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THEMATIC PRIORITY

RESOURCE AND MATERIAL EFFICIENCY

BEST PRACTICE GUIDELINES FOR CULTIVATION OF MICROALGAE ON NUTRIENT RICH DIGESTATE

PART 2 :

BEST PRACTICES FOR OPTIMUM NUTRIENT REMOVAL AND ALGAL CULTIVATION

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Executive summary

This document is an output of the INTERREG North West Europe funded ALG-AD project. The document reports the results and recommendations developed through the operation of ALG-AD technology at three industrial pilot sites in North-West Europe (United Kingdom, Belgium and France).

The ALG-AD technology combines anaerobic digestion (AD) and microalgal cultivation technologies. The technology was developed to take excess nutrient rich digestate waste produced from anaerobic digestion of food and farm to cultivate algal biomass for animal feed and other products. The excess nutrient rich digestate (NRD) being defined as digestate which is prohibited under EU legislation from being returned to land.

The document reports guidelines and best practice, and associated considerations, recommendations and conclusions drawn from the set-up and running of the large-scale cultivation facilities at the three pilot sites. The cultivation of microalgae using NRD, including maximising uptake of nutrients and biomass production, and the downstream processing of the algal biomass are covered.

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Glossary

AD: Anaerobic Digestion

NRD: Nutrient Rich Digestate

OECD: Organisation for Economic Co-operation and Development

PAS110: Publicly Available Specification 110

QP: Quality Protocol

WRAP: Waste and Resources Action Programme

IBC: Intermediate Bulk Container

PPE: Personal Protective Equipment

ICP-OES/ ICP-AES: Inductively Coupled Plasma Emission Spectrometry / Inductively Coupled Plasma-Atomic Emission Spectrometry

XRF: X-ray Fluorescence

TAN: Total Ammoniacal Nitrogen

VFAs: Volatile Fatty Acids

RCF: Relative Centrifugal Force

DAF: Dissolved Air Flotation

1 INTRODUCTION

ALG-AD: AN INNOVATIVE SOLUTION TO TREAT EXCESS DIGESTATE

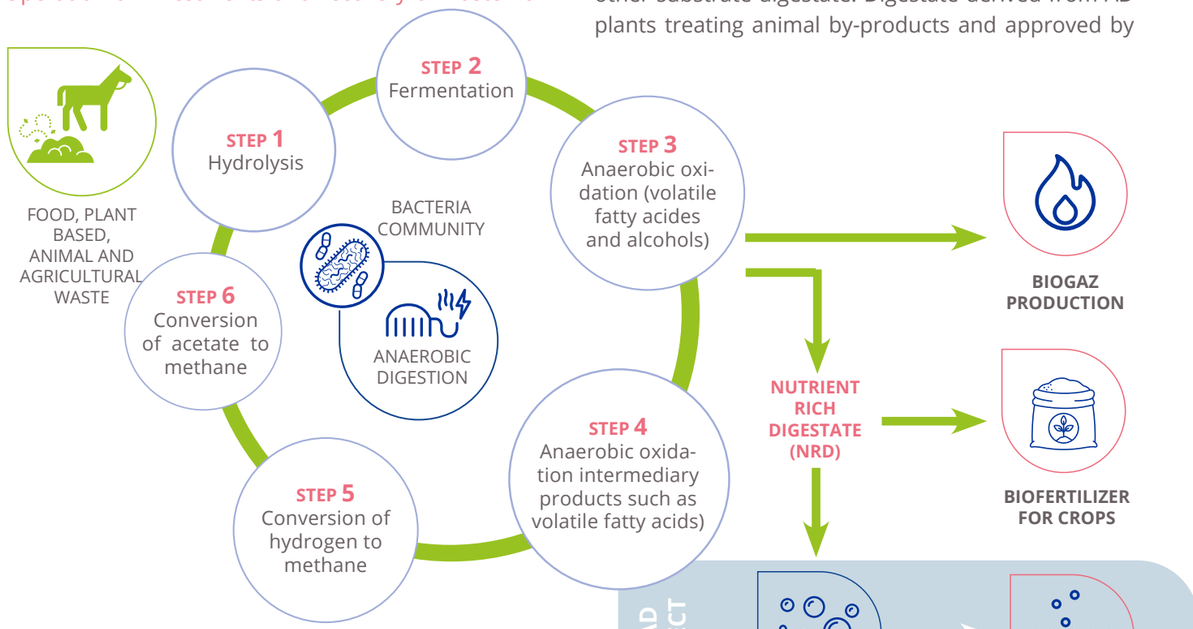
The ALG-AD project is part-funded under the ERDF (European Regional Development Fund) INTERREG North West Europe Programme (2017-2021). The Interreg North-West Europe (NWE) Programme is one of the fifteen transnational cooperation programmes financed by the European Union. These programmes encourage public, scientific, private and civil society organisations to cooperate with a view to improving the economic, environmental, territorial and social development of Europe's regions. The programme co-finances these organisations to work together in transnational projects on specific themes.

ALG-AD is funded under Priority 3: Resource and Materials Efficiency and involves 12 partners from across the UK, France, Germany and Belgium. This report contributes as **Output WP1 A1.2 to Work Package 1**: Operation of investments and recovery of waste nutrients to produce algal biomass. More details can be found at www.nweurope.eu/projects/ALG-AD

ALG-AD is a circular economy project set within North West Europe (NWE) to address resource and materials efficiency. NWE is a highly agricultural area, where high population levels combined with an increasing food consumption generate significant amounts of food and farm waste. Anaerobic Digestion (AD) is one of the most used technologies for biological processing of these organic wastes.

The AD biological process results in the production of Methane and CO_2 (main economic driver of AD plants) and as a by-product produce a nutrient rich digestate (NRD). Most of this this NRD can be applied to agricultural land as a biofertilizer. However, due to nitrogen (N) and phosphorus (P) run-off into natural environments and groundwaters, which can cause eutrophication problems, the spreading of digestate on agricultural land is limited throughout North West Europe with the introduction of Nitrate Vulnerable Zones: NVZs (European Nitrate Directive 91/676/EEC).

The application of this Directive can lead to the production of thousands of tons of excess digestate, which pose an issue. Dealing with excess NRD is an acute worsening issue and solutions are needed. This Nitrate Directive is applied to agricultural-derived and other substrate digestate. Digestate derived from AD plants treating animal by-products and approved by



Schematic representation of the anaerobic digestion process and link to the ALG-AD project

Animal and Plant Health Agency (APHA) is subject to a grazing ban once the digestate is used on land (The Official Information Portal on Anaerobic Digestion by NNFCC 2021). In our project, the pilot installed at the Cooperl site (France) was dealing with this type of material.

Additionally, the legislation for the other AD treated materials is expected to become stricter and include all types of digestate. Microalgal cultivation technology coupled to AD plants using digestate could help resolve the increasing challenges of excess digestate by producing high value products using this waste

The ALG-AD project combines microalgae cultivation with AD technology by using the excess digestate as a waste-based medium for microalgal biomass production. This microalgae biomass can then be processed as an ingredient for animal feed or for other products. The ALG-AD project provides a circular economy solution to the excess digestate currently underused in NWE by enabling digestate remediation using microalgae and providing a new market for the AD industries to valorise their excess digestate.

Within the project, three pilot investment sites have been developed to test microalgal production using digestate as a waste-based medium at large-scale, e.g. more than 1,000 L cultivation. Two of the pilots were directly located at AD industry sites based in the UK (Langage AD) and in France (Cooperl). The third was located at a digestate analysis organisation in Belgium (INNOLAB).

This report brings together best practice recommendations from the operation of the three investments and provides insight and advice on the optimisation of microalgal cultivation and biomass production using digestate as a cultivation medium, and on optimum nitrogen and phosphorus remediation from the digestate using the microalgae. The report includes information on microalgae cultivation, as well as on the photobioreactor cultivation technology used for the development of the ALG-AD technology.

This best practice document is of relevance for the following groups of stakeholders:

- **Anaerobic Digestion Sector (AD plant stakeholders, sectoral agencies, chemical analysis laboratories, research institutes)**
- **Microalgae stakeholders**
- **Farmers (land crops but also animal production and microalgae farms)**
- **Animal Feed Sector (e.g. feed additive producers, compound feed producers; non-governmental organisations and research institutes)**
- **Environmental organisations**
- **Public authorities**
- **Business support organisations or consulting organisations (technical and environmental design offices)**
- **Biofertilizer and biostimulants businesses**

REQUIRMENTS AND CONSIDERATIONS FOR INSTALLING ALG-AD TECHNOLOGY.

1) A large area of flat land is required with a sufficient footprint for the microalgae plant.

2) Given the weather conditions for NW Europe, this area needs to be covered. Ideally, algal cultivation requires greenhouse or polytunnel facilities, with plenty of natural light for successful growth of the photosynthetic organisms. If facilities are sheltered from natural sunlight, installation of artificial light will be required. Temporary structures such as polytunnels are also an option. Ideally the orientation of the greenhouse/polytunnel should be East-West. The temperature regime will need to be maintained, so during summertime, a cooling system is needed, and in winter a heating system is required.

3) An adequate electricity and freshwater supply will be required. AD facilities may be able to produce the electricity required on site, without additional cost for the operation of the microalgae plant.

4) Access to an appropriate drainage system that accepts treated wastewater

5) Access to a CO_2 supply or any other carbon source (bicarbonate or organic carbon) is needed. Potentially this can be also sourced from the AD plant.

6) Prior to installation, planning permission and licensing may be required.

7) Civil and electrical work is required before microalgae plant installation; this additional cost must be assessed beforehand.

8) The algal cultivation process requires water, so the water and electricity supplies need to be easily accessed.

2 MICROALGAE CULTIVATION FOR THE INOCULATION OF LARGE-SCALE CULTIVATION SYSTEMS

Based on practical experience from each pilot site (Devon, Oostkamp, Britanny), this section describes the step-by-step process used to produce microalgae culture in sufficient volume to produce an inoculum for large-scale cultivation. The acclimation to digestate as a waste-based medium is explained.

Upscaling from a master algal culture

Culturing of a single species of microalgae starts by isolating cultures from the local environment or by acquiring a master culture from a culture collection facility (such as the CCAP culture collection, UK, https://www.sams.ac.uk/facilities/ccap/ or the SAG culture collection at Göttingen University, Germany: https://sagdb.uni-goettingen.de/). Master cultures are received in small volume (approximately 20 to 50 mL) in liquid form or in solid form (agar plates). Here, the upscaling of microalgal culture is described for a master culture in liquid form. The master culture is used to inoculate new cultures from which higher volumes of algae can be cultivated, starting in the laboratory and progressing up to the desired cultivation volume at large scale.

From master culture to vials, it is recommended to use a mother inoculum of 10% of the total final inoculated volume (e.g. to inoculate a total volume of 50 mL, use 5mL of the master culture). However, it is advised to use a higher inoculation volume if the master culture is not dense, i.e. less than 1x106 cell mL-1 or OD (750nm) >0.3.

Add the required medium for the inoculated strain at the concentration indicated by the medium provider (suggested to use F/2 medium for standard green algae); the correct medium is usually indicated by the culture collection from which the master culture came.

After inoculation, the vials can be placed under low light (less than 100 μmoles photons m2 s-1), at the temperature required for the inoculated strain (also usually indicated by the culture collection ~18-20°C). Monitor the culture daily and allow growth for a minimum of 5-10 days before inoculating the next volume

Scaling-up of microalgal cultures, from flasks in the laboratory to larger pilot-scale bags and tubular reactors. A1/A2: Flasks from 250ml to 1L; B: 5L flasks; C: 20L carboys; D: 80L bags; E: 800L photobioreactor. (Source : Dr Claudio Fuentes Grünewald, Swansea University)

RECOMMENDATION

At lab scale it is better not to open the flasks in order to keep the risk of contamination as low as possible. From 20L onward the biotic parameters can be recorded by sampling the microalgae cultures every other day.

