

RHIZOSPHERE BACTERIAL COMMUNITIES OF *ARUNDO DONAX* GROWN IN SOIL FERTILISED WITH SEWAGE SLUDGE AND AGRICULTURAL BY-PRODUCTS

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Application of sewage sludge to soil is a potentially inexpensive source of nutrition for plants, but may contain undesirable and toxic substances, e.g. heavy metals. Alterations in microbial communities can serve as an environmental indicator of possible soil contamination. We used two molecular fingerprinting methods (Automated Ribosomal Intergenic Spacer Analysis, ARISA and Terminal Restriction Fragment Length Polymorphism, T-RFLP) to study changes in the genetic diversity of bacterial communities in the rhizosphere of *Arundo donax* L. cultivated in the soil fertilised with additive based on sewage sludge from wastewater treatment plant and agricultural by-products represented by crushed corn hobs and wastes from grain mill industry. The metagenomic DNA extracted from rhizosphere samples were collected in August and November 2014. The amount of mgDNA was statistically higher in samples with additive than in control samples without it in both dates. The Venn diagrams showed that operational taxonomic units which were common to all samples were represented in 32.8% in ARISA and 43.4% in T-RFLP. However, based on Principal component analysis and subsequent PERMANOVA statistical tests did not confirm significant differences in the rhizosphere of control plants and plants grown in the soil supplemented with sewage sludge in dose 5 and 15 t/ha present in the additive.

Key words: genetic diversity, ARISA, T-RFLP, *Arundo donax* L.

Sewage sludge production is growing world-wide due to the industrialization and population increase (Kominko *et al.* 2017). One of its possible utilization is the application to the agricultural soil. Several studies reported that the sewage sludge can be a rich source of nutrients, and moreover, its application can improve soil properties (Kołodziej *et al.* 2016). Because the sewage sludge is considered a waste potentially containing heavy metals or other hazardous chemicals, its usage by farmers is

defined by Council Directive 86/278/EEC and in Slovak legislative by Act no. 188/2003 Z.z. Using of sewage sludge is especially suitable to restore productivity of marginal soils intended for growing of non-food plants. Up-to-date, there is the global demand for renewable energy sources, and cultivation of energy crops on marginal land is one of the possibilities how to meet the requirements. *Arundo donax* L. (giant reed) is considered a promising energy crop from *Poaceae* family. Giant reed is char-

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acterised by vigorous growth and the production of a huge amount of biomass. The species is also known for high phytoremediation potential (Alshaal *et al.* 2014) and ability to growth in saline (Di Mola *et al.* 2018), contaminated or other marginal soils (Pilu *et al.* 2012). Although *A. donax* can grow in poor soils, fertilisation positively affects the yield of above-ground biomass. As it is grown for non-food purposes, sewage sludge can be a rich source of low-cost nutrients there.

The aim of the experiment was to evaluate changes over time in the genetic diversity of bacterial communities in the rhizosphere of *Arundo donax* growing in soil supplemented with additive derived from sewage sludge and agricultural by-products using two molecular fingerprinting methods T-RFLP (Terminal Restriction Fragment Length Polymorphism) and ARISA (Automated Ribosomal Intergenic Spacer Analysis). Both methods use

fluorescently labelled DNA fragments to produce fingerprint of microbial community. However, the difference is in the DNA region used for PCR amplification. T-RFLP uses a highly conserved gene that must be digested with restriction endonuclease(s) and ARISA uses internal transcribed spacer (ITS) region which is highly variable in length. The advantages of these methods are that can be used to quickly detect the genetic diversity of a microbial community in a large number of samples simultaneously. Also, both methods are very useful for monitoring changes in microbial communities over time.

The pot experiment was set up as follows: plants of *Arundo donax* L. were planted individually in nine pots. Three pots were considered as control samples with arable soil only, another six pots were supplemented with the additive composed of sewage sludge from the wastewater treatment plant Pannon-Víz Zrt. (Győr, Hungary) and agricultural

T a b l e 1

Genetic diversity indices of bacterial communities detected in the rhizosphere without and with additive

