Downloaded from orbit.dtu.dk on: Apr 20, 2024

DTU Library

Green bioprinting

Viability and growth analysis of microalgae immobilized in 3D-plotted hydrogels versus suspension cultures

Krujatz, Felix; Lode, Anja; Brüggemeier, Sophie; Schütz, Kathleen; Kramer, Julius; Bley, Thomas; Gelinsky, Michael; Weber, Jost

Published in:
Engineering in Life Sciences

Link to article, DOI:
10.1002/elsc.201400131

Publication date:
2015

Document Version
Early version, also known as pre-print

Link back to DTU Orbit

Citation (APA):
Krujatz, F., Lode, A., Brüggemeier, S., Schütz, K., Kramer, J., Bley, T., Gelinsky, M., & Weber, J. (2015). Green bioprinting: Viability and growth analysis of microalgae immobilized in 3D-plotted hydrogels versus suspension cultures. *Engineering in Life Sciences*, *15*(7), 678-688. https://doi.org/10.1002/elsc.201400131

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Engineering in Life Sciences

Green bioprinting: viability and growth analysis of microalgae immobilized in 3D-plotted hydrogels versus suspension cultures (on "Biomaterials made in Bioreactors")

Journal:	*Engineering in Life Sciences*
Manuscript ID:	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a

Complete List of Authors:	Krujatz, Felix; Technische Universität Dresden, Institute of Food Technology and Bioprocess Engineering Lode, Anja; Technische Universität Dresden, Centre for Translational Bone, Joint and Soft Tissue Engineering Brüggemeier, Sophie; Technische Universität Dresden, Centre for Translational Bone, Joint and Soft Tissue Research Schütz, Kathleen; Technische Universität Dresden, Centre for Translational Bone, Joint and Soft Tissue Research Kramer, Julius; TU Dresden, Institute of Food Technology and Bioprocess Engineering Bley, Thomas; Technische Universität Dresden, Institute of Food Technology and Bioprocess Engineering Gelinsky, Michael; TU Dresden, Institute of Materials Science Weber, Jost; Technische Universität Dresden, Institute of Food Technology and Bioprocess Engineering
Keywords:	flow cytometry, immobilization, microalgae, viability, 3D-plotted hydrogel

SCHOLARONE™
Manuscripts

Research Article:

Green bioprinting: viability and growth analysis of microalgae immobilized in 3D-plotted hydrogels versus suspension cultures

Felix Krujatz[1*],

Anja Lode[2],

Sophie Brüggemeier[2],

Kathleen Schütz[2],

Julius Kramer[1],

Thomas Bley[1],

Michael Gelinsky[2],

Jost Weber[1]

[1] Institute of Food Technology and Bioprocess Engineering, TU Dresden Bergstraße 120, 01069 Dresden, Germany

[2] Centre for Translational Bone, Joint and Soft Tissue Research, University Hospital and Faculty of Medicine *Carl Gustav Carus*, TU Dresden, Fetscherstraße 74, 01307 Dresden, Germany

Correspondence:

Felix Krujatz, M.Sc. (Felix.Krujatz@tu-dresden.de), Institute of Food Technology and Bioprocess Engineering, TU Dresden, Bergstraße 120, 01069 Dresden

Phone: 0049-(0)-35146332727

Fax: 0049-(0)-35146337761

Keywords: flow cytometry, immobilization, microalgae, viability, 3D-plotted hydrogel

Abbreviations: DIBAC, bis-(1,3-dibarbituric acid)-trimethine oxonol, **TAP**, Tris-Acetate-Phosphate, **L/D cycle**, light/dark cycle

For Peer Review

Practical Application

Biotechnological processes using photosynthetic microorganisms are of growing industrial interest for the production of renewable energies, platform chemicals or pharmaceutical drugs. Microalgae can be cultivated in suspension, or immobilized in three-dimensional structures. We analyzed microalgae growth and population dynamics at different temperatures and illumination conditions in suspension and immobilized in 3D-plotted hydrogels.

For microbial processes, the viability of the organisms is essential as productivity depends directly on the number of active catalytic units. Therefore it is important to understand how cultivation conditions influence the population viability. We found that even under non-optimal temperatures, the number of viable microalgae was directly influenced by length of exposure to light for both suspension and immobilized cultures. The cultivation of microalgae in 3D-plotted hydrogels, a highly-organized immobilization technique, affords new fields of applications, e.g. the co-cultivation of different microorganisms in close vicinity but without direct contact.

For Peer Review

Abstract

In this study, we compared the effects of suspension cultures and a structural organized immobilization technique that we called “Green Bioprinting” on the growth and viability of microalgae. *Chlamydomonas reinhardtii* 11.32b and *Chlorella sorokiniana* UTEX1230 were suspended in culture medium or embedded in hydrogels by a 3D-bioprinting process. Culture conditions included temperatures of 26°C, 30°C or 37°C under continuous illumination or a 14/10 hours light/dark cycle. Viability was analyzed by flow cytometry using $DiBAC_4(3)$ dye, which is sensitive to membrane potentials, for suspension cultures, and by fluorescence image analysis for hydrogel-embedded cultures.’

Flow cytometry analyses of microalgae suspension cultures showed that the illumination conditions greatly influenced population homogeneity. Compared to cultures subjected to continuous illumination, a 14/10 hours light/dark cycle significantly increased the number of membrane-polarized cells within a microalgae population. Embedding microalgae in 3D-plotted hydrogels facilitated their viability. Immobilized microalgae cultures attained stable growth rates between 0.4 d^{-1} and 0.7 d^{-1} whereas the growth rate of suspension cultures was directly dependent on the temperature and illumination conditions.

For Peer Review

1. Introduction

Due to their ability to grow photoautotrophic using carbon dioxide as the sole source of carbon and light as energy source microalgae production systems are of growing industrial interest. Significant biotechnological applications include food additives, renewable biofuel production, and synthesis of pharmaceuticals and dyes [1-3]. Current research also describes the ability of microalgae to remove metals and organic compounds from aquatic and urban habitats (phytoremediation) and focuses on their potential functions as toxicity indicators and biosensors [4].

Chlamydomonas reinhardtii and *Chlorella sorokiniana* are widely-used and robust organisms both for photosynthesis research and biotechnological applications due to their high growth rates and temperature resistance [5]. Basically, cultivation of microalgae is performed in open or closed photobioreactor (PBR) systems, varying in size and shape. Light energy for photosynthetic processes is commonly provided by external or internal illumination devices [6]. Self-shading of phototrophic microorganisms and inhomogeneous illumination of PBRs cause heterogeneous light distributions, which subsequently induces the formation of heterogeneous cell populations. It has been proven that population heterogeneity of microbial cultures can significantly influence the productivity of biotechnological processes [7]. Conventional methods to determine the physiological state of microalgae are summarized by [8]: carbon uptake, ATP formation, oxygen evolution, and colony forming units [9]. All these methods provide information about the average cell population and neglect the properties and contributions of subpopulations.

Flow cytometry has been acknowledged as a suitable technique for analyzing the characteristics of microalgae subpopulations [10]. Cells are subjected to hydrodynamic focusing by a sheath fluid before being excited by a laser, and forward and side scattered light as well as fluorescence intensities can be detected. Up until now, single cell analysis of microalgae populations was applied only for the study of the cellular responses of microalgae, cyanobacteria or phytoplankton exposed to heavy metals [11], herbicides [12], contaminants [13], oxidative [14] or shear stress [15], and not for bioprocess monitoring.

Fluorescent dyes must be carefully selected because microalgae/cyanobacteria produce a variety of autofluorescent molecules, e.g. chlorophyll, carotenoids, or

For Peer Review

fluorescent proteins like phycoerythrin and phycocyanin. A sensitive green fluorescent dye should be chosen to prevent spectral interferences with autofluorescent signals when monitoring the physiological state of microalgae during a bioprocess by flow cytometry [10]. Dorsey et al. [16], Prado et al. [12] and Michels et al. [15] used fluorescein diacetate (FDA) to analyze microalgae viability by cellular enzyme activity. FDA enters the cells passively by diffusion. The non-charged substrate is hydrolyzed by intracellular esterase activity to a polar fluorescent product [10]. Certainly, esterase activity and fluorescence intensity are strongly dependent on the intracellular pH and diffusion properties of analyzed cells.

Microalgae are cultivated in suspension for most applications, however immobilization of phototrophic cultures is advantageous as it may facilitate separation of cells from medium [17] or cell protection from high shearing by stirring or aeration. In his review, Moreno-Garrido [18] distinguishes between passive and active immobilization techniques used for microalgae. Passive immobilization involves adhesion on secondary surfaces like biofilms or treatment with flocculent agents, e.g. chitosan. In this study, we focus on active immobilization, that is, the entrapment of living cells in three-dimensional structures.

