Report on the Training School "Bioactives in *Ulva*" in Aveiro, Portugal

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Host of the training school

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Organization Committee of the Training School

Ana Moreira (University of Aveiro, PT), Margarida Martins (ALGAplus, PT), Cláudia Nunes (University of Aveiro, PT), Rosário Domingues (University of Aveiro, PT), Thomas Wichard (University of Jena, DE)

Trainers

Ana Moreira (University of Aveiro, PT), Andreia Ferreira (University of Aveiro, PT), Hermann Holbl (University of Jena, DE), Rosario Domingues (University of Aveiro, PT), Thomas Wichard (University of Jena, DE)

The COST Action SeaWheat, the University of Aveiro, and the Friedrich Schiller University Jena organized a 3-day Hands-On-Training School on "Bioactives in *Ulva*" (Working Group 4) in continuation of the previous workshop. This cooperative effort, which took place from June 26–28, 2024, introduced the participants to the fundamental sample preparation approaches in targeted analysis and omics approaches. The Department of Chemistry at the University of Aveiro kindly hosted the training school and provided the laboratories.

Rational and objectives

Ulva, commonly known as sea lettuce, is a genus of green algae found in marine and brackish environments. *Ulva* species are essential for nutritional value, environmental role, and industrial application. In all cases, chemical analysis, including the entire analytical process, is crucial to identify and quantify chemical compounds. The Training School aimed to train the participants on the fundamental principles and their differences in metabolomics, lipidomics, metabolite profiling, and targeted analysis in seminar classes and hands-on practical sessions in the chemistry laboratory (see the Protocol Bool in the Annex).

Organization

The training school was part of Working Group 4's (WG4) planned list of events for the current grant period. The local organizing committee, led by Prof. Dr. Rosário Domingues, Dr. Ana Moreira, and Dr. Andreia Ferreira, played a pivotal role in ensuring the training school's successful operation. They were supported by Dr. Thomas Wichard and Hermann Holbl (University Jena, DE).
The scientific committee (Rosário Domingues (PT), Ana Moreira (PT), and Thomas Wichard (DE) of the training school selected fifteen trainees from about 25 applicants regarding their defined criteria (such as the quality of the motivation letter, scientific background as well as the applicant's current status). The Protocol Book (Annex of the report) compiled standardized protocols used within the COST Action CA20107 and taught to participants

within the training school. News and information about the training school were posted on the SeaWheat COST Action social media channels (such as LinkedIn) and the Action websites.

Participants

The COST Action training school was well received, so 15 trainees were selected from 7 different countries (including 4 ITC countries). Nine participants were Master's or PhD students, three more advanced researchers, and two employees from the industry. All participants were younger than 40 years old and related to biological sciences (Figures 2 1,2).

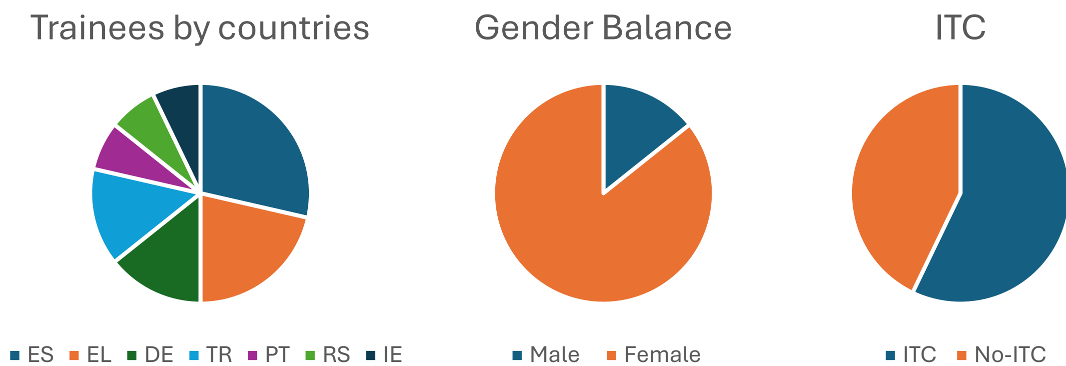


Figure 1: Distribution of the trainees by country, gender, and members of ITC countries

Figure 2. Group photo of 14 Trainees and four trainers of the Seawheat Training School outside the venue, Department of Geosciences, Aveiro, June 26-28, 2024.

Training School design and functioning

By participating in the bioactives training school at the University of Aveiro, researchers and students gained valuable insights and skills, contributing to advancements in bioactive compounds and their applications. The curriculum was split between instructional seminars in the mornings and hands-on laboratory practice in the afternoons. In addition, the participants had their first introduction to data analysis. An introduction to theory and practice was provided in the following subject areas.

- **Lipidomics** workflow and lipid extraction of dried macroalgae (*Ulva*), including an introduction to the in-house mass-spectrometric platform.
  (by Prof. Rosário Domingues and Dr. Ana Moreira**)**
- **Glycomics** workflow and workflow of neutral sugar analysis (by GC-FID).
  (by Prof. Dr. Manuel A. Coimbra and Dr. Andreia Ferreira)
- **Metabolomics** workflow, extraction, and subsequent targeted analysis of thallusin.
  (by Dr. Thomas Wichard and Herman Holbl)

A trip to the aquaculture company (AlgaPlus) enriched the Training School. The company's industrial cultivation was visited, and algae material was taken for chemical analysis in the laboratory the next day (Figure 3).

Conclusions

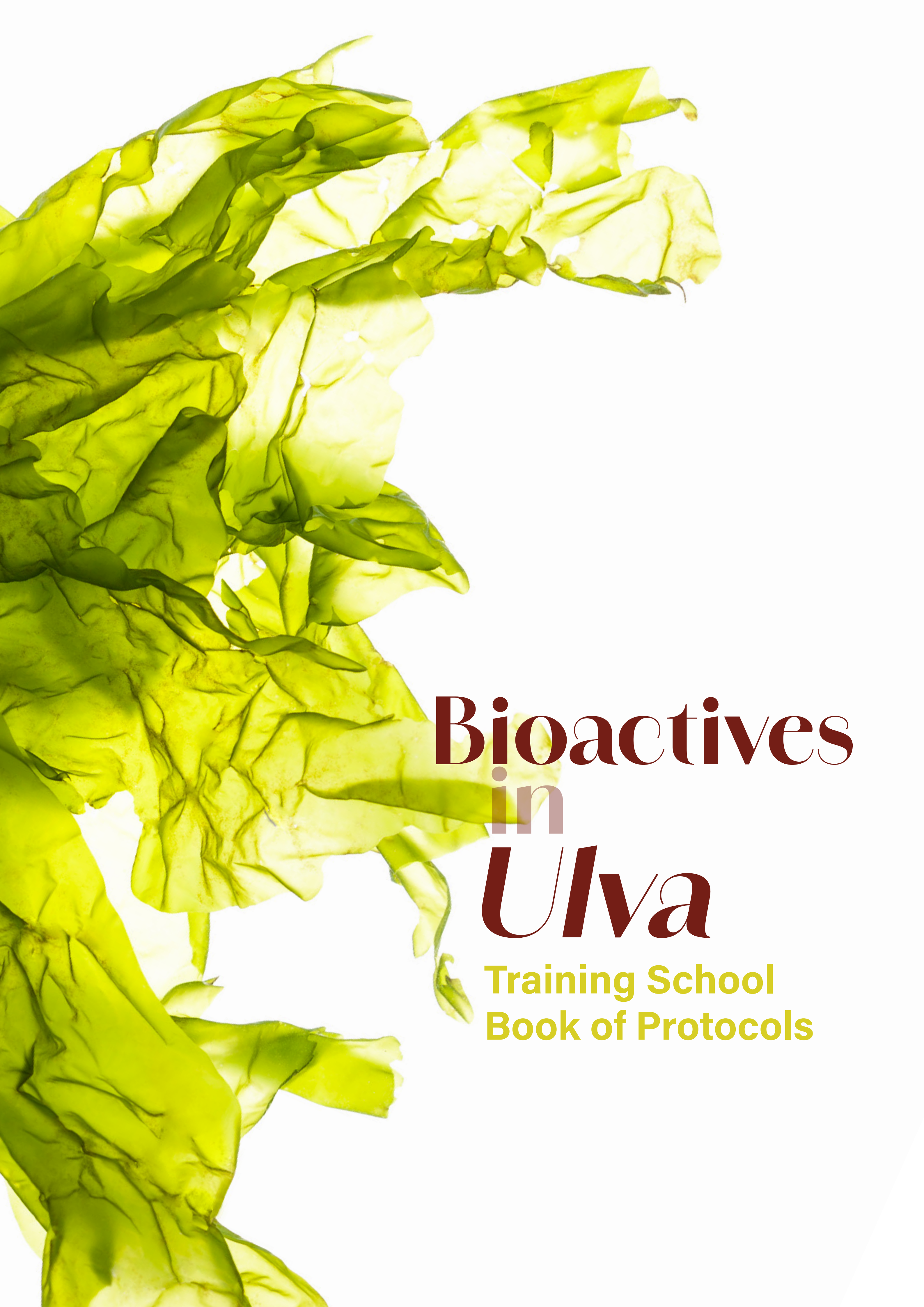
This training school focused on approaches to analyzing bioactive compounds (e.g., lipids, sugars, thallusin) in the holobiont *Ulva*, a genus of green algae commonly known as sea lettuce. The event supported, in particular, newcomers in chemical analysis with a strong background in (marine) biology. The training school taught skills from the algal collection (at the local company AlgalPlus) to sample preparation and extraction of chemicals ready for mass spectrometric analysis. The class applied protocols in lipidomics, sugar analysis, and a specific approach for thallusin quantification, which was developed within the COST Action CA20106 during the first grant period.

