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2. **The Scottish Association for Marine Science LBG, SAMS**
3. **A4F Algafuel SA, A4F**
4. **Karlsruher Institut fuer Technologie, KIT**
5. **Agencia Estatal Consejo Superior de Investigaciones Científicas, CSIC**
6. **Subitec GmbH, SUBITEC**
7. **PROTEUS, PROTEUS**
8. **MICROPHYT, MICROPHYT**
9. **SENSIENT Cosmetic Technologies, SENSIENT CT**

ABACUS

Algae for a biomass applied to the production of added value compounds

BBI 2016.R9 - Exploiting algae and other aquatic biomass for production of molecules for pharma, nutraceutic, food additives and cosmetic applications

Collaborative project

Start date of the project: 01/05/2017

Duration: 36 months

Deliverable D2.1

Protocols of culture characterization for algae screening and selection

WP	2	Adaptation and selection of algae
Task	2.1	Selection and characterization of algae

Dissemination level[1]	PU	**Due delivery date**	04/08/2017
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[1] Dissemination level: **PU** = Public, **CO** = Confidential, only for members of the consortium (including the BBI), **CI** =Classified, information as referred to in Commission Decision 2001/844/EC.

[2] Nature of the deliverable: **R:** Document, report (excluding the periodic and final reports) , **DEM**: Demonstrator, pilot, prototype, plan designs, **DEC**: Websites, patents filing, press & media actions, videos, etc., **OTHER**: Software, technical diagram, etc.

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Disclaimer

This deliverable is a living document and will be updated as project progresses. For the latest version it is recommended to visit the Project's website, where updated versions will published: www.abacus-bbi.eu

Document Version	Date	Author	Comments[3]
V0	02/08/2017	M. Pérez (CEA)	Creation
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[3] Creation, modification, final version for evaluation, revised version following evaluation, final

Deliverable abstract

This deliverable D2.1 reports the methods and protocols that will be used to perform the algae selection and characterization. One of the main issues within partnership projects can be the lack of standardization between laboratories in the protocols/analysis methods undertaken, which may induce misleading conclusions and generate waste of time to sort out reasons for diverging experimental results from one partner to the other. This in turn can lead to issues linked to how cultures have been grown, how biomass has been sampled and problems with the interpretation of the data generated. Several protocols have been proposed in the document as initial standard methods to be used in order to address the issues linked to both inter- and intra-assay variation between the ABACUS project partners. Main aspects covered in this document include microalgal growth determination, genetic fingerprinting and biochemical composition determination, with a special focus on terpenoids which are primary targets of the ABACUS project.

Abbreviations

BSA: Bovine Serum AlbuminCCAP: Culture Collection of Algae and Protozoa

CT: Controlled Temperature

DGGE: Denaturing Gradient Gel Electrophoresis

DMSO: DiMethylSulfOxide

DNA; DesoxyriboNucleic Acid

DW: Dry Weight

FAME: Fatty Acid Methyl Ester

GC-FID: Gas Chromatography- Flame Ionization Detector

OFN: Oxygen Free Nitrogen

PAR: Photosynthetically Active Radiation

PCR: Polymerase Chain Reaction

PPE: Personal Protection Equipment

PUFA: Poly Unsaturated Fatty Acid

RNA: RiboNucleic Acid

RT: Room Temperature

SOP: Standard Operation Protocol

TCA: TriChloroAcetic acid

Table of contents

1 Introduction

Previous studies have highlighted differences in biomass composition in a range of different species (Slocombe et al., 2015). Without the standardization of the methods utilized overestimation of actual biochemical content can occur, this has been particularly true of algal lipids. This has then lead to a certain amount of hype and the industry has suffered from a lack of credibility (Lauren et al., 2012). One of the main issues within partnership projects can be the lack of standardization between laboratories in the protocols/analysis methods undertaken. This in turn can lead to issues linked how cultures have been grown, how biomass has been sampled and problems with the interpretation of the data generated. This document has been designed to try and address the issues linked to both inter- and intra-assay variation between the ABACUS project partners. Lauren et al (2012) demonstrated the merits of this by investigating the analysis of lipids, proteins, carbohydrates, ash and moisture from a single sample at 3 different laboratories by 8 different researchers over a number of days. Their results have suggested that training is key in the procedures to be undertaken and that using detailed documentation of the analytical methods can significantly reduce the variation seen between different laboratories. Standardization should also aid in understanding the potential differences seen in productivity when cultures are scaled up from the laboratory to commercial levels.

In order to ensure standardization of procedures between the partners of ABACUS this document has been created to act as a repository of the standard operating procedures (SOPs) used within the partnership. It needs to be considered as a “living” document and will be updated at regular intervals as improvements and new analysis methods are added. In order to facilitate access for all the partners it will be available in the partners section of the ABACUS e-Room. Moreover, this document will be publicly available through the project website (*www.abacus-bbi.eu)*

2 Prior to the analysis: Sample preparation

Sample stabilization is a key step to ensure the preservation of valuable compounds in microalgal biomass over time and freeze drying is the most gentle and one of the most extended techniques for it[4]. Therefore, as a general procedure it is recommended to perform freeze-drying of the samples as soon as possible after collecting.

- Take a certain volume of microalgae broth and centrifuge it until the supernatant is clear. Then discard the supernatant
- Wash the cell pellet by resuspending it in the same initial volume of water. In the case pf marine species, washing should be performed with an ammonium formate solution containing the same osmolarity as that of the culture medium. This will prevent the cells from swelling, leaching or eventually lysing during the washing step and will replace salts from the culture medium, what can often have an impact in terms of dry weight quantification of the biomass. Ammonium formate completely evaporates during lyophilization and does not leave any residue.
- Centrifuge again the algae suspension and discard supernatant.
- Lyophilize the cell pellet. Grind and homogenize before taking the required weight sample to analysisStandard Operating Procedures (SOPs)

2.1 Media Recipes

2.1.1 Purpose

To show the media recipes for micro-algal and cyanobacterial growth. Recipes include: f/2, f/2+Si, Modified Woods Hole Medium (MWC), Modified Artificial Seawater Medium (MASM), Soil Extract (SE) and modified BG11.

2.1.2 Scope

This SOP covers the media formulations for cultivating micro-algae

2.1.3 Materials and Equipment

- Chemicals listed in each table
- Weigh boats
- Analytical balance
- Autoclave
- Pipettes
- pH meter

2.1.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing with hot samples

2.1.5 Procedure

1. Ensure all glass- and plastic-ware is clean and sterilized.

[4] Handbook of microalgal culture: applied phycology and biotechnology / edited by Amos Richmond and Qiang Hu. – Second edition. ISBN 978-0-470-67389-8

2. Ensure stock solutions are clean and sterile (e.g. no cloudiness, no formation of bacteria/fungus)
3. Ensure media formulations are appropriate for the species/strain to be grown.
4. Stock solutions can be maintained in a fridge for up to 1 year. Always check stocks for any evidence of contamination (*e.g.* cloudiness or hyphae from fungi) prior to use.
5. For best results, use cultivation medium that is ≤ 7 days old.

Part I - f/2 Medium

Table 1. Recipe for f/2 Medium.

Stock A - Nitrogen	**(g L^{-1})**
$NaNO_3$	75
Stock B - Phosphorus	**(g L^{-1})**
$NaH_2PO_4 \cdot 2H_2O$	5.65
Stock C – Trace Metals	**(mg L^{-1})**
Na_2EDTA	4160
$FeCl_3 \cdot 6H_2O$	3150
$CuSO_4 \cdot 5H_2O$	10
$ZnSO_4 \cdot 7H_2O$	22
$CoCl_2 \cdot 6H_2O$	10
$MnCl_2 \cdot 4H_2O$	180
$Na_2MoO_4 \cdot 2H_2O$	6
Stock D - Vitamins	**(mg 100 mL^{-1})**
Thiamine HCl (Vit B_1)	10
Cyanocobalamin (Vit B_{12})	0.05
Biotin	0.05
Media Formulation	**(mL L^{-1})**
Stock A	1
Stock B	1
Stock C	1
Stock D	1

Notes

- To ~ 900 mL of natural filtered seawater, add stocks A-C. Bring volume to 1L and sterilise (*i.e.* 121°C for 15 minutes, in an autoclave). Once cooled to room temperature, add Stock D and adjust pH to 8.0 using 0.1 M HCl or NaOH.
- Keep stock solutions refrigerated
- Stock solutions can be kept up to a year; however they are monitored for any contaminants.

Part II - f/2 + Si Medium

Table 2. Recipe for f/2 + Si Medium.

Stock A - Nitrogen	**(g L^{-1})**
$NaNO_3$	75
Stock B - Phosphorus	**(g L^{-1})**
$NaH_2PO_4 \cdot 2H_2O$	5.65
Stock C – Trace Metals	**(mg L^{-1})**
Na_2EDTA	4160
$FeCl_3 \cdot 6H_2O$	3150
$CuSO_4 \cdot 5H_2O$	10
$ZnSO_4 \cdot 7H_2O$	22
$CoCl_2 \cdot 6H_2O$	10
$MnCl_2 \cdot 4H_2O$	180
$Na_2MoO_4 \cdot 2H_2O$	6
Stock D - Vitamins	**(mg 100 mL^{-1})**
Thiamine HCl (Vit B_1)	10
Cyanocobalamin (Vit B_{12})	0.05
Biotin	0.05
Stock E - Silicate	**(g L^{-1})**
$Na_2SiO_3 \cdot 9H_2O$	30
Media Formulation	**(mL L^{-1})**
Stock A	1
Stock B	1
Stock C	1
Stock D	1

Notes

- To ~ 900 mL of natural filtered seawater, add stocks A-C and E. Bring volume to 1L and sterilise (*i.e.* 121°C for 15 minutes, in an autoclave). Once cooled to room temperature, add Stock D and adjust pH to 8.0 using 0.1 M HCl or NaOH.
- Keep stock solutions refrigerated
- Stock solutions can be kept up to a year; however they are monitored for any contamination.

Part III – Modified WC Medium (MWC)

Table 3. Recipe for Modified Woods Hole Medium (MWC).

