Phyto2Energy Project

Phytoremediation driven energy crops production on heavy metal degraded areas as local energy carrier

WP2: Plant-microbes interactions: improving biomass production and remediation

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 610797.

WP2

The new bioinoculum: some aspects of (1) development and (2) evaluation

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WP2: Results

What has been done ?

Development of the inocula application method

C

Phyto2Energy
4L: 4% Molasses + 1% inulin
Date of preparation: 10.03.2016 r.

The product contains three components: A,B,C which had to be mixed before use.
A,B,C components mixing time _ _ _ _/_ _/_ _
YYYY/MM/DD

INSTRUCTION

Component A: bacterial lyophilisate

⇩

Component B: 0.85% NaCl

⇩

Component C: 4% molasses + 1% inulin

⇩ 24h incubation

Dilution and application of the product

1st step
Add Component B (0.85% NaCl) to Component A (bacterial liophylisate) and mix.

2nd step
Add mix Component A (bacterial liophylisate) + Component B (0.85% NaCl) to Component C (4% molasses + 1 % inulin). Then mix and leave for 24h at room temperature.

3rd step
Use inocula obtained according to the step 2 in 10% solution (4L inoculum + 36L water).

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WP 2:

The overall goal is:

To

Demonstrate the freeze-drying as a promissing method for bioinoculum production on the example of *Pseudomonas putida* strains

The specific aim:

Evaluation of some biological activities on example of *P. putida* after freeze-drying

This aim will be reached by the comparing the activities of freeze-dried and not freeze-dried cells of *P. putida*

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WP 2:
Why the lyophilization process was chosen ?

Freeze dried cultures have some advantages, including:

- their low volume,
- convenience for transportation and storage,
- ease of use.

Disadvantages:

- freeze-drying can cause many types of damage to cells, including a loss of viability,
- reduction of metabolic activity, and
- changes in cell morphology, etc.

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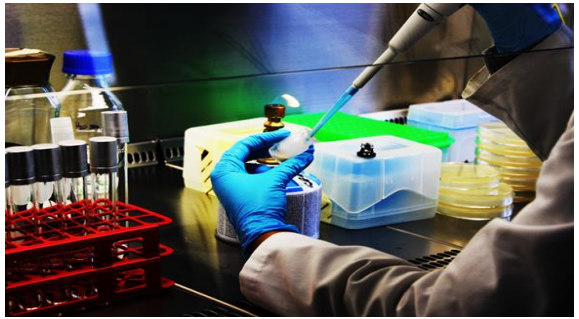
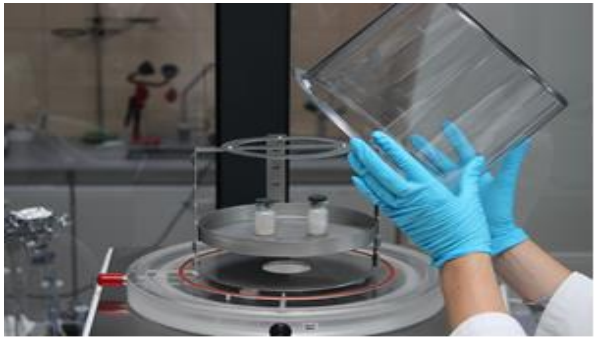
WP2: *Evaluation of lyophilization efficiency – numer of bacteria*



Time (days)	Number of bacteria (after lyophilization - AL) [cfu/ml]	Number of bacteria (before lyophilization - BL) [cfu/ml]	**BSR** = (logAL/logBL) x 100
0	2.4×10^{10}	4.8×10^{11}	**88.87**
3	1.3×10^{10}	4.8×10^{11}	**86.59**
14	5.4×10^{9}	4.8×10^{11}	**83.32**
60	3.0×10^{9}	4.8×10^{11}	**81.14**
180	2.1×10^{8}	4.8×10^{11}	**71.23**
360	6.4×10^{7}	4.8×10^{11}	**58.77**

Time (days)	Number of bacteria (after lyophilization - AL) [cfu/ml]			Number of bacteria (before lyophilization - BL) [cfu/ml]			**BSR** = (logAL/logBL) x 100		
	E41	E42	R85	E41	E42	R85	E41	E42	R85
0	1.9×10^{10}	1.4×10^{10}	3.4×10^{10}	1.0×10^{11}	4.9×10^{10}	2.5×10^{11}	**93.28**	**94.85**	**92.44**
180	2.5×10^{8}	8.2×10^{8}	2.2×10^{8}	1.0×10^{11}	4.9×10^{10}	2.5×10^{11}	**76.36**	**83.34**	**73.16**



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WP2: *Evaluation of lyophilization efficiency*

The following properties of *P. putida* strains were evaluated before and after freeze-drying:

- Enzymes activities – API-ZYM® (BioMerieux) test and the plate method with various media
- Biofilm formation - crystal violet (CV) method
- Biosurfactant production – blood agar, drop-collapse method, surface tension measurements
- Antibiotic sensitivity- disc diffusion method, PMs
- FAME analysis
- Anifungal activity
- Phenotype MicroArrays – metabolic activities (PM1, PM2, PM 3, PM4, PM9, PM10, PM11, PM12 and PM13 plates

