Feasibility of Labile Zinc Phytoextraction Using enhanced Tobacco and Sunflower: - Results of 5- and 1 -Year Field Scale Experiments in Switzerland

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Outline

I. Introduction - Optimisation of phytoextraction

- Å *in vitro* breeding and mutagenesis of high yielding crops
- Å appropriated fertilization and crop rotation scheme

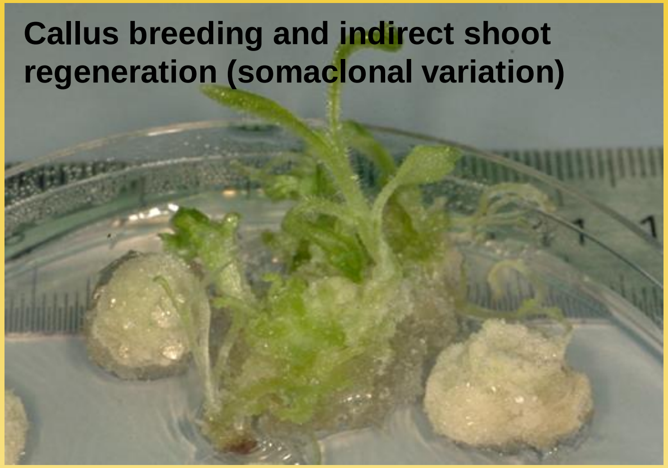
II. Cleaning up time for the phytoextraction of ótotalô and óbioavailableô metals from contaminated topsoil using improved tobacco and sunflower cultivars

III. Main results of a 5- and a 1-year time series of phytoextraction at field scale, aimed at a fast reduction of bioavailable (labile) Zn from topsoil on a metal contaminated site of Bettwiesen in Switzerland

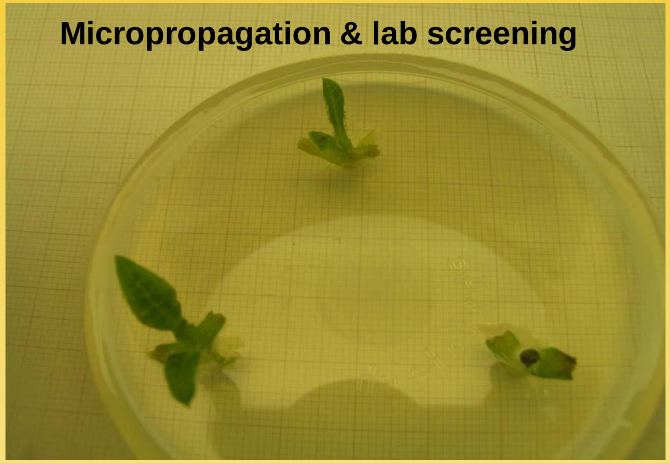
IV. Conclusion and Outlook

Improved metal Phytoextraction of Tobacco by In Vitro Breeding and Assessment by comparative Field Experiments since 1996

1. Tobacco clones with improved metal uptake were obtained from *in vitro* breeding (non-GMOs)



Callus breeding and indirect shoot regeneration (somaclonal variation)



Micropropagation & lab screening

2. *In vitro* bred tobacco were tested since years under real field conditions (PHYTAC, COST 837, 859, GREENLAND...)

Rafz (CH) 23.10.2003

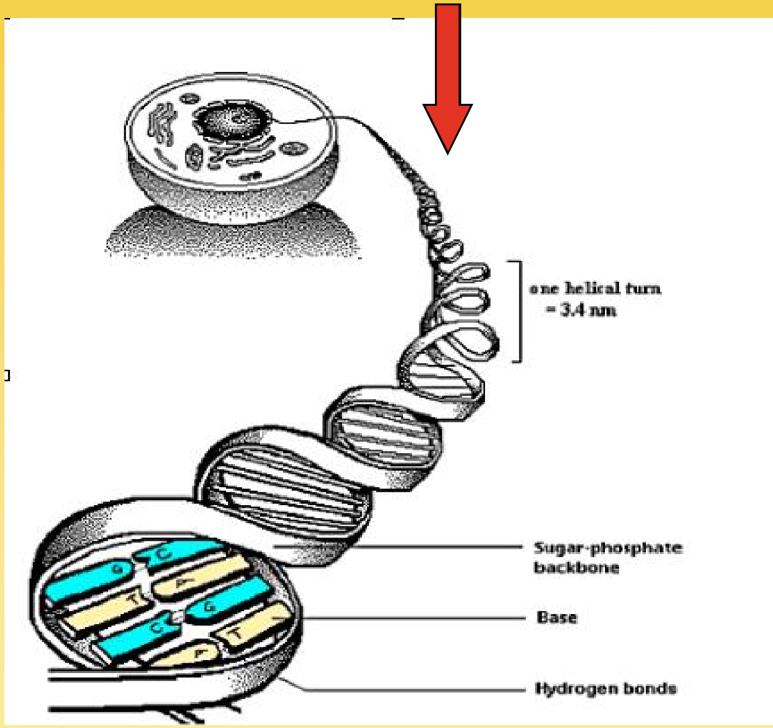
Balen (B) 20.8.2003

- <u>A comparative freeland assessment</u> within the 4th FWP PHYTAC on the <u>acid sandy soil of Balen</u> (Zn smelter) in Belgium showed an <u>enhanced metal extraction up to a factor of 12-15 for Cd, Zn, and Pb</u>.

Guadagnini et al. 1999, Herzig et al. 2003, 2005 & PHYTAC 2005

EMS-Mutagenesis and field-based Mutant Screening of Sunflower M_2-M_4 Generation on the metal-contamianted Site of Rafz (CH)

Mutagenesis with **EMS mutagen**
(alkylating, point mutation)



Goal: Sunflower mutants with enhanced metal tolerance, biomass and shoot Me-concentration and removal...

