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Methods in Environmental Microbiology and Bacterial Taxonomy

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I. INTRODUCTION

Methods applied for the analysis of bacterial strains and also those applied for the study of microbial communities inhabiting different environments have been changed significantly in the last decades, and therefore some of these methods were not introduced in many of general microbiological laboratories. Nevertheless, basic techniques used for the determination of general physiological properties (e.g. cell and colony morphology; basic cell wall structure; temperature, pH and salt tolerance of individual bacterial strains or their carbon source utilization and antibiotics resistance profile) are still in use and play an important role in several fields of microbiology (e.g. in the description of new taxa or in clinical microbiology). The most important ones among these techniques have been incorporated in our recent practical entitled '*Practical Microbiology*' (2013, Edited by: E.M. Tóth and K. Márialigeti). The purpose of that guide was to give a collection of exercises covering the most important fields of general microbiology, and to introduce the students into the work in a microbiological laboratory, to acquire the skills needed for aseptic work, to learn how to handle living cultures and to get familiar with the general techniques of microbiology. Therefore, the major aim to compile this practical was to give a collection of such a set of methods, which are applied currently in bacterial taxonomic analyses (e.g. description of new species and genera or revision of existing taxa) and those applied for the study of microbial communities in various environments.

At first sight, the two above mentioned fields (taxonomy and environmental microbiology) may be unrelated and gathering exercises related to these areas of microbiology may look constrained, although, the basis of many of environmental investigations is the correct identification of bacterial/microbial taxa, which serves as a link between taxonomy and environmental microbiology. Furthermore, many techniques suitable for the study of individual strains can be applied (with some modification and supplementation) also for the analysis of complex microbial communities. Therefore, in this practical book, we provide an overview of the procedures used in environmental microbiological studies and in bacterial taxonomy. Although this collection of methods is not exhaustive, most common classical phenotypic, chemotaxonomic and nucleic acid-based techniques, methods applied for the enumeration of bacteria and a survey of data analysis are presented here with a short note on the integration of these methods in the microbiological study of environmental samples and in bacterial taxonomy.

The Authors

Please consult Chapter 2 of 'Practical Microbiology' (2013, Edited by: E.M. Tóth and K. Márialigeti) for the safety regulations that should be applied during the work in a microbiological laboratory.

II. CLASSICAL PHENOTYPICAL METHODS USED IN BACTERIAL TAXONOMY (Erika Tóth)

II.1. Morphotypes of bacteria

The morphological characteristics of bacteria have diagnostic value.

Many cases, the colony morphology of bacteria strongly depends on the applied medium. In spite, growing in standardised circumstances on a given medium, the size, shape, elevation, margin, colour, surface, density, consistence of the colony or diffusible pigment production can be described **(Fig. II/1.)**.

Fig. II/1. Bacterial colonies on the surface of nutrient medium

All bacteria belong to a certain morphological cell type, though cultivation conditions, age of culture or the physiological state of bacterial cells all influence their appearance (size, shape, etc.), some of them have even morphological cell cycle.

To study the cell morphology of bacteria, most often stained preparations are examined by light microscopy, while details are examined by electron microscopy (fixation can modify cell morphology, therefore to examine cells in native preparations is sometimes also essential). The regular shapes of bacterial cells (coccus, rod, filament, etc.) are well known, but there are also bacteria with special, irregular shape(s) **(Fig. II/2-3.)**.

Simple staining

Simple staining is a direct way of staining. The bacterial cells can be stained with a positively charged dye which binds to proteins and nucleic acids, or with negative staining where the bacteria remain unstained against a dark background. Usually cell morphology is studied using fixed smears. In case of simple staining, only one dye is used (e.g. safranin - red, methylene blue - blue, crystal violet – violet, to stain the cells; India ink and nigrosine, to make visible the capsules in the dark background).
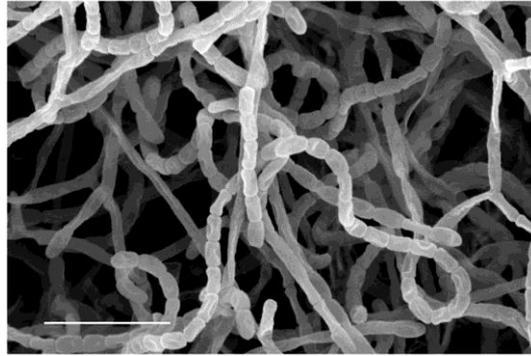
Fig. II/2. Scanning electron micrograph of mycelia of *Streptomyces* sp.

Spores are visible on the image (bar 5 µm).
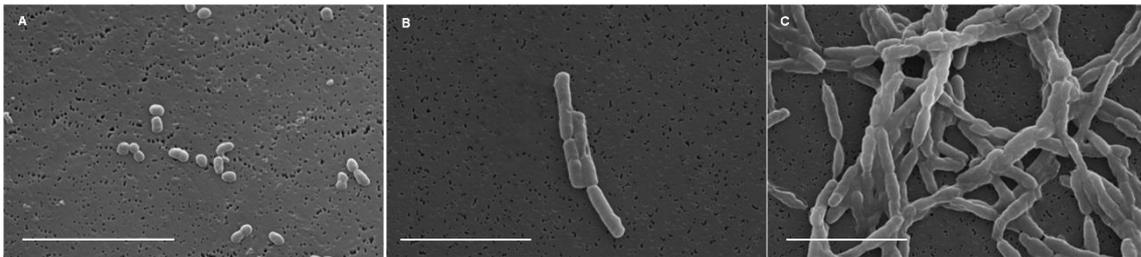
Fig. II/3. Morphological cell cycle of *Aquipuribacter hungaricus*

From coccoid shapes cells develop to rods then filaments are formed which can be carved again into short rods (bar 10 µm).

EXERCISE II/1.: Simple staining of bacterial strains

Object of study, test organism

Microorganisms with different cell morphology: 24 hrs slant culture of *Bacillus cereus var. mycoides*, *Eshcerichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Nocardia asteroides*, *Lactobacillus* spp., and 1 week old culture of *Micromomospora* sp. and *Streptomyces* sp.

Materials and equipment

glass slide and glass dropper dispenser
ethanol
inoculating loop
Bunsen burner
safranin/crystal violet dye solution
light microscope
immersion oil
benzene to clean lenses
wad of paper

Procedure

1. Degrease the surface of a glass slide then put it down on a staining stand with the degreased surface upwards, and let it cool down.

2. When it is cooled, label the degreased slide adequately.
3. Put a small drop of water onto the slide, then suspend a small loopful of bacterial culture in it. Thereafter make a film layer (smear) with the needle of the inoculating loop and let it dry.
4. Fix your preparation with heat over the Bunsen burner: move the slide over the flame carefully 2-4 times, do not warm it up strongly.
5. Drop safranin/crystal violet dye onto the fixed smear until it is fully covered and stain for 1 minute.
6. Wash the smear with tap water to remove excess dye solution.
7. Dry the slide.
8. During microscopy, first use 40×, then 100× objective lenses. In the latter case, use immersion oil.
9. Observe the morphology of the cells and draw the microscopic picture to be able to identify the bacterium after given standard preparations.
10. After finishing microscopic observation, clean all used objective lenses with benzene (do not use ethanol for this purpose as it can dissolve lens adhesives).

Study the Gram staining preparations of pathogenic microbes by using the Gram stain-tutor™

Gram staining preparations can be studied in tutor software, e.g. by Gram stain-tutor™. This software is adequate to check the morphology and possible staining characteristics of pathogenic bacteria. The software presents Gram staining micrographs of different microbes, including pure cultures and also that of complex samples (e.g. human/animal tissues).

II.2. Rapid identification of bacteria - multi-test systems

Many rapid multi-test systems for identifying bacteria are available nowadays **(Table II/1.)**. These systems offer the advantages of miniaturization and are usually used in connection with a computerized setup for the identification of (micro)organisms. Most are based on the biochemical reactions of strains. It is important to know that these systems are designed for the identification of particular taxa though multi-test systems can provide useful information on the physiological characteristics of microorganisms. The reactions may not always agree with the results from classical characterization tests or with the results obtained with other multi-test systems. Using multitest systems care should be taken to adhere to the instructions (e.g. inoculum age and size, incubation temperature) to retain adequate results.

The BIOLOG rapid identification system **(Fig. II/4.)** is based on the utilization of 95 different carbon sources, which could be used as sole source of carbon or energy for the tested microorganism. The result of the test is a kind of metabolic fingerprint of the given bacterium. The microtiter plates contain the carbon sources, a buffer, and also a redox dye (tetrazolium) in lyophilized form: when a compound is utilized, the redox dye is reduced and turns from colourless into violet colour (formazan) indicating the oxidation of the carbon source. The system can be connected to a microplate reader and computer database and analysis software. Based on its oxidation pattern the unknown bacterium strain can be identified.

Table II/1. Some examples for often used, commercial multi-test phenotypic characterization systems for bacterial identification

Manufacturer*	Test	Planned for	No. of tests
Biolog	Biolog test	General use. (Based on the reduction of tetrazolium salts when cells oxidise the given carbon source.)	95
bioMerieux	API 20E	Enteral Gram-negative rods	20
	API 20 Strep	Streptococci, enterococci	20
	API 50 CH	General use. (Based on the acid production from single carbohydrates.)	49
	API Staph	Staphylococci	10
	API ZYM	General use, enzyme tests	19
	Vitek GP I	Gram-positive cocci, coryneform rods	30
	API Coryne	Coryneform rods	20
BD Diagnostic systems	Crystal Gram-Positive ID	Aerobic Gram-positive bacteria	29
	Enterotube II	Enterobacteriaceae	15

*Web sites of manufacturers: Biolog, Hayward, CA. (www.biolog.com); bioMérieux, Marcy l'Etoile, France (www.biomerieux.com); BD Diagnostic Systems, Franklin Lakes, NJ (www.bd.com)
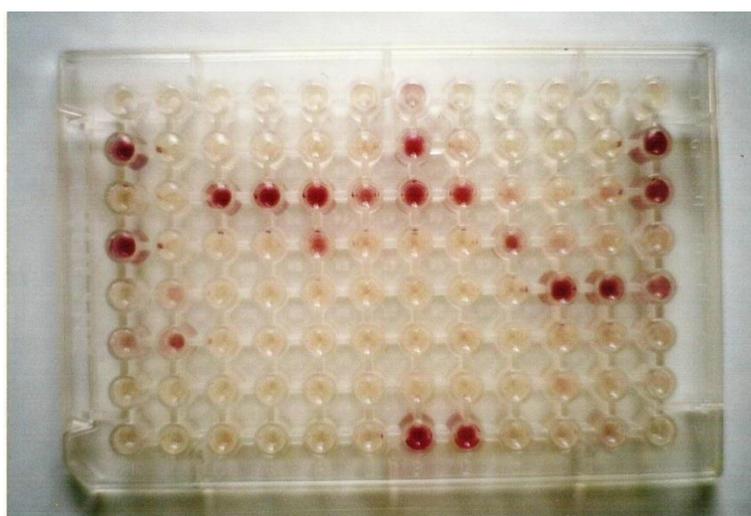
Fig. II/4. Biolog Gram-negative microplate test result after 24 hrs incubation at 37 °C

In the case of the Analytical Profile Index (API) system **(Fig. II/5.)**, different test panels are prepared in dehydrated forms which are used after addition of bacterial suspensions. After incubation, positive test results are indicated by a colour change or gas formation, etc. and scored as a profile. Identification of the bacterium is then easily obtained from the database with the relevant cumulative profile code book or software.

EXERCISE II/2.: API test to determine biochemical activity of different bacteria

Object of study, test organisms
> *Bacillus cereus* 24 hrs slant culture
> *E. coli* 24 hrs slant culture
> *Staphylococcus aureus* 24 hrs slant culture

Proteus vulgaris 24 hrs slant culture

Serratia marcescens 24 hrs slant culture

Pseudomonas fragi 24 hrs slant culture

Micrococcus luteus 24 hrs slant culture

Brevibacterium linens 24 hrs slant culture

or other strains of bacteria

Materials and equipment

API tests strips (API 20E, 20NE, 50CH or API ZYM)

API reaction containers

pipettes, sterile tips

sterile distilled water

5 mL physiological saline/test medium

vortex

turbidimeter

Gram-positive and Gram-negative bacterial OD standards

sterile cotton swabs

incubator

Procedure

1. Fill the wells of the container with sterile distilled water (moisture chamber).
2. Prepare suspension in physiological saline/test medium from 24 hrs slant cultures of bacteria using OD standards offered by the manufacturer.
3. Inoculate 1 mL suspension to the adequate liquid medium (physiological saline or test medium offered by the manufacturer).
4. Fill the wells of the API strips with bacterial suspension to the meniscus.
5. Close the containers and incubate them on the adequate temperature for the proper incubation time (24 hrs for API 50 CH, API E and API 20NE, 6 hrs for API ZYM tests).
6. After incubation check the results, register the results into an Excel table.
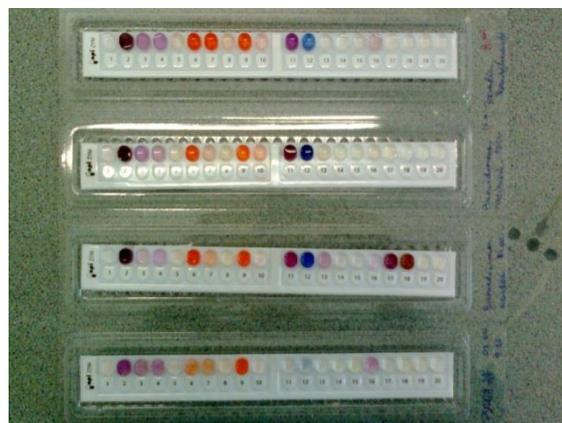
Fig. II/5. Result of API ZYM test of different bacterial strains after 6 hours of incubation at 37 °C

III. CHEMOTAXONOMICAL METHODS USED IN BACTERIAL IDENTIFICATION AND MICROBIAL ECOLOGY (Erika Tóth)

Chemotaxonomy in general is a method in taxonomy based on similarities/differences in the composition of certain compounds among the organisms, based on the analysis of chemical markers of the cell. Chemical data often can be used to define relationships at all levels of the taxonomic hierarchy (qualitative and quantitative analysis). It is possible to view all systematics as 'chemosystematics' since morphological characters, pigmentation, serology or the biochemical properties of organisms reflect their chemical composition.

These studied markers show discontinuous distribution among different taxonomic ranks. They must be characters which are more or less independent from cultivation conditions. At the same time there must exist a practical, simple method for isolation and characterisation of these marker molecules. In case of microorganisms the main chemotaxonomical biomarkers are present in the cell wall (peptidoglycan and derivatives, teichoic acids and derivatives, mycolic acids in many Gram-positive bacteria, etc.) and in the cytoplasmic membrane (lipid soluble pigments, isoprenoid quinones, polar lipids, fatty acids, etc.). Chemotaxonomy also deals with techniques yielding fingerprints or signatures of whole organisms (pyrolysis gas chromatography, mass spectrometry, infrared spectrometry, Raman spectroscopy, etc.).

III.1. The peptidoglycan structure of the cell wall of Bacteria

The basic cell wall structure of Bacteria is presented in **Fig. III/1.**
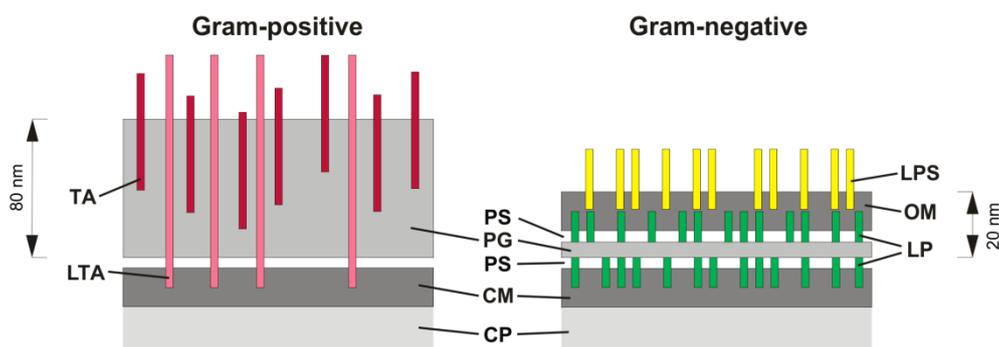
Fig. III/1. Cell wall structure of Bacteria

TA, teichoic acid; LTA, lipoteichoic acid; PG, peptidoglycan; CM, cytoplasmic membrane; CP, cytoplasm; LPS, lipopolysaccharide; OM, outer membrane; LP, lipoprotein; PS, periplasmic space

Both Gram-positive and Gram-negative cell walls of Bacteria contain murein (according to current scientific knowledge it does not exist in Mollicutes and Chlamydiae). A huge diversity is characteristic in the murein of the Gram-positive cell walls. Moreover due to the thickness of

peptidoglycan layer, the whole wall of Gram-positive cells can be isolated and analysed relatively easily. Peptidoglycan constitutes between 40-80% of the wall weight, it is a covalently linked net structure, the remainder is made up of other covalently-bound macromolecules.

Peptidoglycan has a constant amino sugar backbone (as depicted below) and amino acid side chain **(Fig III/2.)**. These oligopeptides of the peptidoglycan can be connected to one another and can form a net-like structure, called as murein. The most important and variable characteristic in cell wall structure is the composition of connecting oligopeptides and the structure of interpeptide bridges within the murein layer.
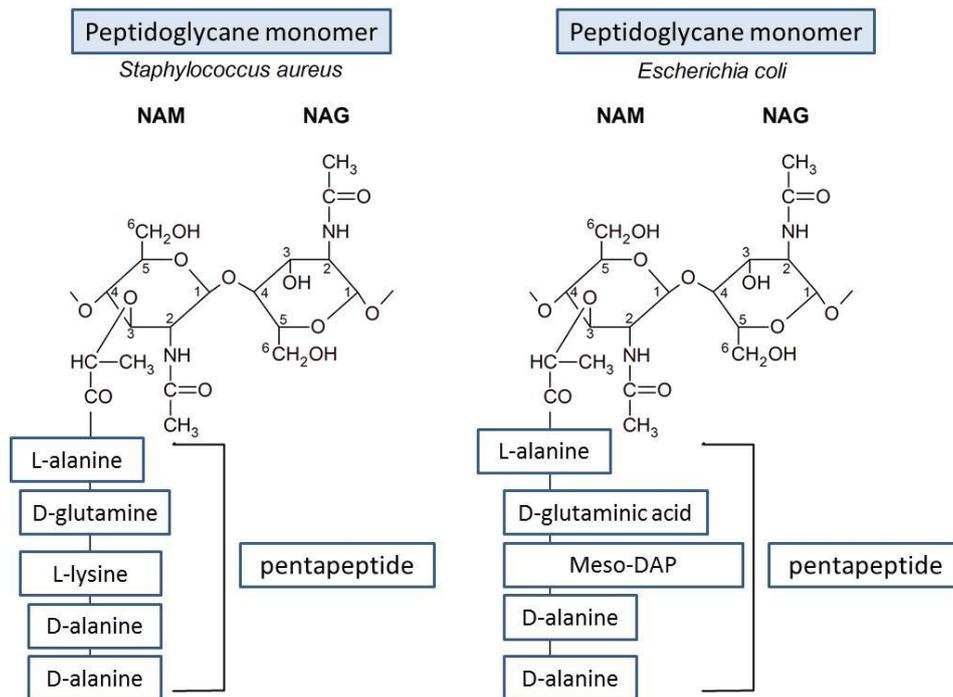
Fig. III/2. Basic peptidoglycan structure of different bacteria

NAM (*N*-acetylmuramic acid), NAG (*N*-acetylglucosamine), DAP (Diaminopimelic acid)

Different taxonomic ranks can be characterised based on that: if the diaminoacid of the cell wall of two bacteria differs, they cannot belong even to the same genus.

There are two basic types of peptidoglycan.

Peptidoglycan **A-type:**

In the 3rd position of the amino acid side chain, the diamino acid binds to a D-alanine in the 4th position of another oligopeptide chain: directly or through a bridge peptide. If there is an interpeptide bridge, it can show also characteristic variations.

Peptidoglycan **B-type:**

A D-glutamic acid of the oligopeptide in the 2nd position binds to a D-alanine of the other oligopeptide in 4th position through an interpeptide bridge.

During the characterisation of the cell wall of a given taxon the following questions are raised:

- ➢ Which diamino acid is present in the cell wall (TLC – thin layer chromatography determination)?
- ➢ Which other amino acids are present (TLC determination)?
- ➢ What is the quantitative ratio of amino acids (GC-MS determination)?
- ➢ Which enantiomers exist in the peptide chains (derivatisation - N-pentafluoropropionyl amino acid isopropyl esters, then GC determination)?
- ➢ What is the sequence of amino acids (determination of N-terminal amino acid)?
- ➢ Glycolyl or acetyl group on muramic acid occur in the molecule (colorimetric determination)?
- ➢ Which diagnostic sugars exist in the cell wall (TLC determination)?

The overall steps of peptidoglycan characterisation are shown in **Fig. III/3.**
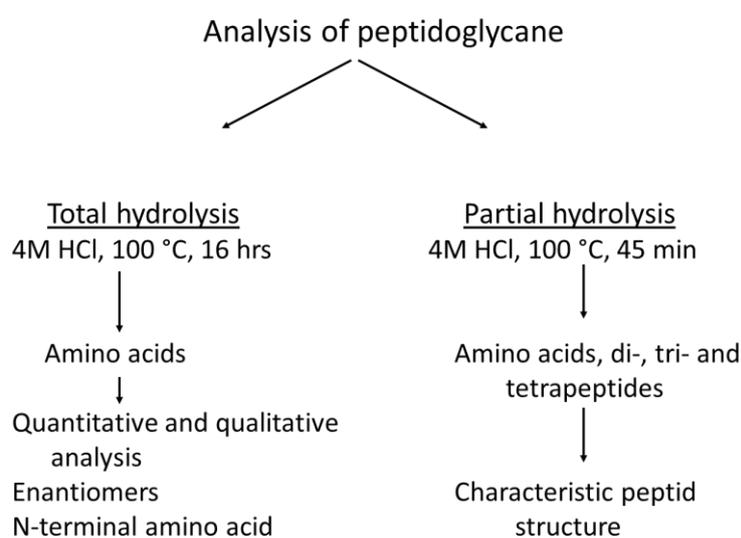
Fig. III/3. Analysis of peptidoglycan in Bacteria

There is no easy procedure available to prepare pure cell wall of bacteria. At the same time the cell wall diaminopimelic acid (DAP) content can be determined from whole-cell lysates as DAP can only originate from the bacterial cell wall (determination of all other amino acids is complicated, requires the preparation of pure cell wall). Usually thin layer chromatography (TLC) is used to investigate the DAP content using heat-treated purified biomass.

EXERCISE III/1.: Determination the DAP content of bacterial cells from whole cell lysate

The DAP content as well as the isomers of DAP of bacterial cell wall is determined during the practical.

Object of study, test organisms
> *Escherichia coli* slant culture
> *Brevibacterium linens* slant culture
> *Nocardioides hungaricus* slant culture

Tsukamurella paurometabolum slant culture
Arthrobacter variabilis slant culture
Bacillus cereus slant culture
Nocardioides sp. slant culture
Pseudomonas fragi slant culture
or other strains of bacteria

Materials and equipment

4 mL glass screw capped sample tubes with Teflon-lined screw cap
  containing 300 μL 6 M HCl solution
dry block thermostat with thermometer
laboratory oven
activated carbon powder
pipette, pipette tips, filter paper
cellulose TLC plate
glass developing tank for running TLC
solvents (methanol, distilled water, 6 M HCl solution, pyridine)
DAP standard solution (0.01 g/mL)
ninhydrin reagent (0.2 w/v% solution in butanol)

Procedure
1. Place 10 loopful of bacteria into a glass sample tube containing 6 M HCl solution, and close it tightly with the Teflon-lined screw cap.
2. For the destruction of cells (and cell walls), incubate it at 121 °C for 20 min in a dry block thermostat.
3. Spill the content into a watch glass and evaporate the remaining HCl at 70 °C in a laboratory oven.
4. Add 100-150 μL distilled water and dissolve the brownish precipitate.
5. To purify the precipitate, prepare a filter from a 1 mL pipette tip (using filter paper and activated carbon powder) and then filter the lysate through this filter tip.
6. Prepare the TLC plate by marking the starting line with a soft pencil, apply 8 μL lysate using a small pipette tip and hairdryer to get as small spots as possible. As standard, use 1% DAP solution (positive control).
7. Run TLC plates (using methanol : distilled water : 6 M HCl : pyridine in 80:26:4:10 ratio as solvent mixture) for 2-2.5 hours in a cooled room or in a refrigerator (at 4-10 °C). (To prepare solvent mixture use rubber gloves!)
8. For the visualisation of the chromatogram, spray it with ninhydrin reagent, and incubate the plates in an oven at 100 °C for 5 minutes. DAP forms greyish-coloured spots.
9. Evaluate the results, compare the DAP content of different bacterial strains. The stereoisomers of DAP will be separated, their retention factors differ strongly.

III.2. Study of isoprenoid quinones

Isoprenoid quinones are found in the vast majority of the aerobic or anaerobic respiring organisms and are located in their cytoplasmic membrane as mobile proton and electron carriers of their electron transport chains. In bacteria the two most important quinone groups are the ubiquinones and menaquinones. Based on their chemical structures, ubiquinones are 2,3-dimethoxy-5-methyl-1,4-benzoquinone molecules or its derivatives while menaquinones have 2-methyl-3-phytyl-1,4-naphthoquinone type structures **(Fig. III/4.)**.

The respiratory quinones have great taxonomic significance as the type of quinone present in an organism and the isoprenoid chain length often reflect the phylogenetic affiliation of a given bacterial taxon. Ubiquinones are widespread in nature, occur in plants, animals and many microbes, among them in most Gram-negative bacteria but never occur in Gram-positives or Archaea. Menaquinones are present both in Bacteria and Archaea: wholly unsaturated forms occur in Bacteria with peptidoglycan B-type and low G+C Gram-positives; partially saturated menaquinones occur in high G+C Bacteria (Actinobacteria) and bacteria with A-type peptidoglycan and also in some Gram-negative bacteria; completely saturated menaquinones appear only in Archaea (e.g. in *Pyrobaculum islandicum*). Cyclic menaquinones are genus-specific markers of genera *Nocardia* and *Skermania*.

Most organisms produce mixtures of either menaquinones or ubiquinones with different polyprenyl lengths, though one is generally the major respiratory quinone. When facultative anaerobic Gram-negative bacteria change their respiration to anaerobic from aerobic, they change also their quinone profile (e.g. *Escherichia coli* produces primarily ubiquinone-8 under aerobic condition, but menaquinone-8 is produced when it grows anaerobically on nitrate).

(a) **(b)**
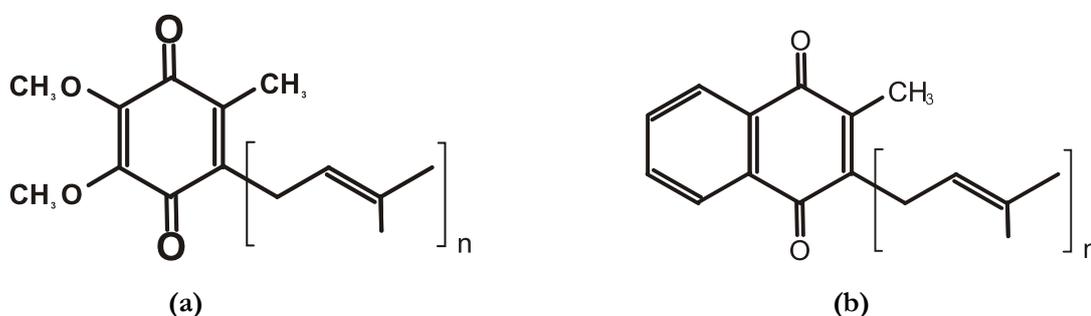
Fig. III/4. Molecular structure of quinones

(a) ubiquinone, (b) unsaturated menaquinone, 'n' is the number of isoprenoid units within the side chain

Isoprenoid quinones are sensitive molecules, can be easily degraded, so all extraction steps must be performed in protection from light, preferably in brown glass tubes to prevent photo-oxidation, and if possible under N_2 atmosphere.

EXERCISE III/2.: Determination the isoprenoid quinone composition of bacterial cells

Object of study, test organisms
  Escherichia coli lyophilised cell mass
  Brevibacterium linens lyophilised cell mass

Nocardioides hungaricus lyophilised cell mass
Pseudomonas fragi lyophilised cell mass
Arthrobacter variabilis lyophilised cell mass
Nocardia asteroides lyophilised cell mass
Tsukamurella paurometabolum lyophilised cell mass
or other strains of bacteria

Materials and equipment
chemical hood
solvents: methanol, chloroform, hexane, diethyl ether, acetonitrile, isopropanol
brown glass sample tubes with Teflon-coated screw cap
filter paper
magnetic stirrer
Silicagel 60 F_{254} TLC plate
pipette, tips
glass developing tank for running TLC
rotary evaporator
K_2 vitamin standard solution (menaquinone MK7)
ubiquinone Q10 standard solution
microcentrifuge
microcentrifuge tubes with filters
HPLC equipment
UV lamp

Procedure
1. Measure ca. 300 mg lyophilised bacterial biomass into a brown colour glass sample tube containing 10 mL chloroform : methanol (2:1) solvent mixture. Stir it overnight with a magnetic stirrer at 4 °C.
2. Filter the mixture through filter paper to remove cell debris.
3. Evaporate the extract in a rotary evaporator, and then collect the residue in 1 mL chloroform : methanol (2:1) solution.
4. Purify the samples with thin layer chromatography using Silicagel 60 F_{254} TLC plates (use K_2 vitamin solution as standard). This way quinones become separated from all other extracted organic materials (other lipids, pigments, etc.) as their retention factors strongly differ, and ubiquinones also separate from menaquinones. The average retention factor (Rf) of menaquinones is 0.8, while that of ubiquinones is 0.3.
5. Place the TLC plates under a UV lamp (254 nm). Quinones appear as greyish spots/stripes on the plates.
6. Scrape off the quinone stripes in a chemical hood and dissolve it in an acetonitrile : isopropanol (65:35) solvent mixture (overnight incubation at 4 °C) for the precise identification of the different quinone types. (To prepare solvent mixture use rubber gloves!)
7. Filter the silica debris using microcentrifuge tubes containing filters. This filtrate can be directly injected to the HPLC column (ODS Spherisorb).

