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Project Officer: Ioannis Fiamekgos
Project Website: www.bowproject.eu
Project e-mail address: bow@csgi.unifi.it

D1.2: Production of microalgal-derived EVs

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Date	**Authors and Contributors**	**Comments**
04/05/2021	Thomas Conlon (ITS), Nicolas Touzet (ITS)	First draft
10/05/2021	Angela Paterna (CNR), Estella Rao (CNR)	Project designing, procedure writing
13/05/2021	Antonella Bongiovanni (CNR), Giorgia Adamo (CNR), Mauro Manno (CNR), Angela Paterna (CNR), Estella Rao (CNR)	Review and editing
18/05/2021	Thomas Conlon (ITS), Nicolas Touzet (ITS)	V2
25/05/2021	Angela Paterna (CNR), Estella Rao (CNR), Mauro Manno (CNR), Antonella Bongiovanni (CNR)	Review and editing
04/06/2021	Thomas Conlon (ITS), Nicolas Touzet (ITS)	V3
20/06/2021	Nicolas Touzet (ITS)	V4
22/06/2021	Andrea Zendrini (CSGI), Paolo Bergese (CSGI)	Review and editing
30/06/2021	Antonella Bongiovanni (CNR), Nicolas Touzet (ITS), Andrea Zendrini (CSGI), Paolo Bergese (CSGI)	Review and editing, final version

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GLOSSARY OF TERMS	
Term	**Definition**
BOW	Biogenic Organotropic Wetsuits
EVs	Extracellular Vesicles
TFF	Tangential Flow Filtration

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1. INTRODUCTION

1.1 OVERVIEW AND APPLICATION

The BOW project is developing a novel approach for the biorefinery and functionalisation of extracellular vesicles (EVs) with biogenic properties. Moreover, the project aims to impart such properties to superparamagnetic nanodevices. As such, it is essential to develop a robust, well-defined, and reproducible protocol for the production and separation of high-grade EVs with biomimetic and organotropic functions.

Microalgae constitute a reservoir of bioactive metabolites and are considered a novel source of biogenic nanovesicles (nanoalgosomes). As such, the BOW project is investigating the potential of scalable microalgal-derived EV production. BOW deliverable 1.2 outlines the standard operating procedures (SOPs) for: 1) the cultivation of microalgal strain/s suitable for the production of EVs and 2) the production and separation of microalgal EVs.

1.2 EXPERIMENTAL DESIGN

The marine chlorophyte *Tetraselmis chuii* (CCAP 66/21b) has been identified as a suitable candidate for biogenic nanoparticle production (Picciotto *et al.* 2021, Adamo *et al.* 2021). A batch cultivation mode is employed using *T. chuii* (CCAP 66/21b) to produce microalgae-conditioned media for subsequent EV separation via tangential flow filtration (TFF).

In batch cultivation, a fixed volume of culture medium is inoculated with a low density of cells (Henley 2019). The cell density increases at a quasi-constant maximum exponential specific growth rate (μmax) until processed at a predetermined end point based on maximum productivity.

For BOW D1.2., *T. chuii* (CCAP 66/21b) batch cultures are maintained at 17 ± 3°C using a light intensity of 80 ± 10 μmol photons m^{-2} s^{-1} with light:dark cycles of 14:10 hours for a 28-day cultivation period. Following batch cultivation, nanoalgosomes (EVs) are separated by size fractionation from the microalgal suspension via multi-step tangential flow filtration (TFF).

1.3 LIMITATIONS AND COMPARISON WITH OTHER METHODS

Alternative cultivation options include continuous and semi-continuous systems. In semi-continuous cultivation (i.e., fixed harvesting frequency), a fixed volume of the culture is harvested at systematic time intervals, and the culture is simultaneously replenished with an equal volume of sterile medium (Quinlan 1986, Benvenuti *et al.* 2016).

In continuous systems, the medium influent and biomass outflow are maintained in equilibrium (i.e. equal and simultaneous input and output) (Nur and Buma 2019). The biomass effluent is equal to the medium influent such that the biomass concentration remains constant (Henley 2019). Algae cultivated in continuous cultures are maintained in exponential growth, balanced at a particular physiological steady state (Henley 2019).

Continuous and semi-continuous cultivation yield a more consistent biomass composition with a higher productivity output over time compared to batch cultures (Henley 2019). However, such cultivation modes are more complex, requiring stabilisation of internal variables to reach steady-state conditions. Batch cultivation is the preferential mode of operation due to the reliability, reproducibility and flexibility of the process.

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TFF facilitates rapid and continuous processing of microalgal suspensions (e.g., > 7.5 L d^{-1}). TFF (or crossflow filtration) is a process of separation in which the feed is passed parallel to the filter (or ultrafiltration membrane) as opposed to conventional systems in which the feed passes perpendicular through the filter membrane. In conventional (terminal) filtration, the particles in the feed stream that cannot pass through the filter build up and subsequently clog the filter pores. In TFF, the crossflow current liberates material that accumulates on the filter membrane and reintroduces this material to the recycled feed stream.

Pore size and filter material determines the constituency of the permeate (i.e., the filtrate or the solution that passes through the membrane) and the retentate (i.e., the recycled portion of the feed stream that does not permeate the membrane). The term clarification is used for applications in which the product (EVs) is a constituent of the permeate. This mode of operation is used to separate nanoalgosomes exocytosed by the microalgal cells. The membrane quantitatively removes solids larger than the pores of the membrane (e.g., 0.65 µm, 0.2 µm and 500 kDa) and allows passage of soluble materials that are smaller than the membrane pores (e.g., large EVs; 0.65 µm-0.2 µm and small EVs; 0.2 µm-500 kDa). The retentate is returned to the feed reservoir, acting as a process reservoir, and more clarified product permeates through the membrane (Spectrum Laboratories, Inc., 2015). TFF is an alternative approach to differential ultracentrifugation (dUC) for EV separation. It may be viewed as a preferential mode of operation as the low shear stress facilitates the recovery of intact nanoalgosomes (Adamo *et al.* 2021). It also constitutes a higher throughput process.
The TFF protocol outlines procedures relating to the MINI-MICROKROS samplers (Repligen). As an alternative, GEHealthcare (CFP-4-E-4MA, CFP-2-E-4MA and UFP-500-E-4MA, or CFP-4-E-4X2MA, CFP-2-E-4X2MA and UFP-500-E-4X2MA) or other cartridges can be utilised, and the procedures adapted by application of the manufacturer instructions.

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2. HEALTH & SAFETY WARNING

In procedure 5.1. step 1, seawater collection should not be carried out alone.

For KrosFlo TFF operation, tubing breakage or fault may result in fluid being sprayed from the pump. Use appropriate measures to protect operator and equipment.

For KrosFlo TFF operation, turn the drive off before removing or installing tubing. Fingers or loose clothing may get caught in the drive mechanism.

For KrosFlo TFF operation, keep fingers away from the rotor while the pump is in operation. Stop the pump before loading or unloading tubing.

In procedures 5.10. and 5.11., isopropyl alcohol is toxic. Wear personal protective equipment. Avoid contact with eyes or skin.

In procedures 5.4. and 5.11., sodium hydroxide is corrosive. Wear personal protective equipment. Avoid contact with eyes or skin.

For the biohazardous liquid waste protocol, sodium hypochlorite and sodium hydroxide are toxic and corrosive. Wear personal protective equipment. Avoid contact with eyes or skin.

In 12.2, bacterial cultures may be pathogenic. Wear personal protective equipment. Use aseptic techniques. Adhere to Safety, Health and Welfare at Work (Biological Agents) Regulations 2013 (Health and Safety Authority, 2014).

3. CAUTIONS/CRITICAL STEPS

Microalgal cultivation is carried out using aseptic technique (i.e., in the sterile field of a Bunsen burner) to minimise microbial contamination.

To avoid any environmental contamination and where feasible: (a) open bioreactors only under laminar flow hood, (b) connect the bioreactors to the TFF system through clamped tubes, (c) use sterile supports and vessels (i.e., collection bottles sterilized at 180° C for 6h, if possible, or by autoclaving for 20') and (d) use PBS sterile for diafiltration.

In procedure 5.1. step 1, seawater collection should not be carried out following heavy rain as it will lower the salinity of surface water (i.e., <24-48 hours prior).

In procedure 5.1. step 4, autoclaved seawater should be allowed to cool (room temperature) prior to nutrient addition.

