

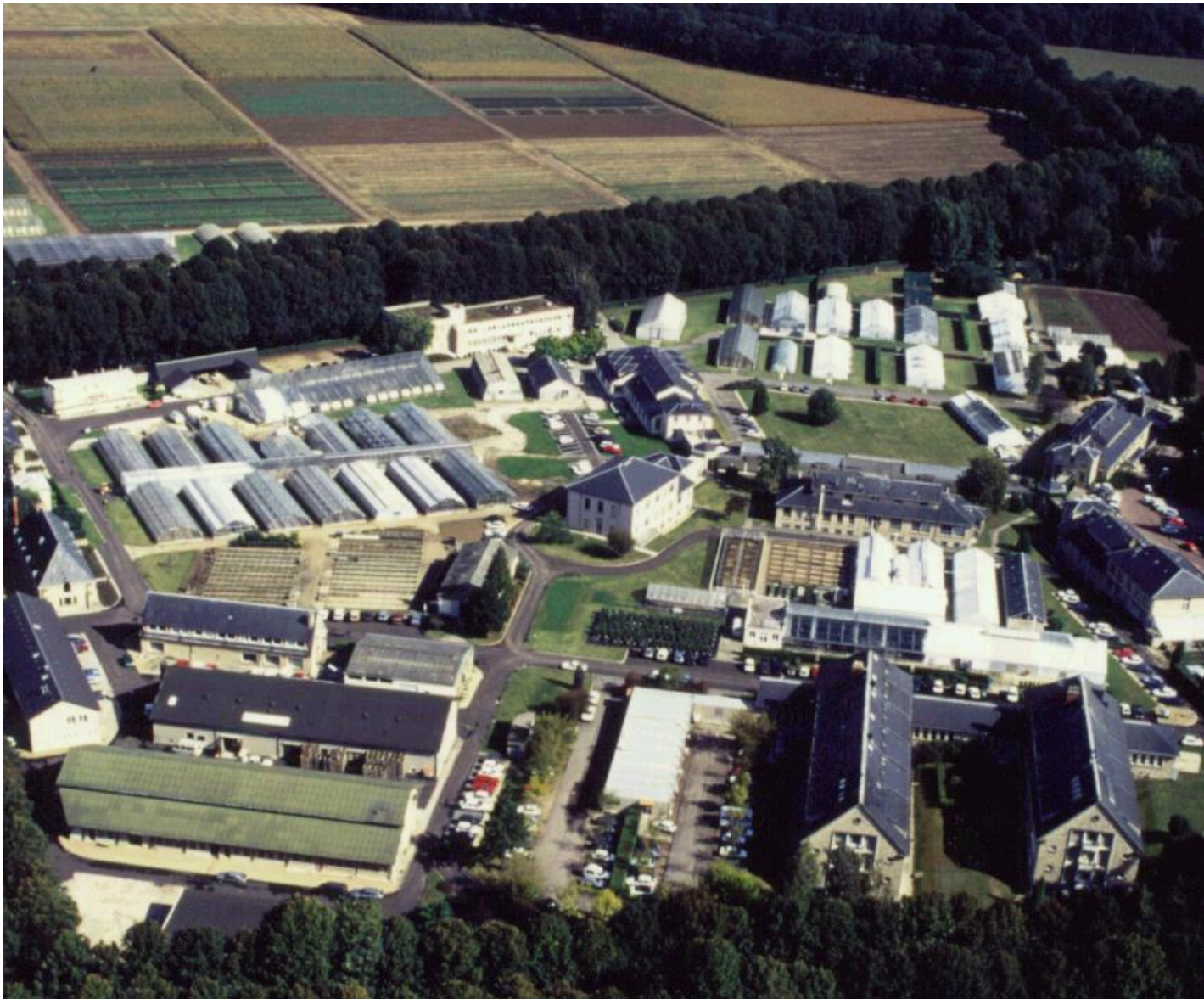
Bioremediation and phytoremediation of PAH-polluted soils

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INRA

- INRA: National Institute for Agronomic Research
- 9500 researchers, engineers, technicians and administrative staff, 1200 students
- Spread over 21 regional research centers, 280 research and 85 experimental units
- Keywords: food, environment, agriculture and society
- Phytopharmacy and Semiochemicals Unit
 - improving pesticide uses within an integrated crop protection context
 - studying intoxication processes in target and non-target organisms, pesticide resistance development and the environmental fate of xenobiotics
 - studying chemical communication between organisms as an alternative methods to classical chemical control
- <http://www.inra.fr>





Outcome of the course

I-PAHs: a family of environmental pollutants with hazardous potential
(PAH properties and hazard, interactions with soil components)

II-Bacteria, fungi and plants: their potential for PAH biotransformation
(principles of PAH transformation, PAH transformation pathways)

III-Bioremediation processes: a way to reduce the environmental hazard of PAHs
(mainly used methods, case studies)

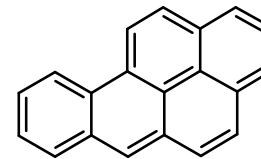
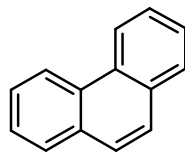
IV-Future prospects
(improving the bioavailability of PAH, as well as the ability of organisms to transform PAHs, phytoremediation)

I

PAHs:

a family of environmental pollutants with hazardous potential

- PAH properties and hazard
- interactions with soil components



Physicochemical properties of PAHs: a basis for recalcitrance

- 2 or more fused benzene rings and/or pentacyclic moieties in linear, angular and cluster arrangement (MW = 128.2 to 278.4)
- thermodynamically stable because of large resonance energy
- very low aqueous solubility ($\ll 1$ mg/l), highly hydrophobic (logKow = 3.3 to 6.75)
- neutral
- strongly associated with particle surfaces in the environment (Koc = 2×10^3 to 3.47×10^6)
- in general, poor volatilization, photolysis and biodegradation

Origin of PAHs

- continuous release into air, soil and water
- contaminate feed and food, and the food chain
- **natural** origin
 - biogenic: components of living organisms
 - geochemical: pyrolysis of organic substances, aromatization of biological compounds during humification processes
 - petrogenic: petroleum, coal, ancient sediments
 - pyrogenic: vegetation fires
- **anthropogenic** origin
 - point sources: spills or mismanaged industrial operations
 - low-level inputs: atmospheric depositions

Biological effects of PAHs on living organisms

- carcinogenicity: increases with ring number and condensation degree
- mutagenicity: form DNA adducts
- teratogenicity
- immunodepressive effects
- high potential to biomagnification

Environmental hazard

- persistent organic pollutants (POP) in the environment
 - long half-lives: phenanthrene: 16-126 d; benzo[a]pyrene: 229-1400 d
 - rate of deposition > rate of degradation → accumulation
- can enter the food chain
- biological adverse effects

⇒ persistence + biological effects ⇒ hazard

⇒ bio- and phytoremediation of soils may reduce this hazard

PAHs/soil interactions (1)

⇒ determine transport, fate and bioavailability of PAHs

- **sorption/desorption kinetics**

- mainly due to binding with natural organic matter (humic acids), affinity expressed as K_{oc}
- function of PAH hydrophobicity (K_{ow}) and molecular size
- PAHs also exist as non-aqueous phase liquids (NAPLs)

- **sequestration**

- time-dependent entrapment within the micropores of soil humic materials
- mechanisms not fully understood

PAHs/soil interactions (2)

- **formation of bound residues**
 - complexes formed from parent compounds or derived metabolites with organic constituents of soils, or living organisms
 - attachment of PAHs to reactive sites (amines, phenols, quinones) on the surfaces or the organic colloids, or incorporation of the compound into the structure of humic and fulvic acids, or cells
- **characteristics of polluted soils**

Characteristics of PAH-polluted soils

	Wood-preserving facility	Manufactured gas plant	Oilfield battery
PAH contamination (mg/kg)	<5000	<25000	<5000
fluoranthene/pyrene ratio	>1	>1	<1
3-ring (%)	50-60	10-30	30-45
4-ring	35-45	40-50	35-45
5-ring	3-5	15-30	10
6-ring	<5	5-15	5-15
Additional chemicals	cresols, phenols, chromated copper arsenate	cyanides, heavy metals, sulfates	methylated PAHs BTEX
Soil organic carbon content	low	high (>10%)	+ high
Aging	30 years	100 years	±
Potential for bioremediation	++	--	+

Polluted soils



II

Bacteria, fungi and plants: their potential for PAH biotransformation

- principles of PAH transformation
- PAH transformation pathways

Agents involved in biotransformation reactions

- All living organisms: micro-organisms, higher plants and animals
- The soil micro-organisms

	Number (/g of soil)	Biomass (ton DM/ha)
Bacteria	10^8	0.5
Actinomycetes	10^7	1.0
Fungi	10^6	1.5

