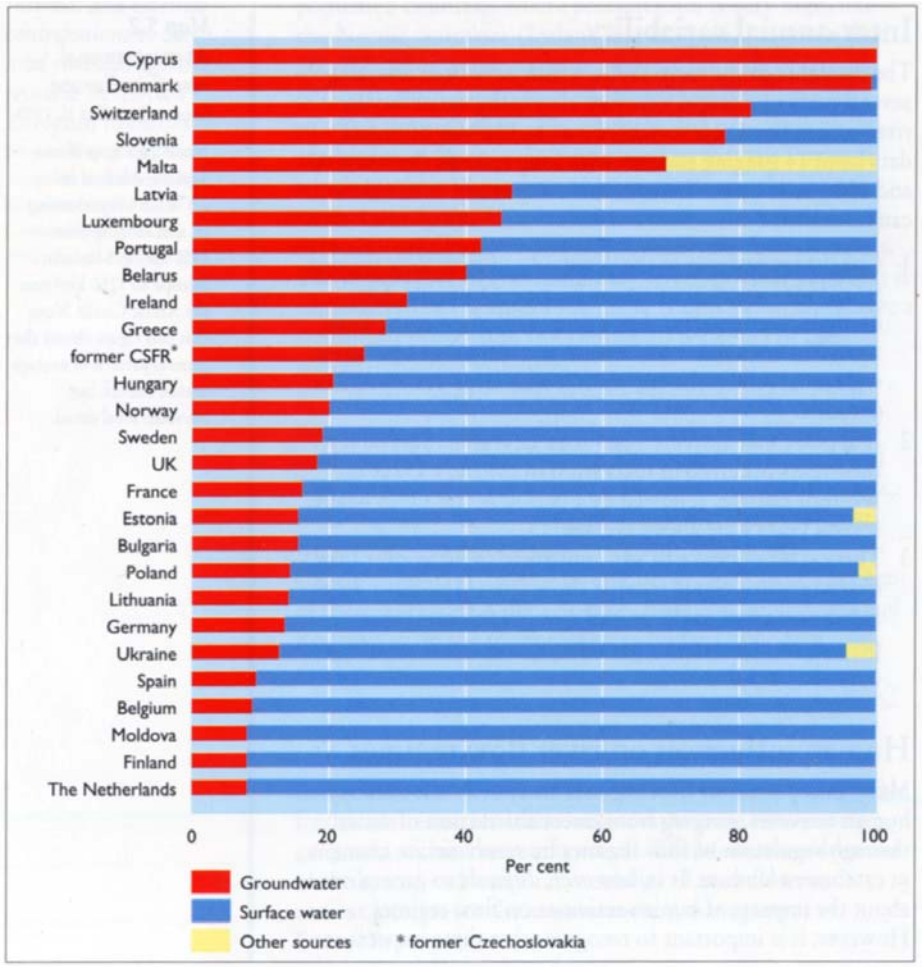
Degradation of 2-methyl-4chlorphenoxyacetic acid (MCPA) in top- and subsoil is quantitatively linked to the class III *tfdA* gene

Carsten Suhr Jacobsen[1, 2 & 3 *] Jacob Bælum[1 & 2]; Trine Henriksen[1], & Hans Christian Bruun Hansen[2]

1) Geological Survey of Denmark and Greenland (GEUS), Department of Geochemistry
2) Royal Veterinary and Agricultural University (KVL), Department of Chemistry
3) Royal Veterinary and Agricultural University (KVL), Department of Ecology



Groundwater as source of drinking water in Europe (red)

GRUNDWATER QUALITY CONTROLE

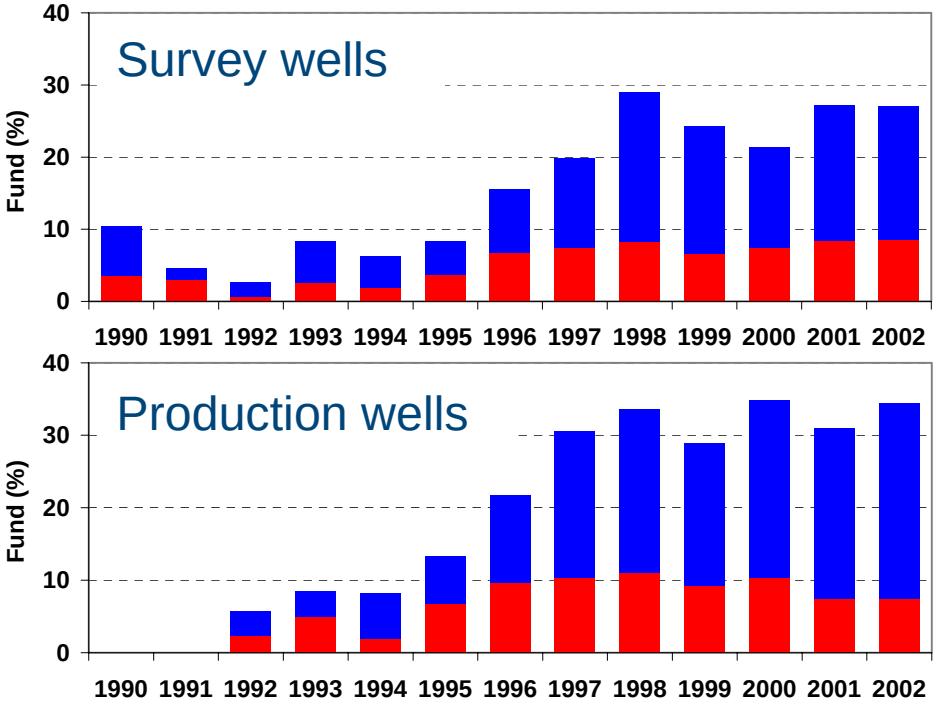
Based on:

- 700 State owned (GEUS) Grumo filters
- 1700 Commercial groundwater abstraction wells
- Pesticide Leaching Assessment Programme (PLAP) (run by GEUS and DJF). See poster B4 by Jeanne Kjaer – and poster B3 by Heid Barlebo for examples.