In procedure 5.3, no automatic stirring mechanism is applied to the bioreactors to avoid shear stress; the suspensions are homogenised manually by swirling (once weekly minimum).

Only use a mild detergent solution or 70% isopropyl alcohol (IPA) to clean the KrosFlo TFF pump head.

For KrosFlo TFF operation, ensure the correct valves are open before beginning a filtration run.

For KrosFlo TFF operation, transmembrane pressure (TMP) should not exceed 0.5 bar.

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In procedure 5.10. step 1 ensure the sensors are removed before cleaning with IPA 100%. The valves remain connected to the lines.

4. MATERIALS, EQUIPMENT AND SUPPLIES

4.1. GENERAL

- Personal protective equipment (PPE)
- Bunsen burner
- Ethanol (≥80%) (VWR, cat no. 85828.440)

4.2. MEDIUM PREPARATION

Seawater collection

- Collection tank
- Conductivity probe (Hach, sens-ion5, cat no. 51800-10)

Preliminary GF/F Filtration

- Seawater
- Membrane filtration unit
- Glass Fibre Filters (GF/F) 47 mm diameter (GE Healthcare Whatman, cat no. 1825-047)
- Autoclavable laboratory bottle (1L) (VWR, cat no. 215-1517)

Autoclaving

- Autoclave (Vapour-line lite) (VWR, cat no. 481-0846)

Filter-Sterilisation and Nutrient Addition

- Sterile Membrane filtration unit
- 0.22 µm pore size filters, 47 mm diameter, cellulose acetate (Merck Millipore, cat no. GSWP04700)
- Sterile laboratory bottle (1L) (VWR, cat no. 215-1517)
- Micropipettes and sterile tips
- Modified f/2 medium stock solutions (see Table 1. Modified f/2 Stock Solutions)

4.3. CULTURE MAINTENANCE

- *Tetraselmis chuii* (SAMS, cat no. CCAP 66/21B)
- Radiometer (Biospherical Instruments Inc. cat no. QLS2101)
- Modified f/2 medium
- Versatile environmental test chamber (Panasonic, cat no. LR-352-PE)
- Monochromatic white LED-array

4.4. BATCH CULTIVATION

Preparation of Photobioreactor

- Autoclavable sterile laboratory bottle (10 L) (VWR, cat no. 215-1520)
- Filtered-airline system
- Syringe filters, 0.22 µm, 25 mm diameter (Fisher Scientific, cat no. 15151499)

Inoculation and Stock Replenishment

- *T. chuii* inoculum (5 $mg \cdot mL^{-1}$ 0.75 L)
- Centrifuge Tube (15 mL) (Sigma-Aldrich, cat no. CLS430052)
- Centrifuge (Eppendorf, cat no. 5702)
- Analytical balance (Acculab, cat no. 210.4)

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- Modified f/2 medium containing modified f/2 stock solution volumes appropriate for a 7.5 L culture
- Monochromatic white LED-array

Preliminary Differential Centrifugation Step

- Centrifuge tube (50 mL) (Sigma-Aldrich, cat no. CLS430290)
- Centrifuge (Eppendorf, cat no. 5702)
- Ammonium formate (0.5 M) (Sigma-Aldrich, cat no. 78314)
- Analytical balance (Acculab, cat no. 210.4)
- Freeze-dryer (Scanvac, cat no. 7.001.000.060)
- Freezer (-20˚C) (Liebherr cat no. GG 4010)

Culture Growth Analysis

- Sampling tube (2 mL) (Fisher Scientific, cat no. 10048419)
- 96-well plate (SPL Life Sciences, cat no. 30096)
- Micropipettes and sterile tips
- FLUOstar OMEGA microplate reader (BMG labtech. Ortenberg, Germany)
- Lugol's iodine (Fisher Scientific, cat no. 12801823)
- Improved Neubauer haemocytometer (Marienfeld Superior, cat no. 0640010)

4.5. TFF ASSEMBLY AND CONTAMINATION TESTING PROTOCOL

- KrosFlo Research II*i* TFF System (Spectrum Laboratories, Inc.) (Repligen, Kr2i)
- Sampler 20CM .65UM MPES .75MM 3/4TCX3/4TC (Repligen, cat no. S02-E65U-07-N) or MINIKROS sampler 41.5CM .65UM MPES .75MM 3/4"TCX3/4"TC (Repligen, cat no. S04-E65U-07-N)
- MINIKROS sampler 20CM 0.2UM PES 1MM 3/4TC X 3/4TC (Repligen, cat no. S02-P20U-10-N) or MINIKROS sampler 41.5CM 0.2UM PES 1MM 3/4"TCX3/4"TC (Repligen, cat no. S04-P20-10-N)
- MINIKROS sampler 20CM 500K MPES 1MM 3/4TC X 3/4TC (Repligen, cat no. S02-500-10-N) or MINIKROS sampler 41.5CM 500 kDa MPES 1MM 3/4"TCX3/4"TC (Repligen, cat no. S04-E500-10-N)
- MICROKROS sampler 20CM 500 kDa MPES 1MM MLLXFLL (Repligen, cat no.C02-E500-10-N)
- Sterile petri dishes (Sigma-Aldrich, cat no. CLS3262)
- BD Difco™ Dehydrated Culture Media: Marine Broth 2216 (Fisher Scientific, cat no. 117535130
- MilliQ H_2O

4.6. PRE-FILTRATION RINSING PROTOCOL

- Sterile deionised water (dH_2O) (1 L)
- Drainage container

4.7. FILTRATION PROTOCOL

- Sterile (autoclaved) permeate collection tank (>7.5 L capacity)
- Sample (e.g., *T. chuii* CCAP 66/21B batch culture)
- Sterile laboratory bottle (1 L) (VWR, cat no. 215-1517)

4.8. EV-RECOVERY PROTOCOL

- Buffer (100 mL) (GF/F, autoclaved 0.2 µm filter-sterilized seawater)

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- Sterile laboratory bottle (1 L) (VWR, cat no. 215-1517)

4.9. CONCENTRATION AND DIAFILTRATION PROTOCOL

- Dulbecco's PBS w/o Calcium and Magnesium
- Sterile laboratory bottle
- Sample (collected retentate from the MicroKros 500 kDa filter module)

4.10. POST-FILTRATION RINSING PROTOCOL

- Sterile deionized water (dH_2O) (1 L)

4.11. POST-FILTRATION CLEANING AND STERILISATION PROTOCOL

- Isopropyl alcohol (IPA) (Sigma-Aldrich, cat no. W292912)
- Sterile deionized water (dH_2O) (1 L)

4.12. SENSOR-CLEANING PROTOCOL

- Isopropyl alcohol (IPA 50 %) (Sigma-Aldrich, cat no. W292912)
- Syringe without needle (10 mL) (Sigma-Aldrich, cat no. Z683582)

4.13. FILTER MODULE STORAGE PROTOCOL

- Drainage container
- 0.05 M Sodium hydroxide (NaOH) (Sigma-Aldrich, cat no. S8045)
- Parafilm (Sigma-Aldrich, cat no. P7793)

5. STEP BY STEP PROCEDURE

Sequential Steps

Microalgal cultivation

1. Medium preparation
2. Culture maintenance
3. Batch cultivation of microalgal strain/s for the production of EVs

EV separation via KrosFlo TFF

1. Assembly and Contamination Testing
2. Pre-Filtration Rinsing
3. Filtration (0.65 µm, 0.2 µm, 500 kDa)
4. EV-Recovery
5. Concentration and Diafiltration (500 kDa, MicroKros)
6. Post-Filtration Rinsing
7. Post-Filtration Cleaning and Sterilisation
8. Sensor-Cleaning
9. Filter Module Storage

Pre-Filtration: steps 1–2
Filtration: steps 3–4
Post-Filtration: steps 5–9

5.1. MEDIUM PREPARATION

Seawater collection (Timing 2h)

1 Seawater (~200 L) is collected at Rosses Point, County Sligo, Ireland (N 54° 18' 24.5"; W 8° 34' 36.8") or Riserva Naturale di Capo Gallo, Palermo, Italy (N 38°12'33.5"; E 13°17'02.4"). The seawater is collected at high tide.

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CRITICAL STEP Collection should not be carried out following heavy rain (i.e., < 24-48 hours prior).

! CAUTION Seawater collection should not be carried out alone.