Stock A	**(g L^{-1})**
$CaCl_2 \cdot 2H_2O$	36.8
Stock B	**(g L^{-1})**
$MgSO_4 \cdot 7H_2O$	37
Stock C	**(g L^{-1})**
$K_2HPO_4 \cdot 3H_2O$	11.4
Stock D	**(g L^{-1})**
$NaHCO_3$	12.6
Stock E	**(g L^{-1})**
$NaNO_3$	85
Stock F	**(g L^{-1})**
$Na_2O_3Si \cdot 5H_2O$	21.2
Stock G – Combined Trace Elements	**(g L^{-1})**
$EDTANa_2$	4.36
$FeCl_3 \cdot 6H_2O$	3.15
$CuSO_4 \cdot 5H_2O$	0.01
$ZnSO_4 \cdot 7H_2O$	0.022
$CoCl_6 \cdot 6H_2O$	0.01
$MnCl_2 \cdot 4H_2O$	0.18
$Na_2MoO_4 \cdot 2H_2O$	0.006
H_3BO_3	1
Stock I	**(g L^{-1})**
Thiamine HCl	0.1
Cyanocobalamin	0.0005
Biotin	0.0005
Media Formulation	**(mL L^{-1})**
Stocks A-I	1

Notes

- To ~ 900 mL of deionised water, add stocks A-H. Bring volume to 1L and sterilise (*i.e.* 121°C for 15 minutes, in an autoclave). Once cooled to room temperature, add Stock I and adjust pH to 8.0 using 0.1 M HCl or NaOH.
- Keep stock solutions refrigerated
- Stock solutions can be kept up to a year; however they are monitored for any contamination.

Part IV – Modified Artificial Seawater Medium (MASM)

Table 4. Recipe for Modified Artificial Seawater Medium (MASM).

Stock A	**(g L^{-1})**
$NaNO_3$	100
Stock B - Phosphorus	**(g L^{-1})**
KH_2PO_4	5
Stock C – Trace Metals	**(mg L^{-1})**
Na_2EDTA	750
$FeCl_3 \cdot 6H_2O$	97
$MnCl_2 \cdot 4H_2O$	41
$ZnSO_4 \cdot 6H_2O$	5
$CoCl_2 \cdot 6H_2O$	2
$Na_2MoO_4 \cdot 2H_2O$	4
Stock D – Vitamin B_1	**(mg 100 mL^{-1})**
Thiamine HCl (Vit B_1)	0.12
Stock E – Vitamin B_{12}	**(mg 100 mL^{-1})**
Cyanocobalamin (Vit B_{12})	0.001
Stock F	**(g L^{-1})**
$MgSO_4 \cdot 7H_2O$	244
Stock G	**(g L^{-1})**
KCl	60
Stock H	**(g L^{-1})**
$CaCl_2 \cdot 2H_2O$	30
Stock I	**(g L^{-1})**
NH_4Cl	26.7
Media Formulation	**(mL L^{-1})**
Stock A	10
Stock B	10
Stock C	6
Stock D	1
Stock E	1
Stock F	10
Stock G	10
Stock H	10
Stock I	1
Soil Extract (See below)	30

Notes

- Trace elements – add the elements listed in Stock C to 1 L of distilled water in EXACTLY the order that they are listed above.
- To make Vitamin B12 stock add 0.1 g cyanocobalamin to 100 mL distilled water, take 1 mL of this and add to 99 mL distilled water.
- Filter sterilize vitamin stocks.
- To ~ 800 mL of distilled water add stocks 1.0 g Tris and 30g NaCl.
- Make up to 1 L with distilled wwater, adjust pH to 8.0 and sterilise (*i.e.* 121°C for 15 minutes, in an autoclave). Once cooled to room temperature, add Stock D & E and adjust pH to 8.0 using 0.1 M HCl or NaOH.
- Keep stock solutions refrigerated
- Stock solutions can be kept up to a year; however they are monitored for any contamination.

Soil Extract

Preparing the Soil

Site selection for a good soil is very important and for most purposes a soil from undisturbed deciduous woodland is best. Sites to avoid are those showing obvious signs of anthropogenic activity and particular care should be taken to avoid areas where fertilisers, crop sprays or other toxic chemicals may have been used.

A rich loam with good crumb structure should be sought. Stones, rootsand larger invertebrates should be removed during an initial sieving through a 1 cm mesh. The seived soil should be spread to air dry and hand-picked for smaller invertebrates and roots. It should be turned periodically and picked over again. When dry it may be sieved through a finer mesh (*i.e.* 2-4 mm) or stored as it is prior to use.

Medium

Soi lis prepeared as above. 105 g of air-dried sieved soil and 660 mL of deionised water are placed in a 1 L bottle and autoclaved once at 15 psi for 15 minutes, then again 24 hours later. The contents of the bottle are left to settle (usually for > 1 week) and then the supernatant is decanted and filtered. The final pH should be between 7.0 – 8.0.

Part V- Modified BG11

Table 5. Recipe for Modified BG11.

Stock A	**(g L^{-1})**
$NaNO_3$	3
K_2HPO_4	0.080
$MgSO_4 \cdot 7H_2O$	0.150
$CaCl_2 \cdot 2H_2O$	0.072
Citric acid	0.012
Ammonium ferric citrate	0.012
EDTA	0.002
Na_2CO_3	0.4
Stock B Combined Trace Elements	**(g L^{-1})**
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$Na_2MoO_4 \cdot 2H_2O$	0.390
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2$ $6H_2O$	0.0494
Media Formulation	**(mL L^{-1})**
Stock A	500
Stock B	1
1.43 g Hepes	
Notes	
• To ~ 500 mL of deionised water, add stocks A and B. Bring volume to 1L and sterilise (*i.e.* 121°C for 30 minutes, in an autoclave). Cooled to room temperature. pH before autoclaving should be 7.3, after autoclaving 7.5.	
• Keep stock solutions refrigerated	
• Stock solutions can be kept up to a year; however they are monitored for any contamination.	

2.1.6 Reporting

- Date, initial, and label the media, include any other relevant info (e.g. pH).
- Take a record in the lab-book.

2.1.7 Reference documents

- R.R. Guillard, J.H. Ryther, Studies of marine planktonic diatoms: I. Cyclotella nana Hustedt, and Detonula confervacea (Cleve) Gran, Can. J. Microbiol. 8 (1962) 229-239.
- Culture Collection of Algae and Protozoa, Recipes: List of media used to maintain strains at CCAP (2014) Accessed on: 07/12/2016, https://www.ccap.ac.uk/pdfrecipes.htm

2.2 Micro-algal Cultivation Regime

2.2.1 Purpose

To standardize the cultivation of micro-algae

2.2.2 Scope

This SOP covers the standard cultivation protocol for micro-algae

2.2.3 Materials and Equipment

- 70% (v/v) ethanol
- Erlenmeyer flasks (100 and 250 mL)
- Foam stoppers
- Tin foil
- Screw-top flask (1 L)
- Thread safety caps (4 vents)
- Silicone tubing
- Whatmann HEPA-VENT filters
- Laminar flow cabinet
- Appropriate cultivation media (*see* Media Recipes for Micro-algal Cultivation)
- Temperature-controlled room (CT) / incubator – set to appropriate temperature (strain dependent), with an appropriate light regime (*e.g.* 16/8 h L/D photoperiod, with 150 µE m^{-2} s^{-1} PAR).
- Air supply
- Bunsen burner
- Measuring cylinder
- Disposable pipettes

2.2.4 AutoclaveSafety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing with hot samples and naked flame

2.2.5 Procedure

Part I – Aseptic technique

1. Perform all cultivation work in a Laminar flow cabinet, clean before and after use with 70% (v/v) ethanol.
2. Prepare cultivation vessels. Using clean Erlenmeyer flasks, place a foam bung in the neck and cover the top with tin foil. Sterilise in an autoclave at 121°C for 15 minutes.
3. Prepare 1 L cultivation vessels. Firstly, make appropriate caps using silicone tubing. Ensure that the inflow tube is long and will be immersed in the culture. Make sure the exhaust tube is attached to the outside only. Seal other vents with plastic stoppers. Loosely replace the cap (complete with tubing), and sterilize as above.
4. Ensure all plastic-ware is hermetically sealed.
5. Make sure cultivation media has been sterilized.

Part II – Cultivation

1. The cultivation regime follows that depicted in Figure 1.
2. Obtain a master culture (*e.g.* 100 mL flask containing 50 mL of culture) / alternatively obtain a starter culture from one of the culture collections.
3. Prepare Erlenmeyer flasks like those labelled B (Figure 1), by transferring 87.5 mL of sterile media, in aseptic conditions (*e.g.* laminar flow cabinet, flaming necks of flasks, sterile equipment, PPE).
4. Transfer 12.5 mL of the Starter culture (denoted A in Fig. 1), equivalent to 25% of the inoculum, bringing the total culture volume up to 100 mL.
5. Flame the neck of the flask, gently swirl, replace the foil and foam bung. Place culture in an incubator or CT room. Leave for 7 days.
6. Prepare Erlenmeyer flasks like those labelled C (Fig. 1), by transferring 75 mL of sterile media, in aseptic conditions (e.g. laminar flow cabinet, flaming necks of flasks, sterile equipment, PPE).
7. Transfer 25 mL of the flask culture created in step 5 (denoted B in Fig. 1), equivalent to 25% of the inoculum, bringing the total culture volume up to 100 mL.
8. Flame the neck of the flask, gently swirl, replace the foil and foam bung. Place culture in an incubator or CT room. Leave for 7 days.
9. Prepare Erlenmeyer flasks like those labelled D (Fig. 1), by transferring 410 mL of sterile media, in aseptic conditions (e.g. laminar flow cabinet, flaming necks of flasks, sterile equipment, PPE).
10. Transfer 90 mL of the flask culture created in step 7 (denoted C in Fig. 1), equivalent to 90% of the inoculum, bringing the total culture volume up to 500 mL. Retain the 10 mL of Flask C for biochemical and water chemistry analysis.
11. Flame the neck of the flask, gently swirl, replace the vented caps. Place filters on the inflow and exhaust tubes. Place the cultures in an incubator or CT room. Connect the inflow tube to an air supply.
12. Monitor algal growth 3 times per week (SOP: Counting micro-algal cells).
13. Allow culture to grow until it enters early stationary phase (~14-21 days)
14. Harvest culture. Retain biomass and water samples.