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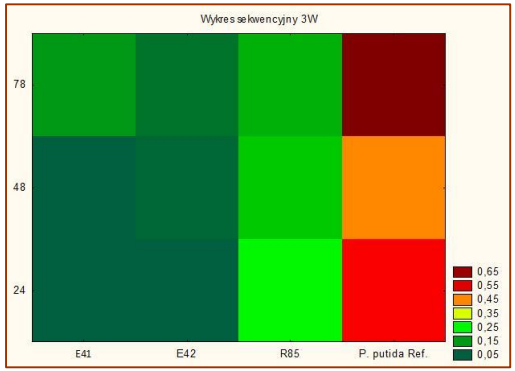
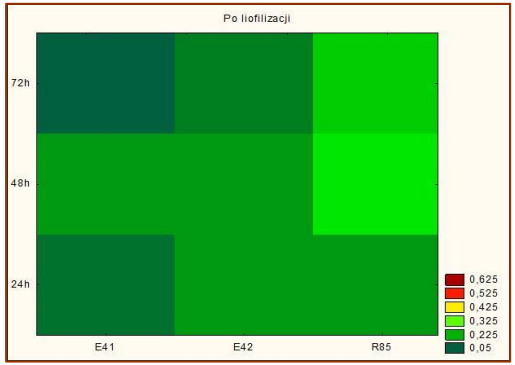
WP2: Results

- **Biofilm formation**





- **Biosurfactant production**

Strains	Surface tension (mN/m)		
	Before lyophilization	After lyophilization (6 months)	After lyophilization (12 months)
P. putida E41	25.96 ± 0.49	35.18 ± 0.28	31.57 ± 0.37
P. putida E42	52.23 ± 0.46	50.93 ± 0.63	51.27 ± 0.67
P. putida R85	54.62 ± 0.46	45.52 ± 0.64	48.74 ± 0.59

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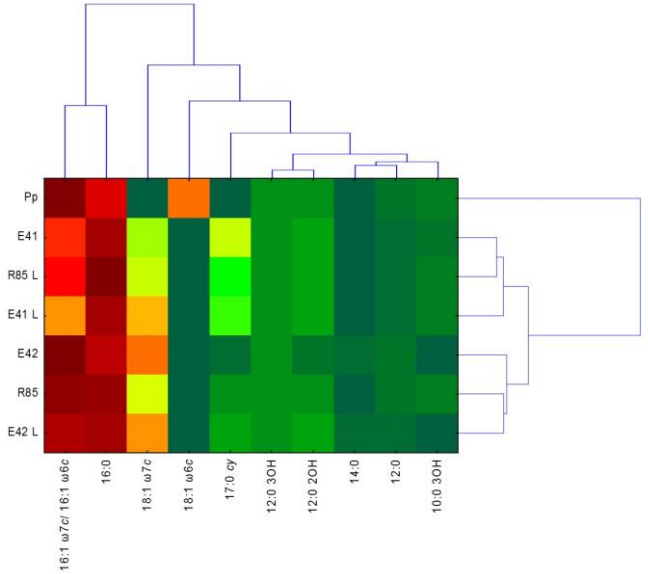
WP2: Results

FAME method proved its applicability for monitoring the changes in the cellular fatty acids composition of microorganisms under stress conditions like lyophilization

FAMEs	Strain						
	Control	Before lyophilization			After lyophilization		
	Pp	E41	E42	R85	E41	E42	R85
saturated (s)	43,51	61,68	41,32	51,30	60,02	49,15	61,03
unsaturated (us)	34,59	38,30	57,56	48,33	39,93	50,54	38,74
s:us ratio	1,26	1,61	0,72	1,06	1,50	0,97	1,58



The FAME patterns showed that the freeze-drying was mostly influenced on the FAME profile of R85 strain.

For this strain the highest ratio saturated:unsaturated FAMEs and decrease the membrane permeability were observed after lyophilization.

The analysis of individual FAMEs showed that the two fatty acids 17:0 *cy* and 16:1 ω7*c*/16:1 ω6*c* were characterized for R85 strain among all studied bacteria.