Total biomass: 2 to 4% of carbon, 5 to 8% of nitrogen

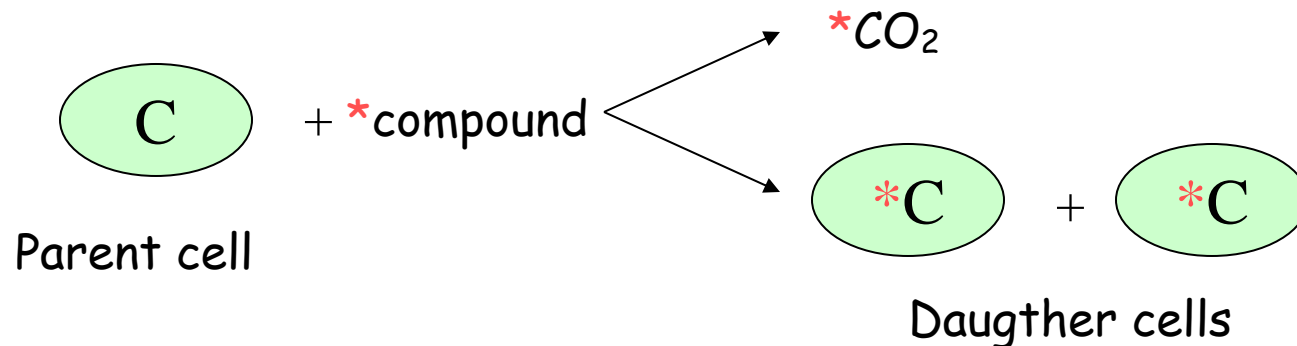
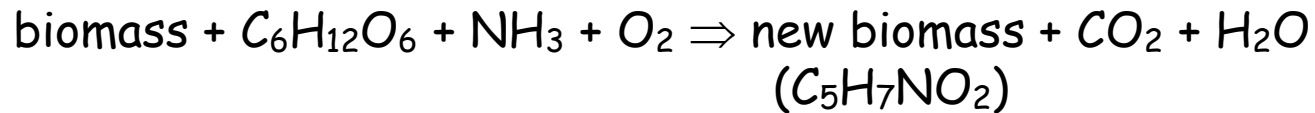
- Functions
 - mineralization and humification of organic matter
 - biogeochemical cycles: C, N, P, S (1 to 2 tons of organic carbon mineralized/year/ha)
 - production of toxins or interesting compounds (antibiotics)
 - degradation of xenobiotics

Soil micro-organisms

	Prokaryotic organisms		Eukaryotic organisms
	Bacteria	Ascomycetes	Fungi
	The most numerous		The largest biomass
Morphology	Unicellular Cocccoidal, rod-shaped, helical, 1-10 μ m	Elongated single cells Branched into filaments or hyphae	Pluricellular Extensive filament forms,
Liquid cultures	+, turbidity	+, pellets	
Cellular organization	A few organelles, no cytoskeleton		Numerous organelles, cytoskeleton
Metabolism	Aerobic or anaerobic	Mostly aerobic	Aerobic (yeasts excepted)
DNA	Cytoplasmic and circular + plasmidic		Chromosomes in nuclear membrane
RNA and proteins	Synthesized in the same cell compartment		Distinct compartments
Reproduction	Division by binary fission	asexual spores: conidia	Sexual spores, except deuteromycetes
Competitive pH	6-8	>8	<5
Competitiveness	All soils	Dominate dry, high-pH soils	Dominate low-pH soils
Interactions	Symbiotic, pathogenic		Mycorrhizal, pathogenic
Common genera	<i>Arthobacter</i> (40%) <i>Pseudomonas</i> (10-20%) <i>Bacillus</i> (10%)	<i>Streptomyces</i> (10% of bacteria)	<i>Penicillium</i> , <i>Aspergillus</i> , <i>Fusarium</i> , <i>Rhizoctonia</i> , <i>Alternaria</i> , <i>Rhizopus</i>
Production	Antibiotics: chloramphenicol,...		Antibiotics: penicillin

Biodegradation = mineralization

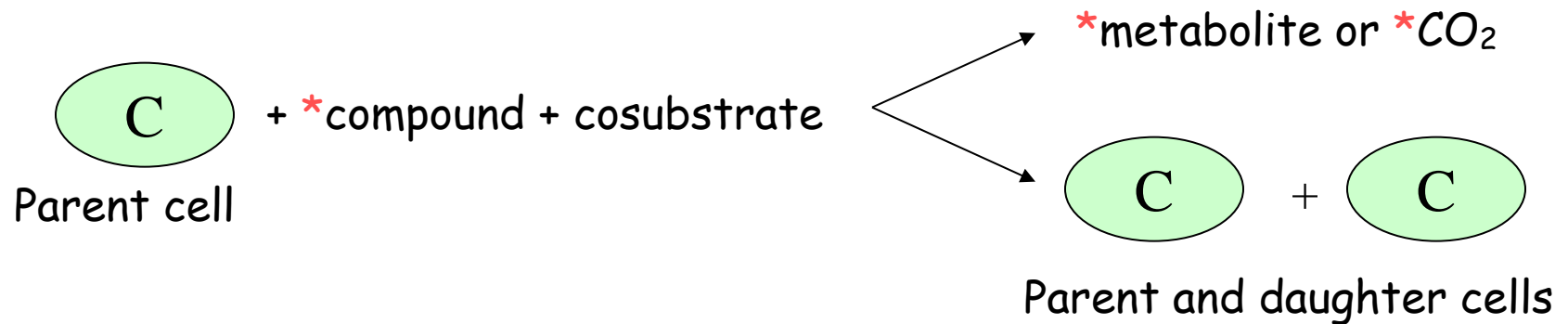
- Complete breakdown of the xenobiotic which become a substrate
- Carbon and energy are used for cellular growth and division



- The most interesting process from an environmental viewpoint

Cometabolism

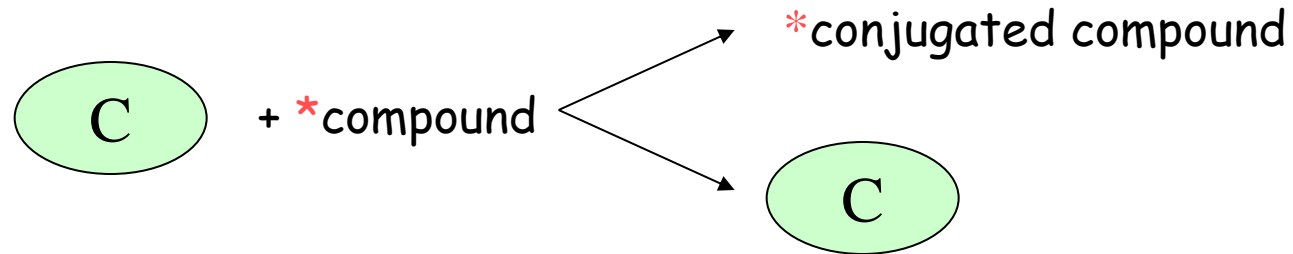
- Partial and fortuitous transformation of the compound
- Carbon and energy are not used for cellular growth and division
- A cosubstrate is needed to supply carbon and energy



- Cometabolism involves enzymes with low substrate specificity
- It induces mainly minor modifications of the compound
- It may lead to the total degradation of the compound during sequential attacks by several organisms
- It represents the main transformation process in the environment, with a possible accumulation of intermediate hazardous products

