MOSSCLONE PROJECT

Creating and testing a method for controlling the air quality based on a new biotechnological tool. Use of a devitalized moss clone as passive contaminant sensor

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7th BIOMAP - June 15, 2015 - Lisbon, Portugal

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Terrestrial mosses & lichens are especially adequate for air quality assessment due to their high efficiency in loading both particulate and gaseous organic and inorganic pollutants.

Why?

- lack of a root system
- high surface/mass ratio
- good ion exchange properties
- scarce seasonality
- absence of cuticles and protection tissues
- micromorphology

Why use transplants?

i) the material can be exposed according to a rational scheme
ii) it is possible to calculate enrichment rates since pre-exposure values are known
iii) the monitoring can be repeated over time

…problems that can arise when using mosses for moss-bags preparation:

i) the environmental impact due to the sampling of native mosses
ii) the natural variability on moss elemental composition causes a certain variation of moss-bag pre-exposure conditions over time
iii) bag preparation is often a home-made affair
iv) standardized protocols for bag use are still missing

Previous results…

- biological matrices are more efficient than various synthetic materials
- devitalized biological samples accumulate more or in a similar way than alive samples
- mosses (e.g. *Hypnum cupressiforme*) are more efficient than lichens (e.g. *Pseudevernia furfuracea*) for their greater exposed surface
- pollutant uptake by moss is mostly due to interception of airborne particulate matter (PM)
- the careful water-washing of moss and lichen materials selected for bag preparation reduces the variability of the results

MOSSCLONE: a consortium of 5 Academic partners and 5 SMEs

Universidade de Santiago de Compostela (USC)

Albert-Ludwigs-Universität Freiburg (ALU-FR)

Analisi e Monitoraggio del Rischio Ambientale Scarl (AMRA)

Universidade da Coruña (UDC)

Centre National de la Recherche Scientifique (CNRS)

Biovía Consultor Ambiental (BIOVIA)

Orion SRL (ORION)

Tecno Ambiente (TecAmb)

T.E. Laboratories Ltd (TeLabs)

Maderas Ornanda S.A. (MadO)

Objectives of the MOSSCLONE project:

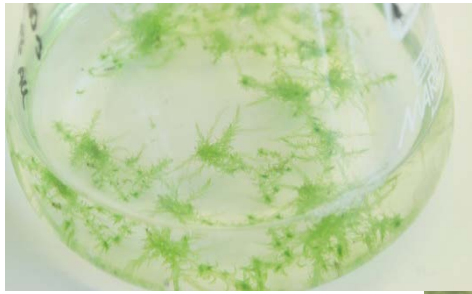
1) Selection of a moss species on the basis of existing knowledge
2) Cultivation of the selected species as a clone
3) Morphological, molecular and physical-chemical characterisation of the selected moss clone
4) Scaling up of the selected clone
5) Design and standardisation of moss-bags in terms of element accumulation through the selection of shape, mesh, ideal ratio moss mass/bag size; exposure: height and time
6) Tool validation: comparison between moss-bags and traditional techniques (i.e. bulk deposition collectors, airborne particles and gaseous pollutants samplers)

COOPERATION

clone MOSS

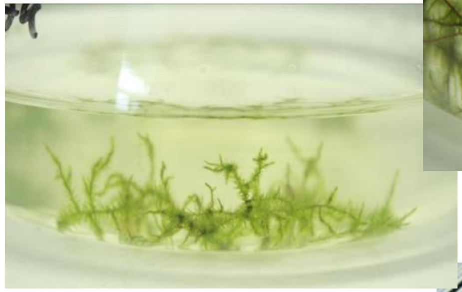
Mosses in axenic *in vitro* culture

- *Physcomitrella patens* & related species
- 17 additional species were established in *in vitro* culture during the last years
- species established during the MOSSCLONE project:
    - *Sphagnum palustre*
    - *Hylocomium splendens*
    - *Hypnum cupressiforme*
    - *Rhynchostegium/Platyhypnidium riparioides*
    - *Pseudoscleropodium purum*

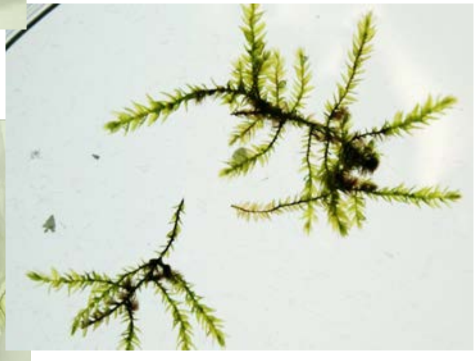


Sphagnum palustre

Hylocomium splendens



Hypnum cupressiforme



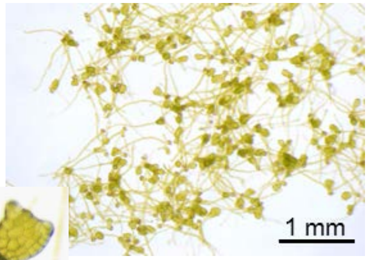
Rhynchostegium riparioides
(picture: David Crespo Pardo)

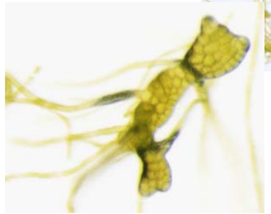
Pseudoscleropodium purum

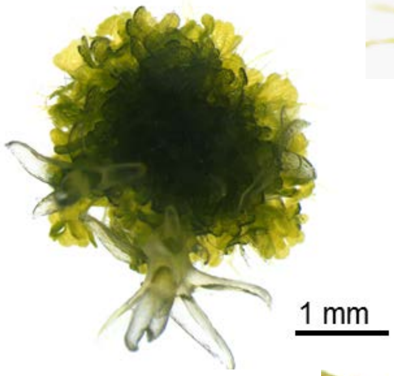
Beike et al. 2014

Sphagnum palustre: from spores to *in vitro* cultivation

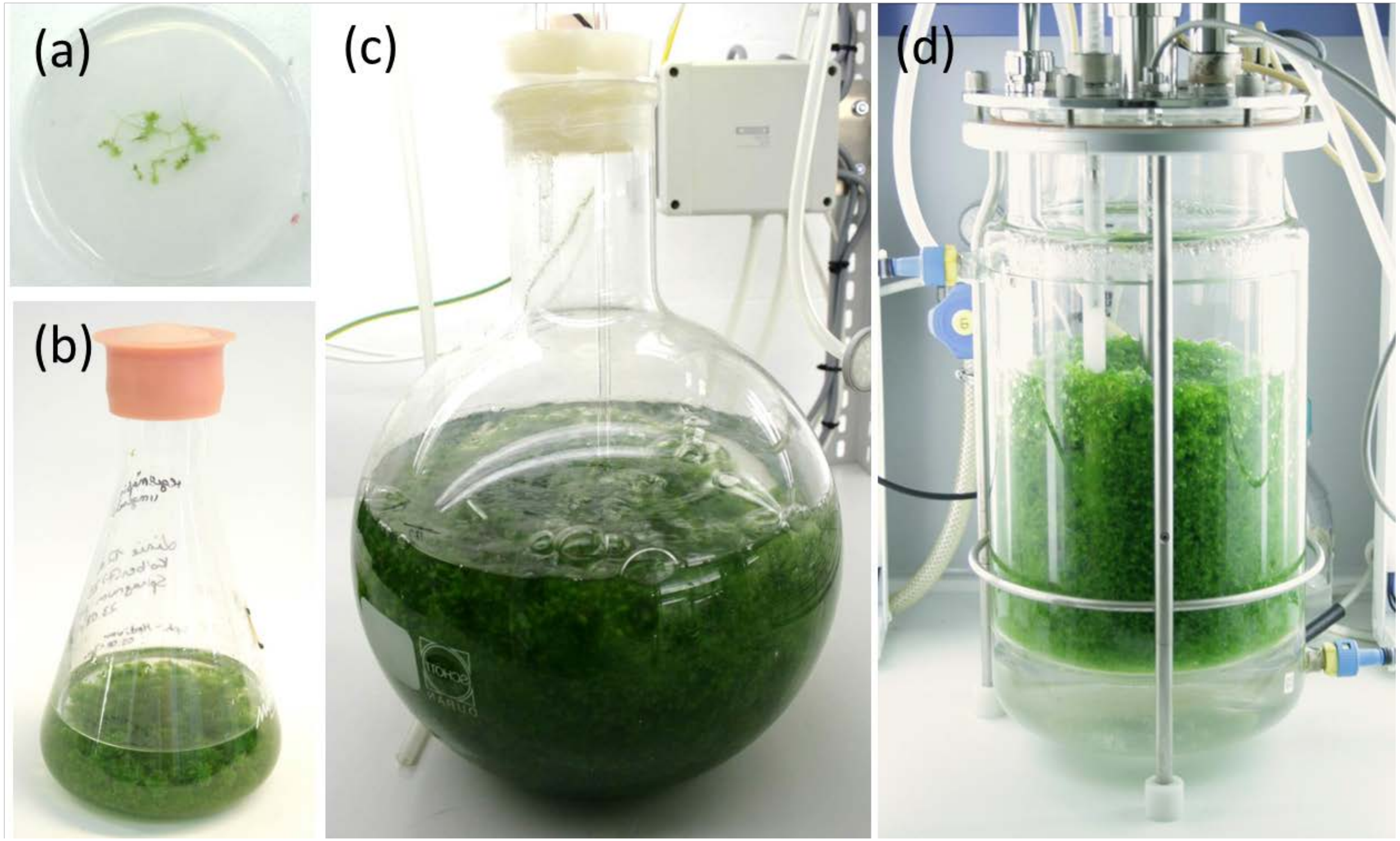
- sterilization of spores from capsules
- spores germinated within a few days
- single protonemata were isolated to establish clonal material
- independent clones were cultivated