Nehnevajova et al. 2007, 2009

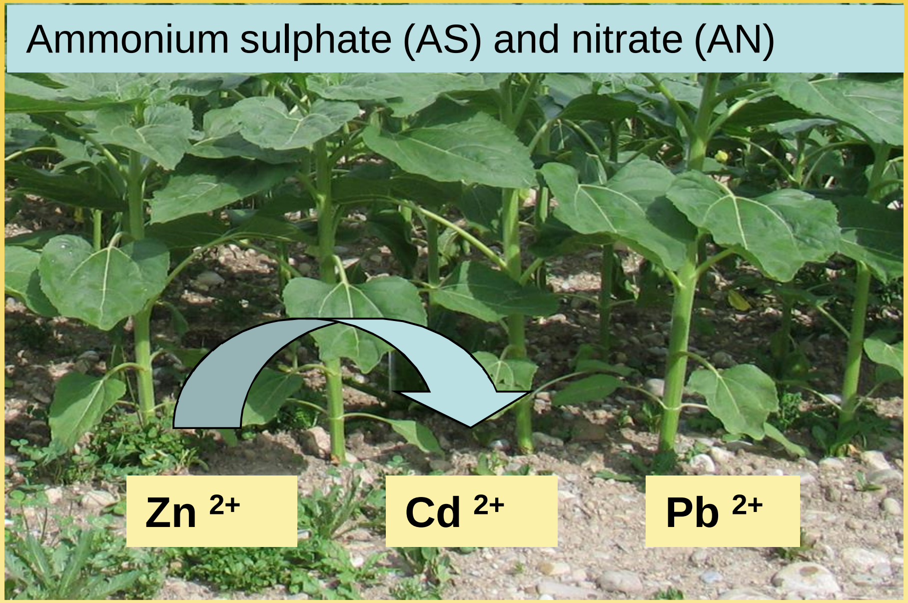
M_{2-4} sunflower mutant screening

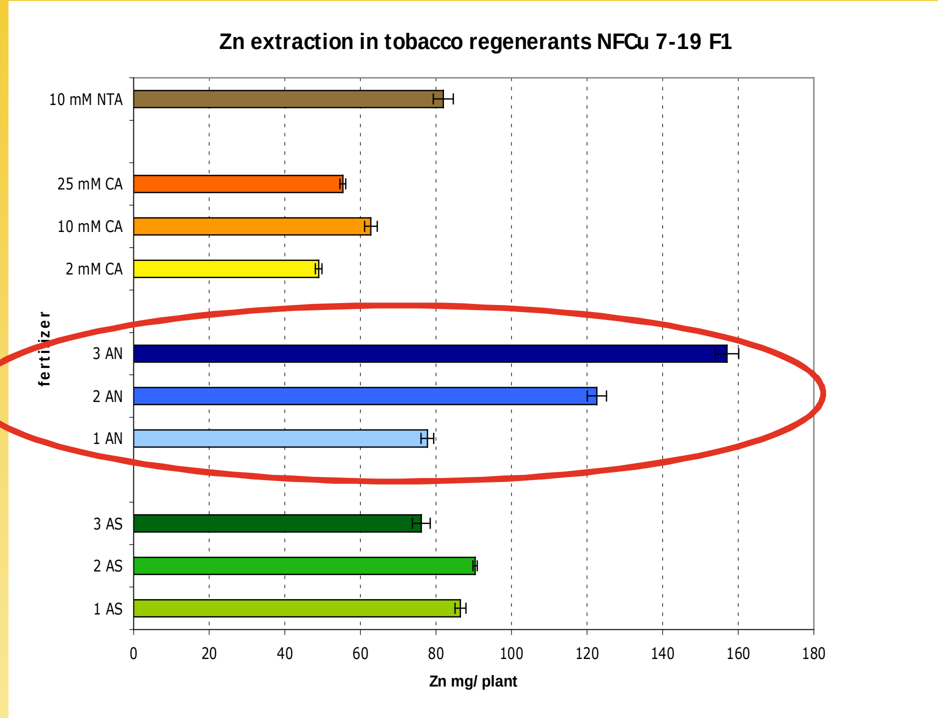
Rafz 2006: Screening of 10 best mutant lines of M_3 generation of 2005; totally 300 mutants

Improved M_4 sunflowers of 2006:
Best mutants produced a 4-5 times higher biomass and improved removal of:
3.5 x more Cd, 4.7 x more Zn,
7 x more Cr, and 8 x more Pb,
as compared to non-mutagenised IBL 04 controls

Appropriate Fertilization enhances Metal Uptake of improved Sunflowers and Tobaccos

The use of fertilisers to gently mobilize metals in the rhizosphere





PHYTAC-QLRT-2001-00429: Development of Systems to Improve Phytoremediation of Metal Contaminated Soils Through Improved Phytoaccumulation

<u>Ammonium Sulphate (AS)</u> is the efficient fertilizer for the sunflower, and - decreases soil pH by 0.6 unit at the Rafz site pH (KCl) $6.0 \pm 0.01 \rightarrow 5.4 \pm 0.02$

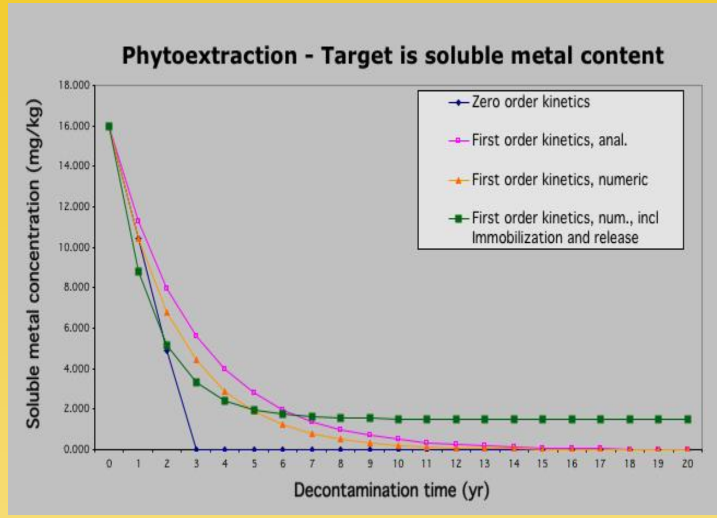
<u>Ammonium Nitrate (AN)</u> is the efficient fertilizer for the tobacco cultivars.
A 3 fold ammonium-nitrate fertilization enhances Zn extraction twice, compared with NTA and Cd extraction 1.5 times compared with single load of AN.

Nehnevajova et al. 2005, PHYTAC 2005

Phytoextraction of the labile Zn Fraction
- a more promising Prognosis ...

Phytoextraction of soluble (bioavailable) Zinc Optimised Szenario Bettwiesen 2005 (CH)	Tobacco	Tobacco	Tobacco	Sunflower	Sunflower	Sunflower
Clone/Mutant Origin of clone Zn uptake mg kg^{-1} Fertilisation	**NBZn7-51F1** in vitro bred 487 1.5AS/AN	**NBCu10-8F1** in vitro bred 293 1AS/AN	**BaG** selection 617 1.5AS/AN	**8-185-04** mutagenese 292 1.5AS/AN	**41-190-04** mutagenese 346 2 AS	**57-19-S** mutagenese 219 2 AS
Biomass t ha^{-1} DW Plant density p ha^{-1}	24.7 40’000	37.5 40’000	32.5 40’000	24.4 70’000	26.8 70’000	19.7 70’000
Zn removal kg ha^{-1} y^{-1}	**12**	**11**	**20.1**	**7.1**	**9.3**	**4.3**
Clean-up time 1 moderate 6 mg kg^{-1} => 0.5mg kg^{-1} Trigger value OIS CH 0.5mg kg^{-1} **Linear decay * years** First order decay * years	**2** 4	**2** 5	**1** 3	**3** 8	**2** 6	**5** 12
Clean-up time 2 high 10 mg kg^{-1} => 0.5mg kg^{-1} **Linear decay * years** First order dacay * years	**3** 9	**3** 10	**2** 5	**5** 15	**4** 12	**8** 25
Clean-up time 3 very high 16 mg kg^{-1} => 0.5mg kg^{-1} **Linear decay * years** First order decay * years	**5** 17	**5** 18	**3** 10	**8** 28	**6** 22	**13** 46

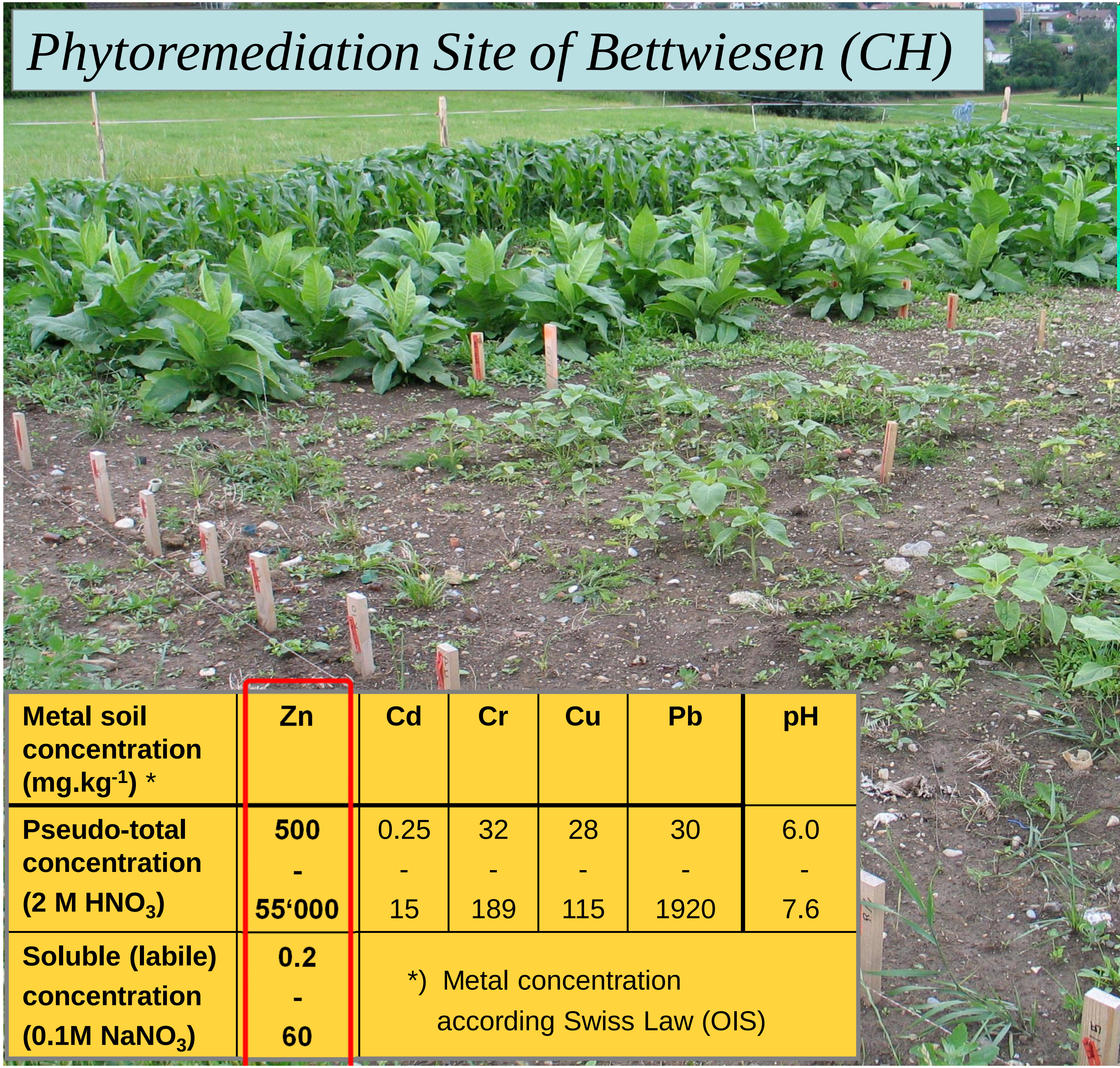
*: calculation based on 25 cm soil depth for selected cultivars, and linear and first order extrapolation of cleaning-up time (decay time).



