UNIVERSITÄT BAYREUTH

Bayreuther Zentrum für Ökologie und Umweltforschung
Bayceer

SCRIPT

Practical course

FUNCTIONAL MICROBIOME RESEARCH

WiSe 2023/24

M.Sc. Students of Bio- and Life Sciences

Supervisors:

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Practical course and seminars take place in the labs of the
Chair of Ecological Microbiology (ÖMIK), Dr.-Hans-Frisch-Str. 1-3

Time frame:
Mo. 04. – Fr. 15. March. 2024, ca. 9:00 – 16:00

To do in advance:

Please print this Script (A4) and bring it along on every day of the practical course.

Bring along your lab coat and laptop. Prepare for each day by reading the respective sections.

Prepare and practice your literature seminars

Get a linux environment working on your computer (section C.2.1)

Generate an SSH key (section C.2.4)

CONTENT

1. Components of the practical course

A. Stable Isotope Probing of methanol-, glucose- and acetate-oxidizing microbes in soil

B. FISH-Microscopy of sewage sludge and biofilm samples

C. Analysis and interpretation of (meta)genomic data

D. Analysis and interpretation of amplicon sequencing data

2. Literature Recommendations

The text books recommended here can help you in preparing for the course and while writing the reports. Support in access to primary literature can be provided by members of the ÖMIK team.

Clark, Stahl, Martinko, Madigan (Eds.) **Brock-Mikrobiologie.** Pearson, 2013

Dumont, Hernández García (Eds.) **Stable Isotope Probing - Methods and Protocols.** Humana Press, New York, NY. 2019. https://doi.org/10.1007/978-1-4939-9721-3

Charles, Liles, Sessitsch (Eds.) **Functional Metagenomics: Tools and Applications.** Springer International Publishing, 2017. https://doi.org/10.1007/978-3-319-61510-3

More literature and references will be provided during the seminars!

3. Lab security

Each student must participate in the **lab security introduction** at the beginning of the practical course. This will be held by the responsible persons of the ÖMIK Chair on **Monday, Mar. 4. at 9:00**. The participation in the introduction must be confirmed by every student by signing the respective security certificate. **Participation is obligatory!**

Do not forget to bring your own lab coat!

Microbes can be pathogenic! They can cause infectious diseases and produce toxins. Each microbial sample and microbial culture handled during the practical course, as long as not certified to be pathogen-free, must be handled as if containing potential pathogens! The following basic security measures are to be implemented:

- Wear you lab coat at all times during the practical course.
- If advised, eye protection must also be worn.
- Long hair are to be worn in a plait, do not carry long pendulous jewellery.
- Shoes must be solid and closed.
- Eating, drinking, smoking, chewing gum, putting on make-up etc. in the labs are not allowed!
- Utmost cleanliness is to be maintained in all labs and on all working materials.
- Apply techniques of sterile sample handling wherever necessary
- Apply mechanical pipetting wherever possible
- Any accidents and injuries must immediately be reported to the course supervisors!

4. Required performance, deliverables and evaluation

A successful participation in the entire module requires:

1. Regular participation: It is required that you arrive on time and participate continuously for all parts of the practical course. Upon previous agreement by the supervisory staff, and if urgent justifications are warranted, it is possible to allow for not more than **one day of absence** during course duration. One second day of absence is possible, but must be justified via a doctoral attestation. Absence for more than two days does no longer warrant the successful completion of the practical course.

2. Final exam: Please register for the final written exam. The examination includes all topics taught during the lecture “Functional Microbiome Research”, during the practical course, during the seminars and presented in the script. Date and time of the final written exam is:
Fri. 22.03.2024, 10:00 – 11:30 in H7 (DHF-Str.)

5. Written report

Each student must submit a written report for the entire practical course. Rationale, experimental procedures and results of all elements of the practical course must be documented in a contiguous manner. The aim is to provide training in putting together a written scientific report. The report should be structured in the following manner:

1. Introduction (scientific background and research questions)
2. Methods (can be kept brief, as long as not significantly extending beyond the procedures described in the script).
3. Results: all steps and results obtained during the course must be put documented. Pay attention to sample naming, proper illustration of axes of graphs and units. Figures (with legends) must be numbered continuously, same as tables (with headers).
4. Discussion of the results and conclusions. Refer to the literature where adequate and avoid simply repeating the results.
5. References

Further, more specific instructions for reporting on the metagenomics part of the course will be given below. The supervisory team can be approached at any time during the course and while preparing the written reports for additional advice on the reporting and illustrations. A joint discussion and presentation of the results of the course will be held on the last day of the practical course.

The written report must be submitted as printed versions. Accompanying E-mails with additional Excel tables etc. containing original data, calibrations, or more extensive metagenomic displays may also be submitted.

Submit your reports latest by Mo., April 15th 2024 in print to the ÖMIK secretary Melanie Nützel or directly to Prof. Lüders. Pdf files can be sent in per mail in parallel.

6. Time plan – Functional Microbiome Research

Timing is always somewhat flexible, updates and changes are possible and will be communicated during the course.
Seminar presentations should be prepared and practiced before the start of the course.

Mo. 4.3.	**Tue. 5.3.**	**Wed. 6.3.**	**Thu. 7.3.**	**Fri. 8.3.**
9^{00} - Welcome and security introduction 10^{00} - Introduction to the SIP experiment (TL) 11^{00} – Setting up of SIP microcosms (TL) 12^{30} – Lunch break 13^{30} – Getting started: Linux and cloud computing (DT)	9^{00} – Metagenomics Introduction, Mock-MG read QC & assembly (DT) 12^{00} – Lunch 13^{00} –CO_2 sampling (TL) 14^{00} – Student seminars (J. Jäger / B. Yegizbay) 15^{00} – Mock-MG binning and refinement (DT) 16^{00} – Biofilm fixation for FISH (DM)	9^{00} – Sample washing and mounting for FISH (DM) 11^{00} –Hybridization (DM) 12^{00} – Lunch 13^{00} – Student seminars (M. Scheer / F.P. Dose) 14^{00} – FISH washing (DM) 15^{00} – First microscopy (DM) 15^{30} –CO_2 sampling SIP microcosms (TL)	9^{00} – Mock-MG taxonomy and pathway annotation (DT/DM) 12^{00} – Lunch 13^{00} – Metabarcoding, 16S amplicon data handling (DT) Flexible - FISH microscopy contd. (DM)	9^{00} - DNA extraction from SIP microcosms, final CO_2 quantification (TL) 12^{00} – Lunch 13^{00} – DNA purification contd., loading of SIP gradients and start of ultracentrifugation (TL)
Mo. 11.3.	**Tue. 12.3.**	**Wed. 13.3.**	**Thu. 14.3.**	**Fri. 15.3.**
9^{00} – SIP gradient fractionation (TL) 12^{00} – Lunch 13^{00} – Preparation of Illumina sequencing amplicons (TL/DM) 15^{00} – 16S amplicon data handling cont. (DT)	9^{00} – IRMS analytics of $^{13}CO_2$ (GEO I, Campus) 11^{00} – SIP amplicon label interpretation (TL) 12^{00} – Lunch 13^{00} – Student seminars (A. Habbachi / L.L. Qiao) 14^{00} – Start of Biofilm-MG workshop (DM)	9^{00} – Biofilm-MG workshop contd. (DM) 12^{00} – Lunch 13^{00} – ^{13}C flux calculations (TL) 14^{00} – Biofilm-MG workshop contd. (DM) Flexible - FISH microscopy contd. (DM)	9^{00} – Biofilm-MG workshop contd. (DM) 12^{00} – Lunch 13^{00} – Biofilm-MG workshop contd. (DM) Flexible - FISH microscopy contd. (DM)	9^{00} – Final joint data interpretation and discussion (all) 11^{00} – Joint Q&A round Mock exam questions (all) 12:00 – Course wrap-up, Joint farewell lunch

Part A: Stable Isotope Probing

A.1 Theoretical backgrounds

Stable isotope probing (SIP) of isotopically labelled RNA and DNA is well-established in environmental microbiology. The concept of a labelling-based detection of process-relevant microbes independent of cellular replication or growth allows for a direct handle on functionally relevant microbiome components. Even over a decade after its introduction, stable isotope probing (SIP) of nucleic acids is still considered as a prime strategy for the targeted identification of microbial key-players in defined environmental processes. Since its conception for the identification of methylo- and methanotrophic bacteria [4], nucleic acid-based SIP has undergone a considerable development and has been applied to a variety of ^{13}C-, ^{15}N- and recently also ^{18}O-labeled compounds. The general strengths of SIP are that it allows for a substrate-based query, for a targeted identification of microbes involved in a specific biodegradation process within a complex community, as well as for the unraveling of involved catabolic genes and carbon flows across microbial kingdoms. All of this is possible in an undirected manner, without essential à-priori probes or genomic information. The basic approach is also a relatively "low-tech" method, which discriminates it from most other technologies capable of tracing isotopic labeling in microbes.

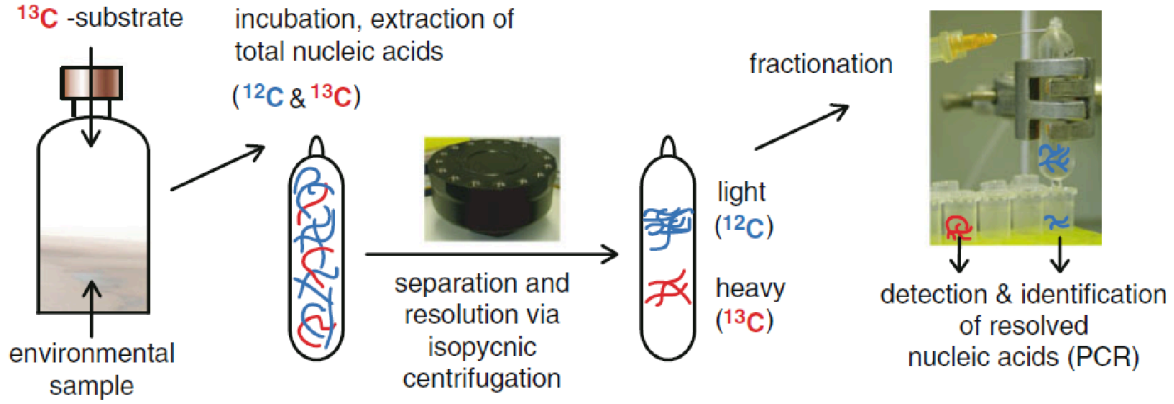


Fig. 1 Schematic view of a typical stable isotope probing (SIP) experiment to identify degraders of ^{13}C-labeled hydrocarbons

A.1.2 Technical considerations

SIP uses ultracentrifugation to resolve isotopically labeled ("heavy") nucleic acids from unlabeled ("light") ones. In this isopycnic centrifugation, freely diffusing cesium salts form a density gradient driven by the delta between minimal and maximal centrifugation force (g_{min} & g_{max}) during the run. Nucleic acids loaded into the gradient then arrange themselves (or "band"), also by diffusion, at the buoyant density (BD) matching their own. CsCl media are commonly used for centrifugation of genomic DNA, which bands at a BD of ~1.70 g ml^{-1} in unlabeled form.