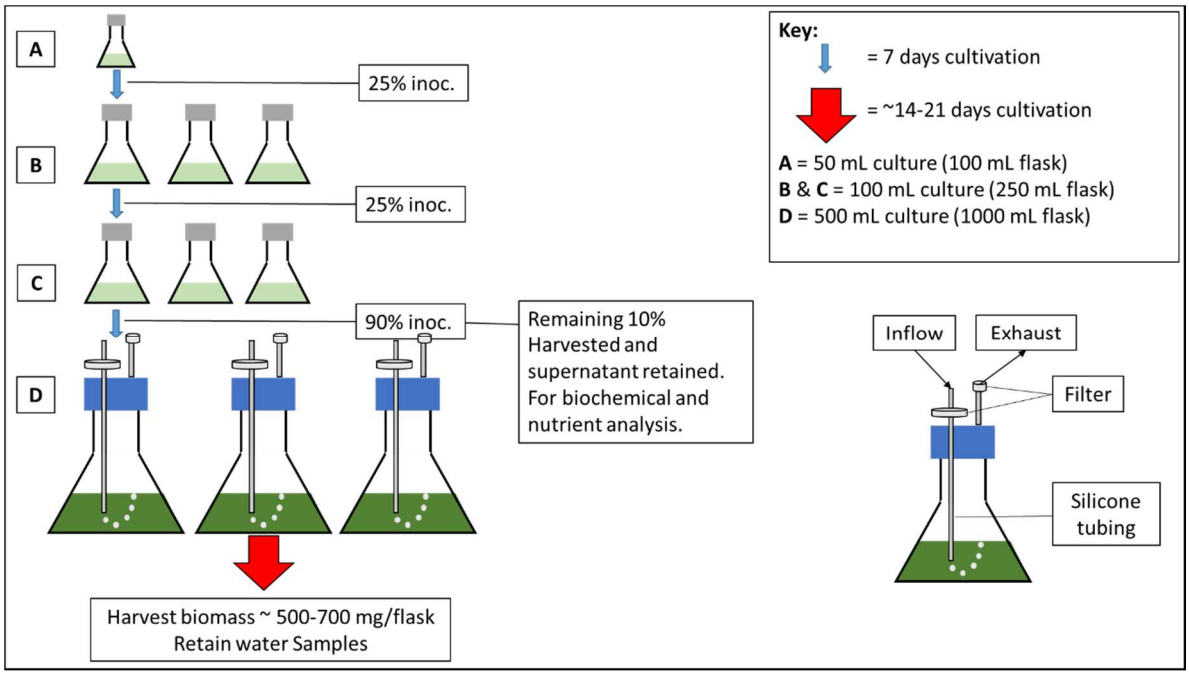

Figure 1. Cultivation schematic.

2.2.6 Reporting

Record data on Microsoft Excel

2.3 Counting Micro-algal cells

2.3.1 Purpose

To show how to count micro-algal cells

2.3.2 Scope

This SOP covers the method for counting micro-algal cells

2.3.3 Materials and Equipment

- Hemocytometer (0.1 mm deep) + cover slip
- Light microscope
- Spectrophotometer
- Coulter counter
- Lugol's Iodine (Dissolve 10g Potassium Iodide in 20-30 mL distilled water, add 5g Iodine and heat gently until dissolved. Dilute to 100 mL with distilled water. Store in an amber glass stoppered bottle in the dark)
- Handheld tally counter

2.3.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE

2.3.5 Procedure

Part I:

1. If possible, determine cell number by using a Coulter counter – or equivalent device from another manufacturer - and following the manufacturer's instructions.

Part II:

1. Gently swirl the cell culture to ensure that cells are uniformly distributed.
2. Aseptically remove an appropriate volume of culture using a pipette and transfer to a clean beaker.
3. Transfer 1mL to an Eppendorf tube. If necessary, dilute the culture with deionized water.
4. If counting motile cells (e.g. *Tetraselmis sp*.), fix with a drop of Lugol's iodine.
5. Make sure the hemocytometer is clean and dry (use 70% ethanol if necessary), and gently place the cover slip on top of the chambers.
6. Transfer 50 µL of the cell suspension to hemocytometer.
7. Using a microscope, focus upon the gridlines of the hemocytometer using a 10x objective lens.
8. Using a hand tally counter, count all of the cells present in one set of 16 squares. Follow a standardized counting regime (e.g. only count cells that are within a square or on the top or left-hand boundaries).
9. Repeat until all four sets of 16 squares are counted.
10. Take the average of the four sets of cell counts and multiply by 10,000 to determine the cell number/mL. Remember to take into account any dilution factors.
11. Please note: when using a 0.2 mm hemocytometer, the cell number should be multiplied by 5,000 instead of 10,000.

Part III:

1. With the same cell culture as used in Part II step 2, generate an appropriate dilution series (*e.g.* 10^5 – 10^7), with approximately 5-10 mL culture volumes.
2. Transfer 1.5-2 mL into a cuvette
3. Read absorbance at 750 nm. This wavelength is selected to avoid interference from photosynthetic pigments
4. A calibration curve can then be generated by plotting Absorbance against cell number/mL.

2.3.6 Reporting

Record data on Microsoft Excel

2.3.7 Reference documents

- R.R. Guillard, M.S. Sieracki, Counting cells in cultures with the light microscope, in: R.A. Andersen (Ed.) Algal culturing techniques, Elsevier Academic Press, London, 2005, pp. 239-252.
- T. Yamaoka, K. Satoh, S. Katoh, Photosynthetic activities of a thermophilic blue-green alga, Plant Cell Physiol. 19 (1978) 943-954.

2.4 Growth of cyanobacteria

2.4.1 Purpose

To determine the growth of cyanobacteria.

2.4.2 Scope

This SOP covers the growth determination of cyanobacteria.

2.4.3 Materials and Equipment

Spectrophotometer

2.4.4 Safety Issues

Standard lab safety should be taken.

2.4.5 Procedure

1. Cell growth should be evaluated twice per day.
2. Transfer 1.5 mL of culture to a cuvette and read absorption at 580 nm and 750 nm.

2.4.6 Additional Information

A calibration curve should be generated which plots the optical density against cell number, biovolume, or dry weight.

2.4.7 Reporting

Record data in Microsoft Excel

2.5 Ash Free Dry Weight/Total Suspended Solids

2.5.1 Purpose

To determine the dry weight (DW) / total suspended solids (TSS) content of the algal biomass.

2.5.2 Scope

This SOP covers the determination of the DW/TSS content of algal biomass only.

2.5.3 Materials and Equipment

- Drying oven
- GF/F filters
- Furnace
- 0.5M ammonium formate
- Analytical balance

2.5.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care should be taken when using the furnace and handling hot samples

2.5.5 Procedure

1. Determine the cell number/biovolume of the culture using the methods outlined in Section 2.3.
2. In triplicate, filter a set volume of culture (*e.g.* 10-50 mL depending upon cell density) using pre-combusted (550°C, 20 mins) GF/F filter (0.7 µm pore) under a low vacuum (< 100 mm Hg).
3. Remove salts by washing filters with an equivalent volume of 0.5M ammonium formate.
4. Dry filters for at least 12 h at 80°C in a drying oven.
5. Allow filters to reach room temperature in a desiccator and then weigh to 4 decimal places.
6. Repeat this process until the filters are a constant weight.

2.5.6 Additional Information

1. Standard curves can be generated to show the relationship between cell number/biovolume and ash free dry weight.
2. Triplicate samples can be taken at a number of time points across batch growth to reflect different cell statuses and biochemistries.
3. A linear relatinship should be found between biovolume (μm^3) and DW.

2.5.7 Reporting

Note values in a lab book and report on Microsoft Excel

2.5.8 Reference documents

- C.J. Zhu, Y.K. Lee, Determination of biomass dry weight of marine microalgae, J. Appl. Phycol. 9 (1997) 189-194.

2.6 Ash Determination

2.6.1 Purpose

To show how to determine the ash content from algal biomass

2.6.2 Scope

This SOP covers the determination of ash content from algal biomass only

2.6.3 Materials and Equipment

- Freeze-drier
- Foil-capsules / tin-foil
- Furnace
- Analytical Balance

2.6.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing with hot samples

2.6.5 Procedure

1. Ensure dry weight (DW) algal biomass is the starting material by freeze-drying (or oven-drying)
2. If necessary, make foil capsules by folding tin-foil into envelope shapes.
3. Label foil capsules with an indelible marker
4. To remove any volatile material, combust foil capsules for 30 minutes at 500°C
5. Once cooled to room temperature (RT), weigh the foil capsules. Record mass.
6. Place in an analytical balance a foil capsule. To this, weigh approximately 10 mg of freeze-dried algal biomass. Record mass.
7. Seal foil capsules to ensure no material is lost.
8. Combust the samples in a furnace for 12h at 500°C.
9. Allow samples to cool to RT, and weigh. Record mass.
10. Ash content can be determined by using the following equation:

$$\%\, Ash = \frac{M2 - M1}{DW} \text{ x } 100$$

Where *M*1 is the mass of the pre-combusted foil capsule, *M*2 is the mass of the foil capsule and ash, *DW* is initial mass of the lyophilised algal sample prior to combustion.

2.6.6 Reporting

Record data on Microsoft Excel

2.6.7 Reference documents

- A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of ash in biomass, National Renewable Energy Laboratory (NREL) Technical Report, 2008, pp. NREL/TP-510-42622.

2.7 Total carbohydrate quantification

2.7.1 Purpose

To show how to perform Total Carbohydrate analysis on a sample

2.7.2 Scope

This SOP covers the extraction and assay for Total Carbohydrate only

2.7.3 Materials and Equipment

- Incubator
- 1M H_2SO_4
- Concentrated H_2SO_4 (>95%)
- Centrifuge
- Glucose (standard)
- 4% (w/v) Phenol
- Spectrophotometer
- Microsoft Excel

2.7.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing with hot samples
- Phenol is a CMR and thus proper lab protocol should be followed when using. This step must be performed in a fume hood. Please refer to safety data sheets (SDS) and control of substances hazardous to health (COSHH) forms prior to use.

