Designing diuron-mineralising consortia by combining the cooperative degradation capacities of different soil bacteria

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Outline of presentation

- Introduction to diuron and associated environmental problems
- Diuron mineralisation by a constructed bacterial co-culture
- Screening known phenylurea-degrading soil bacteria for diuron degradation
- Comparison of diuron-mineralising bacterial co-cultures employing different degradation pathways
- Conclusions

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Diuron – a broadly used phenylurea herbicide

- Agricultural herbicide used in production of fruits, olives, cotton etc

- Total herbicide used in urban and industrial areas and along railways

- Active component in antifouling boat paints

- Algaecide in fountains, artificial ponds and aquaculture

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Unfortunately also a compound detected broadly in the environment

- Rivers, lakes and groundwater
  (Erikson *et al*. 2007; Thurman *et al*. 2000; Lapworth and Goody, 2006; Green and Young, 2006)

- Marine sediments and sea waters
  (Thomas *et al*. 2002)

- Rain in urban and rural areas
  (Scheyer *et al*. 2007)

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One example of the (*possible*) environmental effect of diuron contamination

"Herbicides, particularly diuron, have been correlated with severe and widespread dieback of dominant mangrove species in NE Australia"

Duke *et al*. Marine Poll. Bull. 2005

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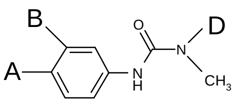
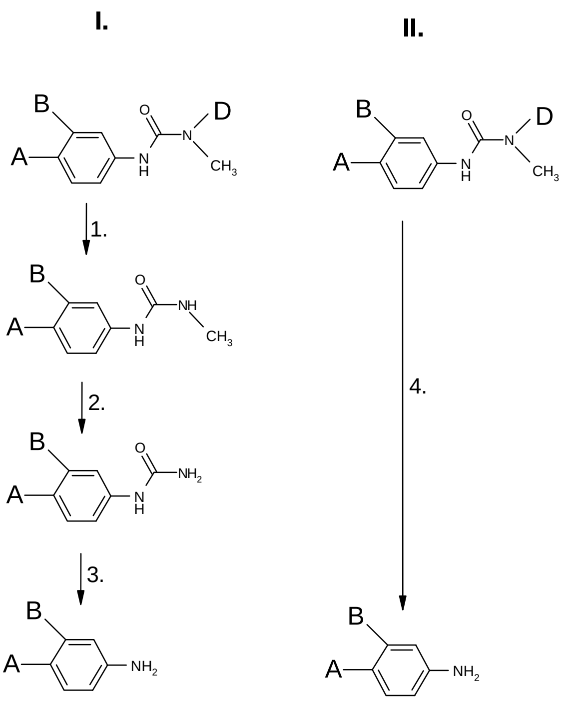
General degradation pathways for phenylurea herbicides



	A	B	D
Isoproturon [N-(4-isopropylphenyl)-N',N'-dimethylurea]	H_3C, H_3C	H	CH_3
Diuron [N-(3,4-dichlorophenyl)-N',N'-dimethylurea]	Cl	Cl	CH_3
Monuron [N-(4-chlorophenyl)-N',N'-dimethylurea]	Cl	H	CH_3
Chlorotoluron [N-(3-chloro-4-methylphenyl)-N',N'-dimethylurea]	CH_3	Cl	CH_3
Fenuron [3-phenyl-N',N'-dimethylurea]	H	H	CH_3
Fluometuron [N-(3-trifluoromethylphenyl)-N',N'-dimethylurea]	H	CF_3	CH_3
Metobromuron [N-(4-bromophenyl)-N'-methoxy-N'-methylurea]	Br	H	OCH_3
Chlorobromuron [N-(4-bromo-3-chlorophenyl)-N'-methoxy-N'-methylurea]	Br	Cl	OCH_3
Linuron [N-(3,4-dichlorophenyl)-N'-methoxy-N'-methylurea]	Cl	Cl	OCH_3



Sørensen *et al*. FEMS Microbiol. Ecol. 2003

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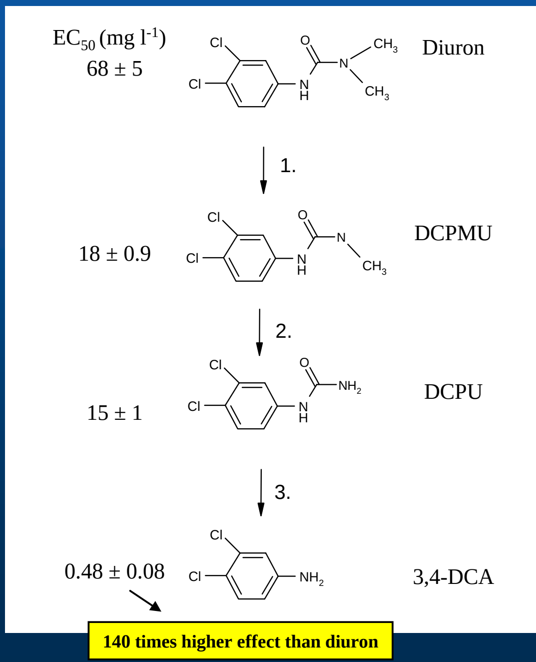
Partial diuron degradation may create metabolites more harmful to non-target organisms than diuron itself

Bioluminescent marine bacterium (*Vibrio fischeri*) as the test organism. Reduction in intensity of light emitted is measured along with standard solutions and control samples. The change in light output and concentration of the herbicide produce a dose / response relationship. The results are normalised and the EC_{50} (concentration producing a 50% reduction in light) is calculated.

