KANSAS STATE UNIVERSITY

JAN EVANGELISTA PURKYNĚ UNIVERSITY IN ÚSTÍ NAD LABEM
Faculty of the Environment

Biological Aspects of Soil Ecology
(implications on phytoremediation with *Miscanthus*)

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Jan Evangelista Purkyně

(1787-1869),

Born in Libochovice, Czech Republic

One of the founders of biology, embryology, histology, pharmacology, anatomy, anthropology, famous in physiology

UJEP

- **~9 000 students, ~900 academic staff**
- **8 Faculties, wide range of disciplines**
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  - **Faculty of Education**
  - **Faculty of Science**
  - **Faculty of Health Studies**


Protected area
České středohoří


Protected area
České středohoří


Protected area
Děčínské stěny


National park
Czech-Saxonian Switzerland


National park
Czech-Saxonian Switzerland

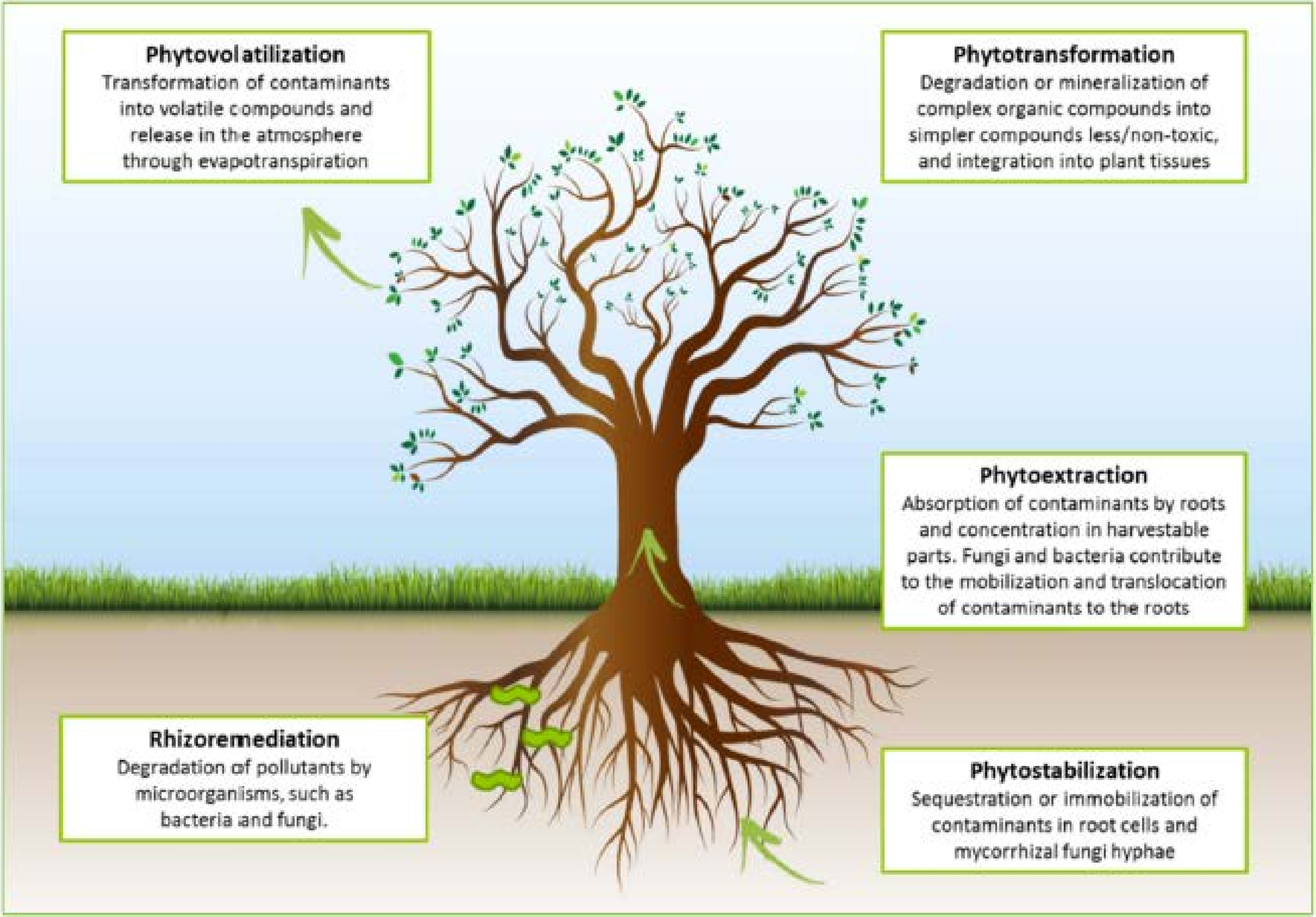

Surface brown-coal mine

Outline

- 0. Motivation
- 1. Ecological roles of soil microorganisms
- 2. Plant-microbe symbioses and interactions
- 3. Rhiosphere and root exudates
- 4. Nitrogen fixation
- 5. Mycorhizza
- 6. Extracellular metabolism
- 7. Overview of methods for study of soil microbial communities
- 8. A few research results

0. Motivation

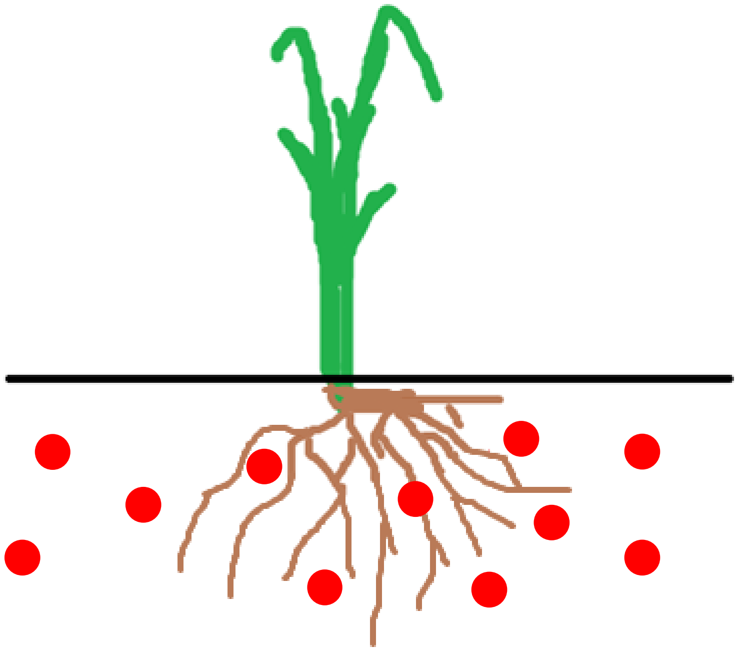
- Recent research on phytoremediation of military sites using second generation biofuel crops
  - *Miscanthus x giganteus*
- Multiple aims:
  - phytoremediation of contamination
  - production of energetic biomass
  - improvement of soil properties
  - ideally together
- Soil organisms play essential roles in these processes



Rohrbacher et al. (2016)

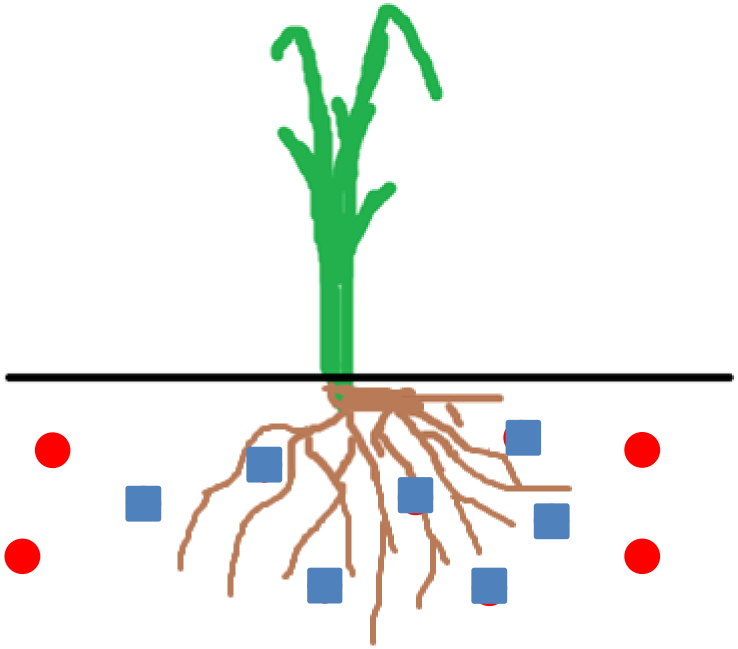
Phytoextraction

- =uptake of pollutants from soil to plants



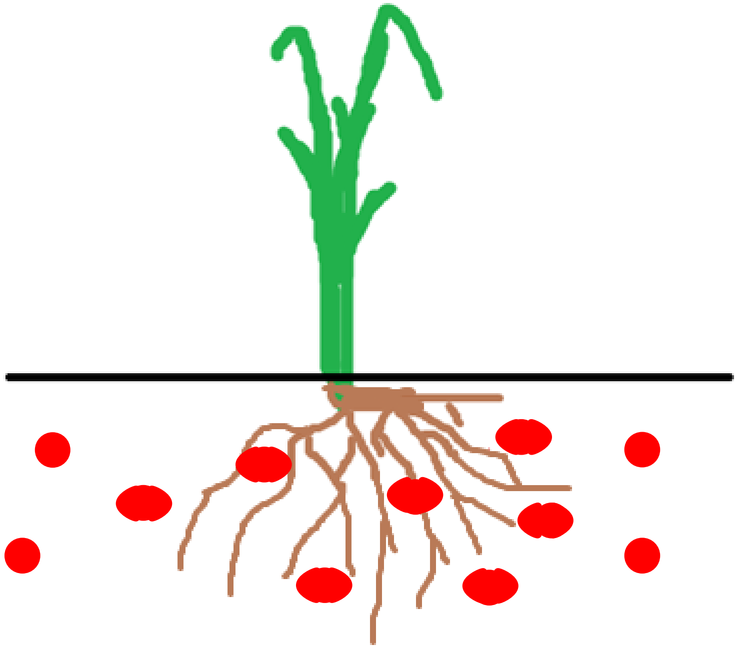
Phytostabilization

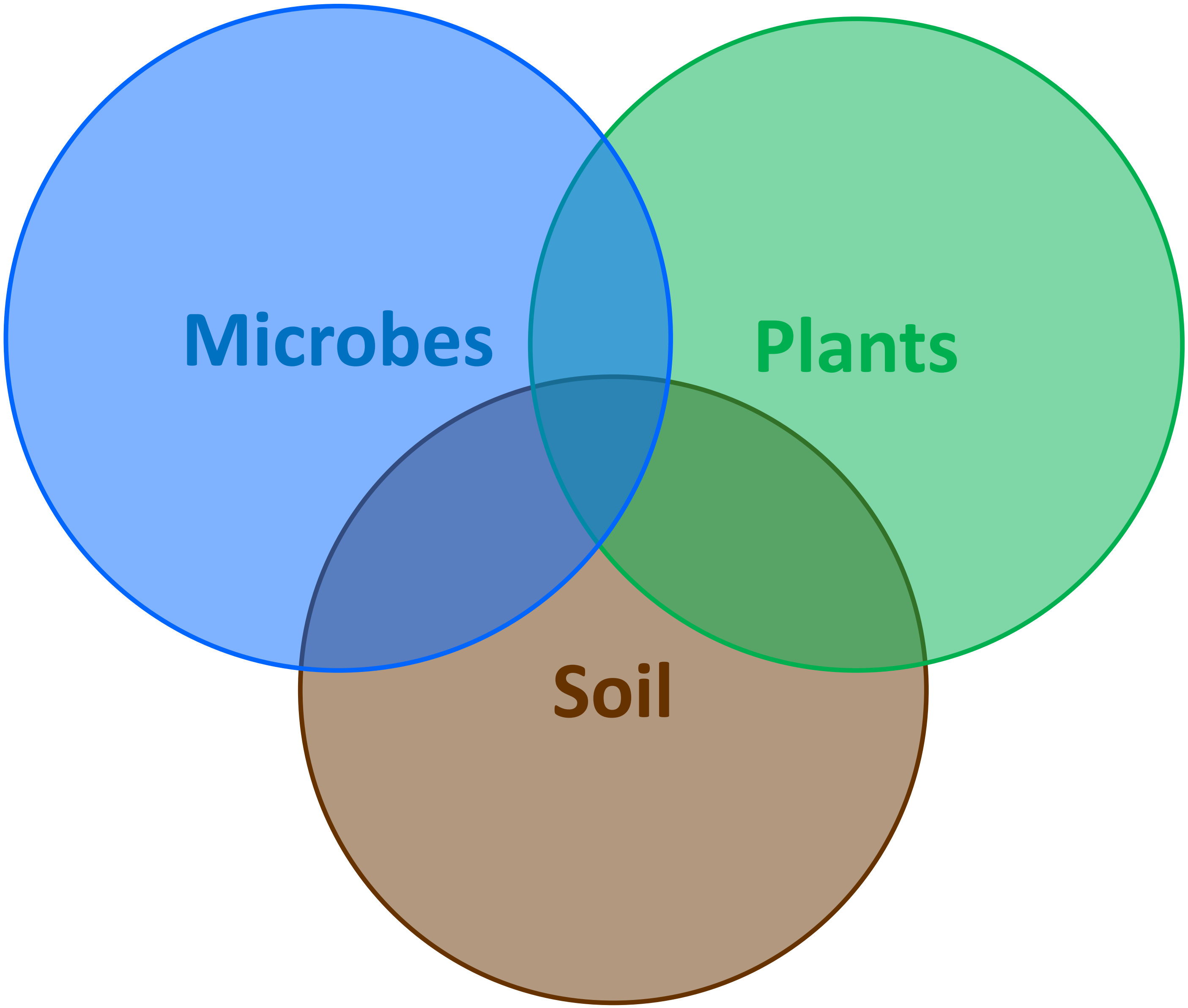
- =changing pollutants to less bioavailable / less toxic