Different *in vitro* cultivation techniques



- 5 - 12 L working volume
- no disruption with a stirrer
- aerated with 0.3 vvm air
- 25 ºC
- 120 µE (µmol/m^2 per second) light intensity
- 16 h light/ 8 h dark photoperiod
- starting pH 4.0 – 4.1, not adjusted during cultivation

The optimized medium for *S. palustre* cultivation

KH_2PO_4	1.84 mM
KCL	3.35 mM
$MgSO_4 * 7\ H_2O$	1.01 mM
$Ca(NO_3)_2 * 4\ H_2O$	4.24 mM
$FeSO_4 * 7\ H_2O$	45 µM
H_3BO_3	50 µM
$MnSO_4 * 1\ H_2O$	50 µM
$ZnSO_4 * 7\ H_2O$	15 µM
KJ	2,5 µM
$Na_2MoO_4 * 2\ H_2O$	500 nM
$CuSO_4 * 5\ H_2O$	50 nM
$Co(NO_3)_2 * 6\ H_2O$	50 nM
Sucrose	68 mM*
NH_4NO_3	1.25 mM

1. Sphagnum palustre was selected to be cloned

- * mo ... imola (1969 ... iol. Plant. 22: 1079-1084
- Simola (1975)
  Physiol. Plant. 35: 194-199

Morphological comparison

	clone		field	
	length	wideness	length	wideness
stem leaves	1756	731	1600	1150
branch leaves	1969	715	2250	1450
hyalocysts	146	27	338	50
chlorocysts	115	11	62	9
weight	2.72 ± 1.13		14.76 ± 7.20	
pore diameter	5-8		10-25	

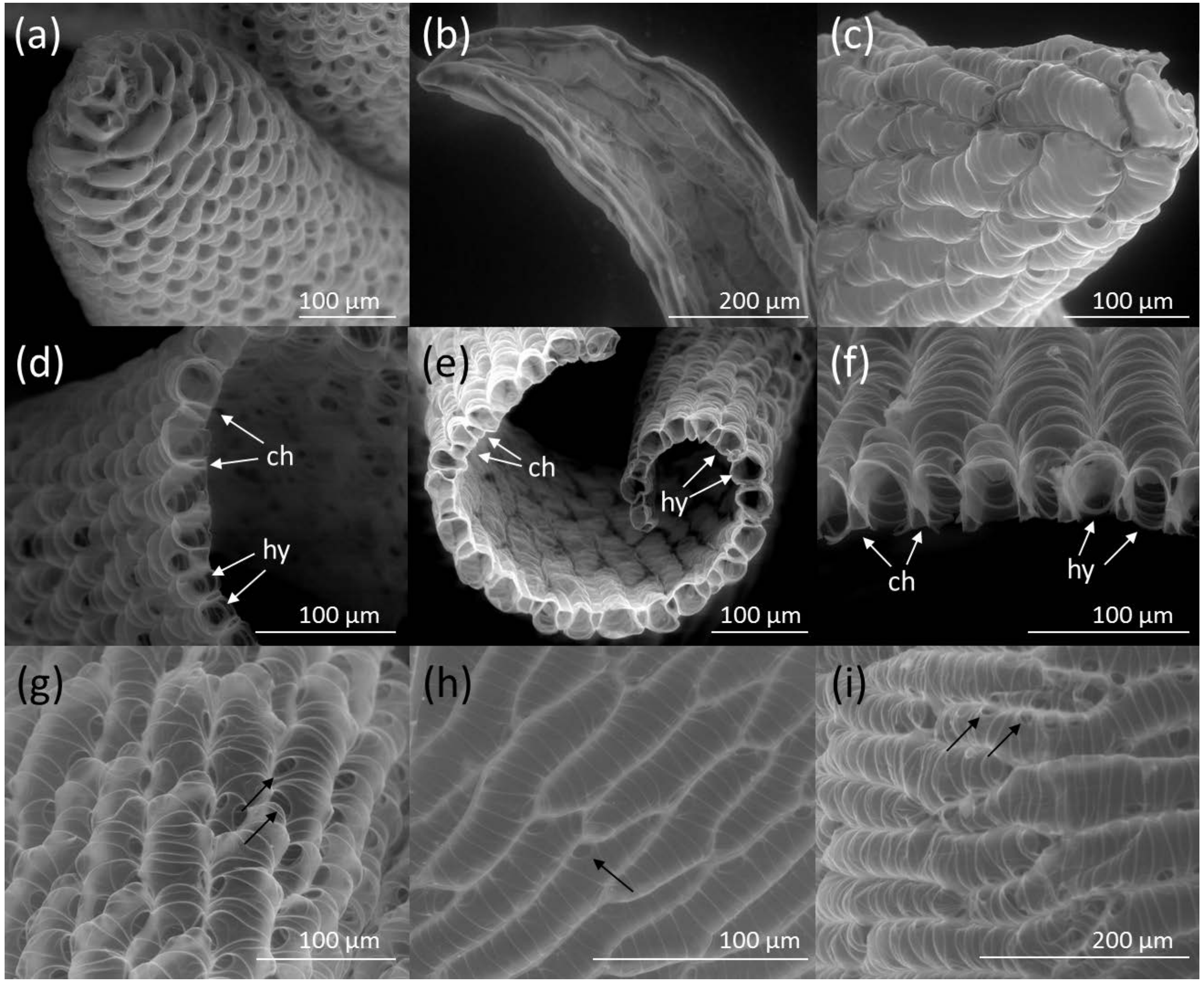
Morphological analysis

Plant Cell Tiss Organ Cult (2015) 120:1037–1049
DOI 10.1007/s11240-014-0658-2

ORIGINAL PAPER

Clonal in vitro propagation of peat mosses (*Sphagnum* L.) as novel green resources for basic and applied research

Anna K. Beike · Valeria Spagnuolo · Volker Lüth · Feray Steinhart · Julia Ramos-Gómez · Matthias Krebs · Paola Adamo · Ana Isabel Rey-Asensio · J. Angel Fernández · Simonetta Giordano · Eva L. Decker · Ralf Reski



Giving the clone a molecular tag

microsatellite analysis

4 polymorphisms detected

	Sample		
Locus (repeat motif)	*Clone 2a*	*Clone 12a*	*Field*
1 (CA)	244-254	244-254	244-254
3 (CA)	169	169	169
5 (GT)	192-198	192-198	188-192
9 (CT)	159-174	159-174	169-184
10 (GA)	233	233	233
14 (AG)	228	228	214
17 (AAG)	159	159	162
19 (AAG)	246-267	246-267	246-267
20 (TTC)	264-289	264-289	264-289
22 (GAT)	99-102	99-102	99-102
28 (AC)	225-237	225-235	225-235
29 (AAG)	194-197	194-197	194-197
30 (GAT)	139-142	139-142	139-142

analysis of RFLP-PCR of the anonymous region RAPDf*

7 polymorphisms detected

2. The clone is slender than field *S. palustre* and was molecularly tagged

*Shaw et al. (2003) American Journal of Botany 90: 1777–1787.

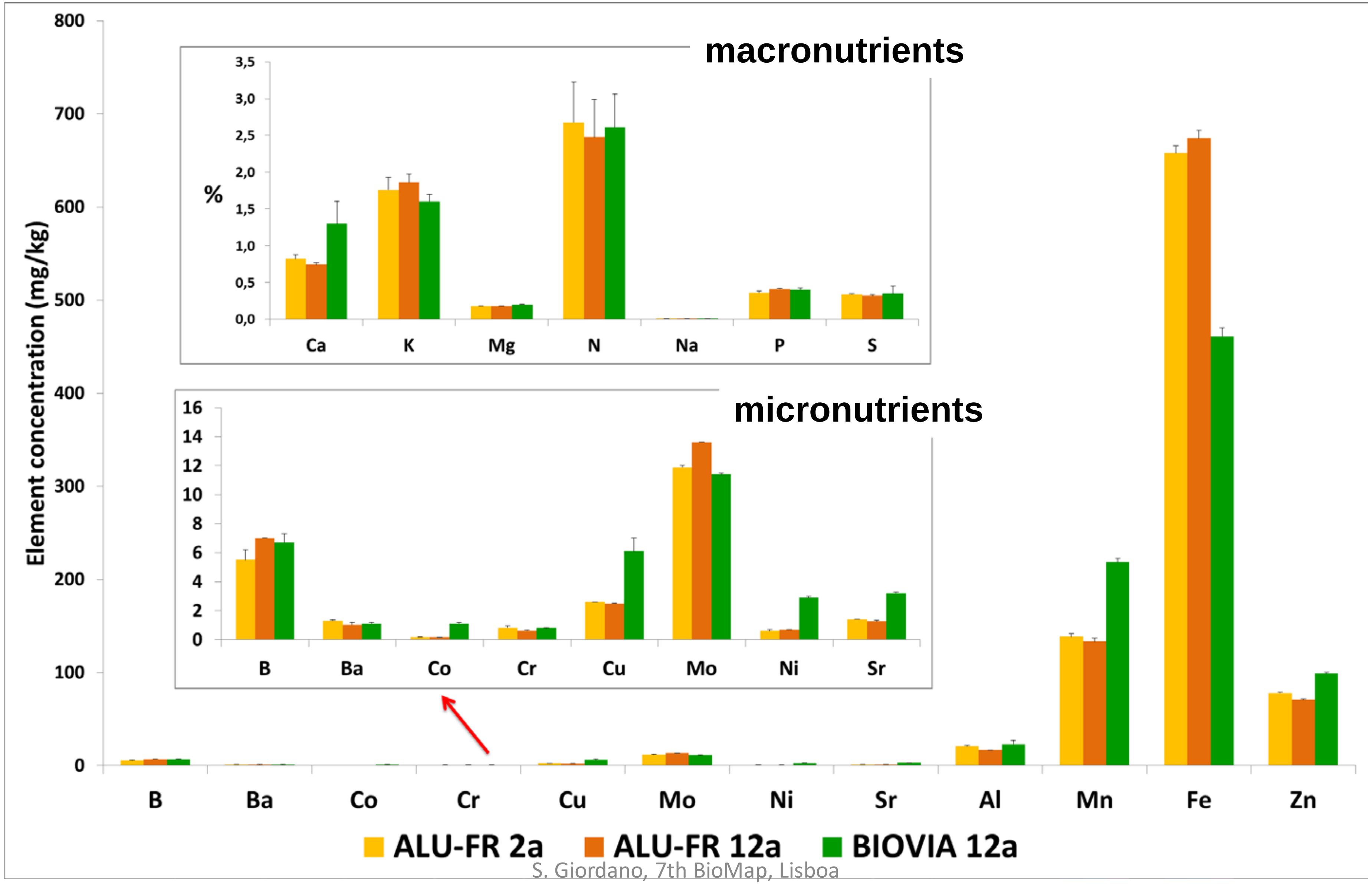
Chemical and physical-chemical characterisation of *Sphagnum palustre* clones