Natural polysaccharides like carrageenan or agar-agar are used to immobilize metabolic active microalgae [19], and alginate has become the preferred material due to the easy handling and bio-compatibility. However, the structural organization of this polymer is commonly limited to spherical beads.

Sing [20] identified increased chlorophyll and carotenoid content in alginate-immobilized microalgae which caused enhanced photosynthetic activity and lipid production compared to suspension cultures. Several other benefits of alginate-immobilization, as opposed to suspension cultures, of microalgae are reported in the literature, e.g. improved glycerol production by *Dunaliella tertiolecta* [21], increased capability for biotransformation of codein to morphine in *Spirulina platensis* [22], and increased resistance of chitosan-immobilized *Synechococcos* ssp. against NaOH toxicity [23]. Recently we have introduces an immobilization technique for microalgae, designated as “Green Bioprinting” [24]. Microalgae were immobilized in alginate-based hydrogel scaffolds by a 3D-plotting process which was first established for bioprinting of mammalian cells in the course of tissue engineering approaches and which allowed the design and fabrication of highly organized

For Peer Review

immobilization structures. We could demonstrate the applicability of the bioprinting technique for immobilization of microalgae e.g. by the measurement of oxygen evolution of hydrogel-immobilized cells. In addition, the option to generate cell-laden scaffolds in which microalgae can be co-cultivated with human cell lines was shown.

In the present study the growth and viability of two commonly used microalgae strains, *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230, within 3D-plotted alginate hydrogels were further analyzed, alongside suspension cultures, at different temperatures (26°C to 37°C) and illumination conditions (continuous illumination or 14/10 hour light/dark cycles). To assess the influence of these parameters on the physiological state of microalgae, analytical methods for determining viability were established for both suspension and immobilized cultures.

For Peer Review

2. Material and Methods

2.1 Microalgae – strains and preculture

Chlamydomonas reinhardtii 11.32b was obtained from the Culture Collection of Algae at Goettingen University (Göttingen, Germany). *Chlorella sorokiniana* UTEX1230 was purchased from the Culture Collection of Algae at the University of Texas (UTEX, Austin, USA).

C. reinhardtii 11.32b and *C. sorokiniana* UTEX1230 were inoculated from Tris-Acetate-Phosphate (TAP) agar plates into 20 mL liquid TAP medium in shake flask cultures (100 mL Schott Duran unbaffeld, Wertheim, Germany) according to [25]. Cultures were incubated for 48 hours in an illuminated incubator (Minitron, Infors, Bottmingen, Switzerland). The photoautotrophic culture conditions were adjusted to 26°C, 100 rpm and 20 µmol m^{-2} s^{-1} of a light emitting diode (warm white LED, Osram QOD panel, München, Germany).

2.2 Suspension cultures – influence of temperature

Above described pre-cultures of *C. reinhardtii* 11.32b or *C. sorokiniana* UTEX1230, respectively (26°C, 100 rpm, 20 µmol m^{-2} s^{-1}) served as inocula for microalgae cultures at 26°C, 30°C, and 37°C. The pre-culture was diluted in 20 mL TAP medium to an initial OD_{750nm} of 0.1 in 100 mL shaking flasks (Schott Duran, Wertheim, Germany). Suspension cultures were incubated at 100 rpm and 150 µmol m^{-2} s^{-1} at the respective temperatures for 144 hours. The illumination was adjusted to either continuous illumination or a 14/10 hour light/dark cycle. All cultivation experiments were conducted in duplicate. Light intensity was measured by a silicon photo diode (deka Sensor + Technologie, Teltow, Germany). At several time points, cultures were sampled to determine cell growth via optical density at 750 nm (helios β spectralphotometer, Thermo Scientific, Germany), and population viability by flow cytometry (see 2.3).

The growth rate µ [d^{-1}] was calculated by:

$$\mu = \frac{\ln(X - X_0)}{t - t_0} \quad (1)$$

For Peer Review

where X and X_0 represent the cell density after 144 hours and at the beginning of cultivation, respectively.

2.3 Determination of microalgae viability in suspension by flow cytometry

Cell broth from the shake flasks was diluted in 0.9% NaCl solution to a cell density of 1×10^6 - 1×10^7 cells per mL (OD_{750nm} ca. 0.01). Viability staining was performed at room temperature using bis-(1,3-dibarbituric acid)-trimethine oxonol ($DiBAC_4(3)$, Enzo Life Sciences Inc., Farmingdale, NY, USA), a probe that is sensitive to membrane potentials, at a working concentration of 2.5 µg mL^{-1} (stock solution of 5 mg mL^{-1} in dimethyl sulfoxide) in the dark. Samples were analyzed by a Cube8 flow cytometer (Partec GmbH, Münster, Germany). A 20 mW 488 nm solid-state laser was used to excite cells subjected to hydrodynamic focusing. $DiBAC_4(3)$ fluorescence was detected using a 590/50 band pass filter (FL2). The red autofluorescence of chlorophyll was used as trigger parameter and was detected using a 675/50 band pass filter (FL3). *C. reinhardtii* 11.32b cells were gated on a FSC-FL3 dotplot to reduce background signals in the FL2 channel.

To evaluate the sensitivity of the staining procedure, microalgae (*C. reinhardtii* 11.32b or *C. sorokiniana* UTEX1230) were diluted in 0.9% NaCl solution and temperature-treated at 50°C and 150 rpm in a lab-shaker for 180 minutes. Samples were collected at 15-minute intervals, stained with $DiBAC_4(3)$ as described above, and analyzed by flow cytometry to follow membrane depolarization.

2.4 Immobilization of microalgae in 3D-plotted hydrogels

The hydrogel plotting material was prepared by dissolving 30 mg mL^{-1} alginic acid sodium salt (Sigma-Aldrich, Taufkirchen, Germany) in water, then adding 90 mg mL^{-1} methylcellulose (Sigma-Aldrich; approx. MW = 88 kDa). After thorough stirring, the mixture was incubated for 2 hours at room temperature to allow swelling of the methylcellulose. The 3D-plotting process was carried out with a BioScaffolder 2.1 (GeSiM, Großerkmannsdorf, Germany) which was operated within a laminar flow box. Immediately prior to plotting, a suspension of either *C. reinhardtii* 11.32b or *C. sorokiniana* UTEX1230 was pelleted. The microalgae were resuspended in the plotting paste with a density of 2×10^6 cells per gram plotting paste which resulted in a final density of approximately 2×10^5 cells within a hydrogel scaffold. The microalgae-hydrogel-mixture was poured into a cartridge which was inserted into the dosing unit

For Peer Review

of the plotting device. Driven by compressed air, the mixture was dispensed through a dosing needle (inner diameter: 250 µm) with a pressure of 1.4 bar and a plotting speed of 10 mm s^{-1} into a 6-well plate. For the investigations described here, 4 layer scaffolds with a length in x- and y-direction of 15 mm and a strand distance of 1.87 mm were built in a 0°/90° configuration (Figure 1). The plotted scaffolds were cross-linked in a 100 mM $CaCl_2$ solution for 10 minutes and thereafter transferred into TAP medium for cultivation.

2.5 Cell number quantification of hydrogel-immobilized microalgae

Hydrogel scaffolds containing microalgae were dissolved in 0.9% NaCl solution containing 55 mM sodium citrate for 30 min at 37°C to recover the microalgae from the alginate matrix. Subsequently, microalgae were collected by centrifugation (15 min at 13,400 × g) and the pellet was subjected to chlorophyll quantification as described by [26]. In brief, after addition of DMSO to the pellets, the cells were lysed by sequential shock freezing in liquid nitrogen**,** and then homogenized using the Precellys® 24 system (Peqlab, Erlangen, Germany). The absorbance of the lysates was measured at 435 nm with a multifunction microplate reader (Infinite® M200 Pro, Tecan, Männedorf, Switzerland) and used to calculate the cell numbers of *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX 1230 according to equations 2 and 3, respectively:

$$y = 636{,}2x \quad (2)$$

$$y = 1674{,}6x \quad (3)$$

2.7 Determination of viability of hydrogel-immobilized microalgae

The algae-laden hydrogel constructs were incubated in 60 mL TAP under the same cultivation conditions described for suspension cultures above. The viability of immobilized algae was estimated by SYTOX Green dead cell staining at 48 hours and 144 hours as described by [27]. The hydrogels constructs were incubated in TAP medium containing 5 µM SYTOX Green (Molecular Probes, Eugene, OR, USA) for 15 min at room temperature in the dark. The total cell number was estimated by the red autofluorescence of chlorophyll. The samples were analyzed by confocal laser scanning microscopy (cLSM) using a Zeiss cLS microscope 510. The excitation was

For Peer Review

performed by an argon laser (SYTOX) at 488 nm (emission 525/10 nm) or DPSS laser (chlorophyll) at 561 nm (emission 605/15 nm).