EU drinking water limit >0,1 µg/l

- Exceeded in 10% of the Grumo-filtres
- Exceeded (since 2000) in decreasing numbers of commercial groundwater abstraction wells

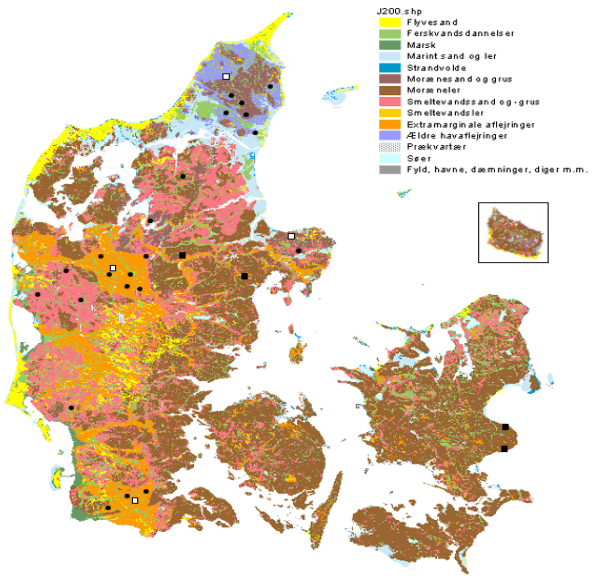
Groundwater contaminated with pesticides



- Red: over the 0.1 ug/l EU-limit
- Blue: detectable but below the 0.1 ug/l EU-limit

Geologic heterogeneity in a small country

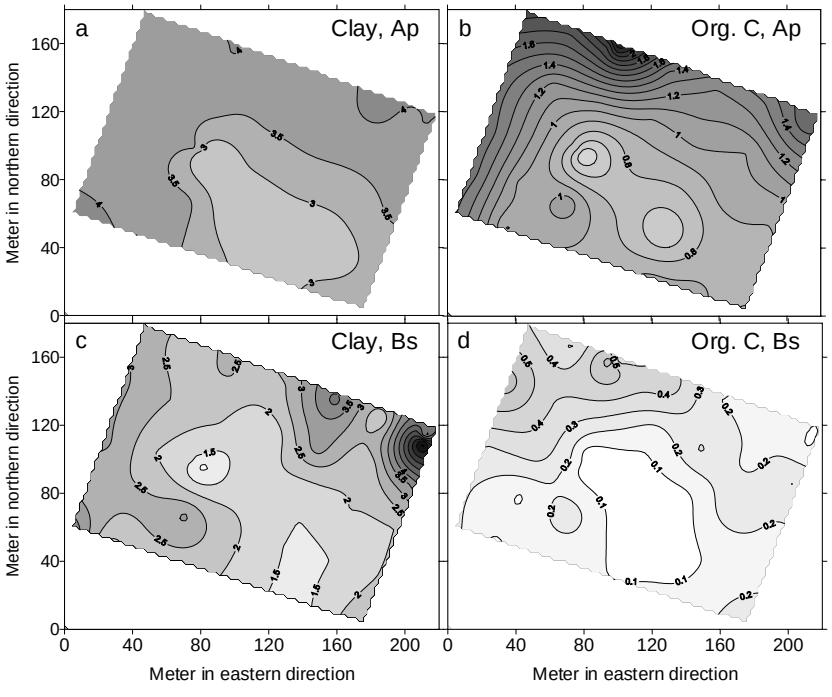
- A large contract with the Danish EPA
- >800 soil samples is described geological and analyzed using geochemical, soil physical, and microbial key-parameters and these are correlated to pesticide sorption and degradation



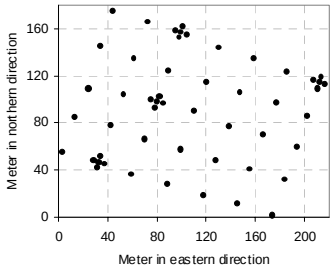
GEUS

MCPA and *tfd*A genes; Warwick, March 2006

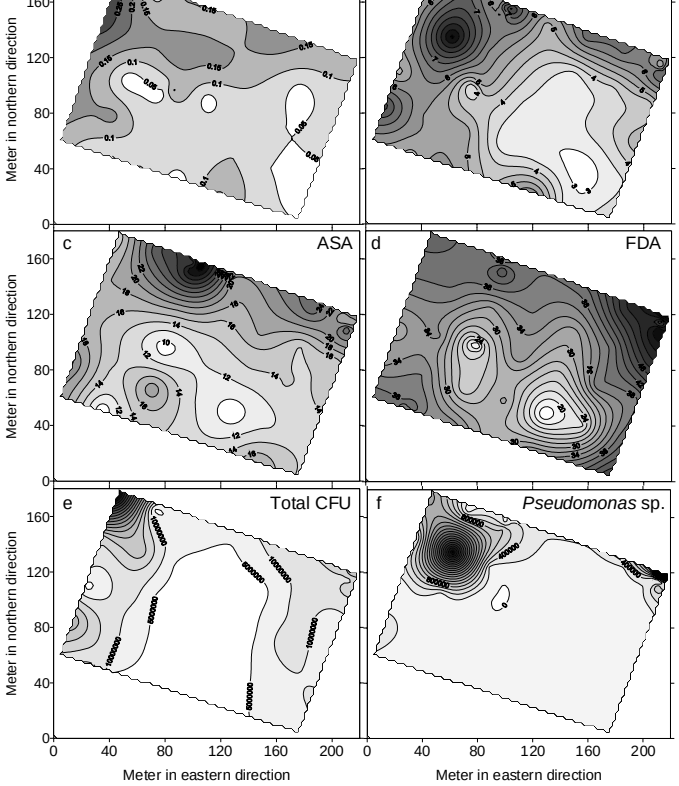
An example on field variability in microbial activity and pesticide mineralisation

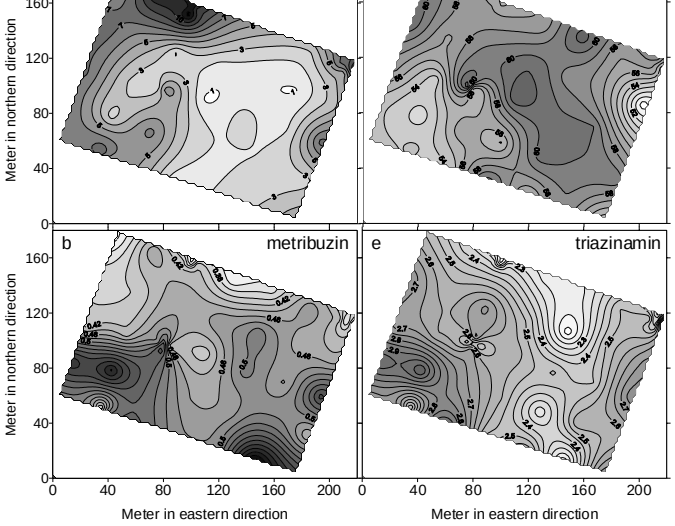


- 50 samples from A horizon and 50 samples from Bs horizon
- Vinther et al in prep.



