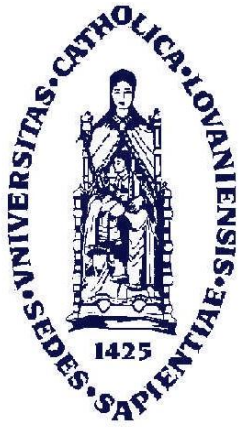
Bioaugmentation for Site Remediation: When Does it Work ?



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Limitations of bioremediation	Measures to overcome the limitations
* Unfavourable conditions for microbial growth and activity	* Biostimulation
* Lack of organisms expressing the appropriate catabolic potential	* Bioaugmentation
* Metabolic misrouting	* Use of engineered strains
* Lack of bioavailability of pollutants	* Use of solubilizing agents

Bioaugmentation

What is bioaugmentation?

Improving the bio-removal capacity of soil or other biotope by inoculating specific competent strains or consortia

What are its premises?

Increasing the metabolic capacities of the indigenous microflora present in the biotope (soil, sediment, sludge, etc.) as a result of an exogenously augmented genetic diversity that leads to a wider repertoire of productive biodegradation reactions

How to evaluate success?

Sustainability, rate and extent of pollutant removal, regulatory compliance

Bioaugmentation Options

Action	Mechanism	Example
Add a pre-adapted strain	Certain sites may not contain adequate pollutant degrading microorganisms	Inoculation of soils and WWTP with chloroaromatic degraders
Add pre-adapted consortia	The presence of the right combination of microorganisms is ensured	Seed sediments with PCB-dechlorinating enrichment cultures
Add genetically optimized strains	Existing degradation pathways may release dead-end or toxic intermediates	Construction of strains effecting complete simultaneous oxidation of chloro- and methyl-aromatics
Add genes packaged in a vector	Genes encoding desirable functions are transferred into microorganisms already present	Degradation of PCBs or pesticides

Table 1 Persistent pollutants

Chemical class	*Examples*	*Application/source*	*Health effects*
Nonhalogenated compounds			
Aromatic hydrocarbons	Benzene	Petroleum co-contaminant; chemical precursor	Toxic, carcinogenic
	Toluene	Petroleum co-contaminant	Irritant
Polycyclic aromatic hydrocarbons (PAHs)	Naphthalene	Mothballs, chemical precursor	Toxic
	Chrysene	Dye production	Toxic, suspected carcinogen
	Benzanthracene	Combustion product	Toxic, carcinogenic
Nitroaromatic compounds	Trinitrotoluene (TNT)	Explosives	Toxic, possible carcinogen
	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	Explosives, rat poison	Irritant, possible carcinogen
Metals	Mercury	Coal combustion product	Toxic
	Uranium	Nuclear power plants, weapons	Carcinogen
Halogenated aliphatics			
Chlorinated ethenes	Perchloroethene (tetrachloroethene)	Degreasing fluid	Depressant, suspected carcinogen
Organochlorines	Lindane	Insecticide	Irritant, neurotoxin, suspected carcinogen
Halogenated aromatics			
Chlorinated benzenes	Dichlorobenzene	Mothballs, chemical intermediate	Irritant, suspected carcinogen
	Dioxins	Combustion product	Chloracne, probable carcinogen
Polychlorinated biphenyls (PCBs)	Aroclor mixtures	Dielectric fluid, flame retardant	Chloracne, probable carcinogen

Source: Lyon & Vogel 2011

Bottlenecks (1)

Ecological background of bioaugmented biotope can be a major barrier in the successful bioremediation performance of an exogenous inoculum

Relations of inoculated microorganisms with their biotic and abiotic environment (survival, activity, migration) can be determining factors in the outcome of a bioaugmentation strategy

Bottlenecks (2)

Soil (and, to a lesser extent, sediment) is particularly recalcitrant to successful bioaugmentation due to the **rapid decline in population size** of active exogenously inoculated cells ("soil microbiostasis" or "obstinacy") generally attributed to both biotic and abiotic factors

Biotic factors are often more consequential (cf. inoculation of sterilized soil): predation, competition with autochthonous microorganisms for nutrients or e-acceptors, presence of root systems releasing organic compounds, etc.

To improve the establishment and efficacy of an inoculant in soil, predictable ecological **selectivity** and/or ecological **protection** must be provided

Factors influencing bacterial survival in soils

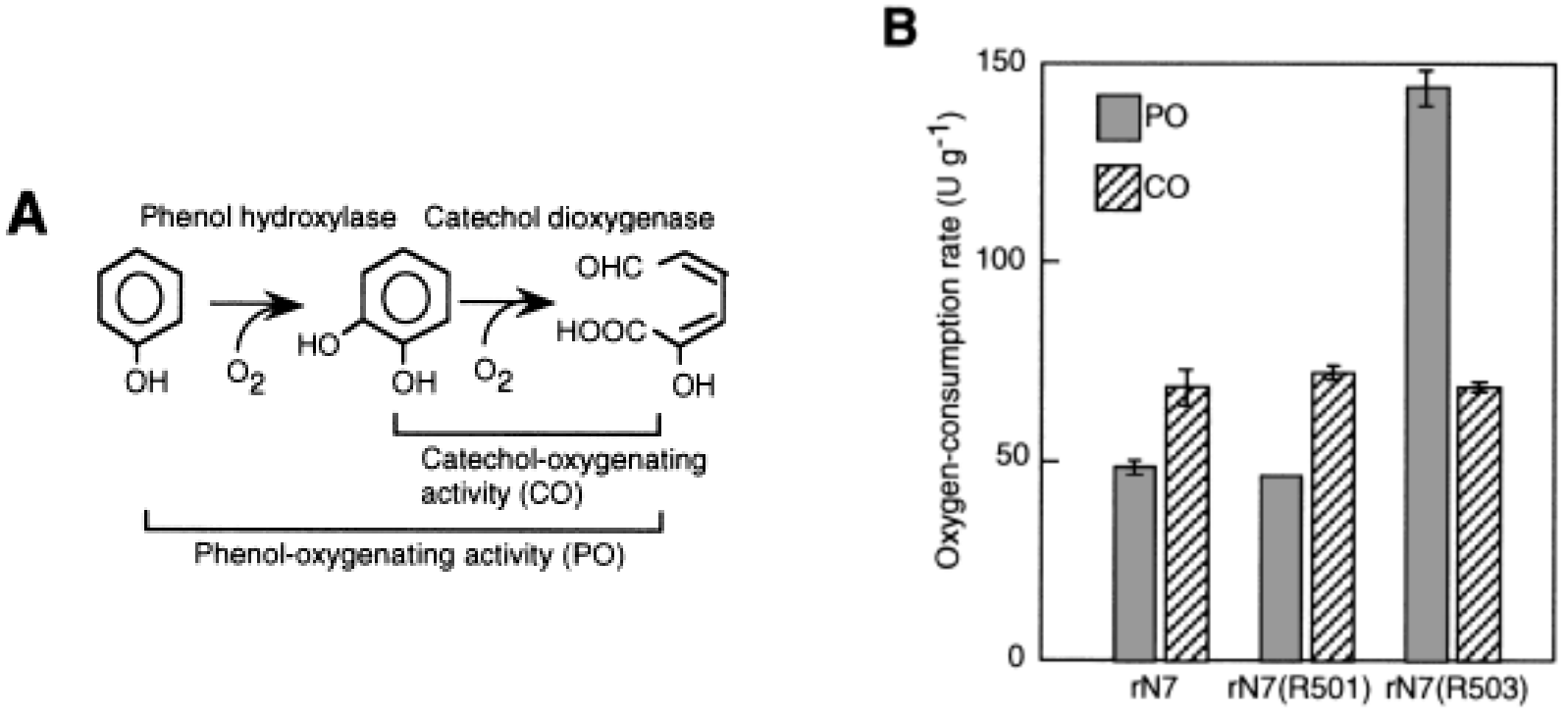
Origin	Factor	Effect
Biotic	Predation	Population size decrease
	Competition	Population size decrease/antagonistic effect on plant pathogens
	Root growth	Release of organic compounds, enhancing survival
Abiotic	Clay minerals	Protection against predation
	Water tension	High tension: water shortage, high osmolarity; low tension: anaerobiosis, increased nutrient availability by diffusion
	Organic carbon	Selection for copiotrophic or oligotrophic species; limited organic carbon results in starvation and reduction in activity
	Inorganic nutrients (N, P)	Limitation results in starvation
	pH	Selection for species; release of nutrients (e.g., P) or toxic compounds (e.g.,Al^{3+}).
	Temperature	Metabolic activity as well as biotic (predatory) pressure affected.
	Chemicals (toxic waste)	Inhibition of sensitive organisms; selection of biodegradative, resistant, or tolerant forms

Van Veen et al. 1997, Microbiol. Mol. Biol. Rev. 61: 121-135

Lessons from ecological studies of activated sludge, a dynamic biotope

Successful case study: stable augmentation introducing foreign catabolic genes into a dominant bacterium (Watanabe *et al.,* 2002)

Unsuccessful case study: instability due to massive augmentation with ‘predable’ bacterium (Bouchez *et al.,* 2000)



Phenol-oxygenating activity of rN7(R503)

A. Initial step of the bacterial phenol-degradative pathway. Strains R5 and rN7 employed extradiol dioxygenase for the degradation of catechol.

B. Phenol- and catechol-oxygenating activities of rN7, rN7(R501) and rN7(R503). The mean of three determinations is shown, and the error bar indicates the standard deviation.

Watanabe et al. (2002) *Environ. Microbiol.* **4**(10): 577-583

El Fantroussi & Agathos 2005 Curr Opin Microbiol 8: 268-275

Table 1

Examples of recent bioaugmentation studies.

Biotope	Microorganism(s)	Pollutant	Reference
Soil	*Pseudomonas* sp. strain ADP	Atrazine	[47]
	Agrobacterium radiobacter J14a	Atrazine	[48]
	Escherichia coli pAtzA	Atrazine	[10]
	Consortia degrading atrazine	Atrazine	[20]
	Alcaligenes eutrophus TCP	2,4,6-Trichlorophenol	[49]
	Desulfitobacterium frappieri PCP-1	Pentachlorophenol	[50]
	Ralstonia eutropha (pJP4)	2,4-Dichlorophenoxyacetic acid	[31]
	Ralstonia eutropha JMP134	2,4-Dichlorophenoxyacetic acid	[51]
	Pseudomonas sp. strain P51	1,2,4-Trichlorobenzene	[52]
	Pseudomonas pseudoalcaligenes POB310	3-Phenoxybenzoic acid	[53]
	Desulfomonile tiedjei	3-Chlorobenzoate	[17]
	Arthrobacter sp. B1B and *Ralstonia eutrophus* H850	Polychlorinated biphenyl	[34]
	Arthrobacter RP17	Phenanthrene	[54]
	Ralstonia basilensis RK1	2,6-Dichlorophenol	[55]
	Encapsulated consortium	Gasoline	[28]
Activated sludge	*Comamonas* sp. RN7(R503)	Phenol	[24]
	Comamonas testosteroni I2	3-Chloroaniline	[56]
	Candidatus Accumulibacter phosphatis	Phosphorus	[36]
	Desulfitobacterium frappieri PCP-1	Pentachlorophenol	[37,38]
Aquifer/groundwater	Methanogenic consortia	BTEX	[19•]
	Pseudomonas stutzeri KC	Carbon tetrachloride	[15]
	Consortium that contains *Dehalococcoides*	Chloroethenes	[2•]
	Consortium that contains *Dehalococcoides*	Chloroethenes	[46]
	Consortium that contains *Dehalococcoides*	Chloroethenes	[16]
	P. putida GJ31, *P. aeruginosa* RHO1 and *P. putida* F1ΔCC	Chlorobenzenes	[27]
	Butane-utilizing enrichment culture	1,1,1-Trichloroethane	[57]
	Hydrogenophaga flava ENV735	Methyl *tert*-butyl ether	[35]
	β-proteobacterium strain PM1	Methyl *tert*-butyl ether	[58•]

El Fantroussi & Agathos 2005 Curr Opin Microbiol 8: 268-275

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	β-proteobacterium strain PM1 **********	Methyl *tert*-butyl ether	[58•]