The organizing team resumed that the Training School promoted approaches to the next generation of scientists and enhanced their technical skills and interests in modern analytical techniques. These techniques are very relevant to entering or continuing in the research field of bioactives in macroalgae. We believe that gaining knowledge and experience in analytical chemistry can open up career opportunities in academia and industry.

Notably, the Training School participants are ready for their individual sample preparation at their home institution and for more efficient networking with chemistry laboratories within the COST Action.

Figure 3. The training school participants visited AlgaPlus (nearby Aveiro) and collected *Ulva* sp. and water samples for chemical analysis in the lab class.

Annex: The report includes the Protocol Book of the Training School "Bioactives in *Ulva*"



Bioactives in *Ulva*

Training School Book of Protocols

Bioactives in *Ulva*

Trainers

Ana Moreira (University of Aveiro, PT)
Andreia Ferreira (University of Aveiro, PT)
Hermann Holbl (University of Jena, GER)
Rosário Domingues (University of Aveiro, PT)
Thomas Wichard (University of Jena, GER)

Local host institution

University of Aveiro (PT)

	WEDNESDAY 26 JUNE	THURSDAY 27 JUNE	FRIDAY 28 JUNE
9:00 - 10:30	**Seminar** Introduction in Lipidomics Rosário Domingues (U. Aveiro)	**Seminar** Introduction in Glycomics Andreia Ferreira (U. Aveiro)	**Seminar** Introduction in Metabolomics Thomas Wichard (U. Jena)
10:30 - 11:00	COFFEE-BREAK	COFFEE-BREAK	COFFEE-BREAK
11:00 - 12:30	**Hands on practice:** Extraction of *Ulva* lipids and fatty acid analysis by GC-MS Ana Moreira (U. Aveiro)	**Hands on practice:** Sugar analysis by GC-FID Andreia Ferreira (U. Aveiro)	**Hands on practice:** Sample preparation: Solvent and solid phase extraction Hermann Holbl and Thomas Wichard (U. Jena)
12:30 - 14:00	LUNCH	LUNCH	LUNCH
14:00 - 15:30	**Hands on practice:** Data acquisition on GC-MS and LC-MS for lipidomic profiling of *Ulva* sp. Ana Moreira and Rosário Domingues (U. Aveiro)	**Visit to ALGAplus**	**Hands on practice:** Sample preparation and data processing in metabolomics Hermann Holbl and Thomas Wichard (U. Jena)
15:30 - 16:00	COFFEE-BREAK	COFFEE-BREAK	COFFEE-BREAK
16:00 - 17:30	**Hands on practice:** Data analysis of lipidomic profiling of *Ulva* sp. Ana Moreira and Rosário Domingues (U. Aveiro)	**Visit to ALGAplus**	Hands on data analysis and concluding remarks (Hermann Holbl, Thomas Wichard and Rosário Domingues)
17:30	FREE TIME	DINNER (19:30)	FREE TIME

HANDS ON LIPIDOMICS - 26TH OF JUNE 6

HANDS ON GLYCOMICS - 27TH OF JUNE 10

HANDS ON METABOLOMICS - 28TH OF JUNE - PART 1 14

HANDS ON METABOLOMICS - 28TH OF JUNE - PART 2 20

NOTES

HANDS ON LIPIDOMICS – 26TH OF JUNE

1. INTRODUCTION

Algae are aquatic organisms that contain essential nutrients and various metabolites with beneficial health properties, important for combating and preventing chronic non-communicable diseases (such as diabetes, hypertension, and cardiovascular diseases) [1,2]. Among the numerous compounds produced by algae, lipids are particularly noteworthy. Omega-3 polyunsaturated fatty acids (PUFAs) are very important for inflammation resolution, cardiovascular healthy and neurological development [3]. These omega-3 PUFAs are often found esterified to complex lipids, including neutral lipids (such as triglycerides) and polar lipids (such as phospholipids and glycolipids) [4]. These lipids have been recognized as possessing bioactive properties, namely antioxidant and anti-inflammatory effects [5]. Additionally, algae provide a sustainable alternative to traditional sources of omega-3 fatty acids, addressing issues related to overfishing and resource depletion, as well as novel compounds with unique properties that offer a vast potential for innovation in the food, nutraceutical, and cosmetic industries [1].

Each algae species has a unique and specific lipid signature [6]. The algae lipidome can also be modulated by manipulating growing conditions, which offers an opportunity to enhance the production of lipids of interest, namely polar lipids esterified to omega-3 PUFAs [7]. The characterization of the algae lipidome using a lipidomics approach with chromatography coupled to mass spectrometry (MS) is a significant opportunity for the discovery and innovation of these alternative and sustainable resources [8].

2. OVERVIEW OF THE LIPIDOMIC WORKFLOW

The lipidomics workflow (Figure 1) involves a series of critical steps for the comprehensive analysis of lipids from biological samples, in this case, *Ulva* biomass. First, sample collection and preparation are performed to ensure uniformity. Collected *Ulva* samples are washed to remove residual medium/salt. For lipid extraction, *Ulva* samples can be dried (either by freeze-drying or oven drying) or used fresh (removing as much water as possible). *Ulva* samples are ground (ideally with liquid nitrogen), followed by the lipid extraction using standard methods like Bligh and Dyer extraction method [9] to separate lipids from other molecules. Once the extracts are obtained, the next step is the chromatography separation using liquid chromatography (LC) for analysis of neutral lipids and polar lipids, or gas chromatography (GC) for analysis of fatty acids, which ensures separation of lipids based on their chemical properties and structural features. Mass spectrometry (MS) online coupled to LC analysis involves ionizing the lipids and analysing them based on their mass-to-charge ratio (*m/z*), often using tandem MS (MS/MS) to obtain structural information. Then, data processing converts raw data into lipid profiles, which are then identified and quantified using specific databases (MzMine, MsDial, LipoStar). Statistical analysis (namely using Metaboanalyst) and biological interpretation help to understand the functions and interactions of lipids, while validation confirms the findings. Finally, results are documented and shared, promoting reproducibility and further research.

The GC coupled with flame ionisation detector (FID) or MS can be used to obtain information about total fatty acids or esterified fatty acids. Both approaches require hydrolysis and derivatization to release the esterified fatty acids and form volatile derivatives as fatty acid methyl esters (FAMEs).