The same procedure is applied for the next up-scaling volumes, using roughly 10% of microalgal inoculum and allowing culture growth for 10 days before up-scaling. Figure 1 shows the entire up-scaling process, from 25mL vials to 20L carboys, 80L plastic bag cultivation column and large-scale cultivation systems from 800L.

Sterilisation: At low cultivation volumes (up to 5L flasks), it is recommended to autoclave all glassware and water used for the up scaling from master culture. Indeed, new cultures received from a culture collection tend to be more fragile and need an acclimation time to their new environment (i.e. laboratory with new conditions of light, temperature, medium), thus additional precautions need to be taken regarding sterilisation processes, avoiding detrimental contamination that could cause the microalgal culture to collapse.

At higher cultivation volumes (5L and up), it is recommended to use bleach (Sodium Hypochlorite) as a sterilisation process: using 0.5mL L-1 of bleach in the water used for cultivation before inoculation allows for a sterilisation of the water and the container used for cultivation. It is recommended to run the bleached water with a gentle aeration or bubbling system for approximately 24 hours, and then neutralise the bleach using sodium thiosulfate at a concentration of 0.2 g L-1. Sodium thiosulfate neutralise the bleach after 20 minutes in lower volumes of water, but for higher volumes (500 L and up), it is recommended to allow one hour for the bleach to be fully neutralised before microalgal inoculation into the cultivation system. It is highly advisable to perform a Chlorine test to verify the bleach neutralization before inoculation.

Acclimation to digestate

Once greater than 250ml of microalgal culture is obtained and the culture is dense and healthy (regular microscopic observation is recommended to assess the health of the culture – see EnAlgae report for detailed advice), the microalgae can be acclimated to digestate step by step.

The acclimation process consists of adding digestate at a low starting concentration and gradually increasing this concentration over time as the microalgae culture grows. It is recommended to start with a 0.5% concentration of digestate (0.5% of the total cultivation volume, e.g. 50 mL of digestate added to a culture of 10L). If the culture is growing well after digestate addition, start increasing the digestate concentration in increments of 0.5%, every 10 days, or according to microalgal growth (i.e. if cultures are growing rapidly, increase the digestate concentration more often).

Once the desired concentration (depending on the ammonium concentration) (~2.5%) is reached in the cultivation system, the acclimation is over, and the culture can be used to start scaling up until a sufficient volume (e.g. carboys or bags) is obtained for the inoculation of the large-scale system. (This recommendation is made according to an average ammonium concentration of 4000 mgL-1 in the NRD)

RECOMMENDATION

The step of inoculum preparation and its cultivation process is very important. It should be worked through carefully, as it gives a successful start for the healthy growth of the culture and helps to prevent microbial contamination. The master stock of the culture should be kept alongside larger scale cultivation, and the large-scale culture should be renewed every 3-5 months (if operating under continuous or semi-continuous cultivation mode.) Ideally, the master stock needs to be kept under laboratory conditions. However, prior to inoculation, an environmental adaptation time is recommended, so the culture should be located at the light and temperature conditions relevant to the larger scale facility for 7-10 days

3 MICROALGAL SELECTION AND CULTIVATION SYSTEMS

Microalgal species selection

Algal species selection should be based on the ability of the microalgal strain to uptake high amounts of nutrients that are available in digestate, as well as their ability to cope with other heavy metals and potentially toxic compounds that can be found in the NRD. Furthermore, strain selection should also consider the microalgal composition in terms of compounds of interest for whatever the target usage is for the microalgal biomass (e.g. pigments for food colouring and dyes or lipids for biofuels). In the case of the ALG-AD project, strains selected were those utilisable as a protein source in the animal feed industry, but also those that show a robust performance e.g. *Chlorella* and *Scenedesmus* genus.

Consideration should also be made regarding the cultivation of monocultures or consortia. Microalgae consortia which are a group of two or more microalgal species, occur in natural environments and can also be engineered at laboratory scale by combining two or more microalgal species. When grown in consortia, microalgae develop symbiotic relationships, helping to prevent the contamination of the cultures and thereby making long-term cultivation possible. It has been suggested that consortium of microalgae could be more efficient at up taking N and P from digestate than monocultures (Gonçalves et al, 2017). Within the ALG-AD project, both approaches have been tested, i.e., the investment sites at Langage-AD and CooperI focused on the cultivation of monocultures, while the operation at INNOLAB opted for a consortium approach. In our work, the monoculture provided a better rate of the nutrients uptake from the waste stream, however, biomass composition in consortia change according to the seasons or the dominant species, therefore biomass composition through the seasons cannot be guaranteed. In contrast monocultures (i.e. one microalgal species) are more predictable, and allow for a higher quality of the produced biomass and associated high-value compounds (Fuentes Grunewald et al 2021).

CONSIDERATION SUMMARY

The right choice of the algal species depends on the purpose for cultivation. Consideration is needed of bioremediation efficiency and/or final use of the biomass. For the best bioremediation of waste streams, the choice of species could be microalgae from local environment. This could be taken from a locally located water source, such as a lake, puddle or any other water reservoir. Additionally, algal species could be in the air, land or water. The use of any of these substrates could be the initial base for the algal species isolation. However, this can be time consuming.

Therefore we recommend identifying the suitable species from the consortium and purchasing the mono-species adapted for mass cultivation from the culture collections mentioned previously. Generally, we can recommend these well-known species for a continuous and effective bioremediation of high nutrient wastes: *Chlorella*, *Scenedesmus* and *Desmodesmus spp*. The use of biomass for higher value products combined with remediation efficiency could alternatively be done with *Spirulina*, and some marine species such as, *Nannochloropsis sp*., *Tetraselmis sp*. and *Phaeodactylum tricornutum*. The Trystrochytrides such as *Aurantiochytrium mangrovei* are recommended for high biomass production in a short period of time, as well as for a high oil (DHA) content.

Open vs closed systems

Microalgae can be grown in a variety of systems, falling within two main categories: open or closed systems. Open systems are exposed to the environment, which is the case for raceway ponds or open tanks. Closed systems or photobioreactors (PBR's) come in many shapes and sizes; they can be tubular (horizontal and vertical), flat plates, membrane-like, etc. Table 1 gathers some of the main commercial suppliers of both open ponds and PBRs in NWE

Both open and closed systems present advantages and drawbacks, the main ones related to cost and contamination. Indeed, open systems are extremely

exposed to contamination from predators and competitive microorganisms that can significantly compromise microalgal cultures. Open systems are also prone to some water loss due to evaporation. In PBR's, microalgae are less exposed to these contaminations and, if occurring, the characteristics of a closed system allows for a level of control over them. PBR's generally allow for the control of the main parameters influencing microalgal growth, including temperature, light intensity and photoperiod (in indoor conditions mainly), pH and CO2 addition or mixing. However, this level of control comes with a cost and PBR's generally have a higher initial cost linked to the purchase and installation of equipment, while open ponds can be installed very inexpensively. Thus, there is a trade-off between the cost of the equipment and the quality of the produced biomass. Microalgal monoculture of species in a closed PBR enables more controlled production, potentially producing algae containing high-value compounds, which would subsequently have higher market value and may justify the higher investment in a PBR. Also, it is important to remark that the location of the microalgae plant is another element to be in consideration for choosing open or closed systems. In general, open system are suggested for pleasant climatic conditions (e.g. Southern Europe), in contrast in high latitudes closed systems are more advisable. There is also a need to consider a land availability and the associated cost of it. Open systems required a vast amount of land compared with the footprint of a closed system.

Within the ALG-AD project, closed systems have been selected to produce microalgal biomass. This selection was based on the requirement to produce biomass as an ingredient for animal feed, where the reliability of the process, traceability and quality of the biomass needs to be ensured, which is guaranteed in closed PBR systems.

RECOMMENDATION

All three of our pilots' sites used a closed PBR system, as the biomass produced is intended for use in a feed product. However, a raceway cultivation system in a greenhouse-covered space does provide a lower cost alternative solution. Our overall recommendation is to use closed PBR systems in the NWE of Europe, as they are more reliable and can be operated with more precision. The ALG-AD project has produced a set of Decision Support Tools designed to support stakeholders to explore options and make decisions based on their own scenario. These can be found on https://www.nweurope.eu/alg-ad

Table 1: Commercial suppliers of PBRs in Europe

Name	Country	Description
Varicon Aqua LTD http://www.variconaqua.com/	UK	Varicon Aqua is the manufacturer of the Phyco range of photobioreactors and the Cell-Hi line of algal nutrients. They are based in the UK and their team has more than 30 years' experience designing, constructing and deploying algae photobioreactors and aquaculture production systems worldwide
LGem	Netherlands	LGem designs and manufactures turnkey microalgae photobioreactors are tailormade, scalable and easy to operate LGem customizes its innovative modular algae systems according to your wishes and budget. They deliver all over the world.
A4F HTTPS://A4F.PT/EN	Portugal	A4F is a biotechnology company, located in Portugal, with more than 20 years of accumulated experience in microalgae Research & Development and microalgae production (up to industrial scale). A4F is specialized in the design, build, operation and transfer (DBOT) of commercial-scale microalgae
SUBITEC HTTPS://SUBITEC.COM/EN	Germany	Subitec is a German technology company headquartered in Stuttgart specializing in process engineering and the supply of microalgae cultivation equipment and turn-key production systems. Established as a spin-off from the Fraunhofer Institute, Subitec has more than 20 years of experience
ALGOSOURCE HTTPS://ALGOSOURCE.COM/EN/EXPERTISES/	France	Algosource offers a range of professional controlled production tools (from a few litres to several cubic metres).
FOTOSINTETICA & MICROBIOLOGICA S.R.L. HTTP://WWW.FEMONLINE.IT/	Italy	F&M commercializes several cultivation systems: glass bubble columns for inoculum production, the Green Wall Panel (GWP®) series for research and large-scale production, race-way ponds

4 BIOREMEDIATION - MAXIMISING UPTAKE OF WASTE NUTRIENTS BY ALGAL CULTIVATION AT PILOT SCALE

The bioremediation capacity of the culture relies on the algal species first being adapted to use the waste nutrients for their growth. The algal culture needs to be acclimated, starting with low concentrations of digestate (e.g 0.5%) with a gradual increase of concentration in the culture, as described in Section 2.

Pre-treatment of the digestate is also recommended, and a detailed explanation of this step of the process is covered in a separate ALG-AD best practice document, Best Practices for the treatment and preparation of nutrient rich digestate for algal cultivation - Output WP1 A1.1. The reader is also referred to the paper by Fernandes et al. (2020).

In order to grow, microalgae require macro and micronutrients. The approximate elemental composition of algal biomass is approximately C:N:P - 50:7:1 (on a weight basis) and based on this, the algal culture needs to get access to these nutrients to grow. The ability of microalgae to use waste containing these nutrients makes them good candidates for bioremediation. The maximum uptake rate of these macro and micronutrients may be calculated prior to cultivation to determine approximate consumption and allow optimum growth (details can be reviewed in the paper from Silkina et al. 2020.

Waste streams such as digestate are very high in ammonium (e.g. > 5,000 mg/L), and this therefore requires dilution, as a concentration of ammonium greater than 200mg/L is toxic for microalgae (Salbitani and Carfagna, 2021). The remediation process can be monitored by checking the waste nutrient uptake efficiency, measured before and after the cultivation process. The following parameter measurements are recommended to quantify remediation

- Optical Density (OD)
- Nutrients (Total N, nitrate, ammonium, Total P, Phosphate -) – See the NRD Best Practice document for analytical methods

Additionally, you can measure:

- Total Carbon, (organic and inorganic TOC and TIC
- Total suspended solids and volatile suspended solids (TSS, VSS)

Culture growth also needs to be monitored. Cell density, in the time frame of 24 or 48 hours, as well as cell dry weight, chlorophyll measurements are recommended. These parameters can be measured manually or automatically, depending on equipment available (these details can be found in the EnAlgae Best Practice document)

Examples of bioremediation from the three ALG-AD pilots are presented in Table 2.