The phytoextraction of total metal topsoil contamination needs a very long cleaning up time ï but we learnt from our first Swiss field data of 2005, that a moderate Ăbioavailableñ or labile fraction of Zn can be removed from topsoil in a few years time span ...

Herzig et al. 2005, 2014

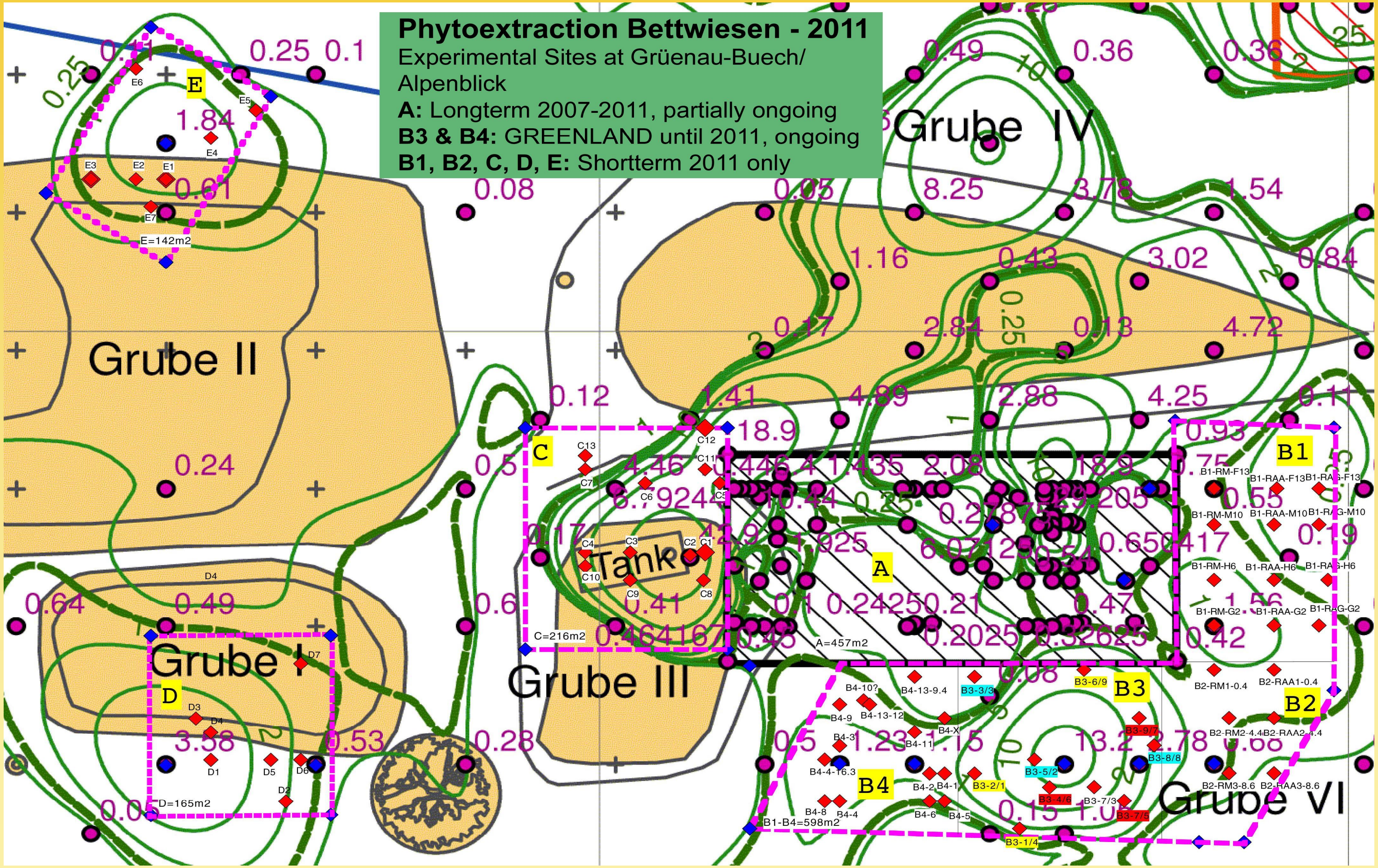
Phytoremediation Site of Bettwiesen (CH)



Metal soil concentration (mg.kg^{-1}) *	Zn	Cd	Cr	Cu	Pb	pH
Pseudo-total concentration (2 M HNO_3)	**500 - 55‘000**	0.25 - 15	32 - 189	28 - 115	30 - 1920	6.0 - 7.6
Soluble (labile) concentration (0.1M $NaNO_3$)	**0.2 - 60**	*) Metal concentration according Swiss Law (OIS)				

Design of the Bettwiesen Phytoextraction Experiments 2007 – 2014 With optimal Spread of labile Zinc Topsoil Contamination

For Phytoextraction Assessment: over 5 years on long-term Plot A - and 1 year on plots B – E



Legend

Orange

Landfill I ï IV (Deposits of a former hot dip Zn plant)

Green lines

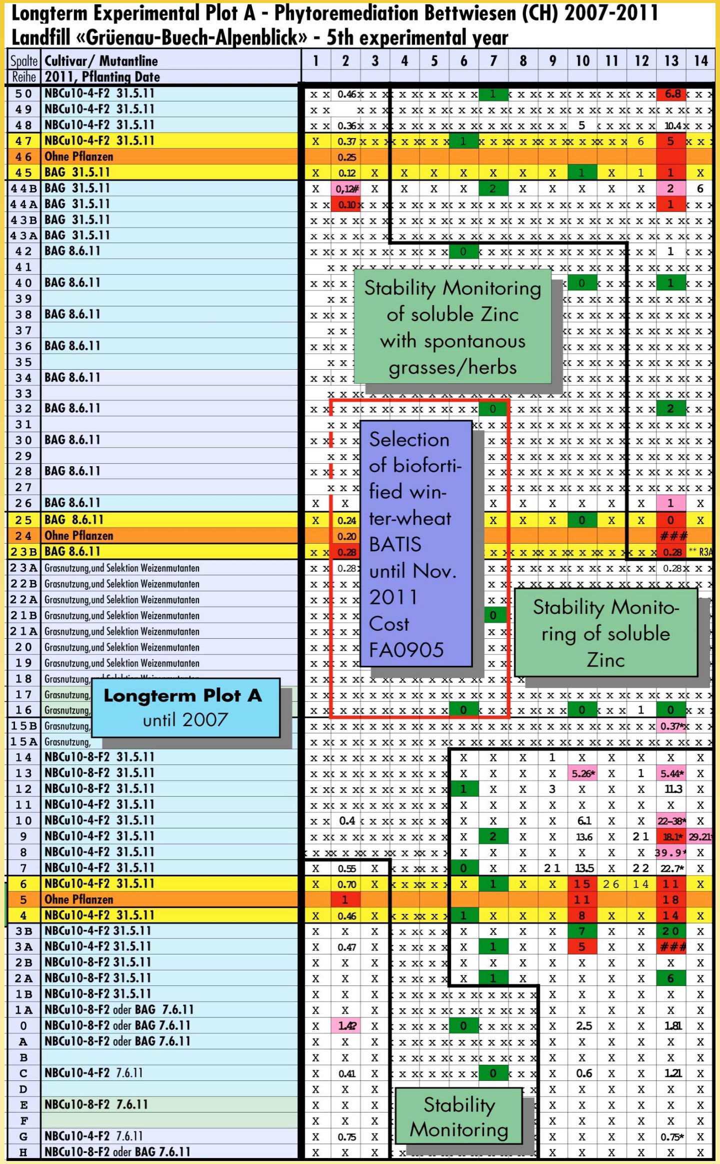
Isopletes of soluble Zn

Bold green

Isoplete of Swiss Trigger value for labile Zn of < 0.5mg/kg

Strongest Clean up Goal for labile Zn

Design of the Bettwiesen Phytoextraction Experiment 2007 – 2011 on Long-term Plot A



A Multi purpose field design allows:

- ➢ continuation of the phyto-extraction experi-ment, including crop rotation scheme
- ➢ assessing of most promising tobacco cultivars and sunflower mutant lines
- ➢ selfing of best sunflower mutants & tobaccos for future...