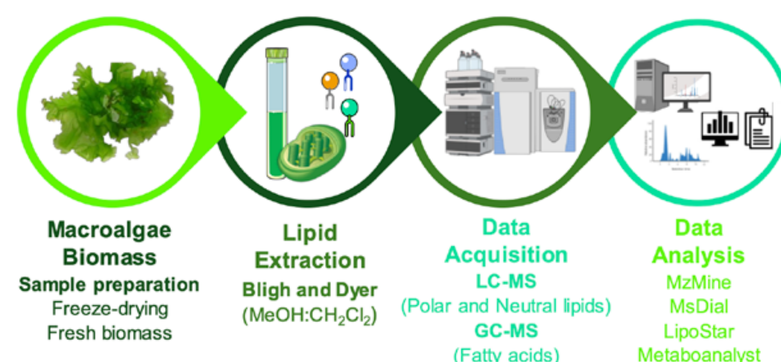


Figure 1 – Lipidomics workflow schematic applied to the analysis of macroalgae (Ulva) lipids.

BOX 1: EXTRACTION SOLVENTS

Other organic solvents can be used for the extraction of lipids, namely food grade solvents, such as ethanol or ethyl acetate. These solvents are associated with low yield of lipid extraction. However, they can be coupled with mechanical processes (e.g. ultrasounds) to increase the recovery of lipids.

3. MATERIALS

3.1. Biological samples
- Dried *Ulva* biomass

3.2. Materials
- Mortar and pestle
- Metal Spatula
- Pyrex tubes (15 mL) with (politetrafluoretileno) PTFE caps
- Glass Pasteur pipettes
- Pipette bulbs

3.3. Equipment
- Analytical Balance
- Vortex
- Orbital Shaker
- Centrifuge
- Nitrogen air stream system
- Gas Chromatography-Mass Spectrometry Instrument (Check section 4.3)
- Liquid Chromatography-Mass Spectrometry Instrument (Check section 4.4)

3.4. Chemicals
- Dichloromethane (CH_2Cl_2)
- Methanol (MeOH)
- Ultra pure water (MiliQ H_2O)
- Hexane 99%
- Nonadecanoate methyl ester in hexane solution (1 µg/mL)
- Methanolic solution of potassium hydroxide (KOH, 2 M)
- Sodium chloride (NaCl, 1 g/100 mL of MiliQ H_2O)

3.5. Consumables
- Amber vials (1.5 mL) with PTFE caps
- Glass vials (1.5 mL) for GC-MS
- Caps with septa for GC-MS
- Glass inserts (200 µL) for GC-MS
- Caps with septa for LC-MS
- Glass inserts for LC-MS

4. PROTOCOLS

(every experimental procedure should be performed using a lab coat and nitrile gloves, and use the fume hood and safety glasses whenever indicated by the trainer)

4.1. Lipid extraction

The lipid extraction protocol is based on the method developed by Bligh and Dyer [9] using a solvent proportion of 2:2:1.8 of methanol:chloroform:water. In this work, we will replace chloroform with dichloromethane for safety reasons.

1. In a glass tube weigh approximately 250 mg of macroalgae biomass.
2. **Add 2.5 mL of methanol** and **1.25 mL of dichloromethane** to the macroalgae biomass (*in the fume hood*).
3. Vortex the mixture for 1 minute.
4. Incubate for 30 minutes in an orbital shaker.
5. Add **1.25 mL of dichloromethane** (*in the fume hood*).
6. Calibrate weight of sample tubes with water tubes before centrifugation. Centrifuge the mixture at 2000 rpm for 5 min.

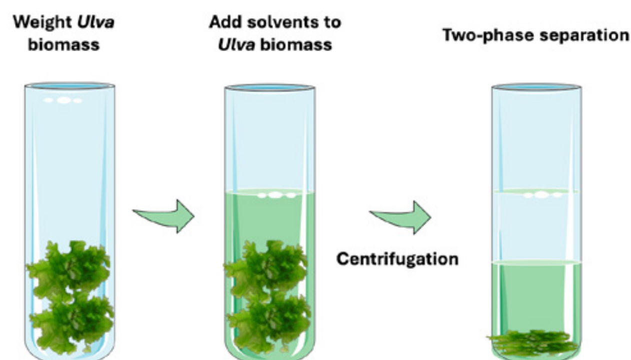


Figure 2 – Lipid extraction of macroalgae (Ulva). Aqueous and organic phase separation after centrifugation.

7. Collect the organic phase to a new glass tube and re-extract **2 more times** the biomass residue with **2.5 mL methanol and 2.5 mL of dichloromethane** (*in the fume hood*), followed by vortex for 1 minute and centrifugation at 2000 rpm for 5 minutes.
8. Dry completely the combined organic phases under a stream of nitrogen.
9. Re-dissolve the dried organic phases with **2 mL of dichloromethane and 2 mL of methanol** (*in the fume hood*). Vortex the mixture for 1 minute and add **1.8 mL of water** (*in the fume hood*), followed by vortex for 2 minutes.
10. Centrifuge the mixture at 2000 rpm for 5 minutes and collect the organic phase (lower) to a new tube (*in the fume hood*).
11. Re-extract the aqueous phase with **2 mL of dichloromethane** (*in the fume hood*), followed by vortex for 2 min and centrifugation during 5 minutes at 2000 rpm.
12. Filter the combined organic phases to a new glass tube using filtered paper in a glass funnel (*in the fume hood*). Clean the filter with **3 mL of dichloromethane** and dry the filtered organic phases under a stream of nitrogen.
13. Transfer the total lipid extract to pre-weighted dark vial (pre-heated at 105 °C and cooled in desiccator for at least 30 min) (*in the fume hood*). Dry the vial, weight it and store at -20 °C until analysis.

4.2. Quantification of lipid extract by gravimetry

1. Dark vials are pre-weighted in an analytical scale. Register each vial weight at least three times.
2. After transferring the lipid extract to the dark vial, dry it under a nitrogen stream.
3. Register the weight of each vial with the lipid extract.
4. Determine the amount of weight by using Equation 1:

Lipid extract (mg) = Vial with Lipid extract (mg) – Empty vial (mg)

4.3. Derivatization and GC-MS analysis

1. Add **30 µg of lipid extract** to a glass tube and dry under a stream of nitrogen.
2. Dissolve the lipids in **1 mL of hexane** with internal standard (nonedecanoate methyl ester, FA 19:0) (*in the fume hood*).
3. Add **200 µL** of a methanolic solution of **potassium hydroxide** (KOH, 2 M) (*in the fume hood*).
4. Vortex for 2 minutes.
5. Add **2 mL of sodium chloride** in MiliQ water (1g/100ml) (*in the fume hood*).
6. Centrifuge at 2000 rpm for 5 min.
7. Collect **600 µL of the organic phase** (**upper part** and **200 µL at a time**) (*in the fume hood*).
8. Dry the total organic phase under a nitrogen stream.
9. To inject, re-dissolve the dried organic phase (Tube 2) in **100 µL hexane**, transfer to a vial with a glass insert inside, and inject 2 µL in a GC–MS instrument (Agilent Technologies 8860 GC System, Santa Clara, CA, USA) equipped with a DB-FFAP column with the following specifications: 30 m long, 0.32 mm internal diameter, and 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA). The running program is described in *https://doi.org/10.1016/j.algal.2023.103218*.

BOX 2: FAMES STORAGE

After fatty acids derivatization, the resulted FAMEs should be analysed as soon as possible. However, these derivatives are stable for one week when stored dried at -20 °C.

BOX 3: COLUMNS FOR LC-MS

The column used in this protocol is a C18 column used for reverse-phase separation. This column allows separation of lipids according to their carbon chain length and unsaturation degree. However, other columns, such as HILIC or normal phase columns can be used for lipidomics through LC-MS. These columns allow separation of lipids according to their polar head groups.

10. After data acquisition by GC-MS, peak areas integration is performed using Agilent Mass Hunter Qualitative Analysis 10.0. The fatty acids identification is performed based on retention time and mass spectra, comparing with a standard mixture of fatty acids (Supelco 37 Component FAME Mix, Sigma-Aldrich, Saint Louis, USA) and the MS spectra from the LipidMaps database *https://lipidmaps.org/resources/lipid_web?page=ms/methesters/me-arch/index.htm*.

