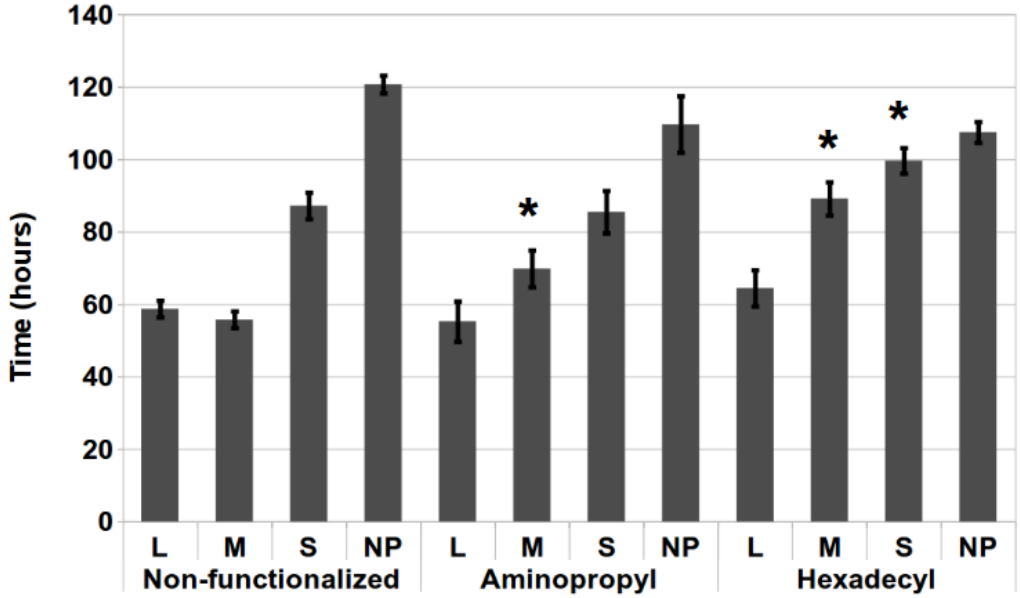

Figure 2.2: Re-growth efficiency measured as time to reach OD=0.15. *Non-functionalized coupons received no surface treatment. The two functionalized surfaces shown in the graph are "aminopropyl" where the surface have been treated with amino groups and "hexadecyl" where the surface has been treated with hexadecyl-groups. The different pore size distributions have been denoted as L=large pore size, M=medium pore size, S= small pore size, NP= non-porous coupon. Each type of coupon was tested in at least 4 biological replicates, with at least 2 technical replicates in each experiment. Error bars represent standard error. Bars marked with an asterisk (*) represent values that are significantly different with a C.I. of 95 % from values of non-functionalized samples of the same pore size.*

If a cell culture had a low starting number of cells, it would take longer to regrow, but longer re-growth times could also mean that the cells are stressed and need a longer lag phase before they start to grow, or that they continue to grow on the coupon surface without moving out in the surrounding liquid culture. Spectrophotometry, while being a straightforward and convenient method for measuring cell-culture density, is not very sensitive to low cell concentrations. In this investigation, with coupons put directly in TAP-medium to allow the culture to regrow, the OD in the first part of the curve was below 0.1. The lag phase in the re-growth curve could therefore be caused either by a "true" lag where the cells were in a low productivity state and needed time before they started to grow, or by the spectrophotometer measurement range where the cell cultures took time to grow to a density that the spectrophotometer can measure.

To distinguish between these causes the PAM yield values were considered.

They were measured on the coupons and the biofilm reactor liquid at the end of the biofilm reactor runs. They all fell in the range of 0.60 to 0.72 (data not shown). There was no significant difference between biofilm reactor liquid and coupons, or between the different coupon types; surface treatments or pore sizes do not affect the PAM yield. Therefore it is likely that the differences in reaching OD=0.15 in fig. 2.8 were a result of differences in starting number of cells and not a result of varying cell health or photosynthetic productivity.

Fig. 2.3 shows three typical examples of surfaces with live and dead cells on them.

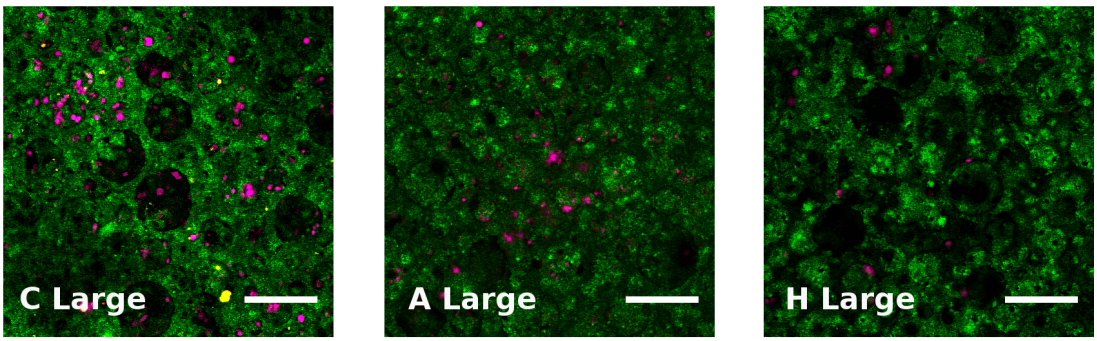

Figure 2.3: Alumina surface with cells. *Example of surface picture z-projection of coupons with large pores. Picture taken with 100x magnification. These pictures are cropped and magnified for greater visibility, they show approximately 25% of the original pictures. C = no surface functionalization, A= amino acid functionalization, H = hexadecyl functionalization. Autofluorescence signal is represented in magenta, dead cells in yellow, and the coupon surface in green. Scale bars are 100 µm.*

Cell counts from the types of pictures shown in fig. 2.3 are shown in fig. 2.4. They were made in two biological replicates and, as can be seen in the figure, there can be large variations between the replicates. This is because cells sometimes grow clumped together.

There was no significant difference in surface cell count between aminopropyl-functionalized coupons and non-functionalized ones in the different pore sized samples, except for the medium pore sized coupons. The hexadecyl functionalized coupons with medium and small pore sizes, however, seemed to have fewer cells attached to them. Hexadecyl is a strong hydrophobe and while its hydrophobicity may promote cell attachment it could also exclude water from the alumina pores, thereby blocking access to them for algal cells. This effect is more pronounced the smaller the pores are, which could explain the results in fig. 2.4.

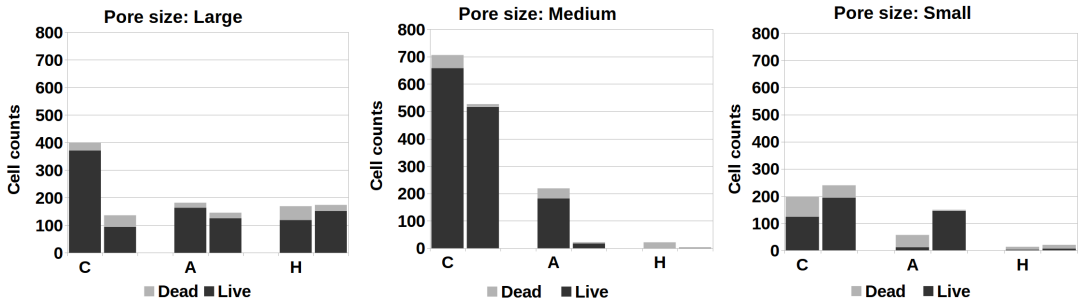

Figure 2.4: Cell counts alumina surface. *Cell counts of live and dead cells from LSM images of surfaces of coupons. C= no surface functionalization, A= amino acid functionalization, H = hexadecyl functionalization. 3 pictures were counted for each surface in two biological replicates. Averages from each biological replicate are shown next to each other.*

But surface counts don't show all the cells that are attached to the coupons. In order to get a better estimate of how many cells are attached inside the ceramic substrate, invisible from the surface, cell counts from pictures of coupons broken in half were also considered. Fig. 2.5 shows images of cut coupons, displaying the cell distribution below the surface of the coupons. The total length of one side of the picture is 0.6 mm.

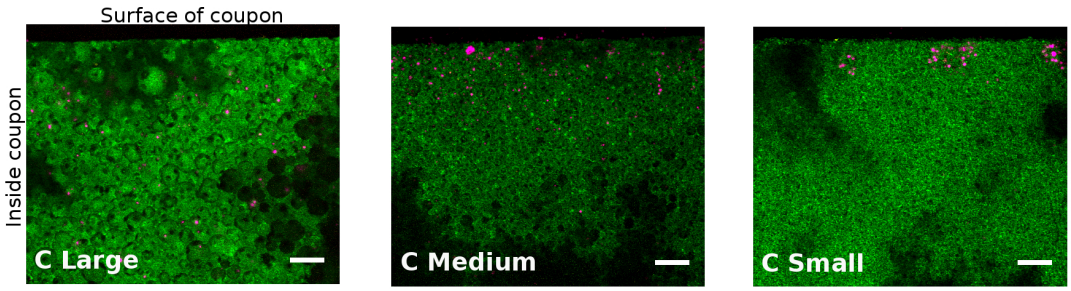

Figure 2.5: Alumina inside with cells. *Examples of cut coupons. The microscopy pictures show cell distribution in the interior of non-functionalized coupons with different pore sizes, magnification 100x. The coupons were cut open and microscopy pictures were taken of the cut side. These pictures are cropped for greater visibility, they show approximately 45% of the original pictures. Autofluorescence signal is represented in magenta, dead cells in yellow, and the coupon surface in green. Scale bars are 100 µm.*

Fig. 2.6 shows cell counts at different depths inside the coupons, made from pictures like the ones shown in fig. 2.5. Cells were fairly evenly distributed in the largest pore size coupons, even more than 1.5 mm from the surface, whereas for the medium and smaller pore sizes most cells resided at or just below the surface. This is probably because the windows between pores were large enough in the largest pore size distribution alumina to let cells move

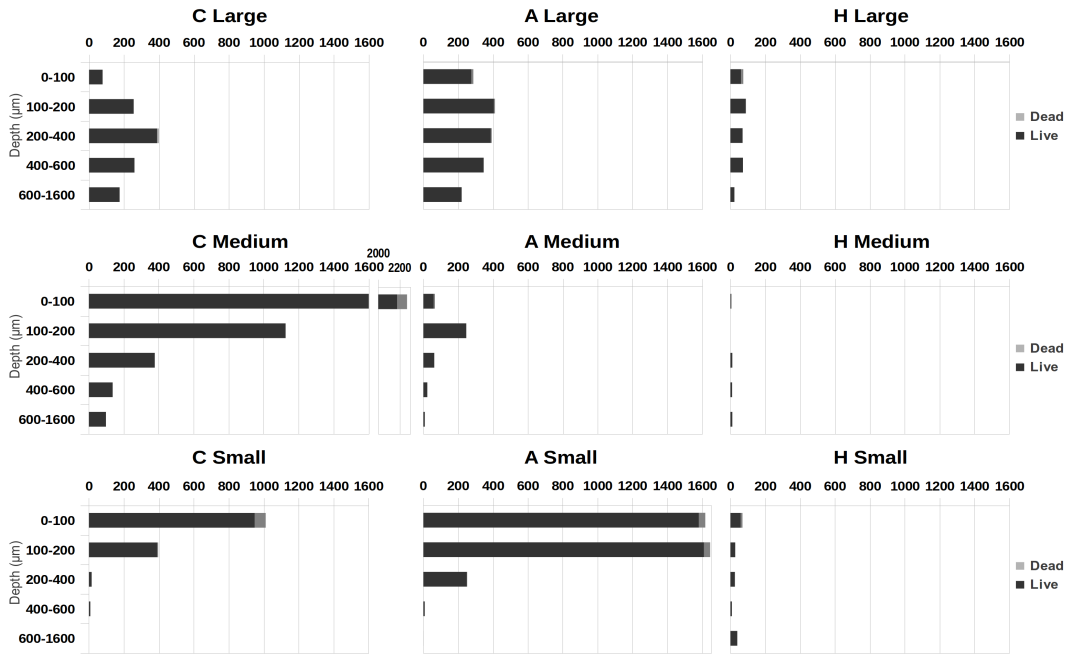

Figure 2.6: Cell counts inside alumina. *Cell counts from LSM pictures of cut coupons. The x-axes show cells/mm^2 cut surface. C= no surface functionalization, A= amino acid functionalization, H = hexadecyl functionalization. Averages of two biological replicates, with 3 separate pictures counted from one replicate, and one picture counted from the other biological replicate. The cells were counted in 100 µm, 200 µm, and 1000 µm intervals at different depths from the coupon surfaces.*

inside the coupon. Since the growth medium contained acetate the cells were not dependent on light, and they could colonize the space below the surface independent of light distribution inside the coupon. In the medium and small pore size distribution alumina there are fewer windows between pores that are large enough to let the cells pass through and so most cells could be found on the surface. It is also clear from the cell count graphs that the hexadecyl treated coupons had fewer cells attached to them than the other coupons, at all depths. The hydrophobicity of hexadecyl excludes water from the interior of the coupons, making it inhospitable for algal cells. As with the cell counts on the surface of the coupons, this effect was stronger for the smaller pore sized coupons.