********** ***In situ***

********** ***Ex situ***

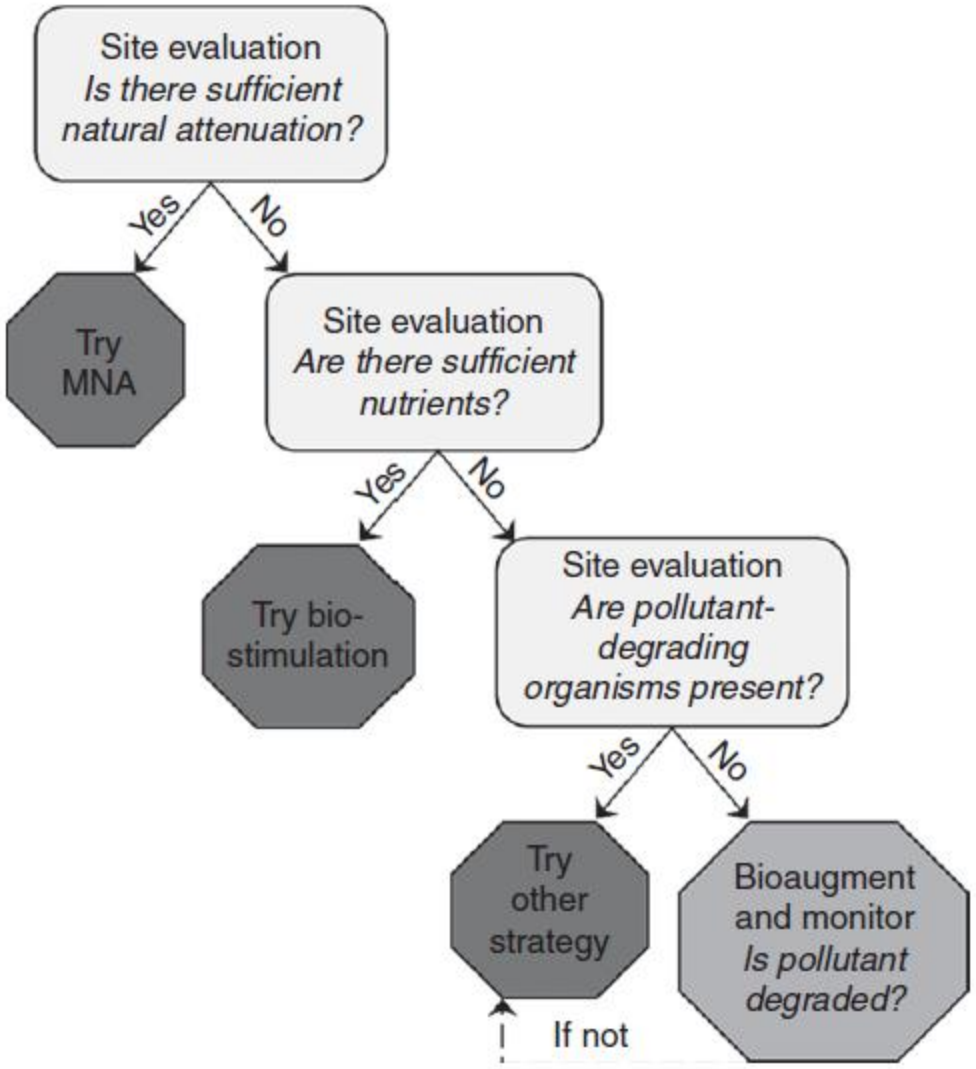


Figure 1 Decision-making process for bioaugmentation.

Source: Lyon & Vogel 2011

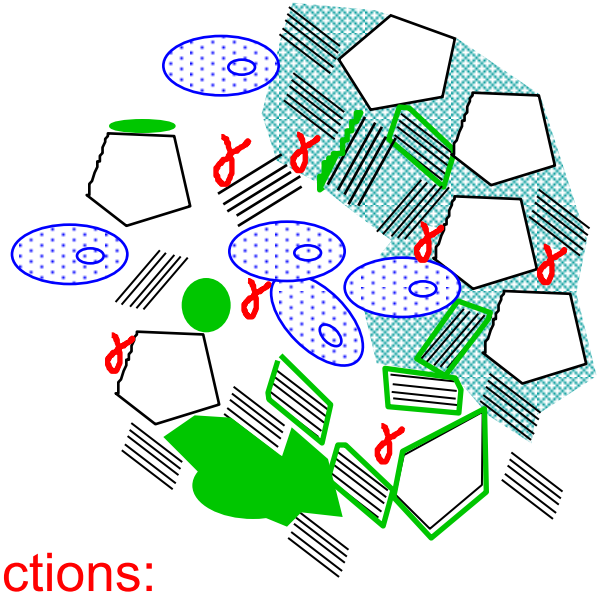
Table 2 Commercially available bioaugmentation inocula targeting chlorinated ethenes

Manufacturer (Web site)	*Product name*
EOS Remediation (www.eosremediation.com)	TCA20™, PJKS-1™, BAC-9™
Regenesis (www.regenesis.com)	Bio-Dechlor Inoculum™ Plus
SiREM (www.siremlab.com)	KB-1®, KB-1® Plus
The Shaw Group, Inc. (www.shawgrp.com)	Shaw Dechlorinating Culture – SDC9™

Source: Lyon & Vogel 2011

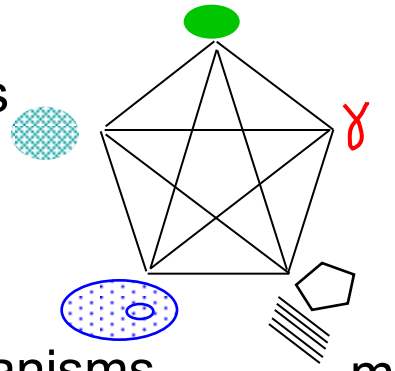
Scientific expertise needed

- Microscopic biological, biochemical and physico-chemical phenomena



interactions:

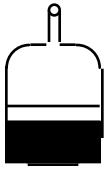
organics & xenobiotics



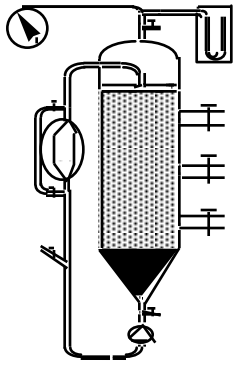
- Bioreactor / bioprocess engineering

laboratory

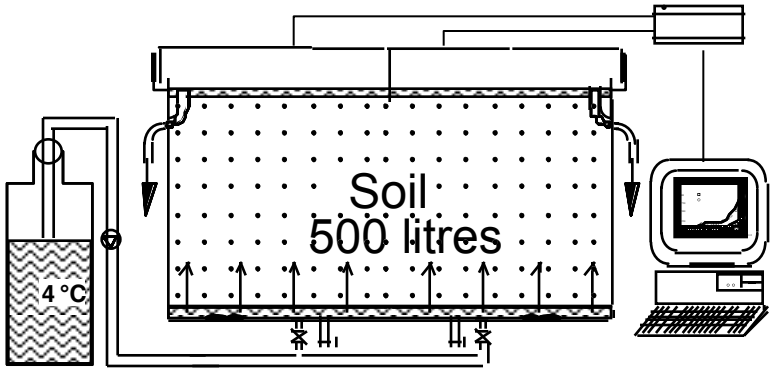
120 ml



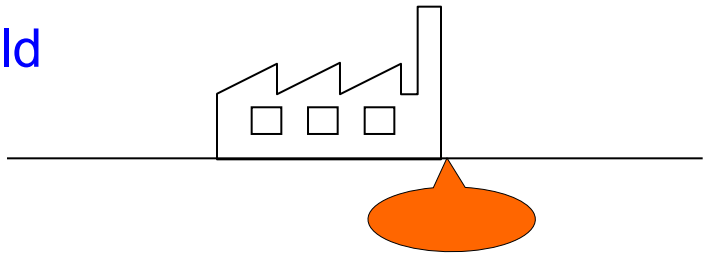
1 litre



pilot

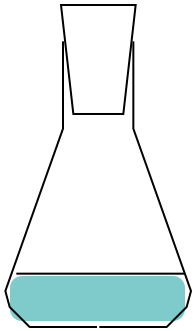


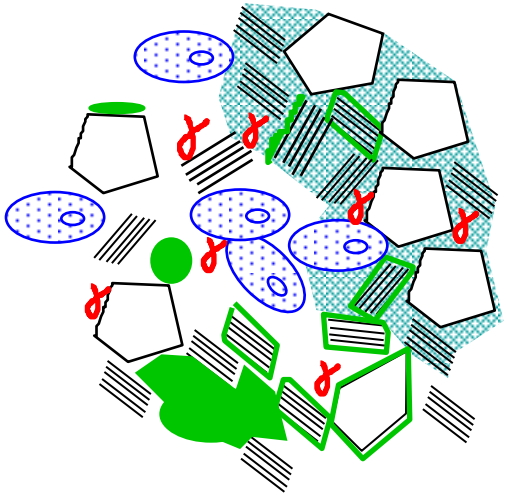
field



Transition in scales and tools

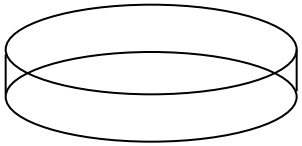
- Defined liquid conditions — Complex environmental matrices