4.4. Analysis by LC-MS and MS/MS

1. Dissolve the lipid extract in dichloromethane to have a final concentration of 2 mg/mL of lipid extract.
2. Transfer 20 µL of lipid extract (40 µg) to an insert containing 72 µL of LC-MS running eluent and 8 µL of internal standard mixture.
3. Mix well and inject 2 µL in the LC-MS instrument by reverse phase liquid chromatography in an Ultimate 3000 Dionex (Thermo Fisher Scientific, Bremen, Germany) using an Ascentis® Express C18 column (Sigma-Aldrich®, 2.1 × 150 mm, 2.7 µm) coupled to the Q-Exactive® hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher, Scientific, Bremen, Germany). The running program is described in *https://doi.org/10.1016/j.algal.2023.103218*.
4. After data acquisition by LC-MS, lipid identification is performed based on typical retention time and MS/MS fragmentation pattern typical for each lipid class as described in [8]. Lipid structures and typical fragmentation patterns can be found in *https://lipidmaps.org/*. Lipids identification, integration and quantification can be performed with the support of bioinformatic tools, such as MsDial, MzMine and LipoStar [10,11]. Statistical analysis of the LC-MS data can be performed on Metaboanalyst [12].

5. REFERENCES

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HANDS ON GLYCOMICS – 27TH OF JUNE

1. INTRODUCTION

Marine algae, sometimes referred as seaweeds, are a valuable source of a variety of bioactive compounds, namely, sulphated polysaccharides. The richness of marine macroalgae in bioactive polysaccharides has attracted great interest for their use in functional foods, cosmetic, and pharmaceuticals applications [1]. The type and amount of polysaccharides differ according to the three major groups of marine algae, Phaeophyta (brown algae), Rhodophyta (red algae), and Chlorophyta (green algae). The major polysaccharides in brown algae include fucoidans, laminarans, and alginates. Red algae usually contain galactans and carrageenans, while ulvans and other sulphated heteropolysaccharides are reported for green algae [2]. Different growth conditions may lead to distinct yields and structural features of polysaccharides produced by marine algae, e.g., they could vary in molecular weight, abundance of monosaccharides, glycosidic linkages, degree and position of the ester sulphates [3,4]. Therefore, the characterization of the carbohydrates using a glycomics approach with gas chromatography-flame ionization detector (GC-FID) and GC-MS is crucial for unveiling their structures and for open opportunities to reveal new applications.

2. OVERVIEW OF THE GLYCOMICS WORKFLOW

The characterization of neutral and acidic carbohydrates of *Ulva* biomass involves different methodologies. Firstly, *Ulva* samples are washed to remove residual contaminants. Milled fresh or dried algae can be used for carbohydrates analysis, as described for lipidomics. Carbohydrates are very polar and hydrophilic molecules, they have lower volatility and different tautomeric forms, consequently, they are not suitable for being analysed by GC. For neutral sugar analysis, several steps are required to obtain volatile sugar derivatives suitable for GC (Figure 3). Thus, the polysaccharides of *Ulva* sample are firstly hydrolysed with sulfuric acid to cleave the glycosidic linkages and release the monosaccharides. Secondly, the aldehyde group of the monosaccharides are reduced to an alcohol with sodium borohydride, avoiding different tautomeric forms. Then, the alcohol groups are acetylated with acetic anhydride to obtain alditol acetates, which are semi-volatile and stable, ready to be analysed by GC. The neutral sugars are identified according to their retention times, determined using standards. They are quantified using an internal standard. The alditol acetates identification can also be confirmed by GC-MS based on their mass spectra.

For samples such as *Ulva*, that contain uronic acids, a colorimetric method with *m*-phenylphenol can be used for their quantification.

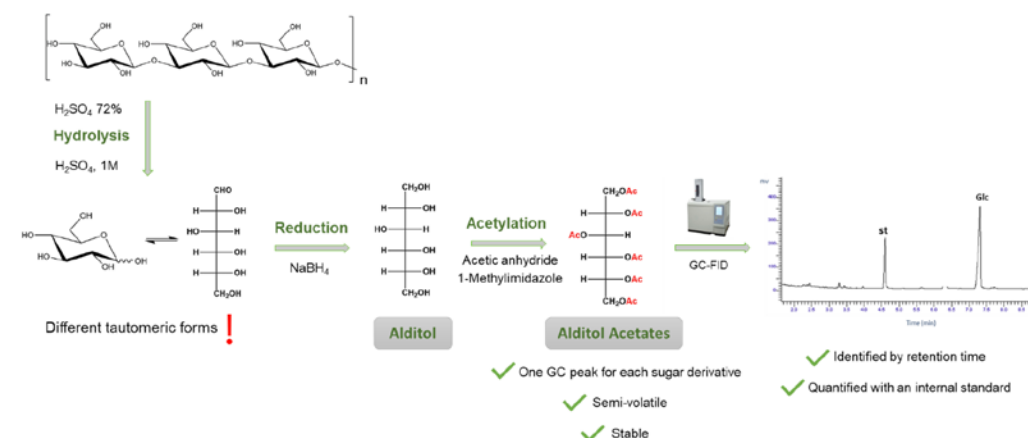

Figure 3 - Workflow of neutral sugar analysis by GC-FID using a glucan as example.

BOX 1: HYDROLYSIS CONDITIONS

Other hydrolysis conditions can be applied for neutral sugar analysis, namely using other acid solutions (e.g. trifluoroacetic acid and hydrochloric acid) and different incubation periods. These conditions should be adjusted to the sample in use, depending for instance on the algae cell wall.

3. MATERIALS

3.1. Biological samples

- Dried *Ulva* biomass

3.2. Materials

- Metal spatula
- Culture tubes
- Pyrex tubes (15 mL) with (politetrafluoretileno) PTFE caps
- Micropipettes

3.3. Equipment

- Analytical Balance
- Vortex
- Block heater
- Centrifuge
- *Speedvac*
- Gas Chromatography-Flame Ionization Detector Instrument
- Absorbance microplate reader

3.4. Chemicals

- 72% Sulfuric acid
- 2-Deoxyglucose (internal standard, 1 mg/mL)
- 25% and 3 M ammonia solution (NH_3)
- Sodium borohydride ($NaBH_4$)
- Acetic acid
- 1-Methylimidazole
- Acetic anhydride
- Dichloromethane
- Distilled water
- Anhydrous acetone
- Galacturonic acid (standard)
- Solution of sodium borate prepared in sulphuric acid
- Solution of *m*-phenylphenol prepared in NaOH

3.5. Consumables

- Glass vials (1.5 mL) for GC-FID
- Caps with septa for GC-FID
- Glass inserts (200 μL) for GC-FID
- Microplates

4. PROTOCOLS

(Every experimental procedure should be performed using a lab coat and nitrile gloves, and use the fume hood and safety glasses whenever indicated by the trainer)

4.1. Neutral sugar analysis

4.1.1. Hydrolysis [5]

1. Weight 1-2 mg of macroalgal biomass/extract in a culture tube (≈ 10 mL).
2. Add **200 μL of 72% H_2SO_4** and incubate for 3 h at room temperature (stir 2 or 3 times).
3. Add **2.2 mL of distilled water** (final concentration of 1 M H_2SO_4) and incubate at 100 °C for 2.5 h (after 1 h of hydrolysis, cool the tubes in a water bath and take out 0.5 mL of the solution for uronic acid analysis. Continue the hydrolysis for more 1.5 h). Cool down the tubes in an ice bath.

4.1.2. Reduction and acetylation [6]

1. Add **200 μL of internal standard** (2-deoxyglucose 1 mg/mL).
2. Neutralize with 200 μL of 25% NH_3 solution (check pH with indicator paper).

3. Reduce with 150 µL of 15% (m/v) $NaBH_4$ in 3 M NH_3 (150 mg $NaBH_4$ for 1 mL 3 M NH_3) and incubate for 1 h at 30 °C.

4. Cool down the tubes in an ice bath and add 2 × 50 µL of acetic acid.

5. Transfer 300 µL to SOVIREL tubes.

6. Put the tubes in an ice bath and add **450 µL of 1-methylimidazole** and **3 mL of acetic anhydride**. Mix and incubate for 30 min at 30 °C.

7. In an ice bath, add **3 mL of distillate water** and **2.5 mL of dichloromethane**. Stir very well for the extraction of the alditol acetates. Centrifuge for 30 s at 3000 rpm (for the separation of the two layers).

8. Remove the aqueous phase using the vacuum.

9. Wash the organic phase with 3 mL of distilled water, stir, centrifuge, and remove completely the aqueous phase, as described previously.