Synthesis

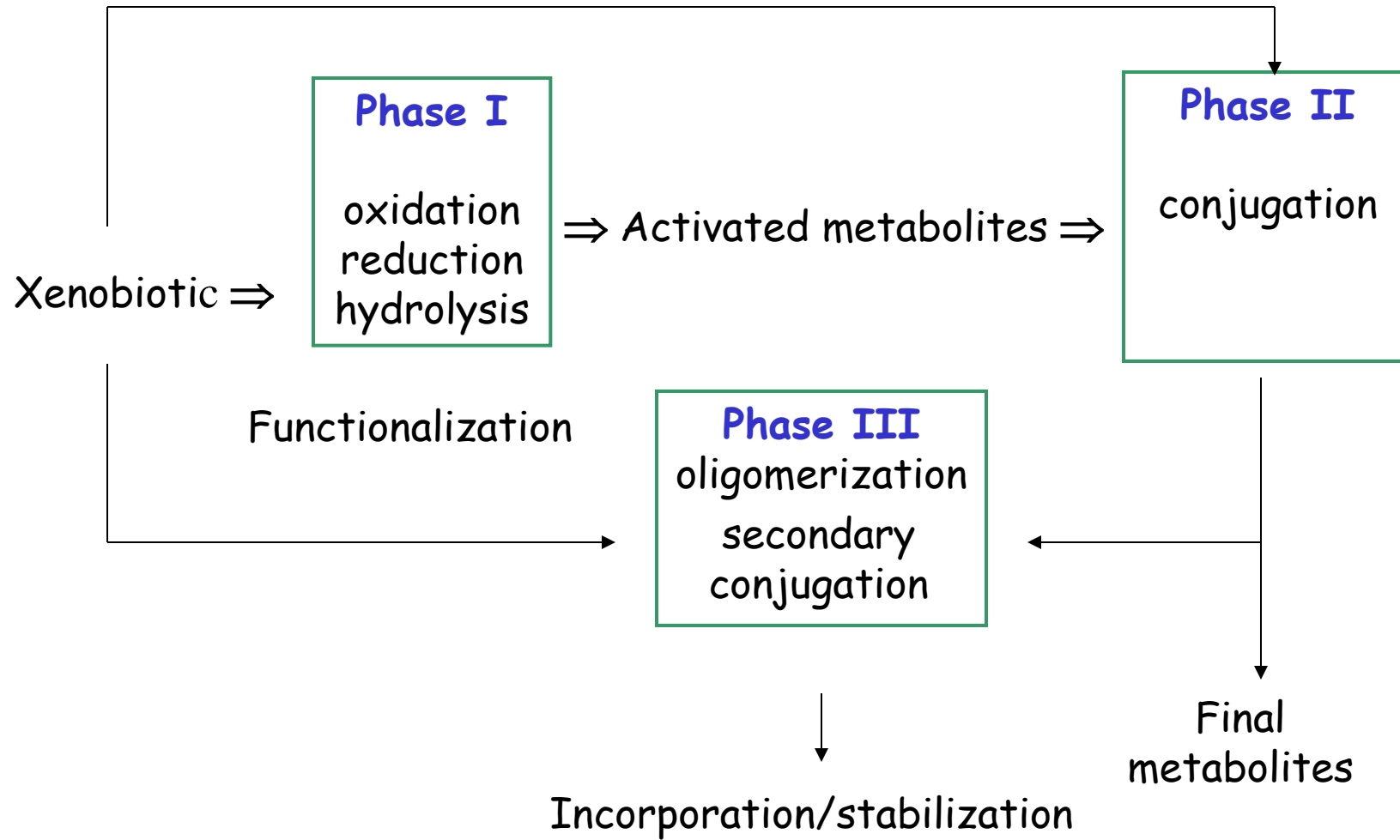
- Partial transformation leading to metabolites more complex and stable than the parent compound
- Conjugation \Rightarrow linkage of the compound with hydrophilic substrates, followed by excretion or storage



- Oligomerization \Rightarrow
 - linkage of the compound with itself, or with residues of xenobiotics or natural products
 - incorporation in cellular components or in soil constituents



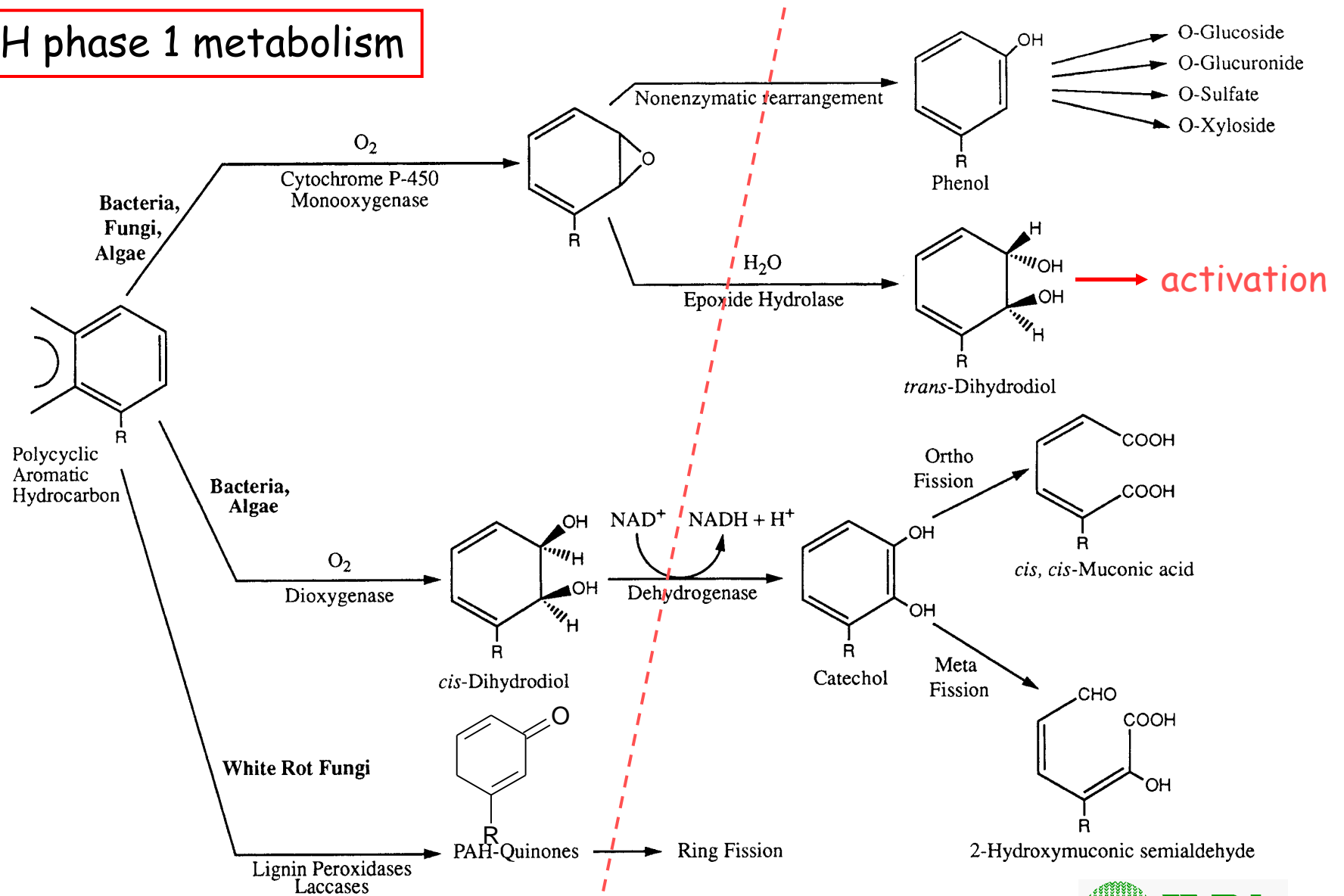
Xenobiotic metabolism



Outcome of the metabolic reactions

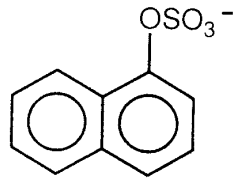
Initial	Phase I	Phase II	Phase III
xenobiotic	oxidation reduction hydrolysis	conjugation	oligomerisation incorporation
lipophilic	amphiphilic	hydrophilic	hydrophilic insoluble
mobile	+/- mobile	little mobile immobile	immobile
toxic	+/- toxic	little or not toxic	not toxic

PAH phase 1 metabolism

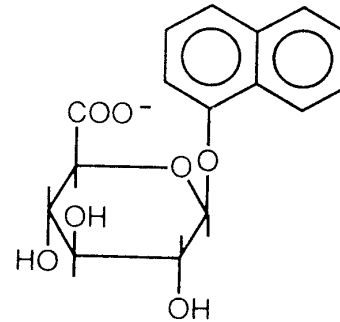


(from Atlas and Cerniglia, 1995)