GEUS





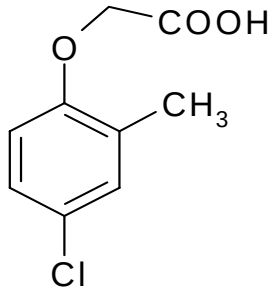
A-horizon samples
Vinther et al in prep
Fredslund et al in prep

- Metribuzine and triazineamine low mineralisation in all samples (higest 3.1% in 90 days)
- Glyphosate mineralisation was found to correlate with microbial activity (SIR and ASA ArylSulfatase Activity) (and with total C).
- MCPA mineralized brilliantly in all A horizon soils with absolutely no correlation to soil parametres of any kind

GEUS

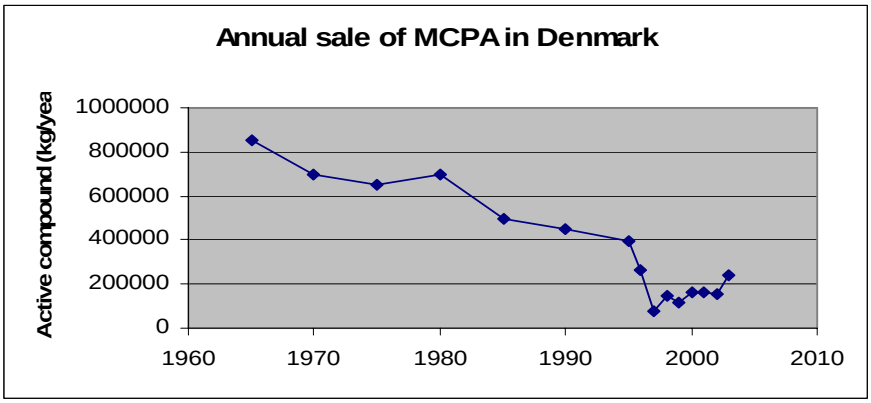
MCPA (a pet herbicide for microbial ecologist)

- Phenoxyacetic acid herbicide (MCPA, 2,4-D og 2,4,5-T)
- Banned (restricted) in Denmark in 1995 but now used in several mixtures against weeds in grasses



4-chloro-2-methylphenoxy aceticacid

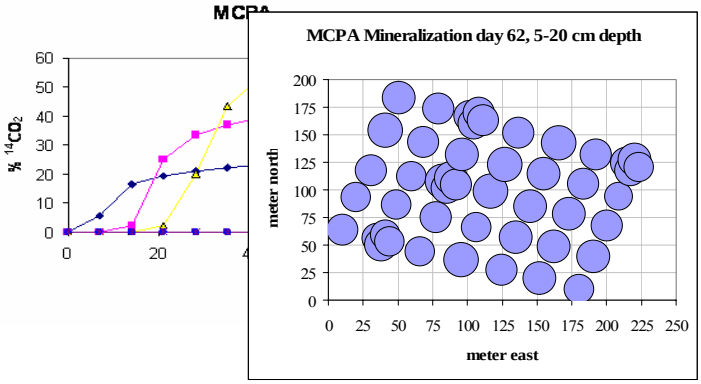
- $C_9H_9ClO_3$
- Mol. weight: 200,6 g/mol
- Log K_{OW}: 2,75 (pH 1)
  0,46 (pH 5)
- pK_a: 3,07

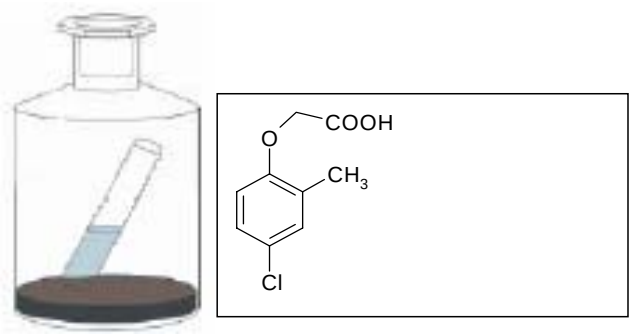


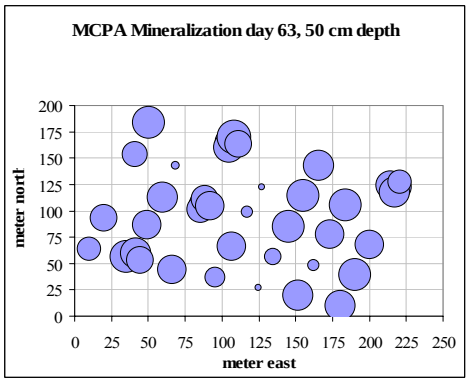
GEUS

Lessons learned from >800 soils in the Danish EPA supported pesticide area zonation project

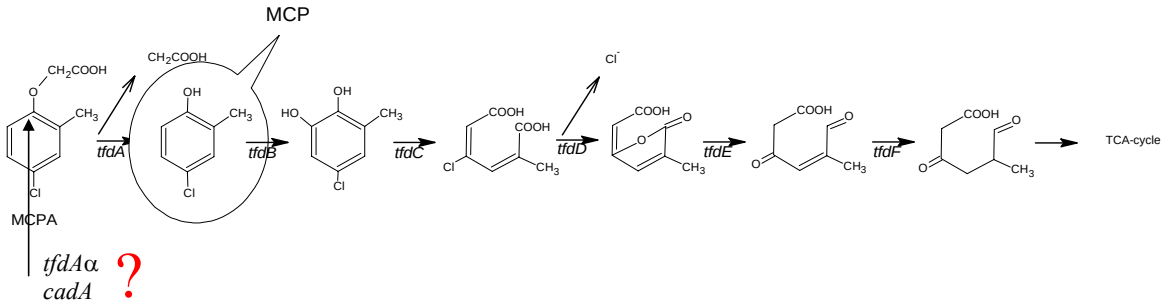
- Higher maximum mineralization of MCPA in subsoils compared to surface soil.
- Metabolite formation in surface soil but not in subsoils ?
- Formation of less bioavailable metabolites?
- Variation in subsoils (does numbers matter ?).







