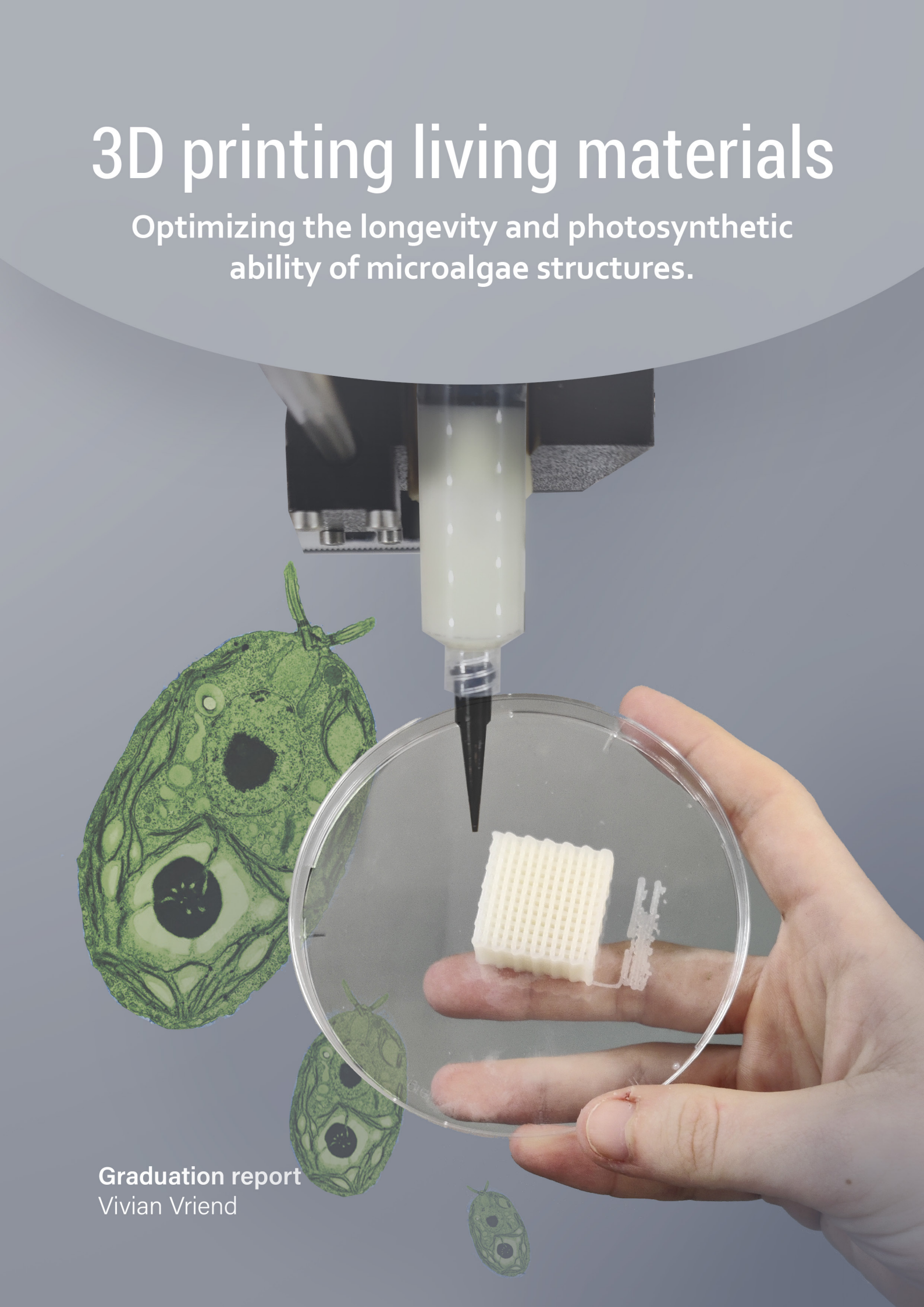
3D printing living materials

Optimizing the longevity and photosynthetic ability of microalgae structures.



Graduation report

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Summary

This graduation report aimed to improve the mechanical properties of a sustainable 3D print material made of microalgae, in order to create an macro-porous 3D structure with enhanced longevity and photosynthetic activity. This research builds upon the alginate based bio-ink from Balasubramanian et al (2021). Their material proposal lacked structural stability and print resolution, which made geometries of more than six layers impossible. Moreover, the bioprints were confined to a nutrient plate. These challenges formed the motivation for the research.

The goal for the optimized bioprinting protocol was to make use of the advantages of additive manufacturing, by creating a macro-porous shape with increased surface ratio. The hypothesis was that the microalgae would have more access to light, air and nutrients which would increase oxygen release and carbon dioxide fixation.

In a series of explorative studies, in close collaboration with nanobiologists, biomaterial designers and 3D printing experts, a direct ink writing approach was selected to construct the microalgae bioprints. Additional components were added to the alginate bio-ink formulation, which concluded in a material with improved print fidelity and resolution, so that more than 25 layers could be bioprinted. With the optimized protocol, the 3D printed construct could be crosslinked so that the print was stable enough to be handled. The microalgae immobilized in the ink formulation survived more than seven days in the 3D printed geometry.

The photosynthetic performance of the material was observed, by building an incubator prototype with oxygen and carbon dioxide sensors. The sensors measured two different bioprints simultaneously, a macro-porous cube and a solid cube. The superiority of the photosynthetic ability of the porous geometry over the solid geometry could not be concluded, due to the limitations of the sensors and the prototype setup. Important insights were found for further research on the improvement of the test setup.

The optimized microalgae bio-ink is a valuable material with sustainable benefits. The material can actively fight carbon dioxide pollution, is carbon neutral in production and is biodegradable. The technical optimization of the bio-ink in this study formed the first steps towards a functional living microalgae artefact.

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Chapter 1

Project description

1.1 Introduction

A contemporary issue in product design is materialism: the notion that consumers buy many products which are often used shortly and thrown away right after use. The careless disposal of fossil-based materials poses a threat to our economy, human health and to the environment.

For instance, the annual economic cost of plastic pollution to the marine ecosystem is $13 billion euros (Raynoud, 2014). Throughout the plastic lifecycle, harmful compounds and toxins are released which are associated with health problems such as types of cancer, diabetes, impact on skin and eyes and birth defects (Azoulay et al, 2019). And lastly, plastic pollution is damaging to human health as well as animal health. According to Jerker Tamelander (2019), head of UN Environment's coral reef unit, "Marine plastic litter pollution is already affecting more than 800 marine species through ingestion, entanglement and habitat change".

Another large problem of the huge production of materials is the emission of carbon dioxide. Atmospheric CO_2 has increased more than 40 percent since the industrial evolution: from 270 to 400 parts per million (Moreia & Pires, 2016). This rise in air pollutants is associated with climate change, noticable by the warming atmosphere and ocean, the loss of Greenland and Antarctic ice sheets mass and a rising sea level (Dawson et al, 2011).

Challenges such as climate change, plastic pollution and the scarcity of fossil materials amplifies the call for novel materials that require less energy and natural resources to produce, do not contain ingredients harmful to nature and can be recycled.

One possible solution is the replacement of common materials with 'living materials'. Materials which are fabricated with the help of organisms such as fungi, algae and bacteria. These natural materials require less energy and no fossil raw materials for production, whilst resulting in materials that are often biodegradable due to their biological origin. Moreover, these materials offer new and unique functions and expressions.

At the Aubin-Tam research group of the TU Delft, scientists are working on the development of a 'living' material with algae cells by combining them with polymers in a hydrogel formulation. The hydrogel traps the living algae cells in a matrix that can house and sustain the encapsulated cells. Although the material is in the early stages of development, it shows huge potential for product application.

The material is made entirely out of biobased ingredients, which saves fossil materials and makes it biodegradable. Most importantly, since it contains organisms capable of photosynthesis, the material can reduce the amount of carbon dioxide in the surrounding air and release oxygen. To summarize, the material proposition of the Aubin-Tam group has air cleaning properties.

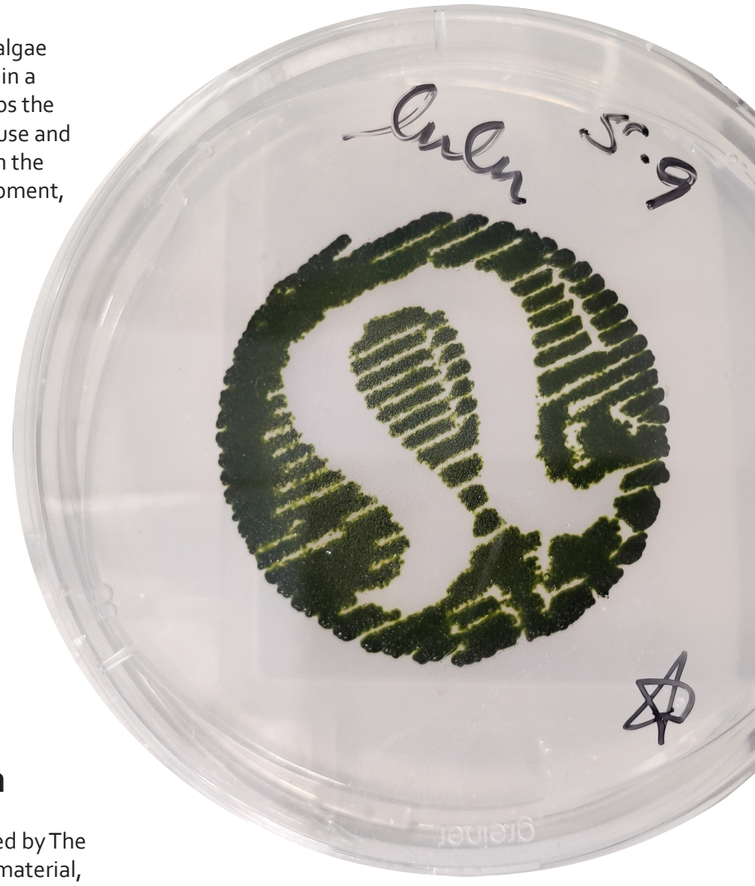
Due to the advantages of this novel material, it was decided to develop the material further.

1.2 Problem definition

Preliminary studies have been performed by The Aubin-Tam group to 3D print the algae material, where they were able to print flat structures on nutrient plates in a sterile environment. The algae 3D prints survived two weeks on the agar plates.

Since the material is relatively new, there are many unexplored challenges. First, the material suffers technical challenges. The algae ink is relatively soft, and unable to form complex 3D structures. At the moment, only flat objects can be 3D printed with the material.

Moreover, the microalgae in the material need a regular supply of fresh water and nutrients to stay alive and thus be able to perform photosynthesis. A method needs to be developed to provide the algae regularly with nutrients, and reach the inner cells within the



structure.
Summarized the technical problems are:

1. Unable to make higher prints, due to the relatively weak mechanical strength of the bio-ink.
2. Unable to cure or solidify multilayer prints.
3. Low lifetime of the algae cells due to finite access to nutrients.

Aside from the technical challenges, there is a lack of understanding about the behaviour of the material. For instance, how much oxygen is the material able to produce? And how do alterations to the shape of the material affect the photosynthetic ability of the algae? Thus, a clear scope and goal were formulated to study the behaviour of the material.

1.3 Stakeholders and scope

This research was a collaboration between two research groups: the Materials Experience Lab at Industrial Design Engineering and the Aubin-Tam group from Applied Sciences. Both are working on the development of sustainable materials enclosing living organisms.

The Materials Experience Lab at IDE is a group of practitioners, who aim to understand and enhance the relationships people have with product materials. Over the last few years, they developed tools and a method to understand how materials are experienced, and inspire designers to develop new and biofabricated materials and applications. Previous work of the lab was, for instance, products made with mycelium, a type of fungus.

At the Aubin-Tam group, scientists are working on the development of biomaterials from a more biological and technical standpoint. The group uses microorganisms to fabricate bio-based and/or living materials.

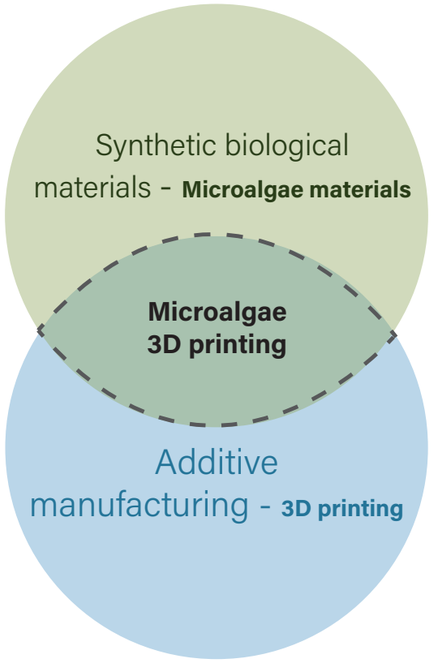


Figure 1. Stakeholder map including goals for each stakeholder and project scope

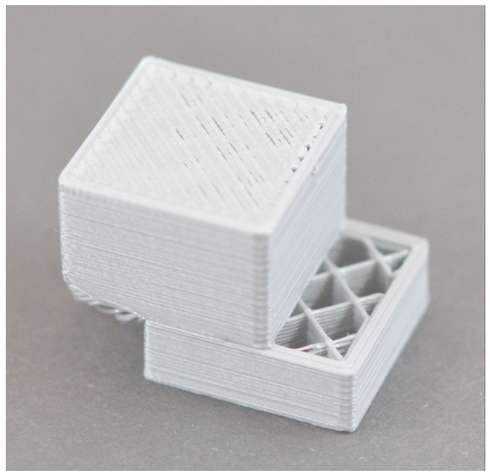


Figure 2. 3D printed cube with a designed inner structure

Since there were two faculties involved in this project, the expertise from both the Materials Experience Lab as well as the Aubin-Tam research group, was used in this research project. Knowledge on microalgae growth and other ink components was combined with information of 3D printing, summarized in Figure 1. These expertises combined forms our scope: microalgae 3D printing.

Since this type of manufacturing was selected, we wanted to use its full potential. The main advantage of 3D printing over other types of manufacturing is that the inner structure of a product can be designed. See Figure 2. This offers new opportunities for the microalgae material to be optimized.

1.4 Goal

The following goal was selected for this research project:

'Increase the longevity and photosynthetic activity of microalgae cells by creating a macro-porous 3D structure.'

The research goal is visualized in Figure 3.

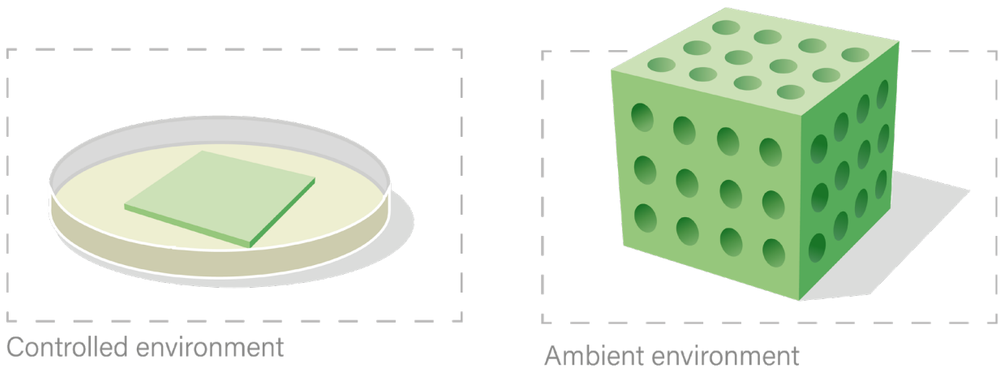


Figure 3. Starting point and goal of the research project

The goal consists of two parts: A technical goal to create a multilayer 3D print which is self-supporting, as well as a vision for the type of shape of the microalgae ink.

The hypothesis was that a micro-porous shape has a positive effect on the photosynthetic ability. By increasing the surface ratio, the microalgae have more contact with the surrounding air and more access to light, which could increase the release of oxygen and uptake of carbon dioxide.

The limiting factors for this project are the time constraint of 20 weeks and unexpected delays due to the Corona virus.

1.5 Approach

In Figure 4 is an overview of the approach is given.

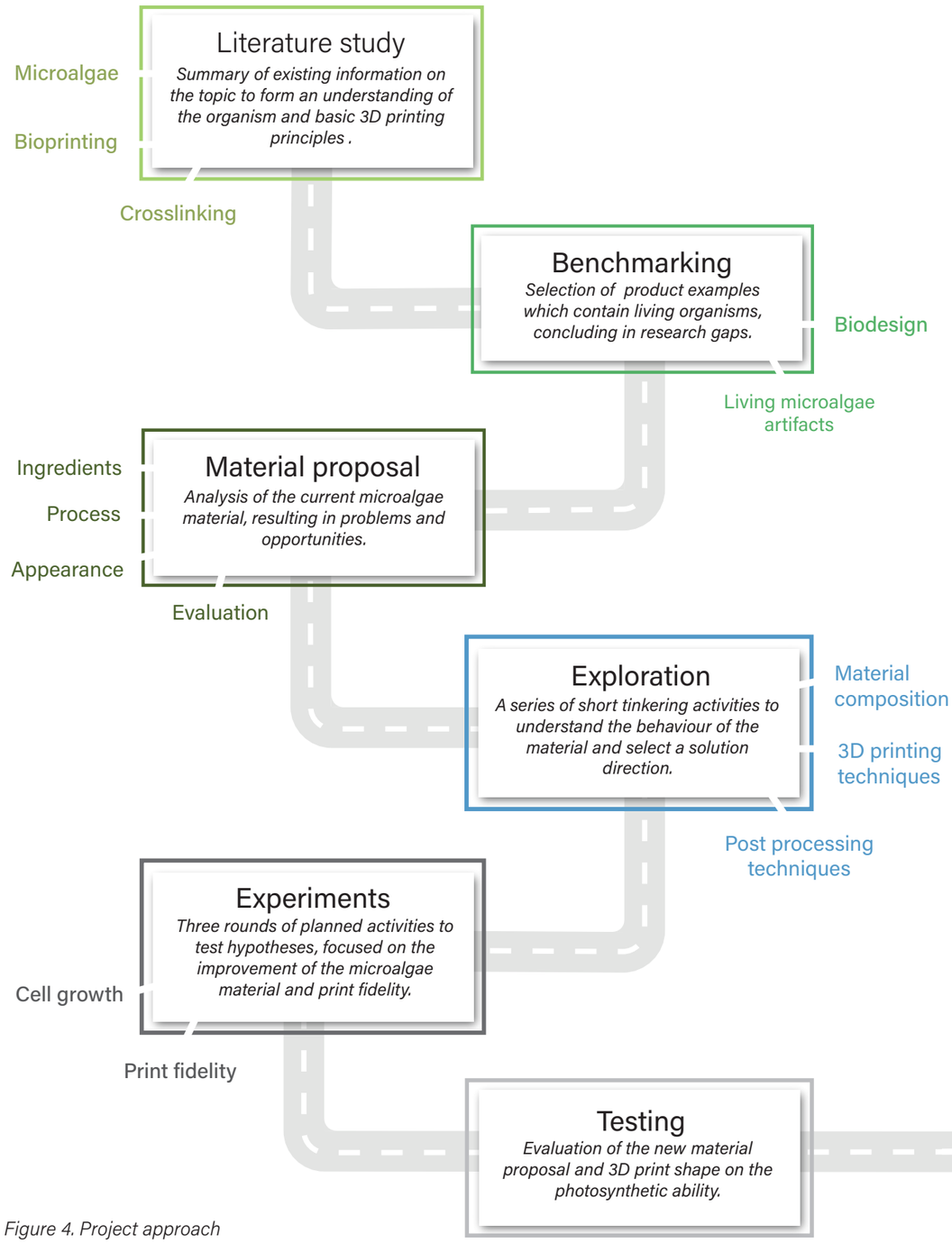


Figure 4. Project approach

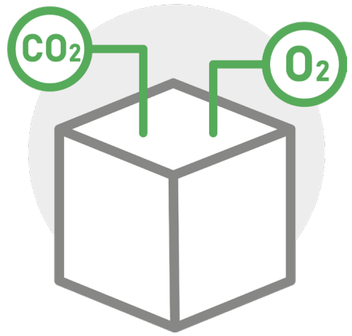
1.5 Deliverables

1. Protocol

Protocol including ingredient list of the optimized bio-ink formulation and a step-by-step guide of the material making, 3D printing and post-processing.

2. Video

Short video showcasing the bioprinting process.



3. Measurement setup

Measurement setup for photosynthetic activity and recommendations for improvement of this setup

1.4 Research questions

The following research questions are answered in this project:

Algae and photosynthetic activity

1. *What advantages can microalgae bring to humans and the environment?*
2. *What is the role of microalgae in carbon capture and oxygen release?*
3. *What is the expected carbon capture and oxygen release of microalgae over time?*
4. *How can photosynthetic activity of algae cells be measured?*
5. *How can cell viability of microalgae be measured?*

Potential of algae in design

6. *What current examples are there of designers collaborating with algae?*
7. *What functions do algae fulfil in these examples?*
8. *What current examples are there of algae bioprinting?*
9. *Based on these current developments, where are research gaps?*

Bioprinting and post-processing

10. *What are the advantages and disadvantages of direct ink writing?*
11. *What characterizes a good bio-ink performance?*
12. *What parameters can be altered to influence the bio-ink performance?*
13. *What is crosslinking and why is it necessary for good bio-ink performance?*
14. *Which crosslinking method is most suitable for microalgae bioprinting?*
15. *What are the key requirements for the optimized bio-ink?*

Material proposal

16. *What is the appearance of the 3D printed algae material and how does it behave?*
17. *Which steps in the material process will be altered, and which steps will stay the same?*
18. *Which growth conditions need to be met for maximum algae growth?*
19. *What are the advantages and disadvantages of the bio-ink composition?*
20. *What are the advantages and disadvantages of the current 3D printing and crosslinking technique?*

Exploration

21. *Which extrusion based cell printing methods are commonly used?*
22. *What are the advantages and disadvantages of each method?*
23. *Which components can be added to the bio-ink to increase the viscosity, without negatively affecting the cell function?*

Experimentation

24. *Which steps should be taken to make the optimized bio-ink formulation?*
25. *Which steps should be taken to 3D print the optimized bio-ink?*
26. *What insights were obtained which led to the new bioprinting protocol?*
27. *How does the optimized bioprint look like and behave?*

Testing

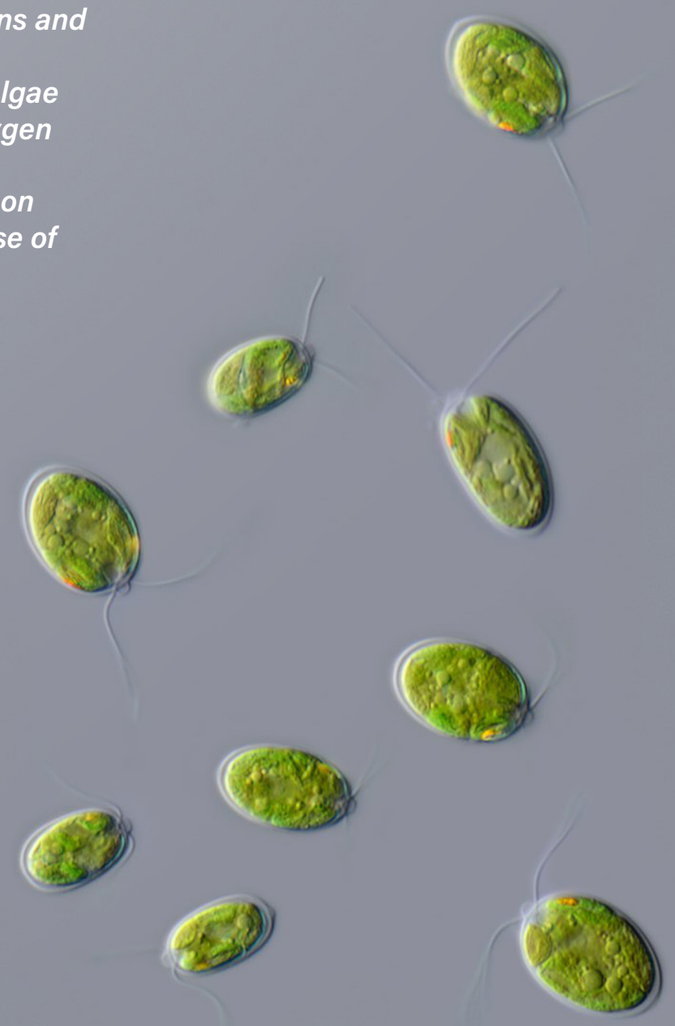
28. *Which sensors can be used to measure the photosynthetic ability of the optimized bioprints?*
29. *How was the measurement setup built?*
30. *Does a macro-porous bioprint show enhanced carbon capture and oxygen release over a solid bioprint?*
31. *What are the limitations of the photosynthetic measurement setup?*
32. *What are the limitations of the incubator?*

Chapter 2

Microalgae and photosynthesis

This chapter introduces the organism who plays the leading role in our project: green microalgae. The following research questions are answered in this chapter:

1. ***What advantages can microalgae bring to humans and the environment?***
2. ***What is the role of microalgae in carbon capture and oxygen release?***
3. ***What is the expected carbon capture and oxygen release of microalgae over time?***
4. ***How can photosynthetic activity of algae cells be measured?***
5. ***How can cell viability of microalgae be measured?***



2.1 Introduction

Algae are organisms that can grow in aquatic habitats, such as lakes, rivers, oceans, and even wastewater. Scientists have estimated that there are around 72,500 algal species in the world (Guiry, 2012). The main characteristic of algae is that they are photosynthetic, which means that they can capture light energy and use it to convert water, carbon dioxide and minerals into oxygen and energy-rich organic compounds (Bassham, 1998). Since algae represent such a large and diverse group of organisms, they contribute to at least 50% of total carbon fixation worldwide (Field et al., 1998) (Chung et all, 2011).

Although algae contain chloroplasts and produce food through photosynthesis, they are not considered plants. Unlike higher plants, microalgae do not have roots, stems, or leaves. Algae are classified as protists: eukaryotes which are not plants, animals or fungi (Figure 5). Algae are broadly classified as Rhodophyta (red algae), Phaeophyta (brown algae), and Chlorophyta (green algae) and classified by size as macroalgae or microalgae (Khan et al, 2018). Macroalgae (seaweed) are multicellular, large-size algae, visible with the naked eye, while microalgae are microscopic single cells. In bio-inks for 3D printing, microalgae are used.

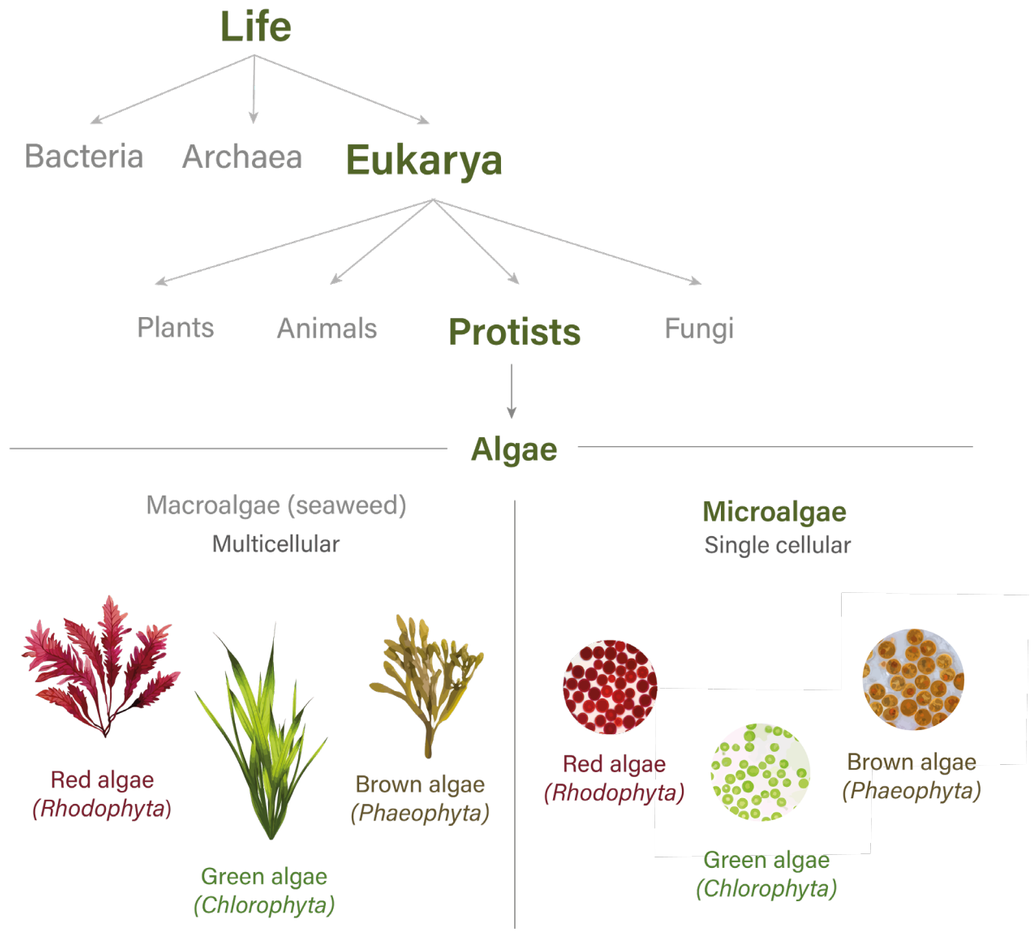


Figure 5. Classification of algae

2.2 Advantages of microalgae

Generally speaking, green microalgae can be pictured as tiny houseplants. Just as plants, they need care in the form of water and nutrients to stay alive. If cared for properly and placed in the correct environment, they grow and increase their biomass. Comparable to plants, they perform photosynthesis, where carbon dioxide and water from their environment are converted to sugar and oxygen. In a way, microalgae act as an air cleaner, where the carbon dioxide is removed and replaced by clean oxygen.

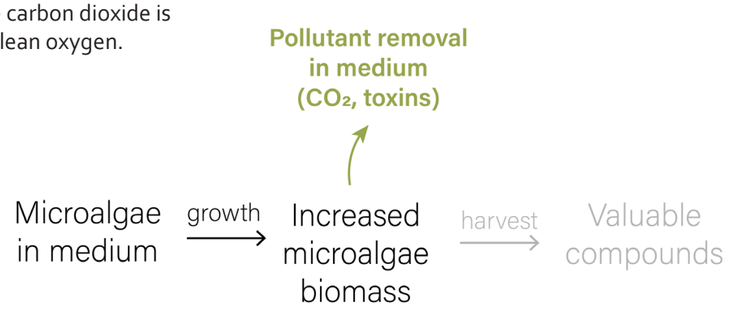


Figure 6. The main advantage of microalgae is the removal of pollutants from its growth medium

2.2.1 Advantages of microalgae over plants

One of the differences with plants is that microalgae are able to grow in various environments. For instance, they can grow excellent on residual water from agro-industrial companies, carbon dioxide from flue gases and diluted digestate from manure (Wolkers et al, 2011). Aside from carbon dioxide, they can also detoxify waste water by removing free metal ions and storing them in the cell (Perales-Vela, Peña-Castro, & Canizares-Villanueva, 2006).

Last but not least, microalgae are more efficient in carbon fixation than plants. While the mechanism of photosynthesis is similar to higher plants, microalgae capture solar energy with an efficiency that is 10^{50} times higher (Li et al., 2008). In other words, microalgae can convert more oxygen for air cleaning purposes than other terrestrial plants. (Moraes et al, 2020)

2.2.2 Focus point of main advantage

Microalgae are useful alive as well as dead. Besides their photosynthetic quality, their biomass can be used in a wide array of applications. From the dead microalgae, polysaccharides, lipids, pigments, proteins, vitamins, bioactive compounds, and antioxidants can be extracted for health supplements, pharmaceuticals, and cosmetics (Das et al, 2011). Burnt, the biomass can even be used as a biofuel to replace fossil fuels. (Milano et al, 2016)

In the future, it could be interesting to extract the micoralgae from a 3D print once they served their purpose. However, this project focused on the ability of microalgae to remove pollutants, especially carbon dioxide. See Figure 6. In the next paragraph, this mechanism is explained in more detail.

2.3 Photosynthesis and respiration

2.3.1 Photosynthesis

Only organisms which contain chlorophyll, a green pigment that absorbs light and converts it into chemical energy, can perform photosynthesis. These organisms are algae, plants and cyanobacteria.

Photosynthesis is the conversion of light energy from the sun to chemical energy that is stored in sugar and other molecules (Campbell et al, 2015). The photosynthesis equation can be seen in Figure 7.

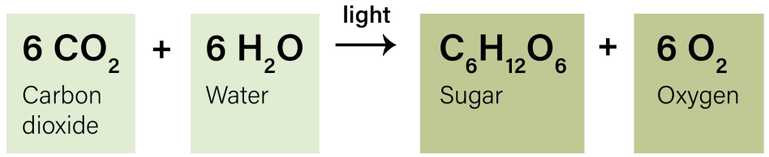


Figure 7. Overall equation of photosynthesis seen in plants, algae and cyanobacteria

Since plants, algae and cyanobacteria can produce their own food, they are called photoautotrophs, which literally means "self-feeders using light" (Campbell et al, 2015). Photoautotrophs are the basis for all food that heterotrophs like animals, fungi, bacteria and humans consume to survive.

They rely on the sugars produced by photosynthetic organisms. Therefore, directly or indirectly, photosynthesis provides most of the energy needed for organisms to live on earth.

In Figure 8, a simplified photosynthetic reaction is shown in microalgae.