The percentage of SYTOX Green-stained cells was determined by particle analysis using the open-source, image analysis tool Fiji (Madison, USA). The fluorescence images were converted to a binary file by applying the Li-algorithm. To identify microalgae cells, the following boundary conditions were used for particle analysis: circularity of 0.25 - 1.00 and area of 90 μm^2 to infinity. Particles that fulfilled these requirements were recorded by the particle analysis tool.

3. Results and discussion

3.1 Quantification of viability in microalgae suspension cultures using $DIBAC_3(4)$-staining and flow cytometry

We first investigated whether $DIBAC_4(3)$ staining was applicable for monitoring the viability of microalgae suspension cultures via membrane polarization status. *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 populations were heat-stressed by incubation at 50°C. $DIBAC_4(3)$-stained cells were detected using the FL2 fluorescence channel (590/50 nm). For both microalgae strains, two populations of cells, stained and unstained, could be detected in a FSC-FL2 dot plot (Figure 2B and 2C). The first quadrant (upper left corner) represent an unstained microalgae subpopulation with intact and polarized membrane; control samples of unstained cells were analyzed to validate this autofluorescence. Non-viable microalgae, which exhibit a dissipated membrane potential, revealed an enhanced green fluorescent signal caused by $DIBAC_4(3)$ staining. These subpopulations were separated within the second quadrant (Figure 2B, upper right corner).

During heat stress, the number of $DIBAC_4(3)$-stained cells increased with duration of time under heat exposure (Figure 2A and 2B). The initial viability, 94% of the *C. reinhardtii* 11.32b population, decreased to 16% within the first 45 minutes of heat treatment. After 60 minutes, almost 92% of cells exhibited a depolarized cell membrane, indicating low heat tolerance of this species.

In contrast, the initial viability of the *C. sorokiniana* UTEX1230 population remained at the constant high value of 97% during the first 45 minutes of heat treatment (Figure 2A). Thereafter, the number of depolarized cells increased slowly. Nevertheless, the

For Peer Review

C. sorokiniana UTEX1230 population revealed a viability of 22% even after 180 minutes of heat treatment.

The membrane potential in microbial cells is generated by the extrusion of protons and the electron transport chain. Due to its negative charge $DIBAC_4(3)$ can only enter cells with a dissipated membrane potential. $DIBAC_4(3)$ was used in several studies to determine the viability of yeast and bacterial populations [28,29]. It is generally accepted that depolarization of a cellular membrane is associated with loss of the cell's metabolic activity. In recent studies, $DIBAC_4(3)$ staining was used to indicate the toxicity of the herbicide paraquat in *Chlamydomonas moewussi* [30] and to stain phytoplankton strains [31]. However, $DIBAC_4(3)$ has not yet been used to monitor viability in microalgae populations during their cultivation.

$DIBAC_4(3)$ staining has demonstrated the capability to determine the physiological status of microalgae cultures with great sensitivity. Thus, membrane depolarization can be used as a viability indicator for microalgae suspension cultures.

3.2. Effects of temperature and illumination on growth and viability of microalgae suspension cultures

Viability of microalgae cultures is important for the productivity of their processes. However, there has been no focus on viability during photoautotrophic microalgae cultivation at the single cell level so far. We investigated the influences of temperature and illumination conditions on population viability during photoautotrophic cultivation of the two most commonly studied microalgae strains *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 using flow cytometry. Shake flask cultures of these microalgae strains were incubated at 26°C, 30°C or 37°C, and subjected to either continuous or 14/10 hours light/dark (L/D) cycle illumination to identify effects on growth rate and population viability.

At 26°C with continuous illumination, the *C. reinhardtii* 11.32b culture exhibited a lag-phase of ca. 20 hours before switching to a linear increase of biomass (Figure 3A, left). The cultures attained a growth rate of $1.15 \pm 0.02\ d^{-1}$ under continuous illumination, but decreased to $0.76 \pm 0.01\ d^{-1}$ when subjected to the 14/10 hours L/D cycle. In general, the initial viability of the *C. reinhardtii* 11.32b populations was between 80% and 90%. At 26°C the viability decreased only slightly over 144 hours of cultivation, independent of the change in illumination conditions (Figure 3A, left).

For Peer Review

Compared to *C. reinhardtii* 11.32b, *C. sorokiniana* UTEX1230 attained a higher growth rate of 1.19 ± 0.03 d^{-1} at 26°C and continuous illumination (Figure 3A, right). The growth rate also decreased to 0.61 ± 0.03 d^{-1} with a change in illumination to the 14/10 hours L/D cycle. The initial viability of *C. sorokiniana* UTEX1230 was between 85% and 97%. At 26°C**,** the high viability was maintained over 144 hours**,** independent of the adjusted illumination conditions (Figure 3A, right).

At an increased incubation temperature of 30°C under continuous illumination (Figure 3, left), the growth rate of *C. reinhardtii* 11.32b decreased to 0.48 ± 0.03 d^{-1}. Only a slight difference in growth rate (μ = 0.44 ± 0.07 d^{-1}) was observed after subjecting the culture to 14/10 hours L/D cycle (Figure 3B, left). More significant differences in population viability were found. For both illumination conditions the viability remained at constant high values of 90% for 45 hours of cultivation. Then, the populations differed according to the amount of illumination provided. After 70 hours of continuous illumination, the viability of *C. reinhardtii* 11.32b fell to only 58 ± 9%, and then to less than 20% after 144 hours. However the viability of the microalgae population subjected to 14/10 hours L/D cycles remained constant at high values of more than 80% for the entire cultivation time.

At 30°C with continuous illumination, the cell density and growth rate of *C. sorokiniana* UTEX1230 (Figure 3B, right) were 55% and 62% higher than those of *C. reinhardtii* 11.32b, respectively. As seen with *C. reinhardtii* 11.32b, the growth rates of microalgae *C. sorokiniana* UTEX1230 at 30°C were comparable under both illumination conditions. Moreover, depending on the illumination conditions, the viability of *C. sorokiniana* UTEX1230 was analogous to that of *C. reinhardtii* 11.32b. The viability of the culture subjected to continuous illumination started to decrease after 60 hours (Figure 3B, right), and finally fell to 40% after 144 hours. The culture subjected to 14/10 hours L/D cycles maintained a high viability of more than 90% over 144 hours of cultivation.

The biomass formation of *C. reinhardtii* 11.32b at 37°C was nearly identical for both illumination conditions (Figure 3C, left). The growth rate at continuous illumination, 0.41 ± 0.03 d^{-1} (Figure 3C, left), was considerably less than it was at 30°C. Viability was again dependent on the illumination conditions provided. Under continuous illumination, the viability decreased linearly, from the beginning of the incubation period**,** to a value of only 5.2 ± 4.8 % after 144 hours. In contrast, the cultures

For Peer Review

subjected to 14/10 hours L/D cycles still maintained a high viability of more than 90% for the entire cultivation time.

At 37°C, the *C. sorokiniana* UTEX1230 cultures (Figure 3C, right) achieved growth rates of 0.81 ± 0.04 d^{-1} under continuous illumination, and 0.37 ± 0.01 d^{-1} under 14/10 hours L/D cycles. Also at 37°C, the viability of the cultures subjected to continuous illumination began decreasing after 70 hours, with a final value of 7.1 ± 0.25 % after 144 hours; cultures subjected to 14/10 hours L/D cycles maintained constant, high viability for the entire duration.

Figures 4A and 4B show the fraction of cells with an intact membrane potential as indicated by $DIBAC_4(3)$ staining after completed cultivations. Under continuous illumination, the number of *C. reinhardtii* 11.32b cells exhibiting an intact membrane potential decreased with increasing temperature (Figure 4A, black bars). A moderate temperature of 26°C and continuous illumination produced the highest number of viable *C. reinhardtii* 11.32b cells. Only 67% of this cell number was obtained from cultures subjected to 14/10 hours L/D cycles at this temperature (Figure 4A, white bar).

The 14/10 hour L/D cycle at temperatures of 30°C and 37°C produced an interesting observation (Figure 4A, white bars). Under these conditions, *C. reinhardtii* 11.32b cultures exhibit high viability, as described above, thus the number of cells with intact membrane potentials was much higher than that of cultures subjected to continuous illumination (Figure 4A, black bars). *C. sorokiniana* UTEX1230 cultures attained significantly higher cell densities than *C. reinhardtii* 11.32b cultures (Figure 4B, black bars). However, we observed the same trend that resulted from the viability caused by different temperatures and illumination conditions (Figure 4B, white bars).

