Studies of metal accumulation and ligand environment in plants by synchrotron radiation techniques

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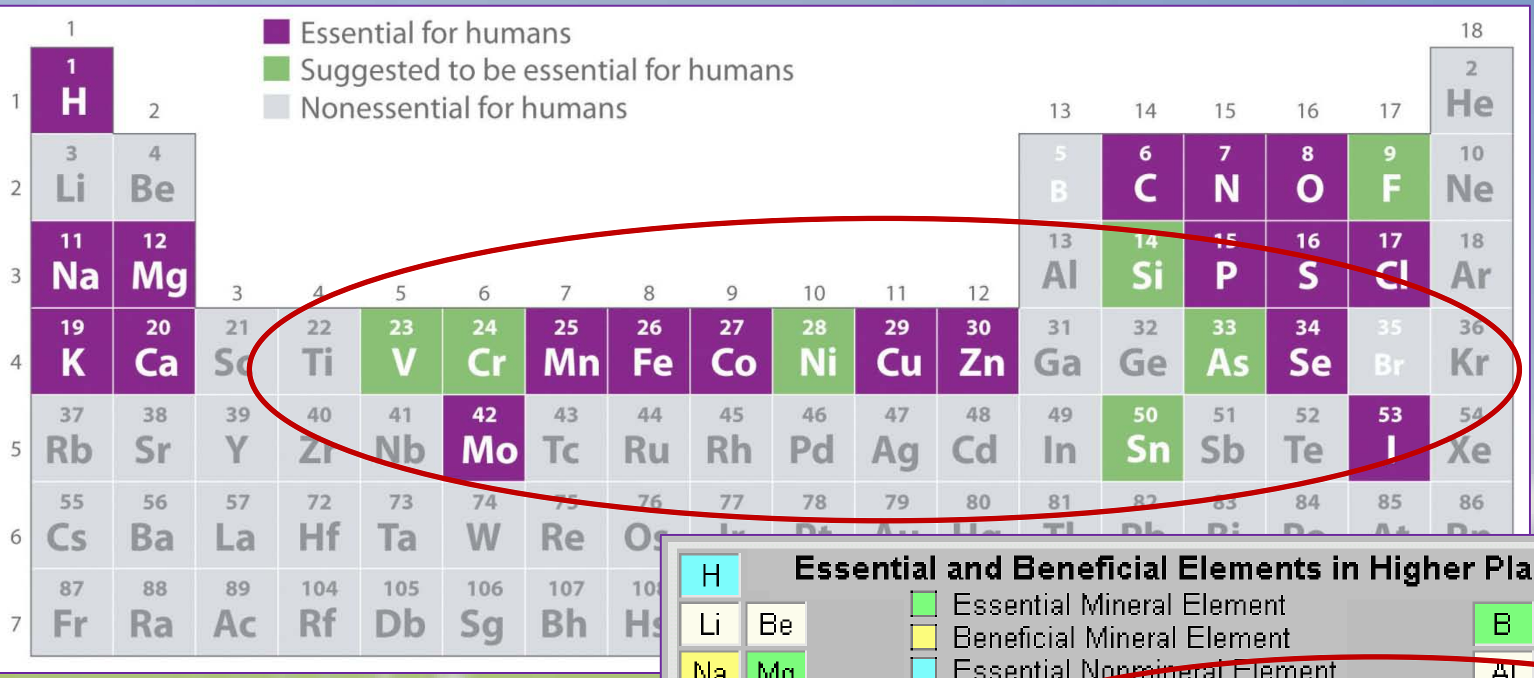
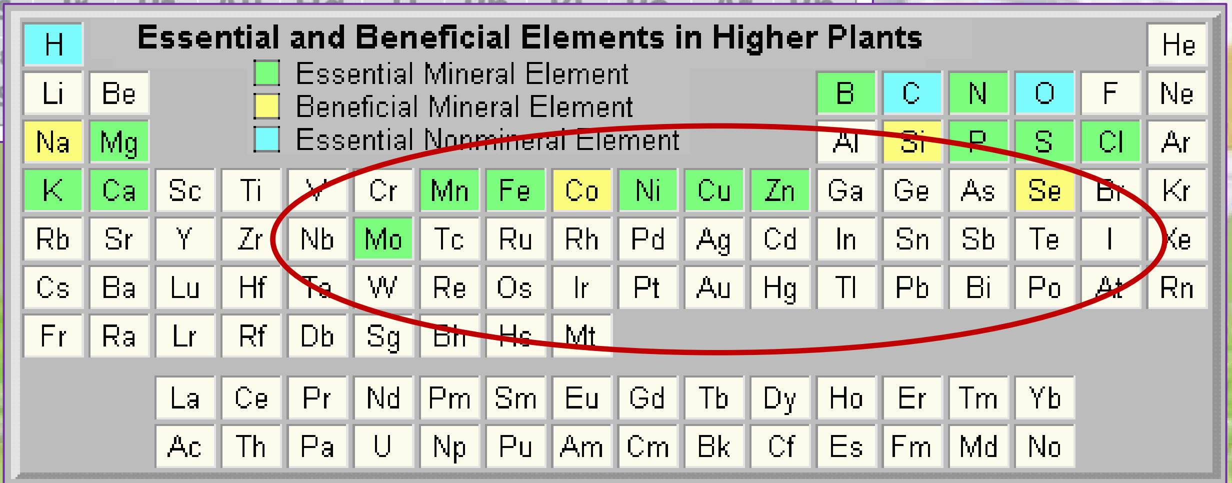
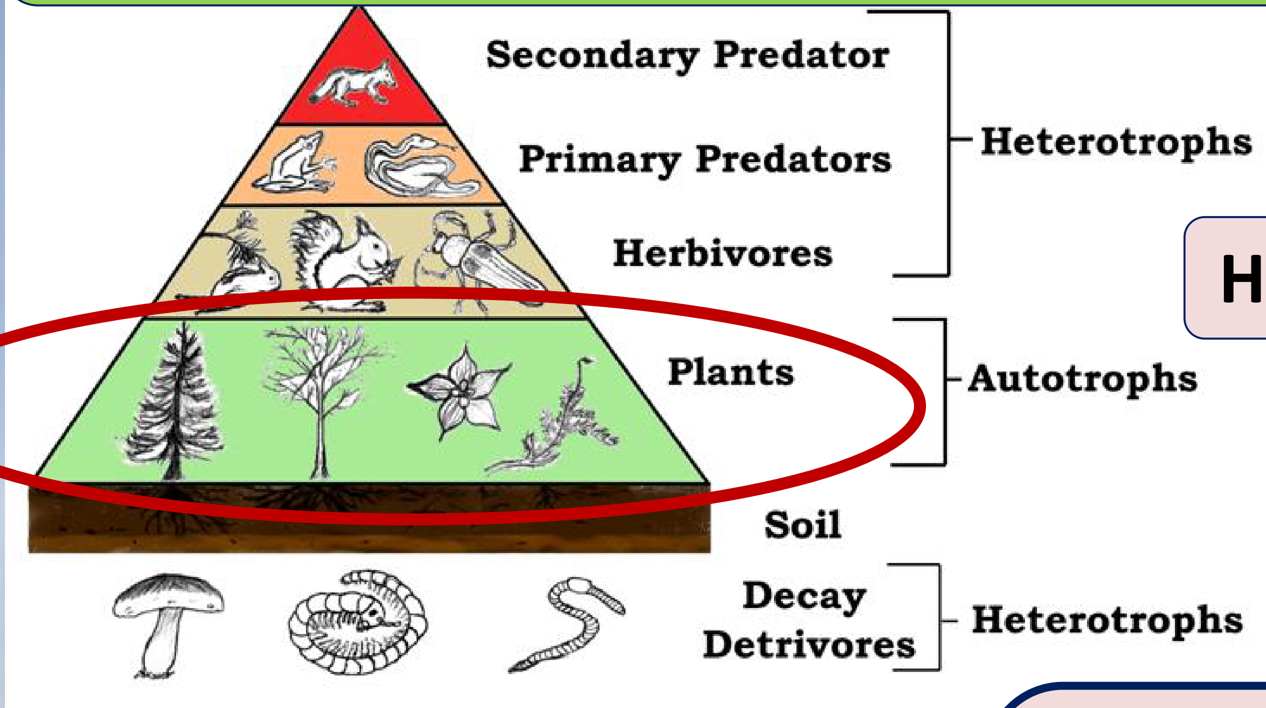
Trace elements

- Concentrations few to few 100 ppm → **toxic**

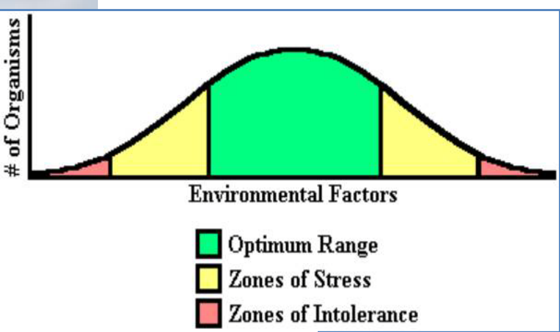




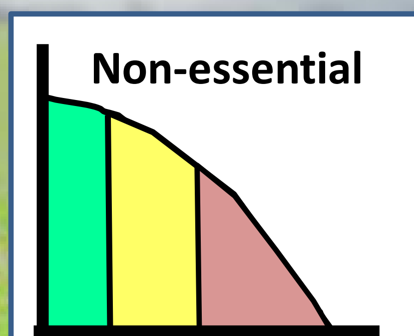
Biomagnification



Hg, Cd, Pb!

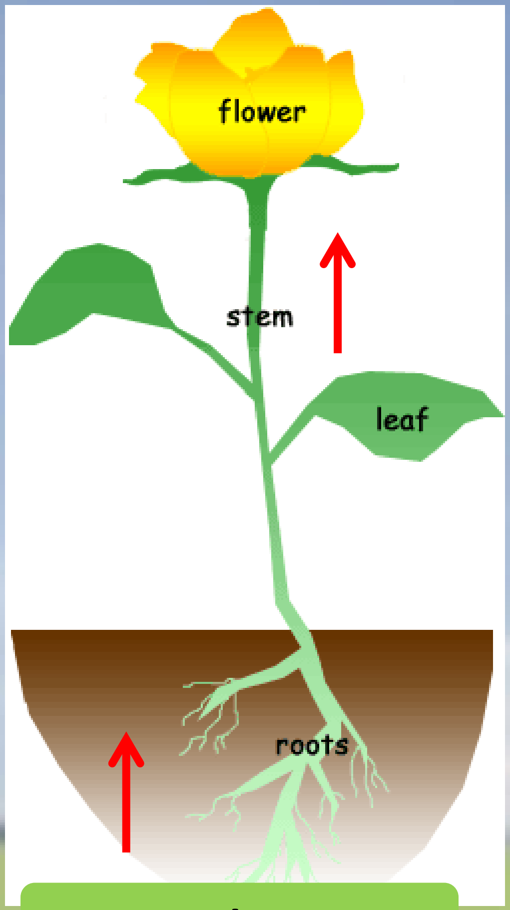


essential



- Metal concentrations in plants have to kept at <u>optimal/ minimal </u>concentrations

- Speciation/ ligand environment of metals



Trace elements

Mechanisms of metal uptake, accumulation, toxicity , ligand environment In plants from organ to cell level

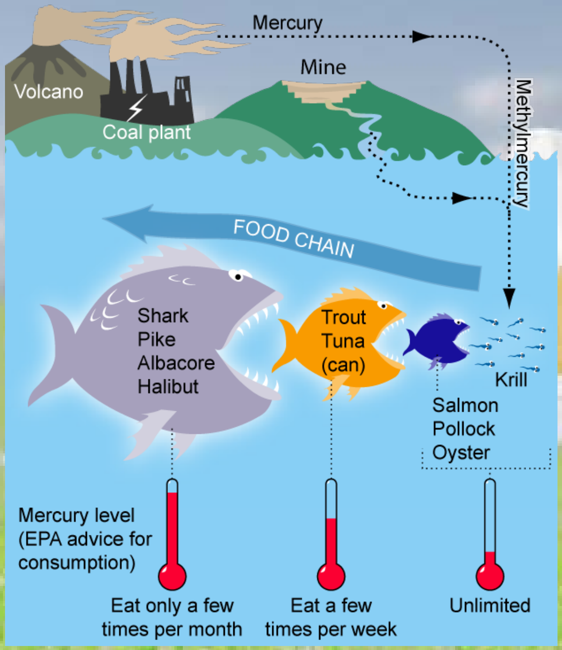
?

Applications

- **Agronomy, food industry**

➢ to improve food nutritional status (Fe, Zn) – *biofortification*

➢ to *decrease* the uptake and bioavailability of unwanted toxic metals (Cd, Hg, Pb…also Al) in food plants and animals



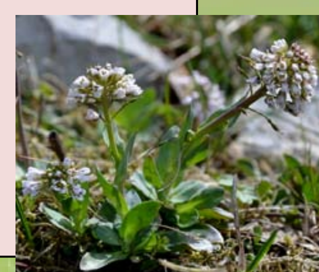
Applications

- **Environment**

➢ to monitor the level of pollution, estimate *bioavailability* of contaminants

➢ To restore polluted areas - *bioremediation*



Main techniques

__Metal accumulation__
*__at **organ level**__*
roots, leaves, seeds

→ Bulk analyses – *XRF, TXRF, AAS, ICP-MS*

__Metal localization at__
__tissue and cellular level__
epidermis, mesophyll, veins; apoplast, symplast

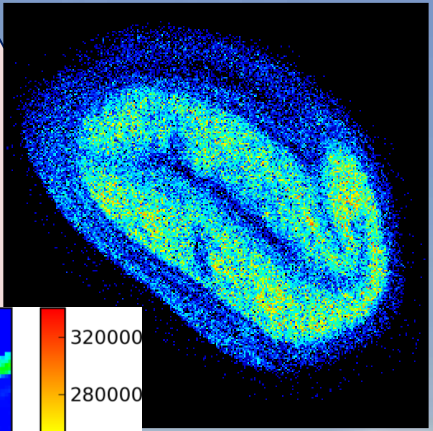
→ Metal localization - *Micro-PIXE,* ***SR-Micro-XRF*** *LA-ICPMS*

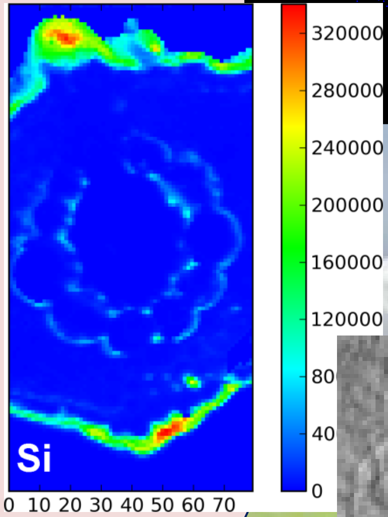
__Metal complexation__
Molecular level

→ Metal ligands - ***(μ)XANES, EXAFS***

Overwiev

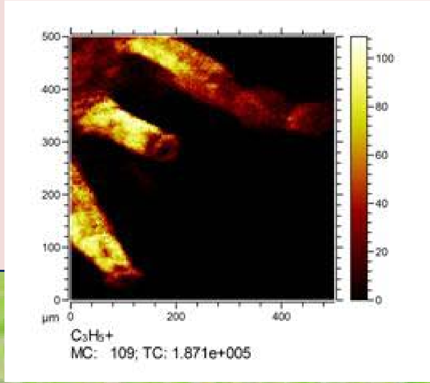
- Techniques for 2D imaging of element distribution in plant tissues
- (μ)XAS – element speciation and ligand environment

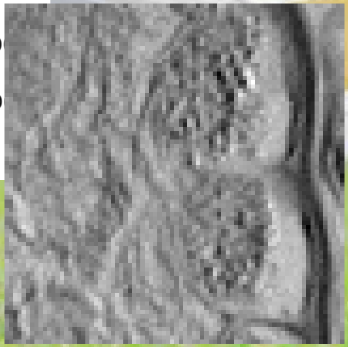




- Sample preparation

- Case studies





Element distribution imaging techniques

- **X-ray fluorescence based techniques**

- ❑ EDX-MA (electron microprobe) – LR-few 10 nm, CS - 0.1 %
- ❑ Micro-PIXE (proton microprobe) – LR-1 µm, CS – 1 ppm

- ❑ XRF spectroscopy, spectromicroscopy
  - ➢ LEXRF – micro beamlines - 1x1 µm spot size, (0.5 -2 keV; 1-9 keV), CS – few 10 ppm
  - ➢ hard X-rays - micro, nano-spectroscopy beamlines (3-35 keV) , CS – 1 ppm
  - ➢ XRF beamline; 200x100 µm spot size, low/middle energy X-rays (1-14 keV), CS – 1 ppm

- **Mass spectrometry based techniques**

- ❑ LA-ICPMS – isotope discrimination; LR 2-5 µm, CS - 0.1 ppm
- ❑ TOF-SIMS - also molecular imaging; LR-few 10 nm, CS - 0.01 %
- ❑ MeV-SIMS – also molecular imaging ;LR-few 10 µm, CS - 0.01 %

