Final Report

The aim of this work was to study the double stress caused by repeated drying and wetting cycles on the microbiota of soil and rhizosphere in salt-affected soils. The planned work was done at three distinct areas in a dry and a following wet seasons in a detailed botanical and microbiological studies. The water level of soda lakes was depleted during the last two year, 2012 and 2013, their margin was strongly shrunken resulted by low precipitation and hot summers. Sampling was done (Böddi-szék, Kelemen-szék, and Zab-szék) at the start of the project on September 16, 2013 year. Water, sediment, soil and root samples were taken from the selected plants (*Bolboschoenus maritimus, Puccinellia limosa, Aster tripolium, Lepidium crassifolium, Astragalus asper*) of the characteristic plant associations.

Bacteria were isolated and their salt and pH tolerance was investigated from selected pure cultures. Ten representative strains form each site was identified according to their 16S rDNS sequences. Basal and substrate induced respiration was also measured. This later was measured by different carbon sources, carbohydrates, amino acids and carboxylic acids. Moreover the catabolic activity pattern was investigated by the newly introduced microrespiration method (www.microresp.com) by using 15 different carbon sources. Comparison of dominant bacteria of the community was made by DGGE profile after amplification of 16SrDNA by bacteria specific primer pair. Colonization indices of the AM fungi was also investigated from the roots of the sampled dominant plants.

In the investigation of the culture independent analysis (at the first sampling, September 16, 2013 year) samples originated from different soda lake environments could be grouped according to the sampling sites in the similarity dendrogram. Bands in the gel represented from the water and sediment samples bacterial community showed significant similarity between water and the adjacent sediment bacterial communities. Rhizosphere samples according to the pattern of bacterial communities created distance groups from the water and sediment samples. The largest separation was found in the case of samples from Zab-szék. These samples were well separated from each other and also from the other sampling locations which could be explained by the geographical distance or the physical and chemical properties which were quite different from the other two sites. DNA isolation was made from 20 bands, others approved to be mixed sequences. During the DGGE analysis representatives of the classes of Betaproteobacteria, Gammaproteobacteria and the phyla of Cyanobacteria, Bacteriodetes and Actinobacteria could be identified.

29 different species representatives could be identified from the 126 bacteria isolated from the rhizosphere of the halophyton plants of the soda lakes of Kiskunság belonging to the phyla of Firmicutes, Actinobacteria, Proteobacteria and Bacteroidetes. Representatives of the Bacillus genus was isolated from the *Bolboschoenus maritimus* rhizosphere from all three sites, while the representatives of the halophylic and alkalophylic *Nesterenkonia* genus were primarily isolated from *Puccinellia limosa* rhizosphere. Typical community members belonged to the Halomonas genus for both plants. In addition, finding again two bacteria Bacillus auranticus and Bacillus alkalisediminis which firstly described from here as new species, representatives belonging probably new undescribed Bacillus species could be also isolated and cultured from here. The isolated bacteria showed the largest growing rate at the 5 and 7 percent NaCl concentrations during the salt tolerance test. In the growth rate at different NaCl concentrations strongly differed according to the plants investigated. According to the pH

tolerance test the average growth rate of the isolated bacteria was balanced, but differences were found in the growth rate at different pH according to the vegetation. Basal and substrate induced respiration values from the traditional gas chromatographic analyses showed strongly significant differences which clearly followed the differences found in humus content. Evaluation of the mirorespiration data by principal component analysis showed that the samples were well separated from each other. The first principal component was responsible of the 77% of the total variance. Here, the samples originated from Böddi-szék separated in largest extent from other samples. The initial results showed that samples could be well separated according to their substrate utilization pattern. Considering the environmental factors the humus content, salt content (EC) and pH proved to be significant effect, but the effect of plant species could be also important, because for example a little difference in physical and chemical properties from two samples from Kelemen-szék but different plant species on it showed large difference in the substrate utilization patterns between these to samples. Occurrence and frequency of the arbuscules of the AM fungi was different both according to the sites and host plant species. The largest colonization frequency was observed in the roots of *Aster tripolium* and *Puccinellia limosa* at Kelemen-szék. Colonization could not be found or it was negligible very low in the root samples originated from Böddi-szék.