8. Use the chromatograms of other authentic strains and standard Q10 as well as MK7 solution for data evaluation. There is a linear correlation between the logarithm of retention times and the number of isoprenoid units in the quinone side chain. This helps to identify the peaks on the chromatograms.
9. Evaluate your data on a semi-logarithmic paper (or with use of Microsoft Excel) and compare the quinone profiles of the different bacterial strains.

III.3. Study of the fatty acid profile of bacteria

Fatty acids can be used as chemotaxonomic markers to distinguish many closely related species by their qualitative and quantitative determination. Fatty acid composition can be used also to identify different bacteria, there are even automatic bacterial identification systems based on it (Sherlock MIDI – Microbial Identification System, http://www.midi-inc.com/pdf/MIS_Technote_101.pdf) **(Fig. III/5.)**.

Fatty acids are useful chemotaxonomic markers as bacteria have a huge variety of fatty acids: straight-chain saturated and unsaturated fatty acids, iso- and anteiso-branched fatty acids, internally branched fatty acids, hydroxy fatty acids, cyclopropane fatty acids, ω-cyclic fatty acids, dicarboxylic fatty acids, ladderane fatty acids, etc. Polyunsaturated fatty acids are rare, occur in bacteria that grow at low temperatures and in cyanobacteria.
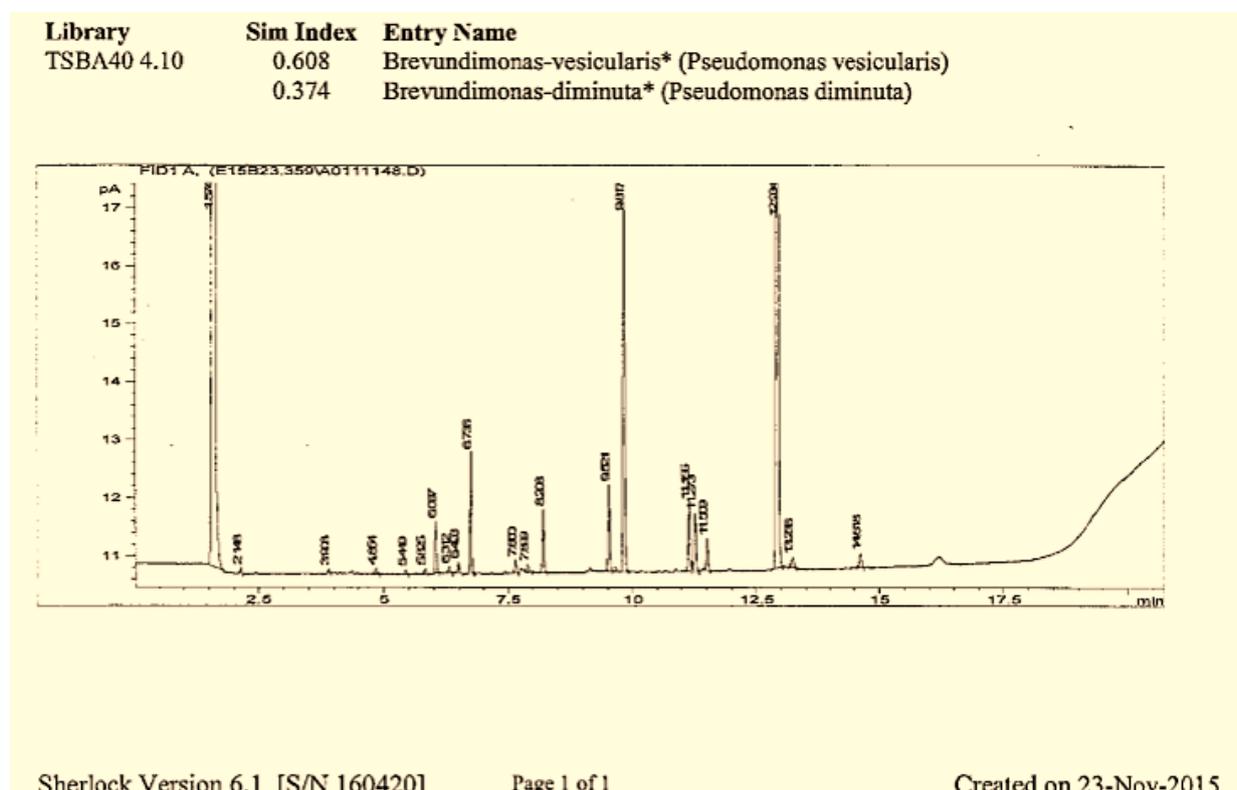
Fig. III/5. Fatty acid profile of a novel species candidate bacterial strain, closely related to the genus _Brevundimonas_

Profile was analysed by the Sherlock MIDI system.

The most important factor to obtain reproducible and comparable fatty acid composition of different bacteria is to standardize the growth conditions of the organisms being examined, since the levels of the different fatty acids vary with the growth temperature, the phase of growth and the medium composition, etc.

Obtaining pure fatty acids contains four steps: after standardised cultivation wet bacterial biomass used directly for saponification (liberation of fatty acids from fats and oils), esterification (to form fatty acid methyl esters), extraction (of fatty acid methyl esters with solvents) and purification of the extract (washing step).

Fatty acid composition is revealed by gas chromatography (GC) at the end and by comparison of the peak retention times of samples with those of known standards (BAME: Bacterial Methyl Ester). If a peak remains still unidentified, mass spectrometry can be used (GC-MS). The use of Sherlock Microbial Identification Systems (MIDI) (www.midi-inc.com) is common, as this system provides a highly standardized methodology.

EXERCISE III/3.: Determination the fatty acid profile of bacteria

Object of study, test organisms

Log-phase cultures (usually 24 hours) grown on TSA medium at 28 °C:
Escherichia coli
Brevibacterium linens
Nocardioides hungaricus
Pseudomonas fragi
Arthrobacter variabilis
or other strains of bacteria

Materials and equipment

10 mL glass sample tubes with Teflon-lined screw caps
Reagent 1 - for saponification (NaOH, methanol, distilled water),
Reagent 2 - for methylation (6 M HCl, methanol)
Reagent 3 – for extraction (hexane, tert-methyl-buthyl-ether)
Reagent 4 – for base wash (NaOH, distilled water)
vortex mixer
overhead mixer
degreased glass tubes
water bath
slush ice
degreased Pasteur pipettes
1 mL glass tubes for sample storage
microliter syringe
gas chromatograph (GC)
BAME (Bacterial Methyl Ester) standard solution

Procedure

1. Add 1 mL Reagent 1 to 2-3 loopful of bacteria in glass sample tubes, close with Teflon-lined screw caps.
2. Vortex and incubate at 100 °C in a water bath for 5 minutes (saponification).
3. Vortex again and put the tubes back to the 100 °C water bath for 25 min, then cool them down suddenly in slush ice.
4. Add 2 mL Reagent 2 and vortex.
5. Incubate it in 80 °C water bath for 10 minutes, then cool them suddenly in slush ice.
6. After this methylation process, add 1.25 mL Reagent 3 and mix with overhead mixer for 10 minutes.
7. Discard the lower phase using Pasteur pipette and add 3 mL Reagent 4 to the upper, 'solvent' phase, mix for 5 minutes. Storage of these fatty acid samples is possible in 1 mL sample tubes at -20 °C for a few weeks.
8. 1 μL from this liquid can be directly injected into a gas chromatograph. [(Conditions of GC analysis: splitless injection, capillary column (SPB-1, 30 × 32 mm id.), FID detector, 280 °C.)] Evaluate the data with the help of BAME (Bacterial Methyl Ester) standard and compare the results of different bacteria.

III.4. Study of the volatile fermentation end products

Short chain fatty acids are the most important intermediates or end-products of different fermentation processes. Fermentation pathways can be mapped with the study of these molecules. Such analyses have importance in the food industry and support also the classification of anaerobic, fermentative bacteria. The different types of fermentations are characteristic to a given taxon.

EXERCISE III/4.: Determination of the volatile fermentation end products (volatile fatty acids) of food samples

Object of study, test organisms
  various cheeses and other dairy products (e.g. yoghurt)

Materials and equipment
  microcentrifuge with tubes
  vortex mixer
  freezer
  50% H_2SO_4 solution
  methyl-t-butyl-ether (MTBE)
  $CaCl_2$ crystals
  microliter syringe
  standard solution (formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, valeric acid, isovaleric acid; **Table III/1.**)
  gas chromatograph

1. Pipette 1-2 g homogenised food product (cheese/yoghurt) into a microcentrifuge tube, and then add 0.05 mL 50% H_2SO_4 and 0.5 mL methyl-t-butyl-ether solution.
2. Vortex for 5-10 sec.
3. Centrifuge the mixture to break the emulsion.
4. Transfer the upper phase (ether-phase) to a clean microcentrifuge tube, then put it into the freezer (-20 °C) to freeze the remaining water.
5. Rapidly transfer the liquid phase to a clean microcentrifuge tube and put $CaCl_2$ crystals into the tube to extract all residual water.
6. Inject 1 μL to a GC from each sample and also from the control solution and determine the volatile acid-composition (GC parameters are given in **Table III/1.**).
7. Evaluate your data and compare the volatile compounds of different food products.

Table III/1. Chromatography parameters during VFA (volatile fatty acid) determination

Chromatography		Standard (in 100 mL distilled water)	
Column type	19001c-003 6 ft 10% FFAP 1% H_3PO_4	formic acid (50%)	0.114 mL
		acetic acid (96%)	0.037 mL
		propionic acid	0.075 mL
Detector type	FID (Flame ionisation detector)	isobutyric acid	0.092 mL
Column temp.	145 °C	butyric acid	0.091 mL
Injector temp.	180 °C	valeric acid	0.109 mL
Detector temp.	190 °C	isovaleric acid	0.109 mL

III.6. Mass spectrometric methods in bacterial identification

Mass spectrometry is the branch of analytical chemistry which runs on the determination of the mass of charged particles. It can be applied for the direct study of organic compounds or can be connected to different separation techniques (e.g. GC-MS, LC-MS). The charged particles are arisen in the ion source then in the mass-analyser they are transferred via an electromagnetic field meanwhile they are separated based on their mass/charge. Ions are detected and from the detected signal a mass spectrum is generated.

Ionisation can be reached on different ways. To study biological material, usually soft ionisation methods are used which do not fragment the studied molecule (e.g. Electrospray Ionisation – ESI; Matrix Assisted Laser Desorption Ionization – MALDI).

Characteristics and steps of mass spectrometry analysis:

➢ Sample transfer to gas phase
➢ Ionization of gas-phase particles
➢ Separation in an electromagnetic field by m/z (mass/charge)
➢ Detection

> ➤ Ion intensity sign transformation into mass spectrum

Parts of a mass spectrometer are shown in **Fig. III/6**.
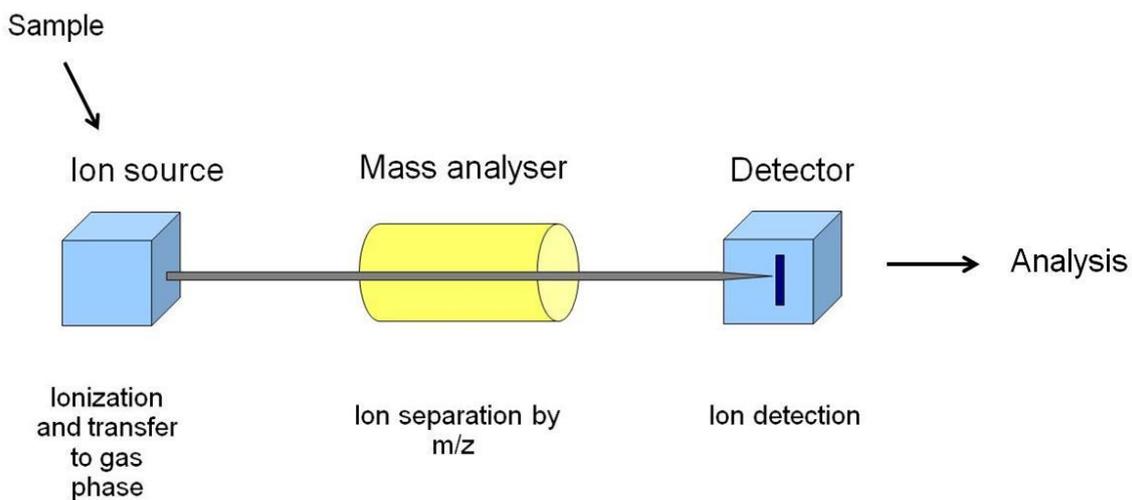
Fig. III/6. Principle of mass spectrometry

Ion source: releases the target ions from the sample. Mass analyser is responsible for the separation of ions. In the detector ions are detected and a mass spectrum is generated.

MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry)

Mass spectrometric methods are often used in microbiology, e.g. in clinical diagnosis, in quality control of food production and in ecological research. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is a technique which is often used in the last few years also in prokaryotic taxonomy. It is used also to assess given types of Bacteria and Archaea in host-associated environments (e.g. the human microbiota). Basic principle of MALDI-TOF MS is shown in **Fig. III/7**.

MALDI-TOF MS basically records the time of flight of proteins from the cell in order to derive a protein profile which can be compared with a database. Therefore it has high taxonomic significance and reproducibility when dominated by the peaks of ribosomal proteins. Ribosomal protein patterns are more or less resistant to changes in cultivation conditions. At the same time cells must be cultivated under optimal conditions, and must be harvested in a growth phase that ensures the highest yield of ribosomes. Shifts from optimal cultivation conditions can cause that protein production shifts toward the formation of stress proteins that may dominate the MALDI-TOF mass spectra. Changes in the physiological state of the cell, as in the case of sporulation of *Bacillus* species, also may drastically alter mass spectra.

The selection of the matrix and the composition of its solution have to fit to the analytical purpose and are crucial for the quality of mass spectra. Matrix which is used in MALDI must be a small molecular weight, relatively volatile compound, having absorption in UV or IR (to be easily excitable by laser), must have conjugated double bonds or an aromatic ring (e.g. alpha-cyano-4-hydroxy-cinnamic acid – CHCA).
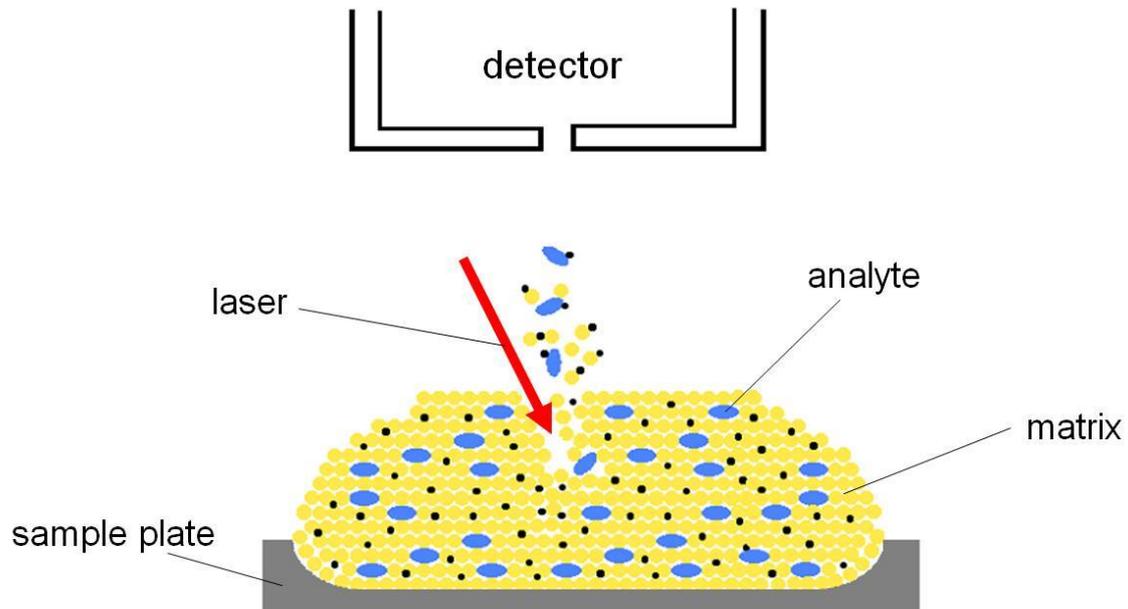
Fig. III/7. Basic principle of MALDI

Most often intact cell MALDI (the pure culture of the given organism is transferred to the sampling plate) is used in diagnosis and also in systematics, being very fast (<<1 minute from culture to identification) and cheap, in the case if the equipment is available in the laboratory. For identification a fingerprint database is needed.

MALDI-TOF MS can be used for:

- Species identification (bacteria, fungi, protozoa, cyanobacteria)
- Strain level identification (typing)
- Detection of toxins and antibiotic resistance, differentiation of pathogenic and non-pathogenic strains

Characteristics of MALDI:

- 'Soft' ionization
- No/minimal fragmentation of macromolecules
- Originally it is a protein analysis method
- Ionization of proteins, oligonucleotides, nucleic acids, lipoproteins, toxins
- Sample is mixed with a matrix
- Ionization by laser

The method is very quick, no preliminary preparation is essential before the analysis, at the same time the equipment is expensive and cultivation of microbes is obligatory, non-cultivable organisms cannot be tested with intact cell MALDI-TOF MS.

The method can be combined also with PCR-based techniques (e.g. detection of viruses, genotyping, SNP, MLST, moreover RNA molecules can be ionized easier than DNA).

III.7. Community level chemotaxonomic analysis

Chemotaxonomic analyses can be used to study also microbial communities of various sources (e.g. soils, wastewaters) where their biomass is high enough, as in chemotaxonomy there is no amplification of the target molecules. Markers are extracted directly from the samples and after purification of the components chromatographic analysis is applied (GC, HPLC). Fatty acids and respiratory quinones can be examined in more details. There are some universal markers, which do not give information about the structure of the community, as they are present also in plants, fungi and other eukaryotic cells. But there are some specific molecules which can be characteristic for different ranks of bacteria (e.g. OH-fatty acids are markers of Gram-negative bacteria, iso and anteiso branching fatty acids are characteristic for Gram-positives). The analysis of quinone compounds can give information also about the activity of a given community: being parts of the respiratory chain of different organisms, in detectable amounts they can be characterised only from actively respiring communities. The overall process of extraction, preparation and analysis are demonstrated in **Fig. III/8.**

Fig. III/8. Steps of community level chemotaxonomical analysis

IV. NUCLEIC ACID BASED TECHNIQUES USED IN BACTERIAL TAXONOMY AND MICROBIAL ECOLOGY (Balázs Vajna, Tamás Felföldi)

Nucleic acids are the information carrying molecules of all living creatures. While DNA stands for long-term information storage within the cell, RNA shows the actually expressed information. In other words, DNA represents possibilities, while RNA represents realisation. But it should be noted that there are several steps from transcribed RNA to functional proteins, which means, not even RNA should be regarded as direct functional information. From most samples both DNA and RNA can be extracted but in this practical guide we will focus mainly on DNA-based analysis.

Nucleic acid based information can be used for two basic approaches. First approach is to identify bacterial strains or community members mainly on the basis of the 16S rRNA gene. Identifying bacteria from communities is extremely important since we know that usually less than 1-10% of bacteria can be cultivated from most of the environments. Second approach is a functional one, when the possible functions of a bacterial community or a strain are checked based on the presence of genes coding the function.

In the following we describe the basic steps from the isolation of nucleic acids till the analysis of sequence information. Most of the steps are applicable both on community and on strain level. We summarize separate pipelines for species description and for studies of environmental samples in Chapter VII and IV.8.

List of materials and equipment necessary for every exercise in Chapter IV. Thus they will not be listed at each exercise again.

micropipettes, sterile pipette tips
microcentrifuge and PCR tubes
microcentrifuge tube rack
microcentrifuge
vortex mixer
molecular biology grade water (marked simply H_2O in this chapter)

IV.1. Separation of nucleic acids

Separation of nucleic acids has several aims. Usually the size of the DNA should be determined and compared with the expected length of the molecule, but these methods can be used also for fingerprint generation and purification. Three main techniques are applied: agarose, polyacrylamide and capillary gel electrophoresis.

Agarose is a polysaccharide polymer built up from agarobiose disaccharides forming helical fibres, which aggregate into supercoiled structure. The resulted three-dimensional mesh forms channels with diameters depending on the concentration of the used agarose. By polyacrylamide and capillary gel electrophoresis the gel is built up from polymerised and partially covalently crosslinked acrylamide and bisacrylamide molecules. In both cases the higher the percentage of agarose or acrylamide is, the higher the resolution of the gel is.

Agarose has the lowest resolution, not more than 10 bp for 1 kb range, but it is the easiest gel to handle. Capillary electrophoresis on the other side has a 1 bp resolution but it needs much more equipment. The required resolution of the electrophoresis usually depends on the question to be answered. For confirmation of the proper size of a desired PCR product agarose gel electrophoresis is generally satisfactory, whereas for high resolution pattern-analysis or separation of cycle sequencing products polyacrylamide (with a resolution of 1 bp) or capillary gel electrophoresis is needed.

Agarose and polyacrylamide gel electrophoresis can also be used for purification of the DNA sample, where the nucleic acid band with the desired size can be excised and purified from the gel. A specific DNA sequence can be identified with agarose gel electrophoresis via Southern blot.

DNA is visualized during or after the agarose and polyacrylamide gel electrophoresis with fluorescent nucleic acid dyes (such as ethidium bromide, SYBR Green, GR safe), usually intercalating into nucleic acids. Ethidium bromide is highly mutagenic, thus it is rarely used nowadays. Nevertheless every dye should be handled with care because their ability of binding to the DNA. At capillary electrophoresis, DNA fragments are fluorescently labelled and detected near to the end of the capillary via laser-induced fluorescence.

It should be noted, that nucleic acids can be separated via column chromatography, as well. This technique is used also as a part of the purification of the isolated nucleic acid and will be detailed in Chapter IV.2.
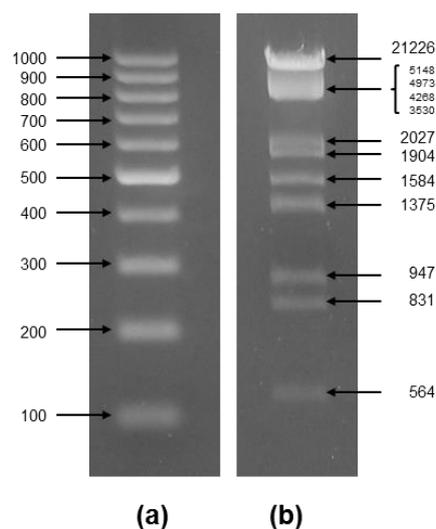
Fig. IV/1. DNA markers used for gel electrophoresis

(a) GeneRuler™ 100 bp (named Marker 100 in the followings), (b) Marker 3 DNA ladder in 1% agarose gel DNA ladder in 2% agarose gel. Sizes are given in base pairs (bp).

EXERCISE IV/1.: Agarose gel electrophoresis

This technique is used for checking the integrity and intensity of isolated genomic or environmental DNA, comparing the size of the PCR product with the desired value, checking its intensity and analysing basic fingerprints (ARDRA, RAPD, etc.).

Object of study
    bacterial genomic DNA, environmental DNA samples, PCR products

Materials and equipment
    laboratory scales
    measuring cylinder
    250 mL flask
    horizontal agarose gel electrophoresis system
    gel casting system
    agarose
    1× TBE (Tris-Borate-EDTA) buffer
    DNA stain (GR Safe nucleic acid stain)
    loading buffer
    Parafilm
    DNA ladder (e.g. Marker 3, GeneRuler™ 100 bp DNA ladder, **Fig. IV/1.**)
    gel documentation system

Procedure
1. The first step of agarose gel electrophoresis is gel preparation and casting. The percentage of agarose depends on the required resolution (see below). In case of 1% agarose gel, add 80 mL 1× TBE solution to 0.8 g agarose. Boil it until the agarose is completely dissolved, cool to approx. 60 °C, and add 1.5 µL DNA stain to the solution and mix. Insert combs into the gel casting system, and pour the agarose solution into it without creating bubbles.
2. When the gel has solidified (requires 30-40 minutes), remove combs, and place the gel into the buffer tank of the electrophoresis apparatus filled previously with 1× TBE solution.
3. Mix 5 µL DNA sample with 3 µL loading buffer on a piece of Parafilm, and load this mixture into the wells of the gel. To achieve a semi-quantitative measurement of the intensity and size of DNA, load 2 µL appropriate DNA ladder next to the samples.
4. Run electrophoresis for the desired time at the desired voltage.

Type of electrophoresis	% of the gel (agarose in 80 mL 1× TBE buffer)	Length of the electrophoresis[1]	Voltage of the electrophoresis[1]
General	1% (0.8 g)	20 min.	100 V
ARDRA	2% (1.6 g)	80 min.	80 V
RAPD, ITS-PCR	2% (1.6 g)	180 min.	80 V

[1]when using 14 cm long gel

5. Detect the presence and quantity of DNA under UV light. Compare the quality (discrete bands or smears; size of the DNA) and quantity (intensity of the signal) of the DNA samples with the ladder. Obtain a digital image if needed for documentation or image analysis.

EXERCISE IV/2.: Polyacrylamide gel electrophoresis (PAGE)

This technique is preferred, when higher resolution than agarose gel electrophoresis is needed (<10 bp), and/or the dominant DNA bands from a sample with many bands are needed to be separated and isolated. Traditionally polyacrylamide gel was used for Sanger sequencing. A modified version, DGGE (Chapter IV.7.) is used for comparing complex environmental fingerprints.

Object of study

      bacterial genomic DNA, environmental DNA samples, PCR products

Materials and equipment

      measuring cylinder
      50-mL Falcon tube
      40% acrylamide solution (acrylamide : bisacrylamide – 37.5:1)
      50× TAE (Tris-Acetate-EDTA) buffer
      ddH$_2$O (double distilled water)
      TEMED (tetramethylethylenediamine)
      20% ammonium-persulfate (APS) solution
      polyacrylamide gel electrophoresis system (Ingeny phorU-2) with gel casting system
      loading dye
      Hamilton syringe
      DNA ladder (e.g. Marker 3, Marker 100, **Fig. IV/1.**)
      gel staining box
      DNA stain (ethidium-bromide solution)
      scalpel
      gel documentation system
      computer with Totallab software

Procedure

1. Assemble the electrophoresis system as described in its manual.
2. Prepare 30 mL of 10% acrylamide solution in a 50-mL Falcon tube: mix 7.5 mL 40% acrylamide solution, 0.6 mL 50× TAE buffer and 21.9 mL ddH$_2$O. Add 60 μL APS solution and 25 μL TEMED to it, load it to the gel casting system and insert combs. Wait 2 hours for the polymerisation. (Acrylamide is toxic! Handle it carefully according to its safety data sheet!)
3. Put the gel casting system into the buffer tank of the electrophoresis apparatus filled previously with 1× TAE solution, and remove gently the combs. Wash the wells with 1× TAE solution using a Hamilton syringe in order to remove unpolymerised acrylamide particles.
4. Mix 10 μL DNA sample with 2 μL loading dye and load it with a Hamilton syringe into the wells of the gel. To achieve a semi-quantitative measurement of the intensity and size of DNA, load 10 μL appropriate DNA ladder next to the samples.
5. Run the electrophoresis at 250 V for 105 minutes.

6. After electrophoresis disassemble the gel electrophoresis system, stain the gel in ethidium-bromide solution for 20 minutes, and destain it for two times with incubation for 15-15 minutes in 1× TAE buffer. (Ethidium-bromide is toxic! Handle it carefully according to its safety data sheet!)

7. Detect the presence and quantity of DNA under UV light and obtain a digital image. The image can be analysed with adequate software (e.g. Totallab).

8. If excision of dominant bands is needed, cut them out with a sterile scalpel, and put them into 30 μL H_2O in an Eppendorf tube. Incubate at 4 °C overnight, thereafter transfer the liquid part into a new tube. Now it is ready for PCR or store at -20 °C for longer time.

EXERCISE IV/3.: Separation of DNA molecules with a model 2100 Bioanalyzer (Agilent Technologies)

Agilent 2100 Bioanalyzer is an on-chip capillary electrophoresis system **(Fig. IV/2.)**, which provides sizing, quantitation and quality control of DNA. Chips are commercially available for the analysis of RNA molecules and proteins, as well. In the case of DNA analysis, usually only 1 μL of sample is required and the resolution of ~5 bp can be achieved.

