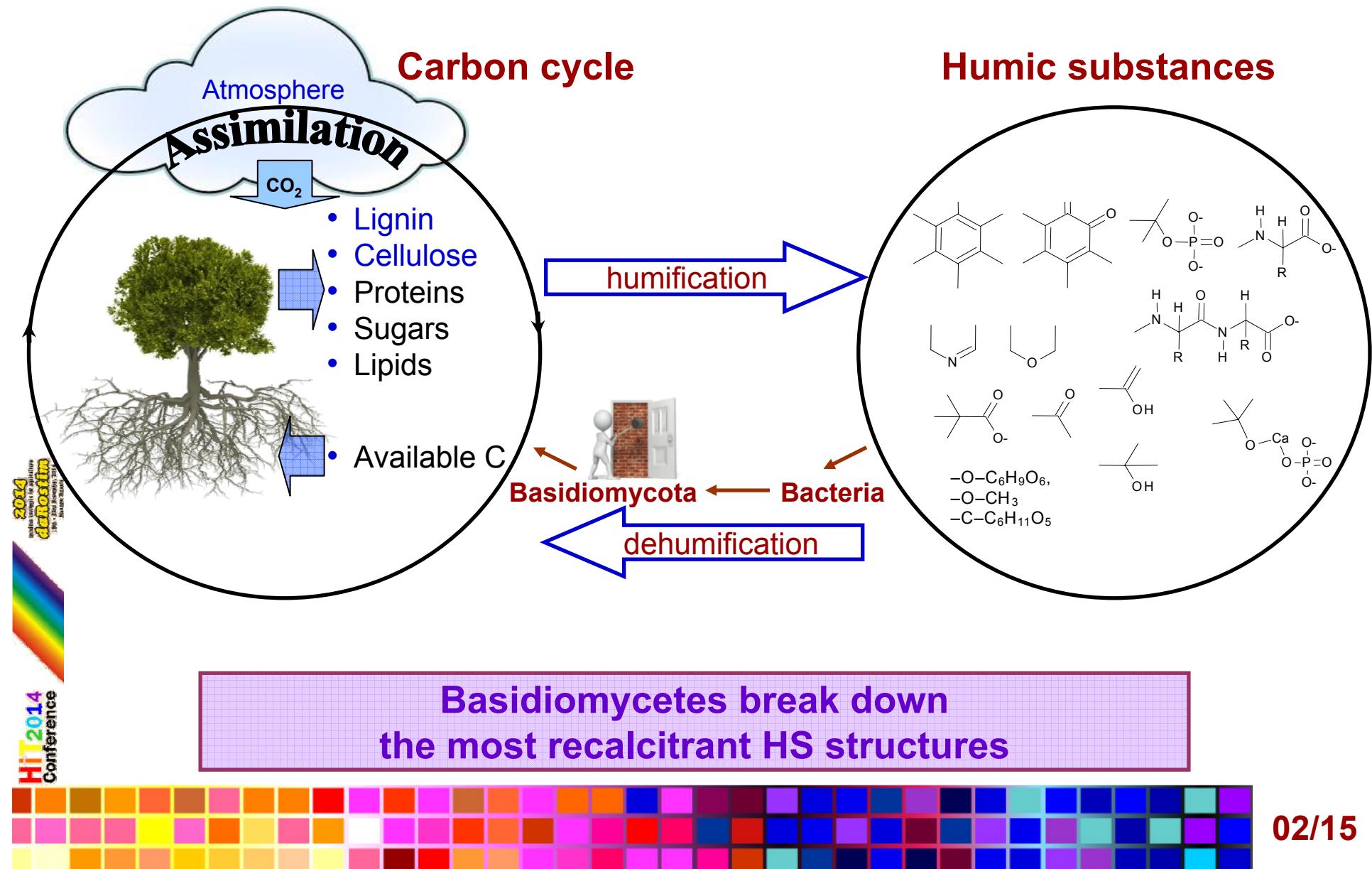


# Molecular Approach to Understanding Mode of Action of Humics on Biota: a Case Study with Basidiomycetes *Trametes maxima*

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# Humics and Basidiomycetes



# Humics mode of action: what is known?

Main approaches to understanding  
mode of action of humics on biota

Searching for  
structure-activity  
relationships

Studying of certain  
physiological  
processes

Properties of humics  
determining activity

- Paramagnetic centers  
(Chukov et al.. 1995)
- Carboxylic groups  
(Canellas et al.. 2008)
- Molecular mass  
(Nardi et al.. 2007)