ARISA					
Season	Dose of sewage sludge in additive [t/ha]	Richness	Gini-Simpson	Shannon	Evenness
August	0	98.000 ± 17.436	0.974 ± 0.002	4.078 ± 0.102	0.892 ± 0.015
	5	95.333 ± 10.693	0.975 ± 0.001	4.066 ± 0.036	0.893 ± 0.020
	15	109.000 ± 12.124	0.975 ± 0.005	4.186 ± 0.089	0.893 ± 0.019
November	0	109.500 ± 2.121	0.971 ± 0.010	4.096 ± 0.192	0.872 ± 0.037
	5	97.333 ± 15.822	0.976 ± 0.003	4.130 ± 0.017	0.905 ± 0.031
	15	96.000 ± 30.414	0.967 ± 0.015	3.971 ± 0.396	0.877 ± 0.023
T-RFLP					
Season	Dose of sewage sludge in additive [t/ha]	Richness	Gini-Simpson	Shannon	Evenness
August	0	83.333 ± 26.502	0.937 ± 0.057	3.490 ± 0.740	0.790 ± 0.110
	5	94.667 ± 37.501	0.960 ± 0.024	3.765 ± 0.584	0.835 ± 0.053
	15	102.333 ± 17.388	0.970 ± 0.005	3.967 ± 0.168	0.859 ± 0.008
November	0	137.000 ± 7.211 ^b	0.980 ± 0.002 ^b	4.303 ± 0.058 ^b	0.875 ± 0.012
	5	97.667 ± 25.423 ^a	0.967 ± 0.010 ^a	3.855 ± 0.306 ^a	0.845 ± 0.027
	15	106.000 ± 12.000 ^{ab}	0.973 ± 0.002 ^{ab}	4.001 ± 0.110 ^{ab}	0.859 ± 0.011

The numbers behind the ± sign represent standard deviation (n = 3). The different letters denote statistically significant differences among sewage sludge doses in additive (LSD, α = 0.05)

by-products based on crushed corn cobs and wastes from grain mill industry (Top Feed & Cargo Hungary Holding Zrt., Hungary) in ratio of 1:1.5 (sludge:agricultural by-products). Three pots from six were with the dose representing of 5 t/ha and three pots with the dose representing of 15 t/ha of sewage sludge in additive. For better characterisation of used sewage sludge and soil additive with their physico-chemical characterisation see article of Šušnovská *et al.* (2013). The metagenomic DNA was isolated twice in 2014 (August and November) from rhizosphere of *Arundo donax*. The methodology of this study with the same additive, molecular fingerprinting methods and ecological diversity indices used were the same as in the study previously published by Ondreičková *et al.* (2016). Venn diagrams were calculated using VENNY 2.1 online program (Oliveros 2007–2015). Permutational multivariate analysis of variance (PERMANOVA) with Euclidean distance measure and 9999 permutation and Principal Component Analysis (PCA) were constructed using the PAST (PAleontological STatistics) software version 3.19 (Hammer & Harper 2006).

The total microbial biomass was statistically higher in the rhizosphere with additive than in

control samples in both dates (Figure 1). Bacterial richness, however, did not copy this trend, and statistical differences were detected only in November between control samples and pots with sewage sludge in additive at the dose of 5 t/ha using T-RFLP method (Table 1). Other diversity indices (Gini-Simpson, Shannon and Evenness), which represent the alpha diversity in ecological studies and provide more information about community composition than simply species richness (Jarkovský *et al.* 2012), are shown in Table 1. Statistical differences were detected only in November samples in the Gini-Simpson and Shannon diversity indices using T-RFLP method (*LSD*, $\alpha = 0.05$). The Venn diagrams show that the operational taxonomic units (OTUs) generated by ARISA and T-RFLP methods that occurred in all three types of samples represent 32.8% in ARISA (Figure 2a) and 43.4% in T-RFLP (Figure 2b). These ubiquitous OTUs were at the same sampling time the most represented among other types of OTUs. PCA analysis represented by PCA graph (Figure 3) was constructed using data of fluorescence intensity in individual OTUs obtained by ARISA and T-RFLP. In ARISA, the bacterial communities from rhizosphere samples collected in