It is not possible to band rRNA in CsCl, as the salt would precipitate during centrifugation at the required BD (>1.9 g ml^{-1}). Instead, rRNA is centrifuged in CsTFA, where it bands at ~1.80 g ml^{-1} in unlabeled form. For both DNA and rRNA, fully ^{13}C-labeled nucleic acids have been observed to be ~0.04 g ml^{-1} "heavier" than their unlabeled counterparts. The maximal effect of full ^{15}N-labelling on nucleic acid BD has been reported to be roughly half of that, and thus less pronounced than the

distinction in BD between a low-GC and a high-GC genome. When conducting isopycnic centrifugation, the reader should be aware of the following principles:

(i) SIP centrifugation runs are very long. As already demonstrated much earlier, genomic DNA requires >24 h of ultracentrifugation for optimal banding. Focusing of bands has been observed to be even less efficient for rRNA, a molecule characterized by extensive secondary structure and self-affinity. Thus, long centrifugation runs of >36 h for DNA, and of 42 – 65 h for rRNA are generally recommended. These long centrifugation times are not detrimental to rRNA, because rRNA is protected during centrifugation by the chaotropic nature of CsTFA.

(ii) Banding is never absolute. Because banding of nucleic acids in SIP is a diffusion process, it never reaches an absolute stage. Even if uniformly labeled pure culture nucleic acids are centrifuged, heavy fractions will always contain backgrounds of light nucleic acids, and vice versa. Thus, the quantitative allocation of specific templates to a certain BD is the most crucial information obtainable from SIP gradients, and not the absolute detectability of certain templates at a given BD. Due to diffusion banding, neighboring gradient fractions will always harbor similar templates. Even if distinctions in template distribution are apparent over entire gradients, they will always occur gradually, over 3-4 neighboring fractions.

(iii) The rotor controls gradient resolution. Because of the small differences in BD between unlabeled and fully ^{13}C-labeled nucleic acids (~0.04 g ml^{-1}), very shallow gradients are required for optimal spatial resolution of light and heavy templates. The steepness of a centrifugation gradient is controlled mainly by the difference between effective g_{min} and g_{max} acting on a gradient, and thus by the difference between the inner and outer radius (r) of centrifugation. For SIP, this difference is optimally as small as possible. In essence, any large vertical, near-vertical or small ("table-top") fixed-angle rotor will produce gradients shallow enough for a good resolution of ^{12}C- and ^{13}C-nucleic acids. In contrast, although they have been occasionally used in the literature, large fixed-angle rotors generate much steeper gradients, where light and heavy nucleic acids band much more close to each other, with an unsatisfactory number of fractions to be resolved in-between. Even more so, SIP is technically impossible in classical swing-out rotors.

A.1.3 Strategies to substantiate label incorporation

As argued above, it is evident that the detection of a given template in a heavy gradient fraction alone does not substantiate label incorporation. For this, it is essential to show that a given template in heavy fractions is absent or less abundant in light fractions of the same gradient, that the appearance of heavy templates in gradients becomes visible over time (coupled to the consumption of the ^{13}C-substrate), and that this appearance is not observed to the same extent in gradients of unlabeled ^{12}C-control treatments. This evidence can be substantiated as follows:

(i) Entire gradients should be evaluated. Although early SIP studies relied on the use of ethidium bromide to visualize bands of unlabeled and labeled nucleic acids, it is now accepted that the fractionation of entire gradients offers a superior means to access the full information of density-resolved nucleic acids in SIP. Especially for intermediate ratios of label incorporation into given populations, either directly or via metabolic cross-feeding, results can be lost without gradient fractionation. After collection, gradient fractions can be screened by both quantitative (qPCR) and qualitative (fingerprinting, gene sequencing) methods to identify labeled taxa. The comparative allocation of specific nucleic acid templates to distinct BDs in gradients of ^{13}C-treatments vs. unlabeled controls provides the most relevant evidence for isotopic labeling. Since the advent of

high-throughput sequencing, the interpretation of SIP gradient fractions is now becoming frequently based on sequencing libraries, or even direct targeted metagenomics of density- resolved DNA.

(ii) Time series incubations should be conducted. The analysis of several successive time points allows monitoring the appearance of labeled templates in 'heavy' fractions over time, and to discriminate primary vs. secondary labeling effects in SIP (i.e., crossfeeding). Primary substrate consumers will always be more directly labeled, whereas metabolites, and even the degraders themselves, may be the basis for secondary label distribution via trophic interactions. Although a potential problem for data interpretation of single time points, crossfeeding can clearly be identified by time series incubations. In fact, this is a strength of SIP, allowing for the unraveling of trophic interactions and food web links in complex microbiota.

(iii) ^{12}C-controls must be regarded. As mentioned above, differences in BD between unlabeled and fully ^{13}C-labeled nucleic acids are very small (~0.04 g ml^{-1}), but can be resolved in SIP. However, BD differences of nucleic acids can be just as large due to distinct GC-content, which is especially problematic in DNA-SIP. Microbial genomes can vary between 35 and 75% GC-content, connected to intrinsic BD variation also of up to ~0.04 g ml^{-1} in CsCl. In effect, fully ^{13}C-labeled DNA of a low-GC bacterium can be of the same BD than unlabeled DNA of a high-GC microbe. This clearly explains the need for including ^{12}C-controls in SIP experiments, especially if labeling efficiency is low. The discrimination of true labeling effects (appearance of labeled nucleic acids) vs. GC-effects (growth of high-GC bacteria) is only possible if gradient results from labeled and unlabeled treatments are compared.

Literature:

Lueders, T. DNA- and RNA-Based Stable Isotope Probing of Hydrocarbon Degraders. In: Hydrocarbon and Lipid Microbiology Protocols; Springer Protocols Handbooks; Humana Press, 2015; p doi: 10.1007/8623_2015_74. https://doi.org/10.1007/8623_2015_74.

A.2 Practical procedures for SIP

A.2.1 Incubation of soil microcosms

- The ÖMIK Team will provide the students with a representative local soil sample named "Grüner Hügel" (GH)
- The soil will be sieved (2 mm) and 5 g of soil will be weighed into 125 ml MK serum bottles (8 x).
- Add unlabelled (^{12}C) or^{13}C-labelled substrates to one each of paired microcosms:
  - 0.2 ml g^{-1} soil of a 50 mM glucose solution
  - 0.2 ml g^{-1} soil of a 100 mM methanol solution
  - 0.2 ml g^{-1} soil of a 100 mM acetate solution optional
  - no amendment controls
- Add by cautious distribution of droplets across soil with a 1-ml syringe, 0.4 mm needle
- Close serum bottles, add 20 ml of air as overpressure reservoir.
- Take starting point gas samples (3 ml in Exetainers) for subsequent CO_2 quantification
- Incubate for 48 h at room temperature
- Take further CO_2 samples after 24 and 48 h.
- Sacrifice microcosms after 48 h and continue with DNA extraction (optional: freeze soil at -20°C)

A.2.2 Quantification of CO_2 by gas chromatography

Gas chromatography (GC) is used to measure soil respiratory activity via the quantification of CO_2 production. Measurements will be done on a Hewlett-Packard 5980 series II gas chromatograph (Palo Alto, California, USA), equipped with a thermal conductivity detector (TCD), a Hewlett-Packard 3396 series II signal integrator and a Chromosorb 102 column (Alltech, Unterhaching). Column details: Length: 2 m; inner diameter: 3.2 mm) with 100% helium as the carrier gas (15 ml per min). The injector temperature is at 150°C, the column temperature at 40°C, detector temperature at 175°C.

To determine CO_2 concentrations, defined standards of gas mixtures are prepared in bottles in a range of 0.5- 5 vol.-% CO_2 with argon as pressure gas. Standards will always be measured at the beginning and end of each measurement series.

- For each measurement, inject 100 µl of the gas phase of each microcosm sampled with a gas syringe (glass) and inject into the GC.
- Measure each microcosm 2-3 times for technical replicates.
- After measurement, the baseline of the peak areas of the gas samples is straightened with the chromatograph editing program (Eurochrom Version 3.05 P5, Knauer Gmbh, Berlin, Germany) for correct output of the actual peak areas.
- Convert peak areas to CO_2 concentrations in Excel.

A.2.3 Soil DNA extraction

- Each student should extract 2 replicates of DNA from one distinct soil microcosm.
- Prepared in advance: Add 0.2 ml (= 1 PCR cup full) of ~1:1 mixed 0.1 mm and 0.7 mm Zirconia/Silica beads (www.biospec.com) to 2 ml bead beating vial with screw cap, autoclave.
- add 800 µl PTN buffer (pH 8) and 100 µl 20% SDS
- add soil or sediment sample (~500 mg, <500 µl), so that vial is filled maximally to below gripping ring
- FROM NOW ON WORK IN FUME HOOD (PHENOL!) AND SWITCH GLOVES REGULARLY
- Add 200 µL Phenol/Chloroform/Isoamylalcohol (25:24:1) pH8.
- Check that vial is not filled to more than 1.75 ml with >250 µl headspace remaining
- Tissue Lyzer: 1 min at 30 Hz
- spin down 4 min at 14000 rpm & 4°C (as all subsequent centrifugation steps)
- take 850 µl supernatant, place in 2 ml “Phase Lock Gel Heavy” tube (Eppendorf)
- Extract by vigorous manual shaking with 1 vol (850 µl) Phenol/Chloroform/Isoamylalcohol (25:24:1) pH8, spin 4 min at 14000 rpm & 4°C
- take 800 µl (or as much as possible) supernatant, place in 2 ml “Phase Lock Gel Heavy” tube (Eppendorf)
- Extract by vigorous manual shaking with 1 vol (800 µl) Chloroform/Isoamylalcohol (24:1), spin 4 min at 14000 rpm
- take 650 µl (or as much as possible) supernatant, mix thoroughly with 2 volumes PEG (1300 µl)
- You can stop working in fume hood now
- Precipitate DNA by spinning at max rpm (16000) and 4°C for 30 min
- remove liquid with pipette (or decant if you dare, best only if pellet is clearly visible).