Rhizodegradation

- =biodegradation of pollutants by microorganisms supported by plant roots




Microbes
Plants
Soil

1. Soil microorganisms

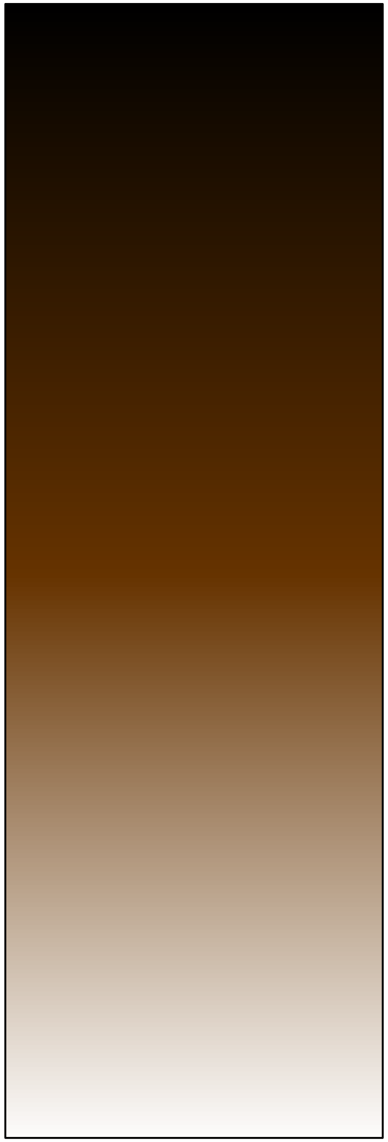
1. Soil microorganisms

- Ecological roles of soil microorganisms
  - **decomposition (biodegradation, mineralization) of organic substances**
    - ~90% decomposed by MO
  - production of organic substances (humus, enzymes, antibiotics…)
  - fixation of nitrogen
  - assimilation of inorganic substances
  - adaptation of environment (pH, temperature…)
  - symbioses
  - …

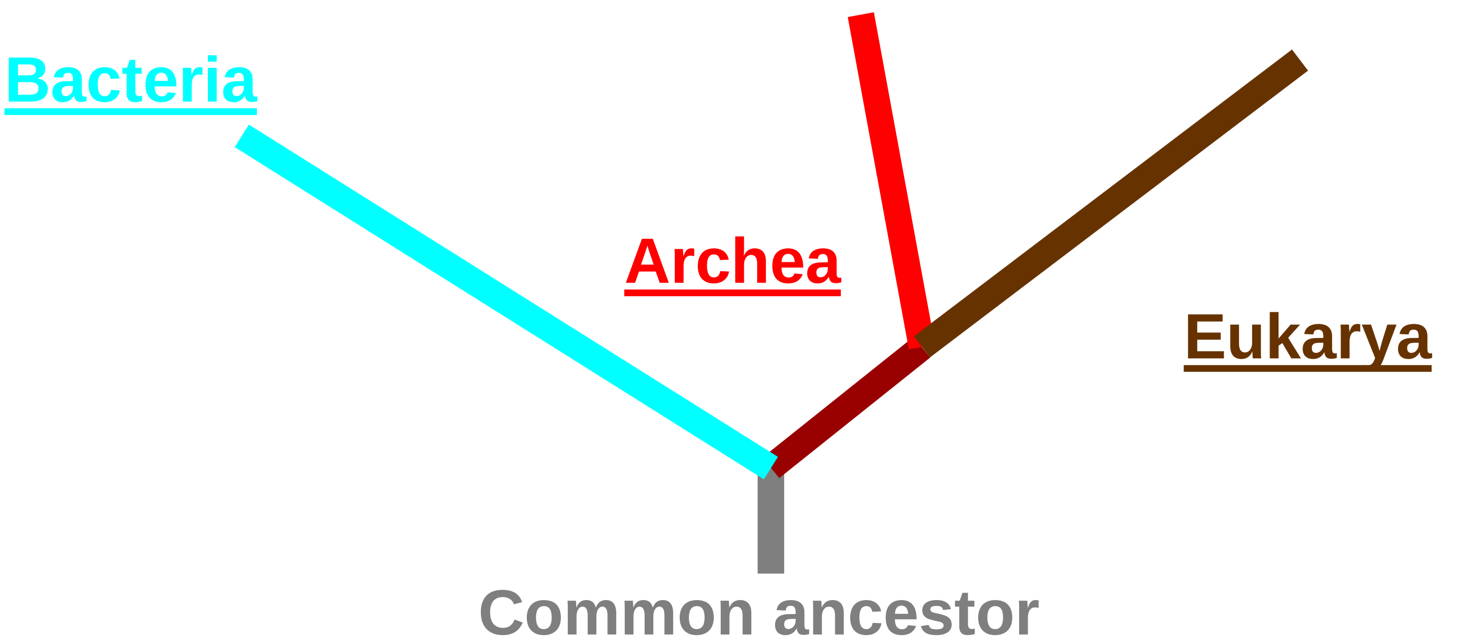
→ microorganisms sustain key soil functions

Soil microorganisms

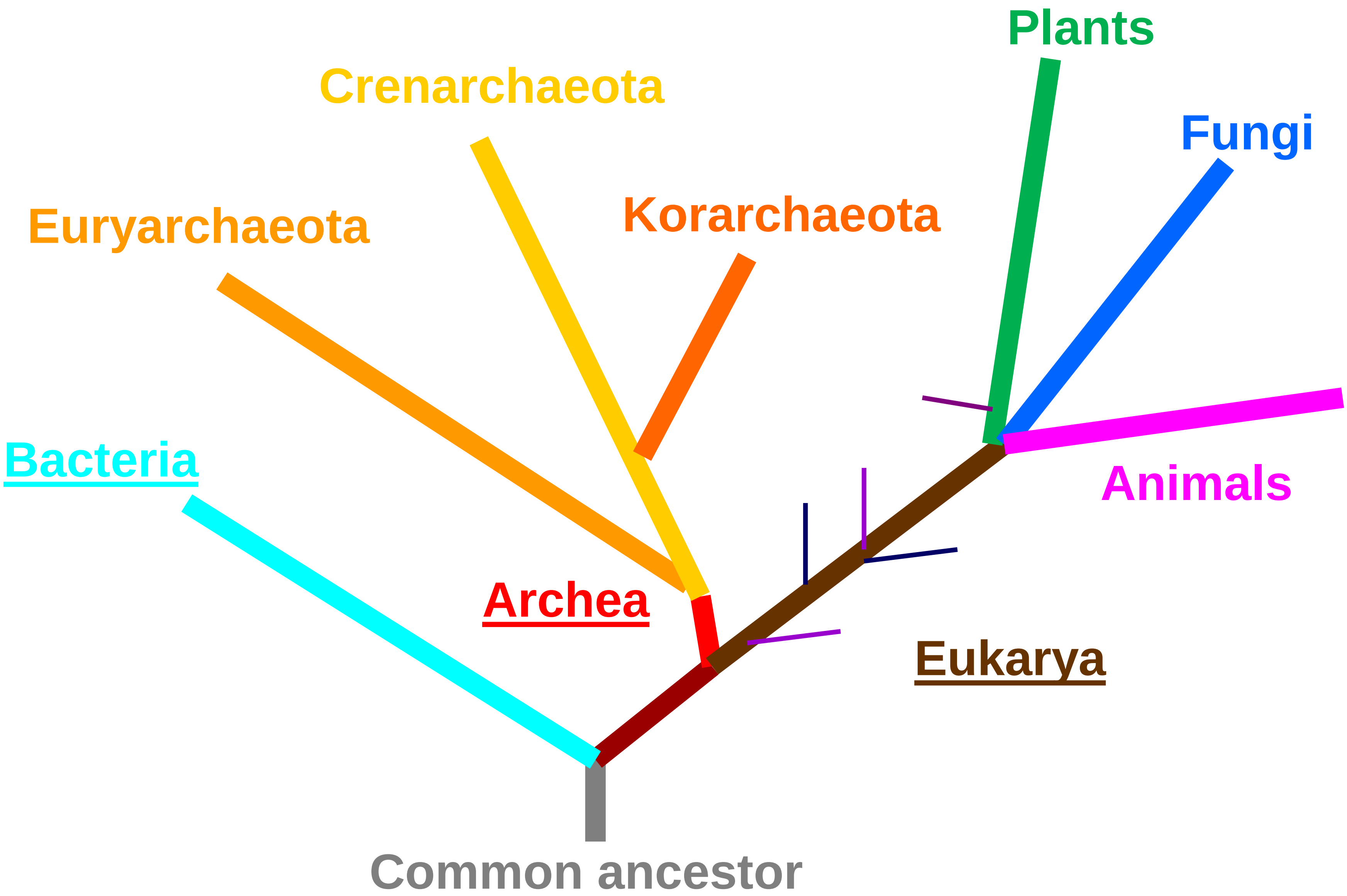
- Abundance is decreased with depth – highest number near surface
  - input of nutrients, oxygen…
- Majority are psychrophiles or mezophiles
- Cumulation around plant roots – rhizosphere
- Bacteria are bound to solid particles (dust…)

