Assessment of chemical and biological methods for determining bioavailability of trace elements in soil

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Outline

- Concept, definitions and disambiguation
- Chemical methods
- (Bio)Sensors
- Methodological assessment
- Implications in ecotoxicology and risk assessment
- and future agriculture
- Conclusions

Bioavailability: concept, definitions and disambiguation

- Sposito, 1989
- Hrudy et al., 1996
- Linz and Nakles, 1997
- NEPI, 1997
- Paustenbach et al., 1997
- Sayler et al., 1998
- ASTM, 1998
- Anderson et al., 1999
- Ruby et al., 1999
- Battelle and Exponent, 2000
- EPA, 2000
- NEPI, 2000a;
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- Casarett and Doulls, 2001
- Lanno, 2001
- Wilkinson, 2001

From Bioavailability of Contaminants in Soils and Sediments: Processes, Tools, and Applications. (USA NRC), Washington, DC: The National Academies Press

- Renella 2019? *no thanks!!*

The bioavailability concept stems from toxicology, and was known since the ancient Egyptian (1550 BC), Greek, pre-Columbian South America natives

More recently in pharmaceutics and nutrition

Bioavailability: concept, definitions and word sense disambiguation

- *For chemists*: bioavailability is the rate and the extent to which an element or a substance is adsorbed from the environment and interact at a biological site of action

- *For biologists*: bioavailability is the capability of an element or a substance to cross the cell membrane and enter into the cell cytoplasm

Bioavailability: concept, definitions and word sense disambiguation

- *For chemists*: bioavailability results from the physical, chemical, and biological interactions that determine the exposure of living organisms to chemicals present in soils and sediments

- It accounts for the ability of a chemical to be absorbed by an organism based on a number of physical processes and chemical mechanisms

Bioavailability: concept, definitions and word sense disambiguation

- *For biologists*: bioavailable elements and substances are absorbed either passively or actively and reach systemic circulation in an organism and are able to elicit a biological response

Bioavailability in soil

- Is the exposure of living organisms to contaminants and nutrients
  - present in the solution
  - to released solid-bound nutrients following direct contact
  - uptake through the cell membrane, and action into the target organism

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Bioavailability: the concept and definitions – what about soils?

- For biologists: bioavailability is the capability of an element or a substance to cross the cell membrane and enter into the cell cytoplasm

- For chemists: bioavailability is the rate and the extent to which an element or a substance is absorbed from the environment and interact at a given site of action

Bioavailability in soil: specificity of the solid phases

- Release of bound nutrients or contaminant is the physical and (bio)chemical solubilization by weathering, chemical processes like redox reactions or chelation, complexation, and biochemical processes through the action of biosurfactant molecules or enzyme activities

Bioavailability: the concept and definitions – what about soils?

- Differently for acquatic ecosystems, a relatively large error occurs if bioavailability is not considered when evaluating the biological impact of soil contamination (Luoma and Jenne, 1977)
- Milestone papers?
  - Alexander, M. 1995. How toxic are chemicals in soil? Environ. Sci. Technol. 29:2713-2717.
  - Alexander, M. 2000. Aging, bioavailability, and overestimation of risk from environmental pollutants. Environ. Sci. Technol. 34:4259-4265.

Bioavailability: current use in management of contaminated soils

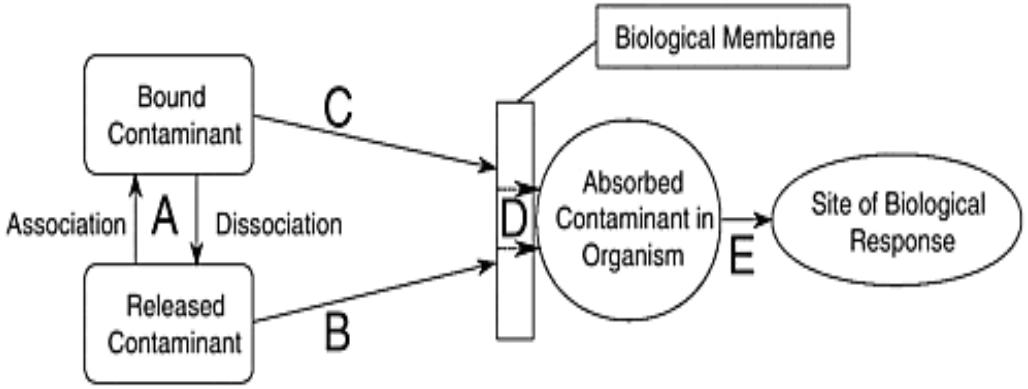
- Bioavailability assessment is increasingly considered in ecological risk assessment

- Site-specific bioavailability tests can be used to determine the potential bioaccumulation of toxic compounds into plants and animals

- In particular bioaccessibility is accounted as a factor in exposure pathways

Bioavailability in soil

- Binding may occur by
  - **adsorption** on solid surfaces
  - **absorption** within a phase (e.g. organic matter)
  - **chemical speciation** (e.g. change in chemical bonding)
  - **Transport** resulting from diffusion and advection to receptors (living organisms) plants, and humans



Bioavailability processes (A, B, C, and D)

Total soil concentration of an element or compound is not equivalent to bioavailable concentration

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integration of chemical and biological processes

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Bioavailability: chemical methods

Kumpiene et al 2017, Pedosphere 27: 389 – 406

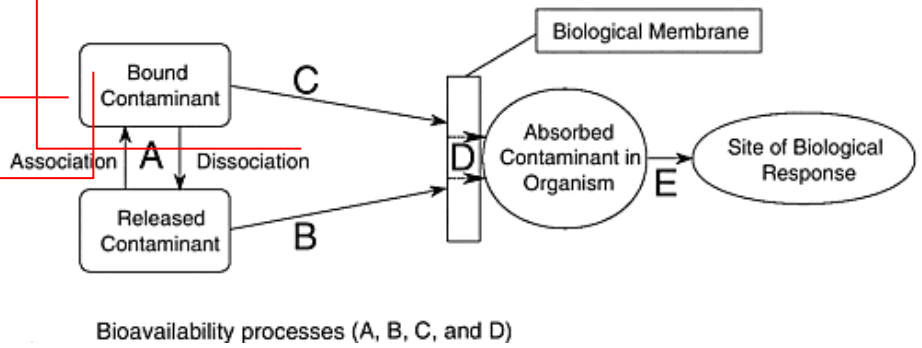
Single solvent extraction protocols commonly used for assessing the environmental availability of trace elements (TEs) in TE-contaminated soils and remediated soils

Extractant[a)]	Extractant concentration	Reference	Country incorporating an extraction protocol in the national legistlation
	mol L^{-1}		
H_2O		McBride *et al.*, 1989	
$NaNO_3$	0.1	Herzig *et al.*, 2014	Switzerland (Bo, 1986)
$CaCl_2$	0.01	Brümmer *et al.*, 1986	The Netherlands
NH_4NO_3	1	Pruess, 1998	Germany (DIN, 1998)
$MgNO_3$	0.01	Ganai *et al.*, 1982	
NH_4Cl	1	Krishnamurti *et al.*, 1995	
DTPA	0.01	Lindsay and Norwell, 1978	
EDTA	0.02	Prüeß, 1992	Austria (Österreichisches Normungsinstitut, 2014)
LMWOA	0.01	Krishnamurti *et al.*, 1997	

[a)]DTPA = diethylenetriaminepentaacetic acid; EDTA = ethylenediaminetetraacetic acid; LMWOA = low-molecular-weight organic acids and amino acids (*e.g.*, acetic, citric, oxalic, malic, and glutamic acids).

Bioavailability: chemical methods

- Water soluble pool
- Exchangeable pool
- Complexed/chelated pool



Bioavailability processes (A, B, C, and D)

Bioavailability: chemical methods

Water

- Extraction by centrifugation of water saturated soils
- Extraction by soil suction probes directly in situ from undisturbed soils
  - repeated collections from the same point without significant soil or plant disturbance
  - Leaching tests using H_2O as leachant have been standardized (e.g., US EPA, 1992; DIN, 1998).

- Ideally, the elemental pool in pore water is in equilibrium with the sorbed TE fraction
- Elemental pool in pore water is directly bioavailable to soil organisms and plant roots
  - water dilute the soil solution depending on the liquid-to-solid ratio which may change the soil
  - various elements are dissolved as organic complexes not necessarily bioavailable

Bioavailability: chemical methods

Ion exchangers, chelators and complexants

- Extractions are used to predict heavy metals bioavailability in soil
- Some standardized protocols have been adopted in environmental legislation of several countries
- Ideally, each salt solution induce the release of the exchangeable and complexed fractions of heavy metals sorbed onto soil solid phases
- Methods are easy to perform, cheap, robust and reproducible
- None of the extraction protocols selectively extracted a single element
- Examples of intereference are:
  - the pH effect of extractants
  - co-solubilization of untargeted soil pools

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Examples

Cadmium availability index: correlation between Cd concentration in grain of durum wheat and 1 M NH_4Cl exchangeable Cd (Krishnamurti et al. 1995)

Plant concentration and exchangeable metals in 0.01 M $CaCl_2$ (Houba et al., 1996; Peijnenburg et al., 2000; Koster et al., 2005; Meers et al., 2007a, b).11

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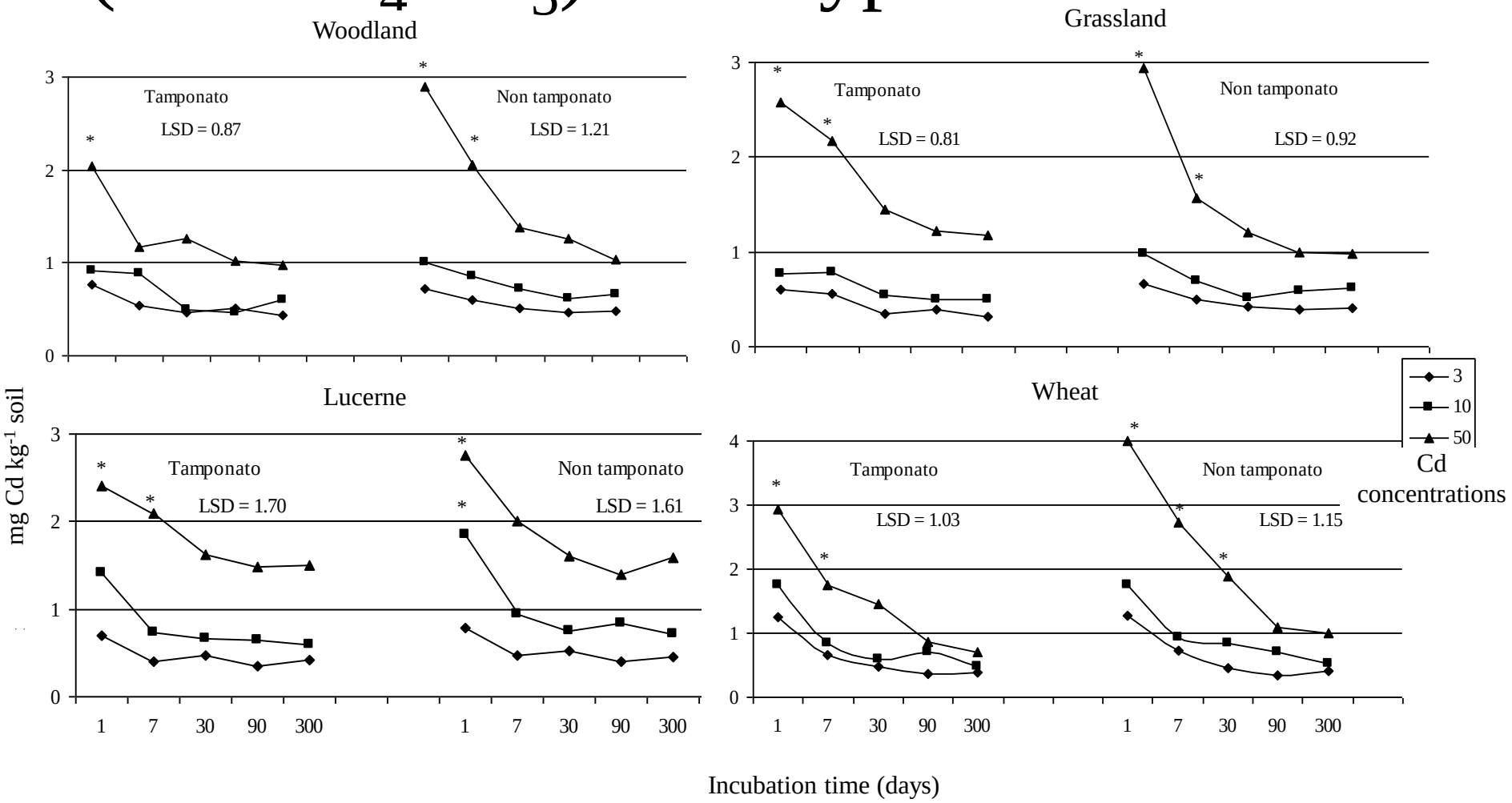
Chemically defined bioavailable Cd (1M NH_4NO_3) in a Hypocalcic calcisol



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Bioavailability: chemical methods

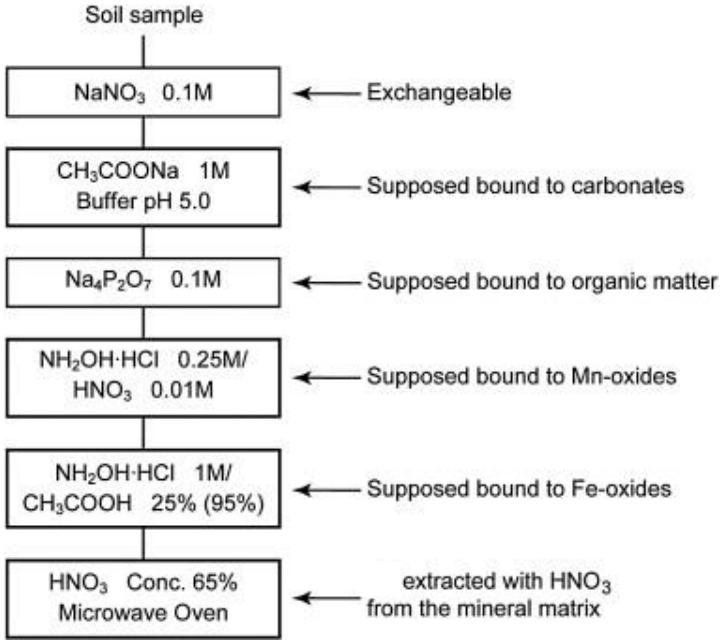
Table 3. Copper and Zn extraction and co-extraction of soil born phases by 1M NH_4NO_3

Reagent	N g/kg	TOC g/kg	Cu mg/kg	Fe mg/kg	Zn mg/kg
NH_4NO_3	432.6	8.38	46	10.54	3.9
H_2O	9.64	3.57	50.6	16.52	4.1
H_2O	0.77	0.55	10.98	3.1	2.76

Bioavailability using chemical methods: single solvent extraction

Tessier et al. 1979

- Sequential extractions
- Are usually used to speciate elements in soil, i.e. quantify the fractions that can be mobilized under different conditions (e.g., acidification oxidation, reduction)
- The former Community Bureau of Reference (BCR) of the EC established a four-step sequential extraction protocol to define:
  - soluble/exchangeable → 0.11M acetic acid
  - Reducible → 0.1M hydrochloride hydroxylamine
  - Oxidizable → 8.8M H_2O_2 /1M CH_3COONH_4
  - residual pool → Concentrated HNO_3



Bioavailability using chemical methods: single solvent extraction

Table 1. The protocol of sequential chemical extraction

Reagent	Time	Volume	Fraction
NH4NO3 1M	2 h	1:20	Exchangeable
Two rinse with H_2O	30 min each	1:20	
CH_3COOH	16 h	1:20	Oxidisable
Two rinse with H_2O	30 min each	1:20	
$Na_4P_2O_7$ 0.1M	16 h	1:20	Organic matter and sulfides
Two rinse with H_2O	30 min each	1:20	
H_3NOHCl 1M	16 h	1:20	Fe/Mn (oxy)hydroxide
Two rinse with H_2O	30 min each	1:20	
CH3COONH4 1M	16h	1:20	Fe/Mn (oxy)hydroxide

Reagent	N g/kg	TOC g/kg	Cu mg/kg	Fe mg/kg	Zn mg/kg
NH_4NO_3	432.6	8.38	46	10.54	3.9
H_2O	9.64	3.57	50.6	16.52	4.1
H_2O	0.77	0.55	10.98	3.1	2.76
CH_3COOH	1.22	23200	53.6	35.42	4.02
H_2O	n.d.*	1539.4	90.6	4.98	5.28
H_2O	n.d.	101.38	11.96	0.34	3.5
$Na_4P_2O_7$	0.09	16.8	159.6	636.6	6.5
H_2O	n.d.	38.2	6.26	155	3.36
H_2O	n.d.	n.d.	4.38	100.18	3.12
H_3NOHCl	25.94	2.11	151	717.6	15.42
H_2O	7.01	1.21	20.8	48.46	6.44
H_2O	0.93	0.75	3.2	10.96	3.52
CH_3COONH_4	215.66	1340.8	44	n.d.	n.d.
H_2O	19.62	96.44	n.d.	n.d.	n.d.
H_2O	0.31	3.56	n.d.	7.88	n.d.
		Total	652.98	1747.58	61.92