- ✓ to know initial values
- ✓ to check homogeneity
- ✓ to understand the mechanisms of pollutants uptake and retention

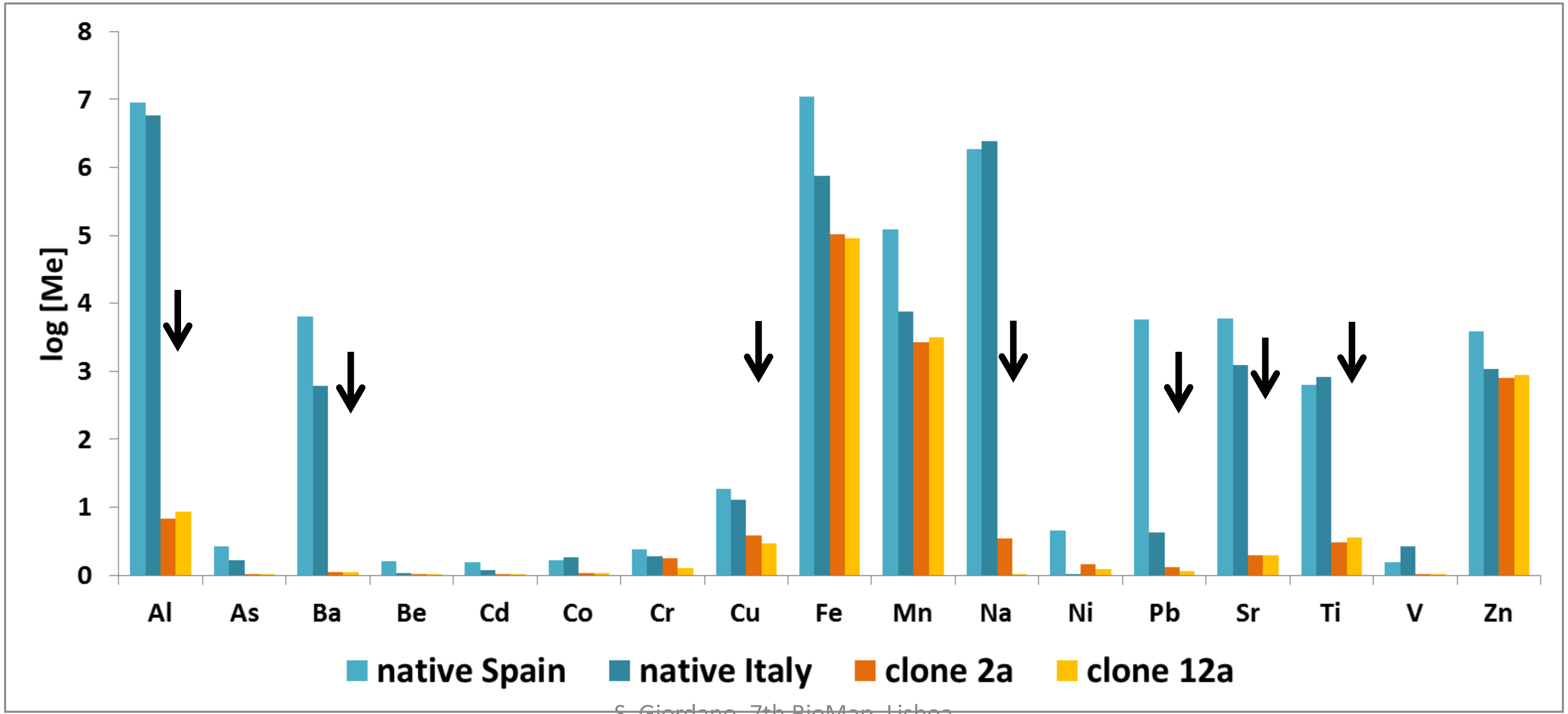
Elemental analysis

- ✓Elemental analysis was performed in TeLabs laboratory
- ✓3 replicates for each sample
- ✓Microwave HNO_3 digestion on powdered moss samples
- ✓ICP MS analysis
- ✓M2 and M3 certified reference mosses from the Finnish Forest Research Institute (Steinnes *et al., 1997).*