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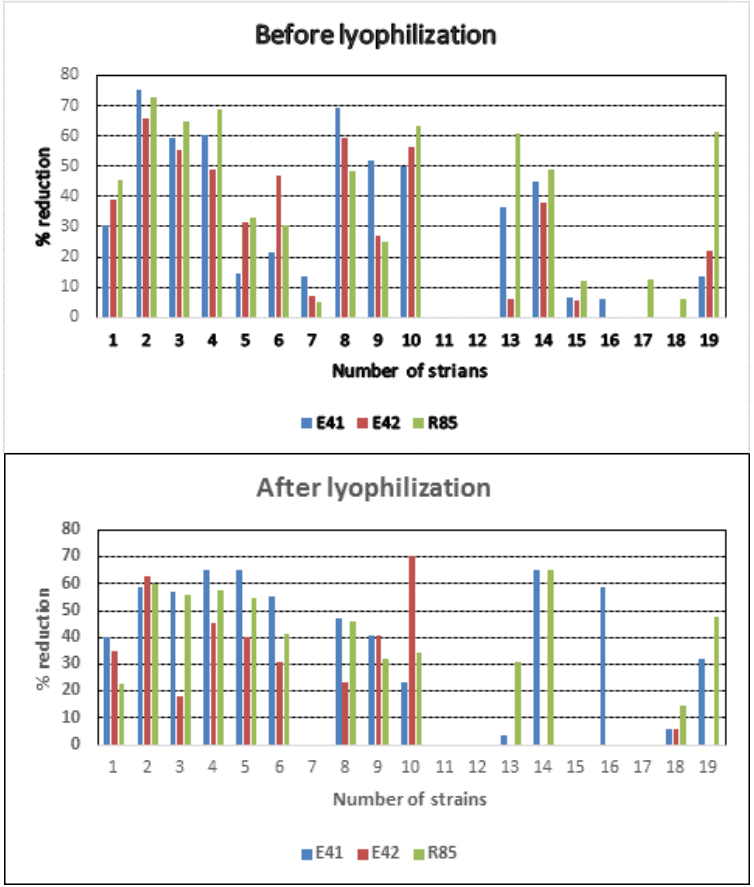
WP 2: Results

1	*Alternaria alternata*
2	*Boeremia strassesi*
3	*Colletotrichum dematium*
4	*Colletotrichum fuscum*
5	*Cylindrocarpon destructans*
6	*Diaporthe eres*
7	*Diplocereus hypericinum*
8	*Fusarium equiseti*
9	*Fusarium oxysporum*
10	*Phylloticta plantagnus*
11	*Rhizoctonia solani*
12	*Sclerotinia sclerotiorum*
13	*Fusarium avenaceum - 2*
14	*Fusarium oxysporum*
15	*Fusarium graminearum*
16	*Fusarium culmorum - 2*
17	*Fusarium graminearum*



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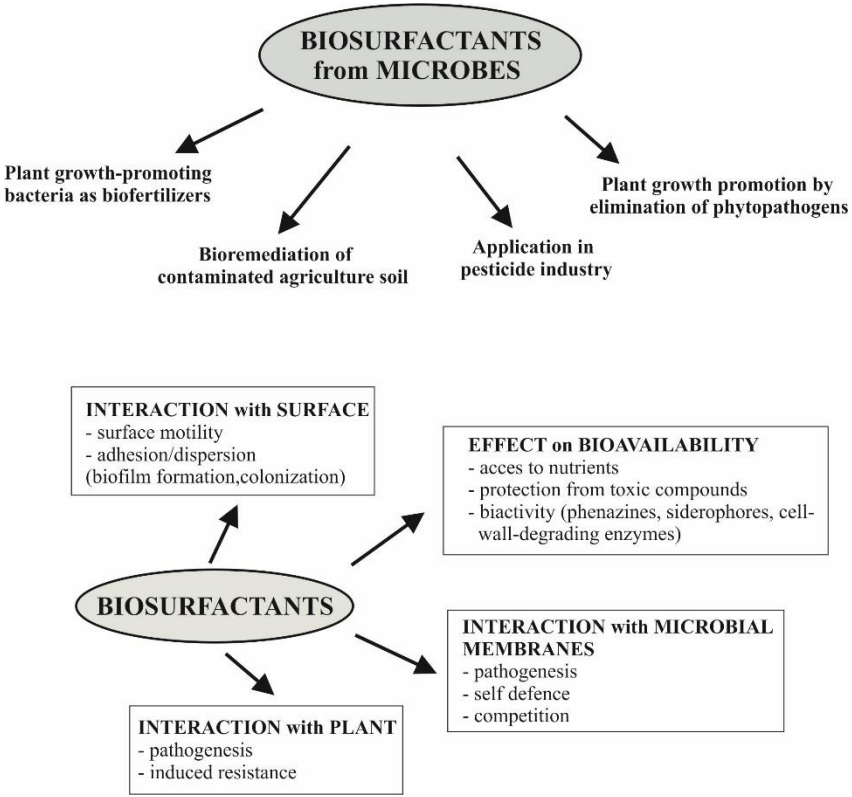
WP 2: Results

BIOSURFACTANTS are synthesized by environmental isolates, and has promising role in the agricultural industry.

Many rhizosphere and plant associated microbes produce biosurfactant; these biomolecules probably play also vital role in plant–microbe interactions.

In agriculture, biosurfactants can be used for plant pathogen elimination and for increasing the bioavailability of nutrient for beneficial plant associated microbes.

Biosurfactants can widely be applied for improving the agricultural soil quality by soil remediation.