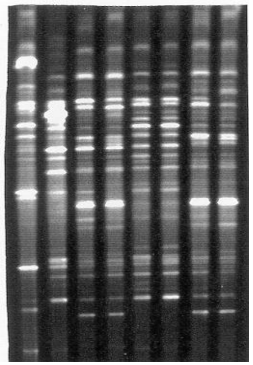


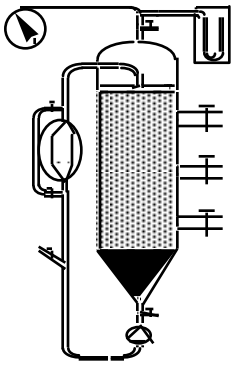


solid
+ liquid
+ gas

- Selection — Monitoring — Exploitation





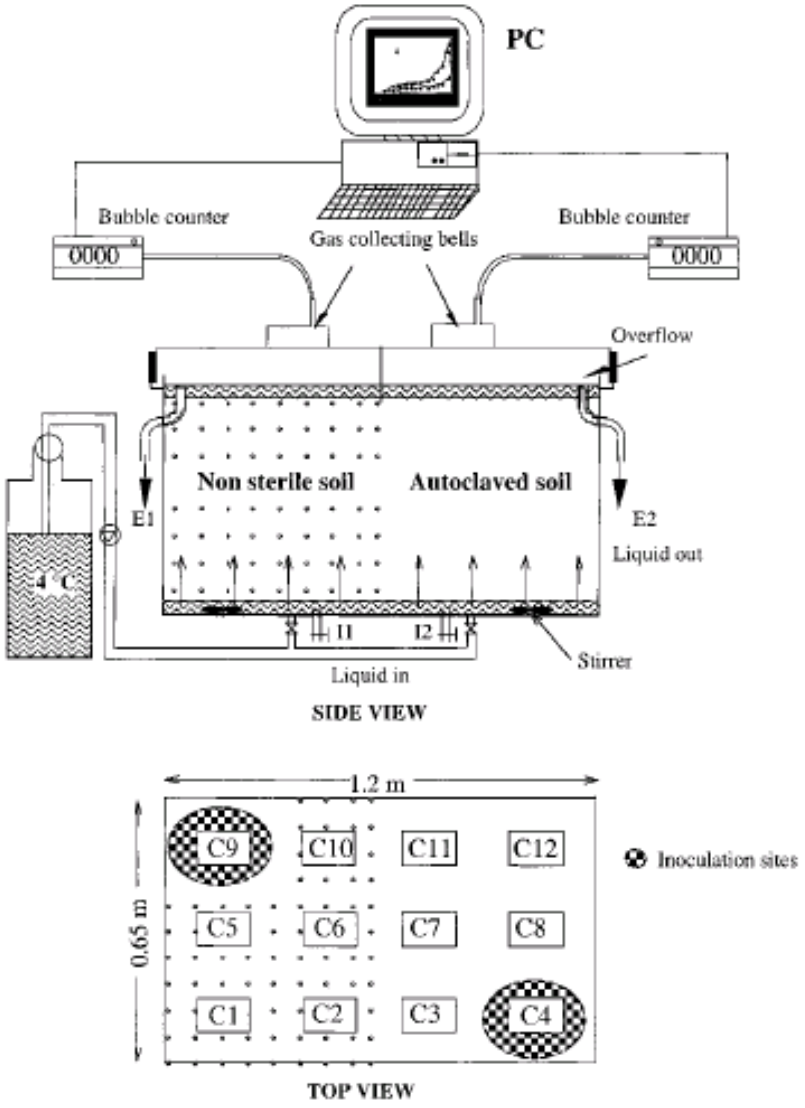


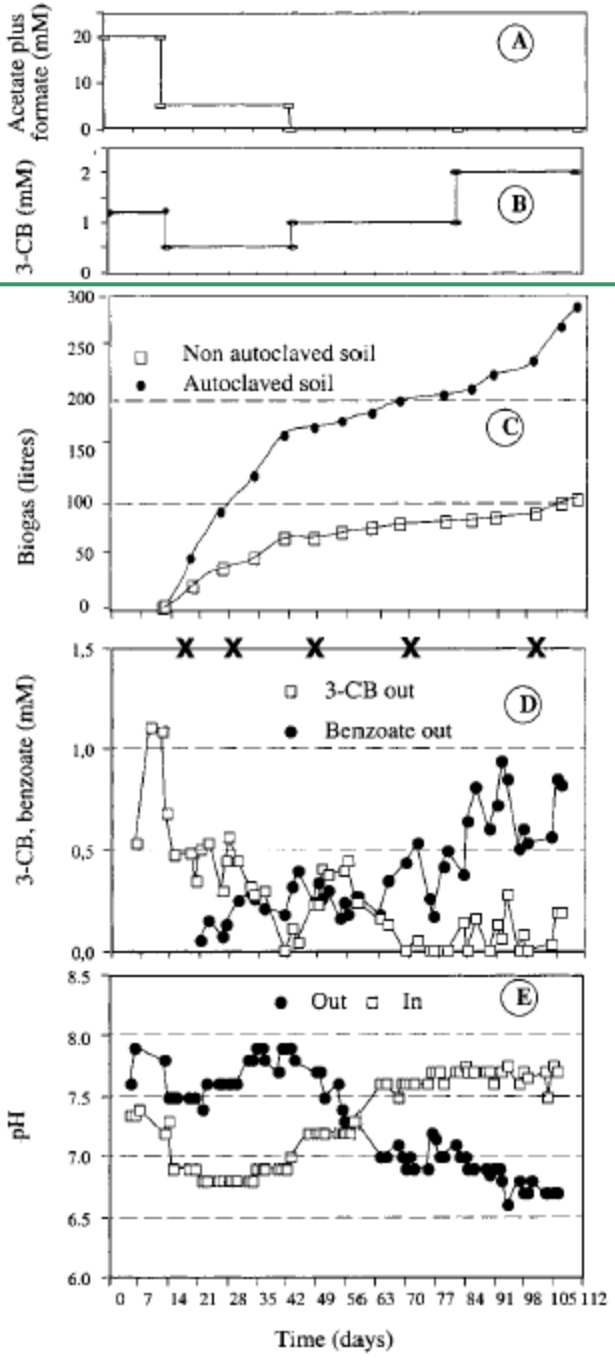
Bioaugmentation of a Soil Bioreactor Designed for Pilot-Scale Anaerobic Bioremediation Studies

Case study: Introduction of a pure strain, *Desulfomonile tiedjei*

El Fantroussi et al. (1999) ES&T

Schematic view of the soil bioreactor



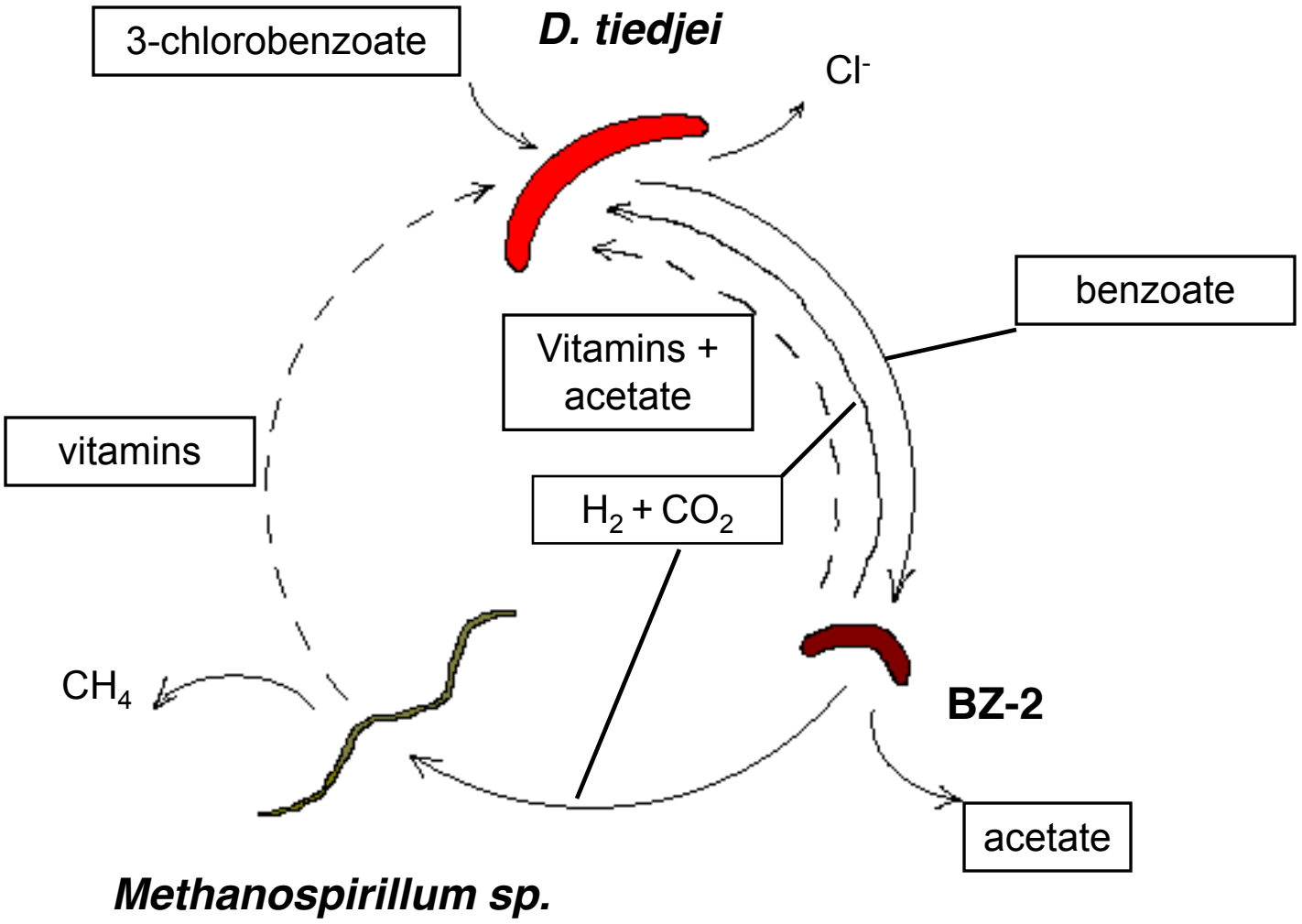


Feeding strategy for bioaugmentation

Time course evolution of the biodegradation capacities

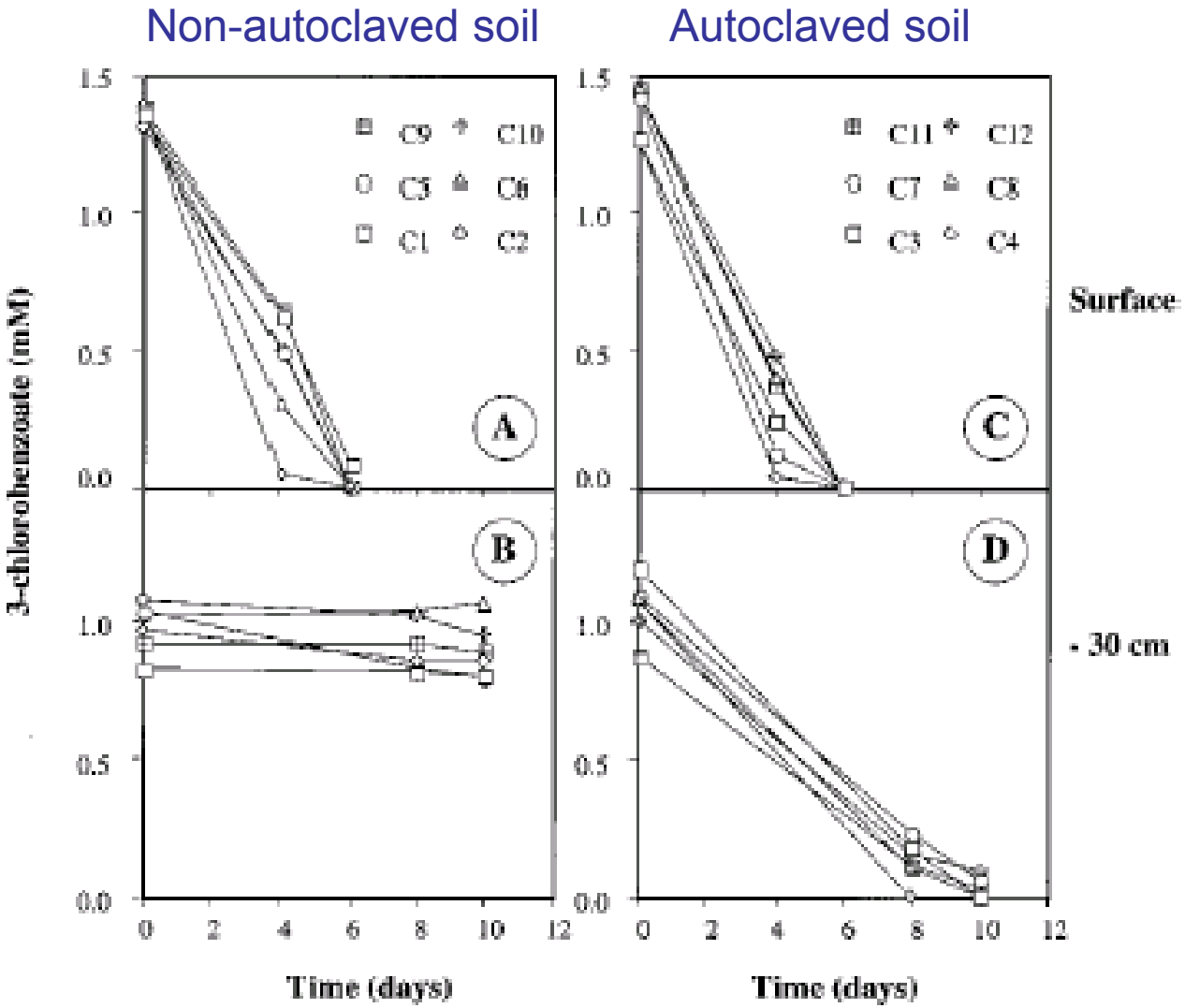
El Fantroussi et al. (1999)
Environ. Sci. Technol. 33:2992-3001

Syntrophic relationships in a defined consortium growing on 3CB



Redrawn from WW Mohn and JM Tiedje (1992) Microbiol. Rev. 56: 482-507

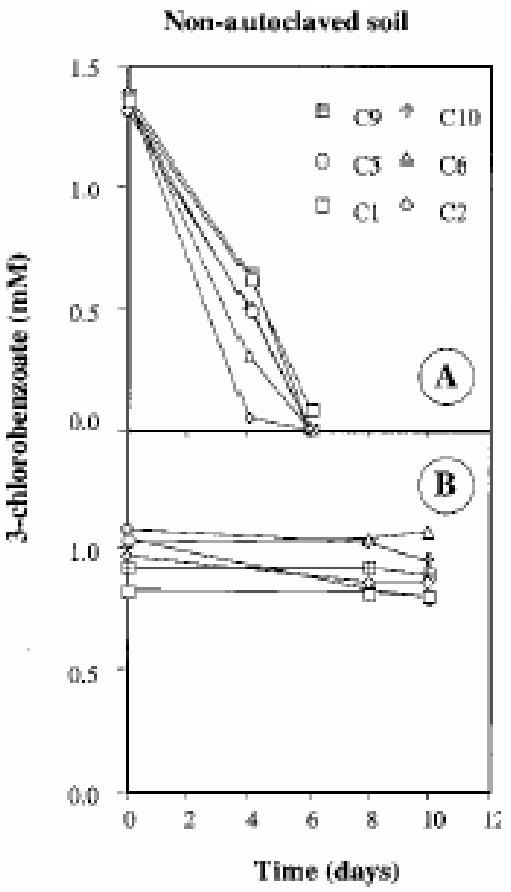
3-CB biodegradation in soil samples taken from different compartments and at different depths



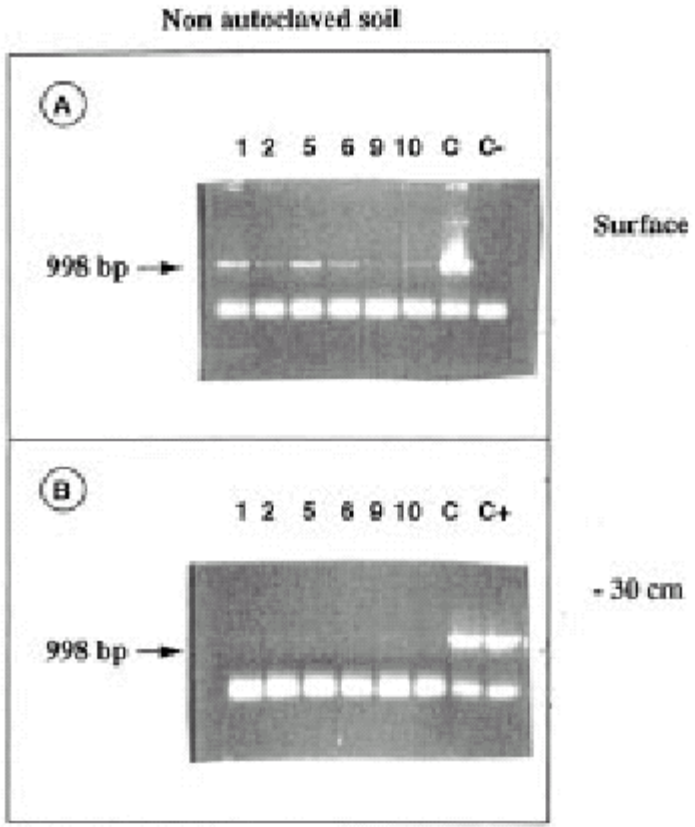
El Fantroussi et al. (1999) Environ. Sci. Technol. 33:2992-3001