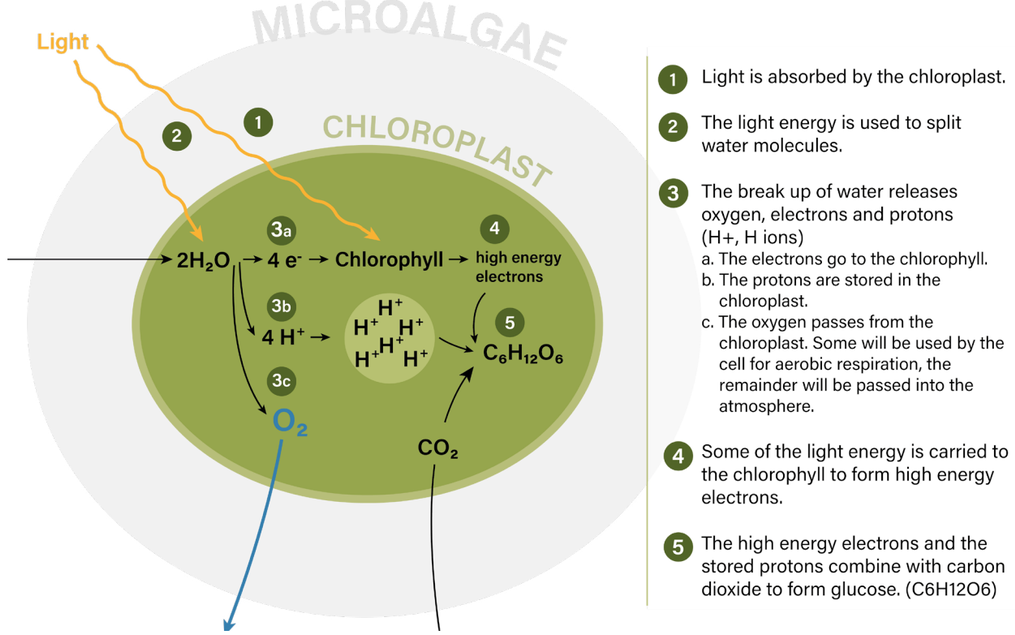


Figure 8. Schematic representation of photosynthesis in microalgae

During photosynthesis, microalgae consume water from their near environment and the chloroplast, the organelle in the cell where photosynthesis takes place, splits the water molecule in hydrogen and oxygen. (step 2) Part of the oxygen is used in the cell (step 3a/3b), the other part is released into the atmosphere (step 3c). The chloroplast also uses carbon dioxide from the environment and the newly formed hydrogen to create glucose. (step 5) Thus, during photosynthesis the carbon dioxide content in the atmosphere lowers, while the amount of oxygen rises.

2.3.2 Cellular respiration

In the dark, microalgae are unable to make oxygen. They need energy to get them through the night, and this is derived from glucose. This process, where sugar is converted into energy, is called cellular respiration (Campbell et al, 2015). The equation is the opposite of the photosynthesis reaction (Figure 9 and Figure 10).

Due to growth, the microalgae accumulate carbon over time. Therefore, the amount of carbon dioxide produced from respiration is less than the amount of carbon dioxide used in photosynthesis, otherwise the microalgae would not be able to grow.

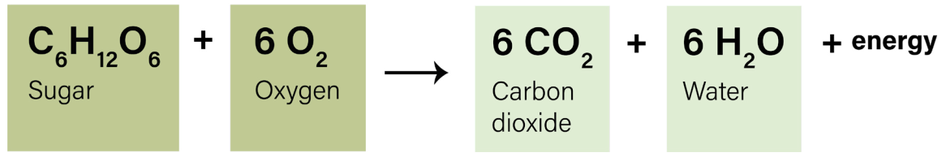


Figure 9. Overall equation of cellular respiration seen in plants, algae and cyanobacteria

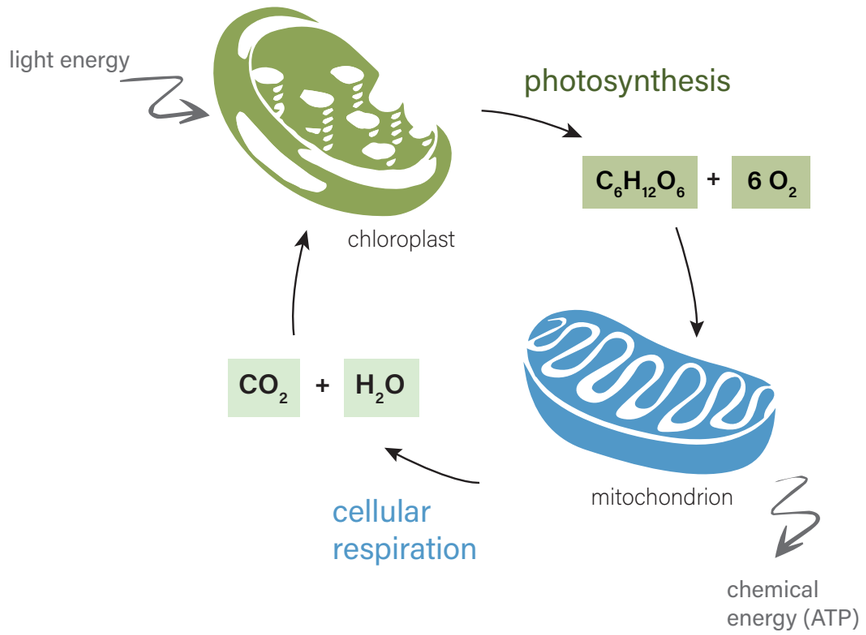


Figure 10. Cellular respiration is the opposite of photosynthesis: sugar and oxygen are converted into carbon dioxide and water.

To summarize, the microalgae can decrease the level of carbon dioxide and increase the level of oxygen in the air during the day, by using photosynthesis. At night, part of this process is reversed due to cellular respiration. Respiration reduces the net amount of oxygen plants produce.

A graph was made to visualize the expected change in oxygen and carbon dioxide levels during the day and night, see Figure 11.

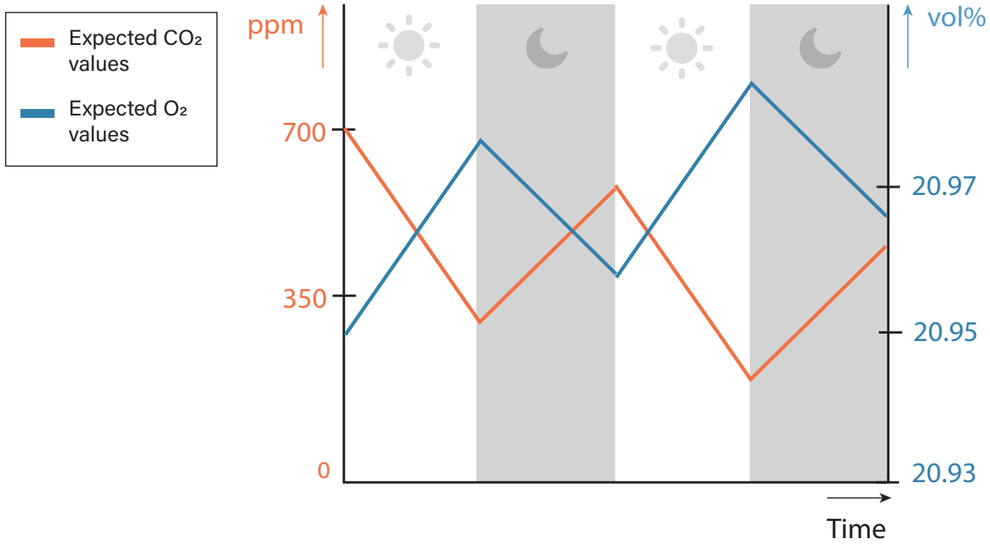


Figure 11. Simplified graph of the expected range and shape of the oxygen and carbon dioxide levels during photosynthesis and respiration in green microalgae for an indoor environment

The average level of carbon dioxide in urban homes is between 400 and 1000 parts per million (ppm) (Abdel-Salam, 2015). That is around 0.04% of the total air volume indoors (Williams, 2020). The hypothesis was that the level of CO2 will start around 700 ppm and decrease during the day. The decrease in carbon dioxide was expected to be twice as high during photosynthesis compared to cellular respiration (Vernier, n.d.). That is why the carbon dioxide level will increase during the night. However, it will reach only half of the carbon dioxide which was used during photosynthesis. Over time, the total amount of carbon dioxide will decline in a waving motion.

The average level of oxygen is 20.95 volume percent (Williams, 2020). If all the carbon dioxide is replaced by oxygen in a specific room or volume, the total oxygen percentage will increase to 21%. The changes in oxygen will thus be relatively small. The hypothesis was that the oxygen will increase over time, in a waving motion similar, but mirrored, to the carbon dioxide graph.

2.4 Potential risks of microalgae

A possible risk of the use of microalgae in a 3D printed structure is leakage of the algae into the surrounding environment, causing contamination. If a significant amount of microalgae would come in contact with nutrient-rich surface waters for example, algal blooms might occur (See Figure 12). This process of progressive enrichment of a body of water with nutrients is called eutrophication (Glibert et al., 2005). Due to human activities such as farming practices, sewage treatment, industrial waste disposal and construction site runoff, the process of eutrophication is accelerated (Chislock et al., 2013). Detrimental effects of dense algal blooms consist of limited light penetration to deeper water layers, resulting in reduced growth and die-offs of aquatic plants (Lehtiniemi et al. 2005).

Furthermore, as a result of high rates of photosynthesis, dissolved inorganic carbon is depleted and the pH of the water rises to extreme levels (Turner & Chislock 2010). When the algal blooms eventually die, the microalgae decompose and consume dissolved oxygen. Lack of oxygen in the water causes so-called 'dead zones', where many aquatic organisms will not survive (Nixon & Fulweiler, 2009).

With the risks of eutrophication in mind, it is essential that the microalgae are placed in a structure in which leakage is not possible. If the 3D print is placed in a medium like water, for example, the cells should stay attached to the bioprint and should not dissolve in the water.

Figure 12. Extreme case of algal blooms in lake Erie in North America. Photo by Zachary Haslick

2.5 Measuring photosynthetic activity and growth

There are different ways to measure if algae cells are alive and if so, how much they photosynthesize. The cells are alive if they move in the medium, and show a green colour caused by chlorophyll. Chlorophyll are pigments that absorb energy of the sun for photosynthesis (Ramaraj, Tsai & Chen, 2013). They absorb specifically red and blue light, and reflect green light which causes the green appearance. (See Figure 13). Without chlorophyll, the algae cannot photosynthesize and thus will perish. In incorrect growth conditions, the microalgae die, causing a colour change from green to brown, yellow or white (See Figure 14). Correct growth conditions to sustain the microalgae cells are further explained in chapter 5.2.1.

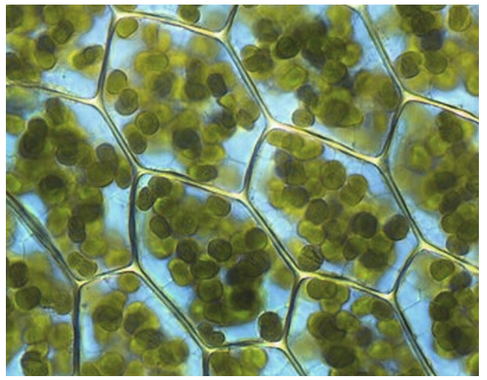


Figure 13. Chlorophyll are green pigments in microalgae

The green colour is an indication if the algae are alive. However, it is not a failproof method to measure the livingness. Once algae die, they can release a burst of colour from the chlorophyll, which will fade over time. To be entirely sure that the microalgae are alive in the material, there are two simple methods to determine the viability:

1. Visual inspection of cell mobility under a microscope
2. Regrowth by taking a microalgae sample and transferring it to a new growth medium

Algae growth is the amount of algae cells in the material. More information on algae growth is given in chapter 5.2.1. There are two methods to measure the algae growth:

1. Measuring chlorophyll content over time with a photospectrometer
2. Count cell number over time with a microscope.

And lastly, the photosynthetic activity can be measured with:

1. Measuring yield (Fv/Fm) of photosynthetic efficiency with an Active Fluorescence measuring device
2. Oxygen and carbon dioxide infrared sensors

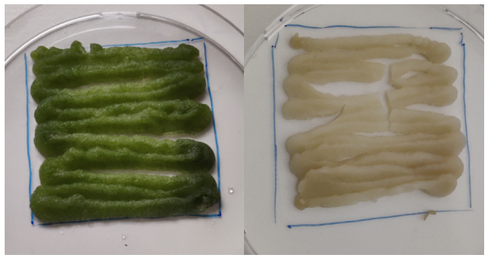


Figure 14. Material including microalgae which changes colour from green to brown

For the phtotosynthetic activity measurement, a test setup was built which included oxygen and carbon dioxide sensors connected to an Arduino. For the test setup, see Chapter 8.3.

In this project, the livingness of the material was assessed by a simple visual inspection of the colour. More advanced methods are preferred for future studies on the material. However, this simple method suited the explorative nature of the project. To specify the colour intensity of a bioprint, a colour guide was made with eight different shades of green which were observed during the experimentation. See Figure 15. Each colour was assigned a number, where a higher number corresponds to a higher intensity of green.

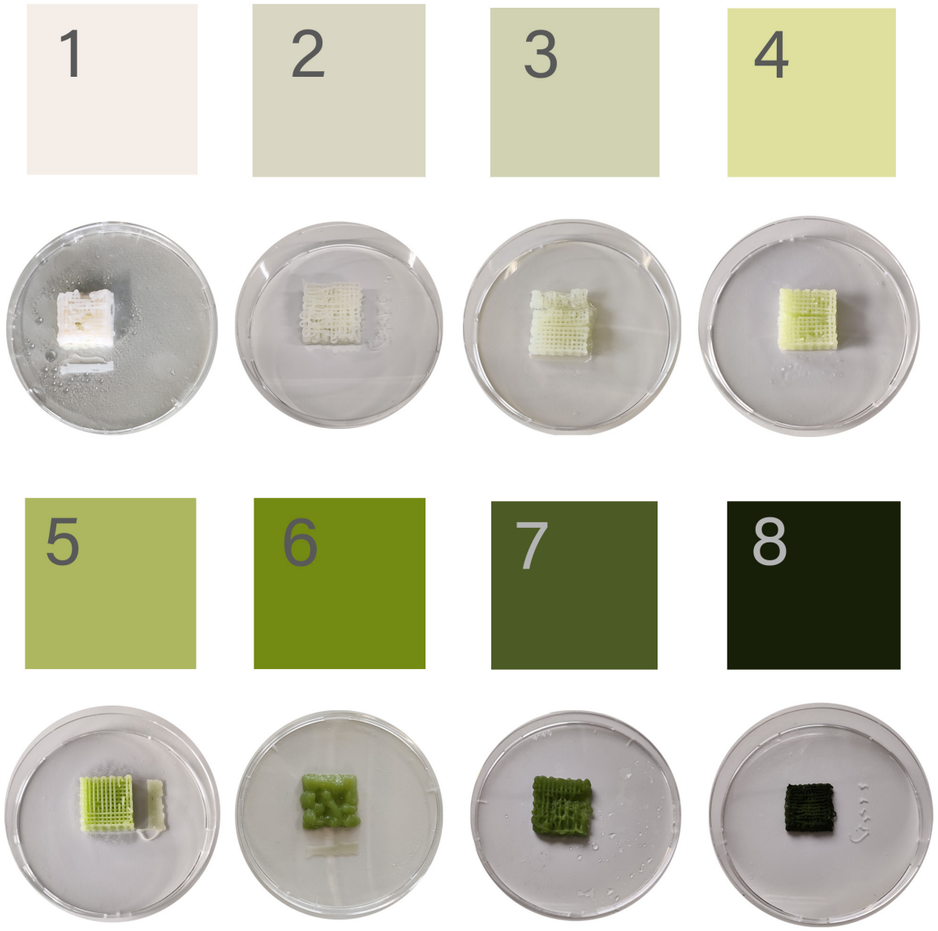


Figure 15. Colour guide to quantify the livigness of the bio-ink

CHAPTER 2

2.6 Conclusion

Microalgae are tiny organisms, invisible to the naked eye. Despite their size, they have a huge impact on the environment and human population. Algae are essential for life on earth, due to their ability to fixate carbon dioxide and to produce oxygen. More than 50 percent of total CO_2 emissions worldwide are captured in microalgae and converted to oxygen (Field et al., 1998) (Chung et all, 2011).

The expectation is that the average CO_2 level in a closed-off space will decline over time, if microalgae are present. CO_2 levels will fluctuate, depending on the amount of light. During the day, the microalgae will convert CO_2, and during the night, CO_2 will be released due to cellular respiration. For oxygen release, the same trend is expected but mirrored. A fluctuating increasing trend should be noticeable. These changes in carbon dioxide and oxygen due to micoralgae activity can be measured with infrared sensors.

From the different approaches to measure the livingness of microalgae, visual inspection of colour intensity was chosen, since it is most time-efficient.

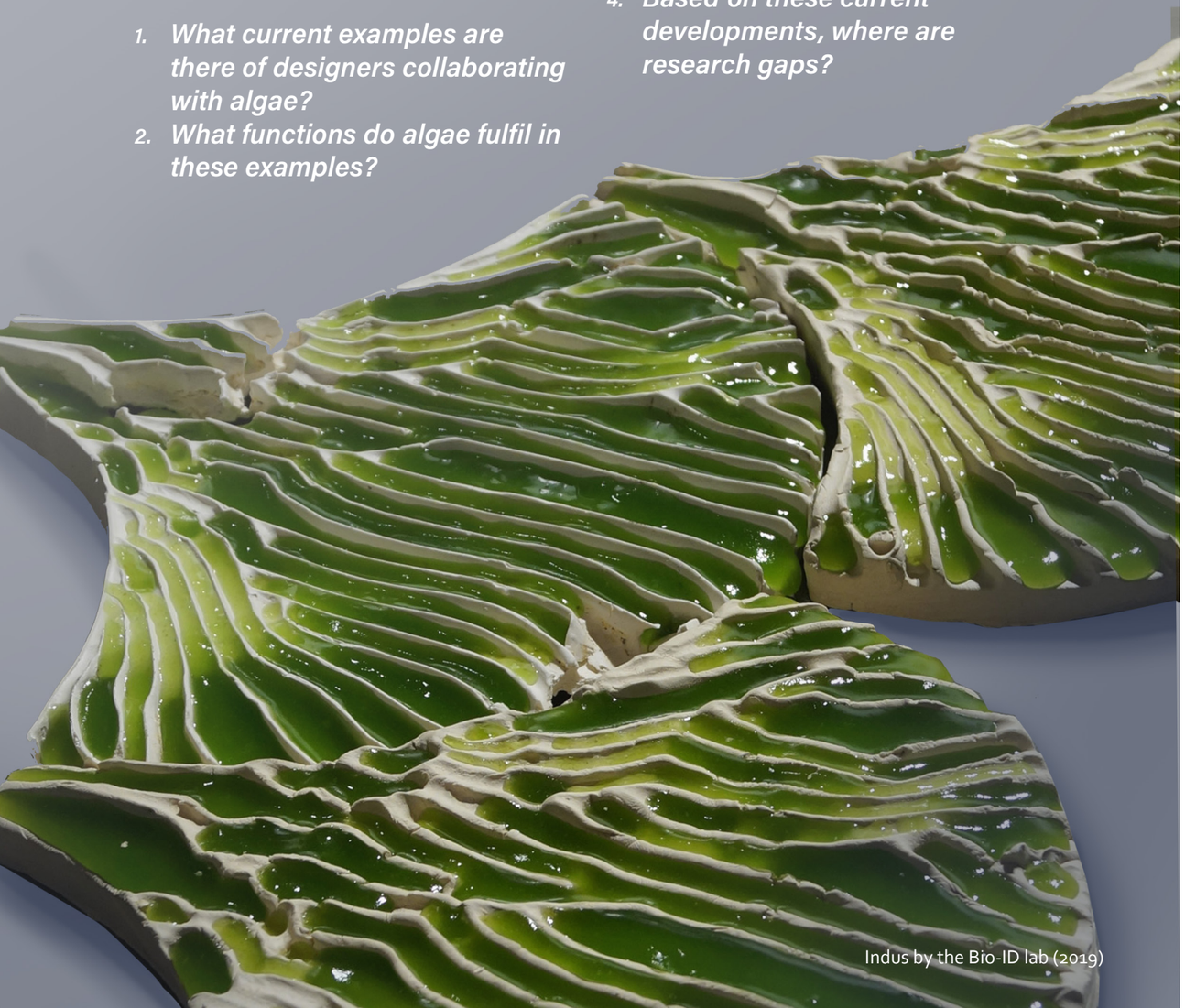
Chapter 3

Living artefacts

In this chapter, the context of growing materials will be explored. The current state of knowledge on the topic will be summarized.

Research questions answered in this chapter:

1. ***What current examples are there of designers collaborating with algae?***
2. ***What functions do algae fulfil in these examples?***
3. ***What current examples are there of algae bioprinting?***
4. ***Based on these current developments, where are research gaps?***



Indus by the Bio-ID lab (2019)

3.1 Introduction

The materials that make up our built environment support our economic growth and enable our urban existence. The fabrication of these materials, however, has a huge impact on our environment. The materials we use are energy intensive, require non-renewable resources and create air- and water pollution (Raynoud, 2014) ,which threatens our health and environmental biodiversity (Azoulay et al, 2019). There is a demand for sustainable materials and production techniques with a lower ecological footprint. Here, designers play an important role in the understanding, selection and development of sustainable materials.

The materials that nature is able to produce are amazingly complex and a rich source of inspiration for materials science. Through evolution, organisms have become capable of dealing with a scarcity of resources and growing materials from a limited selection of components (Wegst et al., 2015). According to Nyuyen et al, 2018, "cells can be considered as nanomaterial factories that constantly sense their environment, draw from various energy sources and simplistic molecular building blocks, refashion these molecules into new structurally and functionally more complex materials, and maintain these materials over time."

By using living cells in materials, characteristics of biological systems can be achieved, such as self-replication, self-regulation, self-healing, environmental responsiveness and self-sustainability. These features are common in biology, but rare in most existing materials. Moreover, these natural materials require less energy and no fossil raw materials for production, whilst resulting in materials that are often biodegradable due to their biological origin. This has led to the emerging field of Growing Design or Biodesign: growing materials from living organisms that achieve unique material functions, expressions, and sustainable solutions for product design (Camere & Karana, 2017).

There are two ways to collaborate with living organisms in product design. Most commonly, designers use the organisms in the early stages of the product design. The growth of the organism is guided to create a living material which is low in material footprint, from which a non-living artefact is created (Karana, Barati & Giaccardi, 2020).

For instance, clothing brand Puma used bacteria to design an alternative for synthetic dyes. Six clothing items, four pairs of running shoes and socks are dyed in collaboration with the bacteria J. lividum (Luchtman, 2017). On the clothing, the bacteria are cultivated and start a live dying process, where they ferment nutrients into a purple pigment. According to the designers, the colours in the clothes "will naturally change and fade over time and with use", (Design to Fade, n.d.) "enhancing the uniqueness of each garment and the activity of the wearer" (Luchtman & Siebenhaar, 2020). In the case of Puma, the bacteria are used during the fabrication of the product to secrete purple pigment. Once the clothing is worn, the bacteria will have died and slowly fade over time. During the product use, the organisms are no longer living, see Figure 16.

The second way to design with living organisms is to collaborate with the organism during the use of the product. Instead of a merely sustainable material, it is now an artefact which can respond and interact with the user. This is called a Living artefact (Karana, Barati & Giaccardi, 2020).

For example, Henk Jonkers, professor of TU Delft, the Netherlands, invented bioconcrete: concrete that heals itself using bacillus bacteria. They are put into capsules together with the nutrient calcium lactate and added to the wet concrete mix. When a crack begins to form, water enters and opens the capsule. The bacteria then feed on the lactate and form limestone, which closes up the cracks (Stewart, 2016). In this example, the bacteria are alive in the material during the product use, See Figure 16, and offer a self-healing material quality to

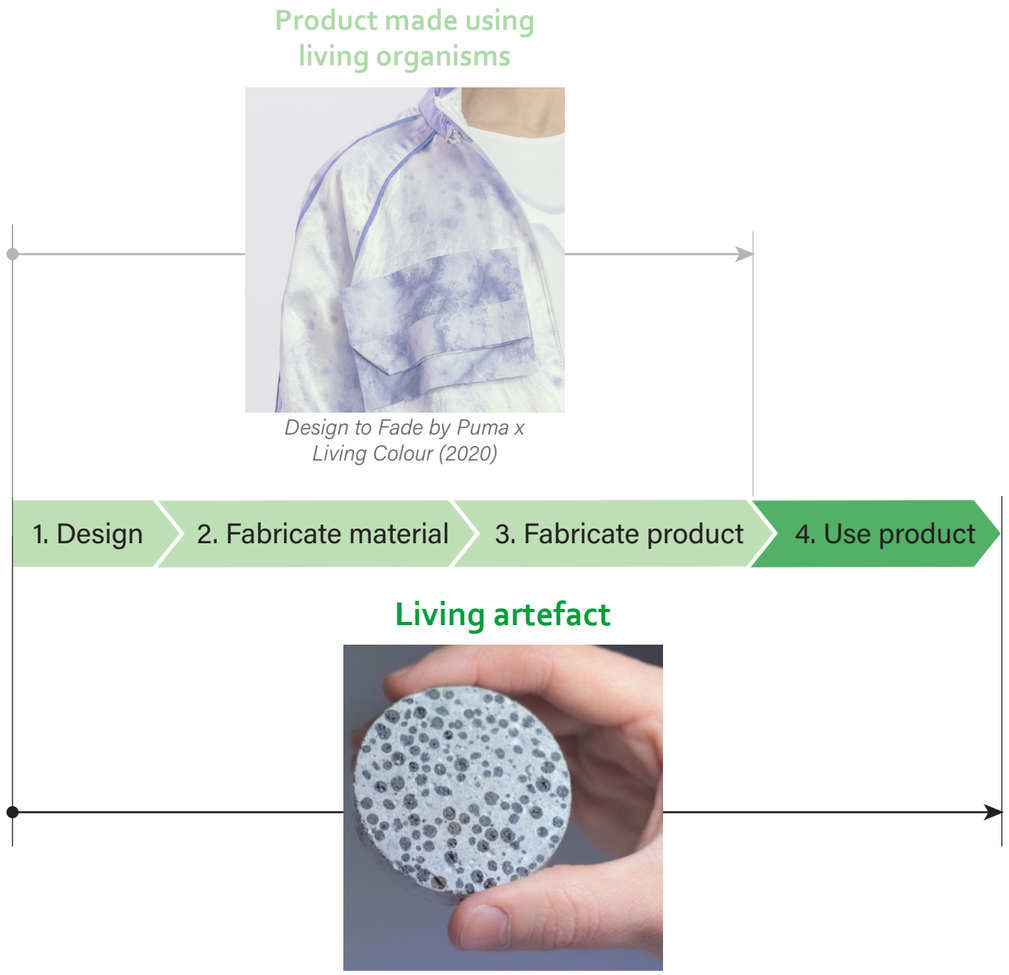


Figure 16. A living artefact contains organisms which are alive during the use of the product

In the case of living artefacts, the question is raised how to design with a material that lives, grows and dies. A product is no longer lifeless, but can be perceived as a pet or companion which needs special care. This revolutionary change in material or product perception requires a more flexible designer mindset, as living materials can show unexpected behaviour compared to more traditional materials. Due to this and the novelty of the material, it might take some time before it will be accepted and adopted by the general public.

3.2 Product examples

In the field of Growing Design, there are numerous product examples which can offer inspiration. A benchmarking analysis was performed, which shows six interesting product examples categorized as living algae artefacts.

Cultures by Lia Giraud (2011)

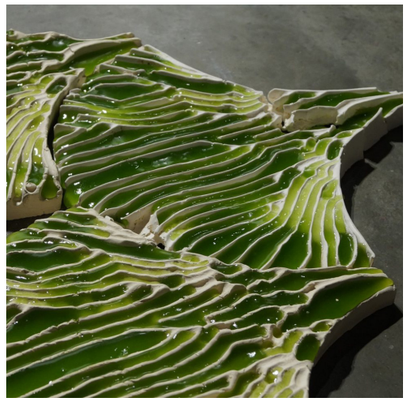
Keywords: *Indoor, light, mobilization, decoration*

The designer called these living microalgae images *Algægraphies*. The principle that is used in this project is the ability of the algae to organise themselves according to light patterns. On top of a grown microalgae culture, a negative image is projected. In the areas where it is lightest, the algae position themselves and form more dense areas, creating a darker green colour. After solidifcation, the cells are trapped in the nutrient medium and over time, the images evolve and wear down. (Giraud, 2011).

Exhale by Julian Melchiorri (2017)

Keywords: *Indoor, furniture, light, photosynthesis*

Julian Melchiorri designed a chandelier which absorbs carbon dioxide and exhales oxygen in indoor environments. The lamp cleverly shows a dual advantage for both humans and organisms: the organisms need light to produce oxygen during the day, and the lamp gives light back to humans at night. The hand-built chandelier consists of 70 leaf modules, which all contain microalgae in a nutrient medium. The leafs are connected to a pump developed by engineers from Arborea, to maintain the microorganisms. (Melchiorri, 2017)



Indus by the Bio-ID lab (2019)

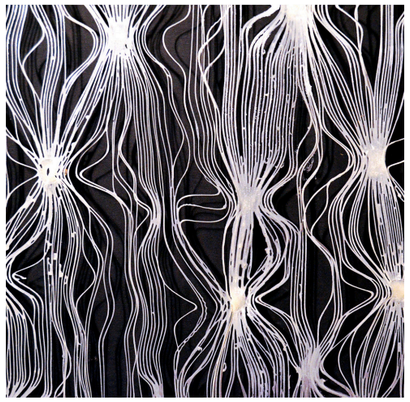
Keywords: *Outdoor, waste water treatment, modular*

This product is a low tech water treatment which empowers citizens of rural India to clean and reuse water locally. It is a wall, consisting of clay tiles inlaid with micro-algae suspended into an alginate hydrogel. Water is poured through inlets from the top, and once it comes into contact with the algae, the algae produce phytochelatins so that the pollutants are stored within the cell. The water collected at the bottom is safe to drink. Individual tiles can be refilled with fresh algae hydrogel once the tile is saturated (Hahn, 2019).

Photosynthetic coating by Post Carbon Lab (2020)

Keywords: *Indoor, textiles, photosynthesis*

A start-up in London designed a treatment for textiles that makes clothing able to photosynthesise. The microalgae are embedded in the textiles. According to the co-founder Dian-Jen Lin, "One large T-shirt generates about as much oxygen as a six-year-old oak tree". The clothes require a different care routine compared to standard clothing and communicate with its owner: a colour transition from green to orange or white shows the user that it is not cared for properly (Chashchyna, 2020).



Living screen by Irina Shaklova (2015)

Keywords: *Architecture, 3D printing, photosynthesis*

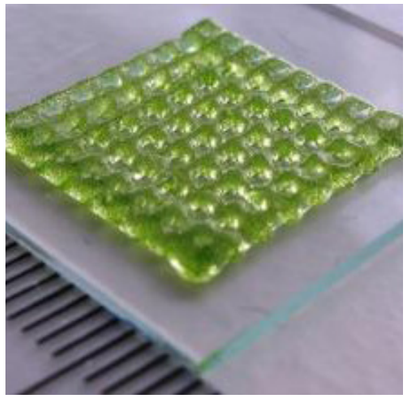
A student of the Institute for Advanced Architecture of Catalonia designed an algae screen of 4x 1.5 metres, entirely 3D printed with a robotic arm. The pattern consists of one continuous line, sometimes intersecting to improve structural strength. The 3D print is made of methylcellulose and sodium alginate ink, mixed with aerial algae. Opposite of aquatic algae, aerial algae do not need a constant flow of water to survive. They do need a humid environment or else they could go in hibernation until the conditions are favorable again (Shaklova, 2015).

Dino Pet by Yonder Biology (2014)

Keywords: *Toy, care, bioluminescence*

This Kickstarter campaign aims to inspire children to explore nature and shape the world around them. The product is a nightlight containing marine algae, Dinoflagellate, which are kept in a transparent container. Activated by shaking, the dinoflagellates respond with bioluminescence, creating a beautiful blue glow which lights up the room. The night light should be kept in a sun lit room, out of direct sunlight during the day. The lifetime is one to three months, and can be extended indefinitely with a supplemental growth medium (Bass et al, 2014).

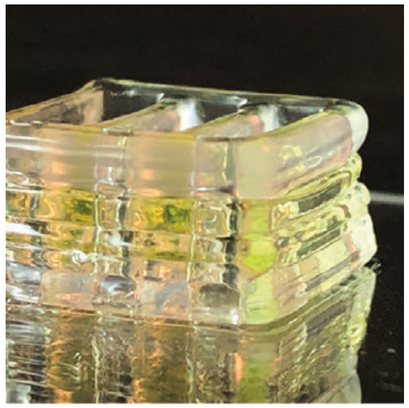
Aside from living microalgae products, there are also some innovative research projects including microalgae 3D printing. Below are three examples relatable to the material proposal of the Aubin-Tam research group.



Fabrication of photosynthetic algae-laden hydrogel by Lode et al (2015)

Goals: *oxygen delivery to human cells*

The aim in this research was to combine microalgae cells with human cells in a 3D print so that the microalgae can provide oxygen to the human cells. The researchers used alginate combined with methylcellulose to create squares of 15 mm width and length. The maximum height that could be achieved was 12.5 mm, 50 layers. The algae survived the 3D printing process and could grow, however the system needs to be optimized in order to achieve long-lasting supply of oxygen of the human cells with oxygen.



Cell-Laden Hydrogels for Multikingdom 3D Printing by Johnston et al (2020)

Goal: *combining algae, yeast and bacteria*

The scientists want to show one type of 3D print ink which can support microorganisms from three different taxonomic kingdoms. A gel ink was made from a synthetic polymer, pluronic F127, with whch squares of 15 mm by 15 mm were printed. The cells in the hardened gel were viable for 90% after a week and did not escape to the neighbour inks. The scientists proved prints thicker than 500 µm are more durable, but do not provide enough diffusion of nutrients to the cells at the core of the hydrogel.

3D Printing of Functional Microalgal Silk Structures by Zhao et al (2019)

Keywords: *microalgae prints with silk*

Alginate hydrogels are relatively unstable in aqueous conditions. The research group from Zhao et al improved the long term stability of the ink by adding silk fibroin. Additionally, hydroxypropyl methylcellulose (HPMC) and hydrogen peroxide (HRP) are added to thicken the hydrogel. HPMC is a thickening agent, HRP a crosslinking agent. The final 3D prints supported cell proliferation for at least 4 weeks and photosynthetic activity for more than 90 days.

CHAPTER 3

3.3 Conclusion

If the material material proposal of the Aubin-Tam group is implemented in a product, it can be characterized as a living artefact, since the microalgae are alive during the user phase. Products which contain living microalgae increase users awareness of biomaterials and showcase a new way of product interaction. This 'living' material requires additional care, and may have a shorter lifetime. As a result, it may take some time before it is fully accepted and can be adopted in society. The added advantage must be clearly communicated to outweigh the disadvantages of these early concepts.

In the product examples which implemented living algae during use, a wide array of functions was shown. The microalgae can function as a decorative element due to the changing colour, or can be of use to clean the air and water. Different types of algae can fulfill different functions. For instance, dinoflagatelles are specifically known for their ability to use bioluminescence, which is not standard for all microalgae.

One large limitation in the current algae product examples is the access to fresh nutrients for the algae cells. Most of the current examples use liquid media in order to pump fresh nutrients and water to the algae cells. The use of algae in liquid media limits the form freedom and functionality of possible product applications, since the products need a liquid container and a pump system. In the case of the Biogarmentry and algae 3D prints, the algae survive on a nutrient plate for about a month. Without access to nutrients, this is much shorter: 7 days. A solution must be found to add nutrients to an algae material to increase its lifetime.