Micro-PIXE
JSI micro-PIXE setup

Micro-XRF
ID21, ESRF

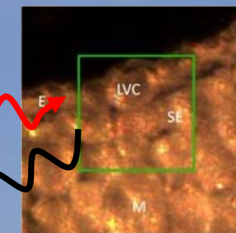
1µm p+ beam
3.0 MeV
100 pA

vacuum

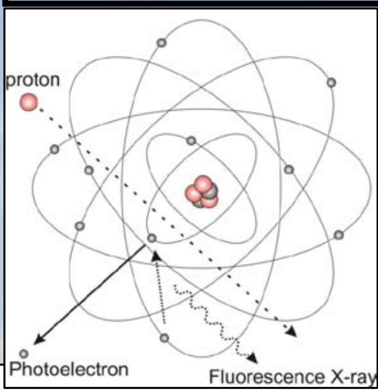
SDD

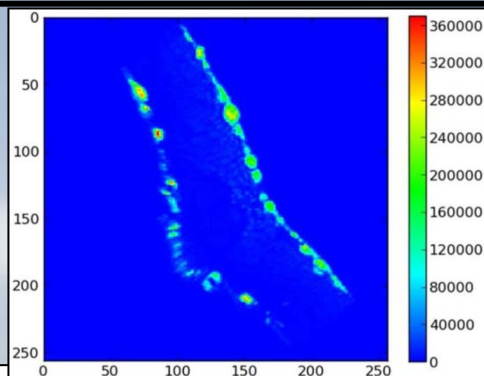
<1µm photon beam
2-9 KeV
10^{10} ph/s

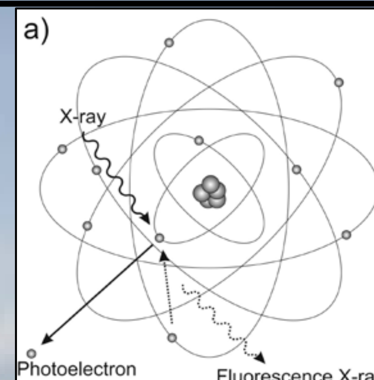
vacuum

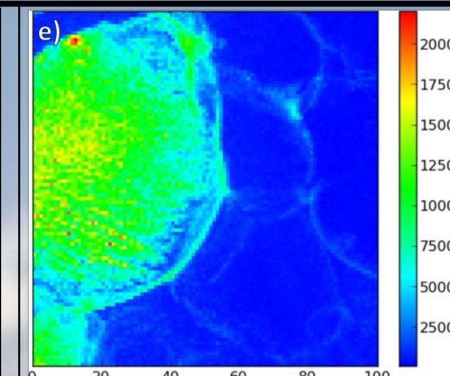


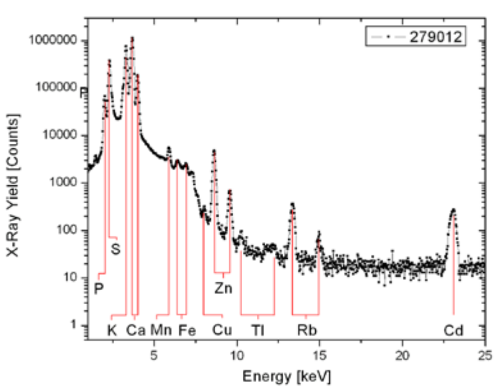
SDD







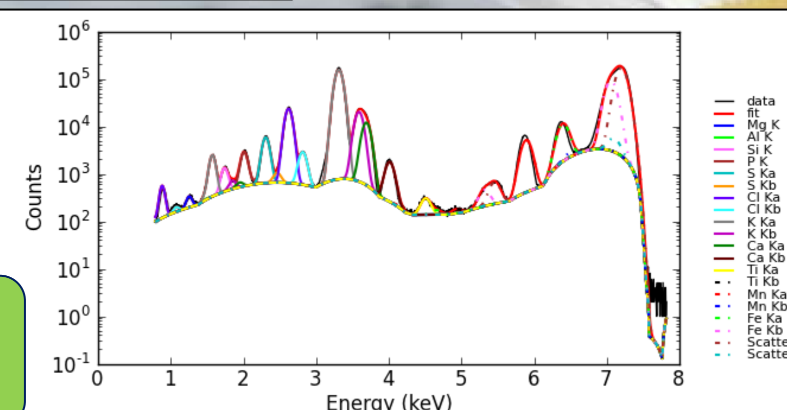




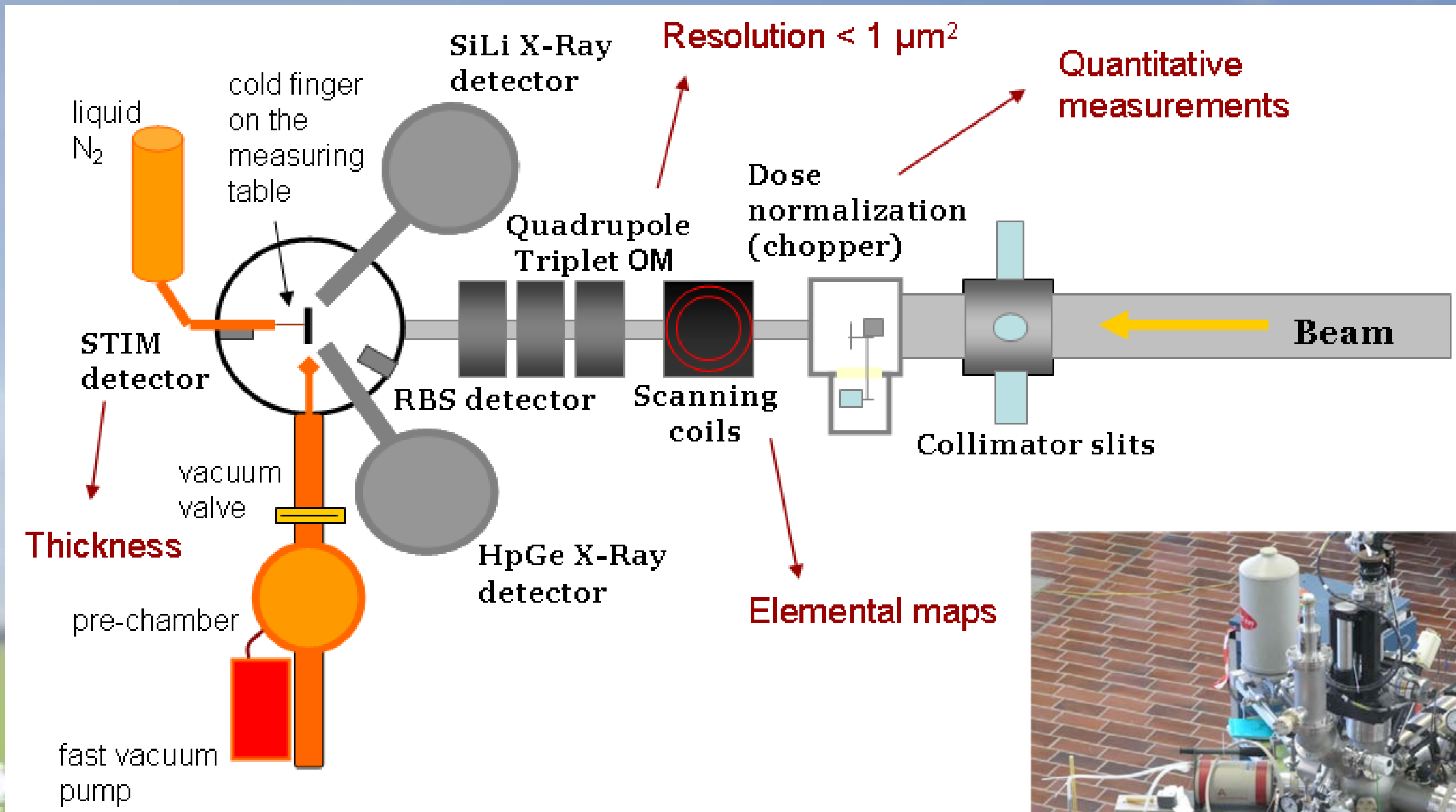
Fully quantitative
GEOPIXE
QA-microXRF
(Kump, IJS)

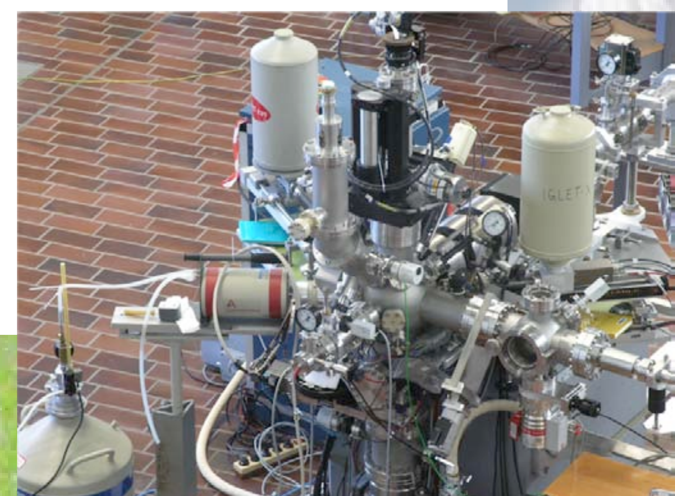
Energy range from Na-U

Soft and hard X-ray beamlines u-XANES



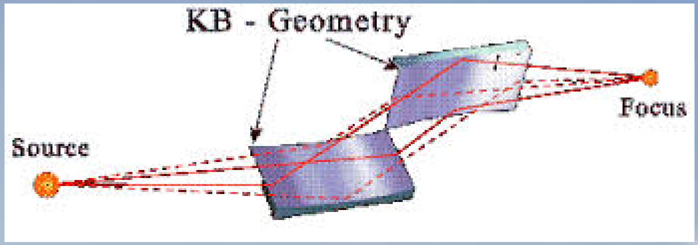
Micro-PIXE
JSI micro-PIXE setup

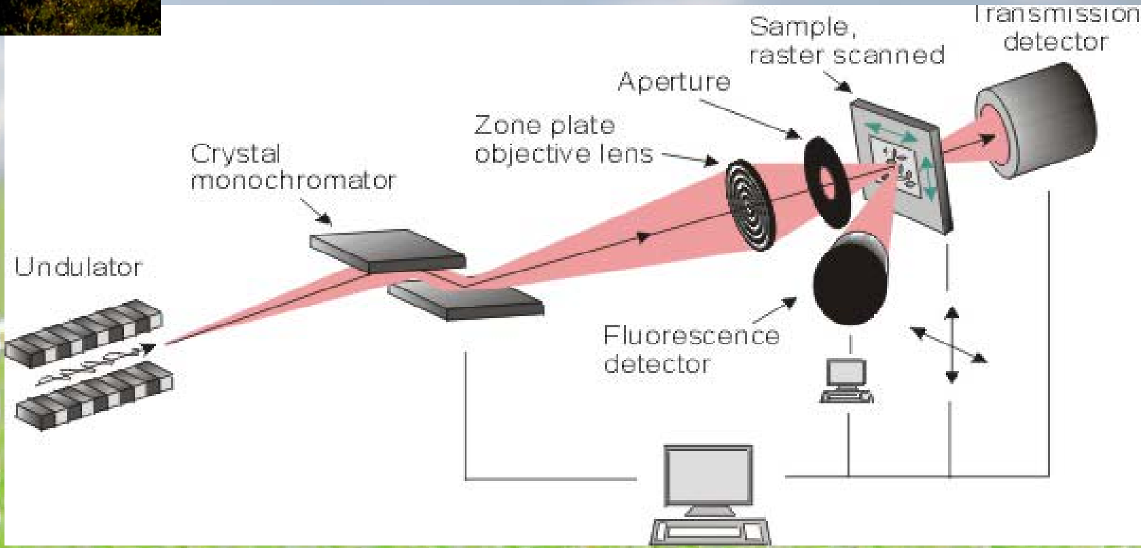




Element localization studies – µ-XRF and µ-XANES; ID 21, ESRF Grenoble

Scanning transmission X-ray microscope (1-9 keV)

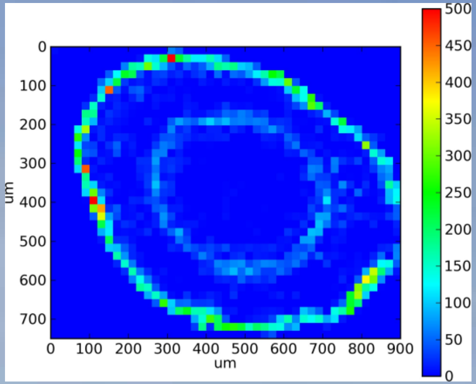


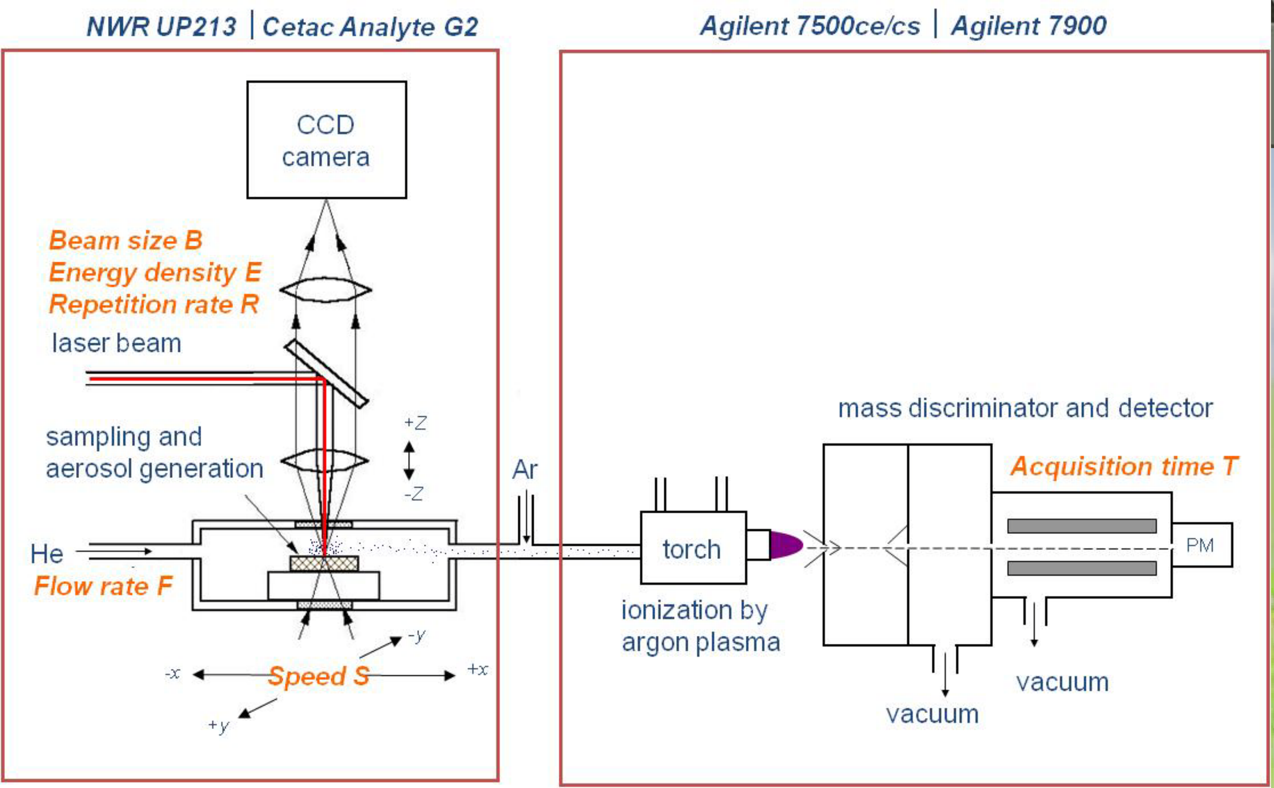


- Localization of elements (LEXRF)
- Resolving sample structure (STM)
- **micro-XANES analysis**

LA-ICPMS, National Institute of Chemistry

Quantitative analysis
Debeljak, van Elteren, Vogel-Mikuš, 2013;
Anal Chim Acta.
2013;787:155-62.



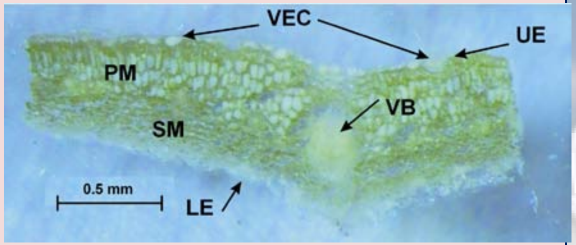


- Isotope discrimination
- High sensitivity – down to 0.1 ppm
- LR >10 µm…slowly reaching 1 µm

Sample preparation for imaging

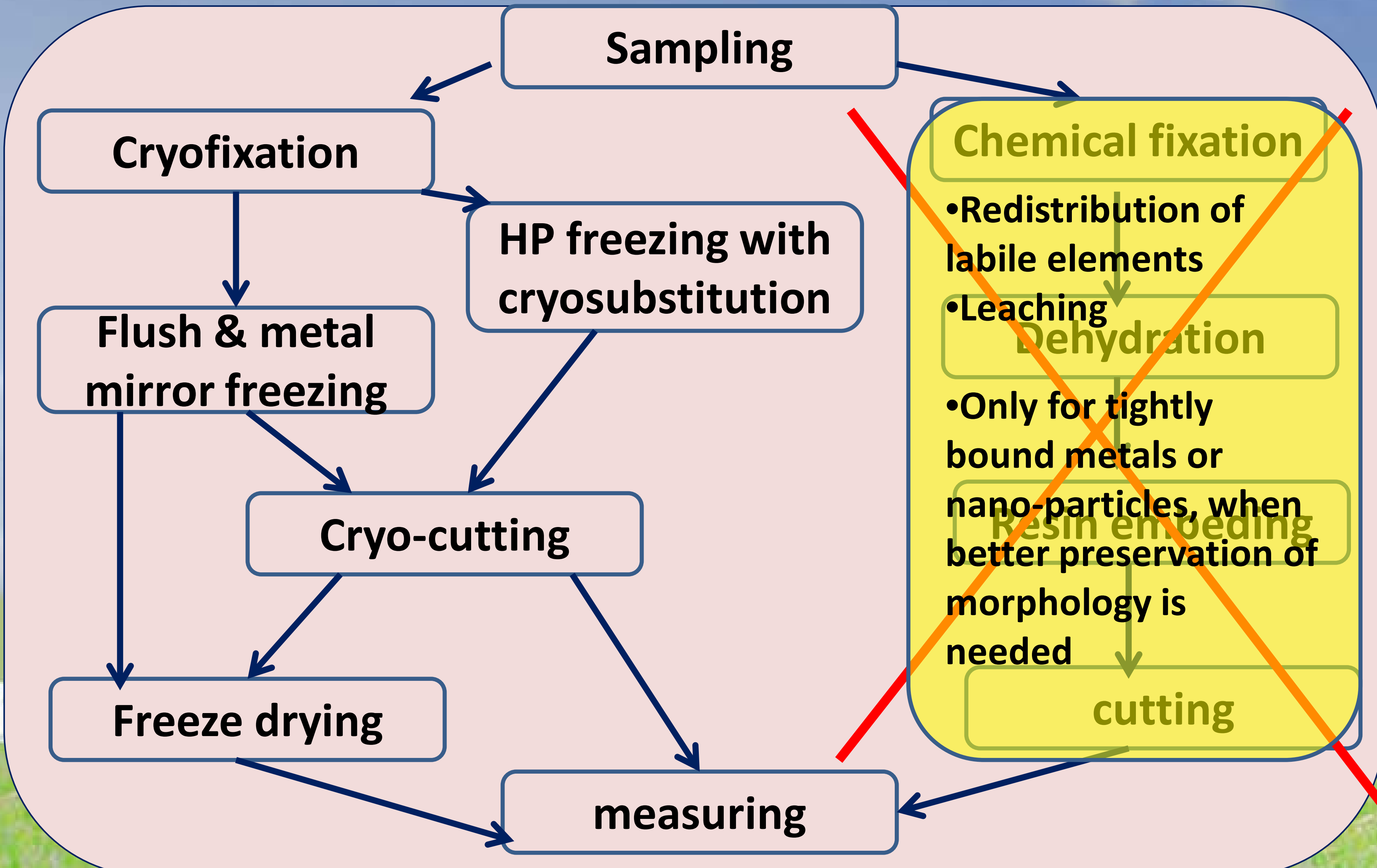
- **Limitations**
  - limited penetration of protons or X-rays and emission of fluorescence X-rays
  - measurement conditions (for PIXE, LEXRF - vacuum compatible samples) $\rightarrow$ dehydration