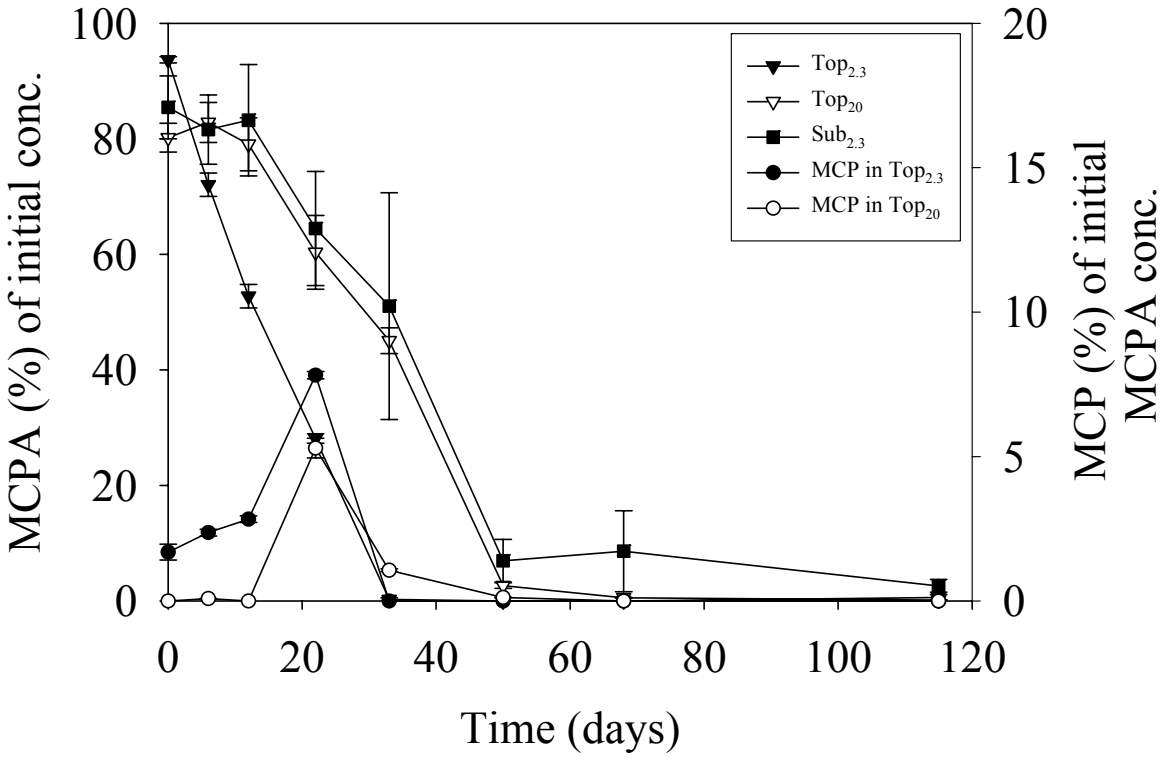
Degradation pathway for MCPA



- The phenol compound MCP is a likely degradation product from MCPA
- Simultaneous and quantitative extraction and detection of MCP and MCPA from soils needed new methods

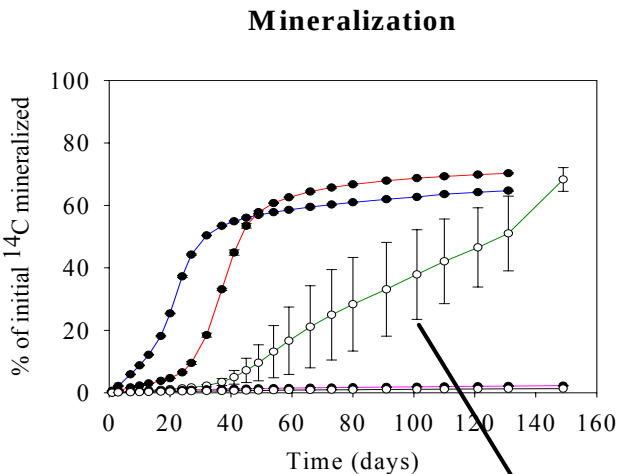
Extraction and quantification of MCPA and MCP in soil: Extraction with "Accelerated solvent extraction" (ASE) (MeOH:H2O 50:50, temp. 50 oC, at 1200 psi) quantification using LC-MS/MS (100×2.1 mm RP18)

Dissipation of MCPA and formation and dissipation of MPC

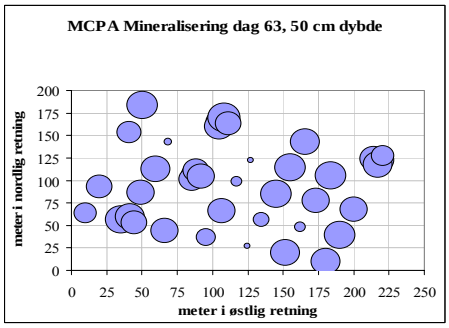


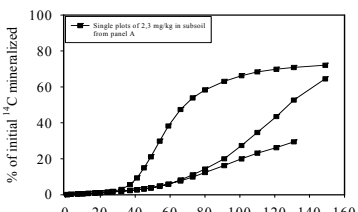
- Semi-quantitative
- Not corrected for extraction efficiency
- Low extraction efficiency

Mineralization of MCPA



2.3 mg/kg MCPA in the topsoil
20 mg/kg MCPA in the topsoil
200 mg/kg MCPA in the topsoil
2.3 mg/kg MCPA in the subsoil
200 mg/kg MCPA in the subsoil





X

Linking microbial degradation activity with analysis of degrader populations

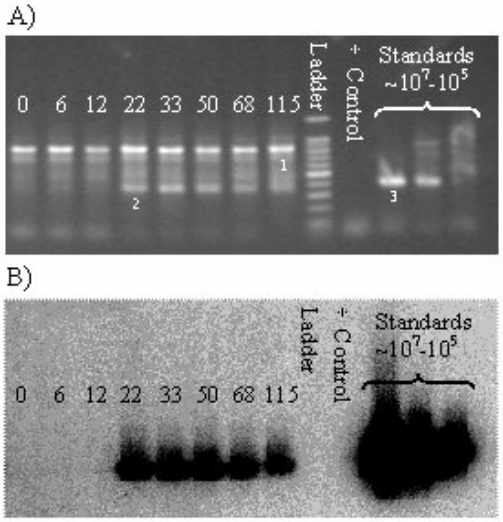
- Microbial degradation activity/kinetics are analyzed using nonlinear regression analysis.
- Brunners & Foghts (1984; Appl. Environ. Microbiol. **47**:167-172) 3/2 models indicates logarithmic growth of degrader populations as validated by F-test analysis of Chi^2 values (Robinson 1985; Adv. Microb. Ecol. **8**:61-114).
- The role of different gene families of *tfd*A and alike genes was investigated.