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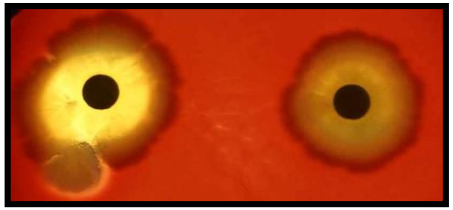
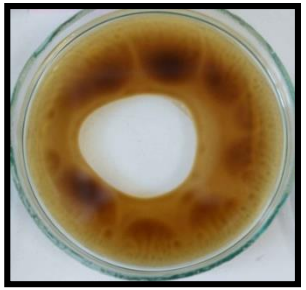
WP 2: Results

Biosurfactant production:

- blood agar
- methylene blue agar
- oil spreading method
- drop-collapse method
- surface tension measurements

E 41





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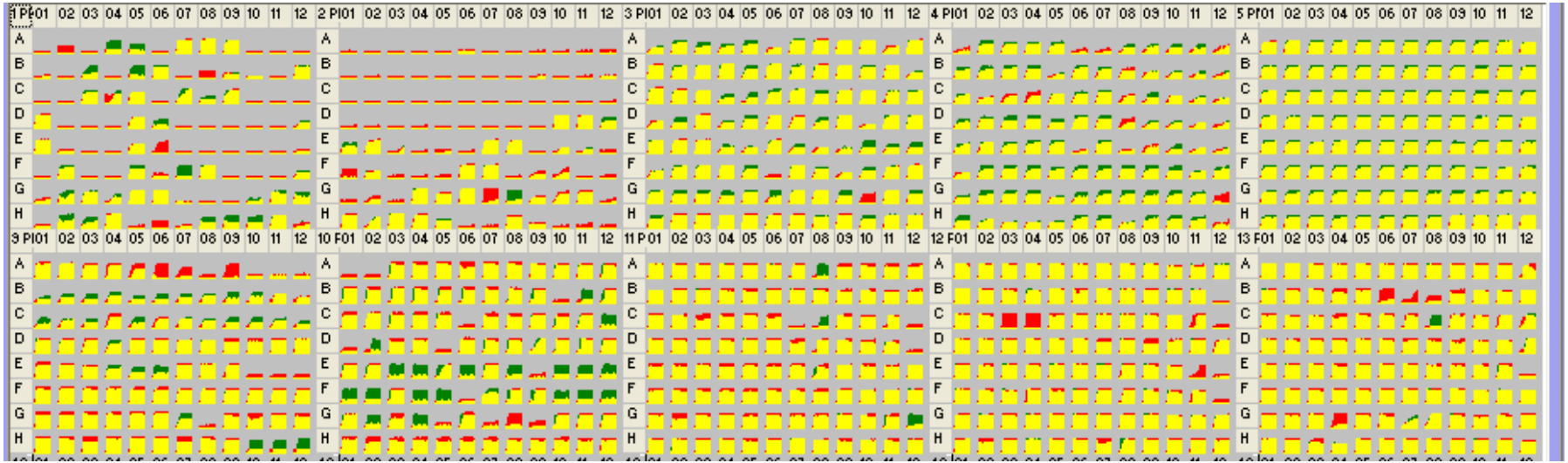
WP 2: Results

Phenotype MicroArrays:

PM1 and PM2 – carbon sources, PM3 – nitrogen sources, PM4 – phosphorus & sulfur sources, PM 5 – nutrient supplements, PM9 – osmolytes, PM10 – pH, PM11 – antibiotics, PM12 – antibiotics, PM13 – antibiotics +chemicals

Green - before lyophilization; red – after lyophilization; yellow – common part (no changes)

E41



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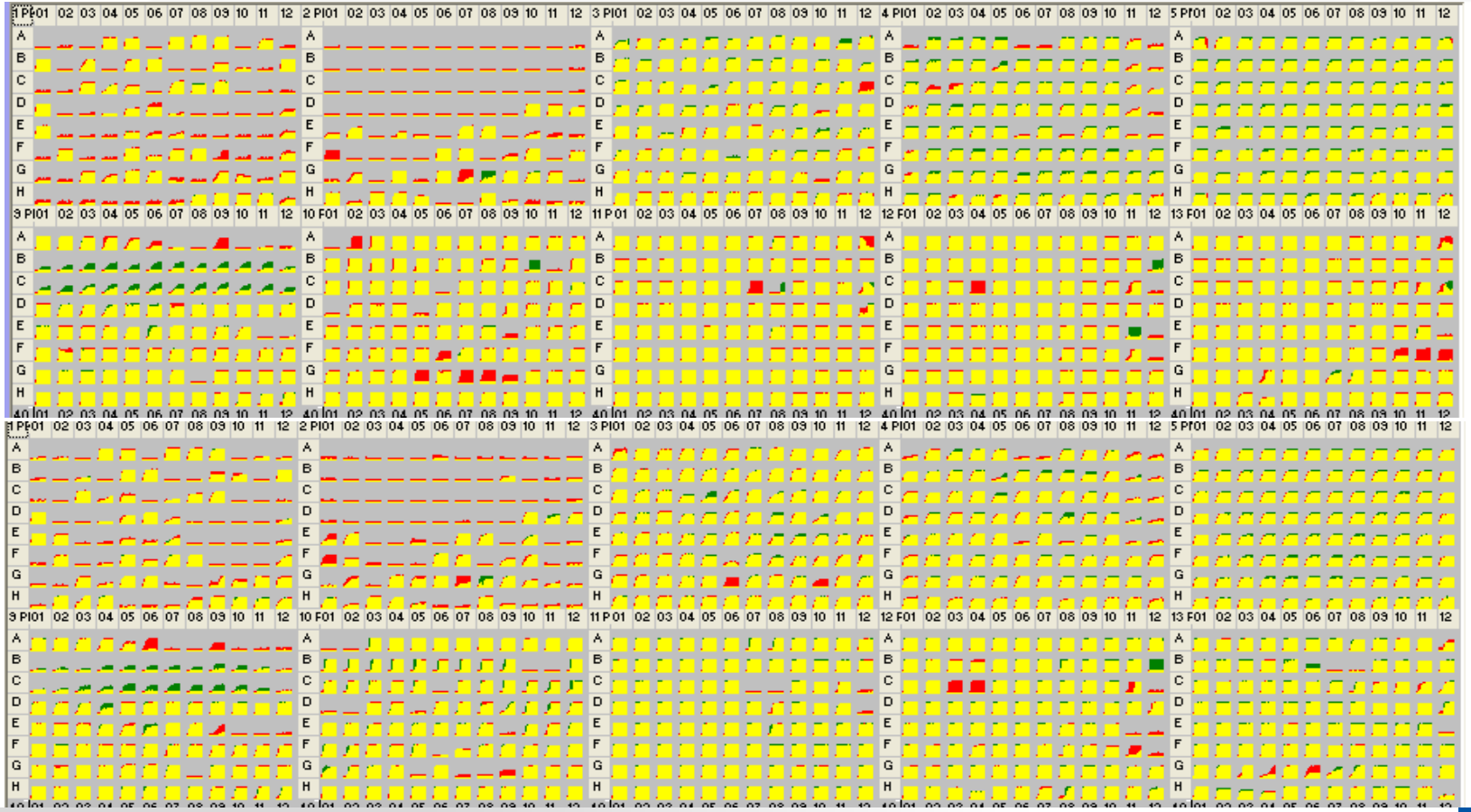
WP 2: Results