Vibrio fischeri

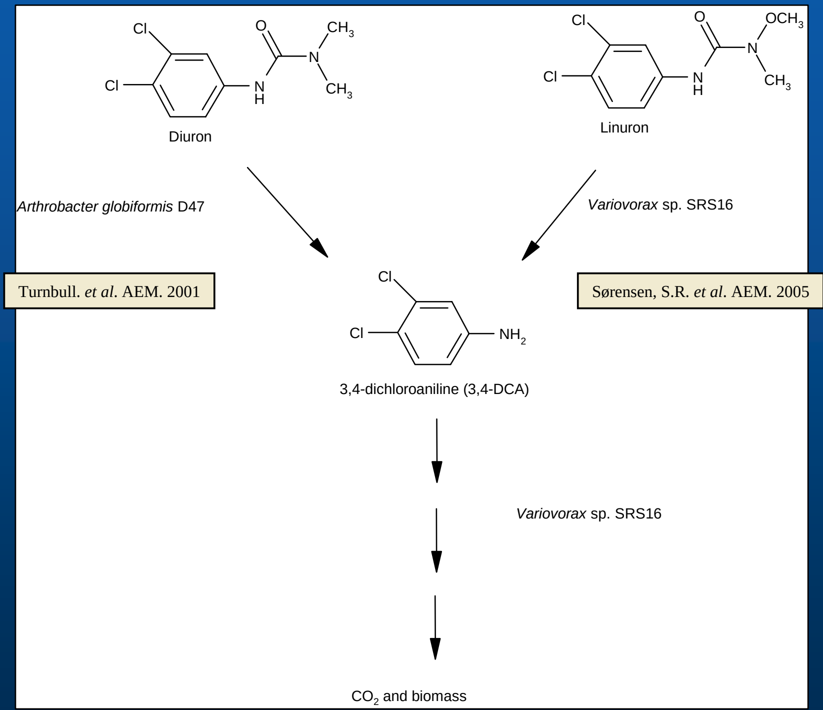


Tixier *et al*. Environ. Tox. Chem. 2001

Aims

- Construct diuron-mineralising bacterial consortia with the vision of developing remediation techniques
- Determine co-culture efficiency in laboratory liquid and soil systems
- Screening soil bacteria degrading structurally similar herbicides for diuron degradation with the aim of finding novel degradation pathways and construct new consortia

The idea : Constructing a diuron-mineralizing bacterial co-culture



Constructed pathway for mineralization of diuron combining the cooperative degradation pathways of the diuron-degrading *A. globiformis* D47 and the linuron-mineralizing *Variovorax* sp. SRS16

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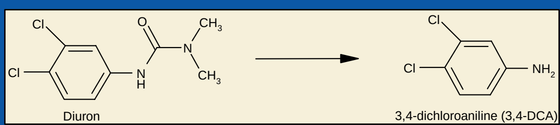
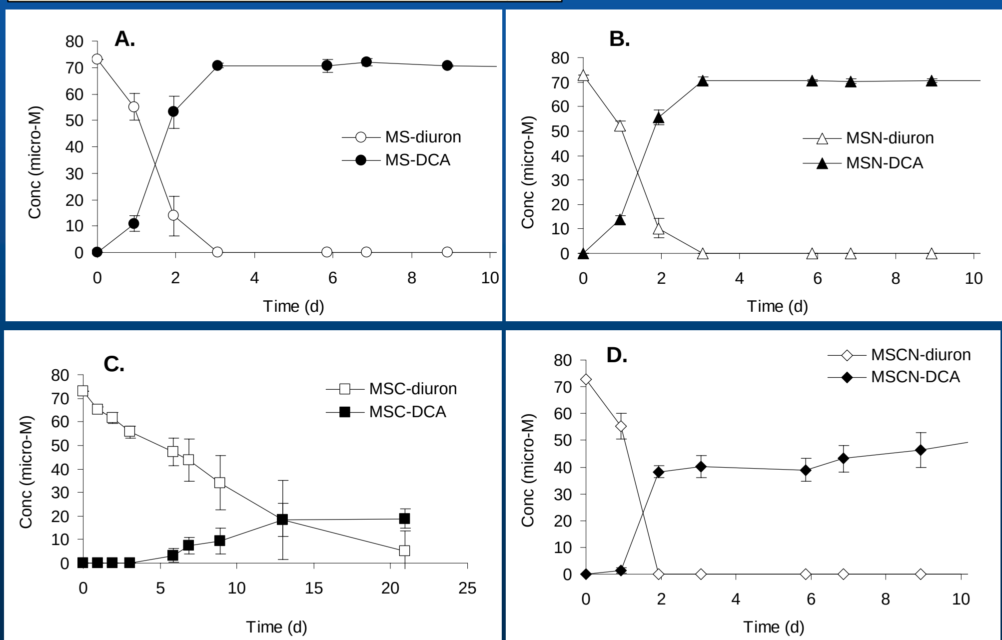
Initial step: Diuron degradation by *Arthrobacter* sp. D47