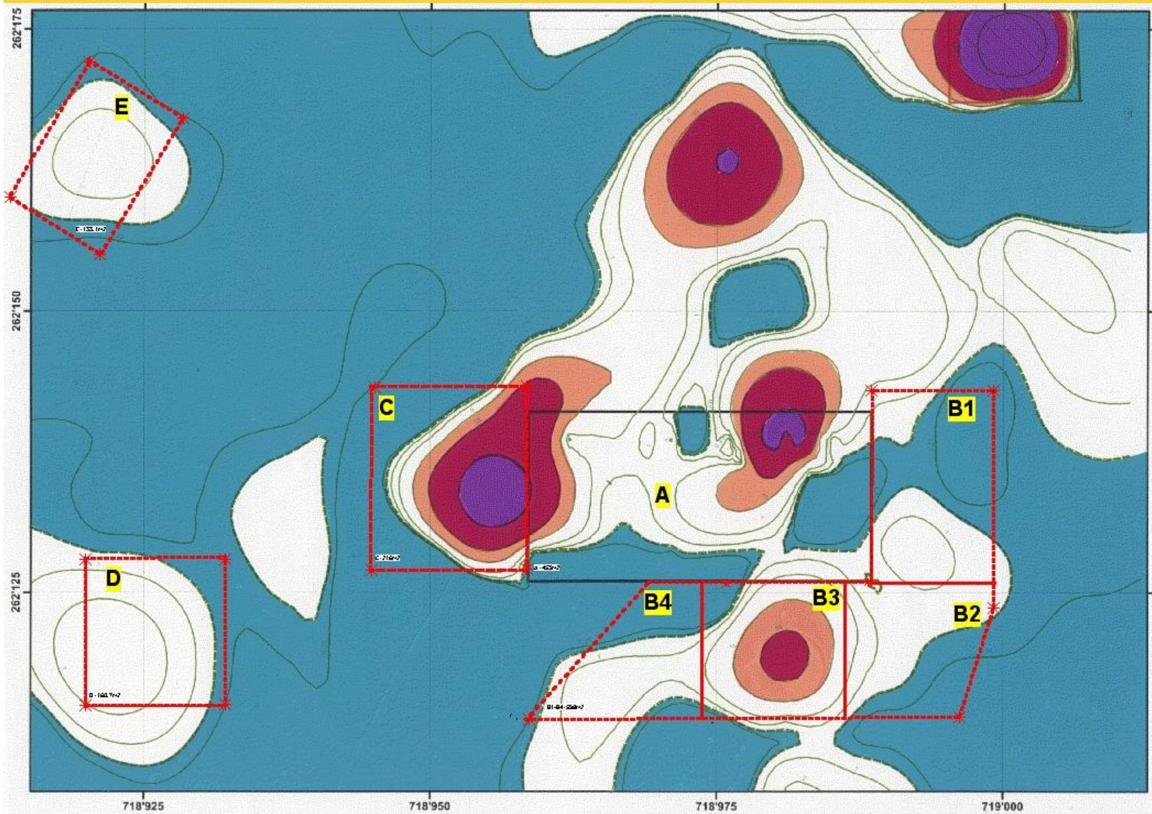
Soil sampling			Duration of Phytoremediation
2007	First Year	Date	Weeks
A-07	start	04.07.07	1
B-07	after fertilisation	26.07.07	3
C-07	flowering	24.08.07	8
D-07	seed ripening	10.10.07	16
E-07	harvesting	07.11.07	21
W-07	winter 07	29.12.07	25
2007	Second Year		
A-08	Start and continued	27.05.08	50
2009...	3. - 5. Year	2009 - 2012	until week 248

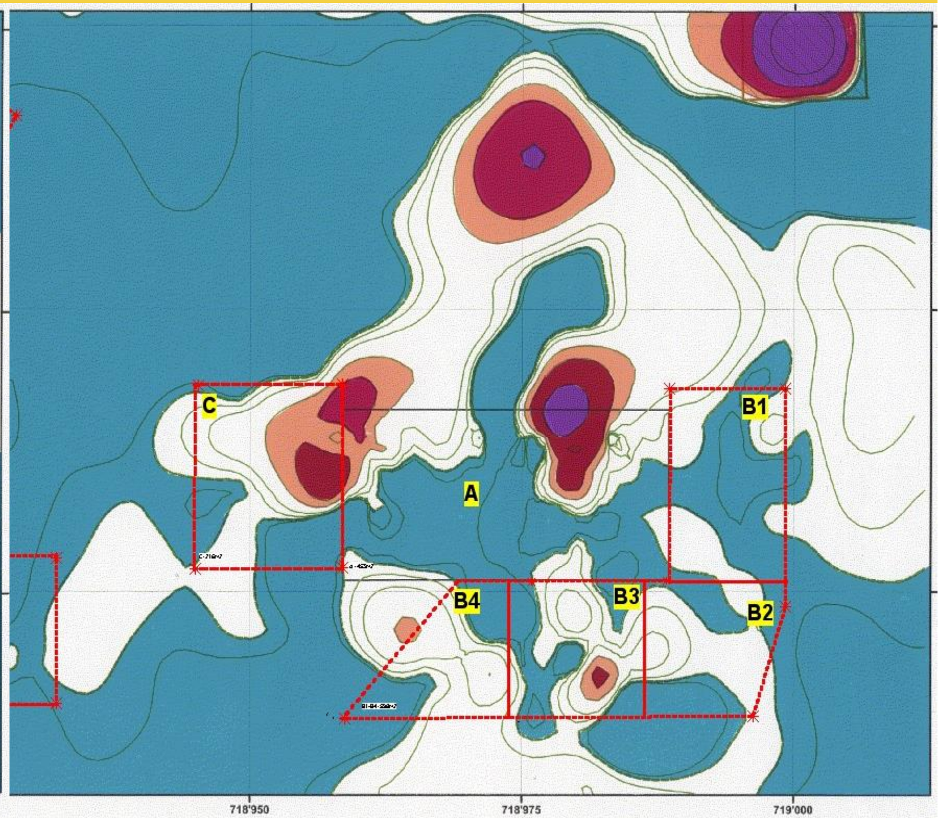
Success Control after 5-Years of Phytoextraction - Long-term Plot A

Site: Landfill Grüenau-Buech-Alpenblick Bettwiesen (CH)