- Add 150 µl ice cold (-20 °C) 70 % EtOH, gently wash pellet, spin down (4 min, 16000)
- remove EtOH carefully by pipetting with a 200 µl pipette tip, dry pellet briefly on lab table (max. 5 min)
- Resuspend each DNA pellet in 50 µl EB buffer (can vary depending on expected yield), pool duplicates
- Suspend by gentle flipping, do not vortex,
- Briefly spin down, transfer to 0.5 ml Eppendorf-Cup, store at -20°C

Buffers etc.:

PTN Puffer: 120mM $NaPO_4$, 125 mM Tris, 0.25 mM NaCl, pH8	16,02 g/l Na_2HPO_4, 0,86 g/l NaH_2PO_4, 11,2 g/l Tris-HCl, 6,6 g/l Tris-Base; 1,46 g/l NaCl, adjust to pH 8 with HCl, filter sterilize, autoclave
20% SDS	20 g SDS in 100 ml H_2O, prepare with sterilized H_2O in baked glassware
30% PEG, 1.6 M NaCl; pecipitation solution	150 g polyethylene glycol 6000 + 46,76 g NaCl in RNAse free water, 500 ml final volume. Prepare in baked glassware, first dissolve PEG in microwave, adjust to final volume, autoclave
EB Buffer	10 mM Tris, pH 8.5, prepare with RNAse free water, filter sterilize, autoclave. Better: take from purchased Kits (Qiagen, etc.).

A.2.4 Optional: Removal of humic acids by Sephadex Gel Filtration

This step is optional and will be decided during the course. Only for impure DNA extracts with much co-extracted humics, iron oxides, etc. Depending on the soil, DNA extracts have to be purified to remove PCR inhibitors like humic acids.

Either use original Sephadex G50 powder (custom-made, higher purification capacity) or ready-to-use Sephadex DyeEx Spin Columns from Qiagen.

Protocol for DyeEx Spin Columns (Quiagen)

- All centrifugation steps at 750g (~2800 rpm), 2 minutes and 20°C (RT)
- gently vortex the spin column to resuspend the resin
- loosen cap a quarter turn, snap off the bottom closure
- place the column in a 2ml collection tube
- centrifuge 2 min (to remove the storage buffer)
- place the column in a fresh sterile 1,5 ml microcentrifuge tube
- fill 50 µl nucleic acids slowly onto resin-bed, do not touch the resin-bed surface with the pipet
- centrifuge 2 min, transfer flow-through DNA into fresh 0.5 ml cup, label, continue

Protocol for custom-made Sephadex Spin Columns

- Sephadex G50 must be pre-hydrated (15 min) in RNAse free water (1g resin in 25 ml H_2O)
- All centrifugation steps at 750g (~2800 rpm), 2 min and 20°C (RT)
- load 500 µl of hydrated Sephadex resin into BioSpin-Columns (BioRad, with collection tubes) and centrifuge 2 min
- Discard flow-through and repeat above step to add more resin
- equilibrate column 2x with 50 µl EB buffer (spin after adding)
- load 50µl of raw DNA extract in EB onto column and spin
- discard column and continue working with DNA effluent

A.2.5 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis is commonly used for visualization, separation and identification of DNA extracts and fragments. When agarose polymerizes (after melting in an electrophoresis buffer), a 'molecular sieve' structure forms within the gel that can be used to separate DNA fragments according to their length. Applying voltage, DNA fragments move through the gel towards the anode due to their negative charge. Short DNA fragments move faster through the 'molecular sieve' than long DNA fragments.

Agarose gels often prepared in a concentration ranging between 0.5% to 2.0%, depending on the desired separation efficiency of the target DNA fragment lengths (see table 1). Commonly used electrophoresis buffers are Tris base, e.g. TBE (Tris Borate EDTA) or TAE (Tris Acetate EDTA). To visualize DNA, a fluorescent dye (GelRed) that intercalates with the DNA double helix is added to the agarose gel. When exposed to ultraviolet light, it will fluoresce with an orange color that strongly intensifies after binding to DNA. Although less toxic than other popular dyes (e.g. ethidium bromide), it should be handled with care.

For security: Apply "good hand bad hand" principle! One glove only!

To increase the density of the DNA samples and make them sink into the wells of the gel, each DNA sample is mixed with a loading buffer that contains 30% Glycerol and a blue dye, Bromphenolblue (BPB, Bromphenolblau). In addition to the samples, a DNA marker / ladder is added into a separate well of the gel. The DNA marker contains DNA fragments of known size and concentration, acting as a reference for all unknown DNA samples.

A gel picture is generated after every DNA extraction or PCR step to visualize success of the step and integrity of nucleic acids.

Reagents (already prepared):

- 1x TAE (4 mM Tris-Acetat, 0.1 mM EDTA)
- 6x Loading buffer [0.1% w/v Bromphenolblue (BPB), 30% v/v Glycerol]
- Sybr Safe Nucleic acid stain dilution (1:1 dilution in TAE, 5000 x working conc.).
- DNA Marker: Gene Ruler 1 kb DNA-Ladder (Thermo)

Gel casting:

- Wear protective glasses and use special “hot-hand” glove!
- Agarose: Serva, 1.5 % gels in 1 x TAE.
- Weigh 0.6 g agarose into 200 ml-Erlenmeyer flask
- Fill up with 40 ml of 1 x TAE buffer, press “zero” on balance
- Boil in the microwave until agarose is dissolved (~2 x 1 min), mix after 1 min by swirling manually using a “hot-hand”
- Check volume by weight, compensate evaporation with MilliQ water
- Cool flask further under tap water
- Add 2 µl of Sybr Safe working solution, mix by swirling manually
- Pour the “not so hot” gel into gel cast tray on a flat surface avoiding bubbles, insert 2 combs

Electrophoresis:

- When gel has fully cooled, remove combs and put gel (in gel tray) into electrophoresis chamber
- Cover gel with 1 x TAE (1-2 mm of buffer over gel)
- Prepare the sample by mixing 5 µl of PCR-DNA with 2 µl 6 x loading dye (spots on parafilm)
- Load 5 µl sample into the appropriate wells carefully.
- Load 5 µl “ready to use” DNA ladder on both sides of each gel
- Run gel at 80 V for 45 min
- Note: DNA and RNA are negatively charged and thus migrate from cathode to anode (or from black to red colour)
- Visualize in UV-Chamber, check the length and intensity of your PCR-amplicons, take photo

A.2.6 Nano Drop quantification of DNA

NanoDrop is a UV-Vis (220 nm to 750 nm) spectrophotometer designed for microvolume (0.5 – 2 µL) analysis of DNA, RNA and protein samples. Besides direct concentration measurement at 260 nm (A_{260}), ratios of sample absorbance at 230, 260 and 280 nm provide information about the quality and purity of a nucleic acid sample. For the ‘260/280’ ratio, ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants, e.g. salts.

Procedure:

1. Start the NanoDrop (ND-ONE), select ‘dsDNA’ measurement on the home screen
2. Make sure that both “Auto-blank” and “Auto-Measure” functions are switched OFF!
3. Clean the surface of the measurement pedestal with 3 µl H_2O and a Kleenex
4. Add 3 µl H_2O, close the arm of the measurement pedestal, initiate blank measurement
5. Remove water blank with a Kleenex
6. Add again 2 µl of water and measure as “zero” sample, check if results and baseline are truly close to zero. If not, repeat cleaning and blank measurement.
7. Remove water with a Kleenex
8. Add 2 µl of DNA-extract and measure DNA. Document measurement, including concentration (ng μl^{-1}) as well as 260/280 ratios
9. Afterwards, again clean the surface of the pedestal with 3 µl ddH_2O and a Kleenex

A.2.7 Centrifugation of DNA-SIP gradients

The protocol and procedures described here are designed for 5.1 ml polyallomer quick-seal tubes to be spun in a VTI 65.2 vertical rotor. Volumes can be easily down- or upscaled for other tubes and volumes. For DNA, gradients should be prepared at an average BD of 1.71 – 1.72 g ml-1 CsCl before centrifugation. This will ensure an optimal resolution of unlabeled and labeled DNA into 'light' and 'heavy' gradient fractions after centrifugation.

- Per gradient, mix in an 15 ml Falcon tube:
  - 5 ml CsCl (~1.84g/ml)
  - up to 1000 µl of GB containing 5 µg of DNA
    (Nano Drop-quantified, volume must be subtracted from that of GB)
- Mix well, measure refractory index (75 µl aliquot) to control pre-centrifugation average density (RI should be 1.4042 ± 0.0002 = 1.72 g/ml CsCl).
  Adjust by adding 100 µl aliquots of GB or CsCl, when necessary.
- Transfer centrifugation medium into 5.2 ml polyallomer QuickSeal tubes (Beckman) using a 10 ml syringe with a 1.2 mm needle. No air bubbles in tube! Balance opposing tubes for rotor to ± 0.02 g.
- Seal tubes by welding with tube topper, put into VTi vertical rotor, don't close empty slots.
- Spin ~36 h at 20°C and 44.500 rpm (184 000 g_{av}).
- Brake setting to "5" to more gently slow down gradients after run.
- Carefully remove tubes from rotor, minimize any mechanical disruption and proceed immediately with gradient fractionation.

Buffers etc:

RI of pure water at 20°C: 1.3330 (check when using refractometer)

CsCl [~1.84 g/ml]. Add 50 g CsCl (Calbiochem) to 30 ml GB. RI should be at ~1.4164

Gradient Buffer (GB): 0.1 M Tris-HCl (pH 8 = 8,88 g/l Tris-HCL & 5,3 g/l Tris-Base), 0.1 M KCl (7,46 g/l), 1 mM EDTA (0,37 g/l). Prepare with RNase-free reagents in nuclease-free water, filter sterilize (0.2 µm), autoclave in baked glassware.