E42

R85



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WP2: Results

Assessment of the ecophysiology in soil using in vitro substrate degradation tests

(Establish various biodiversity indices and functional activities of communities (CLPPs) by the EcoPlates Biolog)

What is the biodiversity response to the different treatments implemented over time ?

- **T0:** 2014
- **T1:** 2015
- **T2:** 2016
- **T3:** 2017

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WP 2: Results

INDICES of biodiversity and biological activity :

- AWCD (average well-color development) - $AWCD = \sum ODi/31$
- Shannon-Weiner functional diversity index - $H = - \sum pi(\ln pi)$, where pi is the ratio of the activity on each substrate (ODi) to the sum of activities on all substrates ∑ODi
- Shannon Evenness (E) index - was calculated from Shannon-Weiner diversity index (H) and substrate richness (S) index as follows: $E = H/\ln S$
- AUC (Area Under the Curve) - $AUC = \sum (A_n + A_{n+1})/2 \times (t_{n+1} - t_n)$

Statistical analysis - principal components analysis (PCA) and cluster analysis (nearest neighbor method with Euclidian distance)

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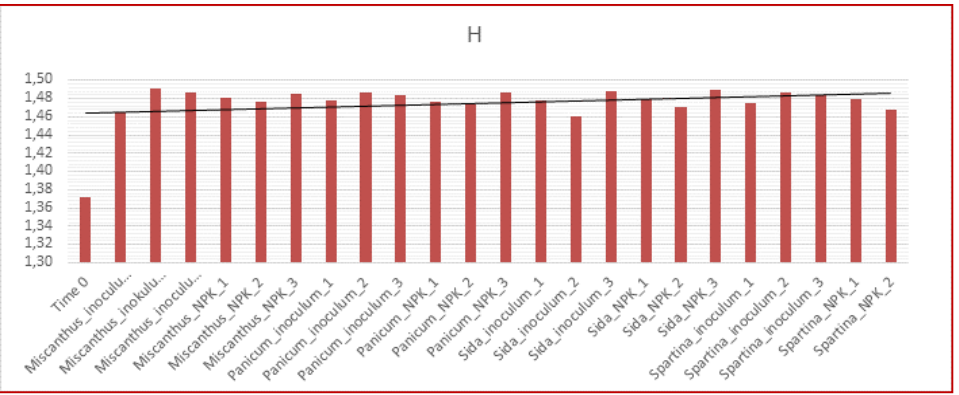
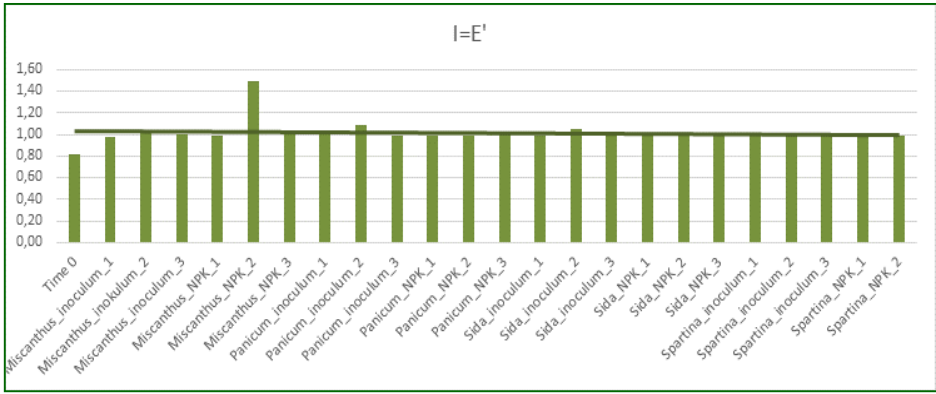
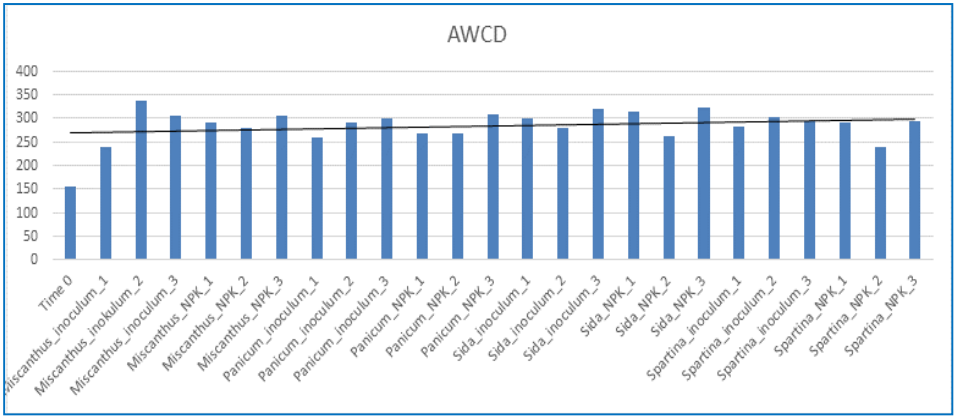
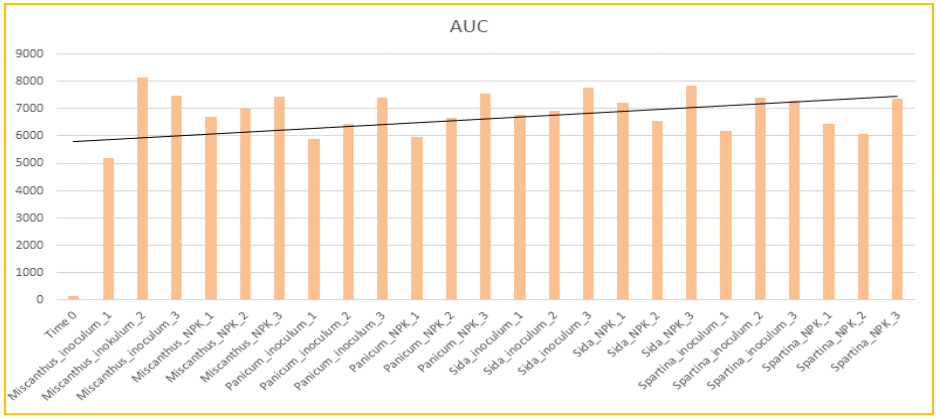
WP 2: Results









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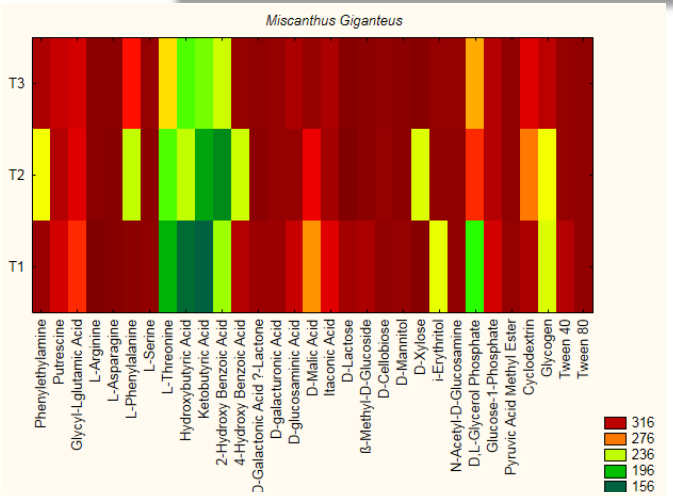