Bioavailability using chemical methods: single solvent extraction

Reagent	Time	Volume	Fraction
$BaCl_2$ 0.1M	16 h	1:10	Exchangeable
Two rinse with H_2O	30 min each	1:20	
HCl 1M	16 h	1:60	Oxidisable
Two rinse with H_2O	30 min each	1:20	
H_3NOHCl 1M, 90^{0}C	3 h	1:20	Fe/Mn (oxy)hydroxide
Two rinse with H_2O	30 min each	1:20	
A. H_2O_2 30%	x*	1:10	Organic matter and sulfides
B. NaOH 0.1M 65^{0}C	1, 4, 16 h	1:10	
Two rinse with H_2O	30 min each	1:20	
CH_3COONH_4 1M	16h	1:20	Fe/Mn (oxy)hydroxide
Two rinse with H_2O	30 min each	1:20	

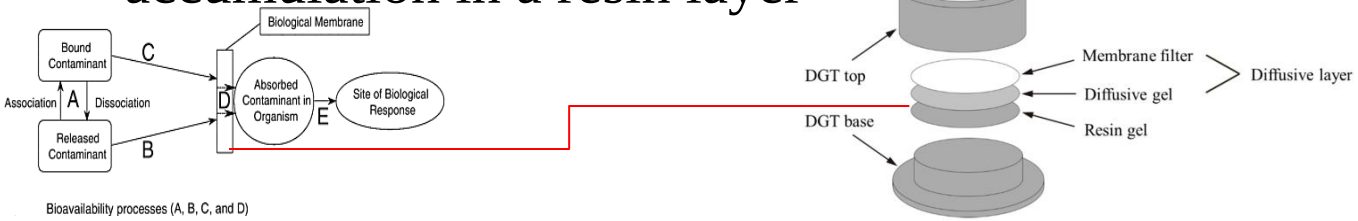
Reagent	N g/kg	TOC g/kg	Cu mg/kg	Fe mg/kg	Zn mg/kg
$BaCl_2$	n.d.	0.52	10.9	4.58	1.4
H_2O	n.d.	0.86	7.64	n.d.	n.d.
H_2O	n.d.	1.26	23.6	6.2	n.d.
HCl	n.d.	576.6	217.2	483.3	19.86
H_2O	n.d.	18.56	13.12	22.34	0.78
H_2O	1.36	38.28	9.64	53.76	3.38
H_3NOHCl	122.84	32.72	344	1203.8	9.16
H_2O	16.08	26.56	9.6	79.36	0.6
H_2O	0.52	23.7	n.d.	n.d.	0.4
H_2O_2	0.14	4.04	9.56	41.21	1.11
H_2O	n.d.	1.7	3.4	6.22	n.d.
H_2O	n.d.	1.73	3.92	12.28	n.d.
NaOH	0.95	19.9	145	83.65	0.03
H_2O	n.d.	n.d.	23.4	16.73	n.d.
H_2O	n.d.	0.80	8.52	61	n.d.
CH_3COONH_4	320.2	2034	46.6	n.d.	n.d.
H_2O	14.46	65.9	0.88	n.d.	n.d.
H_2O	n.d.	20.76	12.82	n.d.	n.d.
		Total	889.1	2163.7	36.72

Bioavailability using chemical methods: single solvent extraction

Diffusive gradients in thin films (DGT)

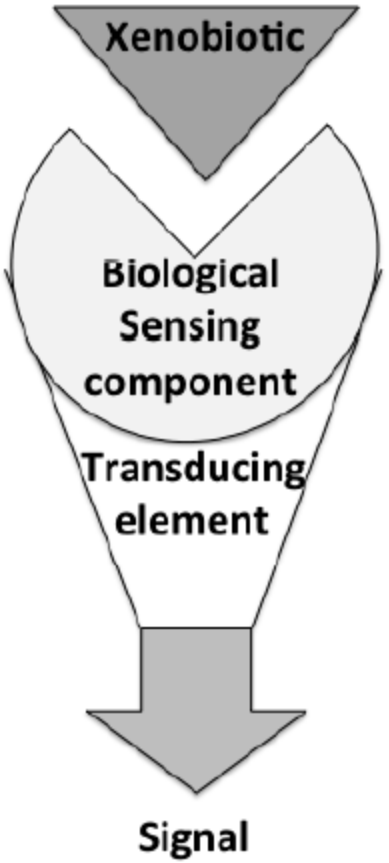
- A devise developed for *in situ* sampling of dissolved TE ions in water, sediments and soils
- The technique is based on diffusion of dissolved TE ions through a diffusive hydrogel layer and accumulation in a resin layer

- Accumulated elements indicate their potential bioavailability and fluxes at the DGTsoil interface.
- It is nowadays considered a standard technique for estimating the phytoavailability, with superior predictability than soil extraction methods



Bioavailability: chemical biosensors (chemosensors)

- **Chemosensors** consist of:
- Receptor and a detector.
- Receptors include enzymes, antibodies, and lipid layers, and are responsible for the selectivity of the sensor.
- The detector is not selective and acts as transducer into an electrical signal
- Detectors can be electrochemical (potentiometric, amperometric, impedance), piezoelectric, thermal or optical (reflectrometry, interferometry, optical waveguide lightmode spectroscopy, total internal reflection fluorescence, surface plasmon resonance)



Bioavailability: chemical biosensors (chemosensors)

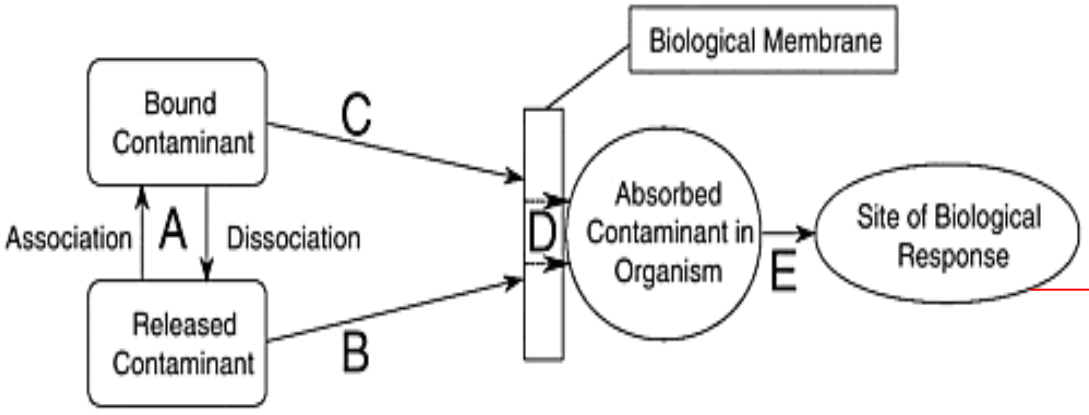
Common chemosensors are:

- piezoelectric detectors (e.g. quartz crystals) that vibrate under the an electric field, and the variations of the resonant frequency of an oscillating piezoelectric crystal in relation to the mass deposited on the crystal surface are used as an index of interactions between the receptor and the analyte.
- The piezoelectric DNA-based biosensors are constructed by immobilizing double stranded DNA (dsDNA), and are then placed in contact with the environmental liquid phase or extracts, allowing the contact between DNA and environmental pollutants
- DNA-based biosensor for the qualitative/semiquantitative detection of genotoxic effects of aromatic xenobiotics such as benzene, naphthalene and anthracene have been used for soil analysis and results were in agreement with standard plant and animal ecotoxicity tests
- Supramolecular chemosensors carry complex multi-molecule aggregates of simple molecules and have proven to be selective for various heavy metals and organic xenobiotics at nanomolar concentration

Chemosensors: quartz microbalance

•Provides information on the bond formation and reversibility of the reactions

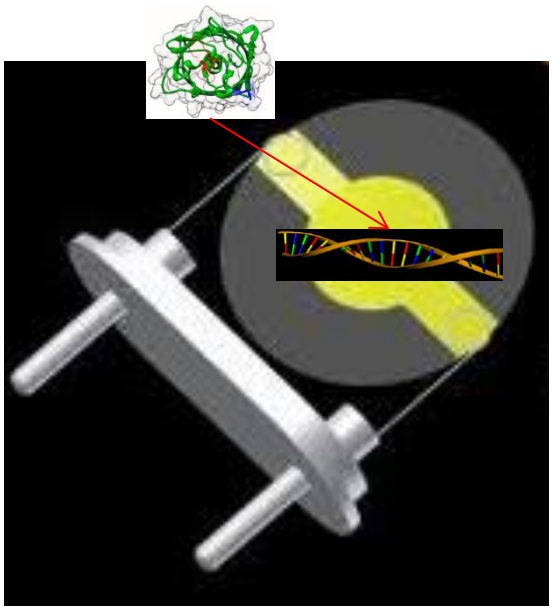
•Detects interactions between partner molecules after immobilization, based on the changes in the vibration of quartz crystals.



Bioavailability processes (A, B, C, and D)

Chemosensors: quartz microbalance

•The quartz microbalance technique is bases on the change of vibration of a quartz crystal vibrating under an electric field after interactions between sorbed molecules and other molecules



Chemosensors: quartz microbalance

Case study: interaction between the β-glucosidase from the soil-borne fungus *Aspergillus niger* because:

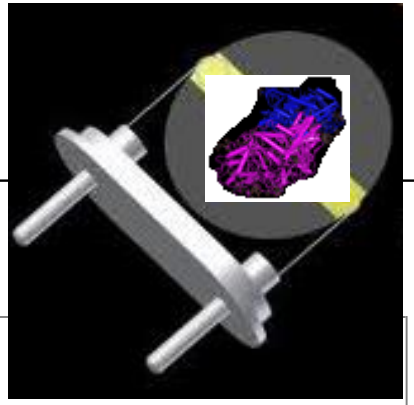
- It is an important enzyme in the soil ecology

- it is well characterized and purified enzymes are commercially available (SIGMA)

- interactions with humic substractes can be detected with other independent techniques (e.g. protein electrophopresis, catalytic activity

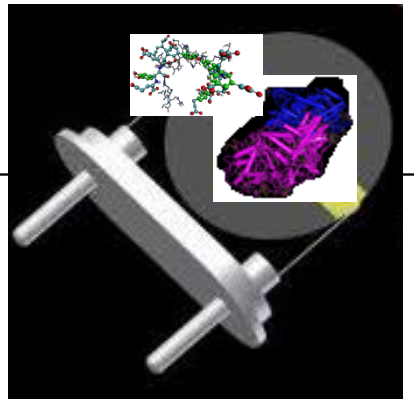
Chemosensors: quartz microbalance

Phase 1: enzyme immobilization on the support



Chemosensors: quartz microbalance

Phase 2: β-glucosidase-humic acids interactions



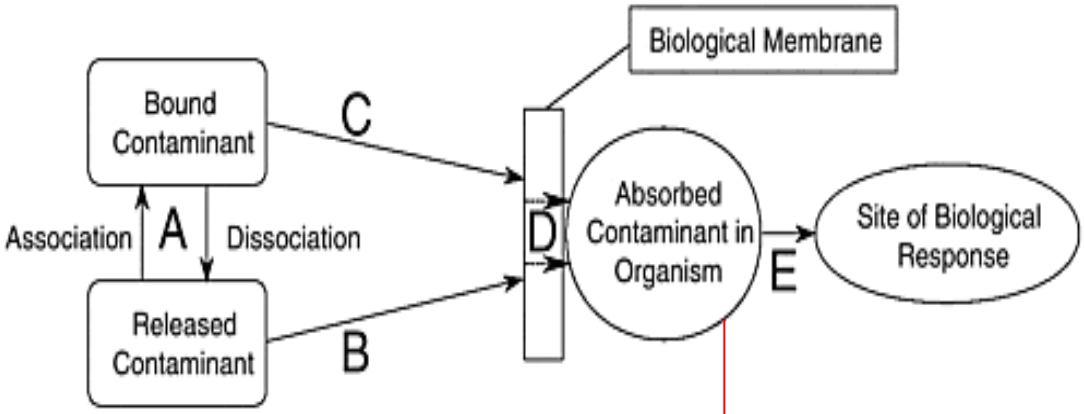
Bioavailability: whole cell biosensors

- Cell biosensors have actracted an increasing interest because they provide immediate biological information (toxicity, cancerogenity, mutagenicity) of the analytical determination

- From 2000s to date, bioreporter data have been interpreted in terms of fluxes of analytes because ot has been understood that gene expression is sustained by discrete quantities of analtes entering the cells, not by single events of membrane crossing

- A reporter system can be already present in a biosensor (natural bioreporter) or it can be inserted in a specific genome region so as to report on the metabolic activity of the host cell

Bioavailability: whole cell biosensors



Indication by the whole cell biosensors

Bioavailability: early bioreporters

lacZ (beta-galactosidase)

xylE (catecol 2,3-dioxigenase)

*tfd*A (2,4-diclorofenoxiacetate oxidase)

Not useful as biorporters for soil because their responses are difficult to distinguish from the strong background in soil

Early bioreporters

lux (bacterial luciferase)

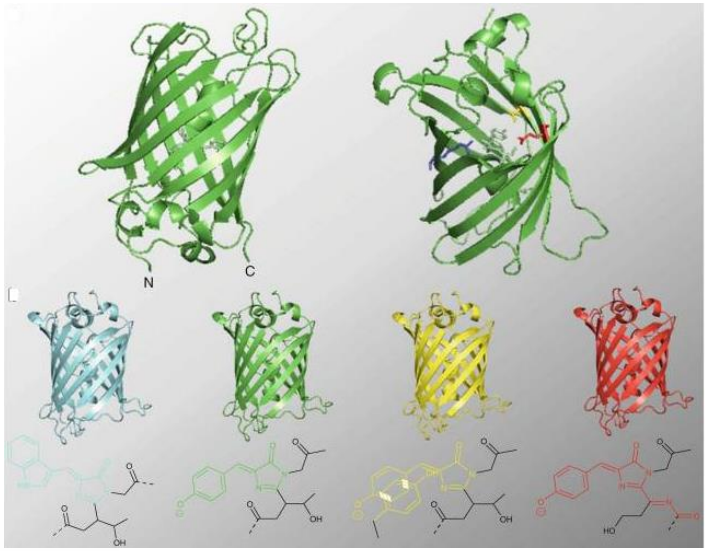
*ina*Z (ice nuclation protein)

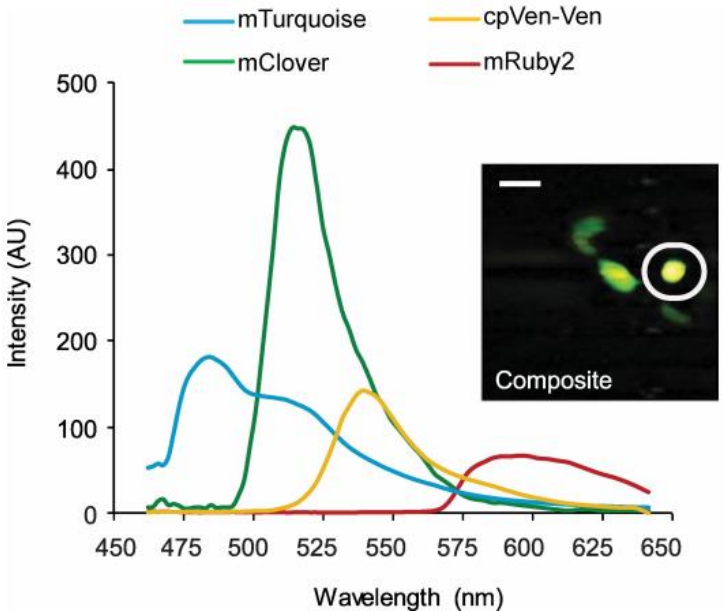
gfp (green fluorescent protein and variants

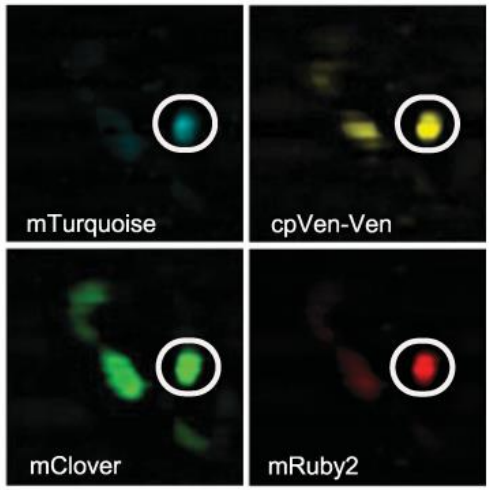
Very useful for determining activity, integrity and impact on soil microorganisms

Early bioreporters

- Variants of the GFP have been produced and inserted to improve its detection in the environmental biosensors and also to create multiple-indicator biosensors







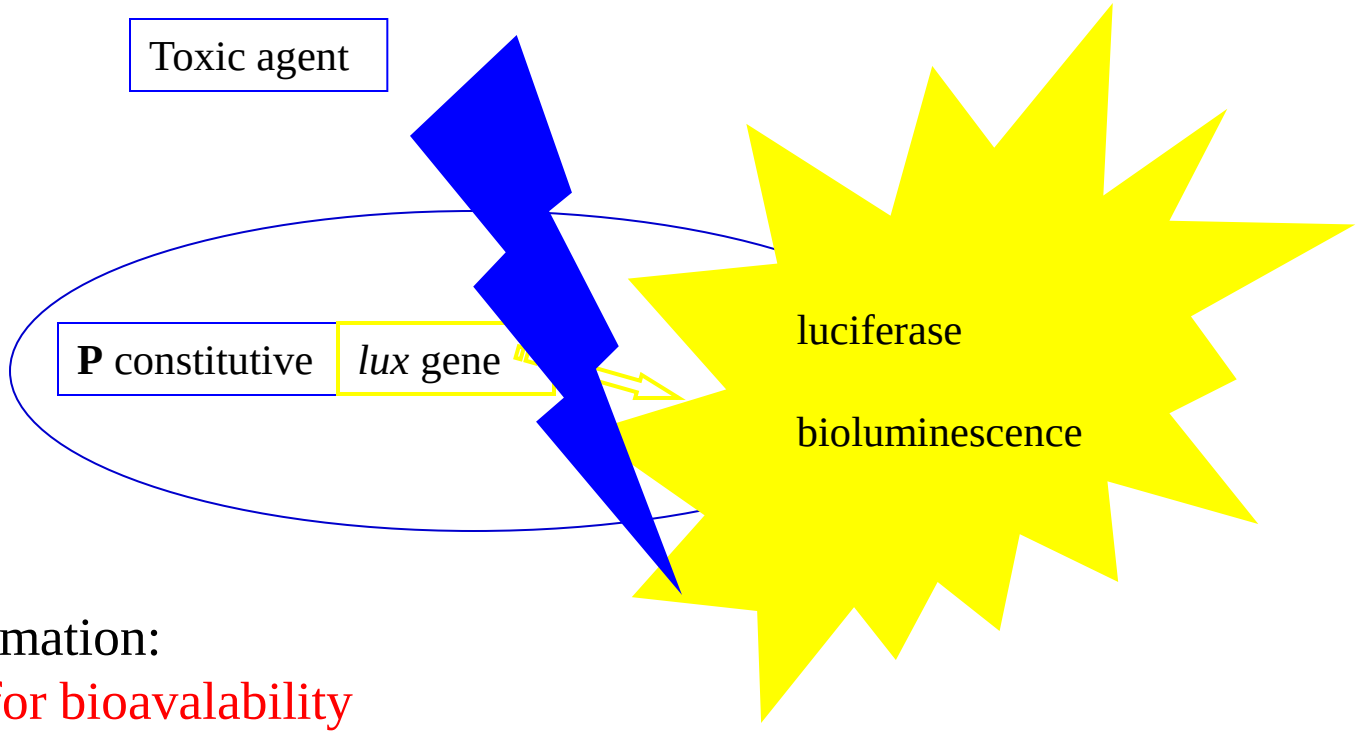
Early bioreporters

- The original idea of using luminescent bacteria as biosensors was of D.L. Isemberg
- Early natural bioreporters: *Vibrio* spp. or *Photobacterium* spp., bioluminescent fungi as *Armillaria mellea* e *Nycena citricolour*

- Luciferase is an enzyme catalyzing the oxidation of $FMNH_2$ and long linear aldheides to FMN e corrisponding fatty acids in the presence of O_2 producing blu-green light (490 nm) termed bioluminescence

- Conservative *lux* operone strucutre allow its relatively ease of transfer to several host cells allowing the construction of bioluminescent lux bioreporters.