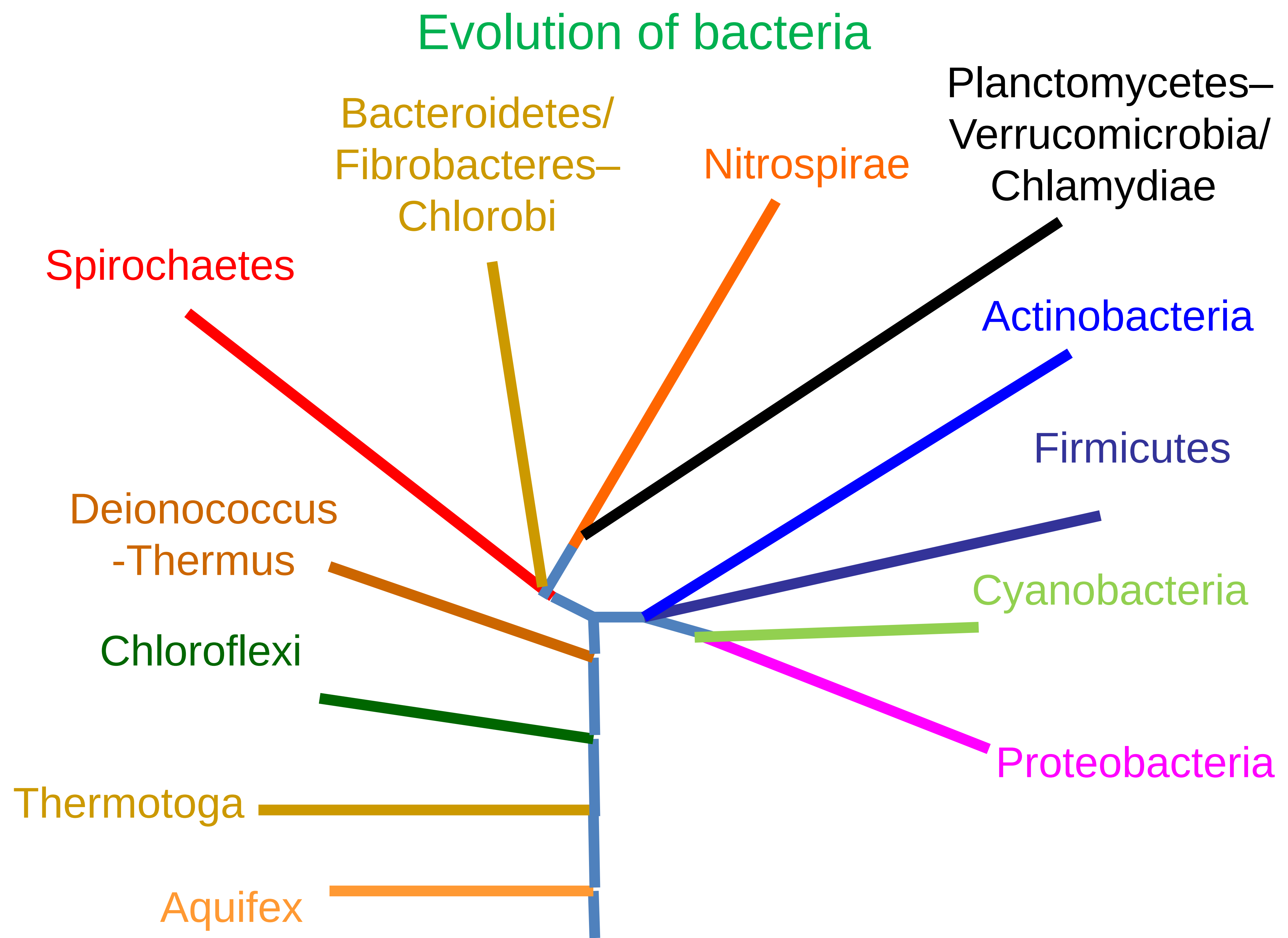


Fylogenetic tree

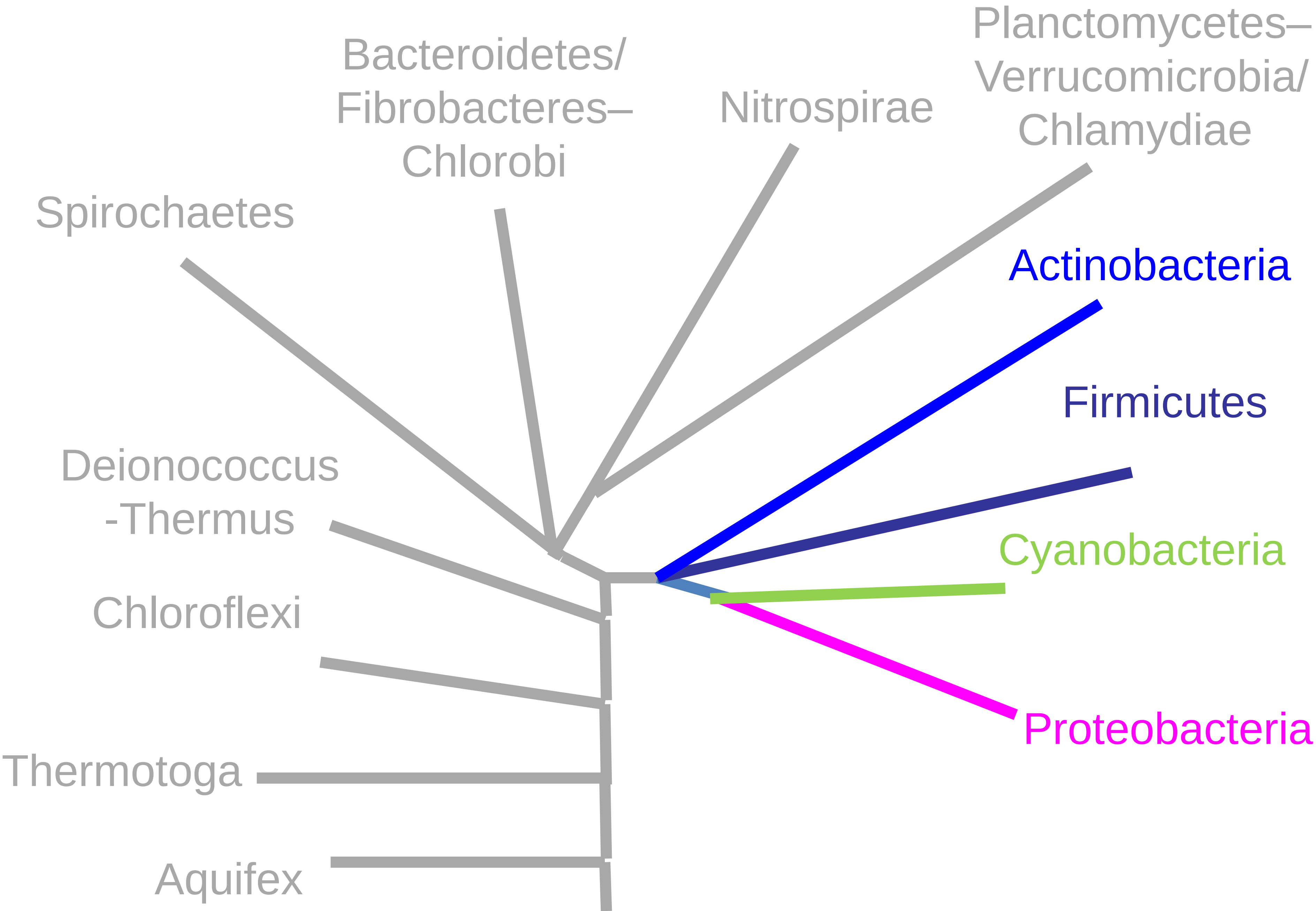


Fylogenetic tree




Evolution of bacteria
Bacteroidetes/
Fibrobacteres–
Chlorobi
Nitrospirae
Planctomycetes–
Verrucomicrobia/
Chlamydiae
Spirochaetes
Actinobacteria
Firmicutes
Deionococcus
-Thermus
Cyanobacteria
Chloroflexi
Proteobacteria
Thermotoga
Aquifex

Evolution of bacteria



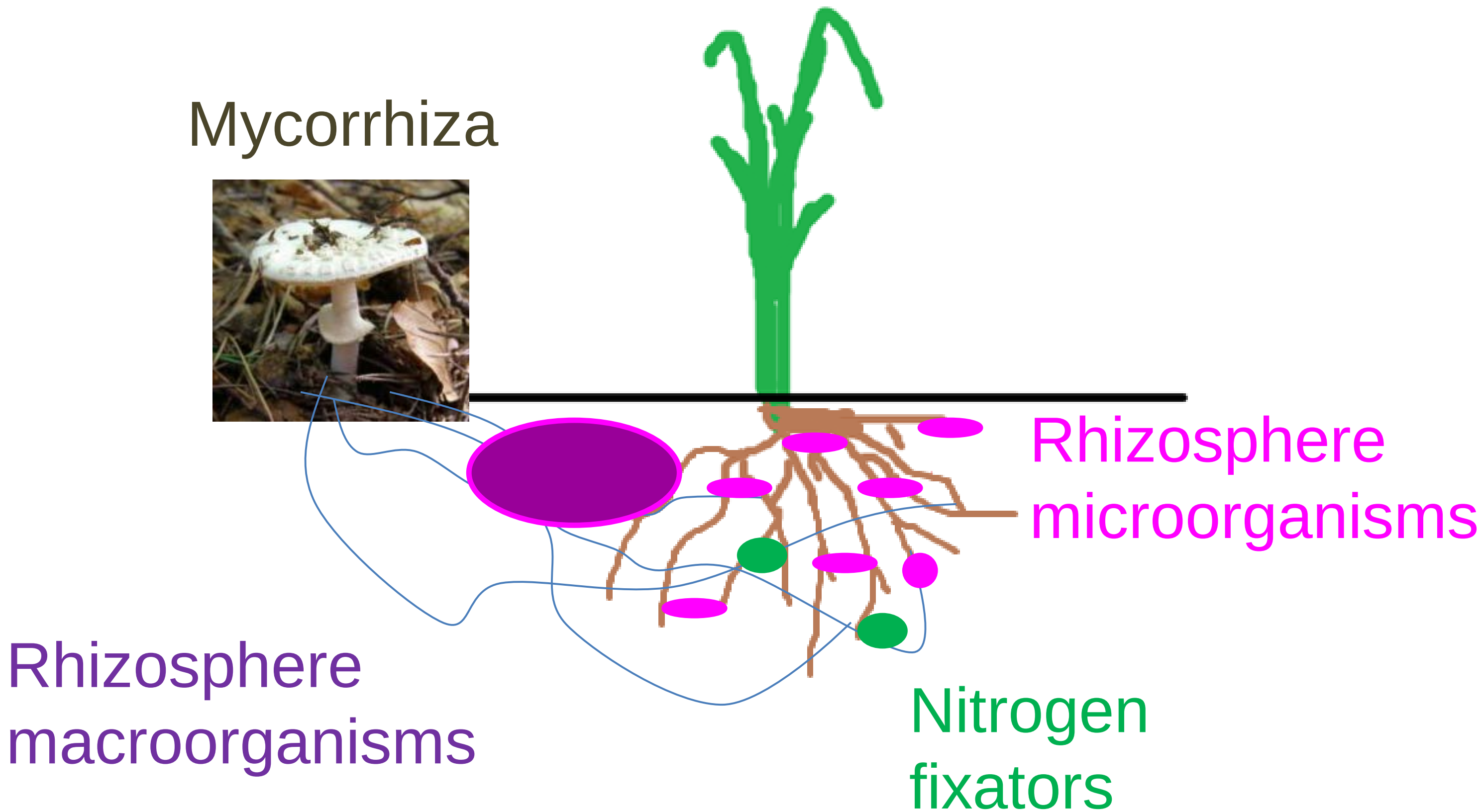
2. Plant-microbe symbioses and interactions

- Common
- Symbiosis = relationship positive or both sides
  - give and take (i.e. ∑ give < ∑ take for both sides)

  → easy shift to parasitism
- Plants are autotrophic → excess of organic matter
- Plants need water and nutrients – provision by partner

Plant microbe interactions

- Majority of plants



Plant-microbe interactions

- Common
- Dominantly around roots – rhizosphere
  - from greek *rhizos* = root

  = soil directly affected by roots
  - includes microorganisms and higher organisms (nematodes, insects…)
  - many different relationships

3. Rhizosphere

3. Rhizosphere

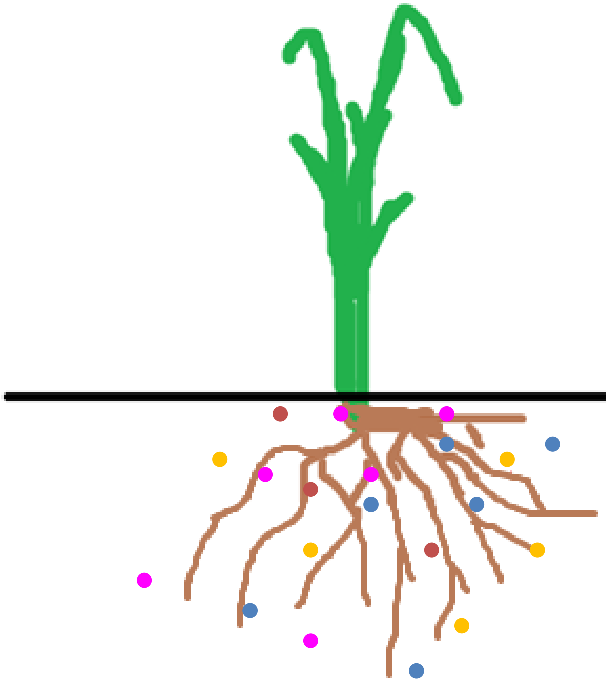
- Usually higher microbial concentrations compared to bulk soil
  - order of magnitude
- More nutrients for organisms
  - dead root cells
  - production of root exudates (organic substances)
  - estimation 17% of C fixed in photosythesis is transerred to the rhizosphere
- Establishment of food chains

Rhizosphere and microbes

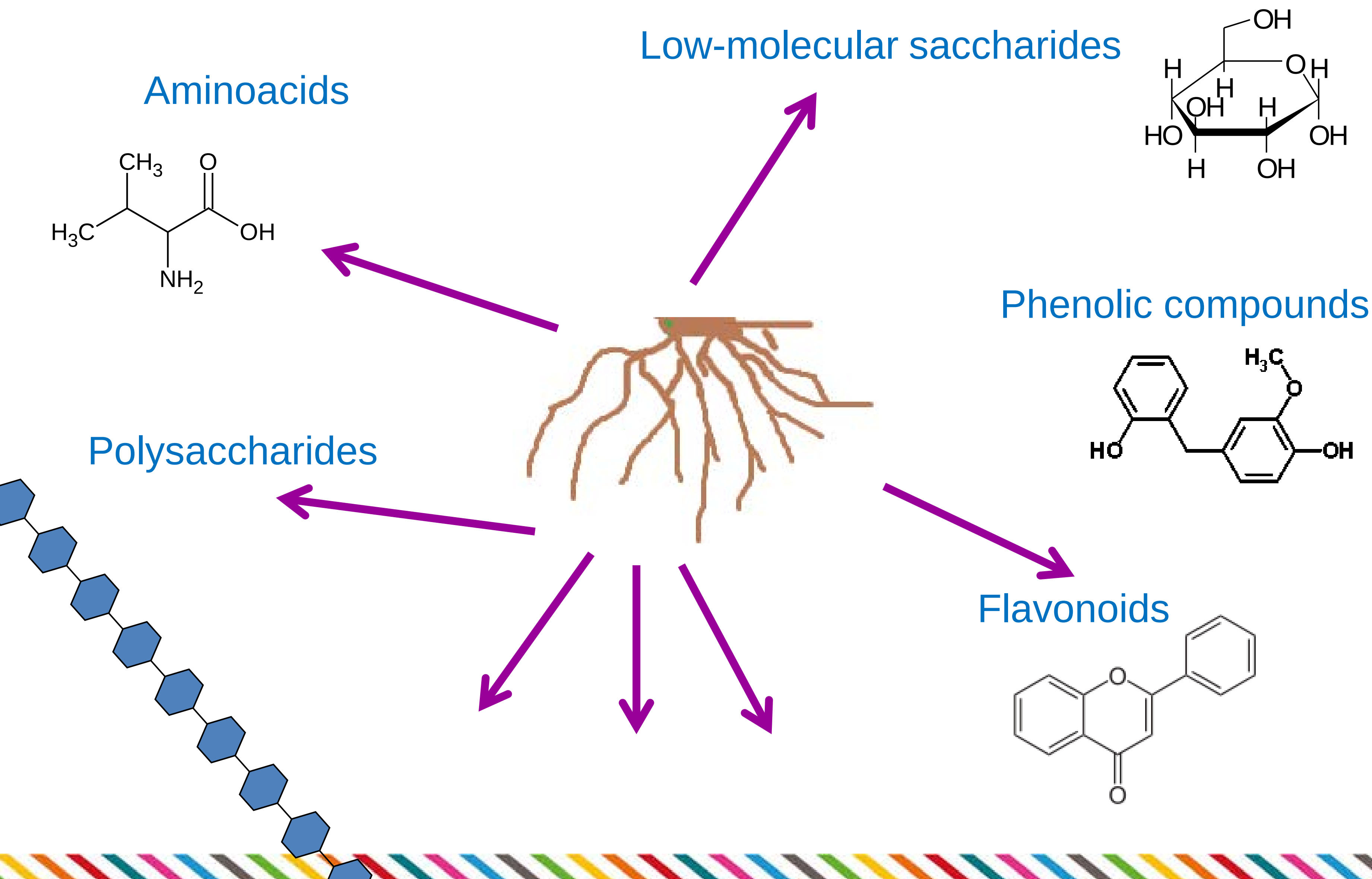
- Soil directly affected by plant roots – in vicinity of the roots
- Interactions with soil microorganisms →
  - predominantly positive relationships (symbioses, metabioses…) between plant and microbes
  - inhibition of pathogenic microorganisms