Temperature and light conditions are the most important elements of microalgae cultivation. Several authors have shown that temperature can significantly influence growth rate [32], carbohydrate and lipid production [33, 34] and the induction of pigment formation [35]. In these studies, temperatures ranging from 10°C to 38°C were considered optimal based on the desired product. The optimal temperatures differ for *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230, the species used in this study. While photoautotrophic growth occurred best between 25°C to 28°C for *C.*

For Peer Review

reinhardtii [36], Ugwu et al. [37] reported high photosynthetic activity of *C. sorokiniana* at temperatures up to 38°C.

The illumination intensity and adjustment of L/D cycles are other important factors because the absorption of photosynthetically usable photons and subsequent electron transport via the eukaryotic photosystems provides the energy for adenosine triphosphate synthesis. In recent studies**,** reduced rates of growth, carbon dioxide fixation, and nutrient uptake could be observed by providing L/D cycles instead of continuous illumination. For example, Tamburic et al. [38] reported reduced growth of *C. reinhardtii* using a 12/12 hours L/D cycle. In *Chlorella vulgaris* an increase in the rates of phosphorus and carbon dioxide with increasing illumination intensities and length of photoperiods was observed [39].

These results are consistent with our findings. Adjustments from continuous illumination to 14/10 hours L/D cycles led to reduced biomass formations and growth rates of *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 caused by decreased photosynthetic activity during the dark periods. The convergence of growth rates for both microalgae strains at 30°C and 37°C is a consequence of the very different population structures resulting from continuous and L/D cycle illumination. The number of depolarized cells increased with time under continuous illumination, whereas cultures under the L/D cycle maintained a very homogeneous population of non-depolarized cells. Thus, the percentage of metabolically active and replication-competent cells was much higher in cultures subjected to L/D cycles.

The high degree of membrane depolarization at continuous illumination might be a result of photoinhibitory mechanisms. Basically, the molecular mechanisms can be classified into donor-side or acceptor-side photoinhibition [40]. Keren and Krieger-Liszkay [41] give an overview on potential mechanisms caused by intense illumination intensity. The authors highlight the effect of excess light on the D1 protein of photosystem II (PSII), the main target of photoinhibition. The loss of D1 activity causes reduced electron transport across the thylakoid membranes and decreased ATP formation. Because most PSII proteins have a very high turn-over rate, it is possible to regenerate the PSII components during dark periods. In cases where the rate of destruction is higher than that of regeneration, the cell loses its metabolic activity.

For Peer Review

3.3. Effects of temperature and light/dark cycle illumination on growth and viability of immobilized microalgae cultures

The immobilization of microalgae in 3D-plotted hydrogels represents a technique for creating highly-organized structures containing embedded microorganisms (see Figure 1). Growth of embedded microalgae was investigated by immobilizing *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 in 3D-plotted hydrogel constructs, then culturing these under the same temperature and illumination conditions as described for suspension cultures. The chlorophyll content was quantified after 48 hours and 144 hours to calculate the cell number and growth rate of embedded microalgae. The viability of hydrogel-embedded microalgae populations was determined using an automated fluorescence image analysis procedure.

Growth rates of hydrogel-immobilized *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 cultured at 26°C, 30°C and 37°C under continuous illumination or 14/10 hours L/D cycles are illustrated in Figures 5A and 5B (red bars). The growth rates of hydrogel-immobilized *C. reinhardtii* 11.32b (Figure 5A, red bars) were stable between 0.4 d^{-1} and 0.6 d^{-1} at all temperatures and under both illumination conditions. In comparison, the growth rates of suspension cultures (Figure 5A, black bars) decreased with increased temperature at continuous illumination. At 26°C and continuous illumination, the growth rate of embedded *C. reinhardtii* 11.32b was 50% lower than in suspension cultures. The difference was only 21% using 14/10 hours L/D cycles. At an incubation temperature of 30°C, the growth rates of hydrogel-immobilized *C. reinhardtii* 11.32b cultures nearly equaled those obtained for suspension cultures at both illumination conditions. Indeed, hydrogel-embedded cultures attained a 44% higher growth rate at 37°C under 14/10 hours L/D cycle illumination compared to suspension cultures.

The growth rates were almost identical, between 0.4 d^{-1} and 0.7 d^{-1}, for hydrogel-embedded *C. sorokiniana* UTEX1230 cultures (Figure 5B, red bars). It is striking that there were no significant differences between the growth rates of hydrogel-embedded cultures subjected to continuous and L/D cycle illumination, whereas suspension cultures cultivated under continuous illumination always attained a higher growth rate than those subjected to L/D cycles.

For Peer Review

This observation was made at all temperatures studied. Identical to *C. reinhardtii* 11.32b, immobilized *C. sorokiniana* UTEX1230 demonstrated a 34% higher growth rate at 37°C under 14/10 hours L/D cycle illumination compared to the corresponding suspension culture.

The quantification of viability for embedded microalgae was done by fluorescence image analysis. The geometry and translucent properties of the 3D-plotted hydrogel constructs enabled fluorescence microscopy analysis. The strong red autofluorescence signal of chlorophyll was used to count the total number of microalgae within the hydrogel according to the predefined exclusion criteria (Figure 6). Afterwards, the green fluorescence channel was used to determine the number of SYTOX-stained dead cells to calculate the viability of the embedded culture. Table 1 compares the viability of suspension and hydrogel-embedded cultures of *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 after 144 hours at the temperature and illumination conditions studied.

Two interesting results were obtained from analyses of viability in hydrogel-embedded microalgae. First, the viability of *C. reinhardtii* 11.32b remained at a constant high value between 80% and 90%, independent on the temperature and illumination conditions. Second, the viability of *C. sorokiniana* UTEX1230 did not improve under continuous illumination with hydrogel-immobilization, as observed for *C. reinhardtii* 11.32b. Immobilized cultures of *C. sorokiniana* UTEX1230 reached between 80% - 90% viability only at 26°C under both illumination conditions. In comparison to suspension cultures under continuous illumination, the viability of immobilized cultures decreased to a lesser extent, but reached only 40% at 30°C and 30% at 37°C after 144 hours cultivation time. By providing the illumination in L/D cycles, the viability of *C. sorokiniana* UTEX1230 remained at 75% - 80% at temperatures of 26°C, 30°C, and 37°C.

Due to its high transparency and easy handling, alginate is the preferred immobilization polymer for microalgae [18]. This conventional immobilization technique has limited opportunities in structural organization and is commonly carried out using Ca-alginate beads. In contrast, 3D-bioprinting approaches offer the opportunity to create highly-organized immobilization structures. This aspect can be very useful in terms of light and nutrient supply because it is possible to achieve

For Peer Review

higher surface-to-volume ratios by organizing the immobilization material in a predefined structure.

Because the 3D-bioprinting immobilization process may affect the physiological state of the cells, we aimed to analyze and compare microalgae growth in immobilized and suspension cultures at varying temperatures and illumination conditions. The photoinhibitory effects observed in microalgae suspension cultures under continuous illumination was diminished by embedding the cells in alginate hydrogels. The illumination intensity was thus attenuated by the translucent properties of the alginate. On the one hand, this effect protects the cells from intense illumination, but on the other hand, the light attenuation can cause limited light conditions within the immobilization structures. This might be a reason for the stable growth rates that were attained for hydrogel-immobilized cultures. A comparable effect was described by Pane et al. [42] who also observed reduced growth rates of *Tetraselmis suecica* cultures entrapped in Ca-alginate beads.

Up until now, viability in immobilized microalgae cultures was estimated using indirect parameters such as oxygen evolution, nutrient uptake rates or qualitative observations. For instance, Fierro et al. [43] assessed the viability of different *Scenedesmu*s strains after the immobilization in chitosan beads to be 46% - 76% by microscopic observations. Santos-Rosa et al. [44] used ammonium-photoproduction capacity and oxygen production as indirect indicators of viability in *Chlamydomonas reinhardtii*.

More accurate quantification of microalgae viability on a single cell scale is possible due to the structural advantages of 3D-plotted hydrogels, compared to beads. Fluorescence image analysis of embedded microalgae in this study revealed facilitated viability up to 90%. *C. reinhardtii* 11.32b attained a higher viability than *C. sorokiniana* UTEX1230 after 144 hours of cultivation within the hydrogels, which could be a consequence of limited light conditions due to the increased growth rates and cell number of *C. sorokiniana* UTEX1230.

For Peer Review

4. Concluding remarks

For biotechnological processes it is essential to have a high number of catalytically active units, i.e. metabolically active cells. Due to heterogeneous growth conditions, microbial populations always consist of subpopulations with different properties. This is exclusively true for microalgae since the most important substrate, light, is generally provided by inhomogeneous external illumination sources. In addition, the self-shading of cells across the reactor radius enhances the formation of light distributions. We investigated the viability of two microalgae strains, *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230, in suspension and hydrogel-embedded cultures subjected to various cultivation temperatures and modes of illumination. Moreover, this study provides methods for quantifying the viability of suspension and hydrogel-immobilized microalgae.