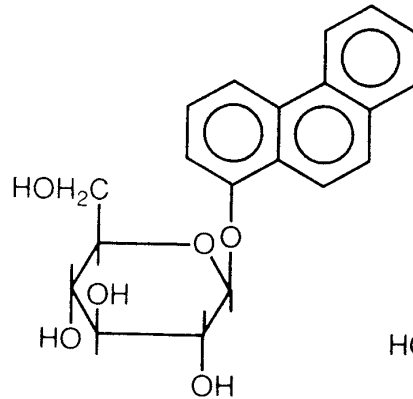
PAH phase 2 metabolism



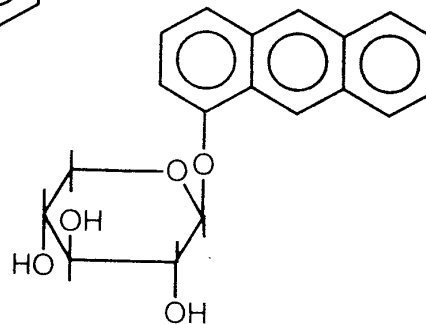
1-Naphthyl
sulfate



1-Naphthyl
 β -D-glucuronide



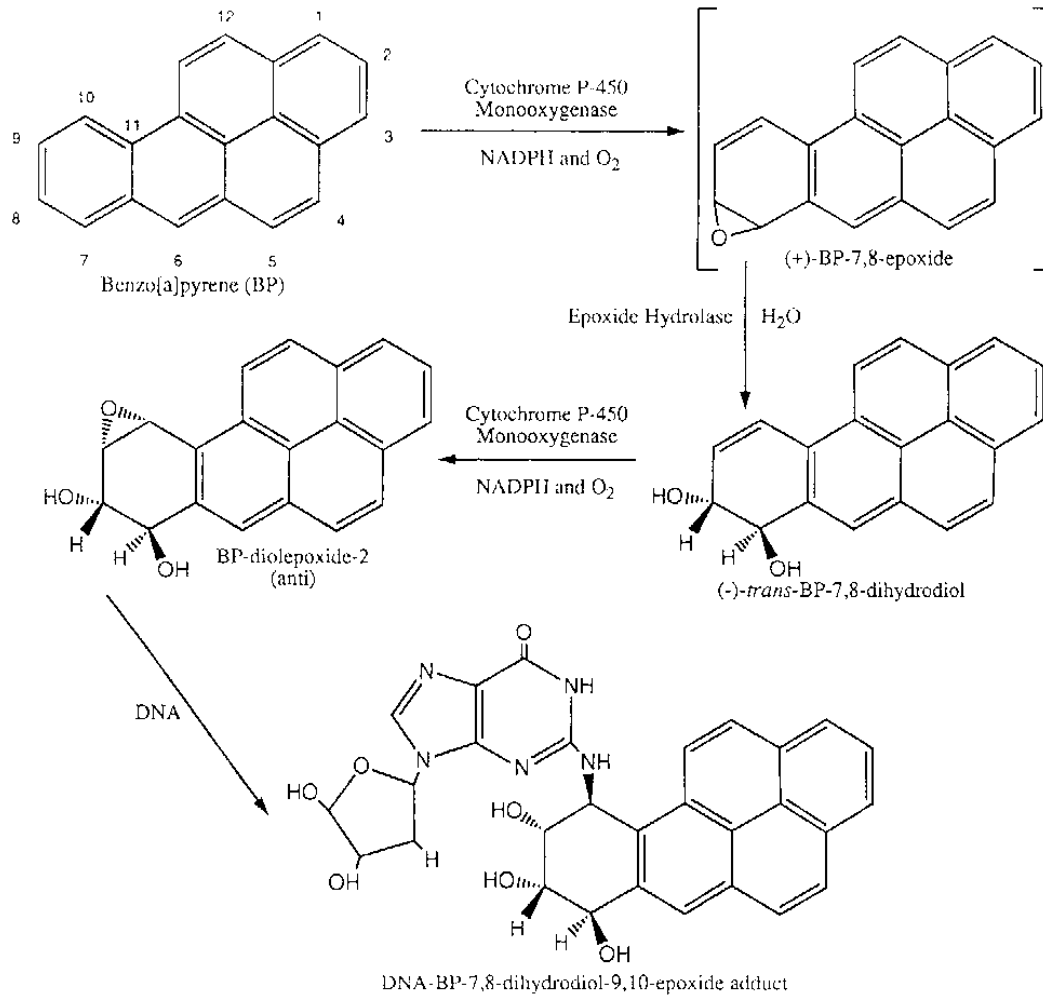
1-Phenanthryl
 β -D-glucopyranoside



1-Anthryl
 β -D-xylopyranoside

(from Sutherland, 1992)

PAH activation



(from Atlas and Cerniglia, 1995)

Actual knowledge on bacterial metabolism of PAHs

David T. Gibson, the pioneer

- **a wide variety of bacteria** metabolize PAHs
- bacteria utilize PAHs as a **sole source of carbon and energy**
- bacteria are able to cleave the aromatic rings and to mineralize them rapidly
- bacteria generally metabolize PAHs to dihydrodiols and to aliphatic chains
- dioxygenases are mainly involved
- the initial ring oxidation reaction is often the rate-limiting step

Actual knowledge on fungal metabolism of PAHs (1)

main data from Carl E. Cerniglia's work

- a **wide variety of fungi** among zygomycetes, ascomycetes and basidiomycetes (white rot or brown rot) metabolize PAHs
- they **need an additional carbon source** to metabolize them
- non-basidiomycetes generally oxidize PAHs without mineralization
- ligninolytic basidiomycetes are able to cleave the aromatic rings and mineralize them
- non-ligninolytic fungi generally metabolize PAHs to:
 - *cis*-dihydrodiols, phenols → conjugated = **detoxification**
 - quinones, dihydrodiol epoxides → bioactive and toxic = **activation**

Actual knowledge on fungal metabolism of PAHs (2)

- **several enzymatic systems** are involved
- fungal metabolism is highly **regio-** and **stereoselective** and is in some cases similar to mammalian metabolism
- fungal metabolism is important as it produced ring-oxidized products, the rate-limiting step for bacterial degradation
- fungal metabolites have high water solubility and enhanced chemical reactivity
- fungi also detoxify PAHs by forming **bound residues** during humification

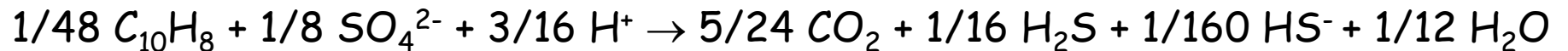
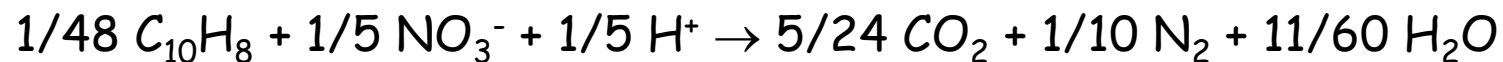
Actual knowledge on higher plant metabolism of PAHs

- our actual knowledge is scarce, but plants may offer a great potential
- whole plants:
 - adsorption onto roots and uptake is significant, but translocation of PAHs with more than 4 rings from roots to leaves is negligible
 - plant germination and growth are strongly inhibited by PAHs with less than 3 rings
 - incorporation of PAHs into cellular components has been reported
- cell suspensions:
 - PAHs are oxidized to phenols or quinones, then conjugated
- actual areas of research:
 - rhizosphere ⇒ bacterial metabolism
 - arbuscular mycorrhizal fungi ⇒ fungal metabolism

Anaerobic degradation of PAHs

- occurs in anoxic environments
- concerns naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene
- terminal electron acceptors: CO_2 , SO_4^{2-} , NO_3^- , Fe_3^+
- theoretical equations for naphthalene

(Rockne and Strand, ESET, 1998, 32 3962-3967)



Dioxygenases

Equation: $R + O_2 + NADH + H^+ \rightarrow R(OH)_2 + NAD^+$

Distribution: bacteria

Function: xenobiotic metabolism

Structure: naphthalene dioxygenase

multi-component system of 3 proteins including a flavoprotein
terminal dioxygenase: iron-sulfur protein of 158 kDa with two
subunits of 20 and 55 kDa

Regulation: ?

inducible by PAHs

Cytochrome P450 mixed-function oxidases

Equation: $\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+$

Distribution: widely distributed among living organisms

Function: xenobiotic metabolism, cellular metabolism

Structure: multi-enzymatic complex (P450 + reductase), mostly microsomal membrane-bound proteins (ER), exposed to the cytosol
P450: heme-thiolate protein of 400 to 525 aa, 45 to 65 kDa
N-terminal segment: anchor signal

Regulation: complex
numerous inhibitors (mechanism-based inactivators)
inducible by physiological, physico-chemical and xenobiotic agents

Molecular data: <http://drnelson.utmem.edu/homepage.html>
superfamily of genes, 500 being cloned

Laccases

Equation: $2RH + \frac{1}{2}O_2 \rightarrow 2R^\bullet + H_2O$

Distribution: fungi, bacteria, higher plants, insects

Function: lignin degradation, morphogenesis, pathogenesis

Structure: dimeric or tetrameric glycoprotein, with 4 Cu,
exocellular, 520 to 550 aa, 60 to 80 kDa
secreting a N-terminal peptide

Regulation:

inhibitors: small anions, metals, fatty acids, chelating agents
inducers: culture medium, lignin and related compounds,
xenobiotics



III

Bioremediation processes: a way to reduce the environmental hazard of PAHs

- mainly used methods
- case studies

How to select a remediation method?