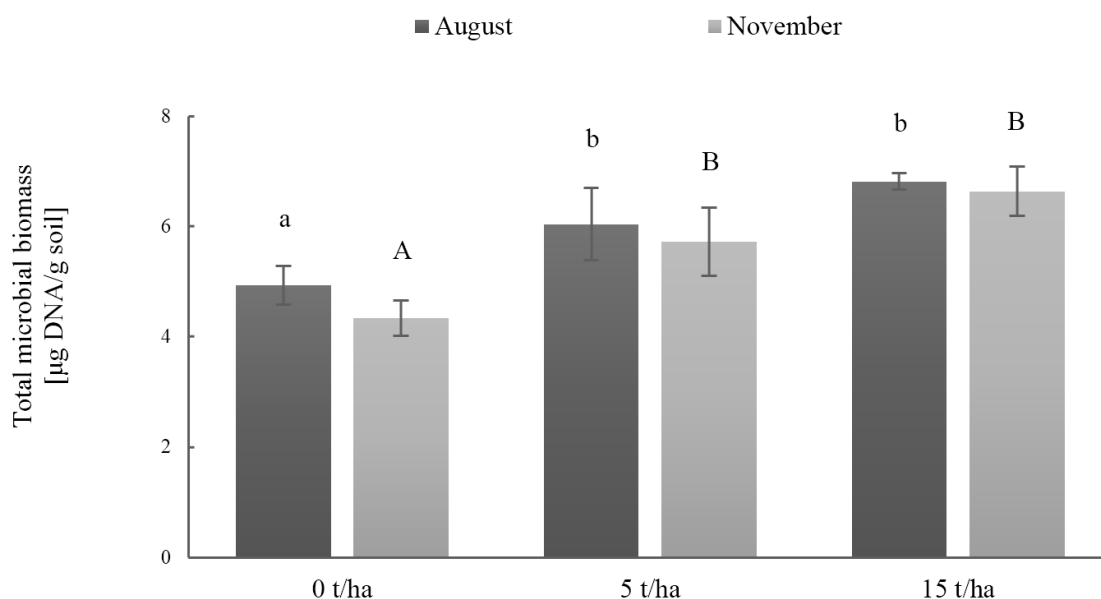


Figure 1. Total microbial biomass expressed as µg extracted DNA per 1 g of soil in the rhizosphere of *Arundo donax* with different dose of sewage sludge in additive. Bar represents standard deviation (n = 3). The different lower- and upper-case letters denote statistically significant differences among sewage sludge doses in August and November (*LSD*, $\alpha = 0.05$), respectively.

T a b l e 2

The results of the Two-Way PERMANOVA derived from the obtained data from PCA analysis

PERMANOVA	P-value	
	ARISA	T-RFLP
Dose of sewage sludge [t/ha]	0.3794	0.6777
Season	0.3840	0.4313
Interaction	0.9625	0.9455

August are located at the bottom of the graph (except one 15 t/ha sample) and are more similar than rhizosphere samples collected in November (Figure 3a). On the other hand, in T-RFLP, rhizosphere samples are more scattered across the plot area. Moreover, the bacterial communities of the control samples show the highest similarity to each other (Figure 3b). Although PCA graphs from ARISA and T-RFLP look different, PERMANOVA did not show statistical differences among doses of sewage sludge in additive and between sampling dates neither in ARISA nor in T-RFLP (Table 2).

These results did not indicate a negative impact of additive application based on the sewage sludge and agricultural by-products to the agricultural soil

on the genetic diversity of rhizosphere bacterial communities. Similar results were obtained by Nakatani *et al.* (2011) after the application of tannery sludge into the soil. Their study did not indicate negative impacts on the microbial properties. Also, Poulsen *et al.* (2013) did not detect major changes in the prokaryotic community composition due to different urban wastes including sewage sludge by using pyrosequencing. They also indicate that contemporary waste products may be used without detrimental effects on the soil microbial diversity in the medium to long-term. Unfortunately, application of sewage sludge to the agricultural soil in Slovak Republic is currently not increasing. Data obtained from Eurostat indicate that in Slovak Republic in 2015 (the last year reported in this statistics) 0% of sewage sludge was applied to the agricultural soil, 44.2% was used as compost, 8.3% for landfill, 30% for incineration and 17.5% of sludge was in “other” category. On the other hand, Ireland, Albania, Bulgaria and Czech Republic have applied in the same year 80%, 77%, 53%, and 48% of the sewage sludge to the agricultural soil, respectively (Eurostat).