2.2.4 Conclusions

Among the alumina variants tested, pore size distribution was the most important factor for cell attachment and re-growth efficiency. The larger pore

size distribution allowed cells to move deeper into the ceramic giving them more surface area to grow on. Had the cells been strictly photosynthetic this effect might have been smaller due to the lack of light inside the coupons. Hydrophobic surface treatment reduced the total cell number, probably because cells couldn't go into the ceramic when water is excluded from the interior of the coupon.

For the largest pore sized alumina the hexadecyl treatment resulted in approximately the same re-growth rate and surface cell counts as the other coupon types. The larger pores in that case might have led water through to the inside of the material, despite the hydrophobicity of the hexadecyl. Overall the coupon types with the large pore sizes show a good colonization and homogeneous distribution of the cells in the measured depths up to 1600 µm, also the hexadecyl treated ones. For the smaller and medium pore size ranges the hydrophobic coupons had very few cells attached on the surface. This might be explained by contact point theory and the fact that several different forces compete in cell attachment - the small pores reduced the number of available contact points to the partly hydrophilic algae, while the hydrophobic interaction did not compensate enough for that effect.

None of the tested samples seemed to decrease cell health, neither by surface treatments, or different pore size distributions. Consequently, these tests showed that alumina porous on micro-scale is a suitable and effective substrate for *C. reinhardtii* cell attachment, and that pore size distributions in the range of 10-50 µm and pore window sizes of around 16 µm ensures good cell attachment. Hexadecyl functionalization had a negative effect on cell attachment, although the negative effect got weaker the larger the pores were. The non-functionalized coupons showed the best overall results.

2.3 *C. reinhardtii* heat tolerance pre-tests

The following chapter is a summary of experiments carried out in the course of the thesis work.

Chlamydomonas reinhardtii heat tolerance pre-tests

Abstract

A thorough investigation of *C. reinhardtii* heat tolerance was done in order to chose a set of experimental conditions that allows detection of small differences in heat stress tolerance between biofilm and liquid culture cells. Tests were done on different sets of temperatures, on different heat stress time periods, and on different heat stress acclimatization time periods. Results confirmed existing literature in that cells were sensitive to heat stress above 40 °C. Cells tolerate stress better when heat stress time is below 30 minutes. An acclimatization time of 180 minutes, whether done to a non-stressed temperature with a subsequent jump to target temperature, or with heating gradually all the way to target temperature, also allow cells to tolerate heat better. For the purpose of further heat tests a set of experimental conditions were chosen; heat stress temperatures of 40 °C, 42 °C, 44 °C, heating period 30 minutes with gradual heating to target temperature giving cells acclimatization time.

2.3.1 Introduction

Chlamydomonas reinhardtii is an important model organism for research on photosynthesis, and as such its temperature tolerance has been tested by many researchers. However, most authors report on tests done using one or two heat stress temperatures and during only one length of the heat stress period. Heat tolerance of any organism is dependent on heat dose, in other words on both temperature and heat stress time. Heat tolerance is also affected by acclimatization. Cells that are experiencing a slowly increasing temperature can activate heat stress protection mechanisms, as has been discussed in section 1.2.6, which can result in survival at higher temperatures than cells that are experiencing a sudden change to a higher temperature.

To design heat stress survival tests of *C. reinhardtii* biofilm compared to liquid culture, it was necessary to find a set of heat doses and acclimatization times that allowed detection of small differences in heat stress tolerance between the two types of cultures. Those small differences were most likely to be found where a small change in heat dose resulted in a large change in heat stress survival. The heat dose would be varied by varying the temperature, while the heat stress time would be kept constant in all tests. Tolerance to different

temperatures was deemed to be more interesting than tolerance to different heat stress time periods, as industrial scale photobioreactors can reach very high temperatures, whereas they can be designed with safe-guards against long term heat stress, for example by shading. See section 1.2.3 for further discussion of the environmental conditions of bioreactors.

A literature study on *C. reinhardtii* heat tolerance gave a starting point for the tests. It showed that 40 °C is an important break-off point. One study showed that *C. reinhardtii* survives heat treatment at 40 °C for 24 hours and that cells that regrow afterwards show little variance in fitness compared to cells in cultures that were never heat stressed [49]. Heat stress above 40 °C, however, have more detrimental effects on the cells: in another study cultures stressed at 41 °C for one hour had a 50 % growth reduction. If cultures were acclimatized to heat stress first they had a somewhat higher re-growth rate, whereas cultures stressed at 42 °C had almost negligible re-growth [57]. Other studies have shown that *C. reinhardtii* cell survival is compromised after a shift to 42.4 °C [72] and 43.5 °C [121].

In the design of the heat stress survival tests small lab bioreactors were used as models for industrial scale photobioreactors. That model included the property of bioreactors to not heat instantly to a new temperature if their environmental conditions change; as they contain a volume of water, temperature change with be slowed and take place gradually. This will mean an acclimatization time for the cells in the bioreactor culture. In the tests described here we were therefore looking for a set of heat stress temperatures were *C. reinhardtii* cells showed a large change in heat stress survival, at an acclimatization time comparable to the heating time of the lab bioreactors in the heat stress setup, at the shortest heat stress time period possible. Cell regrowth ability was used as a measure of heat stress tolerance as the ultimate goal of this work is to find ways to regrow an algal culture from heat stressed cells.

2.3.2 Materials and methods

Algae species, media, and culture maintenance

The alga used in these tests were *Chlamydomonas reinhardtii* wild type SAG 11-32b, maintained on TAP-medium agar plates [50], at 23 °C, under fluorescent cool white light in a day:night cycle of 14:10 hours. Liquid culture for experiments was grown in liquid TAP-medium to exponential phase, under the same light and temperature conditions.

Heat tests

All tests were performed with *C. reinhardtii* liquid culture samples of 1 ml in 1.5 ml Eppendorf cups, in a thermomixer shaken at 600 rpm. Each test was done in three independent experiments, and each experiment was done in biological triplicates. Liquid culture kept at room temperature, approximately 23 °C, for the duration of the test, was used as controls in each experiment. A list of tests performed can be found in Table 2.1. The first test was without acclimatization time and had a range of temperatures covering a large span, with heat stress time one hour ("Sudden heat shock 1"). In the next test the results from the first test was used to hone in on a narrower set of temperatures which was used under the same conditions otherwise ("Sudden heat shock 2"). The proceeding tests used this narrower set of temperatures. In the next test three different heat stress times were tested, and in the two subsequent tests acclimatization time was tested in two different fashions. The "acclimatization with jump" test used gradual heating to a set temperature with a jump to the target temperature, which ensured that all cultures were heated at above stress levels for only 30 minutes. The next test, "acclimatization to target", mimicked conditions in a real photobioreactor by gradually heating the samples all the way to the target temperature.

Test	**Temperatures (°C)**	**Time (minutes)**
Sudden shock 1	20, 30, 40, 50, 60	60
Sudden shock 2	23, 40, 42, 44, 50	60
Stress interval length	23, 40, 42, 44, 50	10, 20 and 30
acclimatization with jump	Gradual heating to 35 °C, then directly to 40, 42, 44, or 50	180 min acclimatization time followed by 30 min at target temperature
acclimatization to target	Gradual heating to target temperature: 40, 42, 44, or 50	Increase 2 °C every 10 min from 23 °C to target (85, 95, 105 and 135 minutes acclimatization respectively) followed by 30 min at target temperature.

Table 2.1: *List of heat tolerance tests for* C. reinhardtii.

Re-growth tests

Cell re-growth abilities were assessed with colony forming unit, CFU, counting; dilution series of the cultures were made and plated on TAP-plates, and CFUs were counted on plates after 7 days.

2.3.3 Results and discussion

The first two tests — the "sudden heat shock" tests where cells were taken directly from room temperature to target heat stress temperature and kept at that temperature for one hour — confirmed the existing literature; heat shock at 40 °C had little effect on re-growth, whereas temperatures above 42 °C decreased re-growth by several orders of magnitude, and no re-growth at all happens for cells stressed at temperatures from 50 °C and above (see Figure 2.7A and B). From these results 40 °C, 42 °C, 44 °C, and 50 °C were chosen as temperatures to test further as they would allow detection of a gradual effect of heat shock.

The next set of experiments tested three stress time intervals for the chosen temperatures. It was concluded from these tests that 30 minutes is the shortest time interval that allow the difference in cell re-growth ability of the chosen temperatures to still be detectable in CFU counts (see Figure 2.7C). With that time interval there is at least one order of magnitude difference in CFU counts between each of the test temperatures for 30 min tests.

The last two experiments tested the effect of acclimatization time on heat shocked cells. In the first test, cells were gradually heated to a temperature below stress level (35 °C) over a period of 180 min, and were then directly transferred to the target temperature. In the second acclimatization test cultures were gradually heated at a slow pace, with 2 °C increase every 10 minutes, all the way to the target temperature. This meant that the cultures stressed at higher temperatures were heated for a longer time. The results showed that acclimatization does have an effect on cell survival, as both cultures stressed at 42 °C and 44 °C had higher survival rate with acclimatization than without (see Figure 2.7D). Cultures stressed at 42 °C degrees showed almost the same survival rate as the controls kept at room temperature, although with variation. The variation in cell heat stress survival at 42 °C when acclimatization or heat stress time varies, indicates that this temperature would allow detection of changes in heat tolerance due to other factors as well. The two different fashions of acclimatization do not yield any difference in heat stress survival.

2.3.4 Conclusions

The overall conclusion from these tests was that using 40 °C, 42 °C, and 44 °C as stress temperatures would allow seeing an effect on cell re-growth and

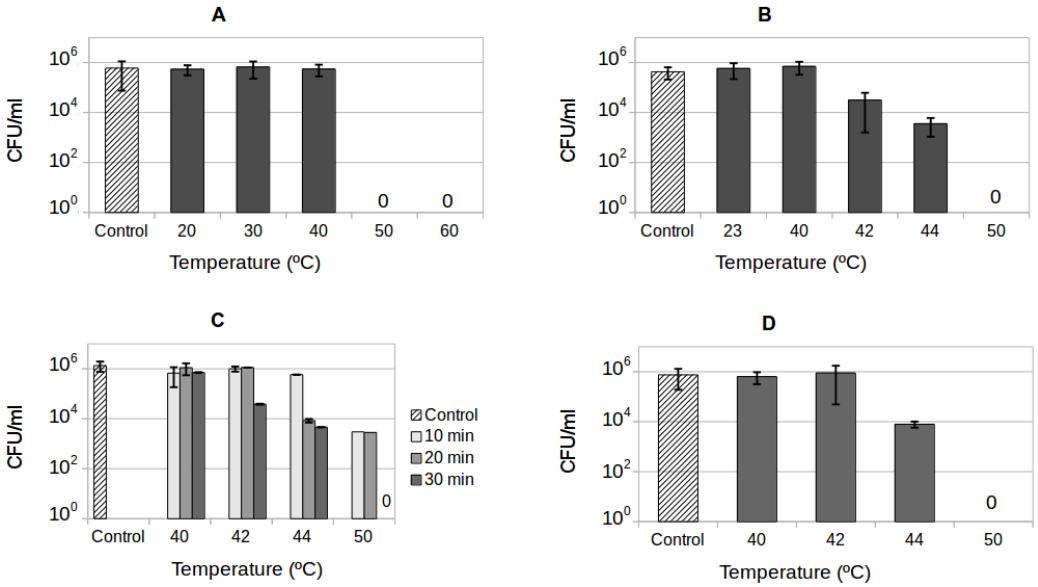

Figure 2.7: Heat tolerance results for *C. reinhardtii*. *Tests show colony forming unit, CFU, counts for cells regrown from heat shocked cultures. A) Sudden heat shock, heat stress period 60 minutes for temperatures up to 60 °C. B) Sudden heat shock, heat stress period 60 minutes, for temperatures between 40 °C and 50 °C. C) Three different heat stress periods at four different temperatures. D) Gradual heating with 2 °C every 10 minutes to target temperature, then 30 min heat stress. All controls are samples kept outside the thermomixer. Bars represent averages of three experimental replicates with four technical replicates in each experiment. All error bars show standard deviation.*

that 30 minutes of stress time would be the shortest time to show an effect. In the heat survival tests on *C. reinhardtii* biofilm cell acclimatization time to heat stress would be a feature that mimics conditions in industrial sized photobioreactors. These tests showed that this acclimatization time will affect the results for cultures stressed at 42 °C and 44 °C. Taken together the chosen experimental conditions — heat stress for 30 minutes at 40 °C, 42 °C and 44 °C, with gradual heating to target temperature — will allow detection of heat stress survival differences of biofilm and liquid cultures.