Linking bioaugmentation to biodegradation

Microcosms



PCR detection of the 16S rRNA of *D. tiedjei* in the bioreactor



El Fantroussi et al. (1999) Environ. Sci. Technol. 33:2992-3001

PCR detection of the 16S rRNA of *D. tiedjei*

TABLE 3. Correlation between 3-Chlorobenzoate (3-CB) Dechlorination Activity and PCR Detection of the 16S rDNA Gene of *D. tiedjei* Using Soil Samples Taken from Different Areas in the Reactor at 30 cm of Depth over Time[a]

	nonautoclaved			autoclaved		
	C1	C6	C9	C4	C7	C12
13 days 3-CB transformation	−	−	+	−	−	−
13 days PCR detection	−	−	+	−	−	−
24 days 3-CB transformation	−	−	+	−	−	−
24 days PCR detection	−	−	+	(+/−)[b]	−	−
43 days 3-CB transformation	−	−	−	+	+	+
43 days PCR detection	−	−	+	+	+	+
62 days 3-CB transformation	−	−	+	+	+	+
62 days PCR detection	−	−	+	+	+	+
93 days 3-CB transformation	−	−	−	+	+	+
93 days PCR detection	−	−	−	+	+	+

[a] The samples were incubated for 10 days in the presence of 2 mM 3-CB. C1−C12 indicate the different compartments in the reactor. [b] Signal not detected by normal one-round PCR but by a nested PCR procedure using primers previously described (*12*).

El Fantroussi et al. (1999) Environ. Sci. Technol. 33:2992-3001

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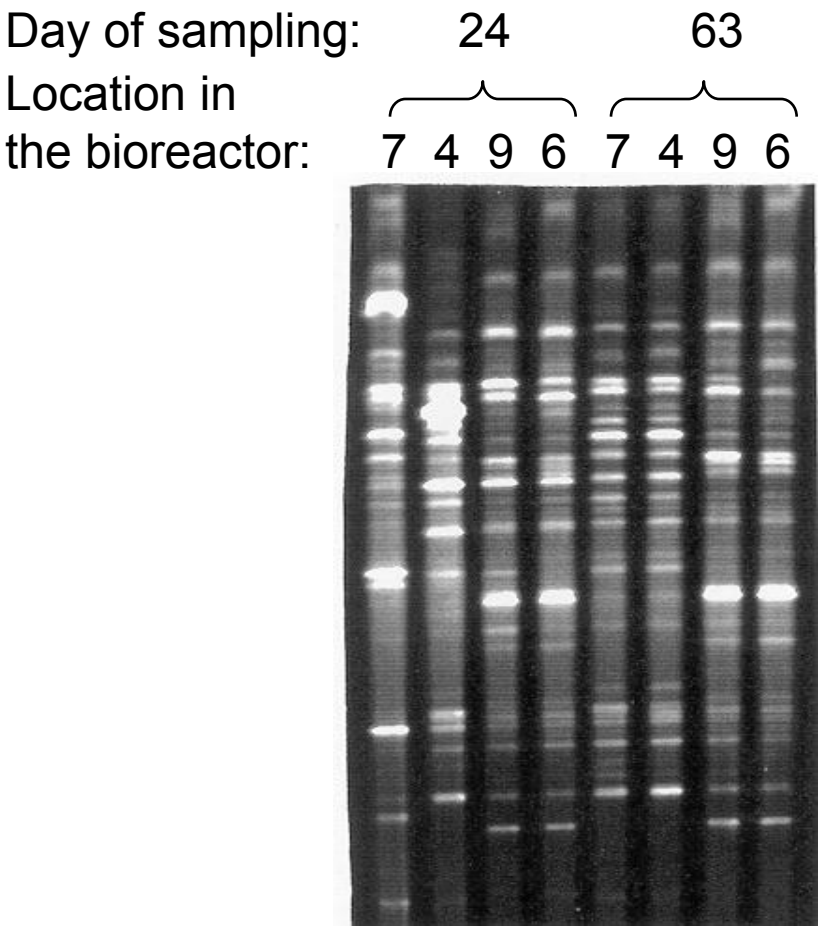
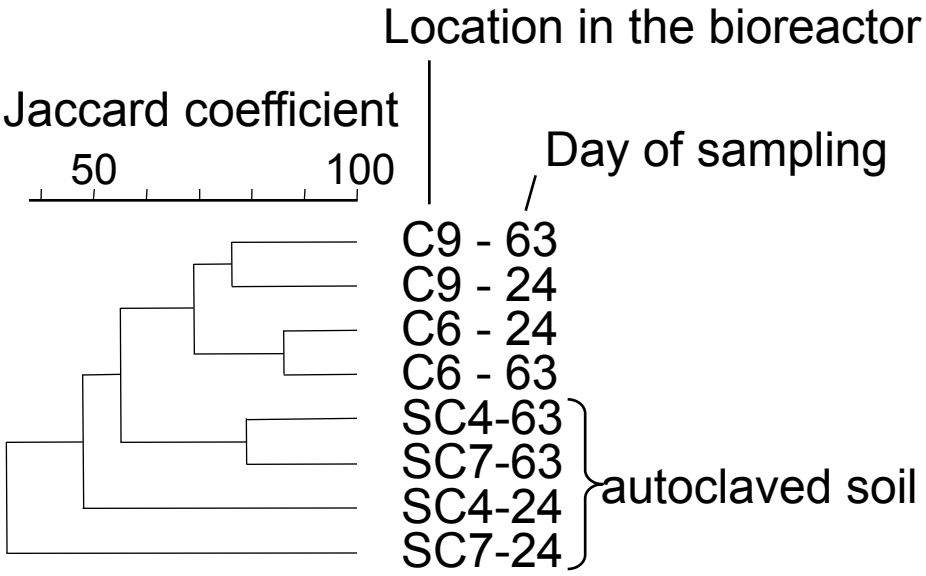
El Fantroussi et al. (1999) Environ. Sci. Technol. 33:2992-3001

Biomolecular monitoring

Fingerprinting of microbial communities at several locations and times in the bioreactor

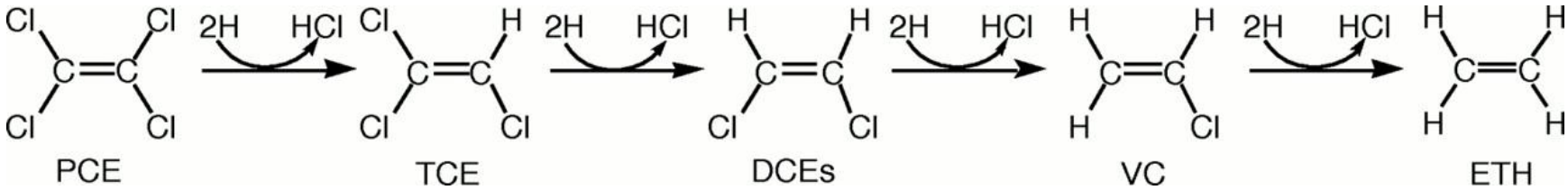
DGGE analysis of 16s rDNA

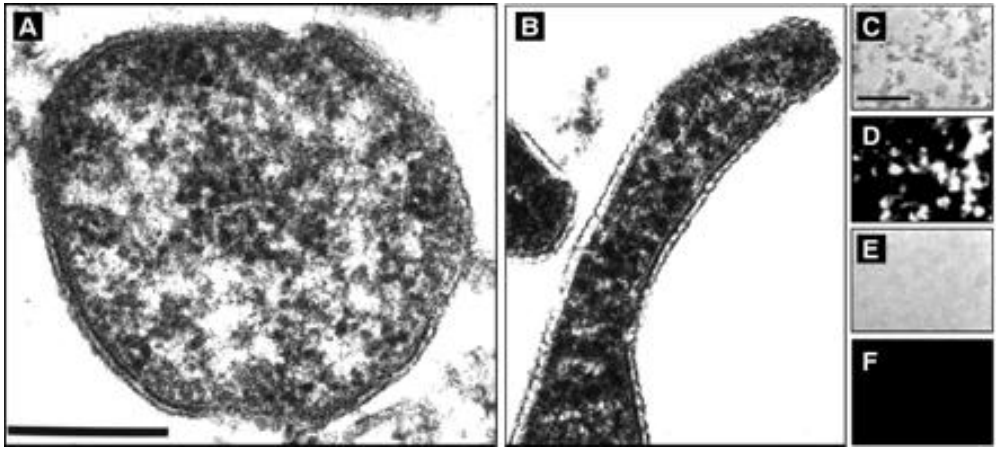




El Fantroussi et al. (1999) Environ. Sci. Technol. 33:2992-3001

Dehalococcoides ethenogenes strain 195





REGENESIS

Leaders in Accelerated Natural Attenuation

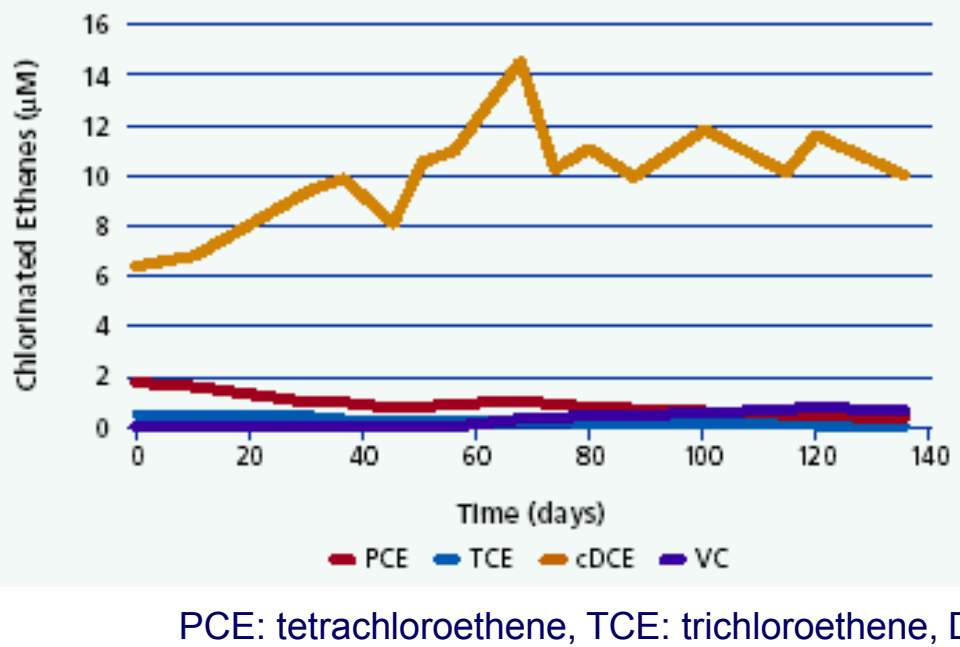
Bio-Dechlor INOCULUM™

- An enriched natural microbial consortium of *Dehalococcoides* sp.
- Offers accelerated dechlorination of PCE, TCE, DCE, VC
- Commercially available in liquid form
- Developed at Dr Frank Loeffler's laboratory, Georgia Institute of Technology

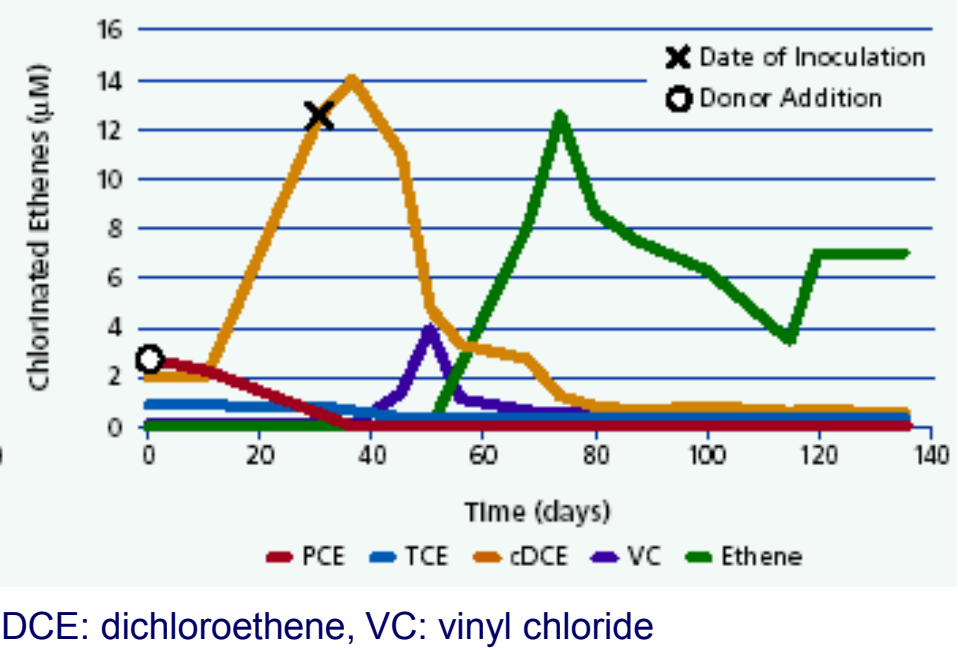
http://www.regenesis.com/products/bd_inoculum/