2.7.5 Procedure

1. Suspend 5mg of lyophilized samples in 0.5ml 1M H_2SO_4
2. Incubate at 121°C for 15mins
3. Let the sample cool to RT
4. Centrifuge sample at 10,000RPM for 10 min
5. Take 30uL of supernatant and gently mix with 0.5ml 4% Phenol
6. Further mix with 2.5ml of concentrated H_2SO_4
7. Allow samples to cool to room temperature
8. Make up glucose standards for Spectrophotometer as with the Spectrophotometer SOP
9. Read absorbance of samples and standards at 490nm
10. Record and plot a standard curve to find Carbohydrate concentration

2.7.6 Reporting

Record data on Microsoft Excel

2.7.7 Reference documents

- Slocombe, S. P., Zhang, Q., Ross, M., Anderson, A., Thomas, N. J., Lapresa, Á., Day, J. G. (2015). Unlocking nature's treasure-chest: screening for oleaginous algae. Scientific Reports, 5(July), 9844. https://doi.org/10.1038/srep09844
- M. Dubois, K.A. Gilles, J.K. Hamilton, P. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350-356.

- P. Schiener, K.D. Black, M.S. Stanley, D.H. Green, The seasonal variation in the chemical composition of the kelp species Laminaria digitata, Laminaria hyperborea, Saccharina latissima and Alaria esculenta, J. App. Phycol. 27 (2015) 363-373.
- E. Fournier, Colorimetric quantification of carbohydrates, in: R.E. Wrolstad (Ed.) Current Protocols in Food Analytical Chemistry, John Wiley and Sons, Hoboken, NJ, USA, 2001, pp. E1.1.1–E1.1.8.

2.8 Direct derivatization of lipids

2.8.1 Purpose

To show the procedure for the direct derivatization of lipids from algal biomass and their analysis by gas chromatography

2.8.2 Scope

This SOP covers the direct derivatization of lipids and their analysis by gas chromatography

2.8.3 Materials and Equipment

- Freeze-drier
- 2 mL screw-top vials
- Teflon-capped tapered vials
- Hexane containing 0.01% (w/v) butylated hydroxytoluene (BHT)
- Internal standard = methyl tricosanoate (5 mg mL^{-1} in hexane)
- External standards = (37 FAMES, PUFA2, PUFA3, methyl 9(Z), 12(Z) Hexadecadienoate Methyl 7(Z) hexadecadienoate
- Anhydrous 1 N methanolic-HCl
- 0.88% (w/v) KCl
- Nitrogen gas
- Incubator/oven
- Helium gas
- -80 °C freezer
- GC-FID (e.g. GC-2014, Shimadzu)
- 30-m, 0.25-mm ID ZB-wax column (Phenomenex)
- GC solution software

2.8.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Work involving solvents should be performed in a fume cupboard
- Exercise care when using gas cylinders

2.8.5 Direct-derivatization Procedure

1. Ensure dry weight (DW) algal biomass is the starting material by freeze-drying (or oven-drying)
2. Weigh 10 mg of freeze-dried algal biomass into a 2mL screw-top vial.
3. To this, add 200 µL hexane containing 0.01% (w/v) BHT.
4. Add 10 µL methyl tricosanoate as an internal standard
5. Add 500 µL anhydrous 1 N methanolic-HCl.
6. Flush vials with nitrogen gas and cap with Teflon lined seals.
7. Incubate for 2 h at 85°C.
8. After cooling to room temperature (RT), add 250 µL of 0.88% (w/v) KCl
9. Transfer the upper hexane phase (containing the FAMES) to a teflon-capped tapered vial
10. Flush this with nitrogen gas before sealing.
11. Samples can be run immediately, or stored under nitrogen at -80 °C.

2.8.6 Gas chromatography

1. Inject 1 µL aliquots of the hexane phase (containing FAMES) into a GC-FID
2. Use helium as a carrier gas at a 1.56 mL min-1 with a split ratio of 100/1
3. Program oven top heat from 160 °C to 240 °C at 4 °C min-1, then run isothermally at 240 °C for 10 minutes.
4. Peak areas can be integrated using GC solution software, with peak area quantified by reference to the internal standard.
5. Peak identities can be ascertained by using external standards.
6. Peaks can be expressed as a proportion of the dry weight of the biomass (% DW), or converted to moles and expressed as a percentage of the total identified fatty acid content mol %)

2.8.7 Record Data

1. Peaks can be integrated and calculated using GC solution software
2. Record data on Microsoft Excel

2.8.8 Reference documents

- T.R. Larson, I.A. Graham, Technical advance: a novel technique for the sensitive quantification of acyl CoA esters from plant tissues, Plant J., 25 (2001) 115-125.
- S.P. Slocombe, Q. Zhang, K.D. Black, J.G. Day, M.S. Stanley, Comparison of screening methods for high-throughput determination of oil yields in micro-algal biofuel strains, J. Appl. Phycol., 25 (2013) 961-972.

2.9 Determination of total lipid and fatty acid profile

2.9.1 Purpose

To show how to determine total lipid and fatty acid content from micro-algal biomass.

2.9.2 Scope

This SOP covers the extraction and quantification of total lipid and fatty acids from algal biomass.

2.9.3 Materials and Equipment

- Lyophilised biomass
- Glass test tubes
- Potassium Chloride – 0.88% (w/v)
- Methanol
- Ultra-turrax homogeniser
- Chloroform
- Oxygen-free nitrogen gas
- Whatmann No. 1 filters (pre-washed in chloroform:methanol)
- Nitrogen evaporator
- Vacuum desiccator
- Quickfit test tubes
- Internal fatty acid standard
- Toluene
- Methylation Reagent (1% concentrated H_2SO_4 in methanol)
- Vortex
- Hotblock
- Potassium hydrogen carbonate (2%)
- Isohexane:diethyl ether 1:1 +BHT (0.01% w/v).
- Isohexane
- 100 µl Hamilton syringe
- 20*20 cm TLC plates
- Isohexane:diethyl ether:glacial acetic acid (90:10:1)
- 1% iodine in chloroform
- Isohexane:diethyl ether
- Ioshexane:diethyl ether + BHT (0.01% w/v)

2.9.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing solvents

2.9.5 Procedure

Total lipid Extract according to Bligh and Dyer (BDE) (Bligh and Dyer, 1959):

1. Weigh out approx. 0.5g of freeze dried algae powder into a glass test-tube. Immediately place the tubes on ice.
2. Add 1ml of 0.88% (w/v) potassium chloride in deionised water to hydrate the powder.

3. Add 15ml of methanol.
4. Homogenise samples using an Ultra Turrax.
5. Add 7.5ml of chloroform and 6.75ml of 0.88% (w/v) KCl in deionised water. Then vigorously mix samples before storing at -20°C for an hour, shake vigorously every 15 minutes.
6. Add an additional 7.5ml of chloroform and 6.75ml of KCl solution and flush the samples with oxygen free nitrogen (OFN) and allow to separate at -20°C overnight.
7. Carefully remove the upper aqueous layer without disturbing the material at the interface
8. Filter the samples into test tubes using prewashed (chloroform:methanol) Whatman no.1 filters.
9. Place samples on N-EVAP nitrogen evaporator and dry the solvent extract under OFN.
10. Re-suspend the total lipid extract in 500µl of chloroform:methanol and transfer to pre-weighed 2ml vials. Repeat with two further washings of 500µl of solvent and add washings to the glass vials.
11. Carefully dry the total lipid extracts were dried under OFN
12. Once dry, place in a vacuum desiccator overnight or for a minimum of 6 hours before being re-weighed.

Total lipid = weight of vial containing lipid – weight of empty vial

% TL = {mass of lipid (g)/mass of sample (g)}*100

13. Re-suspend the total lipid extract in chloroform:methanol+BHT (0.01% w/v) at 1, 10 or 100mg/ml, flush vials with OFN and stored at -20°C.

Fatty Acid Analysis (Ghioni, Bel and Sargeant, 1996 ; Christie, 2003)

1. Using a pipette, place the equivalent of 1mg of total lipid extract into a 15ml Quickfit test tube.
2. Add the equivalent of 1mg of internal standard (if required) into the test tube.
3. Dry the sample under OFN.
4. Add 1ml of toluene to help solubillize neutral lipid and add 2ml of methylation reagent (1% concentrated sulfuric acid in methanol).
5. Whirlimix to ensure sample is well mixed.
6. Flush the tubes with OFN, stopper with a piece of tissue between stopper and tube, to allow release of gas when tube is heated.
7. Place the samples overnight (min. 16 hours – max. 18 hours) on a hotblock at 50˚C.
8. Stop the reaction with 2ml of 2% aqueous potassium hydrogen carbonate.
9. Add 5ml of isohexane:diethyl ether 1:1 +BHT (0.01% w/v).
10. Stopper and shake, periodically releasing the stopper to prevent build-up of CO_2
11. Centrifuge at 1600 RPM for two minutes,
12. Transfer the top solvent layer to a clean 15ml Quickfit test tube
13. Add another 5ml of isohexane:diethyl ether 1:1, stopper and shake once again.
14. Spin as before and transfer the top layer across to the new tube.
15. Dry the solvent layer down under OFN.
16. Re-suspend in 100µl of isohexane and whirlimix.
17. Using a 100µl Hamilton syringe, load the samples onto silica 20x20 TLC plates.
18. Chromatograph the plates in isohexane:diethyl ether:glacial acetic acid (90:10:1).
19. Visualise the FAME bands by spraying the edges of the plate with 1% iodine in chloroform

and mark position of the doublet, leaving a margin about 1.5cm above and below.

20. Scrape the two bands off the plate using a blade and place the silica in a clean 15ml Quickfit test tube.
21. Elute the FAME using 9ml isohexane:diethyl ether and 1ml isohexane:diethyl ether +BHT (0.01% w/v).
22. Stopper and whirlimix.
23. Centrifuge at 1600 RPM for 5 minutes to sediment out the silica.
24. Transfer the solvent across to a clean test tube and dry down under OFN.
25. Re-suspend the sample in 0.9-1.0 ml isohexane and place the sample in autosampler vials.
26. Store the FAME at -20^{o}C under nitrogen before running on GC.