Chapter 4

Bioprinting and bio-inks

This chapter introduces the main principles of 3D printing with living organisms.

The following research questions are answered in this chapter:

1. ***What are the advantages and disadvantages of direct ink writing?***
2. ***What characterizes a good bio-ink performance?***
3. ***What parameters can be altered to influence the bio-ink performance?***
4. ***What is crosslinking and why is it necessary for good bio-ink performance?***
5. ***Which crosslinking method is most suitable for microalgae bioprinting?***
6. ***What are the key requirements for the optimized bio-ink?***

4.1 Introduction

Biofabrication is the design of biologically functional products through bioprinting or bioassembly (Grollet et al, 2016), essentially creating 'living' products. Traditionally, to create a biomaterial, a porous material was created where the cells were mixed into (Dalton et al., 2009). Although it is technically a biomaterial, this simple technique does not allow a precise placement of cells in a controlled manner.

To have more control over the shape of the cell framework, and have a homogeneous distribution of cells, additive manufacturing (AM) techniques are implemented.

Thus bioprinting was introduced: a computer aided process where a digital file is used as a blueprint to print an bioconstruct layer by layer in a prescribed shape. The bio-ink can be stabilized or crosslinked during or immediately after printing to generate the final shape (Gungor-Ozkerim et al, 2018).

In the next paragraphs, the bioprinting process is explained in more detail.

4.2 Direct ink writing

The additive manufacturing or 3D printing technique which is used for bioprinting with microalgae cells is Direct Ink Writing (DIW). The term is defined by Wan et al (2020) as "extrusion through a nozzle under pressure, utilizing a computer-controlled robot to move the dispenser filled with printed ink to construct shapes layer-by-layer". A long hydrogel strand is dispensed on a building platform by a force: either pneumatic, piston-or screw-driven. See Figure 17.

The main advantage of direct ink writing is that it allows thick filaments to be extruded without heat (Lewis, 2006). The filament retains it shape, so that it can be post-processed. Due to the high shape retention, complicated structures can be achieved. Necessary for shape retention is a dispensing material with the correct characteristics, such as a high viscosity (Malda et al, 2013). See pharagraph 4.4 for more in-depth explanations on material properties. Moreover, the fabrication speed is high in comparison to the other methods and the working principle is relatively simple (Lewis, 2002). A printer can be built by labs themselves with a three-axis platform, computer, and dispenser.

The disadvantages of the method are that it can reach a resolution of 200 µm, lower than laser- or inkjet-based systems (Malda et al, 2013).

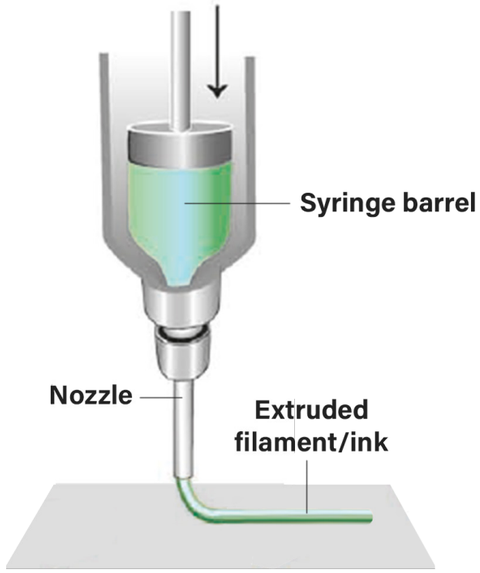


Figure 17. Schematic illustration of direct ink writing. Image derived from Malda et al (2013)

4.3 Bio-ink definition

A bio-ink is a soft biomaterial loaded with living cells (Hospodiuk et al, 2017), used for 3D printing. In our case, the living cells in the bio-ink are algae cells. For the 3D printing of living cells, two types of bio-inks can be made (Ozbolat, 2015). In scaffold-based bio-inks, cells are loaded in a hydrogel which simulates the natural cell environment. Scaffold-free bio-inks print the cells itself, mimicking embryonic development. For the 3D printing of algae cells, scaffold-based hydrogels are used since this process has high cell-viability and is affordable and scalable. (Hospodiuk et al, 2017)

Hydrogels are jelly-like materials in which the liquid component is water. The function of the hydrogel is twofold. First, it physically constrains the cells from escaping to the outside environment by encapsulation (Stanton et al, 2015). According to Lesser and Miller (2001), 'the cell encapsulation allows for precise control over cell attachment and the spatial distribution of the cells and biomolecules within the scaffold'. Secondly, since hydrogels have a high water content and porosity, they allow the encapsulated cells to receive nutrients and remove waste (Stanton et al, 2015). By encapsulating the cells in an aqueous 3D environment, the natural extracellular matrix of the cells is simulated and the cell growth is stimulated.

A general overview of the bioprinting process can be seen in Figure 18. The cells are combined with one or more natural or synthetic polymers to form the bio-ink and printed onto a surface, where the solution is gelled by crosslinking. The curing can be done by polymer crosslinkers, photo activation, or thermal activation while leaving the cells intact and viable.

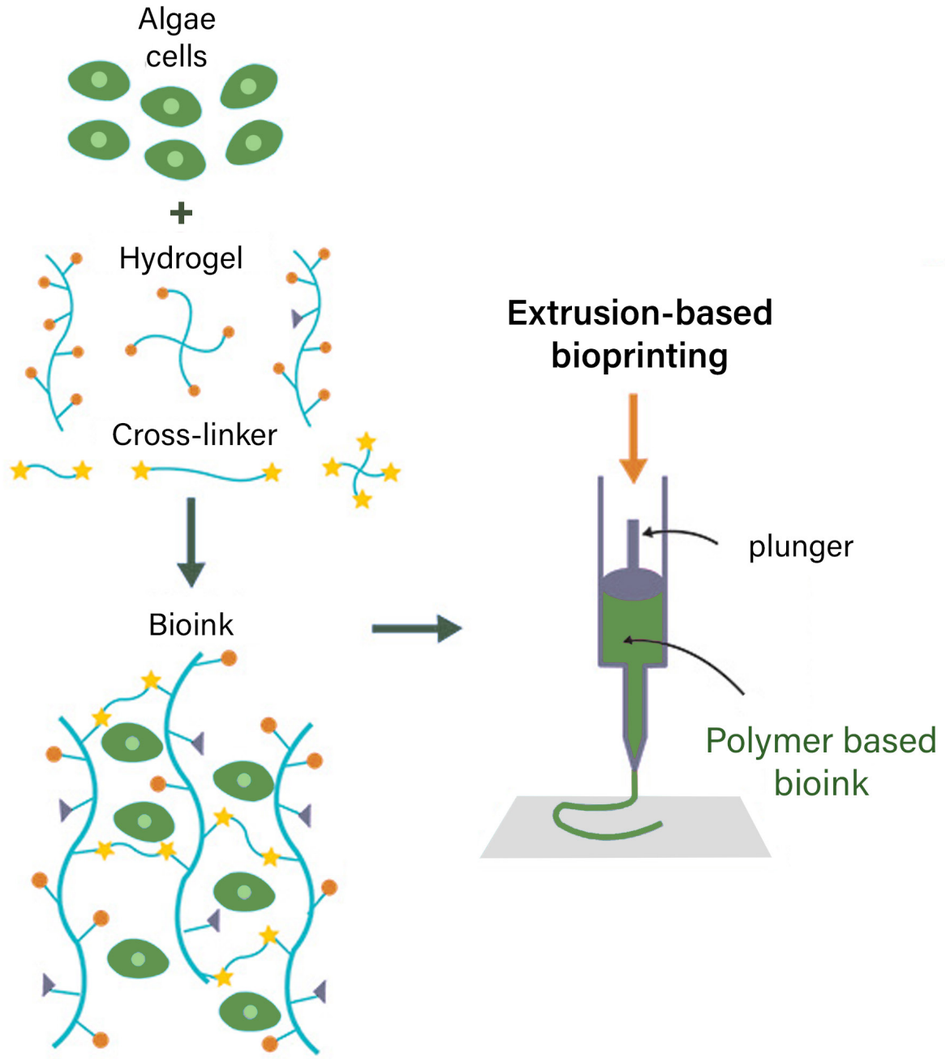


Figure 18. Overview of the bioprinting process. (Merck, n.d.)

4.4 Factors influencing bio-ink performance

A bio-ink 3D print requires two main functions: good cell function and high print fidelity. The hydrogel print should facilitate migration, proliferation and differentiation of the algae cells (Hospodiuk et al, 2017). Additionally, the print requires complex, high resolution prints with good shape stability (Malda et al, 2013). These properties proved in earlier research to be opposing: high printing fidelity results in less cell function and vice versa (See Figure 19).

Traditionally, the printability of hydrogels is improved by making the material more stiff. Either by increasing the polymer concentration, or crosslink density (Khalil & Sun, 2009; Tirella et al, 2009).

However, according to DeForest and Anseth (2012), cells thrive best in an environment in which their migration, growth and differentation is not limited by a dense polymer network. Cells can survive best in hydrogels with a low crosslink density, which allows for the diffusion of nutrients and wastes (Tirella et al, 2011). Unfortunately, hydrogels with a low crosslink density lack the ability to maintain their shape during 3D printing, resulting poor mechanical properties and low shape fidelity (Malda et al, 3013). Therefore, medium crosslinked hydrogels are used which compromise both on biological and fabrication properties.

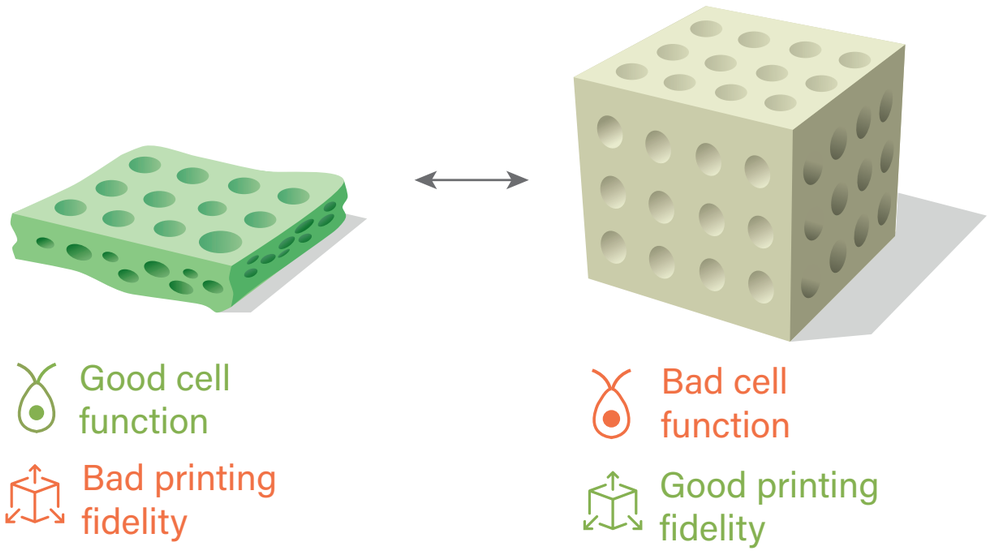


Figure 19. Illustrated trade-off between cell function and printing fidelity

Malda et al, (2013) described six parameters which influence the two main functions of the bioprint: gelation, network properties, fabrication time and shear stress influence the cell function, and nozzle gauge, viscosity and gelation influence the printing fidelity. See Figure 20.

As a designer, there are three variables which can be influenced. The type of hydrogel selected, the nozzle size and the fabrication time.

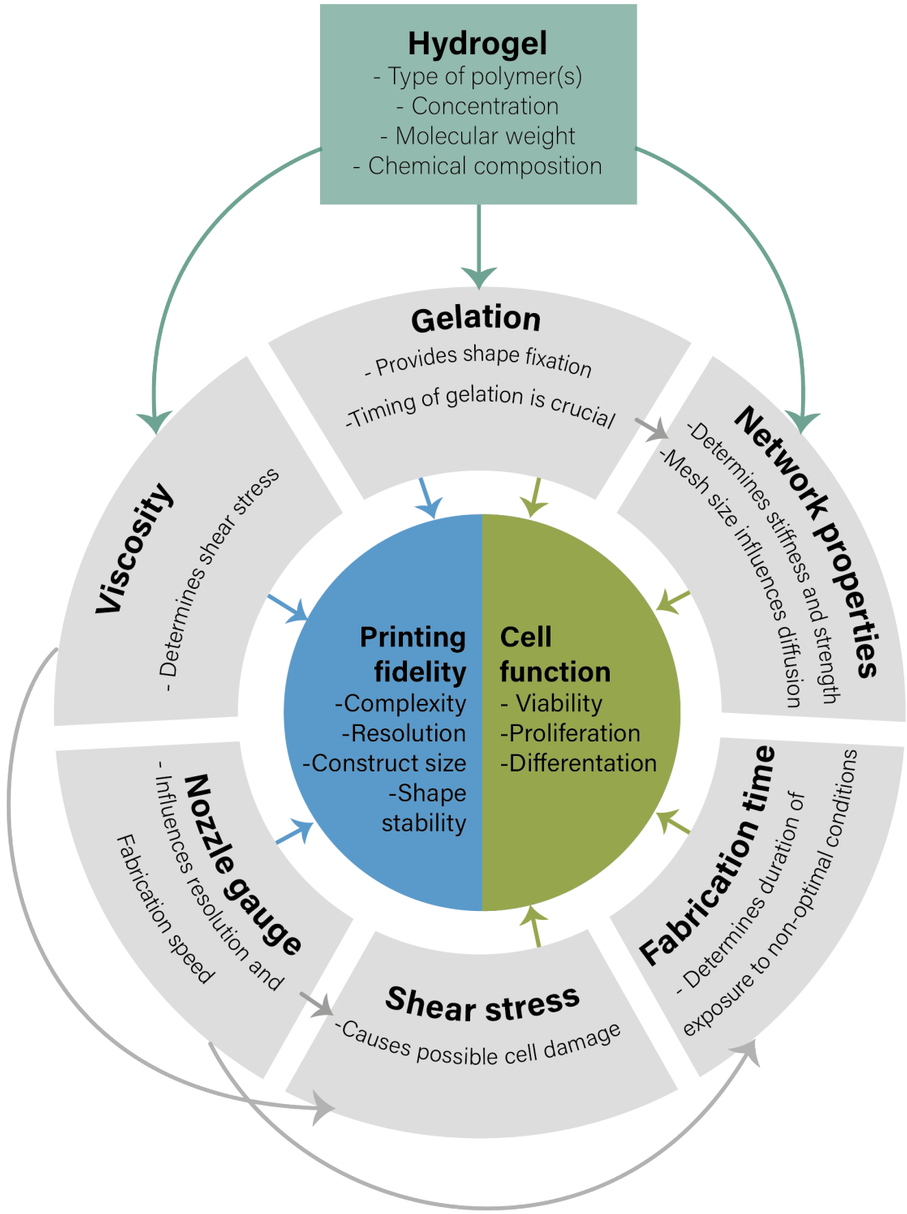


Figure 20. Variables influencing the main outcomes: printing fidelity and cell function. Malda et al, 2013.

1. The hydrogel selection

The type of polymer which is selected for 3D printing defines the viscosity, gelation and network properties of the ink, which in turn influences the printing fidelity and cell function.

Gelation is a form of solidification. It can be defined as the formation of a gel from a mixture of polymers (Mulder, 2000). Gelation is necessary to preserve the shape of the 3D print. The gelation is based on the type of hydrogel that is used. Gelation can influence the printing fidelity as well as the cell function. Less gelation provides less shape stability and complexity of the 3D print.

The viscosity is the resistance of a fluid to flow upon application to stress (Zhang et al, 2001). For example, water has a low viscosity, it flows easily. Honey on the other hand is highly viscous, and does not flow easily. There are two factors that determine the viscosity of a hydrogel: polymer concentration and molecular weight (Malda et al, 2013).

There are various design considerations when it comes to extrusion-based 3D printing of hydrogels, according to Wang et al (2018). First, the viscosity of the material cannot be too low or high. A sufficiently low viscosity is necessary to permit filament extrusion. On the other hand, the viscosity needs to be high enough to hold the shape of the 3D print upon deposition, or the shape needs immediate gelation to assure a stable hydrogel. Preferred is a hydrogel which allows the shape to be maintained over time. A risk of a viscosity which is too high, is that the cells are exposed to damaging shear stresses during extrusion.

2. Nozzle size

The nozzle size defines the resolution and the fabrication speed of the 3D print. A smaller nozzle size allows for more precise 3D printing, and thus a higher print resolution. A risk for a small nozzle is the increase of shear stresses between nozzle and filament, which could cause a negative effect on the survivability of the algae cells (Wang et al, 2018). Under equal print speed, a wider nozzle results in a faster print speed compared to a nozzle with a smaller diameter.

3. Fabrication time

Algae are sensitive organisms, which need certain light conditions, humidity and prevention of contamination. (For a more in-depth explanation of growth conditions, see Chapter 7.2.1). During 3D printing, these growth conditions may be suboptimal for the microalgae. Therefore, a shorter fabrication time is beneficial for the cell function of the microalgae 3D print. The fabrication time can be altered with the print speed or the selection of the 3D print shape. For the 3D print shape applies: less print volume results in less fabrication time.

4.5 Crosslinking mechanisms for hydrogels

Nearly all bioprinting methods use crosslinking after extrusion, since the bio-ink is not not self-supporting (Malda et al, 2013). Crosslinking is the formation of bonds to join two polymer chains together (Jenkins et al, 1996). Once the chains are linked, they become more rigid. Gelation is a form of crosslinking, specific for the formation of a gel (Oliveira & Reis, 2008). Branched polymers form links between the chains, forming one large molecule. At a point in the reaction called the gel point, the solution loses fluidity and the viscosity increases rapidly (Odian, 2004). The large interconnected polymer is called the gel. Gelation is necessary for the 3D print to preserve its shape (Malda et al, 2013).

A crosslinking agent can tie together carbon atoms from different chains of the polymer. The density of the gel and thus the percentage of polymer chains determines the degree of crosslinking (Tolinski, 2015) .

There are numerous crosslinking methods, which can be seen in Figure 21. Not every crosslinking method is suitable for microalgae. For instance, excessive heat or UV light could be harmful to the cells (Balasubramanian, personal communication 2021). Most often, ionic crosslinking is used for microalgae 3D prints, since it does not hinder cell survivability (Lode et al, 2015) (Zhao et al, 2019) (Johnston et al 2020).

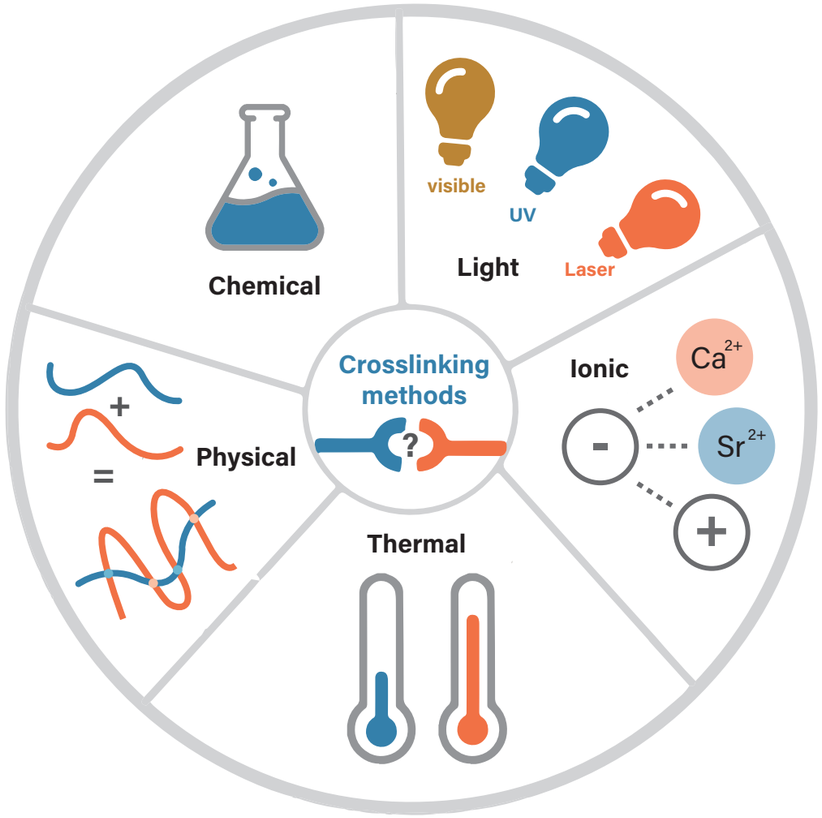


Figure 21. Various crosslinking methods that have been used for 3D bioprinting of hydrogels (GhavamiNejad et al, 2020).

4.6 Bio-ink requirements

The findings on microalgae, bioprinting and crosslinking are summarized in a list of key requirements, which the optimized bio-ink must meet. If the bioprint does not have sufficient cell function or print fidelity, it could be because one of these requirements is not met.

For this project specifically, it was important that the bio-ink could be made and 3D printed with the equipment available at the TU Delft, and could be developed within the time frame of this project, 24 weeks. The following six key requirements were found:

1. Non-toxic
The cells should be able to survive in the ink, otherwise they cannot perform photosynthesis.

2. Insoluble in cell culture medium
The cells should be able to be replenished with nutrient media, without damaging the 3D print shape. Moreover, the bio-ink should prevent the algae cells from escaping, due to risk of algal blooms.

3. Liquid at room temperature
The ink should be able to be extruded through a 3D print nozzle at room temperature.

4. Viscous
This enables the print to retain its shape without spreading post-print.

5. Shear thinning
The shear forces make the bio-ink extrudable by aligning the polymers in a favourable direction.

6. Low adhesion and surface tension
This elaminates the attachment of the bio-ink to the nozzle tip .

CHAPTER 4

4.7 Conclusion

Direct ink writing is the common method to design a biologically functioning product with an additive manufacturing technique. This 3D printing method allows thick, viscous bio-inks to be printed layer by layer without the use of heat, which makes it a cell-friendly approach for microalgae (Lewis, 2006). Since the filament retains its shape after printing, the bioprints can be post-processed. A disadvantage of direct ink writing is that the resolution is lower compared to other laser- or inkjet-based systems (Malda et al, 2013).

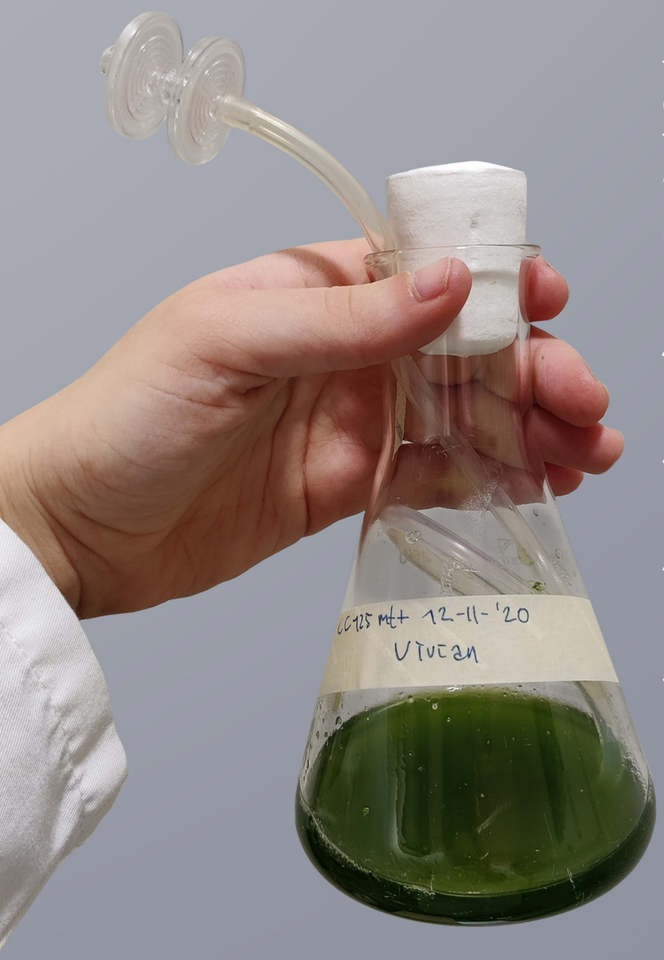
To stabilize the shape of the bio-prints for longer time periods, crosslinking can be performed which joins the polymer chains in the bio-ink, making the material more rigid (Jenkins et al, 1996). The most common method for crosslinking in microalgae bioprinting is the use of ionic crosslinking, since it is cell-friendly (Lode et al, 2015) (Zhao et al, 2019) (Johnston et al 2020).

The two main factors which determine the performance of the 3D printed bioconstruct are the cell function and print fidelity. A high cell function means that the microalgae cells are able to migrate, differentiate and proliferate in the bio-ink, making it a good environment to host the cells (Hospodiuk et al, 2017). Good print fidelity means that the bioprinted shapes have a high resolution and good shape stability, during and after 3D printing (Malda et al, 2013).

Designers can influence the cell function and print fidelity by altering the bio-ink composition, and changing 3D print parameters such as the nozzle size and fabrication time. For the bio-ink, six key requirements were summarized which are essential for a good bio-print performance. The ink should be non-toxic, insoluble in cell culture medium, liquid at room temperature, viscous, shear thinning and should have low adhesion and surface tension.

Chapter 5

Material proposal



This project starts with a 3D printed algae material developed by scientists of the Technical University of Delft. In this chapter, the components and characteristics of the material are explained. The following research questions are addressed in this chapter:

1. ***What is the appearance of the 3D printed algae material and how does it behave?***
2. ***Which steps in the material process will be altered, and which steps will stay the same?***
3. ***Which growth conditions need to be met for maximum algae growth?***
4. ***What are the advantages and disadvantages of the bio-ink composition?***
5. ***What are the advantages and disadvantages of the current 3D printing and crosslinking technique?***

5.1 Introduction to the bio-ink

The Aubin-Tam research group created a material containing living cells which can be 3D printed, called an bio-ink. The bio-ink consists of microalgae cells in an hydrogel made of alginate. Once it is 3D printed, the ink solidifies instantly upon contact with a surface containing calcium. A 3D printed sample can be seen in Figure 22.

The 3D printed structure needs a regular supply of nutrients and water to sustain the algae cells. The nutrients that the algae cells receive is a TAP solution. In the following paragraphs, more detailed explanation is given on the process, components and requirements of the material.

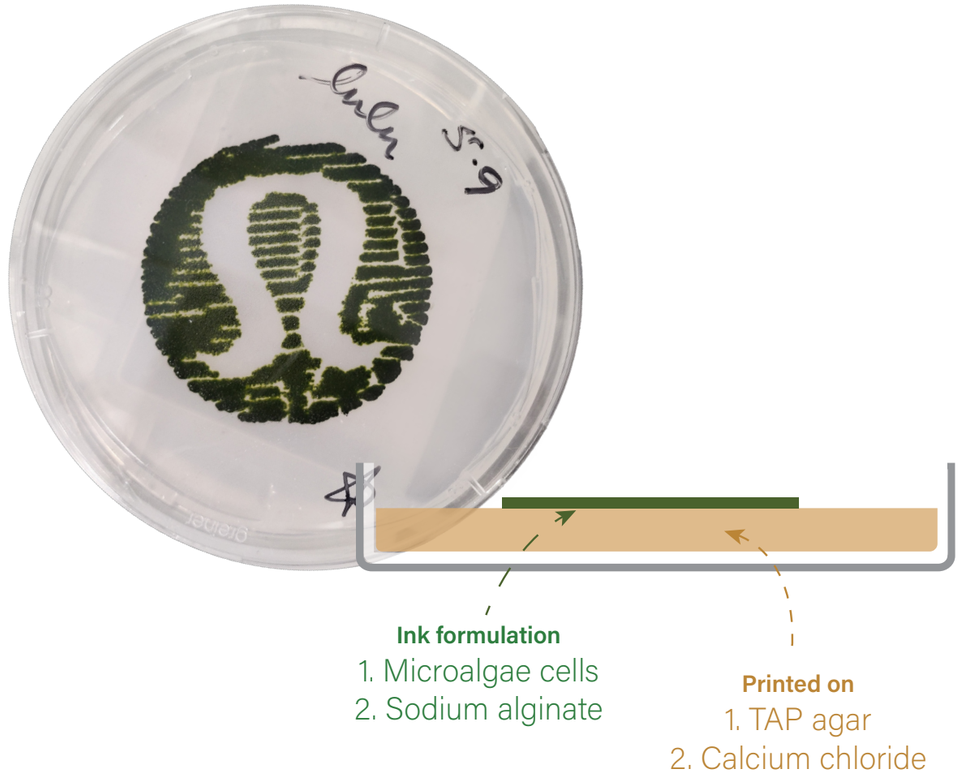


Figure 22. Microalgae 3D print of the Aubin-Tam group of the TU Delft

5.2 Material appearance and behaviour

The alginate bioprints are liquid during 3D printing, and solidify once they are in contact with the agar plate which contains calcium chloride. The bioprinted material has enough mechanical strength after crosslinking to be self-standing, and can be detached and reattached to different surfaces. In Figure 23, the print is attached to a piece of bacterial cellulose. The colour changes from an off-white to a transparant dark green colour in seven days. Specific measurements on cell function and mechanical properties of the bioprint cannot be disclosed, since this is classified information which is yet to be published by the researchers.

Figure 23. Alginate based microalgae 3D print, attached to bacterial cellulose (Balasubramanian, et al, 2021).

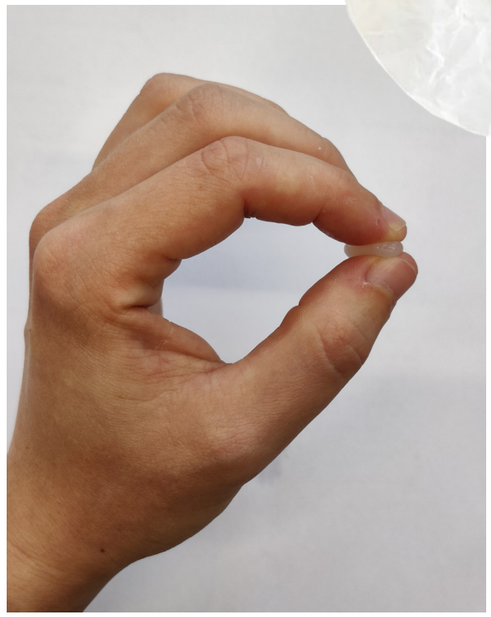


Figure 24. Sodium alginate, crosslinked with calcium chloride (5M) canbe deformed easily.

A maximum print height of six layers could be reached, around 0.5 mm in height. The alginate ink, once crosslinked, can be compared to jelly or cooked spaghetti. See Figure 24. It can be deformed and squeezed, and can easily be cut in half with a blunt tool such as a spoon.

5.3 Process overview

The process which is currently used to prepare and print the microalgae ink is summarized in Figure 25.

For a more detailled explanation, including quantities, see Appendix C.

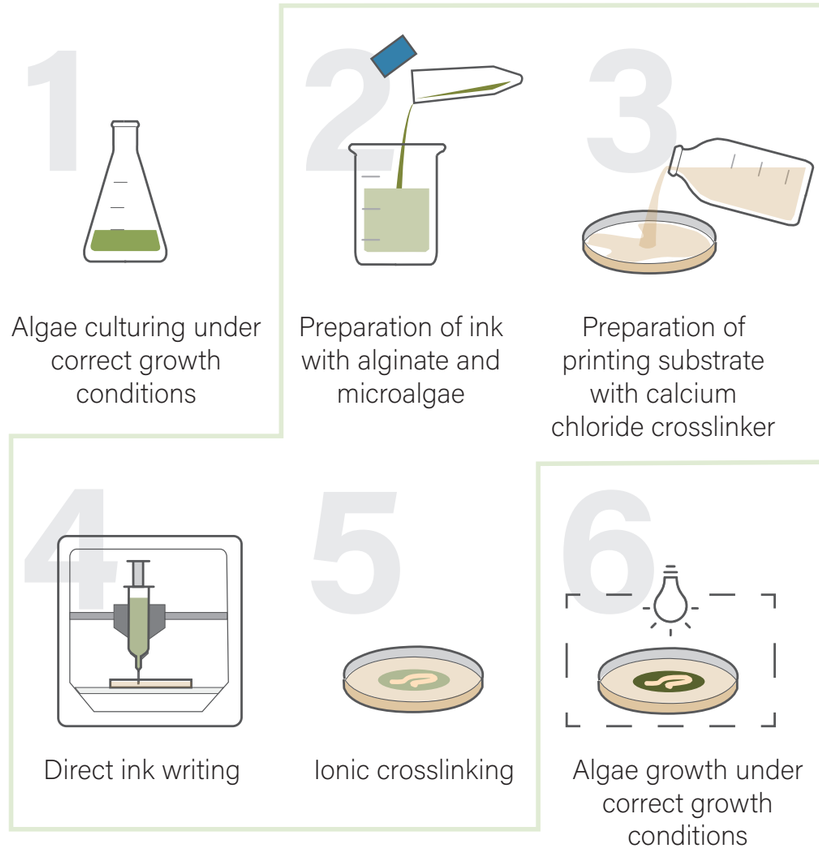


Figure 25. Overview of the current algae printing process carried out by the Aubin-Tam group and the steps in green which will be redesigned.

Due to years of experience with the Chlamydomonas microalgae, the Aubin-Tam research group optimized the culturing and growth conditions of the microalgae. These conditions will not be optimized further. Thus, step 1 and step 6 remained the same. These steps will be explained in the next paragraphs.

The ink composition, 3D printing method and the crosslinking method (step 2 to 5) were optimized in this project.

5.4 Algae culturing

The approach to grow algae was copied from the Aubin-Tam group during this project. For the bio-ink solution, algae cells need to be grown and mixed with the alginate. The growth can be measured by inspecting the amount of algae cells and the size of the cells. There are two ways for algae to increase their number of cells: by asexual reproduction or sexual reproduction. In asexual reproduction, the algae cell basically clones the mother cell to create daughter cells in a process called mitosis. If there are two types of cells with a different mating type in a mixture, sexual reproduction can occur. For this project, Chlamydomonas with mating type mt+ were used. An overview of the reproduction can be seen in Figure 26.

The growth of microalgae cells is in theory defined as the cell volume, and the amount of dry matter, total protein and RNA content (Zachleder et al, 2019).