Early bioreporters: BioTox®, MicroTox®



Information:

Non specific for bioavalability

Cell disruption,

Inhibition of housekeeping enzymes

Specific and inducible bioreporters: determination of actual bioavailability

The first inducible bioluminescent biosensor was constructed by Gary Sayler in 1990 for the detection of naphtalene

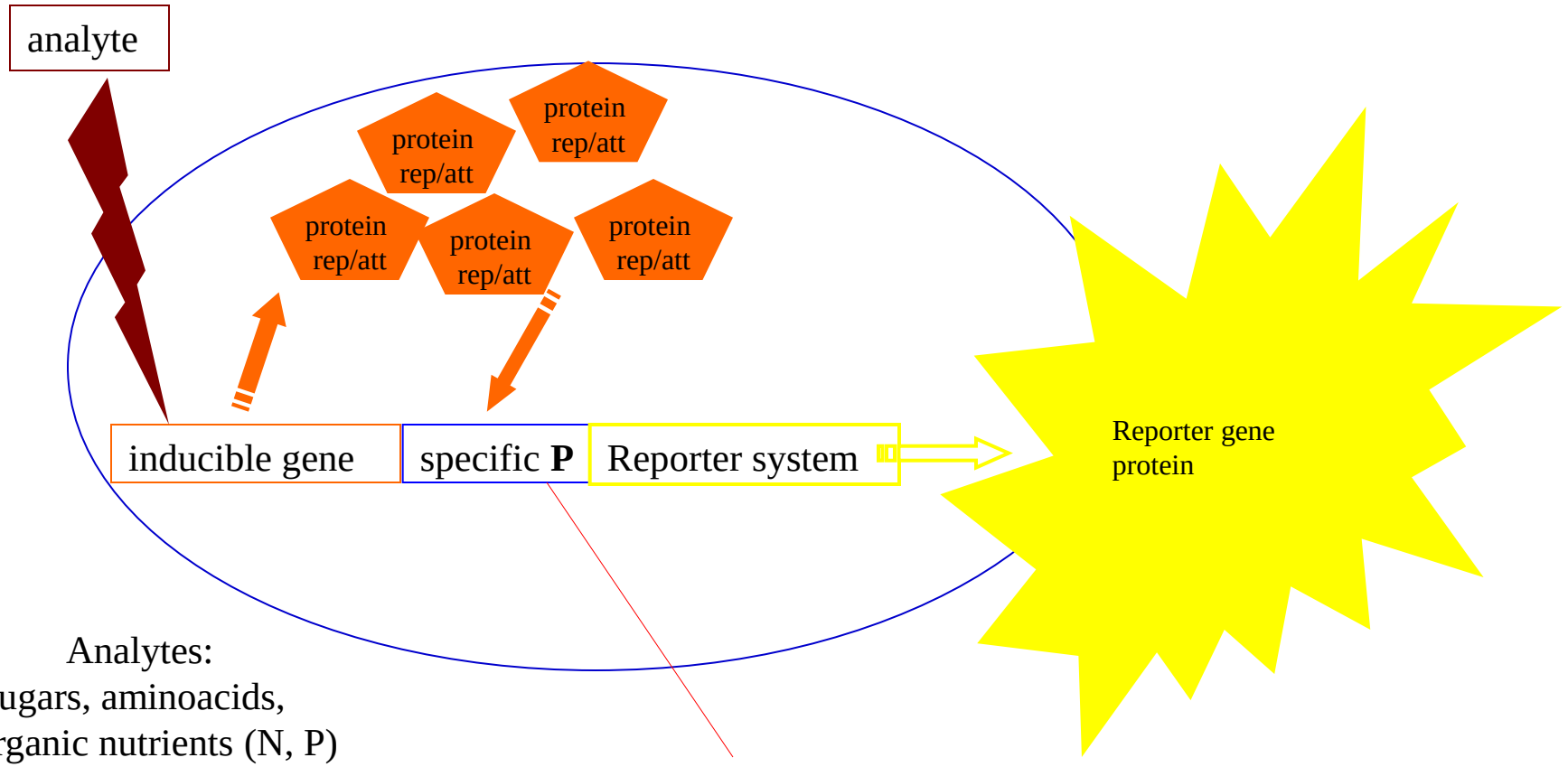
In the original Sayler's scheme the promoterless *lux* gene was under control of the *nah* gene in *P. fluorescens* HK44

This strain became luminescent in the presence of naphtalene and its metabolite salicilate (King et al 1990, Science vol 249, 778–781)

Reporter gene nahG, coding for the salicilate oxidizing enzyme in the catechol pathway
Reporter system: promoterless *lux* operone of *V. fischeri*
Vector: plasmid pUTK21 carrying the *lux*:*nah*G gene fusion
Whole cell biosensor: *P. fluorescens* HK44 bioluminescent in the presence of naphtalene

Was the firs demonstration that a genetic regulatory system could be steadly engineered

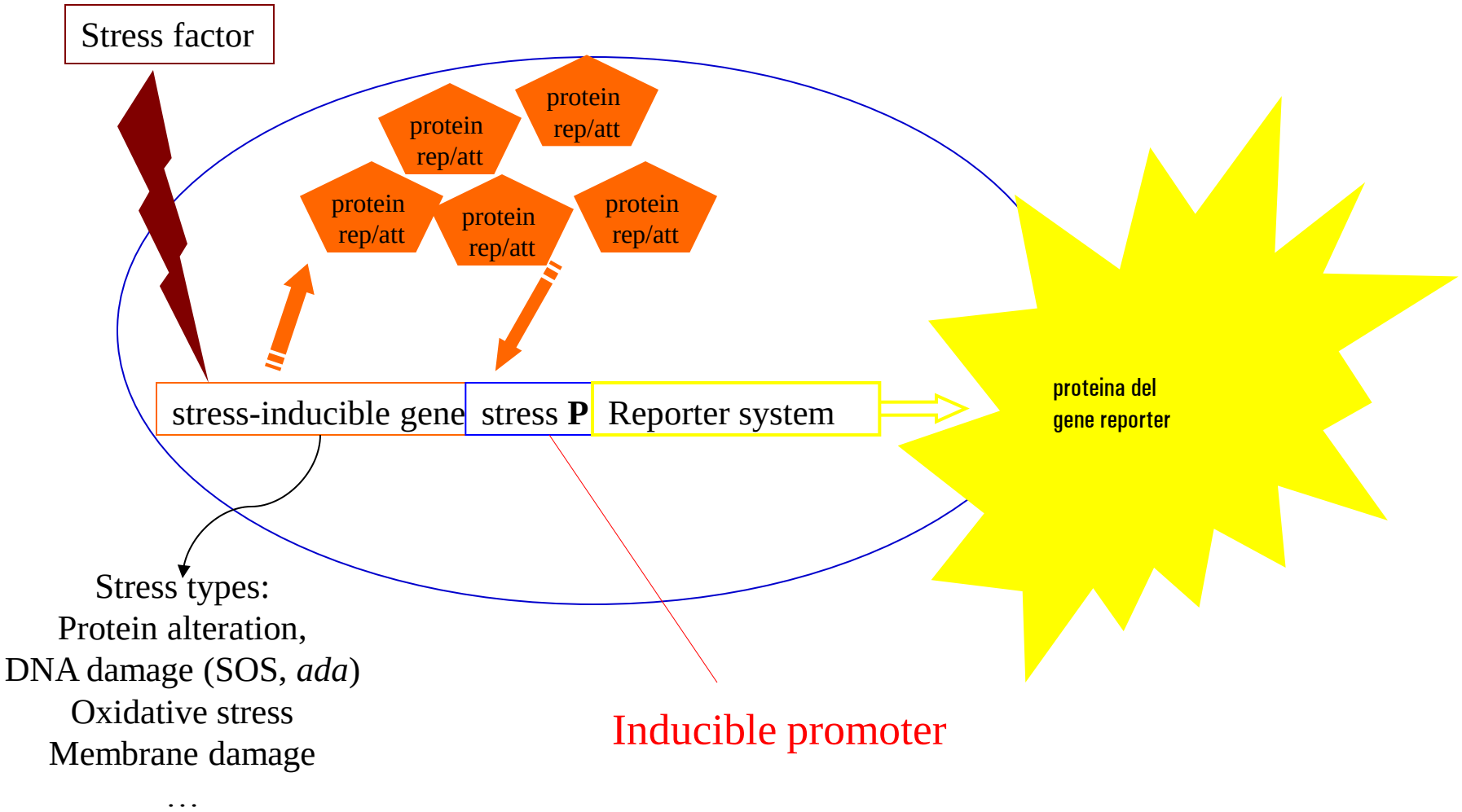
Specific and inducible bioreporters



Analytes:
sugars, aminoacids,
Inorganic nutrients (N, P)
xenobiotics
Heavy metals
Signal molecules

Inducible promoter

Specific and inducible bioreporters



Specific and inducible bioreporters

- The first strains inserted with reoprter systems were enteric strains genetically well characterized
- *E. coli*
- *S. typhimurium*
- The first soil bacteria inserted with reoprter systems were constructed in the 1990s for studying nutrient fluxes and the impact of pollutants in soil microbial communities

Example of strains and plasmids (in brackets) used for constructing whole-cell biosensors

Strains and plasmids (in brackets) used for constructing whole-cell biosensors inserting *lux*, *gfp* and β-galactosidase reporter genes responding to different organic and inorganic compounds and elements

Whole-cell biosensor	Signal	Target analyte
Organic xenobiotics		
P. fluorescens HK44 (pUTK21)	Bioluminescence	Naphtalene
P. putida RB1353/RB1351 (pUTK9)	Bioluminescence	BTEX
P. putida RB1401 (pTOL)	Bioluminescence	BTEX
P. putida TVA8 (pUTK214)	Bioluminescence	BTEX
Stenotrophomonas sp ENV307 (pUTK60)	Bioluminescence	Alkylsulphonates
R. eutropha JMP134 (pUTK220)	Green fluorescence	PCB
Burkholderia sp (pUCD607)	Bioluminescence	PCB
Pseudomonas putida F1 (pUT mini-Tn5 luxCDABE)	Bioluminescence	PCB
N. europaea ATCC 19718(pHLUX20)	Bioluminescence	Alkylsulphonates
P. fluorescens A506 (pTS)	Green fluorescence	BTEX
Heavy metals and metalloids		
S. aureus RN4420 (pI258)	Bioluminescence	As
C. metallidurans CH34 (pMOL30)	Bioluminescence	Cd, Zn, Ni
R. leguminosarum bv *trifolii* F6 (pUCD607)	Bioluminescence	Cu
C. metallidurans AE1239 (pMOL30)	Bioluminescence	Cr
P. putida KT2440 (pUT-mer-lux)	Bioluminescence	Cu
Nutrients and physiologically active molecules		
P. fluorescens 10586 (pP2)	Bioluminescence	C
P. fluorescens DF57 N3 (pP2)	Bioluminescence	N
P. fluorescens DF57 P9 (pP2)	Bioluminescence	P
P. putida KT2440 (pLYS24-davT-lux)	Bioluminescence	C
P. fluorescens WCS365 (pMP5291)	Bioluminescence	Putrescine
R. leguminosarum 4292 (pIJ1737) (pIJ1730)	β-galactosidase	Nodulation factors
R. leguminosarum 3841 (pOT1)	Green fluorescence	Nodulation factors
P. fluorescens F113 SF3 (pLS312), SF5 (pLS52)	Bioluminescence	Ecological interactions

BTEX benzene, toluene, ethylbenzene and xylene; *PCB* polychlorinated biphenyls; *As* arsenic; *Cd* cadmium; *Cr* chromium, *Cu* copper; *C* carbon, *N* nitrogen; *P* phosphorus

Specific and inducible bioreporters: organic pollutants

- *P. fluorescens* HK44 (King et al 1990)
- *P. putida* RB1353 or RB1351(Burlage et al. 1990)
- *P. putida* RB1401 (Burlage et al 1994)
- *P. fluorescens* 10586s (Boyd et al 1997)
- *P. putida* TVA8 (Applegate et al 1998)
- *R. eutropha* ENV307 pUTK60 *R* (Layton et al 1998)
- *Stenotrophomonas* sp (Layton et al 1999)
- *R. eutropha* pJP4(Hay et al 2000)
- *A. chlorophenolicus* (Elvang et al 2001).
- *Burkholderia* sp (pUCD607) (Boyd et al 2001)
- *P. putida* F1 Weitz et al. (2001)
- *Nitrosomonas europaea* (Brandt et al 2002)
- *P. fluorescens* A506pTS (Stiner and Halvorsen 2002)

Specific and inducible bioreporters: selected organic pollutants

- **Linear alkanes**: fusion between the *alk* regulone of *P. oleovorans* with *lux*AB of *V. harveyi*, transformed in *E. coli* DH5R
- **Alkillsulfonates**: fusion between *lux* constructs in *Nitrosomonas europaea* (Brandt et al 2002), *Stenotrophomonas* sp. and *Ralstonia* sp. (Layton et al 1999)
- **Benzene, Toluene and Xylene (BTEX)**: lux constructa in *P. putida* (de Lorenzo et al 1993; Burlage et al 1994; Applegate et al 1998) and *E. coli* (Selifonova et al 1996; Willardson et al 1998)
- gfp constructs inducible by BTEX were carried out in *P. fluorescens* (Stiner e Halvorsen 2002; Casavant et al 2003)
- **Organo-chlorinated and polichlorinated compounds**: lux constructs in *P. fluorescens* 10586s, *Burkholderia* sp. Rasc pUCD607 (Palmer et al. 1998; Boyd et al. 2001), *P. fluorescens* 8866, *P. putida* F1 (Weitz et al. 2001).
- Gfp constructs of ctfdR-tfdDII genes in *Pseudomonas* sp. (Hay et al 2000), tfdCI di *R. eutropha* (Füchslin et al 2003), or gfp fusion with orf0-bphA1 in *P. fluorescens* (Boldt et al 2004)

