Field report

The project “Analysis of effects on microbial biodiversity from tillage practices in soil with next microbial biodiversity from tillage practices in soil with next generation sequencing” took place at AGES, the Austrian Agency for Health and Food Safety, as a cooperation between two of its departments. My internship had two components, first to coordinate the knowledge transfer between those two and second to execute the laboratory work of the molecular biology part.

Since 1988, soil samples have been annually collected by the Department of Soil Health and Plant nutrition from a long term field trial in Lower Austria. The trial examines the effects of different tillage variants on soil organic matter concentration, nutrient availability and microbiota in different soil layers. The fields were all treated equally, only varying the tillage being minimal, reduced and conventional and samples were taken from different soil depths. Kandeler E. et al. (1999) concluded that microbial analyses are more sensitive indicators for field management changes than element contents. Consequently, taking a closer look at the microbiome of the soil by metagenomics is the next step. Metagenomics is the study of genetic material recovered directly from environmental samples. The analyses were conducted at the Institute of Microbiology and Hygiene. Their main tasks are on the one side routine work, where patient samples, like smear tests are examined or batches from food manufacturers are sampled. On the other side, the research division’s scope is molecular and microbiological characterization. Thus, the institute holds the expertise and equipment to conduct genetic analysis of samples carrying microorganisms originating from different habitats.

Thus, soil samples were the subject of microbial analysis and the method of choice was high-through put sequencing with the MiSeq sequencer from illumine. The soil of a compost trial was the original subject for the genetic analysis. However, the compost samples were sieved and dried, and one finding was that these kinds of samples are unusable for DNA isolation, a prerequisite for genetic analyses. In contrast, the soil samples taken from the long-term trial were fresh and wet, also being sieved but stored cooled at -18°C. Therefore, nine samples (triplets from the three different tillage practices) from the year 2012 of the field trial were sent to the analysis institute. The MiSeq is one of the sequencers of choice for the genetic analysis of microbiomes. The illumina guide (2017) was followed with minor changes (see Fig. 1).

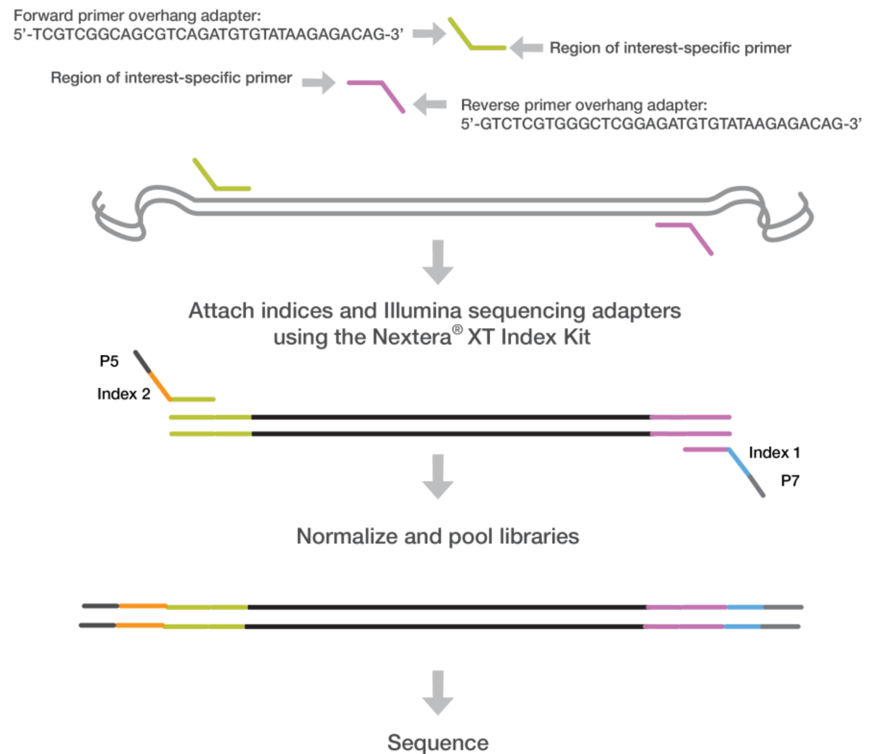

Fig. 1: Schematic illustration of the primers for Amplicon- (green and violet) and Index-PCR (orange blue) with the overhangs. Source: illumina