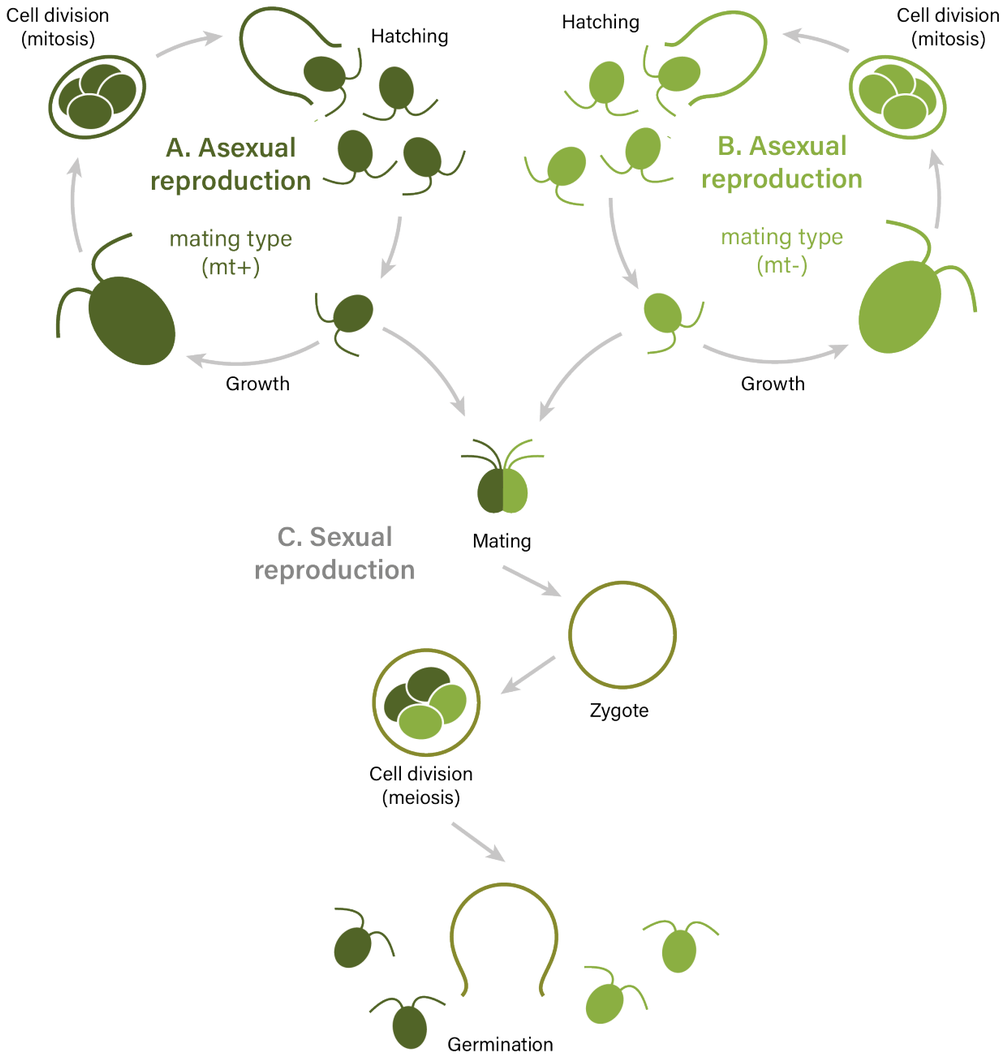


Figure 26. Simplified reproduction of Chlamydomonas. Content from Hallman, 2011.

Algae are able to grow in a culture: an artificial environment which optimizes the algae growth, resembling the natural environment of the algae. The culture consists of three components according to Probert and Klaas (1999):

1. A culture medium contained in a suitable container
2. The algal cells growing in the medium
3. Air, to allow exchange of carbon dioxide between medium and atmosphere.

During visual inspection, the growth of the cells in the culture can be examined by the intensity of the green colour, see Figure 27.

Although most microalgae grow through photosynthesis, by deriving energy for growth from sunlight, some microalgae strains such as Chlamydomonas are able to use organic substrates from their surroundings as a carbon and energy source (Khan et al, 2016). This process is called heterotrophic growth, meaning that the microalgae grow in the dark. Chlamydomonas can do both photoautotrophic as well as photoautotrophic growth, depending on the type of environment.

There are two methods to measure the increase in biomass: either by counting the cells in a sample under the microscope or measuring the optical density of the culture. The optical density measures the ability of an object to slow or delay the transmission of light (Sutton, 2011). In seven days, a culture of 100 ml as can be seen in Figure 27 can reach a cell density of 1,000,000 cells. In order to ensure an adequate increase in biomass, the culture needs to be grown under certain conditions. A summary of the parameters can be found in Figure 28.

Figure 27. Cell growth of C. reinhardtii CC124 (mt+) in seven days

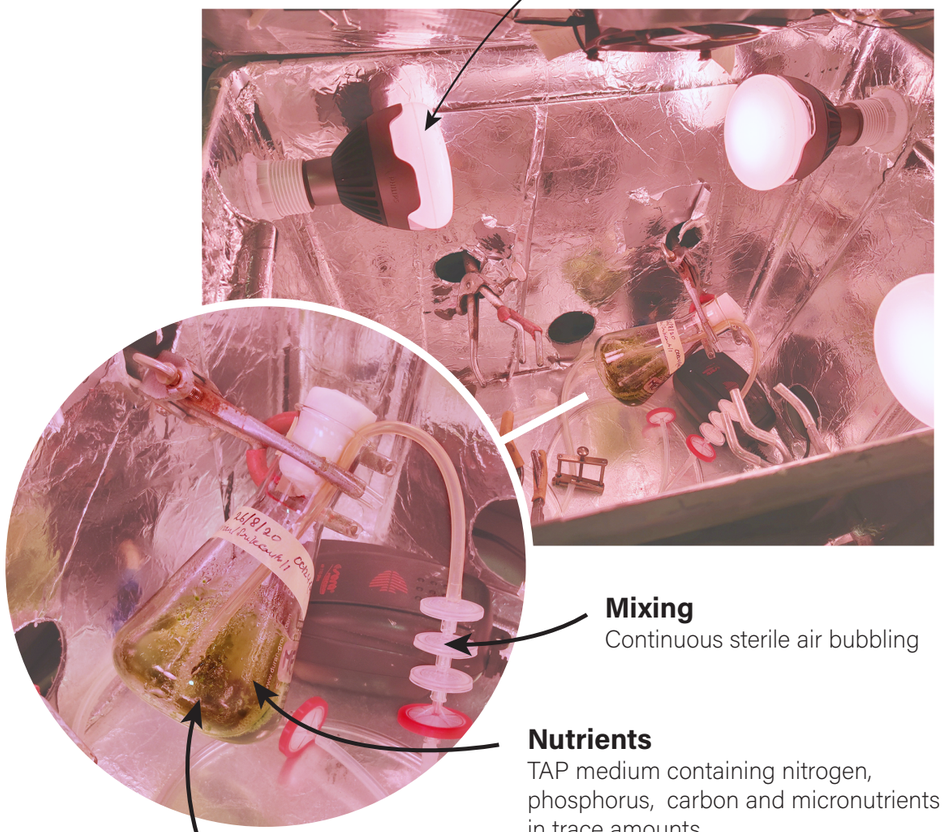


Figure 28. Algae culturing parameters according to Khan et al, 2018

The growth factors are explained in more detail on the next page.

Temperature

Optimal temperature range of most laboratory species of *C. reinhardtii* is between 20-25 °C (Khan et al, 2018). Outside of this range, cell division and growth is still possible, however limited. If the microalgae grow in suboptimal temperatures, there are less cell divisions, leading to a decline in the amount of cells. The cell growth is less affected by the temperature (Zachleder et al, 2019). If temperatures reach below 15 °C or above 35°C, the cells will perish (Harris, 1989). An increase in temperature between the range of 20-24°C is proven to accelerate growth and shorten the cell cycle (Vítová et al, 2011).

Mixing

Recommended is to shake or stir the cultures, to aerate the microalgae. If the microalgae have more access to oxygen, they are proven to grow faster (Mohsenpour & Willoughby, 2016). The Aubin-Tam group managed this by bubbling the algae culture. Another advantage of mixing is to prevent shading in the interior of the glass flask, so that more light can reach the cells (Harris, 1989).

Light

Algae cultures should not be exposed to direct sunlight, since this causes photopigment damage (Harris, 1989). For experimental purposes, fluorescent light bulbs are commonly used. Microalgae are proven not to grow well under constant illumination, so generally light/dark cycles are used (Harris, 1989). The Aubin-Tam group used a photoperiod of 12:12 light:dark with a light intensity of 20 mol m-2 s-1.

Nutrients

The algae cells need specific components to grow well. There are a few standard growth media developed. A standard medium which is used by the Aubin-Tam group to grow *C. reinhardtii* is Tris-acetate-phosphate medium (TAP). The medium contains nitrogen, phosphorus, carbon and micronutrients in trace amounts. The tris buffers the pH (Harris, 1989). The ingredients are described in more detail in the protocol of Gorman and Levine (1965). How TAP medium is made can be read in Appendix A.

Salinity and pH

Salinity is one of the most influential parameters for algae growth (Yeesang & Cheirsilp, 2011). The Aubin-Tam group used a pH of 7 for the microalgae.

5.5 Components

The current bio-ink consists of algae cells and an alginate hydrogel, printed on a substrate of agar, calcium chloride and nutrients. Each of the components are explained below.

5.5.1 Microalgae

For this project, the most widely used laboratory species *Chlamydomonas reinhardtii* was chosen, a genus of unicellular green algae. The strain was purchased from Chlamydomonas Resource Center, USA. The Aubin-Tam group has previous experience with this type of microalgae, and thus this organism was selected for this project.

Figure 29. C. reinhardtii in a culture medium. The individual cells cannot be seen with the naked eye.

Function
C. reinhardtii cells are oval shaped, 10 µm in length and 3 µm in width (Rochaix, 2013), and thus cannot be seen with the naked eye. (See Figure 29). An illustration of the cell can be seen in Figure 30. These are primitive organisms with a simple cellular structure. Algae in this genus have a chloroplast, comparable to an eye which perceives light and two anterior flagella with which they can swim towards a light source (Höög et al, 2014).

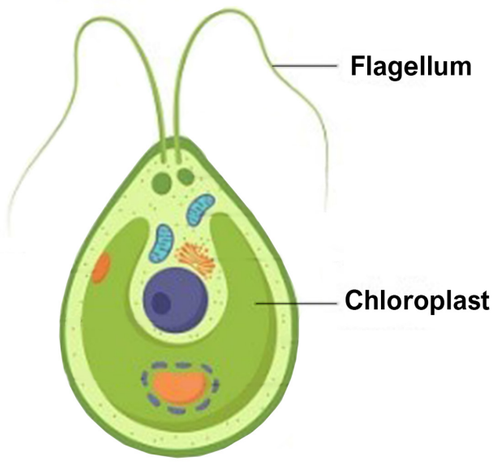


Figure 30. Systematic representation of Chlamydomonas with chloroplast and flagella

The advantages of *C. reinhardtii* over other microalgae are that they are robust: in general they can tolerate a wide range of temperatures, salinities, pH values and light intensities (Barsanti, 2008).

Microalgae grow much more rapidly than classic plant models (Salomé & Merchant, 2019). Under optimal conditions, *C. reinhardtii* grows so rapidly that its numbers can double approximately every eight hours (Harris, 2001). Chlamydomonas have a large surface to volume body ratio, which gives them the ability to take up a large amount of nutrients (Mandall and Mallick, 2014). Moreover, because the cells grow in aqueous suspension, they have more efficient access to water, CO_2 and other nutrients.

5.5.2 Sodium alginate

The other component of the current bio-ink is alginate. The term alginate is used for the salts of alginic acid, but it also refers to all derivatives of alginic acid and alginic acid itself (Alba & Kontogiorgos, 2018). Typically it is obtained from the cell wall of brown seaweed and thus biobased.

The hydrogel is popular in bioprinting processes due to its biocompatibility, low toxicity, low price, ease of use and mild gelation condition (Lee & Mooney, 2012; Jia et al, 2014). Their structural similarity to extracellular matrices of living tissues makes it a popular choice for applications in bioprinting (Lee & Mooney, 2012).

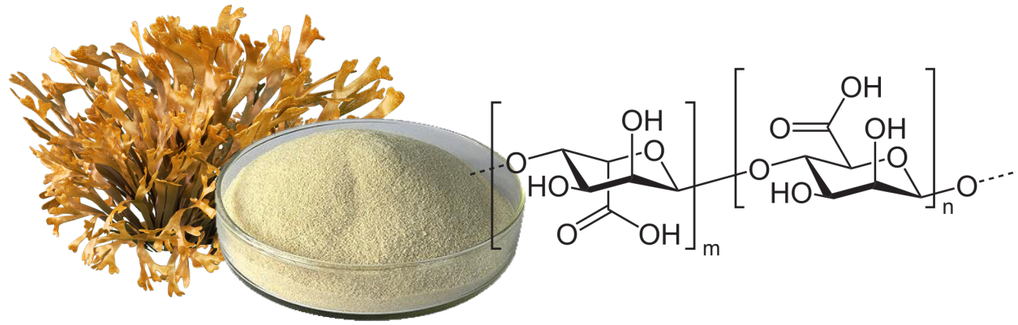


Figure 31. Appearance of sodium alginate and the molecular structure

Function
Alginate is necessary in the bio-ink to encapsulate and protect the algae cells. The polymer consists of two block structures: β-d-mannuronic acid (block M) and α-l-guluronic acid (G block), which are linked by α-(1, 4) glycosidic bonds (Alba & Kontogiorgos, 2018). The chemical structure can be seen in Figure 31. The G subunits are responsible for the forming of the gel phase in alginate, the degree of gelation increases as the number of G units increases.

From experience, the Aubin-Tam research group found that the maximum concentration of alginate in the bio-ink is 5%, more can not be dissolved with the algae cells.

Crosslinking
Alginate can cure or solidify by crosslinking, where two polymer chains join to form one big molecule. In the presence of metal cations, Ca^{2+}, Fe^{2+}, Mn^{2+}, and Al^{3+}, the carboxyl groups crosslink (da Silva Fernandes et al, 2019). In our case, Ca^{2+} ions are used to crosslink the alginate material. According to Phan et al (2013), the sodium ions, Na^{+}, dissociate from the alginate when dissolved in a liquid solution. If calcium chloride, $CaCl_2$, is added, the double charged calcium ions can bind two different alginate strands simultaneously which solidifies the solution. See Figure 32. A more solid gel is created if the calcium concentration and the soaking time is increased.

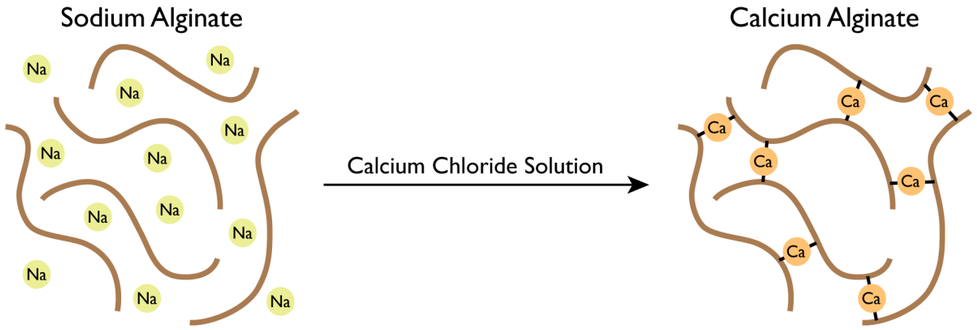


Figure 32. Crosslinking or solidifying of sodium alginate under influence of calcium chloride

5.5.3 Agar with TAP and calcium chloride

Microorganisms are most commonly grown on a petri dish containing solidified agar and nutrients (Zohuri, 2012).

Agar is a creamy white powder, derived from red seaweeds, which can be soluble in water. Agar acts as a gelling agent, and can be used for cooking. See Figure 33.

Figure 33. Japanese dessert made with agar

The advantage of agar is that is liquid at 80 °C and solidifies at 45 °C (Armisen & Gaiatas, 2009). The agar is poured in petri plates while hot, and solidifies to form a jelly surface on which can be 3D printed. This process is reversible with application of heat. Hence, the gel is thermoreversible, meaning that the gel becomes liquid again upon the impact of heat (Li, 2002).

On plain agar, the 3D prints can survive 7 days. By adding the Tris-acetate-phospate medium which is used to culture the microalgae cells to the agar, the cell life can be further extended up till a month.

Lastly, to be able to crosslink the bio-ink, calcium chloride needs to be added to the TAP agar plate. The caclium ions in the plate will react with the sodium alginate to form calcium alginate. The concentration of calcium chloride in the petri plate is 0.05M.

5.6 3D printing and post-processing approach

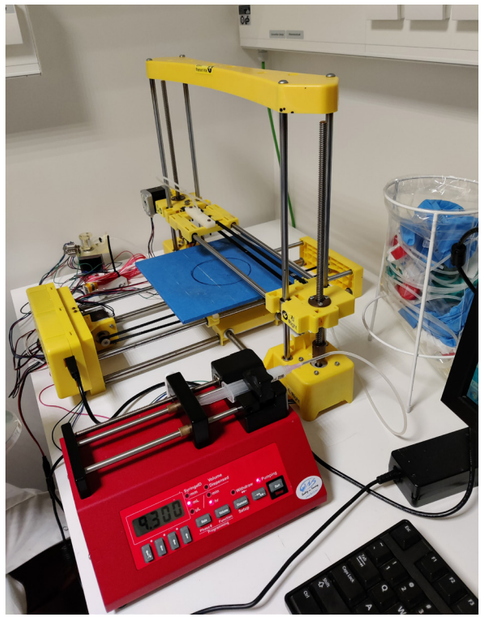


Figure 34. Colido DIY printer used by the Aubin-Tam research group

The material proposal was 3D printed on a Colido DIY printer. The extruder and heater of the standard bioprinter were replaced with a 0.2 mL pipette tip, a silicone tubing system (1 x 1 mm), and a syringe pump. See Figure 34.

Advantages of the 3D print technique is that it is cheap and accessible, the printer can be built for less than 250 US dollars. Disadvantages of the setup are that the printer is not compatible with thicker and more viscous liquids. The ColiDo software is not user friendly, since desired shapes need to be manually programmed with G-codes. For faster prototyping, a different 3D printer should be found.

The 3D prints are crosslinked on petri dishes containing 25 mL of $CaCl_2$ (0.05M). Limitation to this technique is that the crosslinking mechanism is limited to the bottom layers of the bioprint. After more than six layers, the crosslinking agent can no longer reach the alginate. This negatively impacts the print resolution and achievable height of the bioprint.

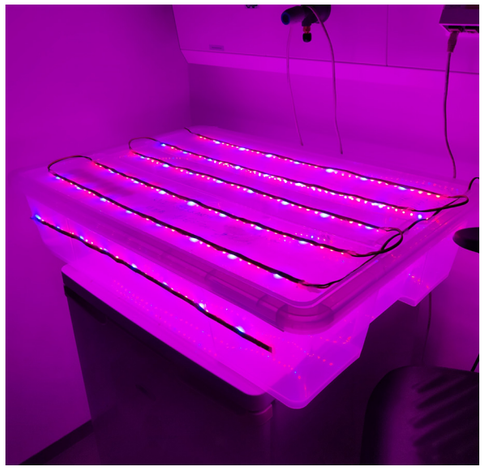


Figure 35. Incubator where microalgae bioprints are placed after they are 3D printed and crosslinked.

Once the ink is 3D printed, the structure can stay alive for two weeks in a sterile lab environment. The samples are placed in an incubator with a light/dark (12:12 hours) cycle. See Figure 35. The LED strip has red/blue lights in a ratio 4:1 with a light intensity of 23 µmol m-2 s-1.

No tests have been conducted with the bio-ink in ambient conditions. However, the research group expects that the bio-ink will have contaminations.

CHAPTER 5

5.7+ Conclusion

The material proposal which was optimized in this project is an alginate-based bio-ink with immobilized microalgae cells of the strain *C. reinhardtii*. The bio-ink was printed with an adapted CoLiDo DIY printer on a plate containing agar, nitrogen, phosphorus, carbon and a crosslinking agent, calcium chloride, where it solidified immediately upon contact.

The material was experienced as a gel-like substance, which could be handled and transferred to different surfaces. The mechanical strength of the bioprint was low, the material could be easily torn apart or cut.

Microalgae cells clone or sexually reproduce under certain growth conditions. This increase in biomass is considered as algae growth. In a week time, a 100 mL solution containing algae cells could reach 1 million cells per mililitre. This was achieved by culturing the cells in a temperature of 20-30 degrees, in a carbon-supplemented medium with a pH of 7. The culture was bubbled continuously and placed under fluorescent light in a light/dark cycle of 12h / 12h for seven days.

The bio-ink formulation showed a good cell function, since the cells could survive three days without nutrients and could survive for more than a month on the nutrient plates. However, the print fidelity was lacking. Up till six layers in height could be achieved, which limited the possibilities for the design of interesting macro-porous inner structures. The limited print fidelity was caused by the bio-ink composition, which was not viscous enough, and the crosslinking approach. In order for the bioprint to have higher print fidelity, nutrients and a crosslinking agent needed to be incorporated in the ink formulation rather than the printing substrate.

Chapter 6 | Exploration

This chapter explores the possibilities of different ionic crosslinking approaches and experiments with a new bio-ink formulation. The chapter concludes with a selection for an optimization approach.

Questions answered within the exploration chapter:

1. ***Which extrusion based cell printing methods are commonly used?***
2. ***What are the advantages and disadvantages of each method?***
3. ***Which components can be added to the bio-ink to increase the viscosity, without negatively affecting the cell function?***

6.1 Introduction

The focus on the experimentation is twofold: how to improve the bio-ink composition and how to crosslink the 3D print. See Figure 36. Two rounds of experiments were done simultaneously. First, methods were explored to ionically crosslink the material. Secondly, a new ink composition was tested.

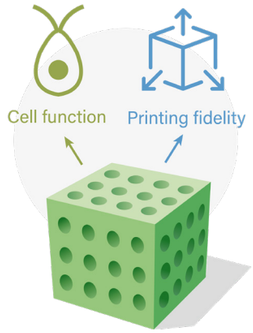
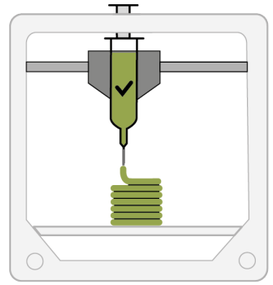


Figure 36. Two focus points in the project goal: improved cell funtion and print fidelity

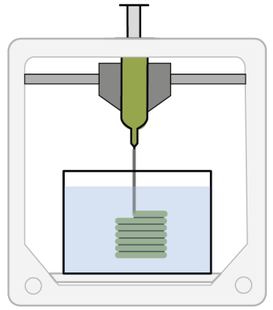
6.2 Testing extrusion based 3D printing methods

Four possible methods were selected which have been used for extrusion based cell printing: direct ink writing with a viscous ink, bath-assisted printing, aerosol spraying (GhavamiNejad et al, 2020) and double-extrusion (Puertas-Bartolomé et al, 2020). See Figure 37.

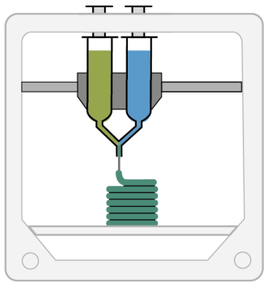
For each approach, a simple experiment was performed with the alginate ink without microalgae cells to test the feasability and possible disadvantages. The results from this exploration can be seen on the next pages.



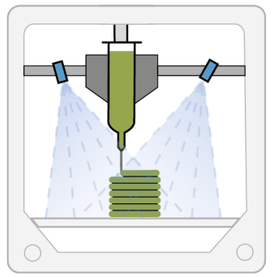
A. Increase viscosity and cure post-print



B. Print in a support bath with crosslinker

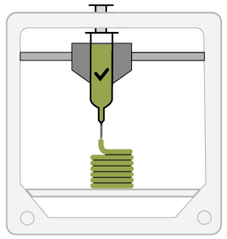


C. Double extrusion printing with crosslinker



D. Aerosol spraying of crosslinker while printing

Figure 37. Four crosslink methods suitable for bio-inks



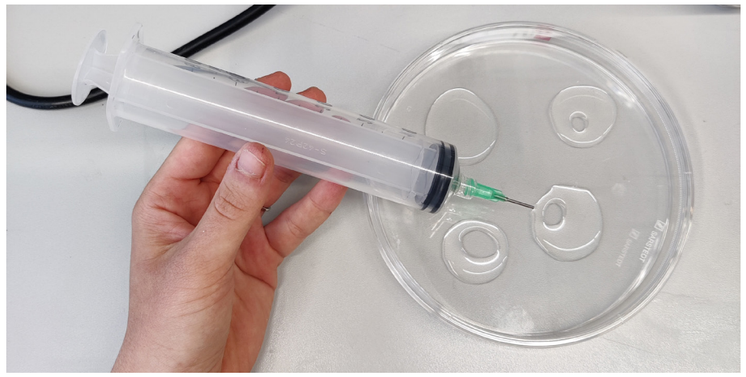


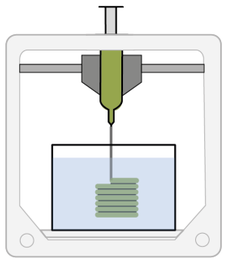
Figure 38. Experimenting with different viscosities of sodium alginate (2.5 w/v% and 5 w/v%)

Can the viscosity of the alginate ink be increased so that multi-layer constructs can be achieved?

The ink composition of the Aubin-Tam group was not sufficient to create a self-standing and viscous ink. The sodium alginate concentration was 2,5% and could be increased to a maximum of 5%, beyond which the concentration becomes too viscous to dissolve in water. (Balasubramanian, personal communication) Figure 38 shows that the highest possible viscosity of sodium alginate, does not retain its shape or allow multiple layers to be stacked on top of each other.

Conclusion

The alginate was not viscous enough and cannot be increased more, since it is at its maximum. For this method to work, one or more ink polymers should be added to increase the viscosity.



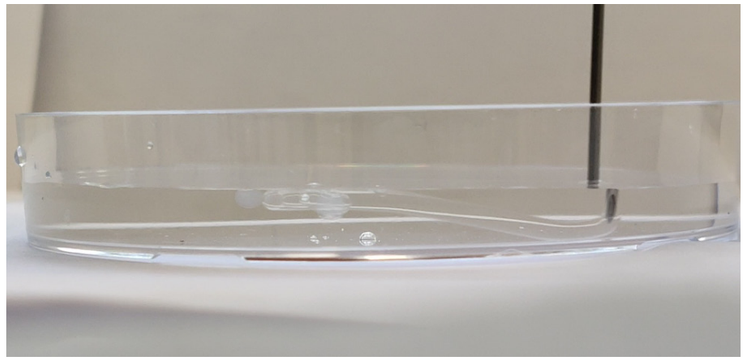


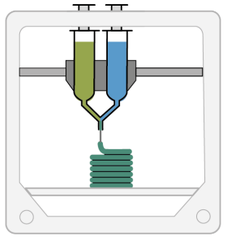
Figure 39. Extruding sodium alginate (5 w/v%) in a calcium chloride bath (5M)

Can multi-layer shapes be created in a crosslinking support bath?

Sodium alginate solidified instantly in a bath of 5M calcium chloride. Specific shapes could not be created, since the alginate did not stick to the bottom of the petri dish, but floated upwards. See Figure 39.

Conclusion

To be able to print in a crosslinker bath with an alginate ink, a more viscous bath could be selected. Commercial print baths are already available which are compatible with sodium alginate, for instance the SunP float from Biotech (SunP Biotech, n.d.).



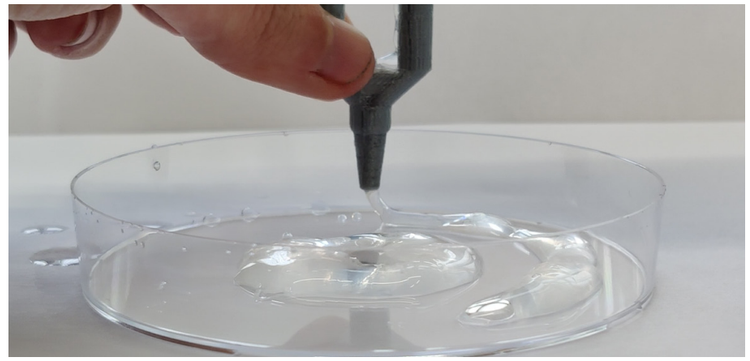


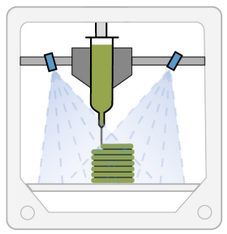
Figure 40. Extruding sodium alginate (5 w/v%) and calcium chloride (5M) simultaneously by using a static mixing nozzle (length 1 cm).

Can multi-layer constructs be achieved with a static mixing nozzle containing alginate and calcium chloride?

Different lengths of static mixing nozzles (8 cm, 5 cm, 1 cm) were tested, and all nozzles clogged quickly due to solidification in the nozzle. Compared to the sodium alginate, too much calcium chloride is extruded. See Figure 40.

Conclusion

In order to print a strand without clogging, less crosslinker should be added. This means that a system needs to be build that can control the extrusion speeds of the syringes seperately.



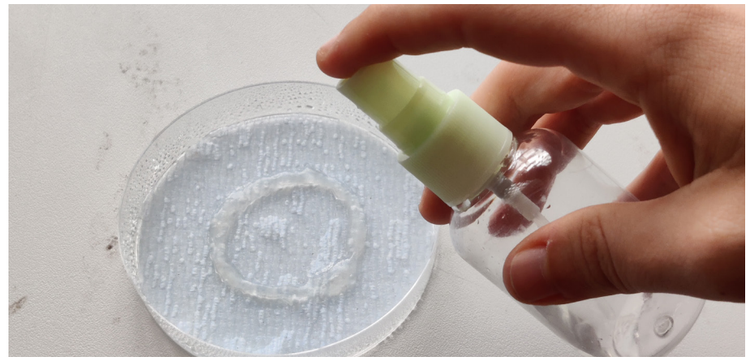


Figure 41. Extruding sodium alginate (5 w/v%) crosslinking it by spraying calcium chloride (5M) each layer.

Can multi-layer constructs be achieved by spraying crosslinker between layers?

With the current alginate composition, creating multiple layer-constructs with aerosol spraying is not possible. The ink flows out before it can be crosslinked. The outer contour is increasing in width, not in height. See Figure 41.

Conclusion

This method could work with a more viscous ink. Since there is no 3D printer at the TU Delft which allows aerosol crosslinking, a new system needs to be built. This method will be time intensive.

6.3 Testing a new ink composition

A new ink composition was made to find possible improvements on cell function and 3D print fidelity. For the selection of ink components, advice was given by Dr. Kunal Masania, head of the research group Aerospace Structures and Materials from the TU Delft. His team had positive previous experiences with an ink composition, compatible with plant cells. The ink composition was developed by Silvan Gantenbein.

The following components were added to the composition:

- Polymer A
- Polymer B
- Agar

The exact procedure for making the ink stock can be found in Appendix 7.1.

The ink was printed on the Colido DIY printer. (See FIgure 42 a and b) The 3D printer extruded with a flow rate of 3.3 mL / h. Four different inks were made to test the survivalibily of the microalgae in the new ink formulation, the ability to crosslink and the print fidelity. The bioprint gcode was manually programmed by Srikkanth Balasubramanian and Kui Yu.

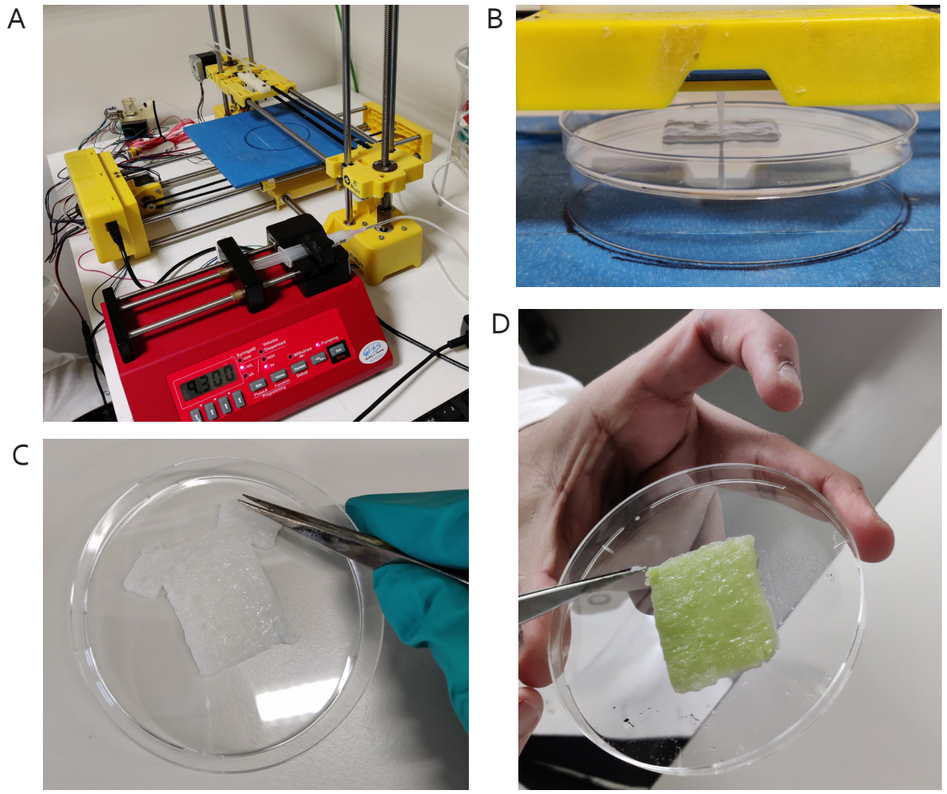


Figure 42. 3D printing of a new ink formulation including Polymer A, Polymer B and sodium alginate on a CoLiDo printer.

Four ink types were made:

1. **Control ink, crosslinked**
   20 mL ink stock + 2.5 mL TAP + 2.5 mL Sodium alginate (5 w/v%)

2. **Control ink, not crosslinked**
   20 mL ink stock + 5 mL TAP

3. **Ink with algae, not crosslinked**
   20 mL ink stock + 5 mL algae culture (1 x 10^6 cells/mL)

4. **Ink with algae, crosslinked**
   20 mL ink stock + 2.5 mL algae culture (1 x 10^6 cells/mL) + 2.5 mL Sodium alginate (5 w/v%)

The inks that included sodium alginate were ionically crosslinked on a agar plate containing $CaCl_2$ for an hour. Afterwards, they were removed with pliers and moved to an empty petri plate. (See Figure 42c)

The results can be seen in Figure 43.

All ink compositions could be printed easily, except for ink composition 4. The ink was too dense, and could not be extruded through the thin tubing of 1 mm diameter. After seven days, the inks with algae show a green colour. Compared to the colour guide from chapter 2.5, the ink which is not crosslinked is a shade 7 and showed a lot of cell growth, the ink which was not crosslinked a mere 5.