Object of study

> bacterial genomic DNA, environmental DNA samples, PCR products

Materials and equipment

> Agilent 2100 Bioanalyzer
> chip priming station
> Agilent High Sensitivity (HS) DNA kit:
>   DNA chips, electrode cleaner, DNA marker (mixture of two marker with sizes of
>   35 and 10,380 bp), dye concentrate, syringe, gel matrix, DNA ladder (mixture of 13 DNA
>   fragments having different size), spin filters, electrode cleaner chip
> vortex mixer (IKA Model MS3 equipped with the proper chip adapter)
> PC equipped with the adequate software (2100 Expert)

Procedure

1. Allow the HS DNA dye concentrate and HS DNA gel matrix to equilibrate to room temperature (this takes approximately 30 minutes, protect from light).

2. Replace the syringe in the chip priming station: unscrew and discard the old syringe, remove the plastic cap of the new syringe and insert it into the clip, then screw the new syringe to the chip priming station.

3. Adjust the base plate in the chip priming station to position C and slide the lever of the clip down to the lowest position. The plunger of the syringe should be positioned at 1 mL mark.

4. Start the 2100 Expert software on the PC, check the connection between the instrument and the PC.

5. Vortex HS DNA dye concentrate for 10 seconds and then spin down.

6. Pipette 15 μL HS DNA dye concentrate into the HS DNA gel matrix and mix by vortexing.

7. Transfer the mixture to the spin filter, centrifuge for 13 minutes at 5500 g. Discard the filter.

8. Take a new chip out of the sealed bag and place it to the chip priming station.

9. Pipette 9.0 μL gel-dye mix into the well marked with a white G letter (surrounded by black background). Close the priming station and press the plunger of the syringe, wait for 60 seconds, then release the plunger to the position at 0.3 mL mark, wait for 5 seconds and release plunger completely (position at 1 mL mark). Open the priming station.
10. Load 9.0 μL gel-dye mix to each well marked with black G letter.
11. Vortex and spin the samples and the HS DNA marker and ladder before loading.
12. Load 5.0 μL HS DNA marker into the well marked with a ladder symbol and also into the 11 wells for the samples.
13. Load 1.0 μL HS DNA ladder into the well marked with a ladder symbol.
14. Load 1.0 μL of sample and 1.0 μL of HS DNA marker into each sample well.
15. Place the chip into the adapter of the vortex mixer horizontally and vortex for 60 seconds at 2400 rpm (with slowly increasing and decreasing vortex speed).
16. Insert the chip into the electrophoresis system, close the lid and start the run within 5 minutes. Select the appropriate assay in the software, enter sample information and click 'Start'.
17. After the run is finished, remove the chip, and clean the electrodes (fill 350 μL H_2O into one well of the electrode cleaner chip, place the chip to the Bioanalyzer, close the lid, wait for 10 seconds, remove the cleaner chip, and allow the water evaporate from the electrodes before closing the lid of the instrument).
18. Evaluate the run according to the guidelines given in the manual provided by the manufacturer.
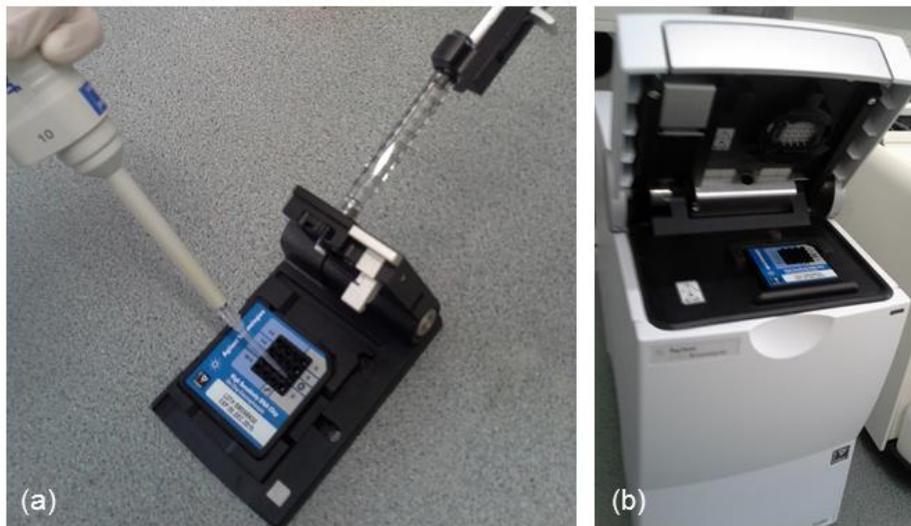
Fig. IV/2.a-b The Agilent 2100 Bioanalyzer on-chip electrophoresis system

(a) Loading the chip on a chip priming station and (b) the set-up of the electrophoresis system with an inserted DNA chip.
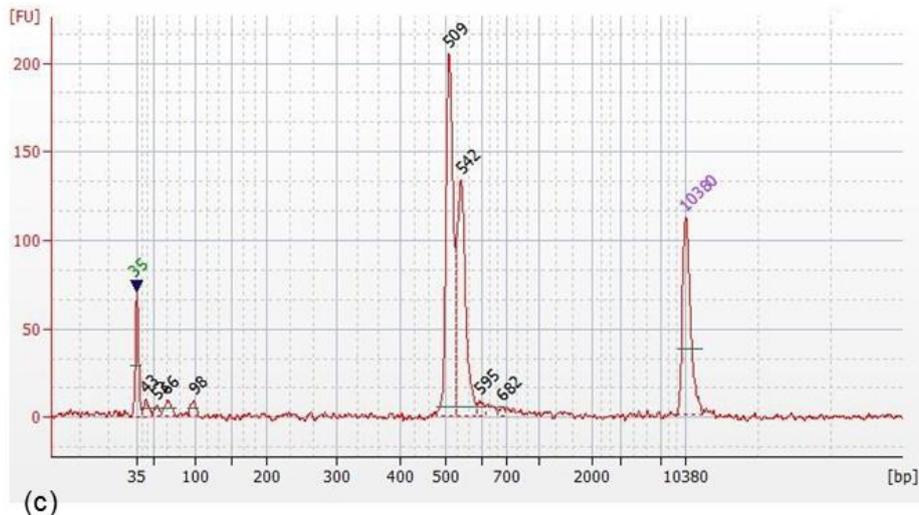
Fig. IV/2.c The Agilent 2100 Bioanalyzer on-chip electrophoresis system

(c) Electropherogram of a Bioanalyzer run (FU - fluorescence units, the size of lower and upper markers is marked with green and purple, respectively).

EXERCISE IV/4.: Capillary electrophoresis of fluorescently labelled DNA

Capillary electrophoresis is the choice, when really high resolution (1 bp) is needed, as for cycle sequencing or fingerprinting techniques (T-RFLP, ARISA, see Chapter IV.7.).

Object of study
    fluorescently labelled DNA (usually PCR product)

Materials and equipment
    genetic analyser used for capillary electrophoresis
      (e.g. ABI Prism™ 310 Genetic Analyser, **Fig. IV/3.**)
    polymer for the electrophoresis (e.g. POP-4® Polymer)
    internal size standard (e.g. GeneScan™ 500 TAMRA™ dye Size Standard)
    electrophoresis buffer (e.g. 10× 310 Running Buffer)
    molecular grade formamide
    sample tubes and septa
    heat thermostat
    slush of ice or chill rack
    adequate software for analysis of the raw data (e.g. GeneMapper® Software)

Procedure
1. Before capillary electrophoresis DNA samples are usually purified with ethanol-precipitation (Exercise IV/7.). Thereafter the pellets are stored at -20 °C.
2. Add 20 μL H_2O to the ethanol-precipitated DNA sample, vortex it and spin it down.
3. Prepare a master mix from 12 μL formamide and 0.3 μL size standard per sample.
4. Pipette 12.3 μL master mix and 1 μL sample into a sample tube, denature at 98 °C for 5 minutes, and put immediately to ice for 5 minutes.

Fig. IV/3. ABI Prism™ 310 Genetic Analyser

IV.2. DNA extraction

DNA extraction has two main steps. The first part covers cell lysis or cell disruption, while in the second step DNA is purified from other molecules and cell debris.

Cell disruption, yielding the so-called crude lysate, can be achieved by physical, chemical or enzymatic methods or with the combination of these. Physical cell disruption can be performed e.g. with a mixer mill (which shakes the cells together with glass beads; **Fig. IV/4.**), blade homogeniser (which homogenises samples with small rotating knives), grinding cells in liquid nitrogen using a mortar and a pestle or with a sonicator (high frequency sound waves break down cellular membranes but may not disrupt cell walls). Disruption of cells with incubation at high temperature (e.g. 98 °C for 5 minutes) is also possible, especially in case of cells with thinner cell walls. Chemical methods include treatment with concentrated alkali (e.g. NaOH, KOH) solution (which helps to open the cell wall), detergents (e.g. SDS – sodium dodecyl sulphate, which disintegrates the cell membrane). Enzymatic methods use proteases (e.g. proteinase K), lysozyme (especially in the case of Gram-positive bacteria), or in the case of yeasts, lyticase. Usually not only one method is applied, but an adequate combination of these, as it can be seen later at the exercises. It is important to find the right protocol for the analysed sample type, which is sufficient for cell disintegration but not too harsh to avoid fragmentation of genomic DNA.

Several techniques are available for DNA purification with different advantages and disadvantages. Their common aim is to remove cell debris, proteins, salts, detergents and other

reagents which were used during DNA isolation. As a starting step proteins and RNA (in case of DNA isolation) are often degraded using proteinase K and RNases.
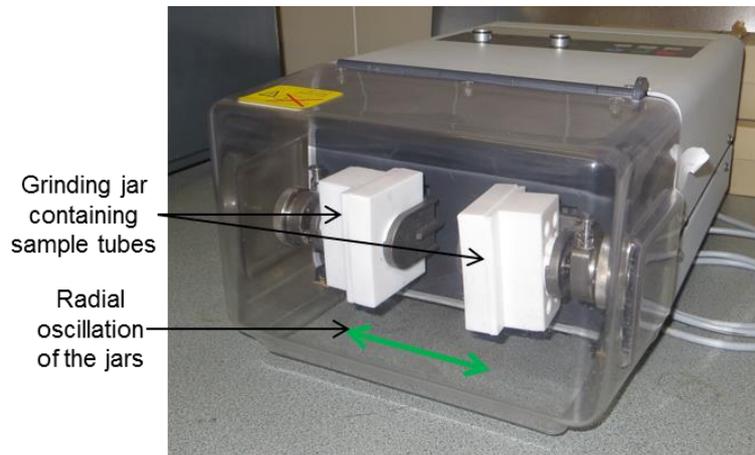
Fig. IV/4. Mixer mill (model MM301, Retsch)

Purification with organic solvents is performed with the addition of a mixture of phenol, chloroform and isoamyl alcohol (e.g. in a ratio of 25:24:1) to the cell lysate in a volume ratio of 1:1, which is followed by centrifugation. Additionally guanidinium thiocyanate can be added, which is a chaotropic agent, destabilizing hydrogen bonds, van der Waals forces and hydrophobic interactions. This causes the denaturation of proteins and nucleic acids, thus taking part in the separation of rRNA and ribosomal proteins, as well. The organic solvents alone are also acting as denaturants for the proteins and causing internal hydrophobic side-chains flip out. After a thorough mixing and centrifugation the organic and the aqueous phases separate. The former contains proteins, cell debris, while the later nucleic acids. Chloroform helps the better separation of the two phases. Isoamyl alcohol reduces foaming and deactivates RNases. The pH of the phenol has an important role. At pH 4-6 DNA partitions into the organic phase, so RNA can be isolated separately from the aqueous phase. At higher pH (7-8) all nucleic acids can be found in the aqueous phase, from there they can be recovered through ethanol precipitation. The method is very effective, and produce high quality nucleic acids, but it is time-consuming, cannot be automated and hazardous waste is produced.

Ethanol precipitation is a supplementary technique, which removes salts and concentrates nucleic acids. Ethanol disrupts H-bond among water and nucleic acids, while increase the electrical attraction between the phosphate backbone of DNA and the added sodium acetate. This causes the neutralisation and thus the precipitation of nucleic acids and salts after incubation and centrifugation. Washing the pellet with 70% ethanol will take salts and small organic molecules away. After drying the pellet can be dissolved in a desired buffer. Instead of ethanol, isopropanol can be used also, as it has higher precipitation efficiency for DNA, but also for salts. For removing dNTPs, ammonium acetate can be used also instead of sodium acetate. Ethanol precipitation is cheap, but quite slow and labour intensive.

CsCl gradient centrifugation was an important technique in the former decades, which has not been used already widely. The ethanol precipitated cell lysate was separated with high-speed centrifugation (450,000 g, 10 hours) through a CsCl gradient using ethidium bromide staining, and subsequently the appropriate DNA- and/or RNA-containing layer was separated. It was a

suitable method to separate nuclear and mitochondrial DNA, as the DNA was protected from fragmentation. After a further ethanol precipitation of the target, the method yielded very pure DNA. On the other hand, the procedure was time-consuming, expertise and special equipment were required, and hazardous waste was produced.

One of the most widespread techniques today is the silicate-based methods applied in many commercial kits as final purification steps. It exploits the selective binding of nucleic acids to a silicate gel at high concentration of chaotropic salts (e.g. sodium persulphate, lithium chloride). In the next steps a selective washing procedure is used. First washing contains low amount of chaotropic salt to remove proteins and coloured contaminants. The second washing is usually ethanol to remove salts. After proper drying of the gel, nucleic acids could be eluted. In case of RNA isolation, elution is achieved by application of a buffer with pH 4-5, while for DNA the pH of the buffer is typically 8-9. This means also, that both nucleic acids can be eluted selectively after each other with the adequate buffers. The silicate-based methods yield pure DNA, free from small fragments, but the method is not suitable for the purification of very large DNA molecules.

New techniques for nucleic acid purification are developed constantly. One approach uses magnetic beads that bind DNA conditionally and can be immobilized on a magnet to separate the DNA from the rest of the sample. The beads are positively charged at low pH, and will therefore bind to DNA, but at high pH they are negatively charged and release the DNA. It is fast, uses no chaotrophic salts or organic washing solutions, and yields high purity DNA. Its disadvantages are that the initial outlay for the magnets is relative high and the procedure is a bit tricky when handling multiple samples.

Further aspects should be considered when nucleic acids are isolated not from cell cultures but from environmental samples. Two basic approaches exist. One is the *a priori* separation of cells from the sample with e.g. Nycodenz® gradient centrifugation. Less fragmented and less contaminated DNA can be obtained, but a fraction of the cells is lost during separation. The second approach is direct lysis of the bacterial cells in the sample matrix, thereafter isolation and purification of the released nucleic acids happen as described above. Sample matrix can cause problem, as e.g. humic substances, clay minerals in soil tend to bind DNA, which inhibit downstream reaction. Therefore the application of different inhibitor removing steps is required. PVPP (polyvinylpolypyrrolidone), which is a highly cross-linked modification of the polymerised N-vinylpyrrolidone, for example binds phenolic compounds, which can be removed thereafter with centrifugation. Another problem is, that direct lysis does not distinguish between cellular and free DNA, the later derive from previously dead cells. Direct lysis yields generally higher amount of DNA, but could be more fragmented.

If using a new type of sample, it is worth comparing the result of different DNA isolation and purification techniques. Often some kind of optimisation or fine tuning of the previously applied protocol is needed. When we handle environmental samples, we should be aware that there is no method, which is equally efficient for all kind of bacteria. Thus every DNA isolation method distorts the original community composition in some degree. As DNA extraction is the first step in every nucleic acid based technique, its accuracy or at least its reproducibility has utmost importance.

Final task of DNA isolation is the quality- and quantity-check. Low quality DNA contains other molecules from the original sample (e.g. proteins, humic substances) or contaminants from the isolation workflow (e.g. salts, phenols, ethanol or detergents). All these substances can hinder subsequent applications. DNA purity can be checked by e.g. spectrophotometric analysis, and described with the ratio of measured absorbance at 260 and 280 nm. If this value is higher than 1.7, the purity of the DNA can be accepted. The result of DNA extraction can be checked with agarose gel electrophoresis, as well, especially if fragmentation is important. For really higher resolution, the Bioanalyzer should be used. To avoid degradation, DNA should be kept in a freezer (at -20 °C).

Different DNA characterisation methods need different DNA quantities. Also the copy number of the target gene is important. rRNA operons (containing 16S rRNA gene) usually occurs in more than one copy in the genome, which means, lower amount of DNA is enough to reach the threshold of detection in a PCR. The quantity of sample used for DNA extraction depends on the DNA content of the individual cells. Recommended cell numbers used for DNA isolation are 10^9 cells for bacteria, 10^7 cells for yeasts and 10^6 cells for animal cell cultures.

EXERCISE IV/5.: DNA extraction from bacterial cells with different techniques

Object of study
      slant cultures of bacteria

Materials and equipment
      inoculating loop
      0.5 M NaOH solution
      1 M TRIS [Tris(hydroxymethyl)aminomethane] buffer (pH 8.0)
      mixer mill (bead beater)
      thermocycler or water bath
      sterile glass beads
      DNA isolation kit (e.g. G-spin™ Total DNA Extraction Mini Kit)

Procedure
Three DNA isolation procedures differing in their cell lysis and purification steps are performed in parallel.
Chemical lysis with NaOH solution:
1. Measure 25 µL 0.5 M NaOH solution into a 1.5 mL microcentrifuge tube that is labelled with the name of the bacterium strain.
2. Suspend a loopful of bacteria in the solution, vortex thoroughly, spin it down and incubate for 15 minutes at room temperature.
3. Add 25 µL 1 M TRIS buffer and 300 µL H_2O.
Physical lysis with thermal denaturation:
1. Measure 100 µL sterile glass beads, 8 µL 1 M TRIS buffer and 100 µL H_2O into a 600 µL microcentrifuge tube that is labelled with the name of the bacterium strain.
2. Suspend a loopful of bacteria and shake the tubes for 1 minute at 30 Hz in a mixer mill.

3. Spin the tubes quickly, and incubate for 5 minutes at 98 °C (in a thermocycler or in a water bath).
4. Vortex for 5 seconds, centrifuge the tubes for 5 minutes at 10,000 g and transfer the supernatant (approx. 70 µL) to a new, labelled microcentrifuge tube.

Using a commercial DNA isolating kit:

This kit is based on enzymatic lysis of the cells followed by a silica-column purification of the DNA.

To complete the procedure, follow the instructions given by the manufacturer at protocol F.

Check the DNA quality of all samples with agarose gel electrophoresis (Exercise IV/1.).

Store isolated DNA at -20 °C for further analysis.

EXERCISE IV/6.: DNA extraction from environmental samples with different techniques

Object of study

environmental samples

Materials and equipment

sterile spatula and tweezer

0.45 µm cellulose nitrate membrane filter

sterile, membrane filtration equipment (Millipore)

DNA isolation kit (e.g. UltraClean® Soil DNA Isolation Kit)

mixer mill (bead beater)

thermocycler or water bath

liquid N_2

sterile mortar and pestle

lysis buffer (CLS-TC or CLS-Y, component of the FastDNA® Kit)

PVPP

DNA isolation kit (e.g. G-spin™ Total DNA Extraction Mini Kit)

Procedure

Two DNA isolation procedures are performed in parallel that differ in their cell lysis and purification steps.

UltraClean® Soil DNA Isolation Kit for soil and water samples:

This kit is based on physical and chemical lysis using ceramic beads and SDS, thereafter patented inhibitor removal techniques and silica-column purification are applied.

1. Soil sample can be added directly to the kit.
2. Cells from water samples (0.1-10 L depending on density) should be concentrated by filtration through a 0.22 µm pore-sized cellulose nitrate membrane filter. Then filters cut into smaller pieces can be added to the bead tube of the kit.
3. Then follow the instructions given by the manufacturer.
4. The only modification is, that instead a vortex adapter, use a mixer mill for 2 minute at 30 Hz.

Physical lysis using liquid N_2:

This procedure can be used as an alternative for the above kit, when we have samples with many endospores or thick cell-wall bacteria.

1. For each sample, pipette 1 mL CLS-TC or CLS-Y lysis buffer into a 1600 μL labelled microcentrifuge tube and add about 50 mg PVPP powder to it. CLS-TC is a lysis buffer developed for bacteria and cells from tissue cultures, whereas CLS-Y for yeast, algae and fungi.
2. Pour liquid N_2 into the sterile mortar. (Handle liquid N_2 always carefully according to safety regulations.)
3. With sterile tweezer and spatula add 1 g sample into the mortar filled with the liquid N_2 and ground it thoroughly.
4. Refill the mortar with liquid N_2 if evaporated and continue the grinding until the sample will form a homogenous powder. (At the first application of the method, the length of the homogenisation should be optimized, to reach sufficient cell disintegration without fragmentation of genomic DNA, as mentioned above in the introduction.)
5. Centrifuge the sample tubes for 8 minutes at 15,500 g and transfer supernatant (the crude lysate) to a new labelled microcentrifuge tube.
6. The crude lysate can be purified in several ways. One option is to use the G-spin™ Total DNA Extraction Mini Kit protocol F from step 4.

Check the DNA quality of all samples with agarose gel electrophoresis (Exercise IV/1.).
Store isolated DNA at -20 °C for further analysis.

EXERCISE IV/7.: DNA purification with ethanol precipitation

Object of study
    bacterial genomic DNA, environmental DNA samples, PCR products

Materials and equipment
    3 M Na-acetate solution (pH 4.6)
    95% ethanol and 70% ethanol
    microcentrifuge (refrigerated)
    vacuum centrifuge

Procedure
1. Prepare a master mix: add 62.5 μL 95% ethanol, 3.0 μL 3 M Na-acetate and 14.5 μL H_2O per reaction into a 1.5 mL microcentrifuge tube, multiply the given amount by sample number plus one.
2. Pipette 80 μL from this master mix to each labelled 0.6 mL microcentrifuge tube containing the DNA sample.
3. Vortex the tubes vigorously, then incubate at room temperature for 15 minutes.
4. Centrifuge the tubes for 20 minutes at 18,000 g at 4 °C, and carefully remove the supernatant by pipetting.
5. Add 250 μL 70% ethanol, vortex the tubes vigorously, and centrifuge at 18,000 g for 10 minutes. Remove the supernatant by pipetting.

6. Dry the precipitate on the bottom of the microcentrifuge tubes in a vacuum centrifuge (this takes ~30-40 minutes).

IV.3. DNA amplification with PCR

DNA fragments from 100 to 10,000 bp can be amplified by PCR (polymerase chain reaction). PCR is used for two major aims. First is to amplify a specific sequence, e.g. in order to identify the bacteria (e.g. 16S rRNA gene), to check whether it contains a given metabolic gene or encodes a disease factor. Second is to create fingerprints in order to group samples (Chapter IV.6-7).

The whole process contains repeating thermal cycles with three steps in each cycle. During denaturation, the two complementary DNA strands separate due to incubation at high temperature (94-98 °C). In the annealing step, forward and reverse primers (oligonucleotides) hybridise to the complementary sites on the single-stranded DNA chains at the primer-dependent temperature. During extension or elongation, DNA polymerase enzyme catalyses the synthesis of the complementary strands with extension from primers using the dNTPs present in the reaction mixture. This reaction amplifies the region located between the two primers, which theoretically results in an exponential increase of DNA molecules **(Fig. IV/5. and IV/6.)**.

In more details. There is an initial denaturation usually for 5 minutes at 98 °C, in order to melt strands of the whole template DNA, which GC-content in case of bacterial genomic DNA can reach 75%. Due to the restricted thermostability of the polymerases, the enzyme should be added after the initial denaturation, or its quantity should be increased to balance for its activity reduction. In case of a nested PCR (see below), the initial denaturation can be reduced to 3 minutes at 95 °C without enzyme activity loss in the second PCR. During the cycles, the length of the denaturation depends on the length of the PCR product. Usually 30 seconds is enough, but for RAPD (Chapter IV.6.), where we do not know exactly the sequence length of the product, it can be increased up to 90 seconds. The annealing step is the core of the PCR, as this step is responsible for the process specificity: specificity for the amplified DNA region (e.g. gene) and specificity for the studied taxa. As a rule of thumb the annealing temperature should be 3-5 °C lower than the melting point of the primers, but many online calculators are available, where e.g. possible secondary structure formation can be checked, as well. If the primers are longer than usual (18-22 bp) or contain more degenerate sites, the length of the annealing should be increased. To increase specificity, touch-down PCR can be also applied, where in the first cycles an elevated annealing temperature is used, and after enough specific templates are synthetized, the annealing temperature can be decreased in the subsequent cycles to increase yield. Temperature of the elongation depends on the enzyme (usually 72 °C), and its length is a function of product length and enzyme processivity (usually about 2 kbp per minute). After the cycles, a final elongation is applied to ensure the extension of all remaining single-stranded DNA. As PCR has high sensitivity, the application of a negative control without any DNA template is compulsory to check whether reagents were free from contaminating DNA or any cross-contamination occurred among the samples. The application of a positive control (a DNA sample from which an adequate product was synthetized in a previous PCR) can help in cases,

where the reaction was assembled correctly, but there is no positive signal due to the absence of the target sequence or due to the presence of inhibitory compounds in the sample.

Sometimes the given specificity or quantity cannot be achieved in a single PCR. In that case, the product of the first PCR can be used as a template for the second PCR, whose primers are positioned or nested within the original primers. Therefore it is called as nested PCR.

Subsequent steps (e.g. sequencing reaction) may require the purification of the PCR product to eliminate the polymerase enzyme, remaining nucleotides and primers, DNA template and produced primer dimers. The principle of purification is similar to the silicate-based DNA purification method described above (Chapter IV.2.), which is used during DNA isolation. In this case, the proper size DNA (e.g. 100 bp - 10 kbp) is retained, while reagents and other DNA fragments (primer dimers and genomic DNA) are removed during the purification steps.
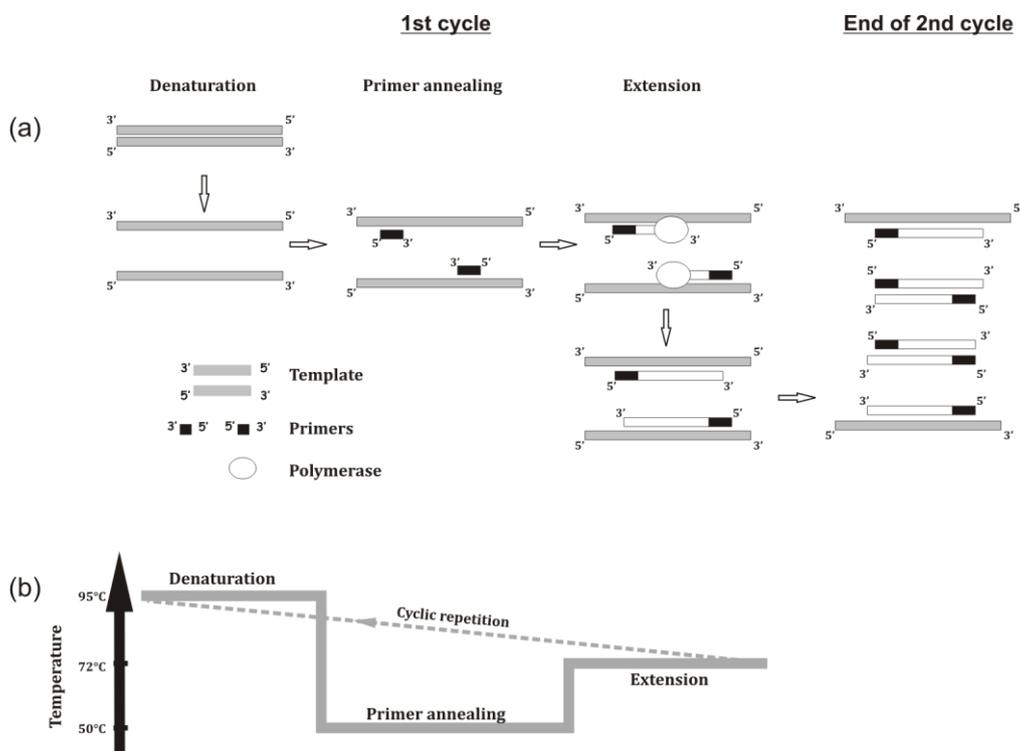
Fig. IV/5. Amplification of a DNA fragment with Polymerase Chain Reaction (PCR)

(a) Outline of a general PCR procedure and (b) its thermal profile.
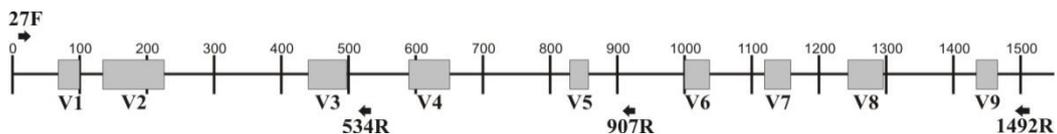
Fig. IV/6. Regions of the 16S rRNA gene and binding sites of selected universal primers

Numbering is according to the 16S rRNA gene of *Escherichia coli*. Primer binding sites are marked with arrows, whereas variable regions (V1-V9) are marked with grey rectangles.