Primer selection and validation

Primer set	Model genes	Primer (5'-3') forward (f) / reverse (r)	Optimal annealing temperature Used (°C) ***	Note
*tfdA**	*R. eutropha* JMP134/ *Burkholderia* sp.RASC	f: AACGCAGCGRTTRTCCCA* r: ACGGAGTTCTGYGAYATG*	58	Unspecific binding-qPCR not possible
tfdA-intern	*R. eutropha* JMP134	f: GCATACGACGCGCTGCCTCG r: CTTCGGCCACCGGAAGGCCT	65	Used for probe preperation to southern blot hybridization
*tfdA***	Sequence alignment of 23 known tfdA-genes	f: GAGCACTACGCRCTGAAYTCCCG*, ** r: GTCGCGTGCTCGAGAAG	64	One band on agarose gel (app. 220 bp.) - qPCR possible
*tfdAα**	Alignment of RD5-C2, HWK12 and HW13 (tfdAα-genes)	f: CCGGCGTCGATCTGCGCAAG r: GTTGACGACGCGCGCCGA	N.D. ****	No clear band on the agarose gel-fluffy PCR products.
*tfdAα***	Alignment of HW13, HWK12 and BTH (tfdAα-genes)	f: ACSGAGTTCKSCGACATGCG* r: GCGGTTGTCCCACATCAC	65	One band (app. 350 bp)-qPCR maybe possible
cadA	HW13 (cadA-gene)	f: AAGCTGCARTTTGARAAY* r: MGGATTGAAGAAATCCTGRTA*	58	No PCR product

* R=(G/A), Y=(T/C), S=(G/C), K=(G/T) and M=(A/C).
** GC clamp added to the 5' end of the primer for the PCR/DGGE, 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCG
*** The optimal annealing temperature is based on gradient ramping (58-69 °C**).**
**** N.D no optimal annealing temperature could be determined.

Primer selection and validation



- **Soil DNA from days 0, 6, 12, 33, 50, 68 and 115 of topsoil amended with 20 ppm MCPA were used as template (panel A left)**
- ***R. eutropha* JMP 134 inoculated into the soil in concentrations of 8×106, 8×105 and 8×104 cells/g soil (panel A right)**
- **Panel B shows Southern hybridization analysis of the agarose gel shown in panel A) using a *tfdA* probe. Similarity can be detected for band no. 2 and 3.**
- **Results from the use of Vallaeys, T. et al. 1996. FEMS Microbiol. Ecol. 20:163-172. primers against *tfd*A genes**

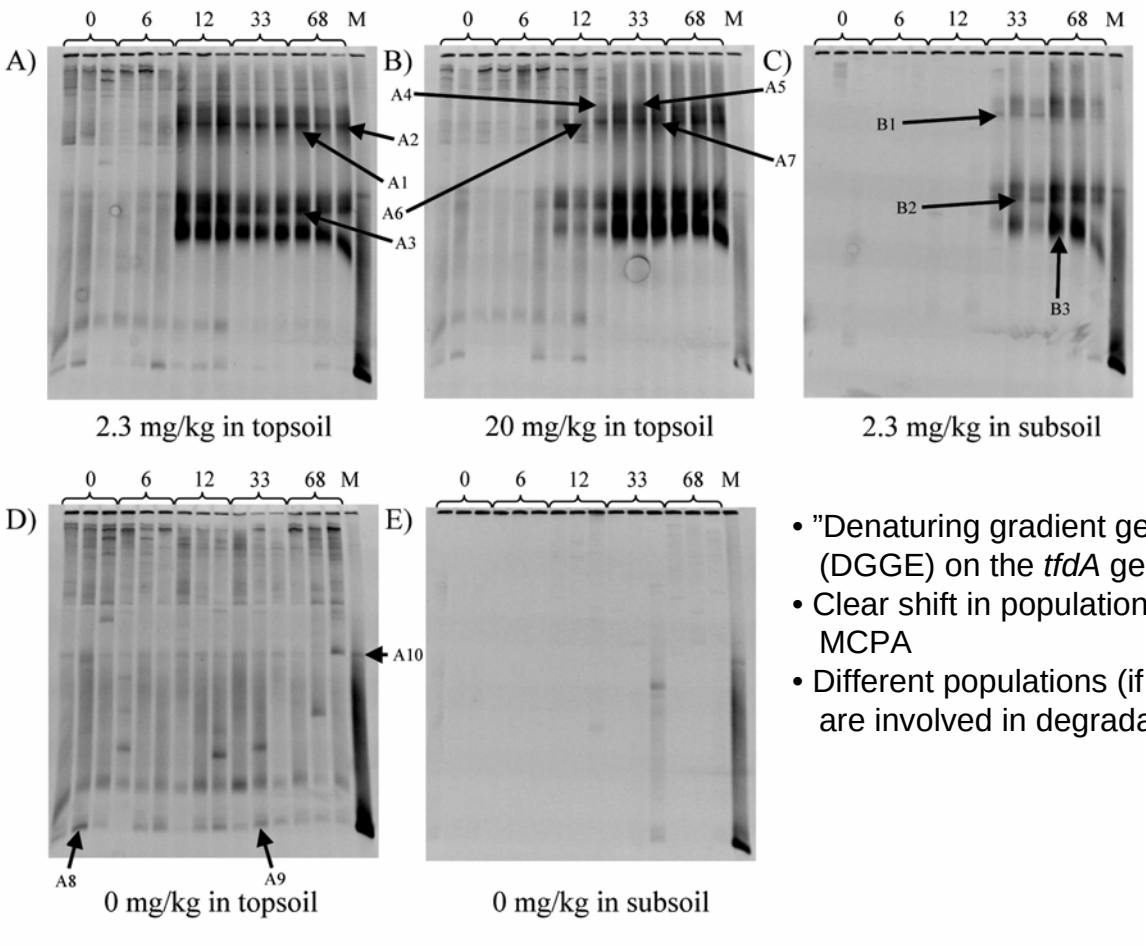
GEUS

New primers where developed

- A BLAST search in 2004 revealed a large group of new *tfd*A genes not covered by the old consensus primers
- Forward primer: GAGCACTACGCRCTGAAYTCCCG
- Reverse primer: GTCGCGTGCTCGAGAAG
- DGGE using GC clamp