Spring 2007 prior to phytoextraction

Winter 2011/12 after 5 years of phytoextractionon plot A





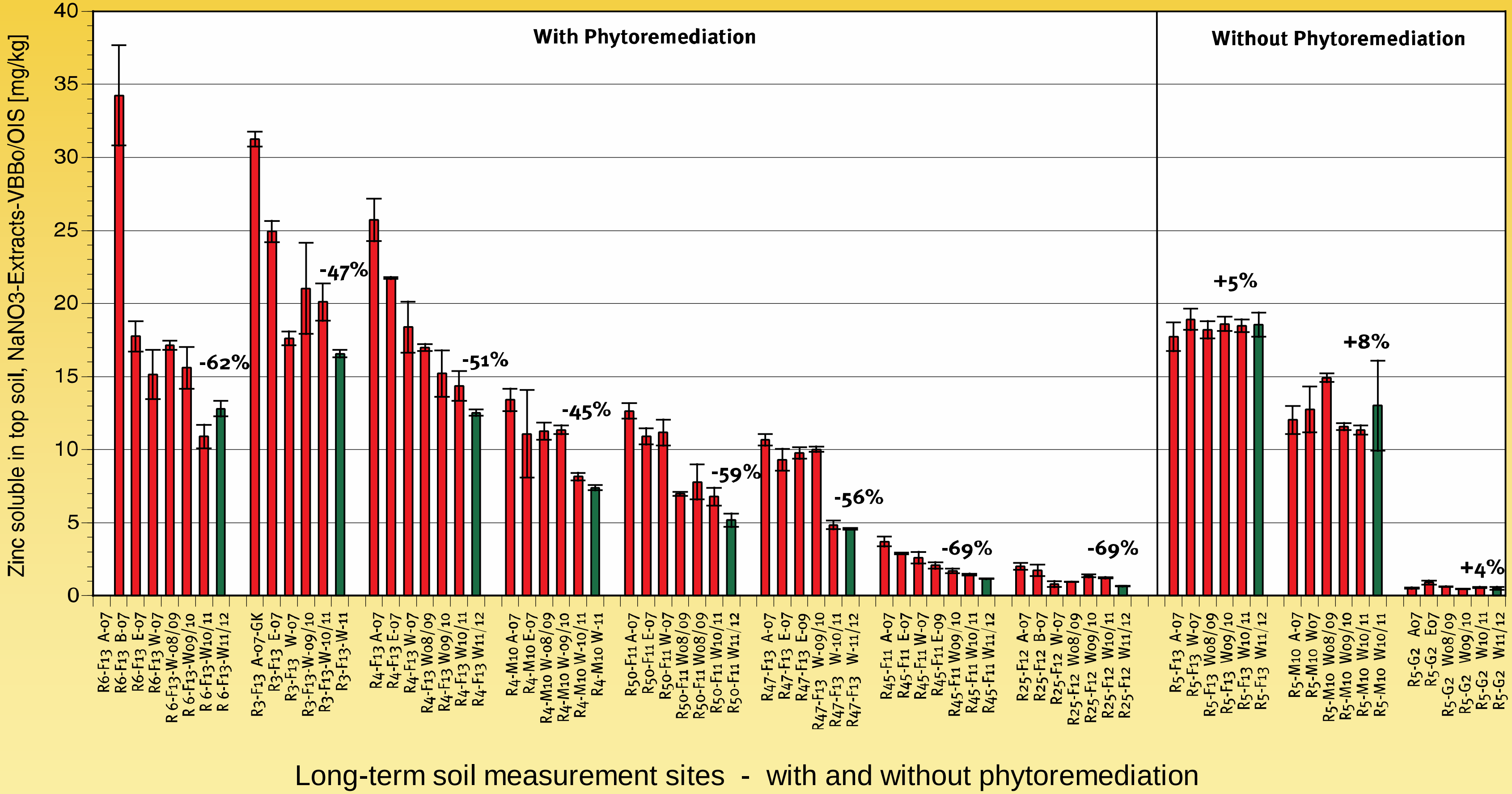
Prior to phytoextraction treatment - 27% of the area of long-term plot A (blue colored) showed a low soluble Zn contamination, below the Swiss Trigger value (Zn-soluble < 0.5mg/kg; $NaNO_3$-extraction, OIS-CH).

After 5-years of phytoextraction on plot A, the area below the Swiss Trigger Value (blue) could be strongly enlarged by 106%. Full soil functionality is recovered by phytoextraction.

Treatment Effect over 5- Years - With and without Phytoremediation

Phytoremediation Bettwiesen on Landfill Grüenau-Buech-Alpenblick 2007 – 2011/12 (CH)

Reduction of labile Zinc in Topsoil



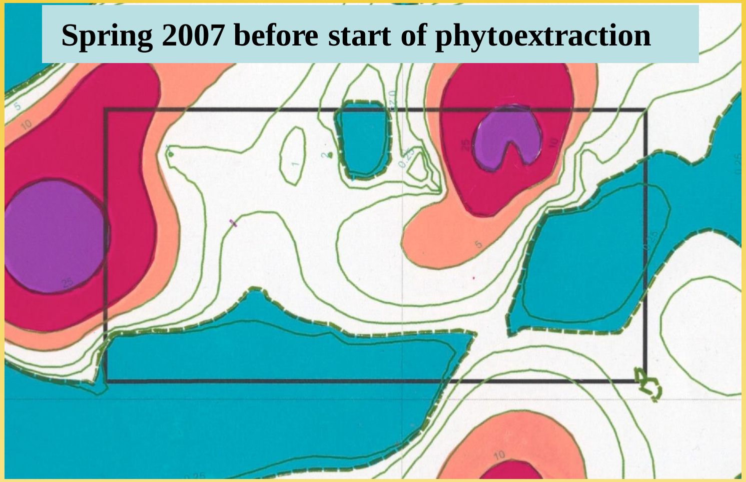
Å Over the five-year phytoextraction period an efficient 45 - 70% decay of the initial labile (soluble) Zn top soil concentration was found.

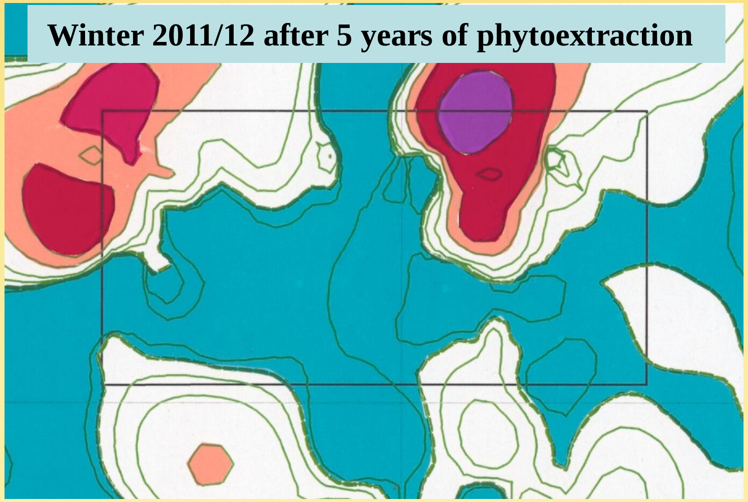
Å Without the phytoextraction the labile topsoil concentration remains almost constant over time...

After 248 Weeks of Phytoextraction with improved sunflower and tobacco in crop rotation scheme - Soil Series June 2007 ï Winter 2011/12 (A07-W11/12)

Treatment Effect of Phytoextraction after 5 Years on Subplot A

Relative change of land area and labile Zn concentration levels after five- and one-year of phytoextraction treatment at Bettwiesen site 2007-2012 (CH). Cleaning up threshold for labile Zn is the Swiss trigger value of 0.5 mg·kg^{-1} (0.1M $NaNO_3$-extraction, OSP 1998).


Spring 2007 before start of phytoextraction


Winter 2011/12 after 5 years of phytoextraction

Relative change of land area *, after five- and one-year of Phytoextraction at Bettwiesen 2007-2012, Switzerland	Blue area Zn < 0.5mg·kg^{-1} %	Orange area Zn 5-10mg·kg^{-1} %	Red area Zn 10-25mg·kg^{-1} %	Purple area Zn > 25mg·kg^{-1} %
Contamination level for labile Zinc #	below Swiss trigger value	elevated	strong	very strong
Long-term plot A - 5 years				
A – Relative Treatment Effect ‡	106	1.7	-20.5	-24.1

Positive Treatment Effect after 5 years in subplot A:

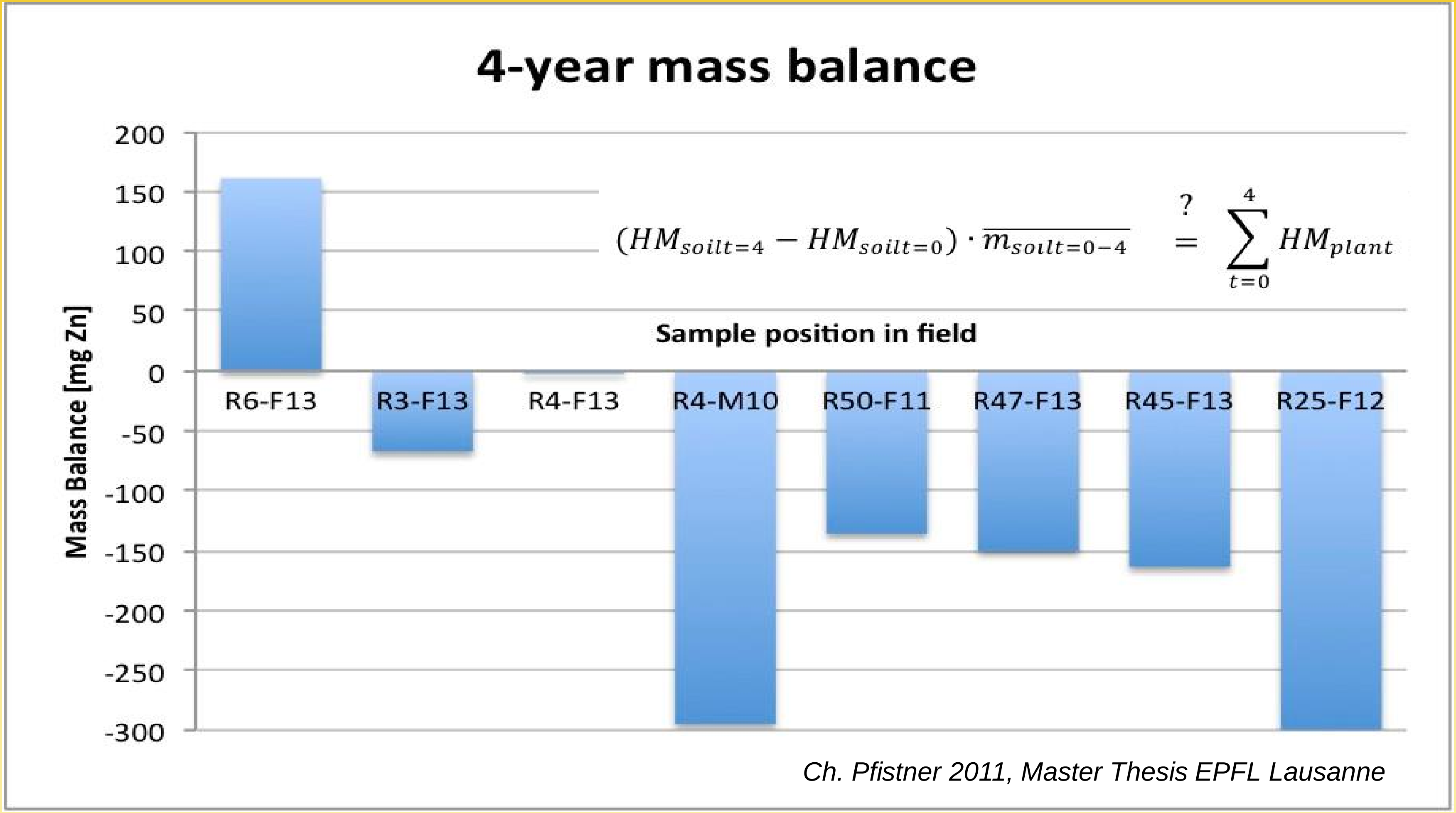
1. the **blue area** with a labile Zn topsoil concentration below the Swiss Trigger Value more than doubled in long-term plot A. ------- > **Entire recover of soil functionality and health due to phytoextraction !**

2. The **red area** with strong labile Zn concentration of 10-25mg.kg^{-1} was reduced by 20%.

3. Also the **purple area** with a labile Zn concentration >25mg.kg^{-1} was reduced by 24%, whereas the **orange area** remained constant.

Herzig et al. 2014

Mass Balance Analysis – 4 Year MBA-Study Bettwiesen 2007 - 2010



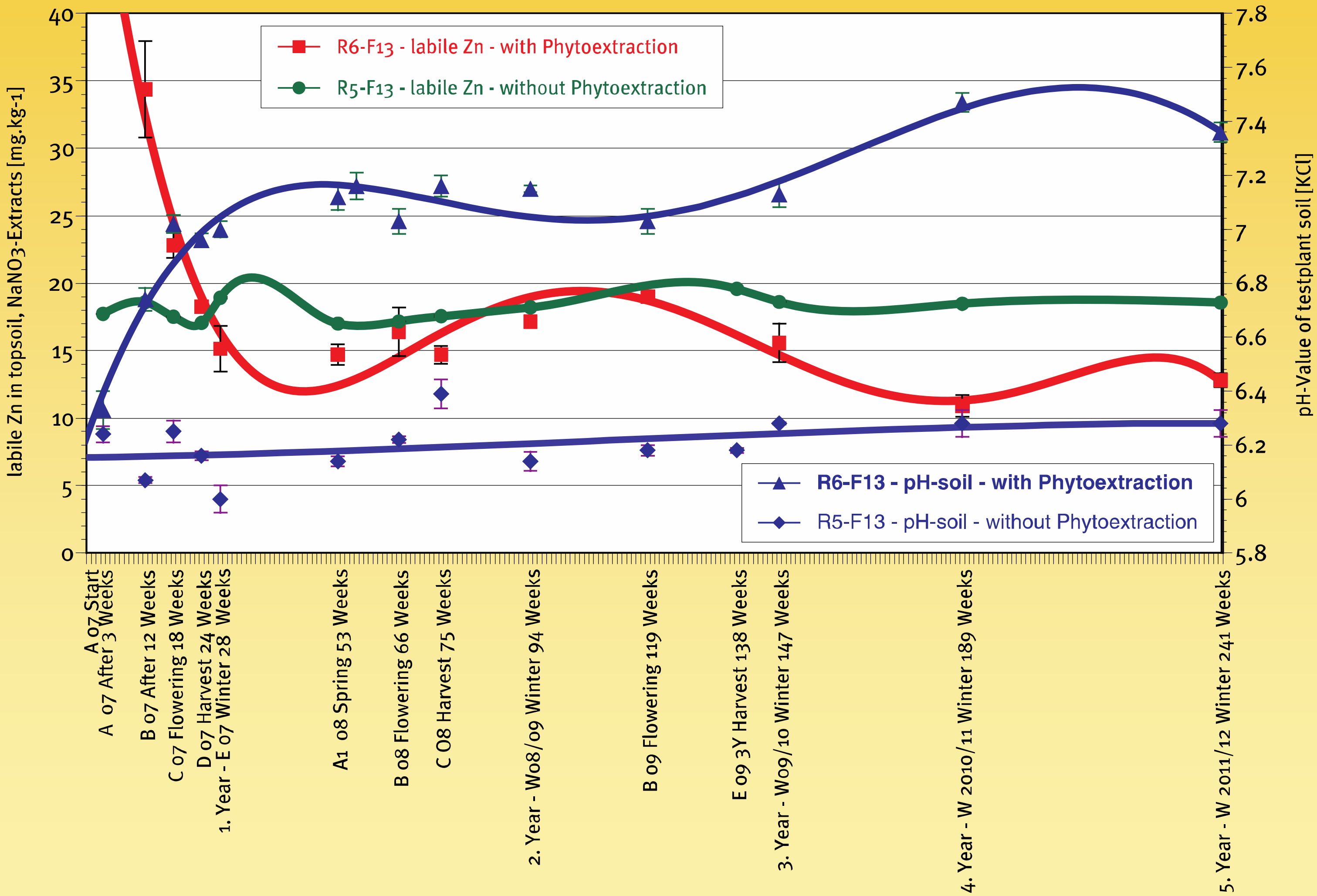
Å **<u>Most of the MBA samples analyzed have negative mass balance, meaning that more is extracted from the sunflower & tobacco plants than the decrease found in the soluble Zn pool in the soil</u>.**

Further explanation: decrease in total soil Zn → re-load the soluble Zn pool → mobilization of the Zn by the root system.. Complexe sample R6-F13 → rosty nails & metal waste were found at the very beginning...

Treatment Effect over 5 Years - Change of soluble Zinc and pH value of topsoil – with and without Phytoextraction

Phytoremediation Bettwiesen on Landfill Grüenau-Buech-Alpenblick 2007 – 2011/12 (CH)