2.4 *C. reinhardtii* biofilm heat stress survival

The following manuscript is in preparation.

Chlamydomonas reinhardtii heat stress survival when growing as a biofilm on porous alumina substrates

Authors:

Maria Johansson*; Christian Soltmann; Klaus Slenzka; Matthias S. Ullrich

*corresponding author

Abstract

Heat stress decrease productivity of photobioreactor cultures and can cause them to break down altogether. Biofilms have been shown to have greater heat stress resistance than planktonic cultures. In this study the heat stress resistance of *C. reinhardtii* biofilm was compared to that of planktonic cultures, to assess if a photobioreactor planktonic culture broken down from heat stress, could be restored from a biofilm, making it a self-restoring photobioreactor. Biofilm growing on alumina with pores in the ranges of 10-50 µm, or 5-20 µm and either functionalized with aminopropyltrimethoxysilane or not modified at all was tested. Differences in heat stress survival can be seen between the small pore range and the large pore range biofilm samples, but overall planktonic samples do not regrow faster from a heat stressed biofilm sample than from a heat stressed planktonic sample. It is possible that this reveals a design conflict, since the nutrient rich and axenic conditions in the photobioreactor is not beneficial to the formation of biofilm. To the knowledge of the authors this is the first study to compare heat stress tolerance of an algal biofilm to that of it planktonic culture.

Key words:

Alumina; *Chlamydomonas reinhardtii*; Heat stress; biofilm temperature tolerance

2.4.1 Introduction

Heat stress is one of the most common stresses in outdoor photobioreactors, PBRs. Heat stressed PBR cultures are less productive than non-stressed ones,

and may break down altogether. It is therefore important to find methods to handle heat stress of PBR cultures. The study reported here tested a method for restoring PBR cultures after heat stress breakdown, using the green alga *Chlamydomonas reinhardtii*, which has been widely studied for its hydrogen producing ability [87, 75].

The effects of heat stress depends on dose, in other words on both the level of heat stress and its duration; the higher the dose the longer the recovery time [62]. Work on *C. reinhardtii* has shown that cells survive heat treatment at 40 °C for 24 hours and can regrow afterwards with little variance in fitness [49]. Heat stress above 40 °C drastically reduce recovery growth of *C. reinhardtii* cultures: one study showed that cultures stressed at 41 °C for either 1 hour or 2 hours had a 50 % growth reduction, although cultures acclimatized to heat for 3 hours prior to heat stress had a somewhat higher recovery growth rate and grew to a higher final concentration than non-acclimatized cultures [57]. The same experiment showed that cultures treated at 42 °C had almost negligible re-growth.

Temperatures in outdoor algal cultures easily reach above 40 °C [77, 93], even when the air temperature is lower. Therefore, cooling systems are used to maintain production levels. Evaporative cooling is common in large scale systems. It works best in dry climates and requires access to a water source. Another possibility is to use heat exchangers, which requires electricity. Both methods therefore contribute significantly to the water and energy use of the PBR systems [25, 94]. Passive, less resource demanding methods, have also been suggested and tested, such as decreasing the amount of heat that reaches the culture by submerging the PBR in water [98, 134], or using plastic or glass that reflects infrared light in the culture vessel. Another research area is to reduce the light-harvesting antenna sizes of photosystem I and II [99]. This is done mainly to reduce self-shading of the culture and increase productivity, but has the added benefit of reducing the amount of solar radiation re-emitted from the cells as heat.

An extreme heat stress event could still damage or kill a PBR culture, even if it is cooled. In remote locations, or where personnel time is costly, restarting the culture will be expensive. An alternative approach for handling damaged cultures in such cases, was suggested by Bartsev and Okhonin [9]: allowing occasional down-time of the PBR during and after heat stress breakdown but making the PBR culture "self-restoring" by using a surviving aliquot of the culture as inoculum for a new culture. With such a method the number of surviving cells with retained reproductive ability would determine the length of the downtime period. Methods which increase the heat resistance of cells therefore support the self-restoring method.

Microorganisms growing in a biofilm have been shown to have increased tolerance to stress compared to planktonic cultures [81], including heat stress. Studies on *E. coli* have shown that genes involved in heat stress response are expressed more strongly in biofilm [112, 118]. A biofilm consists of cells in a matrix of extracellular polymeric substances, EPS. Natural biofilms consist of many different species, are highly structured, and have varying microenvironments with enzyme and oxygen gradients [14]. It is not fully known why biofilms are more stress resistant than planktonic cultures, but hypotheses include cells in biofilm being more slow-growing [12] or that the EPS in itself act as a barrier to a harsh outer environments. The biofilm also retains extracellular enzymes, increasing their concentration near the cells, thus allowing them a higher net gain of nutrients. This rewards efficient cells, and promotes more efficient phenotypes [139].

The heat stress response, HSR, of cells consists of many different mechanisms. Comparing different treatments for increasing heat resistance of cells using methods which assess both early and late HSR allows detection of differences in heat dose. Reduced carbon assimilation in photosynthesis is an early HSR [1, 97], and can be assessed with a chlorophyll fluorometer measuring quantum yield of the photosynthetic machinery in plants and algae. Experiments on photosynthetic cells using chlorophyll fluorometry show that the photosynthetic quantum yield decreases with increasing temperature and is almost non-existent at 45 °C [62], and that cultures stressed at temperatures below 42 °C recover their yield when transferred back to room temperature, whereas for temperatures higher than 42 °C the minimum fluorescence yield, F_0, remained elevated even after transfer, indicating that the photosynthetic machinery had been damaged [58, 62, 92].

The HSR also include increased expression of heat shock proteins such as the cytosolic chaperon Hsp70A, and the PSII reaction center protein, psbA. Hsp70A, has been used in several studies of *C. reinhardtii* as a proxy for heat stress level [121, 130]. Hsp70A protein levels in *C. reinhardtii* increase a few minutes after cells have been subjected to heat shock at 42 °C, reaching a maximum after 2 hours of heat stress [95], which makes *hsp70A* expression an early HSR. *psbA* transcription levels can be used as an indicator of late heat shock response [80, 76, 143]. Maximum transcription of *psbA* occurs at 42 °C [76].

The purpose of the work presented here is to investigate whether a biofilm growing inside a planktonic culture could be used as an inoculum for the planktonic culture after a heat stress event has killed it. In previous work we tested *C. reinhardtii* biofilm growth on a porous alumina material, see section

2.2. This study aimed at comparing the heat stress tolerances of *C. reinhardtii* planktonic cultures to *C. reinhardtii* biofilm on the same alumina materials.

2.4.2 Materials and Methods

Algae-species, media, and culture maintenance

The alga *Chlamydomonas reinhardtii* wild type SAG 11-32b was used for these experiments. Stock cultures were maintained on TAP-medium [50] agar plates at 23 °C under cool fluorescent white light in a day:night cycle of 14:10 hours. Experimental cultures were grown in TAP-medium at room temperature as described in section 2.4.2.

Alumina ceramic

Ceramic substrates for algal growth were manufactured as described in [5], and some substrates had their surface functionalized via silanization. Previous work has shown that alumina with pore size distributions in the range of 10-50 µm, without surface functionalization or functionalized with aminopropyltrimethoxysilane, had the most cells attaching to them. In this study three variants of the ceramic substrates were used: alumina without surface functionalization with "large" (C-L) and "small" (C-S) pores, and alumina functionalized with aminopropyltrimethoxysilane with large pores (A-L). A-L and C-L substrates had pore sizes in the range of 10-50 µm, with and average pore size of 16 µm, and C-S substrates had pores in the range of 5-20 µm with an average pore size of 3 µm. The ceramics were cut in the shape of round coupons 12 mm in diameter with a thickness of a few millimeters, to fit into holders in the culture vessels used in the experiments.

Heat stress experiments

Planktonic and biofilm cultures were grown together in 500 ml glass laboratory PBRs, fitted with holders for the alumina coupons. Each photobioreactor could hold three alumina coupons, submerged in planktonic culture. The PBRs were bubbled with air to mix the culture and provide carbon dioxide. PBR cultures were started by adding 20 ml pre-culture to 330 ml TAP-medium. Cultures were allowed to grow for 5 days, with PBRs immersed

in transparent water baths at room temperature with air constantly bubbled through them, and illuminated by 9000 K fluorescent bulbs regulated to a 12:12 h day:night cycle. During this period cells attached to the alumina coupons' surfaces. At the end of the five day period culture bottles were temporarily removed from the water baths, which were then heated to stress temperatures before bottles were immersed again. Four baths were used: three for stressing samples at 40, 42 and 44 °C respectively, and one for the non-stressed controls that were kept at room temperature, RT. The culture bottles were kept in the water baths for 50 minutes - the time interval had been chosen so that the whole planktonic culture would reach the set temperature and stay at it for at least 30 minutes. At the end of the heating period the bottles were taken out and the biofilm growing on the alumina substrates as well as planktonic culture samples were removed and used for various measurements as described below.

Re-growth

Re-growth rate was tested by starting planktonic cultures from heat stress biofilm samples as well as from planktonic culture samples. Alumina coupons were rinsed with TAP-medium after the heat stress period, and were then put into 15.5 ml wells in a 6-wellplate. 10 ml TAP-medium was added to each well. For planktonic cultures a 250 µl heat stressed sample was added to 10 ml TAP in a well. The well-plates were placed under cool fluorescent light at 23 °C and optical density measurements, OD, were taken 4 days after re-growth cultures were started. OD measurements were normalized to the OD RT-controls for each sample type in order to compare re-growth of heat stressed samples from planktonic culture and from the three substrate types to each other, independent of absolute cell numbers in the re-growth cultures.

Pulse Amplitude Modulation (PAM) fluorometry

PAM-measurements were done within 45 minutes after heat stress, using a "Mini-PAM" portable chlorophyll fluorometer from Heinz Walz GmbH, Effeltrich, Germany. Biofilm samples on alumina coupons were rinsed with TAP-medium. Both biofilm samples and planktonic culture samples were dark-adapted for 30 minutes. Using the saturation pulse method [120] the minimum chlorophyll fluorescence, F_0, and the maximum chlorophyll fluorescence, F_m, were measured. The photosynthetic quantum yield, i.e. the

maximum efficiency of photosystem II, was calculated according to the formula: Yield=$(F_m - F_0)/F_m$ [46]. A decrease in F_m lowers the yield, and indicates increased heat dissipation. A rise in F_0 also lowers the yield and indicates damage in the photosynthetic machinery [86].

Confocal Laser Scanning Microscopy

Alumina substrate coupons were imaged in a confocal laser scanning microscope, CLSM, as previously described in section 2.2, with the difference that the cells were stained with fluorescein diacetate, FDA, prepared according to the protocol in [68]. Cell enzymes hydrolyze FDA into fluorescein and two acetates. Fluorescein fluoresces at around 535 nm. Cells which showed fluorescein fluorescence were assumed to have active metabolism and intact membranes. Cells showing chlorophyll autofluorescence were assumed to be intact. Images were taken in three channels; one for chlorophyll autofluorescence (excitation wavelength 488 nm and emission wavelength filter 660-799 nm), one for fluorescein (excitation wavelength 488 and emission wavelength filter 520-553 nm), and one channel to image the alumina surfaces by using back-scattered light (excitation wavelength 633 nm and emission wavelength filter 628-649 nm). Stacks of images were taken at 4 different random locations on each coupon surface. Cells in each channel were counted using ImageJ software [111] as previously described section 2.2. Cells which showed fluorescein fluorescence were counted as living, and the total cell number was counted as the cell count in the chlorophyll autofluorescence channel.

Quantitative real time PCR

The mRNA expression of *hsp70A* and *psbA* were studied in the heat stressed A-L samples and in corresponding planktonic culture samples using quantitative real time PCR, qPCR. The constituively expressed *cblp2* gene was used as reference gene [119]. Samples taken for qPCR were snap-frozen in liquid nitrogen within 5 minutes of culture bottles being removed from heat baths and were subsequently stored at -80 °C. For mRNA extraction, TRI reagent (Sigma-Aldrich) was added to the frozen A-L coupon samples, which were then shaken with the reagent for 30 minutes. Planktonic culture samples were thawed on ice and centrifuged at 4 °C to pellet the cells before TRI reagent was added. Extraction then proceeded according to the reagent manufacturer's protocol. Samples were treated with RNAse free DNase (Ambion). The samples were run in an Eppendorf realplex thermocycler using the

QuantiTect SYBR green kit following the manufacturer's instruction (Qiagen). The primers for *cblp* were Fwd: GCCACACCGAGTGGGTGTCGTGCG Rev: CCTTGCCGCCCGAGGCGCACAGCG, and for *hsp70A* Fwd: GATCGAGCGCATGGTGC Rev: TCCATCGACTCCTTGTCCG, from [130]. For *psbA* the primers were Fwd: GGCCAAGGTTCATTCTCTGA Rev: CACCGAATACACCAGCAACAC from [133]. The results were analyzed using the software REST 2009 provided by Qiagen [104]. The software calculates the Pfaffl ratios [105] for samples compared to their controls, and also calculates the statistical significance of results using a randomization test.