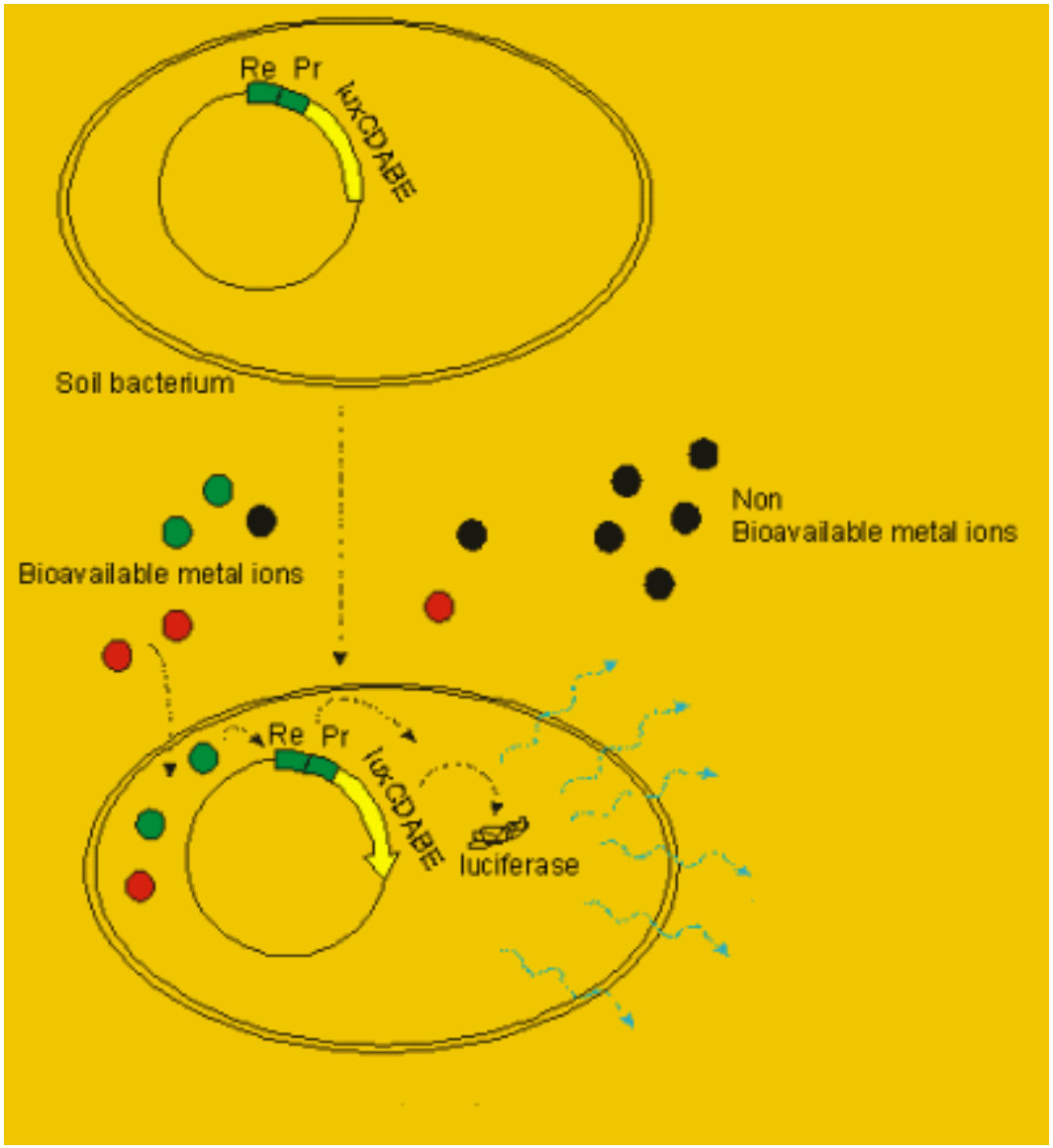
Specific and inducible bioreporters: selected organic pollutants

- **Polichlorinated biphenils (PCB)**: lux constructs with various plasmidial operons of *R. eutropha* coding for degradative nezymes (Fava et al 1993; Layton et al 1998)
- **Bioactive molecules**: lux constructs with genes responding to the bioavailability of structural analogs (e.g. isomers), antibiotics (Bahl et al. 2004), endocrine disruptors (Desbrow et al. 1998), quorum sensing molecules (Andersen et al. 2001) through gene insertions of co-regulatory genes activates by parent molecules

Bioavailability of heavy metals: specific and inducible bioreporters

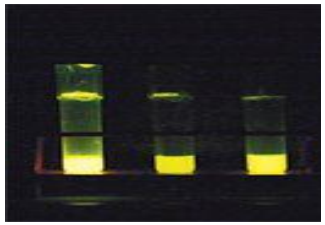
- *E. coli* (Selifonova et al 1993) (Hg)
- *Staphylococcus aureus* (Corbisier et al 1993) (As)
- *R. eutropha* pMOL28 and pMOL30 (Collard et al 1994) (Cd, Zn, Ni)
- R. eutropha (AE104) 607 (Paton et al 1995) (Cu)
- *R. eutropha (AE104)* (Corbisier et al 1999) (Cr)
- *P. putida* KT2440 (Hansen and Sørensen 2000) (Cu)
- *P. fluorescens* (Tom-Petersen et al 2004) (Hg)

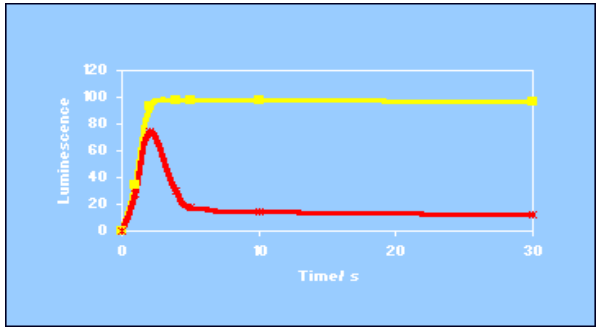
Bioavailability of heavy metals: specific and inducible bioreporters



Heavy metals biosensors: case studies

- Suspension of 2 g of sieved (<2 mm) sediments in 8 ml of 2% NaCl
- Shake for 5min by hand and settling for 30min
- Adjustment of pH and conductivity
- Reconstituition freeze-dried V. fischeri cells
- Addition of 300 ml of the bacterial suspension to 300 ml of samples (solid/supernatant)
- Measure of bioluminescence
- Calculation of the inhibition of bioluminescence index (INH%) after 15 or 30 minutes





INH%

< 20%

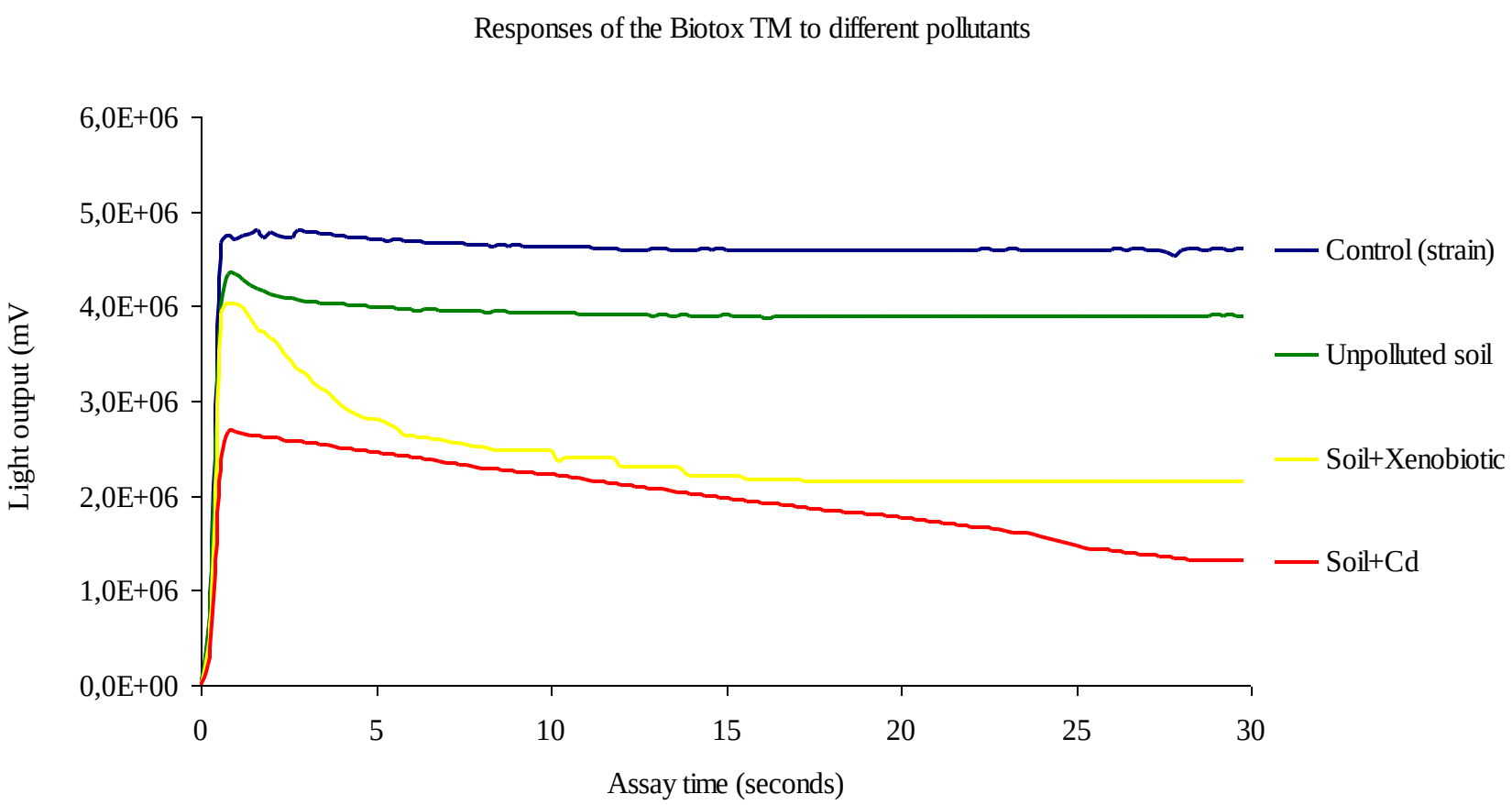
> 20%

Inhibition% =

$100 - [(TL_{15} / K_F * TL_0) * 100]$

$K_F = CL_{15}/CL_0$

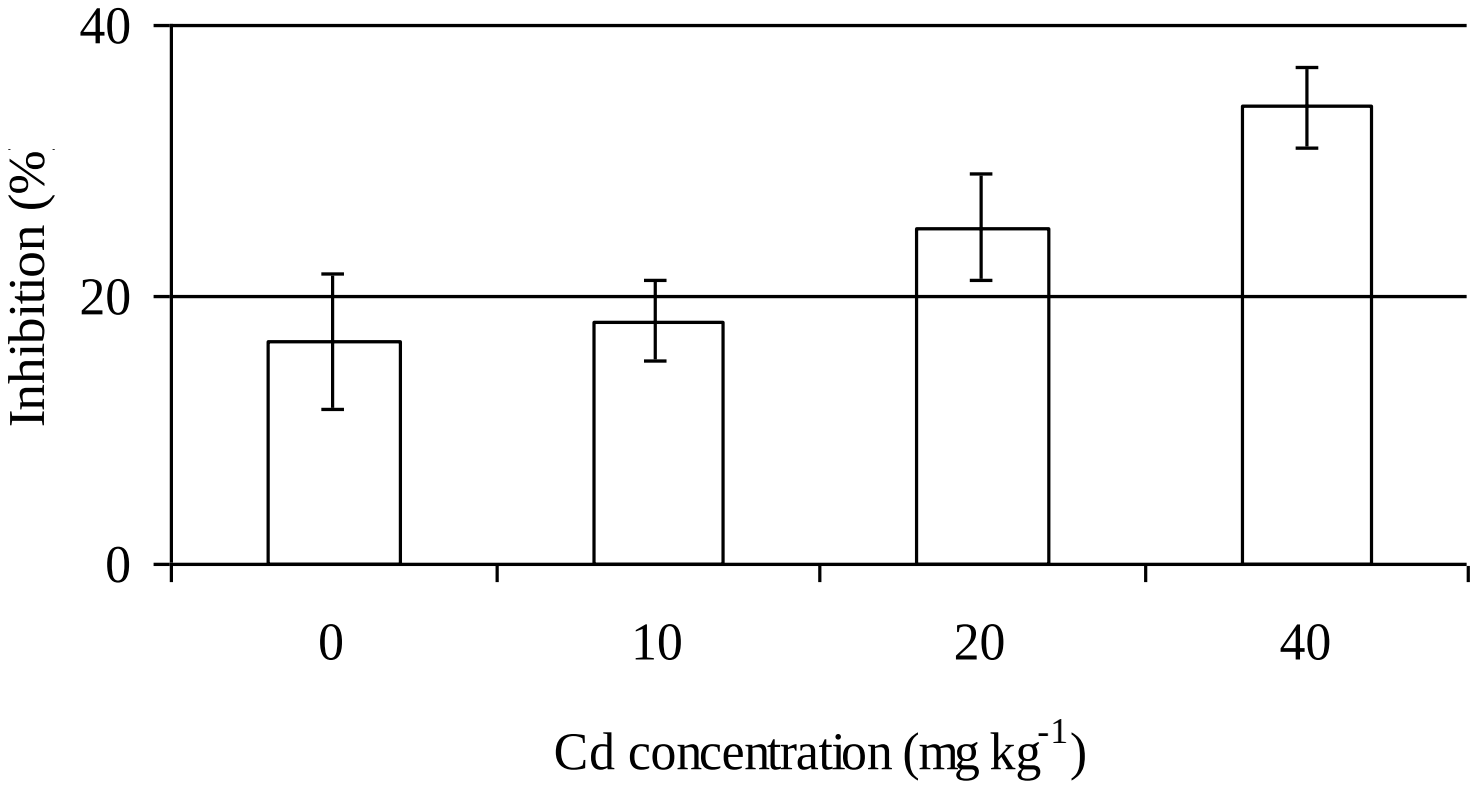
Response of the Biotox (*V. fischeri*) test to soil pollution



Biotox test: the AGIR experiments (Bordeaux, France)

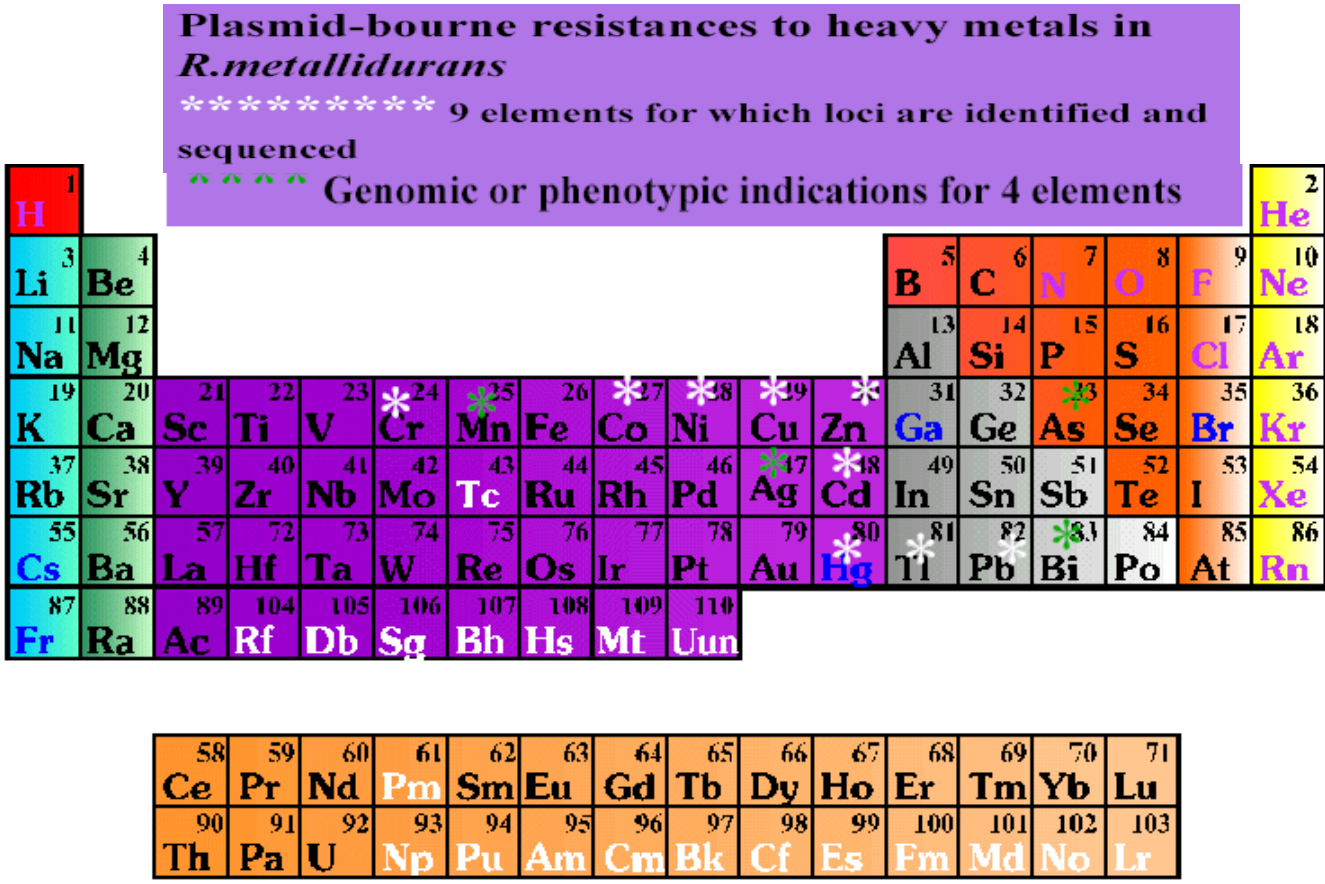
Soil	pH (H_2O)	Clay %	Silt %	Sand %	TOC %	N tot %	$CaCl_2$-extractable Cd (mg Kg^{-1})	Cd	Soil management
Parcel 16	7.5	17.5	15.0	67.5	0.95	0.10	< 0.01	0.7	Miaze
Parcel 17	7.6	17.5	15.0	67.5	0.74	0.08	< 0.01	1.2	Fallow
Parcel 18	6. 7	17.5	15.0	67.5	1.33	0,11	< 0.01	0.7	Miaze
Parcel 19	6.5	17.5	15.0	67.5	0.33	0.03	< 0.01	1.0	Fallow
Parcel 25	6.0	17.5	15.0	67.5	1.20	1.12	< 0.01	0.8	Miaze
Parcel 11	7.5	17.5	15.0	67.5	0.25	0.03	0.10	10.6	Miaze
Parcel 12	6.8	17.5	15.0	67.5	0.23	0.02	0.12	8.0	Fallow
Parcel 13	6.1	17.5	15.0	67.5	0.27	0.03	0.13	8.7	Miaze
Parcel 14	5.5	17.5	15.0	67.5	1.11	1.10	0.14	8.4	Fallow
Parcel 28	6.1	17.5	15.0	67.5	0.29	0.03	0.13	10.0	Fallow
Parcel 6	7.1	17.5	15.0	67.5	0.47	0.04	0.12	18.0	Miaze
Parcel 7	7.1	17.5	15.0	67.5	0.33	0.03	0.12	17.3	Fallow
Parcel 8	6.3	17.5	15.0	67.5	0.35	0.04	0.14	16.0	Miaze
Parcel 9	6.5	17.5	15.0	67.5	0.29	0.03	0.13	16.5	Fallow
Parcel 33	6.1	17.5	15.0	67.5	0.35	0.03	0.16	16.9	Fallow
Parcel 1	7.1	17.5	15.0	67.5	0.49	0.04	0.34	41.0	Miaze
Parcel 2	7.2	17.5	15.0	67.5	0.26	0.03	0.33	31.2	Fallow
Parcel 3	6.3	17.5	15.0	67.5	1.0	0.91	0.40	41.0	Miaze
Parcel 4	6.1	17.5	15.0	67.5	0.94	0.09	0.44	36.0	Fallow