The prints showed improved viscosity, compared to the alginate bio-ink and could reach up till four layers in height.

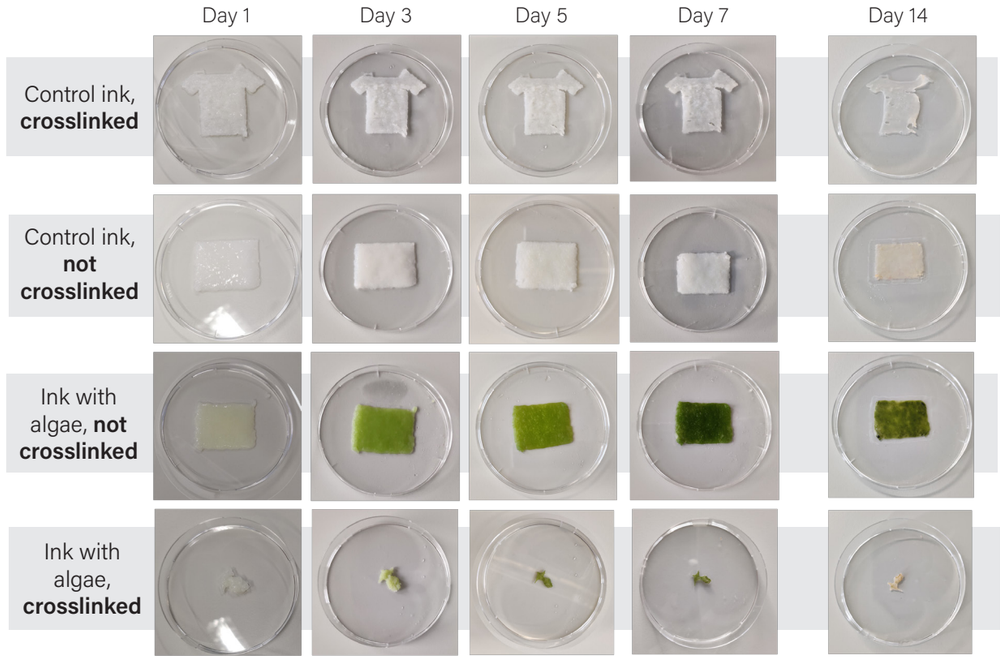


Figure 43. Results of experiment 12, addition of ink components to the material proposition of the Aubin-Tam group

6.4 Evaluation of extrusion based bioprinting methods and bio ink formulation

The exploration concluded with two decisions for the 3D printing approach of a micro-porous 3D structure. First, three additional ink components were selected: Polymer A, Polymer B and agar. Secondly, a selection for a 3D printing method was chosen: direct ink writing. The choices are explained below.

6.4.1 Ink composition

Addition of Polymer A, Polymer B and agar in powder form improved the ink properties tremendously. A more viscous ink was created compared to the material proposal which could be layered without flowing. The components were biocompatible with the microalgae cells, since they survived in the ink and showed growth over seven days. Morover, the ink can be crosslinked with $CaCl_2$ and showed structural stability.

One of the inks could not be extruded with the current Colido printer. The printer does not accomodate viscous materials, since the tubing is too thin. A new 3D printer should be selected so that denser inks can be printed.

6.4.2 3D printing methods

For the selection of a 3D printing approach, the deciding factor was feasability. Due to time constraints, the 3D printing should be possible with the current equipment available at the TU Delft. Although double extrusion and aerosoal printing are interesting 3D printing approaches which could accomodate high resolution prints,they need an adapted print head for crosslink spraying or a pump system which can control two syringes. This was not possible in the time frame of the project. Moreover, the prelimenary experiments showed low resolution prints.

Since a material was proposed by Dr. Masania with promising material characteristics for a high print fidelity, and a 3D printer was available at the TU Delft which could be used directly for this method, extrusion method A was selected. This method is the most feasable for the time frame of the project.Method B can reach high resolutions, and there are already commercial support baths available on the market which could be tested. However, adaptions are necessary to the printer to allow it to print in liquid. This is too time intensive.

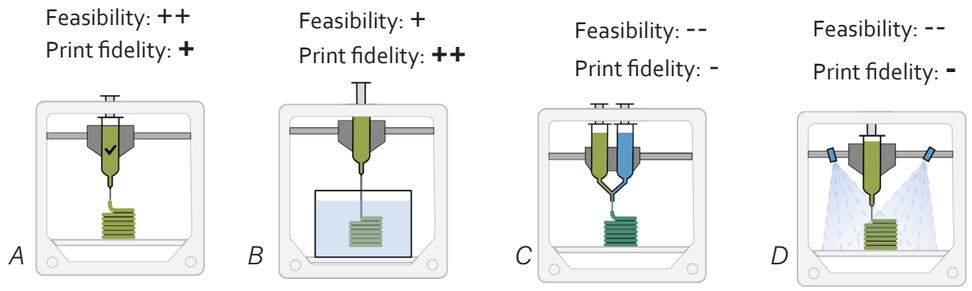


Figure 44. Extrusion method A is chosen for the bio-ink optmization.

CHAPTER 6

6.5 Conclusion

Four extrusion based cell printing approaches were selected: A) increasing the viscosity of the bio-ink, B) printing in a support bath, C) double extrusion with crosslinker and D) aerosol spraying of crosslinker. A method was preferred which was feasable to execute within the time of the project, and resulted in a high print fidelity. Double extrusion printing and aerosol spraying required new 3D printers or adaptions on existing printers, which would have be too time intensive to carry out in this project.

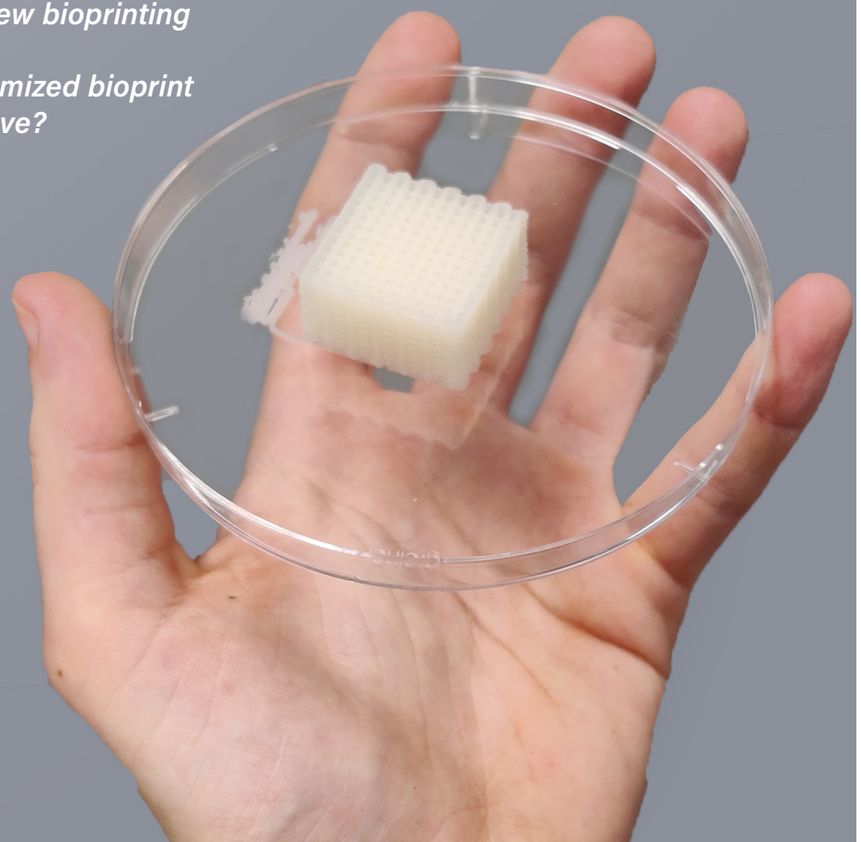
Help in the selection of additional ink components was given by Dr, Kunal Masania of the TU Delft, who had previous experience with the 3D printing of plant cells. He suggested the addition of Polymer A, Polymer B and agar powder to the ink formulation. This composition proved to be promising, since it had improved viscosity and shape retention compared to the alginate material proposal. Furthermore, the ink formulation could be 3D printed on a Direct Ink Writer which was available at the research lab of Dr. Masania.

It was decided that the ink showed enough shape stability to be post-crosslinked, so it was decided that extrusion in a print bath was not necessary.

Chapter 7 | Experimentation

A protocol for the optimized ink formulation and printing approach are presented in this chapter. The findings which led up to this protocol are explained and we conclude with an evaluation of the optimized bioprint.

1. ***Which steps should be taken to make the optimized bio-ink formulation?***
2. ***Which steps should be taken to 3D print the optimized bio-ink?***
3. ***What insights were obtained which led to the new bioprinting protocol?***
4. ***How does the optimized bioprint look like and behave?***



7.1. Introduction

Six rounds of experiments were done to come up with the optimized bioprinting protocol. The following topics were tested:

1. The impact of alginate concentration on the cell function.

2. The impact of crosslinker concentration on the cell function and print stability,

3. The most optimal way to load the 3D printer syringe barrel

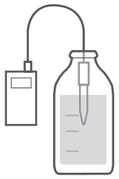
4. The effect of immersion in TAP medium to prolong the cell survivability

The step-by-step approach for the bio-ink preparation, including an ingredient list, can be found in Figure 45. Pictures of the process can be seen in Figure 46. The step-by-step approach of the bioprinting can be found in Figure 47, with accompanying pictures in Figure 48.

The equipment from Figure 46 belongs to the biolab of the Aubin-Tam group from Applied Sciences, TU Delft. The 3D printer belongs to the printing lab at Aerospace Engineering, TU Delft.

In general: All equipment which comes in contact with microalgae must be cleaned thoroughly with ethanol.

Ink stock preparation



1. Prepate 100 mL nutrient solution (TAP media) and check for a pH of 7.

2. Add Polymer A, Polymer B, Agar and Sodium Alginate.

3. Blend with a handheld blender for 2 minutes until the mixture is thick like honey.

4. Autoclave and store at 55 °C.

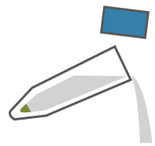
5. Blend with a handheld blender for 2 minutes until there is a smooth, homogeneous mixture.

6. Store ink stock in centrifuge tubes, 30 mL per tube (45g) Wrap the cap with parafilm.

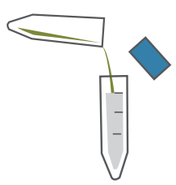
Bio-ink preparation

7. Prepare 100 ml algae culture and grow for 7 days until there are 2.000.000 cells/ml.

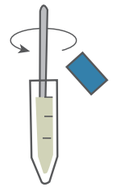
8. Add 10 mL TAP media to 5 mL algae culture in a centrifuge tube so that there are 1.000.000 cells/ml.. Vortex.



9. Centrifuge for 5 minutes at 4000 rpm and discard the supernatant. Add 10 µL TAP media to the pellet and vortex.



10. Add the pellet to the 30 ml ink stock.



11. Stir with a spatula.

Figure 45. Bio-ink preparation step-by-step

Ingredient list for 30 mL ink (one syringe)

Algae culture

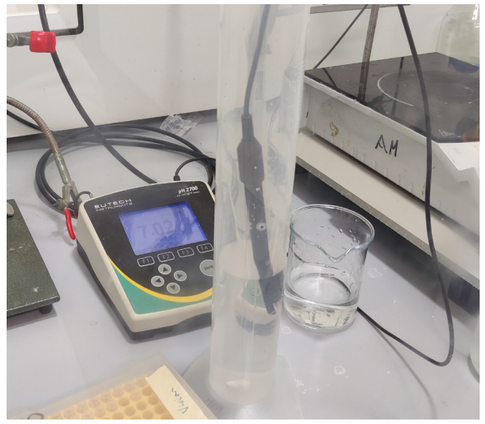
- **100 mL** TAP media
  - » **100 mL** deionized water
  - » **242 mg** Tris base
  - » **2.5 mL** TAP solution ($NH4Cl$ + $MgSO_4$ -7H_2O + $CaCl_2$ - 2H_2O, stored at 4 °C)
  - » **37.5 µL** phosphate buffer
  - » **100 µL** Hutner's trace elements (stored at 4 °C)
  - » **107 µL** Acetic acid
- Chlamydomonas Rheinhardtii CC-125 (mt+) strain streaked onto a petri dish containing TAP agar

Crosslinking

For **100mL 0.1M** $CaCl_2$:
- **1.4701 g** of $CaCl_2.2H_2O$
- **100 mL** deionized water

Bio-ink

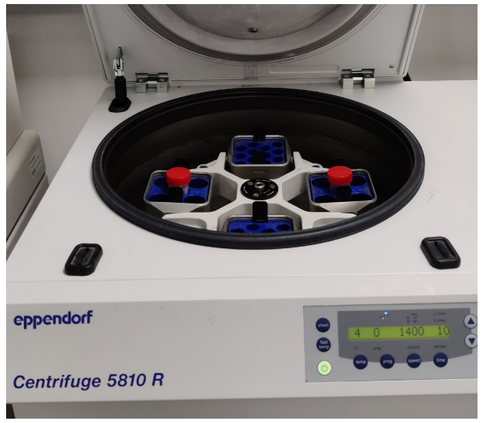
- **37.5 mL** TAP media:
  - » **37.5 mL** deionized water
  - » **90.75 mg** Tris base
  - » **0.94 mL** TAP solution (NH_4Cl + MgSO4 -7H2O + $CaCl2$ -2H_2O, stored at 4 °C)
  - » **14.1 µL** phosphate buffer
  - » **37.5 µL** Hutner's trace elements (stored at 4°C)
  - » **40.1 µL** Acetic acid
- **1.13 g** Polymer A
- **0.56 g** Polymer B
- **0.56 g** Agar
- **0.15 g** Sodium Alginate
- **100 µL** TAP media



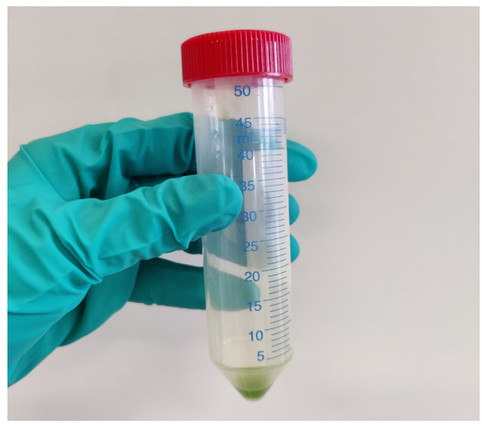
Measuring the pH (step 1)

Blending until it is thick like honey (step 3)

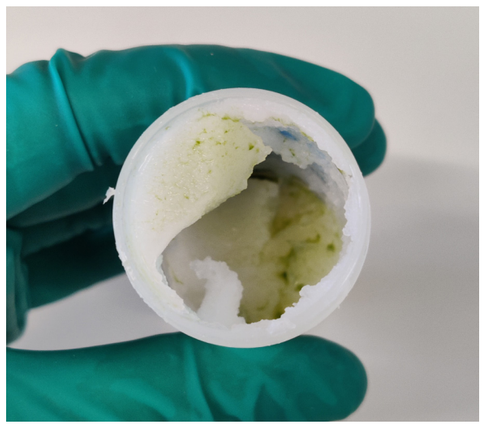
Blend autoclaved ink for two minutes (step 5)



Centrifuge microaglae culture (step 9)



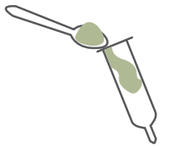
Microalgae pellet, vortexed (step 10)



Ink with microalgae pellet (step 10)

Figure 46. Photos of the bio-ink preparation

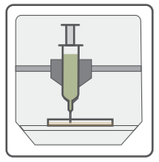
3D printing and crosslinking



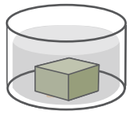
12. Add the bio-ink with a spoon to a syringe with luerlock tip.

13. Add a cap to the syringe tip and wrap the top with parafilm. Centrifuge for 1 minute at 3000 rpm.

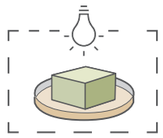
14. Remove the cap and attach a tapered tip (0.86 mm). Create an air gap between barrel and plunger with a long needle and push the plunger in.



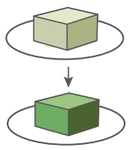
15. Print the desired shape with an adapted commercial 3D printer, containing a syringe pump system.



16. Pour 0.1M $CaCl_2$ over the 3D print to crosslink and wait 5 minutes.



17. Remove the bioprint and place in a sterile environment under correct growth conditions.



18. In 7 days, the colour of the bioprint will gradually change to dark green.

3D printing settings

3D printer

- Ultimaker 2+, adapted with a syringe pump system.
- Syringe 10 mL with a luerlock tip (NewPharma, n.d.)
- Weller taper tip needle, Inner diameter: 0.86 mm (ESD, n.d.)

Print settings in Cura

Print mode: cold extrusion (fan turned off)

- Layer height: **0.5 mm**
- Initial layer height: **0.53 mm**
- Line width: **0.7 mm**

- Print speed: **48 mm/s**
- Travel speed: **100 mm/s**
- Number of slower layers: **2**
- Retraction Minimum Travel: **1.4 mm**
- Travel avoid distance: **0.8438 mm**

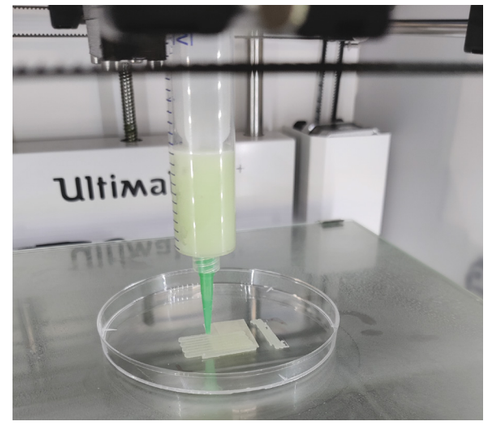
- Build Plate Adhesion Type: **Skirt**
- Skirt line count: **2**
- Skirt distance: **2 mm**
- Skirt Minimum length: **250 mm**

Figure 47. 3D printing and crosslinking step-by-step

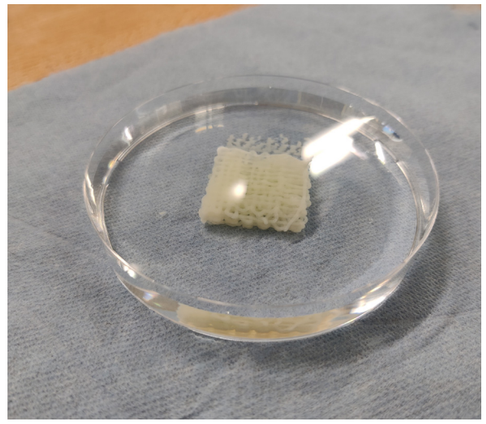
Adding bio-ink to the syringe (step 12)

Centrifuging the syringe with bio-ink (step 14)

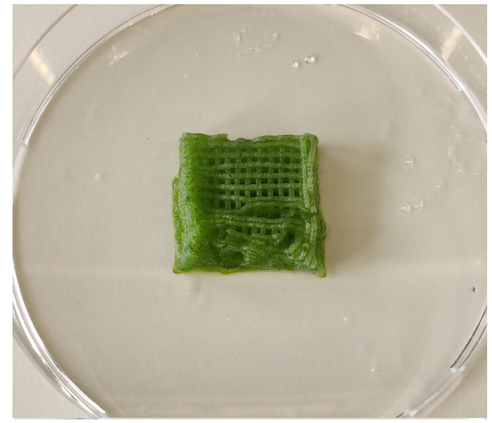
Creating an air gap with a long needle (step 14)



Direct ink writing (step 15)



Bioprint submerged in crosslinker (step 16)



Changing colour of the bioprint (step 18)

Figure 48. Photos of the 3D printing and crosslinking of the bioprint

7.2 Experiment findings

In this paragraph, the six most important research findings will be presented, which led to the improved ink formulation and processing approach.

Experiment 1: Testing an ink formulation with different alginate concentrations

Finding 1: A shape with more weight and thicker lines improved the cell function.

Finding 2: Small changes in alginate concentration did not impact the cell function.

The impact of the alginate concentration on the cell survivability was tested, by preparing three inks with increasing alginate concentrations.

Ink with alginate (0.2 w/v%):
20 ml ink stock + 5 ml alginate (5%) + 5 ml microalgae culture

Ink with alginate (0.4 w/v%):
20 ml ink stock + 2.5 ml alginate (5%) + 2.5 ml TAP + 5 ml microalgae culture

Ink with alginate (0.8 w/v%):
20 ml ink stock + 1.25 ml alginate (5%) + 3.75 ml TAP + 5 ml microalgae culture

An hypothesis was that higher concentrations of alginate would decrease the cell function, meaning that 3D print would be less green. It was found that small changes in alginate concentration do not affect the cell function. All bioprint samples were similar in colour after seven days, a shade 7.

By accident, the ink concentration of 0.4 w/v% was printed slightly thicker than the other two bioprints. After 28 days, the inks dried out except for the middle one, which still showed a dark green colour, shade 8. This proves that a shape with more bio-ink dries out less quickly, and thus results in a bioprint with higher cell function.

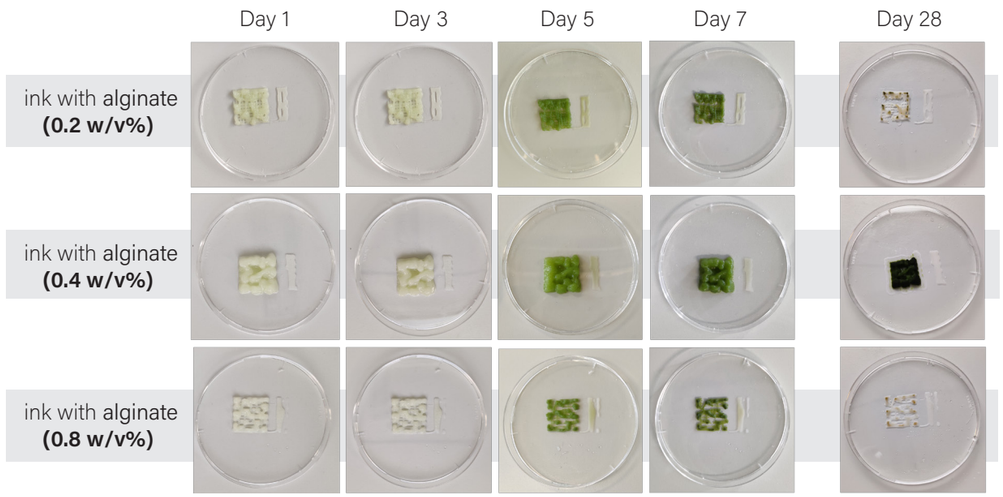


Figure 49. Results experiment 1

Experiment 2: Testing the effect of a crosslinking agent on cell function

Finding 3: Reducing the liquid components in the ink formulation improved the print fidelity.

Finding 4: C. reinhardtii cells did not survive in a bioprint crosslinked with 1M calcium chloride.

In the previous experiment, the bioprints were still rather liquid. On advice from Dr. Masania, the alginate was added in powder form rather than liquid form. Moreover, the algae was added as a pellet rather than a liquid culture.

The goal of this experiment was to find out if the crosslinking agent had a negative incluence on the cell function. Two inks were made, both with the same ink formulation. One was sprayed with a spray bottle containing 1M $CaCl_2$, the other was not. The bioprints were photographed for a week, and again after four weeks.

The results of experiment 2 can be seen in Figure 50. By removing the liquids from the bio-ink formulation, the bioprints showed superior print resolution and height. Now, up till eight layers could be achieved.

On day three, the crosslinked bio-ink lost colour, indicating that the algae cells had died. In comparison, the bioprint which was not crosslinked showed an increase in colour till shade seven. It can be concluded that the concentration of calcium chloride is too high, causing the cells to perish.

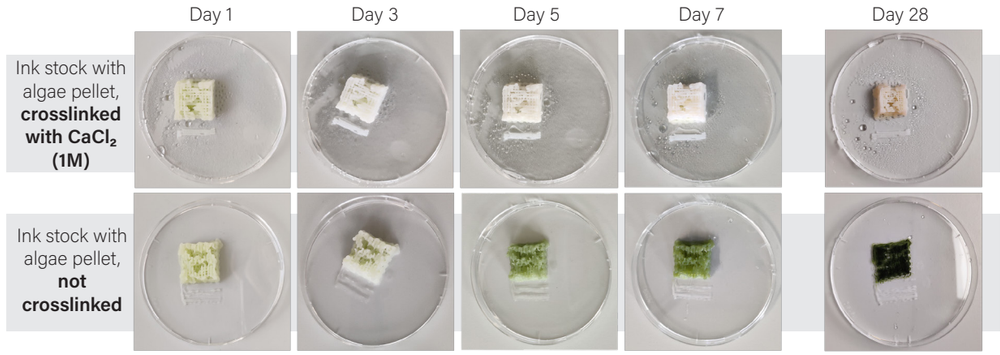


Figure 50. Results experiment 2.

Experiment 3: Testing the effect of different concentrations of crosslinking agent on cell function

Finding 5: For cell growth in the bioprint, the crosslinking concentration of calcium chloride should be less than 0.5M.

This experiment aimed to find a crosslinking concentration which allowed the algae cells to be alive in the bioprint. Moreover, the effect on cell function of a minimal media was researched by excluding the carbon source from the TAP media. The same ink formulation was used as experiment 2, except for the TAP media which was replaced with Tris minimal. Three calcium chloride solutions were made of 0.1M, 0.2M and 0.5M. A control bioprint was made which was not crosslinked as a comparison.

The results can be seen in Figure 51.

All bioprints showed a green colour, except for the bioprint which was submerged in 0.5M $CaCl_2$. This concentration is too high for the algae cells. There is not much difference between the bioprint crosslinked with 0.2M and 0.1M in terms of cell function.

All bioprints grew a less intense colour of green, instead of a shade 7 after seven days, the bioprints are a shade 4. This is caused by the nutrient media. Since the algae cannot grow in the dark, the acquire less biomass and thus a less intense colour.

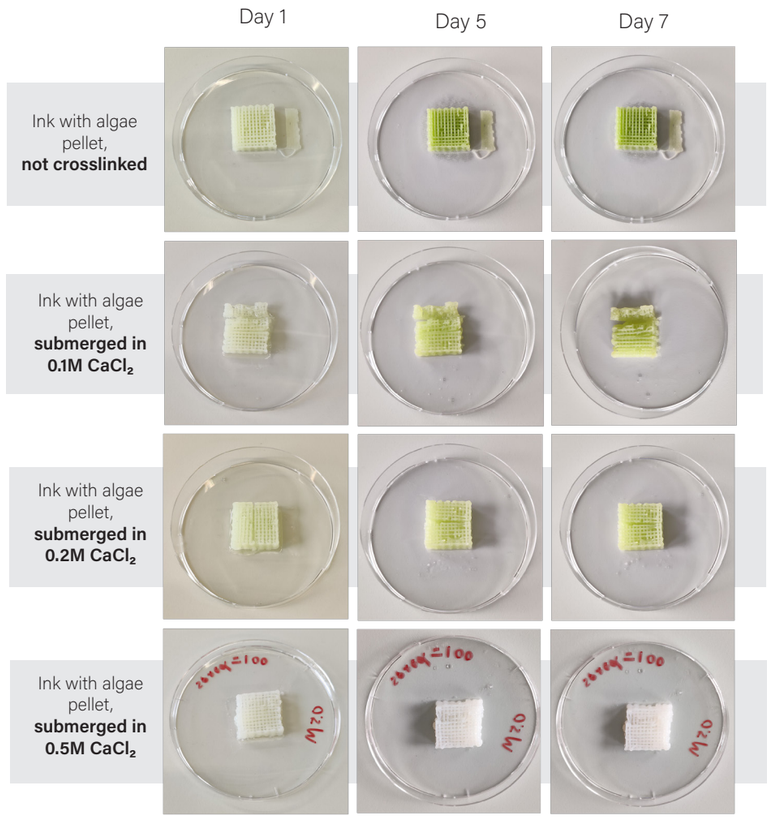


Figure 51. Results experiment 3

Experiment 4: Testing the effect of different concentrations of crosslinking agent on cell function and shape fidelity

Finding 6: Bioprints should be crosslinked with a minimal concentration of 0.1M to be able to remain its shape.

This experiment was done to gain more understanding on the structural stabiltity of the bioprints after crosslinking. Two crosslinking methods concentrations were tried, 0.1M $CaCl_2$ and 0.01M $CaCl_2$. The samples were submerged for five minutes. One sample functioned as the control, and was not crosslinked. The growth of the bioprints was observed over twenty days. There is a gap in the data, since there was a christmas holiday between day 3 and 20 and pictures could not be made during that time period.

The sample which was not crosslinked was a shade 8 after 20 days, one shade darker than the bioprint crosslinked with 0.1M $CaCl_2$. The sample crosslinked in $CaCl_2$ could not hold the shape and fell apart in the crosslinking solution. Therefore, it can be concluded that 0.1M is too low of a concentration, to be able to crosslink the bioprint completely in five minutes.

Experiment 6: How much does the CaCl2 concentration impact the crosslinking and algae growth?

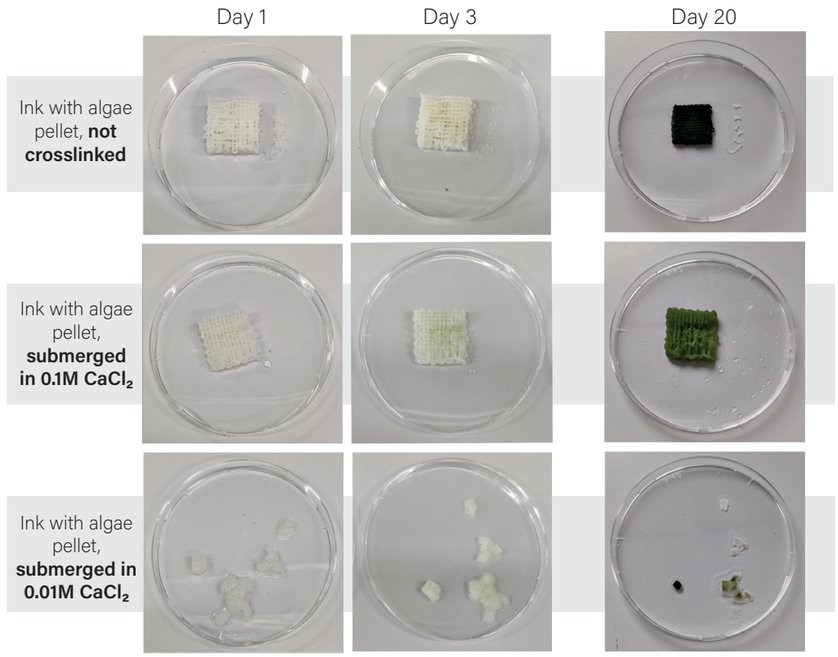


Figure 52. Results experiment 3

Experiment 5: Testing approaches to remove air bubbles in the bio-ink in order to increase print fidelity

Finding 6: Centrifugation proved to be the best solution to remove air bubbles from the bio-ink.

During the loading of the bio-ink in a syringe for Direct Ink Writing, numerous air bubbles appear. See Figure 53. These air bubbles negatively impact the print resolution and amount of layers that can be printed. Due to the excess air, gaps are formed in the printed filament lines, which creates an unstable base for the next line to be printed. An example of such a print can be seen in Figure 54a. Different techniques were explored to load the syringe barrels without air bubbles.

Figure 53. Air bubbles in the syringe barrel

The following three methods were tried: bottom up filling with a coupler, centrifuging and vacuuming. See Figure 55. Out of all three methods, centrifuging was the only one which could remove the air bubbles effectively. Centrifuging at 3000 rpm for 1 minutes gave the best results, see Figure 54b. Longer than a minute is not advised, since the ink formulation and microalgae liquid seperate in the barrel.

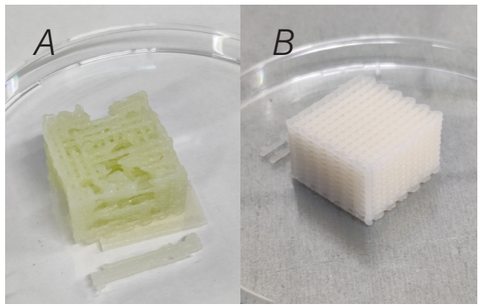


Figure 54. a: bioprint made with ink that is not centrifuged. b: bioprint made with ink that is centrifuged.

Figure 55.Appraoches to remove air bubbles in the syringe barrel. From left to right: Bottom up filling with a coupler, centrifuging and vacuuming.

Experiment 6: Testing enhanced cell viability with immersion in TAP media

Finding 6: Immersion of the bioprints in TAP media after one week does not improve the cell viability

Two solid shapes were 3D printed, and two porous shapes, following the protocol of Chapter 7.1. For each type of shape, one was immersed in TAP after seven days of bioprinting, the other was not. Immersion was done by pouring TAP media in the petri plates, and removing the prints after 5 minutes to clean petri plates with a sterile spatula.

The results can be seen in Figure 56. All bioprints showed a green colour in shade 6. They bioprints immersed in TAP did not show a greener colour. However, the solid print immersed in TAP showed signs of fungal contamination. This was probably caused by the spatula, or a contamination in the TAP media.