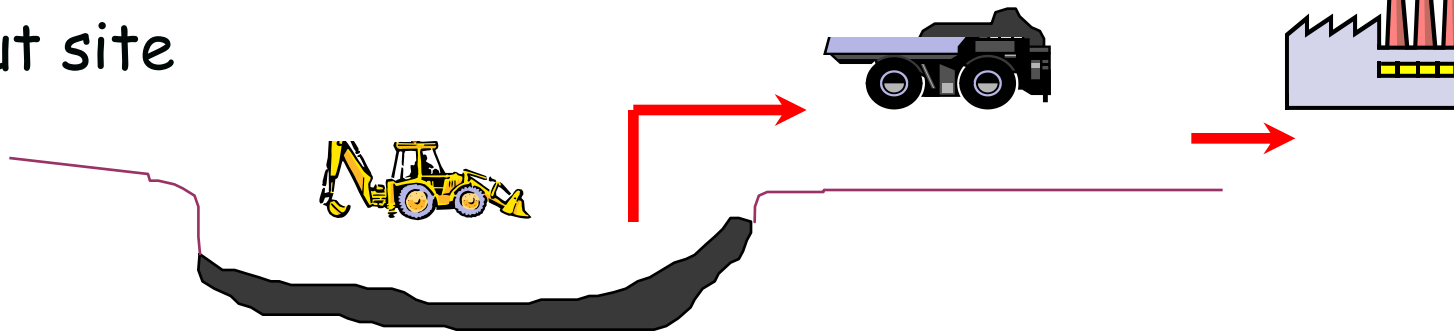
- pollutant: type, concentration, extent, ageing
- type of soil: hydrogeology, texture, permeability
- local constraints: space, noise, smell, dust (urban areas)
- cost
- time limit
- threshold

Bioremediation

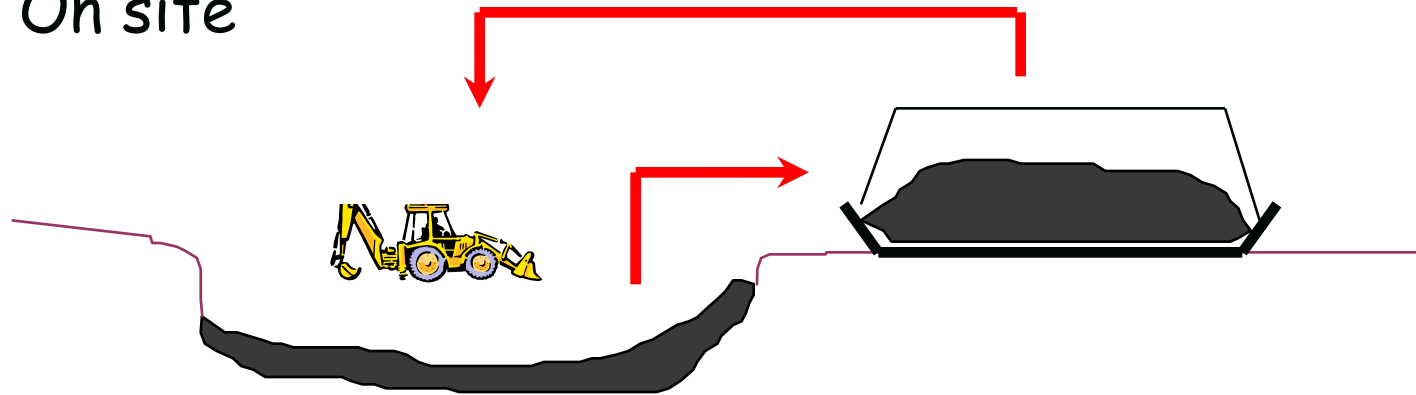
- use of the natural or engineered capacity of micro-organisms to transform PAHs
- incubation conditions are optimized by aeration, agitation, moistening, addition of nutrients
- case studies: Federal Remediation Technologies Roundtable
<http://www.frtr.gov/cost/>