Root exudates

- Chemicals liberated from roots to surrounding soils
  - deliberately
  - from dead root cells
- Inhibitting pathogenes
- Attracting soil-born microorganisms (chemotaxis)
- Inducing degradation pathways



Root exudates



Effect of root exudates on soil organisms

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- Not always clear
- Attracting partners for positive relationships (mycorrhiza, actinorrhiza…)
- “Feeding” rhizosphere microorganisms – plant protection
- Fighting plant pathogenes
- Modulating microbial physiology – quorum sensing, induction of methabolic pathways etc.
- …

Implications for bio- and phytoremediations

- Stimulation of biodegradation potential / activity of microorganisms
  - organic nutrients for heterotrophic microorganisms
  - induction of specific biodegradation pathways
  - co-metabolism
  - …
- Improvement of soil parameters
- Improvement for plant growth / biomass yield
- Compatible partners for plants
- Protection of the plant

4. Nitrogen fixation

Nitrogen fixation

- = diazotrophy
- Important process for fertilization of soils with N
- Significant also for water ecosystems
- N_2 → .... → NH_4^- → organic N
- Nitrogenase complex
- IN≡NI is very stable molecule

→requires a lot of energy (12-16 ATP / N_2)

→ provides significant evolutionary advantage = nitrogen independence

- Even N excess → often symbioses („N for C“) - dominantly with plants

Nitrogen fixing microorganisms

- Bacteria only
- Symbiotic
  - rhizobia + legumes (soya, beans, pea…)
  - rhizobia + non-legumes (*Parasponia*, canabinacea)
  - actinobacteria (*Frankia…)* + actinorhizal plants
  - proteobacteria + non-legumes (*Azospirilium…)*
- Non-symbiotic with plants
  - cyanobacteria (dominantly not soilborn)
  - *Azotobacter*
  - …

Rhizobial symbiosis

- *Rhizobia* with legumes (pea, soya, beans…)
- Positive for both partners
- Plant provides nutrients (organic acids etc.) and O_2
- Bacteria provides N-substances (NH_3 and aminoacids) obtained by fixation of air N_2
- Covers up to 80% of nitrogen in plant biomass

Grasses

- Known symbioses of diazotrophic bacteria with grasses
- Not as well described as rhizobial
- Best described for Brazilian sugarcane
  - grow well without fertilizing with N
  - N balance is positive
- Similar for other grasses
- Research using isotope tracing of ^{15}N

Miscanthus

- Described reports of *Miscanthus x giganteus* undisturbed growth without N fertilizng
- Keymer and Kent (2014): In the first year 16% of N in *M. x giganteus* biomass originates from symbiotic fixation even in N non-limiting soil
- Eckert et al. (2001): Izolation and identification of azotrophic *Azospirillum doebereinerae* from *Miscanthus x giganteus* rhizosphere
- Bourgeois et al. (2015): *M. x giganteus* stimulates various nutrient cycling bacteria and fungi including azotrophic *Rhizobiales*

5. Mycorrhiza

5. Mycorrhiza

- From greek *Mycos* = fungus, *rhizos* = root
- Symbiotic association between plant roots and fungal mycelia
- Estimation of up to 95% plant species
- Positive for both partners

Mycorrhiza

- Fungus serves as prolonging of plant roots
  - better uptake of minerals (limiting for plant)
  - better uptake of water (limiting for plant)
- Protective effect of fungal mycelium
- Stimulation of rhizosphere
- Plant (=autotrophic) provides organic substances (carbohydrates, limiting for heterotrophic fungus)

Mycorrhiza

- Better physiology of both plant and fungus in the working symbiosis
  - young trees grow faster and have higher survival rate
  - fungi grow better
  - common dependence of fungus on the plant (obligatory symbiosis) – common for basidiomycota
  - dependence of plant on fungus is less common

Mycorrhiza

Mycorrhiza

- Endomycorrhiza – fungal mycelium penetrates between plant cell wall and cell membrane
  - Up to 85% plants – mostly arbuscular (through vesicels called arbuscules)
- Ectomycorrhiza – fungal mycelium only wraps the root
  - ~10% plants, dominantly trees → higher economic significance

Mycorhizza and *Miscanthus*

- Sarkar et al. 2015, 2017: Arbuscullar mycorrhizal fungi:
  - – promoted growth of *Miscanthus*
  - – protection against high concentration of metals
  - – increased uptake of metals
  - – increased growth on nutrient-limited soil
- Firmin et al. 2017: AMF
  - – increased accumulation of metals in roots and shoots
  - – descreased oxidative stress of *M. x giganteus* especially in leaves

→ more research needed

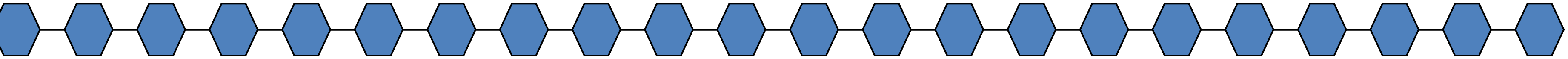
6. Extracellular enzymes

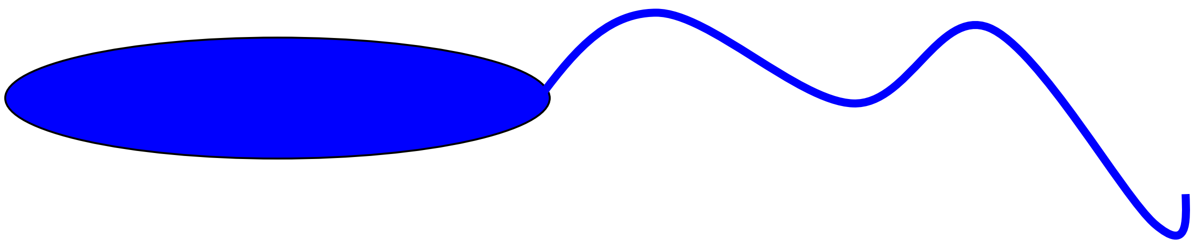
6. Extracellular enzymes

- Significant players in decomposition of organic matter and nutrient cycling
  - Especially for biodegradation of polymers (lignin, cellulose, chitine…) – can not fit inside microbial cells
- Dominant producers of extracellular enzymes in soil:
  - bacteria
  - fungi
  - plant roots
- Extracellular enzyme activities follow
  - overall metabolic activity
  - nutrient scarcity (low P → high phosphatase activity)

Extracellular enzymes

- Activity of extracellular enzymes – decomposition of polymers, involment in nutrient cycling





Extracellular enzymes

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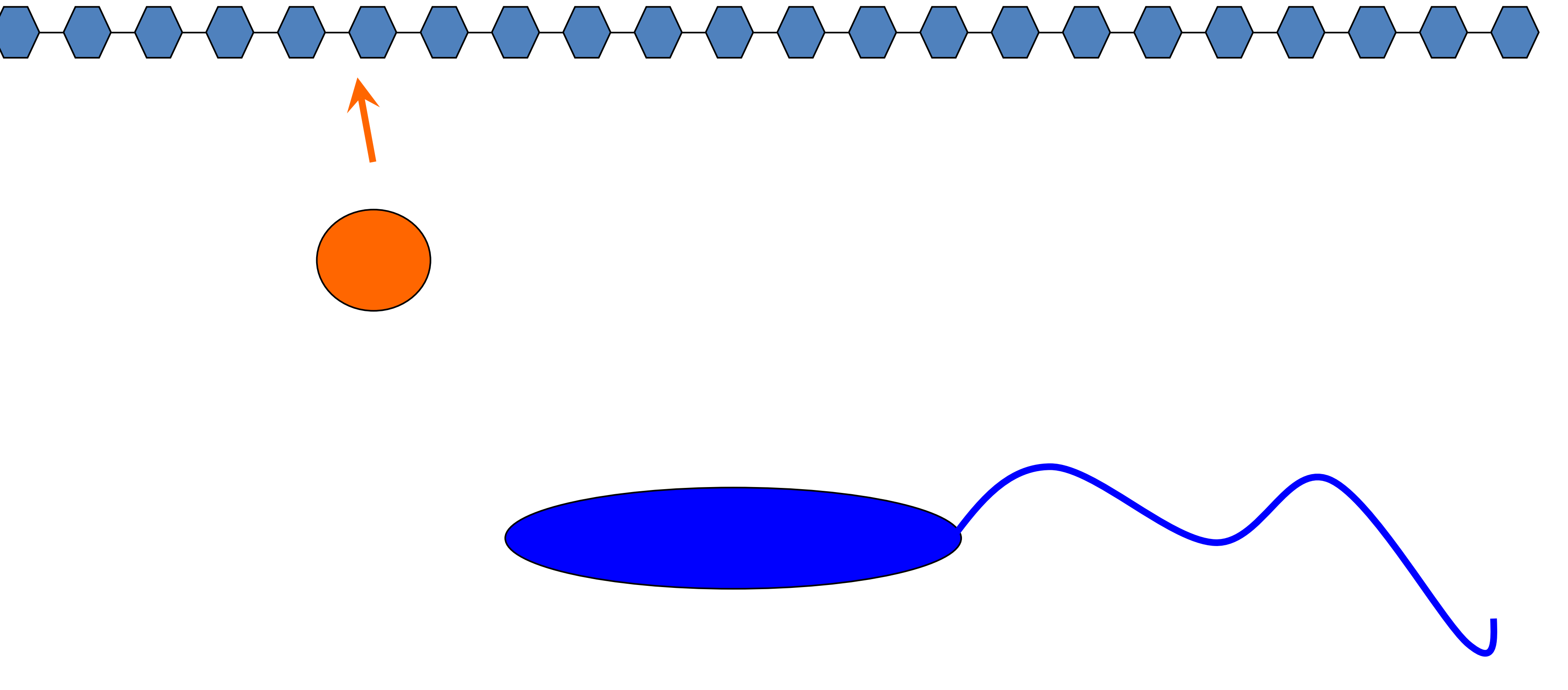
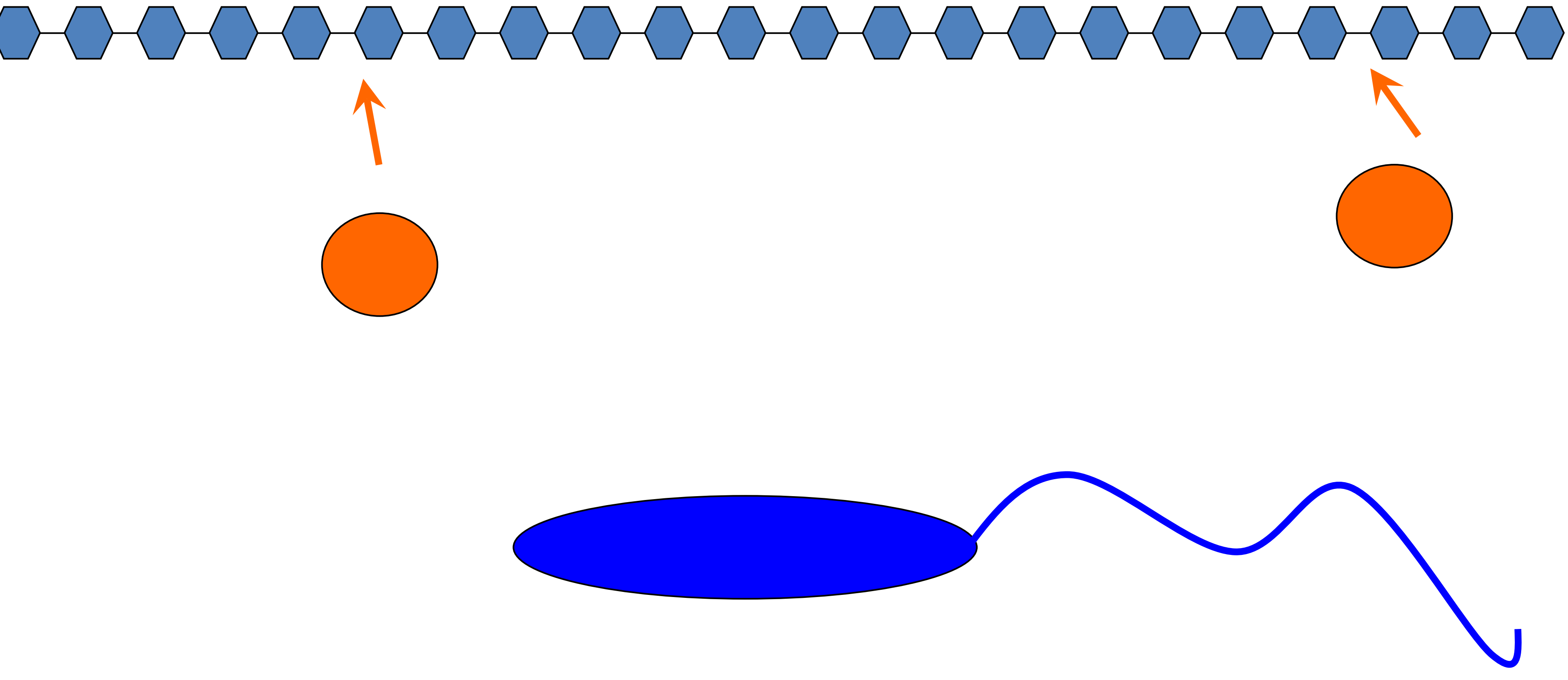