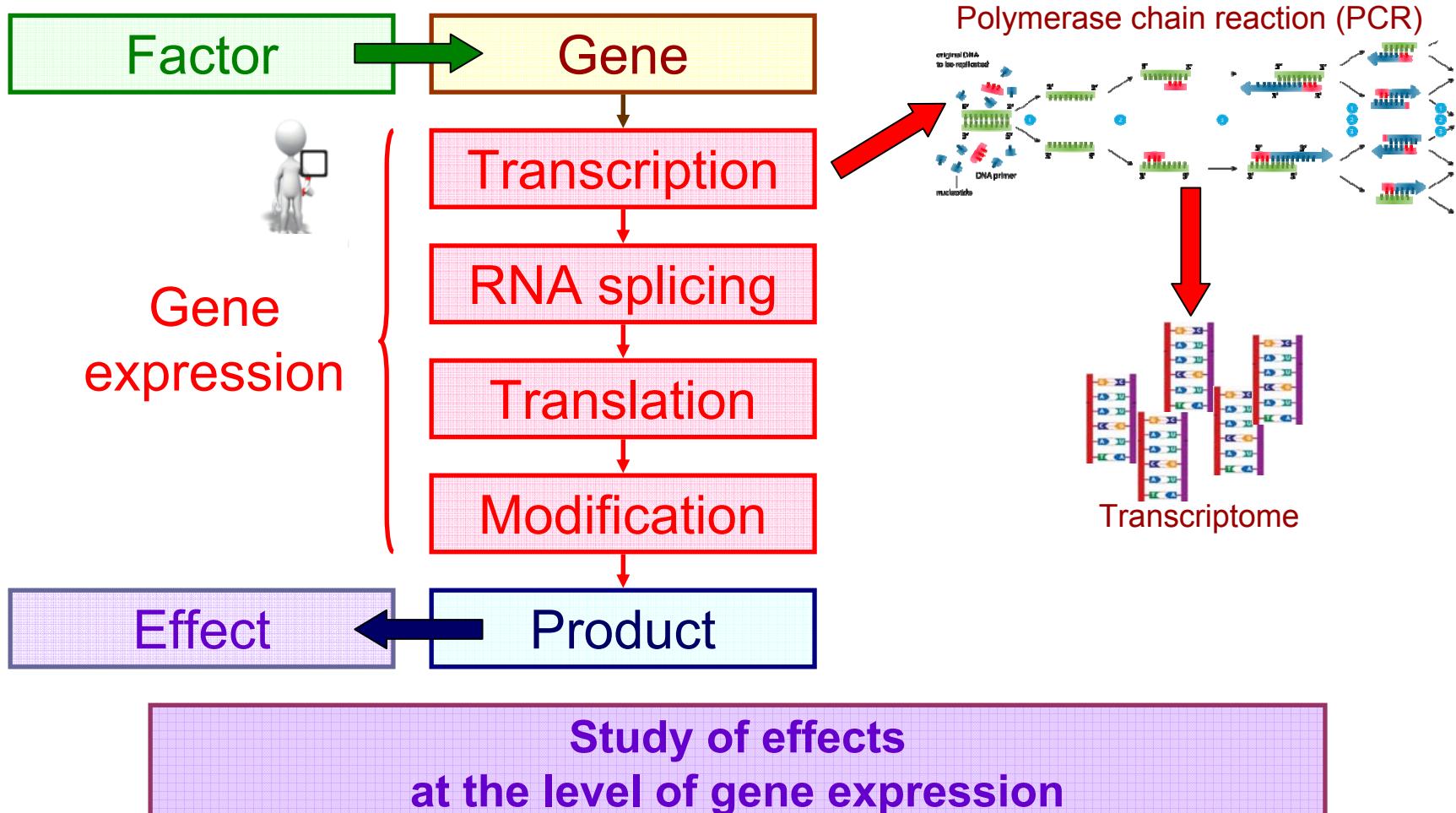


Processes influenced  
by humics

- H<sup>+</sup>-ATPase  
(Canellas et al.. 2002)
- H<sup>+</sup>-pyrophosphatase  
(Zancani et al.. 2009)
- Fe(III) chelate reductase  
(Aguirre et al.. 2009)
- Glycolysis (Nardi et al.. 2007)
- Krebs cycle (Nardi et al.. 2007)

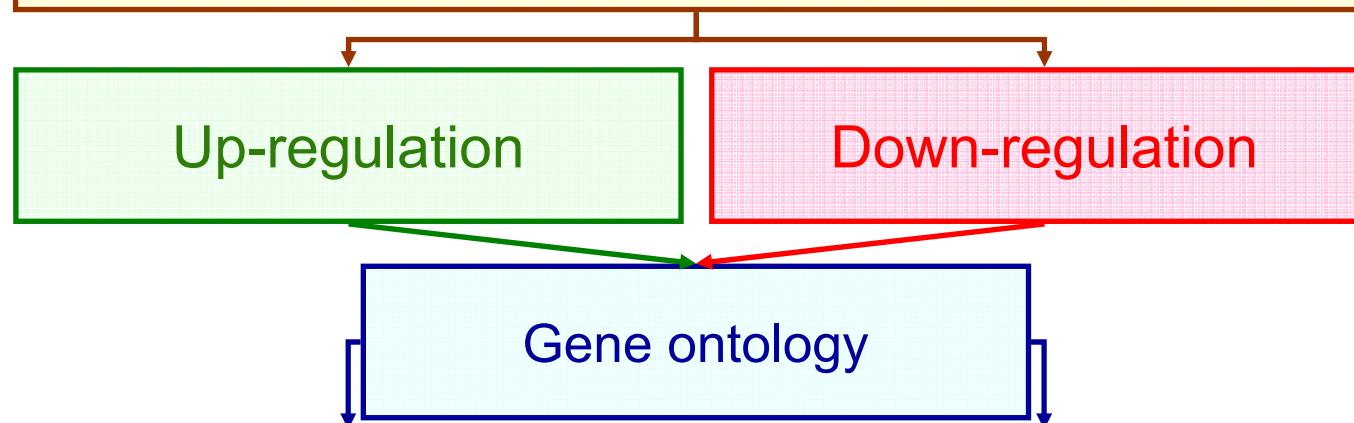
Primary target(s)?