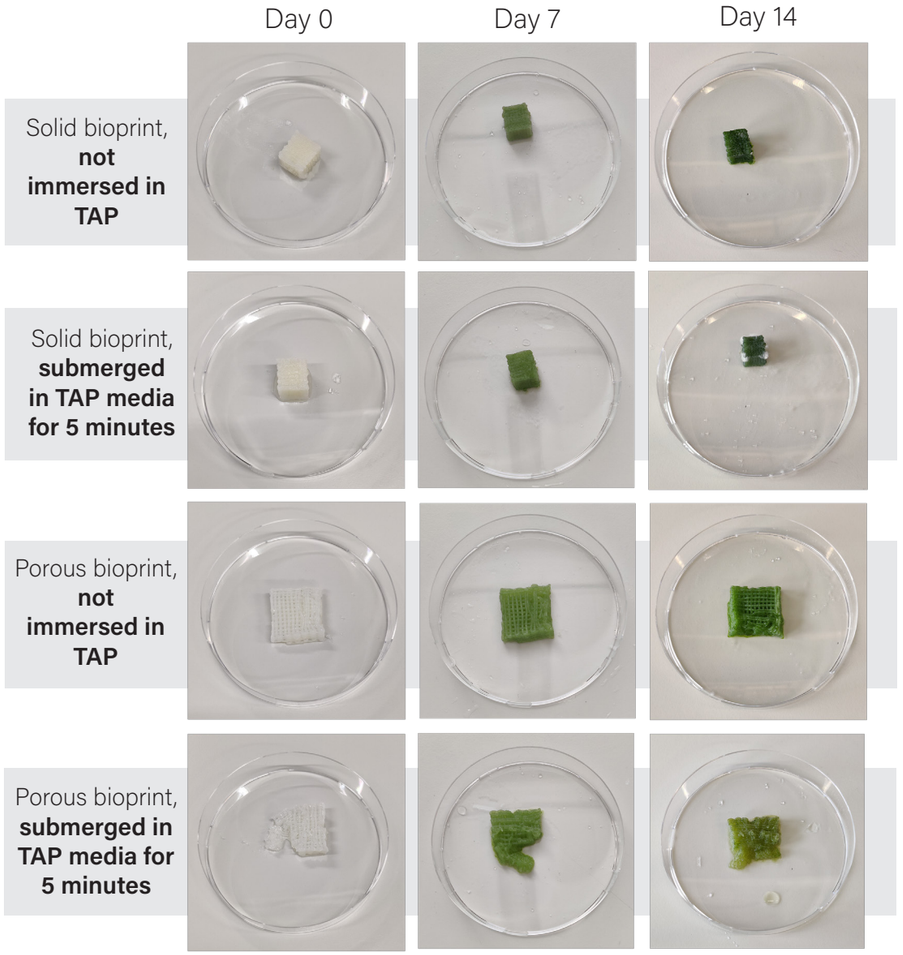
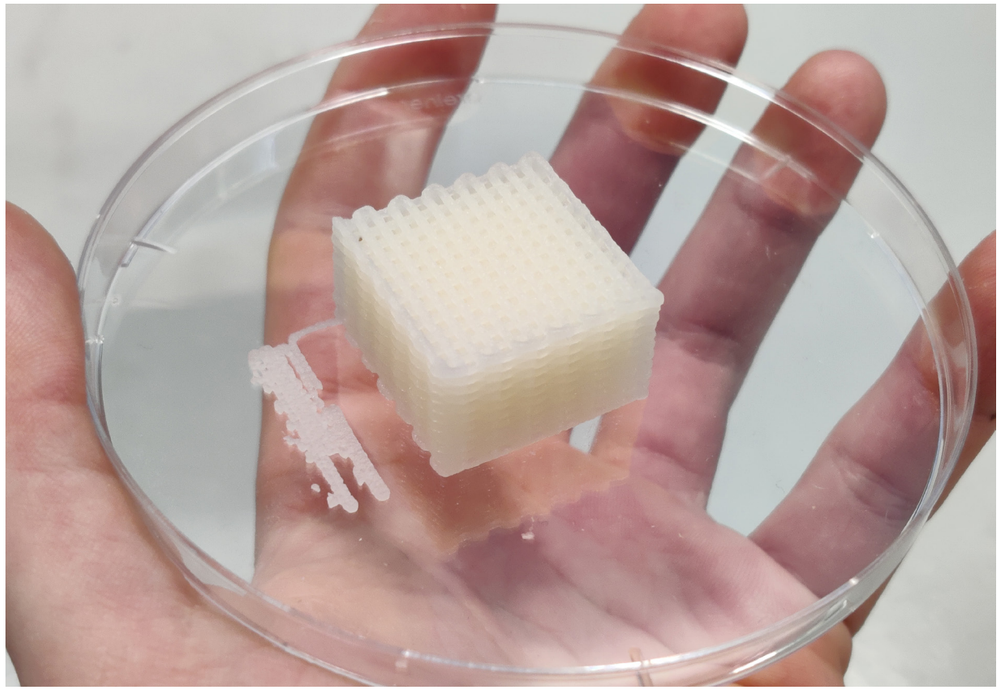
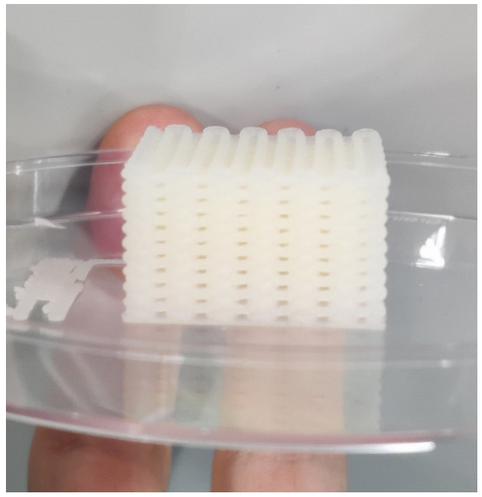


Figure 56. Results of experiment 6

7.3 Bioprint evaluation

Pictures of the final 3D print can be observed in Figure 57.





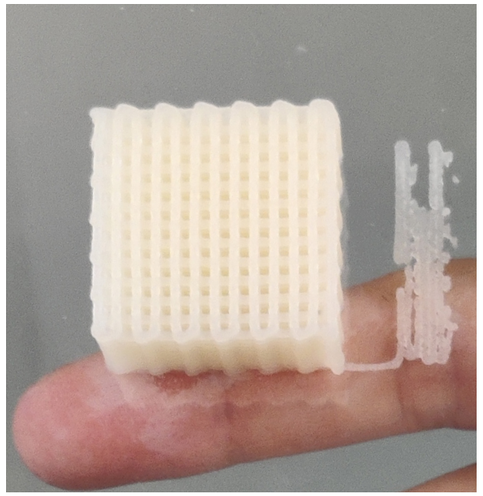
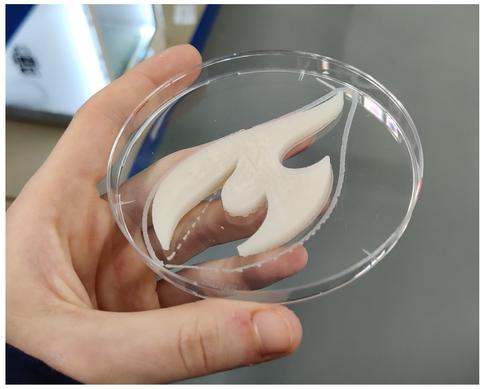


Figure 57. The optimized bioprint seen in different angles. The print is 25 x 25 x 10 mm.



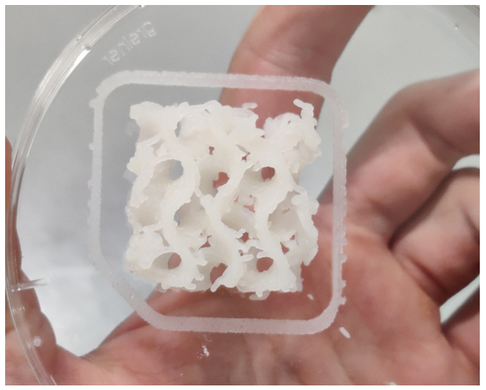
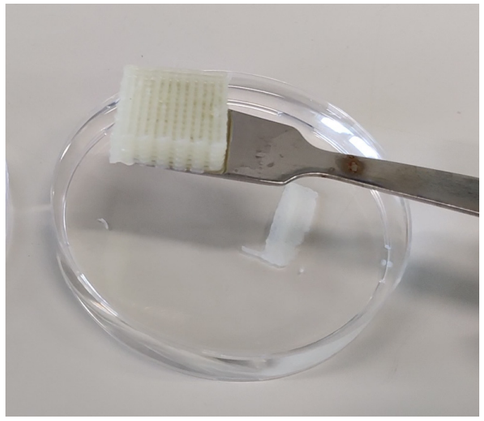


Figure 58. Different shapes were printed with the optimized material. Left: the TU Delft logo. Right: a gyroid



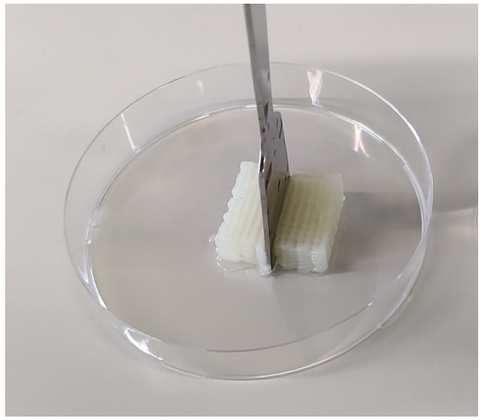


Figure 59. The bioprint is solidified enough to be transferred with a spatula, but soft enough to be cut easily.

The final sample could reach up till 25 layers in height. The print resolution is high, complex shapes can be achieved, such as gyroids. See Figure 58.

Once crosslinked, the material shows enough structural stability to be able to be transferred, but soft enough that it can be easily cut. See Figure 59. The texture is comparable to cooked spaghetti.

The final prints dried out in the incubator, see chapter 8.7 for further explanation, so a green coloured print could not be presented. However, in earlier experiments the ink proved to support cell growth. See Figure 60.

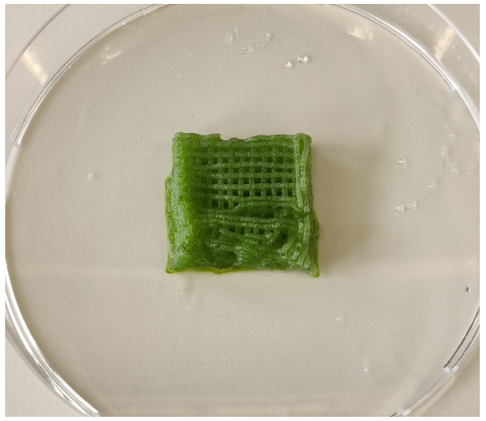


Figure 60: The material proved to grow green after seven daysin earlier experiments.

CHAPTER 7

7.4 Conclusion

During experimentation, the following steps were found to provide the optimal bio-ink formulation. The ink stock was prepared by mixing TAP medium with Polymer A, Polymer B, agar and sodium alginate and mixed with a handheld blender. The bio-ink was prepared by adding the algae culture (*Chlamydomonas rheinhardtii*, strain CC-125, (mt+) to TAP medium. After vortexing, the algae pellet was added to the ink stock and was gently stirred.

By trial-and-error, the most optimal approach to prepare the bio-ink for 3D printing was found. To remove air bubbles, the loaded syringe was capped and centrifuged. An air gap was created between barrel and plunger. A tapered tip of 0.86mm was found to be the right inner diameter for printing. A 3D print was made with the Ultimaker 2+, with an additional syringe pump system. Five–minute immersion of the 3D print in $CaCl_2$ made curing of the material possible.

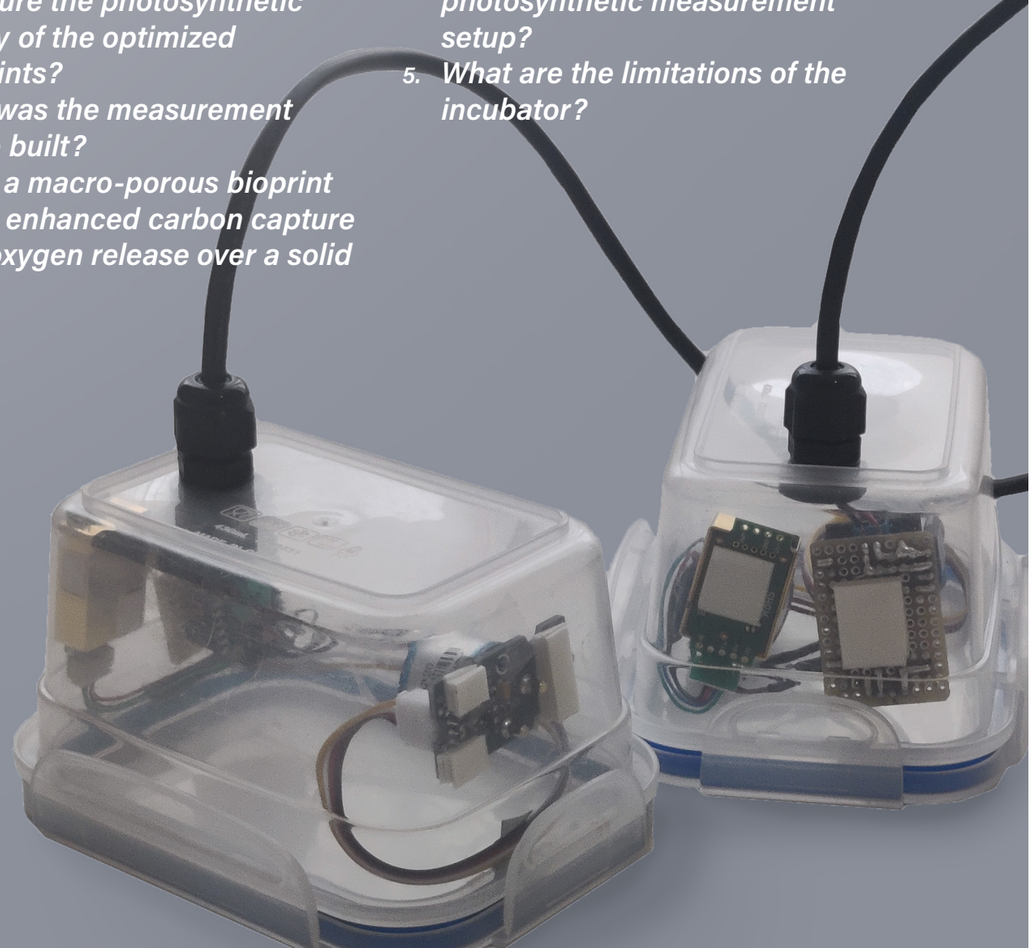
In a series of short experiments, valuable insights were gained which led to the bioprinting protocol explained above. A shape with more weight and thicker lines were found to improve cell function. Print fidelity was improved by reducing liquid components in the bio-ink. Furthermore, a concentration between 0.1M and 0.5M calcium chloride was found to improve cell growth. To remove air bubbles in the bio-ink, centrifuging up to 1 minute was shown to improve the 3D print quality. Longer than 1 minute caused the algae and liquid to separate in the barrel.

The final algae 3D print is up to 25 layers in height and extreemly precise geometries can be printed. In correct lab conditions, the print can grow a green colour in seven days and remain viable for three weeks. The texture of the material can be compared to cooked spaghetti.

Chapter 8 | Testing

The optimized bioprints will be tested on their photosynthetic ability performance in this chapter. The measurement setup is explained, and the sensor data are discussed in this chapter. The measurement setup was evaluated, as well as the incubator used for algae growth. The following research questions are answered:

1. ***Which sensors can be used to measure the photosynthetic ability of the optimized bioprints?***
2. ***How was the measurement setup built?***
3. ***Does a macro-porous bioprint show enhanced carbon capture and oxygen release over a solid bioprint?***
4. ***What are the limitations of the photosynthetic measurement setup?***
5. ***What are the limitations of the incubator?***



8.1 Introduction

Microalgae can influence the level of carbon dioxide and oxygen in their direct environment due to photosynthesis. Carbon dioxide and oxygen are colourless and odourless gasses in the earth's atmosphere. As has been mentioned in Chapter 2, the average level of carbon dioxide in urban homes is between 400 and 1000 parts per million (ppm) (Abdel-Salam, 2015). That is around 0.04% of the total air volume indoors. Oxygen constitutes for 20.95 vol% of the atmosphere (Williams, 2020).

To measure these two atmospheric gasses, two sensors were selected. The working principle of these sensors is briefly explained in the following paragraph.

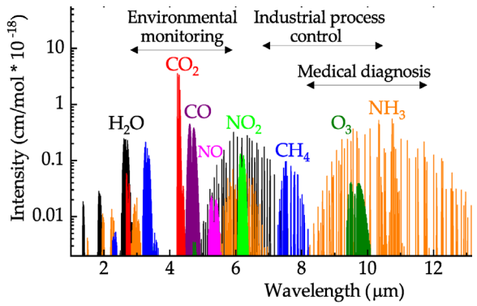


Figure 63. Infrared absorption of CO_2 and other gasses (Popa & Udrea, 2019).

8.2 O_2 and CO_2 sensor

The selected CO_2 sensor is the Winsen MH-Z19B (Figure 61), which makes use of infrared technology. In the sensor is an infrared lamp which directs light towards an optical filter. (See Figure 62) The filter prevents wavelengths other than the gas which will be measured to pass through. Behind the optical filter is a detector,which converts the light intensity measurement to a gas concentration value, expressed in parts per million. The infrared light produces a band of light which is specific for carbon dioxide: 4.26 micron (Popa & Udrea, 2019). See Figure 63.

Infrared sensing is a technique most often used for CO_2 detection (Vaisala, n.d.). The sensors have multiple benefits over other chemical sensors. The sensors are stable and highly selective to the gas. Since the gas does not directly interact with the sensor, it can withstand harsh conditions such as dust, dirt and high humidity.

Figure 61. The CO_2 sensor used to measure the photosynthetic ability of the bio-ink

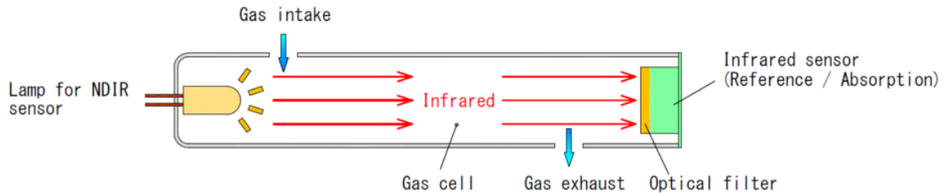


Figure 62. Working principle of the CO_2 sensor

The oxygen sensor which will be used is a Grove (ME2-O2-Φ20) sensor, see Figure 64. The sensor works like an electrochemical cell. A chemical reaction is measured, which creates an electrical output that is in proportion to the oxygen level (Gaslab, 2020).

The advantages of this type of sensor is that they are the least expensive of their kind. Moreover, they have low power requirements, low detection limits, are often less affected by interfering gases and are small in size (Manjavacas & Nieto, 2016).

A possible limitation of the sensor could be that it is temperature dependent, since it relies heavily on temperature compensation to provide a reliable reading (Gaslab, 2020).

Figure 64. The O_2 sensor used to measure the photosynthetic ability of the bio-ink

8.3 Building the measurement setup

The carbon dioxide and oxygen sensor were placed in a transparent box, made of Polypropylene. The box is selected to be as transparant as possible. so that light can shine on the 3D prints. It is important that the boxes are airtight, so that the surrounding environment cannot influence the measurement results and so that the sensors measure the photosynthetic activity only. To ensure that no air from the surrounding can enter the box, the lid is closed with a rubber ring. The cables which stick out of the box, are connected to a cable gland.

The sensors were attached to the walls of the box with double sided tape, which left space in the middle to place a 3D printed sample. Both sensors are connected to a Wi-Fi module, a Photon, which can read the sensor values and plot them in a spreadsheet. See Figure 65. The specific code for the sensors is written by Dr. ir. Doubrovski of the TU Delft. The procedure for setting up the Photon and connecting them to the internet is explained in Appendix D.

Since there is a built-in temperature sensor in the photon, the temperature of the boxes could be measured as well.

Two boxes were made, both containing a photon, CO_2 sensor and O_2 sensor. See Figure 66. This allowed two bio-ink prints to be tested simultaneously under equal light conditions. The interior of the box can be seen in Figure 67. The inner volume is 0.71 m³ and the dimensions are 120 x 85 x 70 mm. The volume is chosen as small as possible on purpose, so that changes in the carbon dioxide and oxygen level are quickly noticable.

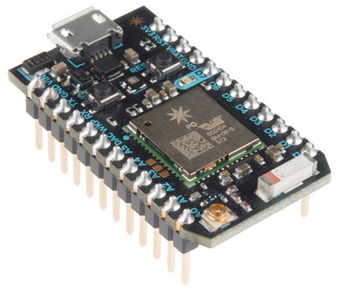


Figure 65. PhotonH by Particle

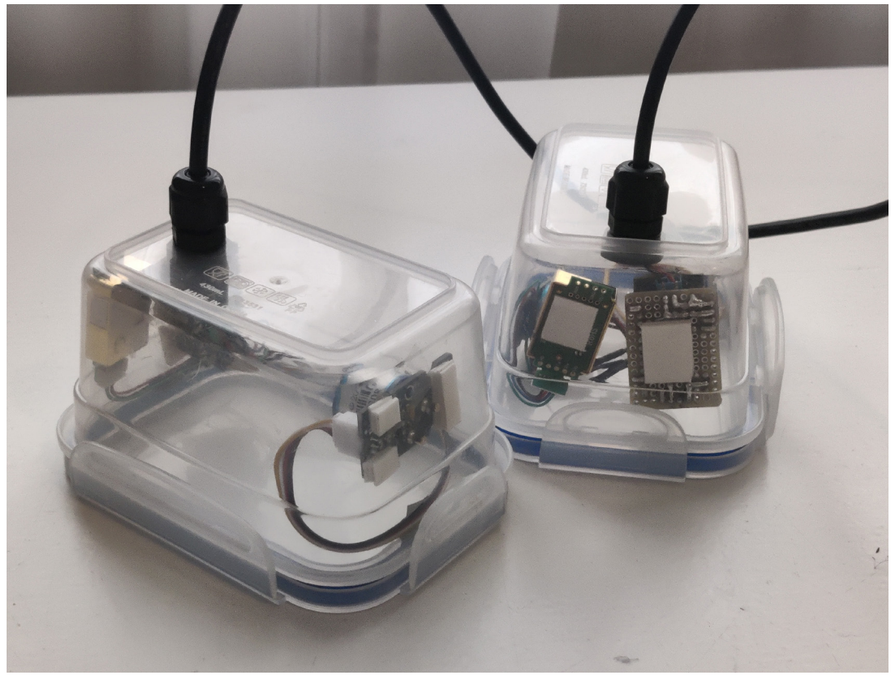


Figure 66. Bioprinted constructs can be placed in these boxes to measure the CO2, O2 and temperature.

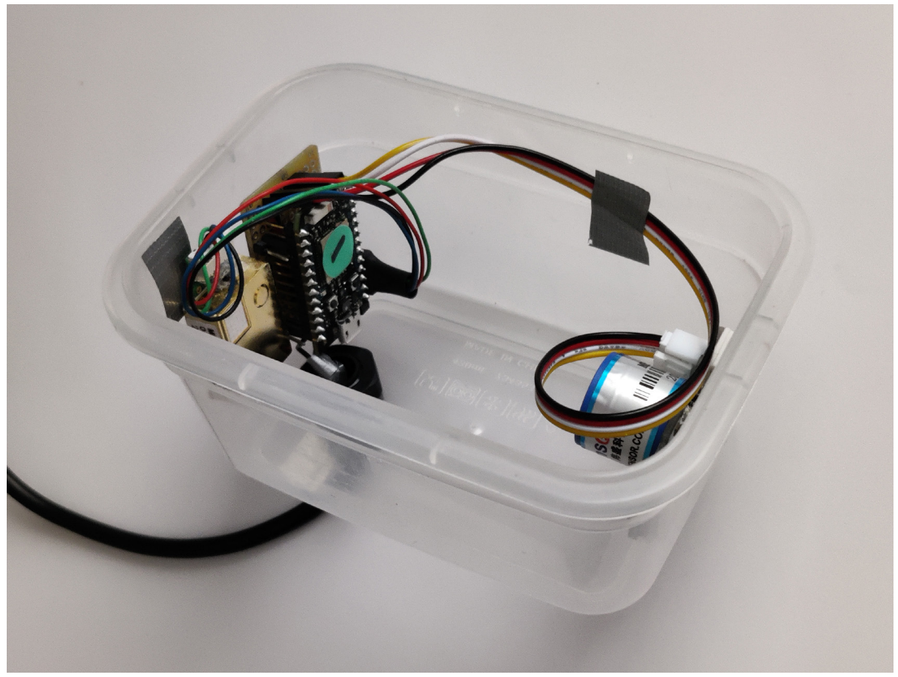


Figure 67. The interior of the measurement box, containing a CO2 sensor, an O2 sensor and a photon.

To ensure that the correct growth conditions were met, the incubator setup explained in Chapter 5.3 was mimicked. LED strips, ratio red/blue 4:1, were bought from webshop LightintheBox (n.d.). Seven strips were cut, five short strips for the lid and two longer strips for the sides. The strips were reconnected by soldering, and glued to the top and sides of a box sized 380 x 380 x 260.

To copy the exact Photosynthetically Active Radiation (PAR) of the incubator used by the Aubin-Tam group, a quantum sensor LI-190R was used. The probe was placed in various positions at the bottom of the incubator, and measured to be 20 m-2 s-1 in all positions. The probe was then placed in one of the small measurement boxes, and the lid was closed. The height of the small box was adjusted by heightening it with a carbon box, see Figure 68, until the quantum sensor measured 20 m-2 s-1.

The small measurement boxes which included the sensors where placed the larger transparent container with LED strips. The boxes were placed symmetrically in the middle, with equal distance to the walls of the outer box. Three beakers were placed in the box, which were filled with salted water. In theory, this solution should increase the humidity to 70 percent.

The whole setup was places in a cupboard, to ensure that the samples received light from the LEDs only. The cupboard was openen once in a while, to check on the bioprint samples. A timer switch was used to switch the LEDs on and off. The measurement box was off during the night for twelve hours, See Figure 69, and turned on during the day for 12 hours, see Figure 70.

After a while, the interior of the measurement box became hot. To solve the problem, a fan was placed on one side of the box. The lid was heightened by 15 mm, which allowed an air gap for ventilation. See Figure 69.

Figure 68. Photosynthetically Active Radiation measurement with a quantum sensor.

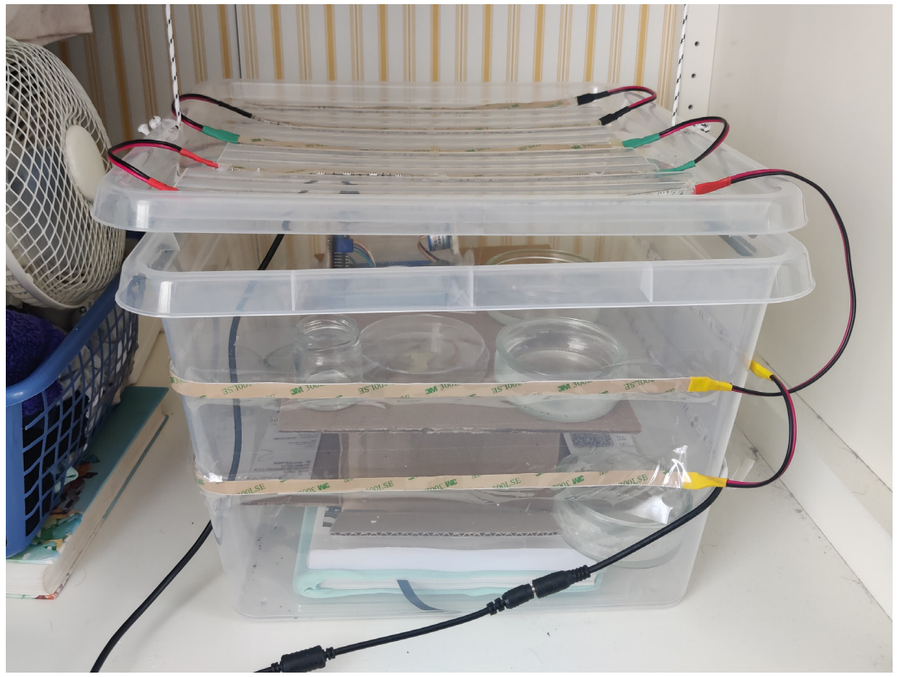


Figure 69. The test setup for the photosynthetic activity during the night, including a fan

Figure 70. The test setup for the photosynthetic activity during the day.

8.4 Experiment setup

In this last experiment, the photosynthetic ability of two 3D printed microalgae prints was tested to see how much influence the infill has on the carbon capture and oxygen release.

Two microalgae prints were 3D printed according to the protocol of Chapter 7.1 The prints were similar in weight, ten grams, and were made from the same bio-ink. They differed in shape, sample 1 was a solid cube of 14 x 14 x 14 mm, (Figure 71) sample 2 a macro-porous rectangular prism of 25 x 25 x 10 mm (Figure 72). The samples weighted equally on purpose, so that there is a similar number of algae cells in the prints. The bio-ink was made with the protocol from Chapter 7.1, with one point of difference. Instead of a carbon-supplemented medium, minimal media was used.

The carbon source is excluded from the medium, so that the microalgae do not use energy during the dark phase to grow. In this way, it is assured that the measurements in carbon dioxide and oxygen can be purely assigned to the photosynthetic activity.

The two samples were 3D printed, crosslinked with $CaCl_2$ (0.1M) and transferred with a spatula to small petri plates. The plates were placed on the lids, and a screwcap with salted water was placed next to the bioprints. The two measurement boxes with sensors were placed over the samples (Figure 73). Both boxes were positioned in equal distance from the walls of the outer box. The sensors were connected to a power source, and the cupboard was closed so that the samples were placed in complete darkness. The samples remained in the measurement setup for 108 hours, four and a half days.

Figure 71. Sample 1, a solid cube of 14 x 14 x 14 mm

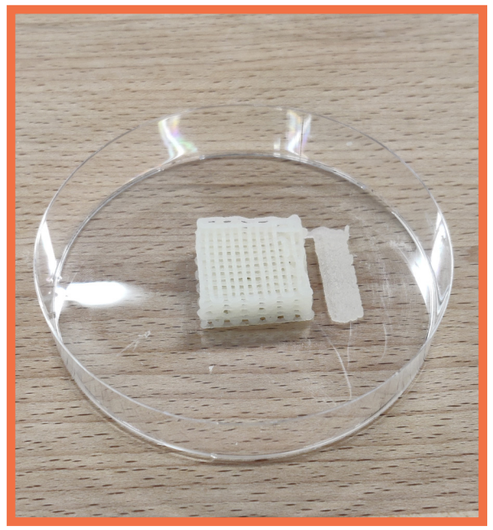


Figure 72. Sample 2, a macro porous rectanglular prism of 25 x 25 x 10 mm.

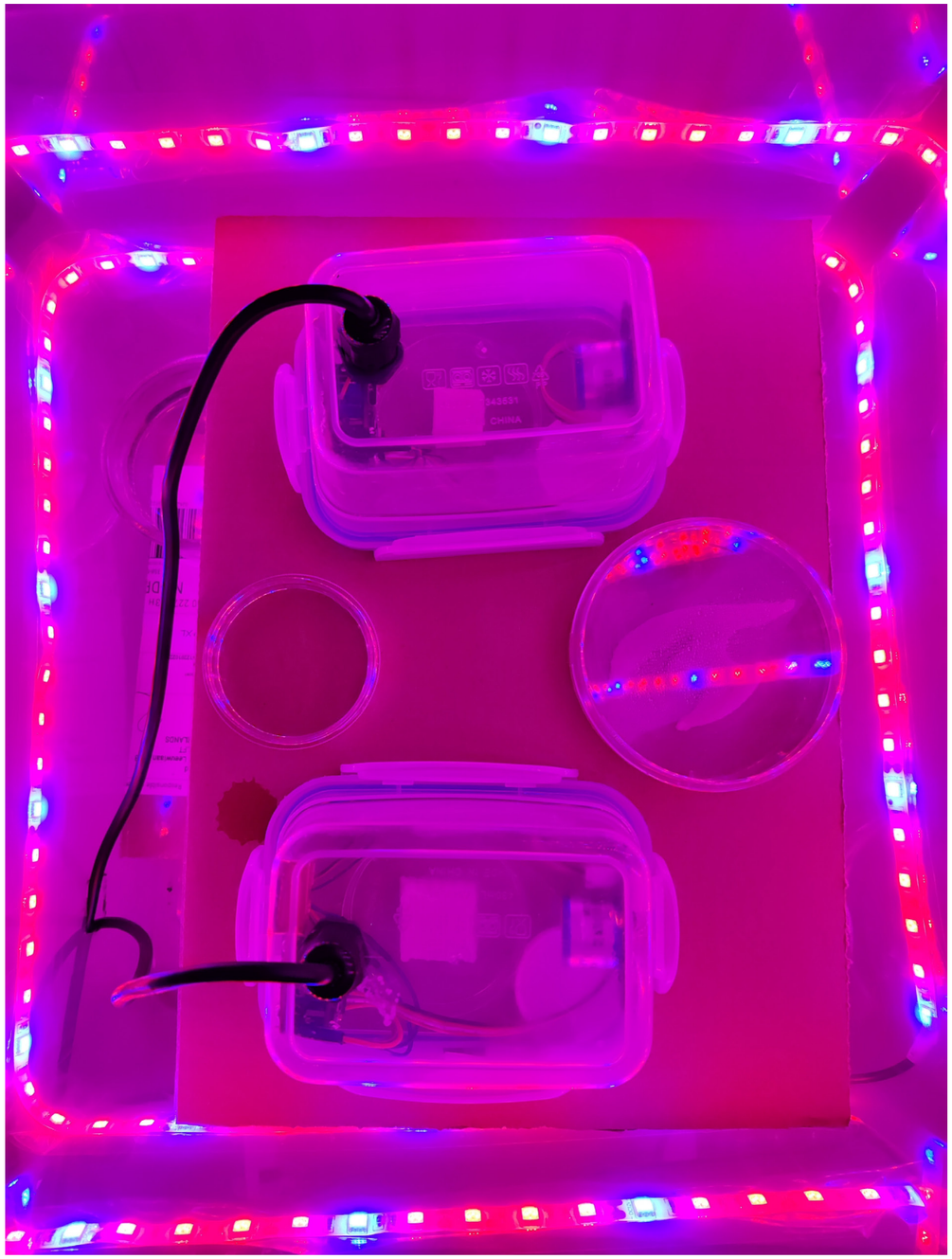


Figure 73. Sample 1 and 2, placed in the measuring setup

8.5 Results

The results of the oxygen sensor can be seen in Figure 74. The first thing that was noticeable was that the graphs of sample 1 and 2 were mirrored. Between 12 PM and 12 AM, the lights were off, and the sensors showed a value around 21 vol%, the average percentage of oxygen indoors. When the lights switched on between 12 AM and 12 PM, sample 1 increased 2 percent in oxygen percentage and sample 2 decreased five percent.

The temperature in the two measurement boxes can be seen in 76. The first day of measurement was missing in the graph since the temperature sensors results were not read out by the Photon. When the lights were off, the temperature is around 28 degrees Celsius. The temperature sharply increased when the lights switched on to an average of 35 degrees Celsius.

Lastly, the carbon dioxide graph can be seen in Figure 75. Again, the graphs of both samples show a large difference in measurement value. The solid bioprint shows an average carbon dioxde level of around 8500 ppm, which increases to almost 1000 ppm over the next four days. A waving pattern can be observed. The carbon dioxide level decreased when the lights were off, and increased when the lights were on. The carbon dioxide graph from the porous bioprint remained a relatively constant value of 3500 ppm. A minimal waving motion can be observed in the graph, a slight increase when the lights are on and a slight decrease when the lights are off.