2.9.6 Reporting

Record data on Microsoft Excel

2.9.7 Reference documents

- E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Canadian journal of biochemistry and physiology. 37 (1959) 911-917.
- C. Ghioni, J. Bell, J. Sargent, Polyunsaturated fatty acids in neutral lipids and phospholipids of some freshwater insects, Comp. Biochem. Phys. B. 114 (1996) 161-170.
- W. Christie, Lipids: their structures and occurrence, Lipid analysis. Isolation, separation, identification and structural analysis of lipids. The Oily Press, Bidgwater, England. (2003) 4.
- W.W. Christie, Preparation of ester derivatives of fatty acids for chromatographic analysis, Advances in lipid methodology. 2 (1993) e111.

2.10 Protein Determination – Micro-algae

2.10.1 Purpose

The purpose is to show how to extract and quantify proteins from algal cultures.

2.10.2 Scope

The scope covers the extraction and quantification of proteins extracted from algae only.

2.10.3 Materials and Equipment

- Lowry Reagent A = 2% (w/v) sodium carbonate (anhydrous) in 0.1N sodium hydroxide solution.

  4 g of NaOH was dissolved in 1 L of deionised water. Then 20 g of Na_2CO_3 was dissolved in the sodium hydroxide solution.

- Lowry Reagent B = 1% (w/v) sodium potassium tartrate

  1 g of $C_4H_4KNaO_6 \cdot 4H_2O$ was dissolved in 100 mL of deionised water.

- Lowry Reagent C = 0.5% (w/v) copper sulphate solution

  500 mg of $CuSO_4 \cdot 5H_2O$ was dissolved in 100 mL deionised water.

- Lowry Reagent D = a 48/1/1 volumetric ratio of A/B/C.
- Lowry Reagent E = Folin Ciocalteu-Phenol solution. This can be purchased from Fisher Scientific, product code 11431092.

  Equal parts of 2N Folin Ciocalteu-Phenol was mixed with deionised water.

- Please Note: Reagents A, B, and C can be stored indefinitely. Reagents D and E should be created fresh at day of analysis.
- 24% (w/v) TCA
- Bovine Serum Albumin(BSA)
- 1.5ml Micro-centrifuge tubes
- Micro-centrifuge
- Refrigerated Centrifuge
- Incubator
- Spectrophotometer
- Microsoft Excel
- Water Bath

2.10.4 Safety Issues

- Caution should be taken when using the 95^{o}C incubator/water bath
- Standard lab protocol should be followed to maintain safety at all times

2.10.5 Procedure

1. In triplicate, weigh 5mg of freeze-dried Microalgae
2. Add 200 µL of 24% TCA and vortex
3. Incubate at 95^{o}C for 15 min
4. Cool to RT
5. Add 600 µL of water to sample
6. Spin at 15,000 RPM for 20min at 4^{o}C
7. Discard the supernatant then re-suspend the pellet in 500 µL of Lowry Reagent D

8. Incubate at 55^{o}C for 3h
9. Spin at 15,000RPM for 20min at RT
10. Collect supernatant and discard the pellet
11. In triplicate, add 25 µL of supernatant to 1.5ml micro-centrifuge tubes
    - At the same time, a BSA calibration curve can be generated using BSA in the concentration range of 0-5 mg/mL.
12. Add 1000 µL of Lowry Reagent D to each tube and mix by inversion
13. Incubate samples for 10mins at RT
14. Add 100 µL of Lowry Reagent E to the tubes and vortex immediately
15. Wait for 30 min at RT
16. Run samples at 600nm and record data
17. Plot data to form a calibration curve to work out protein quantity

2.10.6 Reporting

Record data on Microsoft Excel

2.10.7 Reference documents

- Slocombe, S. P., Ross, M., Thomas, N., McNeill, S., & Stanley, M. S. (2013). A rapid and general method for measurement of protein in micro-algal biomass. *Bioresource Technology*, *129*, 51–57. https://doi.org/10.1016/j.biortech.2012.10.163

2.11 Protein Determination - Cyanobacteria

2.11.1 Purpose

The purpose is to show how to extract and quantify proteins from cyanobacteria cultures.

2.11.2 Scope

The scope covers the extraction and quantification of proteins extracted from cyanobacteria only.

2.11.3 Materials and Equipment

- Lowry Reagent A = 2% (w/v) sodium carbonate (anhydrous) in 0.1N sodium hydroxide solution.

  4 g of NaOH was dissolved in 1 L of deionised water. Then 20 g of Na_2CO_3 was dissolved in the sodium hydroxide solution.
- Lowry Reagent B = 1% (w/v) sodium potassium tartrate

  1 g of $C_4H_4KNaO_6 \cdot 4H_2O$ was dissolved in 100 mL of deionised water.
- Lowry Reagent C = 0.5% (w/v) copper sulphate solution

  500 mg of $CuSO_4 \cdot 5H_2O$ was dissolved in 100 mL deionised water.
- Lowry Reagent D = a 48/1/1 volumetric ratio of A/B/C.
- Lowry Reagent E = Folin Ciocalteu-Phenol solution
- Equal parts of 2N Folin Ciocalteu-Phenol was mixed with deionised water.
- Tris 50mM (pH 8)
- Bovine Serum Albumin(BSA)
- 1.5ml Micro-centrifuge tubes
- Micro-centrifuge
- Refrigerated Centrifuge
- Incubator
- Spectrophotometer
- Microsoft Excel
- Water Bath
- Vacuum Pump
- Eaton Press Chamber

2.11.4 Safety Issues

Standard lab protocol should be followed to maintain safety at all times

2.11.5 Procedure

1. Harvest liquid cultures by rapidly filtering culture with vacuum pump.
2. Re-suspend cells in 1ml Tris buffer in 1.5ml Eppendorf tubes
3. Transfer suspension to a pre-chilled Eaton press chamber and pressed at 250Mpa
4. Spin at 15,000 RPM for 20min at 4^{o}C
5. Discard the supernatant then re-suspend the pellet in 0.5ml of Lowry Reagent D
6. Incubate at 55^{o}C for 3h
7. Spin at 15,000RPM for 20min at RT
8. Collect supernatant and discard the pellet
9. In triplicate, add 25 µL of supernatant to 1.5ml micro-centrifuge tubes

    - At the same time, a BSA calibration curve can be generated using BSA in the concentration range of 0-5 mg/mL.
10. Add 1000 µL of Lowry Reagent D to each tube and mix by inversion
11. Incubate samples for 10mins at RT
12. Add 100 µL of Folin-Ciocalteu phenol to the tubes and vortex immediately
13. Wait for 30 min at RT
14. Run samples at 600nm and record data
15. Plot data to form a calibration curve to work out protein quantity

2.11.6 Reporting

Record data on Microsoft Excel

2.11.7 Reference documents

- Slocombe, S. P., Ross, M., Thomas, N., McNeill, S., & Stanley, M. S. (2013). A rapid and general method for measurement of protein in micro-algal biomass. *Bioresource Technology*, *129*, 51–57. https://doi.org/10.1016/j.biortech.2012.10.163

2.12 Elemental Analysis

2.12.1 Purpose

To show how to determine the total organic nitrogen and carbon content of algal biomass

2.12.2 Scope

This SOP covers the determination of total organic carbon and nitrogen from algal biomass only

2.12.3 Materials and Equipment

- Freeze-drier
- Elemental Analyser (e.g. ANCAGSL 20-20 stable isotope analyzer, PDZ Europa, Sercon Ltd.)
- Tin capsules
- Analytical balance
- L-isoleucine standards

2.12.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE

2.12.5 Procedure

1. Ensure dry weight (DW) algal biomass is the starting material by freeze-drying (or oven-drying)
2. On an analytical balance, tare a tin capsule.
3. Weigh ~2 mg of freeze-dried biomass. Record weight.
4. Fold and press capsule into a small, neat cube shape.
5. Calibrate instrument with L-isoleucine standards ranging from 5-200 µg N and 33-1320 µg C.
6. Samples can be determined using an Elemental analyser.
7. Insert blanks periodically throughout your samples, as well as before and after each sample run.

2.12.6 Reporting

Record data on Microsoft Excel

2.12.7 Reference documents

- S.P. Slocombe, M. Ross, N. Thomas, S. McNeill, M.S. Stanley, A rapid and general method for measurement of protein in micro-algal biomass, Bioresource Technol., 129 (2013) 51-57.
- S.P. Slocombe, Q. Zhang, M. Ross, A. Anderson, N.J. Thomas, Á. Lapresa, C. Rad-Menéndez, C.N. Campbell, K.D. Black, M.S. Stanley, Unlocking nature's treasure-chest: screening for oleaginous algae, Scientific Reports, 5 (2015).

2.13 Carotenoid Determination

2.13.1 Purpose

To show user how to extract carotenoid pigment and analyse the results via HPLC

2.13.2 Scope

This covers the extraction of Carotenoids and pigments only.

2.13.3 Materials and Equipment

- Ethanol
- Microalgae sample
- 500 µm glass beads
- 0.2 µM PTFE filter
- HPLC bottles
- Refrigerated Centrifuge
- Mixer-Mill
- Eclipse XDB-C8 (4.6x150mm, 3,5uM particle size) Column
- Agilent HPLC-UV-DAD series 1200
- Solvent A (70:30 methanol, 28mM aqueous TBAA pH6.5)
- ChemStation Software

2.13.4 Safety Issues

Follow standard lab safety protocol, and wear PPE

2.13.5 Procedure

- Pigment Extraction
  1. Follow cell culture protocol to prepare cells
  2. Take out a pellet of $50.1x10^6$ cells from culture
  3. Disperse pellet in 500 µL Ethanol
  4. Mix with 0.728g of 500 µM glass beads for 30min in the mixer-miller set to 30Hz
  5. Remove the beads then centrifuge the sample for 5 mins at 4500RPM at 4oC
  6. Filter supernatant with a 0.2 µM PTFE filter for HPLC analysis
- HPLC Analysis of Pigment
  1. Separate pigments on the Eclipse column using the Agilent HPLC machine
  2. Measure the elution gradient over 30 min from 95% to 5% Solvent A
  3. After this run has completed, set an isocratic flow of 5% solvent A for 10 min
  4. Measure pigment over the wavelengths 405, 436, 450 and 665nm
  5. Save the results and access via the ChemStation spectral library for analysis

2.13.6 Reporting

All reporting will be performed on the ChemStation Software

2.13.7 Reference documents

- Serive, B., Nicolau, E., Bérard, J. B., Kaas, R., Pasquet, V., Picot, L., & Cadoret, J. P. (2017). Community analysis of pigment patterns from 37 microalgae strains reveals new carotenoids and porphyrins characteristic of distinct strains and taxonomic groups. PLoS ONE (Vol. 12). https://doi.org/10.1371/journal.pone.0171872