Treatments

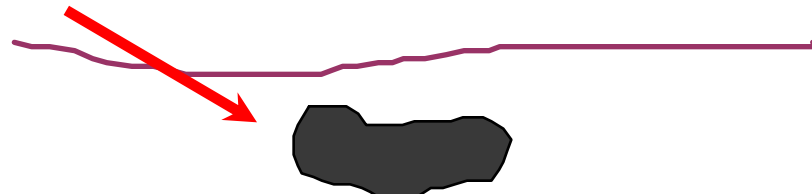
- Out site



- On site



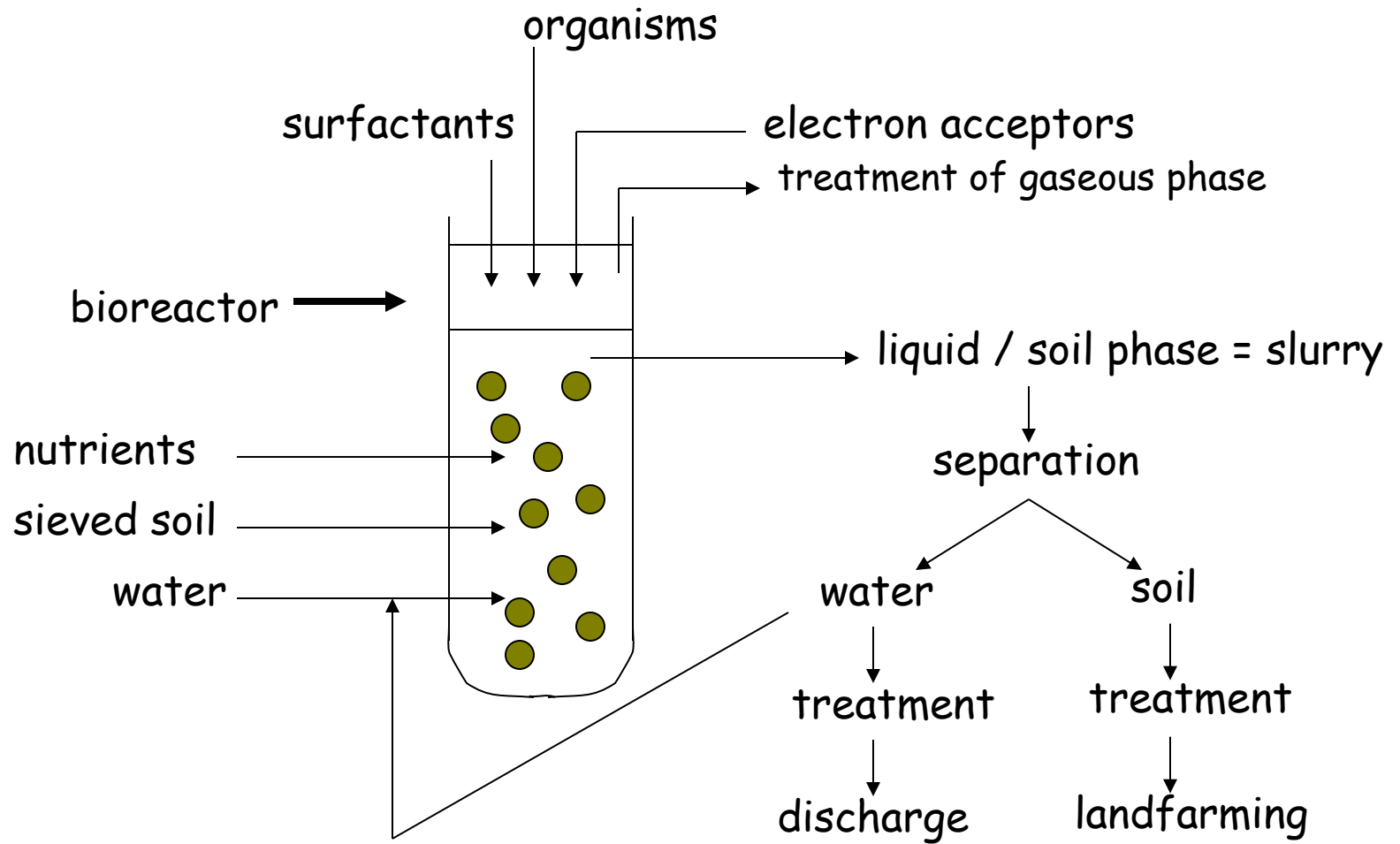
- In situ

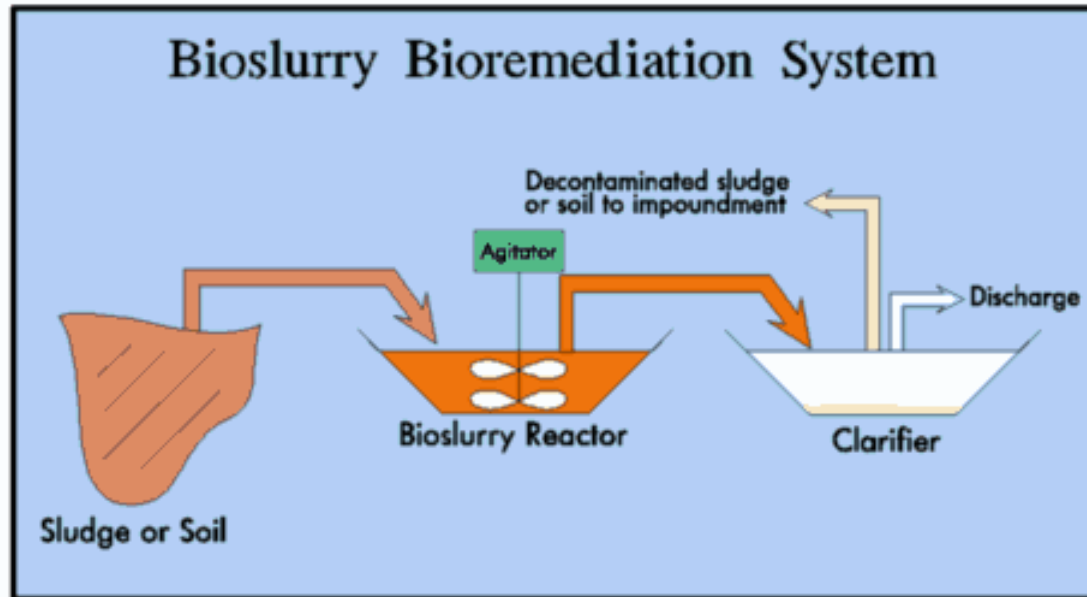


The *ex situ* bioremediation methods

- Many advantages:
 - optimized monitoring ensured by mixing, heating, ventilation and moistening
 - optimized biotransformation due to the supply of electron acceptors and nutrients, (micro-organisms)
- reduction of the heterogeneous structure of the soil
- 3 main methods: bioslurry, biopile, landfarming

The bioslurry



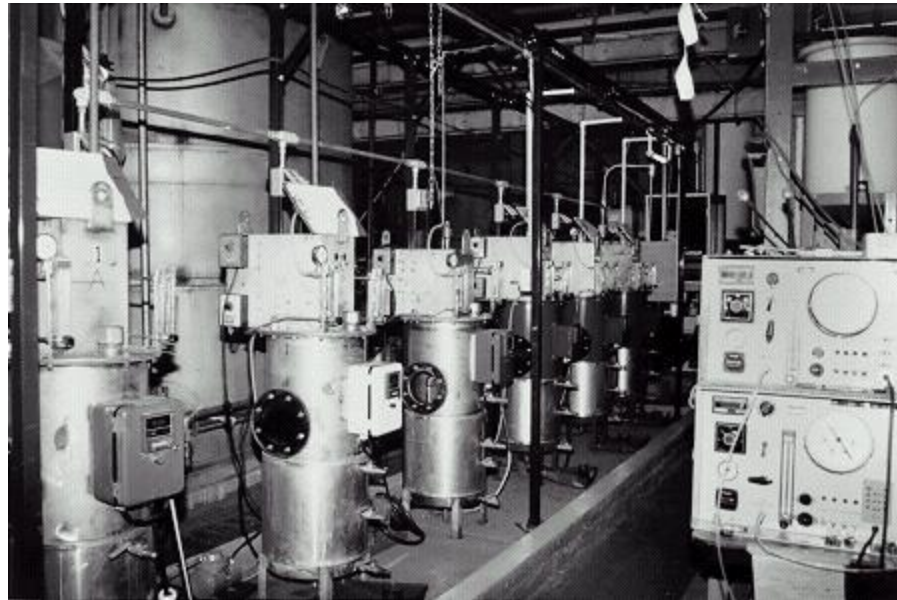


Schematic of a bioslurry bioremediation system. Source: Adapted from the U.S. EPA (8).

Bioslurry

- Pollutants: recalcitrant compounds, PAHs (2,500 to 250,000 mg/kg)
- Type of soil: heavy soils, with high clay content
- Duration of the treatment: 1 to 6 months
- Operating conditions: pH 4.5 to 8.8
temperature 15 to 35 °C
fixed or rotative
nutrients and surfactants
- Soil native microflora or engineered strains
- Cost: 50 to 250 \$/m³
- Efficiency: 3-ring PAHs 98-99%
4-ring PAHs 85-95%
> 4-ring PAHs 55-85%
- Emerging technology: Fenton's reagent to oxidize heavy PAHs
- <http://www.epa.gov/ORD/SITE/reports>

The bioslurry reactor: ECOVA



Bioslurry: case study (1)

- **Operating conditions**

raw materials: 12,630 tons of soil containing up to 10 g PAH/kg
after sifting, wetting and centrifuging: 8,925 tons of slurry
($<80\mu\text{m}$) treated in four bioreactors

- **Parameters of the bioreactors**

Volume	750,000 L
Shaking	900 rpm
Air	$350 \pm 170 \text{ m}^3/\text{h}$
Solid particle ratio	20% by weight
Antifoam	200 mg/L
Dispersing agent	1000 mg/L
Temperature	$30 \pm 10 \text{ }^\circ\text{C}$
pH	7.2 ± 1.0
Dissolved O_2	$> 2.0 \text{ mg/L}$
NH_4^+	$60 \pm 20 \text{ mg/L}$
PO_4^{2-}	$20 \pm 10 \text{ mg/L}$

Southeastern Wood Preserving
Superfund Site
Canton, Mississippi

Bioslurry: case study (2)

- Results**

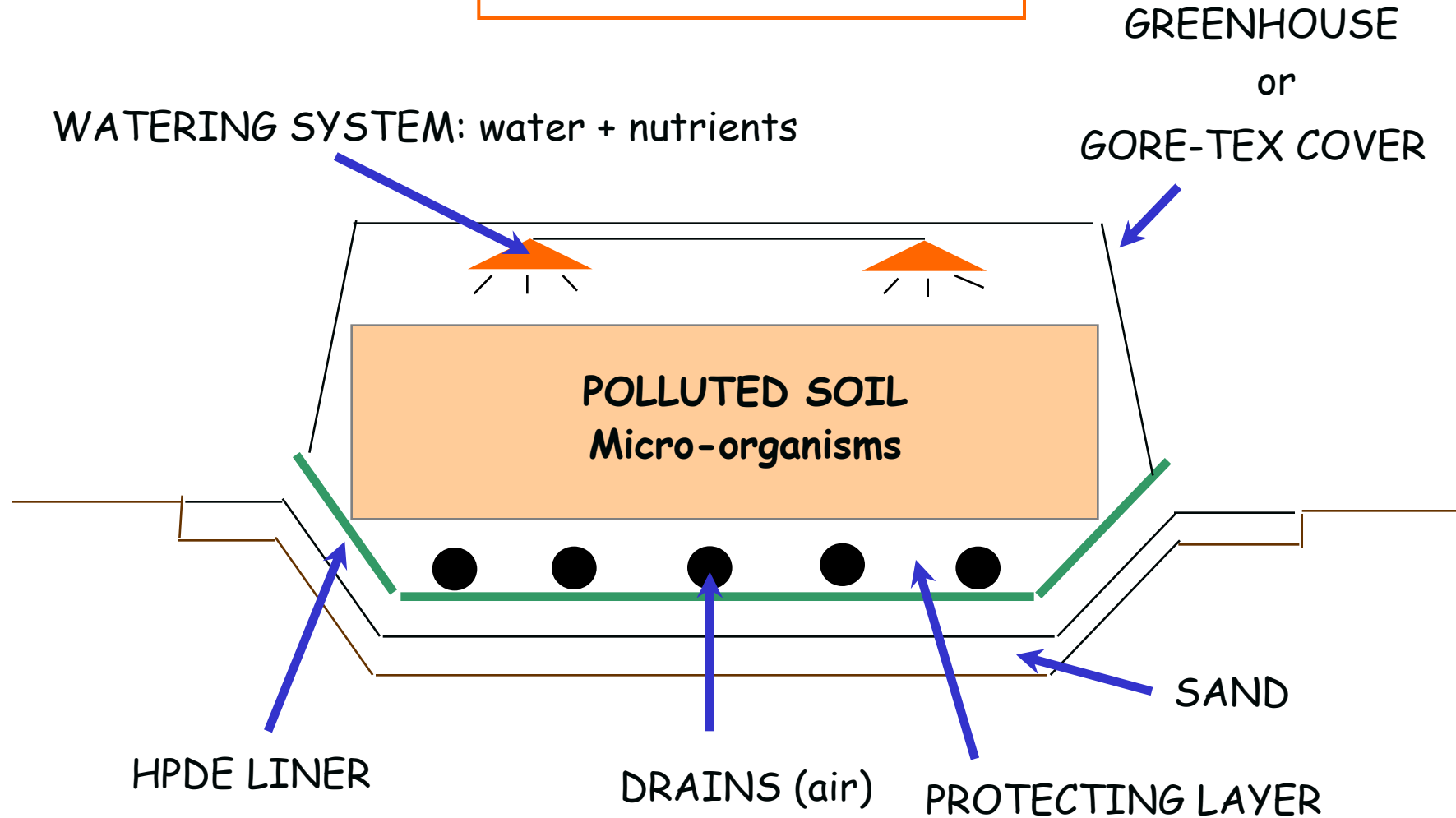
	Initial amount (mg/kg)	Efficiency (%)
3-ring PAH ₁	4613 ± 1785	94
4-ring PAH ₁	3432 ± 113	95
5 and 6 ring PAH ₁	729 ± 329	62