Biomass Production

If the main objective is to maximise biomass production, then the amount of waste nutrients needs to be controlled carefully. It is recommended that the waste stream should be diluted to a lower level to begin with, so that the algal culture can physiologically process the nutrients and does not have a toxic excess. Usually this would be lower than if the purpose of the algae was purely for bioremediation purposes.

RECOMMENDATION

The recommendation for effective bioremediation is based on first identifying the nutrient composition and concentrations of the waste stream. The presence and concentration of essential macronutrients such as N and P for algal growth is important for successful results. It is advisable to perform a full digestate characterization (in order to know the N, P and heavy metal composition) prior to use of this side-streams in microalgae cultivation.
The pre-treatment and dilution of the waste nutrient source is key for a good performance of the culture (refer to the Best Practice on treatment of NRD, available on the ALG-AD website. During algal cultivation using waste nutrients, the monitoring of essential biotic and abiotic parameters is a further key step for a successful and effective bioremediation process.

From the experience of the Langage AD site, we run the culture with 1% of AD waste (with the ammonium concentration below ~50mg/L). This concentration of waste nutrients was optimum for *Chlorella* and *Scenedesmus* maximum biomass production in a semi-continuous mode of cultivation. This was done to allow a stable microalgae culture throughout seasons, and also improve the nutrient and bioremediation capacity compared with those cultures grown at higher dilution.

Bioremediation data

Species	Digestate Origin	N (mg/L)	P (mg/L)	Elemental composition	Cultivation mode	Biomass Productivity/concentration	Nutrient removal efficiency	Year
LANGAGE AD *Scenedesmus sp.* and *Chlorella vulgaris*	Food waste and dairy factory waste	Raw NRD: 183 mg/Kg treated NRD: 135 mg/Kg Raw NRD: 37.4 treated NRD: 33.8		July 2018: Fe: 527; Mo: 0.36; Cu: 2.58; Mn: 4.58; Se: 0.21; Ni: 1.65; Zn: 9.84; Co: 0.21	Working volume 5,000 L tubular PBR autotrophic and 400 L mixotrophic cultivation	*Scenedesmus* - production per batch 4.24 kg; p/y 186.73 kg; *Chlorella* - 2.20kg per batch, 97 kg p/y (considering data obtained from mixtrophic cultivation, please refer to Fuentes-Grunewald et al 2021)	N removal per year (p/y): 10.91 kg; NRD use p/y: 2.55t; Raw digestate use p/y: 5.11 t;	2018-2020
INNOLAB/UGHENT Mixed consortium *Desmodesmus sp.* and *Chlorella sp.*	Agriculture waste (plant based)	Treated NRD (paper filtration): 2370 mg/Kg Treated: 9.61 mg/Kg Treated NRD: 246.6		Zn: 12.6 ; Pb: 2.53; Ni: 0.359; Hg: 0.993; Cu: 2.53; Cr: 1.26; Cd: 0.253; As: 2.53; Ca: 146; Mg: 133; K: 2130	3,200 L vertical and 600L horizontal tubular reactor	Production per Batch 4.34 kg; p/y 47.5 kg	Nitrogen removal p/y: 10.7 kg; Nutrient rich digestate use p/y: 3.52 t; Raw digestate use p/y: 4.58 t;	2018-2020
COOPERL/CNRS/UBO Thraustochytrids microalgae *Aurantiochytrium Mangrovei*	Pig manure	Treated NRD (membrane filtration) 2480 mg/Kg Treated: 138 mg/Kg Treated NRD: 17.9		Ca: 188; Mg: 26.4; Na: 426; K: 1054	Working volume 1,350 L	Production per Batch 5.4 kg; p/y 250 kg	Nitrogen removal p/y: 3.1 kg; Raw digestate use p/y: 1.9 t;	2018-2020

Table 2: Bioremediation of various waste streams using different microalgal species from ALG-AD experimental sites (with the extrapolation for use per batch and per year to compare among the pilots).

5 LARGE-SCALE MICROALGAE CULTIVATION USING TREATED DIGESTATE AT THE THREE INVESTMENTS

Langage AD (England)

Digestate	Ultra-filtered using membranes
Microalgae	*Chlorella vulgaris*/*Scenedesmus obliquus*
Photobioreactor	Vertical tubular - working volume 5000L, 400L mixotrophic module
Cultivation mode	Photoautotrophy and mixotrophy
Cultivation cycle	10 - 12 days each cycle (auto and mixo cultivation in total)

For a detailed description of the growth approach in two steps, please check Fuentes Grunewald et al. 2021

Digestate used and treatment method

The digestate used at the investment site was provided by the anaerobic digestion plant Langage AD (England). Digestate was the result of the treatment of food waste, including kitchen waste and dairy factory waste, and was certified PAS 110. The digestate composition is presented in Table 3.

The digestate was treated using membrane filtration with a pore size of 100 Kda (the membrane filtration rig is shown in Figure 2) details of the pre-treatment process can be found in Fernandes et al. 2020 . Filtered digestate was diluted to 1%, avoiding toxicity linked to high ammonium concentration. The digestate was added to the cultivation system using a peristaltic pump at an initial rate of 100mL/h. Digestate (2.5% of the total working PBR volume) was added at the beginning of each growth cycle and after either each harvest or when the Ammonium content in the culture was below 10 mg/L)

This rate was adjusted according to the nutrient uptake observed during cultivation.

Microalgae cultivated

Laboratory scale and pilot-scale experiments were conducted using the microalgae *Chlorella vulgaris* (CCAP 211/11R) and *Scenedesmus obliquus* (CCAP 276/6A). Results obtained from experimental work and large-scale trials at Langage showed that *S. obliquus* had the best performances while growing on digestate, mainly linked to its robustness against harsh environments. Results of *S. obliquus* (Figure 3) cultivation at the investment site are presented in this document.

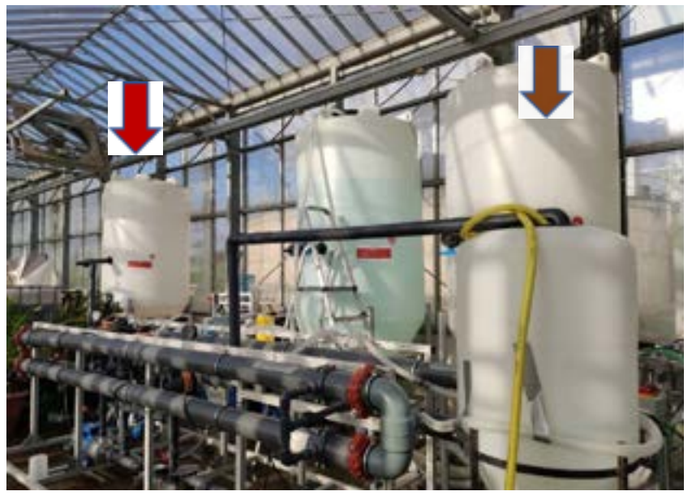


Figure 2: Membrane filtration system showing membrane filtration rig (red arrow) and concentration tank (brown arrow).

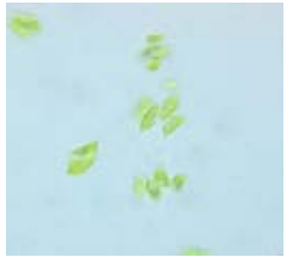


Figure 3: x40 Microscopy image of the microalgae Scenedesmus obliquus

The photobioreactor and cultivation process

The PBR used at Langage AD is a vertical tubular system, with a total capacity of 7,000 L and a working volume of 5,000L (or 5 tons). The PBR relies on an automated system for the control and monitoring of pH and CO2 injections, as well as temperature and oxygen levels. The vertical tubes correspond to the light phase of the system (Figure 4), where microalgae in culture have access to direct natural or artificial light for photosynthesis, and the dark tank corresponds to the phase in which the microalgae have no light access and carry out gas exchanged releasing the O2 producing due to photosynthesis (the retention time in this part are seconds only). The PBR is further equipped with an artificial light system to provide an additional source of energy when natural light is not sufficient (i.e. wintertime and/or cloudy days).

The PBR is used as a photoautotrophic system, where microalgal growth relies on light (from natural or artificial sources) and nutrients from the NRD provided by filtered digestate. For improved biomass production and increase of the digestate absorption by microalgae, a mixotrophy system (400L module) has been put in place following photoautotrophic cultivation. Mixotrophy is a cultivation process during which microalgae have access to an organic carbon source, usually in the form of sugar such as glucose or dextrose, in addition to the light, and the nutrients provided by the NRD digestate.

Microalgae produced photoautotrophically were harvested and concentrated using membrane technology. The membrane filtration system used for the harvesting was the same as the one showed in **Figure 2.** The harvested biomass, was further concentrated up to 6 g/L in the case of Scenedesmus and 2g/L in the case of Chlorella, was fed into a 400L mixotrophic

Table 3: Composition of raw and micro-filtered digestate used for microalgal cultivation.

	Raw NRD (Initial composition)	Ultra-filtered NRD permeate
pH	8.02	8
Dry weight	5.94%	2%
Ammonium (NH4+-N)	4016 mg/L	3146 mg/L
Phosphorus (P)	665 mg/kg	50.7 mg/kg
Calcium (Ca)	6756 mg/kg	37 mg/kg
Potassium (K)	2015 mg/kg	1203 mg/kg
Magnesium (Mg)	113.7 mg/kg	6.04 mg/kg
Sodium (Na)	1150 mg/kg	2146 mg/kg
Aluminium (Al)	109.3 mg/kg	20.96 mg/kg
Copper (Cu)	1.62 mg/kg	0.56 mg/kg
Iron (Fe)	6.22 mg/kg	8.18 mg/kg
Zinc (Zn)	32.9 mg/kg	1.06 mg/kg

reactor. A carbon source (dextrose at a concentration of 10 g/L) under light conditions were provided to the culture for three days. A mixotrophy approach was used as a mean to enhance biomass production and improve the remediation of nitrogen and phosphorus from the digestate by microalgae. Detailed results of the semi-continuous growth of the target microalgae strain can be found in Fuentes-Grunewald et al 2021.