Experimental Row R6-F13 with crop rotation of tobacco and sunflower



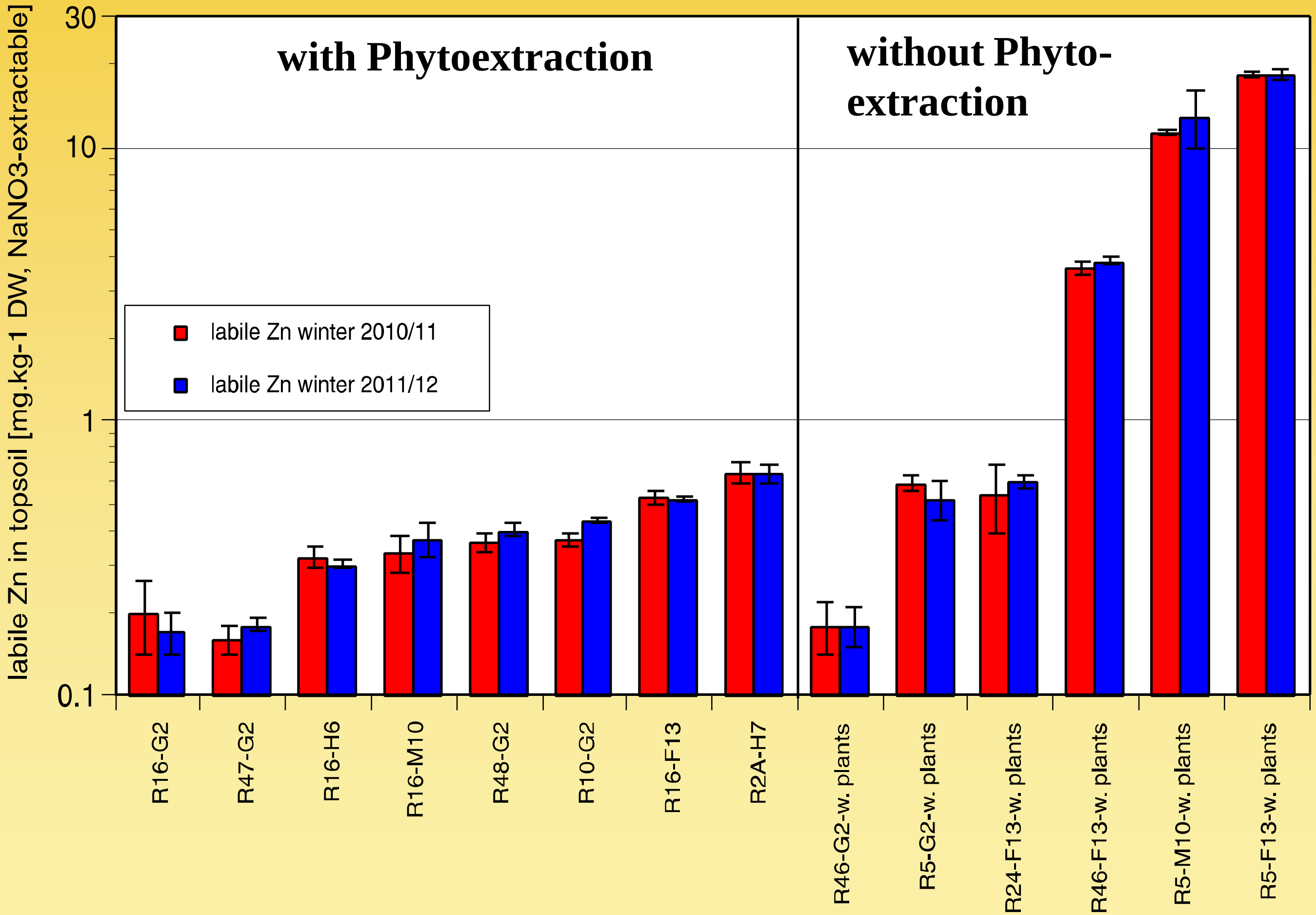
Å **An efficient 60% decay of the initial labile Zn top soil concentration (red) within 5 years is correlated with a strong increase of the soil pH (blue) of 1 unit.**

Å With the help of the plant rhizosphere a strong immobilization results as a welcomed side effect of phytoextraction.

Å **Without phytoextraction the labile Zn remains almost constant, and soil pH shows only little fluctuation.**

Time Stability after Ending 5-Year Phytoextraction Treatment

Site: Landfill Grüenau-Buech-Alpenblick Bettwiesen (CH)



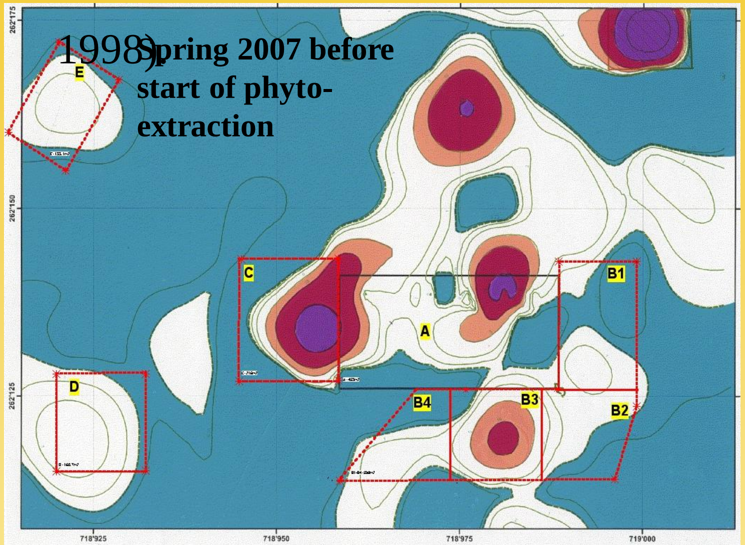
Å The diagram shows the change of labile, 0.1 M $NaNO_3$-extractable Zn concentration at Bettwiesen site, after ending five-year phytoextraction treatment. Samples (R46, R5, R24) represent long-term controls of the experiment without growth of plants.

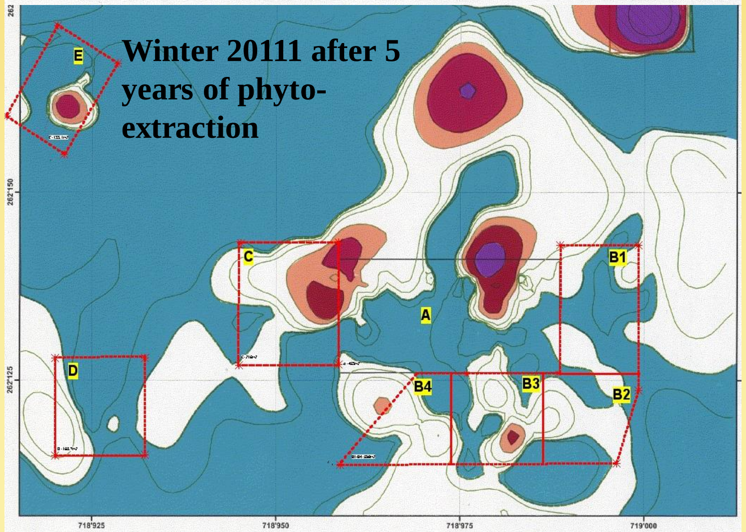
Å The fluctuation over time after one year stop of phytoextraction are in the same order of magnitude as long-term controls..

Å Based on these data, no significant re-supply of the labile, 0.1 M $NaNO_3$-extractable Zn from the non-labile soil fractions was observed. These results also agree with the findings of the Mass Balance Analysis.

Succes Control of Phytoextraction after Five- and One-Year(s) of Treatment

Relative change of land area and labile Zn concentration levels after five- and one-year of phytoextraction treatment at Bettwiesen site 2007-2012 (CH). Cleaning up threshold for labile Zn is the Swiss trigger value of < 0.5 $mg \cdot kg^{-1}$ (0.1M $NaNO_3$-extraction, OSP-CH)