2.4.3 Results and discussion

Re-growth rates of heat stressed samples are shown in Fig. 2.8. Cells attached to A-L and C-L substrates, show the same re-growth pattern as cells in planktonic culture; samples stressed at 40 °C have a similar re-growth rate as samples that were kept at room temperature, samples stressed at 42 °C regrow much slower, and the samples stressed at 44 °C don't regrow at all during the 4 days of the test. Cells attaching to C-S coupons, however, have slower re-growth than non-stressed samples already after having been stressed at 40 °C. These results show that cells growing as a biofilm on alumina substrates did not regrow faster than cells in planktonic samples, but they also show that heat tolerance for attached cells vary with the substrate they are growing on.

The slower re-growth at 40 °C for cells on C-S substrates could have been caused by lowered growth rate, or longer adaptation time compared to C-L and A-L samples. It could also be caused by lowered starting number of cells if many cells were killed during the heat stress period. Previous work showed that C-S substrates have fewer cells attaching to them than on A-L or C-L substrates (see section 2.2), meaning that the absolute value of re-growth at day 4 was smaller for C-S samples than for A-L or C-L samples, but it does not explain why re-growth is lower for C-S samples treated at 40 °C than C-S samples treated at RT. Cells attaching to C-S substrates seem to be more strongly affected by heat stress than cells attaching to substrates with larger pores or in planktonic culture.

The surviving ratio of cells after heat shock, shown in Table 2.2, can be found out from the live/dead cell counts in the CLSM images of substrate surfaces. Living cell ratios show large variations and do not correlate with the re-growth results in Figure 2.8. Rather, they show that the ratios of live cells for all biofilm samples treated at RT, 40 and 42 °C stay within in a range from approximately 20-40 %, except the A-L sample treated at 42 °C which

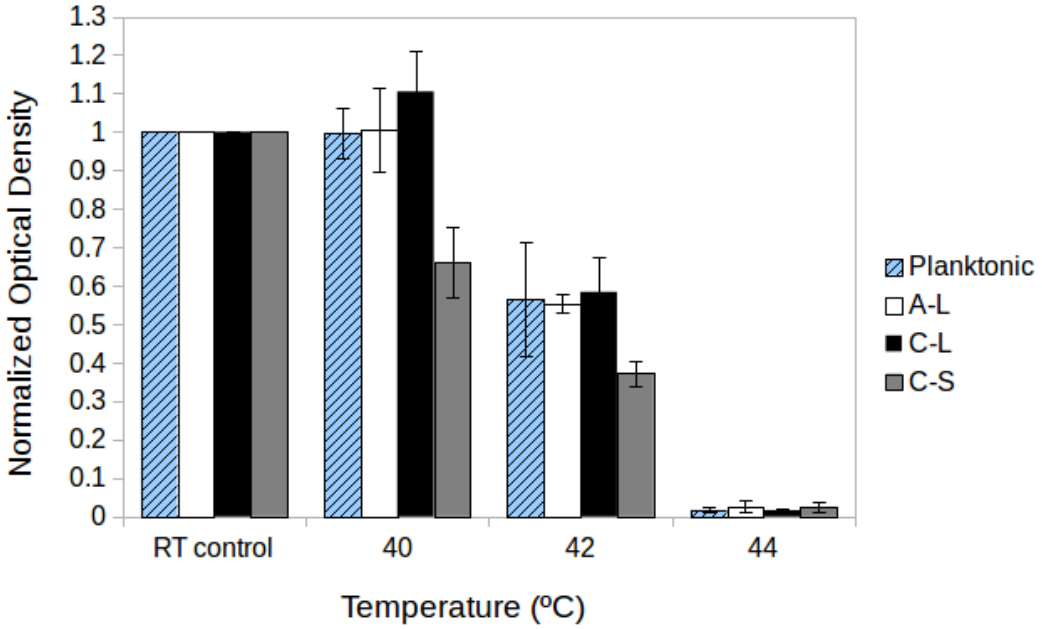

Figure 2.8: Re-growth rate after heat stress. *Optical density of re-growth cultures on day 4, normalized to the optical density of the re-growth on day 4 of the cultures kept at room temperature, RT, for each sample type. Averages of three biological replicates, with two technical replicates in each experiment. Error bars represent standard error.*

	RT	**40 °C**	**42 °C**	**44 °C**
A-L	21 %	35 %	11 %	0 %
C-L	43 %	23 %	19 %	0 %
C-S	24 %	38 %	40 %	1 %

Table 2.2: Percentages of living cells in total cell counts shortly after heat stress. *Cell counts from surface of substrates, and from planktonic samples, imaged with CLSM were made in three or four technical replicates from one experiment. RT=room temperature.*

has a lower ratio of living cells. Few living cells could be detected in the 44 °C samples. The total cell number on substrates also stay the same across temperatures (data not shown) which indicates that the living cell ratios remaining relatively high also after stress at 42 °C is not caused by dead cells disappearing from the surface. These results indicate that it is not the starting number of living cells that cause all 42 °C samples and C-S 40 °C samples to have smaller re-growth than at RT. Rather it seems that either the cells' ability to reproduce is impaired, or adaptation time or re-growth rate has changed, for cells on C-S substrates.

PAM yield values taken shortly after the heat stress period are shown in Fig. 2.9A. As with re-growth values in Fig. 2.8 they are shown normalized to their own sample type at RT. Neither biofilm nor planktonic samples show changed yield at 40 °C. Interestingly, cells on C-S substrates do not show lower average yield than cells on the other substrate types at 40 °C, although the variance is larger for those samples. Yield is still relatively high for all sample types at 42 °C, although cells are affected, and is very low at 44 °C, with no difference between cells on substrate surfaces and cells in planktonic culture at any temperature. Both the PAM and re-growth results are in line with other studies on *C. reinhardtii* planktonic cultures, summarized in the introduction [49, 57, 58, 92].

If a yield value is lower in one sample compared to a similar sample it could be either because of decreased maximum fluorescence, F_m, or increased minimum fluorescence, F_0. The graphs in Fig. 2.9B show F_0 and F_m values for all samples. All sample types have lower F_m for samples treated at 40 °C than for samples treated at room temperature, which indicates increased heat dissipation. In A-L and planktonic samples F_0 is still the same at 40 °C as for room temperature samples, so cells are not yet damaged at that temperature, as also re-growth results (Fig. 2.8) indicate.

In C-S samples at 40 °C both F_0 and F_m is much lower than at room temperature. The effect of both F_0 and F_m being lowered is that the photosynthetic yield for C-S at 40 °C stays more or less the same as for room temperature

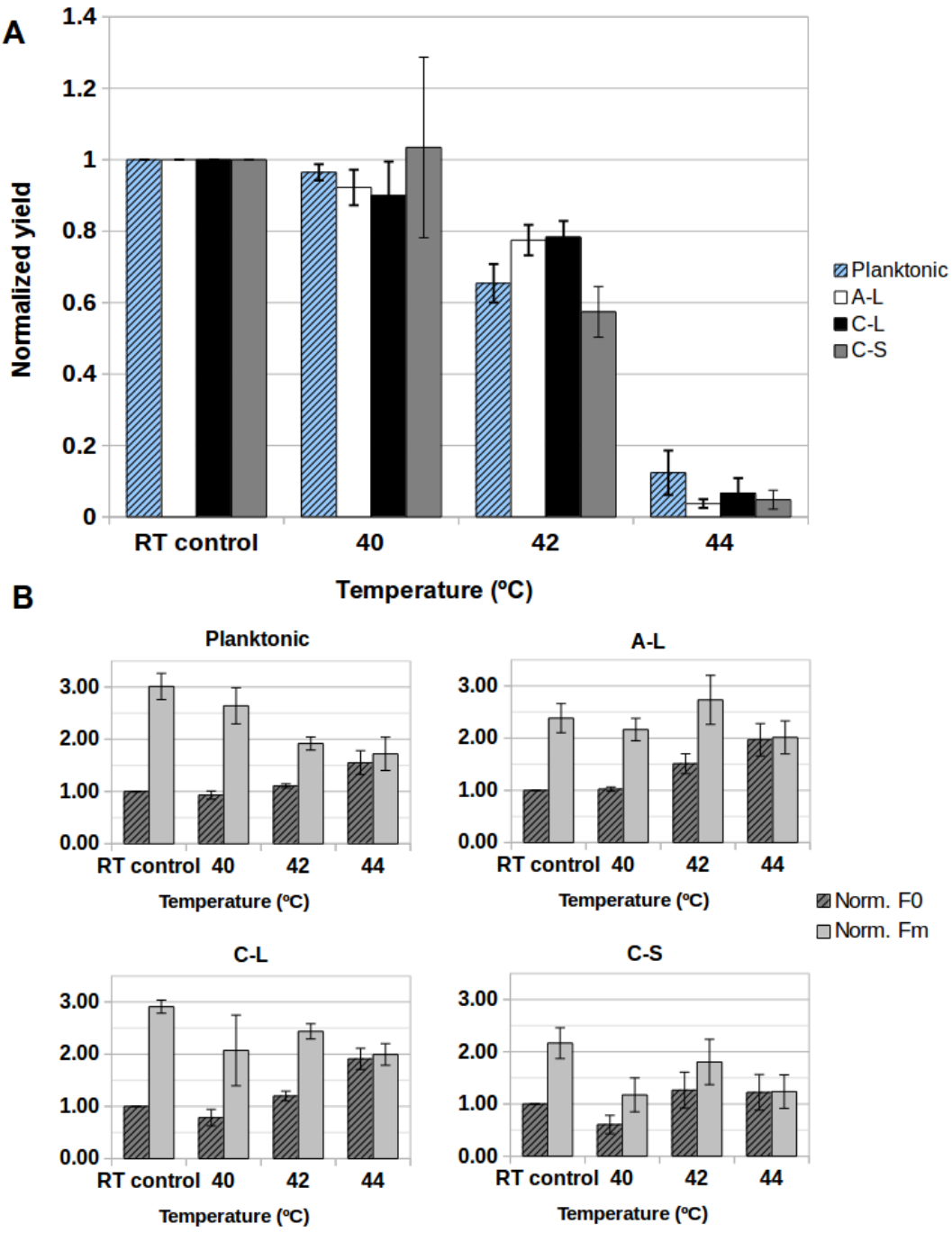

Figure 2.9: Photosynthetic fluorescence. *A) Yield measured by PAM-device and normalized to yield-value at room temperature, RT, for each sample type. B) PAM fluorescence values at start of measurement (F0) and maximum fluorescence after light pulse (Fm). Both Fm and Fo values are normalized to Fo at RT for each sample type. Averages calculated from three or four experimental replicates, with one or two technical replicates in each experiment. Error bars represent standard error.*

samples. The lower re-growth of C-S samples after stress at 40 °C does indicate that some damage is done to cells, the unchanged yield value therefore does not mean that cells are unaffected by the increased temperature. A lower F_0 (while F_m is also lowered) indicates either fewer cells, more efficient photochemistry, or increased heat dissipation. The cell counts do not show a reduced number of cells in C-S 40 °C samples compared to room temperature samples (data not shown), neither do they show a reduced percentage of living cells (Fig. 2.2). The lowered F_0 value is therefore more likely to be the result of processes in the photosynthetic machinery. F_m being dramatically lower indicates increased heat dissipation, rather than increased photochemistry.

The decreased yield at 42 °C seem to be an effect of both decreased F_m and increased F_0 for all samples, although in A-L it is mostly due to increased F_0, and for the planktonic samples the lower yield is mostly due to decreased F_m. This indicates that the cells are protecting themselves with heat dissipation but also starting to get damaged, correlating with the re-growth results in Fig. 2.8. At 44 °C F_0 and F_m stay the same, meaning that of all light absorbed by chlorophyll none is going to photochemistry, but is either fluoresced or dissipated as heat, because the photosynthetic machinery is damaged. The very low re-growth rates for samples stressed at 44 °C indicates that this damage is irreversible.

Fig. 2.10 shows mRNA expression of *psbA* and *hsp70A* for A-L samples. *hsp70A* seem to be regulated at a lower level in substrate samples compared to planktonic samples, for all temperatures. This might be an effect of the relatively high age of the cultures; Schembri et al [118] tested *E.coli* biofilms vs both planktonic exponential growth and planktonic stationary phase and found that DnaK, the bacterial homolog to Hsp70 was up-regulated in biofilm compared to exponentially growing planktonic cultures, but down-regulated compared to stationary phase culture. Compared to RT, however, *hsp70A* is strongly up-regulated for all samples for both 40 °C and 42 °C. *Hsp70A* expression reacts within 30 minutes of heat shock sensation [130], so this was to be expected.

psbA is only slightly up-regulated in the substrate samples as compared to the planktonic samples at 40 °C, and there is no change in regulation of *psbA* at stress temperatures compared to room temperature. *psbA* has been shown to be up-regulated within 1.5 hours at 40 °C [143] and at 42 °C [76], which is almost double the heat stress time that was used in these experiments. *psbA* expression, as a late HSR, might therefore not have started yet.