Flow cytometry is a fast, easily-wielded analytical tool for identifying the subpopulation structure of microalgae suspension cultures. By monitoring the physiological state of microalgae populations during photoautotrophic cultivation, we demonstrated that, despite a higher cell density at continuous illumination, the number of viable cells was significantly higher after applying 14/10 hours light/dark cycle illumination. As a consequence, it was possible to maintain the viability of microalgae suspension cultures over a wide temperature range (26°C to 37°C) even at non-ideal temperatures for *C. reinhardtii* 11.32b. Our findings that the identification of heterogeneous microalgae populations depend on growth conditions, and that light/dark cycle illumination can beneficially affect population homogeneity and viability, are important facts for cultivating microalgae.

Microalgae were embedded within a hydrogel in a highly structural organization by a 3D-bioprinting approach. Because single cell analysis is not applicable to immobilized cultures, a Fiji-based fluorescence image analysis procedure was established to quantify the viability of hydrogel-immobilized microalgae using chlorophyll autofluorescence and a membrane-permeable fluorescent dye. In comparison to suspension cultures, immobilized microalgae attained very stable growth rates over a wide temperature range and independent of the illumination conditions. At 37°C, higher growth rates of hydrogel-immobilized *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 cultures than those of suspension cultures were obtained using a 14/10 hours light/dark cycle. This immobilization technique provides a tool for cultivating

For Peer Review

microalgae in highly-organized, predefined structures even under non-optimal temperatures.

Acknowledgements

The authors gratefully acknowledge financial support from the Free State of Saxony (Project ID: 4-7531.60/29/16). Moreover we would like to thank Ms. Mandy Quade for performing microscopic analyses.

The authors have declared no conflict of interest.

For Peer Review

5. References

[1] Spolaore, P., Joannis-Cassan, C., Duran, E. and Isambert, A., Commercial applications of microalgae. *J. Biosci. Bioeng*. 2006, *101*, 87-96.

[2] Weber, J., Krujatz, F., Hilpmann, G., Grützner, S. et al., Biotechnological hydrogen production by photosynthesis. *Eng. Life Sci*. 2014, *0*, 1-15.

[3] Zhu, L.D., Hiltunen, E., Antila, E., Zhong, J.J. et al., Microalgal biofuels: Flexible bioenergies for sustainable development. Renew. *Sust. Energ. Rev*. 2014, *30*, 1035-1046.

[4] Perales-Vela, H.V., Peña-Castro, J.M. and Cañizares-Villanueva, R.O., Heavy metal detoxification in eukaryotic microalgae. *Chemosphere*. 2006, *64*, 1-10.

[5] Morita, M., Watanabe, Y. and Saiki, H., High photosynthetic productivity of green microalga *Chlorella sorokiniana*. *Appl. Biochem. Biotechnol*. 2000, *87*, 203-218.

[6] Posten, C., Design principles of photo-bioreactors for cultivation of microalgae. *Eng. Life Sci*. 2009, *9*, 165-177.

[7] Müller, S., Harms, H. and Bley, T., Origin and analysis of microbial population heterogeneity in bioprocesses. *Curr. Opin. Biotech*. 2010, *21*, 100-113.

[8] Gallo-Villanueva, R., Jesús-Pérez, N., Martínez-López, J., Pacheco, A. et al., Assessment of microalgae viability employing insulator-based dielectrophoresis. *Microfluid. Nanofluid*. 2011, *10*, 1305-1315.

[9] Vavilin, D.V., Ducruet, J.-M., Matorin, D.N., Venediktov, P.S. et al., Membrane lipid peroxidation, cell viability and Photosystem II activity in the green alga *Chlorella pyrenoidosa* subjected to various stress conditions. *J. Photoch. Photobio. B*. 1998, *42*, 233-239.

[10] Hyka, P., Lickova, S., Přibyl, P., Melzoch, K. et al., Flow cytometry for the development of biotechnological processes with microalgae. *Biotechnol. Adv*. 2013, *31*, 2-16.

[11] Jamers, A., Lenjou, M., Deraedt, P., Bockstaele, D.V. et al., Flow cytometric analysis of the cadmium-exposed green alga *Chlamydomonas reinhardtii* (Chlorophyceae). *Eur. J. Phycol*. 2009, *44*, 541-550.

For Peer Review

[12] Prado, R., Rioboo, C., Herrero, C. and Cid, Á., Screening acute cytotoxicity biomarkers using a microalga as test organism. *Ecotox. Environ. Safe*. 2012, *86*, 219-226.

[13] Stauber, J.L., Franklin, N.M. and Adams, M.S., Applications of flow cytometry to ecotoxicity testing using microalgae. *Trends Biotechnol*. 2002, *20*, 141-143.

[14] Imase, M., Ohko, Y., Takeuchi, M. and Hanada, S., Estimating the viability of Chlorella exposed to oxidative stresses based around photocatalysis. *Int. Biodeter. Biodegr*. 2013, *78*, 1-6.

[15] Michels, M.A., van der Goot, A., Norsker, N.-H., Wijffels, R., Effects of shear stress on the microalgae *Chaetoceros muelleri*. *Bioprocess Biosyst. Eng*. 2010, *33*, 921-927.

[16] Dorsey, J., Yentsch, C.M., Mayo, S. and McKenna, C., Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. *Cytometry*. 1989, *10*, 622-628.

[17] Lam, M.K. and Lee K.T., Immobilization as a feasible method to simplify the separation of microalgae from water for biodiesel production. *Chem. Eng. J*. 2012, *191*, 263-268.

[18] Moreno-Garrido, I., Microalgae immobilization: Current techniques and uses. *Bioresource Technol*. 2008, *99*, 3949-3964.

[19] Thakur, A. and Kumar, H.D., Use of natural polymers as immobilizing agents and effects on the growth of *Dunaliella salina* and its glycerol production. *Acta Biotechnol*. 1999, *19*, 37-44.

[20] Sing, Y., Photosynthetic activity, and lipid and hydrocarbon production by alginate-immobilized cells of *Botryococcus* in relation to growth phase. *J. Microbiol. Biotechn*. 2003, *13*, 687-691.

[21] Grizeau, D., Navarro, J.M., Glycerol production by *Dunaliella tertiolecta* immobilized within Ca-alginate beads. *Biotechnol. Lett*. 1986, *8*, 261-264.

[22] Ramachandra Rao, S., Tripathi, U. and Ravishankar, G.A., Biotransformation of codeine to morphine in freely suspended cells and immobilized cultures of *Spirulina platensis*. *World J. Microbiol. Biotechnol*. 1999, *15*, 465-469.

For Peer Review

[23] Aguilar-May, B., del Pilar Sánchez-Saavedra, M., Lizardi, J. and Voltolina, D., Growth of *Synechococcus* sp. immobilized in chitosan with different times of contact with NaOH. *J. Appl. Phycol*. 2007, *19*, 181-183.

[24] Lode, A., Krujatz, F., Brüggemeier, S., Schütz, K. et al., Green Bioprinting: fabrication of photosynthetic algae-laden hydrogel scaffolds for biotechnological and medical applications.
submitted to Eng. Life Sci. (special issue: Biomaterials Made in Bioreactors)

[25] Harris, E. H., The *Chlamydomonas* sourcebook: a comprehensive guide to biology and laboratory use. San Diego, CA: Academic Press, 1989.

[26] Hopfner, U., Schenck, T.-L., Chávez, M.-N., Machens, H.-G. et al., Development of photosynthetic biomaterials for in vitro tissue engineering. *Acta Biomater*. 2014, *10*, 2712-2717.

[27] Sato, M., Murata, Y., Mizusawa, M., Iwahashi, H. and Oka, S., A simple and rapid dualfluorescence viability assay for microalgae. *Microbiol. Cult. Coll.* 2004, *20,* 53–59.

[28] Boyd, A.R., Gunasekera, T.S., Attfield, P.V., Simic, K. et al., A flow-cytometric method for determination of yeast viability and cell number in a brewery. *FEMS Yeast Res*. 2003, *3*, 11-16.

[29] Papadimitriou, K., Pratsinis, H., Nebe-von-Caron, G., Kletsas, D. et al., Rapid assessment of the physiological status of *Streptococcus macedonicus* by flow cytometry and fluorescence probes. *Int. J. Food Microbiol*. 2006, *111*, 197-205.

[30] Prado, R., Rioboo, C., Herrero, C., Suárez-Bregua, P. et al., Flow cytometric analysis to evaluate physiological alterations in herbicide-exposed *Chlamydomonas moewusii* cells. *Ecotoxicology*. 2012, *21*, 409-420.