10. Transfer the organic phase to tubes specific for the *speedvac* and evaporate the dichloromethane.

11. Add **0.5 mL of anhydrous acetone** and evaporate as described previously.

4.1.3. Neutral sugars analysis by GC-FID

1. Dissolve the alditol acetates in 50 µL of anhydrous acetone.

2. Inject 2 µL in the GC-FID with a capillary column DB-225 (30 m length, 0.25 mm internal diameter, 0.15 µm film thickness). The running program was the same, as previously described in *https://doi.org/10.1016/j.algal.2022.102756*.

4.2. Uronic acids analysis by colorimetry

4.2.1. Hydrolysis

1. Weight 1-2 mg of sample in a tube (≈ 10 mL).

2. Add 200 µL of 72% H_2SO_4 and incubate for 3 h at room temperature (stir 2 or 3 times).

3. Add 2.2 mL of distilled water and incubate at 100 °C for 1 h.

4. Cool the tubes in a water bath and take out 0.5 mL of the solution for uronic acid analysis (this part of the procedure is equal to the first part of the neutral sugar analysis).

5. Add 2 or 3 mL of distilled water, depending on the amount of uronic acids in the sample

4.2.2. Preparation of the standards for the calibration curve

1. Make a solution of galacturonic acid (200 µg/mL).

2. Prepare the standards (0-100 µg/mL) of GalA

Standard	Concentration (µg/mL)	Volume H_2O (µL)	Volume GalA (µL)
P0	0	500	0
P1	10	475	25
P2	20	450	50
P3	40	400	100
P4	60	350	150
P5	80	300	200
P6	100	250	250

4.2.3. Preparation of the standards/samples for analysis

1. Arrange 3 tubes for each sample/standard (1 blank and 2 replica) with 0.1 mL of sample/standard.

2. Put the tubes in an ice bath and add **1 mL of 200 mM sodium borate** in sulphuric acid. Stir very well.

3. Put the tubes in a water bath at 100 °C for 10 min. Cool down the tubes in an ice bath for 5 min.

4. Add **20 µL of MFF** (*m*-phenylphenol 0.15% w/v in 0.5% w/v of NaOH) to 2 of the 3 tubes of each sample and standard.

5. Stir well, transfer 300 µL for the microplates, and read the absorbance at 520 nm.

BOX 2: COLORIMETRIC METHOD

The application of this colorimetric method for analysis of uronic acids only allows quantification of total content of uronic acids. The detailed composition in uronic acids can be achieved using a high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

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HANDS ON METABOLOMICS – 28TH OF JUNE - PART 1

Comparative metabolic profiling of the endo- and exo-metabolome of *Ulva mutabilis*

1. INTRODUCTION

Ulva cultures without associated bacteria develop very slowly and do not form the expected morphology known from nature; instead, they form a mass of undifferentiated cells (callus, the right picture above). Two specific types of bacteria, *Roseovarius* sp. and *Maribacter* sp., are associated with *Ulva* and influence the development of the alga by releasing morphogenetic compounds (= morphogens). *Roseovarius* sp. induces the blade cell division to form the typical thallus, acting similarly to the plant hormone cytokine. *Maribacter* sp. triggers the cell wall formation and the differentiation of basal cells into a rhizoid, which enables the fixation of the alga to the substratum [1,2]. The combination of the two bacteria harboring different functional traits recovers *Ulva*'s morphogenesis completely. The culture supernatant can also replace the bacteria containing the algal growth- and morphogenesis-promoting factors (AGMPFs). For *Maribacter* sp., the AGMPF (−)-thallusin responsible for rhizoid and cell wall formation has been elucidated [3].

Figure 4 - Germlings of Ulva mutabilis with associated bacteria (left, 1 mm) and under axenic conditions (right, 100 μm).

The following protocols describe the extraction, sample preparation and derivatization of primary metabolites of marine macroalgae for the analysis by liquid chromatography (LC-MS) and gas chromatography (GC-MS) coupled to a mass spectrometer (ThemoScientific Orbitrap) [4,5]. The practical part of this training school will focus on extracting endo- and exometabolites of *Ulva* as well as on thallusin released by the associated bacteria into the seawater sampled at ALGAplus (Aveiro, Portugal).

Sample example: Due to time and equipment constraints, the LC-MS results for the data analysis was derived from axenic *Ulva mutabilis calli* incubated with and without AGMPFs for two weeks. The investigation will identify metabolites unique to the two morphotypes, with the aim to identify metabolic pathways that are up- or down-regulated.

2. WORKFLOW

2.1. Experimental Design

- **Objective Setting:** Define the purpose of the study (e.g., discovery, understanding metabolic pathways, etc.).
- **Sample Selection:** Choose appropriate biological samples (e.g., tissue, cell cultures, water samples) considering the study's objectives.
- **Controls and Replicates:** Ensure proper controls and biological/technical replicates are included to account for variability.

2.2. Sample Collection and Preparation

- **Collection:** Collect samples using standardized protocols to minimize variability and degradation.
- **Storage:** Store samples appropriately (e.g., at -80 °C) to preserve metabolite integrity.
- **Preparation:** Prepare samples for analysis, which may involve steps like centrifugation, filtration, extraction (e.g., liquid-liquid extraction, solid-phase extraction), and derivatization (if required).

2.3. Metabolite Extraction

- **Extraction Methods:** Use suitable extraction methods based on the nature of metabolites (e.g., polar vs. non-polar metabolites).
- **Quenching:** Rapidly stop metabolic processes in cells, tissues, media to preserve the current metabolic state.

2.4. Analytical Techniques

- **Chromatography:** Use techniques like gas chromatography (GC) or liquid chromatography (LC) to separate metabolites.
- **Mass Spectrometry (MS):** Utilize MS for metabolite identification and quantification after separation (GC-MS, LC-MS).
- **Nuclear Magnetic Resonance (NMR) Spectroscopy:** Another method for metabolite identification and quantification, useful for detecting a wide range of metabolites without prior separation.

2.5. Data Acquisition

- **Instrument Calibration:** Ensure instruments are calibrated and performance-checked for accurate and precise measurements.
- **Data Collection:** Collect data using software associated with the analytical instruments.
- **Quality Control:** Incorporate quality control samples to monitor the analytical performance.

2.6. Data Processing

- **Preprocessing:** Peak detection, deconvolution, alignment, and normalization are necessary (e.g., XCMS).
- **Annotation:** Match detected peaks with known metabolite databases (e.g., KEGG).

2.7. Data Analysis

- **Statistical Analysis:** Use statistical methods to identify significant changes in metabolite levels (e.g., PCA, PLS-DA, t-tests, ANOVA) using, e.g., Metaboanalyst.
- **Bioinformatics Tools:** Use tools for pathway analysis, network analysis, and functional annotation to interpret the data in a biological context.

2.8. Interpretation and Validation

- **Biological Interpretation:** Link changes in metabolite levels to biological processes.
- **Validation:** Validate findings using independent samples and possibly other analytical techniques.

2.9. Reporting and Visualization

- **Data Visualization:** Use visualization tools to present results (e.g., heatmaps, metabolic pathway maps).
- **Reporting:** Write comprehensive manuscripts detailing the findings including the applied methodologies, and interpretations.

2.10. Data Sharing

- **Repositories:** Deposit raw and processed data in public repositories for reproducibility and further research by the scientific community.

2.11. General security remarks

- **Protect yourself**

The solvents and reagents used are highly toxic. Thus, wear a lab coat, safety glasses, and appropriate gloves, and work under a chemical fume hood. Read the MSDS of chloroform, pyridine, MSTFA, methoxyamine hydrochloride, Iodomethane, DMSO and potassium hydroxide. Follow the recommendations for correct disposal.

- **Protect your samples**

Your samples are complex enough. Do not increase complexity by adding, e.g., your own fatty acid profile or plasticizers. Try to use Teflon whenever possible. For the same reason, always analyse several blank samples through the extraction and/or derivatization procedure.

