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WP4, Deliverable D4.1, D1

A robust analytical workflow for targeted and untargeted metabolite analyses.

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Introduction

Metabolomics is a research field that assesses the metabolome profile and the respective metabolic pathways of an organism or a full microbial community. The approaches can be either qualitatively or semi-quantitively depending on the target question. Overall, there are two main ways to study metabolomics: (a) targeted metabolomics, where known metabolites are analyzed in an approach that is used primarily to prove a specific microbial life cycle hypothesis and (b) untargeted metabolomics, where a wide spectrum of possible metabolites is detected and studied in a single organism or a complex microbial community usually as a function of a changing parameter.

For IceBio, our target is supraglacial and proglacial processes where metabolic processes affect and change as a function of environmental parameters such as light, nutrients, etc. The IceBio metabolomic work will target primarily supraglacial snow and ice field microbes and will be carried out as a combination of non-targeted analyses of daily and or seasonal changes in metabolite profiles combined with laboratory algal culture experiments where more detailed questions are addressed (i.e., nutrient or light stress etc.).

Overall, the target samples will be either from snow, ice, or cryoconite hole communities which are the main biomes in the supraglacial environment (Anesio et al 2017, Fig 1A). In the snow, we usually find bacteria, fungi and variably coloured snow algae (these belong to the C*hlorophyte phylum,* and they can be green, yellow, and dark red) depending on the species and the carotenoid pigments they contain. On bare ice, the dominant glacier ice algae belong to the *Streptophytes*, with *Ancylonema sp* dominating. The glacier ice algae are usually brown to deep purple due to the purpurogallin pigment that they produce. In cryoconite holes, the dominant microbes are prokaryotes (primarily cyanobacteria).

These supraglacial environments change in the summer melt season and when such samples are collected from their natural habitat so that their metabolites are analyzed the following steps workflow has been developed and will be further tested within at least two of PhD projects of IceBio:

Sample Collection and Melting

Frozen surface snow and ice samples once collected must be melted to separate the extracellular and intracellular metabolites by filtration (Fig 1B). Important to note is the fact that thawing of the samples needs to be done under controlled conditions to avoid changes in the metabolome profile due to melting. In our teams' previous work, have shown that the most appropriate temperature and condition for melting are controlled melting at 10°C, as this usually takes only 20 minutes and does not significantly affect the metabolome of microbial cells, while higher temperatures and longer melt times do (Peter et al, 2024, in review).

Filtration and Quenching

Once the samples are melted, they are filtered to separate the cells from the extracellular matter and then quenching of the samples is done to stop any metabolic changes. This is generally performed by either instantly freezing the samples or by quenching the samples with organic solvents (usually methanol) as this inhibits further the enzymatic activities in cells (Fig 1B).

Metabolite Extraction

For cell lysis, ball milling, and liquid nitrogen freeze-thaw cycles in addition to ultrasonication are very efficient in lysing all types of cells including spores, cysts, etc. To extract a wide range of polar and non-polar metabolites, a bi-phasic solvent extraction protocol with the combination of solvents using methanol, Methyl tert-butyl Ether (MTBE) and water could be used compared to the conventional extraction protocol (Sostare et al. 2018). (Fig 1C and D)

Data Acquisition

For a representative metabolite analysis for each sample set a minimum number of biological and technical replicates of at least four or more should be used to account for biological and instrumental variability (Fig 1D). Importantly, blanks are required to identify and remove contaminants in all sample handling and analyzed steps from sample collection, metabolite extraction and mass spectrometric analysis (Fig 1 E/F). In addition, one should use quality control (QC) samples (e.g., pooled aliquots of all the samples), and internal standards (IS, a standard mixture of known chemical compounds used to track the mass spectrometry performance and also to quantify known metabolites) to cross-correlate results.