2 Conductivity is measured *in-situ* using a conductivity probe (Hach, sens-ion5). Measurements are recorded on-site (e.g., typical value 40 ± 5 $\mu S \cdot cm^{-1}$).
• PAUSE POINT. Seawater is stored at room temperature up to 6 months.

Preliminary GF/F Filtration: (Timing 5·min L^{-1})

3 Seawater is filtered using Glass Fibre Filters (GF/F) (47 mm diameter, GE Healthcare Whatman) as a preliminary step to remove large impurities.

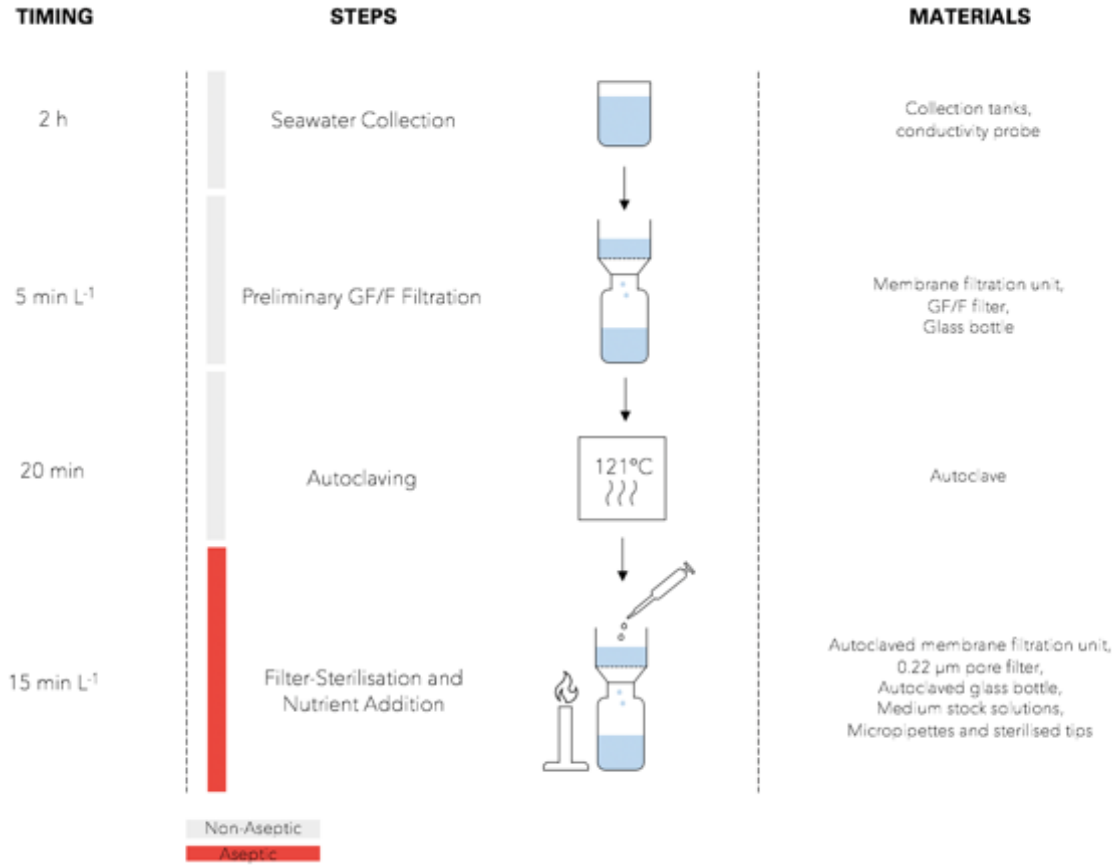

Figure 1. *Illustrative overview of medium preparation.*

Autoclaving (Timing 20 min)

4 The GFF-filtered seawater is subsequently sterilised by autoclaving at 121°C (1.2 atm) for 20 minutes.
CRITICAL STEP Autoclaved seawater is allowed cool to room temperature prior to nutrient addition.
TROUBLESHOOTING If precipitates are visible after autoclaving, GF/F filtration is repeated prior to filter-sterilisation and nutrient addition.

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Filter-Sterilisation and Nutrient Addition (Timing 15 min·L^{-1})

CRITICAL STEP Filter-sterilisation and nutrient addition are carried out using aseptic technique (i.e., in the sterile field of a Bunsen burner) to minimise microbial contamination.

5 The GFF-filtered and autoclaved seawater is filter-sterilised (0.22 µm pore size, 47 mm diameter, cellulose acetate, ~2L membrane^{-1}) into sterile glass bottles.

6 The filter-sterilised seawater is enriched with modified f/2 stock solutions (Guillard, 1975) (for appropriate volumes see Tables 1, 2 and 3).
• PAUSE POINT. medium is stored at room temperature and used within 7 days.

Compound	Stock Concentration (g L^{-1} dH_2O)	Quantity (mL L^{-1} media)	Treatment
$NaNO_3$	75	1	Autoclave
$NaH_2PO_4{\cdot}H_2O$	5	1	Autoclave
f/2 Trace metal solution	See Table 2	1	Filter-sterilise
f/2 Vitamin solution	See Table 3	0.5	Filter-sterilise

Table 1. *Modified f/2 Stock Solutions (-Si + Se) (Guillard 1975)*

• PAUSE POINT Stock solutions are stored at 4˚C up to 12 months.

Compound	Stock Concentration (g L^{-1} dH_2O)	Quantity added L^{-1} trace metal solution
$FeCl_3{\cdot}6H_2O$	-	3.15 g
$Na_2EDTA{\cdot}2H_2O$	-	4.36 g
$MnCl_2{\cdot}H_2O$	180	1 mL
$ZnSO_4{\cdot}7H_2O$	22	1 mL
$CoCl_2{\cdot}6H_2O$	10	1 mL
$CuSO_4{\cdot}5H_2O$	9.8	1 mL
$Na_2MoO_4{\cdot}2H_2O$	6.3	1 mL
$H_2SeO_3^*$	1.29	1 mL

Table 2. *Modified f/2 Trace Metal Solution (Guillard 1975) H_2SeO_3 is a modified additive to the f/2 trace metal solution.*

Compound	Stock Concentration (g L-1 dH20)	Quantity added L-1 vitamin solution
Thiamine HCL (vit B1)	-	200 mg
Biotin (vit H)	1	1 mL
Cyanocobalamin (vit B12)	1	1 mL

Table 3. *Modified f/2 Vitamin Solution (Guillard 1975)*

5.2. CULTURE MAINTENANCE

Subculturing (Timing 5 min per culture)

1 Stock cultures of *Tetraselmis chuii* (CCAP 66/21B) are sub-cultured every 20 ± 5 days using 10-25 % of the original stationary phase culture as the inoculum for the new culture. A fed-batch cultivation process is employed to increase culture volume. Cultures are maintained in sterile 100 mL autoclaved round bottom test tubes or autoclaved culture flasks (1 L or 2.5 L) depending on the culture volume (Fig.2).

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CRITICAL STEP Subculturing is carried out using aseptic technique (i.e., in the sterile field of a Bunsen burner) to minimise microbial contamination.

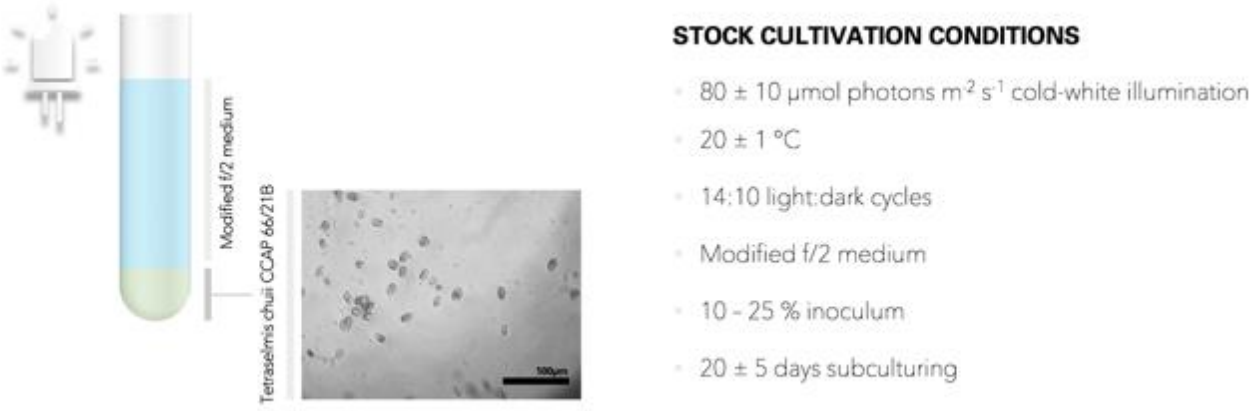

Figure 2. *Illustrative overview of microalgal culture components and stock cultivation conditions. Tetraselmis chuii (CCAP 66/21B) is obtained from the Culture Collection of Algae and Protozoa. The strain was isolated from a marine sample collected in Yorkshire, England (Butcher 1960). Stock cultures of T. chuii are preserved at 20˚C ± 1˚C in filter-sterilised (0.45 µm pore size filter) autoclaved (121˚C, 1.2 atm, 20 min) seawater enriched with modified f/2 medium using cold-white illumination. Light intensity and photoperiod are set to 80 ± 10 µmol photons m^{-2} s^{-1} (model QLS2101, Biospherical Instruments Inc.) and 14:10 light: dark cycles, respectively.*