  - Investigation of element distribution at tissue and cellular levels are usually done on tissue cuttings



- **Main goals to be achieved during sample preparation**
  - **preserve local redistribution of elements in tissues**
  - **preserve sample morphology**
  - **preserve metal ligand environment as similar as "in vivo" stage**

Sample preparation - methodology

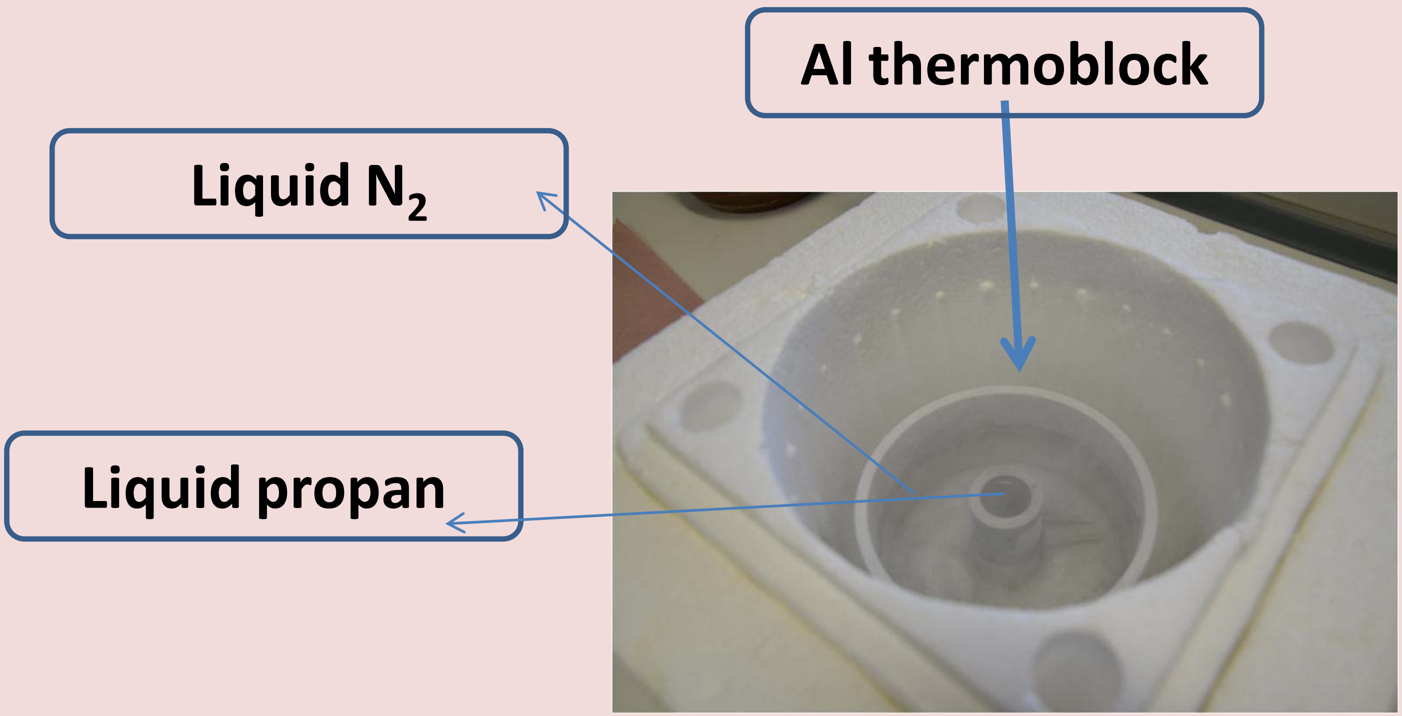


Flush freezing, freeze-drying

- Goal – **to prevent ice crystal formation to a higher extent as possible** (membrane damage)
- **Freezing - cryogens** – enable better contact between the sample and cooled liquid (isopentane, propane) → higher freezing speed → vitrification
- Metal mirror freezing → press the sample against LN_2 cooled metal
- LN_2 does not give good results (vapors act as insulator)

Flush freezing, freeze-drying

- Goal – to prevent ice crystal formation to the highest extent as possible



Flush freezing, freeze-drying

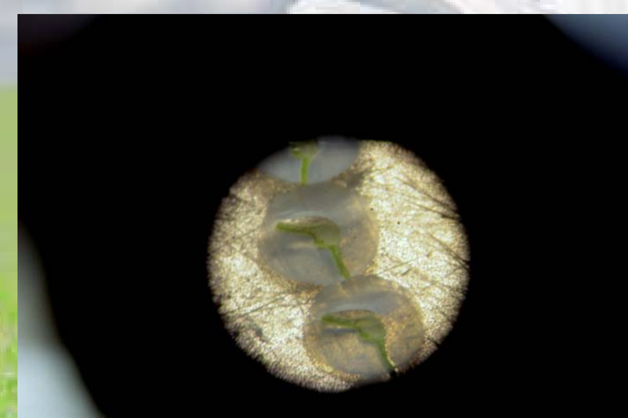
- Good results are obtained only with small pieces of tissues (few mm, thickness 0.2 mm)
- Tissue freezing media – support for cutting; does not penetrate the cells; it may interfere with surface structures, such as waxes, trichomes











Freeze-drying

- Should be performed gradually from -196°C to 25°C to prevent shrinking of the specimens






Freeze-drying

- Should be performed gradually from -196°C to 25 °C to prevent shrinking of the specimens
- Computer assisted
- Improvised

Shelf temperature

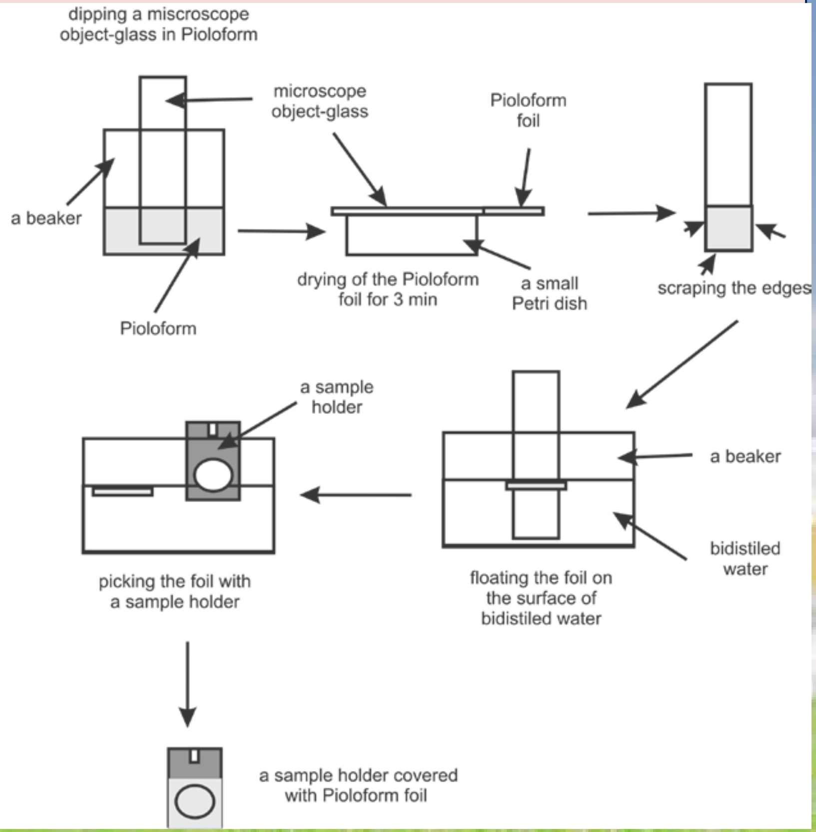
3rd day – transfer the samples to the highest position – adjustment to room temperature, 24 h

2nd day - transfer the samples to the higher position, 24 h

1st day – pour LN2 into the lowest shelf to cool it down, put in the box with samples, leave for 24 h

Mounting of the samples

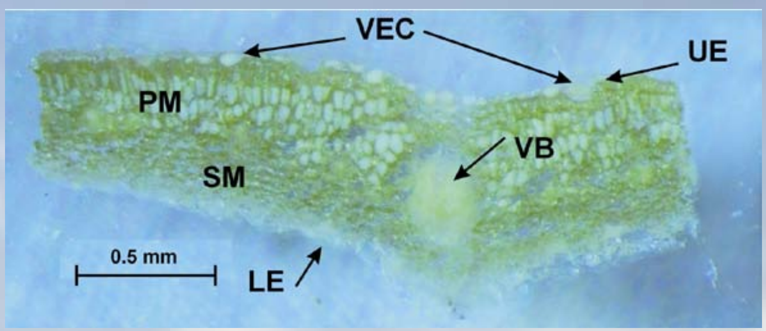
- Sandwich techniqe – between two layers of polymer foils
- Pioloform (~ 300 nm)
- Ultralene (4 µm)

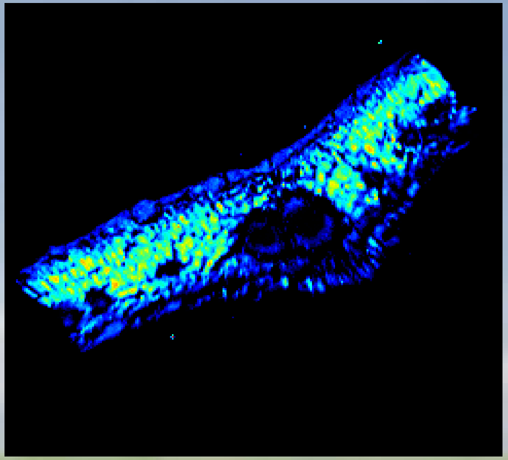


Results

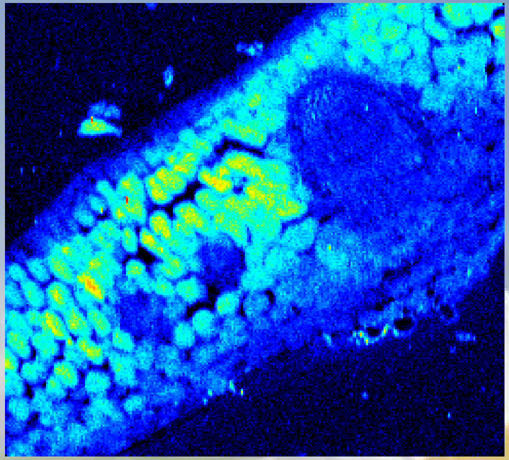
- Well retained morphology and element distribution

µ-PIXE; leaf cross-sections

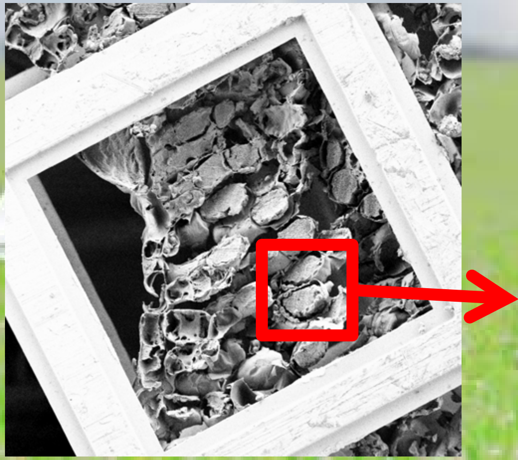




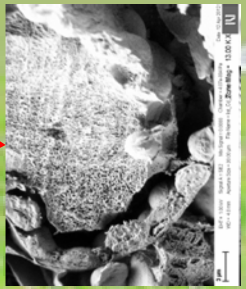
Ca – freeze-dried

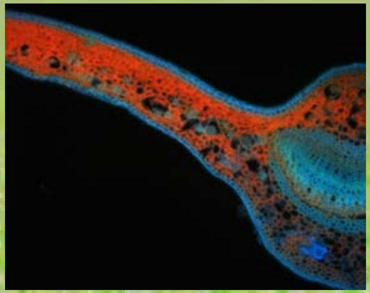


Ca – frozen hydrated



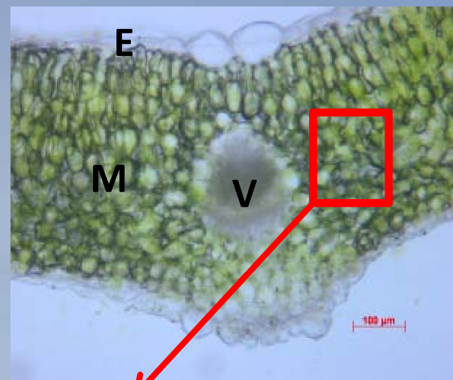
SEM

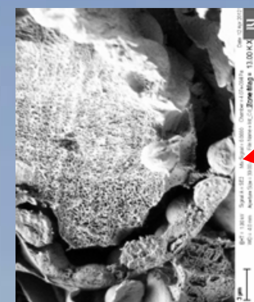


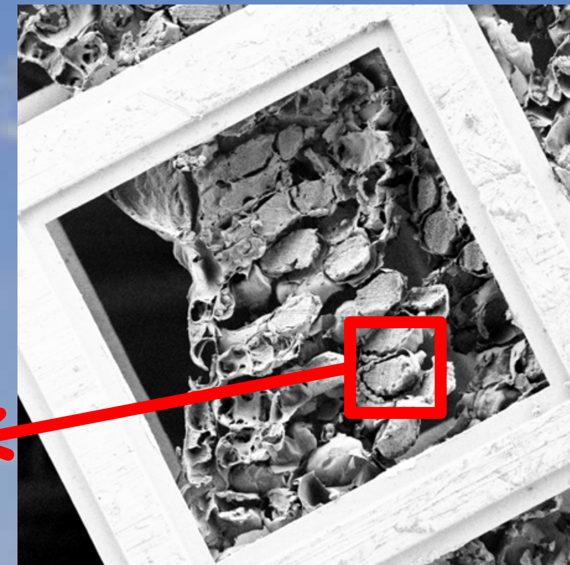


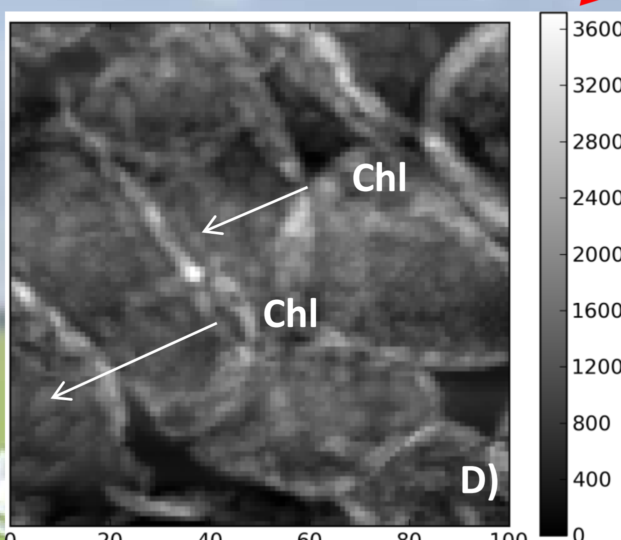
Fluorescence Microscopy (UV)

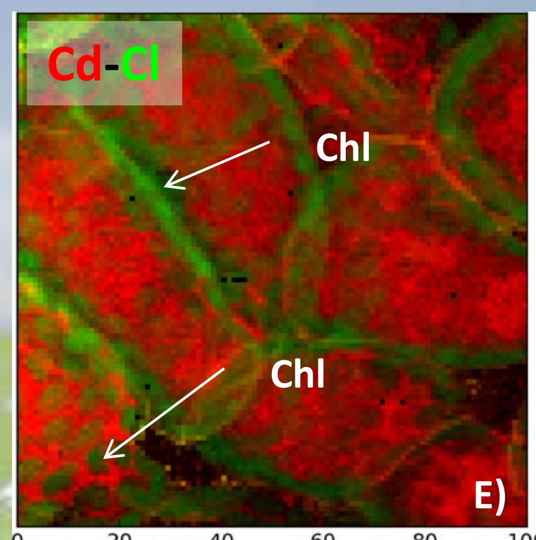
Cd hyperaccumulator *Thlaspi praecox* – *subcellular localization in $CdCl_2$ treated plants*