- Financial Data (\$/ton)**

	Slurry making	Biotreatment	Slurry drying
Plant material and labor	25-30	8-12	4-8
Supplies, electricity,...	16-20	20-25	12-16
Analysis	< 4	4-8	< 4
Total	42-50	32-46	16-25

+ treatment of water in wastewater treatment plant

The static biopile



Biopile

- **Pollutants:** all organic pollutant, PAHs <5,000 mg/kg
- **Type of soil:** permeable
- **Duration of treatment:** 6 to 24 months
- **Operating conditions:** height: 0.9 to 3 m
cover: textile or green house
ambient temperature
moistening 40 to 75 % of MHC
soil piled in mounds
static (forced venting) or dynamic (soil reversal)
nutrients and surfactants
collection of leachates
- **Soil native micro-flora or exogenous micro-organisms**
- **Cost:** \$ 40 to 220 / ton of soil
- **Efficiency:** 80 %
- **Similar method:** composting with addition of fertilizers such as manure
- <http://www.epa.gov/OUST/cat/biopiles.htm>

Biopile

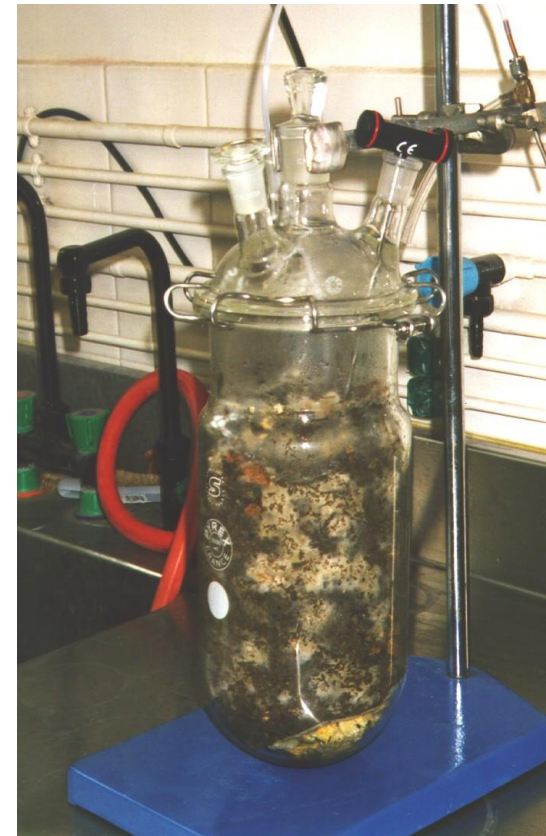


Biopile: case study (1)

- Operating conditions: laboratory scale study
sifted sandy loam (15 mm) containing 2800 mg PAH/kg, 220 mg cyanide/kg
17 g heavy metal/kg, ...
- Parameters of the "biopiles"
Jars containing 6 kg polluted soil
Static in the dark
Air: forced venting 3 L/h
Temperature: 25 ± 5 °C
Moistening: 80% of the MHC
pH: 6.9
C/N/P ratio: 100/5/1, agricultural fertilizers
Inoculation: the fungus *Trametes versicolor* pregrown on wheat
bran pellets (0.6g pellets/jar)
Surfactant: Montanox 80 (non ionic), 2 % by weight
Duration: 50 weeks

GDF Manufactured Gas Plant
Rouen, France

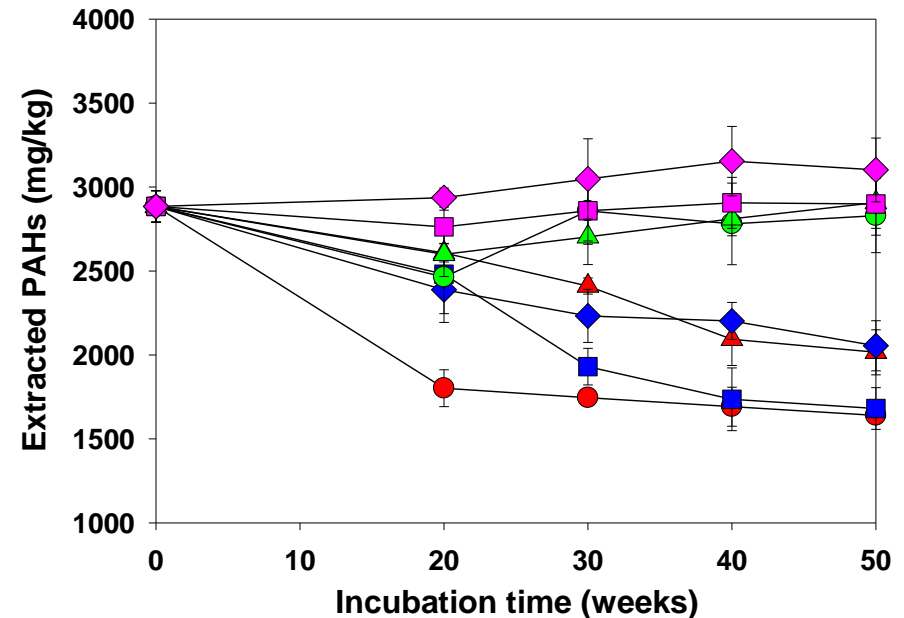
Biopile: case study (2)



Biopile: case study (3)

• Results

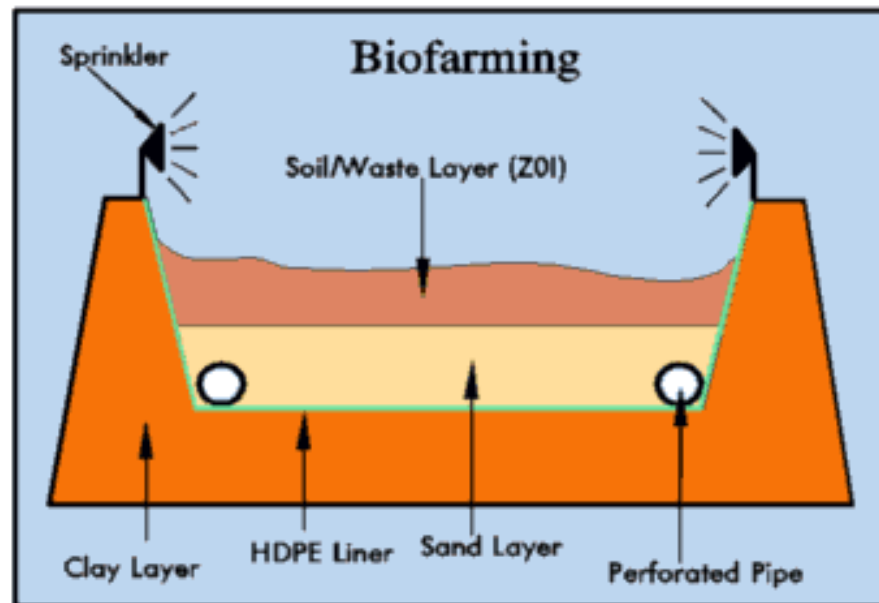
	Pellets	<i>T. vers</i>	C/N/P	Surf.
●	+	+	+	+
◆	+	+	+	-
■	+	+	-	+
◆	+	+	-	-
●	+	-	+	-
◆	-	-	+	-
■	-	-	-	+
◆	-	-	-	-



→ a 40% decrease of PAH amounts extracted from the soil after 50 weeks in the presence of the pellets, the fungus, nutrients and surfactant