Proof of concept and validation of the technology

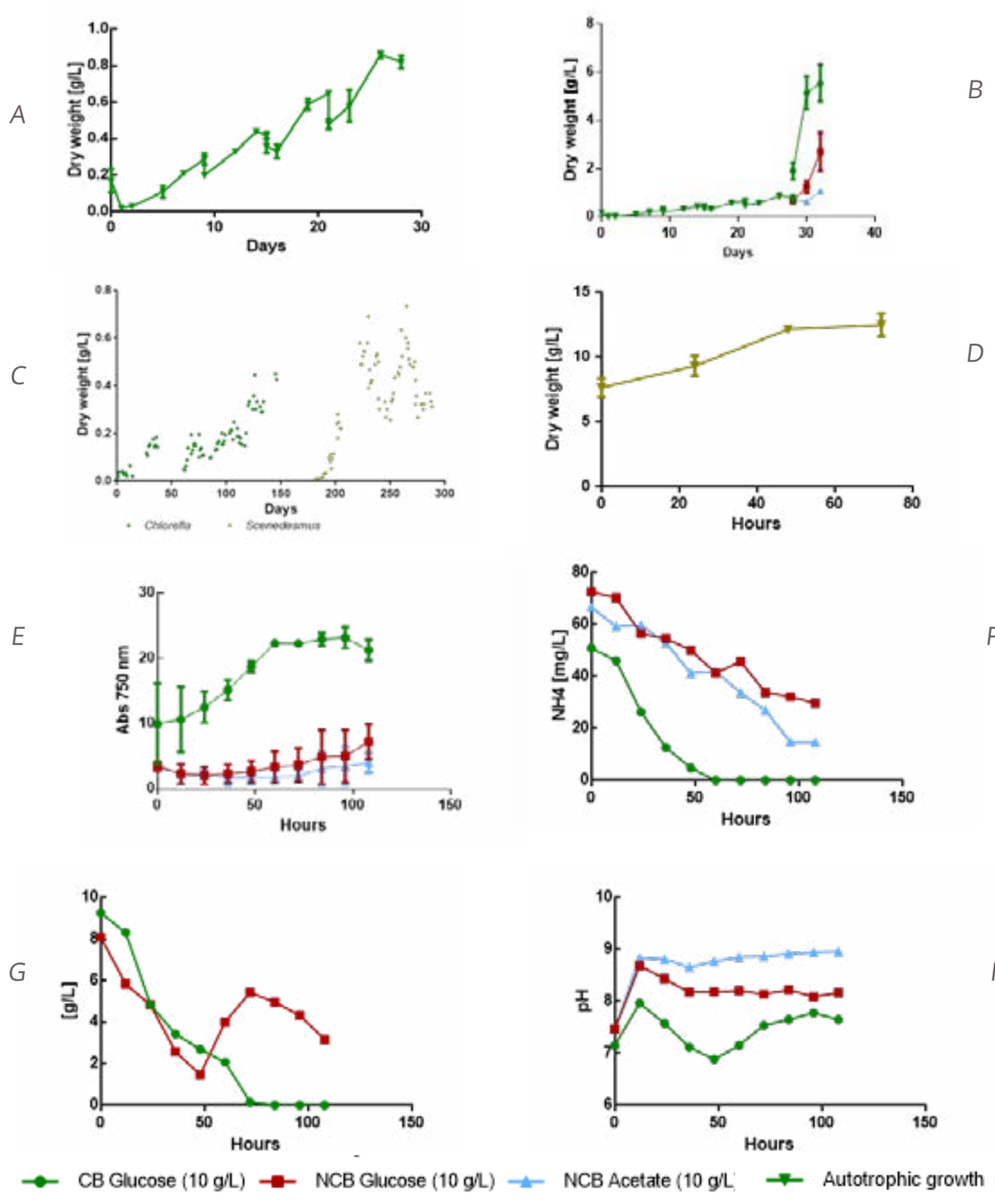
Results of 80 consecutive days of cultivation of *S. obliquus* and *C. vulgaris* in the large-scale photobioreactor under photoautotrophic growth conditions are presented (Figure 5). Biomass harvesting started after 25 days of cultivation when the dry weight reached 0.6 g/L and harvesting occurred every week following the initial harvest. Two mixotrophy trials as described in the previous section are also presented (Fuentes-Grunewald et al, 2020).

The automation of the photobioreactor allowed for the maintenance of an average temperature of 25°C and an average temperature pH of 7.5 in the cultivation system. Oxygen demand averaged at 15.35 ppm/min throughout the cultivation time.

Figure 4: 7 tonnes photobioreactor installed at the Langage AD site. Image shows the light phase of the facility (i.e. vertical tubes) and the control panel allowing for parameter control and monitoring.

Overview of the main biotic and abiotic parameters in autotrophic and mixotrophic cultures at pilot and industrial scale extracted from Fuentes-Grunewald et al 2021:

*Using Chlorella vulgaris and Scenedesmus obliquus. A. Chlorella autotrophic growth at pilot scale (800 L). B. Chlorella autotrophic growth compared with mixotrophic growth (day 28 onward). C. Autotrophic growth at industrial scale (5m3), day 0 to 146 for Chlorella, day 182 to 290 for Scenedesmus. D. Scenedesmus mixotrophic growth. E. Growth (abs 750nm) comparison of three C sources in Chlorella mixotrophic experiments. F. Ammonium uptake in mixotrohic Chlorella experiments. G. Glucose uptake in mixotrophic Chlorella cultures. H. pH trend in mixotrophic Chlorella cultures. * <0.05; ** <0.005; *** <0.001.*



Autotrophic cultures

Pilot-scale autotrophic experiments were performed using *Chlorella vulgaris*, this strain showed interesting results once the algae were acclimatised to 2.5% NRD. In Figure 5A *Chlorella* culture showed a short lag phase (acclimation period) when the inoculum was transferred from the 80L bag indoor culture to the 800L PBR greenhouse conditions. A sharp drop in biomass concentration during lag phase from day 0 to day 2 was recorded, likely due to the higher solar radiation under outdoor conditions. From day 2 (once the algae were acclimatised to new natural light conditions/ photoperiod) until day 27 a constant and continuous growth was recorded, obtaining an average growth rate of 0.32 ± 0.1 d-1 (Figure 5A). A similar growth rate (0.33 ± 0.1 d-1) was obtained when the *Chlorella* cultures were run at industrial-scale (5 tons) at Langage AD (Figure 5C, day 0 to day 146). This similar growth behaviour can be explained because *Chlorella* was already acclimatised to 2.5% NRD in the lab and at pilot-scale, and that is why it was used as a reliable and healthy inoculum for industrial-scale operation. *Scenedesmus* autotrophic growth at industrial scale were similar (no significant difference) in terms of growth rate compared to *Chlorella* culture in the same industrial PBR system. Nevertheless, the average biomass production (g/L) was considerably higher (> 103%) in *Scenedesmus* with an average production of 0.342 ±0.19 g/L compared to *Chlorella* with an average of 0.168 ±0.10 g/L (Figure 5C). It must be noted that industrial cultures of *Scenedesmus* also shows robustness, a reliable growth, and no visible contamination (either predators or competitors) occurred over 108 days of continuous autotrophic growth (Figure 5C).

	Autotrophic		Mixotrophic		
Parameters	*Chlorella*	*Scenedesmus*	*Chlorella*	*Scenedesmus*	units
Growth rate	0.32	0.33	0.50	0.42	d-1
Duplication time	2.16	2.13	1.39	1.67	days
Nitrogen uptake	104.6	134.9	115.5	98.8	mg/L/day
Phosphorus uptake	1.89	0.95	-	-	mg/L/day
Glucose uptake	-	-	2692	-	mg/L/day
Dextrose uptake	-	-	-	4980	mg/L/day
Biomass productivity	84.27	43.69	1621	1591	mg/L/day
Biomass production (average)	0.46	0.35	5.32	12.50	g/L
Maximum biomass production	1.01	0.73	5.51	13.83	g/L
Nitrogen concentration	370.5	97.45	140.8	82.72	mg/L
Maximum Ammonium	910	165.57	254.9	153.82	mg/L
Phosphorus concentration	9.53	5.24	37.4	-	mg/L
N:P (average)	39	18.6	6.8	-	

Table 4: Extrapolation based on experimental results and published in paper of Fuentes Grunewald C., et al, 2021

Main biotic and abiotic parameters in autotrophic and mixotrophic cultures of Chlorella vulgaris and Scenedesmus obliquus.

Mixotrophic cultures

The new combined autotrophic-mixotrophic growth approach followed in this study. From the results obtained at two different scales and in two different locations using the model microalgae *Chlorella*, a significant difference in terms of growth rate (56% increase, p=0.02) in the mixotrophic reactor (CB-Glucose) compared with the autotrophic reactor was analysed. The doubling time in mixotrophic cultures was reduced by 35% and the biomass (g/L) obtained (48 after inoculation in the mixotrophic reactor) was one order of magnitude higher or an improvement of 1097 % (Table 4, Figure 5B). Clearly the results in the mixotrophic cultures were improved and better production parameters were obtained compared to the autotrophic cultures.

For the pilot-scale *Chlorella* mixotrophic experiments, we evaluated different carbon sources with and without the initial concentration of the biomass, where significant differences (p=0.004) were found in terms of growth rate and biomass production. Acetate as carbon source showed slight growth obtaining 1.07 g/L after 48 hours of mixotrophic growth (Figure 5B). Similar results were observed (Figure 5E) using absorbance at 750 nm as a proxy of the dry weight. As shown in Figures 5B and 1E, better growth performance was obtained when glucose was the carbon source elected. However, we recorded a significant difference (p=0.004) between the pre-concentrated microalgae biomass (CB-Glucose) and the non-concentrated biomass (NCB-Glucose). Figure 5B shows an improvement of 104% of the CB-Glucose when harvested (5.51 g/L) compared with NCB-Glucose (2.70 g/L) at the same time. An increase in bacterial contamination was detected in cultures where the initial biomass concentration was low (NCB-Glucose and NCB-Acetate), from 48 hours onward an explosive boost in bacterial contamination was recorded. By contrast, when the initial concentration is higher (CB-Glucose; 2g/L as initial concentration) a negligible bacterial contamination was recorded.,.

RECOMMENDATION

A combination of using firstly photoautotrophic cultivation followed by mixotrophic cultivation allows for maximum uptake of nutrients from the digestate and for maximum biomass production

COOPERL (FRANCE)

Digestate	Ultra-filtered (300KDa)
Microalgae	*Aurantiochytrium mangrovei*
Photobioreactor	Vertical tubular - volume = 1,000L
Cultivation mode	Heterotrophy
Cultivation cycle	2 days

Digestate used and treatment method

The digestate used at the investment site was provided by Cooperl Arc Atlantique (France) and was the result of the anaerobic digestion of pig manure. For microalgal cultivation, the digestate was treated using ultra-filtration at a pore size of 300 kDa and 0.14 µm membrane. The composition of the raw digestate can be found in Table 5.

Table 5: Composition of pre-treated digestate provided by Cooperl Arc Atlantic for microalgal cultivation

	Raw digestate composition	Filtered digestates composition (300kDa)	Filtered Digestate composition (1.14 m)
Digestate origin	Pig manure	Pig manure	Pig manure
pH	8.0-8.1		
Dry Weight	0.6-0.9%		
Nitrogen (N)	2480 mg/Kg	2227 mg/Kg	2286 mg/Kg
Phosphorous	138mg/Kg	36 mg/Kg	38 mg/Kg
Potassium (K)	1054 mg/Kg		
Calcium (Ca)	188 mg/Kg		
Magnesium (Mg)	26.4 mg/Kg		
Sodium (Na)	70-470 mg/Kg		

Microalgae cultivated

To develop nutrient removal from digestate, coupled with the production of high-value compounds, the investment at Cooperl focused on the culture of the DHA-rich Thraustochytrids microorganisms *Aurantiochytrium mangrovei* (A. mangrovei, Figure 6). Thraustochytrids were cultivated in heterotrophic conditions but light can be needed for the synthesis of protective pigments. In this study as only heterotrophic conditions were used, the addition of one or more organic substrates was essential, as well as air mixed with an oxygen supply. This cultivation system allowed for higher productivity, growth rate, and biomass concentration in cultures, and therefore less space was needed for industrial scale production. Ideally, the cultivation process would be carried out in axenic conditions (no contamination), however, the maintenance of axenic culture was not compatible with the scope of the ALG-AD project to produce microalgae at large scale, hence non-axenic cultivation of *A. mangrovei* was developed and scaled-up at the project investment.