Fractionation:

- After centrifugation, carry rotor from centrifuge to bench with minimum eruption, remove tube from rotor and adjust within fractionation device.
- Fit sterile 0.4-mm needle to tubing from syringe pump, pump out air
- Carefully poke needle into centrifugation tube at bottom of nozzle. Poke needle minimally into opposing wall for better fixation. Careful: do not puncture opposing wall!
- Poke hole into bottom of tube with a sterile 0.4-mm needle.
- Start syringe pump at rate "7" (1 ml/min), collect 10-11 fractions (~500 µl) in sterile 2-ml cups by manual shifting rack every 30 sec. (for more fractions: 25 sec)

Time steps in 30 sec intervals:

1	2	3	4	5	6	7	8	9	10	11

0:30	1:00	1:30	2:00	2:30	3:00	3:30	4:00	4:30	5:00	5:30

Density measurement:

- Measure refractory index of fractions (75 µl from each fraction), start with lightest (= 13th) fraction. Take care to have refractometer in "RI" mode!
- Due to fractionation, the 11th fraction will contain some water and the refractory index may be much lower than expected. In this case, it should be discarded. If needed, the density can be estimated from the decreasing densities of the other fractions.
- Densities can be calculated from refractory indices by equation in Excel:
  $y = -11{,}293\ x^2 + 42{,}6513\ x - 35{,}9133$

Beware: this standardization has been empirically generated for the above gradient setup! A change in salt batch, concentrations or stocks make re-standardization necessary!

Nucleic acid precipitation:

- Precipitate DNA from fractions with 2 vol (~1000 µl) PEG each.
- Mix thoroughly; spin 30 min at max speed (16000 rpm) and 4°C.
- Take care that all 2-ml cups are orientated similar within the centrifuge, because pellets will not be visible after centrifugation and might be lost by pipetting during washing if localization is not known.
- Remove supernatant with pipet, don't discard or pellet might be lost.
- Wash with 150 µl 70% ice cold EtOH, spin 5 min at max speed
- Remove supernatant with 200-µl pipet
- Elute by placing 25 µl of clean (RNA-grade) EB Buffer on assumed pellet, shake 1 min in Eppendorf Thermomixer at 1400 rpm and 30°C to dissolve uniformly.
- Spin down 1 min at max speed, Place eluted nucleic acids in 8-cup strips with single caps

A.2.8 Amplification of bacterial 16S rRNA gene amplicons for Illumina-Sequencing

- Each student will continue working with one selected set of gradient fractions.
- Each DNA fraction is amplified in only one replicate. Do not forget negative and positive controls!
- Prepare following PCR Master Mix. Pipetting scheme for one PCR reaction (multiply n + 10 %):

2x NEB-Next PCR Kit	25 µl	
50 µM ilu_515f primer	0.3 µl	
50 µM ilu_806rN primer	0.3 µl	
PCR H_2O	22.4 µl	
DNA-template	2 µl	(not include in Master Mix!)

Thermal profile for Cycler:

95°C	3 min	
95°C	30 sec	}

55°C	30 sec	} 30-32 cycles
72°C	60 sec	}
72°C	5 min	
8°C	hold	

Continue with gel electrophoresis and amplicon purification, as adequate.
Amplicons are sent out for sequencing with a company. Further instructions will be provided.

PART B: FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

B.1 OVERVIEW

Fluorescence in situ hybridization (FISH) is a technique developed in the mid 1990s. FISH uses fluorescent DNA oligonucleotide probes to target taxon-specific sequence patterns in ribosomes (ribosomal RNA). This results in fluorescence signals for microbial cells of a certain group, lineage or taxon, that can be detected using a fluorescent microscope.

FISH of microbial cells always consists of four parts:

1) Fixation of the sample containing the target cells. Fixation stabilizes macromolecules and cytoskeletal structures thus preventing lysis of the cells during hybridization. Fixation also permeabilizes the cell walls for the fluorescently-labeled oligonucleotide probe molecules. . Standard fixatives are aldehydes and alcohols.
2) The fixed cells are incubated (hybridized) in a buffer containing the labeled probe at a specified temperature which favours the specific binding of the probe to the target. Ideally, only those probe/rRNA pairs will form which have no mismatches in the hybrid. Consequently, only target cells that contain the full signature sequence on their rRNA will be stained. The subsequent washing step will remove all unbound probe molecules.
3) Finally, the DNA of the hybridized cells is counterstained with DAPI and embedded in antifade mounting medium.
4) The hybridized and counterstained cells are then analyzed with epifluorescence microscopy or super-resolution microscopy.

***Safety*:** Be aware that most nucleic acid stains are believed to be mutagenic. In addition, the fixatives and formamide, which is used in the buffers, have to be handled with great care - use the appropriate gloves, work under the fume hood if necessary and dispose waste according to the waste management system.

Literature

Pernthaler, Jakob; Glöckner, Frank-Oliver; Schönhuber, Wilhelm; Amann, Rudolf. 2001. **Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes**. Methods in Microbiology, Volume 30, Pages 207-210, https://doi.org/10.1016/S0580-9517(01)30046-6

Fuchs, B. M., J. Pernthaler, and R. Amann. 2007. **Single cell identification by fluorescence in situ hybridization**, p. 886-896. In C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. Marzluf, T. M. Schmidt, and L. R. Snyder (ed.), Methods for General and Molecular Microbiology, 3rd ed. ASM Press, Washington, D.C.

Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer. 1992. **Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions**. Systematic and Applied Microbiology 15:593-600.

Llobet-Brossa, E., R. Rosselló-Mora, and R. Amann. 1998. **Microbial community composition of wadden sea sediments as revealed by fluorescence in situ hybridization**. Applied and Environmental Microbiology 64:2691-2696.

This protocol is a modified version taken from Pernthaler et al. (2001).

https://www.arb-silva.de/fish-probes/fish-protocols/

Table: Properties of the FISH probes. The names, target organisms, sequences, fluorophores and the fluorophore's specific excitation and emission wavelengths (Ex./Em.) of probes and the properties of the 4',6-Diamidin-2-phenylindole (DAPI) counterstain are given.

FISH probes	Target	Sequences (5'→3')	Fluorophore	Ex./Em. [nm]
EUB338	All Bacteria	GCTGCCTCCCGTAGGAGT	Atto-633 or Atto-488	630/651 or 500/520
BET359	*Burholderiales* (ex. β-Proteobacteria)	CCCATTGTCCAAAATTCCCC	Atto-633	630/651
GAM42a	γ-Proteobacteria	GCCTTCCCACATCGTTT	Atto-647	647/667
Ntspa-662	Genus *Nitrospira*	GGAATTCCGCGCTCCTCT	Atto-565	564/590
		Stains		
DAPI	all DNA	-	-	358/461

Table: Filter combinations of the ÖMIK Fluorescence Microscope

Filter set	Exitation [nm]	Emission [nm]	Dyes
"DAPI"	365 - 395	425 - 475	DAPI
"FITC"	450 - 490	500 - 550	Atto 488
"TXRED"	540 - 580	590 - 665	Atto 565
"Cy5"	610 - 650	660 - 735	Atto 633

B.2 Practical procedures for FISH

B.1.1 Fixation

Fixation of fresh sludge or biofilm samples (Llobet-Brossa et al., 1998).

Please also consider bringing an own biofilm sample (kitchen sink, aquarium filter, etc.)!

1) Fix biofilm samples with fresh 4% paraformaldehyde in a 1:1 mixture (250 µl + 250 µl, 2% end conc.). Cut pipet tips for handling sludge. Make sure PFA is not precipitated after thawing. Incubate over night at 4°C.
2) Wash: centrifuge at 16.000 x g for 5 minutes; pour off supernatant and resuspend sample with 1 ml 1 x PBS pH 7.6
3) Repeat step 2 twice.
4) Add 500 µl PBS, resuspend cells well. Stop here and continue with B.1.2 (without EtOH mixing) if fixed samples are not to be stored for a longer time.
5) Stop here and continue with B.1.2. Only for intended extra frozen storage, add 500 ml cold, absolute ethanol, mix well and store at -20°C or -80°C until further processing

Buffers etc.:

PBS Puffer	137 mM NaCl (8 g/L), 2.7 mM KCl (0.2 g/L), 10 mM Na_2HPO_4 (1.44 g/L), and 1.8 mM KH_2PO_4 (0.24 g/L). Autoclave.

B.1.2 Sample preparation

1) Label slides with pencil!
2) Mix fixed sample by flipping and dilute a small aliquot of 5 to 10 µl (depending on cell density and well size) 1:5 or 1:10 in MQ (10+40, 10+90 µl). Spot ~5 µl of diluted samples into wells of a gelatin-coated (multi-wells) microscopy slide.
3) Let air-dry at room temperature or 37°C for 30 - 60 min
4) Dehydrate slides in a 50%, 80% and 100% ethanol series for 3 minutes each, subsequently air dry at 37°C (or max. 46°C)

B.1.3 Hybridization

1) Prepare 2 ml of hybridization buffer (**Table 1**) in 2 ml Eppendorf tube.

Table1: Hybridization buffer, 35 % stringency

Reagent	Volume	Final concentration
5 M NaCl	360 µl	0.9 M
1 M Tris-HCl, pH=8.0	40 µl	20 mM
Formamide, deionized	700 µl	35 %
Sterile H_2O	898 µl	add to 2 ml
10 % SDS (add last)	2 µl	0.01 %

Note: The final formamide concentration depends on the probe used and determines the stringency of hybridization

Details on hybridization probes to be used are given above. Further probes may be selected!
Add 10 µl of probe working solution (10 pmol μl^{-1}) to 90 µl of hybridization buffer in a 0.5-ml microfuge tube; keep probe solutions dark and on ice.