In the second year study we selected another area, Apajpuszta Kiskunság National Park where four typical plant communities were found representing a salinity and sodicity gradient from Lepidium „vakszik" through Puccinelia „mézpázsitos" then Artemisia „ürmöspuszta" to „sziki legelő". The samples were taken in June and September 2014 in dry and wet or even waterlogged conditions thus we could compare the two extremities.

We investigated the composition of plant communities, the bacterial communities from the rhizosphere soil by cultivation using two different media (R2-modified to pH=9.0 and Horikoshi G9 pH=9.0), grouped by ARDRA and the representative isolates were identified according to their 16SrDNA sequences. Comparison of dominant bacteria by cultivation independent method made by DGGE profile and after amplification of 16SrDNA by bacteria specific primer pairs nested PCR was applied. The PCR sequences were investigated by LGC Genomics (Berlin, Germany). Basal and substrate induced respiration was also measured. Moreover the catabolic activity pattern was investigated by microrespiration method - MicroResp(Tm) by using 15 different carbon sources.

The mycorrhizal status of the most abundant plants and the diversity of AMF communities were examined with PCR-RFLP and DGGE methods. Three categories were separated depend on the AMF colonization intensities of roots: high (M = 50-100%), medium (M =10-50%) and low (M=0-10%) colonization intensity. Density of AMF infective propagules were estimated by the amount of mycorrhizal root colonization in most probable number (MPN) test. The extractable glomalin-related soil proteins (GRSPs) were also investigated from the soil samples. To examine the mycorrhizal diversity we performed DGGE analysis on the plant roots of MPN test. In a nested PCR protocol NS1-NS4 and then GC-NS31 -AML2 and GC-AMV4.5NF-AMVDG primers were used on the DNA extracted from roots to amplify mycorrhizal sequences. The reason for using three different primer pairs was that no common primer is known for AMF. The products were separated in a denaturating gel.

Culturable bacterial counts were detected between 3.2 x 10(5) and 8.8 x 10(6) CFU /g soil by the two different alkaline media. There were no significant differences in bacterial counts between the two media used with only one exception. A total of 240 bacterial strains isolated

from rhizosphere samples. 96 representative strains which were previously grouped by ARDRA identified based on their 16S rDNA sequencing. Mainly halotolerant or halophilic and/or alkalitolerant or alkaliphilic members of the phyla Actinobacteria (30 species), Firmicutes (13 species) and Proteobacteria (5 species) were identified. With the exception of one strain they showed more than 97% sequence similarity with any culturable and described strains in the EzTaxon database. The most alkaliphilic bacteria among isolates was the *Nocardiopsis valliformis* coming from the „vakszik" samples appeared in both sampling dates able to grow in the pH range between 8.0 and 14.0. Other halophil and alkaliphil strains of *Bacillus* genus, *B. alkalisediminis, B. aurantiacus* and *B. okhensis*, and *Halomonas* genus, *H. campisalis* and *H. nitrilicus.* Several bacteria could be isolated from only one type of rhizosphere, e.g. the *Nesterenkonia, Nocardiopsis, Halomonas* and *Roseivivax lentus* species appeared only at "vakszik" rhizosphere, the representatives of *Bacillus aurantiacus, Bacillus invictae, Bacillus okhensis, Arthrobacter koreensis, Isoptericola halotolerans and Zhihengliuella halotolerans* could be found only at *„mézpázsit"* rhizosphere; two *Georgenia* species, a *Paenibacillus* and an *Enterobacter* only at *„ürmös"* while *Microbacterium phyllosphaerae* and a non-symbitic *Rhizobium* only at the „sziki legelő". Other bacteria could be isolated from the rhizosphere at least two plant communities.
In the cultural-independent approach the DGGE similarity dendrogram shows that the rhizosphere samples divided into four major groups according to the four plant communities. The Lepidium "vakszik" was more divided from other groups, while Artemisia "ürmös" and "sziki legelő" were somewhat closer but clearly distinguishable. This was corresponded with the physical and chemical differences among sites, mainly the soil pH, salt content and humus content were the most important factors. On the other hand the seasonal differences were also expressed in all four communities. This clearly shows the seasonal differences in bacterial communities. Altogether 89 bands could be detected in the gels, 45 of them could be considered as homogenous community members. From the bands 12 excised bands could be evaluated 8 belonged to the Acidobacteria, 2 to the Bacteroidetes, 1 to the Proteobacteria and 1 to the Actinobacteria phyla. Identification to species level was not possible because of the low level of similarity (83-95%) with the described species in EzTaxon database. Only one genus, *Aridibacter* could be identified which belong to the Acidobacteria phylum. Closer relations (92-99%) could be found with unculturable environmental clones. While Acidobacteria phylum is considered to be the most abundant community constituent of any soils very few of them could be cultivated up to now.