The re-growth, live/dead cell counts, photosynthetic fluorescence and qPCR data together paint a picture where cells in planktonic culture as well as cells

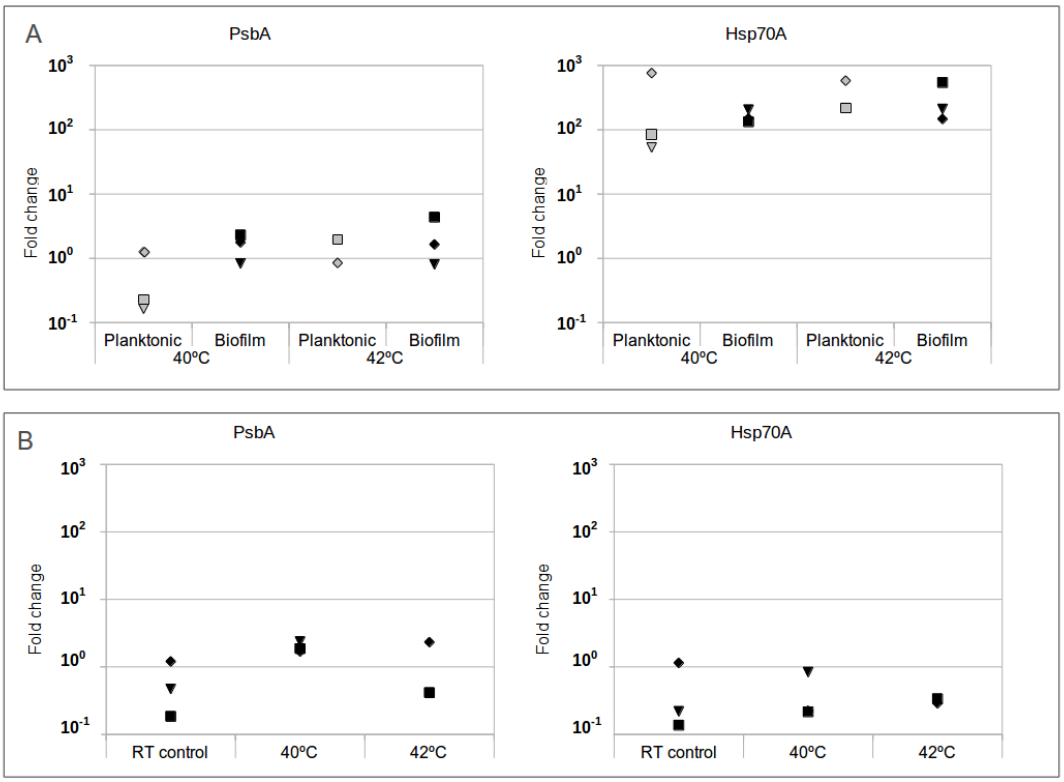

Figure 2.10: mRNA expression in A-L samples and corresponding planktonic culture. *A)* psbA *and* hsp70A *expression in heat stressed samples compared to room temperature controls. All* hsp70A *samples show a statistically significant upregulation compared to room temperature controls using a 95 % confidence interval.* psbA *samples, however, do not show a significant up- or downregulation compared to room temperature controls. B)* psbA *and* hsp70A *expression in A-L samples compared to planktonic samples. No biofilm samples showed a significant up- or downregulation compared to planktonic samples. Two biological replicates were used, one with two technical replicates and the other with one technical replicate.*

attached to a surface, are alive and protecting themselves against heat at 40 °C by up-regulating heat shock proteins and increasing heat dissipation from the photosynthetic apparatus successfully enough to be able to reproduce. Cells attached to C-S substrates however, and their photosynthetic machinery, are affected at 40 °C, so even though they are alive and actively doing photosynthesis after the heat stress event they are not able to reproduce at the same rate as cells in planktonic culture or cells attached to C-L and A-L substrates. Previous tests have shown that C-S substrates have fewer cells growing on them than C-L and A-L substrates, and that those cells are sitting mostly on the surface (see section 2.2). Therefore, a possible explanation for the decreased heat tolerance of cells on C-S substrates at 40 °C is that the surface of the substrate is more exposed to stress than cells in planktonic culture or cells residing inside of the substrate. An alternative explanation is that the alumina substrate is conducting heat more efficiently than water so that the cells attached to them are exposed to a higher heat dose than the samples in the surrounding planktonic culture. If they were exposed to a higher heat dose they would show increased F_0 at lower temperatures than planktonic samples, since the photosynthetic machinery would be damaged earlier. If, additionally, the lower cell number on C-S substrates make them even more exposed than cells attached to the other sample substrates it would explain why C-S sample $F_m$values react at lower temperatures than the other samples; they are already stressed and activate heat dissipation mechanisms. If cells on alumina surfaces are more exposed to heat because of the ceramics heat conduction efficiency, it would mean that it is important to design the ceramic substrates not only to have high cell attachment, but also to provide a beneficial environment of the cells. The type of alumina substrates investigated in this work can also be bi-functionalized to have a hydrophobic interior [73] and a hydrophilic surface, which could help isolate against and delay temperature changes for cells growing on the surface.

Stress at 42 °C results in cell damage in all types of samples. Cells are still alive after the stress periods, but their photosynthetic ability is impaired, and either only a portion of them are able to reproduce, or they have a longer lag phase before they can start to reproduce. At 44 °C practically all cells, regardless of sample type, are irreversibly damaged after the heat stress period.

These tests show that there is no increased stress tolerance for cells attached to the porous alumina surfaces compared to those in the planktonic culture, and that attached cells might even be more affected by heat stress than planktonic cultures. As discussed in the introduction, cells in biofilm express stress response genes more strongly than planktonic cells [112]. Natural biofilms, however, are usually comprised of many species, and form in nutrient-deplete

conditions. Non-sterile conditions were important for the formation of a thick biofilm in a study by Irving and Allen [64]. In nutrient replete and axenic conditions, such as those in a PBR, biofilms tend to be thin and unstructured [30]. The results of these experiments could mean that the *C. reinhardtii* in the culture have attached to the alumina surfaces, but has not formed a protective biofilm. The idea of using biofilms in a bioreactor culture as stress resistant re-growth inocula therefore creates a design conflict between creating an productive PBR with an axenic culture of a highly efficient algae strain under nutrient replete conditions and having an environment that could support a stress resistant biofilm.

2.4.4 Conclusions

Chlamydomonas cells growing on the ceramic surfaces tested in this study do not survive heat shock better than cells in planktonic culture. Cells growing on substrates and in planktonic culture both show heat shock response at 40 °C, but retain their ability to recover and regrow at the same rate as non-shocked cells. At 42 °C cells are severely stressed and their photosynthetic machinery is damaged. Cells are still surviving the heat shock for a while but their ability to reproduce is impaired. At 44 °C most cells are dead. For small pore substrates, however, cells have slower re-growth at 40 °C. The nutrient rich and axenic conditions in the bioreactor are not beneficial for the formation of biofilms, which can explain the lack of heat protection for cells attached to substrates. The design of the substrates is important to provide a protecting environment for attached cells, and there is room for product development and innovations on the materials side. The survival and reproduction rates at different temperatures are line with other work on *C. reinhardtii* cultures, but, to the knowledge of the authors, this is the first study doing a direct comparison between heat stress tolerance of an algal biofilm with its planktonic culture.

2.5 Silica sol-gel encapsulation of algae

The following manuscript is in preparation.

A new process for silica sol-gel encapsulation of algae for storage purposes

Authors:

Maria Johansson*; Matthias S. Ullrich; Christian Soltmann

*corresponding author: m.johansson@jacobs-university.de

Abstract

Photobioreactor culture facilities need reliable, easy to maintain, storage of algal cells for starter cultures. Storing cells encapsulated in a protective material offers advantages such as low risk of contamination as well as short start-up times of new cultures. The work presented in this paper demonstrated the effectiveness of using a novel type of silica sol-gel encapsulation process for storage of *C. reinhardtii* algal cells. The process is designed to separate the technical preparation of the sol-gel from the step when biological cells are mixed in, thus keeping the environment in the gel at favorable conditions for biological cells from the moment they are added. In addition, it uses colloidal silica which results in less gel shrinkage and stress on cells.

Health of encapsulated cells was tested using photosynthetic fluorescence measurements and chlorophyll *a* measurements. Gel stability was monitored through silica concentration measurements. Results show that cell cultures go through an adaptation phase the first two days after encapsulation, during which chlorophyll *a* content and maximum photosynthetic fluorescence, Fm, decreases. Values are stabilized after the adaptation phase and fluorescence measurements show that cells survive and stay productive up until the end of the 8 week tests. The sol-gel also showed good stability over the whole experimental period and no gel shrinkage was observed. The results show that sol-gel encapsulation can be a suitable method for storage of *C.reinhardtii* cells for up to two months.

2.5.1 Introduction

Photobioreactor (PBR) culture facilities rely on storage of micro-algal cells. A good storage solution requires little maintenance and offers survivability over

periods of several months up to several years, as well as fast establishment of liquid cell cultures from stored cells.

The most common methods for long-term storage of cells in biobanks are cryopreservation and freeze-drying [33]. These methods inactivate cells by removing water so that risks inherent to active cell cultures, such as mutations and contamination, can be avoided. Both methods offer viability of microbial cells for decades or more. However, during the freezing process there is risk of harm to cells from ice formation or from changed salinity due to concentrating solutes. Survivability of samples also risk being compromised by temperature fluctuations during storage. Experience has shown that eukaryotic micro-algae are so sensitive to freeze-drying and cryopreservation that other storage methods are preferred in algal culture collections [33, 54].

A common storage method for micro-algae is culturing on agar at sub-optimal temperatures and low light. Under those conditions cells remain active, but grow at a very slow rate. This storage method, however, requires frequent re-streaking of cells, and exposes cells to risk of contamination from other species. Another option is to store cells encapsulated in a gel material. Encapsulated cells have slow growth and their energy is redirected from growth to repair which enhances their survivability [36]. An added benefit is that the encapsulating gel is a barrier to contamination from other species [79]. Silica sol-gel has become a popular encapsulation material [35] because because its manufacturing routes can be made biocompatible.

Silica sol-gel synthetization starts from a stabilized solution of hydrolyzed silica precursors. Gelation of the silica solution takes place when the precursors undergo a polymerization process. The precursors, consisting of monomers or small particles of hydrolyzed silica, connect to each other through hydrolysis reactions and form a gel network. Two different types of precursors can be used: silica alkoxides or aqueous silica. Silica alkoxides are often impractical for the immobilization of biological components, due to the alcohol produced by the hydrolysis reaction. Even after gelation, changes in the gel network continue during aging and can result in further hydrolysis [24]. Therefore research efforts have been directed towards aqueous silica precursors.

Aqueous silica is often used as a subcomponent in the sol-gel process for the entrapment of biological components [115, 114, 100, 28]. By adding acid to aqueous silica, a fast gelation occurs at neutral pH. However, sodium ions are released in the process and the resulting level of salinity is detrimental to many microorganisms. Additives such as glycerol or PEG have been used to protect encapsulated cells from high sodium concentration [115, 34]. However, glycerol can hinder CO_2-molecules to reach the cells [34] and has proven toxic to some species of algae [89].

Alternatively, stable dispersions of silica nanoparticles, colloidal silica, can be used for sol-gel processing. The preparation of this commercially available silica sol is based on the formation of silicic acid by removal of alkali ions from an aqueous solution of hydrolyzed silica by an ion exchange process or dialysis, followed by nucleation, polymerization and growth of silica particles under defined conditions to monodisperse, colloidal, sols [141]. Therefore, defined nanoparticles are already present in the sol, and forms a substantial part of the later gel structure.

The next step of the manufacturing route is condensation of silica monomeres and particles into a gel. Due to the unusual behavior of aqueous silica suspensions, colloidal silica are destabilized as the pH is increased away from the isoelectric point at intermediate, neutral pH values [135, 29, 100]. Condensation takes place when hydroxyl groups react with each other to form oxygen bridges between the silica atoms, releasing water in the process. Polymeres and small particles are formed first and eventually the whole volume is bridged and a gel has formed. An increasing silica content, temperature or salt concentration of the sol reduces the gelation time. The gelation kinetic is also influenced by the size of the counter ions of the silica sol and their ability to structure water molecules [135, 29, 100].

Cells, suspended in a buffered solution, are normally mixed in the silica solution before the condensation step, and are therefore subjected to the chemical and physical environment of the gel during condensation [28]. This gel still contains a large amount of water so hydrolyzation and condensation continues in an aging process, in which the gel could shrink as water is released. Gel shrinkage might affect the cell physiology with mechanical stress [24]. Compared to silica alkoxides the gelation process of colloidal silica sol can be assumed to more like an adhesion between the colloidal particles than condensation reactions of their hydroxyl groups. As a result the mechanical stress for immobilized cells during aging is reduced [21].