EXERCISE IV/8.: PCR amplification of the 16S rRNA gene and its purification

Object of study

bacterial genomic DNA or environmental DNA samples

Materials and equipment

10× PCR buffer

1 mM dNTP mix

forward and reverse primers:

27F primer: 5' AGA GTT TGA TCM TGG CTC AG 3'

534R primer: 5' ATT ACC GCG GCT GCT GG 3'

1492R primer: 5' TAC GGY TAC CTT GTT ACG ACT T 3'

5 U/μL Dream *Taq* polymerase enzyme

positive control DNA

thermocycler

PCR product purification kit

Procedure

1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (the number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (μL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H$_2$O	up to 24	

4. Pipette 24 μL master mix into each labelled PCR tube, and add 1.0-1.0 μL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	30 sec
	Annealing	52 °C	30 sec
	Extension	72 °C	1 min[1]
Final extension		72 °C	10 min
Store		4 °C	∞

[1]The length of extension depends on the length of the PCR product. Using primers 27F and 1492R requires 1 minute, whereas the usage of 27F and 534R requires only 30 seconds.

7. If required by the further methods, purify the PCR product. In general, follow the instructions given by the manufacturer.

Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.). DNA quantity might be slightly lower as compared to the PCR product prior to purification, but this is the cost for eliminating PCR reagents and small-sized DNA fragments (e.g. primer dimers).
Purified and unpurified PCR products can be stored for a few days at 4 °C (or at -20 °C for long-term storage).

Metabolic genes

Metabolic potential of bacteria can be studied with detecting different genes that encode proteins involved in a particular metabolic pathway or with studying proteins that indicate general metabolic activity (e.g. using PCR-based methods or hybridisation). However, the presence of such a gene in the bacterial genome does not indicate unequivocally that the corresponding protein is translated and present in an active form within the cell. Therefore only indirect information on a certain function can be gained through the DNA-based detection of a metabolic gene. RNA-based methods are one step closer to the functionality, since in this case, the presence of mRNA within the cell indicates that the first step on the way reaching enzyme activity (transcription-translation-catalysis) has happened.

Phylogenetic information can also be obtained in some cases using metabolic genes **(Table IV/1.)**, and therefore in environmental microbiological studies the potential metabolic activity of microorganisms can be coupled with the genetic diversity (species composition). Compared with the most widely applied gene, the 16S rRNA gene, metabolic genes have a main advantage. Due to the degenerate character of the genetic code (a particular amino acid is encoded by more than one triplet), protein-coding regions are more variable, and therefore phylogenetic analyses with higher resolution can be performed in the case of closely related taxa. However, it should be noted that in several cases, phylogenetic information may be blurred by horizontal gene transfer.

Some of these genes are referred in the literature as catabolic genes (encoding enzymes which catalyse a particular step in the biodegradation pathways of organic compounds), while others are referred as housekeeping genes (expressed constitutively, since the encoded enzymes are required for basic cellular functions).

Table IV/1. Some metabolic genes used in phylogenetic analyses or in the studies of microbial metabolic activity in various environments

Genomic region	Encoded gene(s)	Major features
cpeB(A)	phycoerythrin	component of the phycobilisomes, present in cyanobacteria and red algae
cpcBA-IGS	phycocyanin operon (coding and non-coding regions)	component of the phycobilisomes, present in cyanobacteria and red algae
psbA	D1 protein of photosystem II	horizontal gene transfer is possible by viral contribution; in some algae, copies with different nucleotide sequences can be found
rbcL	large subunit of the RuBisCO enzyme	a key enzyme in CO_2-fixation is encoded by this gene, horizontal gene transfer among taxonomic groups exists
rpoC1	DNA-dependent RNA polymerase enzyme	this gene is present in a single copy in the prokaryotic genome
amoA	subunit A of the ammonia-monooxygenase enzyme	this enzyme catalyses the rate limiting step of ammonia oxidation
nirS	cytochrome cd_1 type nitrite reductase enzyme	this enzyme catalyses an intermediate step of denitrification, only one of the two types is present within a bacterium strain, lateral gene transfer among taxonomic groups exists
nirK	copper-containing nitrite reductase enzyme	
nosZ	nitrous oxide reductase enzyme	this enzyme catalyses the last step of denitrification, not present in all denitrifying bacteria
dsrAB	dissimilatory sulphite reductase enzyme	characteristic genes of sulphate reducing bacteria
nifH	a component of the nitrogenase enzyme (dinitrogenase reductase)	in the case of fast-growing *Rhizobium* species, these genes are located on plasmids, while in the case of slow-growing *Rhizobium* species, these genes can be found on the chromosome
nodD	protein involved in the first step of nodulation	
catA	catechol 1,2-dioxygenase enzyme	a key enzyme in the degradation of monoaromatic pollutants, which cleaves the aromatic ring between the two hydroxyl groups
pheU	largest subunit of the phenol hydroxylase enzyme	this monooxygenase catalyses the first step of phenol degradation, while in some bacteria a single-component phenol hydroxylase exists
glnII	glutamine synthetase (GS) II enzyme	GS enzyme has a dual role in the cell: glutamine biosynthesis and ammonia assimilation; GSII is typically expressed under nitrogen starvation
atpD	beta subunit of the ATP synthase enzyme	this subunit comprises the catalytic site of membrane-coupled ATP synthesis
recA	recombinase A protein	this protein is involved in genetic recombination and in DNA repair

EXERCISE IV/9.: PCR amplification of the *pheU* gene

Object of study

    bacterial genomic DNA or environmental DNA samples

Materials and equipment

    10× PCR buffer
    1 mM dNTP mix
    forward and reverse primers:
        pheUF primer: 5' CCA GGS BGA RAA RGA GAR GAA RCT 3'
        pheUR primer: 5' CGG WAR CCG CGC CAG AAC CA 3'
    5 U/μL Dream *Taq* polymerase enzyme
    positive control DNA
    thermocycler

Procedure

1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (their number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (μL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H₂O	up to 24	

4. Pipette 24 μL master mix into each labelled PCR tube, and add 1.0-1.0 μL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.

6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
5 cycles	Denaturation	94 °C	1 min
	Annealing	58 °C	1 min
	Extension	72 °C	1 min

5 cycles	Denaturation	94 °C	1 min
	Annealing	57 °C	1 min
	Extension	72 °C	1 min
25 cycles	Denaturation	94 °C	1 min
	Annealing	56 °C	1 min
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Store		4 °C	∞

7. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.) or with another DNA separation method. If required, purify the PCR product accordingly.
8. PCR products can be stored for a few days at 4 °C (or at -20 °C for long-term storage).

EXERCISE IV/10.: PCR amplification of the *nifH* gene

Object of study
    bacterial genomic DNA or environmental DNA samples

Materials and equipment
    10× PCR buffer
    1 mM dNTP mix
    forward and reverse primers:
      nifH-40F primer: 5' GGN ATC GGC AAG TCS ACS AC 3'
      nifH-817R primer: 5' TCR AMC AGC ATG TCC TCS AGC TC 3'
    5 U/µL Dream *Taq* polymerase enzyme
    positive control DNA
    thermocycler

Procedure
1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (their number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H$_2$O	up to 24	

4. Pipette 24 µL master mix into each labelled PCR tube, and add 1.0-1.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
35 cycles	Denaturation	94 °C	30 sec
	Annealing	50 °C	30 sec
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Store		4 °C	∞

7. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.) or with another DNA separation method. If required, purify the PCR product accordingly.
8. PCR products can be stored for a few days at 4 °C (or at -20 °C for long-term storage).

EXERCISE IV/11.: PCR amplification of the *nodD* gene

Object of study
    bacterial genomic DNA or environmental DNA samples

Materials and equipment
    10× PCR buffer
    1 mM dNTP mix
    forward and reverse primers:
      nodD1F primer: 5' CTC GTC GCG CTC GAC GCA TTG A 3'
      nodD1R primer: 5' TGC CCC ATG GAC ATG TA 3'
    5 U/µL Dream *Taq* polymerase enzyme
    positive control DNA
    thermocycler

Procedure
1. Let frozen DNA samples and PCR reagents thaw at room temperature.

2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (their number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).

3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H₂O	up to 24	

4. Pipette 24 µL master mix into each labelled PCR tube, and add 1.0-1.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.

5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.

6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
35 cycles	Denaturation	94 °C	1 min
	Annealing	60 °C	1 min
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Store		4 °C	∞

7. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.) or with another DNA separation method. If required, purify the PCR product accordingly.

8. PCR products can be stored for a few days at 4 °C (or at -20 °C for long-term storage).

EXERCISE IV/12.: PCR amplification of the *atpD* gene

Object of study

    bacterial genomic DNA or environmental DNA samples

Materials and equipment

    10× PCR buffer
    1 mM dNTP mix
    forward and reverse primers:
      atpD-225F primer: 5' GCT SGG CCG CAT CMT SAA CGT C 3'

atpD-782R primer: 5' GCC GAC ACT TCM GAA CCN GCC TG 3'
5 U/µL Dream *Taq* polymerase enzyme
positive control DNA
thermocycler

Procedure
1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (their number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H$_2$O	up to 24	

4. Pipette 24 µL master mix into each labelled PCR tube, and add 1.0-1.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	1 min
	Annealing	55 °C	40 sec
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Store		4 °C	∞

7. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.) or with another DNA separation method. If required, purify the PCR product accordingly.
8. PCR products can be stored for a few days at 4 °C (or at -20 °C for long-term storage).

EXERCISE IV/13.: PCR amplification of the *glnII* gene

Object of study, test organisms

     bacterial genomic DNA or environmental DNA samples

Materials and equipment

     10× PCR buffer

     1 mM dNTP mix

     forward and reverse primers:

       glnII-12F primer: 5' YAA GCT CGA GTA CAT YTG GCT 3'

       glnII-689R primer: 5' TGC ATG CCS GAG CCG TTC CA 3'

     5 U/µL Dream *Taq* polymerase enzyme

     positive control DNA

     thermocycler

Procedure

1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (their number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H$_2$O	up to 24	

4. Pipette 24 µL master mix into each labelled PCR tube, and add 1.0-1.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	30 sec
	Annealing	58 °C	1 min
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Store		4 °C	∞

7. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.) or with another DNA separation method. If required, purify the PCR product accordingly.
8. PCR products can be stored for a few days at 4 °C (or at -20 °C for long-term storage).

EXERCISE IV/14.: PCR amplification of the *recA* gene

Object of study
 bacterial genomic DNA or environmental DNA samples

Materials and equipment
 10× PCR buffer
 1 mM dNTP mix
 forward and reverse primers:
  recA-41F primer: 5' TTC GGC AAG GGM TCG RTS ATG 3'
  recA-640R primer: 5' ACA TSA CRC CGA TCT TCA TGC 3'
 5 U/µL Dream *Taq* polymerase enzyme
 positive control DNA
 thermocycler

Procedure
9. Let frozen DNA samples and PCR reagents thaw at room temperature.
10. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (their number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
11. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H$_2$O	up to 24	

12. Pipette 24 µL master mix into each labelled PCR tube, and add 1.0-1.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
13. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
14. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	1 min
	Annealing	58 °C	1 min
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Store		4 °C	∞

15. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.) or with another DNA separation method. If required, purify the PCR product accordingly.
16. PCR products can be stored for a few days at 4 °C (or at -20 °C for long-term storage).

IV.4. DNA sequencing

Currently two major types of DNA sequencing is applied worldwide, the improved version of the classical Sanger sequencing and the so-called next-generation sequencing (NGS) techniques. The first is still the gold standard in the determination of nucleotide sequences of DNA molecules, while NGS methods have much higher throughput but somewhat higher error rate. By the way, since these techniques provide several magnitudes larger datasets than it was obtained by previous sequencing methods, the resolution of the analyses have been increased significantly.

The most widely applied type of Sanger sequencing, which applies capillary electrophoresis coupled with fluorescent signal detection, operates with a chain-terminating cycle sequencing reaction **(Fig. IV/7.)**. The reaction is similar to a common PCR in several features, but differs in the following: contains only one primer, besides dNTPs fluorescently labelled ddNTPs (dideoxynucleotides) are also present in the reaction (four different fluorescent labels corresponding to the four different nucleotide types) and a special modified polymerase enzyme is applied. The theoretical basis of sequencing is simple: since ddNTP molecules do not allow the incorporation of additional nucleotides, if such a nucleotide is incorporated into the DNA chain during the extension, the reaction terminates. As a result, DNA fragments with different lengths are produced in the sequencing reaction, which are fluorescently labelled corresponding to the terminal nucleotide. After purification, the fluorescently-labelled single-stranded DNA fragments are subjected to capillary electrophoresis: the fluorescent signals of terminal ddNTPs are detected with laser excitation and the size of DNA fragments is measured based on their motility in the gel within the capillary. The created sequencing chromatograms are analysed in two separate steps. First, the software in the genetic analyser performs base-calling **(Fig. IV/8.)**. In the second step, these chromatograms can be analysed individually with another software (e.g. correcting the biases of automatic base-calling and removal of primer sequences).
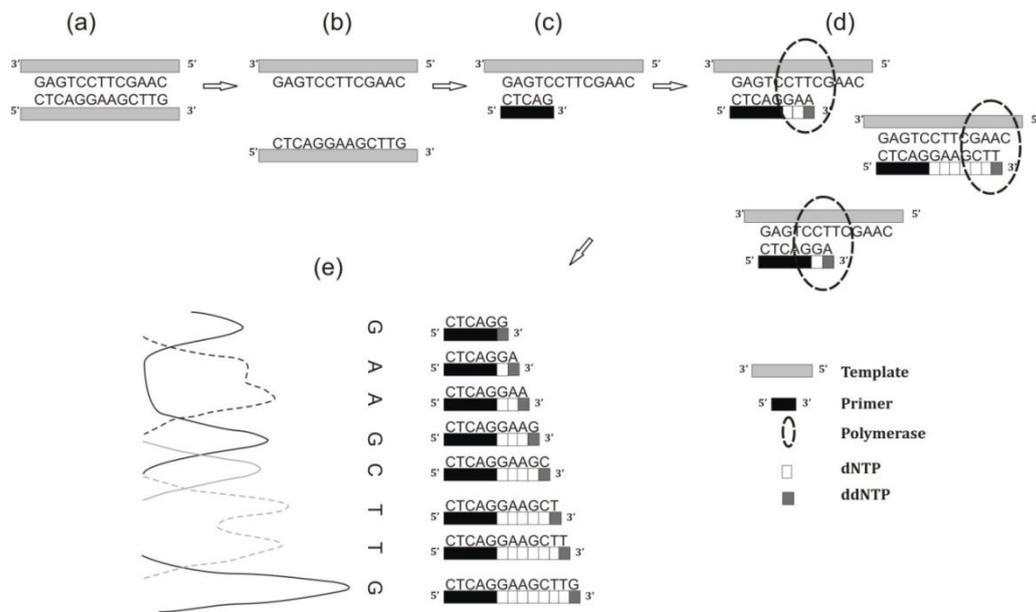
Fig. IV/7. DNA sequence determination based on the Sanger method

(a) PCR product (b) Denaturation (c) Primer annealing (d) Extension: each reaction terminates with the incorporation of a ddNTP (e) Capillary electrophoresis: fragments are size separated and each ddNTP is detected at different wavelength, marked with different lines.
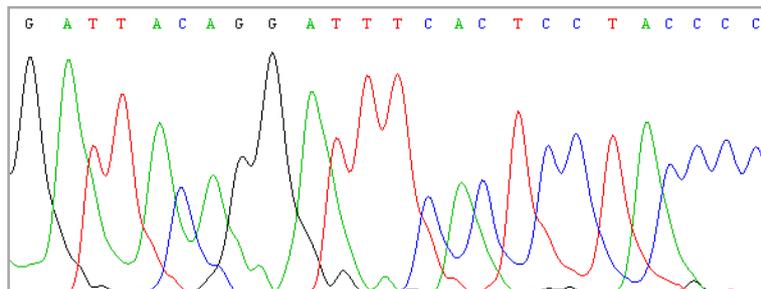
Fig. IV/8. A chromatogram generated after Sanger sequencing of DNA

EXERCISE IV/15.: Sanger sequencing of DNA molecules (and analysis of the obtained chromatograms)

This method allows the determination of nucleotide sequence in DNA fragments shorter than 1000 bp. In this example, the sequencing of the complete 16S rRNA gene is given, but with replacing the primers in the below described protocol, sequencing of other genes/regions could be performed.

Object of study, test organisms
> purified PCR products (or other DNA samples planned to be sequenced)

Materials and equipment
> ABI BigDye® Terminator v3.1 Cycle Sequencing kit:
>   Ready Reaction Mix, 5× sequencing buffer
> primer according the DNA sequence to be determined, e.g. in the case of sequencing the almost complete 16S rRNA gene one of the following primers:

519F primer: 5' CAG CAG CCG CGG TAA TAC 3'
926R primer: 5' CCG TCA ATT CCT TTG AGT TT 3'
thermocycler
formamide
sample tubes and septa for capillary electrophoresis
slush of ice or chill rack
ABI Prism™ 310 Genetic Analyzer
computer with installed MEGA software (version 5 or higher)

Procedure

1. Let frozen DNA samples and reagents thaw at room temperature.
2. Label two 1.5 mL microcentrifuge tubes (reagents required for the two master mixes will be pipetted into these tubes), and some PCR tubes (twice as the number of purified PCR products).
3. Make a master mix in a 1.5 mL microcentrifuge tube as described below (one master mix for each primer), multiply the reagent amounts by the number of DNA samples to be sequenced plus one. (The two master mixes for the two primers should be prepared in separate tubes!)

Name and quantity (μL) of the reagents		In case of X sample
5× sequencing buffer	1.5	
Ready Reaction Mix	1.0	
sequencing primer	0.5	
H₂O	4.0	

4. Pipette 7 μL master mix into each labelled PCR tube, and add 3 μL of DNA sample to the appropriate tubes. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
5. Put all PCR tubes into the thermocycler, and start the reaction with the following parameters:

		Temperature	Time
	Denaturation	96 °C	30 sec
28 cycles	Annealing	50 °C	5 sec
	Extension	60 °C	4 min
Store		4 °C	∞

6. Perform sample purification with ethanol precipitation as described in Exercise IV/7.
7. Add 20 μL formamide to the pellets, vortex and spin them quickly. Transfer the samples to sample tubes used for capillary electrophoresis.
8. Denature samples in a thermocycler at 95 °C for 5 minutes. Put the tube immediately to ice or into a chill rack.
9. Check the genetic analyser for buffer and polymer level, check the capillary injection counter.
10. Put the sample tubes to the rack of the genetic analyser.
11. After performing a test run on the genetic analyser, run the capillary electrophoresis at 12.2 kV using POP 6™ polymer at 50 °C for 110 minutes. After the run is complete, the

12. Start the MEGA software, and open the alignment window (Align>Edit/Build Alignment>Create a new alignment). Open the first chromatogram to be analysed (Sequencer>Edit Sequencer file).

13. Remove low quality data from the beginning of the sequence. In the case of sequencing with a reverse primer, the reverse complement of the sequence should be used for further analysis (Edit>Reverse complement). Find all ambiguous nucleotides (marked with N in the nucleotide sequence, Ctrl+N), and make manual correction, if possible. Remove low quality reads and the primer sequence from the end of the chromatogram. Save data in an '.ab1' format (Data>Save file), and import data to the alignment window (Data>Add to Alignment Explorer). Perform the same steps with all obtained chromatograms.

14. Find the overlapping regions of the partial 16S rRNA gene sequences generated from the same PCR product (Search>Find motif). Use 'Copy – Paste' functions to assemble the 16S rRNA gene, and finally save the result.

15. Save the assembled sequence in a fasta format for further analysis (e.g. taxon identification or phylogenetic analysis).

The most common outcome of Sanger sequencing in microbiological practice is the identification (or characterization) of a bacterial strain or an environmental clone (see below in Chapter IV/7.). If the performed analysis was based on the 16S rRNA gene, the curated database EzTaxon (www.ezbiocloud.net) containing the sequences of type strains of prokaryotic species is recommended. In other cases (i.e. sequencing metabolic genes or non-coding ITS regions), a search in a general DNA database (such as using Blast against GenBank, blast.ncbi.nlm.nih.gov/Blast.cgi) is sufficient.

Table IV/2. Comparison of most common NGS technologies

	Roche 454	Ion Torrent	Illumina	PacBio
Read length (bp)	500-1000	400	200-300	50,000
Amplification before sequencing	Yes	Yes	Yes	No
Error rate (%)	~1-2	~2-3	~0.1	~10-15
Run time	10-20 h	5-7 h	2-6 days	2 h
Prokaryote genome/run	1-20	20-200	20-200	~5
Cost/nucleotide	High	Medium	Low	Medium

Regarding NGS technologies **(Table IV/2.)**, those could be divided into two major types. First types apply single molecule amplification prior to sequence determination (e.g. Roche 454 pyrosequencing, IonTorrent semiconductor sequencing). During this process, amplification of single molecules is required to get proper signal for the sequencing reaction. This can be achieved with emulsion PCR (a clonal amplification method), where small PCR reactions are performed in a water-oil emulsion with DNA molecules bound to a solid surface (e.g. small beads). The second

types of NGS technologies perform single molecule sequencing directly (also termed as third generation sequencing technologies, e.g. PacBio).

The IonTorrent Personal Genome Machine™ (Life Technologies) **(Fig. IV/9.)** sequencing system combines semiconductor sequencing technology with the natural biochemistry of DNA synthesis to directly translate chemical information into digital data. The sequencing technology exploits the process of nucleotide incorporation, since this reaction is coupled with a release of a hydrogen ion. These hydrogen ions are detected by small pH sensors present with extreme density on a semiconductor chip.

Fig. IV/9. The Ion Torrent PGM™ (Life Technologies) next-generation DNA sequencing system

During 454 pyrosequencing, as applied by the GS Junior (Roche) sequencing system **(Fig. IV/10.)**, similarly to the previous instrument the 'sequencing by synthesis' theory is adopted: the nucleotide incorporation into the DNA strand results pyrophosphate release that is subsequently converted into chemiluminescent light signal, which is recorded by a CCD camera. In the case of both NGS methods, the massive parallel sequencing is achieved by the high density of small sequencing wells on a miniaturised plate **(Fig. IV/11.)**.

The third most popular NGS technology is the Illumina chemistry, which is based on reversible terminator sequencing (also a 'sequencing by synthesis' method). In this case, all four dNTPs (labelled with different dye) are added to the reaction and their incorporation is allowed as a next base. After a four-colour imaging, the fluorescent dyes are cleaved, washed away and the cycle repeats.

PacBio applies a single molecule real-time (SMRT) sequencing technique, which is also based on DNA synthesis. In this technology, single DNA template-polymerase complexes are immobilized in the bottom of small illuminated wells, and the four nucleotides labelled with different fluorophores are present in the reaction, their incorporation serves the production of the detected light pulse. Subsequently, during this incorporation, the fluorophore is released and the next nucleotide can be attached to the DNA strand.

Although the huge amount of data generated by NGS technologies are processed after sequencing as a whole dataset, the visualization of individual reads **(Fig. IV/12.)** are incorporated in the analysis software.

Fig. IV/10. The GS Junior (Roche) next-generation DNA sequencing system
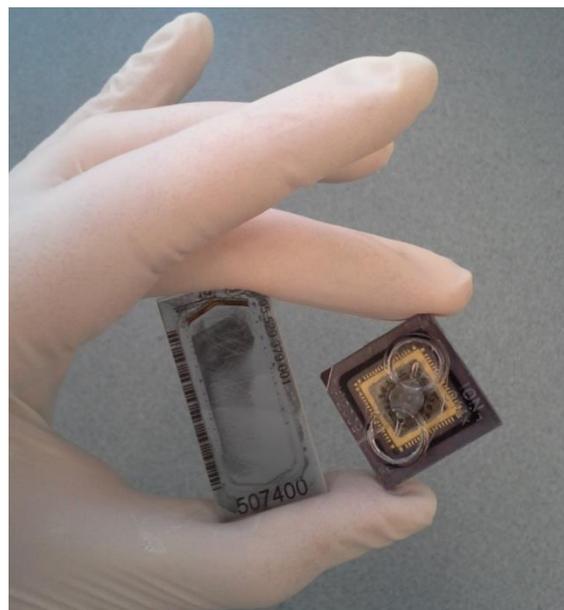
Fig. IV/11. Picotiter plate and semiconductor chip used for next-generation DNA sequencing
Picotiter plate (on the left) of Roche GS Junior containing ~300,000 sequencing wells and a 314™ Chip (on the right) of Life Technologies Ion Torrent PGM™ containing ~1 million wells.

For pure cultures, the most common use of NGS is the sequencing of whole genomes or analysing the different effects (chemical, physical, etc.) on the molecular level via the complete set of transcribed RNAs (transcriptomics). Although, whole genome sequence analysis till now did not get a central role in bacterial taxonomy (it is not obligatory to determine the complete genome of a newly described species), obtaining complete bacterial genomes became available in

many laboratories with the use of NGS technologies **(Table IV/2.)** due to the relatively low cost of these methods.
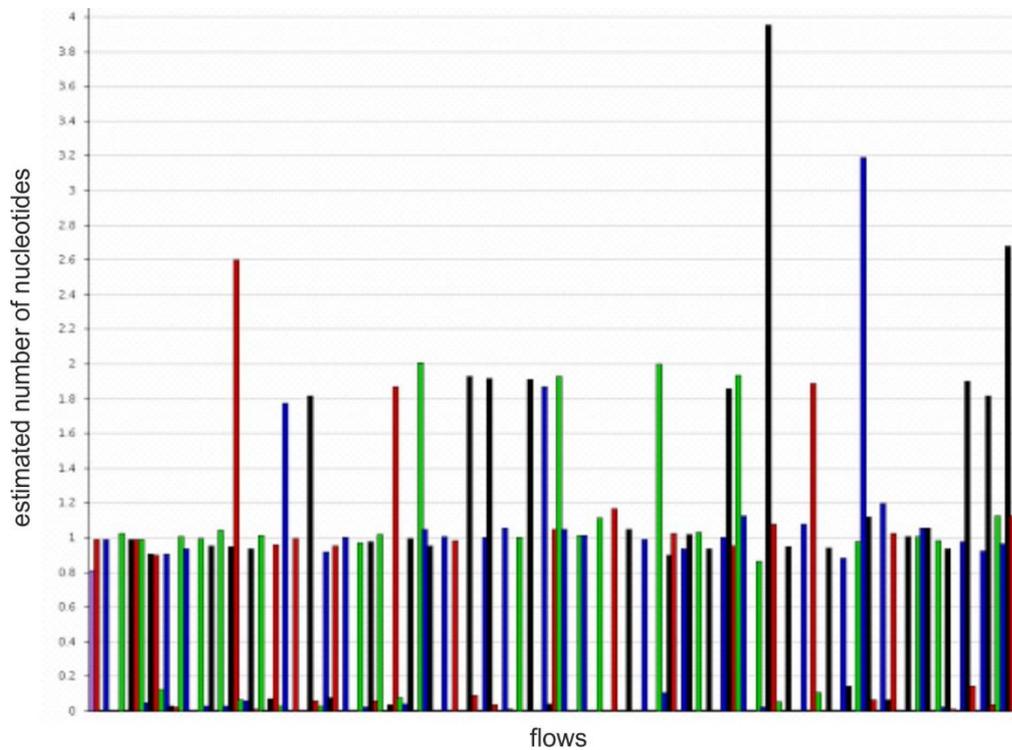
Fig. IV/12. A pyrosequencing flowgram generated on a Roche GS Junior system
Signal intensity corresponds with the number of incorporated nucleotides.

IV.5. Phylogenetic analyses

The aim of a phylogenetic analysis is to reconstruct the evolutionary relationships among the studied organisms. This can be achieved by using DNA sequences encoding genes in which the mutation rate (speed of nucleotide exchange) is suitable for the deduction of events happened in the past (i.e. divergence of taxa). Phylogenetic analyses have currently a central role in the taxonomy (identification, classification and determination) of micro- and macroorganisms, and also in ecological and biodiversity studies.

The general steps of a phylogenetic analysis includes: collecting the DNA sequences to be analysed, sequence alignment, selecting the proper nucleotide substitution model (and other parameters of the analysis), construction the phylogenetic tree, evaluation (and testing) the obtained tree. There are many tools available which could be used in microbiological studies, but probably the most comprehensive phylogenetic tool is the MEGA software (megasoftware.net), which incorporates all the key features required for such analysis and provides a huge diversity of options **(Table IV/3.)**.

Table IV/3. The most widely applied parameter options in the phylogenetic analysis of microorganisms

Method	Description
Nucleotide substitution models	
Kimura 2-parameter	assumes same nucleotide frequencies and that there is no variation in the rate of substitution among sites; considers that the rate of transition and transversion differs
Tamura 3-parameter	also accounts for GC-content bias
Tree construction methods	
Neighbour-Joining	distance-based method; creates a tree based on the pairwise distance values between each analysed taxa; initiates the analysis from a star network with joining taxa having the lowest distance value into a new node; including this newly created node iterates the joining steps until all nodes are resolved
Maximum Likelihood	character-based method; calculates the probability of obtaining the given pattern at each position using a given tree and a given substitution model; the likelihood value of the whole tree is calculated based on all these received probabilities; after a slight parameter change, calculates the new likelihood, which continues until there is no relevant change in the likelihood value

EXERCISE IV/16.: Phylogenetic analysis using the MEGA software

Object of study
   DNA sequences in fasta format

Materials and equipment
   computer with installed MEGA software (version 5 or higher) and with internet access

Procedure
1. Start the MEGA software, and open the alignment window (Align>Edit/Build Alignment>Create a new alignment).
2. Import saved fasta sequences (Edit>Insert Sequence From File). Sequences could be imported from GenBank also, e.g. based on a similarity search (click on a sequence, then Web>Do Blast Search; sequences could be imported from the web browser window by simply clicking on 'Add To Alignment')
3. Align sequences using ClustalW: first, select all sequences (Edit>Select All), then perform a multiple alignment (Alignment>Align by ClustalW).
4. Save your alignment (Data>Save Session).
5. Open your sequences for phylogenetic analysis (Data>Phylogenetic Analysis).
6. Perform a search for the best-fit model parameters (Models>Find Best DNA/Protein Models). In the options, chose the proper 'Gaps/Missing Data Treatment': (i) select 'Use All Site', if the alignment contains only insertions and deletions and no missing data are present (if both are present, sequences should be trimmed back at both ends in the Alignment Explorer window to remove missing data), (ii) select 'Complete Deletion', when only missing

IV.6. Other genotyping methods

Genotyping is a process, when the genetic make-up, the genotype of different individuals, strains is compared with each other. With the introduction of next-generation DNA sequencing techniques it became easier and cheaper to determine the whole genome sequence of organisms, thus to carry out whole-genome genotyping. But in many cases, genotyping is based only on a few genes (e.g. the 16S rRNA gene) or many small parts of the genome (see RAPD technique below).