Biotox test: the AGIR experiments (Bordeaux, France)

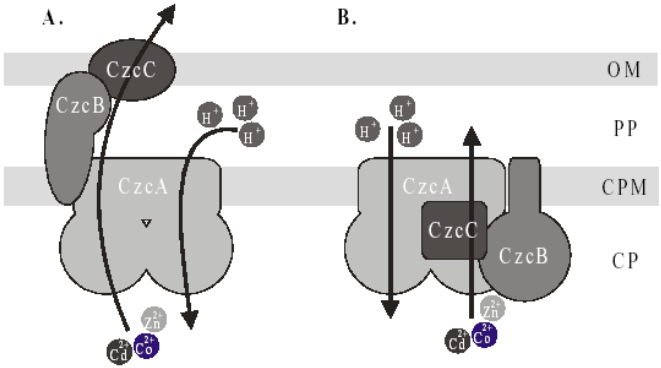


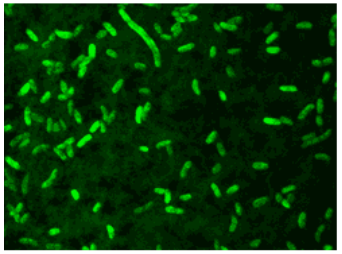
BIOMET® test: the AGIR experiments (Bordeaux, France)

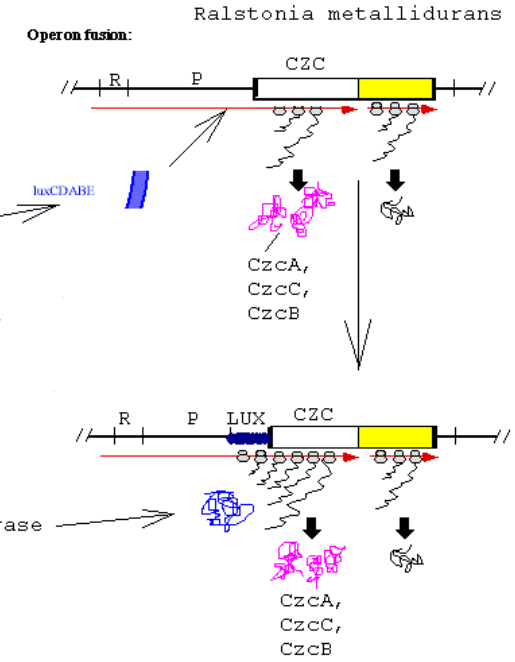
The BIOMET® test is based on the use of a transcriptional fusion between the *lux* gene of *V. Fischeri* and the promoter gene of the czc metal inducible operon of Ralstonia metallidurans, a multi-metal resistant soil borne bacterium



The *czc* operone encodes for the membrane efflux system of Cd, Zn and Co, conferring metal resistance



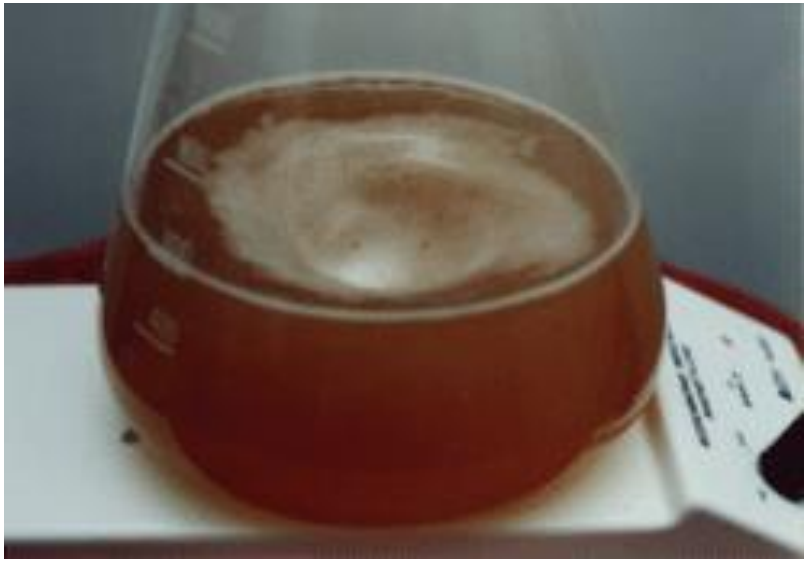




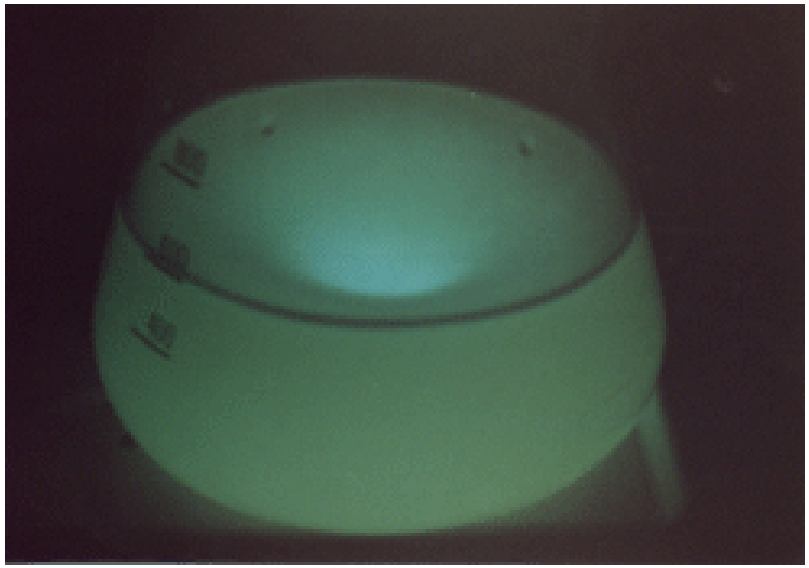
The membrane proteins czcCBAxRS are cation/proton antiport mechanisms against Cd, Zn and Co

BIOMET® test: the AGIR experiments (Bordeaux, France)

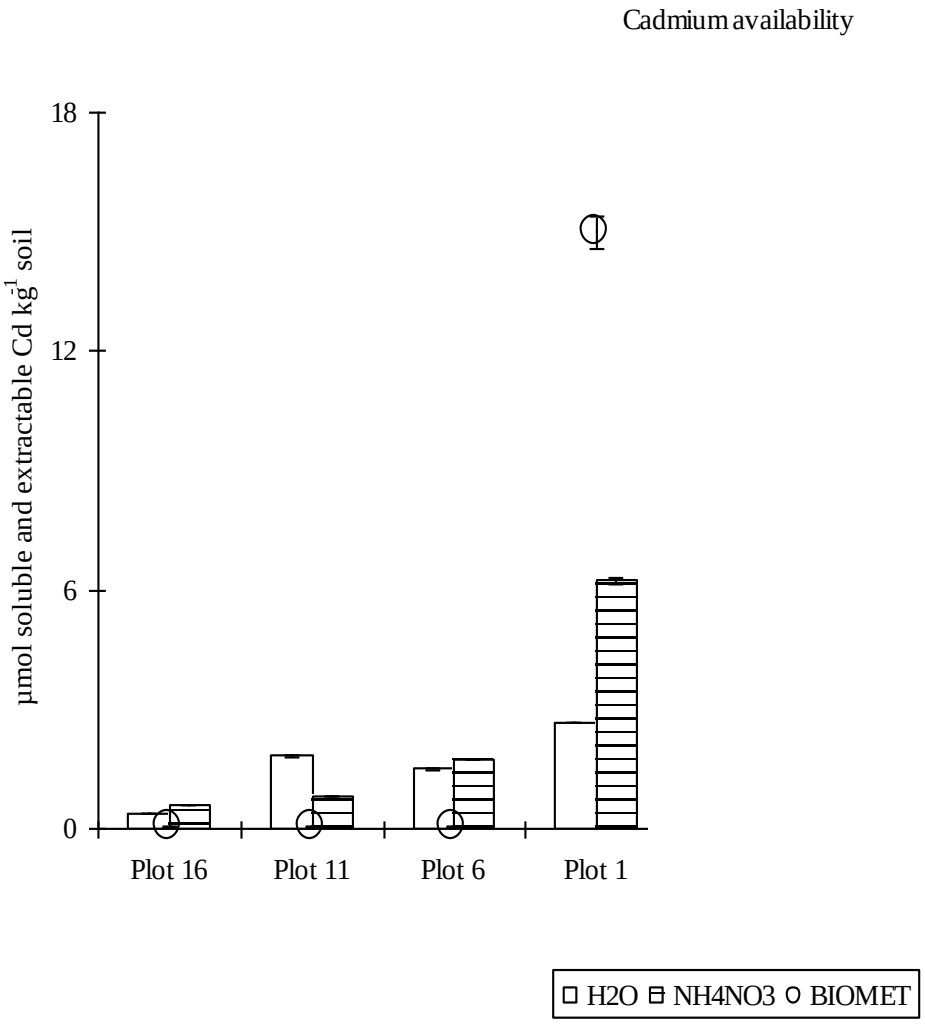
Cd-



Cd+

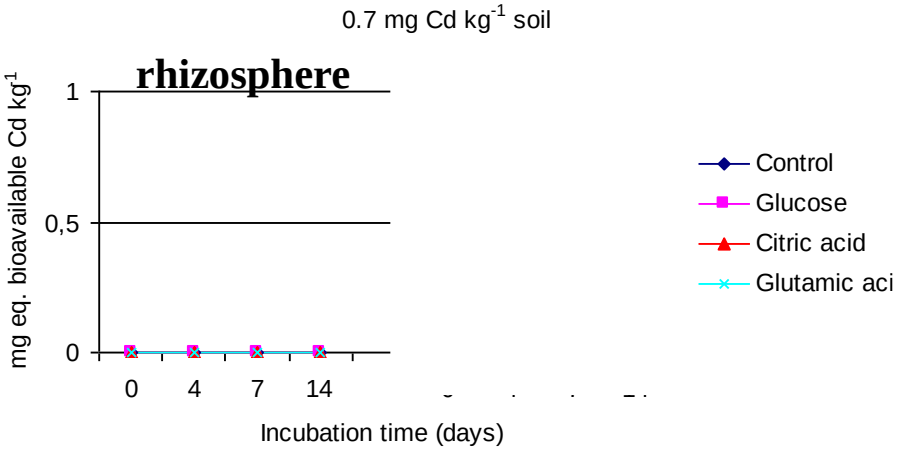


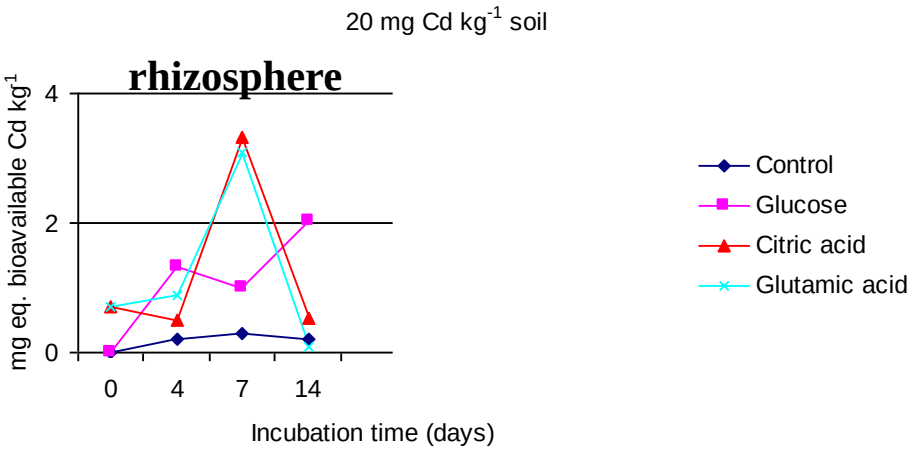
BIOMET® test: the AGIR experiments (Bordeaux, France)

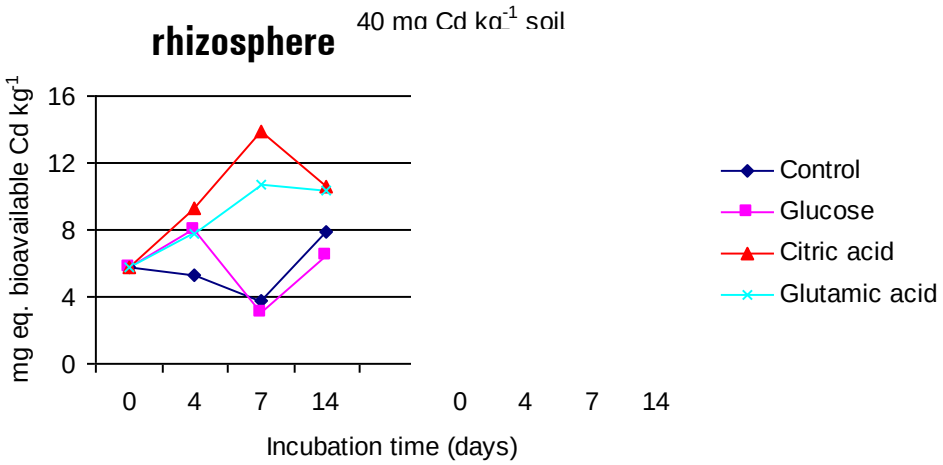


Both the BioTox® and BIOMET® responses were in agreement with those of soil microorganisms, soil enzyme activities and toxicity symptoms in maize plants

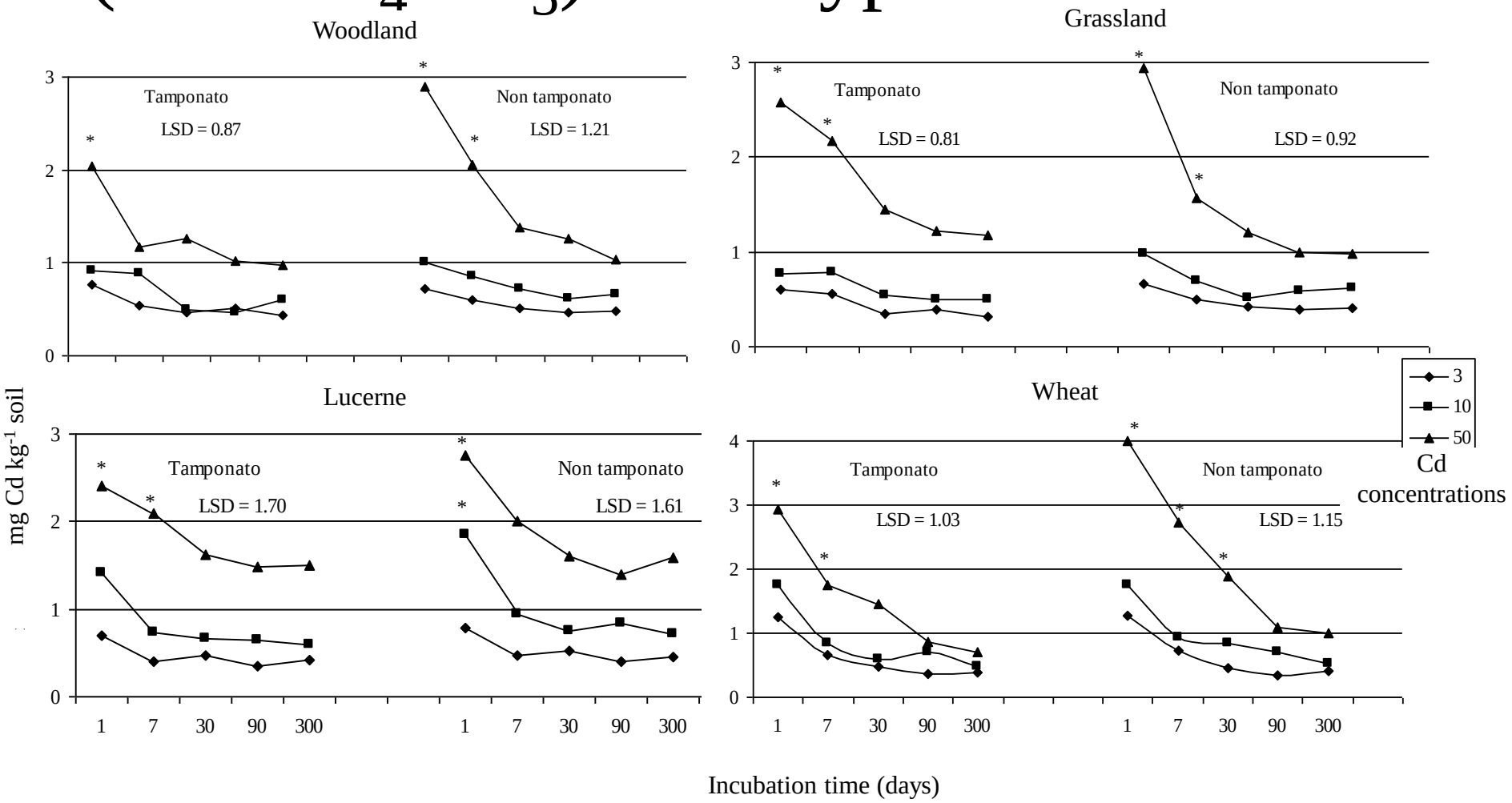
BIOMET® test: the AGIR experiments (Bordeaux, France)