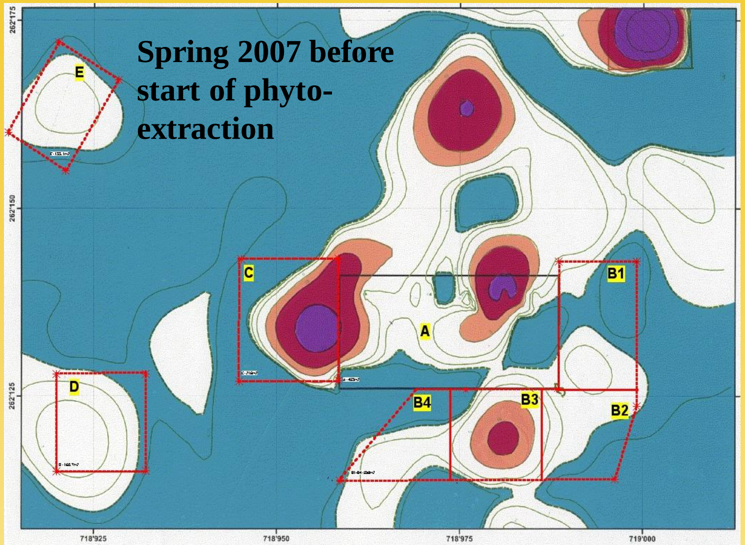


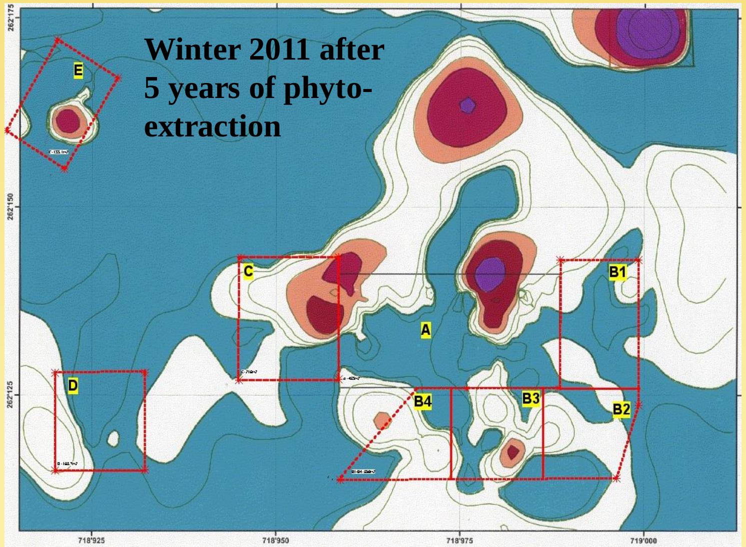


Relative change of land area *, after five- and one-year of Phytoextraction at Bettwiesen 2007-2012, Switzerland	Blue area Zn < 0.5$mg \cdot kg^{-1}$ %	Orange area Zn 5-10$mg \cdot kg^{-1}$ %	Red area Zn 10-25$mg \cdot kg^{-1}$ %	Purple area Zn > 25$mg \cdot kg^{-1}$ %
Contamination level for labile Zinc #	below Swiss trigger value	elevated	strong	very strong
Long-term plot A - 5 years				
A – Relative Treatment Effect ‡	106	1.7	-20.5	-24.1
Short-term plot B-E - 1 year				
B1 – Relative Treatment Effect ‡	27.0	0	0	0
B2 – Relative Treatment Effect ‡	19.8	0	0	0
B3 – Relative Treatment Effect ‡	464	-84.1	-87.8	0
B4 – Relative Treatment Effect ‡	10.2	na	0	0
C – Relative Treatment Effect ‡	-4.0	34.0	-61.4	-100
D – Relative Treatment Effect ‡	310	0	0	0
E – Relative Treatment Effect ‡	848	na	na	0

*) total area land of each subplot A - E is set as 100%
#) labile Zn topsoil concentration, according 0.1M $NaNO_3$-extraction for soluble contents, (OSP 1998).
‡) Relative Treatment Effect: relative change of land area prior and past phytoextraction (A2007-W2011/2012, and A2011-W2011/2012):
- negative scores represent reduction; + scores increase of land area of same Zn contamination class.

Treatment Effect <u>after only 1-year</u> of Phytoextraction - Plots B - E





Relative change of land area *, after five- and one-year of Phytoextraction at Bettwiesen 2007-2012, Switzerland	Blue area Zn < 0.5mg kg^{-1} %	Orange area Zn 5-10mg kg^{-1} %	Red area Zn 10-25mg kg^{-1} %	Purple area Zn > 25mg kg^{-1} %
Contamination level for labile Zinc #	below Swiss trigger value	elevated	strong	very strong
Short-term plot B-E - 1 year				
B1 – Relative Treatment Effect ‡	27.0	0	0	0
B2 – Relative Treatment Effect ‡	19.8	0	0	0
B3 – Relative Treatment Effect ‡	464	-84.1	-87.8	0
B4 – Relative Treatment Effect ‡	10.2	na	0	0
C – Relative Treatment Effect ‡	-4.0	34.0	-61.4	-100
D – Relative Treatment Effect ‡	310	0	0	0
E – Relative Treatment Effect ‡	848	na	na	0

<u>Positive Treatment Effect after only One Year, Subplots B - E:</u>

1. The <u>**blue area**</u> with a **labile Zn** topsoil **concentration below** the **Swiss Trigger Value increased** up to **5-8 times** in plots **B3** and **E**
2. The <u>**orange area**</u> **with elevated Zn load was reduced by 84% in B3, wherease increased by 34% in C.**
3. The <u>**red area**</u> **with strong labile Zn concentration of 10-25mg.kg^{-1} was reduced by 60-90% in plots B3 and C.**
4. Also the <u>**purple area**</u> **with a labile Zn concentration > 25mg.kg^{-1} was doubled in plot C.**

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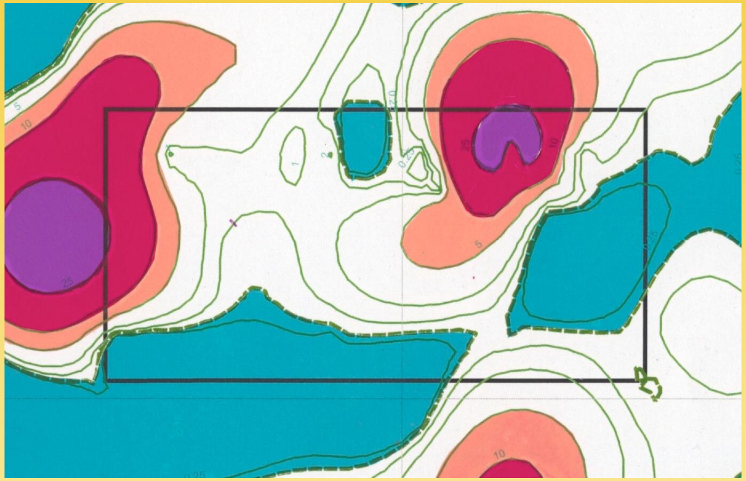
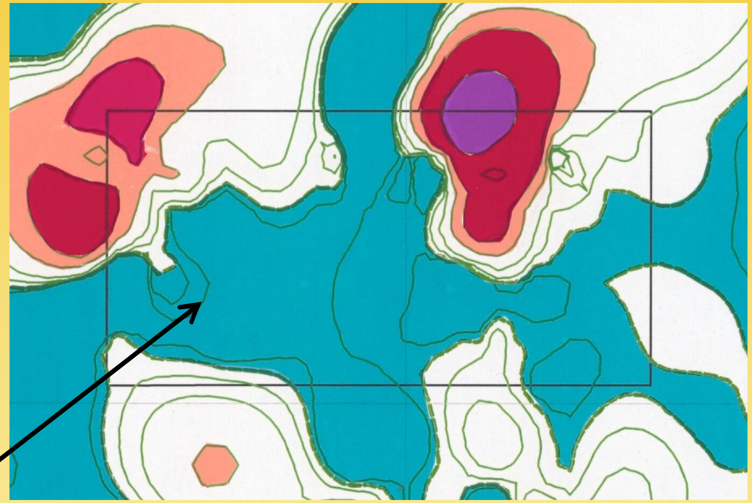
Longer-term Extraction Efficiency of soluble Zinc from Topsoil - Before and after 5 years of Phytoremediation 2007 – 2011/12

Long-term site A: Grüenau-Buech-Alpenblick Bettwiesen (CH)

<u>June 2007 before start of phytoremediation</u>



<u>Winter 2011/12 after 5 years of phytoremediation</u>



<u>Blue:</u> Soluble zinc below the Swiss Trigger Value (OIS) for non-contaminated agricultural land
(Zn-sol. < 0.5mg/kg; $NaNO_3$-extraction)

Å **The positive effect of 5 years of phytoextraction is shown by the enlargement of the blue colored area of factor of 2, where full soil fertility is achieved again.**

Å **On one of these phytoremediated plots a mutant screening of a biofortified winter-wheat variety was started in November 2011, and now confirms the complete recovery of soil fertility.**

2. *progress is possible, when selected and/or improved cultivars of high yielding, metal-tolerant and extracting crops, including appropriate fertilization & multi-cropping techniques were combined...*

Summary / Outlook

1. *phytoextraction of „total“ Zn, Cu and Pb contamination from topsoil with non selected/improved crops may need up to centuries for restauration, that is far away from beeing feasible ...*

3*. the phytoextraction of the bioavailable, metal concentration of contaminated topsoil is more efficient - quenching the main risk of a possible food-chain and groundwater contamination ...*

4*. After 5-year of phytoextraction at Zn contaminated site in eastern Switzerland using efficient tobacco and sunflower cultivars and appropriate cultivation techniques, moderate up to strong bioavailable Zn concentration of topsoil can be reduced by 45-70% of the initial concentration.*

- For moderate soluble Zn concentration, the area of phytoremediated land that fulfills the Swiss Trigger Value (<0.5mg/kg Zn) could be enlarged by 100%.

- the post-harvest bioavailable Zn concentration remains relatively stable over wintertime, and Zn extraction continues in the following years.

A Mass Balance Analyse confirms a higher Zn plant extraction compared to the decay of the soluble fraction. This underlines that plant roots also partially forage the „total“ Zn pool.

The phytoextraction of low until moderate soluble (labile) Zn contamination from topsoil within a few years time span looks feasible - based on these Bettwiesen data ...

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