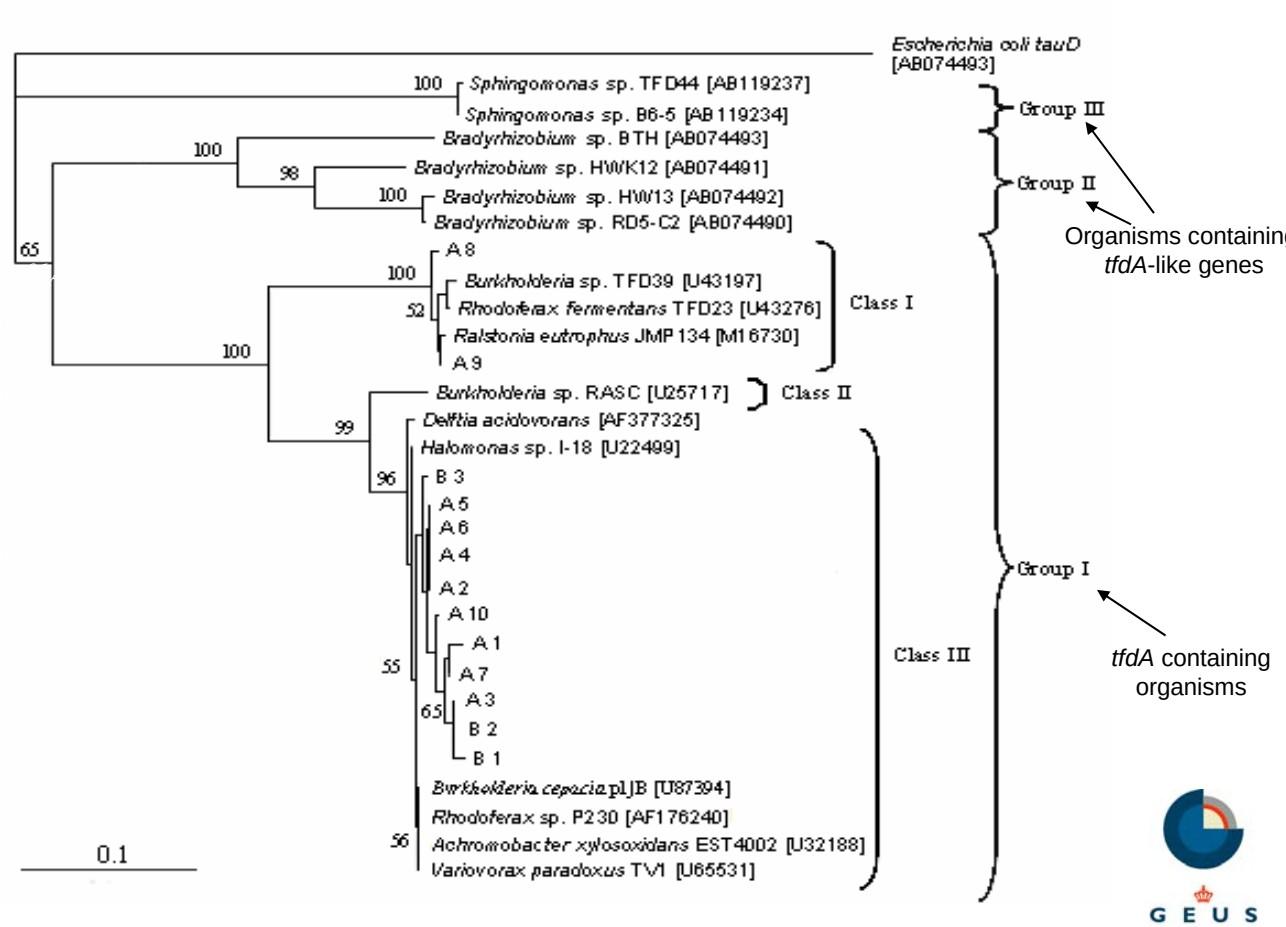
GEUS

DGGE on tfdA genes



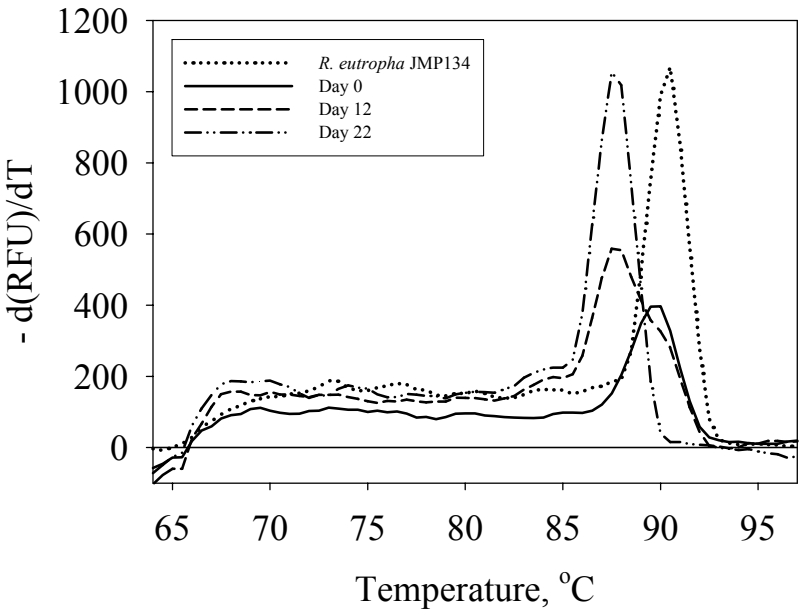
- "Denaturing gradient gel electrophoresis" (DGGE) on the *tfdA* gene
- Clear shift in populations in soils with MCPA
- Different populations (if DGGE are trusted) are involved in degradation.