Determination of microbial activities

- Activity of extracellular enzymes – decomposition of polymers



Determination of microbial activities

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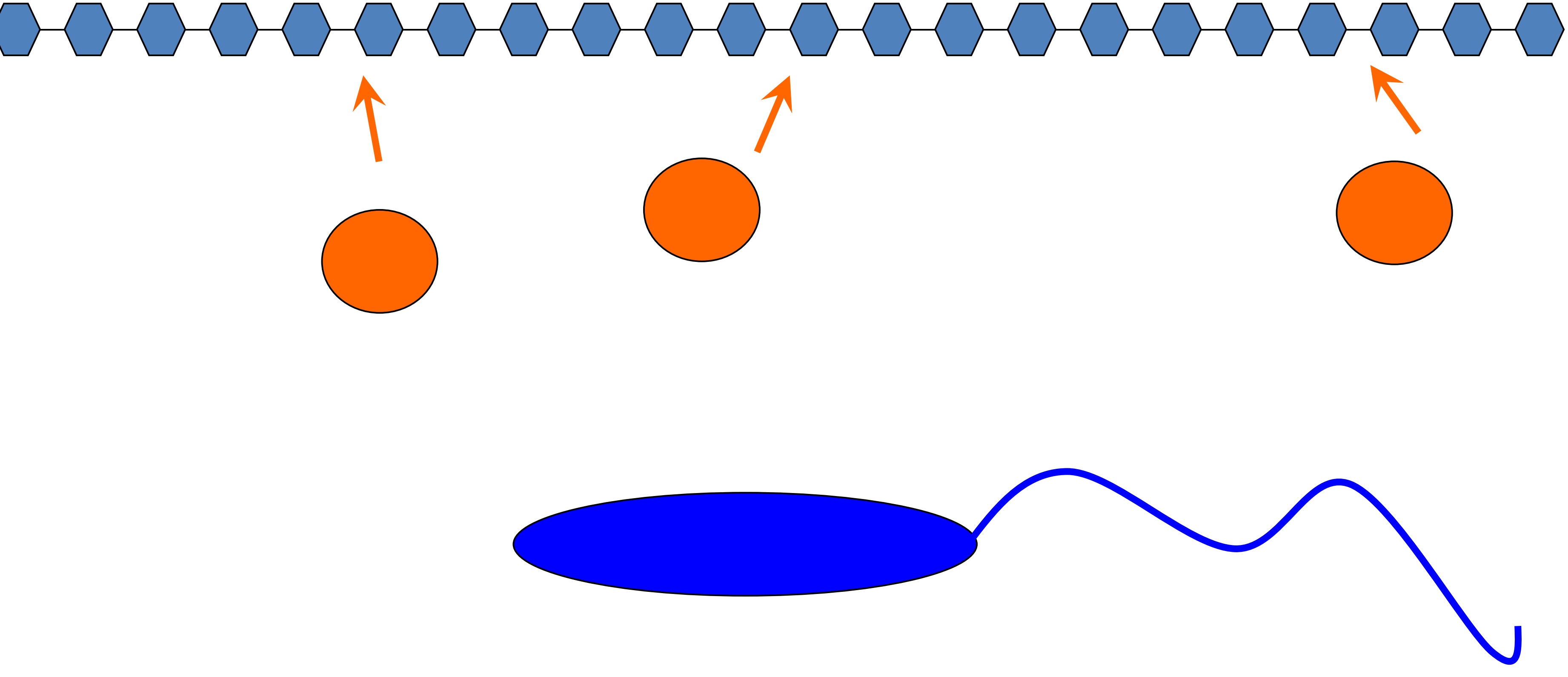

Extracellular enzymes

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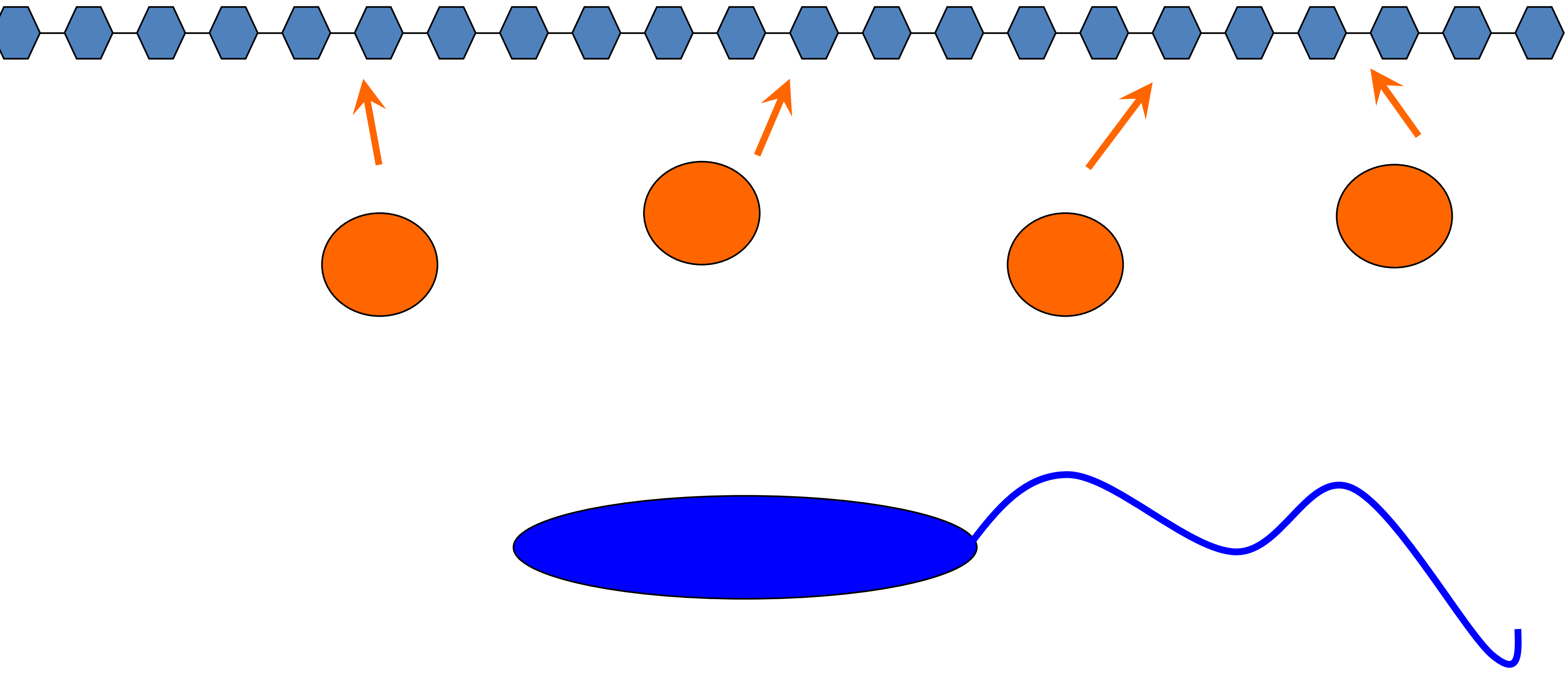

Extracellular enzymes

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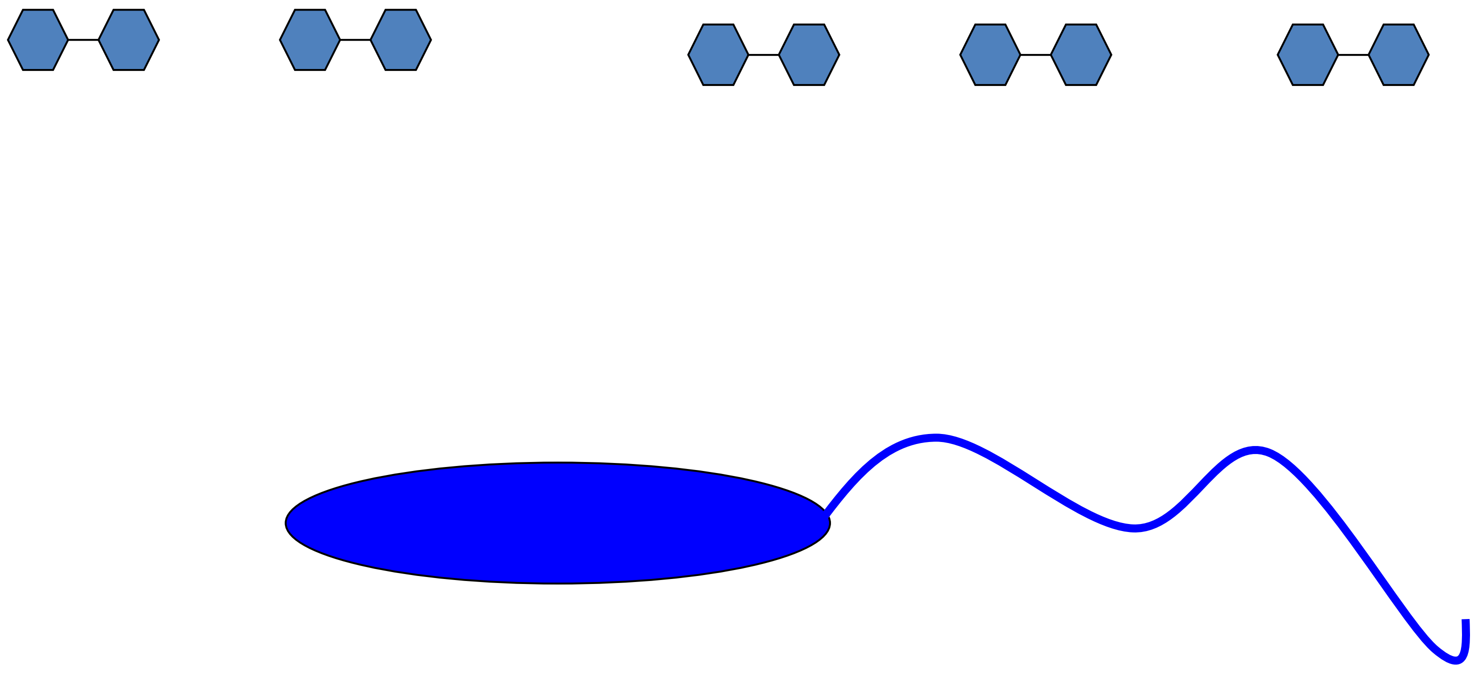

Extracellular enzymes

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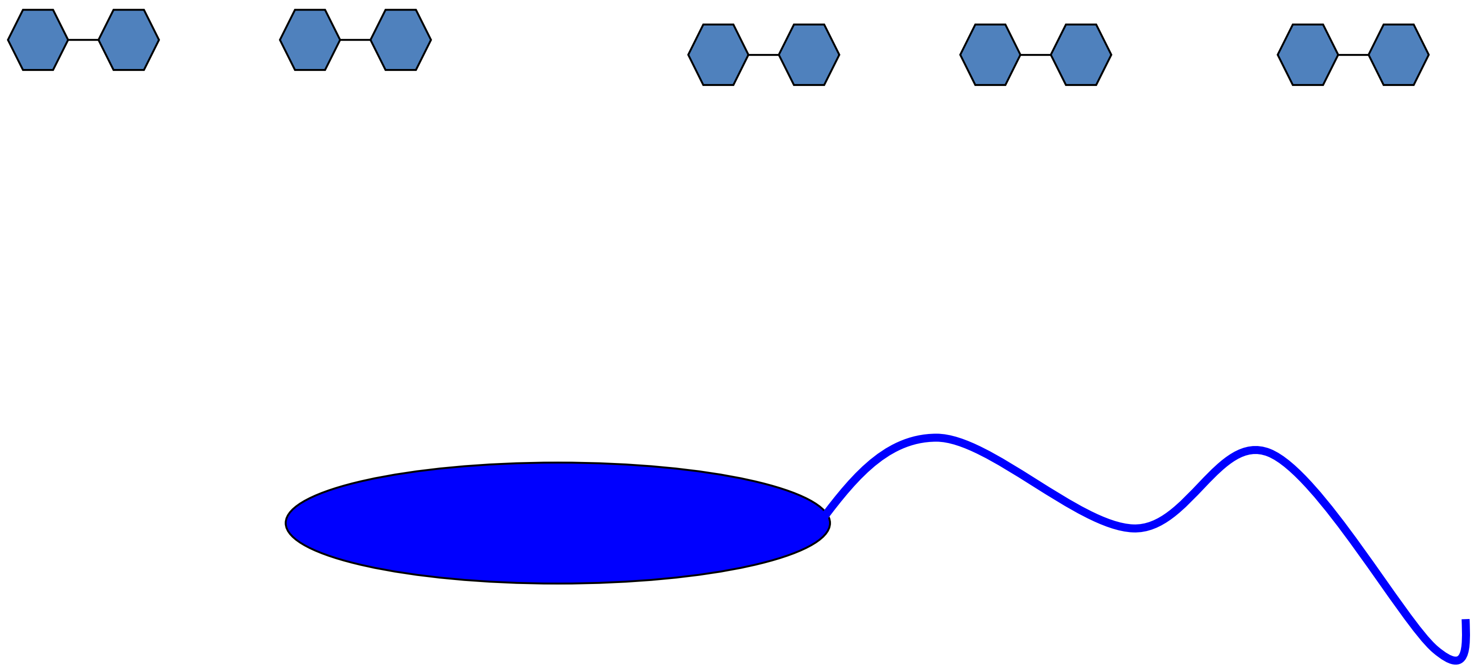
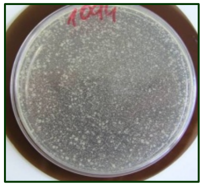


Extracellular enzymes

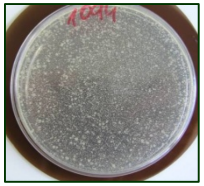
- Hydrolyses
  - phosphatases, sulphatases, proteases, celulases, chitinases…
- Oxidoreductases
  - laccases, peroxidases, oxygenases…

7. Methods of analyses of soil microbial communities



Methods of analyses of soil microbial communities

- Cultivation – determination of Colony Forming Units
  + covers viable microorganisms
  - only 1-2% of soil microbes cultivable