If colloidal silica is mixed in with the precursors the porosity of the gel will be increased. The porosity of the gel is important for nutrient exchange between the biological cells and the medium surrounding the encapsulation, and for allowing cells space to grow.

The study reported here use a novel method for silica sol-gel preparation. Only colloidal silica sol with monodisperse particles are used. To ensure a high optical transparency for photosynthesis, a sol with relatively small silica particles of 8 nm was chosen. The pH value was adjusted by an ion exchange process to ensure a low salt content. Due to the low salt content and the dilution of the sol by the added biomass, the gelation process is relatively

slow [100]. A heat treatment with subsequent cooling, before the biomass is added, enhances the gelation kinetic of the neutral silica sol. The benefits of this novel method are that it is fast, and that pH and salinity can be kept within biologically compatible ranges from the moment the cells are mixed into the gel. The study was conducted to assess the health of the encapsulated cells during a several weeks long time period, as well as to assess the stability of the sol-gel over a longer period of time in a bioreactor environment.

2.5.2 Materials and Methods

Algae species, media, and culture maintenance

The algal species used was *Chlamydomonas reinhardtii,* wild type, SAG 11-32b. It was kept on TAP-medium agar plates [50], from which liquid cultures were started in TAP-medium. Liquid cultures were kept under fluorescent cool white light in a day:night cycle of 14:10 hours, and were grown for 72 hours. Liquid cultures were centrifuged at 3000 rpm for 10 minutes in 50 ml tubes to harvest the biomass. 4 ml wet biomass was harvested from 1 liter liquid culture.

The bioreactors in the experiments were run with TAP-medium with no acetate added, so called Tris-minimal medium, to minimize risk of contamination from other species that, like *C. reinhardtii*, use acetate as a carbon source.

Sol-gel preparation and encapsulation of algal biomass

Sol-gel was prepared from silica sol (Köstrosol 0830, Chemiewerk Bad Köstritz GmbH, 07586 Bad Köstritz, Germany), an aqueous solution of colloidal silicate, stabilized with sodium hydroxide. Particle size was 8 nm and pH of the sol solution was 9.5. All ingredients for the sol-gel were autoclaved, and the preparation process took place under sterile conditions. Two sets of sol-gel were prepared, one for use with alumina foam substrates and one for glass slides substrates (see section 2.5.2). They were prepared using the same protocol, with minor differences as explained below.

The silica sol was divided in two aliquots. From the first aliquot sodium ions were removed by cation exchange. The silica sol was mixed with ion exchange beads and stirred until it had a pH-value of 4. The beads were removed using a sterile filter. The second aliquot was left as delivered by

the manufacturer with pH 9.5. Silica sol with conditions favorable to algae was prepared by adding the following amounts of the Tris-minimal medium components: 0.121 g TRIS, 1.25 ml salt-solution, 18.5 µl KP-solution, and 250 µl NaCl-solution, to a 50 ml test-tube [50, 136]. For the sol-solution to be used with alumina foams 50 µl of Hutners trace solution was also added [61]. 35 ml acidic sol was added and the test tube was then filled up with basic sol to a total volume of 50 ml. The resulting silica sol had a pH of 7 and the same salt composition and concentration as Tris-minimal medium. Silica sol at pH 7 will start to condensate, but the condensation process will take several days. To increase the condensation rate the solution was heated in a water bath at 95 °C for 15 minutes for the solution to be used with alumina foams and 20 minutes for the solution to be used with glass slides. The solution was then cooled down for 15 minutes in a 10 °C water bath. The heating time period was chosen so that the the sol would remain pipettable during the last steps of preparation. Wet algal biomass harvested as described in section 2.5.2 was mixed into the sol-gel at a volume ratio of 20 v/v%. Sol-gel encapsulated algal biomass was pipetted onto substrates, 3.5 ml on each alumina foam and 1 ml on each glass slide. The gel aliquots on their substrates were put in lidded Petri-dishes with a drop of sterile water (foams) or TAP-ac (slides) to avoid drying out the gel surface, and were left to solidify for 24 hours at room temperature.

Substrates and experimental bioreactors

Two different substrates for the sol-gel encapsulated algae were used in the experiments: porous alumina foams and glass microscope slides. They were used in two different types of bioreactors. Both type of reactors were illuminated with cool fluorescent white light in a day:night cycle of 14:10 hours.

Glass microscope slides were used as substrates in order to allow easy removal of samples for measurements. An area of 2 by 2.5 cm was sectioned off the end of each slide using a string of glue. Sol-gel-algae mixture was pipetted into that area, as described in section 2.5.2, to ensure that each sol-gel-algae aliquot had the same thickness. The slides were put in a glass bioreactor filled with 250 ml Tris-minimal medium. The medium volume was chosen so that both bioreactors held the same volume ratio of growth medium to sol-gel. The whole growth medium volume was removed and replaced by fresh medium once a week. The replaced medium was poured into a beaker, which was then filled up to 250 ml with Tris-minimal medium to compensate for evaporation, and aliquots of it was set aside for dissolved silica concentration measurements. Air was bubbled through the reactor through a sterile

filter using an aquarium pump. From the reactor the slides were easily removed temporarily for PAM-measurements. The glass slide reactor was run for 6 weeks. PAM-measurements were done once a day the first three days, the first measurement was made before the start of the reactor. After that, PAM-measurements were done once a week. Additionally, one glass slide was removed each week to be used for chlorophyll extraction. The removed slides were stored at -20 C until extraction.

The second type of bioreactor was a tubular photobioreactor, designed to test biological filters, in which six circular porous substrates could be fitted sequentially. The substrates consisted of alumina foams, as described by Barge et al [5], with pore sizes varying between 0.5 and 3 mm. They were 8 cm in diameter and 1 cm thick. Medium was pumped through the substrates by a peristaltic pump. The gel on these substrates was thereby subjected to more shear stress than the on in the glass slide reactor. Medium volume in the whole reactor system was 500 ml and the medium was exchanged for fresh medium once a week. Aliquots of the replaced medium were set aside for dissolved silica concentration measurements. The reactor was bubbled with air from an aquaria pump through a sterile filter and it was run for 8 weeks. PAM-measurements were taken on foams at the end of the run.

Pulse Amplitude Modulation (PAM) fluorometry

PAM-measurements were made with a "Mini-PAM" portable chlorophyll fluorometer from Heinz Walz GmbH, Effeltrich, Germany, using the saturation pulse method [120]. The photosynthetic quantum yield, i.e. the maximum efficiency of photosystem II, was calculated from the measured minimum chlorophyll fluorescence, F_0, and the maximum chlorophyll fluorescence, F_m, as [46]:

$$Yield = \frac{(F_m - F_0)}{F_m}$$

PAM-measurements on foams were made on samples that had been dark-adapted for 30 minutes. Three measurements of different spots on each foam were made. PAM-measurements on glass slide samples were taken on all slides on the start day, thereafter on 4 slides each measurements day. They were done on samples that had been dark-adapted for at least 15 minutes.

Chlorophyll extraction and spectrophotometry

Sol-gel encapsulated algae from one glass slide was moved into a mortar and liquid nitrogen was poured over them. A pinch of $CaCO_3$ and sand was

added when the nitrogen had evaporated, and the mixture was mortared. 5 ml methanol:acetone mix (3:1) was added and the whole mixture was moved into a 15 ml tube. 5 ml methanol:acetone mixture was added to the mortar to rinse out the last of the algae/sol-gel mixture so that it too could be added to the tube. The mixture was left at room temperature for 30 minutes, after which it was centrifuged for 5 min at 3000 g, and decanted. Optical density, OD, was measured in a spectrophotometer on samples diluted by a factor 10 with methanol:acetone mixture at 647, 664 and 750 nm in a 1 cm cuvette. The OD-measurements taken at 750 nm were subtracted from the measurements taken at 647 nm and 664 nm to correct them for turbidity. Chlorophyll *a*, chl *a*, concentration was calculated using the formula

$$\text{chl}\,a = (11.93 * OD_{664} - 1.93 * OD_{647}) * DF/1000$$

where DF is the dilution factor [107].

Dissolved silica

Silica concentration was measured on samples from the used growth media in the bioreactors to assess the stability of the sol-gel. Blank samples were prepared from centrifuged algae culture in Tris-minimal medium; 400 ml algae culture was centrifuged at 1500 g and decanted to remove cells. All samples were stored at -20 °C until measured.

Silica concentration was measured using test LCW 028 from Hach Lange. Tris-minimal medium contains phosphate and therefore oxalic acid was added to the samples, in accordance with the protocol from Hach Lange. Silica concentration measurements were done in a Xion 500 spectrophotometer, Hach Lange.

2.5.3 Results and discussion

The health of the gel encapsulated cells during their 45 days in the bioreactor was monitored through the three photosynthetic fluorescence parameters F_m, F_0 and photosynthetic yield, as shown in Figure 2.11, and through their chlorophyll *a* content, as shown in Figure 2.12. Yield values give an indication of the efficiency of cells in converting light to photosynthetic products. A change in yield can be a result of failing cell health or of cell adaptation to changes in the environment.

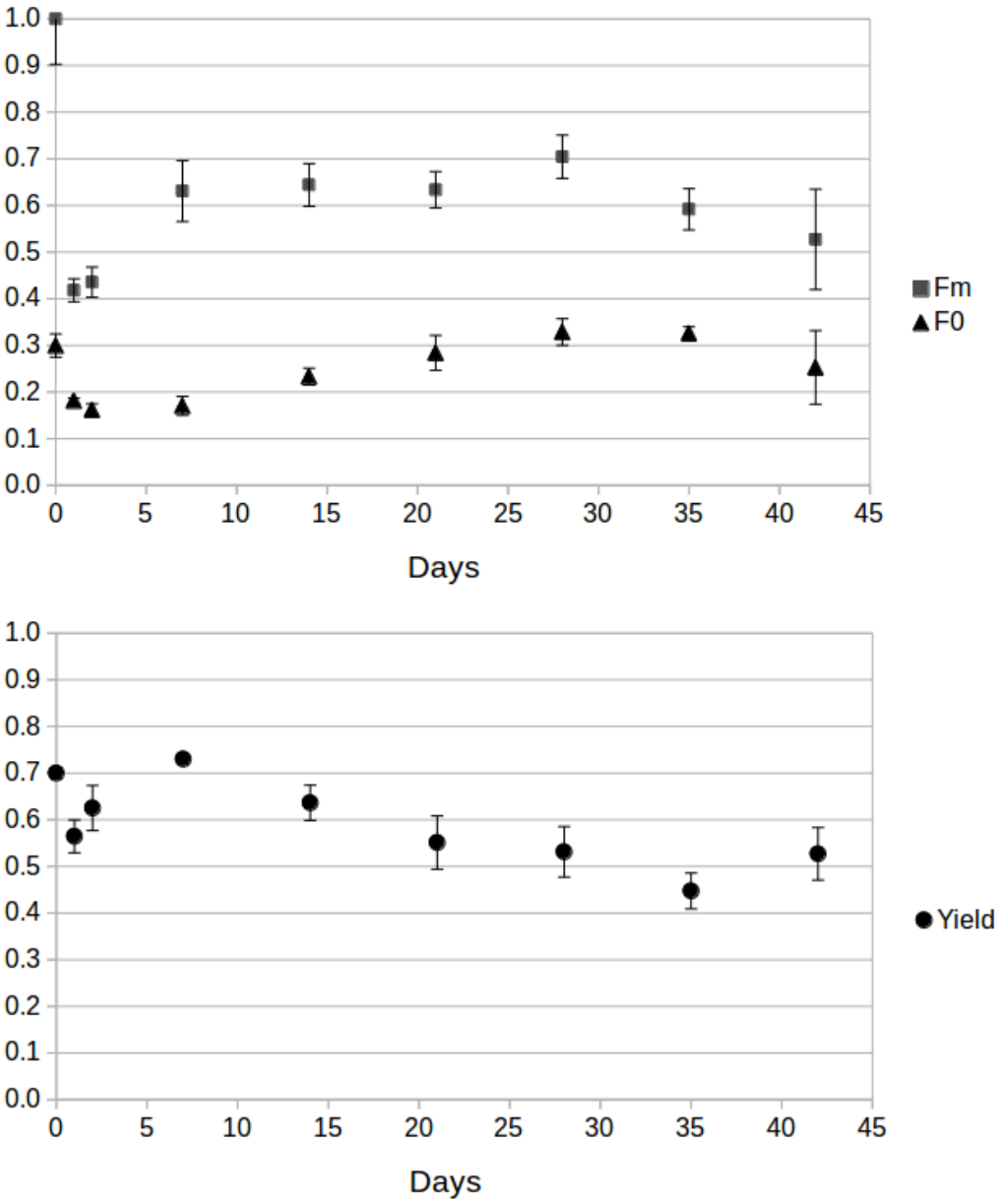

Figure 2.11: PAM-measurements on glass slides. *Values shown on day 0 are averages of measurements on all 12 samples. All other values shown are averages of 4 samples, randomly selected from the original 12 samples in different combinations each measurement day. F_m and F_0-values are shown normalized to F_m at day 0. Error bars represent standard deviation.*

At the start of the experiment both yield and F_m show high values, as can be expected from cells freshly taken from a healthy liquid algal culture. F_m decreased dramatically during the first two days after the start of the experiment, but F_0 also decreases somewhat. This results in a slightly lower photosynthetic yield. The chlorophyll *a* content (Figure 2.12) decrease strongly during the first days of the experiment, as well.