It should be noted that the carbon dioxide graph starts at 2000 ppm, before it sharply increases. This is because a maximum limit was programmed in the code of 2000 ppm. This means that the actual value was higher at the start. On the 30th of January at 12:00, this limit was increased to 1000 ppm, hence the sharp increase.

A graph of the first five hours was made for the carbon dioxide sensor, see Figure 77. It shows an increase from around 700 ppm to 2000 ppm in a matter of an hour.

The reasion why the carbon dioxide levels increased sharply is still a mystery. Is it caused by the algae cells, the ink, or something else? Additional experiments were performed to figure out why the carbon dioxide levels are rising once the boxes are closed.

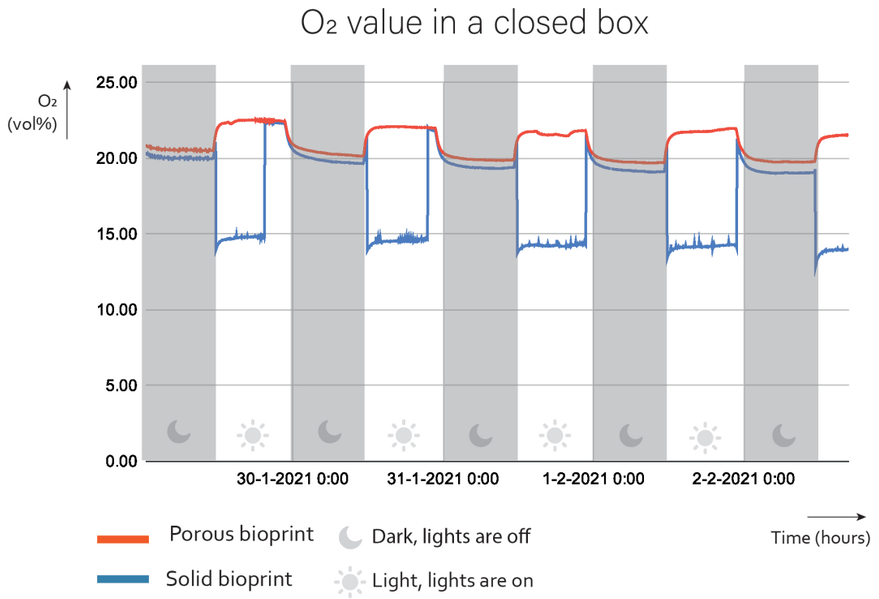


Figure 74. Graph showing the oxygen value in volume percentage in two closed measurement boxes over a timespan of 4.5 days. The red graph represents the sensor which is situated in the box with the porous bioprint (sample 2), the blue graph shows the oxygen level of the box containing a solid bioprint (sample 1).

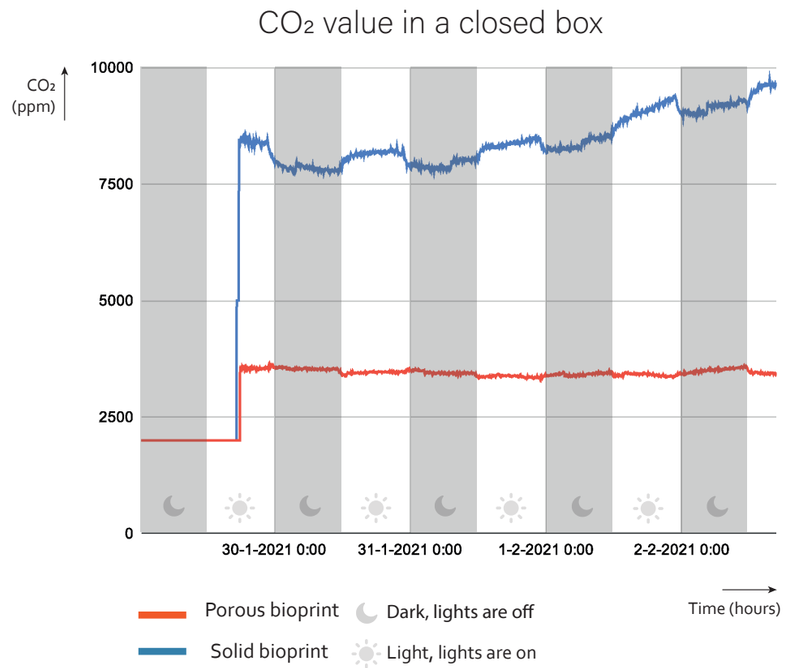


Figure 75. Graph showing the carbon dioxide value in parts per million in two closed measurement boxes over a timespan of 4.5 days. The red graph represents the porous bioprint (sample 2), the blue graph shows the oxygen level of the solid bioprint (sample 1).

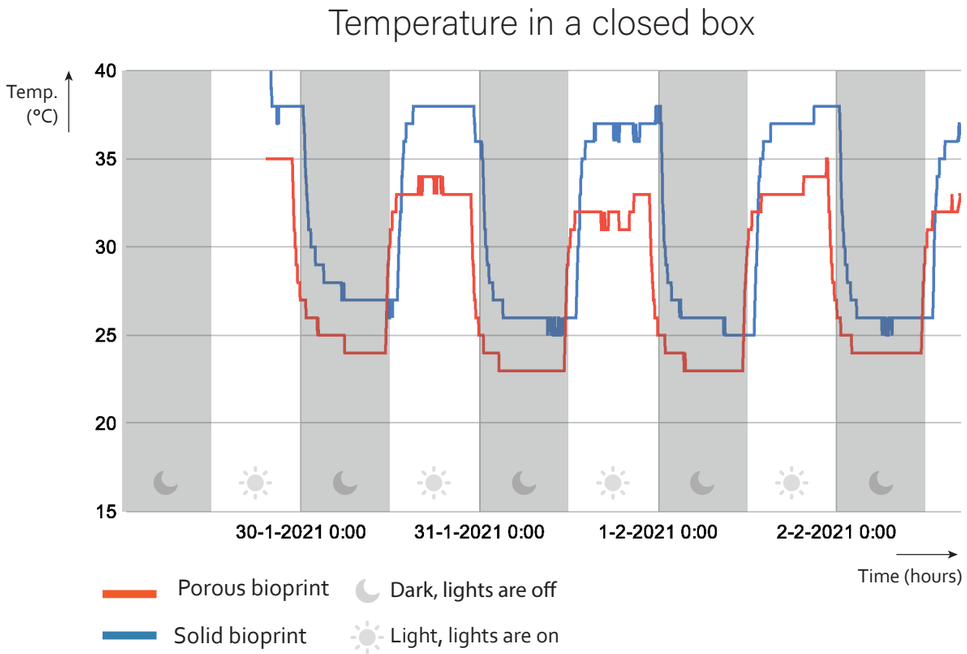


Figure 76. Graph showing the temperature in celcius in two closed measurement boxes over a timespan of 4.5 days. The red graph shows the temperature in the box with porous bioprint, (sample 2), the blue graph shows the temperature in the box with solid bioprint. (sample 1)

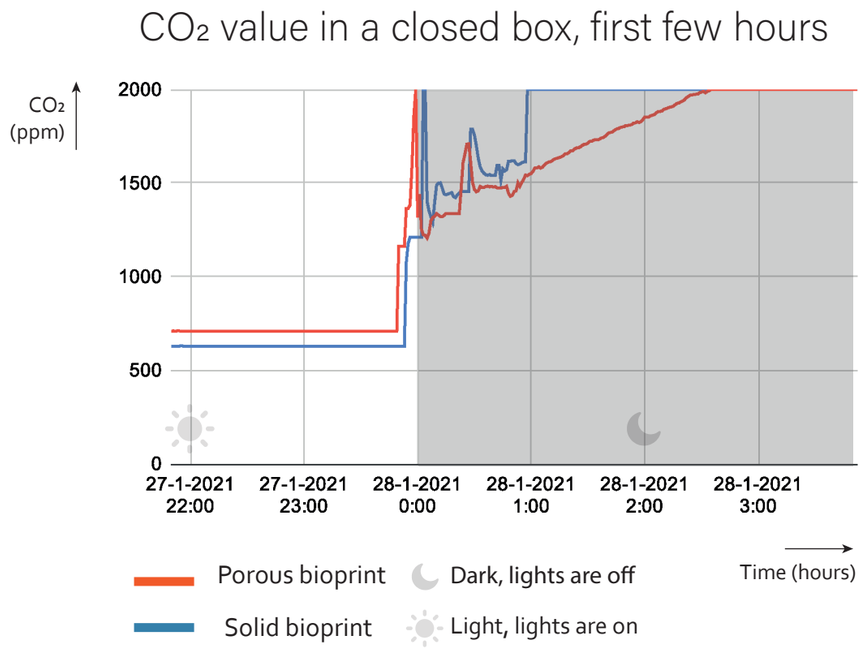


Figure 77. Graph showing the carbon dioxide level in parts per million for the first few hours after the measurement boxes were closed. The red graph shows the CO_2 in the box with porous bioprint, (sample 2), the blue graph shows the CO_2 in the box with solid bioprint. (sample 1)

A control experiment was performed where the increasing value of the carbon dioxide in the first hours was researched in a closed box. To test if the carbon dioxide increased due to the algae, a control ink was made. This ink was made following the protocol of chapter 7.1, and did not include microalgae. Then, an empty box was investigated as well. Both boxes were placed in the incubator, See Figure 78 and 79. Both boxes contained screwcaps with salted water.

The results can be seen in Figure 80. The empty box showed a value of carbon dioxide around 9000 ppm, which slowly increased to 1200 ppm. The control ink showed a larger increase in carbon dioxide.

Figure 78. Empty box except for a screwcap of salt water.

Figure 79. Box containing a control ink without microalgae.

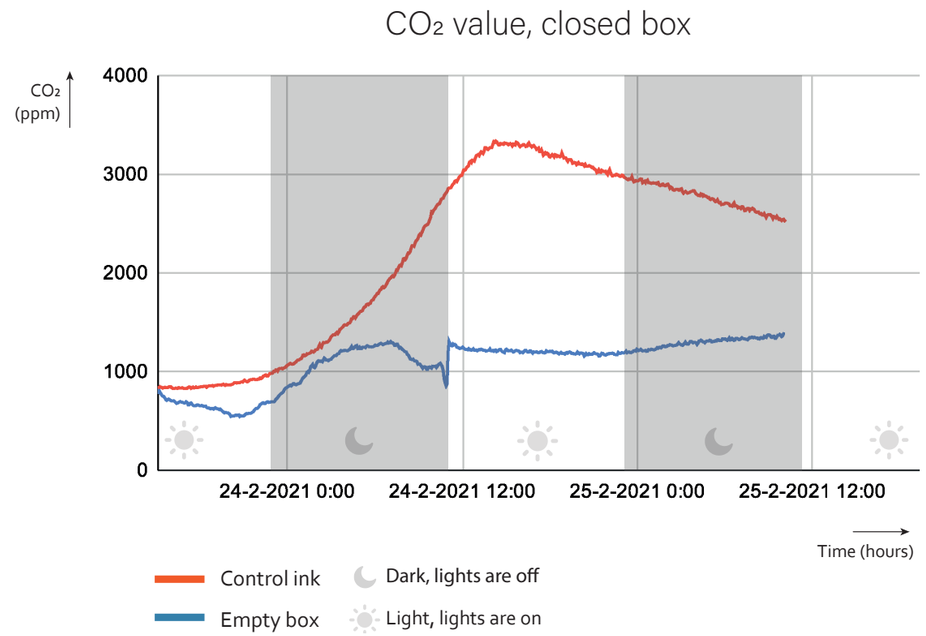


Figure 80. Carbon dioxide increases in a closed box.

8.6 Conclusion

8.6.1 Oxygen measurements

It can be concluded that the oxygen sensor was not a reliable source to measure the oxygen release of the microalgae prints. The hypothesis, which was explained in chapter 2.3, was that the graph would show a general increase in oxygen volume percentage, caused by photosynthesis. Such a trend could not be obvserved in either of the samples. The graphs showed a waving motion, whereas the average level of oxygen stayed 21 percent during the day. The graph was increasing and decreasing in oxygen percentage, in a rhythm which matched the light and dark pattern.

The cause of this pattern was most likely the heat of the LED strips, which influenced the oxygen sensor. When the lights were switched on, the LEDs radiated heat which increased the temperature by seven degrees Celsius. The oxygen sensor thus reacted to the temperature, rather than the oxygen value in the measurement box. Secondly, the lack of ventilation in the measurement box could distort the measurement of the sensor.

The two graphs showed a mirrored image, which is unexpected. The difference in volume percentage, seven percent, is too high to be plausible. Most likely, the sensor reacts to an outlier, increasing or decreasing at random. Therefore, the question whether a porous microalgae bioprint can have a positive impact on the oxygen production of microalgae, cannot be answered with this research setup.

8.6.2 Carbon dioxide measurements

The hypothesis for the carbon fixation of the bioprints was, that the carbon dioxide level would decrease over time. However, the opposite happened. The carbon dioxide quickly increased in a matter of hours, to levels as high as 8000 and 5000 ppm. This number kept increasing over the course of five days. A difference was noticeable between the porous and solid shape. However, the difference is too large to be contributed solely to the macro-porosity of the shape.

The hypothesis was that the carbon dioxide would increase when the lights were off due to cellular respiration, and would increase when the lights were on due to photosynthesis. Again, similar to the oxygen graph, the opposite happens in the porous bioprint graph. Instead of increasing during the day, it decreases and vice versa. The increase of carbon dioxide is in sync with the increase in temperature, which could be explained by the fact that the sensor reacts to the the temperature rather than the carbon dioxide level. Based on the carbon dioxide measurement graph, a difference in carbon capture between the two bioprints cannot be concluded.

Distortions in the carbon dioxide measurement could be caused by the same parameters which influenced the oxygen sensor, the lack of ventilation around the sensors and the temperature.

8.6.3 Control experiment

An increase in carbon dioxide was measured in a closed box containing a control ink without algae. Since the graph which measured an empty box did not result in an increase in carbon dioxide, it can be concluded that one or more of the ink components causes an increase in carbon dioxide. The reason for the increase is not yet known.

8.7 Evaluation functionality of the incubator

It was assumed that the microalgae would grow well if the growth setup from the Aubin-Tam group would be mimicked. However, the microalgae were more sensitive to changes in environment than anticipated. First, it was observed that the bioprints dried out quickly. See Figure 81. An exception were the prints which were placed in the small boxes with sensors. These bioprints did not dry out, and condensation could be observed on the interior walls.

Samples which were left in the large box were observed to decrease in size and turned to a brown colour in a matter of hours. Addition of numerous beakers with salted water did not improve the lack of humidity in the cupboard. It was apparent that the bioprints did not survive in the new setup. The introduction of a fan to the setup decreased the temperature by around four percent, see Figure 82. However, the prints still dried out. Recommendations for improvement of the setup are mentioned in chapter 9.2.2.

Figure 81. Sample dried out after five days in the incubator.

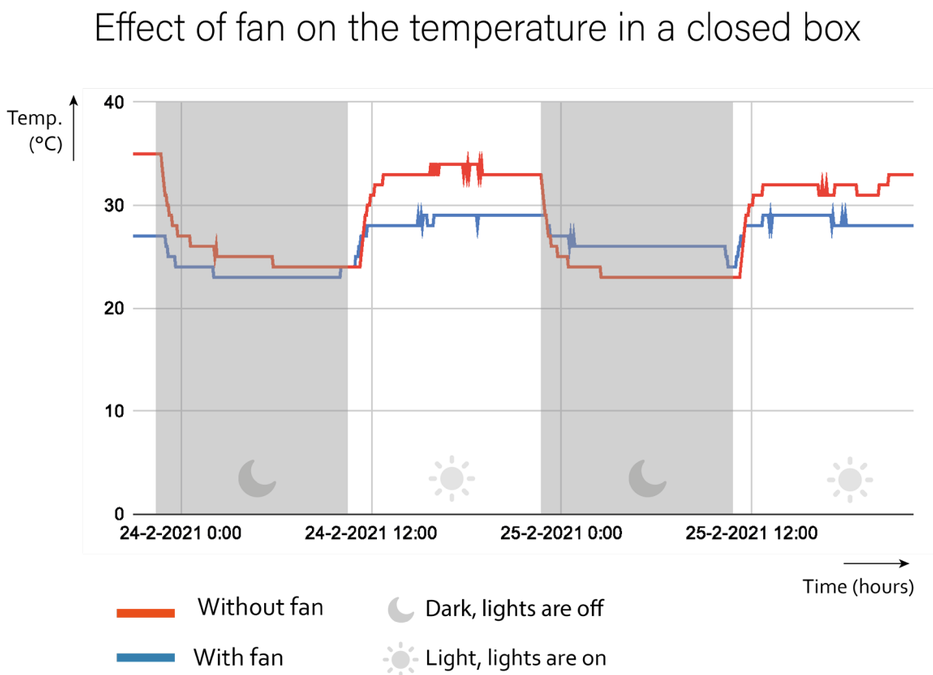


FIgure 82. Temperature in the incubator over two days with and without a fan.

CHAPTER 8

8.8 Conclusion

A final test was performed to compare the photosynthetic ability of a macro-porous bioprinted cube of 10 grams with a solid cube of 10 grams.

A prototype was built, which consisted of two transparent airtight boxes with a volume of 0.71 m^3, placed in a larger transparant outer box. The outer box contained LED strips with a photosynthetically active radiation of 20 m-2 s-1, ratio red/blue 4:1. Each sample was placed in the airtight box, together with a Winsen MH-Z19B infrared carbon dioxide sensor, a Grove oxygen sensor and a photon connected to the internet. The code, written by Zjenja Doubrovski, ensured that the measured data was plotted in a Google spreadsheet.

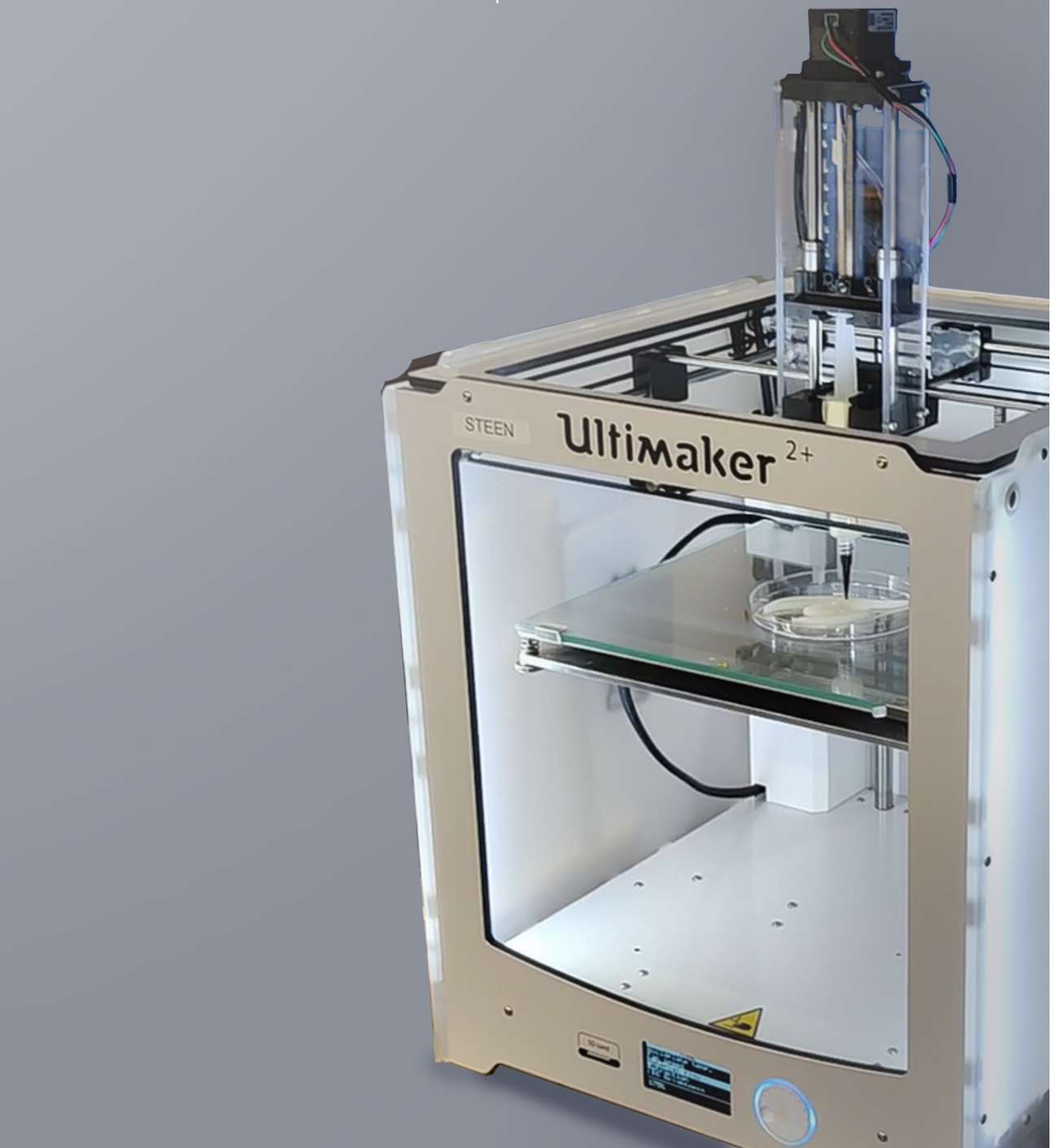
Better photosynthetic activity of macro-porous structures over solid structures could not be concluded from the measurement data. The carbon dioxide graphs of the porous and solid bioprints showed a sharp increase in carbon dioxide, which kept rising far beyond 3000 ppm. Both oxygen and carbon dioxide graphs showed a waving pattern according to the dark/light cycle, which were in sync with the increase and decrease of temperature in the boxes.

It is assumed that the sensor measurements are distorted by the high temperature of around 30 degrees celcius and lack of ventilation in the cupboard.

The incubator was meant for the growth of samples as well as measurements for photosynthetic performance. The microalgae bioprints showed unexpected loss of cell function due to a lack of humidity in the cupboard. The dryness of the prints could not be observed if they were kept in the small measurement containers. It can be concluded that the microalgae are used to the Aubin-Tam lab conditions, and are sensitive to slight changes in growth environment.

Chapter 9

Conclusion and recommendations



9.1 Conclusion

In this study, a bio-ink formulation based on work from Balasubramanian et al (2021) was optimized. The developed ink has improved print fidelity and resolution compared to previous work on microalgae bioprints, done by Lode et al (2015), Zhao et al (2019) and Johnston et al (2020), and can now reach up till 25 layers in height. With the optimized protocol, the 3D printed construct could be crosslinked so that the print was stable enough to be handled.

A large improvement in the new ink composition is the addition of nutrients to the bio-ink. This allows the bioprints to be self-supporting, without the need for an additional nutrient plate. The microalgae immobilized in the ink formulation survived more than seven days in the 3D printed geometry. Since high resolution bioprints can be achieved, there are more possibilities to create intricate shapes that can positively impact the cell growth and photosynthetic ability of the microalgae, such as macro porous structures or shapes with channels for nutrient distribution and waste removal. This way the product can be shaped in such a way to accommodate the organism that it is housing.

In addition, a test setup prototype was developed to measure the effect of macro-porosity on the photosynthetic performance. Within this study, it could not be confirmed that macro-porous structures have better photosynthetic performance over solid structures. Presumably, the sensor measurements were distorted by external factors such as temperature and ventilation. In order to reject or confirm the hypothesis, further research is needed, in which these variables are controlled for. Despite the inconclusive results, additional parameters were found which may prove valuable angles for further research.

The positive impact that microalgae materials can bring to the field of product design is huge. The optimized bio-ink is sustainable during the whole product life cycle. The production is carbon neutral, since it generates oxygen and the composition is completely biobased, made of living organisms and biopolymers found in nature. Waste is no longer an issue, since the material is biodegradable. The biomaterial can be especially useful in heavily populated areas, where higher levels of carbon dioxide are observed. Here, a piece of nature can be brought to your living room.

However, before this material is ready for large scale application, a couple of issues need to be overcome. Future research should be focused on the improvement of the robustness and prolongation of the microalgae cell viability. Currently, this material is suitable for short term applications only, such as artworks, installations and decorations. With further improvements, this biomaterial can be used in other disciplines such as interior design for its air cleaning properties, or in the fashion industry for its aesthetic appeal.

The optimized biomaterial offers a sustainable alternative to common materials. It could effectively decrease material pollution and battle the rising level of atmospheric carbon dioxide.

9.2 Recommendations

The recommendations can be divided in four subjects: recommendations to measure ink performance, recommendations for improvements on the algae incubator, bio-ink improvements and recommendations for further research on the photosynthetic measurement setup.

9.2.1 Bio-ink performance

More insights are necessary to test the performance of the bio-ink, in order to optimize it further.

First of all, the livingness of the bio-ink needs to be tested. In the project, the livingness was measured by the colour intensity of the bio-ink. More indepth analyses are necessary to see how long the cells can survive in the ink, how much the cells perish over time and if the cells can be regenerated. This could be done with the following tests:

1. Measurement of chlorophyll content and cell density

The amount of chlorophyll or cell density shows how many cells are alive in the material. These two factors can be tested with a photospectrometer. Since the cells are immobilized in the material, they need to be extracted from the ink first before they can be measured. The measurements must be repeated for a few days to be able to conclude if the material is still living.

2. Visualisation of cells in the 3D print

With microscopy, the movement of the cells can be seen in the ink and visualised in a photograph.

3. Measurement of cell number over time

A small portion in the form of a square can be taken with a knife and viewed under the microscope. The cell number can be counted for this specific area, and multiplied by the whole area of the 3D print to estimate the total amount of cells in the 3D print.

4. Measure regeneration of cells

An experiment can be done to test if the cells can be regenerated. To do so, the ink matrix needs to be dissolved and centrifuged. The supernatant is thrown away, and the remaining cell pellet is re-dissolved in fresh media. This culture can be used to make a bio-ink, which can then be checked for algae growth with microscopy or a photospectrometer.

Then, the photosynthetic activity can be further researched.

4. Measurement of photosynthetic efficiency

The efficiency of the microalgae can be measured with an Pulse Amplitude Fluorometer (PAM) The machine measures how many photons are actually used for photochemistry of photosynthesis. (Ritchie & Bunthawin, 2010).

Regarding risks, it is important to measure the stability of the bioprint. This assures that microalgae cells will not escape the 3D print and cause algal blooms in surrounding water bodies.

5. Measurement of cell immobilization

The prints can be immersed in sterile water and retrieved. The remaining liquid can be viewed under the microscope to check escaped algae cells.

Lastly, the impact of the cells on the mechanical strength of the bio-ink can be tested to see if the cells have a negative impact.

6. Tensile test

Two samples can be made, one with algae and one without. The difference in tensile strength can be measured with an universal testing machine.

9.2.2 Algae incubator

If the performance tests from the previous paragraph are carried out, more understanding is gained on the advantages and limiting factors of the material. Aside from knowledge on the performance, more insight is necessary in the behaviour of the microalgae. Before the bio-ink can be used in a product design context, it is necessary to understand the exact parameters necessary for growth.

Earlier, temperature, salinity, light and nutrients have been discussed as parameters impacting the microalgae growth. Humidity was not mentioned, however it is believed that this parameter plays a role in the survivability of algal cells. In the incubator, see Figure 83, a drying bio-ink has been observed, see Figure 84. The drying process happened quickly in a matter of hours. Samples placed in the small measurement boxes containing the sensors did not dry out.

The lack of humidity could be caused by the temperature of the LED lights, or the lack of ventilation in the cupboard. Simply placing a fan in the box is not enough, the samples probably need an input of fresh air.

To figure out the correct growth environment, the following tests could be done:

1. Placing bioprints of the same weight and shape in rooms of different sizes.

2. Subjecting bioprints to different LED strips with varying light intensities.

3. Spraying the microalgae prints with sterile water in time intervals.

Since the C. reinhardtii is specifically trained for the lab environment at the TU Delft, it could be possible to train the algae cells to accomodate better to the indoor home environment. This has been done for the Photosynthetic coating by Post Carbon Lab, which was mentioned in chapter 3.2

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Figure 83. Incubator with fan

Figure 84. Bioprints dry out after a few hours in the incubator

9.2.3 Bio-ink improvements

The optimized bio-ink composition meets six of the seven ink requirements mentioned in chapter 4.6. The ink is non-toxic, and shows microalgae cell growth. Moreover, it can be 3D printed at room temperature, does not stick to the nozzle tip and shows viscous and shear thinning behaviour, seen by a good shape retention after printing.

The bio-ink formulation has one limitation, namely that it falls apart in liquid cell culture medium. See Figure 85. It would be favourable if the shape remains stable in liquid medium, since the lifetime could be prolonged. Risk of algal blooms would be lessened as well if the 3D print stays intact in liquid. Now, the leftover liquid cannot be thrown away by users since it contains algae cells.

Moreover, the microalgae print is still relatively fragile. it can be deformed with little effort, see Figure 86,which limits the product application possibilities. It would be favourable if the ink was more robust, and retained its physical integrity against physical distortions.

Here, two methods are mentioned which could optimize the insolubility of the bio-ink

1. Add components to the ink to improve the strength and toughness

Since the selection of components for bio-inks is relatively complex, it is advised to ask experts on possible polymer candidates.

2. Test different crosslink durations

Now, only the cell function was researched for different crosslink concentrations, even though the duration of the crosslinking time is also an influencing factor on the degree of crosslinking.

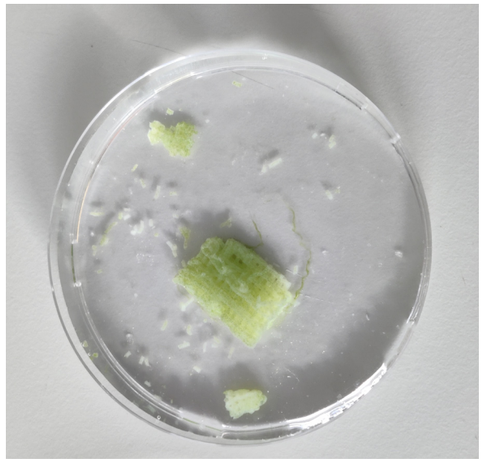


Figure 85. The bioprint falls apart after ten minutes in TAP medium.

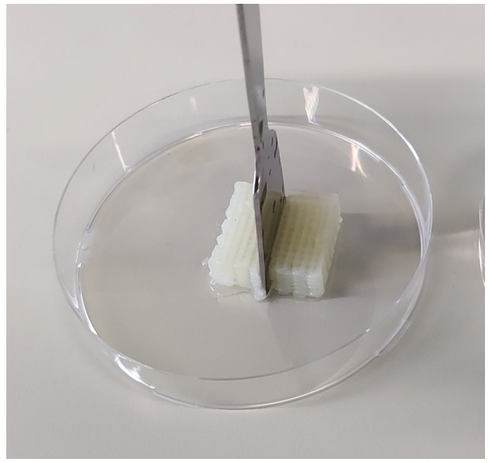


Figure 86. The bioprint can be deformed easily.

9.2.4 Photosynthetic measurement setup

The distorted measurements observed in chapter 8 were probably caused by the high temperature in the measurement box, and the lack of ventilation.

1. Mitigating temperature effects

In order to mitigate the effects of temperature on the experiment results, it is recommended to either select measurement instruments which are less sensitive to high temperatures, or to decrease the overall temperature of the measurement box. This can be done by selecting LEDs which radiate less warmth, or installing a ventilation or cooling system. Since the algae were initially cultivated at room temperature, it is advised to use a similar temperature for the measurement setup.

2. Mitigating effects due to lack of ventilation

The sensors could also have been influenced by the lack of ventilation in the measurement boxes. Since the measurement boxes were airtight, fresh influx of air was not possible. Instead, larger box sizes could be tested to see whether the initial volume of air within the boxes affects the research results.

The current measurement setup can fit two different shapes of microalgae prints. It is adviced for further reseach to expand the number of samples which can be tested simultaneously. For instance, in a new setup four samples could be made with the same weight but with increasing macro-porosity. This would give more insight into the effect of macro-porosity levels on oxygen production in microalgae 3D prints.

Chapter 10 | References

Abdel-Salam, M. M. (2015). Investigation of PM2. 5 and carbon dioxide levels in urban homes. *Journal of the Air & Waste Management Association, 65(*8), 930-936.

Aivan. (n.d.) *Korvaa – the world's first microbe-grown headset*. Retrieved on 02-11-2020 from https://www.aivan.fi/portfolio/korvaa-headset-made-by-microbes/

Alba, Katerina and Kontogiorgos, Vassilis (2018). Seaweed polysaccharides (agar, alginate Polymer B). *Encyclopedia of Food Chemistry.* Amsterdam, Netherlands: Elsevier.240-250.

Aouf, R. S. (2019). *Aivan grows headphones from fungus and yeast.* Retrieved on 02-11-2020 from https://www.dezeen.com/2019/05/24/korvaa-headphones-bioplastic-fungus-yeast-materials-aivan/

Armisen, R., & Gaiatas, F. (2009). Agar. *In Handbook of hydrocolloids*, pp. 82-107. Woodhead Publishing.

Balasubramanian, S., Yu, K., Meyer, A.S., Karana, E., & Aubin-Tam, M.E. (2021). *Bioprinting of Regenerative Photosynthetic Living Materials [manuscript submitted for publication].* Department of Bionanoscience, Kavli Institute of Nanoscience, Delft University of Technology.

Barsanti, L., Coltelli, P., Evangelista, V., Frassanito, A. M., Passarelli, V., Vesentini, N., & Gualtieri, P. (2008). Oddities and curiosities in the algal world. *Algal toxins: nature, occurrence, effect and detection*, 353-391. Springer, Dordrecht.

Bassham, J.A. (1998). *Photosynthesis*. Retrieved on 25-09-2020 from https://www.britannica.com/science/photosynthesis

Camere, S., & Karana, E. (2017). Growing materials for product design. *In Proceedings of the International Conference of the DRS Special Interest Group on Experiential Knowledge and Emerging Materials*, 101-115.

Campbell, N. A., Reece, J. B., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. V., & Jackson, R. B. (2015). *Biology: a global approach (tenth edition*) (pp. 261). New York, NY: Pearson.

Chashchyna, A. (Interviewer). (2020, 27 of February). *Sustainability Explored. [Podcast].* Retrieved on 12-2-2021 from https://sustainabilityexplored.medium.com/your-clothes-can-breathe-interview-with-post-carbon-lab-on-living-algae-containing-fabrics-b16a5438365d

Chislock, M. F., Doster, E., Zitomer, R. A., & Wilson, A. E. (2013). Eutrophication: causes, consequences, and controls in aquatic ecosystems. *Nature Education Knowledge, 4*(4), 10.

Chung, I. K., Beardall, J., Mehta, S., Sahoo, D., & Stojkovic, S. (2011). Using marine macroalgae for carbon sequestration: a critical appraisal. *Journal of applied phycology, 23*(5), 877-886.