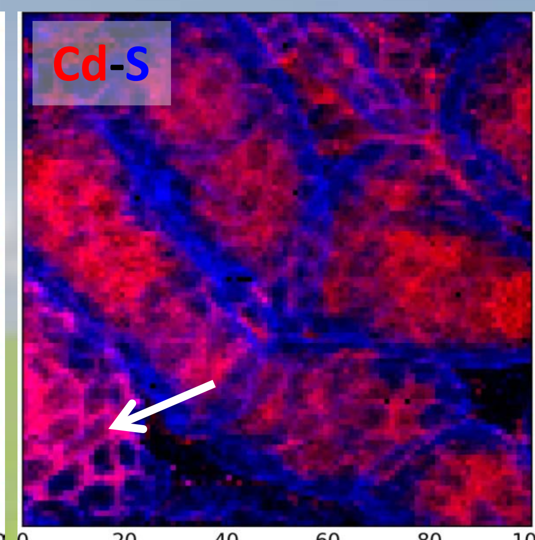






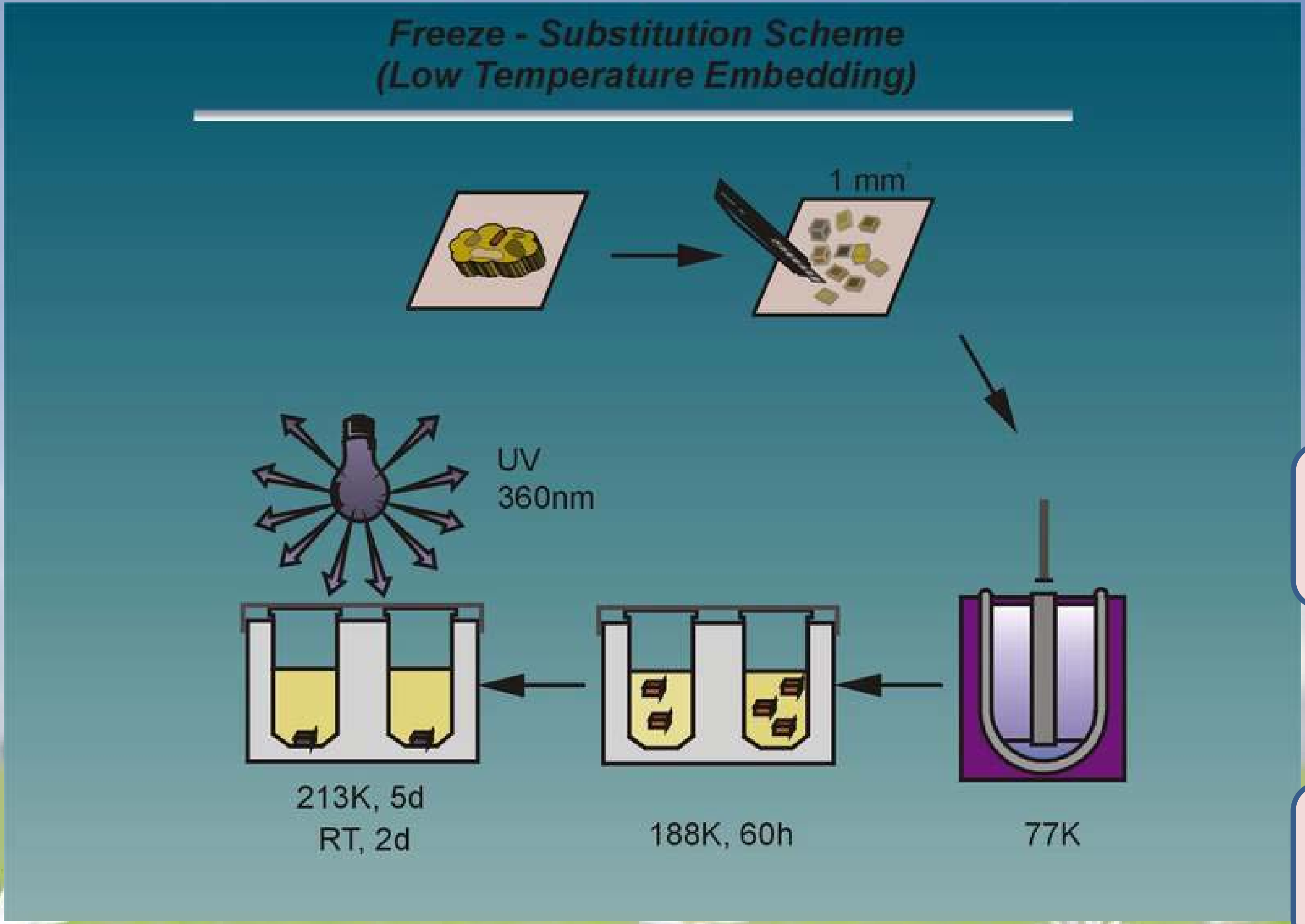






(µXRF, E=3.55 keV, 0.3 x 0.7 µm beam), ID 21, ESRF

High pressure freezing and cryosubstitution



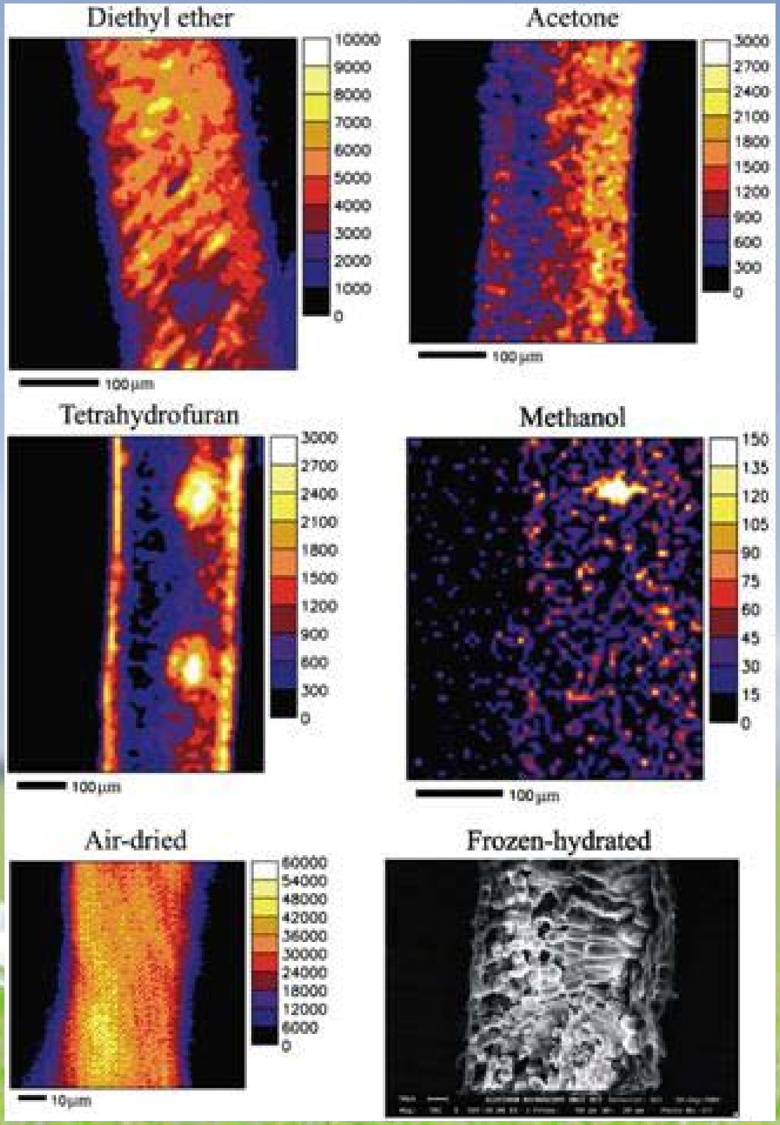
Flush or HP freezing

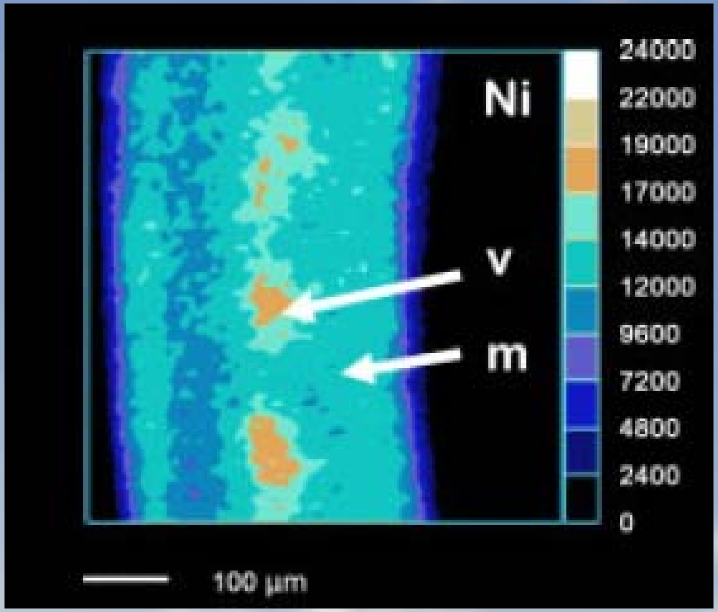
Cryo-substitution

embedding

http://www.mardre.com/homepage/mic/tem/freeze_substitution/freeze_substitution_scheme.html

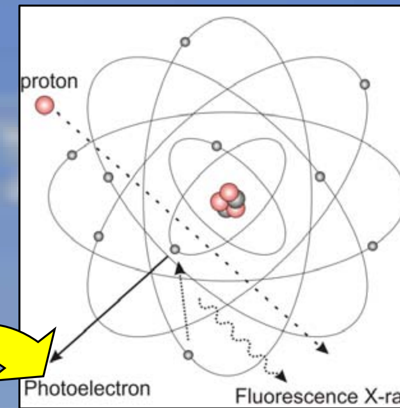
Results



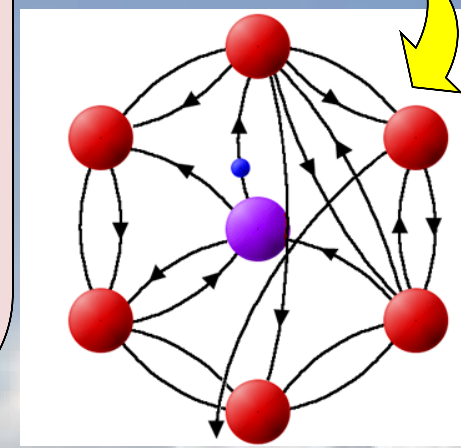


- Budka, et al. Nuclear Instruments and Methods in Physics Research B 231 (2005) 338–344
- Mesjasz-Przybylowicz, Przybylowicz. X-Ray Spectrom. 2011, 40, 181–185

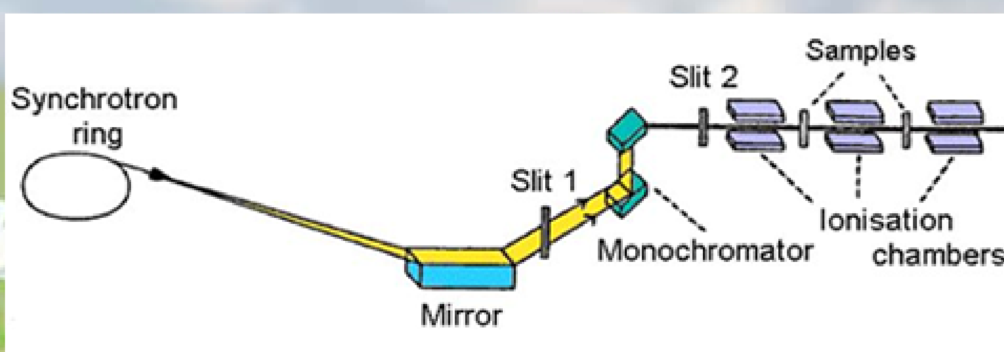
X-ray absorption spectroscopy (XAS)

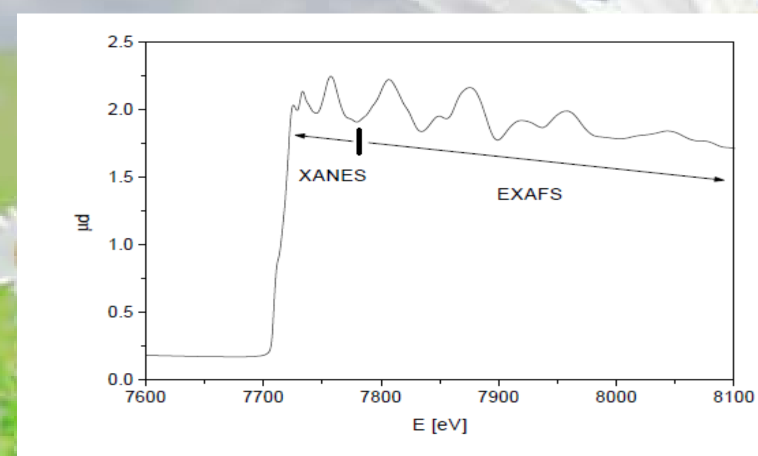


- information about the local coordination environment around absorbing atom.→ BIOVAILABILITY
   - ➢ **X-ray absorption near edge structure (XANES)**
   - ➢ **Extended X-ray absorption fine structure (EXAFS)**



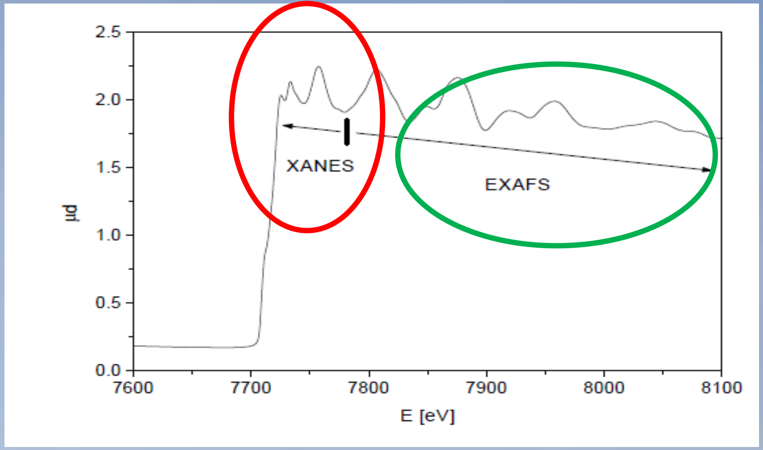
- When excitation energy exceeds binding energy of electrons in atom, photo-effect may occur
- Wave of the ejected photoelectron is then scattered on atoms surrounding the absorbing atom





Can only be performed ad synchrotron facilities!

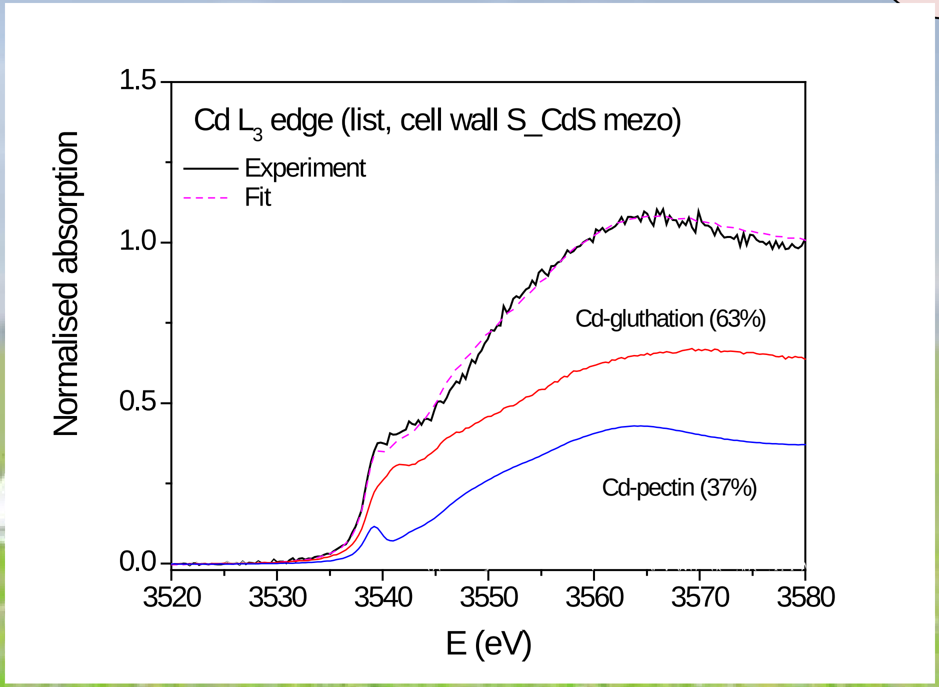
XANES (X-ray absorption near edge structure)



Mathematical approach :

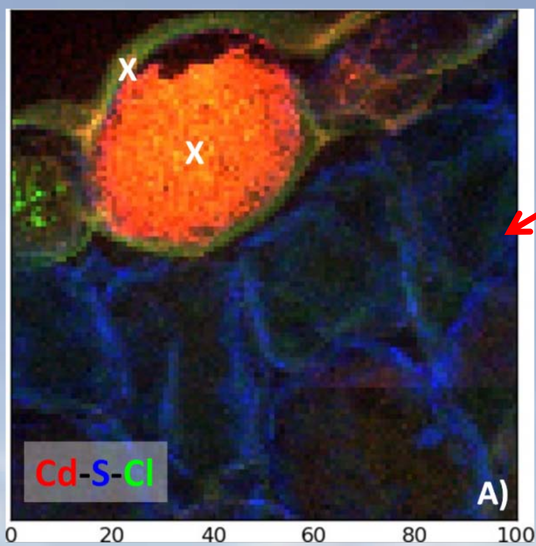
-Linear fit combination of measured standards

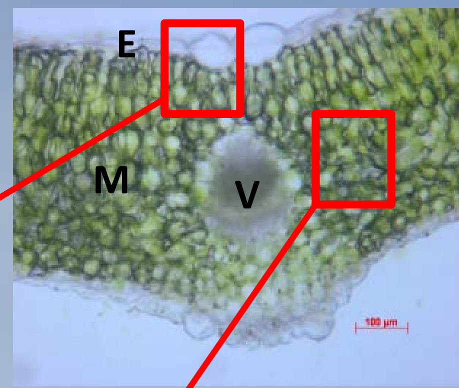
– standards?, radiation damage

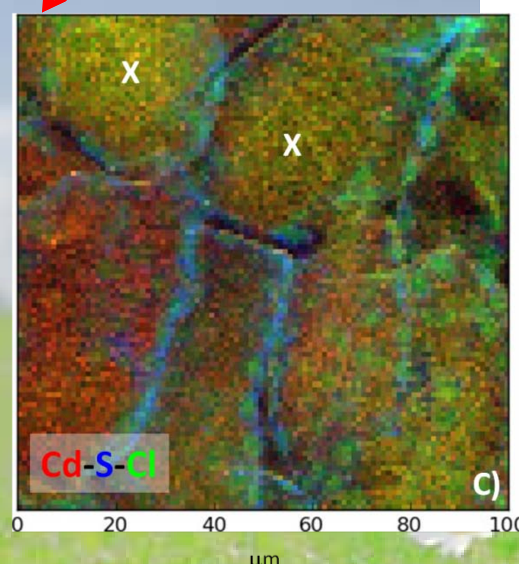


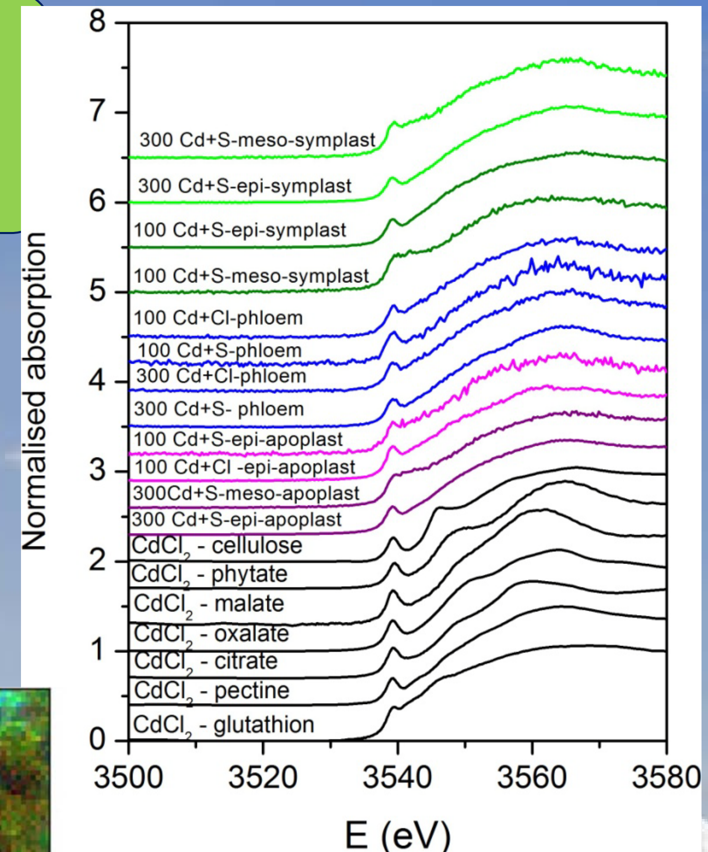
Epidermis - vacuole	
Standard	Contribution (%)
Cd-pectinate	37%%
Cd-GSH	63%%