- **Activity – determination of microbial activities**
  – enzyme activities, respiration, production / consumption of chemicals…
  + covers viable microorganisms
  - dependent on conditions

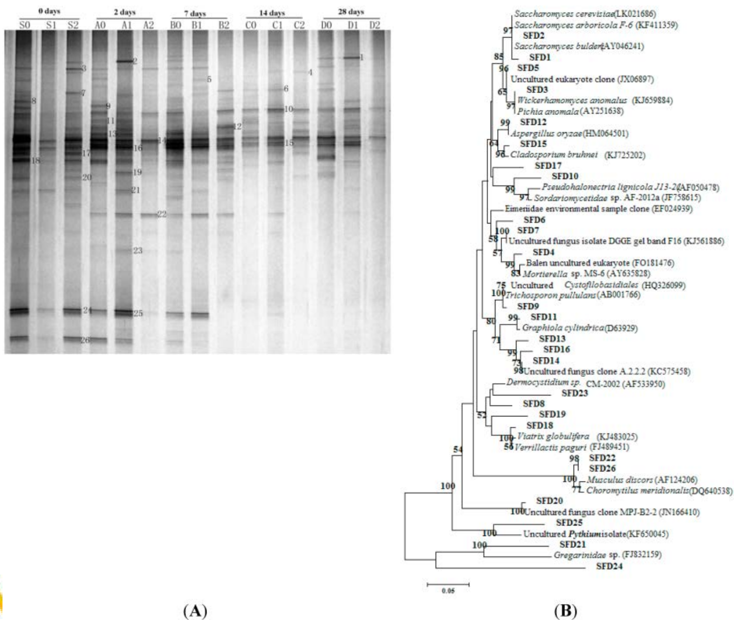
Methods of analyses of soil microbial communities

- Genetic analyses – extraction of DNA / RNA, sequencing, comparison with databases

  + detailed information (taxonomy, abundance of taxons, metabolic potential, transcriptomics – expressed genes, stress genes…)

  - costly and not as spread equipment

  - sometimes too detailed data (limited database data, laborious evaluation)



(Ling et al. 2015)

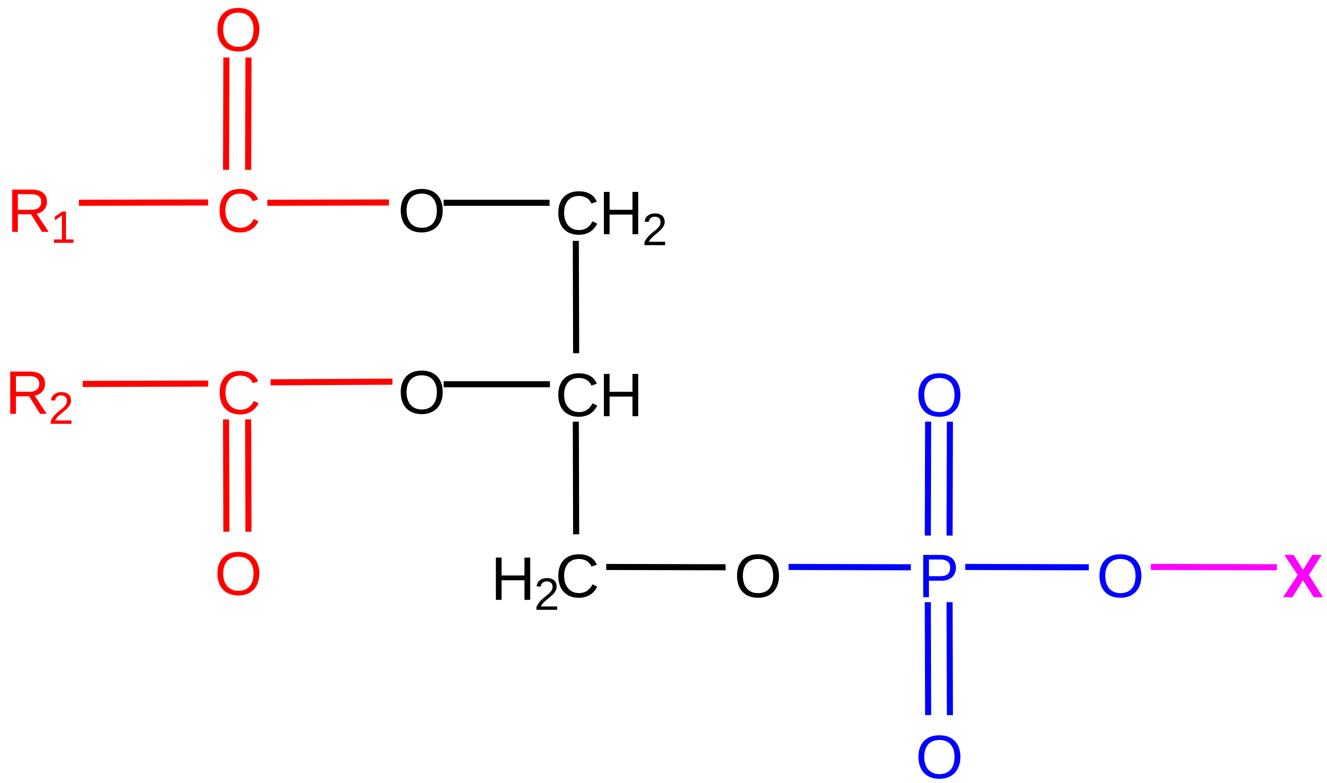
Methods of analyses of soil microbial communities

- Chemical analyses – determination of biomarker molecules / profiles
  - + in general simpler
  - + widespread and cheaper equipment
  - - not as detailed information
  - - possible interferences – need of careful interpretation

Biomarker molecules

- Respiration isoprenoids – chinons, length of side chain
- Polysacharides – surface, sheat
- Polyamines
- Mycolic acids
- Sterols – eucaryotic membrane (ergosterole in fungi)
- **Membrane lipids – especially phospholipid fatty acid profiles**
- …

Phospholipids



R_1, R_2 – fatty acid acyls

X – hydrophilic groups

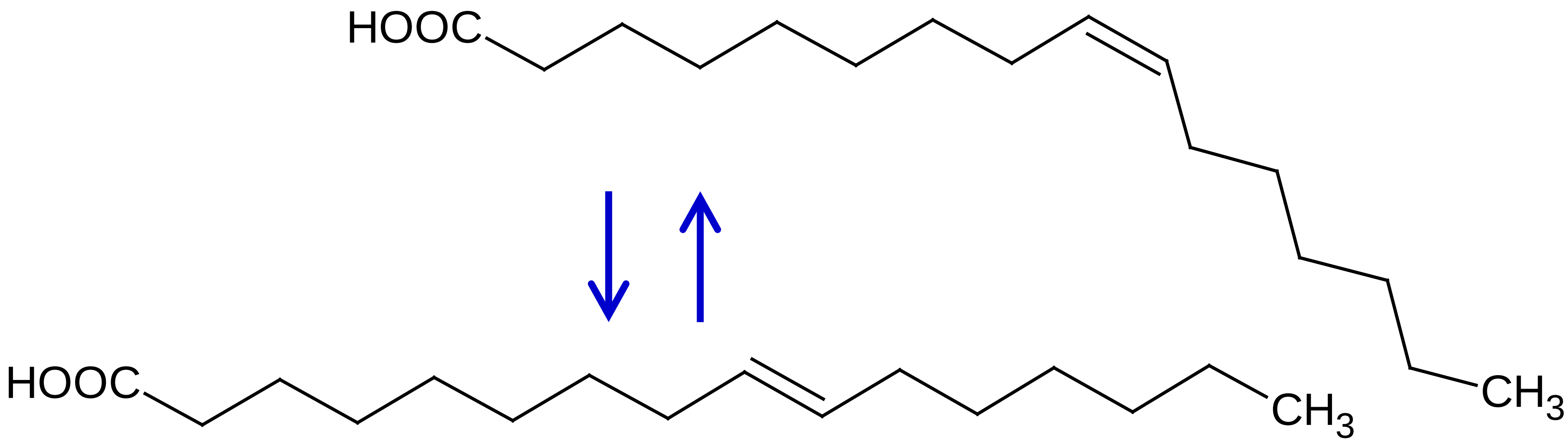
Phospholipids

- *In vivo* in membranes only
  - **cytoplsmatic**
  - outer membrane of G- bacteria
  - inner membranes of eukaryotes
- Never storage compounds → ~proportional content to biomass
- Fast decomposition after cell death → estimation of living biomass

Community biomarker fatty acids

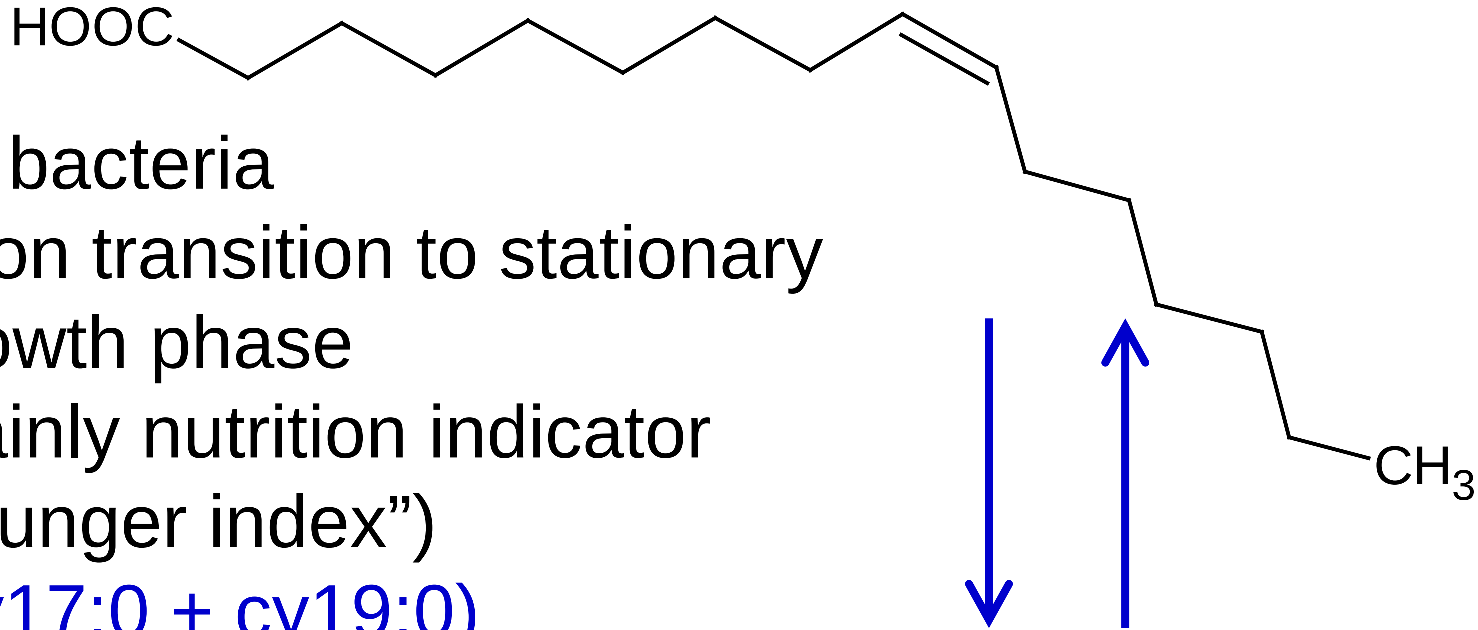
Group	subgroup	Biomarker fatty acids
Bacteria	G+	i14:0, i15:0, a15:0, i17:0, a17:0
	G-	cy17:0, cy19:0, 18:1w7
	Actinobacteria	10Me-16:0, 10Me-17:0, 10Me-18:0
	Other	16:1ω7t, 16:1ω7, 16:1ω9,
Fungi		18:2ω6,9
Protozoa		20:4ω6

cis / *trans* isomeration

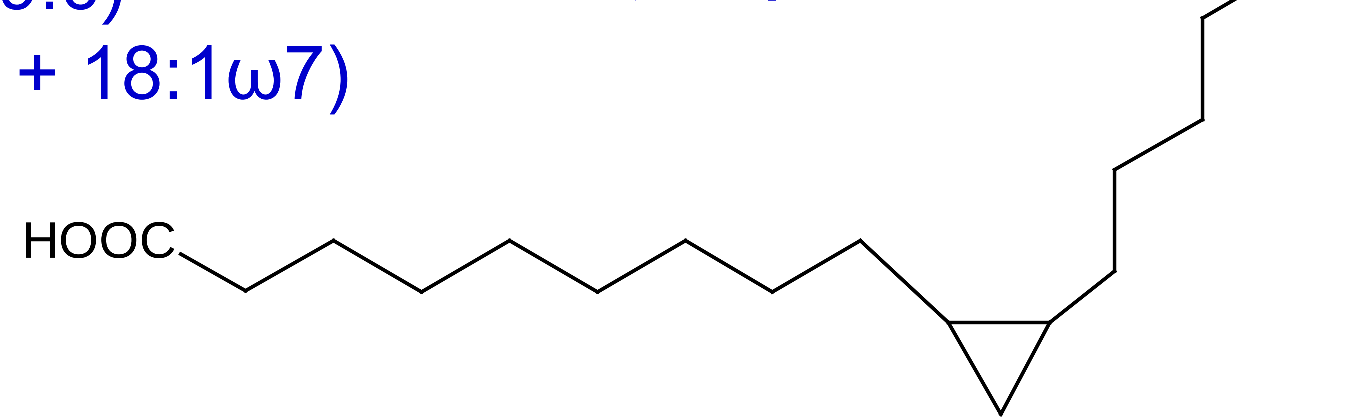


- Bacteria
- Changes directly in membrane
- *trans* / *cis* index
- general stress indicator
- (18:1ω7+16:1ω7) / (16:1ω7t+18:1ω7t)
- >0.1 → soil disturbation and stress