GEUS



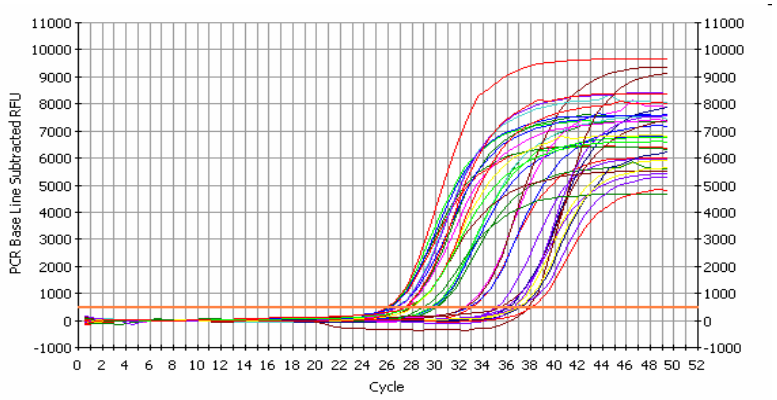
GEUS

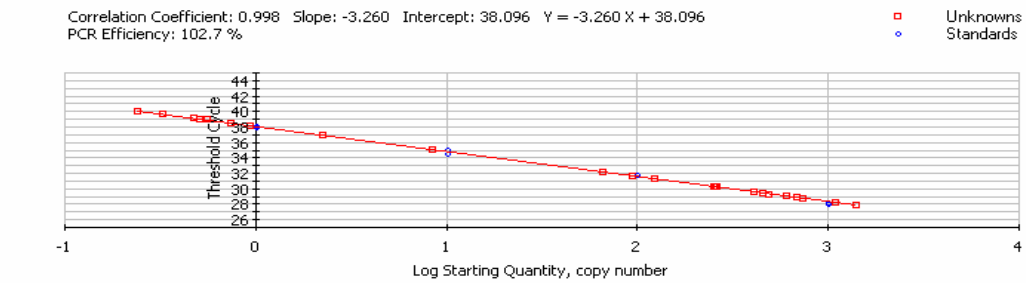
Melting profiles used in analysis of changes in functional diversity of *tfd*A genes



- **Days 0, 12 and 22 in topsoil amended with 20 ppm MCPA using the *tfdA*-II primer set.**
- **Compare to dotted line *R. eutrophus* JMP 134 (pJP4).**

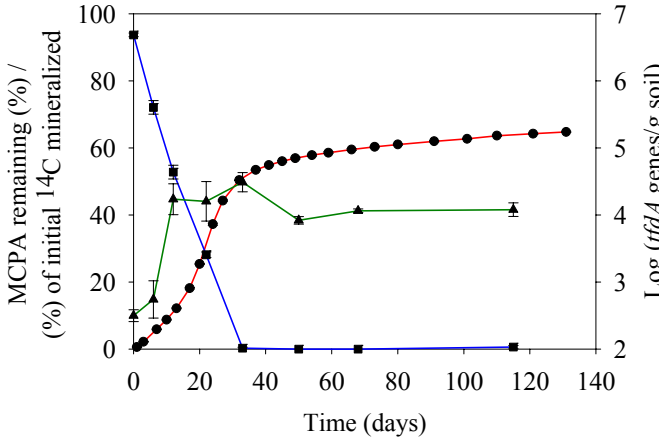
Quantitative PCR on *tfd*A genes in soil



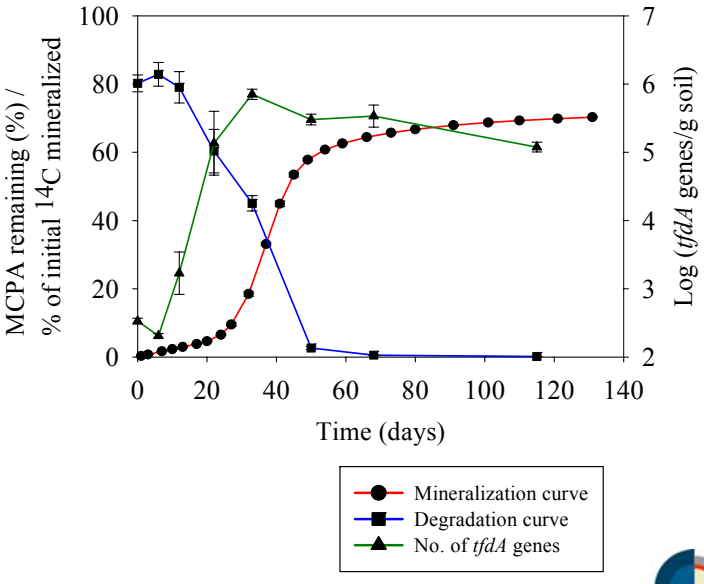


Linking function and *tfd*A gene quantification in top soils

2.3 mg/kg in topsoil



20 mg/kg in topsoil

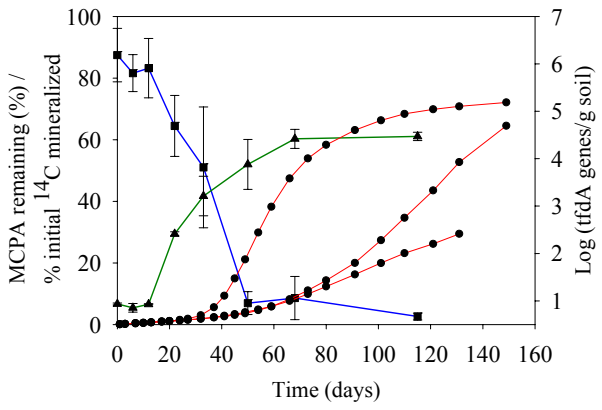


GEUS

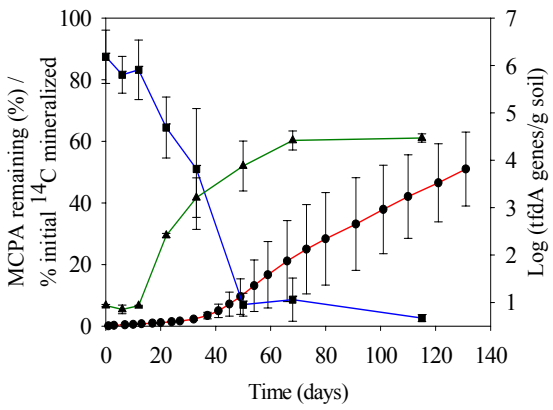
MCPA and *tfd*A genes; Warwick, March 2006