2.14 Phycoerythrin Determination

2.14.1 Purpose

To show users how to perform Phycoerythrin extraction and quantification

2.14.2 Scope

This covers Phycoerythrin extraction and quantification only

2.14.3 Materials and Equipment

- Porcelain Pestle and Mortar
- Sand
- Centrifuge
- Refrigerator
- Purified R-Phycoerythrin (R-PE)
- 0.1M Phosphate Buffer (16.73% NaH_2PO_4 (anhydrous) and 83.27% $NaHPO_4.12H_2O$, pH 6.8)
- Dual Beam UV Visible Spectrophotometer
- 1.5 ml Micro-centrifuge tube
- Microsoft Excel

2.14.4 Safety Issues

Follow Basic lab safety protocols

2.14.5 Procedure

1. Grind 75mg of Microalgae in a 145ml Pestle and Mortar with 30mg of sand
2. Add 0.5ml 0.1M phosphate buffer and rinse into a 1.5ml Micro-centrifuge tube
3. Repeat rinsing twice making solution up to 1.5ml
4. Leave for 24hr at 4°C
5. Centrifuge samples at 17,000RPM
6. Absorbance measured at species specific value against a blank of phosphate buffer
7. Centrifuge sample at 17,000RPM at sample times 3, 5, 10, 20, 30 and 60 mins and record absorbance
8. Spin the samples down until spectrum remains unchanged, then replace supernatant with fresh Phosphate buffer
- Repeat previous centrifugation step 7. for this new buffer solution
9. Scan solutions of R-PE at different dilutions and make a standard curve to find out quantity of PE in solution.

2.14.6 Reporting

Reporting to be performed on Microsoft Excel

2.14.7 Reference documents

- Sampath-Wiley, P., & Neefus, C. D. (2007). An improved method for estimating R-phycoerythrin and R-phycocyanin contents from crude aqueous extracts of Porphyra (Bangiales, Rhodophyta). Journal of Applied Phycology, 19(2), 123–129. https://doi.org/10.1007/s10811-006-9118-7

2.15 Determination of Pigments

Please note that there are three methods described for the extraction and quantification of pigments from biomass. The optimal method will vary on a species-by-species basis. For example, pigments are readily extracted from fragile cells such as cyanobacteria using methanol. However, for more recalcitrant species, such as *Chlorella* or *Nannochloropsis*, solvents such as DMSO may be required.

2.16 Determination of Pigments 1 - DMSO

2.16.1 Purpose

To show how to determine the pigments from algal biomass

2.16.2 Scope

This SOP covers the spectroscopic determination of pigments from algal biomass only

2.16.3 Materials and Equipment

- Freeze-drier
- 2 mL Screw-cap micro-centrifuge tubes
- Vortex
- Dimethyl sulfoxide (DMSO)
- Water bath
- Bench centrifuge
- Quartz cuvette
- Spectrophotometer

2.16.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing with hot samples

2.16.5 Procedure

1. Ensure dry weight (DW) algal biomass is the starting material by freeze-drying. Ensure material is kept in the darkness during this procedure (e.g. wrap in foil).
2. Weigh 1.5 ± 0.5 mg of DW algal biomass into screw-capped micro-centrifuge tubes.
3. Add 2 mL of DMSO and tightly replace the screw-cap.
4. Vortex to ensure a uniform suspension.
5. Incubate overnight at 60°C in a covered water-bath.
6. Allow samples to cool to room temperature (in darkness) and then vortex.
7. Centrifuge for 2 min at 15,600 g.
8. Transfer supernatant to a quartz cuvette.
9. Read absorbance at 480 nm (OD480), 649 nm (OD649), and 665 nm (OD665).
10. The values obtained can be used to determine the concentration of pigments as mg L-1, using Equations 1-4:

Equation 1: Concentration of chlorophyll *a* (mg L^{-1})

$$Chl\ a\ (mg\ L^{-1}) = \frac{(12.47 \text{ x } OD_{665}) - (3.62 \text{ x } OD_{649}) \text{ x } V}{M} / 1000$$

Equation 2: Concentration of chlorophyll *b* (mg L^{-1})

$$Chl\ b\ (mg\ L^{-1}) = \frac{(25.06 \text{ x } OD_{649}) - (6.5 \text{ x } OD_{665}) \text{ x } V}{M} / 1000$$

Equation 3: Concentration of carotenoids (mg L^{-1})

$$Carotenoid\ (mg\ L^{-1}) = \frac{((1000 \text{ x } OD_{480}) - (1.29 \text{ x } Chl\ a) - (53.78 \text{ x } Chl\ b))/220 \text{ x } V}{M} / 1000$$

Equation 4: Total concentration of pigments (mg L^{-1})

$$Total\ Pigments\ (mg\ L^{-1}) = Chl\ a + Chl\ b + Carotenoids$$

Where Chl *a* and Chl *b* are chlorophyll *a* and *b*, respectively. OD_{480}, OD_{649}, and OD_{665} are the values obtained from the spectrophotomer (see 6.9), *M* is the DW biomass in mg, and V is the volume of DMSO in mL.

11. Values can be expressed as a proportion of DW (% DW) by multiplying by 100.
12. If required, incomplete extraction (cells are still pigmented), repeat steps 3 onwards. Extracts should be pooled.

2.16.6 Reporting

Record data on Microsoft Excel

2.16.7 Reference documents

- M.J. Griffiths, C. Garcin, R.P. van Hille, S.T. Harrison, Interference by pigment in the estimation of microalgal biomass concentration by optical density, J. Microbiol. Meth. 85 (2011) 119-123.

2.17 Determination of Pigments 2 – Acetone

2.17.1 Purpose

To show how to determine chlorophyll from algal biomass.

2.17.2 Scope

This SOP covers the solvent extraction and spectroscopic determination of pigments from algal biomass only

2.17.3 Materials and Equipment

- Acetone
- Freezer
- Water Bath
- 2 mm Glass Beads
- Bench centrifuge
- Thermoshaker block
- Sonicator (probe or ultrasonic bath)
- Spectrophotometer

2.17.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing solvents
- Exercise caution when operating a sonicator

2.17.5 Procedure: Acetone

- Extraction:
  1. Always be sure to protect the tubes from direct light (e.g. aluminium foil or light-protected holders).
  2. Centrifuge 0.5mL of culture at 14000RPM, discard the supernatant
  3. Freeze the pellet and defrost to room temperature in a water bath
  4. Repeat step 6.2 twice
  5. Add 0.75 mL of acetone and some 2 mm glass beads (3 to 5)
  6. Place into the thermoshaker block for 10 min at 1400 rpm at 40°C.
  7. Let it cool to room temperature (RT) (optionally in a water bath)
  8. (Proceed to sonicator [probe or ultrasonic bath] for about 2 minutes)
  9. Centrifuge and pass the supernatant to the spectrophotometer at the following wavelengths : 470 nm, 645 nm, and 662 nm.
  10. Spectrophotometric Analysis :
  11. Spectrophotometric measurements: make a 1 mL sample for the measurement in a clean bowl (white with acetone)
  12. If the measured absorption exceeds 1, dilute with acetone
  13. Analysis (Dilution factor = 3, correpsonds to the dilution of 0.5 mL of culture in 1.5 mL of acetone)

$$Chlorophyll\ A\ [\mu g.mL^{-1}] = Dilution\ factor \times (\ 11.75 * A_{662} - 2.350 * A_{645})$$

$$Chlorophyll\ B\ [\mu g.mL^{-1}] = \ Dilution\ factor \times (18.61 * A_{645} - 3.960 * A_{662})$$

$$Total\ Carotenoids\ [\mu g.mL^{-1}] = \frac{Dilution\ factor \times 1000 * A_{470} - 2.270 * Chl_a - 81.4 * Chl_b}{227}$$

2.17.6 Reporting

Record data on Microsoft Excel

2.17.7 Reference documents

- Sükran Dere, Tohit Günes, Rıdvan Sivaci; Spectrophotometric Determination of Chlorophyll - A, B and Total Carotenoid Contents of Some Algae Species Using Different Solvents. Tr. J. of Botany 22 (1998) 13-17

2.18 Determination of Pigments 3 - Methanol

2.18.1 Purpose

To show how to determine chlorophyll from algal biomass.

2.18.2 Scope

This SOP covers the solvent extraction and spectroscopic determination of pigments from algal biomass only

2.18.3 Materials and Equipment

- Methanol
- Water Bath
- Ice machine
- Bench centrifuge
- Ultrasound bath
- Freezer
- Spectrophotometer

2.18.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing solvents. Exercise caution when operating a sonicator with methanol. Methanol is a flammable solvent. Sonication may create sparks which will ignite the solvent.

2.18.5 Procedure: Methanol

1. Take 1 mL of culture (depending on its density, will be needed more or less sample volume) and put it in a 15 mL plastic vial.
2. Centrifuge at 4400 rpm for 8 minutes at 4°C
3. Add 5 mL of methanol 100% to the pellet
4. Put in an ultrasound bath to disregard the pellet with methanol
5. Incubate the cell suspension at 60°C for 15 minutes
6. Incubate the cell suspension at 0°C for 15 minutes
7. Centrifuge the suspension at 4400 rpm for 8 minutes
8. Add more methanol if the pellet is not white after centrifugation and repeat the extraction
9. Samples in MeOH should be stored in the freezer
10. Measure chlorophyll and carotenoid content in a spectrophotometer at 470 nm, 652 nm, and 665 nm in a quartz cuvette (blank is methanol).
11. Calculations
12. Use Arnon's equations to determine the chlorophyll and carotenoid content:

$$Chl_a = \left(16.72 \cdot A_{665.2} - 9.16 \cdot A_{652.4)}\right) \cdot \text{dilution factor}$$

$$Chl_b = \left(34.09 \cdot A_{652.4} - 15{,}28 \cdot A_{665.2}\right) \cdot \text{dilution factor}$$

$$Chl_{tot} = Chl_a + Chl_b$$

$$Carot_{tot} = \frac{\text{dilution factor} \cdot 1000 \cdot A_{470} - 1.63 \cdot Chl_a - 104.96 \cdot Chl_b}{221}$$

2.18.6 Reporting

Record data on Microsoft Excel

2.18.7 Reference documents

- María Cuaresma, Marcel Janssen, Carlos Vílchez, René H. Wijffels, Horizontal or vertical photobioreactors? How to improve microalgae photosynthetic efficiency, Bioresource Technology, Volume 102, Issue 8, 2011, Pages 5129-5137, ISSN 0960-8524,
- Arnon's equation based on: Liechtenthaler: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes (1987). Methods on enzymology 148: 350-382
- Temperature shock based on: Leu, K.L., Hsu, B.D.: A programmed cell disintegration of Chlorella after heat stress. (2005). Plant Science, 168: 145-152

2.19 Determination of Phycobiliproteins

2.19.1 Purpose

To show how to determine phycobiliproteins from algal biomass.