2) Prepare hybridization vessels from 50 ml Falcon tubes: insert a piece of tissue paper into tube and soak it with the remaining hybridization buffer. Label tube.
3) [Use separate tubes for each concentration of formamide (not relevant in course)]
4) Add 10 µl of probe mix to the samples in each well and place the slide into the polyethylene tube (in a horizontal position. Act slow and carefully, so that the drops stay on samples!
5) Incubate at 46°C for 2 - 3 hours (also longer incubation possible)

B.1.4 Washing

1) Prepare 50 ml of washing buffer (**Table 2**) in a polyethylene tube and preheat to 48°C in a water bath

Table 2: Wash buffer	
Reagent	**Volume**
5 M NaCl	700 µl
1 M Tris / HCl	1 ml
0.5 M EDTA	500 µl
MilliQ	add to 50 ml
10% SDS (added last to avoid precipitation)	50 µl

2) Quickly yet carefully transfer the slides into preheated washing buffer (**work in fume hood: hot formamide !!**) and incubate for 25 min at 48°C (water bath)
3) Rinse slides gently with distilled H_2O, do not purge to keep fixed samples attached to slides. Let air-dry in the dark. **The wells have to be completely dry before embedding, otherwise a fraction of cells will detach during inspection**
4) For counterstaining cover each well with 10 µl of DAPI solution (1 µg ml^{-1}), incubate for 3 min; rinse slide first with distilled H_2O, then with pure ethanol and let air-dry in the dark.
5) Mount samples in Citifluor Anti-fade mounting medium (Citifluor Ltd, London, U.K). Add 8 µl per well. Wells have to be completely dry before embedding, otherwise a fraction of cells will detach during inspection.
6) Add a cover slip to the slide. Seal with clear Nagellack. Double stained and air dried slides, as well as mounted slides can be stored in the dark at -20°C for several days without substantial loss of probe fluorescence.
7) Probe-conferred fluorescence fades much more rapidly than DAPI fluorescence in the microscopic image, and UV excitation needed for DAPI will also bleach the probe signal. For counting, it is, therefore, safer to first quantify probe stained cells and subsequently all cells from the same field of vision in the DAPI channel;

Part C & D: Analysis and interpretation of (meta)genomic data

C.1 Introduction

Modern microbial ecology projects typically rely, at least in part, on culture-free sequencing of DNA or RNA extracted from complex substrates such as soil, water, or animal/plant tissue. In our lectures we have talked extensively about two broadly defined DNA-based methods for doing these culture-independent surveys of microbes in the environment: metabarcoding (also known as "amplicon sequencing") and metagenomics. In this sequence analysis section of the module, we will examine both methods, using some popular software packages. In order to do this, we must also learn about using remote computing resources, and how to work in a Linux command line environment.

Throughout the course, you have been given several theoretical lectures on bioinformatic methods, including some explanations of fundamental algorithms. You have also been given numerous examples of microbiomes in nature. Finally, you are creating data from a microbiome right now, during this practical course. In this section of the module, you will bring your new knowledge and your data together, to conduct a metabarcoding study on your soil samples, and a rudimentary metagenomic analysis of two public data sets.

We will cover three large topics, each of which will have a script associated with it that we as a class will generate together:

1. Scientific computing and Linux – script here
2. Metagenomic methods – script here
3. Metabarcoding methods – script here

Each night we will update the scripts to structure the discussion for the next day, and we will run through them in class together. You will have to adapt them to your own computing environment. Together they will become a record of our activity as a class, as we work through the bioinformatic pipelines. The living versions will be kept in the github repository links above.

C.2 Scientific computing

Many algorithms that we will use require large reference databases and examine large portions of experimental data in real-time. Modern studies generate sequence data that can also be quite large in size, sometimes multiple terabytes in size. We can safely say that bioinformatic analyses are very "memory hungry", sometimes requiring hundreds of gb of RAM to conduct more intensive calculations. Our data will be relatively modest in size, but even our datasets and databases will sometimes require enough memory to overwhelm the (random-access) memory resources of standard home or office computers.

Scientists address these issues of "big data" in two ways:

(1) Scientists typically minimize the non-essential parts of the programs that they write to implement their algorithms. This means that you will see very few Graphical User Interfaces (GUIs) used for bioinformatic pipelines, though some high-quality bioinformatic GUIs do exist. Instead, software for bioinformatics are usually called from text-based environments called command-line interfaces (also called "terminals" or "shells"). Because they are so minimal, these programs are also more flexible - they can be wrapped into other programs, including your own simple programs (scripts) that you write. Our first sessions will focus on learning BASH to use these programs, probably the most popular command-line interface for scientific computing.

(2) Scientists do a lot of remote computing, using computing clusters and other shared computing resources to conduct memory-intensive operations on their data. In order to conduct modern research, universities, governments, and corporations have invested in shared computing centers with the hardware necessary for this kind of research. For most of these very large computing resources, Linux/Unix-like operating systems are the most efficient and universal work environment. In our case, we will be using Ubuntu Linux virtual machines in the cloud computing resources of the German Network for Bioinformatics Infrastructure (de.NBI), who have kindly offered their support for this course.

C.2.1 Getting linux working locally

The first step to learning about scientific computing is to get access to a working Linux environment. You could install Linux (e.g. Ubuntu or Linux Mint) on your own computer. However, the step of getting Linux working on your computer can be complicated and your computer probably would not have enough resources to perform the required computations (> 64Gb of RAM), anyway. As we will perform the computations on remote computers made available by deNBI, we just need a way to connect and open a Linux terminal on these machines. There are several ways of doing it listed below. If you are a Windows user and don't want to experiment with a Linux installation, **please install Moba Xterm on your computer before the first day of the course**. It will allow you to open a Linux command line terminal on a remote machine as well as upload and download files to this machine. If it fails for you, we understand, and we will spend much of our first session debugging this step and the following so that everyone can get to their computing resources.

Mac users:

You may not know it, but you have been running something very close to Linux every time you opened your computer! Mac OS is in fact a commercial Unix-based operating system (fun fact: Android is another one). So leave your computer at peace, and just open the terminal application: use finder to go to the /Applications/Utilities folder, and double click "terminal".

PC users, Windows version >=10 :

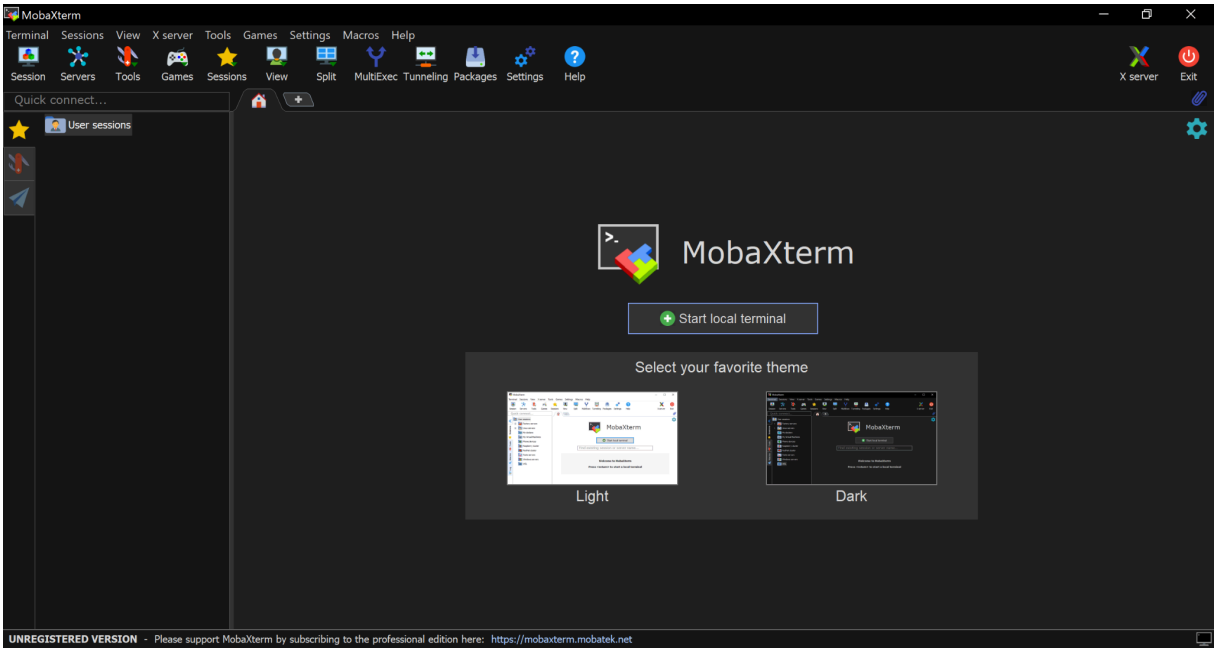
For most windows users in this class, we recommend the use of MobaXterm. To install this software:

1. Use the official download site. (https://mobaxterm.mobatek.net/download-home-edition.html)
2. Download the "installer" version, not the "portable" version.

3. Unzip (extract) this folder, click on the “MobaXterm_installer_23.6” file to start the windows installer.
4. You will be asked about firewall settings. For firewalls, I give MobaXterm all permissions, even public settings.
5. Find it in your applications, or on your desktop.

Note: we have not tested the more protective firewall settings for use with De.NBI servers from campus wifi, so if you choose the more secure firewall options, let us know how it works!

Start MobaXterm by clicking on the icon on the desktop or finding it in your applications. Then choose a color scheme and “Start local terminal”. This starts a local, linux-like terminal on your home computer. This is useful for playing with most linux commands, and handling your own windows files in a linux-like way.



If MobaXterm is running well for you, then you should be ready to try out some linux commands (see “Basic shell commands and symbols” below) and to make an ssh key pair (see “Remote computing section below”). Please try both before the start of the class.

(Note: in the MobaXterm terminal, Copy/paste can sometimes misbehave. If Strg-c and Strg-v don’t work, try Strg+Einfg for copy and Umschalt+Einfg for paste.)

PC users, Windows version <10 :

Finally, if you are running a really old version of Windows, let us know. We will find another solution.

C.2.2 Text Editors:

Much of the work of scientific computing is in setting up complicated commands. It often takes many tries to get the syntax correct for a single command. Because of this…

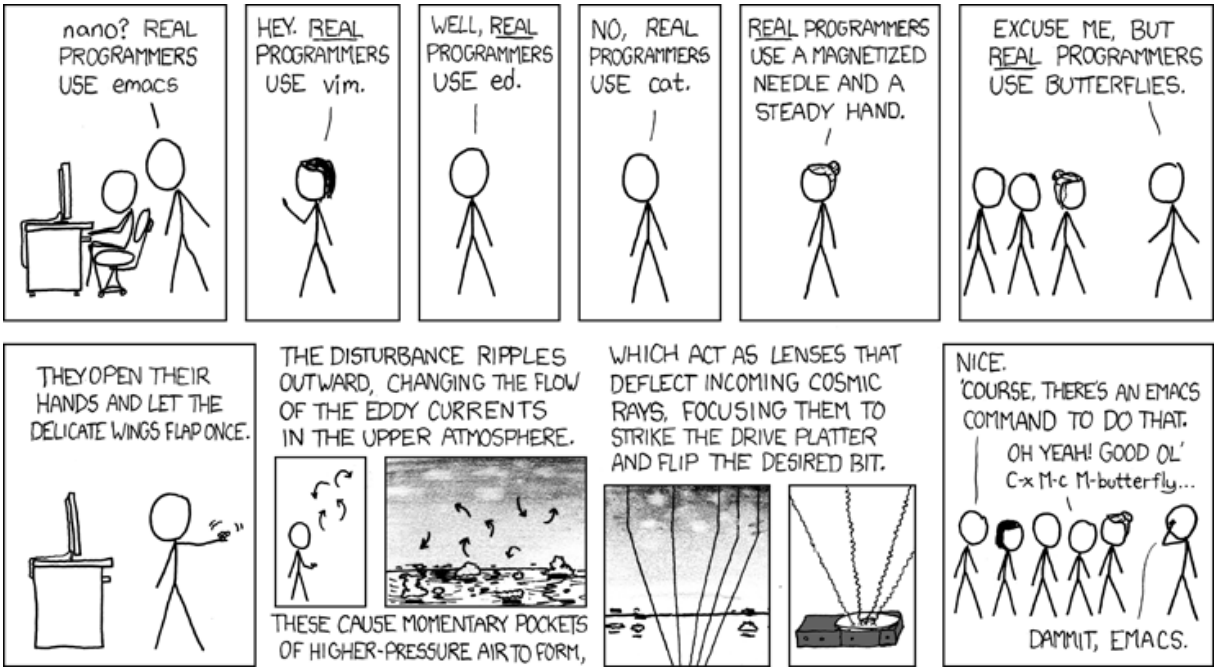
You need a text editor!!!