- **Avoid H_2O and O_2**

Ensure all solvents (pyridine), reagents (MSTFA), and your samples are completely dry before starting derivatization. This is especially challenging for saltwater samples. Solvents should be stored over a molecular sieve (pyridine: 4 Å) and under an argon atmosphere. MSTFA is very hygroscopic and thus stored in a vial closed by a septum. Re-cap your sample whenever possible to avoid air exposure.

3. MATERIALS

3.1. Biological material

Ulva sample (20 mg dry weight or ~200 mg fresh weight) (*n* = 3-8) from different treatments or under different conditions

3.2. Equipment

- Eppendorf pipettes (1000 µL, 200 µL, 10 µL; recently checked and calibrated).
- Glass syringes (100 µL, Hamilton).
- Vortexer (VortexGenie 2, Scientific Industries).

- Tissue Lyzer II (QIAGEN, max. speed: 30 frequencies s^{-1}) or mortar and pestle.
- Microcentrifuge (1-16K, Sigma).
- Automated Evaporation System (TurboVap® LV, Biotage) or any desiccator with vacuum pump.
- Heating block (SBH200D, Stuart).
- Manifold (compatible with luer lock, e.g., Visiprep, Supelco)

3.3. Chemicals

- Liquid nitrogen.
- Methanol (Chromasolv® Plus, Sigma-Aldrich).
- Ethanol (LiChrosolv®, Merck, filtered through 0.2 µm)
- Chloroform (HiPerSolv, VWR, filtered through 0.2 µm, packed under N_2, stabilized with 0.6% ethanol).
- Ultra-pure water
- Extraction mix: Methanol: Ethanol: Chloroform, 2:6:2 (v:v:v), daily prepared, pre-cooled to -20 °C
- (**Box 1**).
- ^{13}C-Sorbitol (4 mM in water, > 99%, Sigma-Aldrich).
- Pyridine (Chromasolv® Plus, Sigma-Aldrich, stored over 4 Å molecular sieve under argon).
- Retention index (RI) mix: C7-C40, 100 µg/ml in hexane (diluted 1:10 from the 1 mg/mL standard from Sigma-Aldrich). (all > 99%, Sigma-Aldrich).
- *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA, in 1 mL vials, Macherey-Nagel).
- Methoxyamine hydrochloride (Sigma-Aldrich).

3.4. Consumables: wear gloves

- Metal beads (ø 3 mm or ø 1.5 mm, stainless steel) or mortar and pestle
- Pipette tips (1000 µL, 200 µL, 10 µL, Eppendorf).
- Centrifuge tube (1.5 mL/ 2 mL, PCR clean grade or higher, Eppendorf).
- Glass screw neck vials (N9, 1.5 mL, Macherey-Nagel).
- Glass inserts for N9 vials and springs (200 µL, wide opening, Macherey-Nagel).
- PP screw caps for N9 vials (Macherey-Nagel).
- Tough Tag labels for microcentrifuge tubes and vials
- Chromabond Easy (Marcherey-Nagel) or Oasis HLB (Waters) SPE cartridges
- GF/F filters (47 mm, 0.7 µm, Whatman)
- Syringe (5 mL)

4. PROTOCOL

The following protocol describes the all steps from sampling to LC-MS and GC-MS based analysis. Preceding steps, such as the design of the experimental set-up or subsequent data analysis, are not the focus of this protocol. In general, setting up at least four biological replicates is recommended. Further, sampling blanks that should undergo identical treatment as the biological samples are essential for the later identification of contaminants.

4.1. Sampling and metabolic quenching

As metabolites underlie diurnal fluctuations, the sampling time should be identical for all replicates. Until metabolic "quenching" (arresting the metabolic activity) occurs, all steps must be conducted as rapidly as possible to prevent metabolic alterations.

1. Collect *Ulva* (more than 200 mg fresh weight per sample) and medium or seawater (1 L) separately.
2. Wash the algae three times with autoclaved ultra-pure water.
3. Collect about 200 mg fresh weight of a specific tissue (cut with a scalpel) in a 2 mL centrifuge tube and immediately freeze in liquid nitrogen (**Box 2**).

This is the chance to normalize all the samples by cell number or biomass. The best way to have a quantitative comparison or differential screening is to start the measurements with the same biomass among samples. The differences between treatments are often found just in the differential concentration of a specific metabolite.

4. Remove the remaining water by lyophilizing at 0.001 bar at -50 °C until completely dry. If no lyophilizer is available, the samples can be used without freeze-drying. Weigh-out is advised after this step in pre-weighed microcentrifuge tubes.

*Afterward, you can store your samples at -20 °C for short-term storage and -80 °C for extended periods (**Box 3**).*

BOX 1: EXTRACTION MIX

The choice depends on what kind of compounds you aim to find. With the present composition we open the spectrum of polarity in order to be as non-selective as possible in a reproducible way.

BOX 2: QUENCHING

The aim here is to stop any metabolic process inside the cell at the time of interest. Flash freezing is common but difficult for microalgae. Thus, we apply rapid filtration and quenching in extraction mix.

BOX 3: STORAGE

For longer periods it is advisable to store the sample at -80 °C. If there is some water left in the sample, metabolites get degraded by oxidation, thus the storage period has to be as short as possible.

BOX 4: INTERNAL STANDARD

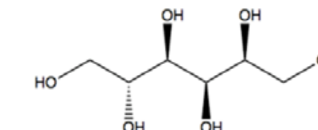
Sorbitol is an alcohol with 6 OH groups (see below). It has to be derivatized to be analyzed by GC-MS. It is added to the sample to check the derivatization efficiency. It has to be added already at the beginning of the extraction step.

4.2. Extraction of algae: Randomize samples from now on and cool them in ice if not completely dry.

1. Place the centrifuge tubes in a pre-cooled (-80 °C) TissueLyser II tube support and let them sit for at least 30 minutes at -80 °C. Add 2 metal beads per tube. Disrupt the cells twice, 30 s at a frequency of 30 s^{-1}. During this procedure, the sample will remain frozen.

If no TissueLyser is available (training school), a mortar with pestle and liquid N_2 can generate powder. However, carefully clean the mortar and pestle after each sample.

2. Add 5 µL of the ^{13}C-sorbitol solution (**Box 4**).
3. Calculate the volume of the extraction mixture proportionally to the biomass weight. As a reference, 1.5 mL of extraction solution should be used for the sample with the highest weight.



4. Add extraction solution to each sample and vortex vigorously to homogenize the sample and allow a more uniform extraction.
5. Place the samples in an ultrasonic bath for 10 min.

During ultrasound treatment, the membrane or cell wall is disrupted. An alternative method can be more suitable for different tissues.

6. Centrifuge the samples at 30.000×g or maximum speed for 15 min.

Remove debris, such as the cell wall, from your supernatant.

7. Transfer the supernatants into 1.5 mL glass vials sealable with PTFE septa.

Glass is preferred to plastic ware because it is inert to organic solvents. Be careful not to transfer particles into the samples for LC-MS because LC is especially susceptible to particles and can damage the system.

1. Transfer 300 µL to a 1.5 mL vial for GC-MS analysis.
2. Transfer 300 µL to a 1.5 mL vial for LC-MS analysis.
3. Prepare quality control (QC) samples:
   i. 2 QCs per run for GC in 1.5 mL vials.
   ii. 1 QC sample for LC in 1.5 mL vials.
   iii. Mix equal and sufficient sample volumes and transfer 300 µL for each QC in total. Solvent blanks are not necessary in the QCs.
   iv. Remaining solutions can be handled as a backup (note down the remaining volume)

8. Evaporate to dryness under nitrogen for ~ 1 hour. If a desiccator is applied, use the following scheme:
   i. Check the solvents for spontaneous boiling after each pressure decrease.
   ii. Atm → 600 mbar → 300 mbar in 50 mbar steps → 270 ± 10 mbar in 10 mbar steps. Wait for 30 min to evaporate $CHCl_3$.
   iii. 270 mbar → 175±5 mbar in 10 mbar steps. Wait for 1.5 h to evaporate MeOH.
   iv. 175 mbar → 85±5 mbar in 10 mbar steps. Leave overnight to evaporate EtOH.
   v. 85 mbar → 0 mbar. Wait for 1 h.

To start the derivatisation process, all the solvents from the extraction mix must be removed. This can be done under a flow of nitrogen or dry air, with a vacuum desiccator, or equivalent methods. You should prevent any contamination. You can then return your samples to -20 °C for short-term storage. In that case, re-dry them directly before derivatization.