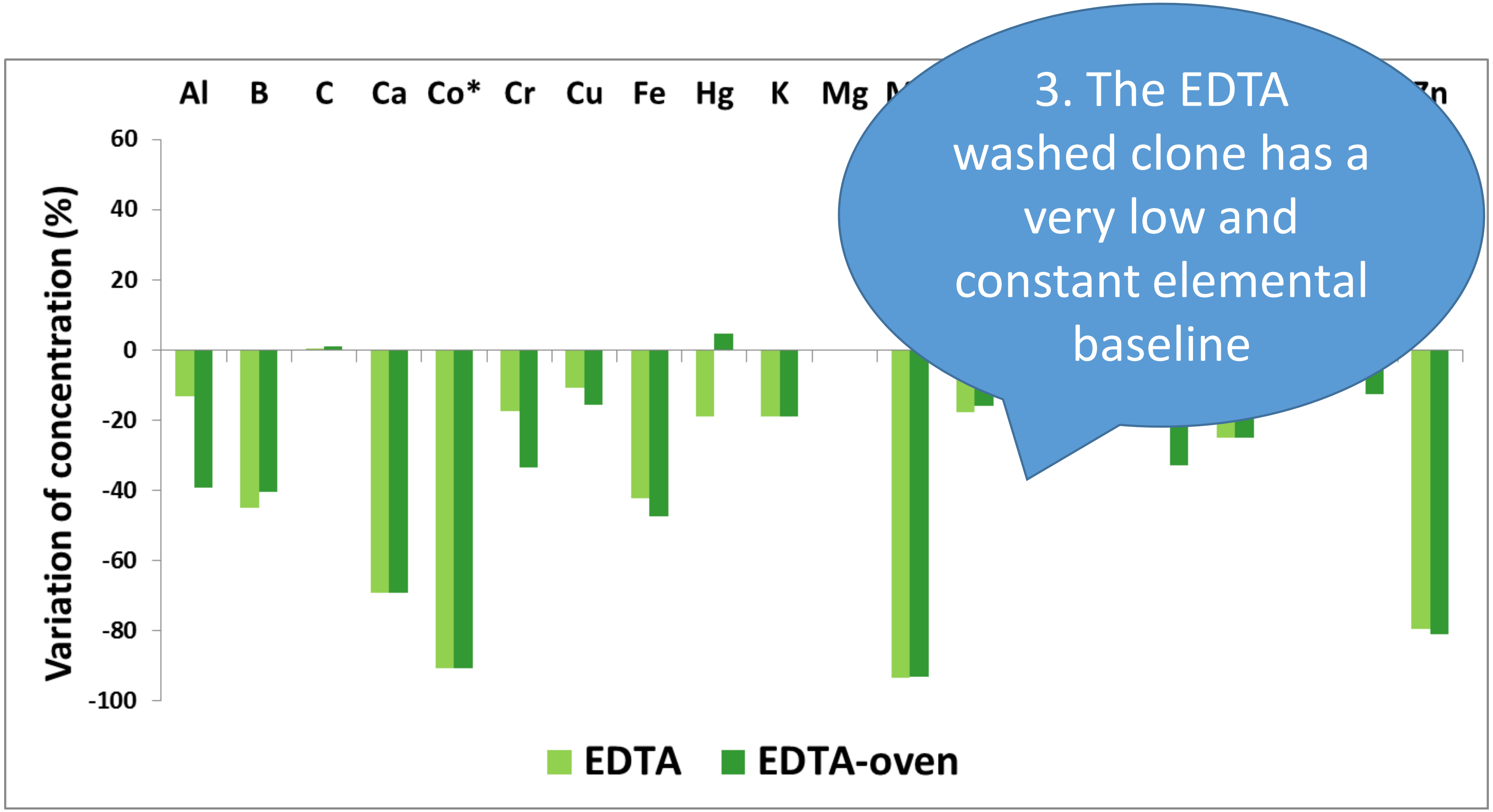
Chemical composition of clones



Chemical Composition of clones *vs* field *Sphagnum palustre*

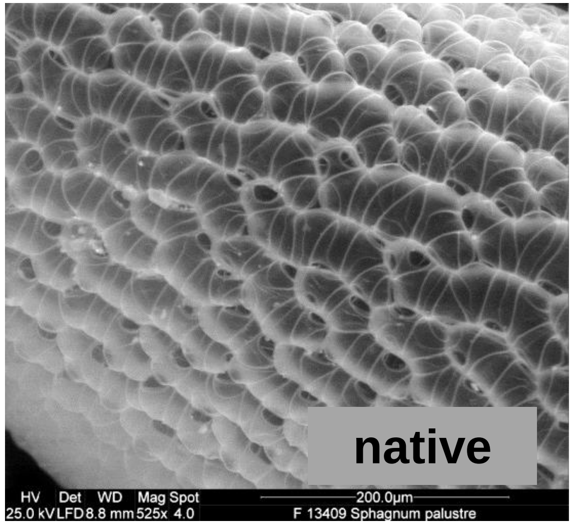


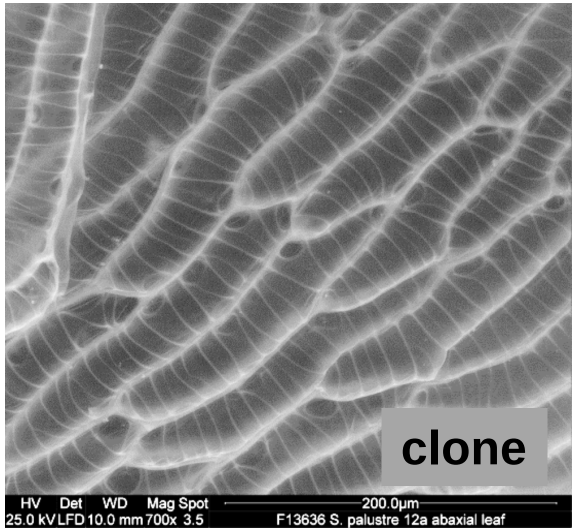
Variation (%) of element concentration in clone after EDTA washing



*Co, As, Be, Cd, Pb, V under detection limit after treatments

Specific Surface Area using B.E.T. N_2 multipoint adsorption technique

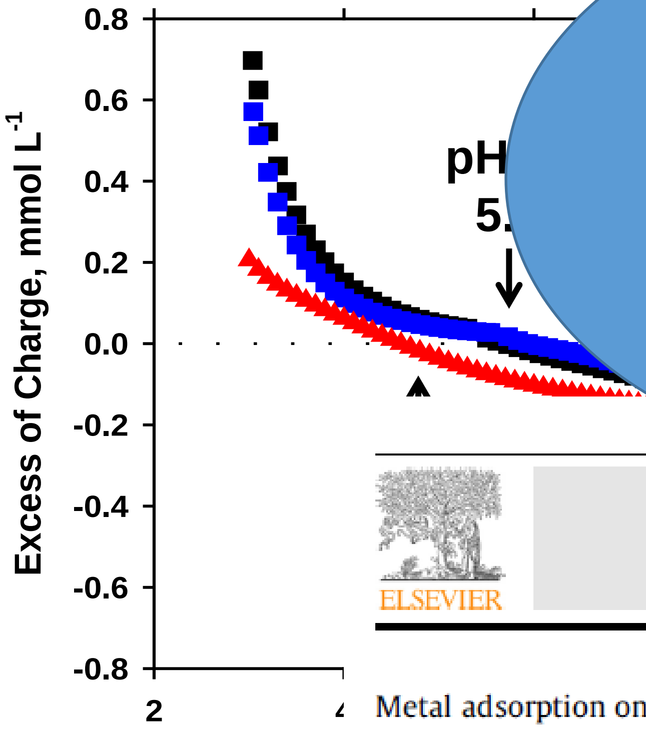




Native	Clone-2A	Clone-12A
10.8 ± 0.3 m^2/g	24 ± 1 m^2/g	28 ± 1 m^2/g

Danish peat (13.3 $m^2\ g^{-1}$) and Heilongjiang peat (9.67 $m^2\ g^{-1}$) *Qin et al., 2006*

Surface acid-base titration



4. higher SSA and charge in the clone than in field *S. palustre*

	Total binding sites (mmol g^{-1})
Clone 2a	1.56
	1.31
	0.65

lists available at ScienceDirect
Journal of Colloid and Interface Science
www.elsevier.com/locate/jcis

Metal adsorption on mosses: Toward a universal adsorption model

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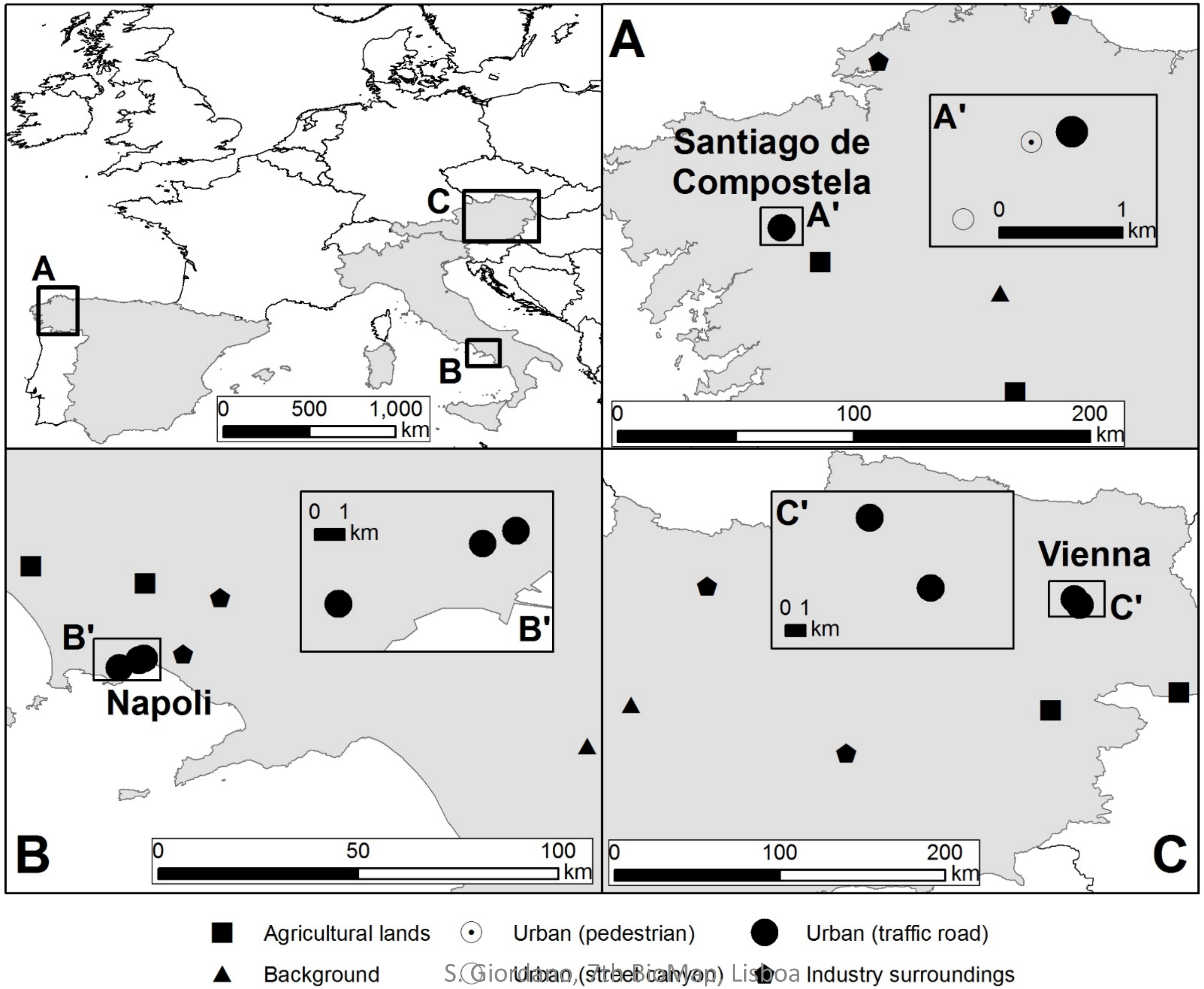
Possible functional groups: phosphodiester (pK_a=3.6–3.7), carboxyl (pK_a = 4.7–5.7), phosphoryl (pK_a = 5.9–7.4), amine (pK_a = 7.7–9.2) and polyphenols (pK_a = 10.1–10.4)

Standardisation assay Task:

- **Country**
  - Austria
  - Italy
  - Spain
- **7 exposure sites**
  - 2 Urban
  - 2 Industrial
  - 2 Rural
  - 1 Background level
- **Standardisation**
  - Shape effect (Sphere, Bag, Envelope)
  - Mesh effect (1,2,4 mm)
  - Weight effect (15,30,45 mg/cm^2)
  - Height effect (4, 7, 10 m)
  - Exposure Time (3, 6, 12 weeks)
- **3 replicates for each item**

Standardisation assay Task



Species used for the standardisation assay

204 kg f.w. harvested!

14 kg d.w. 1134 bags prepared!