The basal respiration values (µg CO2-C/ g soil/hour) were 0.01 from the lowest at "vakszik" followed by 0.24 at "mézpázsitos" then 0.74 at "sziki legelő" to the highest 1.08 "ürmös" in June, while they were 0.01 at "vakszik", 0.15 at "ürmös", 0.17 at "mézpázsitos" and 0.22 at "sziki legelő" in September.

Soil catabolic activity profiles showed clear separation according to the four sampling sites both in June and September samples. The average SIR values for 15 substrates were 0.19, 0.89, 1.59 and 1.42 µg CO2-C/ g soil/hour for "vakszik", "mézpázsitos", "ürmös" and "sziki legelő" respectively in June. While they were 0.13, 0.28, 0.36 and 0.74 µg CO2-C/ g soil/hour for "vakszik", "mézpázsitos", "ürmös" and "sziki legelő" respectively in September. Although the values were lower in September than in June the former shows better separation according to the soil properties.

Mycorrhizal status of plants in June and September 2014: In "vakszik" plant community mostly non-mycotroph plants were found. In "ürmöspuszta" and "sziki legelő" communities

mycotroph plants were in a higher ratio and in "mézpázsitos" the mycotroph and less mycorrhiza dependent *Puccinellia limosa* could be found in more than 90 % abundance.

Puccinellia limosa was collected from "mézpázsitos", and it was observed as poorly colonized plant in both season. From "ürmöspuszta" *Artemisia santonicum, Plantago maritima, Festuca pseudovina* and *Tripolium pannonicum* were analysed. All of them had high colonization intensities in June (50-70%) and high or medium in September (M=70-77%: *P. maritima* and *T. pannonicum*; M= 40-45%: *A. santonicum* and *F. pseudovina*). In "vakszik" *Tripolium pannonicum* was high colonized (M= 52% in June and 80% in September) and *Lepidium crassifolium* contained no mycorrhiza. *Achillea setacea* ("sziki legelő") was low colonized (9% in June and 36% in September and *Plantago lanceolata* ("sziki legelő") were 36% colonized in June and 71% in September.

Our investigations bring into focus the mycorrhizosphere characterization of two abundant plants- *Puccinellia limosa* and *Tripolium pannonicum*- described with different mycorrhizal dependence.

Similar tendencies of microbial functionality were shown by the fluorescein diacetate test, the soil catabolic activity pattern and the measurement of glomalin-related soil proteins (GRSPs). The "vakszik" community always had the lowest values; while the other three variously differed significantly from each other. Glomalin is commonly produced on AMF hyphae in soils and in colonized roots. GRSPs are a significant component of soil organic matter and act to bind mineral particles forming soil aggregates.

We observed remarkable differences between the AMF infectivity in the soils with MPN test. The "vakszik" and "mézpázsitos" plants had very low root colonization and no colonization in the MPN test. The "mézpázsitos" soil was flooded which completely blocked AMF infection. In "ürmöspuszta" and "sziki legelő" 40-70 % (M%) colonization intensities were found and soils contained low density of infective propagules in the MPN test. The mycorrhizal diversity performed by DGGE had to repeat several times to get optimal results for evaluation. The final analysis of the band pattern and the sequences is in progress.