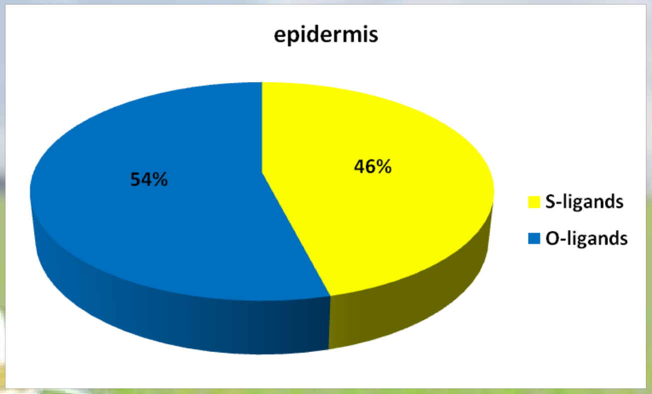
Cd-L3 µ-XANES
Cd ligands in $CdCl_2$ treated plants at sub-cellular level

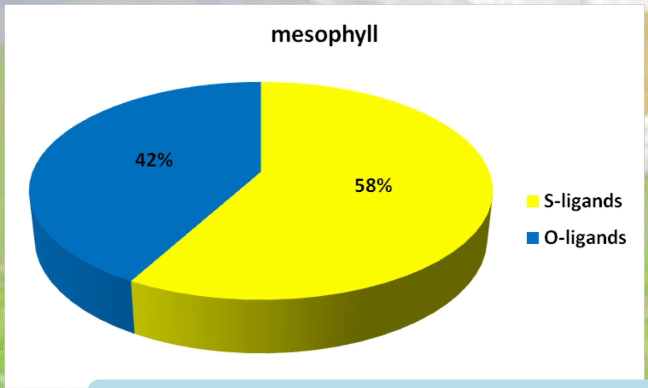






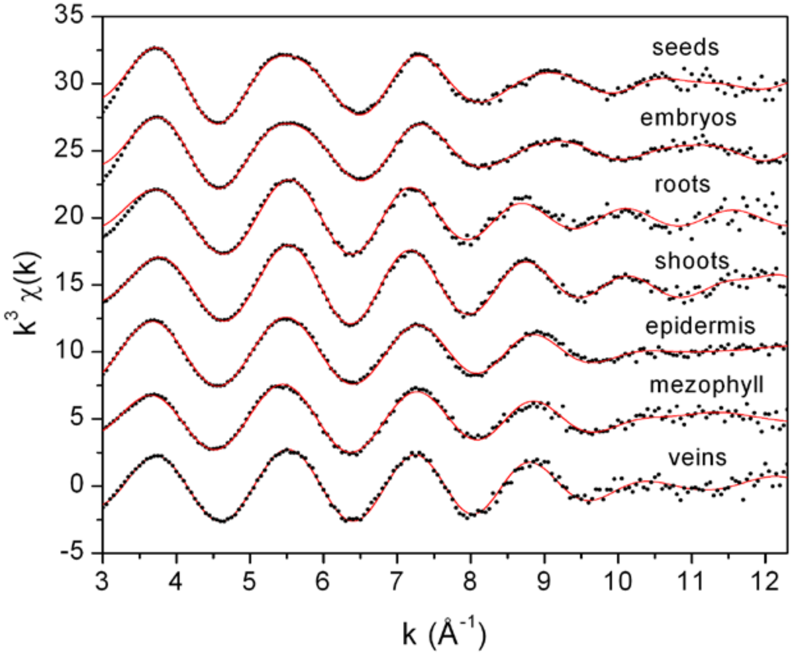




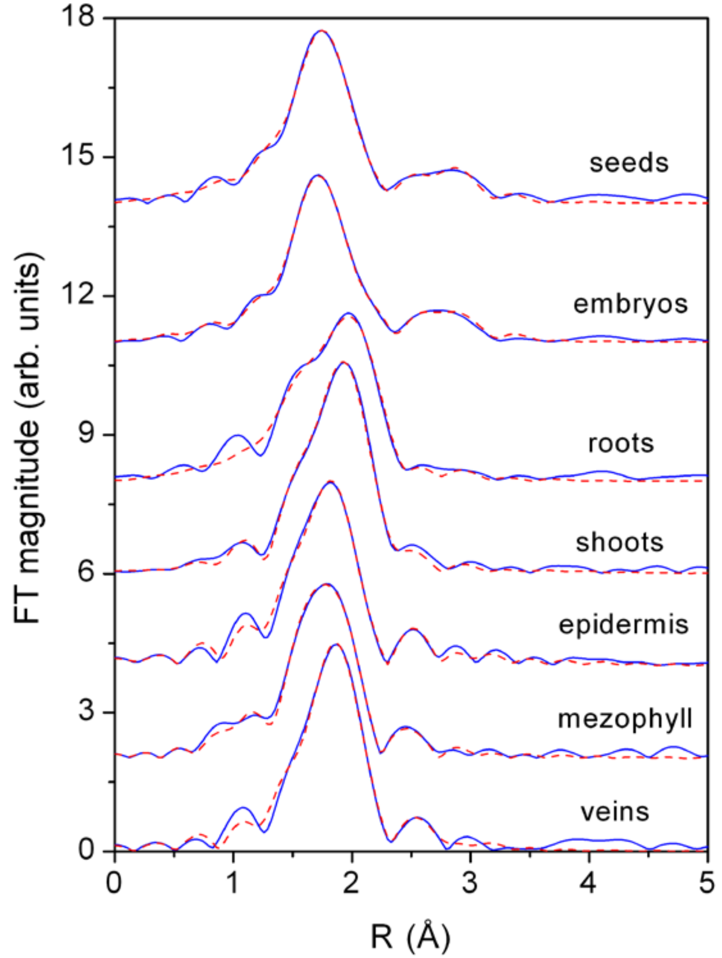


Koren et al. 2013. *Plant and Soil*

EXAFS (Extended X-ray fine structure)
Cd-K EXAFS - Cd ligands in Cd hyperaccumulating plant



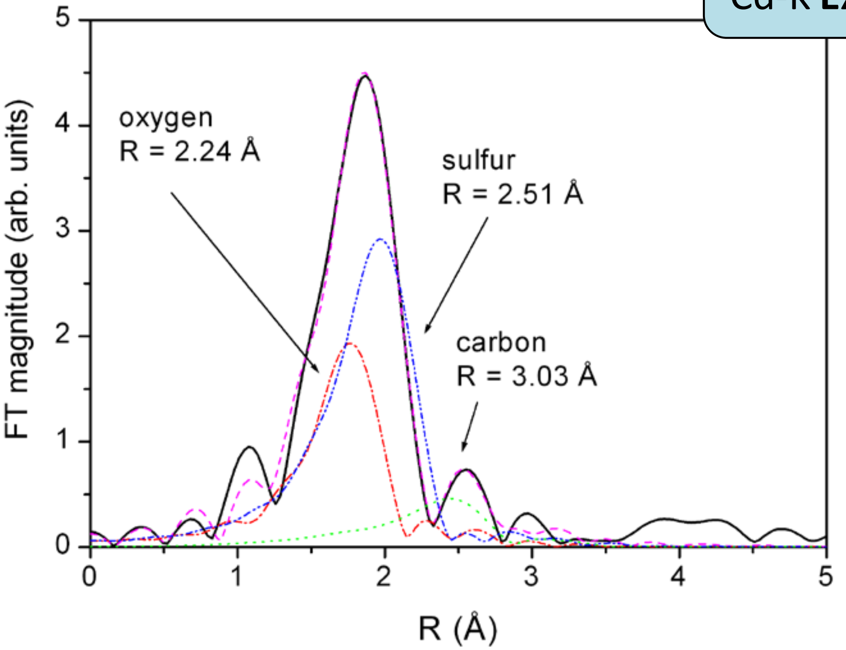
Fourier transform magnitudes of EXAFS spectra



Cd-K-edge EXAFS spectra

EXAFS (Extended X-ray fine structure)

Fourier transform magnitudes of the k^3 weighted Cd-K **EXAFS spectrum**



<u>Neighboring atoms:</u>

First shell:

-O at distance 2,19-2,24 Å **(Cd-O-C)**

-S at distance 2,49-2,51 Å **(Cd-S-C)**

Second shell:

-C at distance 2,82-3,03 Å

<u>Mathematical approach :</u>

-to fit EXAFS spectrum we build **mathematical model**, taking into account a certain number of neighboring atoms at certain distance

-Standardless technique;

-**sometimes difficult to guess the correct combination**

-Information about the first and second shell neighbors but not on the molecule

Vogel-Mikuš et al. 2010. *Plant and Soil* 331, 439-451

Sample preparation for XAS

- **To preserve metal speciation and ligand environment similar to "in vivo"state**

- **Standard XAS beamline – beam size 4 x 1 mm**

➢ Roots, shoots (high water content) - **rapid freezing of <u>fresh biological material</u>** in liquid nitrogen → homogenization →freeze-drying → or measuring in cryo conditions

➢ Grains – intact (Fe, Zn, Se, Cd, As,…) – low water content, avoid oxidation

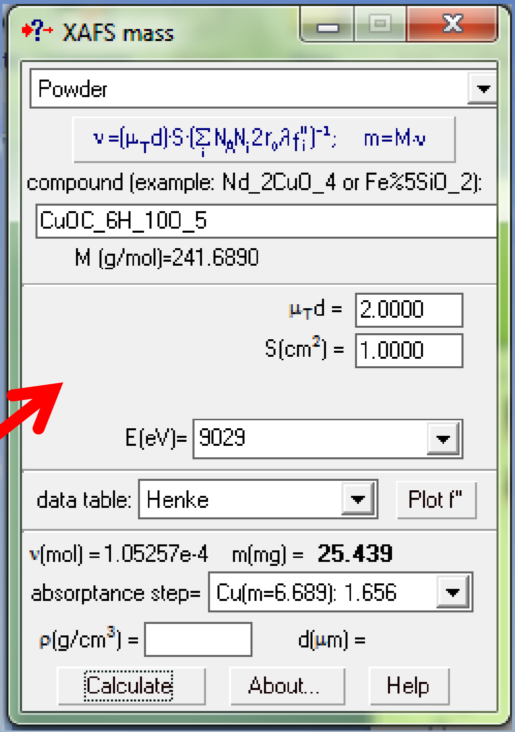
- **μ-XAS**
- Tissue cuttings; the same sample preparation procedures as for imaging

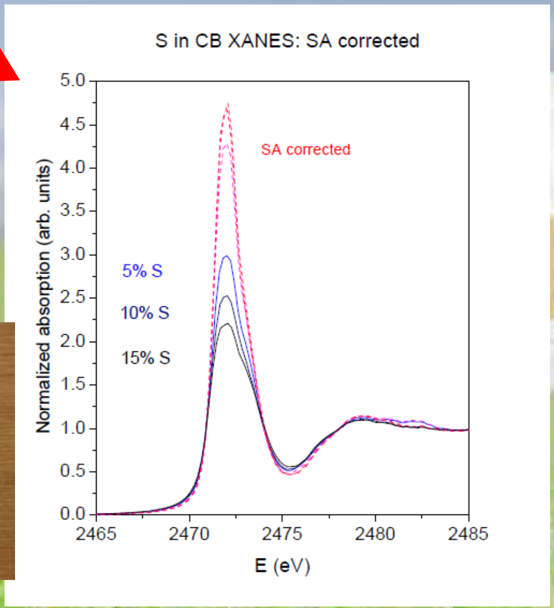
Mounting of the samples

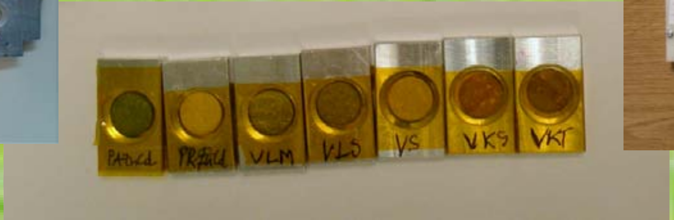
- Press pellet from **homogenized material**….the **proper amount** can be calculated with XAFSmass program (freeware) – this is essential to have **good signal** in transmission mode

- For Fluorescence mode - diluted samples (self – absorption)

- Stick to the holder pellets or intact grains with kapton tape
- Tephlone holders for transmission measurements at high energy (e.g. Cd-K edge)







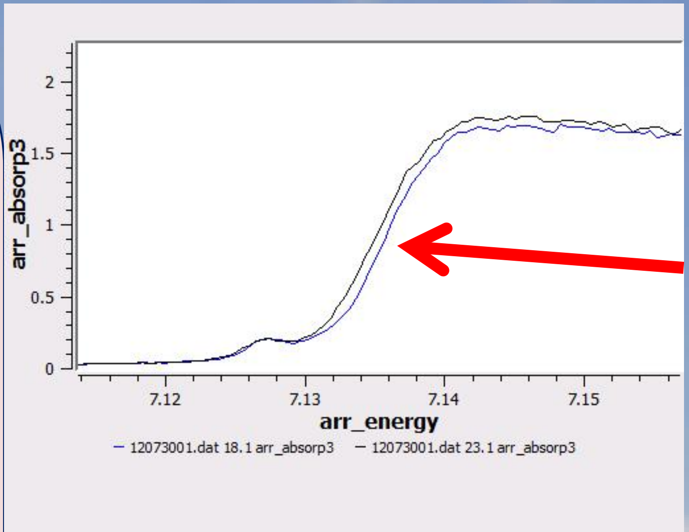
XAS problems & tricks

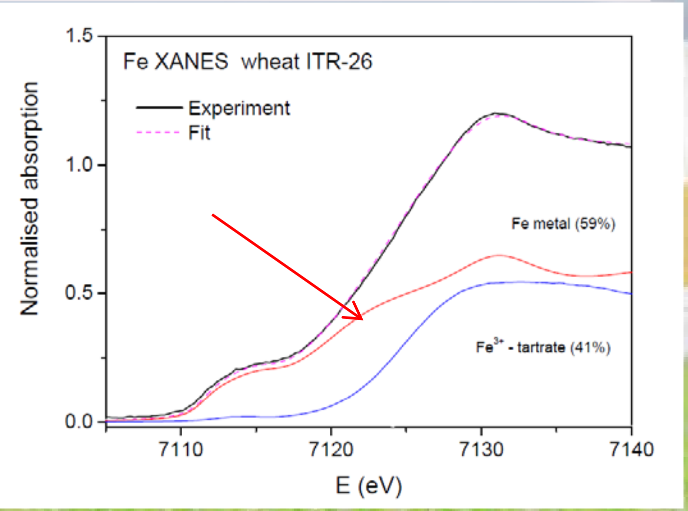
- **Oxidation** during milling/grinding – need to take care with transitional metals, especially Fe → always check for Fe2+/Fe3+ ratio on intact plant organs

- **Reduction during measurements** → measure for short period of time per energy step

- Fluorescence mode – impurities in SDD window; when metal concentrations in the sample are very low (50-100 ppm) (scattering!)