For data acquisition, the samples are most often analyzed using gas- or liquid-chromatography mass spectrometers (GC-MS and LC-MS) that help to identify the compounds in the samples (Fig 1E/F). Both polar and nonpolar metabolites (see Fig 1C) can be analyzed using such MS approaches. For the GC-MS analyses, samples have to be derivatized to make the compounds more volatile to analyze polar metabolites; for analyses using LC-MS, one additional option would be to change the stationary and mobile phases and analyze these polar metabolites by hydrophilic interaction liquid chromatography (HILIC). To detect non-polar metabolites, a combination of LC-MS and reverse-phase liquid chromatography (RPLC) or using non-polar GC columns and GC-MS analyses could be another approach (Fig 1E/F). GC-MS targets mostly the volatile compounds and thus also works in the lower mass range, while LC-MS works with bigger molecules. Therefore, these methods could be complementary. Ultimately, it always depends on the question set by the study which approach is the most appropriate.

Data Analysis

Once the raw data sets are acquired from the instrument, the complex mass spectrometry data can be analysed using several possible bioinformatics tools (Fig 1G). For example, peak picking, peak annotation, and raw data processing could be done using programs like MS-DIAL, MZmine, XC-MS, CAMERA, Compound Discovery, etc. These bioinformatic tools combined with robust statistical data processing packages like omu, metabolomicsR,

Metaboanalyst [Table 1], etc. are some of the possible options that can be applied to analyse the metabolite data, metabolome composition, changes in the pattern or trend of specific metabolites due to variable conditions and related metabolic pathways.

Tool Name	Tool Type	Availability	Raw Data Processing	Blank Removal	Matrix Transformations	Uni-Variate Statistics	Multi-variate Statistics	Export for Downstream Tools	Customizable	URL
GUI										
MetaboAnalyst	Web App (GUI)	Open Source	Y	Y	Y	Y	Y	N	N	www.metaboanalyst.ca/
Workflows										
Galaxy-M	Workflow	Open Source	Y	Y	Y	Y	Y	N	N	github.com/Viant-Metabolomics/Galaxy-M
Workflow4Metabolomics	Workflow	Open Source	Y	Y	Y	Y	Y	N	N	github.com/workflow4metabolomics
UmetaFlow	Workflow	Open Source	Y	Y	Y	Y	Y	N	N	github.com/biosustain/snakemake_UmetaFlow
Chemometrics Tutorials	Workflow / Tutorial	Open Source	N	N	Y	Y	Y	N	Y	github.com/Gscorreia89/chemometrics-tutorials
QIIME2 metabolomics plugin	Language	Open Source	N	N	N	Y	Y	N	N	library.QIIME2.org/plugins/q2-metabolomics/10/
R Libraries										
mixOmics	Package	Open Source	N	N	Y	Y	Y	Y	Y	mixomics.qfab.org
MetaboanalystR	Package	Open Source	Y	Y	Y	Y	Y	Y	Y	www.metaboanalyst.ca/docs/RTutorial.xhtml
omu	Package	Open Source	N	N	Y	Y	Y	Y	Y	cran.r-project.org/web/packages/omu/vignettes/Omu_vignette.html
metabolomicsR	Package	Open Source	N	N	Y	Y	Y	Y	Y	cran.r-project.org/web/packages/metabolomicsR/index.html
MAIT	Package	Open Source	N	N	Y	Y	Y	Y	Y	www.bioconductor.org/packages/release/bioc/html/MAIT.html
ropls	Package	Open Source	N	N	Y	Y	Y	Y	Y	bioconductor.org/packages/release/bioc/html/ropls.html
MSStats	Package	Open Source	N	Y	Y	Y	Y	Y	Y	github.com/Vitek-Lab/MSstats
Python Libraries										
TidyMS	Package	Open Source	Y	Y	Y	N	N	Y	Y	github.com/griquelme/tidyms

Table 1- Tools for statistical metabolomics data analysis (Pakkir Shah et al. 2023)