Lower chlorophyll content would lead to decreased F_m-values, but F_m decreasing more strongly than F_0 indicates that an additional mechanism is at play. Lowered F_m indicates increased heat dissipation of the algal cells photosynthetic machinery [86]. This is a likely effect of the changed environment of the cells; compared to the dense liquid culture the gel encapsulated cells immersed in clear medium receive stronger light which could induce heat dissipation as a protection mechanism.

The Tris-minimal medium in which the gel encapsulated cells are immersed contain no acetate, unlike the TAP-medium in which the liquid culture they were harvested from was grown. The acetate promotes growth, so the change to Tris-minimal medium would also decrease the growth rate. Furthermore, encapsulated cells grow slower than cells suspended in liquid [36]. The slower growth rate of the gel encapsulated cells would mean that dying cells are not replaced as fast as they would be in liquid culture, and the reduced chlorophyll content and F_0-values could be results of a lowered cell concentration.

Taken together the results from fluorescence and chlorophyll *a* content measurements show the cells adapting to their new environment during the first days of the experiment.

F_m has stabilized at the end of the first week, and then keeps at a steady level for several weeks. F_0 increases slowly during the same time period and, as a result, the yield decreases. Reduced F_0 values indicates damaged photomachinery [86]. The slowly increasing F_0-values could therefore indicate that damages to the photosynthetic machinery are accumulating, at the same time as new cells are replacing dying or damaged cells at a lower rate. This is supported by the chlorophyll *a* measurements, which show slowly decreasing chlorophyll content during the same period. This could indicate that the conditions in the bioreactor are not optimal for the encapsulated cells, either due to strong competition for nutrients or because of the light conditions. Weaker light, higher medium turnover or lower biomass concentration in gels might have resulted in a more stable F_0-value. Even though the F_0-values decrease, the results show that many cells are surviving and stay productive for 6 weeks in this environment.

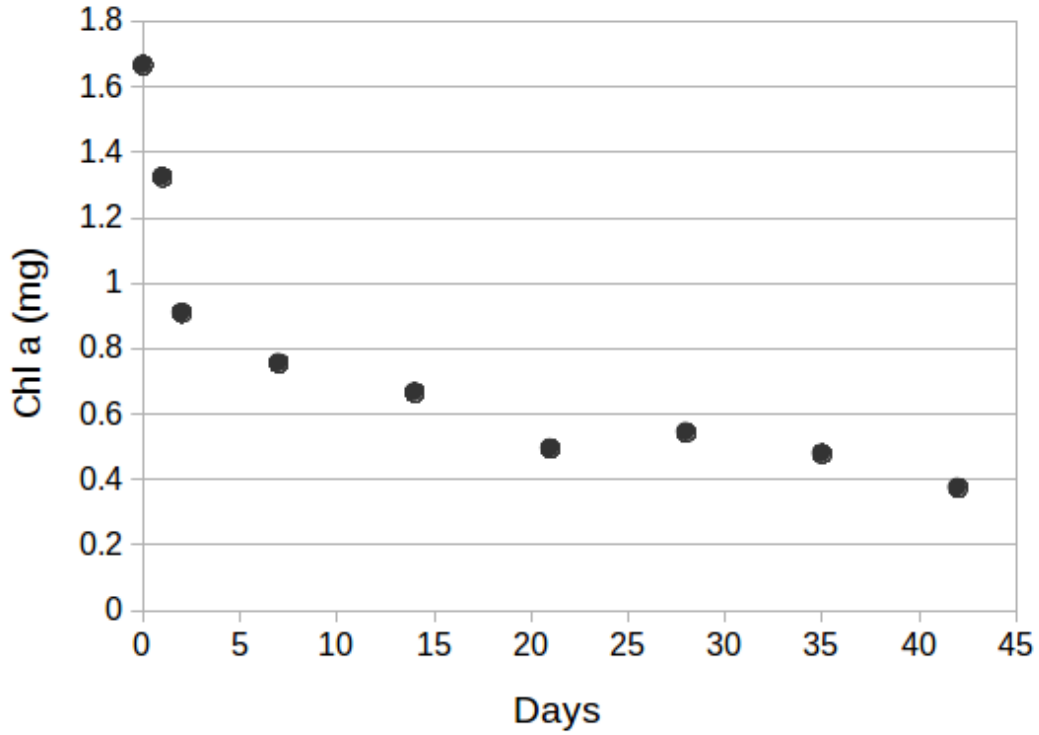

Figure 2.12: Chlorophyll *a* content per sample. *Chlorophyll* a *was extracted from one glass slide sample each week. Values shown represent one experimental run.*

Yield measurements were also taken on the encapsulated cells on foams substrates in the tubular bioreactor, after 8 weeks of experiment. The average yield on all foams were 0.62 (data not shown), which is similar to the yield values from the samples on glass slide substrates. These values are in line with results from studies on healthy algae in natural environments [38], where the yield of phytoplankton in nutrient-replete environments were shown to be around 0.65.

Figure 2.13 shows that the silica release to the medium kept below 5.5 µg/l per day. In the early stage of condensation a high number of remaining silanol groups are present and there is still a non-polymerized silica sol within the gel network [24]. The preference for depolymerization at more weakly condensed sites [32, 138, 23] and an easier dissolution of silica from positive curvature surfaces like small particles [63] result in a higher silica release in the first weeks. During aging condensation continues within the network and the re-precipitation of silica at negative curvature surfaces like the necks between touching particles results in further polymerization and coarsening [32] and silica release decrease. The experiments show that the gel is stable over the tested time periods. Colloidal silica was used in the gel preparation in order to avoid gel shrinking after preparation, and no shrinking of the gels on either type of substrate could be observed. Cell release to the medium was not observed during the experiment.

2.5.4 Conclusions

C. reinhardtii cells encapsulated in sol-gel prepared with the herein described method survive and stay healthy for several weeks. The cells undergo an adaptation phase when first encapsulated during which photosynthetic yield values and chlorophyll concentration decrease rapidly, but these values will then stabilize. The longest experiment was ended after 8 weeks, but the results indicate that cells would be able to survive for some additional time. However, conditions are not optimal for the encapsulated cells; encapsulated cell culture life time might be extended by using less intense light or higher medium turnover rate. Silica is released from the gel, but it stays stable and does not shrink or release cells throughout the experiments. Silica sol-gel encapsulation could therefore be used as a storage method for *C. reinhardtii* cells, for time periods of a few months.

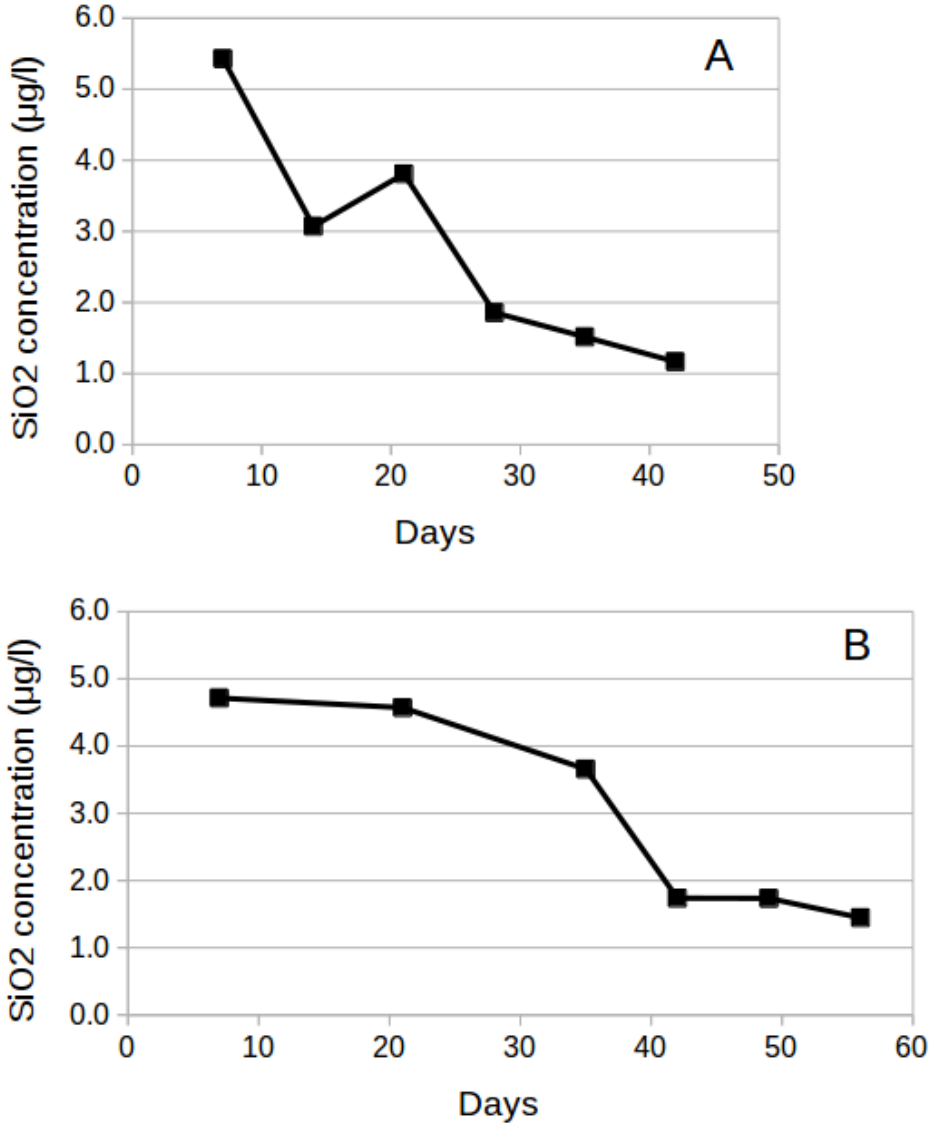

Figure 2.13: Silica concentration in medium. *(A) shows measurements form the bioreactor containing glass slide samples, (B) shows samples from alumina foam bioreactor. Silica concentration values shown here are divided by number of days that elapsed since medium was last exchanged. Values shown represent one experimental run with one sample measured for each point.*

Chapter 3

Discussion, conclusions and future scope

It is of utmost importance that photobioreactors to be used in life support systems are reliable and resilient. Biological life support system design is a research area with comparatively small research budgets. Work is ongoing in programs such as ESA's MELiSSA or DLR's ModuLES, but there is still need of more design ideas and work aimed at increased reliability and resilience. The space PBR research field benefits from general PBR research, and from development of commercial PBRs, by adapting commercial PBR designs to life support systems. Fortunately, PBR engineering is an active research field, wherein several design ideas are being pursued. Reliability and resilience are important also for commercial PBRs, but not as crucial as for PBRs in life support systems. This is therefore a sub-field in PBR research which life support system designers should prioritize, and where their research can complement and make important contributions to general PBR research.

The overall aim of the work presented in this thesis was to investigate methods to increase resilience and decrease vulnerability of PBR cultures. Two avenues to increased bioreactor resilience were explored, as formulated in the two main research questions: "Can a subset of a *C. reinhardtii* culture grown as a biofilm be used to restore the culture after it has broken down due to heat stress?" and "Is immobilization in a sol-gel a possible method for storage of viable *C. reinhardtii cells*?" Below follows a summarizing discussion of how these research questions were answered, organized according to the specific aims that guided the work with the research questions.

3.1 Summarizing discussion of results

3.1.1 *C. reinhardtii* attachment to alumina supports of varying pore size and surface functionalization

The first specific research aim was "To explore attachment and cell viability status of *C. reinhardtii* grown as a biofilm on alumina supports with micro-scale pores, with respect to different pore sizes and surface properties of the alumina". Alumina manufactured with the HAPES-method was used as a substrate in biofilm formation experiments because it is an inert material whose pore sizes and surface properties can be varied in a controllable manner, through the manufacturing process and through surface functionalization using silanization, respectively.

Alumina with various combinations of pore sizes and surface functionalization were chosen based on current knowledge on algal biofilm formation found in literature. Algal attachment on the chosen alumina was tested in a biofilm reactor, to create the best possible conditions for biofilm formation. Attachment and cell viability were then tested with three different methods: re-growth tests, chlorophyll fluorescence measurements, and live/dead staining with imaging in a confocal laser scanning microscope, CLSM. Re-growth tests, i.e. taking out alumina substrates with biofilm growth on them from the biofilm reactor and restarting cultures from them, were central since the wider aim of this work was to test if the biofilms grown on alumina could be used to restart a PBR. The tests give information not only on cell viability, but also on cell reproductive ability. Live/dead staining and imaging in a CLSM was used to test viability of cells as well as to map the distribution of attached cells on the surface of, and inside, the alumina. Photosynthetic ability, used as an indicator for cell health, was tested with chlorophyll fluorescence measurements, using a PAM-device.