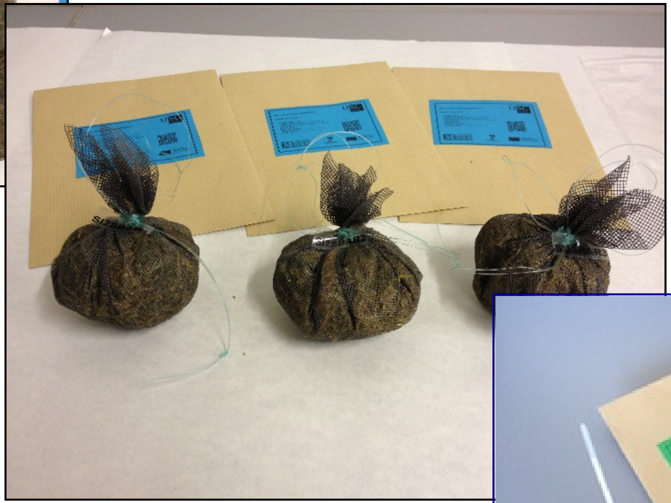
Pseudoscleropodium purum
(Hedw.) M.Fleisch.

Standardisation assay Task

RESULTS “Shape Effect”

Mesh size: 2 mm

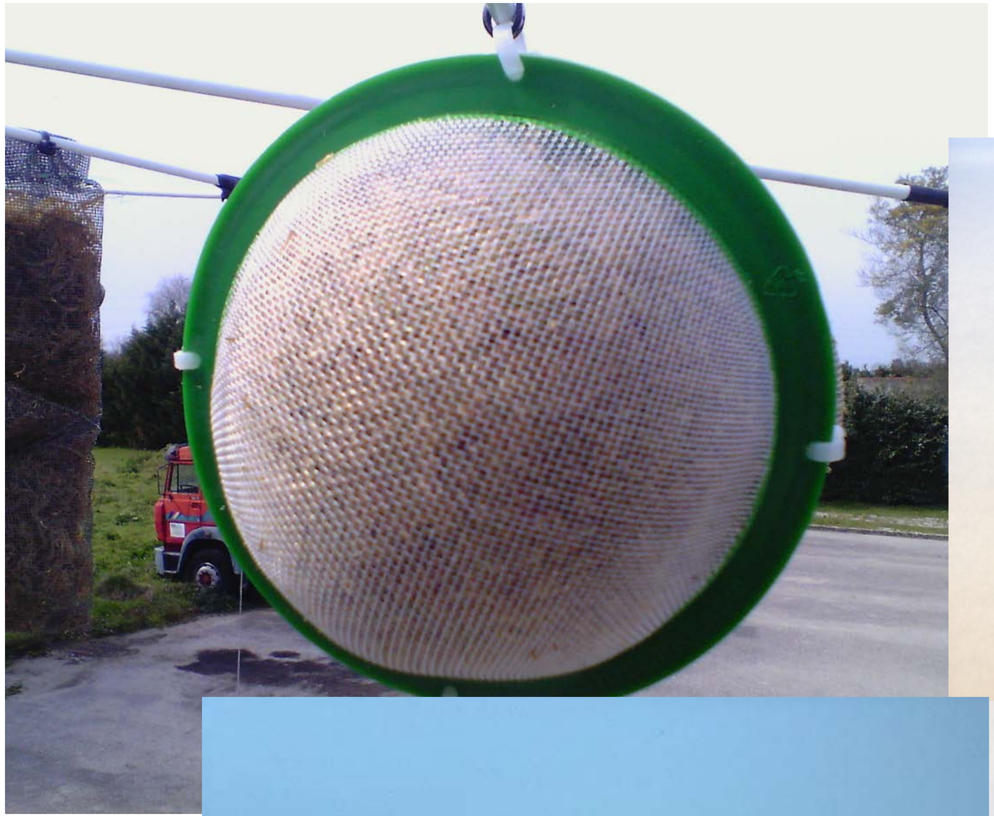
Envelope

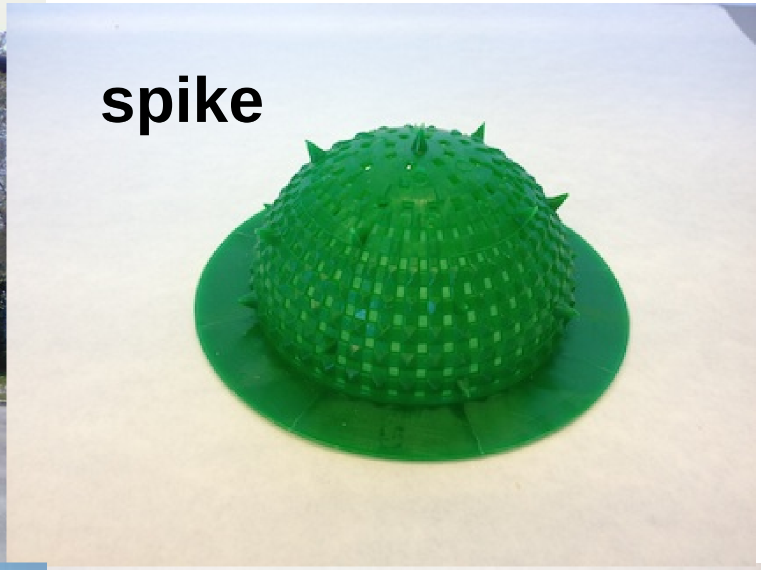


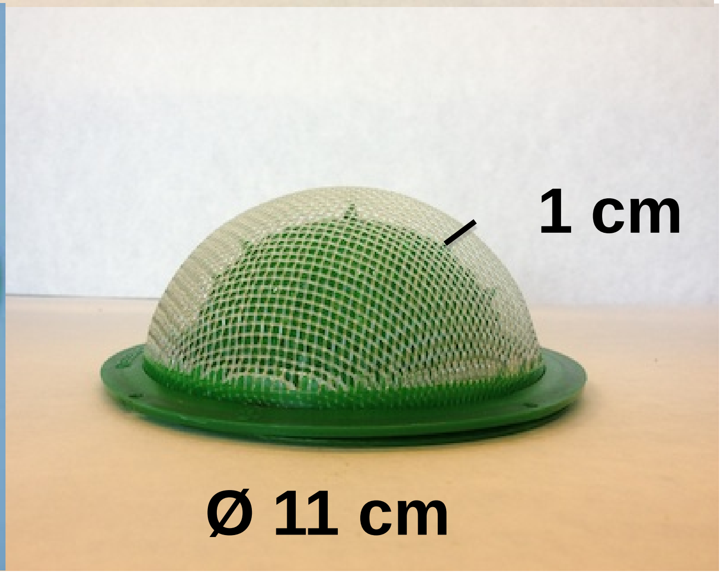
Bag

Sphere

The new device







RESULTS “Shape Effect”

RESULTS “Shape Effect”

Shape	Al	Ba	Cr	Cu	Fe	Hg	Pb	Ni	Sr	Zn	Tot
Envelope Vs Sphere 15	**E*>S**	E=S	E=S	E=S	**E<S**	E=S	E=S	E=S	**E<S**	**E>S**	**=**
Bag Vs Sphere 30	B=S	B=S	B=S	**B>S**	**B<S**	**B<S**	B=S	**B>S**	B=S	**B>S**	**=**

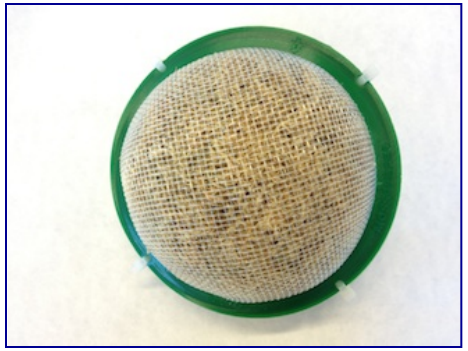
*E=envelope; S=Sphere; B=bag

The **Mossphere** should be preferred because it secures an improved standardisation of bag preparation

Wilcoxon matched pairs test **p-value<0,05**

RESULTS “Mesh Effect”

Weight: 30 mg/cm^2



1 mm   2 mm   4 mm

RESULTS "Mesh Effect"

Mesh	Al	Ba	Cr	Cu	Fe	Hg	Pb	Ni	Sr	Zn	Mesh size	Tot
M01 Vs M02	M01=M02	M01=M02	**M01>M02**	**M01>M02**	**M01>M02**	M01=M02	M01=M02	M01=M02	M01=M02	M01=M02	**M01**	**25%**
M01 Vs M04	M01=M04	**M01<M04**	**M01>M04**	**M01>M04**	M01=M04	M01=M04	**M01<M04**	M01=M04	**M01<M04**	**M01<M04**	**M02**	**0%**
M02 Vs M04	M02=M04	**M02<M04**	M02=M04	M02=M04	M02=M04	M02=M04	**M02<M04**	M02=M04	**M02<M04**	**M02<M04**	**M04**	**40%**

The mesh size does not show any clear pattern affecting the accumulation of elements

The selection of the proper mesh size must take into account the loss of material occurring during the exposure

Wilcoxon matched pairs test **p-value<0,05**

RESULTS "Weight Effect"