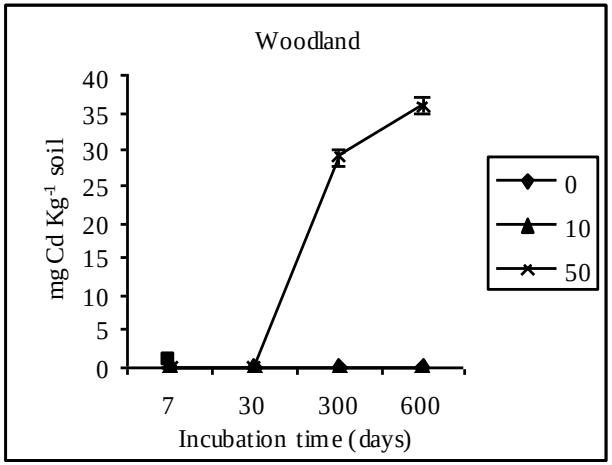


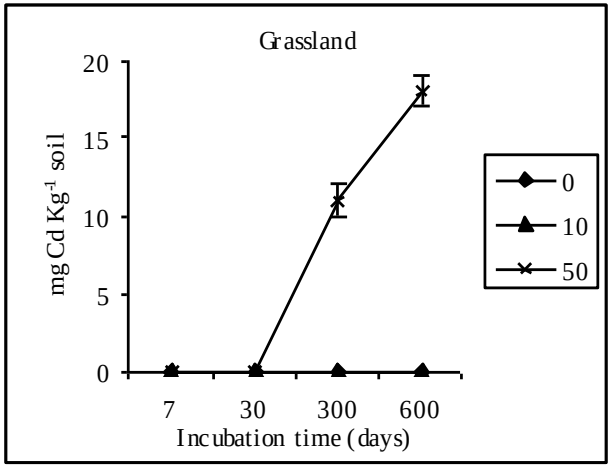
Chemically defined bioavailable Cd (1M NH_4NO_3) in a Hypocalcic calcisol

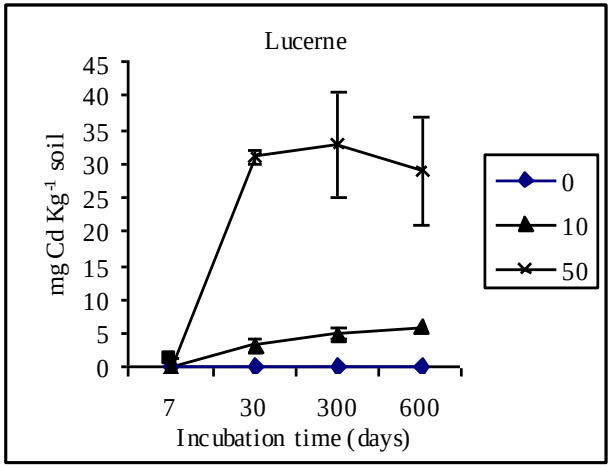


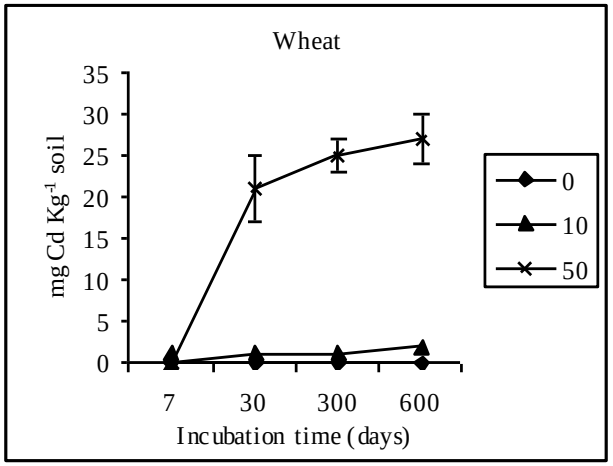
…BIOMET® indicated increasing Cd bioavilability over time…

don't asks why, please

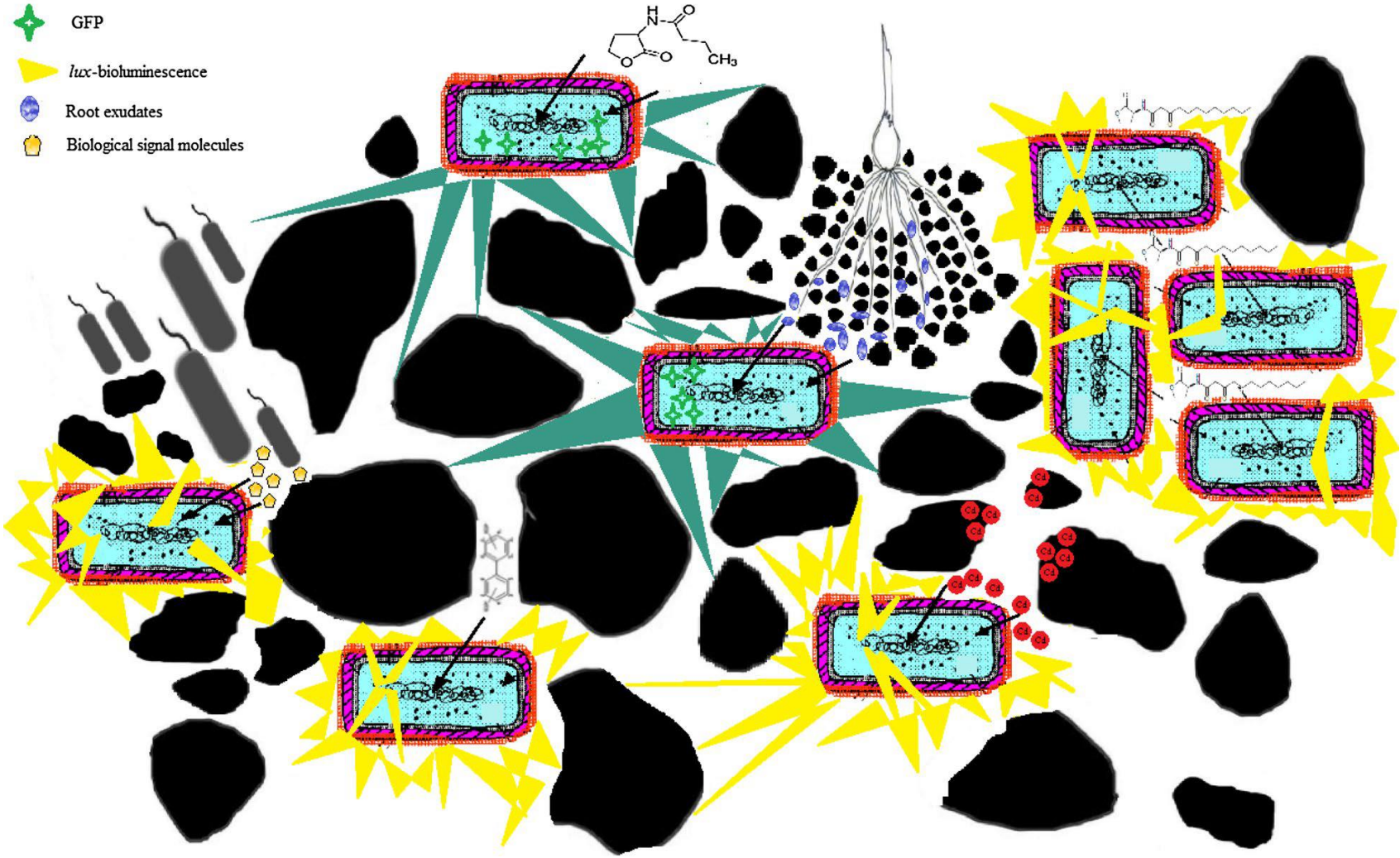








Standard ecotoxicological tests



Bioavailability: implications for ecotoxicology and risk assessment

- Current regulation worldwide for characterizing the level of soil contamination generally does not include measures of the bioavailability of contaminants to humans as ecological receptors

Bioavailability: implications for ecotoxicology and risk assessment

- In tiered risk-based management of contaminated soils bioavailability is considered in the **initial screening-level** step
  - Leaching tests, water soluble, exchangeable fractions

- Soil screening levels for the protection of **human health** (residential or industrial) can enter **bioaccessibility** human exposure via incidental ingestion

- Availability to microbes, plants and fauna are not considered (e.g. RISKNET)

Bioavailability: the human body

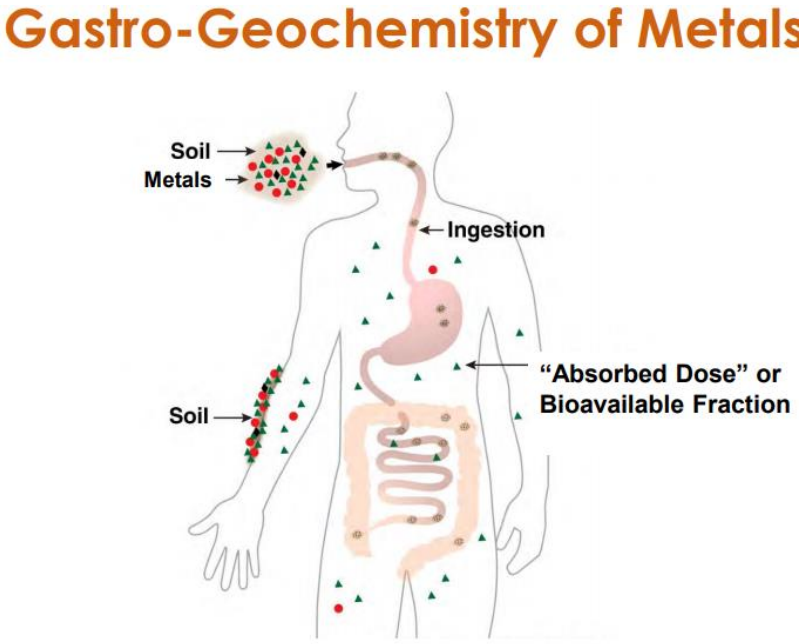
Bioaccessible contaminants:

pools **free to *move*** from soil into the human body

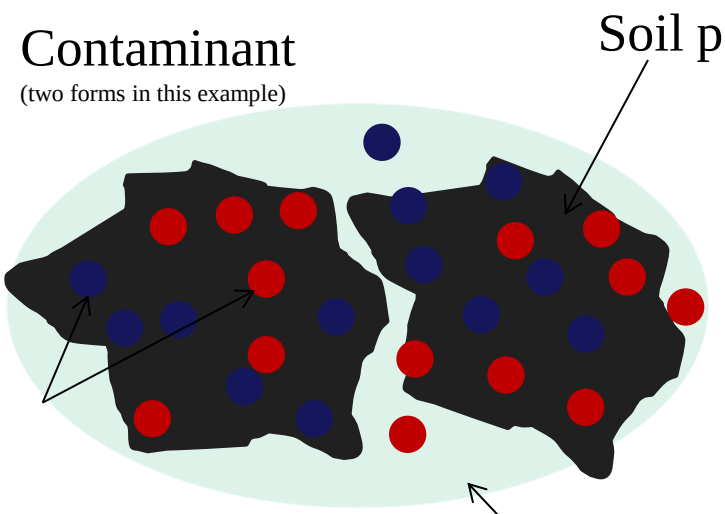
The main route is ingestion

Major controllers:

clay content and type, pH value, presence of other contaminants



Bioavailability: the human body



Contaminated soil can contain different forms of contaminants

Standard protocol: BARGE pools **free to *move*** from soil into the human body

The main route is ingestion

Major controllers in the human gut:

- clay content and type
- pH value
- presence of other contaminants

Bioaccessibility: the methods

PBET = physiologically based extraction test;

SBET = simplified bioaccessibility extraction test; DIN = German Institute for Standardisation 00 19738;

RIVM = Dutch National Institute for Public Health and the Environment batch method;

UBM = unified BARGE method

Overview of methods to assess the trace element (TE) bioaccessibility to humans in polluted soils

Method[a)]	TE(s)	Extraction procedure	Simulated digestive compartment	pH	Temperature °C	L/S ratio[b)]	Residence time	Reference
PBET	As, Pb	Batch	Stomach	2.5	37	100:1	1 h	Ruby *et al.*, 1992,
			Small intestine	7.0	37	100:1	4 h	1996
SBET	As, Cd, Pb	Batch	Stomach	1.5	37	100:1	1 h	Drexler, 1999
IVG	As	Batch	Stomach	1.8	37	150:1	1 h	Rodriguez and
			Small intestine	5.5	37	150:1	1 h	Basta, 1999
USP	Pb, Cr, As, Cd, Ni	Batch	Stomach	1.0	37	1 000:1	2 h	Hamel *et al.*, 1998
MB&SR	Pb, Cr, As, Cd	Batch	Mouth	6.4	37	160:1	5 s	Hamel *et al.*, 1999
			Stomach	2.0	37	2 160:1	2 h	
			Small intestine	7.5	37	4 770:1	4 h	
DIN	As, Cd, Pb, Cr, Hg	Batch	Mouth	6.4	37	15:1	0.5 h	Hack and
			Stomach	2.0	37	50:1	2 h	Selenka, 1996
			Small intestine	7.5	37	100:1	6 h	
SHIME	As, Cd, Pb	Batch	Stomach	5.2	37	2.5:1	3 h	Molly *et al.*, 1993
			Small intestine	6.5	37	4:1	5 h	
RIVM	As, Cd, Pb	Batch	Mouth	6.5	37	15:1	5 min	Sips *et al.*, 1998
			Stomach	1.5	37	37.5:1	2 h	
			Small intestine	5.5	37	97.5:1	2 h	
TGM	As, Cd, Pb	Dynamic	Mouth	5.0	37	5:1	5 min	Minekus *et al.*, 1995
			Stomach	2.0	37	30:1	1.5 h	
			Small intestine	7.0	37	51:1	6 h	
AOACPD	Cu, Zn, Mn, Fe, Al	Batch	Stomach	1.1, 2.0	37	150:1	16 h	AOAC, 2000
UBM	As, Cd, Sb, Pb,	Batch	Mouth	6.5	37	15:1	20 s	BARGE-INERIS,
	Montana		Stomach	1.2	37	37.5:1	1 h	2010
	NIST[c)] 2711 soil		Small intestine	6.3	37	97.5:1	4 h	

Bioavailability: the mistery of the man's (politicians) body

A singular experiment

WWF and The Co-operative Bank took and analysed the blood of 47 people from all over Europe in December 2003, including 39 members of the European Parliament, 4 observers from accession countries, 1 former MEP and 3 WWF staff, representing 17 countries in Europe. The results were released on 21 April 2004: every person was contaminated with a cocktail of persistent, bio-accumulative and toxic chemicals (including cocaine metabolites)

Bioavailability: implications in ecotoxicology and risk assessment

Incorporating bioavailability considerations in the calculation of risk can:

- Optimize management and remediation interventions
- Improve site decision-making, and make interventions more protective
- Balances the risks caused by remedial action with the risks addressed by remedial interventions

Methodological assessmete: chemical *vs* biological methods

Chemical methods

pros

- can be employed in complex natural matrices collected from the field with minor or without preliminary manipulation (e.g. drying, sieving), is mostly used for the analysis of the soil solid phases
- assess physico-chemical processes in the solid and liquid phases and each method measure a single process related to a a specific mechanism through chemical speciation
- are widely accepted because highly standardized

cons

- actually, they measure a mixture of processes from which it is often difficult to determine the prevailing ones, as chemical extractions mobilize and solubilize contaminants from multiple and unknown sites and information is extrapolated from empirical correlations

Biological methods

pros

- provide the key information of biological uptake of elements and molecules (soil bioassay, bioaccumulation)
- with the suitable instruments can be applied to the analysis of the solid, liquid and gaseous phases,

cons

- need specialized biological laboratories, are influenced by the physiological responses of the biosensor to the properties of the , can suffer from biouptake plus other processes that influence toxicity
- are not widely accepted because (with few exceptions) they have been not standardized