# Polymerase chain reaction: molecular level of study



# Humics mode of action: a case with *Arabidopsis thailana*

About 133 genes are involved in the response



- Binding (proteins, nucleotides et al.)
- Catalytic activity
- Transferase activity
- Activity of transporters



- Catalytic activity
- Binding (proteins, nucleotides et al.)
- Activity of transporters

Trevisan S., 2009

A combination of different approaches  
are needed

# Humics and *Trametes maxima*

Submerged liquid cultivation

## C-limited media

(g/L)

NaNO<sub>3</sub> 3.0  
KH<sub>2</sub>PO<sub>4</sub> 0.6  
ZnSO<sub>4</sub>×7H<sub>2</sub>O 0.001  
K<sub>2</sub>HPO<sub>4</sub> 0.4  
FeSO<sub>4</sub>×7H<sub>2</sub>O 0.0005  
MnSO<sub>4</sub> 0.05  
MgSO<sub>4</sub>×7H<sub>2</sub>O 0.5  
CaCl<sub>2</sub> 0.25  
CuSO<sub>4</sub> 0.25  
pH 6.0

*T. maxima*  
is a highly effective  
decomposer of HS



Coal HA or coal [3H]HA  
50 mg/L

## Complete media

(g/L)

**glucose 10.0**  
NaNO<sub>3</sub> 3.0  
KH<sub>2</sub>PO<sub>4</sub> 0.6  
ZnSO<sub>4</sub>×7H<sub>2</sub>O 0.001  
K<sub>2</sub>HPO<sub>4</sub> 0.4  
FeSO<sub>4</sub>×7H<sub>2</sub>O 0.0005  
MnSO<sub>4</sub> 0.05  
MgSO<sub>4</sub>×7H<sub>2</sub>O 0.5  
CaCl<sub>2</sub> 0.25  
CuSO<sub>4</sub> 0.25  
pH 6.0

## Changes in HA structure

- Elemental composition
- <sup>13</sup>C NMR analysis

## HA association with fungus

- penetration of humic material into the cell interior was studied using tritium labeled preparation

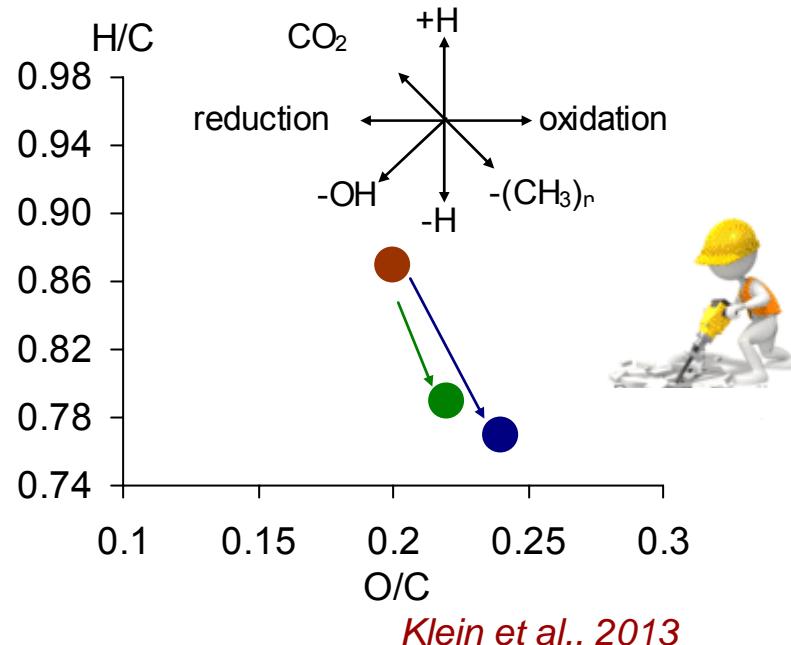
## Effects of HA on fungus

- Morphology
- Biochemical
- Physiological
- Expression of genes related to carbohydrates



# Humics and *Trametes maxima*: transformation of HA

Van Krevelen diagram  
for initial (●) and transformed HA  
in the C-limited (●) or complete (●) media

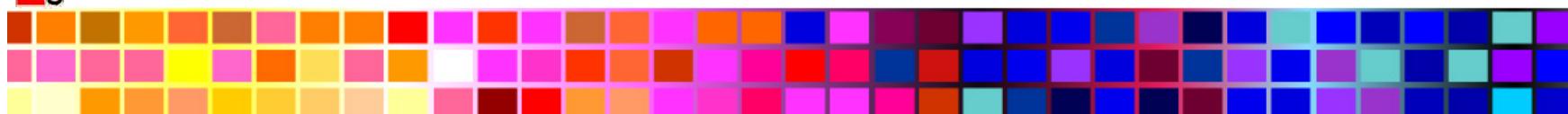


C contents in different structural fragment of initial and transformed HA  
in the C-limited or complete media, %