The aim of genotyping is usually to group samples (strains, clones) by generating more or less complex fingerprints. The taxonomic level of grouping (e.g. genus, species, strain) depends on the resolution of the applied method. If it is fast, cheap and effective enough, the redundant sequencing of the same bacterial species from the same original sample can be also avoided. Occasionally samples can be identified directly during genotyping, as sequencing can be regarded also as a genotyping technique. Technically it can be a stand-alone one-step PCR (e.g. RAPD) or a combination of many steps (see ARDRA technique below).

ARDRA (Amplified Ribosomal DNA Restriction Analysis)

In ARDRA, the amplified 16S rDNA molecules are digested with restriction enzymes. The number and position of restriction sites within this region is variable among different bacterial species. After enzymatic digestion, gel electrophoresis results in patterns that are characteristic for bacterial genera or species, and could serve as a basis of grouping **(Fig. IV/13.)**.
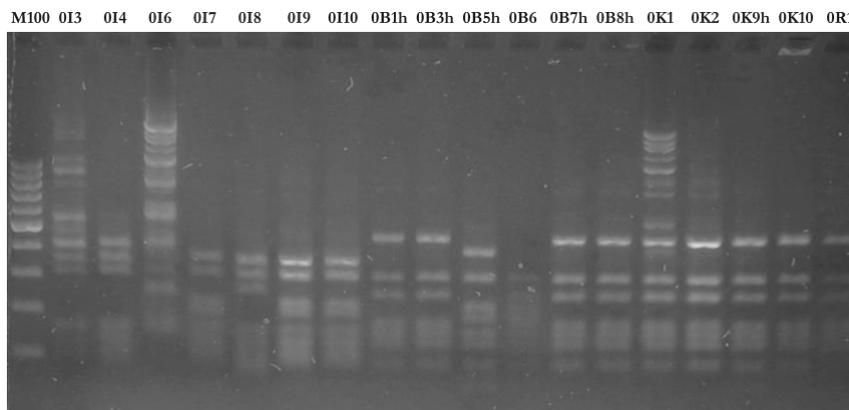
Fig. IV/13. Amplified Ribosomal DNA Restriction Analysis (ARDRA) pattern of PCR products

Image of the agarose gel. The 16S rRNA gene PCR products from bacterial pure cultures (0I3-0R1) were digested with the *Msp*I enzyme. The first lane contains DNA ladder, Marker 100 (M100).

The patterns are determined only by the position of the restriction sites, and it does not account for sequence variations outside the recognition positions. Hence it might occur that sequences without closer relation end up in the same ARDRA group. This bias can be reduced by the application of more restriction enzymes. During enzymatic digestion, many smaller DNA fragments are produced, whose separation requires higher resolution than in the case of normal PCR products. This could be achieved by increasing the electrophoresis time and gel concentration, and lowering the voltage applied for electrophoresis (see Chapter IV/1).

EXERCISE IV/17.: Restriction digestion of PCR products and pattern analysis

Grouping samples based on the restriction patterns of their PCR products

Object of study
      PCR products (e.g. 16S rRNA gene)

Materials and equipment
      10× buffer of restriction enzymes
      restriction enzymes
      water bath
      computers with adequate software

Procedure
1. Let frozen PCR products and enzyme buffers thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some 0.6 mL microcentrifuge tubes (the number is equal to the number of PCR products).
3. Prepare a master mix as follows without the addition of PCR products. For calculating the amount of reagents, multiply the values below with the number of samples plus one (for pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× enzyme buffer	2.0	
enzyme (10 U/µL)	0.15	
H_2O	9.85	

4. Pipette 12 µL master mix into each labelled 0.6 mL microcentrifuge tube, and add 8-8 µL PCR product to the appropriate tubes.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Incubate the samples in a water bath at the given temperature for 3 hours.

Enzyme	Buffer	Recognition and restriction site	Optimal temperature
*Bsu*RI	Buffer R	5' GG↓CC 3'	37 °C
*Hin*6I	Y+/Tango	5' G↓CGC 3'	37 °C
*Msp*I	Y+/Tango	5' C↓CGG 3'	37 °C

7. Prepare a 2% agarose gel (see Exercise IV/1.).
8. Mix the total amount of digested samples (20 μL) with 5 μL loading buffer, and load this mixture into the wells of the gel. To achieve a semi-quantitative measurement of the intensity and size of DNA, load 5 μL appropriate DNA ladder next to the samples.
9. Run the electrophoresis for 80 minutes at 80 V, and detect the presence and quantity of DNA under UV light. Make a digital image using a gel documentation system.
10. Perform the pattern analysis with the adequate software. Create groups based on the number and position of DNA fragments.

RAPD (Random Amplification of Polymorphic DNA)

The speciality of the RAPD technique is that the generated pattern is based on the whole genome and not only on one selected gene. It uses one or more short primers for amplification. Low annealing temperature applied in the PCR allows the random attachment of primers to different regions within the genome. PCR products are generated if two primers anneal to the genome towards each other and within a 'PCR distance', that is the length which could be synthetized by the enzyme in one elongation step. The process will result in PCR products of various lengths.
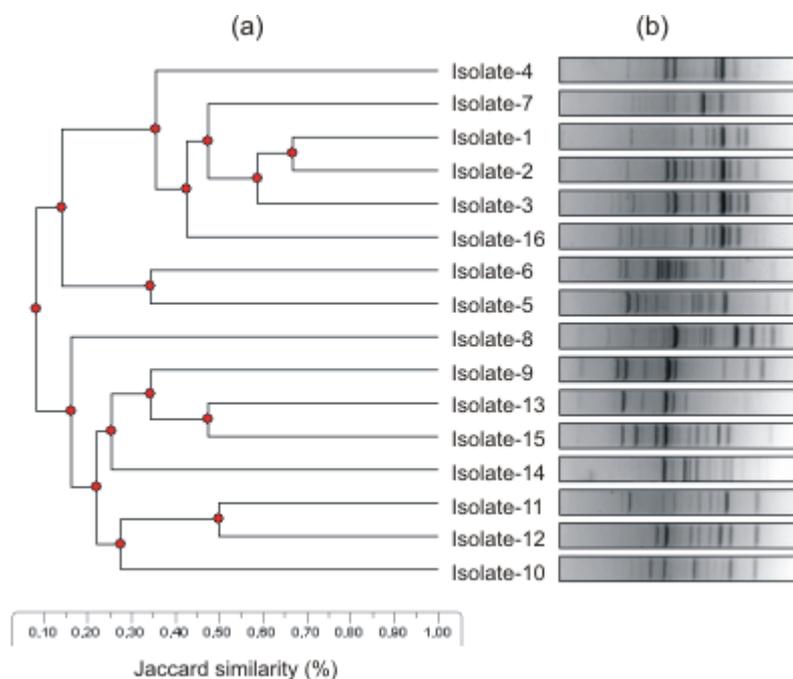
Fig. IV/14. Clustering of strains with the RAPD fingerprinting technique.

(a) UPGMA dendrogram (Chapter VI.2.) of *Synechococcus* isolates using Jaccard similarity values based on RAPD fingerprinting. (b) Image of the agarose gel.

The prerequisite of a RAPD analysis is an intact genome, therefore such a DNA extraction procedure must be chosen that does not significantly fragment genomic DNA (e.g. extraction based on enzymatic lysis). To achieve the appropriate result, PCR should be performed in three replicates, since some intra-sample variability may be observed due to the random annealing of primers to the genomic DNA. After the electrophoresis of the PCR products, dendrogram can be constructed from the resulting patterns using an image and data analysis software **(Fig. IV/14.)**.

EXERCISE IV/18.: Distinguishing bacterial strains using the RAPD fingerprinting technique

Object of study
    bacterial genomic DNA

Materials and equipment
    10× PCR buffer
    1 mM dNTP mix
    M13 primer: 5' GAG GGT GGC GGT TCT 3'
    5 U/µL Dream *Taq* polymerase enzyme
    positive control DNA
    thermocycler
    computers with adequate software

Procedure
1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (the number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	6.0	
M13 primer	2.0	
Dream *Taq* polymerase	0.25	
H₂O	up to 24	

4. Pipette 24 µL master mix into each labelled PCR tube, and add 1.0-1.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.

6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	1.5 min
	Annealing	56 °C	2 min
	Extension	72 °C	2 min
Final extension		72 °C	10 min
Store		4 °C	∞

7. To achieve the appropriate result, PCR reaction should be performed in three replicates (steps 2-6.), since some intra-sample variability may be observed due to the random annealing of primers to the genomic DNA.
8. Prepare a 2% agarose gel (see Exercise IV/1.).
9. Mix the total amount of digested PCR samples (25 µL) with 5 µL loading buffer, and load this mixture into the wells of the gel. To achieve a semi-quantitative measurement of the intensity and size of DNA, load 5 µL Marker 3 and Marker 100 next to the samples.
10. Run the electrophoresis for 140 minutes at 80 V, and detect the presence and quantity of DNA under UV light. Make a digital image using a gel documentation system.
11. Perform the pattern analysis with the adequate software: recognize lanes, identify bands, find bands corresponding to each other in the different samples, and create data matrix. Finally group samples with cluster analysis (see Chapter VI.2.).

LH-PCR (Length-Heterogeneity PCR)

In some cases, PCR amplification of a selected region within the genome of different bacterial strains results in products having different size, which could be applied also as a genotyping tool. Length heterogeneity sometimes is coupled with phylogenetic information, as in the case of the phycocyanin operon **(Fig. IV/15.)** of the genus *Synechococcus* (phylum Cyanobacteria). For the non-coding region between bacterial rRNA genes, similar length heterogeneity could be observed, which is useful for distinguishing some taxa.

(a)   (b)
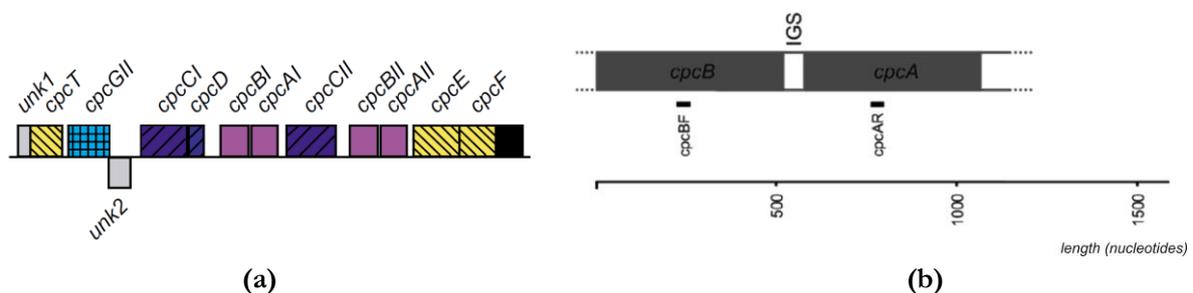
Fig. IV/15. Position of genes encoding phycobilisome proteins

(a) Location of phycobilisome genes within the genome of a *Synechococcus* strain (b) and the binding sites of the PCR primers applied in Exercise IV/19. IGS stands for intergenic spacer.

EXERCISE IV/19.: Genetic characterization of *Synechococcus* isolates with LH-PCR

Prepare phycocianin operon LH-PCR from DNA isolated from *Synechococcus* isolates and/or from lake water samples. The expected size of the PCR product will be determined for different *Synechococcus* groups *in silico* using computational data analysis. Subsequently, the identity of the unknown isolates and environmental samples will be determined based on the length of the obtained PCR product.

Object of study

> DNA from *Synechococcus* isolates and lake water samples

Materials and equipment

> 10× PCR buffer
> 1 mM dNTP mix
> forward and reverse primers:
> > cpcBF primer: 5' TAG TGT AAA ACG ACG GCC AGT TGY YTK
> > > CGC GAC ATG GA 3'
> > cpcAR primer: 5' TAG CAG GAA ACA GCT ATG ACG TGG TGT
> > > ARG GGA AYT T 3'
> 5 U/μL Dream *Taq* polymerase enzyme
> positive control DNA
> thermocycler
> computer with internet connection

Procedure

1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (the number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (μL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H_2O	up to 24	

4. Pipette 24 μL master mix into each labelled PCR tube, and add 1.0-1.0 μL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.

5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	45 sec
	Annealing	45 °C	45 sec
	Extension	72 °C	45 sec
Final extension		72 °C	10 min
Store		4 °C	∞

7. Prepare 1 % agarose gel (see Exercise IV/1.), and check the amount and quality of PCR products in the same way as for general PCR samples.
8. If there are visible PCR products, perform the electrophoresis in a 2% agarose gel to get better separation of the DNA.
9. Mix the total amount of PCR products (25 µL) with 5 µL loading buffer, and load this mixture into the wells of the gel. To achieve a semi-quantitative measurement of the intensity and size of DNA, load 5 µL Maker 100 next to the samples.
10. Run the electrophoresis for 140 minutes at 80 V, and detect the presence and quantity of DNA under UV light. Make a digital image using a gel documentation system.
11. Calculate the expected size of PCR products of the following reference genotypes. First, find primer binding sites in the genome of *Synechococcus* sp. CC9605 (GenBank Acc. No. NC_007516) and make calculation for the size of the *in silico* PCR product. Identify and calculate the size of the overhanging region (which does not contain the IGS) of this virtual PCR product. Fill the following table with IGS length data retrieved from GenBank in the case of all reference isolates. Calculate the expected size of *cpcBA*-IGS PCR products.

Phylogenetic group	Isolate	Acc. No.	Origin	IGS length (bp)	Length of PCR product (bp)
A	LS0590	DQ526403	Lake Superior, USA		
	ACT 1025	HQ859453	Lake Fertő, Austria		
	ACT 9801	GQ888571	Lake Balaton, Hungary		
	ACT 9804	GQ888574	Lake Balaton, Hungary		
B	BO8807	AY224202	Lake Constance, Europe		
	PS725	AF223446	Lake Kasumigaura, Japan		
	MW4C3	AY151213	Mondsee, Austria		
	MW74D3	AY151220	Hallstattersee, Austria		
	ACT 9807	GQ888576	Lake Balaton, Hungary		
	MI0507A	FJ763790	Lake Mikolajske, Poland		
C	PS684	AF223454	Lake Abashiri, Japan		
	BE0807F	FJ763817	Lake Beldany, Poland		
D	PS673	AF223434	Lake Harutori, Japan		
	PS678	AF223441	Lake Kojima, Japan		
M	PS724	AF223436	Lake Tsukui, Japan		
	MA0607I	FJ763810	Lake Majcz, Poland		
	MA0607J	FJ763811	Lake Majcz, Poland		

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12. Calculate the expected size of PCR products for each phylogenetic group. Finally, try to identify the unknown isolates (and the composition of environmental samples) based on the size-distribution data.

Phylogenetic group	Length of PCR product (bp, from… to…)	Code of unknown isolates
A		
B		
C		
D		
M		

EXERCISE IV/20.: Grouping bacterial strains based on the length heterogeneity of the ribosomal ITS region

Object of study

bacterial genomic DNA

Materials and equipment

10× PCR buffer

1 mM dNTP mix

forward and reverse primers:

926F primer: 5' GGT TAA AAC TYA AAK GAA TTG ACG G3'

115R/23S primer: 5' CCG GGT TBC CCC ATT CGG 3'

positive control DNA

5 U/μL Dream *Taq* polymerase enzyme

thermocycler

Procedure

1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (the number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (μL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5.0	
forward primer	0.25	
reverse primer	0.25	
Dream *Taq* polymerase	0.25	
H$_2$O	up to 24	

4. Pipette 24 μL master mix into each labelled PCR tube, and add 1.0-1.0 μL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.

5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	30 sec
	Annealing	56 °C	1 min
	Extension	72 °C	1.5 min
Final extension		72 °C	10 min
Store		4 °C	∞

7. Prepare 1 % agarose gel (see Exercise IV/1.), and check the amount and quality of PCR products in the same way as for general PCR samples.
8. If there are visible PCR products, perform the electrophoresis in a 2% agarose gel to get better separation of the DNA.
9. Mix the total amount of PCR products (25 µL) with 5 µL loading buffer, and load this mixture into the wells of the gel. To achieve a semi-quantitative measurement of the intensity and size of DNA, load 5 µL appropriate DNA ladder next to the samples.
10. Run the electrophoresis for 140 minutes at 80 V, and detect the presence and quantity of DNA under UV light. Make a digital image using a gel documentation system.
11. Estimate the size of the ribosomal ITS region.

IV.7. Analysis of bacterial communities based on nucleic acids

Many of the hitherto described techniques are suitable for the analysis not only of individual bacterial strains but also that of environmental samples. For example, if we are interested in the nitrogen fixing potential of a bacterial community, we can perform a multitemplate PCR for the *nifH* gene. But in case we are interested in what kind of *nifH* gene sequences can be found in the environmental DNA, how high their diversity is, what kind of bacteria carry those *nifH* genes, additional steps are required in our analysis. Thus we have to separate the different *nifH* genes from each other. In the following chapter we present some of the main techniques used in community analysis.

DGGE (Denaturing Gradient Gel Electrophoresis)

The aim of DGGE is to generate a pattern from different environmental DNA samples based on differences in the melting point of the analysed gene sequences (in practice, the multitemplate PCR product). The first step is a PCR, where one of the primers contains an approximately 40 bp long GC-rich clamp on the 5' end, thus the GC-clamp is incorporated into the PCR product. Thereafter the product is run in a polyacrylamide gel containing a gradient of urea and formamide as denaturing agents. During the electrophoresis, the DNA molecules in the PCR product are melted at different denaturing concentrations, the two strands of a molecule are separated from each other except at the site of the GC-clamp, which holds together the two

single-stranded DNA. Therefore the progress of the molecule is slowed down or even stopped **(Fig. IV/16.)**.
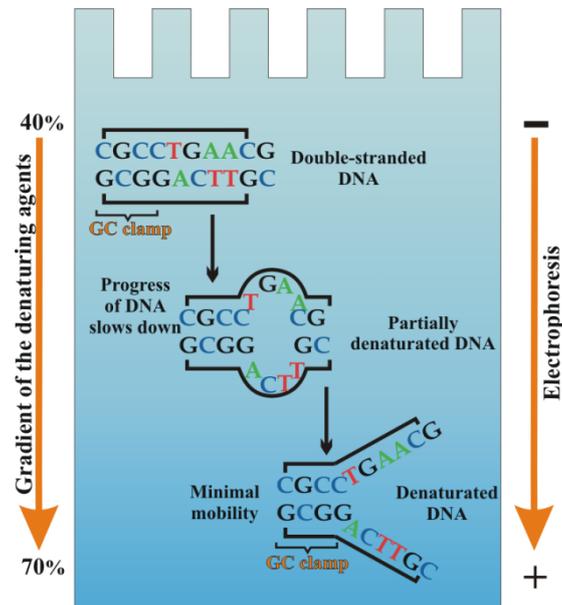
Fig. IV/16. Outline of the DGGE technique

Double stranded DNA molecules are separated in a polyacrylamide gel containing a linearly increasing denaturing gradient of formamide and urea at high temperature (cc. 60 °C). The molecules are melted according to their sequences, but the GC-clamp holds together the two single-stranded DNA, therefore their progress slows down.

Melting point is sequence-dependent, but it might occur that sequences without closer relation are melted at the same point of the gel. The resulted pattern can be analysed as described in Chapter VI.2. **(Fig. IV/17.)**, while separated bands can be excised, and their DNA can be reamplified. Moreover if the band contains only one type of DNA, it can be sequenced, whereas if the band is a mixture of different DNA molecules, it should be cloned (see later) before sequencing.

EXERCISE IV/21.: DGGE analysis of environmental DNA samples

Create a fingerprint from environmental DNA samples and compare their pattern using cluster analysis.

Object of study
    environmental DNA samples

Materials and equipment
    10× PCR buffer
    1 mM dNTP mix
    forward and reverse primers:
      GC-27F primer: 5' <u>CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG
                     CCC G</u>AG AGT TTG ATC MTG GCT CAG 3'
                     (underlined part is the GC-clamp)

534R primer: 5' ATT ACC GCG GCT GCT GG 3'

5 U/µL Dream *Taq* polymerase enzyme

positive control DNA

thermocycler

measuring cylinder

50-mL Falcon tube

40% acrylamide solution (acrylamide : bisacrylamide – 37.5:1)

50× TAE (Tris-Acetate-EDTA) buffer

ddH$_2$O (double distilled water)

TEMED (tetramethylethylenediamine)

20% ammonium-persulfate (APS) solution

electrophoresis system (Ingeny phorU-2) with gel casting system

loading dye

Hamilton syringe

gel staining box

DNA stain (ethidium-bromide solution)

scalpel

gel documentation system

computer with Totallab software

Procedure

PCR using one primer with GC-clamp

1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (the number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	5	
dNTP mix	10	
GC-27F primer	0.5	
534R primer	0.5	
Dream *Taq* polymerase	0.5	
H$_2$O	up to 48	

4. Pipette 48 µL master mix into each labelled PCR tube, and add 2.0-2.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

	Temperature	Time
Initial denaturation	98 °C	5 min
32 cycles — Denaturation	94 °C	30 sec
32 cycles — Annealing	52 °C	30 sec
32 cycles — Extension	72 °C	1 min
Final extension	72 °C	10 min
Store	4 °C	∞

7. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.). The 50 µL volume of the PCR is needed to get strong enough signals during the DGGE.

Preparation of the gel and running the electrophoresis in the Ingeny phorU-2 system

8. Assembly the electrophoresis system as described in its manual.
9. Prepare 7% acrylamide solution for 40%, 60% denaturing gel and stacking gel in 50-mL Falcon tubes.

	40% denaturant gel	60% denaturant gel	Stacking gel
40% acrylamide solution	4.2 mL	4.2 mL	1.05 mL
50× TAE buffer	480 µL	480 µL	120 µL
Urea	4 g	6 g	-
Formamide	3.84 mL	5.76 mL	-
ddH$_2$O	up to 24 mL	pu to 24 mL	up to 6 mL

10. Assembly the gradient maker as described in its manual.
11. Add 50 µL APS and 5 µL TEMED solution to 40% and 60% denaturant solutions and pour them into the gradient maker. Wait 1.5-2 hours for the polymerisation.
12. Meanwhile prepare 5 L fresh 1× TAE buffer using ddH$_2$O, pour it into the buffer tank of the electrophoresis system and preheat it to 60 °C.
13. After the polymerisation of the gradient gel, add 60 µL APS and 6 µL TEMED solution to the stacking gel solution, load it above the gradient gel and insert combs. Wait 0.5 hour for the polymerisation.
14. Put the gel casting system into the preheated buffer tank, and remove gently the combs. Wash the wells with 1× TAE solution using a Hamilton syringe in order to remove unpolymerised acrylamide particles.
15. Add 9 µL loading dye to each of the 45 µL PCR products and load them with a Hamilton syringe into the wells of the gel.
16. First, run the electrophoresis at 200 V for 150 minutes, so the samples will concentrate at the bottom of the stacking gel. Thereafter run it at 120 V for 14.5 hours at 60 °C.
17. Finishing the electrophoresis disassemble the gel system, stain the gel in ethidium-bromide solution for 20 minutes, and destain it for two times with incubation for 15-15 minutes in 1× TAE buffer. (Ethidium-bromide is toxic! Handle it carefully according to its safety data sheet!)
18. Detect the presence and quantity of DNA under UV light and obtain a digital image.

19. If excision of dominant bands is needed, cut them out with a sterile scalpel, and put them into 30 µL H$_2$O in an Eppendorf tube. Incubate at 4 °C overnight, thereafter transfer the liquid part into a new tube. Now it is ready for PCR or store at -20 °C for longer time.

Image analysis
20. Use the Totallab software for pattern analysis: recognize lanes, identify bands, find bands corresponding to each other in the different samples, and create data matrix. Finally group samples with cluster analysis (see Chapter VI.2.).
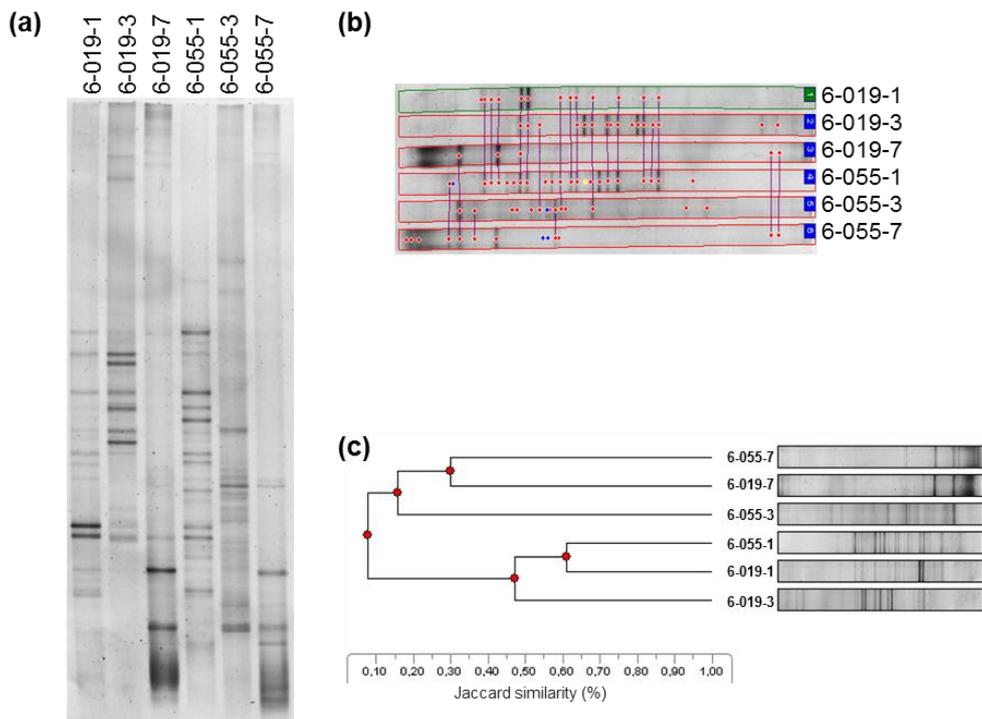
Fig. IV/17. Result of DGGE analysis of environmental DNA samples based on the partial 16S rRNA gene

(a) Image of DGGE gel. For sample names see Chapter VI. (b) Bands alignment: bands corresponding to each other in different samples are connected with lines. (c) UPGMA dendrogram (Chapter VI.2.) using Jaccard similarity values based on the DGGE band patterns.

T-RFLP (Terminal Restriction Fragment Length Polymorphism)

The aim of T-RFLP analysis, similarly to DGGE is to generate a pattern from different environmental DNA samples based on the position of the first restriction site of the endonuclease in the sequences of the multitemplate PCR product. The first step here is also a PCR, where one or both of the primers are fluorescently labelled. Thereafter the PCR product is digested with one or more restriction endonuclease, purified with ethanol precipitation and run on capillary electrophoresis. As only the terminal fragments are labelled with the fluorescent dye, the laser will detect only those molecules (**Fig. IV/18.** and see Chapter IV.1.). Due to the properties of capillary electrophoresis, T-RFLP has much higher resolution than DGGE, and comparison of samples is more precise owing to the applied internal standard during

electrophoresis **(Fig. IV/19.)**. The resulted pattern can be analysed as described in Chapter VI. On the other hand, it is not possible to excise and reamplify the dominant peaks just as in the case of DGGE bands. There is a way to compare fragment sizes with *in silico* digested reference databases, but it has many technical limitations.
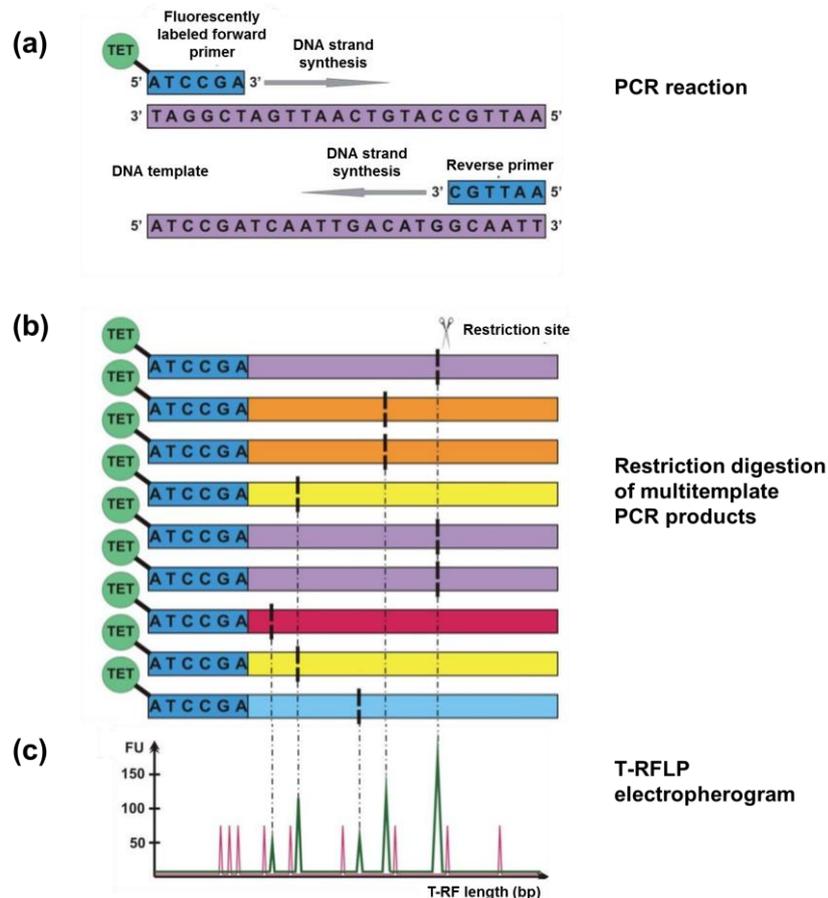
Fig. IV/18. Outline of the T-RFLP technique

EXERCISE IV/22.: T-RFLP analysis of environmental DNA samples

Create a fingerprint from environmental DNA samples and compare their pattern using different techniques.