5.3. BATCH CULTIVATION OF MICROALGAL STRAINS FOR THE PRODUCTION OF EVS

Preparation of photobioreactor (Timing 35 min per photobioreactor)

1 Glass bottles (10 L) are autoclaved and fitted with a sterile filtered airline (syringe filters, 0.22 µm, 25 mm diameter).

CRITICAL STEP Ensure to work in the sterile field of a Bunsen burner or within a Class 2 microbiological safety cabinet to minimise microbial contamination.

Inoculation and Stock Replenishment (Timing 30 min per photobioreactor)

CRITICAL STEP Ensure to work in the sterile field of a Bunsen burner or within a Class 2 microbiological safety cabinet to minimise microbial contamination.

2 Prior to inoculation, the wet biomass concentration of the culture is determined. As such, 10 mL of stock is transferred into a pre-weighed 15 mL Falcon tube. After centrifugation (2,500 *g*, 5' at room temperature), supernatants are discarded. Tubes are re-weighed, and the wet biomass concentration is calculated.

3 Following gravimetric quantification of the wet biomass concentration, the *T. chuii* stock is diluted to 5 mg mL^{-1} to a volume of 0.75 L (10 % of the final culture volume in reactor for EV production) with autoclaved filter-sterilised seawater and is used to inoculate the photobioreactor (Fig.3).

4 The remaining *T. chuii* stock (~250 mL) is transferred to a sterile 2.5 L flask and replenished with medium (1 L culture volume) for the next cycle.

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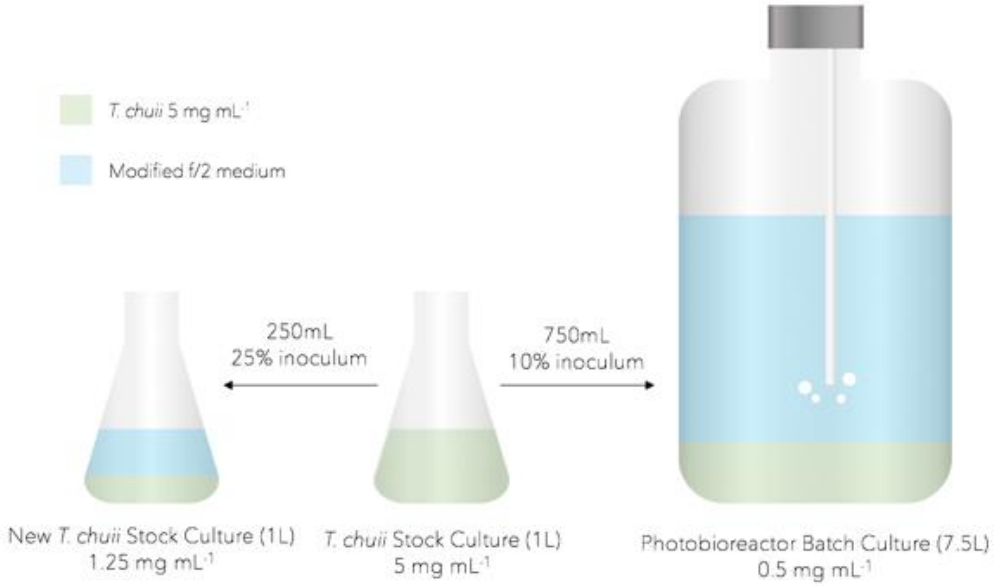

Figure 3. *Illustrative overview of T. chuii photobioreactor and T. chuii stock inoculation volumes and cultivation concentrations. T. chuii stock cultures (1 L, > 5 mg wet biomass mL^{-1}) maintained in 2.5 L flasks are used to inoculate the 10 L autoclaved glass bottles. Photobioreactors are maintained at 17 ± 3˚C using a light intensity of 80 ± 10 µmol photons m^{-2} s^{-1} with light: dark cycles of 14:10 hours for a 28-day cultivation period.*

5 The *T. chuii* inoculum (5 mg mL^{-1}, 0.75 L) and modified f/2 medium (6.75 L autoclaved filer-sterilised seawater containing modified-f/2 stock solution volumes appropriate for a 7.5 L culture) are added to the photobioreactor in the sterile field of a Bunsen burner.

NOTE The final culture volume of 7.5 L (0.5 mg mL^{-1}, ~3×10^{6} cells mL^{-1}) ensures sufficient space in the 10 L glass bottle photobioreactor to facilitate gas exchange via a filtered-airline system (syringe filters, 0.22 µm, 25 mm diameter, ~200 mL min^{-1}).

! CAUTION No automatic stirring mechanism is applied to the bioreactors to avoid shear stress; the suspensions are homogenised manually by swirling (once weekly minimum).

Preliminary Differential Centrifugation Step (Timing 50 min)

6 On day 0 and day 28 of photobioreactor batch cultivation, a 50 mL sample from the culture is subjected to a preliminary differential centrifugation step (300 *g*, 10' x 2; 2000 *g*, 10' x 2; CNR Palermo protocol).

• PAUSE POINT. The supernatant is stored at -20˚C and the wet biomass is washed with ammonium formate (1-5 mL, 0.5 M), freeze-dried, weighed, and stored (-20˚C).

Culture Growth Analysis (Timing 1 h)

7 The growth of *T. chuii* is monitored weekly (days 0, 7, 14, 21 and 28) by analysis of optical density and by microscopic cell counting. Absorbance of 300 µL aliquot samples is determined at λ600 nm using a FLUOstar OMEGA microplate reader (BMG labtech. Ortenberg, Germany). For cell counts, 30 µL of Lugol's iodine is added to a 300 µL sample of the culture, of which 100 µL is transferred to an Improved Neubauer Haemocytometer for cell numeration.

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5.4. TFF ASSEMBLY AND CONTAMINATION TESTING PROTOCOL (Timing 15 min)

1 KrosFlo TFF is switched on (the power buttons are located at the back of the instrument and on the top of the monitor).

2 The 0.05 M NaOH storing solution in the filter module is discarded and adaptors are attached to the module. Valves are attached to the retentate and permeate adapters. Sensors are attached to the retentate, permeate and feed ports. Tubing (size 16 or 25 depending on the cartridge used) is attached to the retentate, permeate and feed ports (Figs.4-5).
! CAUTION Sodium hydroxide is corrosive. Wear personal protective equipment. Avoid contact with eyes or skin.

3 Contamination testing is carried out using marine medium 2216. M2216 agar plates are inoculated with 100 µL of culture sample and permeate, incubated in the dark for up to 1 month at room temperature (20˚C ± 5˚C) and examined periodically for bacterial colony formation. Furthermore, to monitor the integrity of the cartridges, each column is flushed with 1 L of 0.2 µm filtered MilliQ H_2O for every 10-15 L of processed culture, simulating a vesicle isolation process, and both the permeate and the retentate are analysed by NTA and/or light microscopy.