First bioaugmentation study

Chlorinated ethens at control plot effluent



Chlorinated ethens at test plot effluent



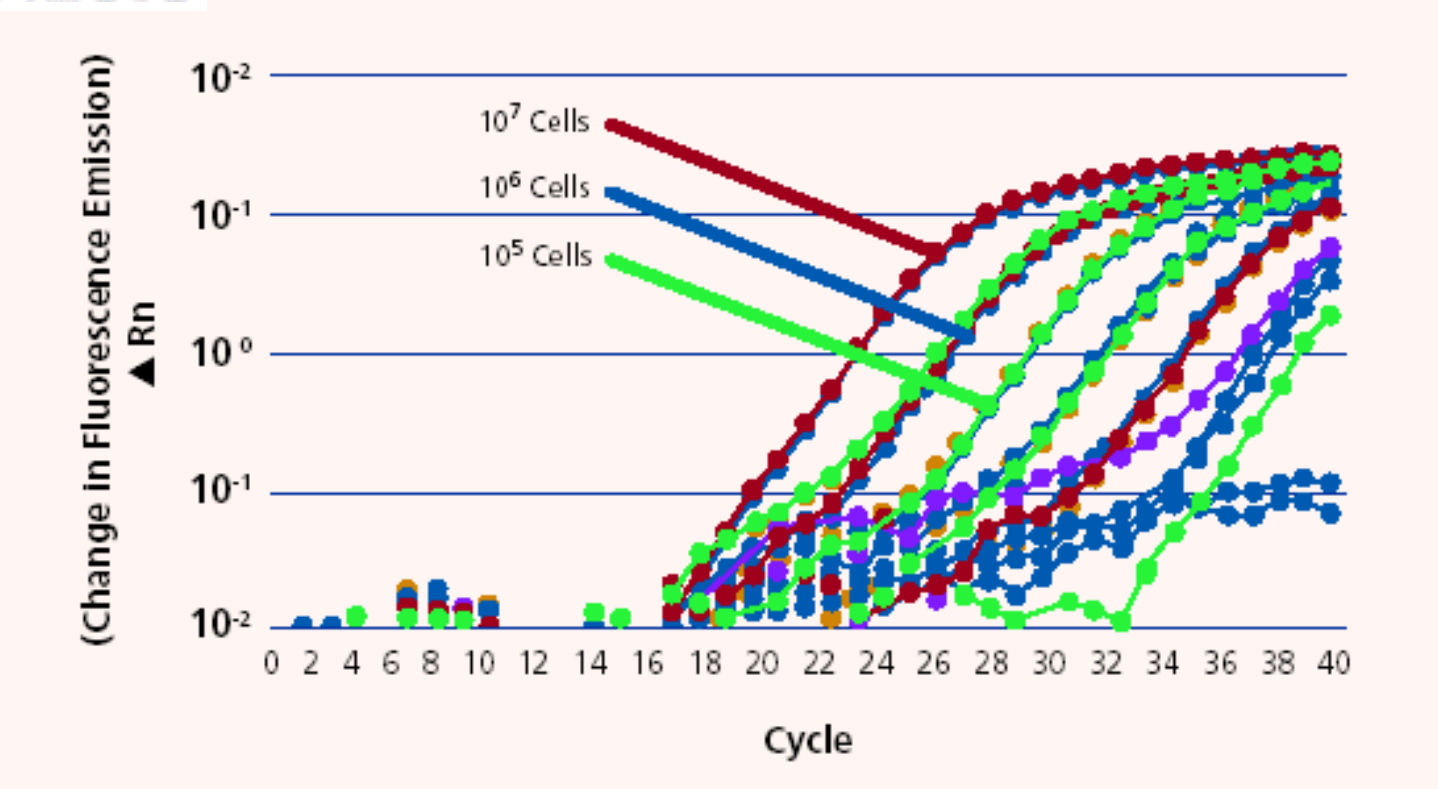
PCE: tetrachloroethene, TCE: trichloroethene, DCE: dichloroethene, VC: vinyl chloride

The bioaugmented test plot clearly indicates complete dechlorination immediately following inoculation, while DCE levels are consistent in the non-bioaugmented control.

Lendvay, J.M. et al. Preventing contaminant discharge to surface waters: plume control with bioaugmentation. Bioaugmentation, Biobarriers and Biogeochemistry, *Proceedings from the Sixth International In Situ and On-Site Bioremediation Symposium.* 19-26 (200

Bio-Dechlor CENSUS℠

- Quantitative detection of *Dehalococcoides* by use of real time PCR
- Allows proper assessment of sites regarding natural biodegradation and helps define the degree of bioaugmentation required



Real-Time PCR Amplification of 10-fold dilutions of genomic DNA derived from *Dehalococcoides*

Controversy (1)

• E.K. Nyer, F. Payne & S. Suthersan (2003) *Ground Water Monitoring & Remediation* 23, 1, 36-45, based on ARCADIS field data on >125 site dechlorination projects:

1• Biostimulation (proper biogeochemical management of native microbial communities) can achieve complete reductive dechlorination

2• Bioaugmentation may shorten the lag phase within a short distance from the injection wells but is not required to assure full in situ dechlorination

3• Because it is not necessary to constrain hydrogen levels for the benefit of inoculated *Dehalococcoides* sp., it is also not necessary to limit rates of e- donor consumption by using "designer" slow release substrates

Controversy (2)

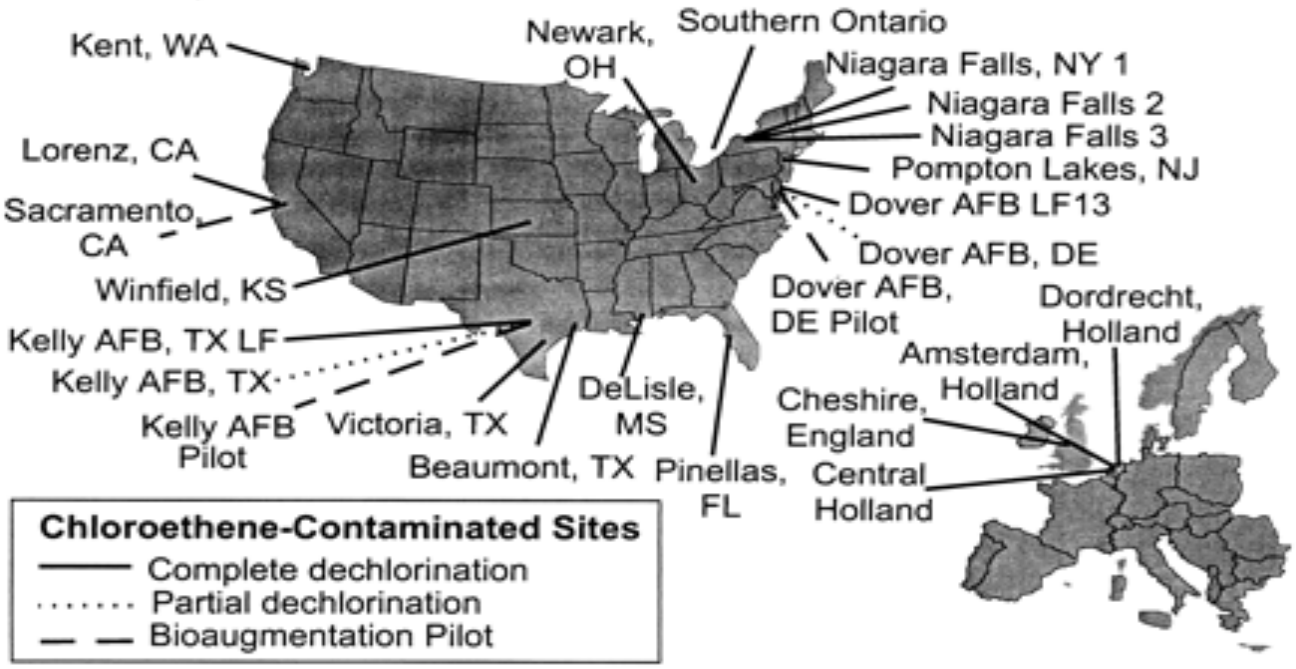
• D. Major, E. Edwards, P. McCarty, J. Gossett, E. Hendrickson, F. Loeffler, S. Zinder, D. Ellis, J. Vidumsky, M. Harkness, G. Klecka, E. Cox (2003) *Ground Water Monitoring & Remediation* 23, 2, 32-48:

1• All mixed cultures dechlorinating PCE or TCE beyond cDCE to ethene contain organisms in the *Dehalococcoides* phylogenetic group

2• Bioaugmentation with *Dehalococcoides* was required to further dechlorinate cDCE to ethene and the introduced organism could migrate through the aquifer in field-scale demonstrations (Dover and Kelly AFB)

3• Microbial consortia are involved in complete dechlorination and in creating conditions for growth of halorespirers, but the latter are not present at every site, hence addition of cultures containing *Dehalococcoides* is a must.

Molecular Analysis of *Dehalococcoides* 16S Ribosomal DNA from Chloroethene-Contaminated Sites throughout North America and Europe



Hendrickson et al. 2002. *Applied and Environmental Microbiology* 68:485-495.