- XANES - Standards? → monitor structure change with FTIR or RAMAN



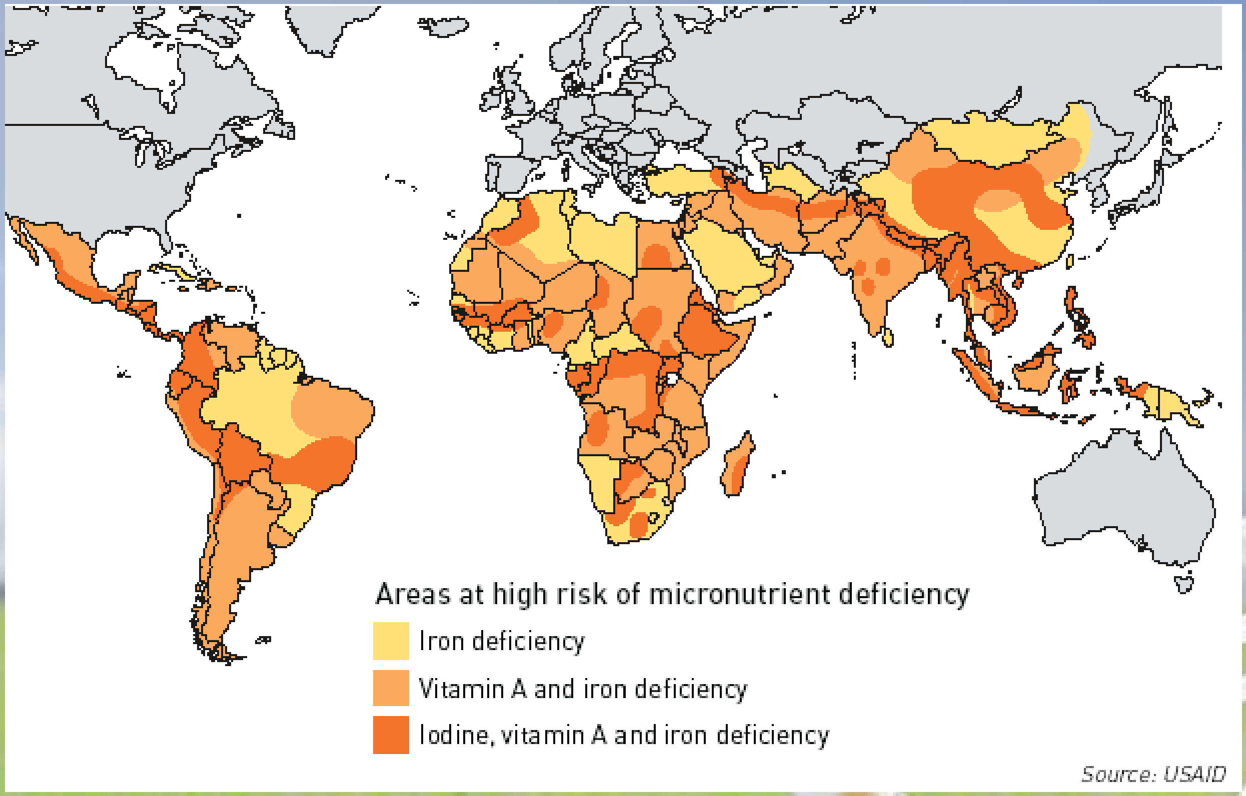


Fe-XANES of wheat grain

Case studies

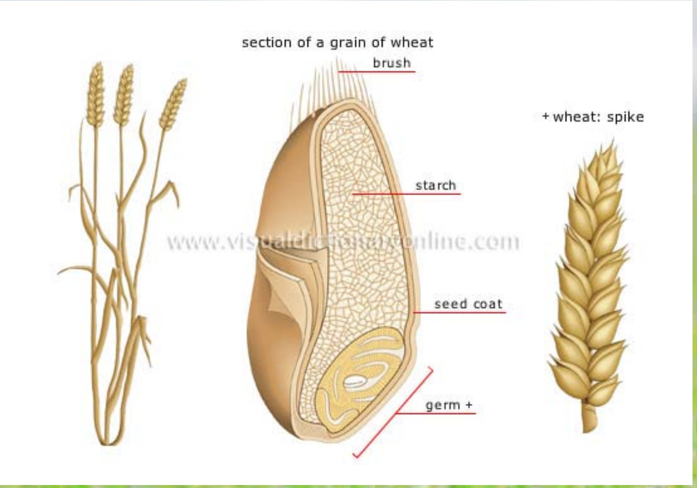
- Fe localization and speciation in wheat and pearl millet (PIXE, Fe-K XANES)
- Al localization in tea (PIXE, LEXRF – TwinMic)
- Se localization and speciation in mushrooms (SR-XRF)

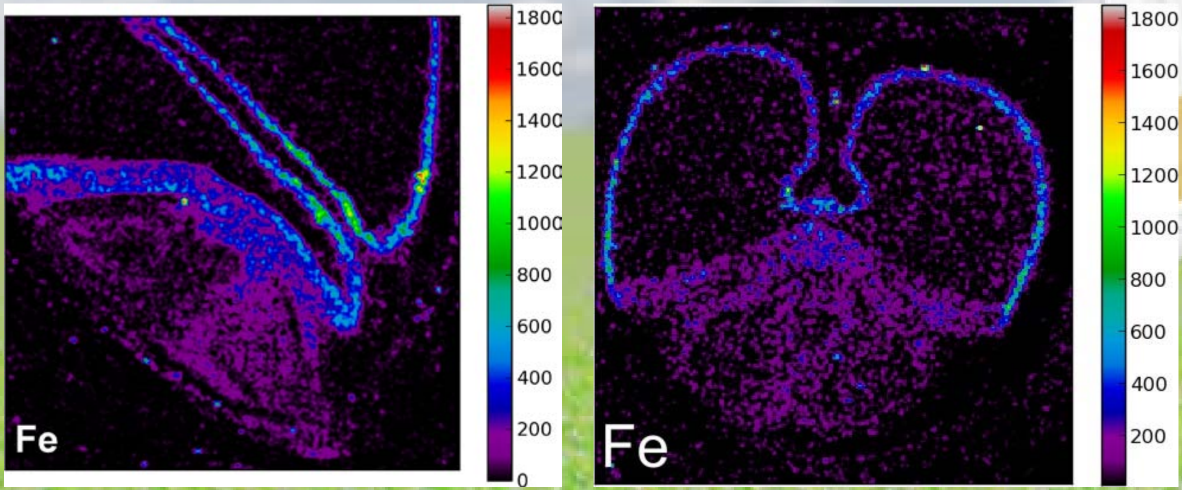
Biofortification
- Iron in wheat-



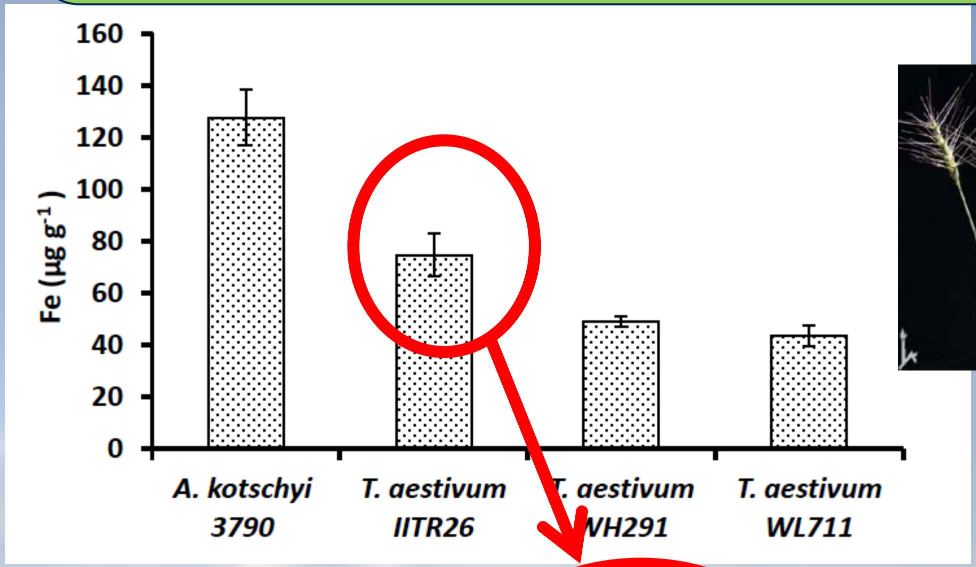
Iron deficiency affects more then 30% of world's population.

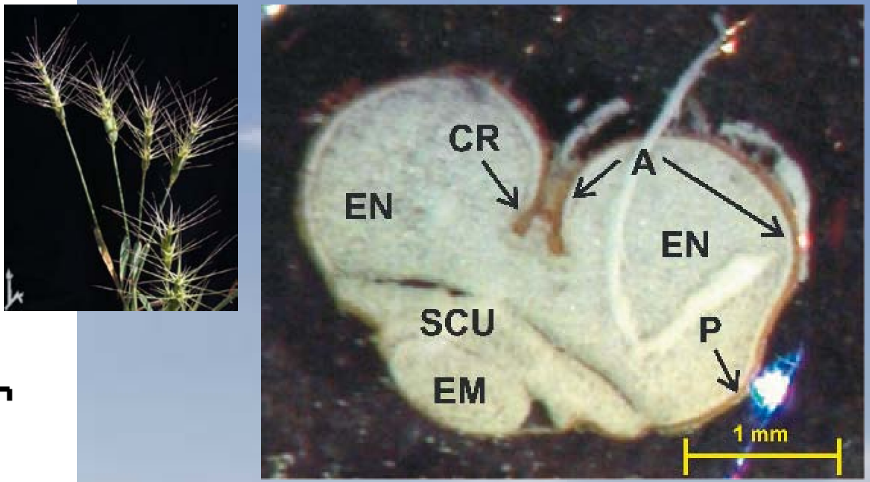
To enhance Fe concentrations in cereals – is this enough?
PARTITIONING ?
BIOAVAILABILITY?

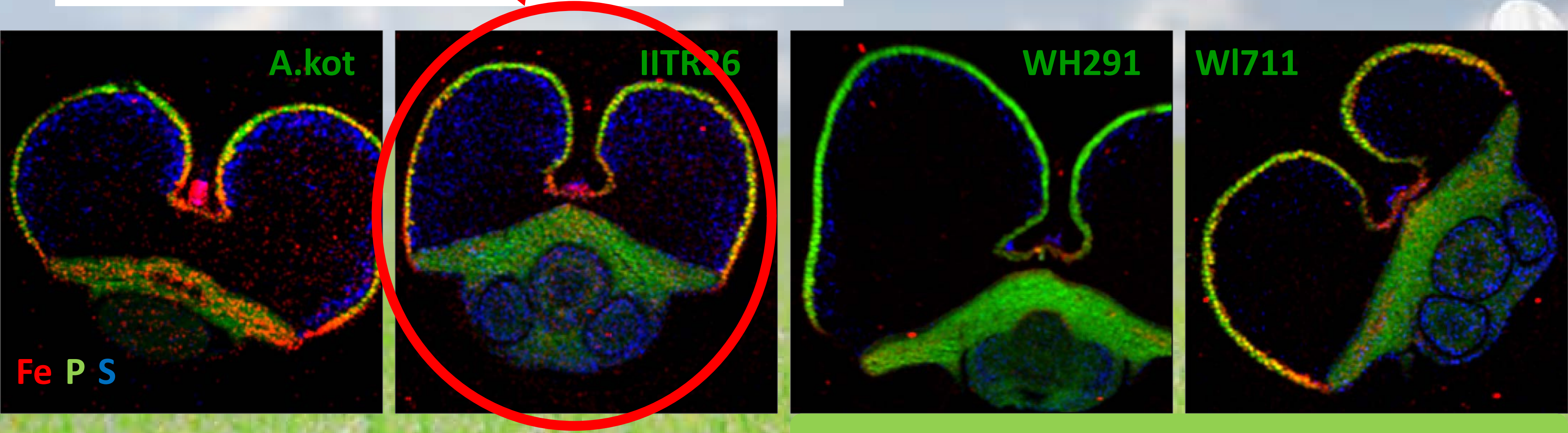




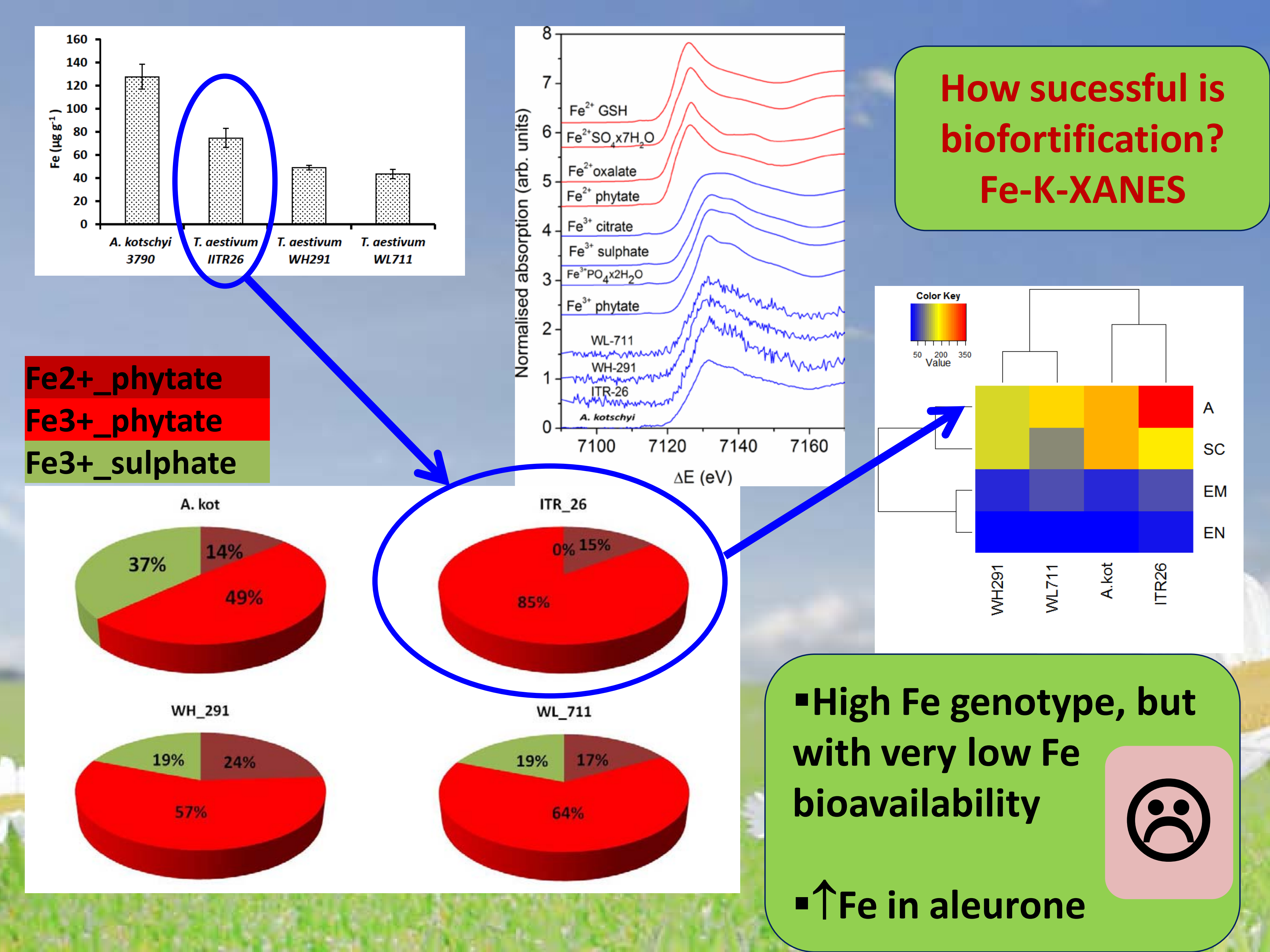
Fe distribution and Fe ligand environment in *Aegilops* and 3 wheat genotypes – micro-PIXE, Fe-K XANES study







Singh et al., 2013, 2014



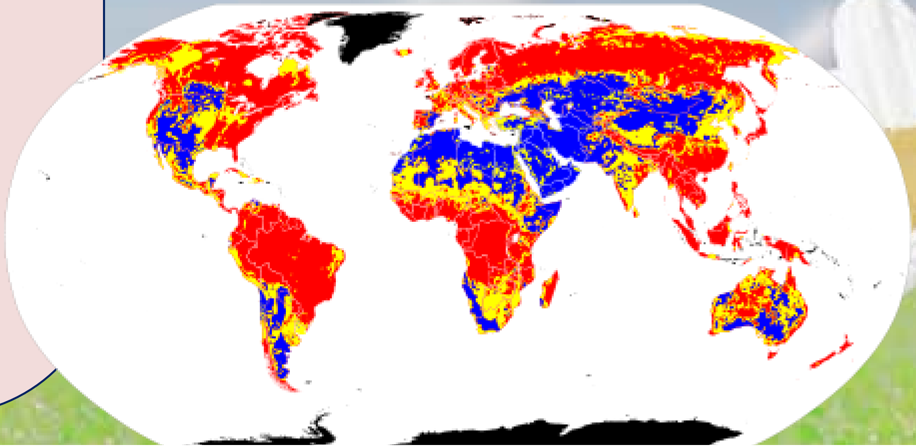
How sucessful is biofortification? Fe-K-XANES
Fe (µg g-1)
160
140
120
100
80
60
40
20
0
A. kotschyi 3790
T. aestivum IITR26
T. aestivum WH291
T. aestivum WL711
Normalised absorption (arb. units)
ΔE (eV)
7100
7120
7140
7160
Fe2+ GSH
Fe2+SO4x7H2O
Fe2+oxalate
Fe2+ phytate
Fe3+ citrate
Fe3+ sulphate
Fe3+PO4x2H2O
Fe3+ phytate
WL-711
WH-291
ITR-26
A. kotschyi
Color Key
50 200 350
Value
A
SC
EM
EN
WH291
WL711
A.kot
ITR26
Fe2+_phytate
Fe3+_phytate
Fe3+_sulphate
A. kot
14%
49%
37%
ITR_26
0%
15%
85%
WH_291
19%
24%
57%
WL_711
19%
17%
64%
▪High Fe genotype, but with very low Fe bioavailability
▪↑Fe in aleurone

Food safety
- Aluminum in tea -

Al is not a trace metal from geological point of view, but in organisms it can be found in trace amounts.

Tea (*Camelia sinensis*)

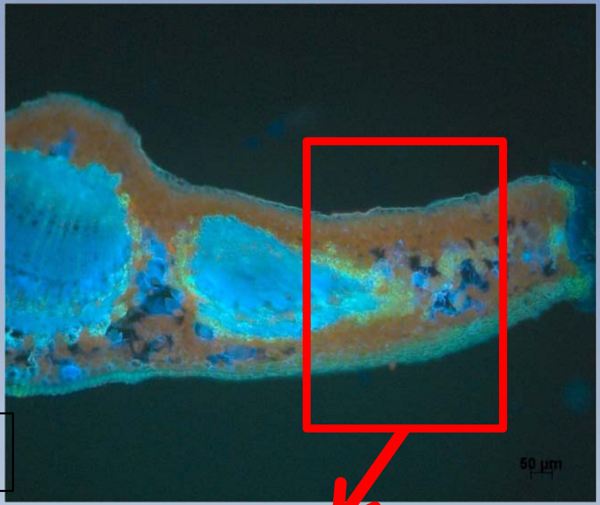
- Grows in (sub)tropical regions - acid soils
- Intense rainfalls, leaching of Ca, K, Na, Mg
- $Al^{3+} + H_2O \rightarrow Al(OH)^{2+} + H^+$
- Tea leaves can contain up **to 3% of Al**
- Toxic for plants and humans?

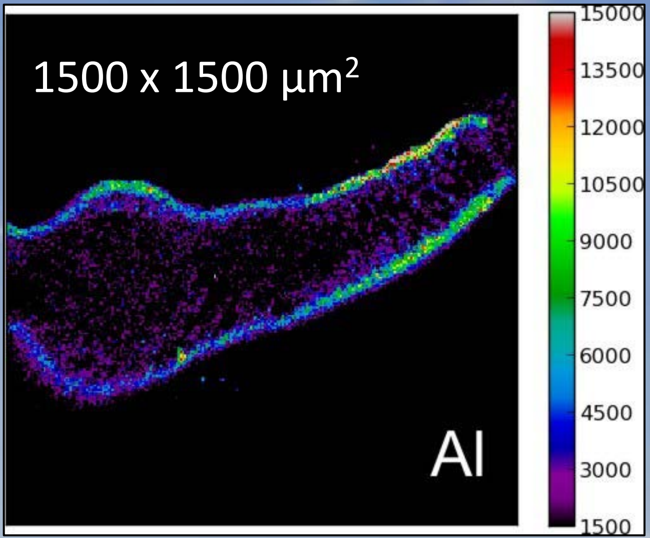


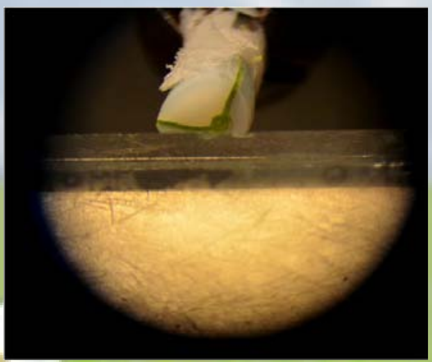
Global variation in soil pH. **Red** = acidic soil. **Yellow** = neutral soil. **Blue** = alkaline soil. **Black** = no data.