Linking function and *tfd*A gene quantification in B horizon soils

2.3 mg/kg in subsoil

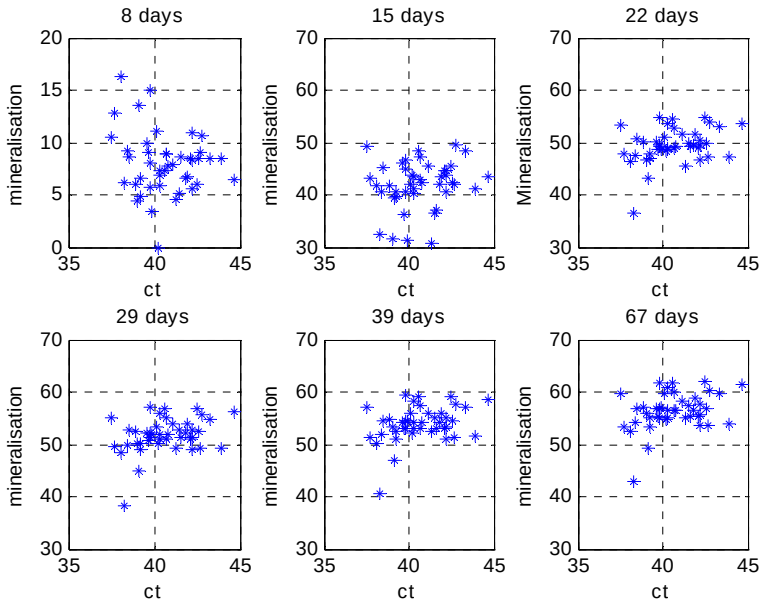


2.3 mg/kg in subsoil



GEUS

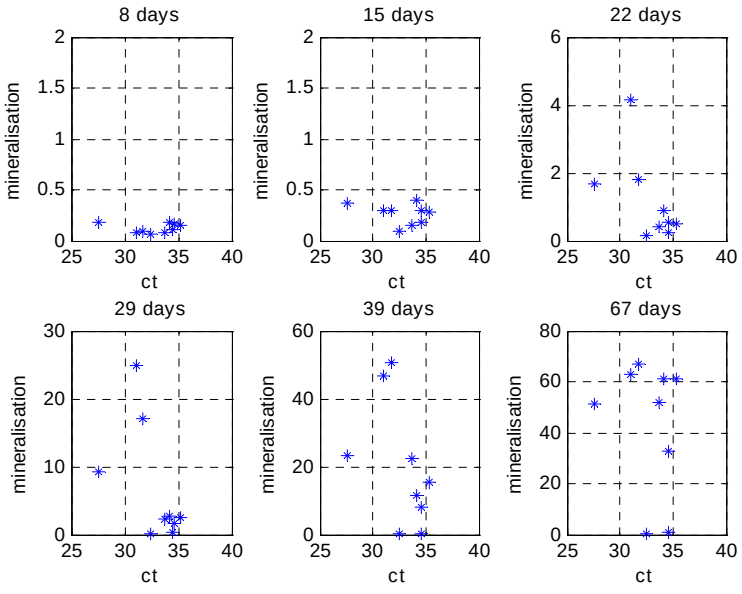
*tfd*A gene analysis of 50 field variation samples plotted against MCPA mineralisation



- High *tfd*A gene copy number (low Ct) is coupled to fast onset of MCPA mineralisation.
- But after 2 weeks the picture disappear.

Fredslund et al in prep.

Bs horizon samples



- Fever soils produced Ct values
- High gene number is associated with faster onset for mineralization.
- But some microbes never wake up ☺

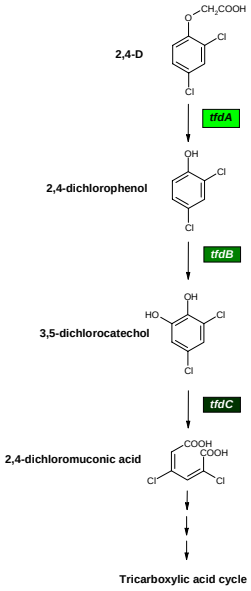
Conclusions

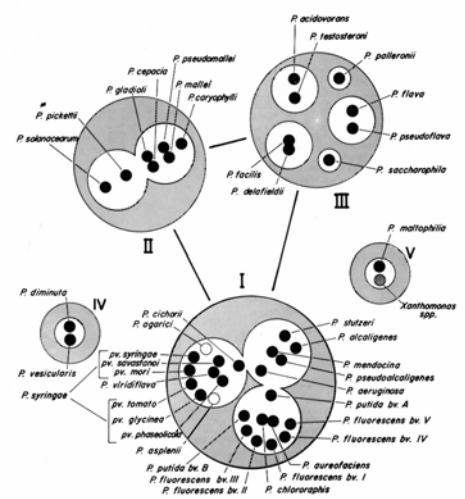
- Fieldvariation of some pesticides can not be explained by soil microbial activity.
- Analysis of MCPA mineralization kinetics revealed growth dependent microbial mineralization in top-soil as well as B-horizon soil.
- New PCR-primers shows sensitive and highly reproducible quantitative results linked to degradation.
- Functional diversity analysis of *tfd*A genes shows population shifts during degradation. The detected *tfd*A-I populations present in the top-soil from the beginning of the experiment where not associated with growing microbial populations. The growing populations where associated with tfdA-III class genes.
- LC- MS/MS analysis revealed formation of MCP in top-soil but not in B-horizon soil. MCP formations is likely associated with *tfd*A-I class genes.
- *tfd*A gene quantification can predict the speed of degradation but the growth kinetics of MCPA is really fast

Acknowledgments

- Mette Andersen for skilled technical support on Real Time PCR.
- Line Fredslund Nielsen for work with the *tfd*A gene in relation to fieldvalidation.
- Finn Vinther, Lars Elsgaard and Ulla Brinch for analysis of soil microbial activity and pesticide mineralization.
- Kaare Johnsen and Mette Nicolaisen for help with phylogeny of tfdA sequences data.
- René Juhler for discussions on MCP and MCPA quantification.
- The Danish research Council (STVF) for financial support through the “SOUND” research center.
- The Danish EPA for founding the Danish pesticide zonation project.

Thank you for your attention !





GEUS