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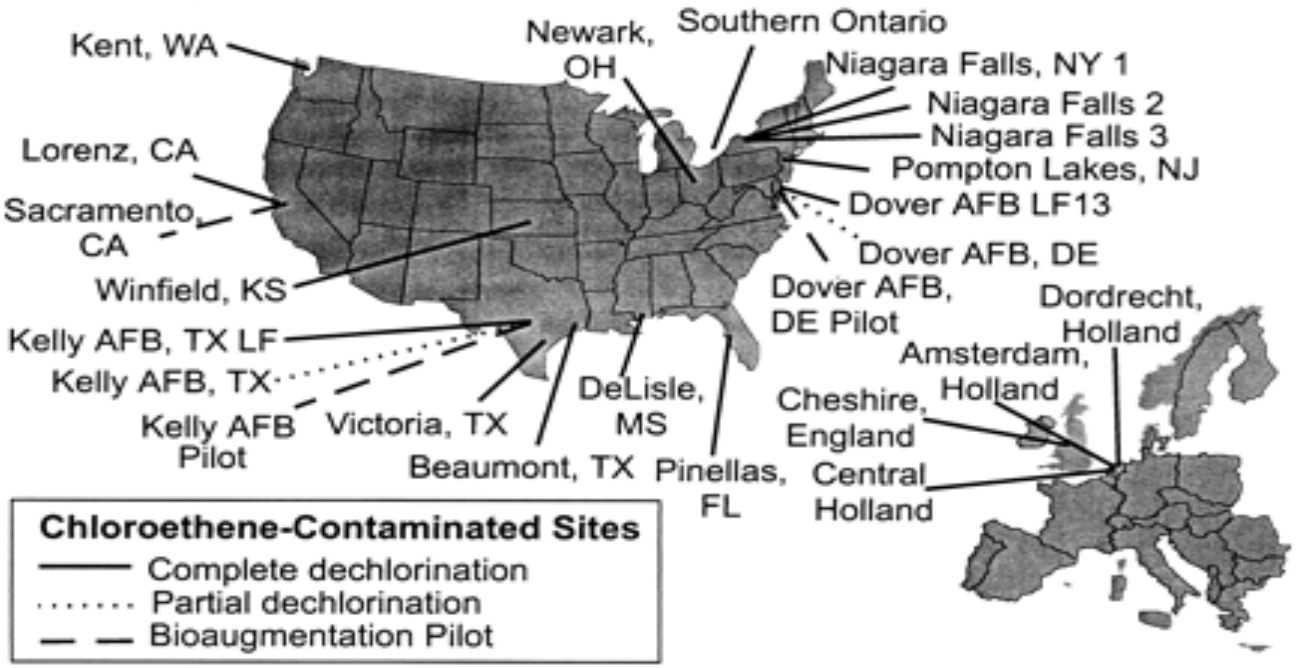
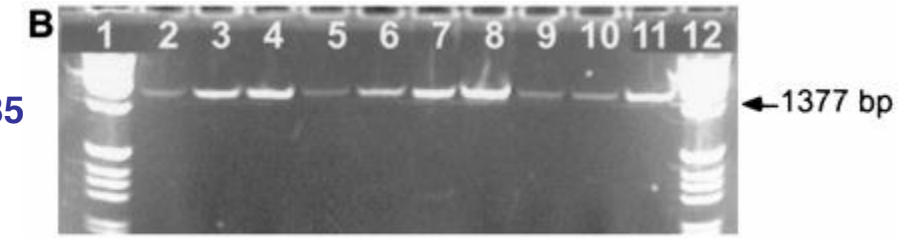
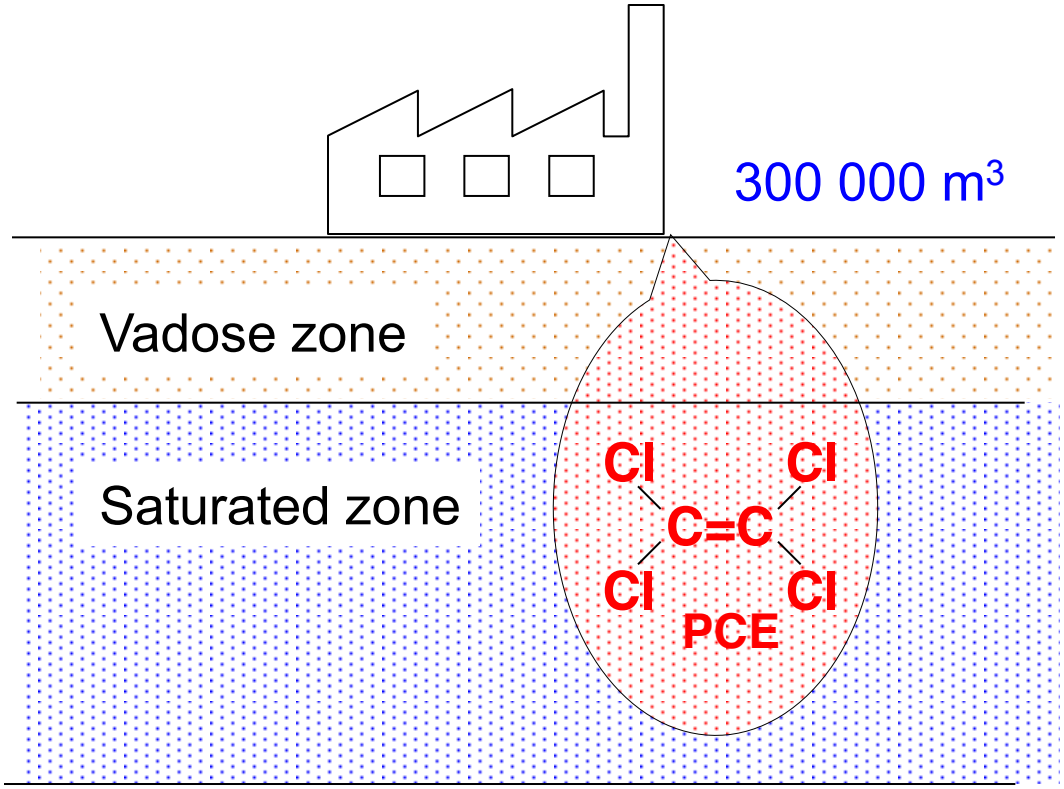


PCR with primers Fp DHC 1 and Rp DHC 1385 targeting 16S rRNA of *Dehalococcoides*



Hendrickson et al. 2002. *Applied and Environmental Microbiology* 68:485-495.

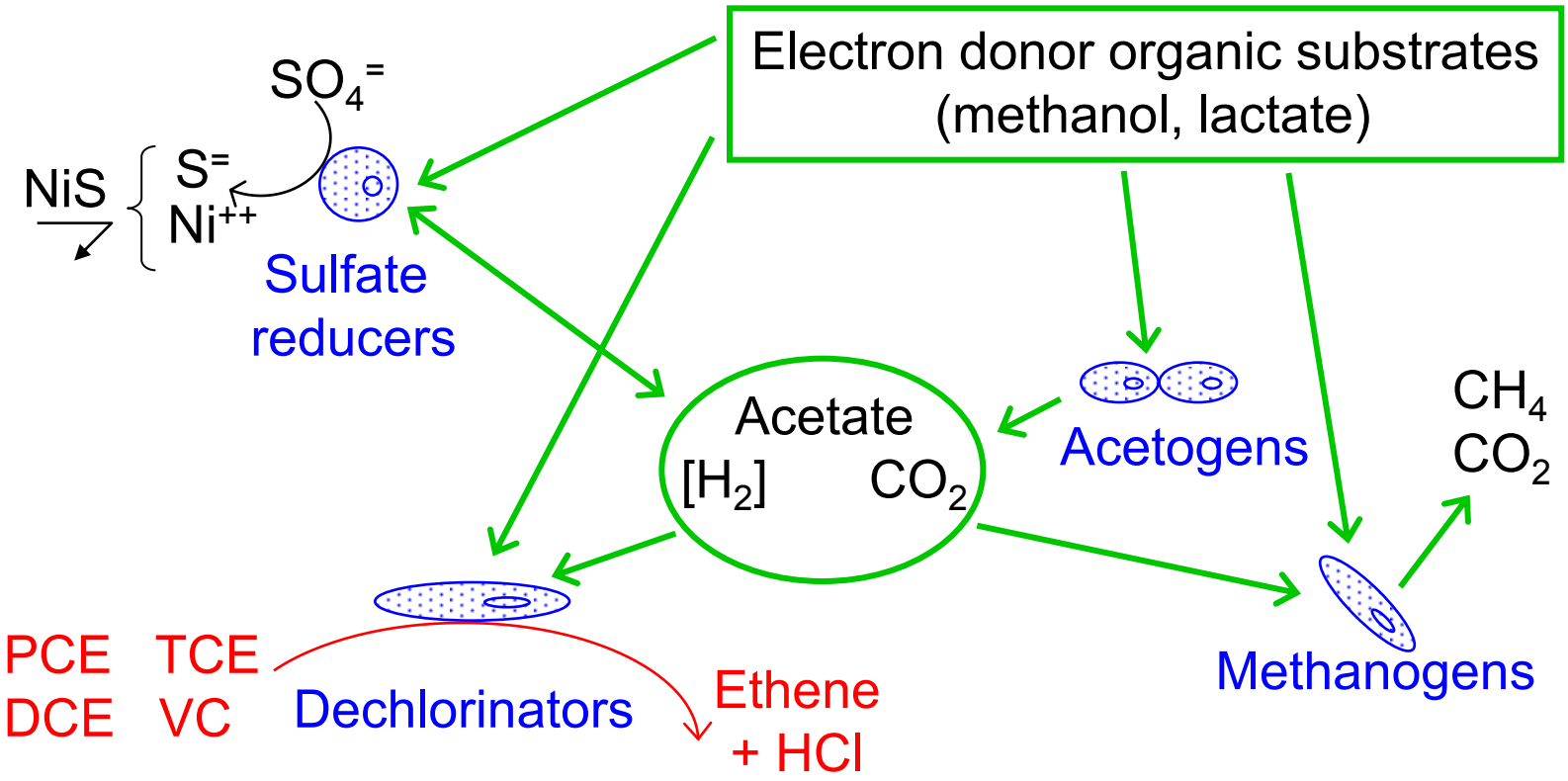
Bioremediation of sediments contaminated by chloro-organic solvents: Indigenous consortia and biogeochemical management or bioaugmentation?



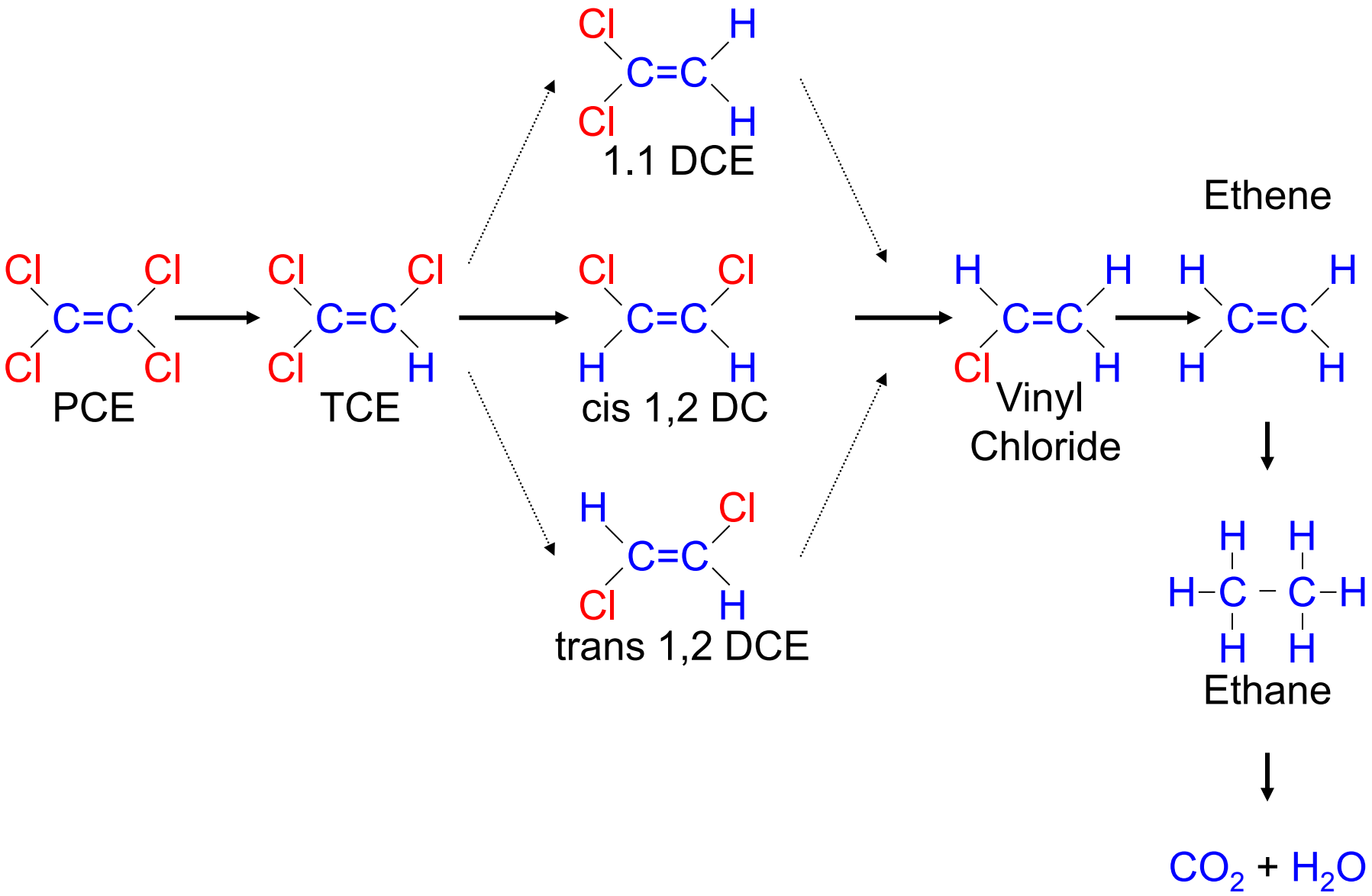
Case story: The former « Dravo » site at Bunnik, The Netherlands

Microbial ecology & physico-chemical environment

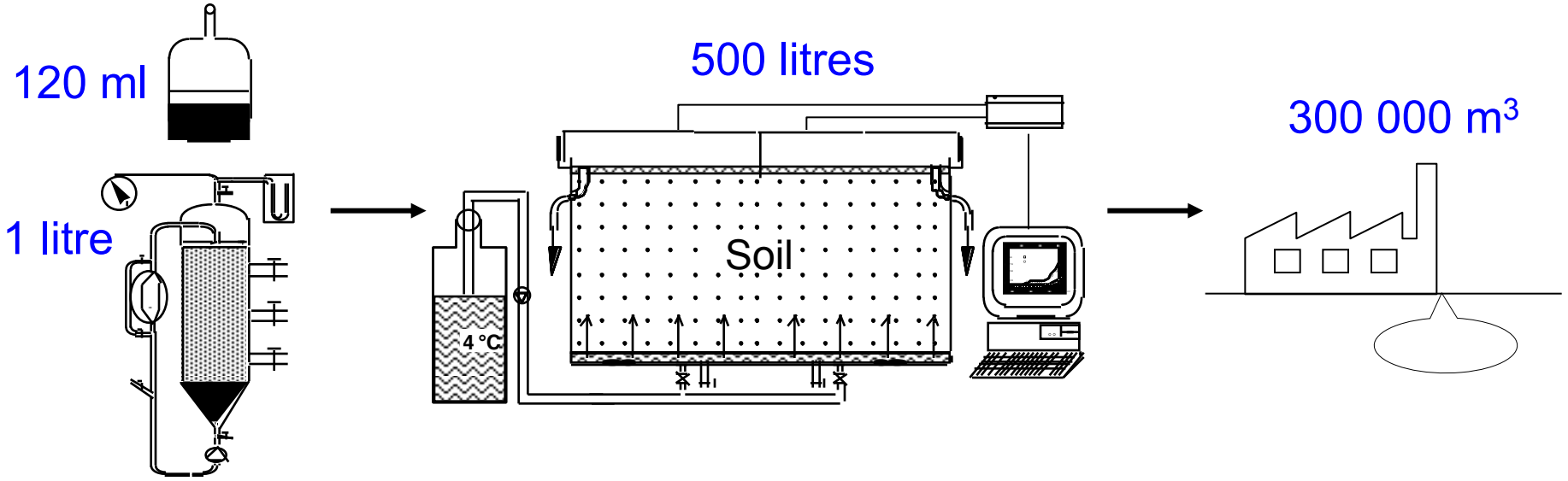
- Consortium of anaerobic microorganisms
- Low redox potential
- Neutral pH
- Cometabolic substrates
- Potential for biostimulation & bioaugmentation