Localization of Al in leaves of tea plant
micro-PIXE, E=2.5 MeV, 1x1 µm^2

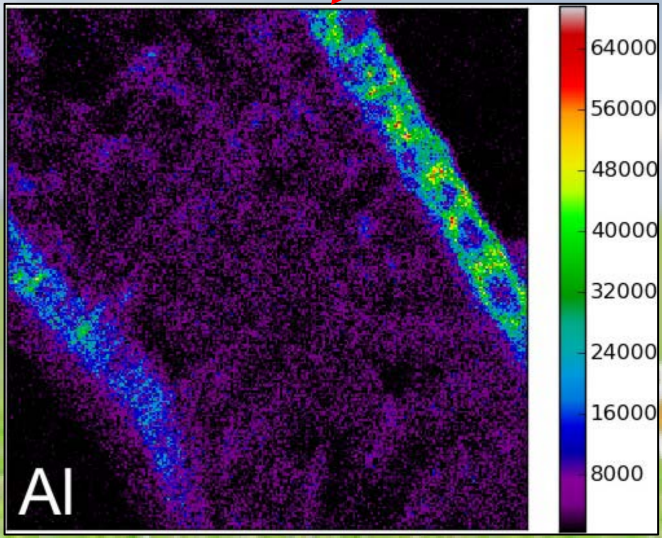
1500 x 1500 µm^2

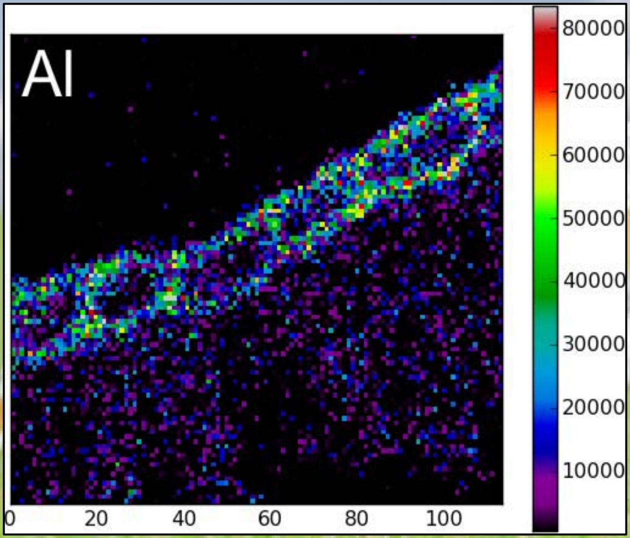






200 x 200 µm^2

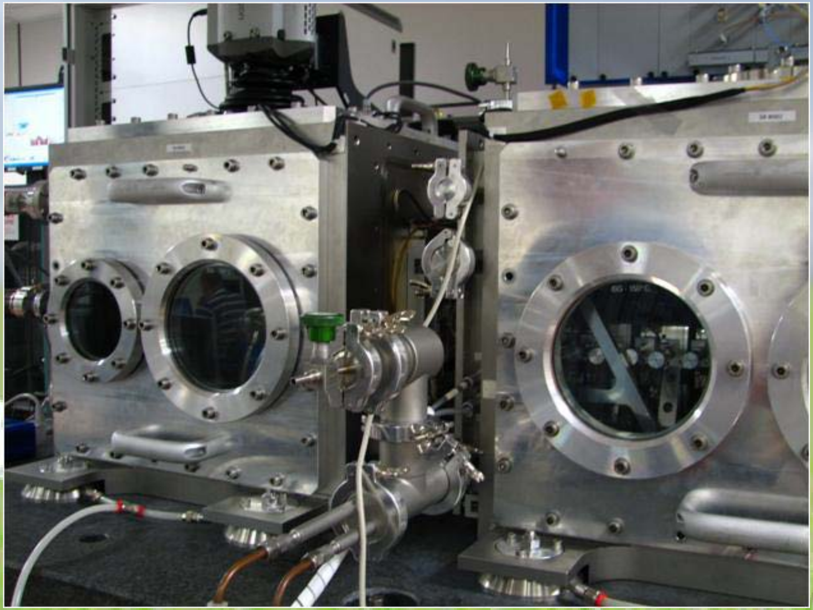


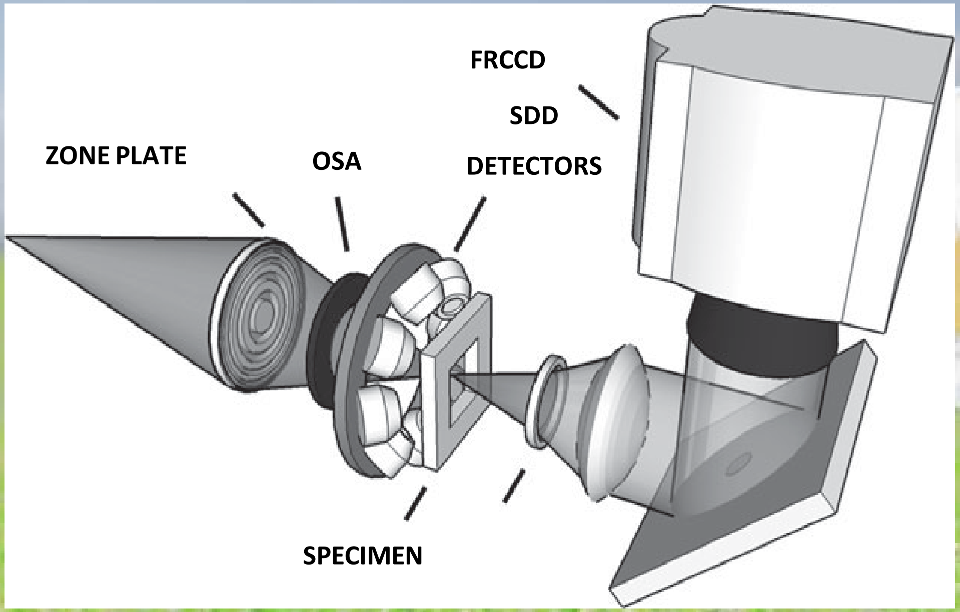


120 x 120 µm^2

TwinMic, Elettra;
X-ray absorption microscopy
Low energy X-ray fluorescence

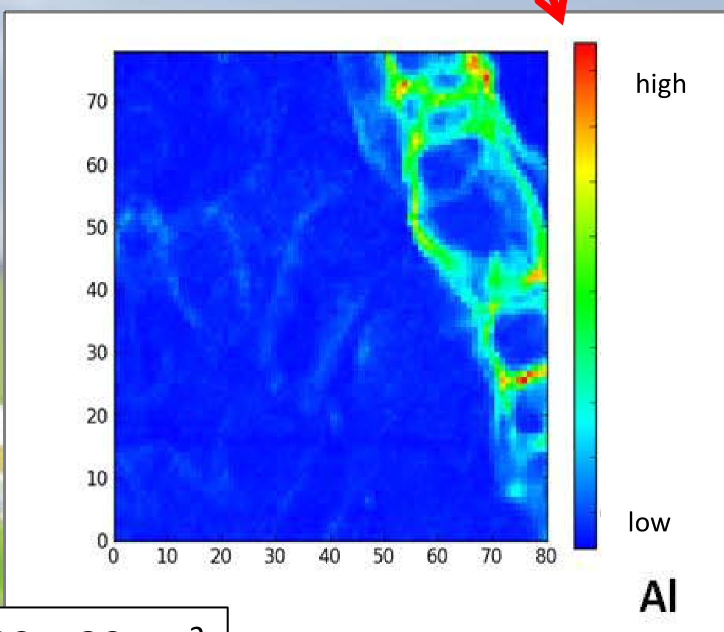
E= 0.5 – 2.0 keV,
beam size 0.8 x 0.8 μm^2
photon flux at 2.0 keV = 2 x 10^7 ph/s/100 mA

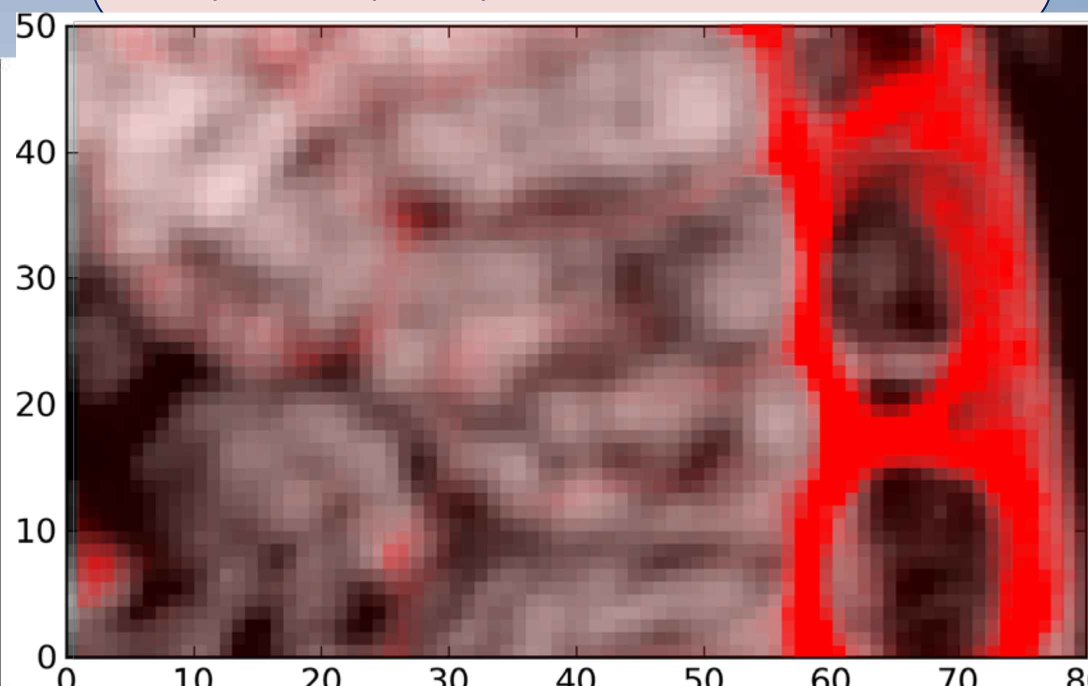




LEXRF, TwinMic
Elettra

TOLRÀ, R., <u>VOGEL-MIKUŠ, K.</u>, <u>KUMP, P.</u>, et al. Localization of aluminium in tea (*Camellia sinensis*) leaves using low energy X-ray fluorescence spectro-microscopy. *J. plant res.*, 2011, vol. 124, no. 1, str. 165-172.





80 x 80µm^2

Food safety
-Mercury and selenium in food plants and mushrooms

Idrija – the second biggest world mercury mine. Wider area is contaminated highly contaminated with Hg.

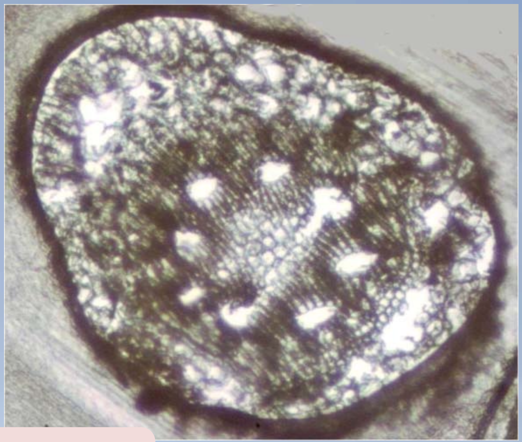
Hg and Se

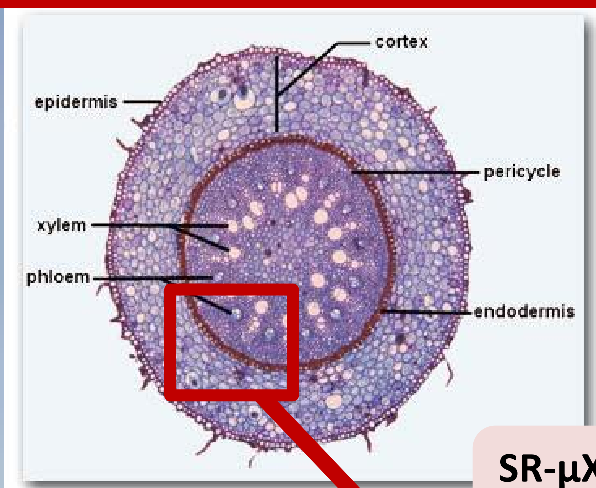
- In plants present in very low concentrations; In Idrija (few – few 10 ppm of Hg, Se not det.)
- μ- PIXE is not sensitive enough to image Hg or Se distribution

- Hg is toxic for organisms already in small amounts – therefore conc. in plants should be kept at minimum

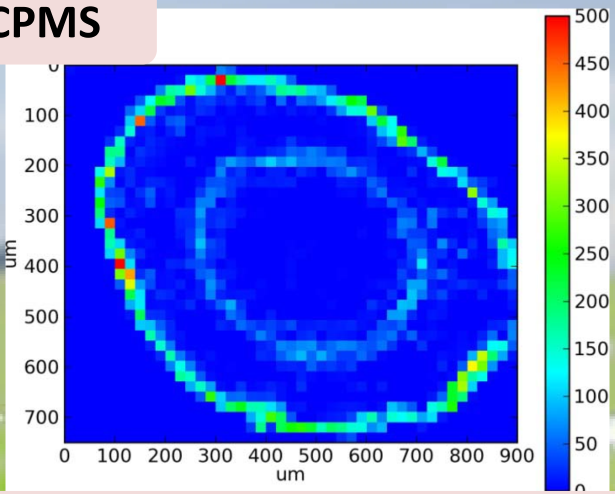
- Toxicity may be alleviated with Se?

Complementing LA- ICPMS and SR-µXRF (ID 22, ESRF)
Hg localization in maize roots



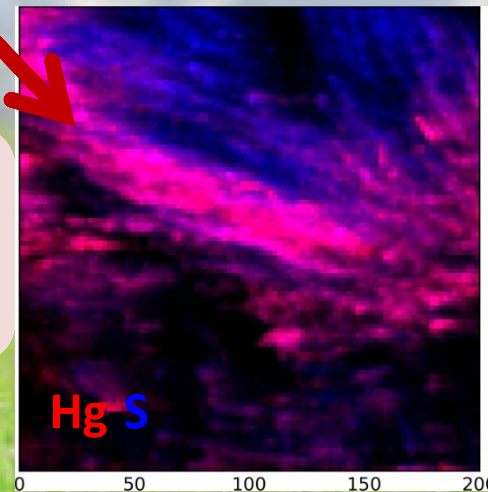


LA-ICPMS



beam diameter, 15 m; laserf luence, 2.5 J cm^{-2}; repetition rate, 20 Hz; dwell time, 1 s; acquisition time, 0.1 s

SR-µXRF, 1 µm x 1 µm beam, frame 200 µm x 200µm



Hg is bound to sulphur in plant roots

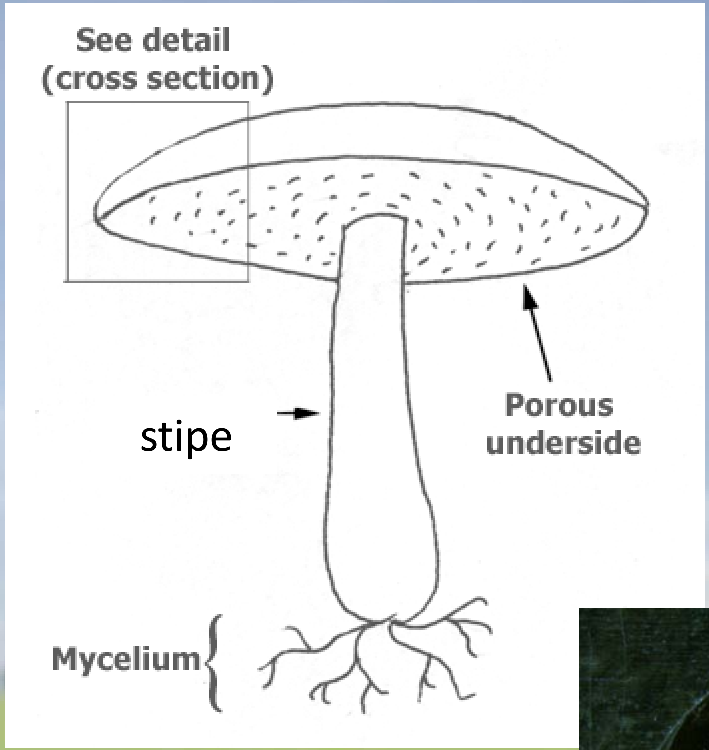
SR-μXRF
(XRF beamline, Elettra - IAEA)
Se and Hg localization in mushrooms

Boletus edulis

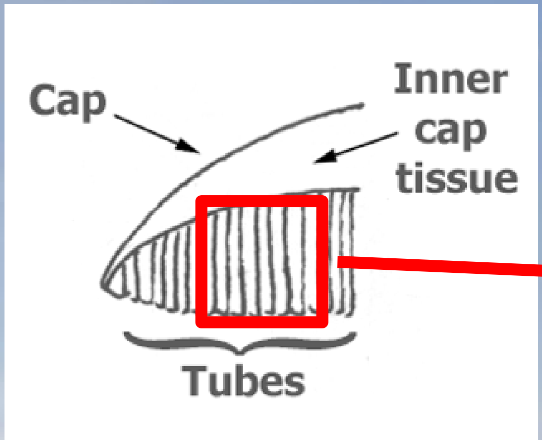
Scutiger (albatrellus) pes-caprae

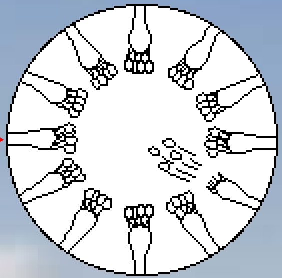
Selenium cocnetrations
Boletus ~ 50 μg/g
Scutiger ~ 500 μg/g