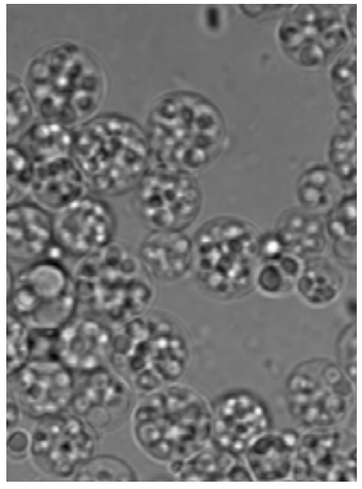


Figure 6: x100 microscopic image of A. mangrovei after cultivation

The photobioreactor and cultivation process

The medium for cultivation:

As mentioned earlier, *A. mangrovei* needed an additional source of carbon for its growth. Hence the cultivation medium consisting of 3 different solutions was added for *A. mangrovei* growth:

The culture medium was prepared from three solutions as follow:

- Industrial glucose syrup (for concentration in culture medium: 20g/L glucose)
- Sterilised concentrate of Yeast extract and peptone at 90g/L each (for concentration in culture medium: 2g/L Yeast extract and 2g/L peptone)
- Digestate (for concentration in culture medium: 2,5 % digestate), previously filtrated on a pore size of 300 KDa, and 0.14 µm membrane
- 15 g/L sea salt diluted in tap water.

Silicone based Anti-foam is added (1 mL/L of culture medium) as soon as the yeast extract and peptone solution was poured into the cylinders.

The Photobioreactor:

Due to the fast-growing characteristics of *A. mangrovei*, the photobioreactor used for large-scale cultivation was different to the one used at the investment site of Langage AD.

The bioreactor was a set of PMMA cylinders of increasing size from 10 to 800L (see Figure 7). Agitation and gas transfer in each cylinder was provided by air flow bubbling from the bottom of the cylinder, at a rate of 0.4 Volume of air per volume of culture per minute (Air-lift system) (Xu et al, 2017). The gas was supplied through a ring of 16 mm diameter PVC tubing with 1.5 mm diameter holes (1 hole for each two litres of culture).

Medium addition and water (for process and for cleaning) were supplied by pumps and delivered at the top of the cylinders. The system was designed with the option of providing light using red LEDs lined around each cylinder (Figure 7 and 8). Light was not an absolute necessity; however preliminary laboratory experiments showed that its use allows the increase of the light protective carotenoid pigment

Figure 8 - The photobioreactor with LED lights

in cells.

The temperature of culture was maintained either by controlling the room temperature (air heater 15 Kw) or by immersing a stainless-steel electric heater (2Kw for 500L of culture). As culture temperature was always higher than room temperature, no cooling system was needed.

A manual valve for sampling and an electrovalve and a pump for medium transfer were connected to the bottom of each cylinder.

Culture medium compounds were supplied automatically to the top of each cylinder, through tubing from 3 containers, using software driven pumps and electrovalves. One pump supplied the digestate + sea salts + water, another pump supplied glucose syrup (kept at 65 °C), and one pump supplied the 50X previously sterilized concentrate of Yeast extract and peptone)

After each addition of medium compounds, the tubes that supplied the medium to the system were automatically flushed with tap water.

Before inoculation, the photobioreactor was filled up with a sodium hydroxide solution (or bleach) at a concentration of 18 ppm. Air was supplied in the bleached water for 1-3 days and cylinders were then rinsed twice with tap water. All tubes and containers were also bleached (except glucose syrup container) by running the pumps with bleached water added in the containers.

The processes of cultivation:

Two processes were used.

1- A batch mode cultivation process was run in 800L cylinders filled up to 500L with culture.

This process can eventually also include the addition of concentrated medium during the exponential growth phase (see details in the part “proof of concept”).

2 -A fed batch mode process was using plug-flow cultures. This process was run in a set of successive cylinders of increasing size (10 to 800L). In this process, the cultures were firstly inoculated in a 10L vertical tube and when desired density was reached the culture was transferred to the next, bigger, cylinder and successively into cylinders of increasing size to incrementally increase the cultivation volume (Figure 7 and Figure 8). The final cultivation volume was of 1000 L (2 x 500L). Every 6.5h, the culture was automatically sent to the next cylinder (Plug-flow). Once the culture had reached the final cylinders, it was cultivated for a further period before harvesting.

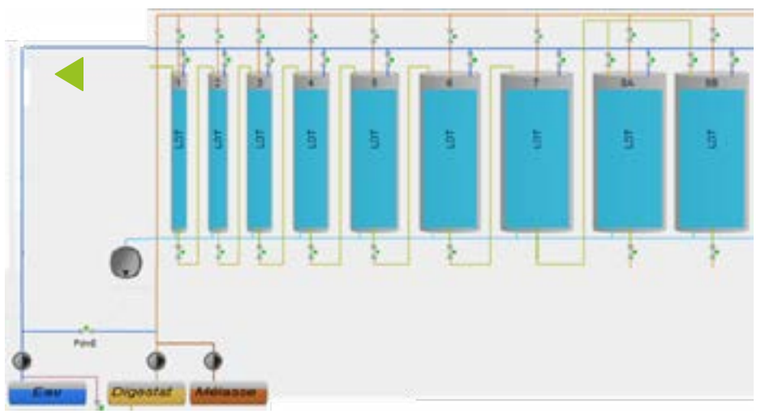


Figure 7 - Schematic of the photobioreactor showing the increasing volume cylinders (numbered 1 to 8B)

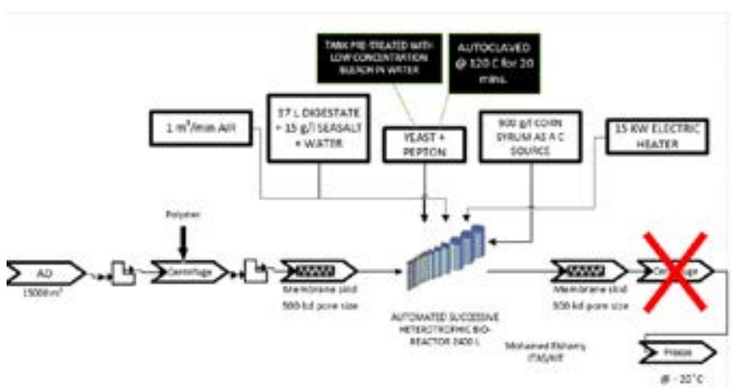


Figure 7 - Schematic image of cultivation process

This cultivation was designed to allow automated continuous operations: In every cylinder, after the culture was transferred to the next cylinder, the cylinder was filled up with water to the top, and air bubbling was maintained for one or two cycle time (6.5 or 13 h) in order to clean and rinse. After draining water and rinsing the cylinder, the next batch was transferred to this cylinder. Successive batches can therefore be processed in successive cylinders. Please note that fed batch cultivation under this continuous mode had not yet been validated.

Inoculation:

A culture of *A.mangrovei* (8L) in late exponential phase, was firstly produced at lab scale:

The culture medium was 250 mL, in a 500mL flask and maintained on a shaking table (100 RPM) for 48 h at 23-25 °C. Two mL from these flasks were inoculated in a new similar flask, and cultivated in similar conditions for 64 h. The content of this new flask (250 mL) was inoculated in 8L of the same medium, and in a 20 litres carboy. This carboy was maintained for 24 hours on the shaking table, with air supply (3.2 L/min through a 4mm diameter tubing) at the bottom of the carboy. The carboy content was transferred to the cylinder for inoculation of the fed batch or batch process.

Culture conditions (related to the strain cultivated):

-Temperature was maintained at around 28 °C,

-pH in the range 4.5-7.5,

-air mixed with the oxygen was supplied by air bubbling, in order to maintain value above 0%. However, as cells were fragile, excessive shearing could be a problem.

Fed batch plug-flow experiment medium supply:

The 3 medium solutions (i.e. cultivation medium) were supplemented to the cultures at a continuous increasing feeding rate for 42 hours, feeding rates (hourly addition) were continuously calculated by the software from following assumptions: growth rate: of 2.8 day-1, constant biomass/substrate yield whose value was calculated from previous cultures. Substrate exhausted at the end of culture in order to boost the lipid production in cells, glucose syrup was added separately to reach a final glucose concentration of 50g/L in the cultivation system.

Batch experiment medium supply:

Water was added up to approximately 400L and heated up to 26-28 °C (needs 5 to 6 hours). Just before inoculation, solutions were poured directly manually, through an aperture at the top of the cylinder. Sea salt was added as 10X concentrate, and 0.46 g/L of Mg SO4 7H2O is also added in these batch as a 250X solution. The culture volume is then adjusted with tap water to 440 or 490 L before inoculation.

Culture assessment and analysis:

Temperature, pH and eventually dissolved oxygen must be checked regularly over the culture period, and corrections applied when needed.

Due to the nature of the medium used and its richness in sugar, such cultures under non axenic conditions can exhibit microbial contaminations.

Controls of process can include:

-Microscopic check: cell form, size, number and integrity, yeast contamination, bacterial contamination

-Flow cytometry: cell counts and cell characteristics (size, morphology, lipids content with Bodipy staining) can be monitored and yeast contamination are checked rapidly (WGA staining)

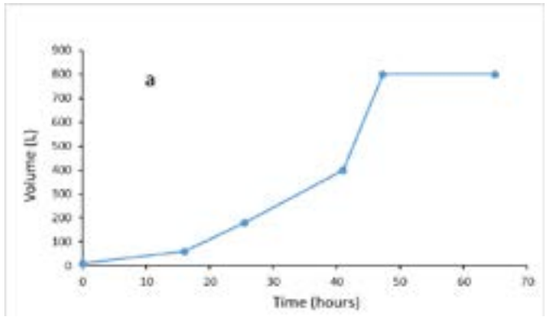
-if specific equipment is available, contamination could eventually be monitored using molecular techniques, (including DNA extraction). PCR analysis could be performed to assess the level of contamination occurring during the cultivation (“semi-quantitative” assessment).

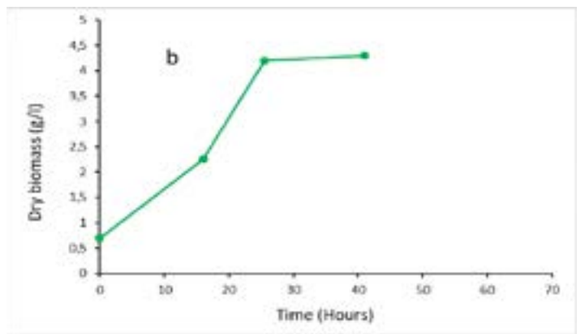
FED BATCH PLUG-FLOW TECHNOLOGY proof of concept from first results

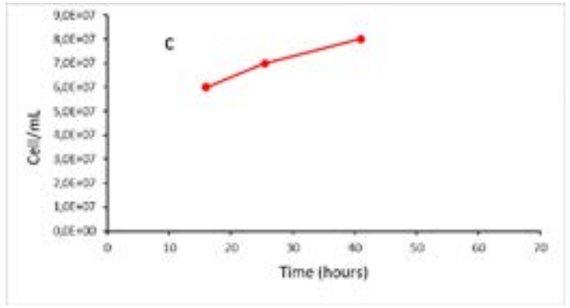
Only one conclusive experiment was run with this fed – batch technology. The automated medium supply, culture transfers and tubing and cylinder rinsing processes at pilot scale were demonstrated to be operative. However, due to Covid pandemic constraints (5 months cultivation was stopped in 2020), fed-batch experiments had to be stopped and repeatability could not be assessed. The developed software allowed significant adjustments, which was of great value as process specifications evolve.

Cultivation volume was increased as described in the previous section and a final volume of 800L (2 x 400L) was reached after 47 hours (Figure 9, a). However, genetic sequencing and flow cytometry of samples showed that yeast contaminations occurred during the time of cultivation and became significant when the high volume of cultivation was reached.