Cyclization of monounsaturated FA



- G- bacteria
- upon transition to stationary growth phase
- mainly nutrition indicator ("hunger index")
- (cy17:0 + cy19:0) / (16:1ω7 + 18:1ω7)
- *cy / pre*

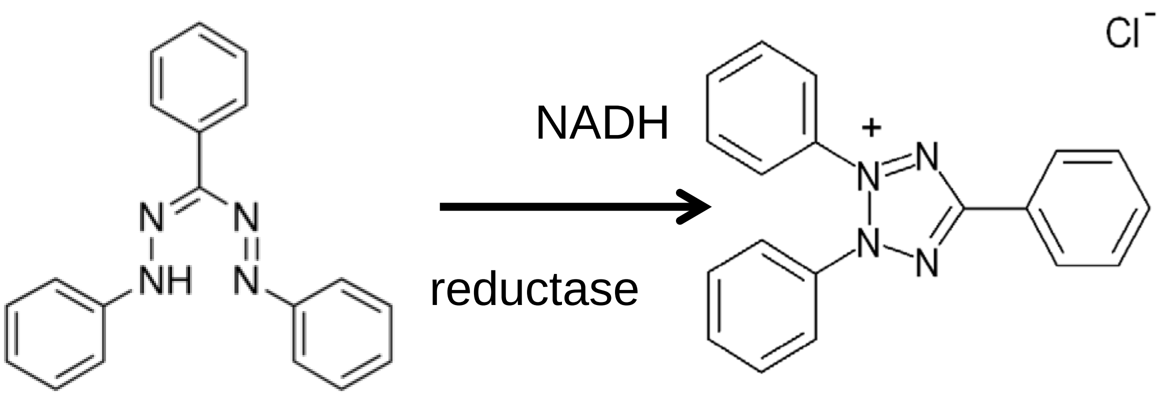


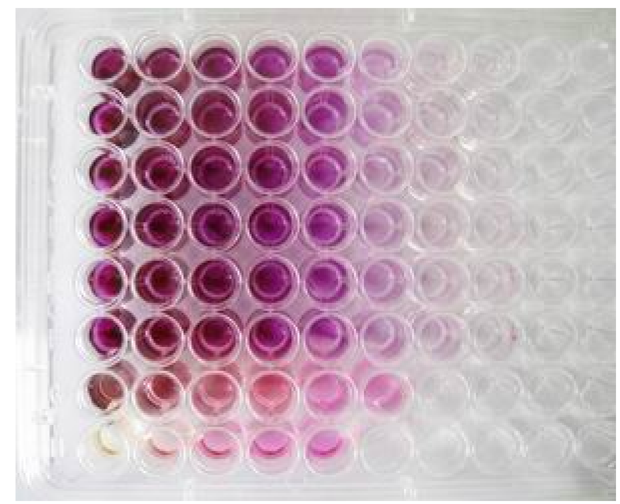
Determination of microbial activities

- Completes biomass data with indication of activities
  - comparison of living biomass vers. activities gives useful information about overall state of community
- Activity of extracellular enzymes – decomposition of polymers
- Respiration – analyses of O_2 consumption or CO_2 production

Tetrazolium assay

- Based of reduction of triphenyltetrazolium to triphenylphormazan
  - pink – spectrophotometric determination at 546 nm)
- Substrate of many oxidoreductases

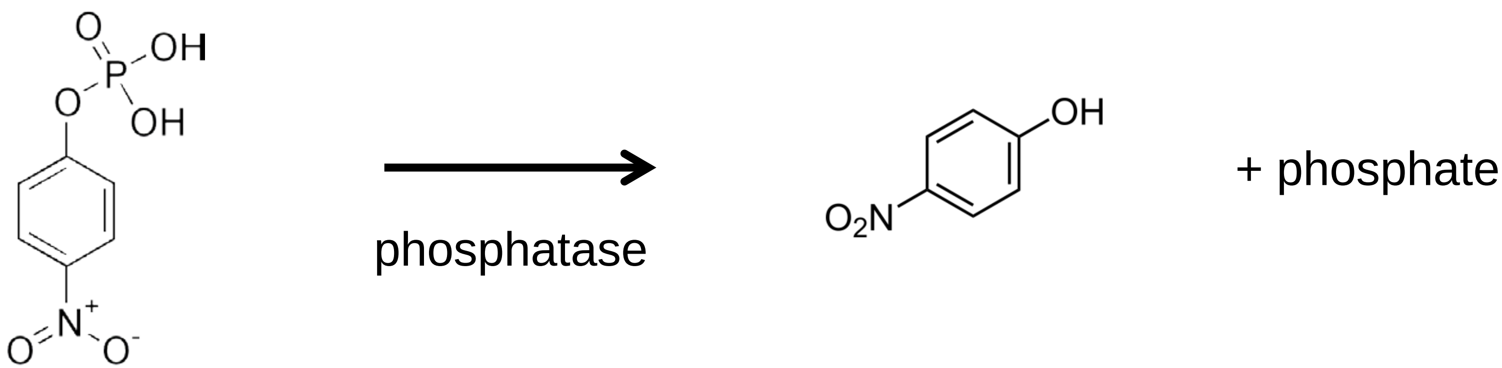




- determines overall activity of soil or sludge

pNP assay

- Hydrolysis of pNP-X to p-nitrofenyl phosphate and X
- Determination of yellow pNP (pH >7, 400 nm)
- Many variants – phosphatases, sulphatases, proteases, glucosidases, chitinases…



8. A few research results

Aim of our research

- **Phytoremediation** = use of plants for elimination / detoxification of pollutants from soil
- Relation of microbial communities to soil cover = use of plants for elimination / detoxification of pollutants from soil
- Evaluation of human effects on soil microorganisms

Pot experiment Sliač, Slovakia – soil parametres

Parametr		2014	2015
Available nutrients (Mehlich III) [mg/kg dry soil]	P	89 ± 67	78 ± 67
	K	375 ± 110	329 ± 94
	Ca	3283 ± 214	3269 ± 274
	Mg	666 ± 66	695 ± 72
Elementary analysis [% dry soil]	N_{tot}	0.24 ± 0.06	0.20 ± 0.04
	H	0.57 ± 0.05	0.56 ± 0.05
	S	0.040 ± 0.014	0.028 ± 0.009
TOC [% dry soil]		2.83 ± 0.68	2.91 ± 0.82
Humus [% dry soil]		4.12 ± 0.94	3.70 ± 0.82

Pot experiment Sliač, Slovakia – PLFA

Indicator	Rhizosphere soil			Bulk soil		
	2014	2015		2014	2015	
$PLFA_{tot}$ [mg/kg]	5.95 - 7.07	4.53 - 7.38	-	6.73 - 8.01	5.79 - 7.90	-
F/B []	0.00 - 0.01	0.04 - 0.08	↑	0.00 - 0.01	0.04 - 0.16	↑
G+ [%]	31.6 - 34.2	37.7 - 40.3	↑	32.8 - 34.6	30.9 - 40.1	-
G- [%]	32.7 - 37.4	37.0 - 30.9	↓	32.27 - 36.44	26.8 – 36.9	-
Actinobacteria [%]	18.8 - 21.6	5.16 - 6.49	↓	18.2 – 20.6	3.9 – 6.3	↓
Other bacteria [%]	11.1 – 11.9	23.1 - 24.2	↑	11.4 – 12.5	22.1 - 24.0	↑
Fungi [%]	0.1 - 0.6	1.6 - 3.4	↑	0.3 – 0.9	1.5 - 7.5	↑
trans/cis	0.38 - 0.43	0.37 - 0.41	-	0.40 - 0.48	0.38 - 0.43	-
cy/pre	0.44 - 0.50	0.30 - 0.37	↓	0.51 - 0.57	0.30 - 0.39	↓

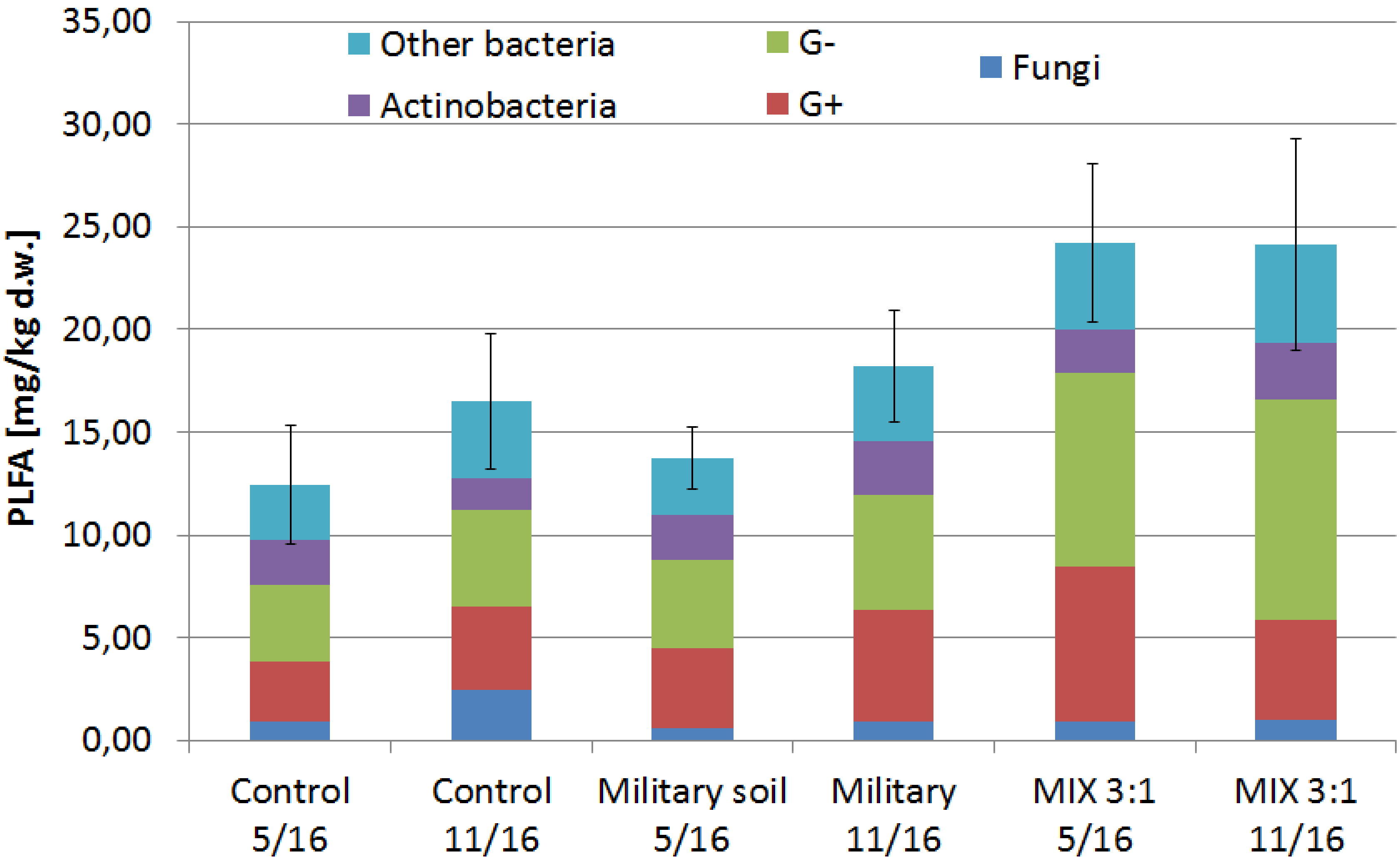
confidence intervals, significant (t-test, α<0.05) increase ↑ decrease ↓ between years

Pot experiment Sliač, Slovakia – activities

Indicator	Rhizosphere soil			Bulk soil		
	2014	2015		2014	2015	
Phosphatases [U/g]	0.056 - 0.063	0.039 - 0.048	↓	0.059 - 0.070	0.038 - 0.051	↓
Arylsulphatases [U/g]	0.0035 - 0.0041	0.0029 - 0.0034	↓	0.0031 - 0.0037	0.0029 - 0.0039	-
Proteases [U/g]	0.028 - 0.036	0.035 - 0.049	-	0.030 - 0.035	0.034 - 0.050	-
Oxidases [U/g.10^{-5}]	2.18 - 2.87	1.50 - 1.95	↓	2.07 - 2.63	1.55 - 1.94	↓
Peroxidases [U/g.10^{-5}]	0.71 - 1.27	2.21 - 2.69	↑	0.71 - 1.08	2.26 - 2.71	↑
Respiration [nmol/g/min]	1.47 - 2.30	2.98 - 4.66	↑	1.66 - 2.57	1.71 - 2.92	-