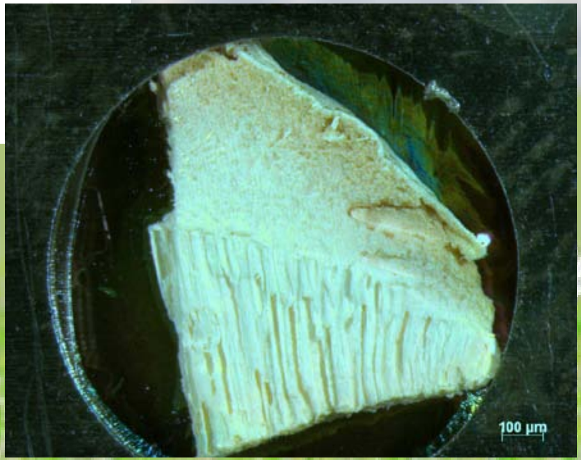
Msuhroom structure

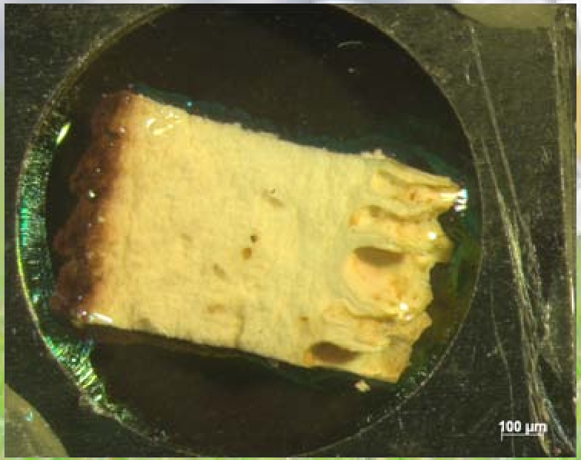


mycorrhiza





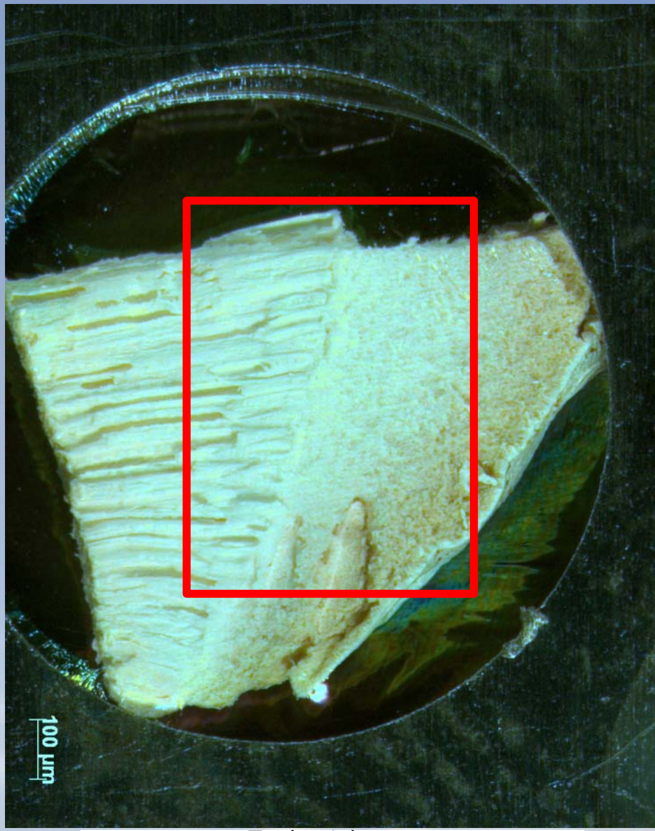


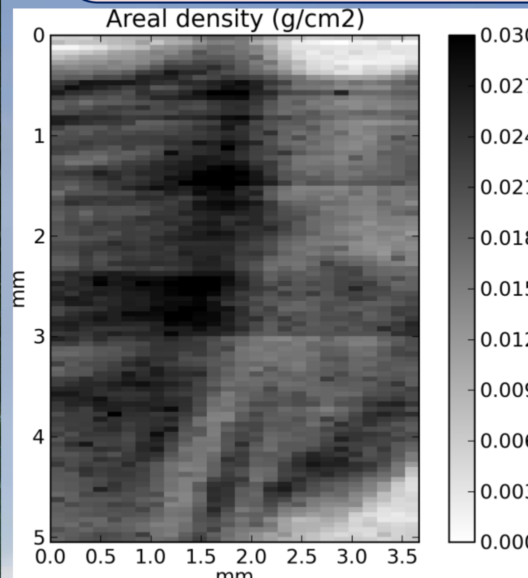


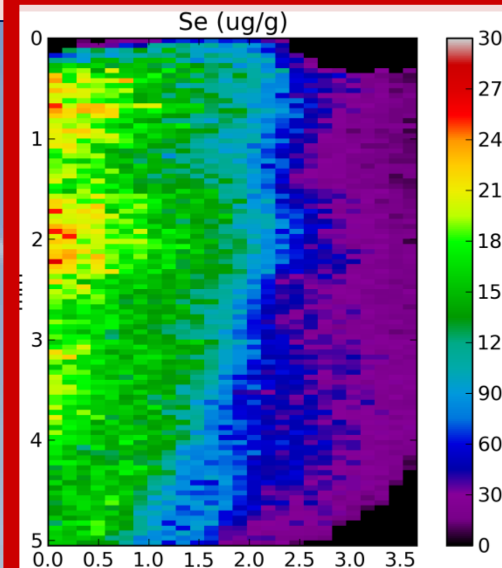
Boletus edulis, cap

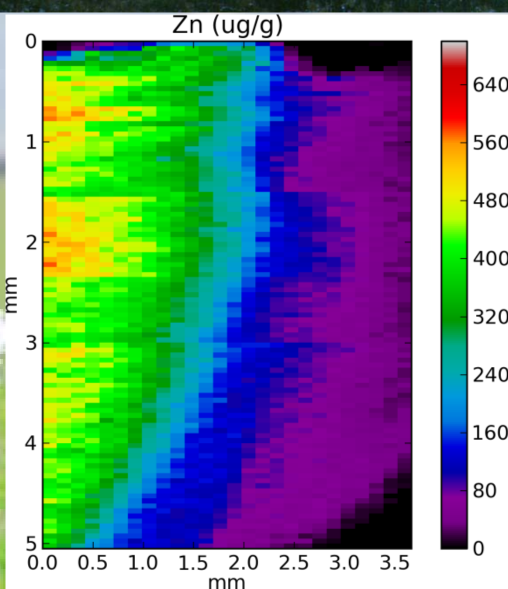
2.5 x 5 mm

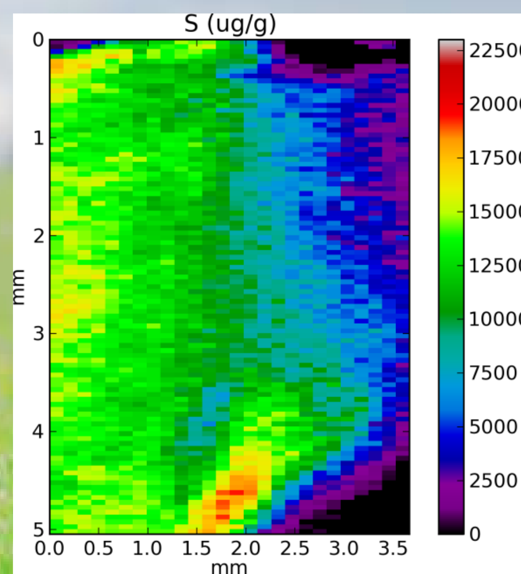
26x101 px, 141x 50 µm, 13 keV

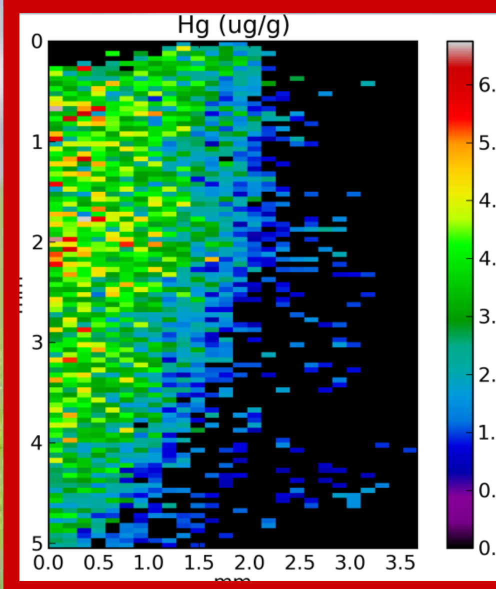




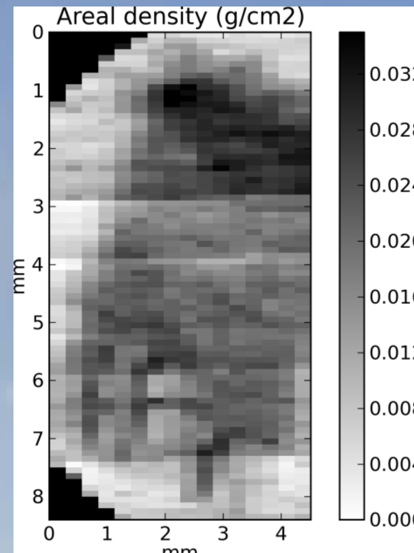


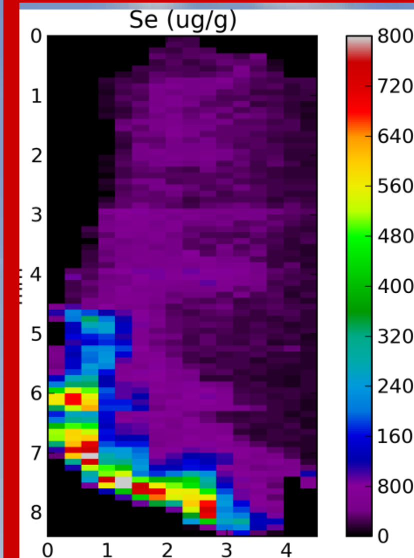


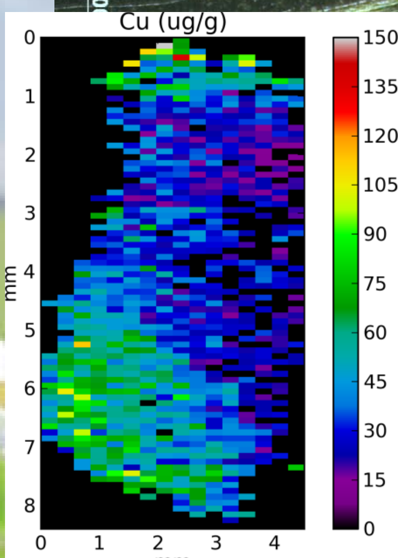


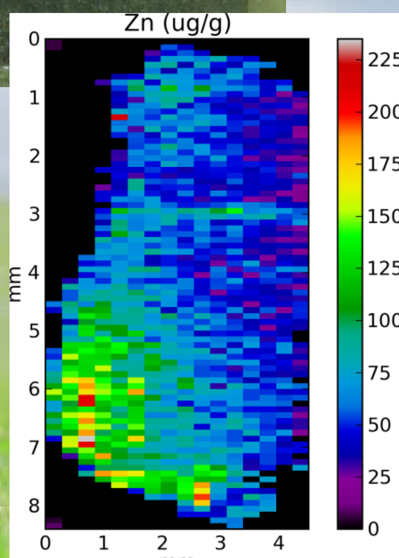


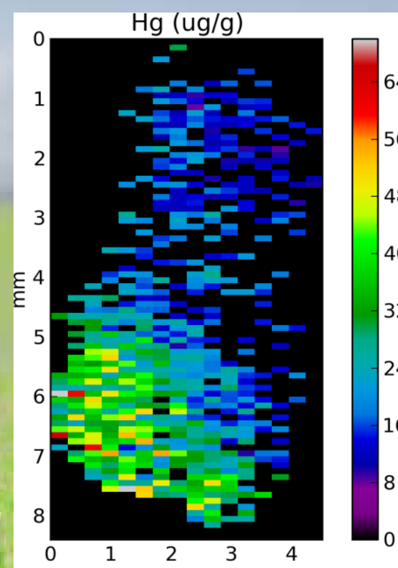
Albatrellus pes-capreae, cap
3x9 mm; 16x92 px; 282x100 µm

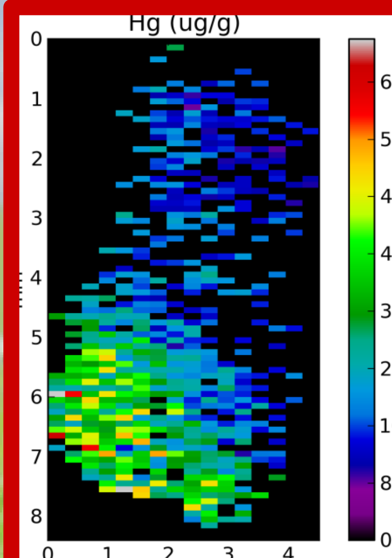




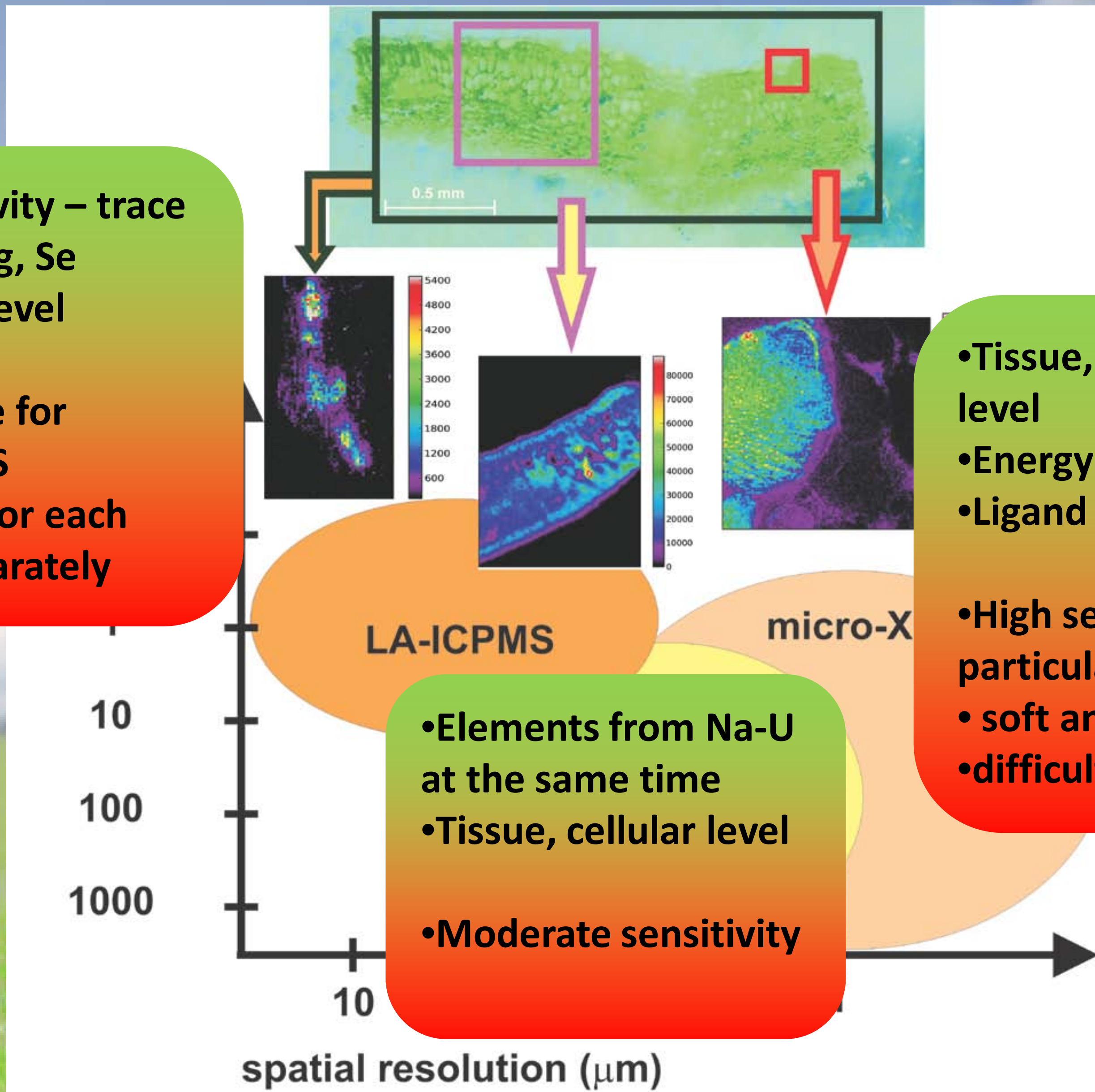








Conclusions



- High sensitivity – trace elements - Hg, Se
- Tissue/cell level

- not suitable for mapping Cl, S
- calibration for each element separately

- Elements from Na-U at the same time
- Tissue, cellular level

- Moderate sensitivity

- Tissue, cellular, subcellular level
- Energy tuning
- Ligand environment (XAS)

- High sensitivity only for particular elements
- soft and hard X-ray regime
- difficult to access

Acknowledgements

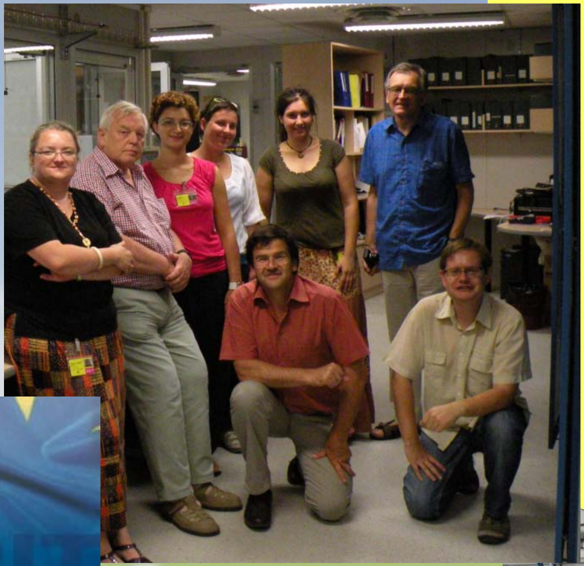
ESRF

elettra

HASYLAB

DESY

IAEA
International Atomic Energy Agency



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