First at all, the DNA had to be extracted from the soil. 0,25g of each sample was weighted in. A kit, Qiagen PowerSoil DNeasy, for DNA extraction specifically for soil and environmental samples was used. After measuring the concentration of the DNA isolates an Amplicon-PCR assay was prepared. The forward and reverse primers were selected from the Klindworth A. et al. (2013) publication and are designed for a region-of-interest specific amplification, the 16S rRNA gene regions V3-V4 of bacteria. The 16S rRNA gene codes for the RNA of the small ribosomal subunit and is used for phylogenetic reconstructions. Due to costs and time constraints, only the regions V3-V4 (~550 base pair) were amplified and examined. The amplicons allowed later on a pair-end read (MiSeq average read length: 300bp) with an overlap of ~50bp. A gel electrophoresis run was conducted to verify the amplicons' length. The amplicon samples were cleaned-up with magnetic beads to remove any remaining polymerase, primers and other PCR reagents. As third, another PCR-assay was conducted using the Nextera XT DNA Library Preparation Kit by illumina. This step is also called Index-PCR, because dual indices and adapters are attached to the previously generated 16S rRNA amplicons. The indices are 8bp long and have a unique sequence. By adding different indices to the two ends of the sequences-of-interest, an individual barcode of those two indices is generated per amplicon. Therefore, the sequencer MiSeq is able to differentiate the later on pooled samples during the sequencing process. The forth step is the normalization and denaturation before loading the pooled samples in the sequencer MiSeq. Normalization describes the procedure of diluting DNA samples to a certain uniform concentration taking into account the different genome sizes of the organisms. Then the normalized samples were mixed with NaOH solution in order to denaturize the DNA resulting in single strands necessary for the subsequent sequencing reaction. The denaturation was stopped by neutralization with a hybridization buffer. The prepared DNA samples are pooled by mixing them all together. Finally, the sequencer MiSeq was loaded. The sequencing reaction takes place on the flow cell. The latter is an optical membrane**,** where the DNA of the samples attaches to. In a high-throughput sequencing, clusters form and are sequenced one nucleotide at a time, with the color at each round indicating the next nucleotide in the sequence. The sequencing takes 56 hours, another couple of hours are needed for processing and saving the generated sequence data into files in the FASTQ format. This format includes the registered sequences per read with quality values. Those files are imported in the online software BaseSpace Sequence Hub by illumina. The output was in the form of tsv-files with the percentage of reads being classified to a specific taxa, as well as Krona charts and bar graphs illustrating the distribution of those classifications.

The whole analysis, including preparation, took 3 weeks. Unfortunately, a first comparison of the data on the phylum level did not indicate any significant variations among the triplets of minimal, reduced and conventional tillage samples. I was also involved in two other projects.

At the Department of Soil Health and Plant Nutrition, I assisted in the Tea Time for Schools project. In summary, tea bags get buried in soil by school classes from all over Austria and dug out after three months. Two kinds of plastic bagged teas are used, Rooibos and Green tea from Lipton. Therefore only the tea material decomposes, faster for the leafy than the woody tea. From this a decomposition rate, named teabag index, is evaluated. On the one hand I localized the closest weather stations to the participating schools to add climatic data to the experiment. On the other hand, I assisted in producing explanation material for the school pupils.

The Institute of Microbiology and Hygiene is conducting a project to establish a mosquito database for subsequent monitoring of invasive species in Austria. An expert is collecting mosquitos through all of Austria by taking water samples containing larvae and eggs from the wild. After hatching and metamorphosis to the adult form, the species and sexes are determined. The adults are then stored as singles in Eppendorf tubes at -80°C before further analysis. For the analysis, pairs of legs are taken from individuals, the chitin is dissolved with formic and chloric acid so that only biomolecules, mostly proteins,

are left. The used method for molecular characterization of mosquitos was MALDI-TOF. Hence, the mosquito solutions were applied on a sample carrier, overlaid with the matrix and followed by a laser shooting on the matrix. The resulting spectra showed patterns being unique for the different species and used to generate reference spectra for the database.

References:

illumina (2017) 16S Metagenomic Sequencing Library Preparation. (Accessed online: 27.10.2017)

Kandeler E., Tscherko D. and Spiegel H. (1999) Long-term monitoring of microbial biomass, N mineralization and enzyme activities of a Chernozem under different tillage management. Biology and Fertililty of Soils 28:343–351. https://link.springer.com/article/10.1007/s003740050502

Klindworth A., Pruesse E., Schweer T., Peplies J., Quast C., Horn M. and Glöckner F. O. (2013).Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research, 41(1), e1. http://doi.org/10.1093/nar/gks808