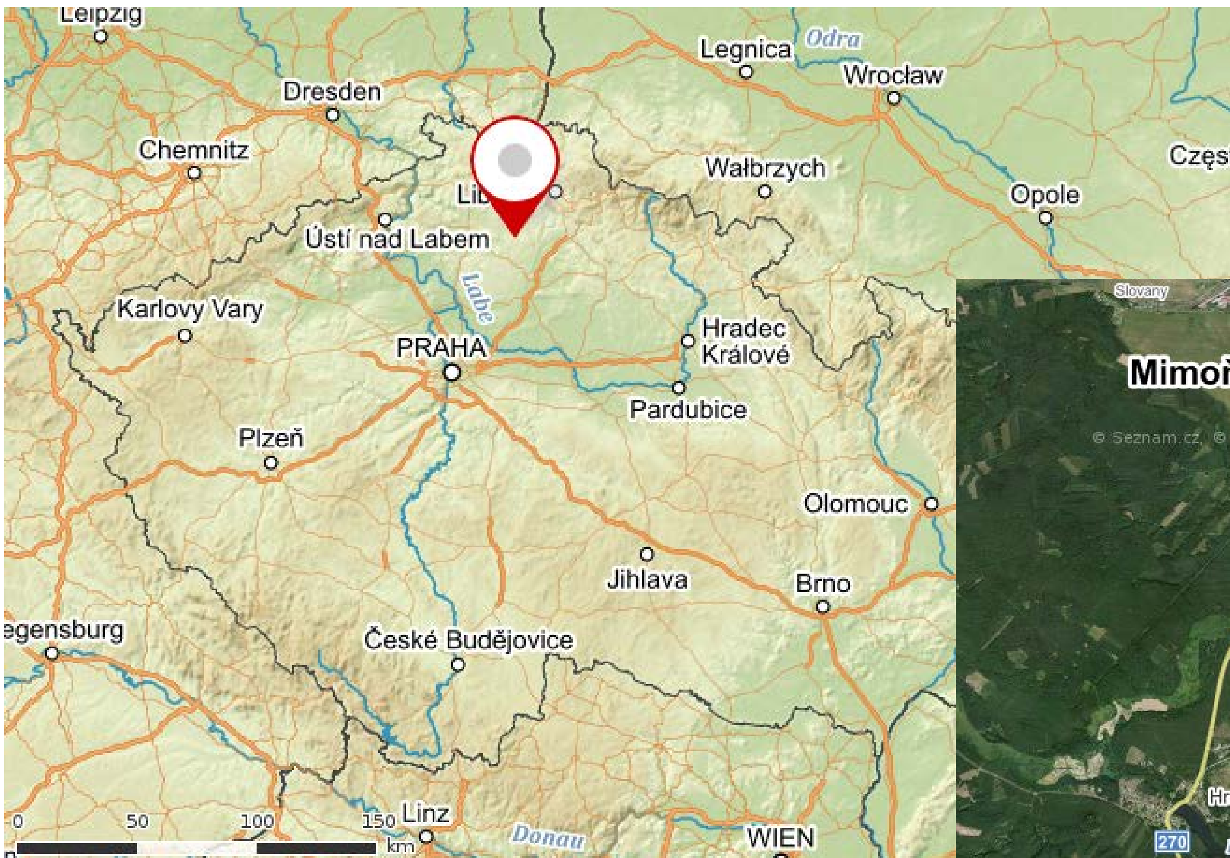
confidence intervals, significant (t-test, α<0.05) increase ↑ decrease ↓ between years

PLFA (spring/autumn)



Pot experiment

- Post-military soil + oil contaminated soil



Airport Mimoň
50°37'2.941"N, 14°43'7.884"E
50.6174839N, 14.7188567E

Control field
50°37'56.657"N, 14°45'25.008"E
50.6324050N, 14.7569467E

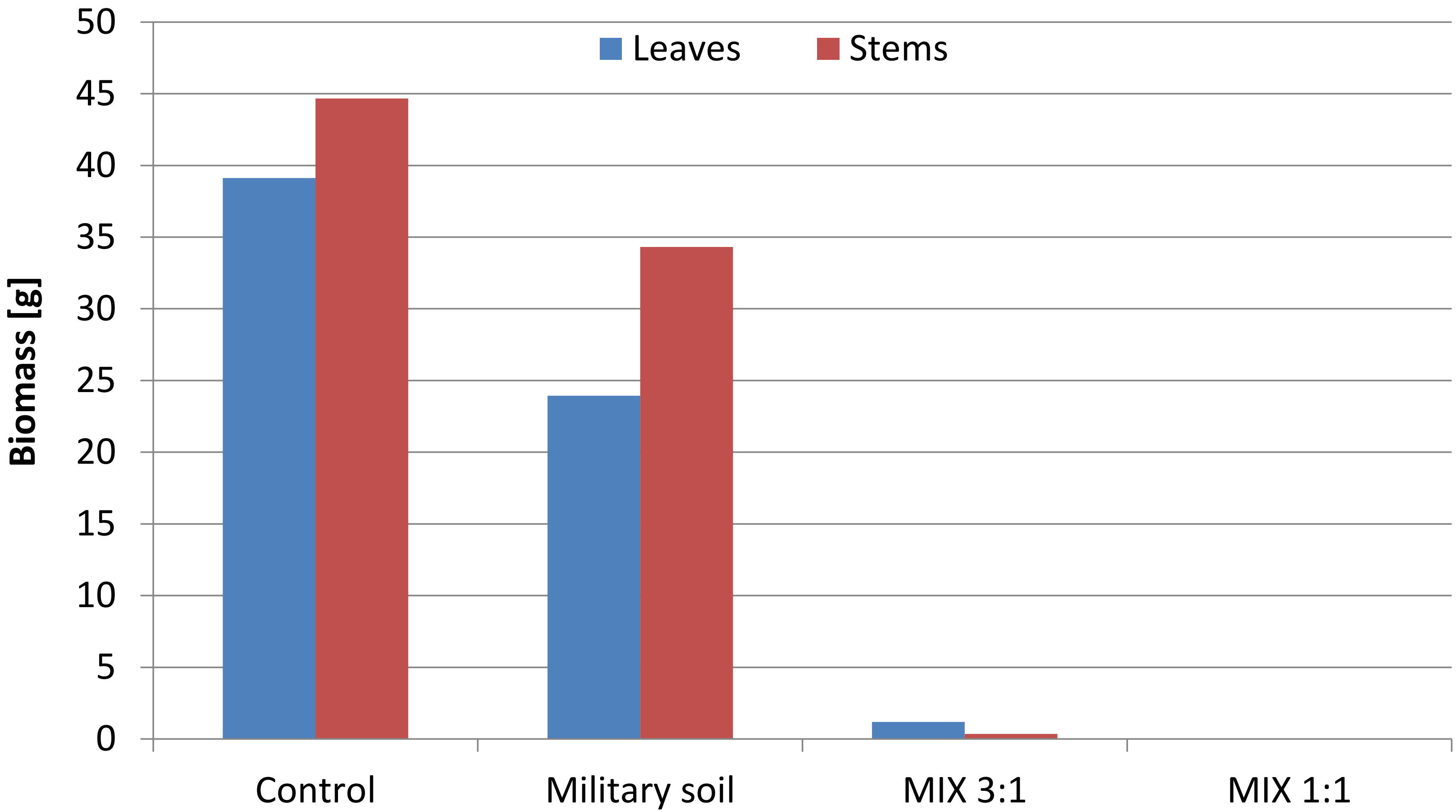
Soil parametres

- ICP-OES, GC-FID

	Prev. v.** [mg/kg]	Control		Military soil		MIX 3:1		MIX 1:1	
		[mg/kg]	BF [%]*	[mg/kg]	BF [%]*	[mg/kg]	BF [%]*	[mg/kg]	BF [%]*
C_{10}-C_{40}	100	<LD	x	<LD	x	8537	x	21290	x
Cd	0,4	1,7	<LD	0,9	<LD	2,1	<LD	2,9	<LD
Co	20	19,1	<LD	25,2	<LD	41,0	<LD	56,9	<LD
Cu	45	<LD	<LD	19,4	26,3	52,9	10,6	84,1	10,0
Pb	55	15,2	<LD	73,2	<LD	86,1	<LD	91,8	<LD
Zn	105	38,8	25,5	308,5	39,9	312,0	30,4	292,1	32,5

* Bioavailable fraction (fraction I according to BCR - extraction 0,11 M CH_3COOH)
** Preventive value in agricultural soil according to Act n. 153/2016 Sb. (CZ)

Results – biomass (1st year)



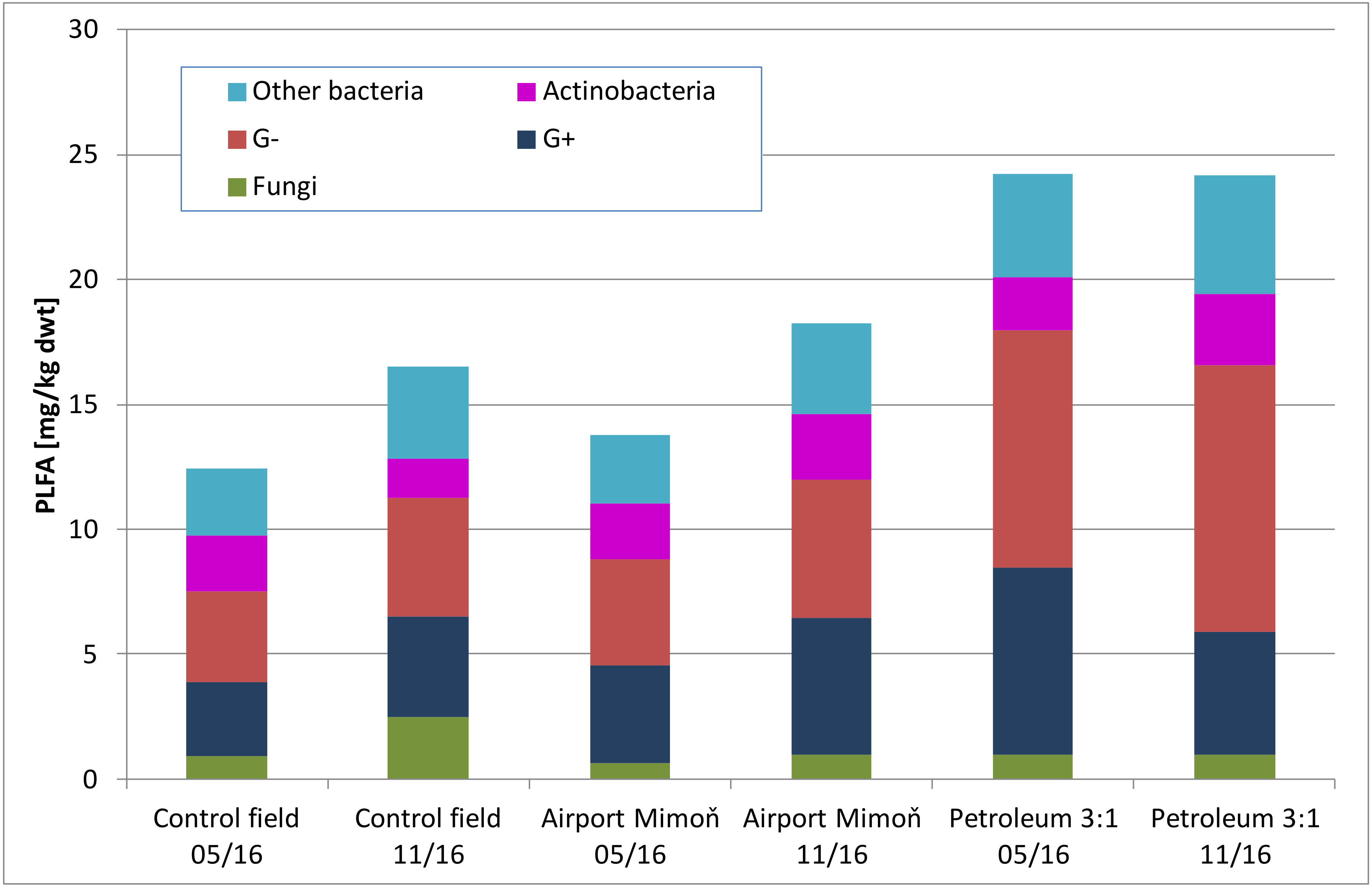
Control

Military soil

MIX 3:1

MIX 1:1

UJEP preliminary greenhouse experiment – PLFA



References

Bourgeois, E.; Dequiedt, S.; Lelievre, M.; van Oort, F.; Lamy, I.; Ranjard, L.; Maron, P.A. Miscanthus bioenergy crop stimulates nutrient-cycler bacteria and fungi in wastewater-contaminated agricultural soil. *Environmental Chemistry Letters* **2015**, *13*, 503-511.

Eckert, B.; Weber, O.B.; Kirchhof, G.; Halbritter, A.; Stoffels, M.; Hartmann, A. Azospirillum doebereinerae sp nov., a nitrogen-fixing bacterium associated with the c-4-grass miscanthus. *International Journal of Systematic and Evolutionary Microbiology* **2001**, *51*, 17-26.

Firmin, S.; Labidi, S.; Fontaine, J.; Laruelle, F.; Tisserant, B.; Nsanganwimana, F.; Pourrut, B.; Dalpe, Y.; Grandmougin, A.; Douay, F.*, et al.* Arbuscular mycorrhizal fungal inoculation protects miscanthus x giganteus against trace element toxicity in a highly metal-contaminated site. *Science of the Total Environment* **2015**, *527*, 91-99.

Keymer, D.P.; Kent, A.D. Contribution of nitrogen fixation to first year miscanthus x giganteus. *Global Change Biology Bioenergy* **2014**, *6*, 577-586.

Ling, J.; Zhang, Y.Y.; Wu, M.L.; Wang, Y.S.; Dong, J.D.; Jiang, Y.F.; Yang, Q.S.; Zeng, S.Q. Fungal community successions in rhizosphere sediment of seagrasses enhalus acoroides under pahs stress. *International Journal of Molecular Sciences* **2015**, *16*, 14039-14055.

Rojrbacher F.; St.-Arnaud. Root Exudation: The Ecological Driver of Hydrocarbon Rhizoremediation. *Agriculture* **2016**, 6, 19.

Sarkar, A.; Asaeda, T.; Wang, Q.Y.; Kaneko, Y.; Rashid, M.H. Response of miscanthus sacchariflorus to zinc stress mediated by arbuscular mycorrhizal fungi. *Flora* **2017**, *234*, 60-68.

Sarkar, D.; Ferguson, M.; Datta, R.; Birnbaum, S. Bioremediation of petroleum hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. *Environmental Pollution* **2005**, *136*, 187-195.

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Thank you for your attention