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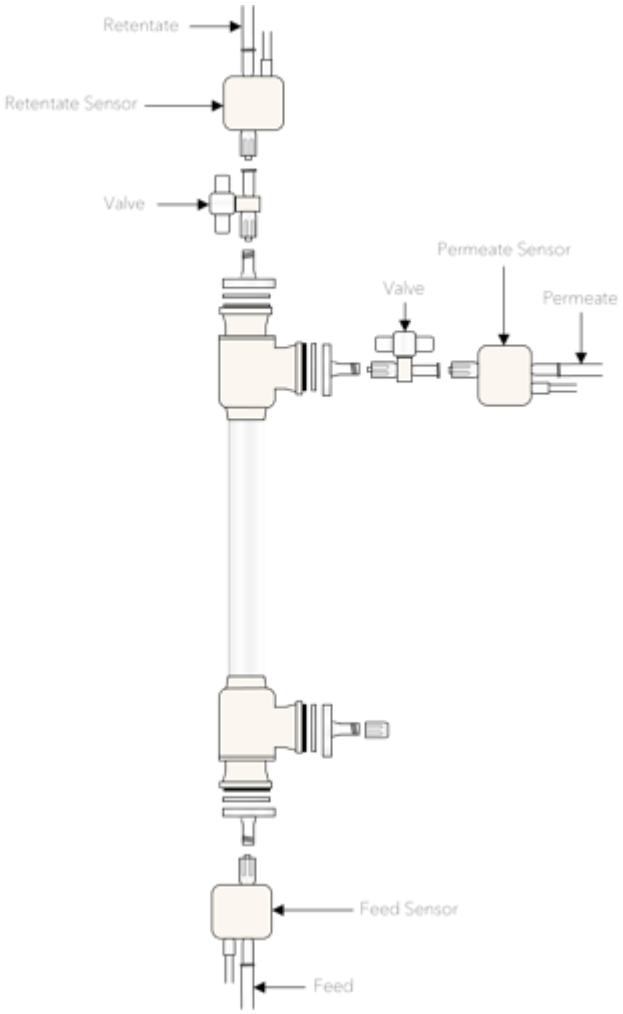

Figure 4, *Primary TFF filter module configuration.*

4 The Feed tube is stretched securely around the pump (Fig.5).

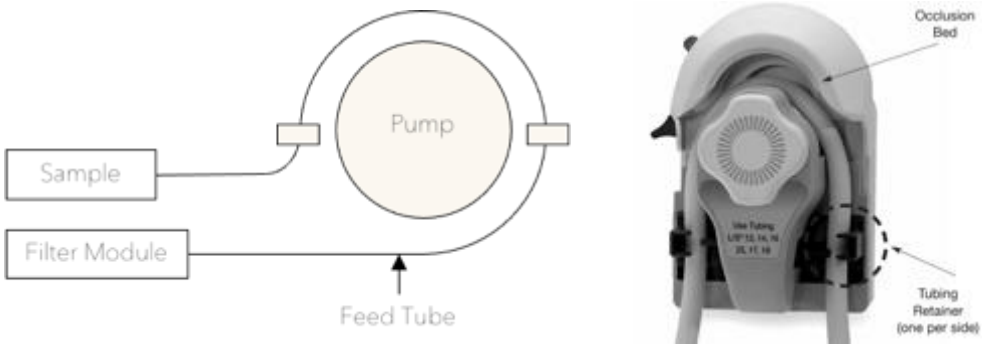

Figure 5. *Feed and pump configuration during loading. (Spectrum Laboratories, Inc., 2015)*

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5.5. PRE-FILTRATION RINSING PROTOCOL (Timing 5 min)

1 In data collection, the module serial number (#) (i.e., the pin number of the cartridge) and tubing size (dependent on cartridge used) are inserted (the keyboard option is located on the bottom right side of the display).

2 The permeate and retentate tubes are directed to waste (e.g., the drain to the sanitary sewer).

3 The pump mode is set to M mode and the maximum flow for the cartridge used is applied: 150 mL/min for MINIKROS 20 CM; 480 mL/min for MINIKROS 41.5 CM; 1000 mL/min for MICROKROS. The filter module is rinsed using 1 L sterile deionised water (dH_2O). Approximately 1 L sterile dH_2O is filtered using the preliminary filter module configuration (Fig.4) and the run is stopped.
• PAUSE POINT.
! CAUTION Ensure the valves are open before pressing start.

4 Residual dH_2O in the cartridge is drained (Fig.6). Close data collection (saving the pre-filtration data is not required).

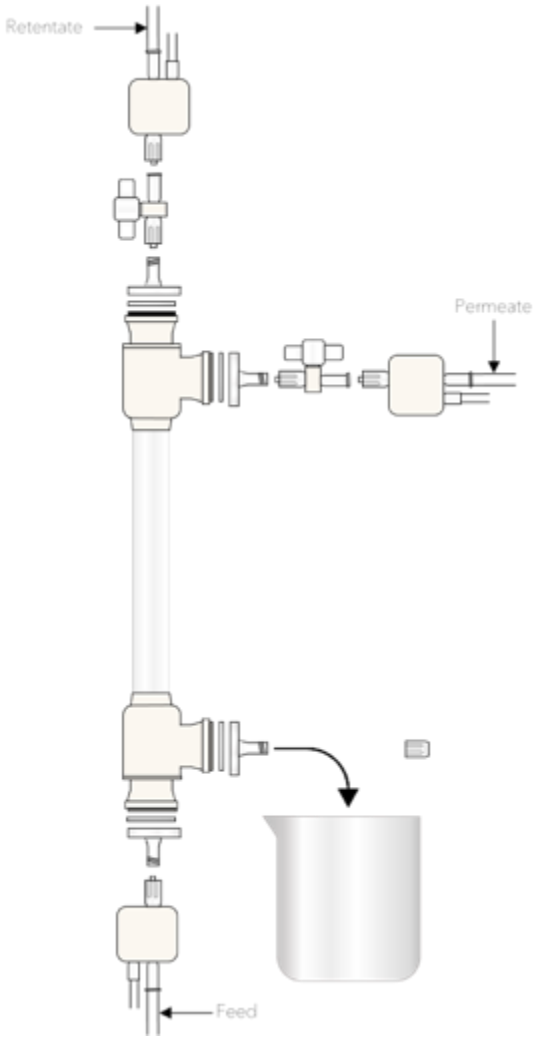

Figure 6. *Draining the filter module.*

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5.6. FILTRATION PROTOCOL (Timing >8 hours depending on the culture volume and permeate flow rate regulation)

1 The sterile (autoclaved) permeate collection tank is placed on the scale. Press T to tare the balance (hold the T-button to tare and wait for the second beep).

2 The feeding tube is inserted into the sample (e.g., photobioreactor/culture) and the feeding valve is opened. The permeate tube is inserted into the permeate collection tank. The retentate tube is inserted into the sample (Fig.7).

3 Data collection is reopened, and the filter code and tubing size (16 or 25) are entered. C pump mode and flow rate (450 mL/min) are selected.

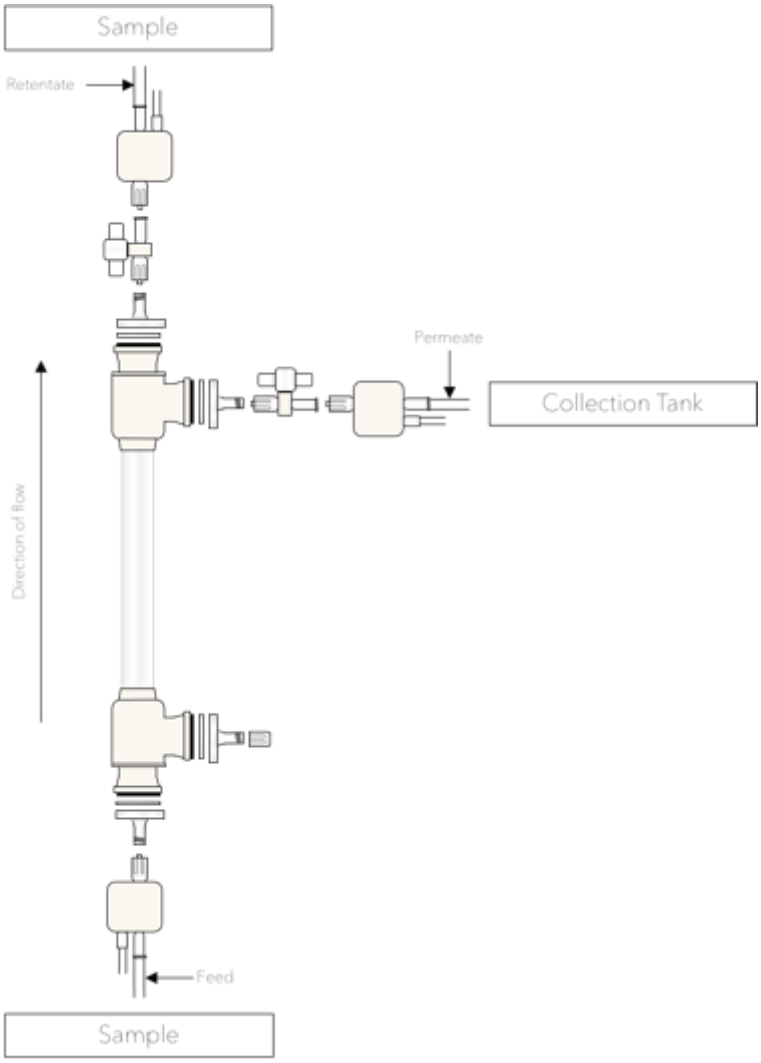

Figure 7. *Filtration protocol configuration (clockwise flow).*

4 In alarm settings, apply feed weight (e.g., 7,500 g for 7,500 mL sample), concentration factor (CF) (e.g., feed weight/100 (75)), alarms perm Hi stop (e.g., an initial stop of 6000 g for a 7,500 g sample) (mass on scale).
Note The run will stop when the perm Hi stop mass is reached.