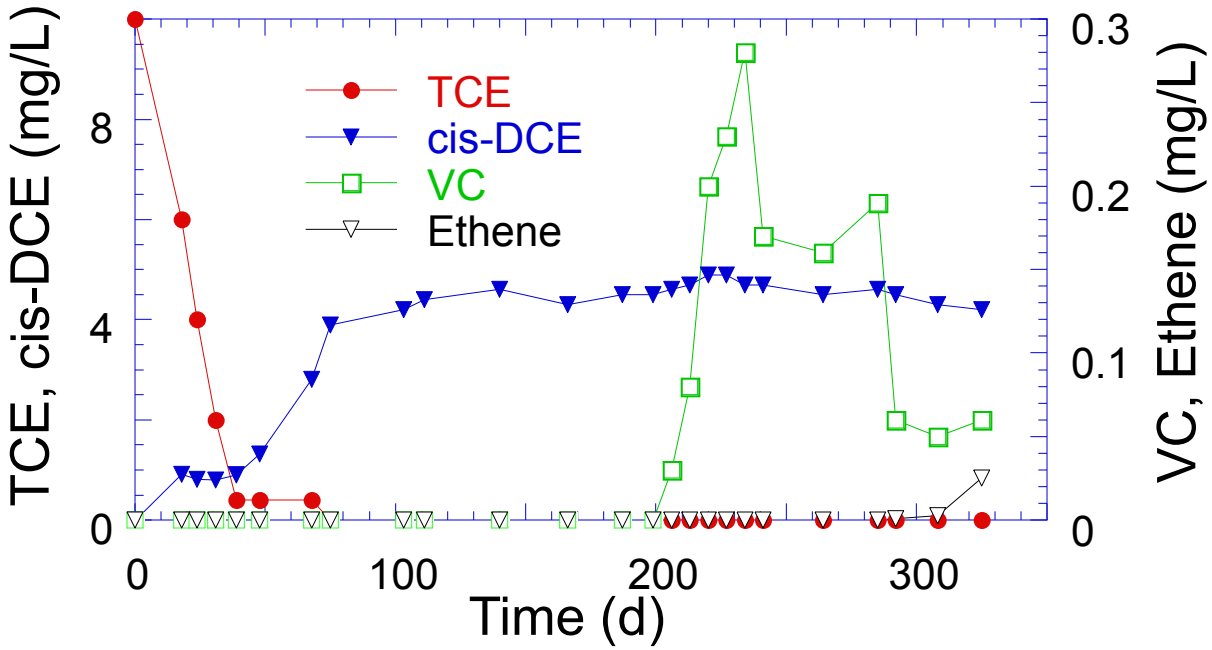
Metabolic pathway of PCE anaerobic dechlorination



Scale-up from the laboratory to the pilot to the field

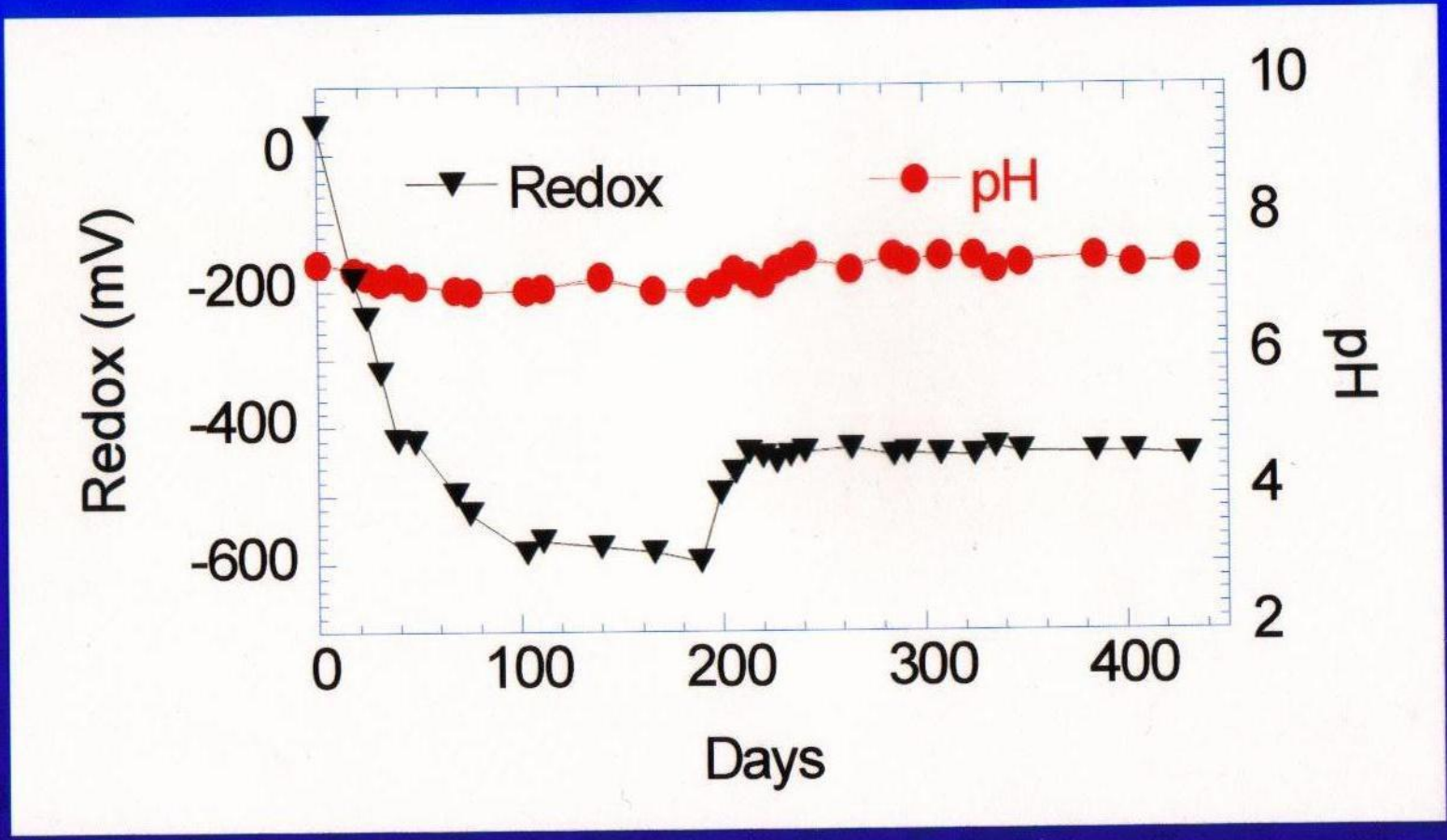


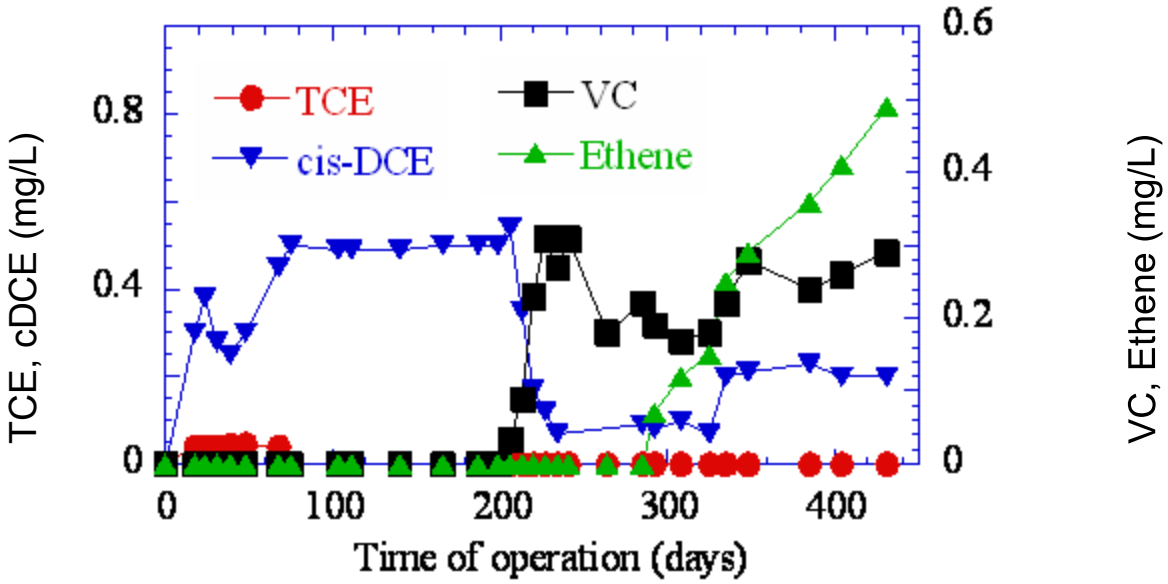
Transformation of chloro-ethenes in the pilot bioreactor

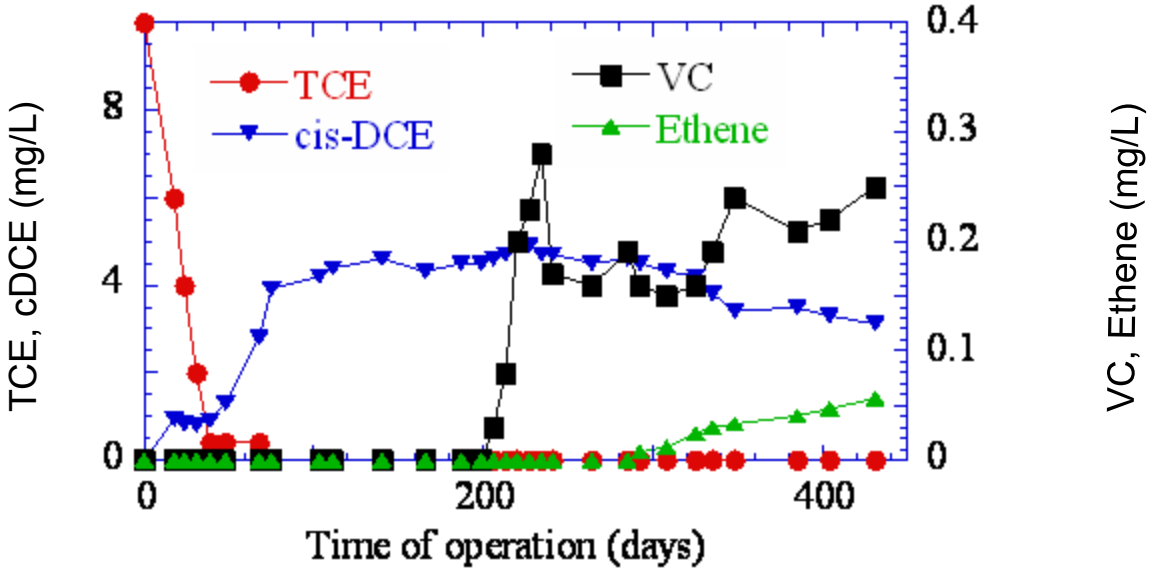


El Mamouni et al (2002)
Wat Sci Technol 45, 49-54

pH and Redox Potential




TCE
cis-DCE
VC
Ethene
TCE, cDCE (mg/L)
VC, Ethene (mg/L)
Time of operation (days)
0
0.4
0.8
0
0.2
0.4
0.6
0
100
200
300
400


TCE
cis-DCE
VC
Ethene
TCE, cDCE (mg/L)
VC, Ethene (mg/L)
Time of operation (days)
0
4
8
0
0.1
0.2
0.3
0.4
0
200
400

Bioaugmentation of Soil with Atrazine Degrading Microbial Communities

Goux et al. AMB (2000); WASP: Focus (2003)