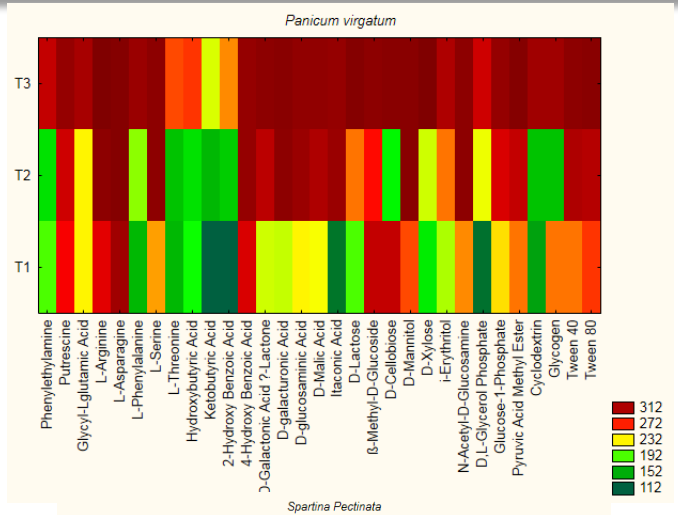

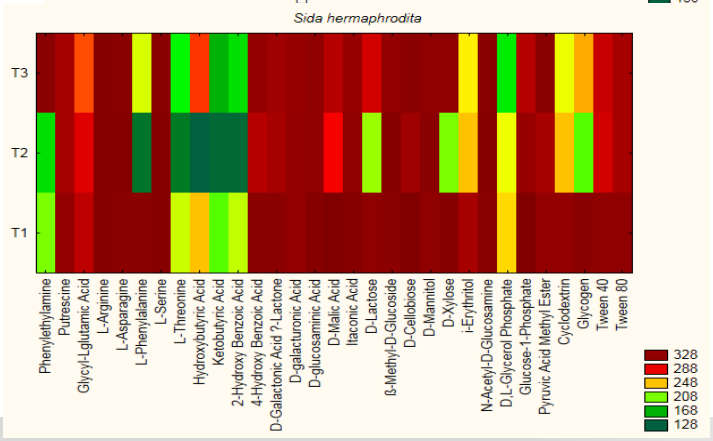

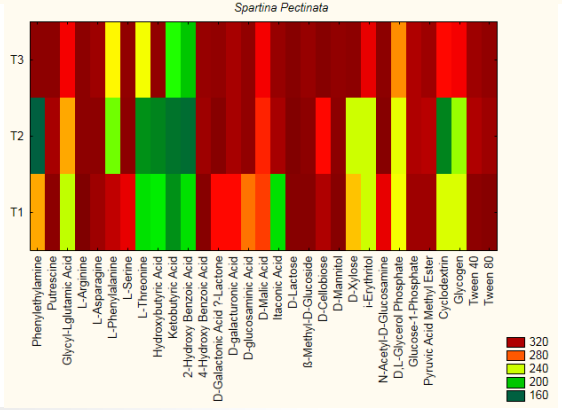

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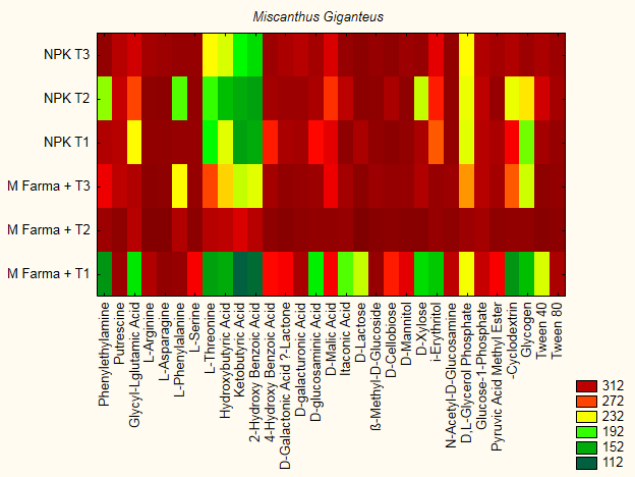