Chemosensors: fifty shades of... bioavailability

Chemosensors

pros

- Inform on the potential interactions and stregnht between analytes and biological molecules
- Ease of use, quick, specific and cheap

cons

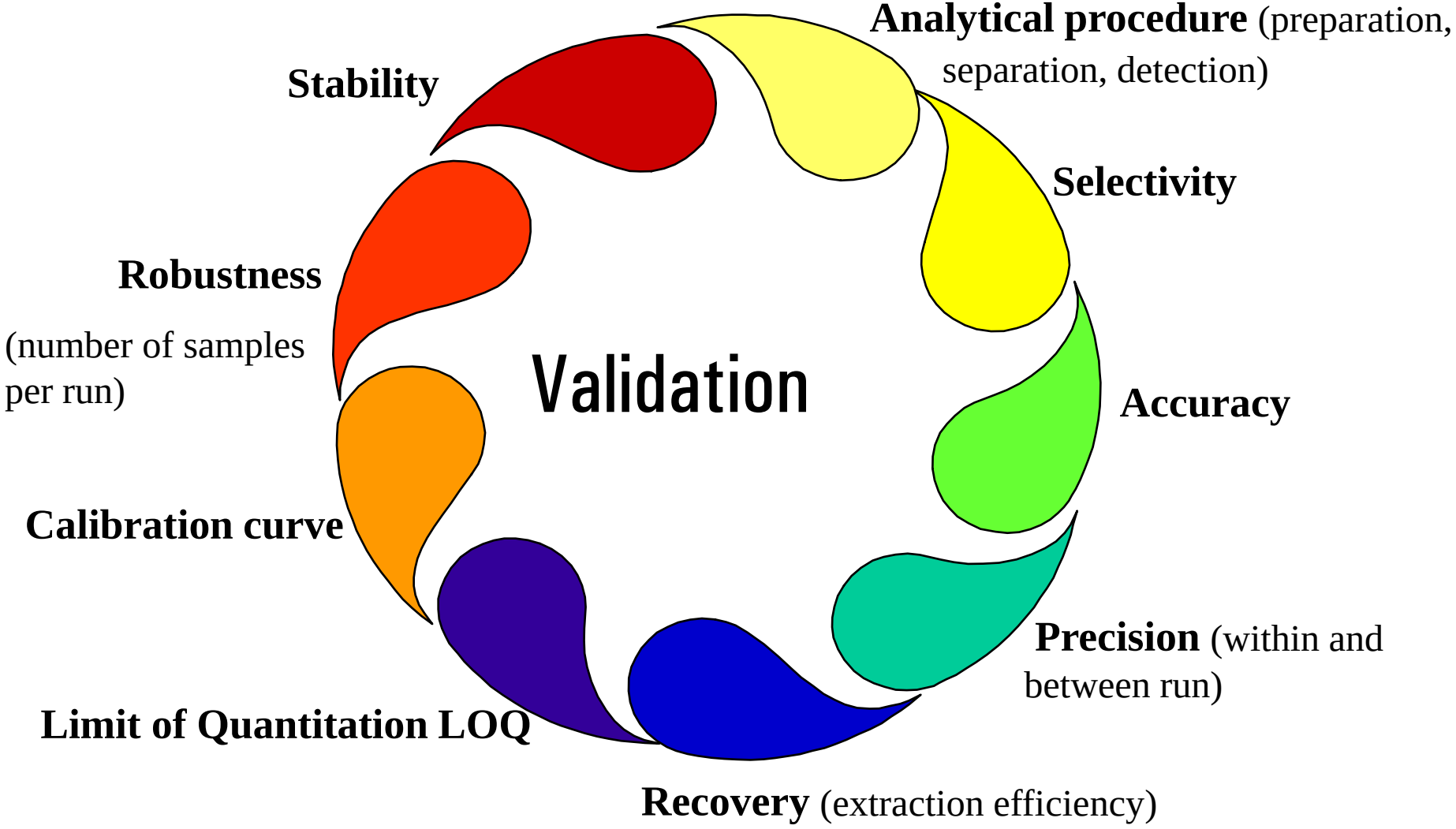
- Only chemical interactions can be described
- Whole soil samples are often too complex to be analyzed and physico-chemical fractionation may be needed to prevent interf

Bioavailability: analytical uncertainities

The analytical techniques must produce consensus results

- Test plan (analytical protocol)
- Sample analysis traceability
- Documentation, possible to reconstruct the study
- Analytical report must be conducted according to the principle of Good Laboratory Practice (GLP)
- **Analytical reportes must be validated**

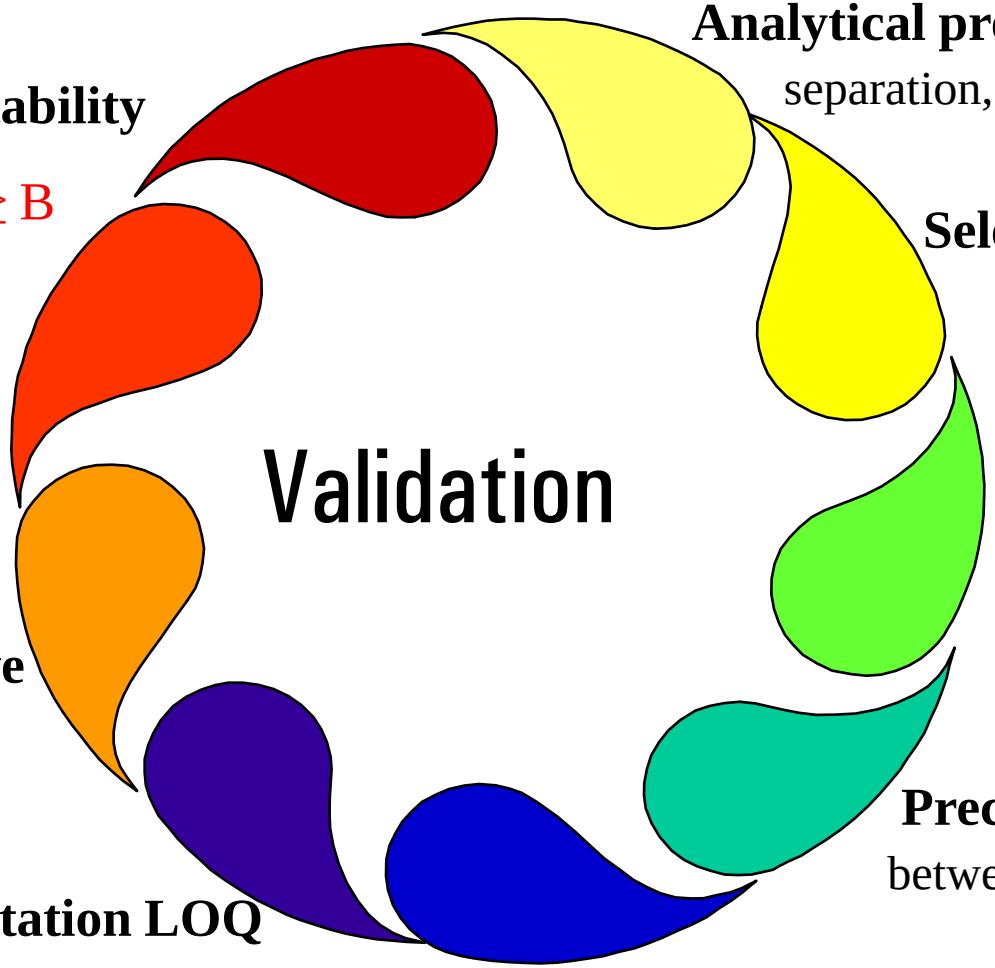
Chemical *vs* biological methods: validation and guidance



Chemical *vs* biological methods: validation and guidance

C = chemical methods

B = biological methods



Analytical procedure (preparation, separation, detection) C > B

Selectivity B >> C

Accuracy C ≈ B

Precision (within and between run) C ≥ B

Recovery (extraction efficiency)

C ≥ B

Limit of Quantitation LOQ

B > C

Calibration curve

C ≥ B

Robustness

(number of samples per run) C > B

Stability

C ≥ B

Whole cell biosensor requirements

- *E. coli* HB101 (pUCD607) rehydrated with 0.1M KCl
- *P. fluorescens* DF5740E7 is cultivated on Luria-Bertani media and resuspended in 0.9% NaCl
- *P. fluorescens* HK44 (pUTK21) cultivated on yeast–peptone–glucose media and suspended in 0.1M KCl
- *V. fischeri* (BioTox™ test) is reconstituted in 2.0% NaCl
- *R. metallidurans* (BIOMET® test) is reconstituted in glucuronic acid
- *P. aureofaciens* PGS12 is cultivated on Ayer minimal broth+ 25 mM HEPES
- *N. europaea* (ATCC 25978) is resuspended in standard NH_4^+-N solution
- *A. tumefaciens C58 GMI 9023* rehydrated with minimal salt media
- *P. fluorescens* DF57-N3 luxAB or DF57-11D1 is cultivated on Luria-Bertani medium and resuspended in 0.9% NaCl
- Need for standardization (see ISPRA note 2015)

Bioavailability in agriculture

- The bioavailable nutrient pools vary significantly by soil type and by plant species due to
  - different complexing capacities of different soils
  - source and forms nutrients
  - different plant mechanisms for accessing soil nutrients

Bioavailability: the potential for improving tomorrows's agriculture

- Chemical methods are currently used to predict the plant available fraction (phytoavailability) of macro- and micronutrients and contaminants

- Prediction using chemical methods is relatively poor, based on empirical correlations, due to the plants metabolic flexibility capable to alter the rhizosphere environment to facilitate nutrient uptake

Bioavailability in agriculture

- *Pseudomonas* sp. (Kragelund et al 1997)
- *Pseudomonas* sp. (Yeomans et al 1999)
- *P. putida* (Espinosa-Urgel e Ramos 2001)
- *P. fluorescens* sp. (Kuiper et al 2001)

- Also used in ‘multi reporter’ systems (Standing et al 2003)

- *Rhizobium* with nodC-lacZ (Bolanos Vasquez and Warner 1997)
- *P. fluorescens* F113 (Smith et al 1999) inhibit Fusarium infections
- *Rhizobium* with promoterless *gfp* (Allaway et al 2001)

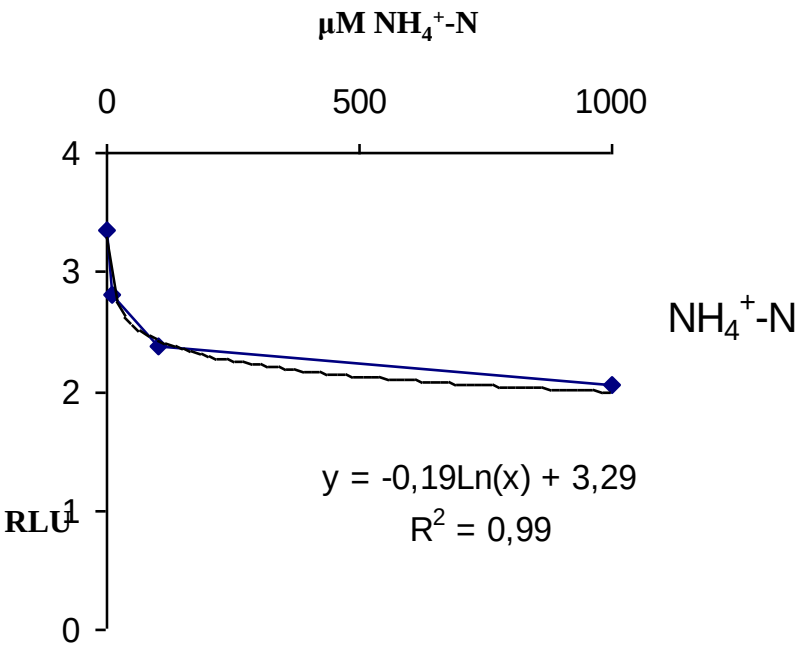
Bioavailability in agriculture

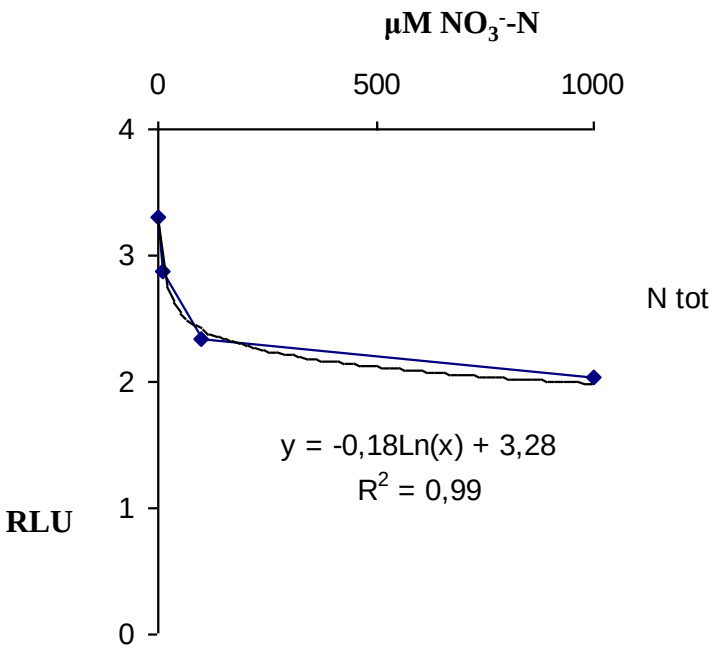
- Improved assessment of plant-available (phytoavailable) fraction of macro- and micronutrients can
  - maximize crop yields (e.g. optimize economic return) defining *biologically* the sufficient/deficient nutrient status of soils
  - minimize the crop environmental footprint
  - detect the specific nutrient uptake (biofortification) and inadvertent plant access of soil-bound contaminants

Bioavailability: C, N and P

Biosensors for N, P and C

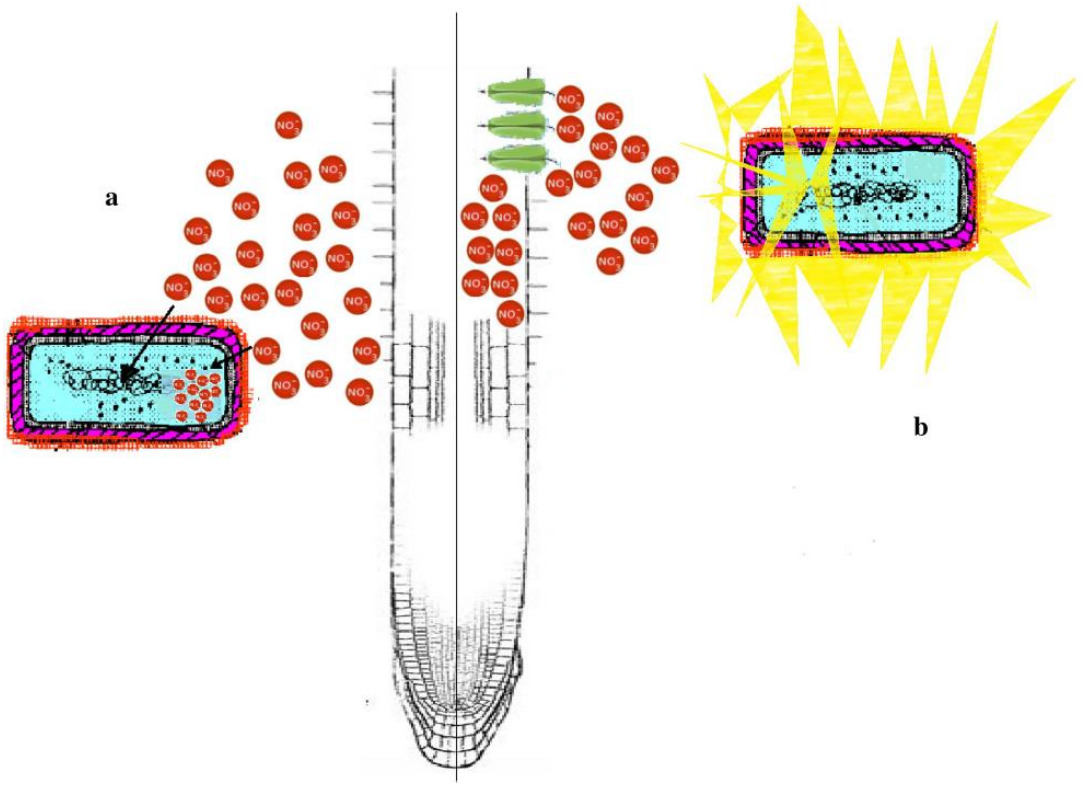
Pseudomonas fluorescens 10586 pUCD607 (*lux*CDABE da *Vibrio fischeri*)
Pseudomonas fluorescens DF57 N3
Pseudomonas fluorescens DF57 P9





Example of experimental results of soil analysis using whole-cell biosensors

- Responses of the *P. fluorescens* DF57 N3 *lux*-inserted constitutive biosensor in function of NO_3^--N availability in the rhizosphere
- When biosensor takes up NO_3^--N bioluminescence decreases (a), when plant absorbs NO_3^--N biolouminescence increases (b)



Direction of the biosensor development and environmental application

- Innovative whole cell biosensors are devised for:
  - emerging inorganic and organic pollutants
  - chemical communication between microorganisms and between plants and microorganisms

Direction of the biosensor development and environmental application

- **Nanosensors** in environmental analysis, based on the relationship between the property monitored and the type of nanomaterial used
- Sensor devices built with: nanoparticles, nanotubes, nanorods, embedded nanostructures, porous silicon, and self-assembled materials.
- Already tested biosensing mechanisms: Ag pyramidal nanoparticle arrays using Localized Surface Plasmon Resonance (LSPR) based on changes in the refractive index of the Ag nanoparticles
- Au nanostructures smaller size than the de Broglie wavelength with absorption peaks in visible/near-UV region and LSPR properties:

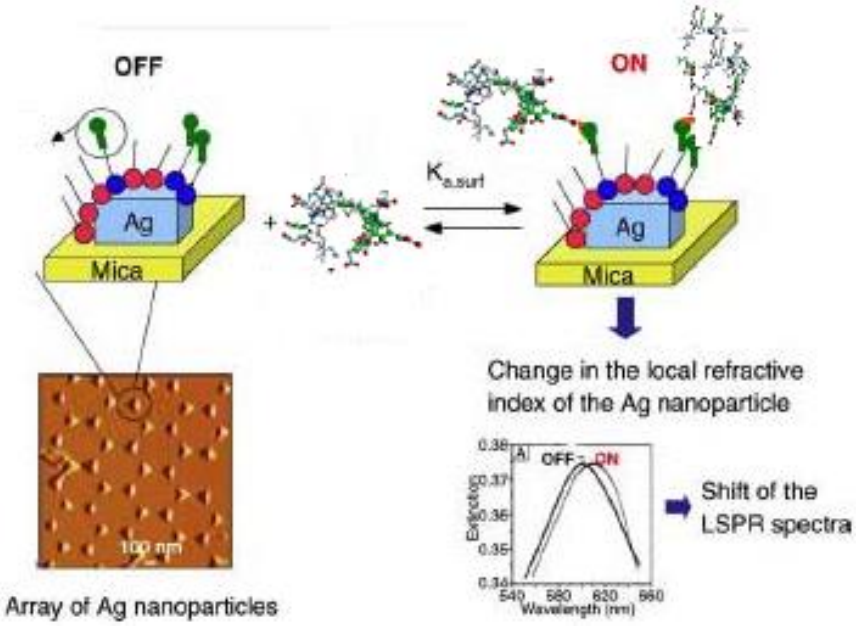
  Semiconductor quantum dots (PEBBLEs: Probes Encapsulated by Biologically Localized Embedding) for intracellular sensing