Despite this contamination in the last cylinders, the







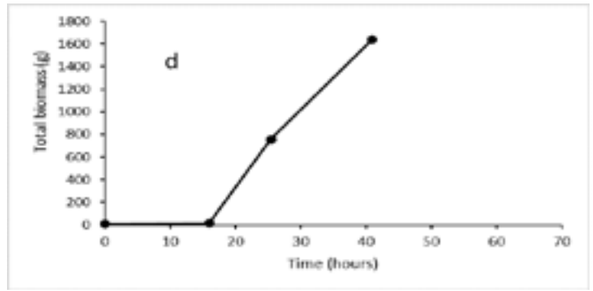


Figure 9: Fed batch plug-flow cultivation : Culture volume (a) ; dry biomass concentration (b) ; Cell count (c) ; Total dry biomass (d), (data from analysis only shown before contamination occurs .

culture showed promising results up to the cultivation volume of 400 L (cylinder 7). Indeed, after 41 hours of cultivation, dry weight reached 4.3 g/L (Figure 9, b). From 15 to 42 hours, the dry biomass weight nearly doubled from 2.25 to 4.3 g/L when the total cell count was only increased by 33% (Figure 9, c), showing that cell average weight increased at the end of culture. This can be related to the increase in cell size observed by microscopy and to the related lipid accumulation. As culture volume increased, the total biomass produced continued increasing until 42 h (Figure 9, d).

Improvements could be put in place to tackle the yeast contamination; such improvements were successful in the batch production experiments:

1) Raise the temperature during cultivation. Experiments demonstrated that at least 25 °C is recommended for healthy, continuous growth of culture.

Optimum temperature is 28°C. Further batch experiments at pilot scale (see below) showed that culture at these temperatures exhibit higher growth rate, and drastically reduced contamination. Lab experiments showed deleterious effect on cell morphology and growth rate at lower than 25 °C.

2) pH control, by manual monitoring and addition of NaOH, is required to maintain pH levels between 4.5 and 7.5.

3) Further improvements could also be made on the medium, as laboratory experiments revealed that magnesium and sulphur could be limiting factors for the growth of A. mangrovei on digestate. Magnesium concentration in this digestate was relatively low (10-16 mg/Kg, Table 5) in comparison to other digestates used in the project. Thus, in batch experiments at pilot scale (see below), sulphur and magnesium were added to the cultivation medium (i.e. digestate).

BATCH TECHNOLOGY proof of concept: A set of ten successful productions over 6 months

A set of ten successive experiments were run using the medium as described above. These productions were run in a sum of 22 cylinders, from February to September 2020. Each cylinder containing 450L or 500L of medium (See Table 6 below).

Cultures were prepared, inoculated and run as described above. Cultures were run over 23-26 or 38-41h (Table 5). Temperature were maintained between 23 and 32 °C, and when low pH occurred during working hours, pH was maintained over 4.5 by addition of an NaOH 10N solution.

The flow cytometry analysis using WGA dye for yeast fluorescence showed no significant yeast contamination in cultures. Main results from the 22 batches were summarized in the Table 6 and 7.

For cultures (P20G-7; P20H-7; P20H-8A; P20H-8B), the

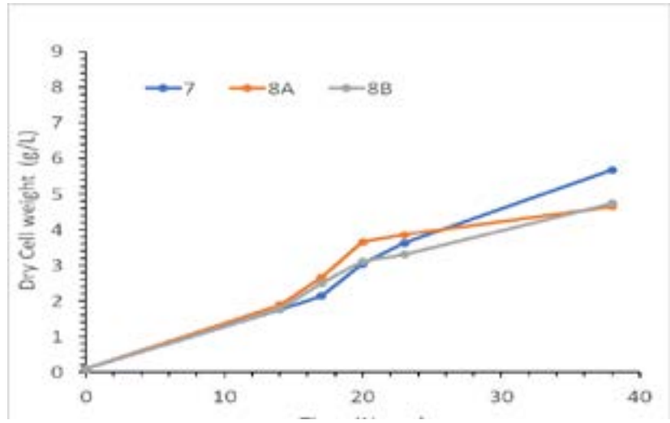


Figure 10Dry Cell weight kinetics in experiment P20G. Cylinder 7 was fed after 15 hours of cultivation with addition of 50% of initial substrate.

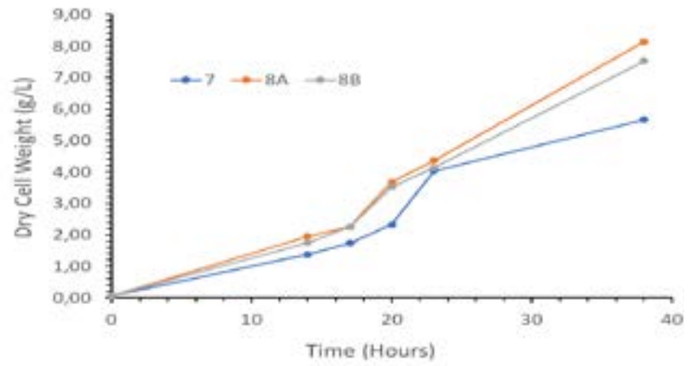


Figure 11.Dry Cell weight kinetics in experiment P20H. The 3 cylinders 7, 8A and 8B were fed after 15 hours of cultivation with addition of 30% of initial substrate.

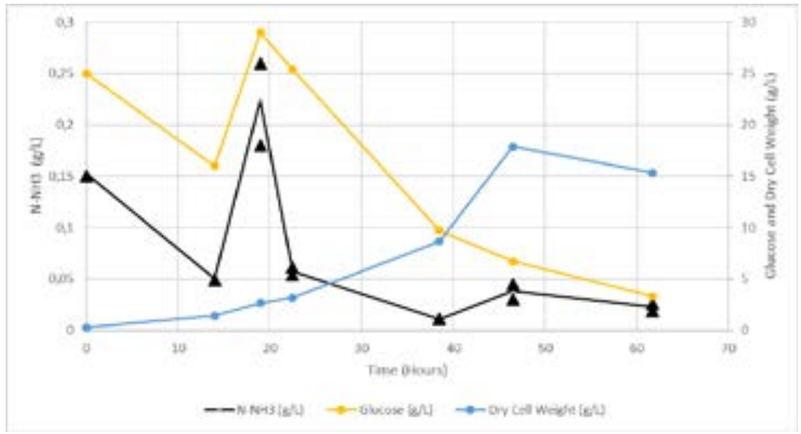


Figure 12: Dry cell weight, glucose, and N-NH4 in culture P20P. Feeding was operated at 16h, with 100% addition of Glucose Syrup, Y.E., peptone and Digestate. Rm: N-NH4 values observed are cumulated NH4 from added substrates (digestate, YE and peptone) and NH4 re

Substrate feeding (glucose syrup, peptone, Yeast extract and digestate) was tested after 15 hours of cultivation.

Examples of batch kinetics (with and without feeding) were shown in Figures 10 and 11. The dry cell weight increased with feeding, and these batches exhibit the highest productivities (Table 6).

Results showed the potential of *A. mangrovei* to produce biomass at a fast growth rate, up to 4 day-1 over 24h cultivation time, and average productivity of up to 4.4 g/L / day. Indeed, in these trials, a significant amount of biomass was obtained in only 41 hours (Figures 11 and 12).

Furthermore, *A. mangrovei* being very rich in lipids (DHA), the strain showed potential to produce high-value products in small cultivation volumes and at high production rate.

NH4 concentration was not measured in most of these large-scale trials. However, experimental results at laboratory scale in similar conditions (10 litre cylinder, non-axenic cultivation) showed that A. mangrovei could recover more than 90% of NH4+-N from digestate when grown on 2.5% of digestate, demonstrating the potential of A. mangrovei for digestate remediation (Figure 12).

In order to assess the remediation rate at pilot scale, NH4 analysis was obtained on samples from an experiment run in January 2021 (experiment P20P). In this experiment (Figure 14 below) feeding of nutrients (100% of initial substrate) was operated at 16h of cultivation and the cultivation was maintained for 61,5 hours. The final N-NH4 value was 0.022g/L, thus , as compared to the 0.11g/L of N-NH4 from 5% digestate added, the remediation rate was estimated to be 80%.

RECOMMENDATION

The main breakthrough of this process development is the production of a high value product (DHA and protein rich organism), in non-axenic (therefore low-cost) culture conditions.

The process proposed here is still under development. The medium composition should be improving by the reduction or suppression of peptone and yeast extract, and the use of a lower cost minerals and vitamins medium.

The carbon source used here (glucose syrup) is a low-cost industrial product, however, as this substrate has an important impact on the process and cost, other low-cost carbon substrates should be tested.

The biomass concentration could be probably significantly improving by increase of the substrate concentration (Carbon source and digestate) and increase of the time of cultivation.

At industrial scale, the choice of PMMA as material for cultivation tanks should be questioned: probably stainless steel would be a better choice.

Experiment	Date	Comments	Culture volume	Culture duration	biomass concentration	Final Culture volume	Biomass at end of culture	Yield (substrate/biomass)	biomass productivity
			(L)	(Hours)	(g/L of washed filtrate)	(L)	(g)	(W/W)	(g/L/day)
P20C 8A	11/02/2020		450	41,75	3,65				2,10
P20D 8A	18/02/2020		450	40,75	3,68				2,17
P20E 7	16/06/2020		450	41,5	4,9				2,83
P20E 8A	16/06/2020		450	41,5	5,18				3,00
P20E 8B	16/06/2020		450	41,5	4,7				2,72
Average P20E	16/06/2020		1350	41,5	4,93	1050	5173,00	0,21	2,85
P20F 7	23/06/2020		500	38	4,20				2,65
P20F 8A	23/06/2020		500	38	4,40				2,78
P20F 8B	23/06/2020		500	38	4,50				2,84
Average P20F	23/06/2020		1500	38	4,37	1350	5895,00	0,18	2,76
P20G 7	30/06/2020	substrate: 1,5X	500	38	5,67				3,58
P20G 8A	30/06/2020		500	38	4,65				2,94
P20G 8B	30/06/2020		500	38	4,73				2,99
Average P20G	30/06/2020		1500	38	5,02	1350	6772,50	0,18	3,17
P20H 7	07/07/2020	MgSO4 0,25X	500	38	5,66				3,57
P20H 8A	07/07/2020	end	500	38	8,14				5,14
P20H 8B	07/07/2020	substrate	500	38	7,25				4,58
Average P20H	07/07/2020	1,5X	1500	38	7,02	1350	9472,50	0,22	4,43
P20I 7	22/07/2020		500	25,25	2,66				2,53
P20I 8A	22/07/2020		500	25,25	3,55				3,37
Average P20I	22/07/2020		1000	25,25	3,11	930	2892,30	0,13	2,96
P20J 7	29/07/2020		500	25,25	3,32				3,16
P20J 8B	29/07/2020		500	25,25	3,22				3,06
Average P20J	29/07/2020		1000	25,25	3,27	930	3041,10	0,14	3,11
P20K 7	05/08/2020		500	26	3,14				2,90
P20K 8B	05/08/2020		500	26	3,28				3,03
Average P20K	05/08/2020		1000	26	3,21	930	2985,30	0,13	2,96
P20L 7	12/08/2020		500	25	2,95				2,83
P20L 8B	12/08/2020		500	25	3,65				3,50
Average P20L	12/08/2020		1000	25	3,30	930	3003,00	0,14	3,17

Table 6: Data from the 22 batch experiments run from February to August 2020

	Average in one batch 41h cultivation in a 500L cylinder	Extrapolated per week (2 batch productions in 1 cylinder)	Extrapolated per week for 3 cylinders (1350L)	Extrapolated to 1 year of operation
Average Biomass produced	5.3 g/L	5.3 kg	15.9 kg	820 kg
Digestate used (filtered)	12,5L	25 L	75 L	3900 L
Nitrogen uptake *	40 mg/L of culture/ batch	80 mg/L/week	80 mg/L/week	4.16 g/L/year
Total nitrogen remediated*	g	40 g	120 g	6.2 kg

Table 7: Extrapolation of pilot production, based on experimental results from experiments P20E to P20 H (38 h to 41.5 h batch cultures)

INNOLAB (BELGIUM)

Digestate	Paper-filtered (10 µm)
Microalgae	*Chlorella sp.* & *Desmodesmus sp.*
Photobioreactor	Vertical & Horizontal tubular - volume = 3000L
Cultivation mode	Photoautotrophy
Cultivation cycle	14 days

Digestate used and treatment method

The raw liquid fraction of digestate (LF) was collected from INNOLAB's outsourced AD plant (Pittem) fed on food and farm waste. The LF was produced on-site after processing the NRD using a centrifugation-dissolved air flotation unit. The received LF was then pre-treated using a lab-scale paper filter (Holland filter bag) with a pore size of 10 µm to remove larger particles. The physical and chemical characterization of LF and paper filtered LF (PLF) were performed using standard analytical methods. Composition of raw and paper-filtered liquid fractions of digestate are presented in Table 8.