In biofilm research bacterial biofilm formation has been tested extensively with the purpose of avoiding bacterial biofilm on for example medical equipment, and multi-species biofilms has been tested with the purpose of improving waste-water treatment processes [19, 3], but little work has been done on axenic microalgal biofilms. However, this research field is growing in importance as more work on biofilm photobioreactors is being done. Alumina is used in medical research as supports for growing human cells, but has not been tested extensively with algal cells. The HAPES-alumina has been tested with biological organisms as spawning plates for fish breeding [73].

The results showed that *C. reinhardtii* attaches well to HAPES-alumina. No toxic effects from the alumina could be detected, indicating that alumina can be used as successfully with algal cells in biotechnological applications, as it can be used with human cells in medical applications. Furthermore, no toxic effects were detected from the surface functionalization. Being able to functionalize the alumina surface through silanization without harming attaching algal cells opens up many opportunities for photobioreactor designs using alumina as a design element. The manufacturing process of HAPES-alumina gave us the research opportunity to test algal attachment to substrates with a set of relatively narrow micro-scale pore size distributions. It was clearly shown that pore size is important for cell attachment; the optimal pore size was 30-50 µm with windows between pores of 16 µm, in other words pore and window sizes between 2 and 5 times the average *C. reinhardtii* cell size. This result is in line with contact point theory [131], saying that surface repositories of 1 to 5 times the cell size gives the best conditions for cell attachment. Pore size also strongly affected distribution of attached cells; with a pore size distribution average close to or just below the average cells size cells will attach well to the alumina surface, but will be growing sparsely inside the alumina as few windows between pores are big enough for cells to pass. When given the opportunity, due to larger windows between pores, cells will also distribute themselves further inside the alumina material. Cell concentration was highest close to the surface, however, where light was available.

The results with varying surface properties showed that alumina without surface functionalization, had the most cells attaching to them. In itself the alumina surface is slightly hydrophobic. Neither positive or negative surface charge, nor varying levels of hydrophobicity, gave a positive effect on cell attachment in these tests. Cell attachment to surfaces is controlled by a combination of repelling and attractive forces [27]. Other work, unlike ours, has shown a positive effect of electrostatic interaction [4]. Hydrophobic interaction has given mixed results in work reported in literature [101, 122]. In our work we have shown that the combination of pore sizes below 30-40 µm and hydrophobic treatment results in a surface with almost no cell attachment. Although not useful for the application researched in this thesis a porous material on which cells have difficulty attaching could find uses in other biotechnological applications, especially since the bulk of research on biofilm is dedicated to questions on how to avoid biofilm formation on processing and medical equipment.

The results from our work on *C. reinhardtii* attachment to HAPES-alumina has resulted in an overview of cell attachment to substrates with pore sizes ranging from 5 to 50 µm and with varying surface charge and hydrophobicity.

HAPES-alumina was shown to be a versatile material with suitable properties for cell attachment.

3.1.2 Heat stress survival of *C. reinhardtii* biofilm

The second specific research aim was "To test heat stress survival of *C. reinhardtii* when growing as a biofilm on alumina support compared to survival as a liquid culture". As preparation for this work a *C. reinhardtii* heat stress survival was mapped, using different combinations of heat stress temperatures, stress time periods, and cell acclimatization times. *C. reinhardtii* heat stress survival and tolerance has been reported in literature before, see for example Goho and Bell or Hema et al [49, 57], but our tests were more detailed on stress time periods and also tested more stress temperatures points in the same set of experimental conditions, than previously reported research. The results were consistent with earlier research: *C. reinhardtii* tolerate stress up to 40 °C, at 42 °C the majority of cells have died but some survive and retain their reproductive ability, and at 44 °C most cells have died. But the results also showed that cell survival varies with heat stress time. With stress times below 10 minutes most cells survive treatment at 42 °C, and when acclimatized to heat by being subjected to progressively higher temperatures during 90 minutes cells survive 42 °C just as well as they survive 40 °C.

The heat stress mapping results were used in the design of the heat stress experiments, to ensure that the algal cultures were tested under a set of heat stress conditions in which small changes in heat stress response would be possible to detect. Heat stress tests of alumina biofilm were conducted in an experimental setup, designed and built for the purpose, in which temperatures could be controlled to an accuracy of -0.1/+0.4 °C. The setup used lab scale PBRs in which algal biofilm on alumina substrates and liquid algal culture could be grown simultaneously. The purpose of these lab reactors was to mimic conditions of a full-scale heat stressed PBR containing a subset of the culture grown as a biofilm, in line with the intention of the first research question.

In these experiments re-growth tests were central, as they mimicked the restart of a heat stressed reactor from a biofilm. Viability and reproductive ability of cells after heat stress were tested in the re-growth tests, and photosynthetic ability was tested with chlorophyll fluorescence measurements. Viability was also controlled with live/dead staining and imaging in a CLSM.

Research on bacterial biofilms have shown that these are more resistant to some stresses than liquid cultures [81, 112, 118], from which we hypothesized

that an algal biofilm would be more tolerant to heat stress than its liquid culture. No reports on heat stress tolerance studies on algal biofilms could be found in literature. We could not confirm our hypothesis, as no added heat stress tolerance of cells growing in biofilm compared to those in liquid culture could be detected in these experiments.

Research on the bacterium *Pseudomonas aeuroginosa* has shown that its biofilm was more tolerant to a tested biocide than planktonic cells in logarithmic phase. However, the biocide tolerance of steady-state planktonic cells were greater than that of the biofilm [126]. The liquid cultures in our experiment had also reached a steady-state. It is possible that their resistance to heat stress had increased as a result. If future research can confirm a growth phase dependence on heat stress tolerance for *C. reinhardtii* it would imply that a biofilm-based restart device can be useful in the start-up phase of a bioreactor. Another possible explanation for our results is that we used axenic cultures. Multi-species biofilms, in nutrient deplete conditions, grow thicker and are more complex in structure than single-species biofilms [64], and might therefore tolerate stress better. The negative results in these experiments could therefore point to a design conflict: Algal culturing is often done in axenic cultures of one species selected for its high production ability of a certain metabolite. One of the reasons often mentioned in earlier studies of closed PBRs is so that it will be possible to legally culture genetically modified algae. In such a reactor only the selected species should be present, so as not to risk a reduction in productivity, and for the same reason the culture is done in nutrient rich conditions. In other words, conditions which are optimal for production efficiency are far from optimal for biofilm growth. A restart device based on biofilm will not be useful in such bioreactors. It could however possibly be applicable in multi-species bioreactors, such as in the waste-water treatment systems proposed in [96, 108].

3.1.3 Stability and survival of *C. reinhardtii* immobilized in sol-gel

The third specific research aim was "To test long term stability and survival of *C. reinhardtii* immobilized in a sol-gel". Storing back-up encapsulated cells would be an obvious measure to increase the resilience of a life support system PBR. Most established cell storage methods, however, are not practical for LSSs since they require specialized equipment, such as freezers that can keep very low temperatures at exact levels. Furthermore, the established methods do not work well for eukaryotic micro-algae [33].

Sol-gel encapsulation was evaluated as a possible storage alternative. Silica sol-gel encapsulation of cells has been shown to work for both bacteria and algae, but the manufacturing process can be harmful to cells [35]. In this work silica sol-gel manufactured with a new method was used. The method separates the sol-gel preparation process from the cell encapsulation process so that the encapsulated cells do not come into contact with harmful chemical by-products from the sol-gel reactions, or other unfavorable conditions.

Encapsulated cells were mounted on supports and put in nutrient medium for up to eight weeks. The sol-gel stability was evaluated through measurements of dissolved silica in the medium. The health of encapsulated cells was estimated through chlorophyll fluorescence measurements as well as chlorophyll concentration measurements.

The results showed that the sol-gel encapsulated cells survived and stayed healthy during the whole experimental period. A significant cell concentration reduction took place during the first few days after encapsulation, but chlorophyll concentration levels stabilized after that. At the end of the experimental periods chlorophyll levels and fluorescence measurements were still stable and it is therefore likely that the encapsulated cells would be able to survive even longer than eight weeks. The encapsulated are slower growing than cells in liquid culture, but are still active. This means that there is a risk of replication errors during meiosis or loss of genes that do not contribute to cell fitness, harming the productivity of cultures started from the cells, especially if they were designed to produce a specific metabolite. However, in algal culture collections today cell strains are stored as low activity cultures on agar in low light conditions, as freeze-drying or freezing methods used for bacterial cells do not work well for micro-algae. Other methods, where the cells have been completely inactivated, would be more suitable for long-term storage.

The new sol-gel manufacturing method will be applicable not only for cell storage in life support systems, but other types of cell encapsulation as well. Due to the relative ease of manufacturing and the beneficial conditions for cells it could be used for example in immobilized cell bioreactors or as medium-term storage in biobanks.

3.2 Major conclusions

3.2.1 Using cells grown in biofilm to restore PBR culture after heat stress

The first research question was "Can a subset of a *C. reinhardtii* culture grown as a biofilm be used to restore the culture after it has broken down due to heat stress?". The work presented here has led to the conclusion that an axenic biofilm of *C. reinhardtii* cells do not tolerate heat stress better than liquid culture, and therefore can not be used to restore a PBR culture if the biofilm is also grown inside the PBR.

However, it was also possible to conclude that the HAPES-alumina tested is a versatile substrate for *C. reinhardtii* cells. There was significant cell attachment to the non-functionalized alumina, and surface functionalization and pore size control in the manufacturing process makes it possible to control how well cells attach and how well cells are distributed inside the substrate.

Increased reliability of PBRs and protection against heat stress is the focus of many previous studies, as outlined in section 1.2.3, but there has been little previous work on self-restoring bioreactors, apart from the work of Bartsev and Okhonin, see [9]. For commercial land-based PBR-facilities personnel would always be on hand to restart reactors and occasional down-time would not be as harmful as it is in a life support system. For space applications, however, reliability is crucial and personnel time expensive. The methods discussed and tested here would have great benefits for space life support systems if a future version of them would be more successful than the one tested. Reducing personnel time and increasing reliability could also open new possibilities for commercial Earth-based PBR-facilities, such as placing them in more inaccessible locations close to resources needed to grow cells.

3.2.2 Storing sol-gel encapsulated *C. reinhardtii* cells

The second research question was "Is immobilization in sol-gel a possible method for storage of viable *C. reinhardtii* cells?". The conclusion is that sol-gel encapsulation is a viable method for storage of *C. reinhardtii* cells. The new manufacturing method which separated sol-gel preparation from cell encapsulation was relatively easy to handle and encapsulated cells survived for the eight weeks of the longest experiment, in room temperature. No

special equipment was needed for storage, apart from that equipment which is used for algal culturing. Cells were also more protected when encapsulated in the sol-gel than they would have been if they were grown under low-light conditions on agar which is common for algae culture collections.

3.3 Future scope

Even though the axenic *C. reinhardtii* biofilm on micro-porous alumina supports did not have better heat resistance than liquid culture the work conducted for this thesis points to possibilities worth exploring regarding autonomously restarting photobioreactors.

Stress resistance of multi-species biofilms: The tests were done with axenic algal biofilms, as the type of PBRs that would use biofilm as a restart device were expected to have axenic cultures. However, some types of bioreactors, as well as water treatment systems, use multi-species biofilms. Multi-species algal reactors might also offer interesting benefits to LSS designs, such as simultaneous waste-water treatment and air regeneration. Researching the resistance to heat and other environmental stress of multi-species biofilms would therefore be an interesting future task.

Controlling cell environment via alumina properties: The micro-porous alumina substrates did not only enable *C. reinhardtii* cells to attach, but changing their pore sizes and surface properties also changed how many cells attached and how they were distributed. Small pore alumina treated to be hydrophobic had no cells attaching to them and also had no water penetrating into them. This has been used in work by Kroll et al [73] to make spawning plates for fish. By changing the surface properties of the alumina and connecting them with other equipment the environment for algal cells could be controlled and the attached cells could be protected from stress. Examples include: bubbling cool air through the alumina to protect the attached cells from temperature change or using alumina with larger pore sizes as a low light environment protecting cells from combined stress of high light conditions and heat.

Demonstration scale test of sol-gel: Encapsulation of *C. reinhardtii* with sol-gel manufactured with the novel method described in chapter 2.5 was shown to work in lab-scale. The next step would be to test sol-gel encapsulation in demonstration scale in a PBR-facility. Tests would include cell health after longer time periods of storage and productivity of cultures started from cells stored for longer time periods in sol-gel.

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