Structural fragments	Initial HA	Transformed HA	
		C-limited	Complete
$C_{C=O}$	5	4	4
$C_{COO}$	13	17	15
$C_{Ar-O}$	15	8	8
$C_{Ar}$	43	43	39
$C_{Alk-O}$	12	18	20
$C_{Alk}$	13	10	15
$\Sigma C_{Ar} / \Sigma C_{Alk}$	2.4	1.8	1.3

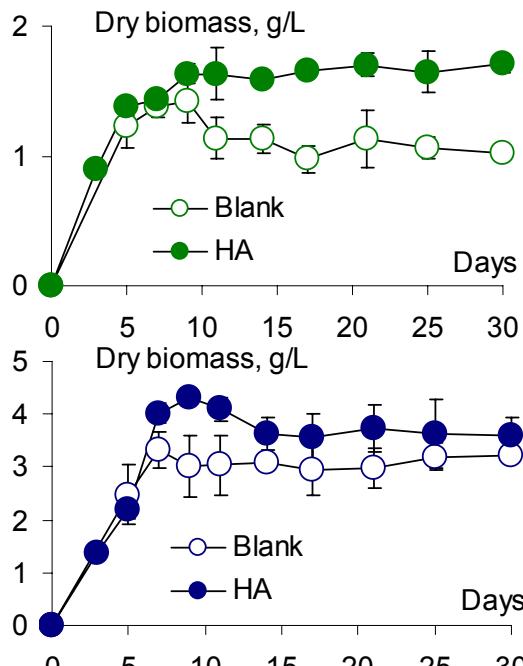
The assignments (ppm):  $C_{C=O}$  (220-178),  $C_{COO}$  (187-165),  $C_{Ar-O}$  (165-145),  $C_{Ar}$  (145-108),  $C_{Alk-O}$  (108-48),  $C_{Alk}$  (48-5)

C-limited media: mainly aliphatic structures  
Complete media: aliphatic and aromatic structures

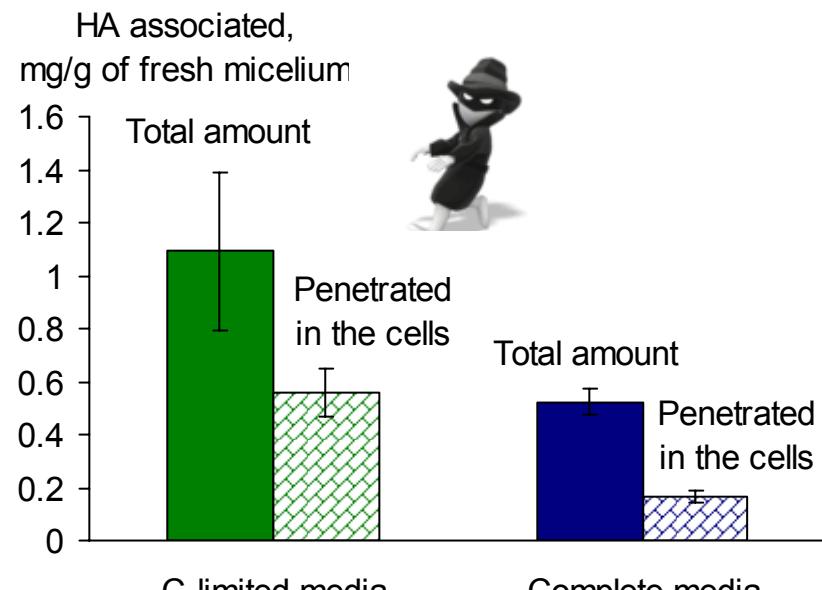


# Humics and *Trametes maxima*: penetration of HA

Effects of HA  
on *Trametes maxima* biomass  
in the C-limited (●) or complete (○) media



Intra/extracellular distribution of [<sup>3</sup>H]HA in  
*Trametes maxima* cells  
in the C-limited or complete media



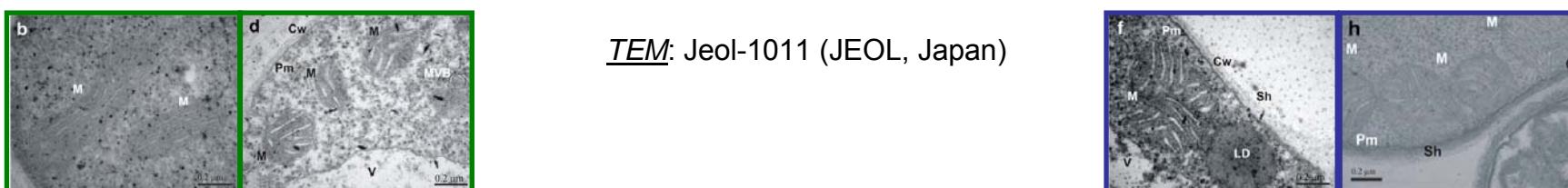
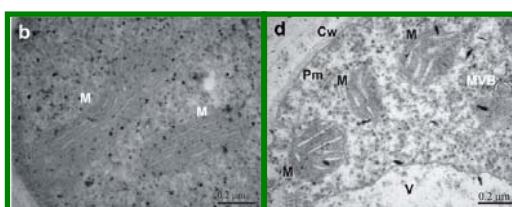
Klein et al., 2014