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5 To confirm the concentration factor, select Reference Calc. and insert the starting volume and CF, then click *Calculate* and the final volume is displayed (the final retentate volume target is 100mL).
Note Insert the appropriate concentration factor if using two scales for the filtration procedure.

6 Apply transmembrane pressure (TMP) if applicable. For MINIKROSS 20CM columns, pass the retentate line through the pressure valve. For MINIKROSS 41.5CM columns, regulate the pressure manually, using a plastic cable tie. Select pressure and valve settings. Ensure pressure unit (bar) and tubing size are selected. Set valve mode: Auto, control: TMP, start position: Custom, custom start position: 25%.
MINIKROSS 20CM TMP recommendation: Apply 0.4 bar valve 1 for 0.65 µm filtration, 0.2 bar valve 1 for 500 kDa filtration and no TMP for 0.2 µm filtration.

CRITICAL STEP

7 The run is started with the retentate valve open and the permeate valve closed to ensure there are no issues e.g., leakage. Open the Excel file to view filtration parameters in real time. If no issues are encountered, the permeate valve is opened in order to obtain a permeate flow rate ranging from 6 to 60 mL/min. The permeate flow rate affects the yield of vesicles isolation: a lower permeate flow rate can lead to higher vesicle yields.

8 At <1 L retentate, the run is paused and the retentate sample is transferred to a 1 L autoclaved bottle.
• PAUSE POINT.

9 Filtration is continued until 100 mL of the retentate remains, the perm Hi stop alarm will need to be modified to reach this end point.
• PAUSE POINT.

5.7. EV-RECOVERY PROTOCOL (Timing 2 min)

1 Move the feed line into a sterile bottle containing 100 mL Buffer (GFF-filtered autoclaved 0.2 µm filter-sterilised seawater). Retentate and permeate lines are maintained in the sample and in the permeate collection tank, respectively.
! CAUTION Ensure the retentate and permeate valves are open.

2 Press start with the retentate valve and the permeate valve open. Stop the run when the 100 mL are filtered.
• PAUSE POINT.

5.8. CONCENTRATION AND DIAFILTRATION PROTOCOL (Timing 2 hours)

The filter module MICROKROS sampler 20CM 500 kDa MPES 1MM MLLXFLL (Repligen, cat no.C02-E500-10-N) allows to concentrate and diafiltrate the sample to a final volume of ~5 mL.

1 Connect the column to the system, as described in section 5.1, inserting the retentate line in the back-pressure valve.

2 Place a sterile (autoclaved) retentate collection tank on the scale. Press T to tare the balance (hold the T-button to tare and wait for the second beep). Fill the tank with the sample (collected retentate from the MiniKros 500 kDa filter module, ~100 mL).

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3 Insert the feed and the retentate tubes into the sample and the permeate tube into the permeate collection tank (waste) placed on the second scale.

4 Switch on the auxiliary pump and load the diafiltration tube into the rotor. Insert an extremity of diafiltration tube into the sample and the other one in a sterile (autoclaved) bottle containing Dulbecco's PBS w/o Calcium and magnesium.

5 Data collection is reopened, and the filter code and tubing size (16) are entered, selecting the C/D pump mode. Set the concentration factor (~6, if you want a final volume of ~15 mL), the diafiltration factor (7) and the flow rate (75 mL/min).

6 Press the start button. The run will stop when the process is completed.

5.9. POST-FILTRATION RINSING PROTOCOL (Timing 5 min)

1 The permeate and retentate lines are directed to waste (e.g., the drain to the sanitary sewer).

2 The filter module is rinsed using 1 L sterile deionised water (dH_2O). The pump mode is set to M mode and the maximum flow rate is applied based on the cartridge type (150 mL/min, 480 mL/min and 1000 mL/min).
! CAUTION Ensure the valves are open before pressing start.

3 Approximately 500 mL sterile dH_2O is filtered using the preliminary filter module configuration (Fig.4) and the run is paused.
• PAUSE POINT.

4 The feed assembly is changed to lateral inflow (Fig.8). Approximately 250 mL sterile dH_2O is filtered using this setup, and the run is paused.
• PAUSE POINT.
Note The permeate tube may be manually pinched in the lateral setup to increase the pressure, thereby providing a more vigorous rinse.

5 The setup is returned to the preliminary configuration (Fig.4) to filter the remaining 250 mL of sterile dH_2O. The run is stopped.
• PAUSE POINT.

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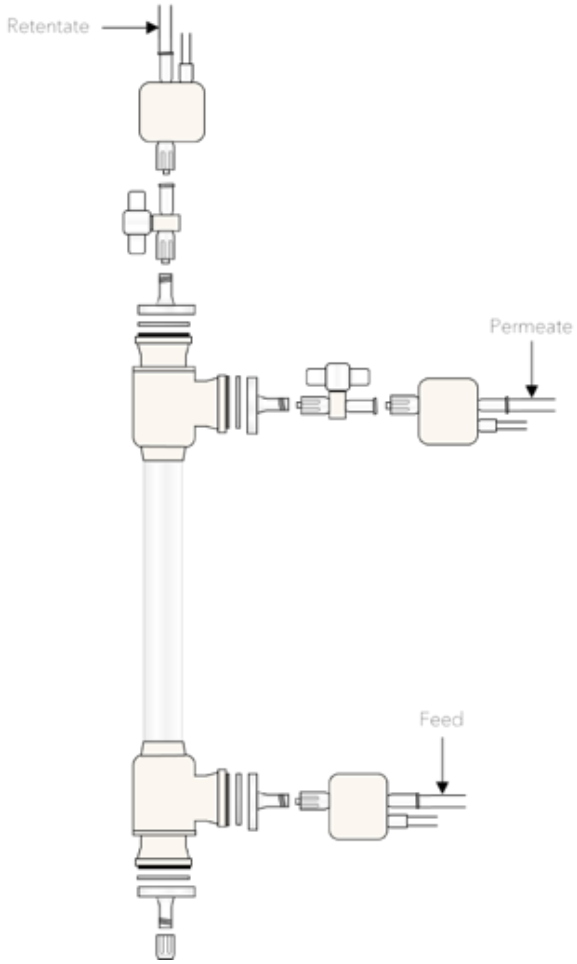

Figure 8. *Lateral inflow configuration*

5.10. POST-FILTRATION CLEANING AND STERILISATION PROTOCOL (Timing 2 hours + overnight)

CRITICAL STEP

1 Remove the sensors when cleaning with 100% IPA. Valves remain connected to the lines. ! CAUTION Isopropyl alcohol is toxic. Wear personal protective equipment. Avoid contact with eyes or skin.

2 Set the pump mode to M mode and the feed flow to 150 mL/min. The permeate and retentate tubes are directed to waste. The feed line is inserted in the IPA bottle.

3 Start the run and stop it after 200 mL of IPA pass through. Keep the cartridge wet for 1 hour.
• PAUSE POINT.

4 After 1 hour, repeat previous step.
• PAUSE POINT.

5 After 1 hour, start the run and stop it after 200 mL of IPA pass through. Keep the cartridge wet overnight.
• PAUSE POINT.

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6 Move the feed line to sterile deionised water (dH_2O). The permeate and retentate tubes are directed to waste. Maximum feed flow rate is applied.