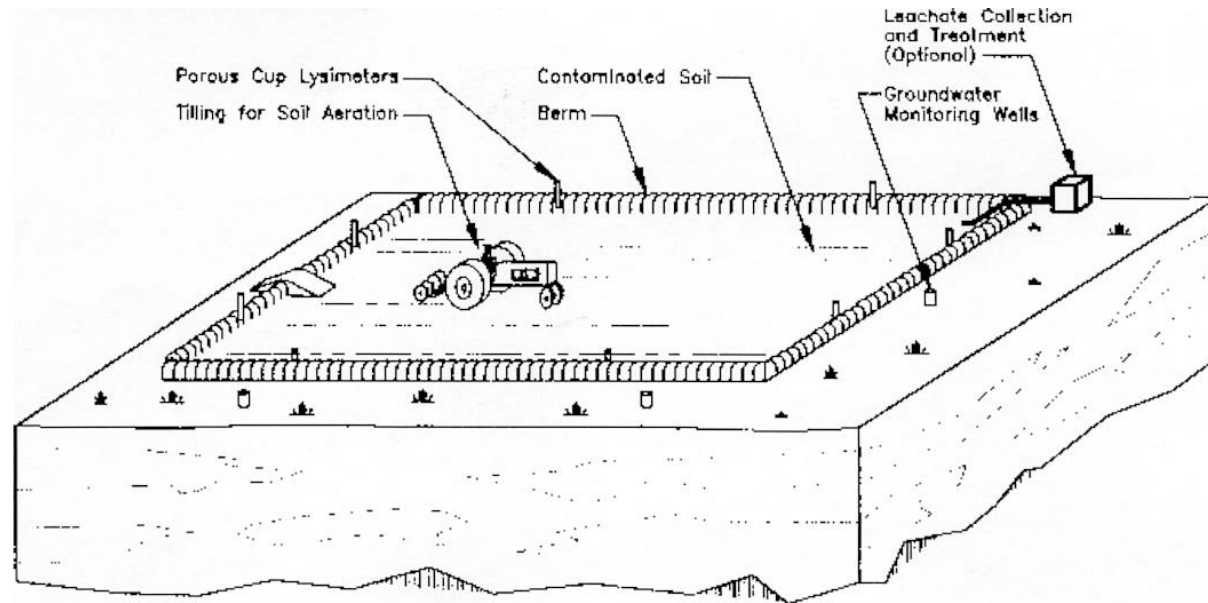
Rama *et al.*, PAC, 2001, 18(4), 397-414

Landfarming



In land farming, waste or contaminated soil is aerobically treated above ground to biodegrade, transform, and immobilize contaminants. Source: Adapted from the U. S. EPA (8).

Landfarming



Landfarming

- **Pollutants:** all organic pollutant, PAHs <10,000 mg/kg
- **Type of soil:** all
- **Duration of treatment:** 6 to 24 months
- **Operating conditions:** soil layered on a specific area, sometimes incorporated in the top layer of an agricultural soil
soil periodically tilled
ambient temperature
nutrients and moisture may be added
collection of leachates may be necessary
- **Soil native micro-flora or exogenous micro-organisms**
- **Cost:** \$ 30 to 60 / ton of soil
- **Efficiency:** 90 %

Landfarming: case study

Treatek, Blackburn, United Kingdom

- **Operating conditions**

30,000 m³ of soil polluted with tar, phenolics, cyanides, heavy metals, PAHs
sifting < 30 mm
soil treated in several layers
soil moistened by a solution of micro-organisms and nutrients

- **Results**

	Initial amount (mg/kg)	Removal efficiency (after 30 months)
PAHs	22,050	99
Phenolics	205	98

- **Cost: 45 \$/m³**

IV

Future projects

- improving the bioavailability of PAHs
- improving the ability of organisms to transform PAHs
- phytoremediation

Improving the bioavailability of PAHs

- Use of synthetic surfactants and biosurfactants to facilitate the partitioning of PAHs from the solid phase of the soil or from NAPL to the water phase
- A considerable literature has been obtained at a laboratory scale, but a few results are usable in industrial processes
 - a variety of ionic and nonionic surfactants enhance the solubilization of PAHs
 - the enhancement varies with surfactant structure, concentration in solution, hydrophobicity of the PAHs, degree and age of contamination
 - cationic agents, adsorbed onto soil, solubilize PAHs at supra-CMC levels, but they are toxic for micro-organisms
 - nonionic compounds disperse the soil particles, and also solubilize PAHs, with a reduced toxicity
 - limitations: high concentrations of PAHs are required to solubilize low amounts of PAHs, in the presence of a high water-content of the soil
 - most successful results are obtained in bioslurry reactors

Improving the ability of organisms to transform PAHs

The problems

- The complete breakdown of a pollutant requires a sequence of metabolic reactions
- Xenobiotics often include unusual chemical bonds or substitutions
- Organisms have not had enough time to evolve appropriate pathways

The solutions

- Development of a microbial community
- Development of the capabilities of given strains by genetic construction
 - genetically modified organisms: *GMOs*
 - engineered micro-organisms: *GEMs*

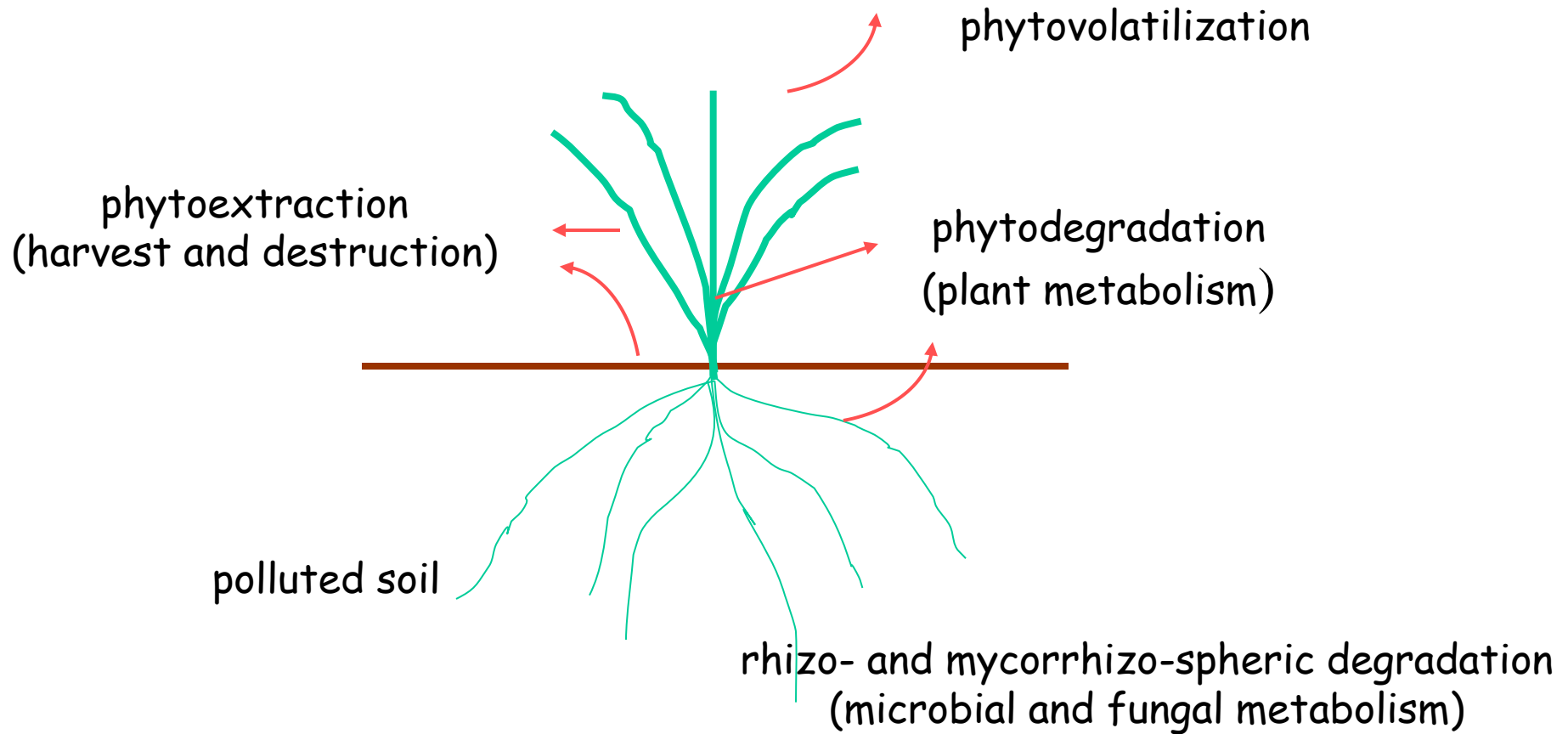
The limitations

- Ecological problem: risk of rambling genes
- World-wide regulation by legislation

The ways of research

- Improvement of the metabolic properties of the organism (Abstract N°58)
- Development of suicidal elements for biological containment of organisms

Phytodecontamination



Phytodecontamination ex planta

A chemical present in the soil induces a plant response, which increases or changes its exudation, modifying then rhizospheric microflora composition or activity

Other effects:

- improvement of physical and chemical soil conditions
- increase of humification and adsorption

→ dissipation of PAHs
impact of each process not elucidated

Plant species: fescue (*Festuca*), alfalfa (*Medicago*), sudangrass (*Sorghum*)
ryegrass (*Lolium*), corn (*Zea*), soybean (*Glycine*),...