Object of study
    environmental DNA samples

Materials and equipment
    10× PCR buffer
    1 mM dNTP mix
    forward and reverse primers:
      HEX-27F primer: 5' HEX-AGA GTT TGA TCM TGG CTC AG 3'
      534R primer: 5' ATT ACC GCG GCT GCT GG 3'
    5 U/µL Dream *Taq* polymerase enzyme

positive control DNA

thermocycler

10× buffer of restriction enzymes

restriction enzymes

water bath

genetic analyser used for capillary electrophoresis (e.g. ABI Prism™ 310 Genetic Analyser)

polymer for the electrophoresis (e.g. POP-4® Polymer)

internal size standard (e.g. GeneScan™ 500 TAMRA™ dye Size Standard)

electrophoresis buffer (e.g. 10× 310 Running Buffer)

molecular grade formamide

sample tubes and septa

heat thermostat

slush of ice or chill rack

computer having internet connection and with adequate software for raw data analysis (e.g. GeneMapper® Software) and for subsequent data analysis (see introduction for Chapter V).

Procedure

PCR using fluorescently labelled primer

1. Let frozen DNA samples and PCR reagents thaw at room temperature.
2. Label a 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube) and some PCR tubes (the number is equal to the number of DNA samples plus two additional tubes for the positive and negative controls).
3. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus three (for controls and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	5	
dNTP mix	10	
HEX-27F primer	0.5	
534R primer	0.5	
Dream *Taq* polymerase	0.5	
H₂O	up to 48	

4. Pipette 48 µL master mix into each labelled PCR tube, and add 2.0-2.0 µL DNA sample to the appropriate tubes. Do not add any DNA to the negative control tube, but to the positive control tube add a DNA sample, which had been amplified successfully in a previous PCR.
5. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.

6. Put all PCR tubes into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		98 °C	5 min
32 cycles	Denaturation	94 °C	30 sec
	Annealing	52 °C	30 sec
	Extension	72 °C	30 sec
Final extension		72 °C	10 min
Store		4 °C	∞

7. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.). The 50 µL volume of the PCR is preferred to have enough templates for multiple restriction reaction.

Restriction reaction and purification of its product

8. Let enzyme buffers thaw at room temperature.
9. Label 1.5 mL microcentrifuge tubes (in this will be measured all the reagents for the master mix) according to the number of used restriction enzymes and some 0.6 mL microcentrifuge tubes (the amount is determined by the number of PCR products multiplied by the number of used restriction enzymes).
10. Prepare master mixes as follows for each restriction enzyme, but do not add PCR products to it. For the amounts multiply the below amounts with the number of samples plus one (for pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× enzyme buffer	2.0	
enzyme (10 U/µL)	0.15	
H₂O	4.85	

11. Pipette 7 µL master mix into each labelled 0.6 mL microcentrifuge tube, and add 13-13 µL PCR product to the appropriate tubes.
12. Vortex the tubes gently, and spin them quickly to collect the liquid on the bottom of the tubes.
13. Incubate the samples in a water bath at the given temperature for 3 hours.

Enzyme	Buffer	Recognition and restriction site	Optimal temperature
*Alu*I	Y+/Tango	5' AG↓CT 3'	37 °C
*Bsu*RI	Buffer R	5' GG↓CC 3'	37 °C
*Hin*6I	Y+/Tango	5' G↓CGC 3'	37 °C
*Msp*I	Y+/Tango	5' C↓CGG 3'	37 °C

14. Perform sample purification with ethanol precipitation as described in Exercise IV/7.

15. Add 20 µL H$_2$O to the ethanol-precipitated DNA sample, vortex it and spin it down.
16. Prepare a master mix from 12 µL formamide and 0.3 µL internal size standard per sample.
17. Pipette 12.3 µL master mix and 1 µL sample into a sample tube, denature at 98 °C for 5 minutes, and put immediately to ice for 5 minutes.
18. Check the genetic analyser for buffer and polymer level, for capillary injection counter.
19. Put the sample tubes to the rack of the genetic analyser.
20. After test runs, load samples for 8 seconds at 15 kV, thereafter run the electrophoresis for 30 minutes at 15 kV and at 60 °C.

Data analysis

21. Start the Genemapper software for raw electropherogram analysis: detect peaks, check quality of internal size standard, signal-to-noise ratio and export peak table. If the electropherogram has low quality, run the sample again (steps 16-20.) with modified electrophoresis parameters according to the first run, so change the amount of sample volume added to the master mix or change the duration of sample loading.
22. Use T-REX (trex.biohpc.org/index.aspx) or scripts in program R for noise filtration and alignment of electropherograms. Analyse the created data matrix as described in Chapter VI.

Sample Name	Peak size in bp	Peak height in FU	Peak Area
6-055-1 Alu I	202.8	105	860
6-055-1 Alu I	205.51	230	2102
6-055-3 Alu I	202.84	718	7784
6-055-3 Alu I	207.16	2671	33273
6-055-7 Alu I	202.25	2152	23680
6-055-7 Alu I	206.78	2052	26680
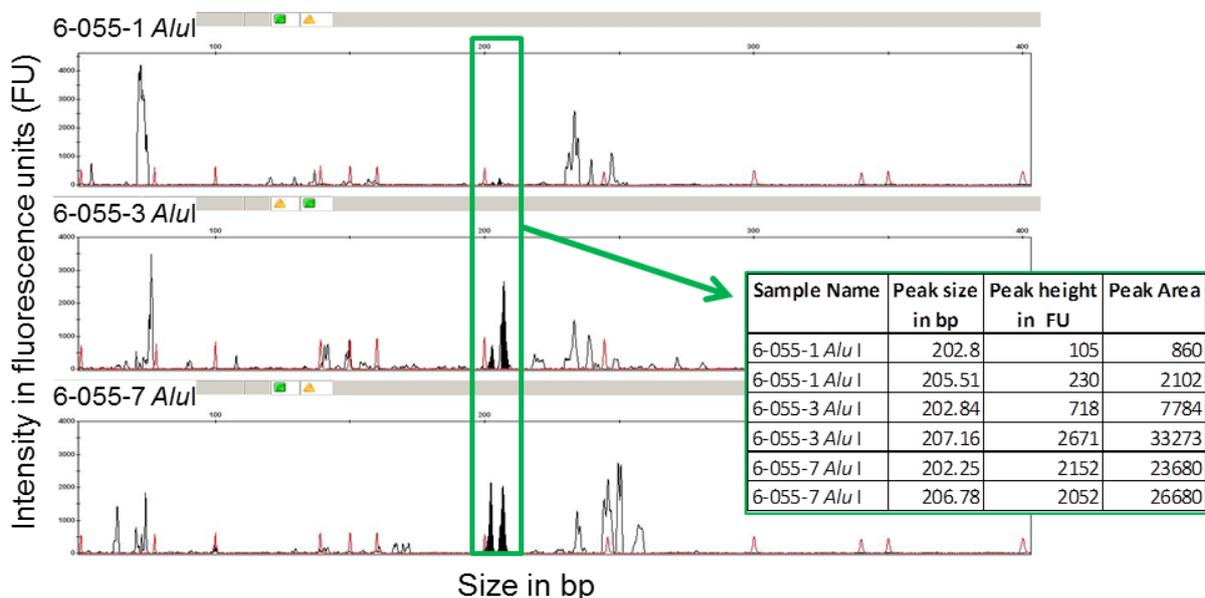
**Fig. IV/19. T-RFLP electropherogram of environmental DNA samples
based on the partial 16S rRNA gene**

Black curves denote the samples, while the red curves are the internal size standards. Properties of the marked peaks are given in the table on the right. For sample names see Chapter VI.

Preparing clone libraries

Molecular cloning enables us to separate mixed PCR product into homogeneous DNA molecules **(Fig. IV/20.)**. DNA molecules (insert DNA) are ligated into plasmid vectors, then introduced

into competent cells with transformation. Ideally only one plasmid will enter into each cell. The transformed cells are plated on antibiotic containing media. As antibiotic resistance is carried by the plasmid but not by the original competent cells, only transformed cells will grow on the plate. Usually the plasmid contains another marker for indicating successful ligation (e.g. the blue-white screening technique). During incubation competent cells and plasmid vectors within them proliferate. Thereafter plasmid-containing insert DNA are isolated from the colonies, the insert DNA molecules are reamplified and grouped e.g. with ARDRA (Exercise IV/17.). Finally representative sequences are subjected to sequencing (Exercise IV/15.).
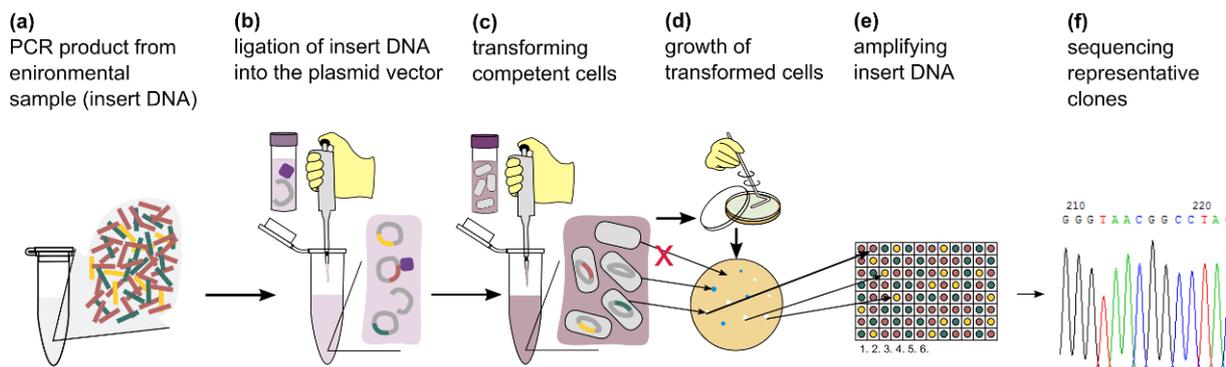
Fig. IV/20. Outline of preparing and analysing clone libraries

EXERCISE IV/23.: Preparing and analysing clone libraries

Object of study
      purified PCR product (insert DNA) from environmental DNA sample

Materials and equipment
      96-well PCR plates with plate adhesive cover
      heat thermoblock
      slush of ice
      LB plates containing ampicillin
      vector, ligation buffer, ligase
      competent cells
      SOC medium
      IPTG (Isopropyl β-D-1-thiogalactopyranoside; 100 mM)
      X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 50 mg/mL)
      sterile toothpicks
      10× PCR buffer
      1 mM dNTP mix
      forward and reverse primers specific for the plasmid vector:
        M13F primer: 5' GTA AAA CGA CGG CCA GT 3'
        M13R primer: 5' GGA AAC AGC TAT GAC CAT G 3'
      insert DNA specific primers

5 U/µL Dream *Taq* polymerase enzyme

positive control DNA

thermocycler

Procedure

Preparing clone library

1. Pipette 5 µL ligation buffer, 1 µL vector, 3 µL purified PCR product (insert DNA) and 1 µL ligase into a PCR tube. Mix it gently with pipetting and incubate at 4 °C overnight.
2. Spin down the PCR tube containing the ligation reaction.
3. Pipette 2 µL ligation reaction product into a new 1.5 mL microcentrifuge tube, and to put into ice.
4. Add 25 µL competent cells to it and incubate for 20 minutes on ice.
5. Put the cells for 50 seconds into thermoblock at 42 °C, and immediately put back to ice. This heat and cold shock helps transformation of the cells.
6. Add 500 µL SOC medium to the cells, and incubate them at 37 °C for 1.5 hour.
7. During incubation, spread 20 µL X-Gal and 100 µL IPTG onto the ampicillin-containing LB plates.
8. After the 1.5 hour incubation at 37 °C, spread 50, 100 and 200 µL cell suspension onto 3 LB plates.
9. Incubate the plates for 24 hours at 37 °C.
10. Next day transfer white colonies to new LB plates and incubate them for 24 hours at 37 °C.
11. Next day pipette 50 µL H₂O to each well of a 96-well PCR plate and transfer the new colonies into it with sterile toothpick. Let the two last wells empty, as these sites will be used in the following PCRs for negative and positive controls. Close the plate with adhesive cover.
12. Vortex the plate, and lyse the cells in a thermocycler at 98 °C for 5 minutes.
13. Centrifuge the plate for 3 minutes at 4,000 g in order to settle down cell debris.
14. Store the clones in the plate at -20 °C if not processed shortly.

Analysing clone library

15. Let frozen clones in 96-well plate and PCR reagents thaw at room temperature.

Often insert DNA itself is a partial 16S rDNA sequence. If one would try to amplify insert DNA directly, coamplification of 16S rDNA from the competent host cell could not be avoided. Thus, in a first PCR, the insert DNA with short flanking region from the vector DNA is enriched using vector specific primers. In that case the orientation of the insert DNA within the flanking vector regions is unknown, which is important if subsequent grouping of the insert DNA is required e.g. with ARDRA (step 23).

16. First a vector-specific PCR with primers M13 is prepared. Label two 1.5 mL microcentrifuge tube (all the reagents required for the master mix will be pipetted into this tube), as the volume of the master mix will exceed 1.5 mL, and a 96-well PCR plate.
17. Prepare a master mix as follows without the addition of DNA samples. For calculating the amount of reagents, multiply the values below with the number of DNA samples plus seven (for negative, positive control and pipetting errors).

Name and quantity (µL) of the reagents		In case of X sample
10× PCR buffer	2.5	
dNTP mix	5	
M13F primer	0.25	
M13R primer	0.25	
Dream *Taq* polymerase	0.125[1]	
H₂O	up to 24	

[1]As the plasmid DNA is much smaller than a genomic DNA, moderate initial denaturation is sufficient, thus the amount of polymerase can be reduced.

18. Pipette 24 µL master mix into each well of the PCR plate, and add 1.0-1.0 µL DNA sample from the clone library plate to the appropriate well of the PCR plate with multichannel pipette. Do not add any DNA to the negative control site, but to the positive control site add a DNA sample, which had been amplified successfully in a previous PCR. Close the plate with adhesive cover.
19. Vortex the plate gently, and spin it quickly to collect the liquid on the bottom of the wells.
20. Put the PCR plate into the thermocycler and start the reaction with the following parameters:

		Temperature	Time
Initial denaturation		95 °C	3 min
32 cycles	Denaturation	94 °C	30 sec
	Annealing	52 °C	30 sec
	Extension	72 °C	1 min
Final extension		72 °C	10 min
Store		4 °C	∞

21. Check the amount and quality of PCR products with agarose gel electrophoresis (Exercise IV/1.).
22. Prepare a second, insert DNA specific PCR using the M13 PCR product as template in the same way as above (steps 16-21.), with the following modifications: use insert DNA specific primers and PCR thermal profile and only 20 cycles during the amplification. This latter helps avoiding the coamplification from minute amounts of carryover DNA from the host competent cells.
23. Group clones e.g. with ARDRA (Exercise IV/17.), than subject representative clones to sequencing (Exercise IV/15.).

Microbial community analyses with NGS technologies

Next-generation DNA sequencing technologies has been also introduced in the analysis of complex microbial communities. There are two main forms of such approaches. The so-called amplicon sequencing (i.e. sequencing a PCR product) is used for deciphering the taxonomic composition of the studied community. It is usually based on one selected gene (most commonly the 16S rRNA gene), for which a massive parallel sequencing is performed (sequencing >5,000-10,000 reads/sample). If the read length is around 200 bp, only family-level identification is possible, while using reads around 400-500 bp, genus-level identification of community members

can be achieved. In the case of amplicon sequencing, Illumina and 454 pyrosequencing are the most commonly used NGS techniques in microbial community ecology studies.

The other type of NGS-based investigations performed in the study of microbial communities is termed as the 'shotgun' metagenomic approach. In this case, the whole environmental DNA (which contains the genomes of hundreds of organisms, including viruses, bacteria, protists, algae and also macroscopic species) is fragmented, and subsequently this fragmented DNA library is sequenced. Based on the obtained data, large genome fragments could be assembled. These genome fragments are suitable not only for the taxonomic identification of community members, but also for predicting their metabolic potential based on the detected protein-coding genes, and therefore assessing the functional profile of the whole community. Such metagenomic studies require large sequence data (> several 100,000 reads/sample) and are performed with the Illumina MiSeq and IonTorrent sequencing systems. It should be noted, that although single molecule NGS techniques (e.g. PacBio) promise great potential due to the long sequence reads and the lack of amplification prior to sequencing, their application in environmental microbiological studies is currently relatively sparse.

IV.8. Study of environmental samples in practice

Many methods were described in Chapter IV. **Fig. IV/21.** shows, how they are connected to each other, when environmental samples are examined. The very first step is the creation and strict formulation of a hypothesis. The hypothesis and later the first experiences and results will determine which methods should be applied. Just arbitrary increasing the number of the applied methods will not improve the quality of the results!
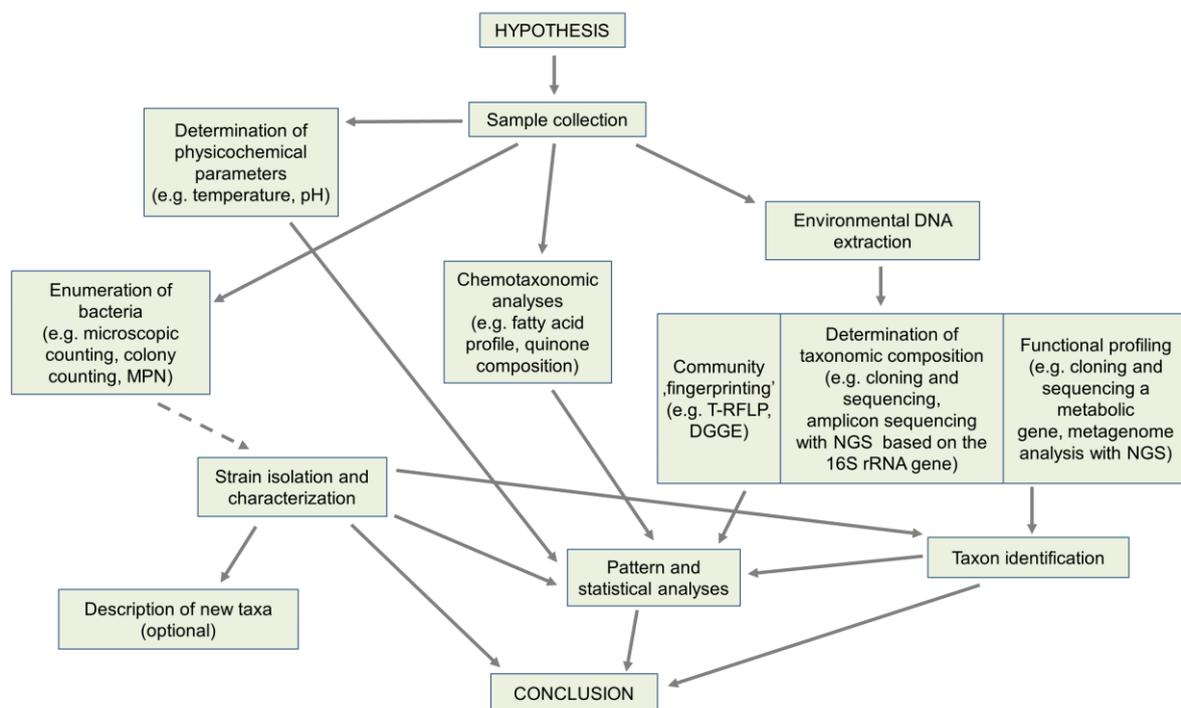
Fig. IV/21. Methods in the analysis of microbial community structure
The collection of methods is not exhaustive, the figure depicts an example of analysis workflow.

On **Fig. IV/22.**, three approaches suitable for deciphering the microbial community structure are summarized as example.
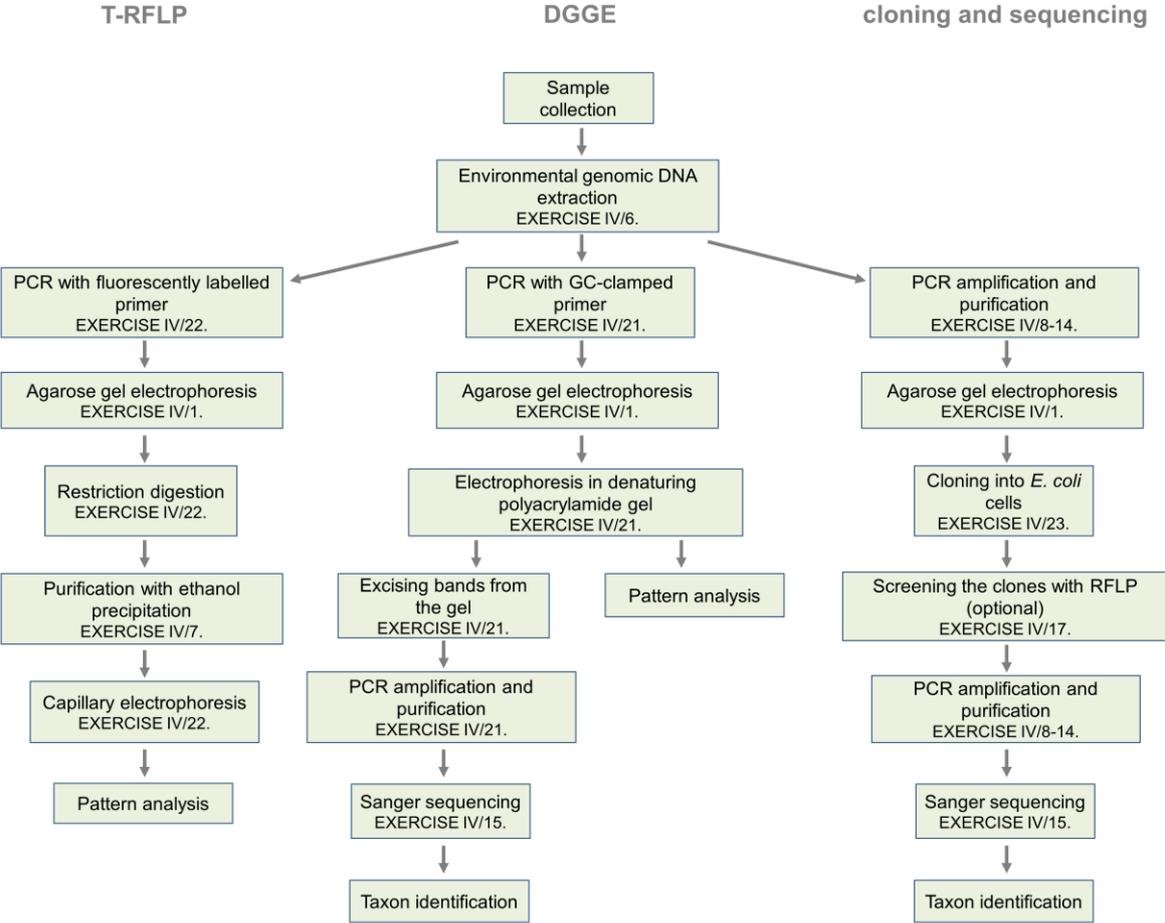
Fig. IV/22. General process workflow of T-RFLP-, DGGE- and cloning and sequencing-based environmental microbiological studies

V. QUANTIFICATION OF BACTERIA (Erika Tóth)

Enumeration of microbes is often essential in ecological studies. Cultivation and cultivation independent methods are both used to count them but it is well known from literature that usually less than 1-10% of bacteria are cultivable (depending on the studied environment) called as the 'grate plate count anomaly'. Several reasons are responsible for this phenomenon, e.g. the cultivation medium is not adequate or the bacteria can be in VBNC (viable but non-culturable) state. This anomaly is also represented by the observation, that microscopic cell counts are often magnitudes higher than CFU (colony forming units) numbers. In this practical guide the number of bacteria is determined from aquatic samples.

V.1. Microscopic cell counting

For direct cell counting, most often fluorescent/epifluorescent microscopic techniques are used on fixed and stained samples (sometimes the autofluorescence of microbes is examined).

In case of fluorescence microscopy the specimen is illuminated with light of a defined wavelength which is absorbed by the fluorophore(s). Then the specimen emits light of longer wavelengths (different in colour than the absorbed light) which is detected by the microscope.

Many fluorescent stains are known, one of the most common is DAPI (4',6-diamidino-2-phenylindole), which is able to penetrate cell membranes and binds to the double helix of the DNA. DAPI has an absorption maximum at a wavelength of 358 nm (UV) and its emission maximum is at 461 nm (blue), so in fluorescence microscopy DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. Cells can be easily counted, if a known volume of fixed water sample is filtered through a membrane and, after staining the cells, the filter is investigated by an epifluorescence microscope **(Fig. V/1.)**.
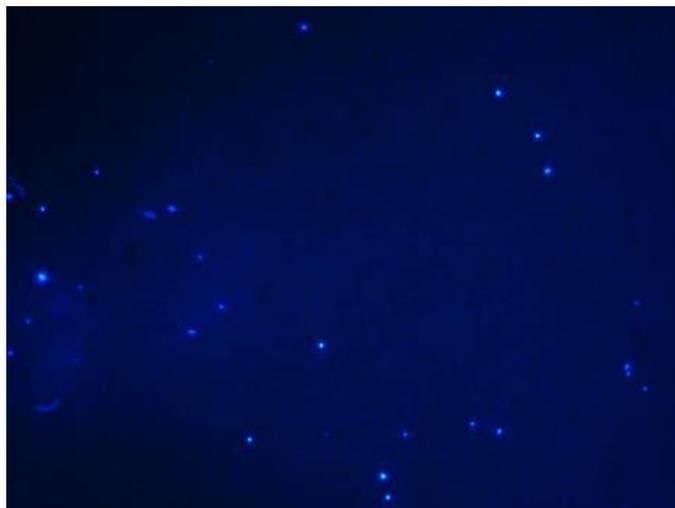
Fig. V/1. Epifluorescence microscopic image of DAPI stained cells from a water sample

EXERCISE V/1.: Enumeration of microbes with DAPI staining

The description of the procedure applies already fixed and filtered samples and lacks the previous steps of sample filtration and fixation.

Object of study

      paraformaldehyde fixed (overnight at 4 °C) water samples filtered on a nitrocellulose filter
        (Millipore, 0.22 μm)

Materials and equipment

      small size (25 mm in diameter) plastic Petri dishes
      scalpels
      pipette with pipette tips
      DAPI solution (1 μg/mL)
      80% ethanol
      distilled water
      glass slide and cover slip
      Vectashield Mounting Medium (H-1000, Vector Laboratories Ltd.)
      immersion oil
      paper towel
      epifluorescence microscope with a mercury lamp
      digital camera connected to the microscope and PC
      computer with adequate software

Procedure
1. Transfer the filter (with the known volume filtered and fixed sample) from its sample holder to an empty Petri dish.
2. Cut out a small piece (ca. 0.5 cm^2) from the filter with a scalpel or scissors, and pipette 30 μL DAPI solution onto its surface (avoid light as DAPI is sensitive for it!).
3. After 2 minutes, transfer the filter into 80% ethanol (hold in a next Petri dish) for a few seconds.
4. Take out the filter from the alcohol and put it into double distilled water for a few seconds.
5. Dry the stained piece of filter.
6. Place the filter onto the surface of a degreased glass slide, put a drop of Vectashield Mounting Medium onto the filter and then cover it with a cover slip. With the help of a paper towel gently press the cover slip to remove any excess of mounting medium.
7. Examine the slide with epifluorescence microscope using a 100× objective and immersion oil under UV excitation and using a blue filter.
8. Record images from at least 20 different microscopic fields with a digital camera.
9. Count the cells on each picture with the help of the given program package, determine the mean values and enter your results in an excel spreadsheet.
10. Determine the cell count for one mL water sample based on the amount of filtered water.

V.2. Quantification of different bacteria based on MPN method

MPN (Most Probable Number) is often used to quantify different types of bacteria from natural waters. The test is able to estimate the number of viable (and growing) microorganisms in a sample after borderline dilutions, After the incubation period, estimation of microbial germ count is possible using statistical tables based on the number of positive tubes/wells (showing characteristic microbial growth: change in optical density, shifting of pH, changing of redox values and subsequently colour, etc.). This method presumes that in the liquid medium the distribution of cells is uniform, cells do not clump together and that there is detectable growth even if only one viable microbial cell is present in the tube/well. The applied medium determines which type of bacteria can be enumerated (total germ count, coliform bacteria, etc.).