With it you should record all the linux scripting you do in a text file, even if it doesn't work at first. When using a command line program, it is usually best to write out a command in your text editor, then copy-paste to the terminal. If it works, you have the functioning code for re-use. If it doesn't, you can change it in the text file without having to re-type everything again.

Don't use a word processor as it adds 'invisible' format-related characters to make text look pretty. On the command line, every character counts and these formatting-related characters might have a meaning. For the windows environment, we recommend one of the following:

1. Notepad (which comes with windows)
2. Notepad++ (can recognise the scripting language and color command elements according to their function)
3. Text editor that comes with MobaXterm.
4. VIM (only if you want to keep scripting after this class, see next).

To understand the full importance of the issue of text editors for the programming and scientific computing communities, study the following graphic:



(Here is a full explanation of this graphic.)

C.2.3 Getting around a Linux Environment:

Basic shell commands and symbols

These are the universal BASH commands we will be using to get around and to manipulate our files, in addition to specialized bioinformatic programs. We will now try out each in our local BASH shells, so you can get accustomed to them.

pwd – print the current working directory

ls – list the contents of the current directory

cd – change directory

cp – copy a file

mv – move or rename a file

mkdir – make a new directory

echo – print something out to the display

cat – concatenate two or more files, or print the content of a file

rm [-r] – remove a file. Essentially deletes a file forever. Be careful!!

sudo – execute another command that requires root privileges

apt – access Ubuntu's native software package management system, for updating and upgrading software.

top – opens up a real time display that shows you the "busiest" processes ongoing in your machine. After you start the program, press `i` to see only active processes, press `e` to make memory units more readable, and press `1` then `t` to keep a close eye on your cores.

man – print the reference manual for a utility, command, or program, if available. If not, try the `--help` or `-h` flag for any given program without a manual.

head – show top several lines from a text file

tail – show last several lines from a text file

less – interactively open a text file for reading

Important symbols for BASH environment

We will also play with the following special characters:

~ home directory (try with **cd**)

. current directory (try with **cd**). Also filenames that start with "." are "hidden".

.. parent directory (try with **cd**)

> direct the output of a process to a file

$ variable "expansion". Put this in front of a variable after declaring it.

| pipe, connects the output from one command to another

; command separator

/ directory separator (compare to Windows!)

**** escape a character, so that it is no longer a "special character"

& send a process to run in the background

= variable assignment

***** wildcard for multiple characters (try with **ls**)

? wildcard for single character (try with **ls**)

comment (place it before a command and see what happens)

If a process needs to be repeated in the exact same way for different items, we can put these commands and symbols together to make loops. Loops repeat the same process several times, with only the variable changing. The different values for the variable are usually read from some sort of list:

for , **in** , **do** , **done** – commands for constructing a loop. Like this:

```
for i in *;
do
   echo $i
done
```

i is the variable.
* is a wildcard, makes a list of everything in our directory.
The **for** statement tells where to find values for the variable
The **do** statement tells what commands to execute for each of the variable values
done signifies the end of the loop.

Advanced programs

These are more complicated utilities that we will be needing quite frequently:

ssh – secure shell. This opens an encrypted terminal, usually on a remote machine, so you can use that computer as if it were right in front of you.

wget - a file downloader, using standard file transfer protocols.

conda – a software package and environment program that is used to handle the complex installation environments necessary for our kind of work. Conda should activate immediately when you log in to your de.NBI virtual machine. We will learn about the intricacies of Conda as we go.

nohup - a helper utility that we wrap around our big jobs, which tells our remote computer not to stop the process even if the connection breaks.

We will use numerous other programs and features of BASH. I will try to explain them as we use them. Use all the above to explore the terminal, and ask me questions as you wander. Be independent: remember that your computing environment will not look exactly like mine, you will have your own directory names, etc. Part of scientific computing is learning to adapt other's scripts to your own setup!

C.2.4 Remote computing

Now that your local Linux environment is working, let's use it to talk to your de.NBI virtual machine. We will use SSH to communicate with our de.NBI machines. To do this, you need to create an SSH keypair.

SSH Key generation

Secure login systems usually rely on an asymmetric, public/private keypair scheme. In such systems, you as a user create both a public key that you can give to servers, and also a complementary private key that only you control. When logging into a server that has your public key, you can prove your identity by providing the only key in the world that "fits" the public key, your very own private key.

We need you to generate your keyset, and following this we need to add your public key to your de.NBI virtual machine. To do this, in your local terminal generate a key pair, we'll use ssh-keygen. Start up your mobaXterm local terminal, stay in your home directory, and type…

```
mkdir .ssh/
cd .ssh
ssh-keygen -t rsa -f <nameOfYourKeyHere>
```

-t tells the algorithm which type of key generation algorithm to use.

-f is the name you want to give your key file

You will probably be asked to generate a password associated with your key. Keep this password, if you lose it you may need to set a new key.

This process will generate two keys. The public key should have a ".pub" extension, and can be shared freely. The private key will not have a file extension or ".ppk". If you are not sure, look at the file with **less** or **cat** or **head**, and it should say -----BEGIN OPENSSH PRIVATE KEY----- in the first line. Note that you can also use the MobaKeyGen utility in MobaXterm, under "tools". Either way, put both keys in somewhere you can find in your windows. Guard the private key with your life, and send us a copy of the public one in an email, with your name clearly in the email somewhere.

Logging into your de.NBI virtual machine

Once we have placed your public key into your assigned de.NBI machine, it should be ready to accept your login.

To get the login for your machine, you will use SSH. The correct SSH login command will be different for each of you, because your virtual machine has its own unique port number through which it is accessed. **As soon as we receive your public key**, we can activate your personal virtual machine (VM) from de.NBI. We can then generate your login information, and we will send you this information in an email. This login information will include your username, ip-address, and port number.

For example, when logging into my de.NBI VM from my home computer, I combine my username, ip-address, location of my private key file, and port number into the following command:

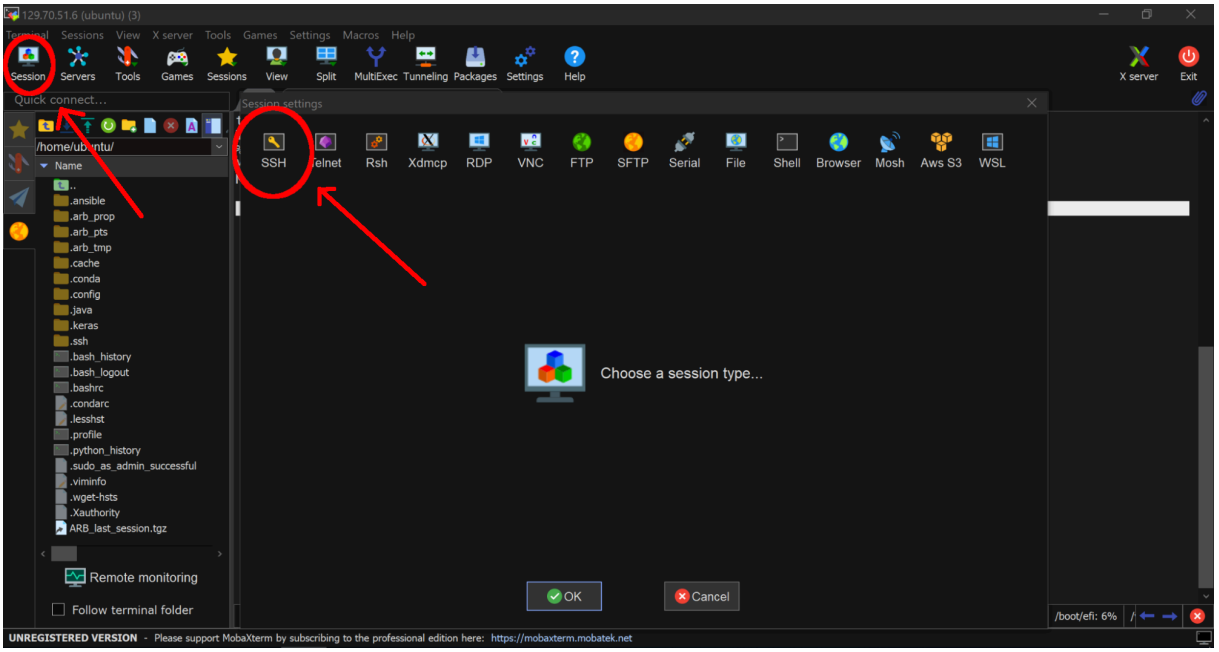
ssh -p 30192 -i </path/to/your/ssh/private/key> ubuntu@129.70.51.6

-p is the port number. Use only this port to interact with on your virtual machine.