Effect of HA is more pronounced  
under C-limited conditions

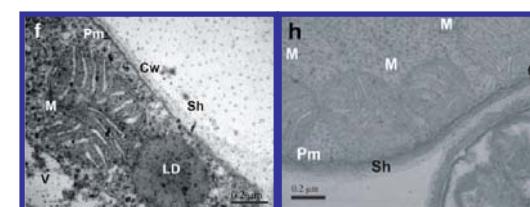
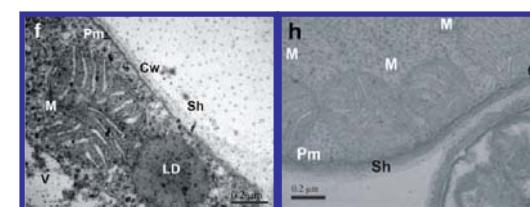
# Humics and *Trametes maxima*: ultra-structural changes



C-limited media		Parameters of ultra-structure		Complete media	
Blank	+HA	Blank	+HA	Blank	+HA
2.0±0.4	2.2±0.2	Diameter of hyphae, µm		2.0±0.2	2.2±0.3
59±7	41±6	Thickness of the cell wall, nm		45±9	57±8
108±29	51±6	Hyphal sheath thickness , nm		52±9	73±5
0.42±0.05	0.29±0.03	Diameter of mitochondria, µm		0.38±0.04	0.36±0.02
0.70±0,10	0.47±0.07	Length of mitochondria, µm		0.60±0.10	0.45±0.03

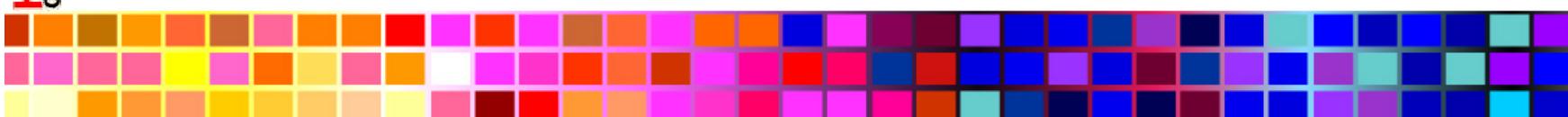


TEM: Jeol-1011 (JEOL, Japan)



Klein et al., 2014

HA induced ultra-structural changes in mitochondria



# Humics and *Trametes maxima*: effect on cellular respiration

Respiration rate, ng atom O/g dry weight

C-limited media		Variant	Complete media	
Blank	+HA		Blank	+HA
291±37	1507±111	Without inhibitors	398±570	956±169
207±6	708±90	Cytochrome pathway inhibition KCN, 2mM	151±20	736±48
84±29	527±15	Alternative pathway inhibition Salicylhydroxamic acid, 2mM	175±28	277±96
0.4	0.7	Cytochrome / Alternative pathway ratio	1.2	0.4



↓  
ROS↓  
Carbohydrates ↑

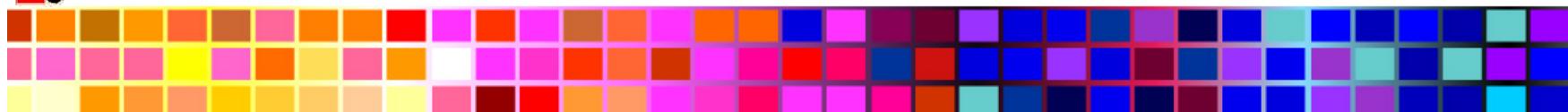
Respiration analysis: Polarograph,  
Analyse Record4usb



↑  
ROS↑  
Carbohydrates ↑

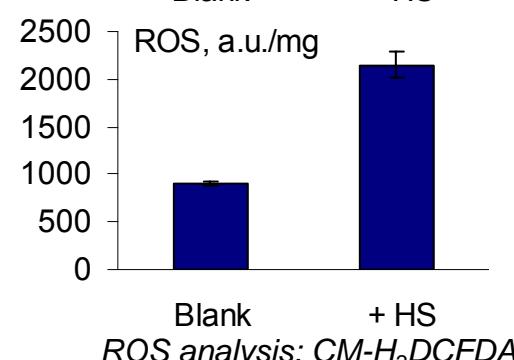
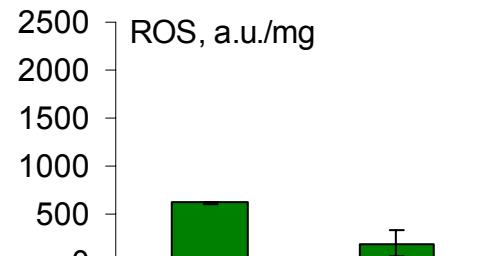
Klein et al., 2014