In the third year we made a pioneer work in soil metagenomics, as in our knowledge, no soil metagenomic investigation have been done in Hungary. Altogether 8 samples from the 4 sites of Apaj in two samplings (June and September 2014) were analysed. Soil metagenomic study focused only to bacteria by amplicon sequencing of the 16S RNA gene using Roche Junior NGS platform. 320 basepair long sequences were selected to reveal phylogenetic relations of the soil bacterial assemblage and were classified to genus level. Altogether 33 phyla were detected of which sequences related to Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Planctomycetes and Proteobacteria were identified in the highest proportions. Both seasonal and vegetation related differences were observed. Members of phylum Gemmatimonadetes were identified in significantly higher numbers in autumn than in summer and pointed out mainly from soils dominated by Puccinellia and Lepidium. Seasonally, Actinobacteria related sequences were notably more abundant in June than in September, while regarding the different vegetation types members of phylum Acidobacteria were found mainly from Artemisia and Achillea dominated habitats in both seasons. Furthermore, Artemisia and Achillea type vegetation showed the highest bacterial diversity based on both Shannon and Simpson indices.

The diversity and functionality of indigenous arbuscular mycorrhizal fungal (AMF) communities in relation to vegetation type were studied with Glomalin Related Soil Protein (GRSP) content and colonization intensity analysis, most probable number (MPN) test and PCR-RFLP methods. Colonization intensities in September 2015 were very similar to the previous year, only Festuca pseudovina ("ürmöspuszta") and Achillea setacea ("sziki legelő")

were more colonized. GRSP content was higher in every plant communities in 2015 year than in 2014, but still "vakszik" had the lowest values. The other three communities were not differed significantly.
MPN test with soils from 2014 year showed that there are only a low amount of infective propagules in the four plant communities of Apajpuszta and the DGGE patterns were the same in all communities. MPN tests with soils from 2015 and 2016 are in progress. With molecular analyses based on AMF 18S rDNA region taxa like Claroideoglomus lamellosum, Funneliformis caledonium, F. geosporum, F. mosseae, F. fragilistratum, Glomus indicum, Rhizophagus fasciculatus, R. intraradices, R. iranicus, R. irregularis, R. vesiculiferus, Septoglomus constrictum and other unidentified Glomus spp. were found. Occurrence of some AMF species have been described first in Hungary, while others were reported earlier from semiarid sandy soils.

Flooding experiment was performed with Tripolium pannonicum. Plants were grown in three treatment such as (1) flooded, (2) control (optimal irrigation) and (3) alternation of wet-dry cycles (2 weeks flood, 2 weeks drought and 2 weeks flood again) with sterilized (AMF-) and non-sterilized (AMF+) soil from "ürmöspuszta" as inoculum in quartz sand. Biomass was highest in AMF+ control group and in AMF- flooded group, but the chlorophyll content was not different. The vitality of roots was the highest booth in AMF+ and – group at flooding treatment. Tripolium pannonicum can develop aerenchyma with numerous and very large air-storage lacunae in the root cortex and so the flooded environment during the experiment was still optimal for the plants.
Plants from control group had the highest colonization intensities and GRSP content. In AMF+ GRSP content and the microbial activity (FDA test) was significantly higher than in AMF- group. In the mixed and flooded treatment colonization intensities were still high and in the mixed treatment GRSP content was also significantly higher in AMF+. In the flooded group the microbial activity was low, the GRSP content was the same in AMF+ and AMF-. The AMF inoculation didn't increase the amount of the GSRP. We suggest that in this case flooding prevented the extraradical growth of AMF.