Mesh size: 2 mm

Weight	15 mg/cm^2	30 mg/cm^2	45 mg/cm^2

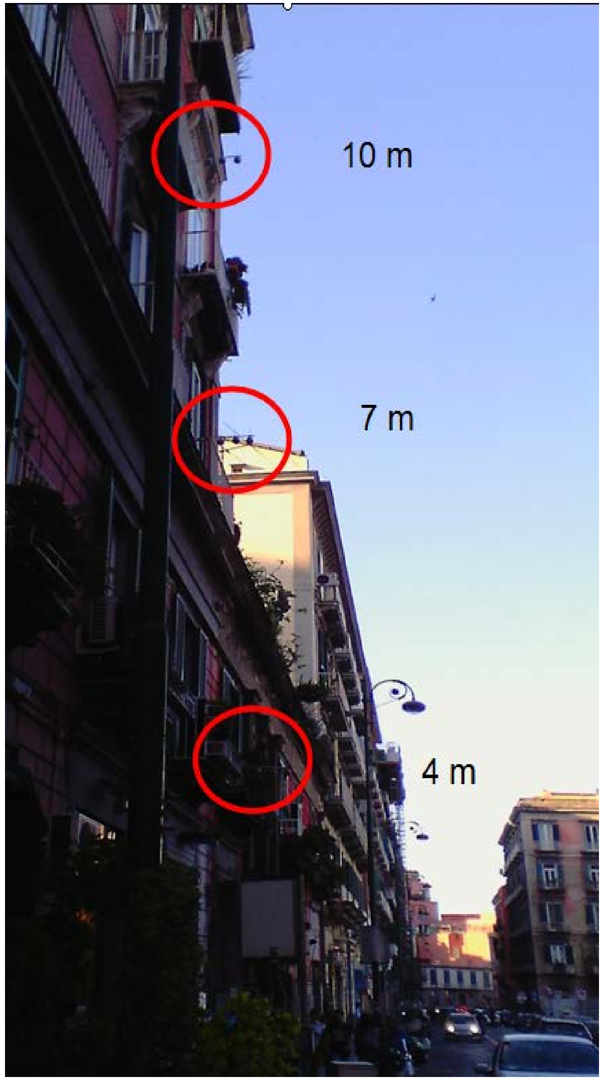
RESULTS “Weight Effect”

Weight	Al	Ba	Cr	Cu	Fe	Hg	Pb	Ni	Sr	Zn	Weight	Tot
15 Vs 30	**15>30**	**15>30**	15=30	**15>30**	**15>30**	15=30	**15>30**	**15>30**	**15>30**	**15<30**	15	80%
15 Vs 45	**15>45**	**15>45**	15=45	**15>45**	**15>45**	15=45	**15>45**	**15>45**	**15>45**	**15>45**	30	20%
30 Vs 45	**30>45**	30=45	30=45	**30>45**	**30>45**	30=45	30=45	30<45	30=45	**30>45**	45	0%

The smallest amount of moss seems to ensure an improved accumulation performance

Wilcoxon matched pairs test **p-value<0,05**

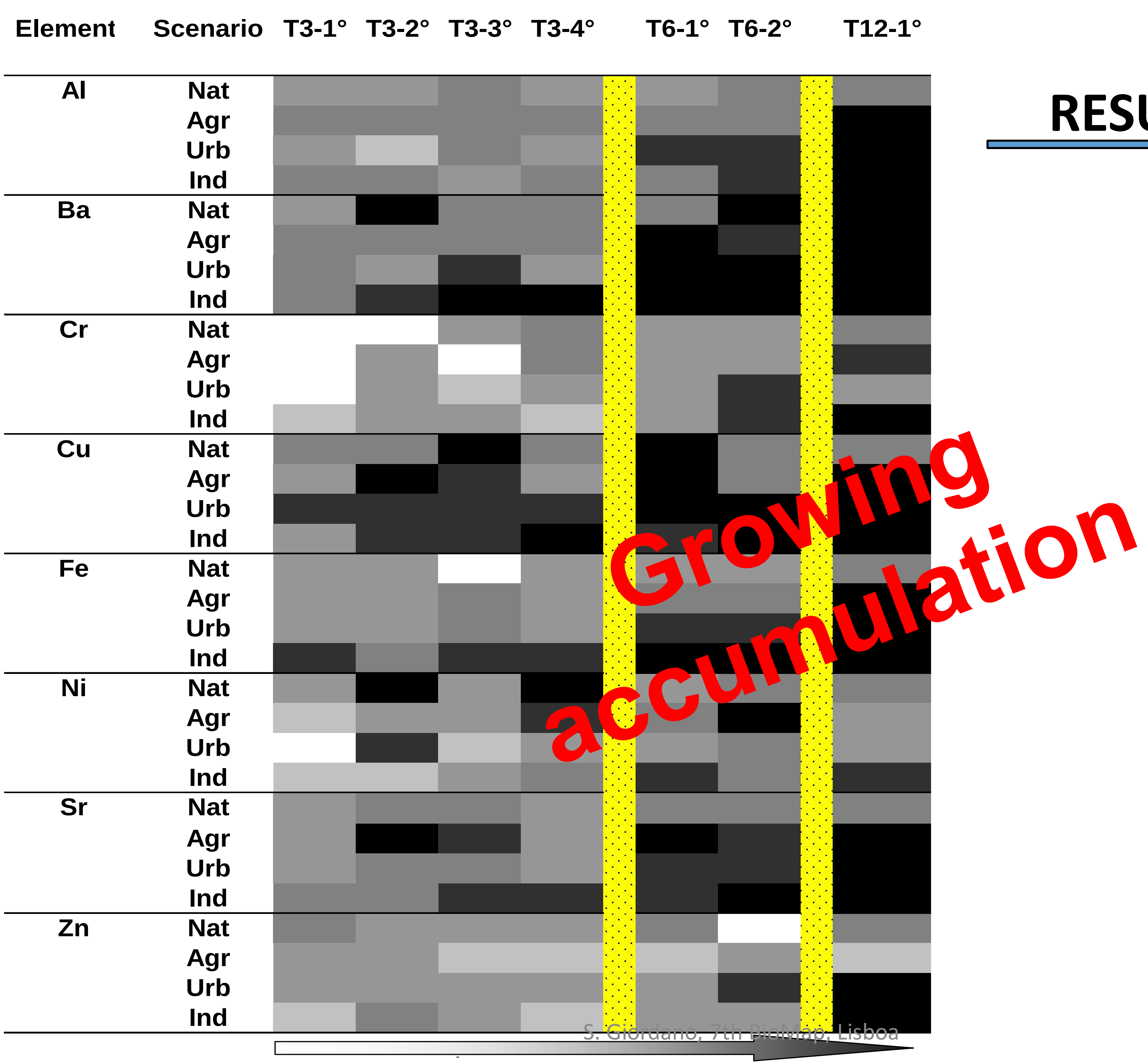
RESULTS "Height Effect"



The height range investigated (4-10 m) did not show any pattern related to the enrichment of the post-exposed moss

The selection of the exposure height may depend on practical questions

RESULTS “Time Effect”



5. Exposure was standardised

Shape	Mossphere
Mesh	2 mm
Weight	15 mg/cm^2
Height	4 m
Exposure period	6 weeks

SCALING UP MOSSCLONE

Growth condition

- *Culture medium*: 12 L ml knop + microelements + **2 % sacarose** + amonium nitrate
- *Initial pH*: 4 (before autoclaving)
- *Temperature*: 25 ºC
- *No Photoperiod*
- Agitation with air flow
- *Time of incubation*: 31 days

- *Initial culture : 6 flask*
- *Minimum weight: 100 g f.w./12 L (≈2 g d.w.)*
- *Mean weight: 222 ± 34 g f.w. (4.45 d.w.)*

- *4 Bioreactor*
- *12 L culture medium*

- *Final fresh weight: 1404 ± 285 g*
- *Final dry weght: 118 ± 25 g*

Production rate: 3,8 g d.w./day

Annual production (4u): 4 kg d.w.

MOSSPHERE PRODUCTION

Bioreactor culture

↓

Washed
1 EDTA (10 mM)
(20´ ; 1 L/12,5 g d.w.)
3 Distilled water
(20´; 1 L/10 g d.w)

↓

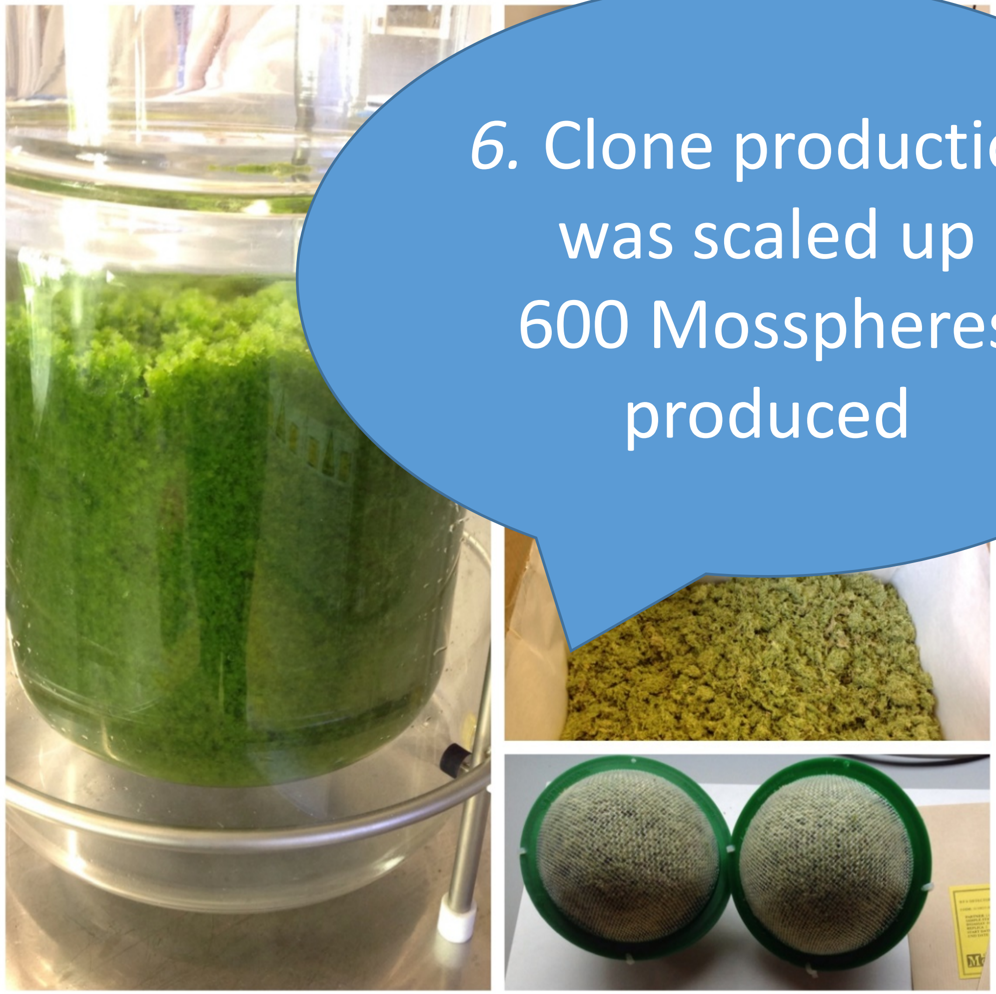
Devitalized
8 h – 50 ºC
8 h – 80 ºC
8 h – 100 ºC