Physico-chemical characteristics of the soils tested

Soils	Type	pH (Water)	% N	% Organic Carbon	C/N
BIO1	Sandy soil, compost & straw (50:25:25)	5.7	0.33	9.59	29.06
BUZ3	Loamy	8.5	0.10	0.88	8.8
BUZ4	Loamy	7.9	0.14	1.22	8.71
LLN1	Loamy	6.1	0.15	1.66	11.07

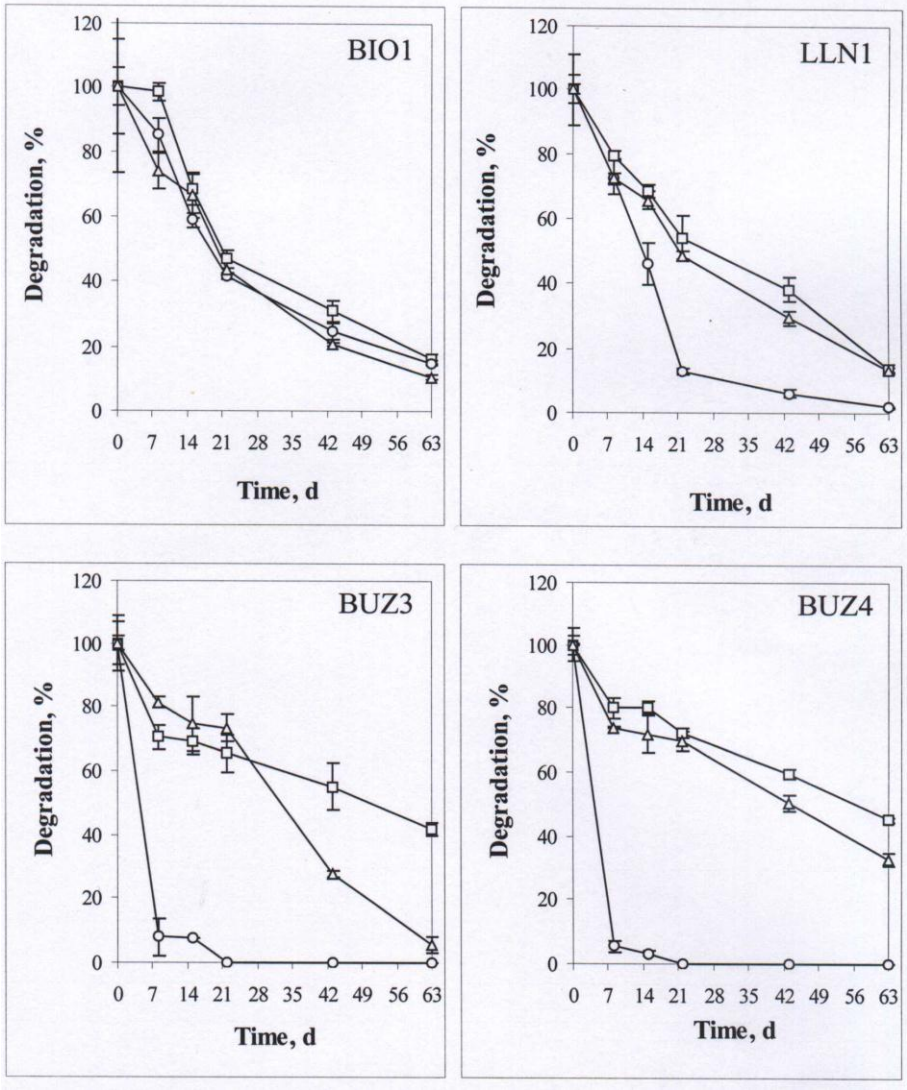


Figure 1. **Degradation of atrazine in the soils immediately after inoculation.**
Squares = non-inoculated soils; circles = soils inoculated with COM1; triangles = soils inoculated with COM15. With the exception of two BIO1 samples (see error bars), 95% confidence intervals were always lower than 5%.

Goux et al. (2003) WASP: Focus 3, 131-142

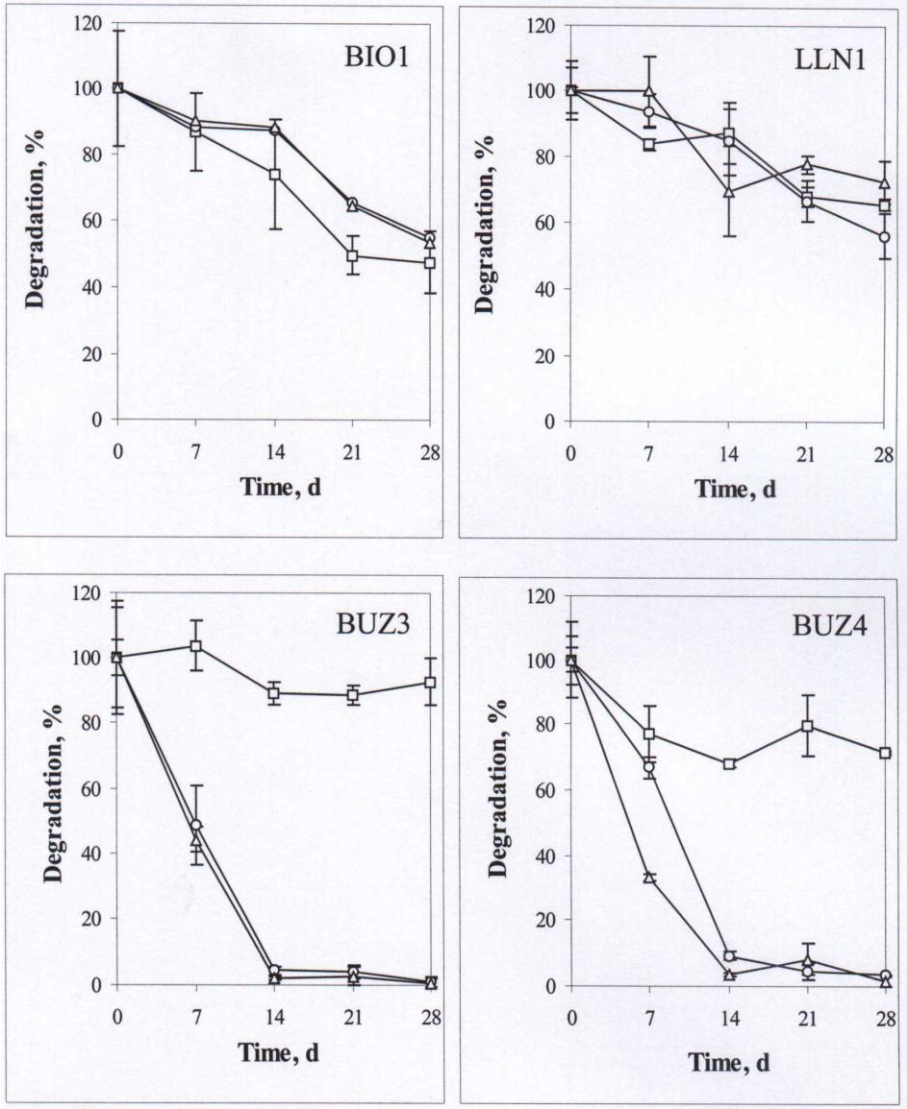


Figure 2. **Degradation of atrazine in the soils nine months after inoculation.** Squares = non-inoculated soils; circles = soils inoculated with COM1; triangles = soils inoculated with COM15. Error bars were 95% confidence intervals.

Goux et al. (2003) WASP: Focus 3, 131-142

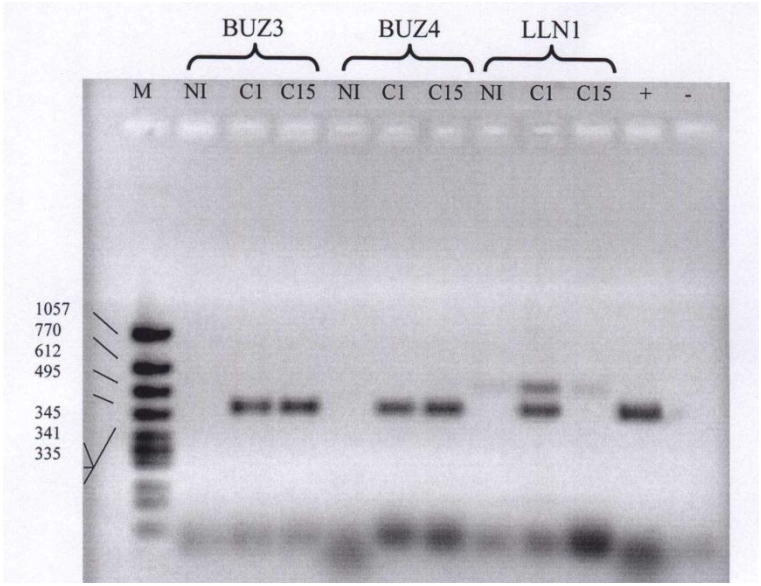


Figure 3. ***atzA* detection in soils nine months after inoculation and 28 days after second atz addition.**
Lanes: (M) size marker, fragment sizes are given in bp; NI = non-inoculated; C1, C15 = inoculated with COM1 or COM15, respectively; (+) positive control; (-) negative control. Expected size of the amplified fragment: 528-bp.

Goux et al. (2003) WASP: Focus 3, 131-142

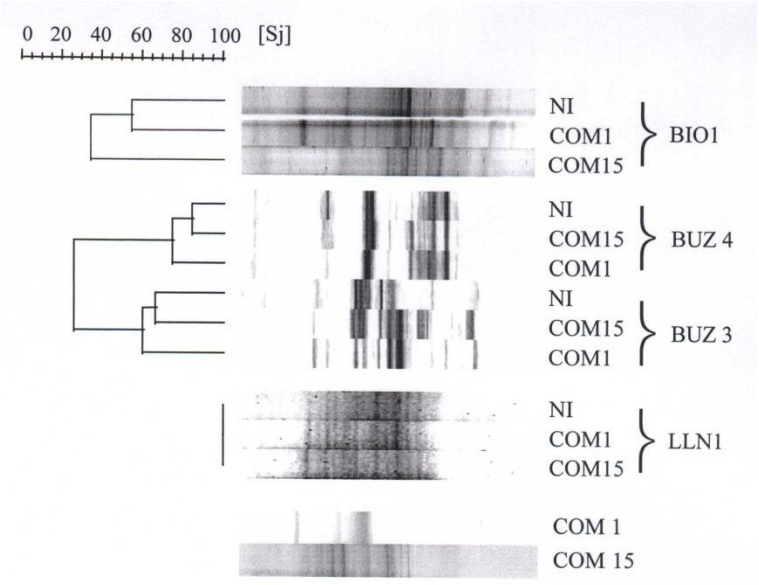


Figure 4. **DGGE analysis of 16S rDNA fragments from the soils inoculated with COM1 and COM15 and from pure cultures of the communities.**
The dendrogram of relatedness is based on the Jaccard coefficient (Sj). NI = Not Inoculated.

Goux et al. (2003) WASP: Focus 3, 131-142

Strategies (1)

- If target pollutant is very recalcitrant, a GEM, pure isolate or defined consortium capable of mineralizing it may gain a strong competitive advantage (ecological selectivity) upon inoculation in situ, provided the local abiotic conditions are favorable

- If a temporal effect is required, e.g., batchwise removal of pollutants aided by physicochemical interventions (landfarming, bioventing, etc.) the introduced strain need not have a long-term survival

- A GEM acting as donor of catabolic genes to unknown autochthonous recipients can enhance the mineralizing capability of the indigenous flora, but care must be taken against uncontrolled HGT (e.g., resistance markers)

- A GEM inoculant constructed using as host a bacterium representing the dominant population in the biotope may be a good candidate for successful survival, growth and activity

Strategies (2)

- Spatially organized habitats (granular sludge, riverbank sludge, biofilms) can promote enhanced HGT from an inoculant to the indigenous microflora
- Introduction of inoculant immobilized in appropriate carrier material or encapsulation matrix for long-term protection and slow release may prove advantageous in situ
- Dual bioaugmentation (e.g., metal-detoxifyer plus organic-degrader) or, more generally, multiple inoculation for co-contaminated sites holds promise
- ✓ **Bottom line: Extensive, stringently validated field-scale studies of these (& other!) bioaugmentation strategies are key to success**

Perspectives

- **Adaptation of "superbugs" into a robust and versatile suite of quasi-universal "heirloom microorganisms" (Singer et al. 2005) for complex polluted sites (*Deinococcus radiodurans*, *Sphingomonas* EPA 505, *Burkholderia xenovorans* LB400, *Pseudomonas* sp. ADP, etc)**

- **Enhancement of the resilience of biodegradative strains by rhizo-directed strain selection (Kuiper et al. 2004)**

- **Use of "activated" soil or sediment with the same characteristics as the target site by "priming" the strain or consortium of interest to site conditions (biostimulation with the pollutant(s), Gentry et al., 2004)**

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