	Raw liquid fraction	Paper filtered liquid fraction
Digestate origin	Plant and food waste	Plant and food waste
pH	7.87	7.87
Dry Weight	1.23%	1.22%
Nitrogen (N)	2430 mg/Kg	2370 mg/Kg
Phosphorous	25.30 mg/Kg	9.61 mg/Kg
Potassium (K)	2130 mg/Kg	1720 mg/Kg
Calcium (Ca)	146 mg/Kg	35.8 mg/Kg
Magnesium (Mg)	133 mg/Kg	92.1 mg/Kg
Zinc (Zn)	12.5 mg/Kg	1.19 mg/Kg
Lead (Pb)	2.53 mg/Kg	0.239 mg/Kg
Nickel (Ni)	0.359 mg/Kg	0.09 mg/Kg
Mercury (Hg)	0.993 mg/Kg	0.024 mg/Kg
Copper (Cu)	2.53 mg/Kg	0.239 mg/Kg
Cadmium (Cd)	0.253 mg/Kg	0.024 mg/Kg
Dry organic matter	-	24.19%
Acetic acid	-	361 mg/Kg
Propionic acid	-	29 mg/Kg

Table 8: Composition of digestate provided by INNOLAB (Pittem) for microalgal cultivation

Microalgae cultivated

The operation at INNOLAB cultivated a mixed culture (or consortium) of the two green microalgae *Chlorella sp.* and *Desmodesmus sp.* (Figure 13). The mix was obtained from the culture collection of BCCM/DCG group, based at the Faculty of Sciences, Ghent University, Belgium.

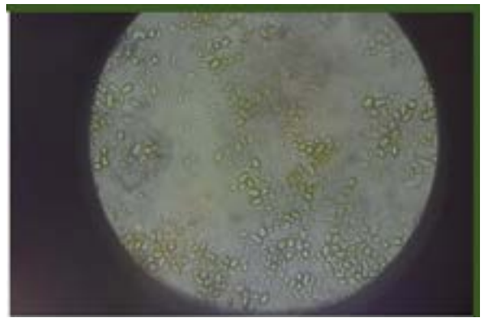


Figure 13 : x40 Microscopic image of the Chlorella sp. and Desmodesmus sp. consortium cultivated at Ghent

The photobioreactor and cultivation process

The investment at INNOLAB (Belgium) was installed in January 2020 and the facility is composed of two separate photobioreactors. The first photobioreactor is similar to the one at Langage AD, with a smaller total volume of 2,500L. The second photobioreactor is a horizontal system with a volume of 500L, totalling 3,000L of cultivation volume (Figure 14).

Prior to cultivation, the entirety of the photobioreactor was cleaned by running bleached water through the system for a minimum of 24 hours (at a concentration of 5mL/L). The bleach was then washed away until no chlorine was detected. The water used for algal cultivation was passed by an UV-light system prior to being fed to the reactors.

Proof of concept of the technology: the results so far

Results of 47 consecutive days of cultivation of Desmodesmus sp. in the pilot horizontal photobioreactor (500L) under photoautotrophic are presented. Biomass harvesting started after 15 days of cultivation

Figure 14 Cultivation system at INNOLAB, left: horizontal system, right: vertical system

when the dry weight reached 1.7 g/L and harvesting occurred around every 2 weeks following the initial harvest. The photobioreactor was not controlled for temperature, but a pH control around a value of 7-8 was achieved with CO2 feeding.

Photoautotrophic Cultivation: growth performances and nutrient uptake

Dry weight results show that *Desmodesmus* grew consistently during the 57 days of cultivation (Figure 15). It was possible to harvest around 1.7 g/L every two weeks, amounting to 1.7 kg of dry biomass per month.

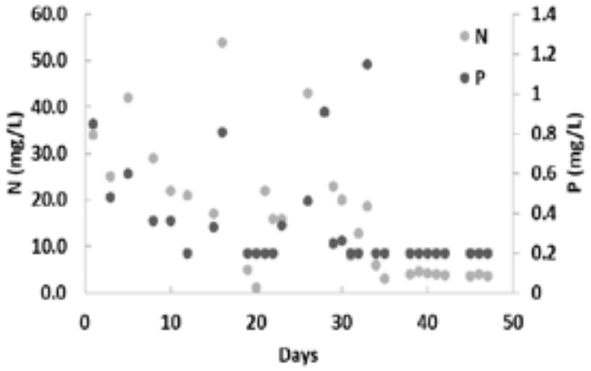
Nitrogen and phosphorus levels decreased during the cultivation and both nutrients were always low upon every harvesting (20 mg/L for N and 0.3 mg/L for P); the fine tuning of nutrient feeding could result in the almost complete consumption of these nutrients, as shown for the final run (4 mg/L of N and <0.2 mg/L for P). Remediation of excess nutrients from the digestate by the microalgae was successful in the photobioreactor under photoautotrophic conditions.

Experimental results Here, results of laboratory scale experiments are presented.

Three concentrations of paper-filtered digestate (5%, 10 % and 20%) were tested for the growth of a consortium of *Chlorella sp.* and *Desmodesmus sp.*. Cultivation trial was conducted in a 3L photobioreactor (Figure 16) were temperature was maintained at 17- 23 °C and the photobioreactor was equipped with LED light strips to provide sinusoidal light with maximum light intensity of 100 µmol/m2/s and photoperiod of 16: 8 hr (light: dark). Microalgal cultivation occurred for 14 days.

Results indicated that *Chlorella sp.* and *Desmodesmus sp.* growth was best when using a concentration of 10% of paper-filtered digestate (21,821 cells/µL, Figure 17).

Experimental results are promising, and initial successful cultivations have been obtained in the large-scale photobioreactor, thus it can be said with confidence that results of growth and nutrient uptake obtained at laboratory scale are likely to be found at large scale.



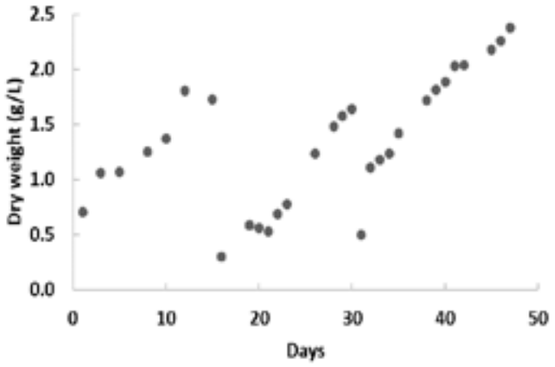


Figure 15 : Results of dry weight measured for 47 days of Desmodesmus sp. cultivation (top graph) and growth performances results (table). Nitrogen concentration (light grey dots, left axis) and phosphorus concentration (dark grey dots, right axis) for 47 days of De

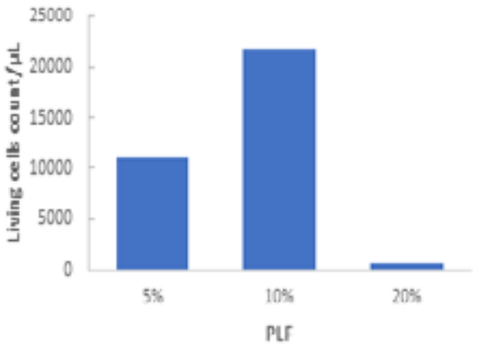


Figure 17 Living cell count per µL on Day 14 during substrate optimization experiments at increasing concentrations of PLF (paper-filtered fraction of digestatefor 47 days of De

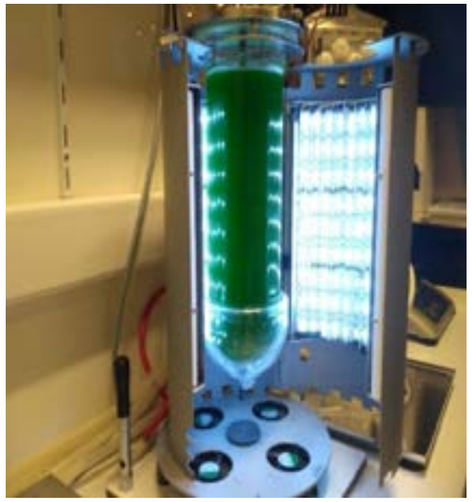


Figure 16 3L photobioreactor used for experimental cultivation of Chlorella sp. and Desmodesmus sp. for 47 days of De

6 DOWNSTREAM PROCESSING: HARVESTING, STORAGE AND TRANSPORTATION

The following section explains the procedures followed at each investment to harvest and store the microalgal biomass in a way that is efficient, practical, and in which the properties of the biomass are optimally retained (e.g. cell structure, nutrient content and compound characteristics).

The aim of harvesting microalgae is to concentrate the biomass from 50-200 fold (5-15% total solids) for subsequent drying/extraction or direct extraction of wet biomass in the shortest period to avoid spoilage of the biomass. The dilute cultures of photobioreactors require a large volume of water to be processed (typically 0.05-0.5% solids content). It is essential for the harvesting technique to be quick, >90% harvesting efficiency, inexpensive, to leave no toxic residues, and to not affect the quality of the biomass.

The aim is to produce high-value products from algal biomass grown on digestate. The identified and targeted high-value compounds/products include;

1) DHA for *Thraustochytrids*

2) Proteins (Total proteins)

3) Peptides (After hydrolysis)

4) Carbohydrates (Total carbohydrates and Sulphated polysaccharides)

5) Pigments (Astaxanthin)

6) Squalene

Partners at Cooperl are using *Thraustochytrids* and are mainly interested in DHA, Squalene, Astaxanthin, and Peptides. The strains grown at Langage and Innolab are mainly targetting peptides.

In addition, recommendations are shared for how to store and/or transport the biomass, for example for delivery to third party processors of the biomass, in order to retain the optimal properties of the microalgae.

Langage AD (England)

Membrane filtration has been the preferred technology to process biomass by separating particles (i.e. microalgal cells) and water. Harvesting (or biomass dewatering) was performed using a pore size of 100KDa at Langage AD (Figure 20).

Membrane filtration was very efficient for the harvesting of microalgal biomass. Indeed, at Langage AD, three to four hours of operation of the micro-filtration membrane allowed for biomass concentration from 0.4 g/L (dry weight) to 7g/L (dry weight). Culture volume was reduced from 2500 L to 200 L with a solid concentration of 2%.

At Langage AD, after micro-filtration, the concentrated culture is then centrifuged and a thick biomass paste is obtained (2% solid content), reducing further the culture volume, facilitating storage of the processed biomass (Figure 20).