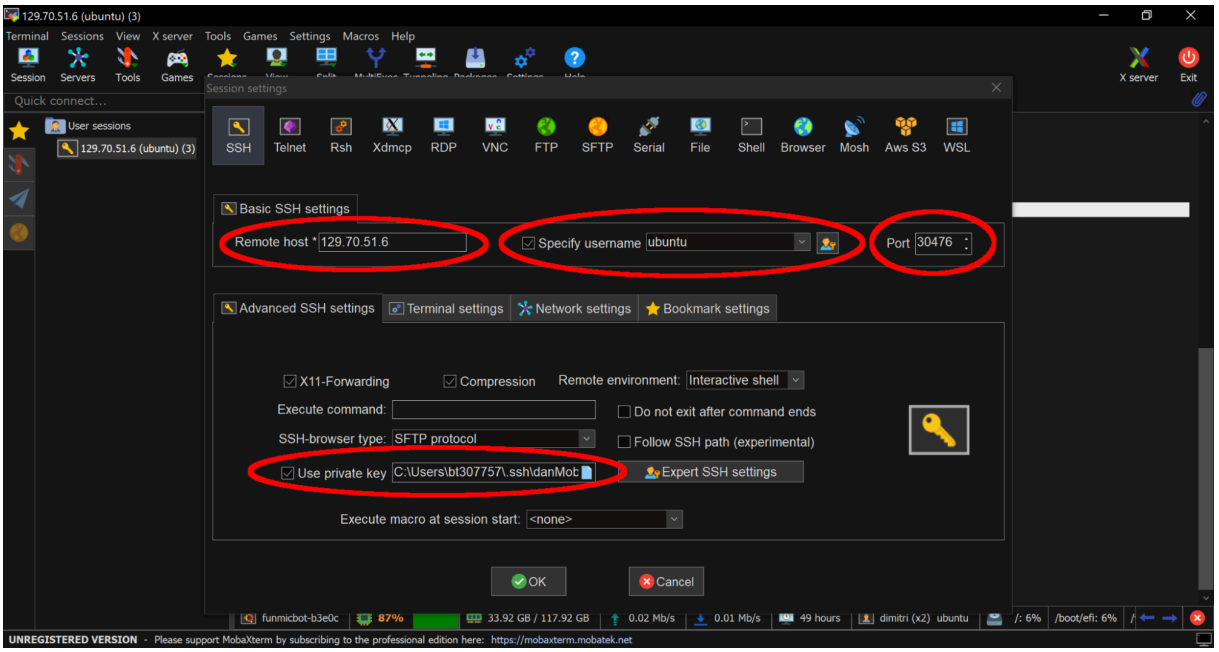
-i is where you have stored your private ssh key. If you followed the instructions above, this will be in your .ssh folder in your home directory.

ubuntu is my username, and probably will be yours too (assigned by de.NBI).

If you are using MobaXterm, instead of directly typing the **SSH** command, you can create a dedicated SSH session that you can use whenever you want to log into your de.NBI virtual machine. This is really convenient! To do this, start a new SSH session:



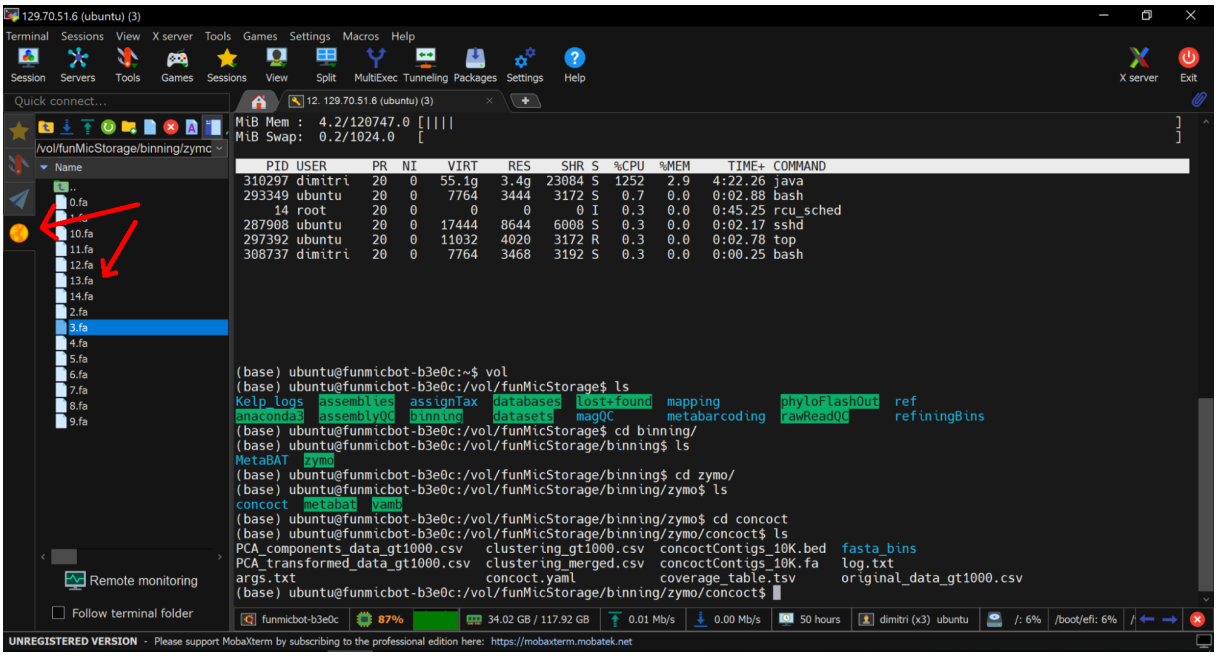
Copy the IP address, username, and port of your de.NBI virtual machine into the appropriate boxes. Open “Advanced SSH settings” and click “Use private key”. Put the path to your private key in windows here.



If all keys are in place, this should allow you to log into your de.NBI VM. This session can be re-used everytime you want log in with MobaXterm. Go ahead and explore it in just the same way you explored your local Linux filetree above, using your new knowledge of BASH commands (**ls**, **cd**, etc).

Getting files to your home computer from your de.NBI machine

In MobaXterm, when you are in an ssh session, you can download files with a few clicks, by selecting “SSH browser” in the left side-bar and navigating to the file you would like. You can drag this file icon over to an open folder in your Windows file navigator, or right-click on it and select “download”:



If you are using a Mac or older windows machine, let us know and we will use alternative ways to download your files from your de.NBI, such as **scp** or **sftp**.

Part D: Analysis and Interpretation of Sequencing Data

D.1: Metagenomic Methods

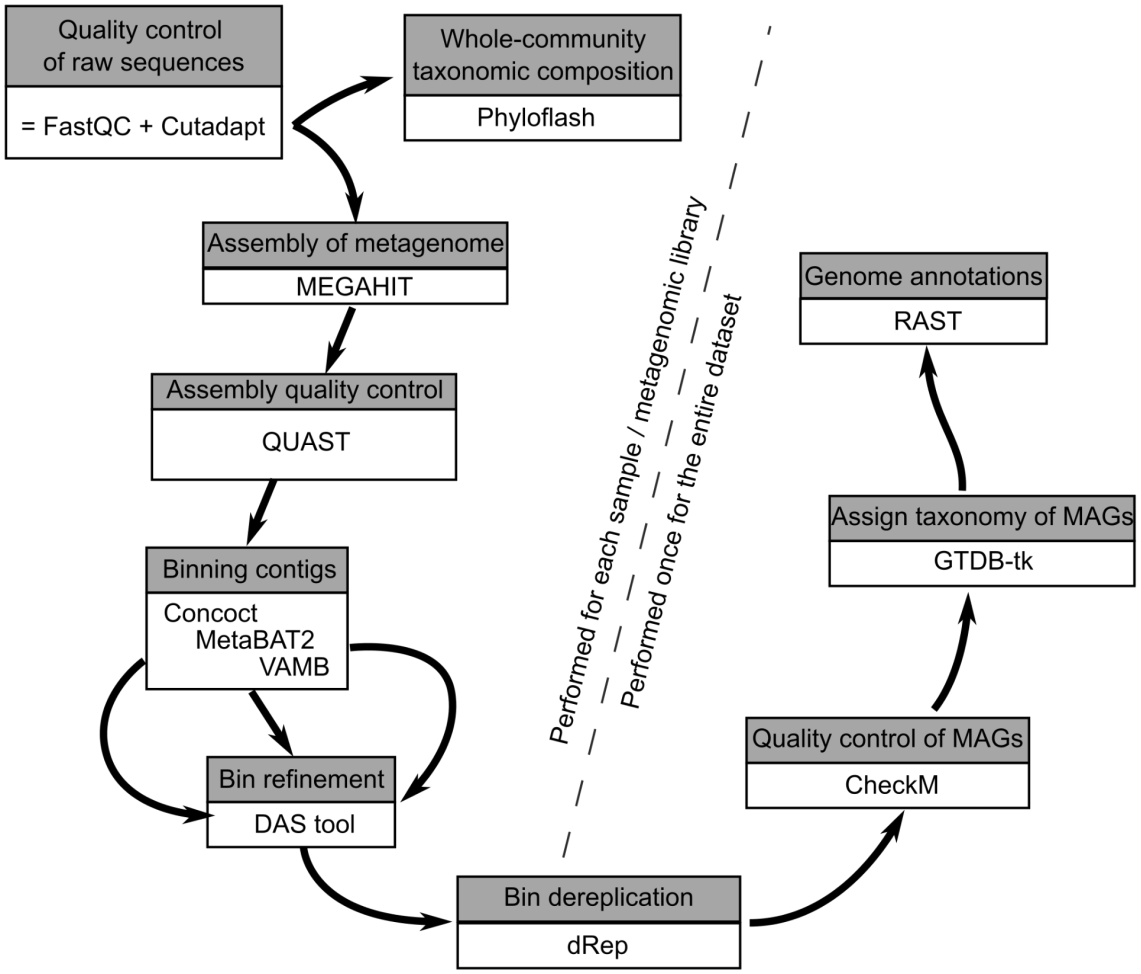
When direct sequencing of environmental DNA and RNA became possible, scientists developed two broadly-defined DNA-based methods for exploring the diversity in a given biological sample: metagenomics and metabarcoding (=“amplicon sequencing”). We’ve talked at length about both in lecture, so we won’t dive into intricacies here. Briefly, however, metagenomics involves randomly fragmenting all of the DNA available in a sample, and sequencing as many of these DNA fragments as possible, hopefully without bias. This generatess millions of reads that can be considered a statistical sample of the genetic material of all the organisms present in the biological sample, i.e. the metagenome of the sample. This sequenced sample of the metagenome can be handled in two ways.

Option 1 is to treat a metagenome like a single genome, and observe the metabolic pathways that are present in this “super-genome”. We often don’t need to know exactly which organisms are responsible for the presence of a metabolic pathway in which we are interested, and often numerous organisms are responsible anyway. From this method we can quickly mine our biological samples (soil, water, plant tissue, etc) for interesting genes and pathways. This was the classic method of handling metagenomic data, but it has some important limitations: i) attribution of a metabolism to a microbial taxon can be challenging (many functional genes are bad phylogenetic markers), ii) the analysis might identify “chimeric pathways” (e.g. all genes are present in the community, but never

together in the same genome), iii) the analysis might wrongly estimate the abundance of metabolisms in the community (e.g. cannot differentiate if 4 genes encoding a function belong to 4 different genomes or are found in just a single one, in 4 copies) among others.. All these limitations can be overcome if we know what DNA fragments and genes encoded on them belong to the same genome. This is why we will use a genome-centered approach to metagenomic analysis (see option 2).