7 Start the run and stop it after 3 L of dH_2O has passed through.
• PAUSE POINT.

5.11. SENSOR-CLEANING PROTOCOL (Timing 5 min)

1 50% isopropyl alcohol (IPA) is syringed through the sensors 3 times into the 50% IPA storage bottle for reuse.
! CAUTION Isopropyl alcohol is toxic. Wear personal protective equipment. Avoid contact with eyes or skin.

5.12. FILTER MODULE STORAGE PROTOCOL (Timing 10 min)

1 Residual dH_2O in the cartridge is drained (Fig.5).

CRITICAL STEP

2 The sensors are removed when cleaning with 0.05 M NaOH. Valves remain connected to the lines.
! CAUTION Sodium hydroxide is corrosive. Wear personal protective equipment. Avoid contact with eyes or skin.

3 The pump mode is set to M mode and a flow rate of 450 mL/min is applied. The permeate and retentate tubes are directed to waste. The feed line is inserted in the 0.05 M NaOH. The run is commenced and stopped after 300 mL of NaOH passes through.
! CAUTION Ensure the valves are open before pressing start.

4 The filter module is disassembled. The module is positioned upside down when disconnecting the feed line. The adaptors are removed, and the openings are sealed using parafilm. Store at 4˚C.

6. TIMING

Medium Preparation

Seawater collection: Timing 2 h

Preliminary GF/F filtration: Timing 5 $min \cdot L^{-1}$

Autoclaving: Timing 20 min

Filter-sterilisation and nutrient addition: Timing 15 $min \cdot L^{-1}$

Culture Maintenance

Subculturing: Timing 5 min per culture

Batch Cultivation of Microalgal Strains for the Production of EVs

Preparation of photobioreactor: Timing 35 min per photobioreactor

Inoculation and stock replenishment: Timing 30 min per photobioreactor

Preliminary differential ultracentrifugation step: Timing 50 min

Culture growth analysis: Timing 1 h

Pre-Filtration

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Assembly and Contamination Testing: Timing 15 min

Pre-Filtration Rinsing: Timing 5 min

Filtration

Filtration (0.65 µm, 0.2 µm, 500 kDa): Timing typically >8 hours (timing is dependent on the sample volume and permeate flow rate regulation to obtain maximum EV yields).

EV Recovery: Timing 10 min

Concentration and Diafiltration (500 kDa): Timing 2 h

Post-Filtration

Post-Filtration Rinsing: Timing 5 min

Post-Filtration Cleaning and Sterilisation: Timing 2 h + overnight

Sensor-Cleaning: Timing 5 min

Filter Module Storage: Timing 10 min

7. TROUBLESHOOTING

Precipitates Visible After Autoclaving Seawater

If precipitates are visible after autoclaving, GF/F filtration is repeated prior to filter-sterilisation and nutrient addition.

Compromised Culture

Stock cultures are maintained in the case of irreparable culture compromising (e.g., excessive bacterial contamination, culture senescence etc.). Serial periodic subculturing is carried out to maintain supplementary stocks of *T. chuii*. Stocks are preserved both on modified f/2 nutrient agar and in modified f/2 medium. A fed-batch cultivation process is employed to increase stock culture volumes. Such supplementary stocks are utilised to inoculate new batch cultures if complications arise during the cultivation process.

KrosFlo TFF Integrity Test (Spectrum Laboratories, Inc., 2015)

Membrane integrity tests measure the capillary pressure of wetted pores. It is recommended to integrity test Hollow Fiber membrane modules prior to use (Fig.9). Dry modules must be fully primed to ensure complete membrane wetting, otherwise a false integrity failure will be indicated due to non-wetted or incompletely wetted pores. The Integrity Test worksheet measures the inlet pressure at set time intervals and calculates the ΔP over time. If the psi/min change is not > 0.5 psi/min then the filter is fit for use. A sharp decrease in pressure over time indicates either a broken filter or incompletely wetted filter (Spectrum Laboratories, Inc., 2015).

KrosFlo TFF Leak Test (Spectrum Laboratories, Inc., 2015)

The flow path connections should first be pressure tested with air prior to filling the systems with fluids.

1. Close the pinch clamp on the permeate line. Close the backpressure control valve on the retentate line.
2. Turn on the KRII*i* and ensure that it is tared to zero.
3. Open the vent on the processing reservoir and run the pump until a feed pressure of ~5 psi is reached and maintained.
4. If there is no significant pressure drop (<0.5 psi/min), the system has no leaks and is ready for use.
5. If the pressure reduction is >0.5 psi/min, then check to make sure all the seals and fittings are secure. Adjust connections as necessary to eliminate pressure reduction.

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6. After assuring system is free of air leaks, relieve the pressure by opening the backpressure clamp.

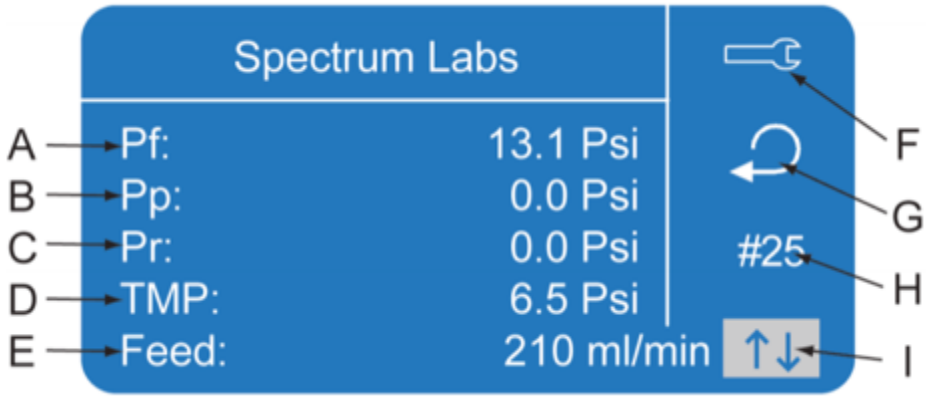

Figure 9. *Home Screen Guide (Spectrum Laboratories, Inc., 2015).*

A. Feed Pressure – Measurement from pressure sensor on the inlet or feed of the TFF filter. Units can either be psi, mbar, or bar.

B. Permeate Pressure – Measurement from pressure sensor on the permeate of the TFF filter. Units can either be psi, mbar, or bar.

C. Retentate Pressure – Measurement from pressure sensor on the outlet or retentate of the TFF filter. Units can either be psi, mbar, or bar.

D. TMP (Transmembrane Pressure) – Calculated measurement of the average of the Feed and Retentate pressures minus the Permeate pressure.

E. Feed Flow Rate – Recirculation flow rate based on calculation of pump rpm and selected tubing size. Press the ENTER key when this is highlighted to change pump speed. When the Feed Flow Rate screen is shown, the four pressures will still display on the right side of the screen. Units are ml/min.

F. Setup – Pressing the ENTER key on this icon goes to the Setup screen. The Setup screen has calibration functions, alarm settings, hardware setup and diagnostics.

G. Pump Direction – Pressing the ENTER key on this icon toggles between clockwise and counterclockwise flow direction.

H. Tubing Size – Pressing the ENTER key on this icon will select the tubing size (#13, #14, #16, #25, HP15, #17, HP24, #18, HP35, #36, HP36).

I. Cursor Idle Place Holder – Place the cursor on this icon when cursor is idle to avoid any unintended changes.

8. ANTICIPATED RESULTS

8.1. CULTIVATION OF MICROALGAL STRAIN/S SUITABLE FOR THE PRODUCTION OF EVS

The batch process should follow a typical growth profile (i.e., lag, exponential, stationary etc.) (Fig.10). The day-0 biomass range of the starting culture (0.5 mg wet biomass mL^{-1}) is 1.5 ± 0.1

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mg dry weight mL^{-1}. The day-28 biomass yield range is 4.6 ± 0.1 mg dry weight mL^{-1}. Acceptable bacterial enumeration (Day-28) is within 10^3 CFU mL^{-1}.