HA stimulated either the cytochrome or  
the alternative pathway of respiration

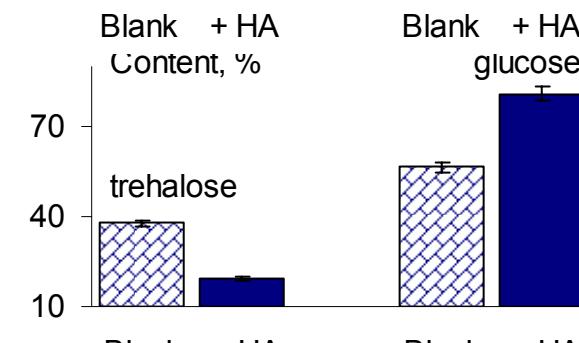
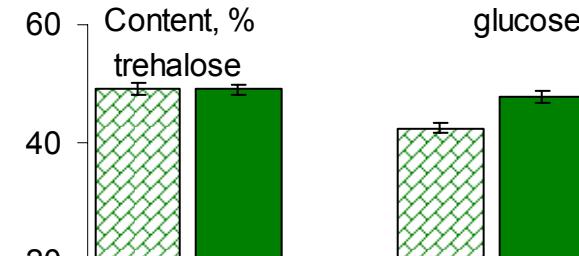


# Humics and *Trametes maxima*: effect on ROS and carbohydrates

Effect of HA on  
Reactive Oxygen Species (ROS) formation  
in the **C-limited** or **complete** media



Effect of HA on  
carbohydrates profile  
in the **C-limited** or **complete** media



Carbohydrates analysis: Kristall 5000.1  
(Chromatech, Russia)

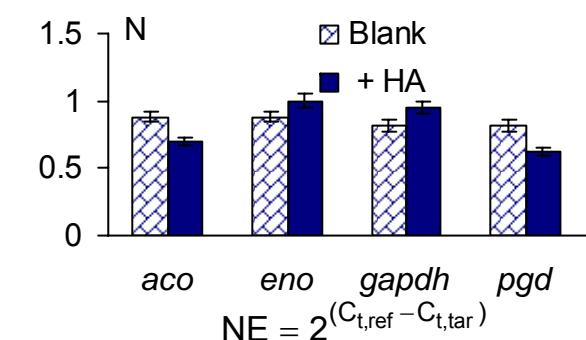
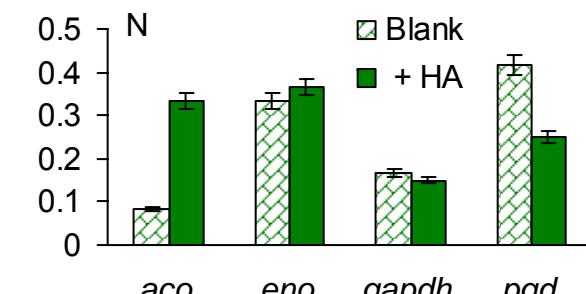
HA induce ROS formation in the complete media  
and increase in glucose concentration in both media

# Humics and *Trametes maxima*: effect on gene expression

Metabolic pathway	Enzyme	Gene
Glycolysis	Phosphoglucoisomerase	<i>pgi</i>
	Phosphofructokinase	<i>pfk</i>
	Glyceraldehyde 3-phosphate dehydrogenase	<i>gapdh</i>
	Enolase	<i>eno</i>
	Pyruvate kinase	<i>pyk</i>
Pentose-phosphate pathway (PPP)	6-phosphogluconate dehydrogenase	<i>pgd</i>
Gluconeogenesis (GNG)	Phosphoenolpyruvate carboxykinase	<i>pyck</i>
Krebs cycle	Aconitase	<i>aco</i>
	Fumarase	<i>fh</i>

Reference gene:  $\beta$ -actine, primers: Beacon Designer

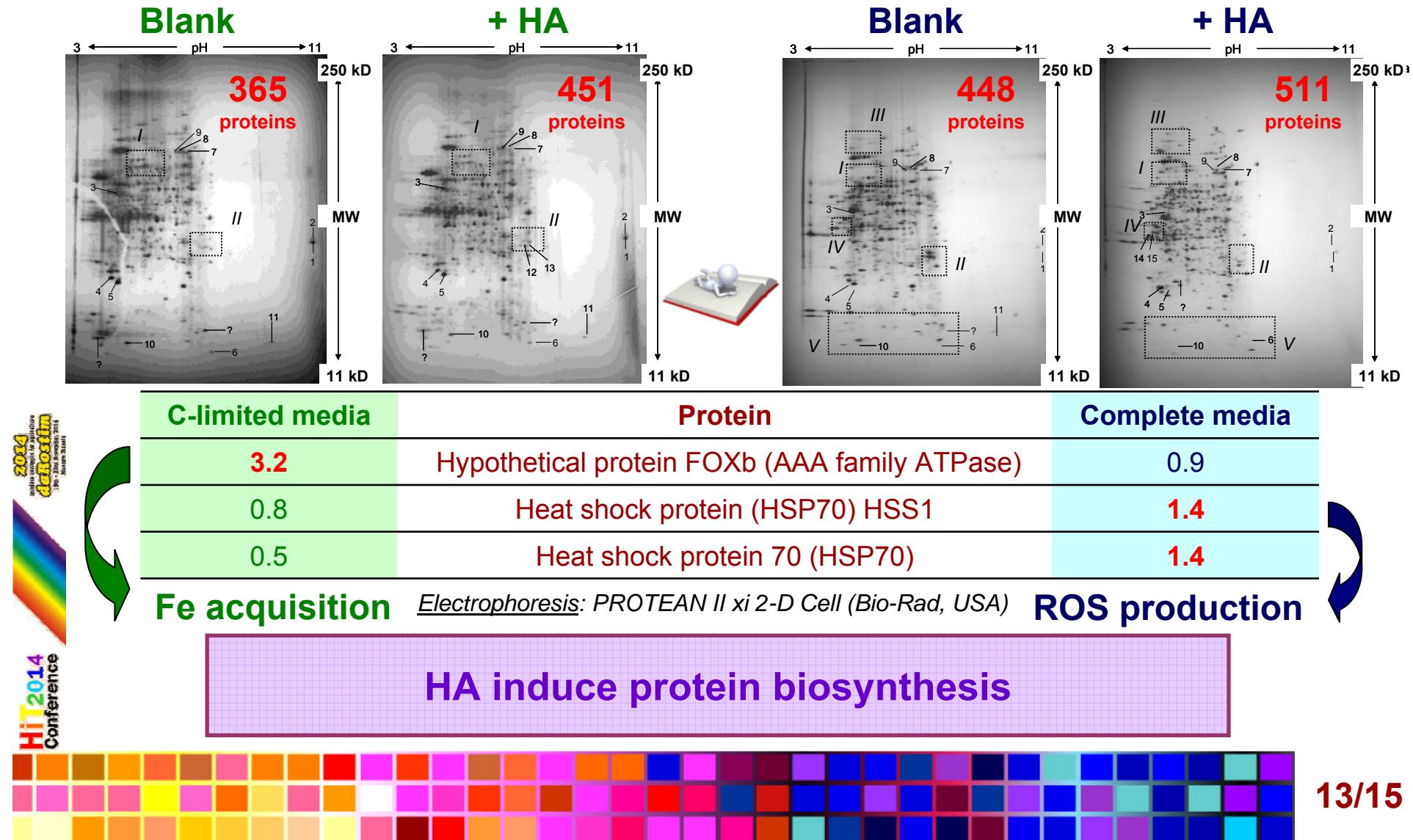
Effect of HA on gene expression  
in the **C-limited** or **complete** media