Option 2 is to try to recover individual genomes from the soup of our metagenomic reads. This approach can give you some very-fine-grain information about the taxonomy and function of the most abundant species of microbes in your sample. Quality **metagenome-assembled-genomes** (MAGs) are also rapidly increasing our ability to resolve the functions of microbial groups that have never been cultured (see for example Nayfach et al. (2021)). However, this approach requires deep sequencing, meaning fewer samples per sequencer run, and more computational steps needed. Additionally, limited numbers of genomes are typically recovered, especially in very microbially diverse samples, and especially when sequencing depth is insufficient to power the de-novo methods of assembly used to discover genomes.

We will first explore our MAG-creation pipeline with publicly-available mock community dataset, taken from Sereika et al. (2022). You will then independently repeat it with a "wild" microbiome from a study on kelp decomposition causing growth of impressive massive white filaments of microorganisms (van Erk et al. 2020). The exact steps of our analysis will be in the code that we produce each day. Here is an overall schematic of the approach, with the particular software packages we will use:



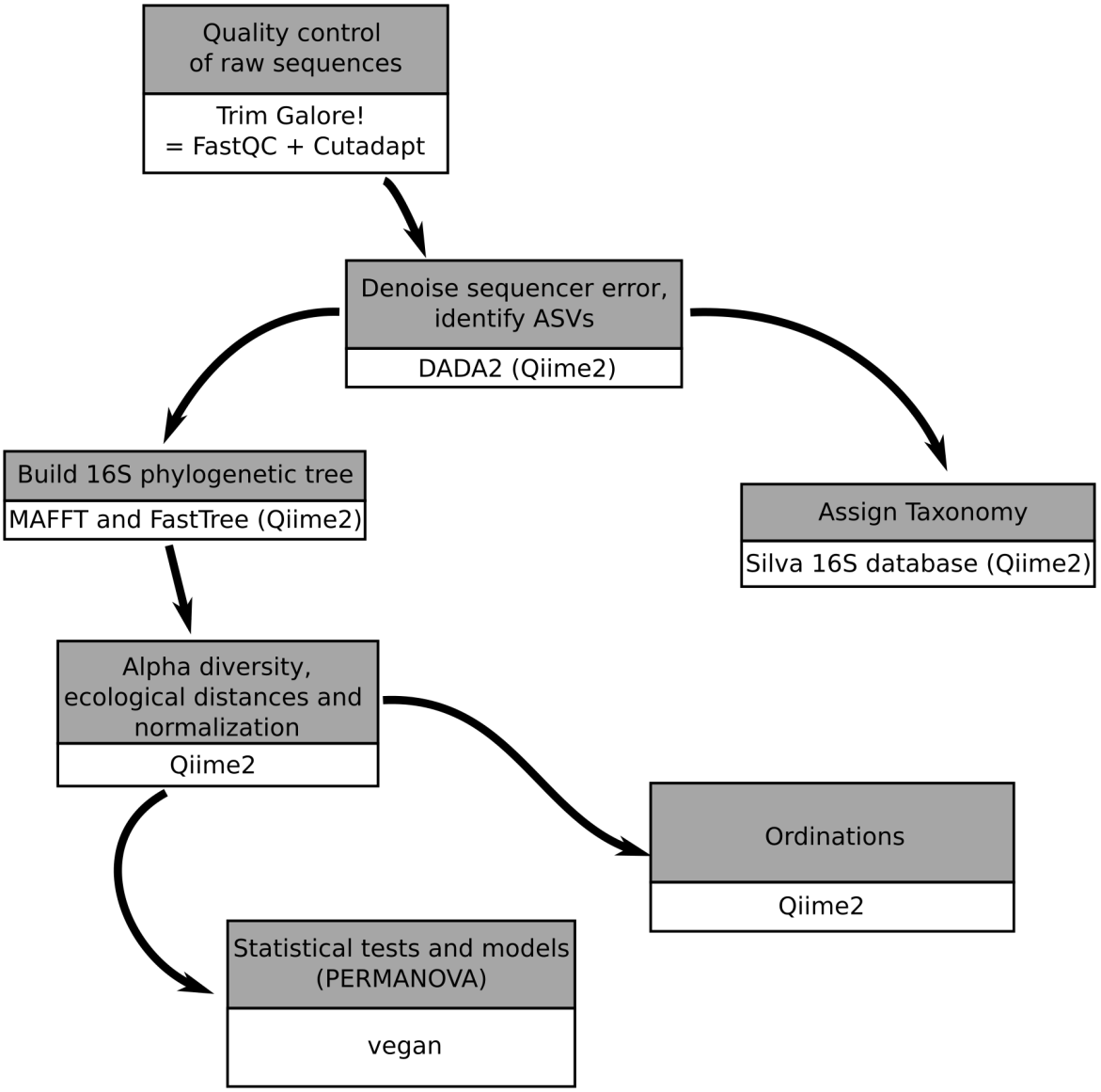
D.2: Metabarcoding ("amplicon sequencing") methods

In contrast to metagenomics, metabarcoding uses selective PCR or bioinformatic methods to target only a particular loci of the genome for all of a group of organisms in a biological sample. If taxonomy of microbes (e.g. prokaryotes and fungi) is of interest, ribosomal genes are typically targeted. These genes are useful barcodes because they are theoretically present in all life, in multiple copies, and differences in their sequences can often predict evolutionary relationships (taxonomy). By targeting these genes, we can draw a general picture of who is present in our sample, and perhaps even relative abundances (careful!!!!). We also begin to model changes in the microbial community structure by ecological predictors such as pH or moisture, if we have this data. In the case of prokaryotes, the 16S small subunit of the rRNA is commonly used. For your soil study with Dr. Lüders, you will be using primers that target the variable region 4 (16S-V4).

Other genes are possible, if your question is not taxonomy but function. For example, Nif genes can be used as a marker for nitrogen fixation, or secondary metabolic gene clusters can be sampled using different backbone synthase genes.

Generally, metabarcoding studies do not require the same level of deep-sequencing that metagenome studies require. Many more samples can sequence in a single sequencer run, sometimes as few as several thousand reads is sufficient to saturate diversity curves for a single

sample. As such, they are often a good “first step” to understanding your study system. As above, the exact steps of our analysis will be in the code that we produce each day. Here is an overall schematic of the approach, many of which we will execute within the Qiime2 pipeline:



Part E: Writing your Reports for sequence analysis section

Once you have run the Kelp-decomposition reads through the metagenomic pipelines, it’s time to think about what you have done, and look for some interesting results. Your report will show me that you had a conceptual understanding of each of the steps that we performed. For your report on the bioinformatic/sequence analysis section of the practical, there is no specific page requirement, but you should address all of the following:

E.1 Metagenomes:

E.1.1 Quality control of raw reads:

- How was the quality of the raw read library?
- How long were your reads on average?
- Were these single or paired-end reads?
- Was trimming necessary? And/or enforcing a minimum read length?
- Were 16s primers included in these reads? How do you know?
- Include figures that support your statements.

E.1.2 Metagenome assembly and community composition

- Give a general picture of the quality of your assembly. For example…
- How many contigs?
- N50?
- From your phyloflash results, are you able to say anything about the general bacterial/archeal community composition of your sample, from 16S data?

E.1.3 Binning and refinement of bins

- How did your binning process go? What worked, what did not?
- How many bins did you have before refinement by DAStool, and how many after? If there is a difference, tell me why that might be.
- Did you observe replicated bins? Were bins from some samples more complete or of higher quality than those from other samples? Why might this be?
- How is the quality of your refined bins? Use completeness and contamination metrics to explain your answer.

E.1.4 MAG taxonomic assignments

- Are your candidate MAGs closely related to anything that has been sequenced before? Do you think it is the same genus/species/strain? Support your answer using statistics from GTDB-tk.
- If not, what is the closest known MAG or reference genome? And do you think you have discovered an important new genome? You can address this more in your comments about the metabolic pathways of your organism below, if you like.
- If so, what is known about the ecology and metabolism of this closest relative?
- Do the genomes you find generally match your Phyloflash 16S community composition results above (section 1.2 above)? What mismatches between these results do you observe, if any? How do you explain this?

E.1.5 Genome annotation

- Given your results from RAST, what metabolic pathways do you observe in your MAG? How does this organism generate energy (electron donors, electron acceptors)? Where does it get it’s carbon from? etc.
- What role does this organism play in the ecosystem? You can review your 16S community composition results, or talk to other students about their MAGs. Do you find any interesting genes that indicate an important ecological function (e.g. primary production), or are of human interest (e.g. pathogenicity, antibiotic resistance, mutualistic/complementary function like essential amino acid synthesis, etc, etc)?

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- Have you encountered limitations of RAST annotation? Pathways that were not identified, genes that were wrongly annotated?
- Feel free to discuss any other interesting data points that popped up in your pathways analysis or in general in the metagenomics pipeline.

E.2 Metabarcoding

E.2.1 Quality control of raw sequences

- Were these single or paired-end reads?
- Was trimming necessary? And/or enforcing a minimum read length?
- How does the read length compare our metagenomics data? If it is different, why would this be?

E.2.2 Denoising

- What were the results of applying a denoising algorithm to your data? More specifically:
- Were many reads lost? If yes, why?
- How many genetically different organisms are predicted in your total data set?
- How deep is your sequencing, by sample, before normalizing (rarefying) your read depths?
- Do you feel good about this depth, or do you think more is needed? Support your answer with figures.

E.2.3 Community analyses of metabarcoding data

- After normalization (rarefying), can you compare alpha diversity among your samples?
- Show me ordinations from (1) a simple taxon-based dissimilarity coefficient and (2) a phylogenetically-weighted dissimilarity coefficient.
- Can you find any interesting groupings in these ordinations?
- If so, what explains these groupings - an experimental treatment? Or maybe a co-variate/confounding variable of some kind?
- What effect did changing the dissimilarity coefficient have on your two ordinations?
- Extra credit - what is the algorithm used to make your ordination? Can you find this information?
- How does this kind of beta-diversity-focused, community-wide analysis compare to the methods we used to detect ^{13}C-enriched species from your SIP experiment?
- Generalize your answer to the previous question a bit: what is the conceptual difference between community analysis methods like ordinations versus population-specific methods like the SIP approach? When is one appropriate, and when is the other useful instead?