TROUBLESHOOTING see section 7.2. Compromised culture

8.2. SEPARATION OF MICROALGAL EVS

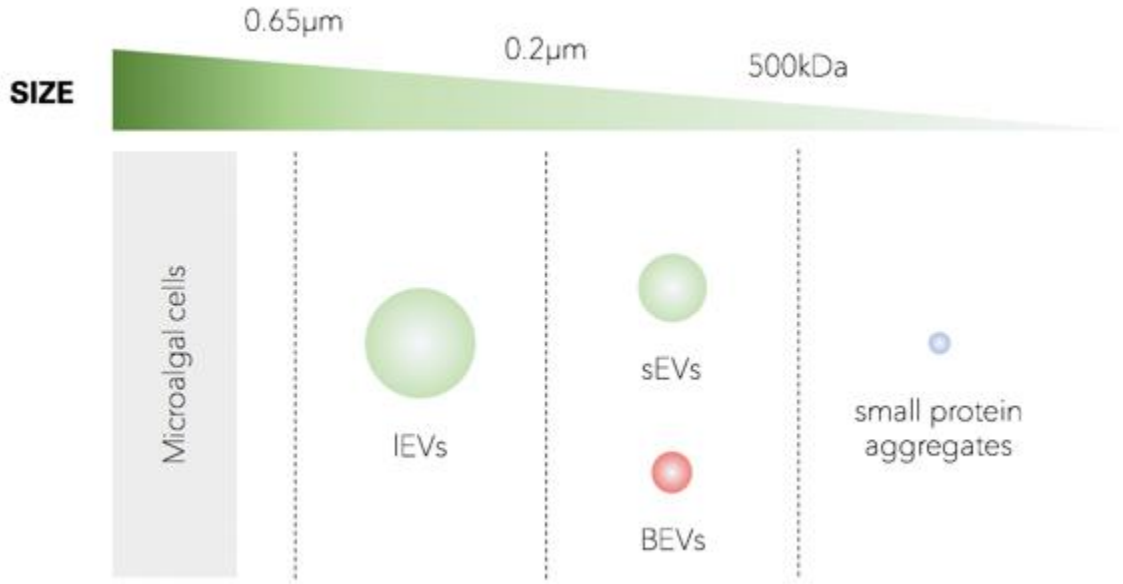

Figure 10. *Schematic size indication of large EVs (lEVs), small EVs (sEVs), bacterial EVs (BEVs) and small protein aggregates. The size ranges of components obtained by Tangential Flow Filtration (TFF).*

In KRII*i* TFF batch clarification, the membrane quantitatively removes solids larger that the pores of the membrane and allows passage of soluble materials that are smaller than the membrane pores. As such, pore size (e.g., 0.65 µm, 0.2 µm and 500 kDa) determines the constituency of the permeate and the retentate.

Product Fractions

1 >0.65 µm retentate should contain the microalgal cell harvest.

2 <0.65 µm >0.2 µm microalgae-conditioned media retentate product should contain the large EV (lEV) fraction.

3 <0.2 µm >500 kDa microalgae-conditioned media retentate product fraction should contain the small EV (EV) fraction.

4 <500 kDa permeate should contain no desired products and comprise of small protein aggregates.

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9. DATA AND RECORD MANAGEMENT

9.1. CULTIVATION OF MICROALGAL STRAIN/S SUITABLE FOR THE PRODUCTION OF EVS

All sub-culturing and batch operations are documented in designated lab books. Moreover, a data worksheet is used to document batch process parameters (Table 4).

Batch Identification	Date of inoculation	
	Operator	
	Reactor Code	
TFF	Date of TFF processing	
	Notes TFF	
Shipment and Analysis	Date of shipping/ arrival	
	Date of analysis	
Seawater	Date of seawater collection	
	Starting salinity of water (mS/cm)	
Culture Stock	Source of *T. chuii* cells	
	Date cell stock was last sub-cultured	
Inoculum	Concentration of inoculum (cells/mL)	Concentration of inoculum (wet biomass mg/mL)
	Volume of seawater in reactor (mL)	
	Volume of inoculum in reactor (mL)	
Cultivation Conditions	Light intensity level outside reactor on day 0	
	photoperiod (light: dark)	
	Temperature variance	
	Growth medium	
	Air flow	
Growth Analysis	Day 0: Absorbance (600 nm)	Day 0: cells/ mL
	Day 7: Absorbance (600 nm)	Day 7: cells/ mL
	Day 14: Absorbance (600 nm)	Day 14: cells/ mL
	Day 21: Absorbance (600 nm)	Day 21: cells/ mL
	Day 28: Absorbance (600 nm)	Day 28: cells/ mL
Dry Biomass Concentration (mg/mL)	Day 0: Dry Biomass (mg/mL)	Day 28: Dry Biomass (mg/mL)
Contamination	MacConkey	
	Sabouraud	
	Marine 2216	
	Black Membrane	

Table 4. *Data collection parameters for the batch process. Batch process parameters include batch identification, TFF operations, cultivation conditions, growth analysis and contamination testing.*

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9.2. SEPARATION OF MICROALGAL EVS

The Trial Data worksheet is used to log, calculate, and collect filtration run data (Table 5). Data for each filtration run is saved.

Column Abbreviation	Description
Time	Time (H:MM: SS)
P_{inlet}	Inlet Pressure
$P_{retentate}$	Retentate Pressure
$P_{permeate}$	Permeate Pressure
TMP	Calculated Transmembrane Pressure
DP	Pressure Drop through filter
Q_{inlet}	Feed Flow (calculated from Pump RPM and tubing size)
$Q_{permeate}$	Permeate Flow (measured and entered by user at specific times)
$Q_{retentate}$	Retentate Flow
$M_{permeate}$	Total mass of permeate (user inputted). This can be changed to total volume of permeate by clicking on the 'By Volume' button on the header.
LMH	Filtrate rate in liters per m^2 of membrane per hour
VT	Volumetric Throughput (a measure of the permeate per cm^2 of SA)
Pump	RPM of the pump
Temp	Temperature (user entered)
Conc Factor	Concentration Factor
Shear	Shear rate of the fluid through the fibers (s^{-1})

Table 5. *Trial data collection worksheet (Spectrum Laboratories, Inc., 2015).*

10. WASTE MANAGEMENT

Non-Biohazardous Waste

Liquids are not disposed in the municipal waste. Chemical waste, including stock containers with unused product are not disposed in the municipal waste. Empty or rinsed container must be free of any hazardous residue. Sharps are disposed in an appropriate, puncture-resistant container.

Biohazardous Waste

Biohazardous waste: cultures, stocks, or any other item contaminated with a biohazard or pathological waste.

Biohazardous liquid waste

An appropriate disinfectant (e.g., sodium hypochlorite or sodium hydroxide) is added to biohazardous waste.

! CAUTION Sodium hypochlorite and sodium hydroxide are toxic and corrosive. Wear personal protective equipment. Avoid contact with eyes or skin.

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Following sufficient contact time, the waste is disposed of by pouring down the drain to the sanitary sewer or in accordance with the disinfectant requirements.

Biohazardous solid waste
Biohazardous solid waste is disposed in a leak-proof autoclavable biohazard bag that is clearly labelled with the universal biohazard symbol prior to decontamination via autoclaving.

11. RELATED PROTOCOLS OR SOPS

Nanoalgosome separation by differential ultracentrifugation protocol (Picciotto *et al.*, 2021; Adamo *et al.*, 2021).

12. QUALITY CONTROL AND QUALITY ASSURANCE

12.1. CULTURE GROWTH ANALYSIS

The growth of photobioreactor cultures is monitored weekly (days 0, 7, 14, 21 and 28) by microscopic observation, optical density analysis and by microscopic cell counting. Absorbance of 300 µL samples is determined at λ600 nm using a FLUOstar OMEGA microplate reader (BMG labtech. Ortenberg, Germany).

12.2. DETECTION OF CULTURE CONTAMINATION

1 Bacterial enumeration using the Black Membrane technique, SYBR gold staining and epifluorescence microscopy.

2 Spread plate 100 µL (neat) of the culture in agar media: M2216 (marine), seawater with agar or Nutrient broth in seawater. Plates are incubated in the dark for up to 1 month at room temperature (20°C ± 5°C) and examined periodically for bacterial colony formation. If colony formation is too numerous to count, serial dilutions of the culture are conducted for subsequent spread plating. Colonies are streaked in selective media and incubated at 37°C o/n to identify potential pathogenic colony formation. Selective media used: MSA (micrococcus, detected in *T. chuii* cultures), MC (for Pseudomanidaceae), BEA, and Sabouraud (important to detect the presence of fungi).
TROUBLESHOOTING
! CAUTION Bacterial cultures may be pathogenic. Wear personal protective equipment. Follow aseptic techniques. Avoid contact with eyes or skin. Adhere to Safety, Health and Welfare at Work (Biological Agents) Regulations 2013 (Health and Safety Authority, 2014).

13. REFERENCE SECTION

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