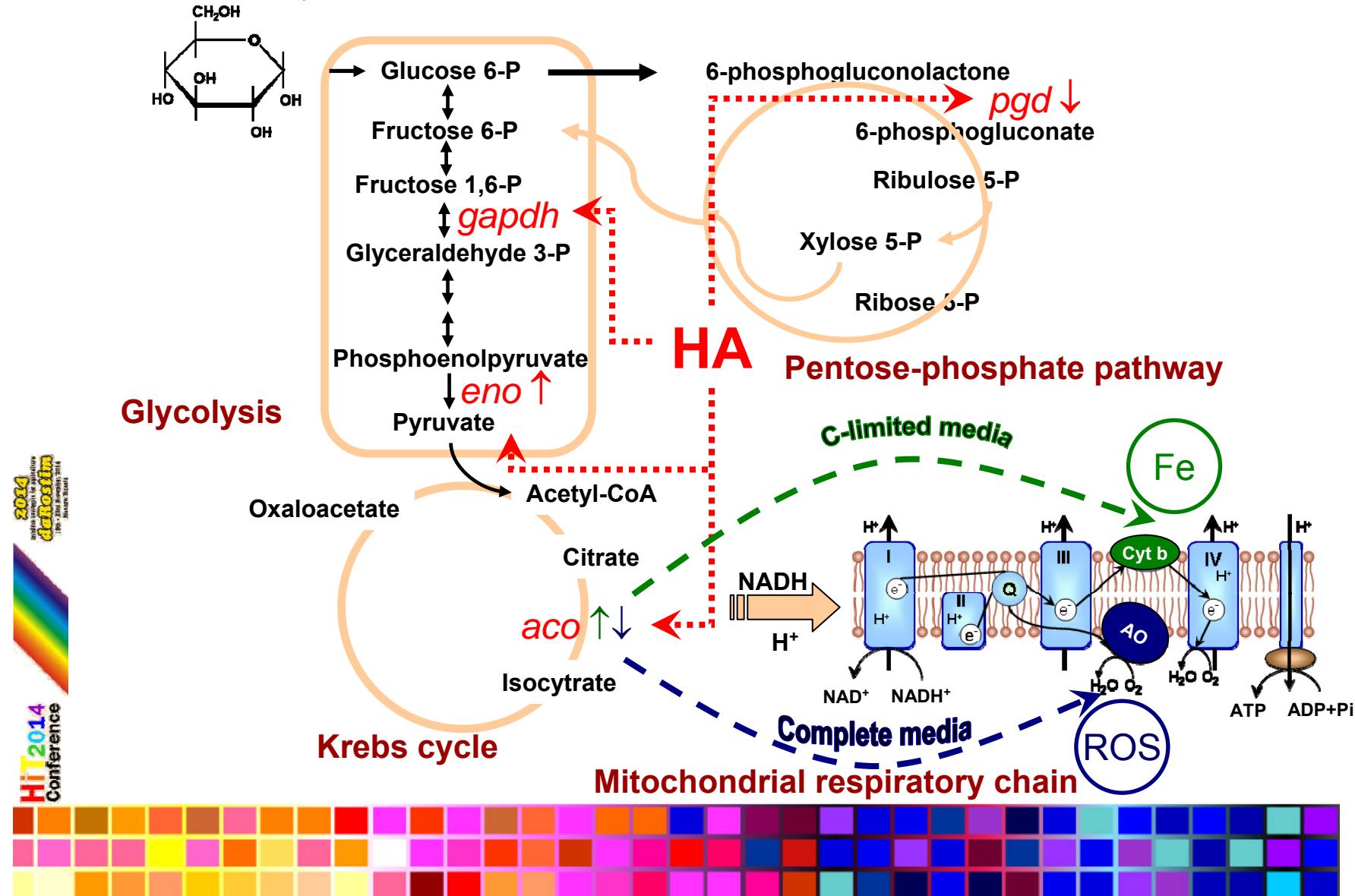
Real-time PCR: C1000 (BioRad, USA)