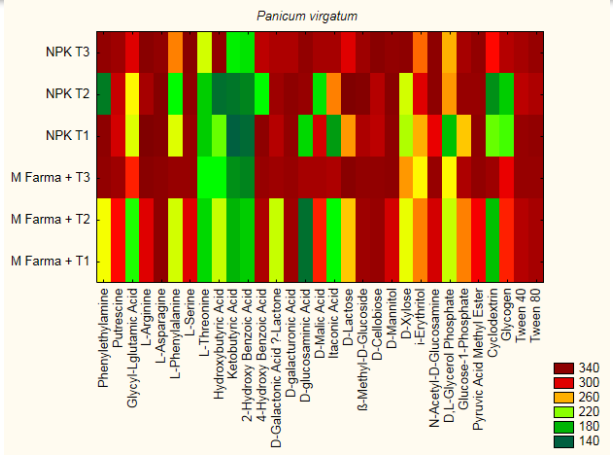

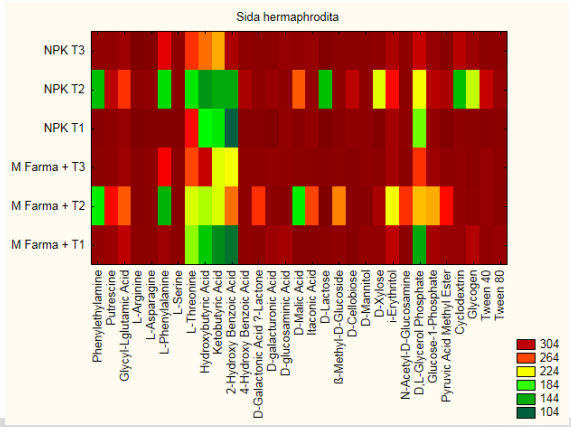

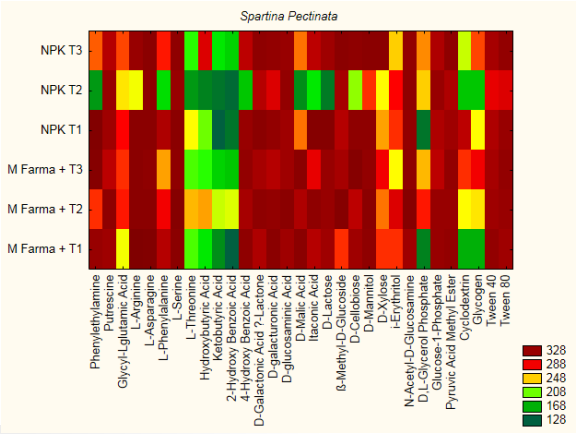

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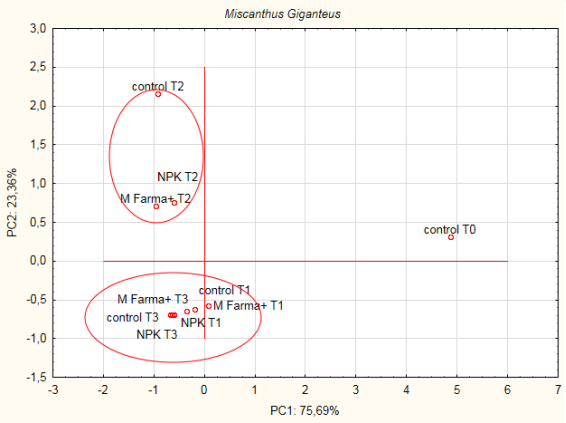

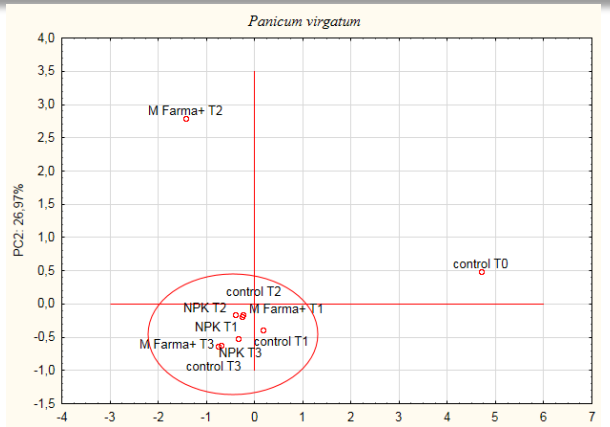

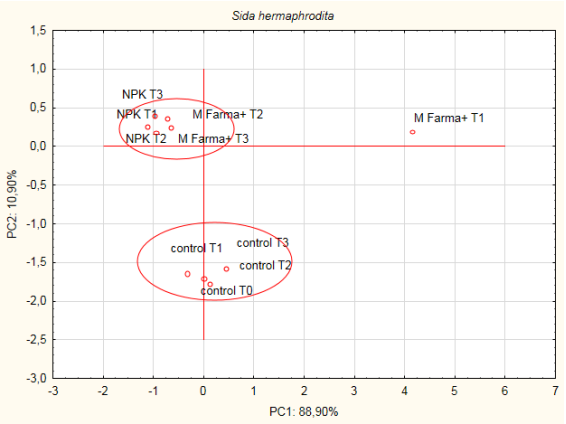

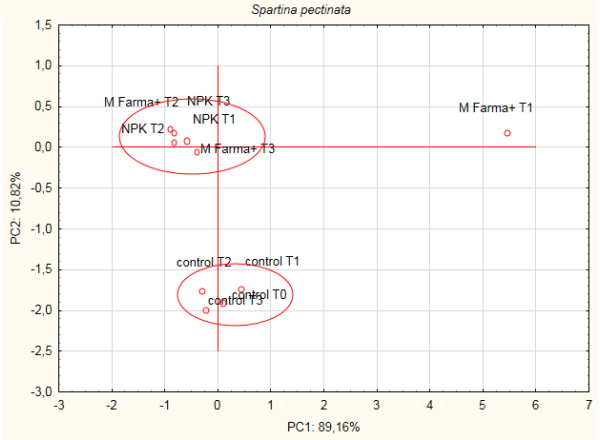

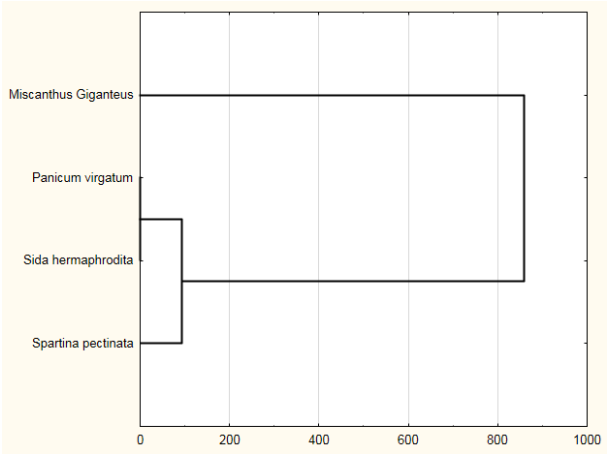

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