EXERCISE V/2.: Determination the total germ count of surface waters with the MPN method

Object of study

water sample from a natural aquatic habitat (e.g. Danube)

Materials and equipment

pipette, sterile tips
sterile Eppendorf tubes
sterile microtiter plate
medium for cultivation (adequate to cultivate heterotrophic bacteria, e.g. nutrient, R2A medium)
microplate reader (Tecan Sunrise)
MPN table to evaluate data

Procedure

1. Measure 1350 µL medium to 8 different Eppendorf tubes.
2. Make a dilution series in the medium (1350 µL medium and 150 µL sample).
3. From all dilutions pipette 250 µL to the wells of the microtiter plate in 5 parallels in each case.
4. Incubate it at room temperature for several days.
5. Check the optical density (OD) of the wells at 620 nm with a microplate reader after 24 and 48 hrs (if possible even later).
6. Calculate the cell number values with Garthright and Blodgett's MPN-calculator (www.fda.gov/downloads/Food/FoodScienceResearch/UCM164329.zip).

Quantification of hygienically relevant bacteria – study of faecal indicator organisms

In hygienic practise, indicator organisms are used to qualify the pollution of different waters (natural waters, drinking waters, etc.). Their presence in the water indicates whether the water is contaminated by faeces or not. Indicator organisms must be present everywhere where pathogens

are also present, they cannot be obligate pathogenic microbes and they must be characterised by longer survival time than the general enteral pathogenic microbes. Moreover, the quantity of indicator organisms must show direct correlation with the degree of faecal contamination and there should be a simple test method to check them.

The most common faecal indicator organisms are coliform bacteria (including *E. coli*), faecal enterococci and clostridia.

Coli-count determination by Colilert system

Coliforms in hygienic practice are defined as facultative anaerobic, Gram-negative, non-endospore-forming, rod-shaped bacteria that ferment lactose by producing acid and gas within 48 hours at 37 °C. Coliforms are members of the family Enterobacteriaceae.

The Colilert method can be used to test faecal contamination of different waters by coliform bacteria. It is a commercially available enzyme-substrate liquid broth medium, which simultaneously detects and quantifies both total coliforms and *Escherichia coli*. It is adapted to the MPN method. Coliforms (including *E.coli*) metabolise ortho-nitrophenyl-galactopyranoside using the enzyme β-galactosidase to produce ortho-nitrophenyl. This results in a yellow colouration of the test well **(Fig. V/2.a)**. *E.coli* will also metabolise 4-methyl-umbelliferyl-glucoronide using the enzyme β-glucuronidase to produce 4-methyl-umbelliferone which fluoresces under long wave (365 nm) ultra violet light. **(Fig. V/2.b).**

EXERCISE V/3.: Enumeration of coliform bacteria and *Escherichia coli* with the Colilert system

Object of study
    water sample from a natural aquatic habitat (e.g. Danube)

Materials and equipment
   sterile 100 mL bottles
    sterile distilled water
    sterile measuring cylinder
    pipettes, sterile tips
    Colilert growth substrates (kept in dark)
    Quanti-Tray bags
    Quanti-Tray sealer
    37 °C incubator
    UV lamp
    MPN table to evaluate data

Procedure
1. Two dilutions (1:10 and 1:100) are used for each sample except those, where extremely high pollution can be expected.

2. For the 1:10 dilution, take 10 mL from the original sample and dilute it with 90 mL sterile distilled water in a sterile 100 mL bottle.
3. For the 1:100 dilution, take 1 mL from the original sample and dilute it with 99 mL sterile distilled water in a sterile 100 mL bottle.
4. Add growth substrate (stored at 4 °C in the fridge) and shake it vigorously. Wait for approximately 15 min for complete dissolution.
5. Fill the samples into the Quanti-Tray bags and carefully seal them with Quanti-Tray sealer avoiding bubble formation.
6. Sign Quanti-Tray bags with sample ID number and degree of dilution.
7. Incubate the bags at least for 18 hours (when possible, up to 24 hours) at 37 °C.
8. Count positive wells of total coliforms (yellow compartments) and compare with MPN table. Give the coliform values for the sample.
9. Count positive wells of *Escherichia coli* (blue compartments under UV-light at 365 nm) and compare with MPN table. Give *E. coli* values for the sample.
10. Register the results into an Excel table.
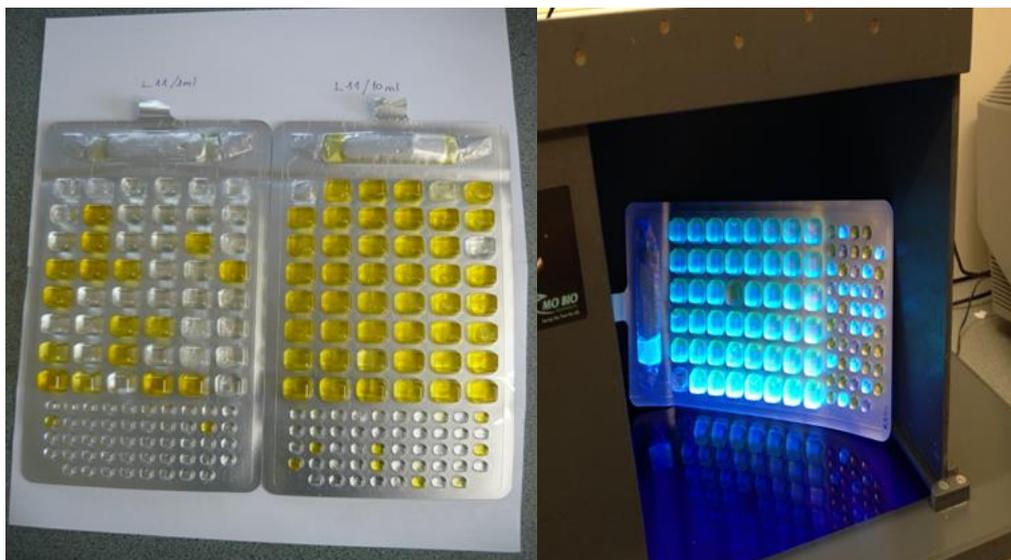
(a) coliform (b) *E. coli*

Fig. V/2. Detection of coliform and *E. coli* in Quanti-Tray bags

Determination the number of enterococci from surface waters

Enterococcus faecalis (formerly named as *Streptococcus faecalis*) is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of mammals (including humans). It is considered as one of the most suitable indicators of faecal pollution in aquatic habitats, as this bacterium does not reproduce in non-faecal environments, and often perishes in natural waters. Thus its presence confirms the results of coliform testing and indicates the fresh faecal contamination of water.

Detection is based on the fact that the enzyme β-glucosidase of *E. faecalis* breaks the compound 4-methyl-umbellipheryl-β-D-glucoside in the medium and in case of positive reaction it can be visualised under UV lamp.

EXERCISE V/4.: Determination the number of enterococci with the MPN method

Object of study

water sample from a natural aquatic habitat (e.g. Danube)

Materials and equipment

pipettes, sterile tips (1 and 5 mL)
multichannel pipette
18 mL DSM broth (ready to use, DSM 3F0085, Bromphenol Blue Tubes)
  (stored in the lab at room temperature)
microplates
sterile plastic wells
closing foils
UV lamp
43±1 °C incubator
MPN table to evaluate data

Procedure

1. Two dilutions (1:2 and 1:20) are used for all samples.
2. Add 18 mL water sample to 18 mL DSM medium (= 1:2 dilution).
3. Take 2 mL from 1:2 dilution and add to 18 mL DSM medium (= 1:20 dilution).
4. Sign 96-well microplates (stored at 4 °C in the fridge) with sample ID number and degree of dilution.
5. Put 1:20 dilution in sterile plastic well and pipette 200 μL into each microplate well with multichannel pipette equipped with sterile 300 μL tips (fix them slightly after taking them from the box!) in 4 respective rows.
6. Discard the rest of the sample/DSM mixture.
7. Put 1:2 dilution in the same plastic well and fill 200 μL in each microplate well with multichannel pipette equipped with sterile 300 μL tips (fix them slightly after taking them from the box!) in 8 respective rows.
8. Seal microtiter plate with closing foil (stored also in the fridge).
9. Incubate at 43±1 °C for 48 hours, check under UV-light, count positive wells and compare with MPN table. Give the *Enterococcus* values for the sample.
10. Register results into an Excel table.

Quantification of clostridia from surface waters

Obligate anaerobic bacteria multiply in habitats with low oxidation-reduction potential. They have been categorized based on their O_2 requirements: usually strict anaerobes grow in $\leq 0.4\%$ O_2, and aerotolerant anaerobes grow in $\geq 2.5\%$ O_2 concentration of the atmosphere. The metabolism of obligate anaerobic chemoorganotrophic heterotrophic bacteria may be based on

fermentation or anaerobic respiration. The appropriate method for the cultivation of these bacteria should be chosen with considering the sensitivity of the given organism to oxygen concentration and/or redox value within the media.

C. perfringens is a Gram-positive, rod-shaped, anaerobic, pathogenic bacterium. Due to its endospores it survives in surface waters for a relatively long period, therefore its presence can indicate old faecal pollution of surface waters.

EXERCISE V/5.: Determination of *Clostridium perfringens* from surface waters

Object of study
　　water sample from a natural aquatic habitat (e.g. Danube)

Materials and equipment
　　250 mL sterile bottle
　　60 °C water bath
　　0.45 μm pore-size cellulose nitrate membrane filter
　　sterile filtration equipment
　　distilled water
　　pipettes, sterile tips
　　tweezers
　　TSC (Tryptose Sulphite Cycloserine) agar medium (ready to use)
　　anaerobic indicator strip
　　anaerobic bags with stick

Procedure
1. Pasteurise 120 mL water sample in a sterile 250 mL glass bottle in a water bath (60 °C, for 15 min).
2. Filter 100 mL, 10 mL and 1 mL (amended with at least 10 mL of sterile distilled water) through sterile 0.45 μm pore-size cellulose nitrate membrane filters in separate sterile filtration units.
3. Put filters with sterile tweezers onto pre-dried TSC agar plates. Put the agar plates into anaerobic bags, add an anaerobic indicator strip dipped in water and oxygen-binding sachet (open carefully - there are different types of sachets, the one which is needed to fill with water, you have to add 8 mL distilled water).
4. After it immediately close the bag with stick. Incubate at 44 °C for 21 hours.
5. Count light brown, brown, black colonies immediately after opening the anaerobic bag.
6. Take photographs of appropriate dilutions **(Fig. V/3.)**. Give the number of clostridia in the sample.
7. Register results into an Excel table.
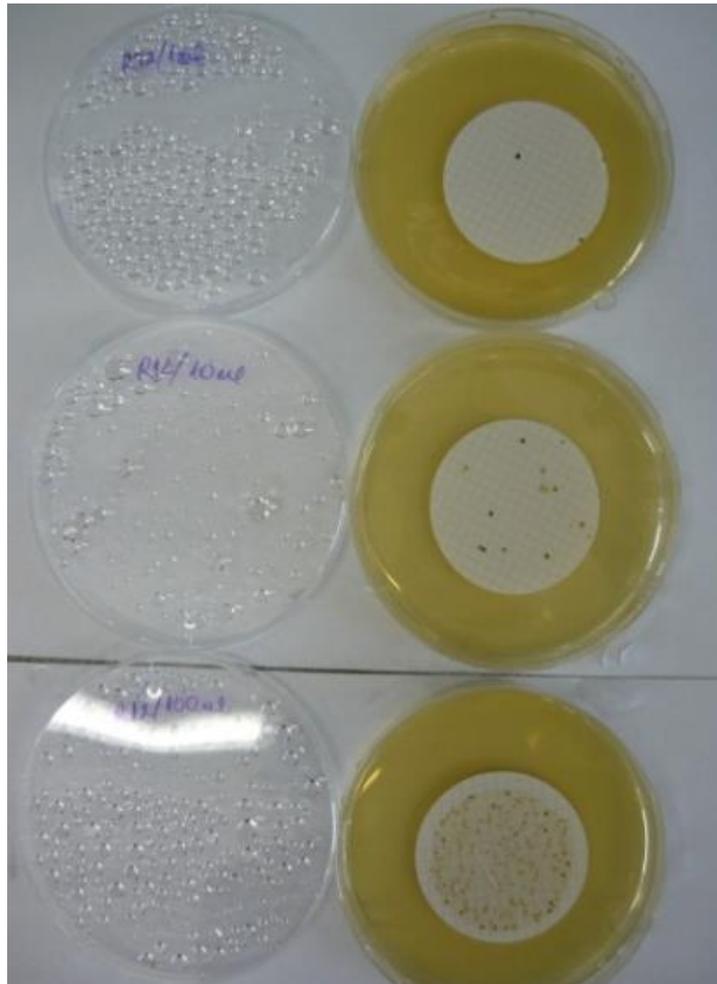
Fig. V/3. Enumeration of *C. perfringens* on the surface of TSC medium

Different filter volumes: 1, 10 and 100 mL water sample. *C. perfringens* forms brownish and black colonies.

VI. DATA ANALYSIS (Balázs Vajna)

Many methods described in the previous chapters generate patterns to characterize the microbes from a sample. These patterns are occasionally complex, especially if we consider other environmental data from the same samples. Although it is easy to visualize the result of a PCR with an agarose gel electrophoresis but the primary results of many other analyses are difficult to interpret. Perhaps from a RAPD gel image with only ten samples we can tell which samples are similar to each other but further steps are required for quantitative analysis (e.g. cluster analysis). In case, we have hundred samples, not even assigning the samples into the main groups is easy.

In general raw results from different methods (RAPD, DGGE, T-RFLP, chemotaxonomic analyses) are collected into data matrix. It is a common spreadsheet where each sample is represented in a separate column and each band, T-RF peak, etc. from a sample can be found in a separate row. In data analysis samples (columns of the data matrix) are called as objects, while band, T-RF peak, etc. are called as variables **(Fig. VI/1.)**. It is a special case when more than one type of variable (e.g. T-RF peaks and environmental data) is present in one dataset. We will come back to this situation in Chapter VI.4. The main problem with large datasets (called multivariate datasets) is that only the great mess of numbers can be seen and so the visualization and real evaluation of data is difficult.

Each **column** of the matirx corresponds to one **sample**, called **object**

	6-019-1	6-019-7	6-055-1	6-055-3	6-055-7
Alu-050	0	1.21	0.34	0	0
Alu-053	0	0	0	0	0.47
Alu-054	0	0	2.87	0.27	0
Alu-056	0	0.78	0	0	0
Alu-057	0	0	0	0	0.21
Alu-059	0	0.69	0	0	0
Alu-060	0	0	0	0	0
Alu-062	0.75	0	0	0	1.61
Alu-063	0	0	0	0.66	6.19
Alu-065	0	7.49	0	0	0
Alu-066	0	4.31	0.51	1.5	0
Alu-067	0	0	0	0	0
Alu-070	22.41	3.99	0	1.29	2.94

Each **row** of the matrix corresponds to one **T-RF**, called **variable**

Fig. VI/1. Part of our example T-RFLP data matrix

Column headers stand for sample name, whereas row headers for length of the respective T-RF. E.g. the last value from the 6-019-1 sample ('22.41') shows, that the length of this T-RF resulted from the *Alu*I digestion of the PCR product is 70 bp and the relative abundance of this T-RF within the electropherogram is 22.41%.

In this chapter we present different analyses through the same dataset **(Fig. VI/1.)**, which is the result of a 16S rRNA gene based T-RFLP fingerprint obtained from monitoring the bacterial community composition during oyster mushroom substrate preparation (Vajna et al., 2012). The samples (objects) were denoted with a three-parted-code (e.g. 6-019-1), where the first number indicated the year of sampling (e.g. 2006), second set of numbers stood for the factory production series (e.g. 019), and the third for the main stages of substrate preparation (e.g. 1st). Based on our assumption the last numbers are important, as bacterial community composition of the samples is thought to group according to stages (1, 3 and 7).

Often a scientific question requires more than one type of analysis because of more reasons. First, some statistical analyses need certain assumptions to evaluate them, so those assumptions have to be tested on the dataset before the analysis. If the analysis requires a specific distribution of the dataset (e.g. assume a normal distribution of variables), the test is called as parametric statistical analysis. The other type of statistical analysis does not require so hard assumptions (e.g. it requires only that the dataset should be symmetric). These statistical analyses are called as non-parametric tests. However the parametric approaches usually have more power that is smaller difference could be detected significantly. In other words smaller sample size should be investigated to detect the same effect than in non-parametric approaches. Second reason for using different analyses is that they often could answer for different aspects of the question complementing each other.

In this chapter we use exploratory data analysis tools where we try to interpret data structure of large datasets (Chapter VI.1.), and we identify groups of samples (Chapter VI.2.). Then we continue with formulating strict null hypotheses performing statistical tests and interpret them (based on the test results the null hypothesis will be accepted or rejected). Without details we will introduce some useful techniques (Chapter VI.3.). Finally we present a study in which we are looking for possible correlations among different types of datasets in a multiple dataset for the same samples (Chapter VI.4.).

There is not any exercise in this chapter, since the aim of this part of the practical guide is to get a general overview in data analysis.

Finally let us give a few words about the applied software. A simple diagram can be created by Microsoft Excel but it is not a dedicated program for detailed data analysis. Past (**Pa**leontological **St**atistics) is a free, user friendly program for scientific data analysis with functions for data manipulation, plotting, univariate and multivariate statistics, ecological analysis (folk.uio.no/ohammer/past). The last recommended program to perform statistical analysis is R (www.r-project.org), which is a free software environment for statistical computing and graphics. It is a command line operated program which makes its use a bit more difficult. One partial help is offered by RStudio, a powerful and productive user interface for R (www.rstudio.com). The main advantage of R is that it is an open-source software supported currently by 7814 (in 2016) supplementary packages and with exercises almost every task can be converted into R commands, the so-called scripts. For special analysis, package *vegan* can be used involving ordination methods, diversity analysis and other functions for community and vegetation ecologists (cran.r-project.org/web/packages/vegan/index.html).

VI.1. Visualizing relationship among samples

Hundred-percent stacked column chart

One of the easiest way for visualization is creating a 100% stacked column chart **(Fig. VI/2.)**. In case of many samples and variables it is still quite complicated. However at first sight, several main trends can be noticed by it, especially if some variables can be interpreted alone (e.g. in case of T-RFLP the presence of a T-RF and its size represents the abundance of a genotype on the community, but this is not the case in RAPD).
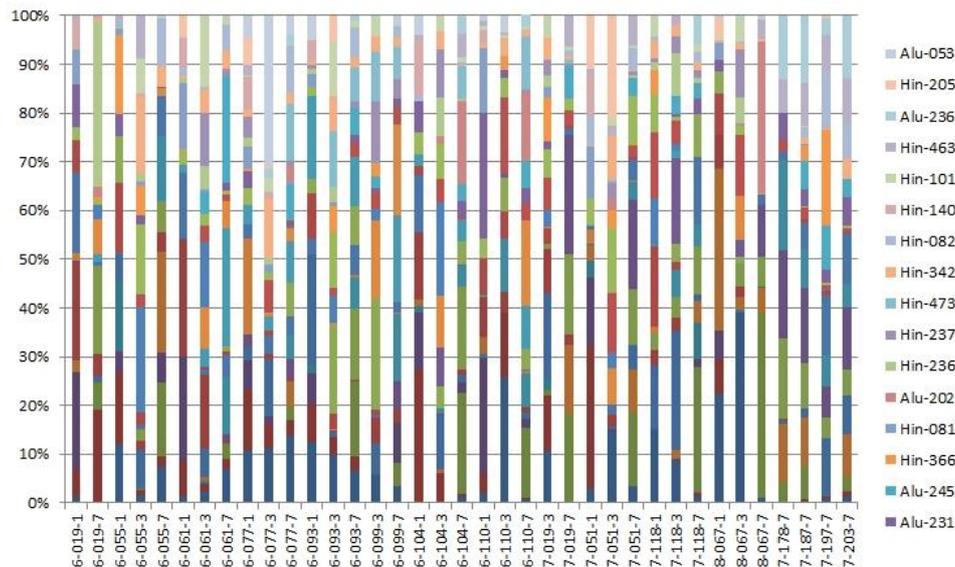
Fig. VI/2. Hundred-percent stacked column chart **of our example T-RFLP dataset**
Each column represents a sample, while different colours correspond to different T-RF peaks. Some of the peak names are given in the legend on the right side.

Diversity indices

Diversity indices compress large datasets (with more samples) into a single value representing something about the attributes of samples but losing the majority of the information. Even so sometimes we need them as additional information, hence some analyses are not independent from the diversity of samples. Extremely low or high diversity values can cause outlier sample settings and can bias the result of the analysis. The two most traditional indices are the Simpson and the Shannon indices **(Fig. VI/3.)**.

The Simpson index ($D=1-\sum[n_i/n]^2$, where 'n_i' is the abundance of the nth variable, whereas 'n' is the abundance of all variable together) measures the evenness of the community. Its minimum value is zero: the community consists of only one type of object. Whereas the Simpson index tends to the maximum ($D=1$) when all objects in the community have the same abundances.

The Shannon index ($H=-\sum[n_i/n]*ln[n_i/n]$) shows the 'entropy of the community'. Its minimum is zero like at the Simpson index: there is only one type of object in the community. Whereas its maximum value has no upper limit: there are many types of community members.

Generally, the values of the two indices show similar trend across samples **(Fig. VI/3.)**. By their usage, we have to be aware, that the Simpson index overestimates, whereas the Shannon index underestimates dominant variables.
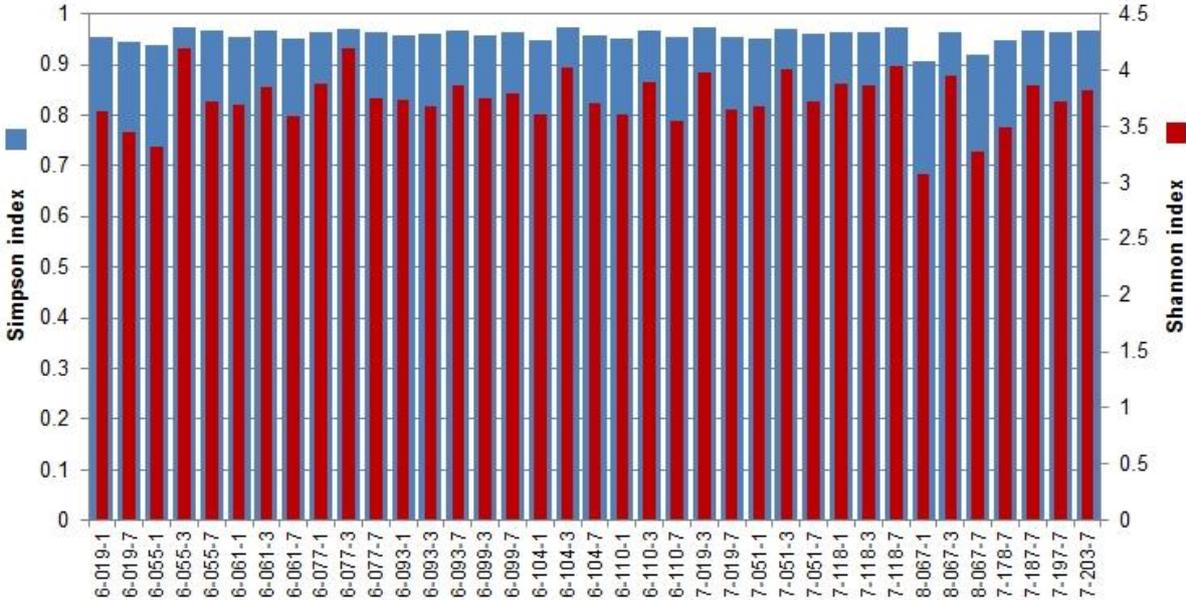
Fig. VI/3. Simpson and Shannon indices of the samples based on our example T-RFLP dataset

Ordination

The main problem with large datasets is that it is impossible to represent them graphically in more than three dimensions. The aim of the ordination methods is to reduce the number of dimensions of a multivariate dataset without losing too much information (i.e. the hidden structure within the results).

Principal Component Analysis (PCA)

The most common method of ordination is **p**rincipal **c**omponent **a**nalysis (PCA). During PCA the original variables are converted into linearly uncorrelated new variables, called as principal components (PCs). Therefore PCs reflect the original data structure in fewer dimensions than the original dataset. The first principal component accounts for as much of the original data variability as possible. All subsequent PCs account for as much of the original data variability as possible under constraints that they are uncorrelated with previous PCs. The new sample coordinates in the ordination, according to them the samples can be plotted on the ordination diagram, can be calculated as the linear combination of the values of the original variables, while PCs are linear combinations of the original variables. The major advantage of PCA is to combine the joint display of samples and the original variables in a single diagram (called as biplot) **(Fig. VI/4.a)**. PCA has two prerequisites: multinormal distribution and linear relations among the original variables. Albeit PCA can tolerate small deviations from the prerequisites, since it is a quite robust analysis. The efficiency of this method can be reviewed through the relative contribution of each PC to the original variability as seen on a special diagram, called screeplot **(Fig. VI/4.c)**. An ideal PCA covers more than 50% of the original variability with the first 2-3 PCs. An alternative approach is to overlay the result of a classification (see Chapter VI.2.) onto

the ordination **(Fig. VI/4.b)**. When classification derived groups nicely separated on the plot, the result of the PCA is confirmed.
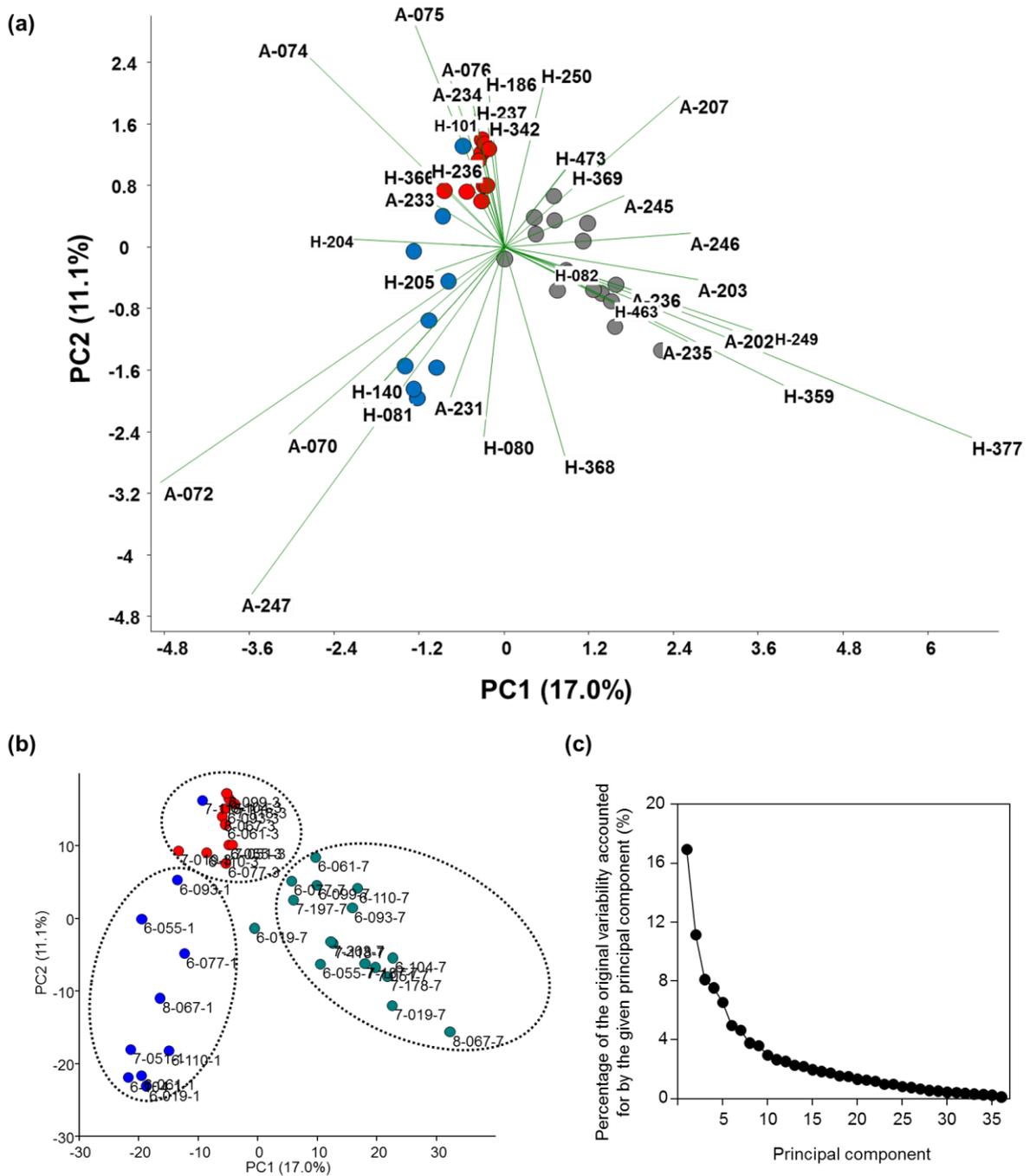
Fig. VI/4. Principal component analysis (PCA) of our example T-RFLP dataset
(a) PCA with biplot function. Points represent samples, where blue, red and grey dots mark stage 1, 3, and 7 samples. Lines represent the dominant peaks obtained by T-RFLP. In peak names 'A' and 'H' stands for *Alu*I and *Hin*6I enzymatic digestions and the number thereafter for the length of the respective T-RF.
(b) PCA with overlay of cluster analysis result. Dotted lines encircle samples with Bray-Curtis similarities higher than 0.25 (Fig. VI/6.). (c) Screeplot of PCA.