[31] Peperzak, L. and Brussaard, C.P.D. Flow cytometric applicability of fluorescent vitality probes on phytoplankton. *J. Phycol*. 2011, *47*, 692-702.

[32] Xin, L., Hong-ying, H. and Yu-ping, Z.m Growth and lipid accumulation properties of a freshwater microalga *Scenedesmus* sp. under different cultivation temperature. *Bioresource Technol*. 2011, *102*, 3098-3102.

[33] Converti,A., Casazza, A.A., Ortiz, E.Y., Perego, P. et al., Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis*

For Peer Review

oculata and *Chlorella vulgaris* for biodiesel production. *Chem. Eng. Process*. 2009, *48*, 1146-1151.

[34] Venkata Subhash, G., Rohit, M.V., Devi, M.P., Swamy, Y.V. et al., Temperature induced stress influence on biodiesel productivity during mixotrophic microalgae cultivation with wastewater. *Bioresource Technol*. 2014, *169*, 789-793.

[35] Markou, G. and Nerantzis, E., Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. *Biotechnol. Adv*. 2013, *31*, 1532-1542.

[36] Fadaghi,H., Jayasundera, K., Krishnan, D. and Liu, X., The Effect of Temperature on Cell Density of Wild Type and Mutant *Chlamydomonas reinhardtii*. *The Expedition*. 2012, *1*.

[37] Ugwu, C.U., Aoyagi, H. and Uchiyama, H., Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. *Photosynthetica*. 2007, *45*, 309-311.

[38] Tamburic, B., Zemichael, F.W., Maitland, G.C. and Hellgardt, K., Effect of the Light Regime and Phototrophic Conditions on Growth of the H_2-producing Green Alga *Chlamydomonas reinhardtii*. *Energy Procedia*. 2012, *29*, 710-719.

[39] Gonçalves, A.L., Simões, M. and Pires, J.C.M., The effect of light supply on microalgal growth, CO_2 uptake and nutrient removal from wastewater. *Energ. Convers. Manage*. 2014, *85*, 530-536.

[40] Han, B.-P., Virtanen, M., Koponen, J. and Straškraba, M., Effect of photoinhibition on algal photosynthesis: a dynamic model. *J. Plankton Res*. 2000, *22*, 865-885.

[41] Keren, N. and Krieger-Liszkay, A., Photoinhibition: molecular mechanisms and physiological significance. *Physiol. Plantarum*. 2011, *142*, 1-5.

[42] Pane, L., Feletti, M., Bertino, C. and Carli, A., Viability of the marine microalga *Tetraselmis suecica* grown free and immobilized in alginate beads. *Aquacult. Int*. 1998, *6*, 411-420.

[43] Fierro, S., del Pilar Sánchez-Saavedra, M. and Copalcúa, C., Nitrate and phosphate removal by chitosan immobilized *Scenedesmus*. *Bioresource Technol*. 2008, *99*, 1274-1279.

For Peer Review

[44] Santos-Rosa, F., Galván, F. and Vega, J.M., Biological viability of *Chlamydomonas reinhardtii* cells entrapped in alginate beads for ammonium photoproduction. *J. Biotechnol*. 1989, *9*, 209-219.

For Peer Review

Table 1: Viability of microalgae cultured for 144 hours in suspension and embedded in hydrogels by 3D-bioprinting under varying temperature (26°C, 30°C and 37°C) and illumination conditions (150 µmol m^{-2} s^{-1} continuous illumination or light/dark cycles of 14/10 hours).

	Chlamydomonas reinhardtii 11.32b		*Chlorella sorokiniana* UTEX1230	
Light/Dark cycle 26°C	**24/0***	**14/10**	**24/0**	**14/10**
Viability suspension [%]	63.6 ± 12.8	71.3 ± 7.1	98.3 ± 1.0	96.3 ± 2.3
Viability immobilized [%]	≈ 90 %	≈ 85 %	≈ 90 %	≈ 80 %
Light/Dark cycle 30°C	**24/0**	**14/10**	**24/0**	**14/10**
Viability suspension [%]	14.6 ± 14.0	86.0 ± 2.2	40.6 ± 4.7	94.5 ± 3.1
Viability immobilized [%]	≈ 80 %	≈ 80 %	≈ 40 %	≈ 75 %
Light/Dark cycle 37°C	**24/0**	**14/10**	**24/0**	**14/10**
Viability suspension [%]	5.2 ± 4.8	93.0 ± 0.24	7.1 ± 0.25	99.4 ± 0.06
Viability immobilized [%]	≈ 80 %	≈ 85 %	≈ 30 %	≈ 75 %

*Light/Dark cycles of 24/0 hours represent continuous illumination.

For Peer Review

Figures

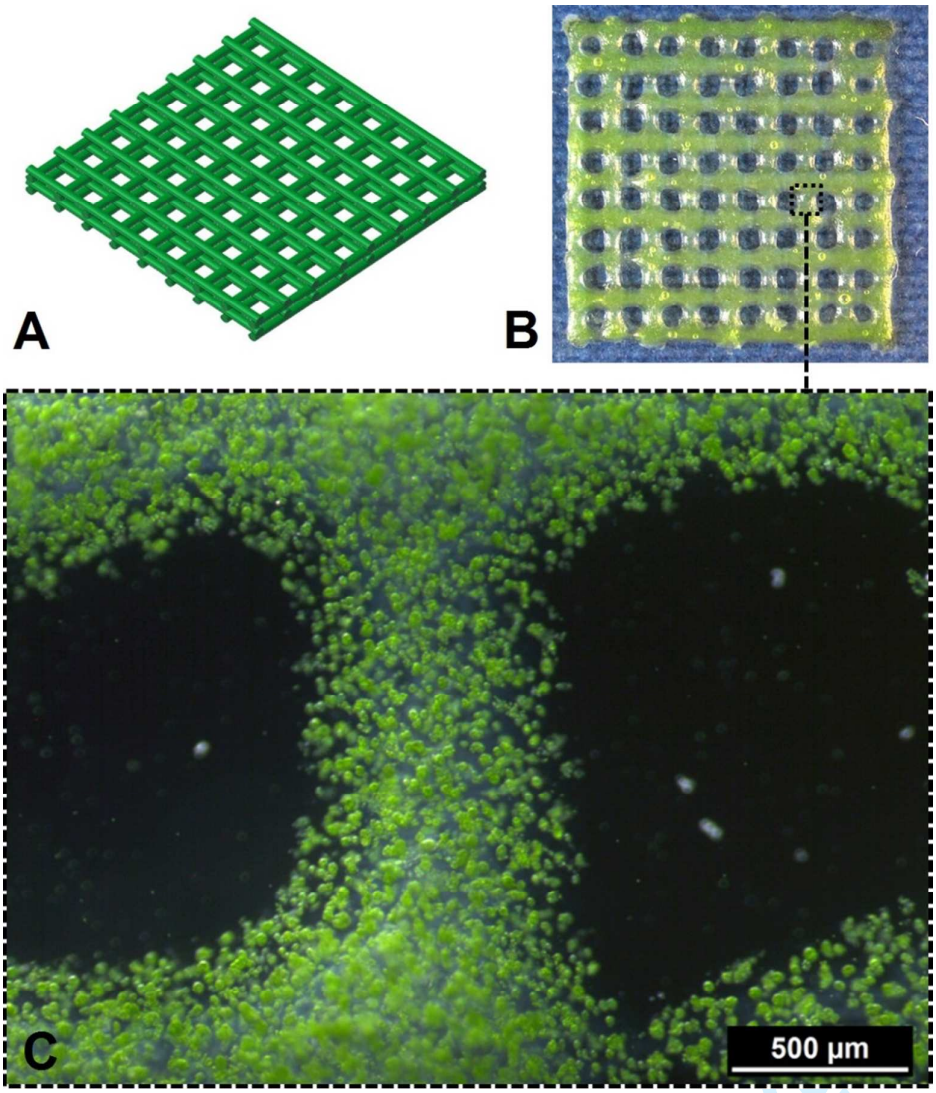

Figure 1: (A) Hydrogel CAD-model, (B) manufactured scaffold by 3D-bioprinting. (C) *C. reinhardtii* 11.32b cells were mixed into the plotting paste at 2×10^5 cells per mL and cultured in TAP medium at 100 rpm, 150 µmol m^{-2} s^{-1} (LED light) for 144 hours.