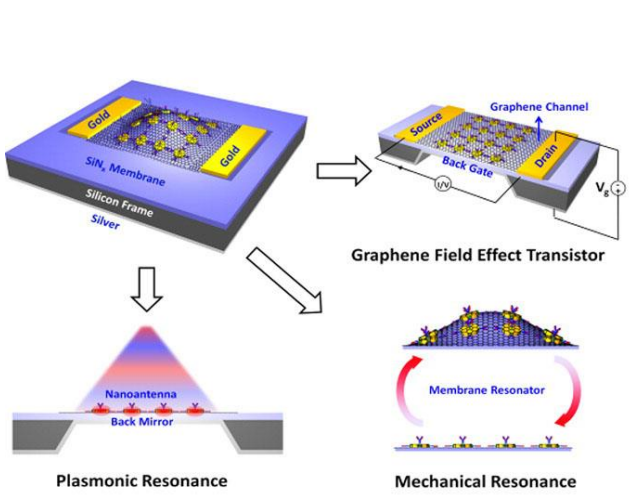
  Nanoparticle films have been as gas sensors because the increased surface area of the sensor increase its sensitivity

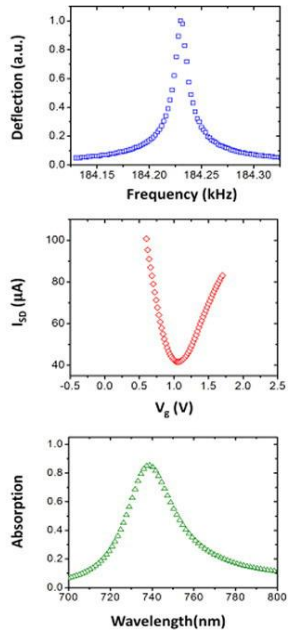
Direction of the biosensor development and environmental application

- Magnetic nanoparticles bound to biorecognitive molecules (e.g. DNA, enzymes) can be used to enrich the analyte to be detected an therefore the sensitivity of the sensors can be substantially improved.
- Sensors based on nanowires and carbon nanotubes (CNTs) with field effect transistors (FET) have been widely used to detect gases such as greenhouse gases in environmental applications
- Sensors based on bulk nanostructured materials such as Pt and Au can be used to construct new electrochemical specific sensors
- Nanoporous Si network (2-5nm thickness) is semiconductor material with an internal surface area-to-volume ratio of up to $500 m^2/cm^3$ are used for gas sensors that induce a change in color.
- Self-assembled nanostructures with biomolecules (e.g. liposomes, protein, nucleic acids) convert the biochemical interaction into an electrical signal, can be constructed in nanoarray (up to 400 spots). Can detect environmental pathogens

Nanosensor development and environmental application

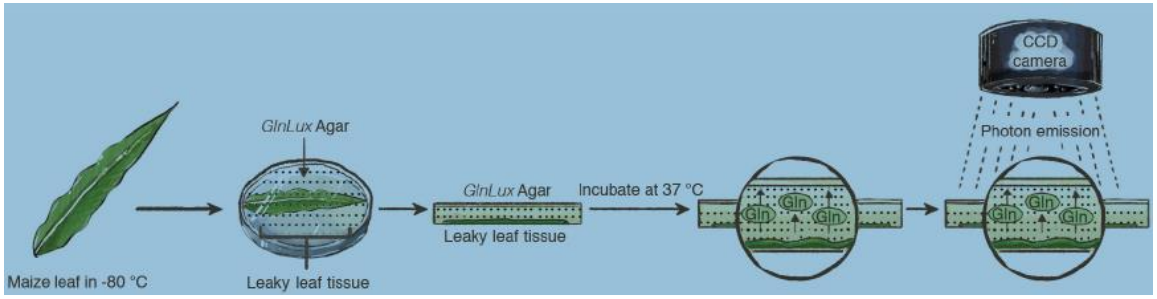


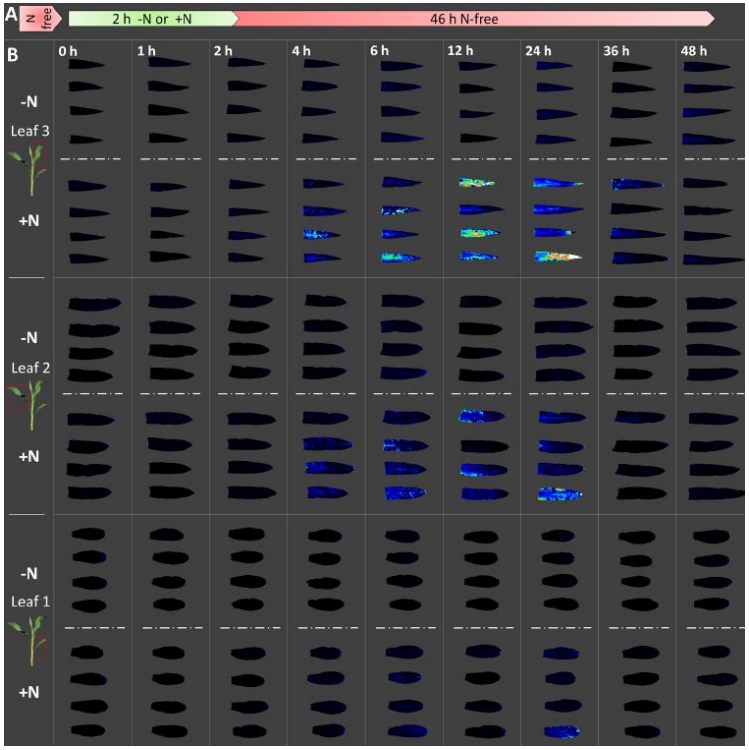




Plant biosensors development for agro-environmental applications

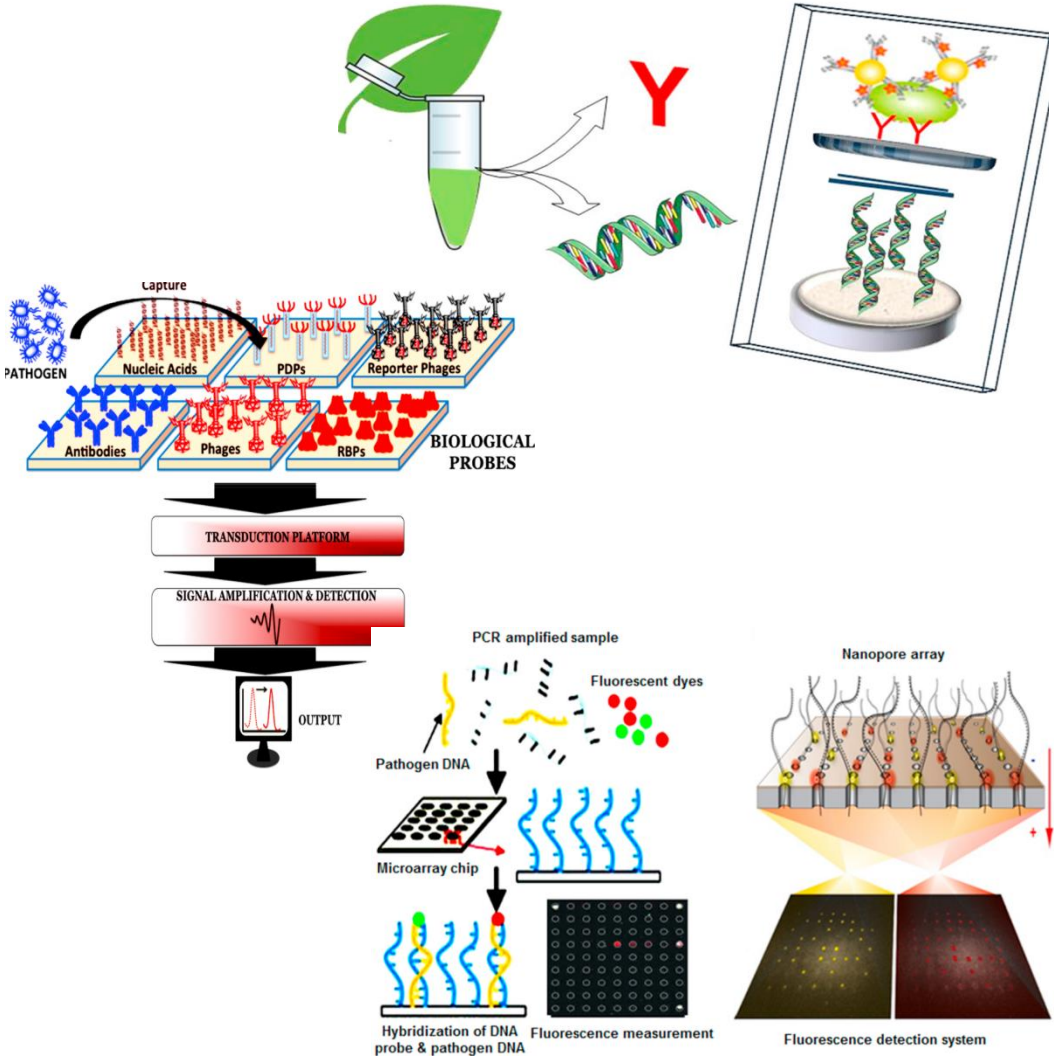
- Based on a *Escherichia coli* biosensor auxotrophic for Gln (GlnLux) engineered with a constitutive *lux* operon to emit luminescence upon bioavailability of Gln
- The biosensor cells can be embedded into agar (GlnLux agar) and freeze-thawed leaf tissue can induce Gln leakage
- Bioluminescence is proportional to N fertilization
- N organication can be localized by photon-capture camera





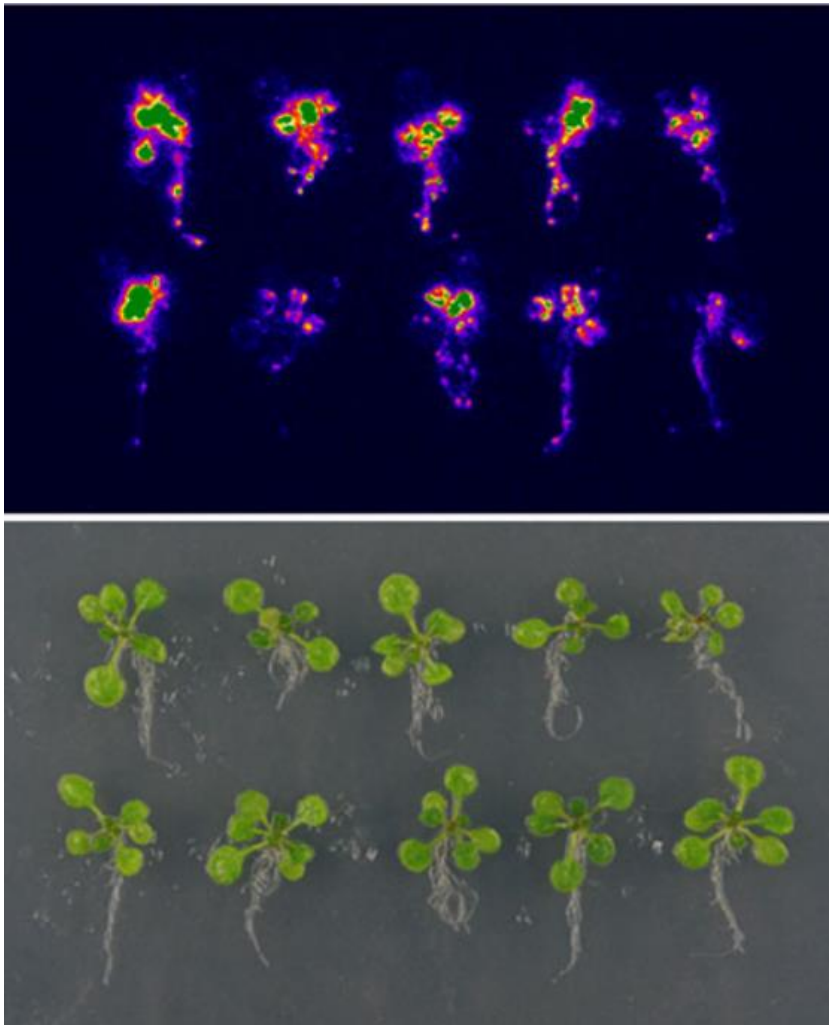
Plant biosensors development for agro-environmental applications

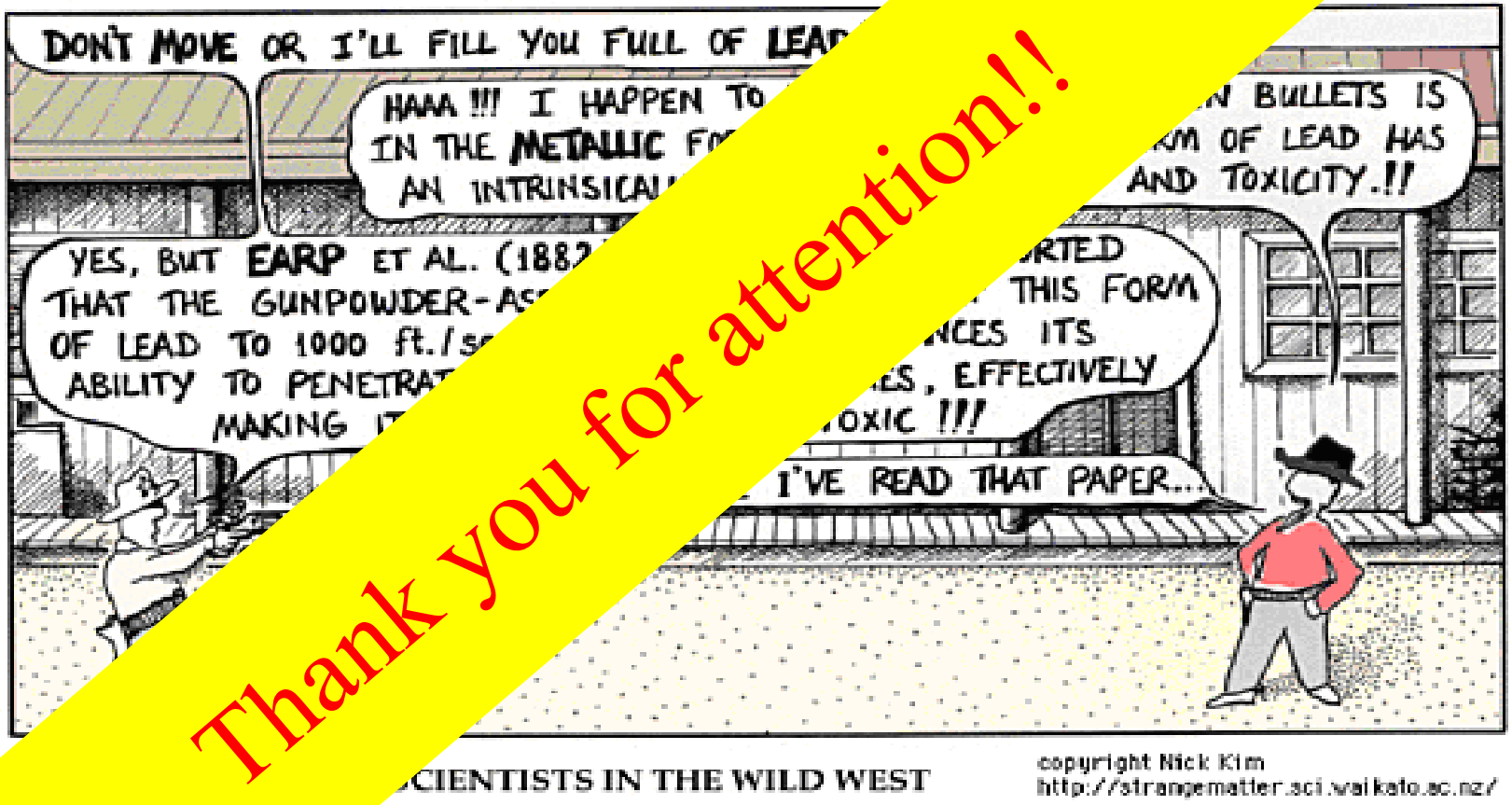
- Detection of plant phytohormone and plant/pathogens interactions using antibody-based and nucleic acid- based biosensors
- Different nanomaterials (e.g. cabon nanochannels, metallic nanoparticles are used for devise rapid in lab and on-site detection biosensors
- Recent developments integrate nanotechnology for enhancing detection sensitivity/specificity using simple low-cost methods for early identification of plant phytohormones and pathogens



Plant biosensors development for agro-environmental applications

- *Arabidopsis thaliana* plants have been successfully ingeneered to act as multicellular botanical biosensors To test their new method, the team experimentally engineered yeast, plant, and mammalian cells to contain customizable ligand-binding domains (LBDs), receptors for several small molecules and emit light after ligand binding
- Arabidopsis plants exhibited a 50-fold increase in luminescence in the presence of target molecules





Thank you for attention!!