Non-metric multidimensional scaling (NMDS)

Another method is the **n**on-**m**etric multi**d**imensional **s**caling (NMDS). The advantage of NMDS over the PCA is that it does not need so strict prerequisites. The first step is to create a distance matrix from the original dataset where Bray-Curtis distance is preferred in the case of biological data. Bray-Curtis distance is calculated as follows $BC = 1 - \sum min[n_{ji}; n_{ki}] / \sum [n_{ji} + n_{ki}]$, where '$n_{ji}$' and '$n_{ki}$' are the abundance of the ith variable in sample 'j' and 'k'. Shortly the minimum and the sum of all variables at both sites are summed and their ratios are subtracted from 1. The values of the distance matrix are ranked and thereafter NMDS uses only the ranks of distance values. Finally, it arranges samples in a two- or three-dimensional plot so that the rank order of ordination distances on the plot is as close to the rank order in the original distance matrix as much as possible **(Fig. VI/5.a)**. Differences among the original rank of distance values in the distance matrix versus the obtained ranks of distance values in the NMDS ordination can be reviewed on the Shepard stress plot **(Fig. VI/5.b)**. In ideal case, all points should be placed on a straight ascending line with a slope equal to 1. The deviation from the ideal case is expressed with a stress value calculated using point distances from the ideal line. In our example dataset it is 0.213. There is no general rule, but stress values under 0.15 are preferred. So our ordination is not perfect, but its goodness is confirmed by overlaying results of cluster analysis. Samples from different stages are nicely separated **(Fig. VI/5.a)**. The relative high stress value however, denotes that we should avoid drawing detailed conclusions about relationship among samples within each stage based on this NMDS ordination.
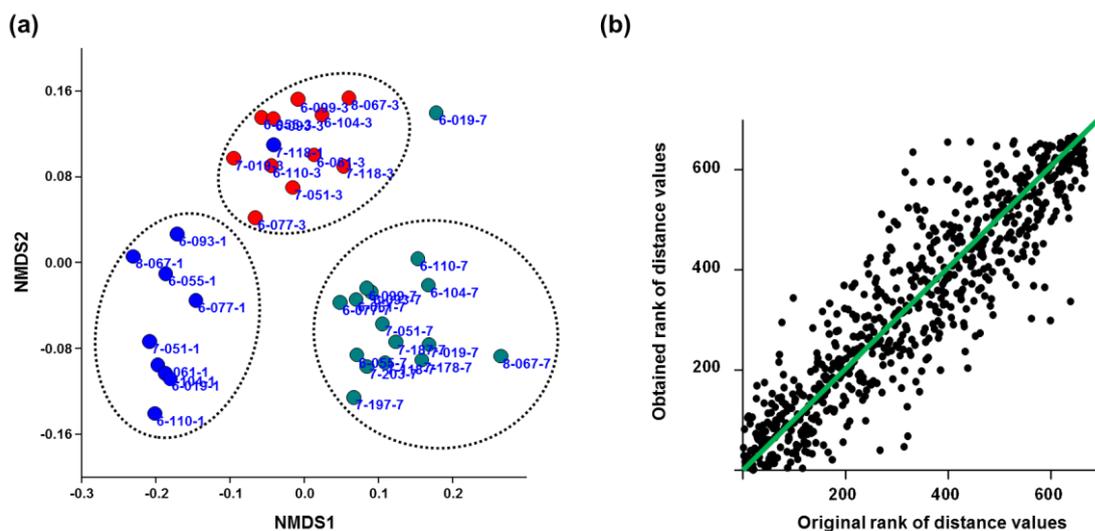
Fig. VI/5. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances of our example T-RFLP dataset

(a) NMDS ordination with overlay of cluster analysis result. Points represent samples, where blue, red and grey dots mark stage 1, 3, and 7 samples. Dotted lines encircle samples with Bray-Curtis distances lower than 0.75 (Fig. VI/6.). (b) Shephard plot for the NMDS ordination. Data point distribution is deviated from the straight ascending green line with a slope equal to 1.

VI.2. Identifying group of samples

Previous approaches gave nice representation about relations of samples to each other but the direct classification of samples needs further methods – as mentioned already at overlaying groups on the ordinations. One popular method for classification is cluster analysis in which the first step, similarly to NMDS, is the calculation of a distance matrix. Then dendrogram can be created with different algorithms. For our purpose, the **u**nweighted **p**air **g**roup **m**ethod using arithmetic **a**verages (UPGMA) is appropriate. The robustness of the dendrogram is tested by bootstrapping methods. In that case, the original variables are re-sampled for many times (e.g. 1000 times) and the percentage of replicates in which the position of a given group (a node on the dendrogram) is the same in the replicates and in the original dendrogram is calculated **(Fig. VI/6.)**. In our example the three major groups formed more or less by the samples of the three stages can be observed as a result. These groups were used for overlaying on the ordinations **(Fig. VI/4-5.)**. Group of stage 1 and 3 samples are separated from the other samples except one sample from both stages. Group of stage 7 samples (except sample 6-019-7) is supported by higher bootstrap value than 50%.
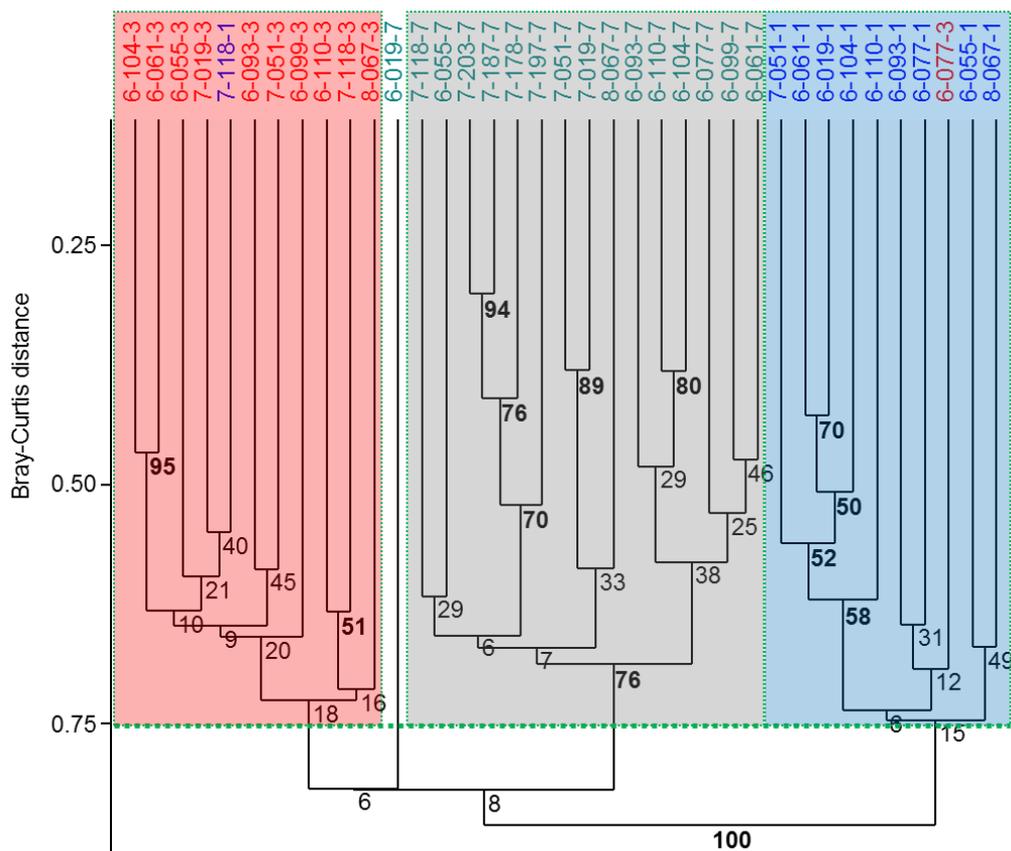
Fig. VI/6. Dendrogram resulting from Bray-Curtis distances based UPGMA cluster analysis of our example T-RFLP dataset

Groups (coloured rectangles) are formed from samples having lower Bray-Curtis distance value than 0.75 (dotted green horizontal line). Bootstrap values are shown at the nodes with bold font when having higher value than 50%.

VI.3. Hypothesis testing

Testing the separation of groups in multivariate dataset

Our example dataset consists of three main groups according to stage 1, 3 and 7 (that is the last number of sample names). As we have already seen above the samples are separated according to the stages. But the question is whether it is a statistically significant separation or not?

One way is to make a distance matrix among the samples and to tag distance values **(Fig. VI/7.)**, whether they represent the distances within one group (orange on the figure) or distances between samples belong to different groups (green on the figure). We will end up in two lists of distances which are plotted for better understanding. It shows that the range of distance values overlaps in our example but the distance values are higher between groups than within groups. Therefore our null hypothesis, what can be checked by two-sample t-test, is that 'the means of these two lists are the same' (that is, means of the samples – within a group and between groups – have the same distances from each other). The first step, in the evaluation of the test is to define the null hypothesis. The second step, is to calculate the statistical p-value beside the truth of the null hypothesis. In the case of the t-test it calculates the difference of means of the lists and produce a probability value (p, which in our case is $p = 1.7532 \times 10^{-89}$). p value promotes to reject or accept the null hypothesis: if p value is very low we make a small mistake if we reject the null hypothesis, which denotes that the means of the two groups are quite different. We have to note that variable thresholds (levels of significance, e.g. $\alpha < 0.01$) are used for marking limit under which p value is regarded as a statistically significant result. We always have to be aware that at which level was the significance stated.
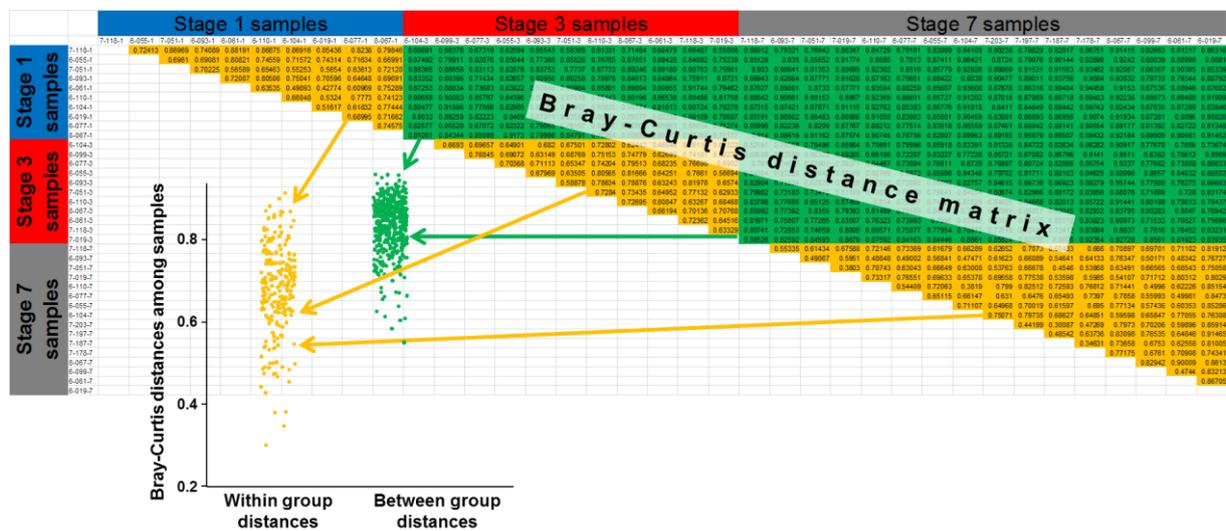
Fig. VI/7. Bray-Curtis distances among samples in our example dataset

The upper right triangle shows the distance matrix based on Bray-Curtis distances. Orange background denotes within, while green background denotes between group distances. The two type of distance values are plotted on the lower left graph.

Another analysis is the Anosim (**an**alyis **of sim**ilarity) which is based on similarities or distances among samples (within and between groups as well). These values are converted to ranks to compare the groups in a non-parametric statistical method. The null hypothesis is that 'there are

no differences among within and between group distances' (which fails with p < 0.0001 in case of our example dataset). Additionally a pairwise comparison is performed where the null hypothesis (equal distances) fails with p < 0.0003. It shows that all groups are statistically different from each other.

Testing differences among data with less dimensions

Many times we have additional variables in the dataset, such as pH, temperature, nitrogen content, etc. Occasionally new variables can be calculated, such as the above mentioned diversity indices. We could be interested in the following: (i) whether the variable is significantly different in groups, (ii) whether there is significant correlation among variables, (iii) whether there is any relationship among these variables and our multivariate dataset. The first two cases will be described here, whereas the third one in the next section (Chapter VI.4.). It should be noted, that the previous techniques (Chapter VI.1-2.) can be applied for these variables, as well.

For comparing values of one variable, more correctly their means, among different groups we use **an**alysis **o**f **va**riance (ANOVA). The null hypothesis of ANOVA is that 'all means of one variable in different groups are equal to each other'. We use first only the stage 7 samples which are the mature mushroom substrate where we have measured pH, moisture, ash and nitrogen content. For example as the first part of the substrate production takes place on the open air we can test the effect of seasons on the aforementioned physicochemical parameters. In our example dataset none of the variables are season-dependent (e.g. for moisture content p = 0.6286; **Fig VI/8.**).

(a)

|	Nitrogen content (% w/w)	Moisture content (% w/w)	Ash content (% w/w)	pH	Sampling date
6-019-7	0.76	77.3	7.6	8.38	17.02.2006
6-055-7	0.76	75.7	8.6	7.93	15.05.2006
6-061-7	0.76	75.1	7.4	8.40	31.05.2006
6-077-7	0.64	75.1	7.4	8.41	10.07.2006
6-093-7	0.79	74.3	8.4	8.94	21.08.2006
6-099-7	0.72	73.0	6.8	8.60	01.09.2006
6-104-7	0.97	77.2	10.0	7.92	12.09.2006
6-110-7	0.90	75.8	8.4	8.52	23.09.2006
7-019-7	0.75	75.7	8.8	8.41	01.02.2007
7-051-7	0.67	74.5	6.7	8.57	17.03.2007
7-118-7	0.78	72.9	10.6	8.94	24.04.2007
7-178-7	0.88	75.4	10.7	8.42	03.11.2007
7-187-7	0.96	72.6	9.4	7.98	14.11.2007
7-197-7	0.89	77.5	11.9	8.82	24.11.2007
7-203-7	0.95	75.4	12.8	8.27	05.12.2007
8-067-7	1.11	75.4	9.6	8.50	18.04.2008

(b)

Fig. VI/8. Physicochemical parameters of the Stage 7 samples in our example dataset
(a) Data matrix and (b) moisture content grouped according to stages

Second example, changes in the Shannon index among different stages was tested. Here we got significant differences (p = 0.006; **Fig VI/9.)**, thus the null hypothesis ('group means are equal') was rejected. This result does not denote that all group means are significantly different from each other, but a special test is needed to reveal the difference among groups. These can be performed after evaluating of ANOVA test, therefore they are called as *post hoc* ('after' in Latin) tests. One of them is Tukey's **h**onestly **s**ignificant **d**ifference (Tukey-HSD) test which compares

group means pairwise. Not surprisingly in our example case only the Shannon indices from stage 3 are significantly different (at significance level, $\alpha = 0.05$) from stage 1 ($p = 0.028$) and from stage 7 ($p = 0.009$), whereas indices from stage 1 and 7 are not significantly different from each other at the same significance level ($p = 0.896$).
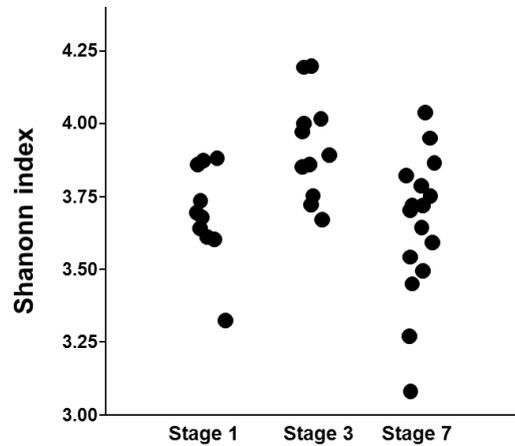
Fig. VI/9. Shannon index of samples in our example dataset grouped according to stages

To show overlapping points from the same group more clearly, points within each group
are separated from each other horizontally using a random 'jitter' value.

For analysing relationship among different variables linear correlation can be used. The null hypothesis is that 'there is no correlation among variables'. In our example case the correlation coefficient is significant ($p = 0.008$, at significance level, $\alpha = 0.05$) only between the ash and nitrogen content **(Fig. VI/10.)**.

(b)

|	correlation coefficient	p value
N - Moisture	0.186	0.491
N - Ash	**0.634**	**0.008**
N - pH	-0.238	0.375
Moisture - Ash	0.224	0.404
Moisture - pH	-0.198	0.462
Ash - pH	-0.004	0.987
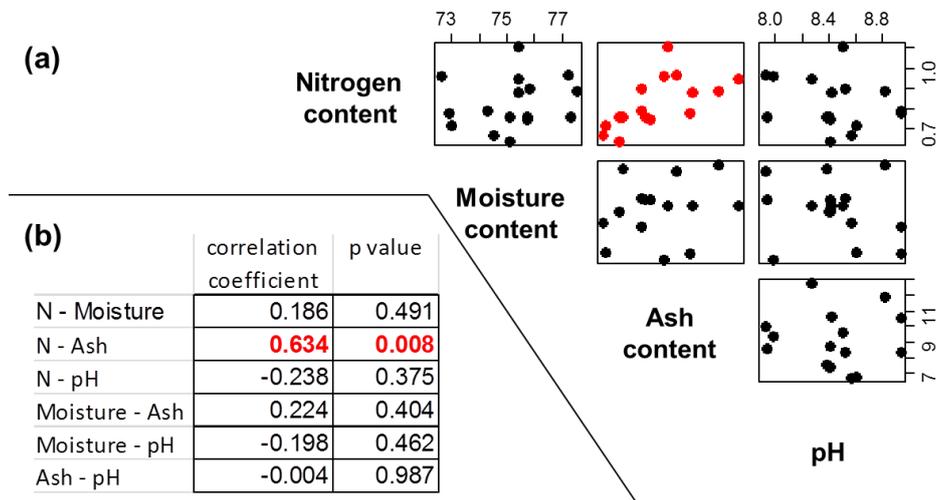
Fig. VI/10. Analysing linear correlations among physicochemical variables in our example dataset

(a) Scatter plots of variables. (b) Linear correlation among variables.
Significant relationship is marked red. 'N' stands for nitrogen.

VI.4. Linking different datasets to each other

When we have more than one dataset for the same sampling, it is not the best way to put them into a joint data matrix. For example the T-RFLP dataset has 482 variables compared to the 2 variables of diversity indices. If we put them into a joint dataset, the two diversity indices would be strongly underweighted next to the 482 T-RFs. Therefore it is better to leave them in separate datasets. We show here two methods to test the relationship among different datasets for the same samplings: here T-RFLP fingerprints and diversity indices. In the first option the values of diversity indices can be linearly correlated with the sample coordinates on the principal components of PCA. Both indices show weak significant correlation with the second principal component **(Fig. VI/11.a)**. The second option is to prepare PCA ordination for T-RFLP dataset, as above, and try to fit diversity indices onto the plot. Significance of fits can be tested with random permutations **(Fig. VI/11.b)**. While the second option is easier to interpret, the advantage of the first one is that the correlations can be tested with not only the first two principal components.

(a)

		Shannon index		Simpson index	
		correlation coefficient	p value	correlation coefficient	p value
PC1		-0.126	0.459	-0.092	0.587
PC2		**0.323**	**0.051**	**0.304**	**0.068**
PC3		0.098	0.566	0.145	0.393
PC4		-0.176	0.298	**-0.337**	**0.042**
PC5		0.064	0.707	-0.074	0.665
PC6		**0.367**	**0.025**	**0.366**	**0.026**
PC7		-0.054	0.752	0.010	0.952
PC8		0.060	0.722	0.061	0.722
PC9		**0.505**	**0.001**	**0.428**	**0.008**
PC10		0.019	0.912	-0.005	0.978

(b)
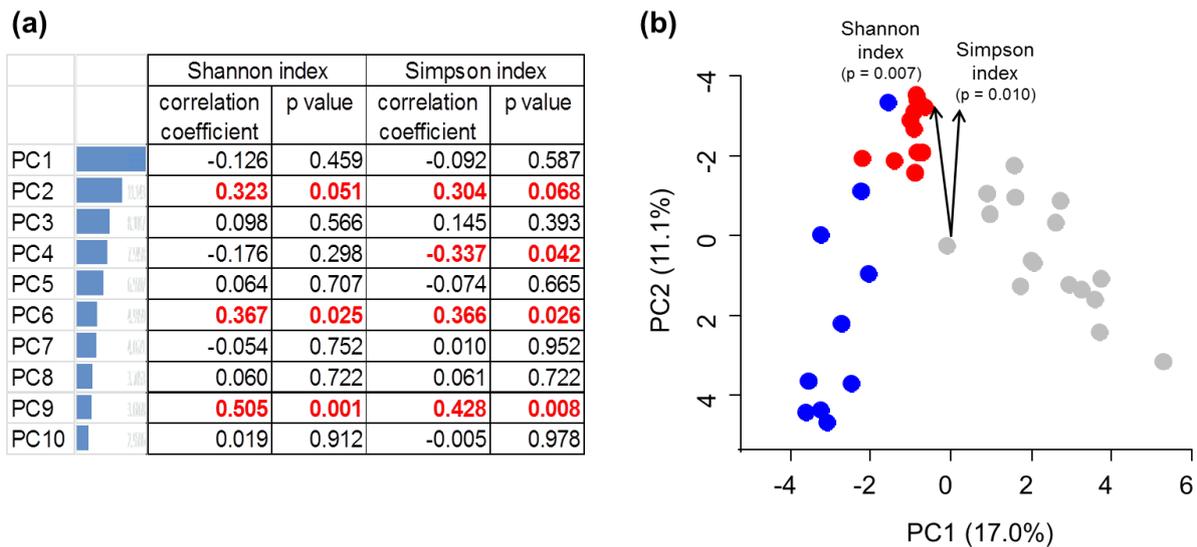
Fig. VI/11. Comparing T-RFLP fingerprints and diversity indices in our example dataset

(a) Linear correlation between diversity indices and the first ten principal components. Significant relationship is marked red. Blue bars in the second column denote the relative contribution of each principal component to the original variability.
(b) Diversity indices fitted as vectors with 'envfit' function onto the PCA ordination of T-RFLP fingerprints (Fig. VI/4.). Significance of fitting is given in parentheses.

VII. SPECIES IDENTIFICATION IN PRACTICE (Erika Tóth)

Systematics works with grouping, describing and nominating living organisms. There are three separate but related disciplines in taxonomy:

- ➢ Classification: groups living organisms into taxa based on their similarity and evolutionary relationship
- ➢ Nomenclature: nominates living organisms
- ➢ Identification: places a given organism into an existing taxonomical group

In phenetic systems taxonomists group organisms based on mutual similarity of phenotypic characteristics (numerical taxonomy). In phylogenetic systems groups are created based on shared evolutionary relatedness.

In case of prokaryotes even to define the 'species' is not univoque:

A species at higher level eukaryotes is defined as the group of organisms capable of interbreeding and producing fertile progeny, which is reproductively separated from other groups.

Bacteria of the same species must share many phenotypic properties – though many phenotypic data are variable even *within a species*! Bacteria belonging to the same taxon must have similar mol % G+C but two strains having the same mol % G+C are not necessarily belonging to the same species. The DNA from organisms of the same species should show a minimum of 70% reassociation (total genome DNA-DNA hybridisation value). Moreover 16S rRNA gene sequence similarity between the two strains should be above 98% in case they belong to the same species (but there are also some exceptions!). Describing a novel taxon in microbiology must be done on a polyphasic approach - which means complete phenotypic characterisation (including chemotaxonomy) and also genotypic studies.

Characters to be studied in case of description a novel taxon:

1. **Phenotypic characters**
   - ➢ Cell and colony morphology - light microscope and TEM (Transmission Electron Microscopy) (cell size, shape, cell cycle, heterocyst, etc.); appearance in liquid culture, on solid medium (colour, colony size, elevation, shape, consistency, mycelium production, etc.), motility (flagellation type), presence of endospores, capsule, inclusions, etc.
   - ➢ Staining properties (Gram staining, acid fast staining, endospore staining, etc.)
   - ➢ Physiological characteristics (temperature, pH tolerance, ecophysiological markers, pathogenicity, etc.)
   - ➢ Biochemical properties (enzyme reactions - classical tests and fast kits)
   - ➢ Complete chemotaxonomy (cell wall type, lipoquinones, fatty acids, polar lipid profiles, MALDI-TOF MS, etc.)
   - ➢ Immunological characterisation (when necessary)

2. **Nucleic acid studies**
   - ➢ Determination of the G+C content of DNA

- 16S rRNA gene full sequence, creating phylogenetic trees
- DNA-DNA hybridisation
- RiboPrint analysis
- Sequencing other housekeeping genes, MLST (Multilocus Sequence Typing)

Often side by side analysis with closest relatives is essential. Adequate nomination must follow the characterisation and depositing the type strain of the novel taxon in 2 different culture collections is also needed. Basic steps of description of novel taxa are summarized in **Fig. VII/1.**
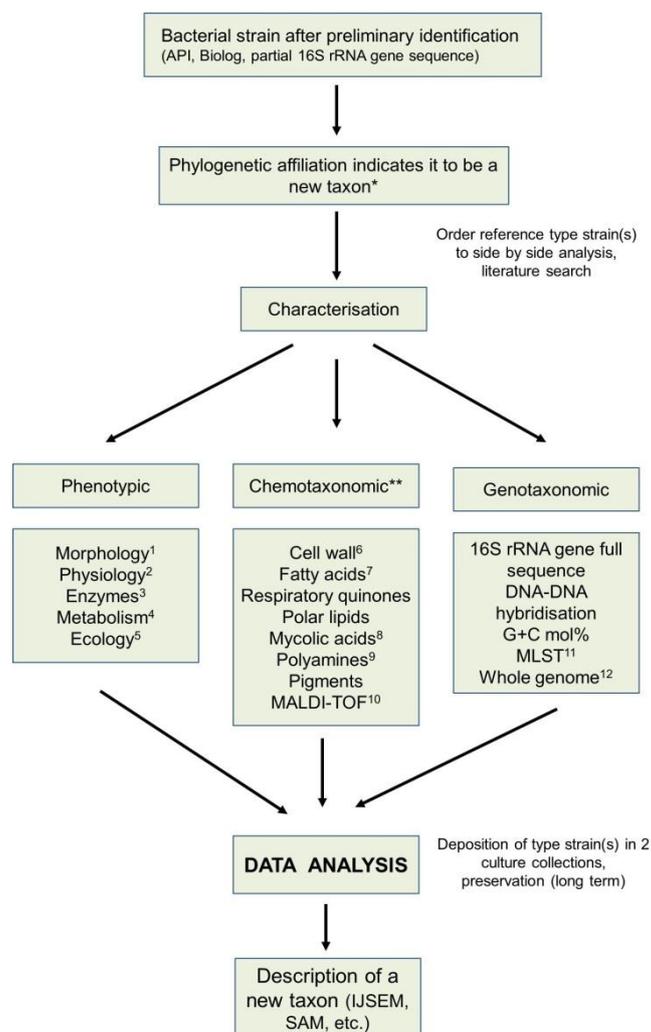
Fig. VII/1. Basic steps of description of novel taxa

*16S rRNA sequence similarity is low (usually lower than 98% but must be checked to a given taxon); **Chemotaxonomic markers also belong to phenotypic characterisation; [1]Cell size, shape, colony morphology, staining characteristics, etc.; [2]Salt, pH tolerance, temperature ranges, etc.; [3]Enzyme activities, API ZYM; [4]Energy source, relation to oxygen, substrate utilisation, etc.; [5]Pathogenicity, habitats, resistance to antibiotics; [6]Complete structure analysis where necessary; [7]Cytoplasmic membrane; [8]Determined only for high G+C Gram-positives; [9]Important marker in case of Gram-negatives; [10]Differentiate also on the strain level; [12]Not yet obligatory (Journal abbreviations: IJSEM - International Journal of Systematic and Evolutionary Microbiology; SAM - Systematic and Applied Microbiology)

Source of figures

Adapted from **Tóth and Márialigeti, 2013**: III/1-4., IV/5-7., IV/14.

Attila Szabó: IV/2., IV/9-12.

Balázs Vajna: IV/1., IV/3-4., IV/13., IV/17., IV/19., VI/1-11.

Erika Tóth: II/1., II/4-5., III/8., V/1-3., VII/1.

Judit Makk and Erika Tóth: II/3.

Judit Makk: II/2.

Márta Vargha: III/6-7.

Rita Sipos: IV/16., IV/18.

Sauer and Kliem, 2010: III/5.

Six et al., 2007: IV/15.a

Tamás Felföldi: IV/8., IV/15.b, IV/21., IV/22.

Tamás Felföldi and Balázs Vajna: cover

Zsuzsanna Pohner: IV/20.

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