2.19.2 Scope

This SOP covers the extraction and quantification of phycobiliproteins from algal biomass.

2.19.3 Materials and Equipment

- Centrifuge
- Freezer
- Water Bath
- Enzymatic Lysis solution (3.3 g L lysozyme, 0.1 M $NaPO_4$ and 0.1 M EDTANa) (pH = 7)
- 2 mm Glass beads
- Thermoshaker block
- Refrigerator
- Spectrophotometer

2.19.4 Safety Issues

- Standard lab safety should be taken
- Wear PPE
- Care taken when dealing solvents
- Exercise caution when operating a sonicator

2.19.5 Procedure

Always protect the tubes from light with aluminum foil as the phycobiliproteins rapidly deteriorate

- Extraction:
  1. Centrifuge 1 mL of culture (with known DW concentration) at 4000 rpm for 10 minutes in a 2 mL micro-centrifuge tube, discard the supernatant.
  2. Freeze the pellet
  3. Defrost and let it return to room temperature (RT) in a water bath at RT
  4. Repeat steps 6.2 and 6.3 twice
  5. Add 1.5 of enzymatic lysis solution and some 2 mm beads (3 to 5).
  6. Digest in a thermoshaker block at 37°C for 4 hours at a high agitation rate
  7. Place the tubes in a refrigerator at 4°C for 12 hours
  8. Centrifuge and pass the supernatant to the spectrophotometer at the following wavelengths: 565 nm, 620 nm, and 650 nm.
- Analysis:
  1. Dilution factor of the samples is 3

$$RPC = R - phycocyanin\ [mg.mL^{-1}] = \ Dilution\ factor/7.38 \times (A_{620} - 0.7 \times A_{650})$$

$$APC = Allo - phycocyanin\ [mg.mL^{-1}] = \ Dilution\ factor/5.65 \times (A_{650} - 0.19 \times A_{620})$$

$$BPE = B - phycoeryhtrin\ [mg.mL^{-1}] = \frac{Dilution\ factor \times A_{565} - 2.8 \times RPC - 1.34 \times APC}{12.7}$$

2.19.6 Reporting

Record data on Microsoft Excel

2.19.7 Reference documents

- Reis et al., 1998. Production, extraction and purification of phycobiliproteins from Nostoc sp. Bioresource Technology Volume 66, Issue 3, December 1998, Pages 181-187.
- R. Bermejo Roman, J.M. Alvarez-Pez, F.G. Acien Fernandez, E. Molina Grima, Recovery of pure B-phycoerythrin from the microalga Porphyridium cruentum, J. Biotechnol. 93 (2002) 73–85.

2.20 DNA Barcoding

2.20.1 Purpose

To extract and purify DNA for barcoding purposes.

2.20.2 Scope

This SOP outlines the extraction and purification of DNA from microalgae.

2.20.3 Materials and Equipment

- Ensure all equipment used has been cleaned and sterilised.
- DNeasy Plant Mini Kit (Qiagen)
- TissueLyser
- Ice
- Liquid Nitrogen
- Bench centrifuge
- Micro-centrifuge tubes
- Glass Beads (0.25-0.5 mm).
- PCR tubes
- PCR Cycler
- Microwave
- Gel electrophoresis equipment
- UV light box
- Fluorescence camera

2.20.4 Saftey Issues

- Standard lab saftey should be followed.
- Exercise care when handling liquid nitrogen and using lab equipment.
- Be careful when handling hot samples.

2.20.5 Procedure

Part I – DNA extraction

1. Refer to pages 24-27 of the DNeasy Plant Handbook (can be downloaded from: https://www.qiagen.com/gb/resources/resourcedetail?id=95dec8a9-ec37-4457-8884-5dedd8ba9448&lang=en)
2. Prior to starting, ensure buffers are appropriately made and completely dissolved (*i.e.* AP1). Preheat a water bath or heating block to 65°C.
3. Transfer 1.5 mL of culture into a micro-centrifuge tube. Centrifuge for 10 minutes at 14,000 rpm.
4. Remove most of the supernatant, leaving behind approximately 50 µL and resuspend the pellet.

5. Add 0.25-0.5 mm sterile glass beads to just below the volume of the algal suspension.
6. Flash freeze samples in liquid nitrogen.
7. Place samples into a TissueLyser or bead beater. Ensure that the samples are balanced and properly aligned. Beat samples for 30 s at 30 MHz.
8. Repeat steps 4 and 5 a further two times.
9. Add 400 µL of Buffer AP1 and 4 µL of RNase A (100 mg/mL)(Keep RNase A stock refrigerated) and vortex vigorously.
10. Incubate for 10 minutes at 65°C. Mix the samples 2 or 3 times during this period by inverting the tube.
11. Add 130 µL of Buffer AP2 and immediately vortex.
12. Incubate on ice for 5 minutes.
13. Centrifuge for 5 minutes at 14,000 rpm.
14. Transfer supernatant to a QIAshredder mini spin column (lilac), placed into a 2 mL collection tube.
15. Centrifuge for 2 minutes at 14,000 rpm.
16. Transfer 400 µL of the flow-through fraction to a new micro-centrifuge tube. Be careful not to disturn any of the sediment at the bottom of the tube.
17. To this, add 600 µL of Buffer AP3/E and mix by pipetting.
18. Pipette 650 µL of the mixture from step 15 to a DNeasy Mini spin column housed in a 2 mL collection tube.
19. Centrifuge for 1 minute at 8000 rpm. Discard the flow through.
20. Repeat steps 16 and 17 with the remaining mixture from step 15.
21. Place the DNeasy Mini spin column to a new 2 mL collection tube. To this add 500 µL of Buffer AW.
22. Centrifuge for 1 minute at 8000 rpm, discard the flow-through.
23. Add a further 500 µL of Buffer AW to the DNeasy Mini spin column.
24. Centrifuge for 2 minutes at 14,000 rpm, discard the flow-through.
25. Transfer the DNeasy Mini spin column to a new 1.5 mL micro-centrifuge tube and pipette 100 µL of Buffer AE directly onto the membrane.
26. Incubate for 5 minutes at room temperature.
27. Centrifuge for 1 minute at 8000 rpm.
28. Repeat steps 23-25.
29. For your DNA extract, if freezing: keep at -20°C. If running PCR on the same day: store in a fridge and follow Part II.

Part II – PCR & Gel Electrophoresis

30. To PCR tubes, add either 0.5 or 2 µL of the extracted DNA.
31. To this, add 50 µL of Master Mix (Table 6), tap on bench to mix
32. Place tubes in the PCR machine and run the PCR programme (Table 7). If possible, avoid the outer spaces.
33. Use 1% Agarose gel (10g L^{-1} Agarose in 0.5% TBE Buffer {50 mL TBE Buffer + 950 mL deionised water}).

34. Melt in microwave until clear (~ 2 minutes).
35. Clean gel chamber and combs with 70% ethanol.
36. Assemble the gel chamber and pour in liquified agar. Place combs and allow to set.
37. Remove gel loading dye and smart mix (for ladder) from the freezer.
38. Once the gel is solid, cover with 0.5% TBE Buffer.
39. Onto a petri dish, aliquot 2 µL volumes of loading dye (14 per gel).
40. To one of the loading dyes, add 4 µL of the smart ladder and mix by pipetting. To the remaining loading dyes, add 4 µL of PCR product.
41. Pipette the combined 6 µL into each gel pocket, starting with the ladder.
42. Operate the gel electrophoresis equipment; 100 V for 35 minutes.
43. Depending upon the loading dye used, the gel may need to be stained (*e.g.* ethidium bromide, midori green, promega diamond).
44. Place gel onto the UV light box platform, equipped with a flourescence camera.
45. Turn on UV and acquire image of the gel.
    a. If well defined bands appear for both the 0.5 and 2 µL samples, then the PCR products are ready to be sent off for sequencing. ALternatively, the PCR products can be purified prior to sending off for sequencing (see Part III).
    b. If no bands appear, you may need to repeat extraction or perform some other troubleshooting steps.

Part III – PCR Product Purification

46. If both the 0.5 and 2 µL samples worked, combine them into a 1.5 mL micro-centrifuge tube (final volume = 90 µL). However, if only one of the PCR products, use only one (45 µL final volume).
47. To this, add 5 times the volume of PB Buffer (*i.e.* 225 µL or 450 µL) and vortex.
48. Tranfer the mixture to a QIAquick column. Centrifuge for 1 minute at 13,000 rpm.
49. Discard the flow-through and replace the column in the collect tube.
50. Add 750 µL of PE Buffer to the column. Centrifuge for 1 minute at 13,000 rpm.
51. Discard the flow-through and replace the column in the collection tube. Centrifuge for 1 minute at 13,000 rpm.
52. Place each column into a new 1.5 mL micro-centrifuge tube.
53. Add 50 µL EB Buffer and centrifuge for 1 minute at 13,000 rpm.
54. Discard column. Place sample in the fridge or make the sequence plate ready for sending away.

Table 6. Master Mix composition.

Component	**Volume**
Taq PCR Master Mix (Qiagen)	25 µL
H_2O	24 µL
Forward Primer (10 µM)	0.5 µL
Reverse Primer (10 µM)	0.5 µL

Table 7. An example of a PCR cycle programme.