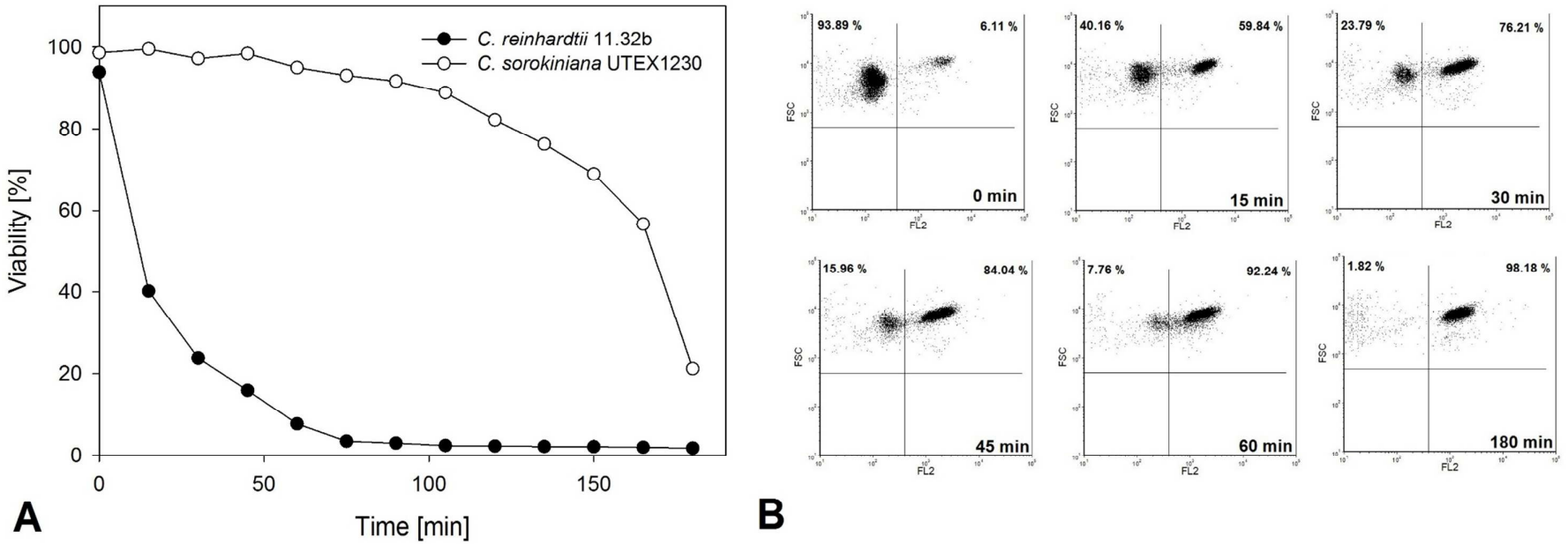

Figure 2: (A) Flow cytometry analysis of the cell membrane depolarization process in 50°C heat-stressed *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 (staining conditions: 2.5 µg mL-^{1} $DiBAC_4(3)$, 5 minutes in the dark). (B) Exemplary course of 50°C heat-stressed subpopulation structures of *C. reinhardtii* 11.32b at several time points; unstained viable cells (quadrant 1, upper left corner) and $DiBAC_4(3)$-stained dead cells (quadrant 2, upper right corner).

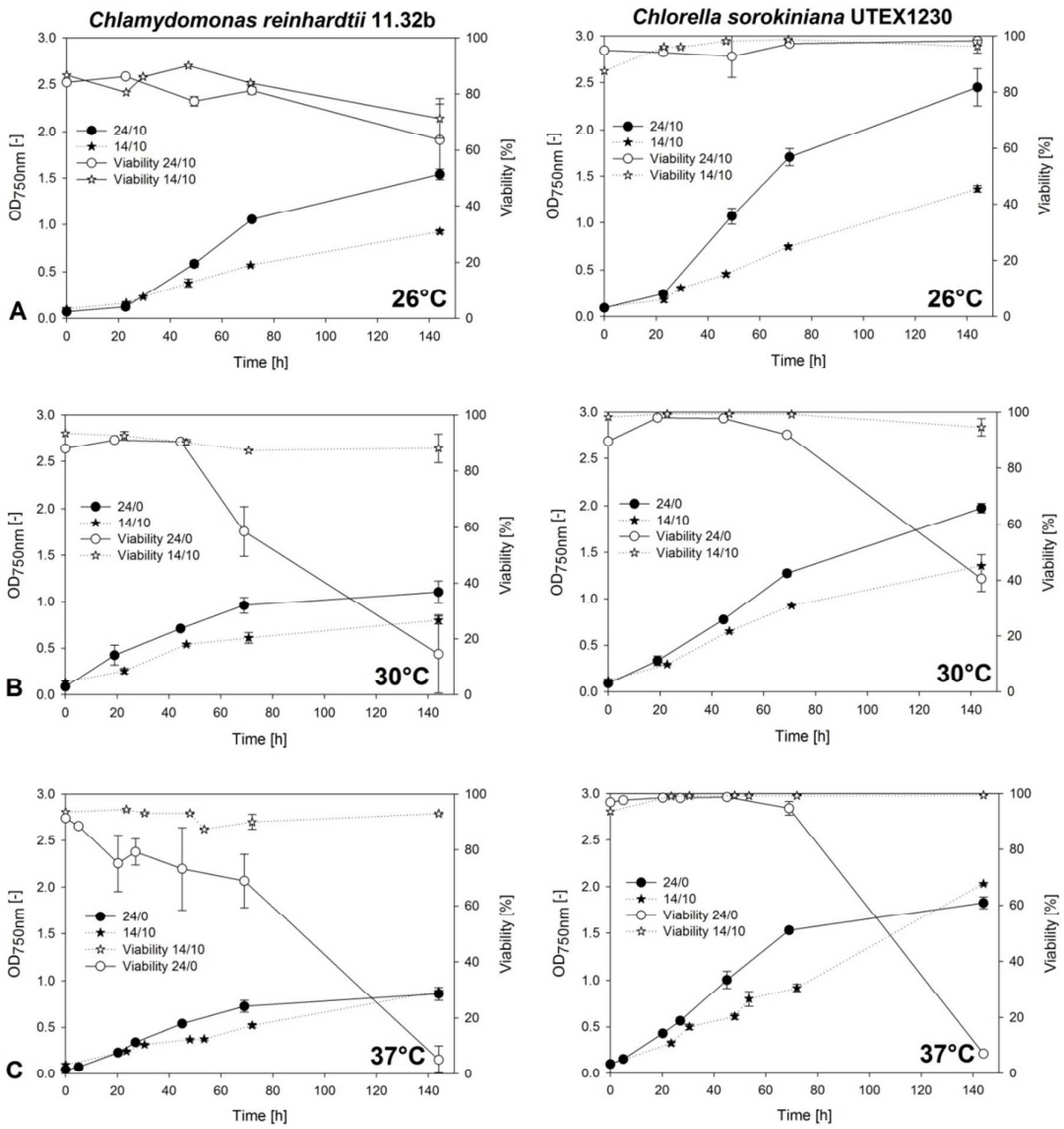

Figure 3: *C. reinhardtii* 11.32b and *C. sorokiniana* UTEX1230 were cultivated in suspension (TAP medium, 100 rpm, 150 µmol m^{-2} s^{-1}) under varying durations of illumination (24/0 represents continuous illumination, 14/10 represents light/dark cycles of 14/10 hours, starting with light period) and temperature conditions: (A) 26°C, (B) 30°C and (C) 37°C; error bars represent standard deviation. All experiments were conducted in duplicate.