In the 4th year, we asked a one-year prolongation before closing the project.
Soil samples were taken in two different seasons (July and October) in 2016 at the same four locations at Apajpuszta as in previous years, but from three different sampling depths (0-10 cm, 10-30 cm, and 30-60 cm). Major physical and chemical properties of the samples were determined along with micronutrient contents. The physiological state of the soil microbiota was evaluated by FDA enzyme activities of the samples and by basal respiration. Substrate induced respiration method was used to estimate the microbial biomass of soil samples. Catabolic activity profiles of the samples were measured by the MicroResp method, using 23 organic carbon sources. We found that the physiological properties were influenced by both vegetation type and soil depth, the latter having a stronger effect on catabolic profiles.
The structure of the soil bacterial communities was investigated by cultivation based methods and direct soil DNA extraction analysis. The isolated bacterial strains (188) maintained in pure cultures, they were identified; characterization of another 180 strains is still in progress. New candidate species were also found and they are under detailed examination.
Soil DNA was extracted from the samples and DGGE was carried out based on the 16S rRNA gene to examine the genetic fingerprints of bacterial communities. We found that vegetation type had a significant effect on the genetic fingerprints, while soil depth had lesser, but still clear influence on the structure of bacterial communities. A much more detailed analysis of community DNA by metagenomic methods will also be carried out.

We investigated the mycorrhizal aspect with a third autumn sampling in Apajpuszta. The diversity and functionality of indigenous arbuscular mycorrhizal fungal (AMF) communities were studied with Glomalin Related Soil Protein (GRSP) content, root colonization intensity analysis, and PCR-RFLP methods. Colonization intensities were similar to the previous year, but we sampled more plant species. GRSP content was lower than the previous year. Still „vakszik" community had the lowest values, and the other three community had very similar values. In the most probable number (MPN) test with soils from 2015 we found no colonization in plants inoculated with soil from „vakszik" community. „Mézpázsitos" had very low values, „ürmöspuszta" and „legelő" communities had almost the same infective AMF propagule content as soils from 2014. MPN test with soils from autumn 2016 is in progress. With molecular analyses we found the same species so far as in previous years, but we completed „sziki legelő" community with more plant species.
We performed a pot experiment to study the effect of different AMF inocula under drought- and salt stress in 2017 year. The model plant of this experiment was sea aster (*Tripolium pannonicum*). More than 1000 seed from different years and different communities of Apajpuszta were sterilized and germinated to have the sufficient amount of plant, because sea aster seeds have low germination rate and germinate slowly. We used three different inocula: (1) pure strain of *Funneliformis geosporum*, (2) soils from „ürmöspuszta" and (3) soils from „sziki legelő" both soil from Apajpuszta. For modelling drought stress polyethylene glycol (PEG) was used, and Na_2CO_3 for salt stress. Drought-salt combination and control treatments were also present. The plants got the stressors week by week and were processed in four status with growing stress conditions. We measured plant biomass, leaf area, Relative Water Content of leaves (RWC), Membrane Stability Index (MSI) of leaves and roots, root vitality with Triphenyl Tetrazolium Chloride (TTC) test, AMF colonization intensity and the pH and conductivity of the culture media (pumice). The analysis of the result is still in progress. It seems so far, that the symbiosis with the specialist *Funneliformis geosporum* can be profitable for the plants to better tolerate drought and salt stress. Preferred indigenous AMF species can also be found in the soil from „ürmöspuszta". The plants inoculated with soil from „sziki legelő" community were colonized later, and in some cases the colonization was not beneficial. The sea aster is not present at „sziki legelő" community at Apajpuszta. It can be explained the lack of the ecotype of the same AMF species in this soil with which the sea aster can easily form mycorrhiza.

Publication progress:
We have published 2 papers in the Agrokémia és Talajtan journal (Q4), another 3 papers in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) journal (Q1) and one other has been submitted to IJSEM journal and close to the acceptance, and 2 papers published in the Acta Microbiologica et Immunologica Hungarica (Q3) journal.
At least two papers are under preparation we intended to submit before closing the project, but unfortunately could not completed:
- The status and functional characterization of AMF communities and AM fungi diversity detected by direct extraction of DNA from the roots of host plants.

- Genetic diversity and catabolic activity profiles of rhizosphere bacterial communities during dry and wet seasons in a saline sodic grassland, Hungary