Stage	Step	Temperature (°C)	Time (minutes)	No. of Cycles
1	1	95	2	1
2	1	95	1	30
2	2	55	2	
2	3	68	3	
3	1	4	Hold	

3 On-going activities

3.1 Low molecular weight terpenes analysis

The development of this SOP, currently ongoing, will be completed based on bibliographic references and work performed in the project. There are two main strategies available for sampling and quantification of light molecular weight terpenes: 1) Head space collection methods, followed by GC and 2) Solvent based extraction methods, followed by GC. Both options will be evaluated and optimized standard protocol for the Project will be set.

3.2 Strategies for genetic fingerprinting of microorganisms below the species level

The ABACUS Project involves research activities from lab to large scale where several public and proprietary microalgal and cyanobacterial organisms are considered. In order to achieve the research objectives, some of these organisms will be transferred between partners along the Project. Due to the fact that even within the same organism significant genetic differences might be found, it is required to set up a strategy for accurate identification of the microorganisms we will work with. Additionally, genetic and even phenotypic changes could be involuntary induced by means of differences in cultivation strategy or conditions. Therefore, it becomes also important to have some reliable tools to trace these potential changes over the Project. In Section 3.2 some alternatives are proposed.

Regarding distinction between cyanobacteria (prokaryotes) and eukaryotic microalgae, it can be verified on the basis of several criterions such as their selective capability to grow in some conditions (Hagemann, 2013), the cell morphology (Cassier-Chauvat and Chauvat, 2014) or spectrophotometry (Liu, 2016). But when it comes to genetic differences, PCR will be useful when addressing the presence of phycocyanin-encoding gene or the presence of cyanobacterial or algal rRNA (16S or 18S, respectively, see Section 3.2). In the case of the two cyanobacteria studied in ABACUS (*Synechocystis* PCC6803 and *Synechococcus* PCC7942), since their full genome sequence is known (http://genome.microbedb.jp/cyanobase/) it is very easy to use PCR to discriminate between *Synechocystis* PCC6803 and *Synechococcus* PCC7942 on one hand and the eukaryotic algae on the other hand.

3.2.1 Denaturing Gradient Gel Electrophoresis (DGGE) + PCR

Denaturing Gradient Gel Electrophoresis (DGGE) is a technique used to separate short- to medium-length DNA fragments based on their melting characteristics. Typically it has been used for identifying single-nucleotide polymorphisms without the need for DNA sequencing and as a

genetic fingerprinting method for complex ecosystem communities, usually in conjunction with amplification of microbial genes (16S rRNA for prokaryotes, 18S rRNA for eukaryotes). Detailed information about this technique is described in literature (Danilo, 2004) as well as examples of successful applications of it (Patel *et al*., 2015).

In order to increase the resolution of this method, and hopefully be able to detect potential differences within the same species, several primer pairs could be used when performing the PCR. Some commonly used primers depending on the type of microorganism are shown in Table .

Table 5. Examples of commonly used primers for PCR amplification

Group	Primer name	Sequence	bp	Reference
Cyanobacteria	Cya781Ra	GAC TAC TGG GGT ATC TAA TCC CAT T	25	NÜBEL ET AL 1997
	Cya781Rb	GAC TAC AGG GGT ATC TAA TCC CTT T	25	NÜBEL ET AL 1997
	Cya106F	CGG ACG GGT GAG TAA CGC GTG A	22	NÜBEL ET AL 1997
Prokatiots in general	533F	GTG CCA GCA GCC GCG GTA A	19	WEISBURG ET AL 1991
	1492R	GGT TAC CTT GTT ACG ACT T	19	LANE ET AL 1991
Eubacteria	895F	CRC CTG GGG AGT RCR G	16	HODKINSON & LUTZONI 2009
	902R	GTC AAT TCI TTT GAG TTT YAR YC	23	HODKINSON & LUTZONI 2009
Chloroficeae	ChloroF	TGG CCT ATC TTG TTG GTC TGT	21	Valiente Moro et al 2009
	ChloroR	GAA TCA ACC TGA CAA GGC AAC	21	Valiente Moro et al 2009
Baciliaroficeae	BaciF	AGA TTG CCC AGG CCT CTC G	19	Valiente Moro et al 2009
	BaciR	CCA TGC TAG TCT TAA CCA TAA AC	23	Valiente Moro et al 2009
Prasinoficeae	EUK528F	CCG CGG TAA TTC CAG CTC	18	ZHU ET AL 2005
	PRAS04R	CAAGCG TAA GCC CGC TTT	18	ZHU ET AL 2005
SSU primers	NS1F	GTA GTC ATA TGC TTG TCT C	19	White et al., 1990
	NS2R	GGC TGC TGG CAC CAG ACT TGC	21	White et al., 1990
ITS primers	ITS1	TCC GTA GGT GAA CCT GCG G	19	White et al., 1990
	ITS2	GCT GCG TTC TTC ATCG ATG C	19	White et al., 1990

3.2.2 Internal transcribed spacer (ITS) Sequencing

Internal transcribed spacer (ITS) is the spacer DNA located between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome or the corresponding transcribed region in the polycistronic rRNA precursor transcript.

For eukaryotes, we find two ITS', ITS1 is located between 18S and 5.8S rRNA genes, while ITS2 is between 5.8S and 26S (in plants, or 28S in animals) rRNA genes. In bacteria and archaea, ITS is located between the 16S and 23S rRNA genes. ITS1 in eukaryotes corresponds to the ITS in bacteria and archaea, while ITS2 originated as an insertion that interrupted the ancestral 23S rRNA gene.

Currently; the ITS region is maybe the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species, for example, to identify geographic races).

Because of its higher degree of variation than other genic regions of rDNA (small and large subunit (SSU and LSU)), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. ITS1+ITS4 primers are the most commonly used by different labs, but there are also several taxon-specific primers described that allow selective amplification of fungal sequences (Gardes and Bruns 1993, White *et al*. 1990, Martin and Rygiewicz 2005) as shown in Figure .

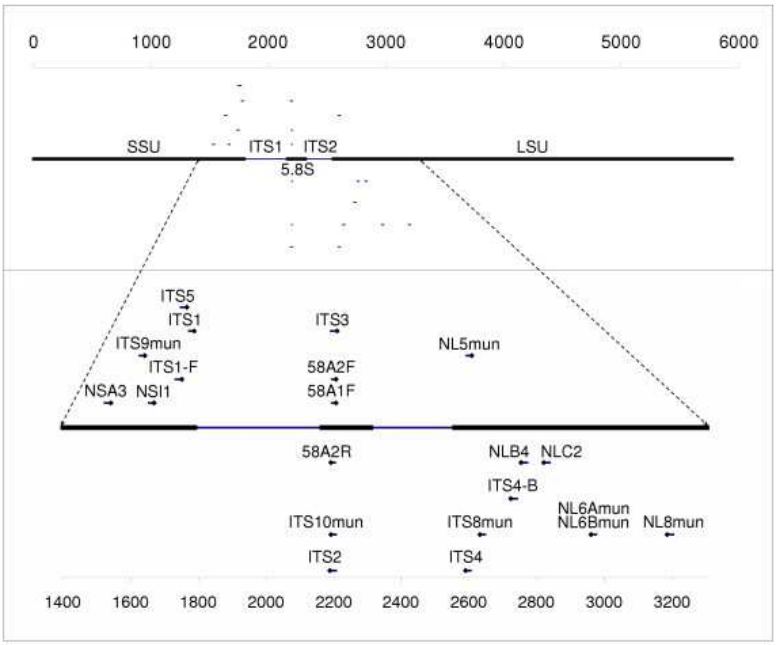

Figure 2. Diagram of primer locations in the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA (Martin and Rygiewicz 2005)

In Table the sequences of common ITS primers are described.

Table 6. ITS region primer sequences[5]

Primer name	Sequence (5'→3')	Comments	Reference
ITS1	TCCGTAGGTGAACCTGCGG		White et al, 1990
ITS2	GCTGCGTTCTTCATCGATGC	(is similar to 5.8S below)	White et al, 1990
ITS3	GCATCGATGAAGAACGCAGC	(is similar to 5.8SR below)	White et al, 1990
ITS4	TCCTCCGCTTATTGATATGC		White et al, 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	(is similar to SR6R)	White et al, 1990
ITS1-F	CTTGGTCATTTAGAGGAAGTAA		Gardes & Bruns, 1993
ITS4-B	CAGGAGACTTGTACACGGTCCAG		Gardes & Bruns, 1993
5.8S	CGCTGCGTTCTTCATCG		Vilgalys lab
5.8SR	TCGATGAAGAACGCAGCG		Vilgalys lab
SR6R	AAGWAAAAGTCGTAACAAGG		Vilgalys lab

3.2.3 q-PCR

Real-time polymerase chain reaction (Real-Time PCR), also known as quantitative polymerase chain reaction (qPCR), is based on the polymerase chain reaction (PCR) and allows monitoring the amplification of a targeted DNA molecule during the PCR. Indeed, qPCR allows a real time monitoring, it contrast with conventional PCR where we only see the end state of the amplification. Real-time PCR can be used quantitatively (quantitative real-time PCR), and semi-quantitatively, i.e. above/below a certain amount of DNA molecules (semi quantitative real-time PCR)[6] [7]

By performing Q-PCR with PCR products obtained with several primer pairs, we will obtain specific melting curves for each microorganism that we could use as a reference for identification without the need for sequencing. Figure 1 shows a tipical melting curve obtained during qPCR.

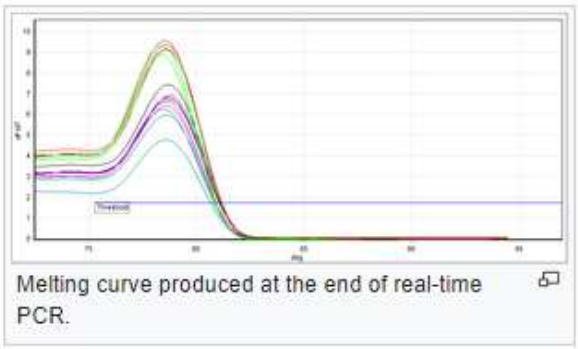

Figure 1. Melting curve obtained with RT-PCR

[5] https://sites.duke.edu/vilgalyslab/rdna_primers_for_fungi/

[6] https://en.wikipedia.org/wiki/Real-time_polymerase_chain_reaction)

[7] http://www.bio-rad.com/en-fr/applications-technologies/what-real-time-pcr-qpcr

3.2.4 Bibliography

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