↓

Stored
Bag vacuum bag

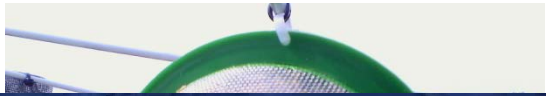
↓

Mossphere (3 g d.w.)

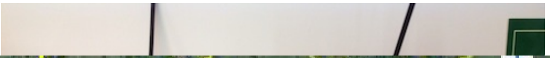


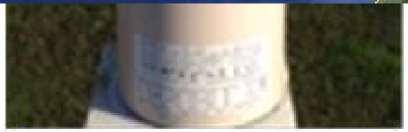
6. Clone production was scaled up
600 Mosspheres produced

UNSHELTERED MOSSPHERE

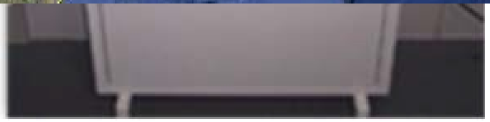


SHELTERED & UNSHELTERED MOSSPHERE





BULK DEPOSIMETER FOR METALS

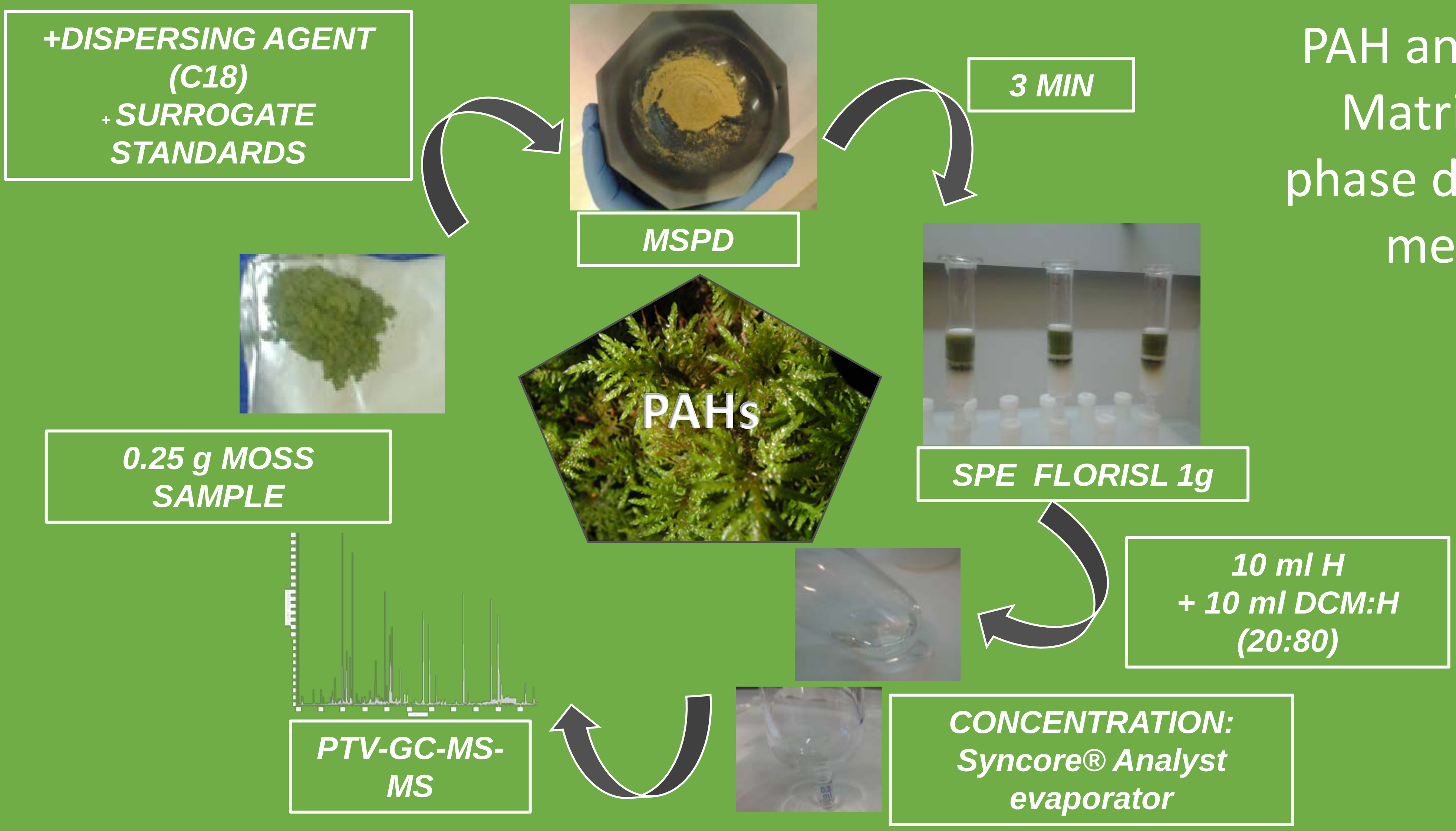


ATMOSPHERIC PARTICULATE MATTER SAMPLER < 10µm (PM10)

7. Mossphere vs. deposition

Correlation	Deposimeter vs. unsheltered Mossphere	PM10 vs. sheltered Mossphere	PM10 vs. unsheltered Mossphere
OK	Al, Ba, Cu, Pb, Ni, Sr	Al, Ba, Pb, Ni	Al, Ba, Cr, Cu, Fe, Pb
NO	Fe, V, Zn	Cr, Cu, Fe, Zn	V, Ni, Zn