4.3. Extraction and sample preparation of culture medium or seawater

1. Attach Chromabond Easy (Macherey -Nagel, Germany) or Oasis HLB (Waters, UK) cartridges to a vacuum manifold and label them.
2. Condition with 4 mL MeOH directly before usage with a syringe
3. Wash columns with 4 mL of ultra-pure water.
4. Connect PTFE tubing in line with sterile-filtered (GF/F, Whatman, USA) seawater or medium sample (1 L): Alternatively, sample can be sterile-filtered before hand
5. Apply vacuum on manifold and adjust flow to ~ 1 L·h^{-1}.
6. Disconnect columns from the tubing and wash with 4 mL of water.
7. Air dry columns with a vacuum
8. Elute columns with 4 mL MeOH
9. Add 5 µL of ^{13}C-Sorbitol Internal standard and vortex briefly
10. Transfer 1.5 mL into a 1.5 mL vial for LC-MS and GC-MS measurements.

11. Evaporate to dryness under nitrogen for ~ 1 hour. If a desiccator is applied, use the following scheme:

i. Check the solvents for spontaneous boiling after each pressure decrease.

ii. Atm → 600 mbar → 200 mbar in 50 mbar steps → 175±5 mbar in 10 mbar steps. Wait for 1.5 h to evaporate MeOH.

iii. 175 mbar → 0 mbar in 10 mbar steps. Wait for 1 h.

To start the derivatization process, all the solvents from the extraction mix must be removed. This can be done under a flow of nitrogen or dry air, with a vacuum desiccator, or equivalent methods. You should prevent any contamination. You can then return your samples to -20 °C for short-term storage. In that case, re-dry them directly before derivatization.

4.4. LC-MS sample preparation (not performed at the training school)

1. For measurement on C^{18} columns: resolve extracts in 100 µL pure MeOH or 80% v/v (preferred), vortex samples, and transfer into new 1.5 mL vials with 200 µL inserts.
2. **Optional:** Samples can be remeasured on HILIC by preparing mixtures of MeOH:ACN:H_2O (5:9:1)
3. **Attention:** Samples should be particle-free and vortexed before they are subjected to chromatography to prevent air bubbles.

4.5. Derivatization for GC-MS and sample preparation (not performed at the training school)

Intracellular metabolites exhibit a broad range of chemical properties. To analyse as many of them by GC-MS, most must be more volatile. Therefore, chemical derivatization approaches will provide derivatives of the substance of interest that are sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition.

Several derivatization methods exist, such as alkylation, acylation, and silylation. In this protocol, we will use the methoximation and silylation reactions which introduce protecting group (Figure 5). For example, the functional groups such as -OH, -NH, -NH_2, or –COOH are silylated by a trimethylsilyl group (TMS; Si[CH_3]$_3$; Figure 6). However, MSTFA will also react with water and alcohol first, thus making sure that samples are water-free. A common silylation reagent is MSTFA, which is a strong TMS donor. However, many reagents are available, e.g., BSTFA, BSA, TMS-DEA, or MTBSTFA [4,5].

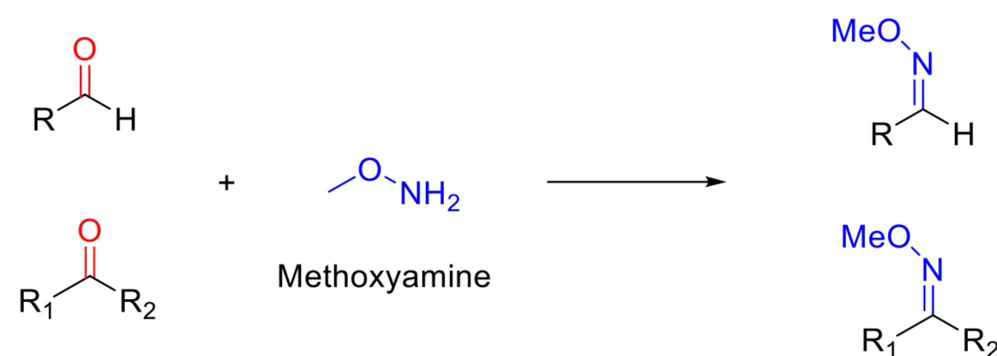


Figure 5 - Schematic methoximation reaction using methoxyamine as a reagent.

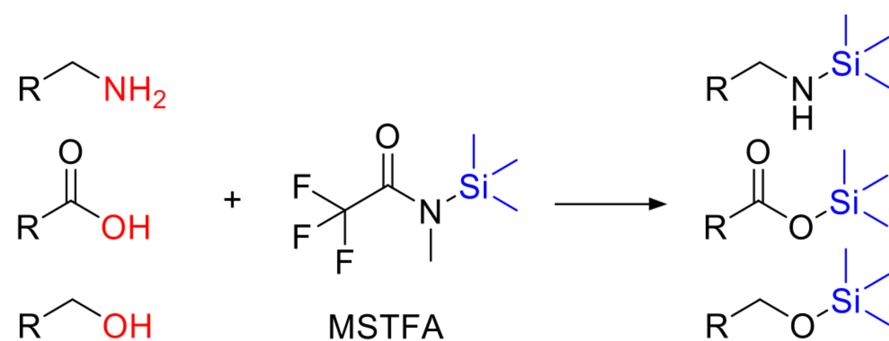


Figure 6 - Schematic silylation reaction using MSTFA as a reagent.

1. Dissolve 20 mg methoxyamine hydrochloride (stored dry) in 1 mL anhydrous pyridine by vortexing and 5 min in an ultrasound bath.

Pyridine is often used as a solvent and reaction catalyst due to its acid-scavenging properties.

2. Add 50 µL of methoxyamine solution to each sample and vortex for 1 min to resolve all metabolites.
3. Incubate at 60 °C for 1 hour and additionally at room temperature for 9 to 16 hours.
4. Transfer 40 µL of samples into 1.5 mL vials with 200 µL glass inserts and close the vials.

Glass inserts will allow the GC-auto sampler to take an aliquot (1 µL) of this small volume (80 µL at the end). If you have some condensation along the glass during the derivatization process, briefly centrifuge your tubes (~ 5 s) before transferring the volume.

If precipitation is seen, centrifuge samples at 8.000 × g for 5 min and transfer the supernatant into a new insert.

The GC-MS system can only measure samples in a gaseous phase. Solid particles such as precipitates will be trapped by the glass wool in the injection liner but will contaminate your system and interact with the analytes of subsequent injections.

5. Add 10 µL of the diluted RI mix to one QC sample and vortex the sample.

The RI mix (retention index mix) used here comprises 34 alkanes with carbon chain lengths of 7-40. Retention indices are used as a reference to calculate system-independent retention times. This allows you to refer to your compound of interest even in measurements after changing analysis parameters such as column flow and column length or to compare it with externally measured reference standards. The volume can be increased or decreased depending on your QC intensity.

6. Wait until the MSTFA reaches room temperature, then add 40 µL of MSTFA to each sample *(in batches not bigger than 20 samples) with a glass syringe or pipette.*

The batch limitation of < 20 is due to the stability of the derivatization, which is reported to be at a maximum of 24 hours. One GC-MS run lasts about 45 min. This allows for a maximum of 24 samples.

7. Incubate at 40 – 60 °C for 1 h and vortex afterwards briefly.

The oxidative reaction breaks down hydrogen bonds. The silylation time has a huge impact on reproducibility and recovery. Long silylation times can only achieve complete silylation of amine groups. However, other compounds tend to degrade.

8. Analyze the batch of samples immediately (< 24 hours) by GC-EI-MS (e.g., Orbitrap or Time of Flight Analyzer (ToF)).

The silylated compounds are not stable over time.