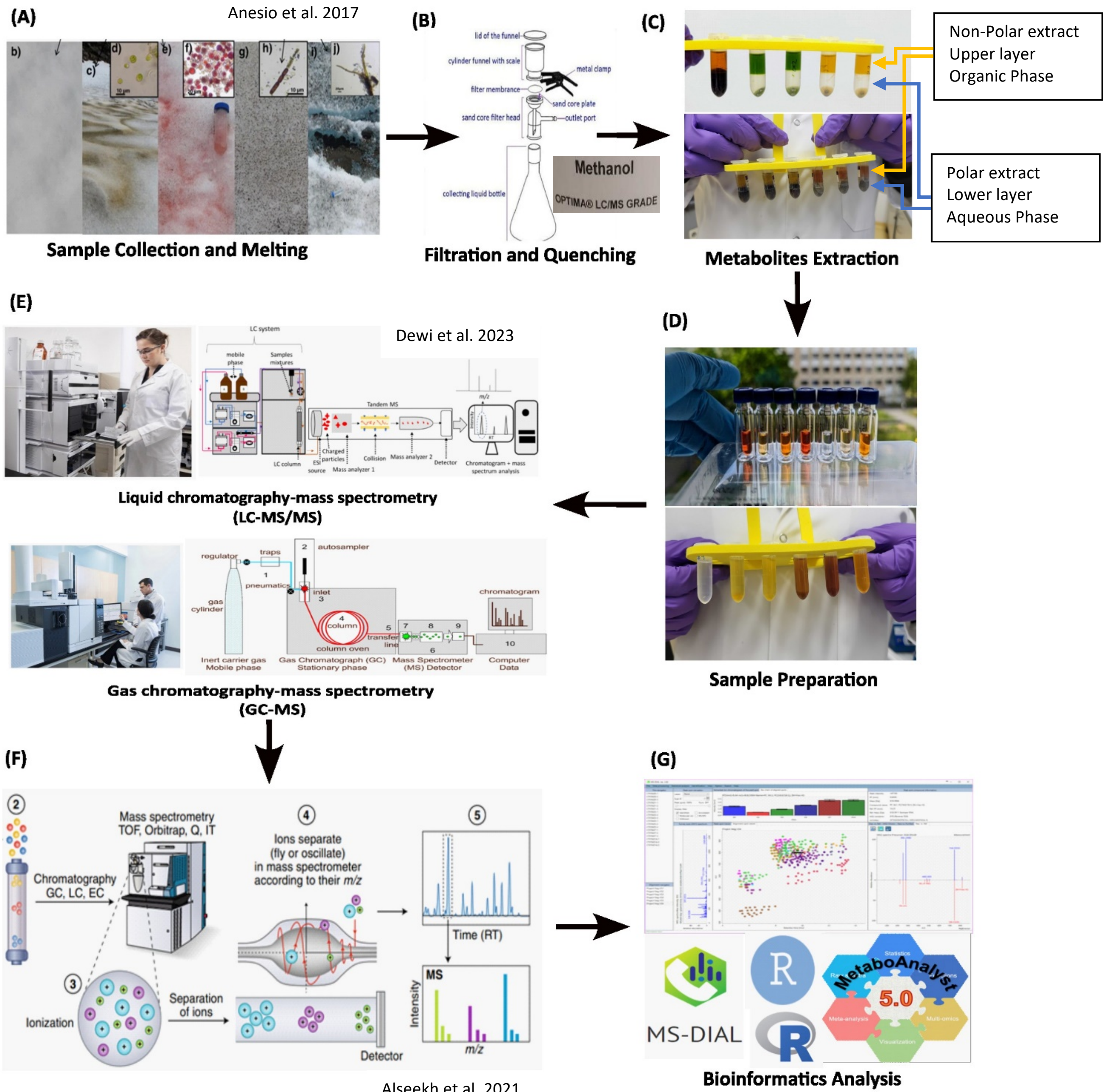

Figure 1- Metabolomics workflow for cryosphere microbial sample analyses **(A)** Snow, ice and cryoconite sample collection; this can include snow algae that are green or red, ice that is dark grey to purple or cryoconite samples (modified after Anesio et al. 2017); **(B)** after melting the samples at 10°C they have to be immediately filtrated and preserved by quenching the samples to stop enzymatic or metabolic activity with 50% methanol; **(C)** Metabolite extraction using bi-phasic solvent extraction; **(D)** further sample handling (eg., derivatization etc.) before them being analyzed (photograph in C and D copyright @Elisa Peter and Pamela Rossel images linked to work in Peter et al, 2024, in review); **(E & F)** LC-MS and GC-MS methods to separate metabolites by chromatography, ionize and analyze using mass spectrometry (figure F is after Alseekh et al. 2021; figure E modified from Dewi et al. 2023). **(G)** Different bioinformatics tools for analyzing the data.

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