Da Silva Fernandes, R., Tanaka, F. N., de Moura, M. R., & Aouada, F. A. (2019). Development of alginate/starch-based hydrogels crosslinked with different ions: Hydrophilic, kinetic and spectroscopic properties. *Materials Today Communications, 21*, 100636.

Das P, Aziz S.S., Obbard J.P. (2011). Two phase microalgae growth in the open system for enhanced lipid productivity. *Renew Energy, 36*(9):2524–8.

Dawson, T.P., Jackson, S.T., House, J.I., Prentice, I.C. & Mace, G.M. (2011). Beyond Predictions: Biodiversity Conservation in a Changing Climate. *Science, 332*(6025), 53-58.

DeForest, C. A., & Anseth, K. S. (2012). Bioactive hydrogels for regenerative medicine. *Annu. Rev. Chem. Biomol. Eng, 3*, 421-444.

Design to Fade. (n.d.) *Design to Fade.* Retrieved on 03-11-2020 from https://designtofade.puma.com/project/living-colours

ESD. (n.d.). Weller *KDS18TNP. Taper tip needle, Gauge: 18.* Retrieved on 25-2-2021 from https://esd.equipment/en/weller-kds18tnp.html

Farell, A. (2017). T*he light that never goes out: Julian Melchiorri's 'Bionic chandelier'*. Retrieved on 03-11-2020 from https://www.driving.co.uk/six-senses/sight/

Feiner, G. (2006). Meat products handbook: *Practical science and technology.* Elsevier.

Field, C. B., Behrenfeld, M. J., Randerson, J. T., & Falkowski, P. (1998). Primary production of the biosphere: integrating terrestrial and oceanic components. *Science, 281*(5374), 237-240.

GhavamiNejad, A., Ashammakhi, N., Wu, X. Y., & Khademhosseini, A. (2020). Crosslinking Strategies for 3D Bioprinting of Polymeric Hydrogels. *Small, 16(*35), 2002931.

Giraud, L. (2011). *Algægraphic installation: 2011-2014*. Retrieved on 12-2-2021 from http://www.liagiraud.com/cultures/

Glibert, P. M., Seitzinger, S., Heil, C. A., Burkholder, J. M., Parrow, M. W., Codispoti, L. A., & Kelly, V. (2005). Eutrophication. *Oceanography, 1*8(2), 198.

Gorman, D. S., & Levine, R. P. (1965). Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi. P*roceedings of the National Academy of Sciences of the United States of America, 54*(6), 1665.

Guiry, M. D. (2012). How many species of algae are there?. *Journal of phycology, 48*(5), 1057-1063.

Hahn, J. (2019). *Bio-ID Lab designs DIY algae-infused tiles that can extract toxic dyes from water.* Retrieved on 28-02-2021 from https://www.dezeen.com/2019/09/21/bio-id-lab-indus-algae-tiles-water/

Hallmann, A. (2011). Evolution of reproductive development in the volvocine algae. *Sexual plant reproduction, 24*(2), 97-112.

Harris, E. H. (1989). *The chlamydomonas sourcebook (Vol. 2).* D. B. Stern, & G. B. Witman (Eds.). San Diego: Academic Press.

Harris, E. H. (2001). Chlamydomonas as a model organism. *Annual review of plant biology, 52*(1), 363-406.

Höög, J. L., Lacomble, S., T O'Toole, E., Hoenger, A., McIntosh, J. R., & Gull, K. (2014). Modes of flagellar assembly in Chlamydomonas reinhardtii and Trypanosoma brucei. *Elife, 3*, 1479.

Hospodiuk, M., Dey, M., Sosnoski, D., & Ozbolat, I. T. (2017). The bioink: a comprehensive review on bioprintable materials. *Biotechnology advances, 35*(2), 217-239.

Jenkins, A. D., Kratochvíl, P., Stepto, R. F. T., & Suter, U. W. (1996). Glossary of basic terms in polymer science (IUPAC Recommendations 1996). *Pure and applied chemistry, 68*(12), 2287-2311.

UN Environment. (2019. *Research highlights true impacts of plastics on our planet, ecosystems, people.* Retrieved on 01-03-2021 from https://www.unep.org/pt-br/node/24584#:%7E:text=%E2%80%9CMarine%20plastic%20litter%20pollution%20is,are%20on%20the%20receiving%20end.

Jia, J., Richards, D. J., Pollard, S., Tan, Y., Rodriguez, J., Visconti, R. P., ... & Mei, Y. (2014). Engineering alginate as bioink for bioprinting. *Acta biomaterialia, 10*(10), 4323-4331.

Khalil, S., & Sun, W. (2009). Bioprinting endothelial cells with alginate for 3D tissue constructs. *Journal of biomechanical engineering, 131*(11).

Khan, M., Karmakar, R., Das, B., Diba, F., & Razu, M. H. (2016). Heterotrophic growth of micro algae. *Recent Advances in Microalgal Biotechnology,* 1-18.

Khan, M. I., Shin, J. H., & Kim, J. D. (2018). The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microbial cell factories, 17*(1), 36.

Lee, K. Y., & Mooney, D. J. (2012). Alginate: properties and biomedical applications. *Progress in polymer science, 37*(1), 106-126.

Lehtiniemi, M., Engström-Öst, J., & Viitasalo, M. (2005). Turbidity decreases anti-predator behaviour in pike larvae, Esox lucius. *Environmental Biology of Fishes, 73*(1), 1-8.

Lesser, C. F., & Miller, S. I. (2001). Expression of microbial virulence proteins in Saccharomyces cerevisiae models mammalian infection. *The EMBO journal, 20*(8), 1840-1849.

Lewis, J. A. (2006). Direct ink writing of 3D functional materials. *Advanced Functional Materials, 16*(17), 2193-2204.

Li, L. (2002). Thermal gelation of methylcellulose in water: scaling and thermoreversibility. *Macromolecules, 35*(15), 5990-5998.

Luchtman, L. (2017). *Biodesign research project - Living colour.* Retrieved on 03-11-2020 from https://issuu.com/kukkadesign/docs/living_colour-ibook

Luchtman, L. & Siebenhaar, I. (2020). *Design to fade.* Retrieved on 03-11-2020 from https://livingcolour.eu/design-to-fade/

Malda, J., Visser, J., Melchels, F. P., Jüngst, T., Hennink, W. E., Dhert, W. J. A., ... Hutmacher, D. W. (2013). 25th Anniversary Article: Engineering Hydrogels for Biofabrication. *Advanced Materials, 25*(36), 5011–5028.

Manjavacas, G., & Nieto, B. (2016). Hydrogen sensors and detectors. *Compendium of Hydrogen Energy* 215-234. Woodhead Publishing.

Mandal, S., & Mallick, N. (2014). Microalgae: The Tiny Microbes with a Big Impact. *Bioenergy Research: Advances and Applications*, 171-184. Elsevier.

Merck. (n.d.) 3D *Bioprinting: Bioink Selection Guide.* Retrieved on 01-10-2020 from https://www.sigmaaldrich.com/technical-documents/articles/materials-science/3d-bioprinting-bioinks.html

Melchiorri, J. (2017). *Bionic chandelier*. Retrieved on 03-11-2020 from https://www.julianmelchiorri.com/Bionic-Chandelier

Milano, J., Ong, H. C., Masjuki, H. H., Chong, W. T., Lam, M. K., Loh, P. K., & Vellayan, V. (2016). Microalgae biofuels as an alternative to fossil fuel for power generation. *Renewable and Sustainable Energy Reviews, 58*, 180-197.

Mohsenpour, S. F., & Willoughby, N. (2016). Effect of CO_2 aeration on cultivation of microalgae in luminescent photobioreactors. *Biomass and Bioenergy, 85*, 168-177.

Moraes, L., da Rosa, G. M., Santos, L. O., & Costa, J. A. (2020). Innovative development of membrane sparger for carbon dioxide supply in microalgae cultures. *Biotechnology Progress*, 2987

Moreira, D., & Pires, J. C. (2016). Atmospheric CO_2 capture by algae: negative carbon dioxide emission path. *Bioresource technology, (215)* 371-379.

Mulder, M. (2000). Membrane preparation – Phase Inversion Membranes, *Encyclopedia of Separation Science*. Academic press, 3331-3346.

NewPharma. (n.n.). *BD Plastipak Spuit Luer-Lok 10ml Stuks 100 (300912)*. Retrieved on 25-02-2021 from https://www.newpharma.nl/bd/67091/bd-plastipak-spuit-luer-lok-10ml-stuks-100-300912.html

Nixon, S. W., & Fulweiler, R. W. (2009). Nutrient pollution, eutrophication, and the degradation of coastal marine ecosystems. *Global loss of coastal habitats: rates, causes and consequences*, 23-58.

Odian, G. (2004). *Principles of polymerization*. John Wiley & Sons.

Oliveira, J.T.; Reis, R.L. (2008). Hydrogels from polysaccharide-based materials: Fundamentals and applications in regenerative medicine. *Natural-Based Polymers for Biomedical Applications,* 485–514.

Ozbolat, I. T. (2015). Scaffold-based or scaffold-free bioprinting: competing or complementing approaches?. *Journal of Nanotechnology in Engineering and Medicine, 6*(2).

Perales-Vela, H. V., Peña-Castro, J. M., & Canizares-Villanueva, R. O. (2006). Heavy metal detoxification in eukaryotic microalgae. *Chemosphere, 64*(1), 1-10.

Phan, S., Yang, K. & Diego, A. (2013). *Deconstructed apple pie*. Retrieved on 10-01-2021 from https://www.scienceandfood.org/deconstructed-apple-pie/

Popa, D., & Udrea, F. (2019). Towards integrated mid-infrared gas sensors. *Sensors, 19*(9), 2076.

Probert, I. & Klaas, C. (1999). *Microalgal culturing.* Retrieved on 17-12-2020 from http://ina.tmsoc.org/CODENET/culturenotes.htm

Puertas-Bartolomé, M., Włodarczyk-Biegun, M. K., Del Campo, A., Vázquez-Lasa, B., & San Román, J. (2020). 3D Printing of a Reactive Hydrogel Bio-Ink Using a Static Mixing Tool. *Polymers, 12*(9), 1986.

Ramaraj, R., Tsai, D. D., & Chen, P. H. (2013). Chlorophyll is not accurate measurement for algal biomass. *Chiang Mai Journal of Science, 40*(4), 547-555.

Ritchie, R. J., & Bunthawin, S. (2010). The use of pulse amplitude modulation (PAM) fluorometry to measure photosynthesis in a CAM orchid, Dendrobium spp.(D. cv. Viravuth Pink). *International Journal of Plant Sciences, 171(6),* 575-585.

Rochaix, J.D. (2013). *Brenner's Encyclopedia of Genetics (Second Edition)*. Massachusetts: Academic Press

Salomé, P. A., & Merchant, S. S. (2019). A series of fortunate events: Introducing Chlamydomonas as a reference organism. *The Plant Cell, 31*(8), 1682-1707.

Stanton, M. M., Samitier, J., & Sanchez, S. (2015). Bioprinting of 3D hydrogels. *Lab on a Chip, 15*(15), 3111-3115.

Stewart, A. (2016). T*he 'living concrete' that can heal itself*. Retrieved on 03-11-2020 from https://edition.cnn.com/2015/05/14/tech/bioconcrete-delft-jonkers/index.html

SunP Biotech. (n.d.) *SunP Float*. Retrieved on 01-03-2021 from https://sunpbiotech.com/index.php/sunp-float/

Sutton, S. (2011). Measurement of microbial cells by optical density. *Journal of Validation technology, 17*(1), 46-49.

Syn.De.Bio. (2016). *Bio-Concrete – Henk Jonkers*. Retrieved on 03-11-2020 from http://syndebio.com/bio-concrete/

Tirella, A., Orsini, A., Vozzi, G., & Ahluwalia, A. R. T. I. (2009). A phase diagram for microfabrication of geometrically controlled hydrogel scaffolds. *Biofabrication, 1*(4), 045002.

Tirella, A., Vozzi, F., Vozzi, G., & Ahluwalia, A. (2011). PAM2 (piston assisted microsyringe): a new rapid prototyping technique for biofabrication of cell incorporated scaffolds. *Tissue Engineering Part C: Methods, 17*(2), 229-237.

Tolinski, M. (2015). *Additives for polyolefins: getting the most out of polypropylene, polyethylene and TPO.* William Andrew.

Turner, A. M., & Chislock, M. F. (2010). Blinded by the stink: nutrient enrichment impairs the perception of predation risk by freshwater snails. *Ecological Applications, 20*(8), 2089-2095.

Vaisala. (n.d.). *How to measure Carbon Dioxide.* Retrieved on 27-02-2021 from https://www.vaisala.com/sites/default/files/documents/VIM-G-How-to-measure-CO_2-Application-Note-B211228EN-C-LOW-v1.pdf

Vernier. (n.d.). *Photosynthesis and respiration – Experiment 31C.* Retrieved on 21-2-2021 from https://education.ti.com/-/media/032F57CEADA64A169ECA7EDD706BBC44.

Vítová M., Bišová K., Hlavová M., Kawano S., Zachleder V., Čížková M. (2011). Chlamydomonas reinhardtii: Duration of its cell cycle and phases at growth rates affected by temperature. *Planta, (234),* 599–608.

Wan, X., Luo, L., Liu, Y., & Leng, J. (2020). Direct Ink Writing Based 4D Printing of Materials and Their Applications. *Advanced Science, 7*(16), 2001000.

Wang, L. L., Highley, C. B., Yeh, Y. C., Galarraga, J. H., Uman, S., & Burdick, J. A. (2018). Three-dimensional extrusion bioprinting of single-and double-network hydrogels containing dynamic covalent crosslinks. *Journal of Biomedical Materials Research Part A, 106*(4), 865-875.

Wegst U.G.K., Bai H., Saiz E., Tomsia A.P. & Ritchie R.O. (2015) *Bioinspired structural materials. Nature Materials (14)*, 23-36

Williams, D. A. (2020). *Earth Fact Sheet.* Retrieved on 21-2-2021 from https://nssdc.gsfc.nasa.gov/planetary/factsheet/earthfact.html

Wolkers, H., Barbosa, M., Kleinegris, D. M., Bosma, R., & Wijffels, R. H. (2011). *Microalgae: the green gold of the future.* Wageningen UR.

Yeesang, C., & Cheirsilp, B. (2011). Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. *Bioresource technology, 102*(3), 3034-3040.

Zachleder, V., Ivanov, I., Vítová, M., & Bišová, K. (2019). Cell Cycle Arrest by Supraoptimal Temperature in the Alga Chlamydomonas reinhardtii. *Cells, 8*(10), 1237.

Zhang, Z. X., Yu, J., Kou, S. Q., & Lindqvist, P. A. (2001). Effects of high temperatures on dynamic rock fracture. *International Journal of Rock Mechanics and Mining Sciences, 38*(2), 211-225.

Zohuri, G. (2012). Polymer science: a comprehensive reference.
.

Chapter 11

Appendices

Appendix A. Making of TAP medium and TAP agar

Ingredients for 1L TAP media:

1. 2,42g Tris base (Sigma-Aldrich)
2. 0,375 mL phosphate buffer (stored at 4°C) (Chlamydomonas Resource Center, USA)
3. 25 mL TAP solution (NH_4Cl + $MgSO_4$ -7H_2O + $CaCl_2$-2H_2O) (stored at 4 °C)
4. 1mL Hutner's trace elements (stored at 4 °C) (Chlamydomonas Resource Center, USA)
5. 1070 µL Acetic acid (Sigma-Aldrich)
6. 1L miliQ

- Graduated cylinder of 1L
- Weighing plates
- Scales
- Pipette aid
- Pipette pen
- pH meter
- Magnetic stirrer
- Stirring bar

Procedure

1. Place a stirring bar at the bottom of a graduated cylinder and add 1L of miliQ. Place the cylinder on a magnetic stirrer.
2. Weigh the correct amount of tris base on a weighting plate and add to the mixture.
3. Take 25mL of TAP solution with a pipet aid and pipet and add to the mixture.
4. Add phosphate buffer and trace elements with a pipette pen.
5. Insert the probe of the pH meter in the mixture and measure the pH.
6. Collect acidic acid from the fume hood and add 1070 µL, and wait until the pH is between 6.95 and 7.05.
7. Wash probe and put the acidic acid back in the fume hood.
8. Pour the mixture in two bottles of 500 mL and autoclave.
9. Store the TAP medium in a heated stove at 55 °C. At room temperature it solidifies.
10. For TAP agar: add 7,5 g agar per 500mL TAP medium. The TAP agar can be used for 7 days.

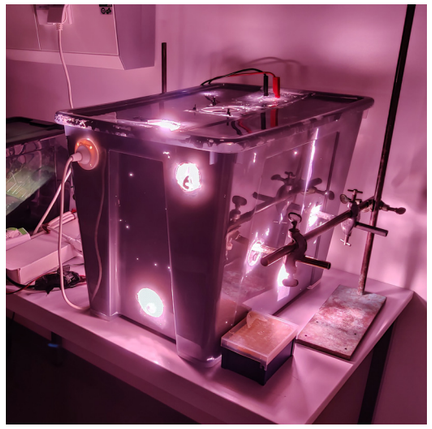
Appendix B. Making of C. rheinhardtii culture

Set-up

- Laminar flow hood
- Algae light chamber with sterile air bubbling
- 100 mL TAP medium
- Chlamydomonas Rheinhardtii CC-125 (mt+) strain streaked onto a petri dish containing TAP agar
- 2x Sterile loops
- Sterile conical flask with tube and Styrofoam cork
- 2x filter 0.2 µm

Procedure

1. Collect the algae culture from the growth chamber and bring with the other equipment to a cell culture room with a laminar flow hood.
2. Disinfect the flow hood, wait for the flow to stabilize and put the equipment in the middle of the chamber.
3. Remove the tube from the flask with a sterile loop and connect two filters on the end of the tube.
4. Pour 100mL of TAP medium in the flask.
5. Scrape with a new sterile loop a small amount from the algae culture in the petri dish and add it to the TAP medium in the flask. Close the flask with the styrofoam cork, making sure the tube with filters sticks out of the flask.
6. Remove the equipment from the laminar flow hood and disinfect it. UV the laminar flow hood for at least an hour.
7. Bring the algae culture to the light/dark chamber and put it an clamp with the tube opening facing down. Attach the filter to the system and adjust the air inflow until it the speed of the bubbling is around one bubble per second.
8. Over the course of seven days, the microalgae required a biomass of 2.000.000 cells/mL.



Appendix C. Bioprinting of alginate hydrogels

Setup

- TAP agar plate (25 mL) with CaCl2 (0.05M)
- 10 mL of 7-day microalgae culture
- 10 mL TAP media
- 10 mL Sodium alginate solution (5 w/v%)
- 1 mL Pipette tip

- CoLiDo DIY printer

Procedure

1. Centrifuge the microculture at 4000 rpm for 5 min, 4 degrees celcius.
2. Discard the supernatant and resuspend the cells in 10 mL TAP medium. Vortex.
3. Add the sodium alginate and vortex.
4. Load a sterile syringe with 10 mL bio-ink. Mount the syringe in the syringe pump.
5. Connect the syringe to a 1 mL pipette tip, which is connected to a silicone tube (1 x 1 mm)
6. Place the agar plate on the print bed, and level the print bed to the syringe tip with the CoLiDo software. Set the extrusion rate to 0.5 mL/h

Appendix D. Photon setup

Instructions

1. Download the Particle app.
2. Create a Google Drive account
3. Connect the Photon to a power source via an usb cable.
4. In the app, click on the + sign and select 'Set up a Photon'. Select the Photon which should be connected and enter the Wi-Fi password.
5. Deconnect the Photon from the power source.
6. Connect the carbon dioxide sensor and the oxygen sensor to the Photon.
7. Connect the Photon to a power source with an usb cable. 2 LEDs will turn on: one slow blinking cyan, one constant blue. Wait for two minutes until the blue LED is switched off.
8. Check the values in the Google spreadsheets. If a value of -1 is given, push the rest-button on the Photon. Wait again for two minutes.
9. The carbon dioxide, oxygen concentration and temperature will be plotted in the Google Spreadsheets every four minutes.

DESIGN FOR our future

TU Delft

IDE Master Graduation

Project team, Procedural checks and personal Project brief

This document contains the agreements made between student and supervisory team about the student's IDE Master Graduation Project. This document can also include the involvement of an external organisation, however, it does not cover any legal employment relationship that the student and the client (might) agree upon. Next to that, this document facilitates the required procedural checks. In this document:

- The student defines the team, what he/she is going to do/deliver and how that will come about.
- SSC E&SA (Shared Service Center, Education & Student Affairs) reports on the student's registration and study progress.
- IDE's Board of Examiners confirms if the student is allowed to start the Graduation Project.

USE ADOBE ACROBAT READER TO OPEN, EDIT AND SAVE THIS DOCUMENT
Download again and reopen in case you tried other software, such as Preview (Mac) or a webbrowser.

STUDENT DATA & MASTER PROGRAMME

Save this form according the format "IDE Master Graduation Project Brief_familyname_firstname_studentnumber_dd-mm-yyyy".
Complete all blue parts of the form and include the approved Project Brief in your Graduation Report as Appendix 1 !

family name: Vriend
initials: V.D. given name: Vivian
student number: 4450205
street & no.:
zipcode & city:
country: the Netherlands
phone:
email:

Your master programme (only select the options that apply to you):
IDE master(s): ★ IPD ○ DfI ○ SPD
2nd non-IDE master:
individual programme: - - (give date of approval)
honours programme: ○ Honours Programme Master
specialisation / annotation: ○ Medisign ○ Tech. in Sustainable Design ○ Entrepeneurship

SUPERVISORY TEAM **

Fill in the required data for the supervisory team members. Please check the instructions on the right !

** chair: Elvin Karana dept. / section: SDE / MM
** mentor: Zjenja Doubrovski dept. / section: SDE / MM
2nd mentor: Marie-Eve Aubin-Tam, Srikkanth Balasubramanian
organisation: Bionanoscience Department TU Delft
city: Delft country: the Netherlands

comments (optional): Chair and mentor were originally of department EM and MD, which recently merged. We believe that their background and expertise differ enough to form a heterogeneous team.

Chair should request the IDE Board of Examiners for approval of a non-IDE mentor, including a motivation letter and c.v..

Second mentor only applies in case the assignment is hosted by an external organisation.

Ensure a heterogeneous team. In case you wish to include two team members from the same section, please explain why.

Procedural Checks - IDE Master Graduation

TU Delft

APPROVAL PROJECT BRIEF

To be filled in by the chair of the supervisory team.

chair Elvin Karana date 22 - 10 - 2020 signature

CHECK STUDY PROGRESS

To be filled in by the SSC E&SA (Shared Service Center, Education & Student Affairs), after approval of the project brief by the Chair.
The study progress will be checked for a 2nd time just before the green light meeting.

Master electives no. of EC accumulated in total: _____ EC

Of which, taking the conditional requirements into account, can be part of the exam programme _____ EC

List of electives obtained before the third semester without approval of the BoE

YES all 1st year master courses passed

NO missing 1st year master courses are:

name _____ date - - signature _____

FORMAL APPROVAL GRADUATION PROJECT

To be filled in by the Board of Examiners of IDE TU Delft. Please check the supervisory team and study the parts of the brief marked **.
Next, please assess, (dis)approve and sign this Project Brief, by using the criteria below.

- Does the project fit within the (MSc)-programme of the student (taking into account, if described, the activities done next to the obligatory MSc specific courses)?
- Is the level of the project challenging enough for a MSc IDE graduating student?
- Is the project expected to be doable within 100 working days/20 weeks ?
- Does the composition of the supervisory team comply with the regulations and fit the assignment ?

Content: APPROVED NOT APPROVED

Procedure: APPROVED NOT APPROVED

comments

name _____ date - - signature _____

Personal Project Brief - IDE Master Graduation

TU Delft

Optimizing nutrient distribution and airflow in microalgae 3D prints — project title

Please state the title of your graduation project (above) and the start date and end date (below). Keep the title compact and simple. Do not use abbreviations. The remainder of this document allows you to define and clarify your graduation project.

start date 24 - 08 - 2020 22 - 02 - 2021 end date

INTRODUCTION **

Please describe, the context of your project, and address the main stakeholders (interests) within this context in a concise yet complete manner. Who are involved, what do they value and how do they currently operate within the given context? What are the main opportunities and limitations you are currently aware of (cultural- and social norms, resources (time, money,...), technology, ...).

This research is a collaboration between two research groups: the Materials Experience Lab at Industrial Design Engineering and the Aubin-Tam group from the Bionanoscience department. Both are working on the development of sustainable materials made with living organisms.

The Materials Experience Lab at IDE is a group of researchers/practitioners, who aim to understand and enhance the relationships people have with product materials. Over the last years they developed tools and a method to understand how materials are experienced, and inspire designers to develop new and biofabricated materials and applications. Previous work of the lab was for instance products made with mycelium, a type of fungus.

At the Aubin-Tam group, scientists are working on the development of biomaterials from a more biological and technical standpoint. The group uses microorganisms to fabricate bio-based and/or living materials. One of the topics of the research group is on 3D printing of micro-algae. This material proposition was a starting point for this graduation project.

Microalgae are a large group of single cell organisms that are capable of photosynthesis. They can be found all over the world, in soil, fresh water, oceans, and even in snow on mountaintops. Microalgae are well known for their robustness and sustainability, as they grow rapidly in harsh conditions with low energy consumption. They are most commonly used in biomass production, providing a carbon dioxide neutral biofuel with a high energy content. Other applications are in food production, (source of protein, vitamins and minerals) and pharmaceuticals (antibacterial, antifungal and antiviral substances). They can also act in biofiltration by removing pollutants from wastewater.

The field of product design is beginning to recognize the advantages of microalgae, mostly focused on their air cleaning properties. Examples of applications are Cloud Collective's urban algae farm, EcoLogicStudio's Urban Algae Folly, and Julian Melchiorri's bionic chandelier Exhale. See figure 1. Microalgae-based structures like Urban Algae Folly can reportedly clean ten times as much carbon dioxide from the surrounding air than large trees, making this concept a perfect fit for polluted urban centers around the world.

In research, current attempts are made to create a 'living material' with algae cells by combining them with polymers in a hydrogel formulation. The hydrogel traps the living algae cells in a matrix that can house and sustain the viability of the encapsulated cells.

The goal of the research on this algae material is to develop the material and possible applications in order to:
-Offer an alternative sustainable material which can be used to design products without using unrenewable resources
- Make products which can clean the air by converting carbon dioxide into oxygen.

This new material can compete with current material manufacturers of for instance plastics, textiles or ceramics. In order to achieve this, the material needs to be developed further and collaborations are needed with bio-technologists to upscale the production. In Figure 2, all the stakeholders and goals mentioned above are summarized.

space available for images / figures on next page

introduction (continued): space for images

Urban algae farm (Cloud collective, 2014)

Urban Algae Folly (EcoLogicStudio, 2015)

Exhale (Julian Melchiorri, 2017)

image / figure 1: Product examples containing algae

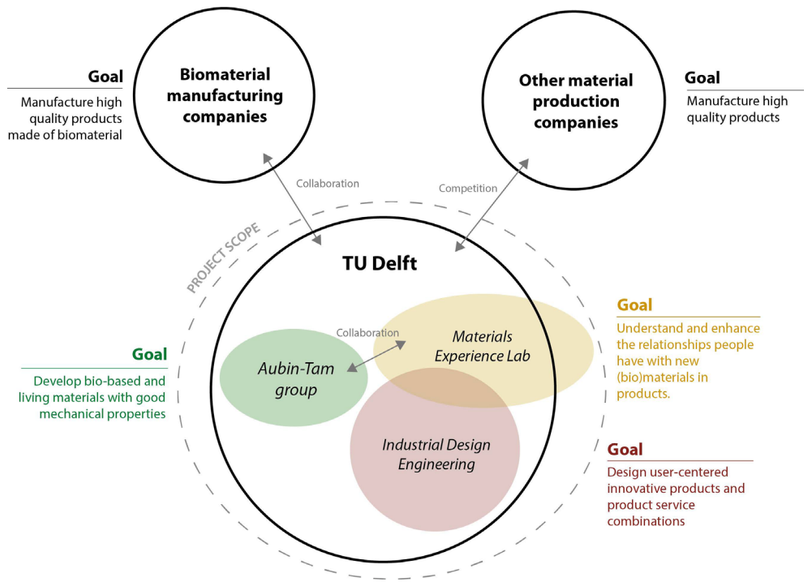


image / figure 2: Stakeholder map including goals for each stakeholder and project scope

PROBLEM DEFINITION **

Limit and define the scope and solution space of your project to one that is manageable within one Master Graduation Project of 30 EC (= 20 full time weeks or 100 working days) and clearly indicate what issue(s) should be addressed in this project.

A contemporary issue in product design is materialism: the notion that consumers buy many products which are often used shortly and thrown away right after use. Commonly-used materials in these products, for instance plastics, threaten our economy, health and environmental biodiversity. Facing challenges such as climate change, CO2 emissions and the scarcity of fossil materials amplifies the call for novel materials that require less energy and natural resources to produce, do not contain ingredients harmful to nature and can be recycled. One possible solution is the replacement of common materials with 'growing materials'. Materials which are fabricated with the help of living organisms such as fungi, algae and bacteria. These natural materials require less energy and no fossil raw materials for production, whilst resulting in materials that are often biodegradable due to their biological origin. Moreover, these materials offer new and unique functions and expressions.

Products containing living microalgae need a regular supply of fresh water and nutrients to stay alive and thus be able to perform photosynthesis. One way to do this is by keeping the algae cells in a liquid medium which can be refreshed by a pumping mechanism, as seen in the products in Figure 1. In the case of a microalgae material, another method is needed to provide the algae nutrients and water. The challenge here is to reach the inner cells within the structure. Our hypothesis is that micro-porous structures containing nutrient channels could increase the longevity and photosynthetic activity of microalgae structures. 3D printing is selected as the processing method, since it can accurately create complex geometries.

Preliminary studies have been performed by The Aubin-Tam group to 3D print with algae material, where they were able to print small structures on nutrient plates in a sterile environment. Before larger porous microalgae structures can be 3D printed, two additional challenges need to be addressed: lowering the contamination in ambient conditions and achieving prints with higher layers by altering the bio-ink formula and/or testing various curing methods.

ASSIGNMENT **

State in 2 or 3 sentences what you are going to research, design, create and / or generate, that will solve (part of) the issue(s) pointed out in "problem definition". Then illustrate this assignment by indicating what kind of solution you expect and / or aim to deliver, for instance: a product, a product-service combination, a strategy illustrated through product or product-service combination ideas, ... In case of a Specialisation and/or Annotation, make sure the assignment reflects this/these.

Create a porous 3D printed microalgae structure which improves the nutrient distribution and airflow to the cells.

The algae bio ink from the Aubin-Tam group will be used as the starting point of the experiments. Alterations to the bio ink will be made, in order to improve the printability and reduce the contaminations in ambient environments. The printing process will also need to be altered, machine settings such as extrusion speeds and paths will be modified. Small scale prints will be made to check the algae growth and accuracy of the prints.

Once the bio ink and the 3D printing process are optimized, three different porous geometries including nutrient channels will be created. The best structure will be selected by testing the algae growth and CO2 reduction.

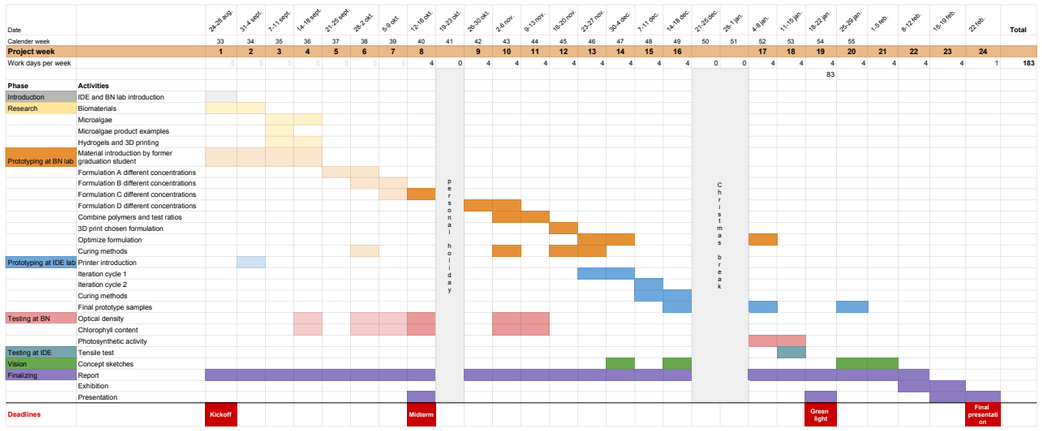
The result will be an optimized bio ink formulation, a 3D printing process, and a set of 3D printed algae samples with different microstructures, and a recommendation for the microstructure with the best nutrient distribution and airflow

PLANNING AND APPROACH **

Include a Gantt Chart (replace the example below - more examples can be found in Manual 2) that shows the different phases of your project, deliverables you have in mind, meetings, and how you plan to spend your time. Please note that all activities should fit within the given net time of 30 EC = 20 full time weeks or 100 working days, and your planning should include a kick-off meeting, mid-term meeting, green light meeting and graduation ceremony. Illustrate your Gantt Chart by, for instance, explaining your approach, and please indicate periods of part-time activities and/or periods of not spending time on your graduation project, if any, for instance because of holidays or parallel activities.

start date 24 - 8 - 2020

22 - 2 - 2021 end date



MOTIVATION AND PERSONAL AMBITIONS

Explain why you set up this project, what competences you want to prove and learn. For example: acquired competences from your MSc programme, the elective semester, extra-curricular activities (etc.) and point out the competences you have yet developed. Optionally, describe which personal learning ambitions you explicitly want to address in this project, on top of the learning objectives of the Graduation Project, such as: in depth knowledge a on specific subject, broadening your competences or experimenting with a specific tool and/or methodology, ... Stick to no more than five ambitions.

My interests are in sustainable (new) materials and prototyping.

I have enjoyed the practical nature of the Integrated Product Design master and I wanted to incorporate rapid prototyping into the project, similar to my approach during my Advanced Prototyping minor. That is why look forward to work in the Applied Sciences lab to create material samples and learning more about 3D printing. Since the algae need some time to grow, I think the most challenging part of the project is time management.

In the bachelor I followed the course 'Towards Circular Product Design', which I enjoyed a lot. So much so, that I did my final bachelor project on sustainable packaging. To complete my master degree, I want to do another sustainable project. The knowledge I obtained in the project can hopefully be used as a stepping stone in my future career in the sustainable design industry.

To summarize: I want to learn:

- To execute an individual design project with good time management
- Gain more in-depth knowledge on biofabricated materials and 3D printing

FINAL COMMENTS

In case your project brief needs final comments, please add any information you think is relevant.