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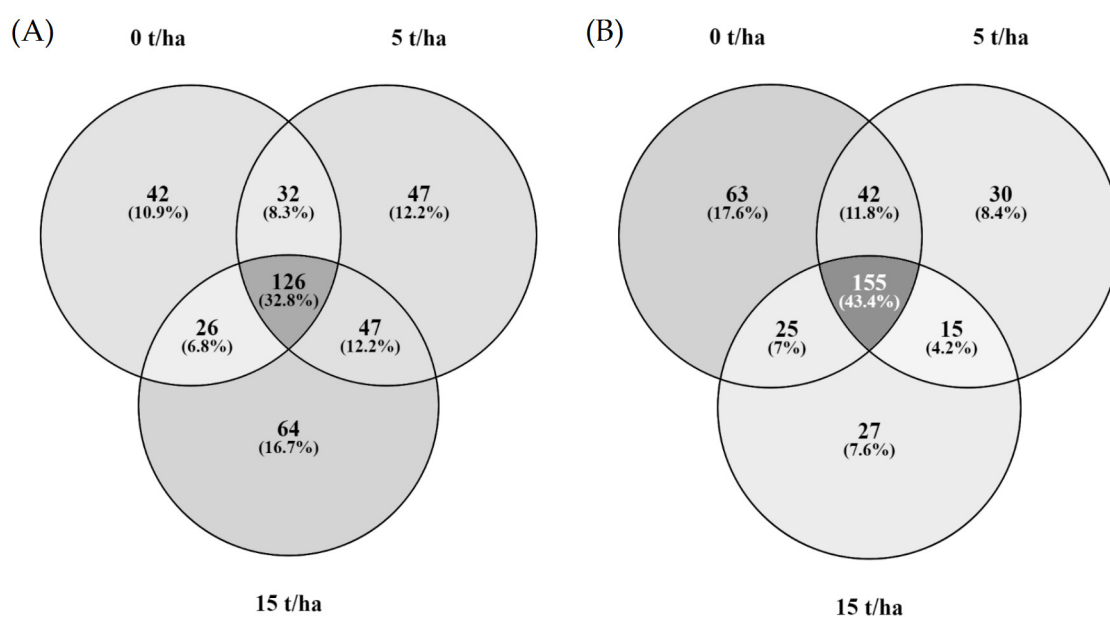


Figure 2. The Venn diagrams constructed from (A) ARISA and (B) T-RFLP data representing the number of shared and unique OTUs among bacterial communities at the different sewage sludge doses in additive.

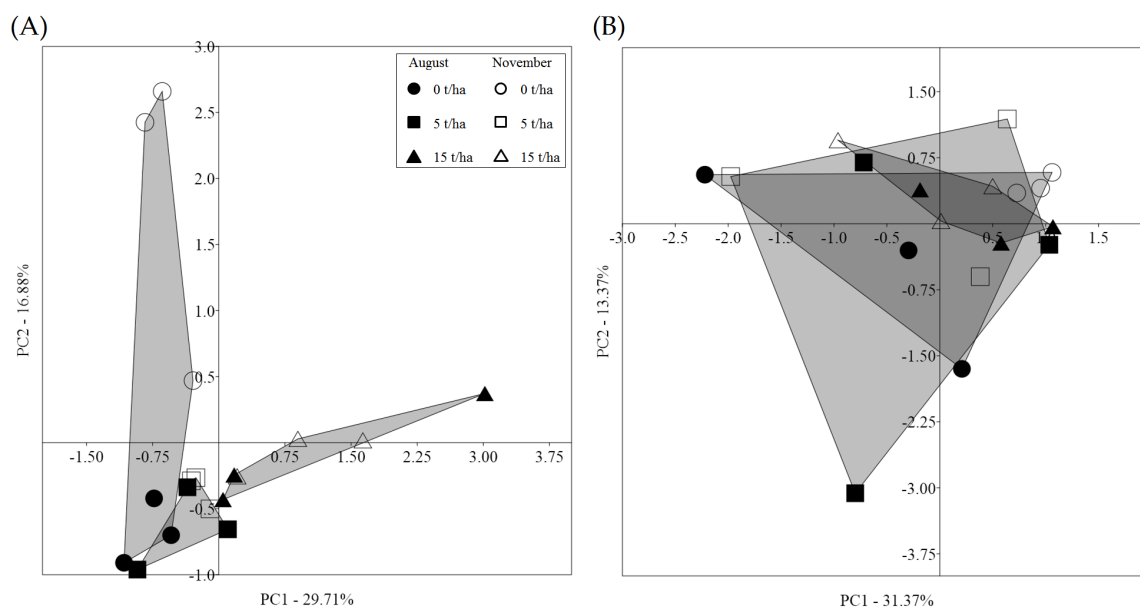


Figure 3. Principal Component Analysis constructed from (A) ARISA and (B) T-RFLP fluorescent data of bacterial communities from rhizosphere of *Arundo donax* with different sewage sludge doses in additive and two sampling times (August and November)

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