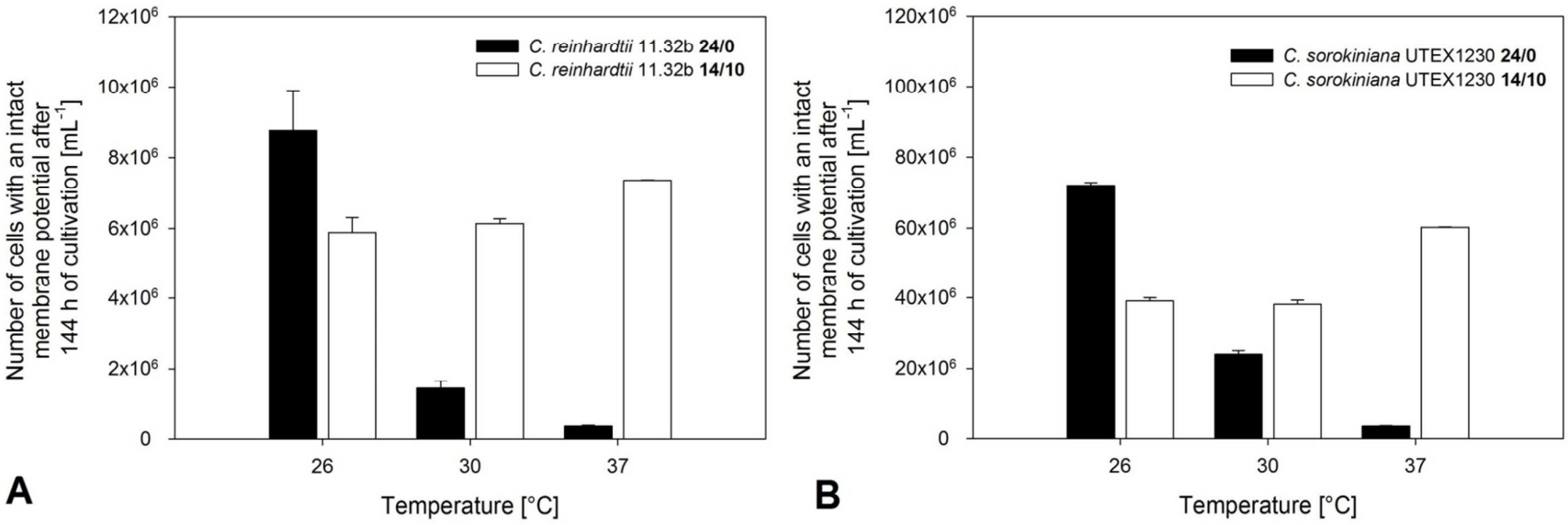

Figure 4: Number of microalgae with intact membrane potential after 144 hours cultivation under varying temperature (26°C, 30°C and 37°C) and illumination conditions (24/0 represent continuous illumination, 14/10 represent light/dark cycles of 14/10 hours). (A) *C. reinhardtii* 11.32b. (B) *C. sorokiniana* UTEX1230. Note the y-axis scaling is tenfold higher for *C. sorokiniana* UTEX1230.

Review

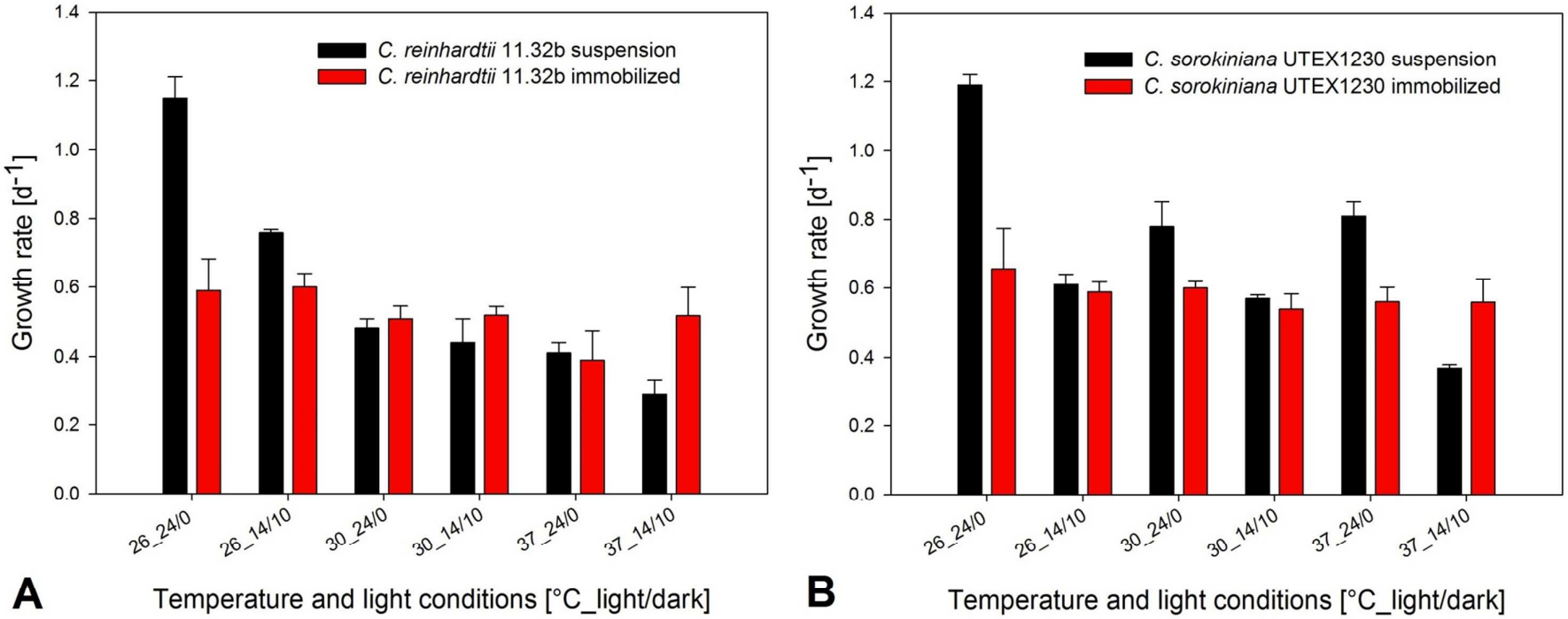

Figure 5: Growth rates of microalgae in suspension (black bar) and hydrogel-embedded (red bar) cultures under varying temperature (26°C, 30°C and 37°C) and illumination conditions (continuous illumination: 24/0 hours and light/dark cycles of 14/10 hours), Error bars represent standard deviation (n=2). (A) *C. reinhardtii* 11.32b. (B) *C. sorokiniana* UTEX1230.

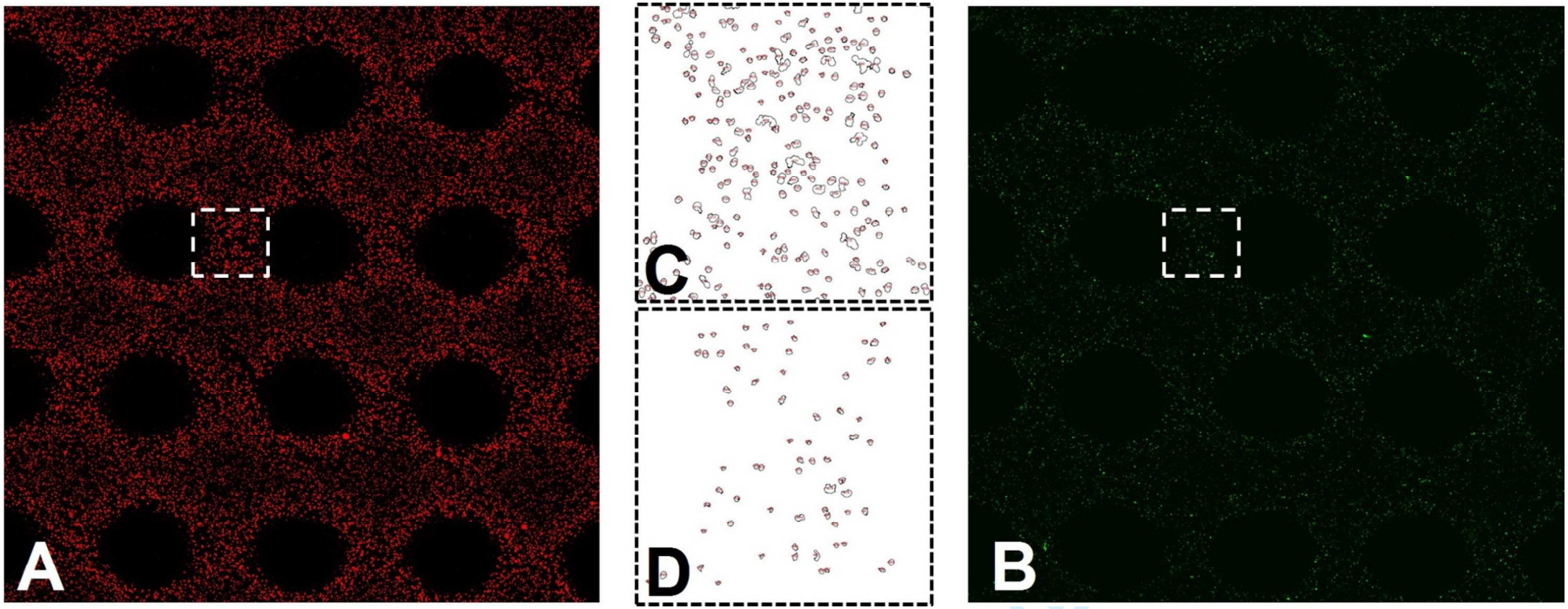

Figure 6: Fiji-based viability image analysis of hydrogel-embedded microalgae. (A) Red fluorescence channel (605/15 nm, chlorophyll). (B) Green fluorescence channel (525/10 nm, SYTOX-stained dead cells). Magnified section of red (C) and green (D) fluorescence, which were analyzed by a Fiji particle-analyzing module (parameter: circularity 0.25 – 1; size 90 µm - infinity).