PAH analysis by Matrix solid phase dispersion method

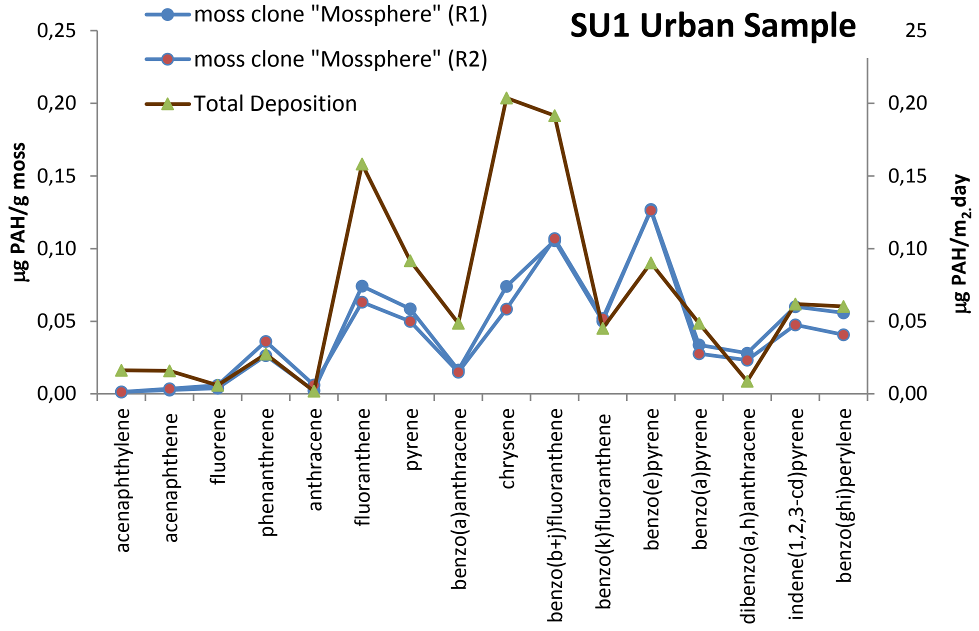


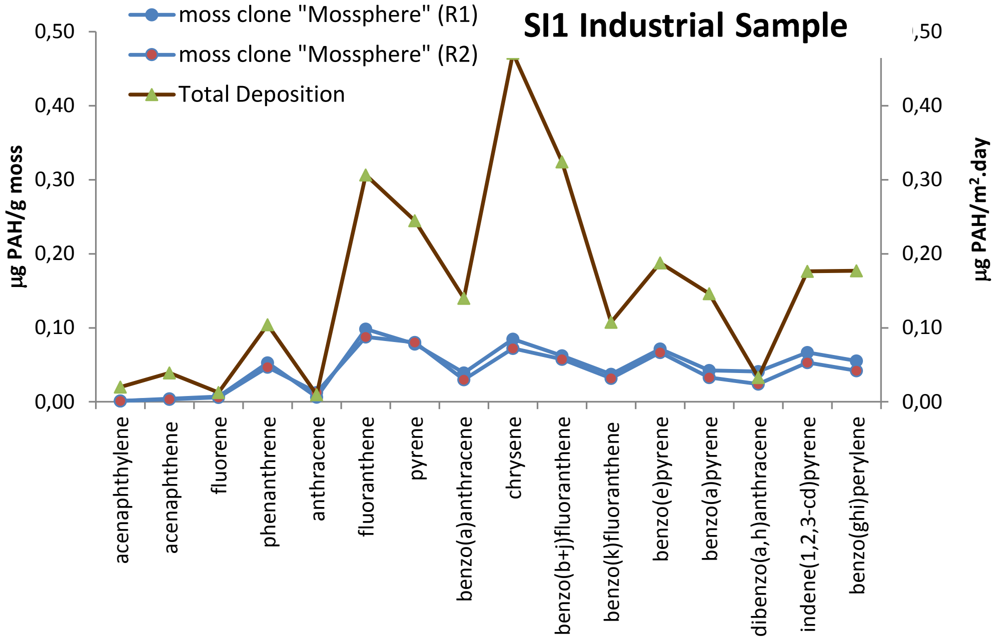
METHOD QUANTITATION LIMITS: 0.09-2.00 ng g^{-1}

TRUENESS: 83-112%

PRECISION: RSD <11%

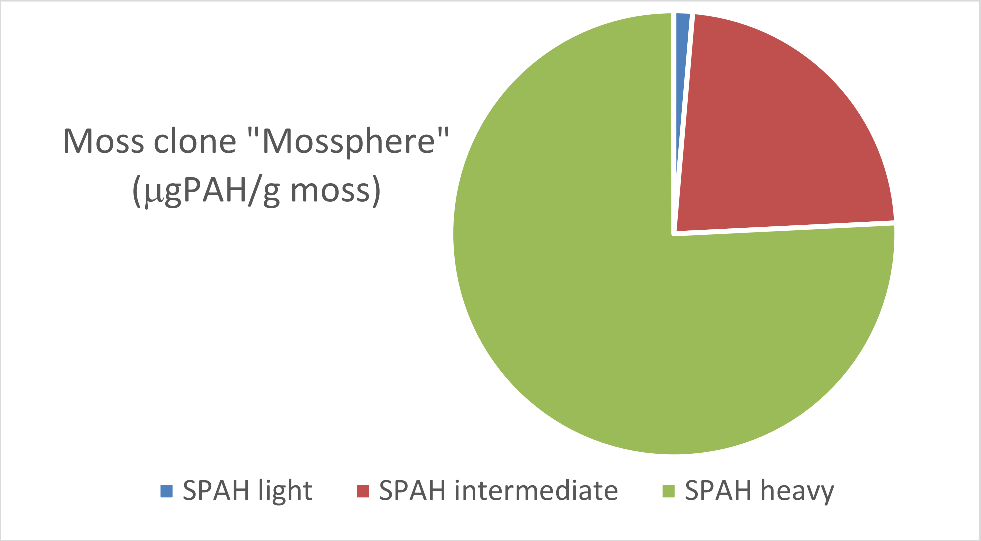
UNIVERSIDADE DA CORUÑA

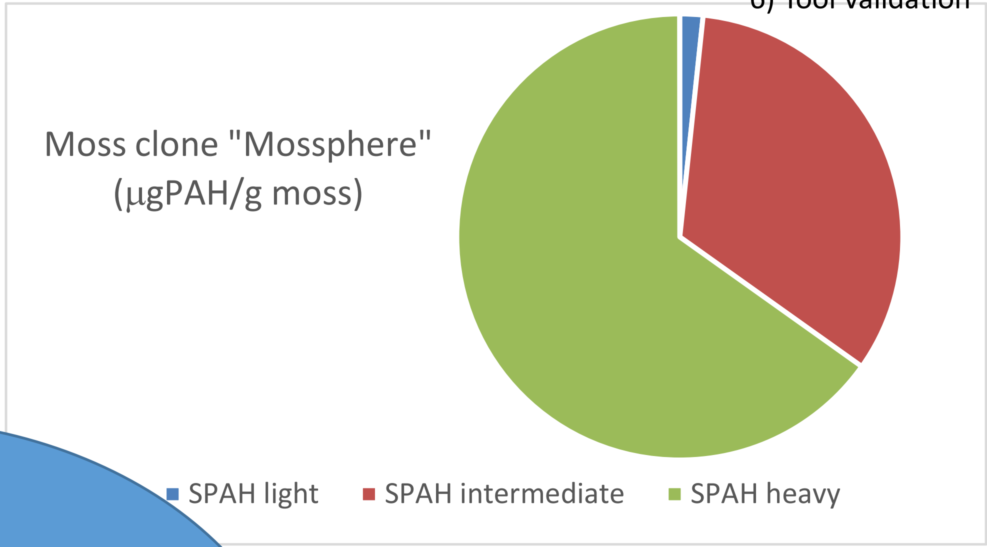




Mossphere

Total deposition

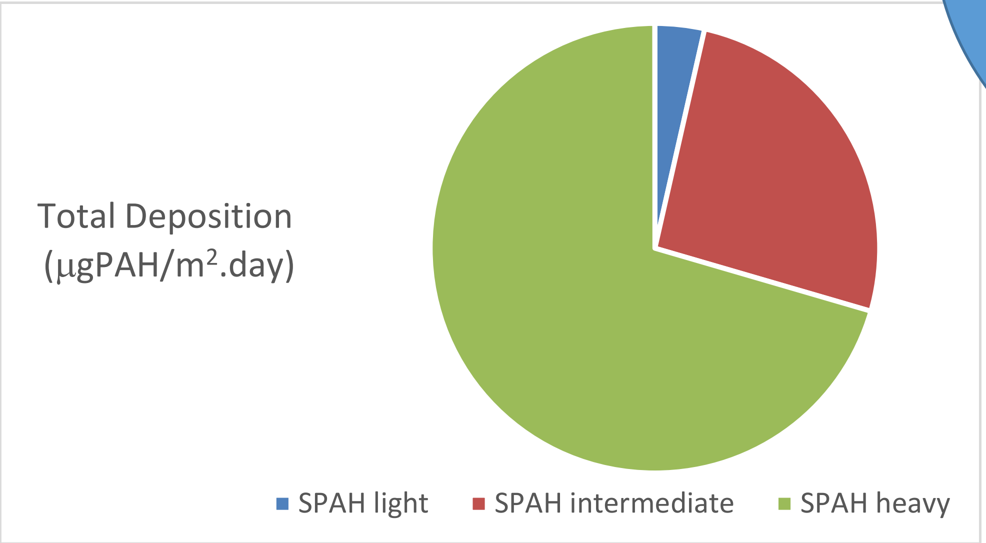


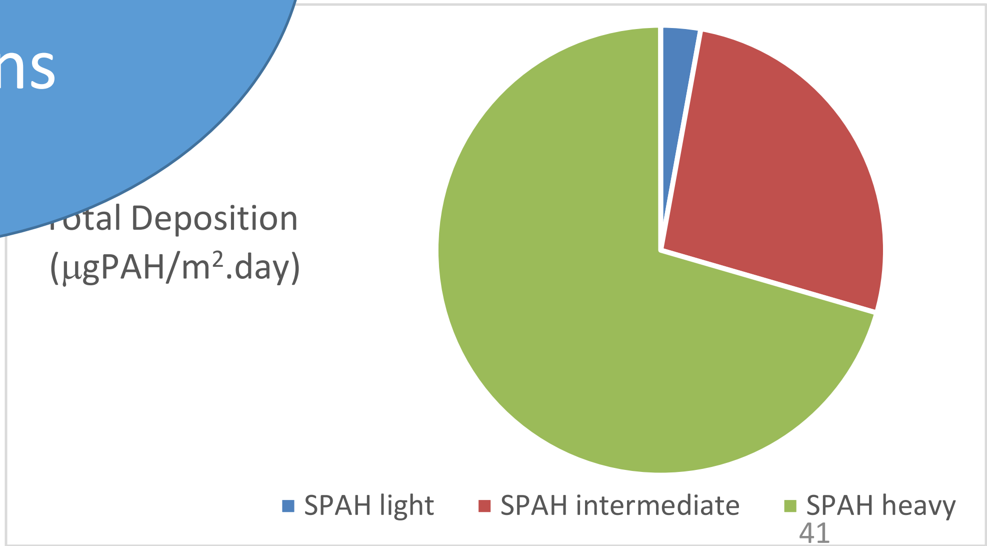


SU1 Urban Sample

8. PAHs profiles are very similar in moss and total depositions

Industrial Sample





Conclusions

- ✓ selection, culturing, scaling up and characterisation of a *Sphagnum palustre* clone
- ✓ the clone has a very low and constant elemental baseline (3 to 100 times lower content)
- ✓ design of a new device: MOSSPHERE®
- ✓ exposure protocol standardisation
- ✓ direct comparability of biomonitoring data
- ✓ patenting: in progress

TO DO

- large scale test over EU
- implementation of EU air quality legislation

clone MOSS

Creating and testing a method for controlling the air qualitybased on a new biotechnological tool
Use of a devitalized mossclone as passive contaminant sensor

Thanks for the attention!

Final meeting
Naples, 2015, March 9-11

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