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HANDS ON METABOLOMICS – 28TH OF JUNE - PART 2

Thallusin quantification in growth medium or seawater by LC-MS

1. INTRODUCTION

Thallusin, a highly biologically active, phytohormone-like and bacterial compound-inducing morphogenesis of the green tide-forming macroalga *Ulva* (Chlorophyta), was determined in bacteria and algae cultures. A sensitive and selective method was developed for quantification based on ultra-high-performance liquid chromatography coupled with electrospray ionization and a high-resolution mass spectrometer. Upon C_{18} solid phase extraction of the water samples, thallusin was derivatized with iodomethane to inhibit the formation of Fe−thallusin complexes interfering with the chromatographic separation. The concentration of thallusin was quantified during the relevant phases of the bacterial growth of *Maribacter* spp., ranging from 0.16 ± 0.01 amol cell^{-1} (at the peak of the exponential growth phase) to 0.86 ± 0.13 amol cell^{-1} (late stationary phase), indicating its accumulation in the growth medium. Finally, we directly determined the concentration of thallusin in algal culture to validate our approach for monitoring applications. Detection and quantification limits of 2.5 and 7.4 pmol L^{-1}, respectively, were reached, which allow for quantifying ecologically relevant thallusin concentrations. Our approach will enable the surveying of thallusin in culture and in nature and will thus contribute to the chemical monitoring of aquaculture [1].

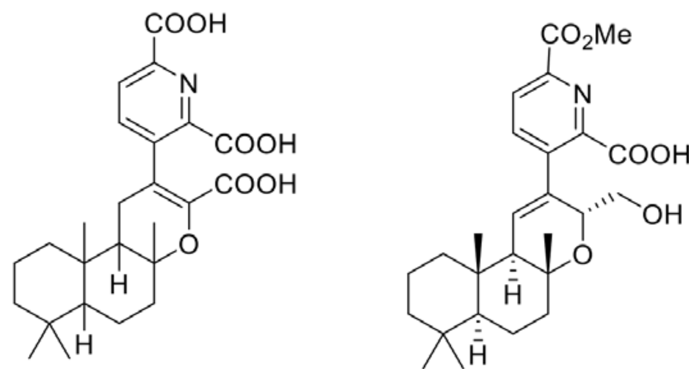

Figure 7 - Structure of thallusin (left) and internal standard 46 (right)

2. WORKFLOW

The quantification approach involved filtration, solid phase extraction of thallusin and its derivatisation with iodomethane. This is followed by a clean-up, also using solid-phase extraction. A high-resolution mass spectrometer operated in single ion mode is recommended for analysis. For reliable quantification, it is necessary to assign an internal standard (Figure 7) that goes through the same steps of the analytical process [1].

3. MATERIALS

3.1. Biological material

- Medium or seawater sample (V ≥ 250 mL, *n* = 3) or bacteria supernatant (V ≥ 50 mL, *n* = 3)

3.2. Equipment

- Eppendorf pipettes (1000 µL, 200 µL, 10 µL; recently checked and calibrated).
- Vortexer (VortexGenie 2, Scientific Industries).
- Automated Evaporation System (TurboVap® LV, Biotage) or any desiccator with vacuum pump.
- Manifold (compatible with luer lock, e.g., Visiprep, Supelco)
- Centrifuge (capable of 9500 rpm)
- Glass vessels for samples in appropriate dimensions (Schott)
- 0.4 mm FKM protective gloves for handling Iodomethane
- Heating plate

3.3. Chemicals

- Methanol (Chromasolv® Plus, Sigma-Aldrich, Germany).
- Ultra-pure water
- Internal standard 46 (IS-46, thallusin derivative, 1 µM in water, synthesized)
- Iodomethane (Sigma-Aldrich, Germany)
- Formic acid (Thermo Fisher, Germany, ≥ 99%)

3.4. Consumables: *only manipulated with gloves*

- Pipette tips (1000 µL, 200 µL, 10 µL, Sarstedt, Germany).
- Glass screw neck vials (N9, 1.5 mL, Macherey-Nagel, Germany).
- Glass screw neck vials (N13, 4 mL, Macherey-Nagel).
- Glass inserts for N9 vials and springs (200 µL, Macherey-Nagel).
- PP screw caps for N9 and N13 vials (Macherey-Nagel).
- Screw cap tubes for centrifugation (50 mL, Sarstedt)
- Tough Tag labels for microcentrifuge tubes
- C_{18} SPE cartridges (Sep-Pak plus short, Waters)
- Cellulose nitrate filters (0.45 µm, Sartorius)
- MIllex GV PVDF filters (0.22 µm, Merck)
- Syringes (1 mL, 5 mL and 50 mL)

4. PROTOCOL

4.1. Sampling and solid phase extraction

1. Centrifuge culture medium or seawater at 10 °C (if possible), up to 9,500 rpm (if possible) for 10 min (time needs to be adapted to the available centrifuge).
2. The centrifuged solid part (algae or bacteria) can be discarded and decant culture supernatant.
3. Filtrate (cellulose nitrate filter, pore size 0.22 µm; Ø = 47 mm) culture supernatant; if necessary, change the filter several times after blockage

4. Add 10 µL internal standard (IS-46, Fig. 3) to the filtered culture supernatant. Measure the exact volume of the filtered supernatant.
5. Install C_{18} SPE cartridges (Luer lock) with vacuum manifold and syringe without piston (50 mL) and label cartridges.
6. Condition cartridge: load 5 mL of methanol (MeOH for HPLC analysis) with a clean beaker and apply low vacuum (~ 950 mbar) for slow, steady, dropwise flow of MeOH (applies to all solvents passing through SPE, if the flow is a faster-adjusted vacuum)
7. **IMPORTANT:** The C_{18}-SPE cartridge should never fall dry during the whole extraction!
8. Load 10 mL Millipore water for equilibration and apply vacuum (~ 900 mbar or lower, depending on position and individual cartridge)
9. Solid phase extraction of filtered culture supernatant or seawater: load sample stepwise and apply vacuum (also ~ 900 mbar or lower)
10. Washing the sorbent of the C_{18} SPE cartridge for desalting: load 10 mL Millipore water on the C_{18} SPE cartridge with a vacuum
11. Second washing step of the C_{18} SPE cartridge: load 4 mL 25% MeOH (75% Millipore water) (v/v) and apply vacuum (~ 900 mbar)
12. Elution of sample: remove the syringe from the C_{18} SPE cartridge and place the cartridge on top of a clean 4-mL-glass vial. load 4 mL 75% MeOH (25% Millipore water) (v/v) with a clean 5-mL syringe (avoid air bubbles in the syringe) and slowly push solvent through the cartridge into the vial; discard the cartridge.
13. Close the glass vial and vortex eluate.
14. Evaporate the eluate to dryness (use the nitrogen flow at 20 - 25 °C or desiccator).

4.2. Methylation with Iodomethane (not performed at the training school)

1. Prepare DMSO/KOH solution (0.2 g KOH powder partially dissolved in 4 mL DMSO, stirred at 100 °C on a heating plate).
2. Add 150 µL of DMSO/KOH solution to the dried sample and vortex for dissolving.
3. Add 30 µL of iodomethane **under the fume hood with special care and equipment** (attention to high viscosity!) and take special care until it is inactivated with formic acid.
4. Shake samples for 2 hours, e.g., with a vortex adapter sponge for glass vials.
5. Add 50 µL of formic acid to inactivate iodomethane and shake for 1 hour. Samples can be stored at room temperature until the following day for clean-up.

4.3. Clean-up of samples (not performed at the training school)

1. Dilute samples by transferring them via pipette into 40 mL ultra-pure water filled in screw cap tubes, respectively. Wash the 4 mL glass vial several times with water.
2. Fill the tube up to 50 mL with ultra-pure water.
3. Repeat the SPE procedure from **4.1** for the clean-up of samples.
4. Dry the 75% MeOH solution with nitrogen flow or a desiccator.
5. Resolve samples in 100 µL of 75% MeOH and vortex samples
6. Filter samples with Millex GV syringe filters (transfer the sample onto the filter by pipetting and pulling the piston up while connecting the filter and 1 mL syringe). Carefully press the sample through the filter into a 1.5 mL glass vial with an insert and spring.
7. Prepare a solvent blank of the 75% MeOH used for evaluation.
8. Samples are ready for measurement in LC-MS (see reference for further information).

5. REFERENCES

[1] J.F. Ulrich, M.S. Gräfe, S. Dhiman, P. Wienecke, H.D. Arndt, T. Wichard. Thallusin quantification in marine bacteria and algae cultures. *Marine Drugs*, 20, (2022).

NOTES

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