HA up-regulate *eno* and down-regulate *pgd*  
Regulation of *aco* depends on glucose presence

# Humics and *Trametes maxima*: effect on transcriptome

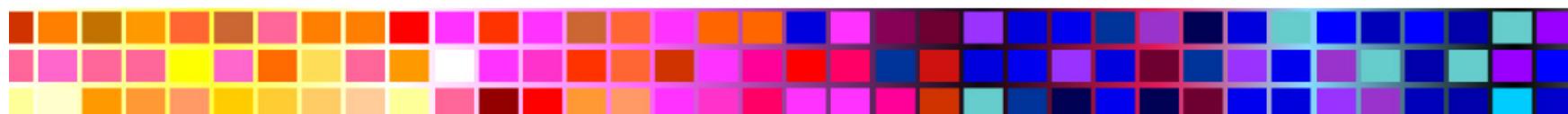


# Humics and *Trametes maxima*: hypothetical scheme

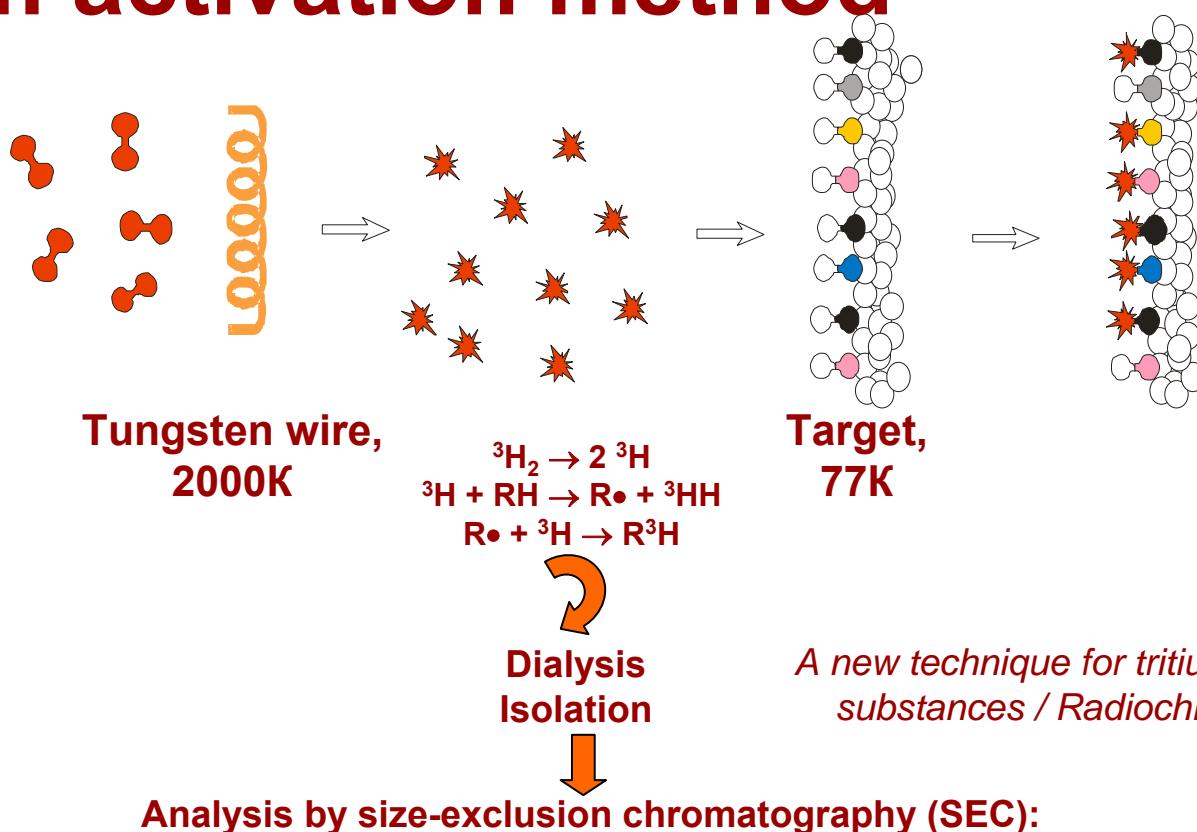




Thank you for your attention!



# Tritium labeled humics: tritium activation method



Badun et al., 2010  
A new technique for tritium labeling of humic substances / Radiochim Acta 98: 161-166

- to verify identity of initial humics to labeled – SEC-profiles of initial must coincide to labeled
- to check tritium distribution – UV and radioactivity profiles must coincide one to another

## Tritium labeled HS