The biomass is immediately frozen at -20°C after harvesting.

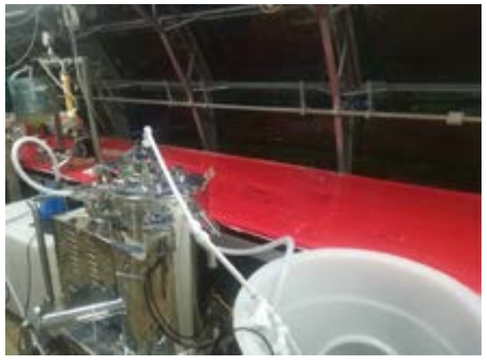


Figure 20: Harvesting (or dewatering) systems used at Langage AD, left: micro-filtration rig; right: continuous flow centrifuge, biomass paste obtained after centrifugation (c. 20% solid)

COOPERL (FRANCE)

Cultures from the two or three cylinders are pooled, and then filtered through a tangential flow filtration system. The filtration system used at Cooperl is from SIVA TM, and this supports 3.5 m2 using a 300 Kd ceramic membrane, in two cartridges supporting 77 channels each (internal diameter 4.6 mm).

The recommended filtration conditions applied at Cooperl are :

- Velocity for retentate on membrane: 4m/S

- Recirculation rate for retentate 2500 L/H

-Transmembrane pressure, 0.6 to 0.9 bars

-Permeate flow-rate: 300 to 180 L/H

-Final retentate volume 50 to 70 L (concentration ratio 15 to 28 X)

-Retentate temperature: 28-35 °C

Harvested retentate 60 to 130 g/L dry weight, are immediately transferred to 5 to 20L containers and the biomass is frozen at -20°C.

Eight hours are needed for filtration treatment of 1350L of culture, including the rinsing and cleaning procedures of the tangential flow filtration equipment. The cleaning procedure also includes NaOH and nitric acid treatments of the filter after each filtration, following SIVA recommendations.

Characteristics of the collected retentate from experiments P20E to P20L are summarized in the table below.

Following the micro-filtration process, clean and sterile water is obtained, and this can subsequently be re-used in the cultivation system for the next cultivation cycle. This approach could limit water consumption during operation and reduce energy and cost.

Figure 21: Harvesting (or dewatering) systems used at Cooperl

Table 9: Characteristics of retentates collected from batch productions using tangential flow filtration.

Collected Retentate	Dry matter in retentate including solutes	Volume retentate	Total dry matter	Total fatty acids
	(g/L)	(L)	(Kg)	(mg/g DW)
Average P20E	103,59	60	6,22	3,6
Average P20F	132,7	45	5,97	100
Average P20G	127,24	52	6,62	74,7
Average P20H	123,66	45	5,56	82,5
Average P20I	60,5	50	3,03	34,7
Average P20J	70,2	73	5,12	41
Average P20K	76,15	72	5,48	53,2
Average P20L	69,33	72	4,99	41,4

UGENT-INNOLAB (BELGIUM)

Harvesting (or biomass dewatering) of the microalgal cultures after photoautrophic cultivation (about 0.7 – 2.2 g/L DW) was also done via (micro)membrane filtration, similar to the other two investment sites. However, a different configuration of filtration system was implemented albeit with the same pore size (0.2 micron) as of the Langage AD system. A Liqo-Vibro (Liquoflux, Rijnen, The Netherlands) unit fitted with free flow vibrating membrane plates was used to concentrate the cultures (Figure 22).

These systems are already employed in chemical processing and manure dewatering companies and can process low and high volumes of liquids. The operation of the unit is PLC (programmable logic controller) controlled and the membrane flux can be adjusted as per users' requirement. This technology was preferred in order to minimize the possibility of both organic fouling (due to biomass, cell debris and digestate particles) and scaling (due to calcium and magnesium precipitates) due to the vibratory shearing action at the membrane surface. Also, the filtered water is free from algae and bacteria can be reused for subsequent batches.

A membrane surface area of 7.5 m2 stacked as a three-module tower was finetuned to suit the culture volumes (0.5 – 3 m3) produced at UGent-INNOLAB site and required final concentration (5 - 7 % DM). A permeate flow rate of 400 L/h was maintained during the trials conducted to optimize the up concentration of the biomass, and the system was operated at room temperature 15 – 18 °C.

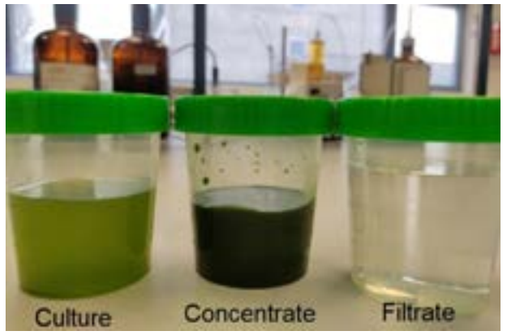
On average, a sample volume with 500 L with initial culture concentration of 2.5 g/L was dewatered to reach 63 g/L. The concentration factor was 25 times and the final volume of concentrated cultures of 20 L was obtained. Effective removal of algae in the permeate was achieved with N and P values of 3 mg/L and 0.2 mg/L, respectively, enabling both reuse and discharge into sewers (Figure 22).

Additionally, the membrane stack with residual cultures (concentration of 7 g/L) was flushed with tap water and stored as inoculum for subsequent bioreactor trials.The concentrated biomass is immediately frozen at - 20 °C to preserve its quality for characterization and hydrolysis. The flushed/diluted cultures are stored in a refrigerator (4 °C) and regularly homogenised for use as inoculum. This technique of preserving a fraction of final harvest to inoculate the following batch has been checked and validated during several 3-L bioreactor experiments and a few runs in the 500-L system.

Prior to membrane filtration, the possibility of single-step centrifugation was also explored. The cultures were harvested in a cubitainer and sent to an external facility (Tomalgae CVBA, Nevele, Belgium), which performed dewatering via centrifugation. The concentration was much higher, between 70 – 120 times, reaching a final concentration of 140 – 240 g/L.

Figure 22: Harvesting (or dewatering) system used at Innolab

Figure 23: Streams before (culture) and after (concentrate and filtrate) harvesting with the LiqoFlux system



Transportation and further downstream processing

In order to transport the biomass, a few approaches have been utilised, and there are advantages and disadvantages of each.

Transportation of biomass using frozen (preferably) or chilled transportation methods is lower cost, however, there is a risk of defrosting and cell damage if there are delays, or if the biomass is retained for longer than 24 hours in chilled conditions, before returning to the -20 frozen storage conditions.

Dry ice transportation has been utilised for smaller amounts of biomass, however, this is a higher cost option, and particularly in the case of transport between the UK and the EU, this is not recommended as very few suppliers offer this service, and it is prohibitively expensive.

Small samples of dry biomass can be transported relatively simply through standard postal agencies.

Once transported to a specialist third party, the biomass from all three investments was then processed at a large scale using hydrolysation.

The hydrolysate is subsequently spray-dried (for biomass amounts over 10 kg) or freeze-dried if < 10 kg, to be transformed into an ingredient for animal feed.

This method enabled the production and preservation of compounds of interest (i.e. peptides) for the animal feed industry. Details on this stage of the process are reviewed in further ALG-AD reports on the Enzymatic Hydrolysation approach developed for *Thraustochytrids*, and for *Scenedesmus*, *Chlorella* and *Desmodesmus*. These reports will be made available on the ALG-AD website.

RECOMMENDATION

Harvesting, storage and transportation approaches need to be sensitive to the specific strain of microalgae cultivated. Membrane technology and centrifugation is recommended for species such as *Chlorella* and *Scenedesmus spp*., and tangential flow filtration for less robust species such as *A. mangrovei.*

Biomass needs to be stored at -20°C to retain optimal biomass characteristics for a maximum of 3 – 6 months depending on a preliminary assessment of biomass stability (including oxidation).

To transport the biomass safely and to minimise the risk of any damage to the biomass, it is crucial to consider:-

i) biomass chemical and biochemical stability in various states (liquid, concentrated, paste, dried) at various storage conditions (room temperature, chilled, frozen),

ii) available capacity at both production and downstream processing sites

iii) duration of the storage, and

iv) targeted ingredients.

7 OVERALL CONCLUSIONS AND RECOMMENDATIONS

Recommendation on cultivation system and trophic mode

Data from the operation of the three investments has shown promising results in terms of microalgae growth using digestate as a waste-based medium and remediation of this medium by microalgae. Different cultivation systems were used across the investments, and vertical and horizontal tubes were both demonstrated to be efficient for microalgal production. Photobioreactors remain the preferred cultivation system to produce specific microalgal strains, allowing for production of high-value biomass and the targeting of high-value compounds for the animal feed market. However, cheaper options such as raceways should be tested specially in those countries where the weather conditions allow the use of open systems

Results showed that combining photoautotrophic and mixotrophic cultivation at the Langage AD operation produced higher biomass concentrations compared to photoautotrophic cultivation alone. The cost of adding a carbon source to the cultivation process can be reduced by using carbon waste from industrial processes. For example, the operation at Langage AD used a carbon waste source from a chewing-gum processing factory. At the Cooperl pilot, using a heterotrophic cultivation of microalgae also showed promising results with a short cultivation time allowing for high biomass production yields. Using mixotrophy and heterotrophy are promising techniques to produce high amounts of biomass and remediate higher quantities of digestate. Furthermore, additional carbon and sugar sources associated to these trophic modes can be supplied by other waste streams, limiting cost.

Recommendations on contamination control

Results demonstrated that, while contaminations are bound to occur in large-scale cultivation systems (especially when mixotrophy and heterotrophy are used), they can be minimised by controlling the system, thus allowing the microalgae to dominate. Improvements can be made to ensure contamination limitation, such as using a higher initial microalgae cell concentration. A bacterial analysis of cultures was carried out within the scope of a safety analysis, and results revealed that no harmful to human health bacterial species (including *Campylobacter*, *Salmonella, Yersinia enterocolitica, Listeria monocytogenes, Clostridioides difficile, Clostridium botulinum* and *Mycobacterium paratuberculosis*) were detected during the entire process from upstream to downstream process. However, this degree of safety is only achieved when micro (0.2 microns) or ultrafiltration (300 KDa) process was applied. Additionally, the presence of pathogens could compromise this process. This information shows that contaminations occurring in microalgal cultivation systems are not of concern to human health, but should still be controlled and monitored to allow efficient production of biomass. Furthermore, downstream processing and treatment of biomass, involve drying and hydrolysis processes, which further remove any contaminations occurring during cultivation.

Recommendations on digestate improvements/supplementation

Digestate is a valuable source of nutrients for microalgae cultivation, and adjustments to composition can be made at a low cost to improve microalgal growth. A detailed explanation of pre-processing techniques and recommendations is explored in another best practice guide - Best Practices for the treatment and preparation of nutrient rich digestate for algal cultivation - Output WP1 A1.1. and Fernandes et al, 2020. In some cases, the filtration process used for treating raw digestate may remove valuable nutrients such as phosphorus. In these circumstances, the nutrient should be supplemented back into the cultivation system to ensure a suitable N to P ratio for microalgal growth. Nutrient addition can incur additional cost, however this cost can be offset by the resulting improvements in biomass production and quality.

Recommendation for harvesting, storage and transportation

Membrane filtration is a rapid and efficient way to dewater cultures, allowing concentration of the biomass and significant reduction of the initial cultivation volume. Furthermore, this method allows for water recycling, bringing further environmental benefits. Continuous flow centrifugation is also recommended as an additional process following membrane filtration. This produces a thick algal paste, which is easier to store, transport and process. However, this approach is not recommended if cultivating Traustochytrids, as cells are broken and content lost in the supernatant if this technique is applied.

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