Degradation of diuron (open symbols) and production of 3,4-DCA (filled symbols) by *A. globiformis* D47 in different liquid media. Initial cell density of 10^6 D47 ml$^{-1.}$ The data are means (n=3).

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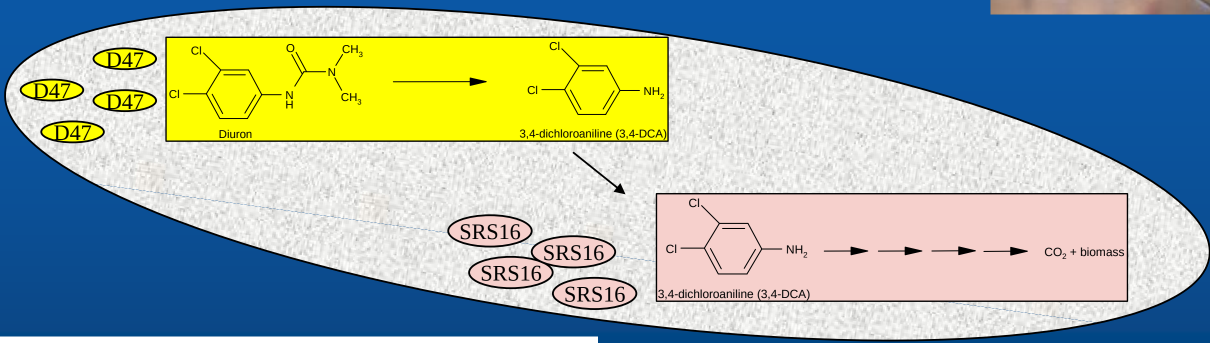
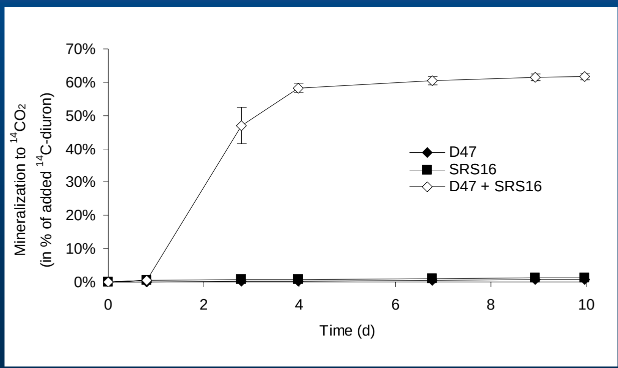
Testing the idea: Consortial diuron mineralization





Mineralization of ^{14}C-ring-labeled diuron to $^{14}CO_2$ in MS. Initial diuron concentrations were 10 mg l^{-1} and initial cell densities approx. 10^6 cells ml^{-1}. The data are means (n=3).

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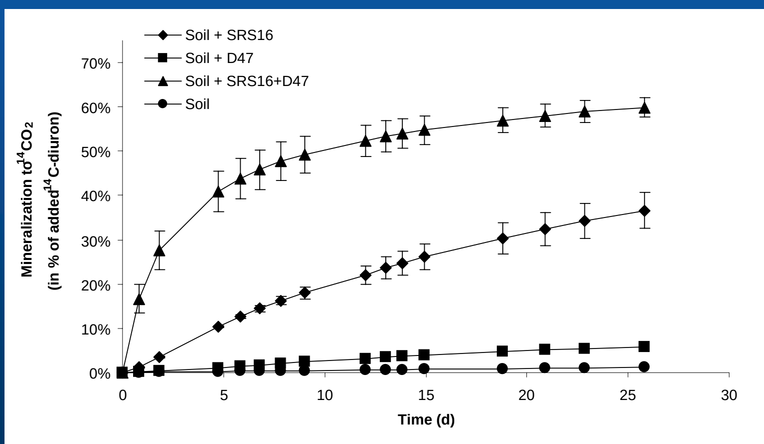
Moving into a more complex system: Augmentation of soil artificially contaminated with diuron



Mineralization of ^{14}C-ring-labeled diuron to $^{14}CO_2$ in soil amended with diuron (2 mg kg^{-1}). Natural soil was used as uninoculated control. The data are means (n=3).

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Soil batch experiments

^{14}C-residues following soil remediation experiment

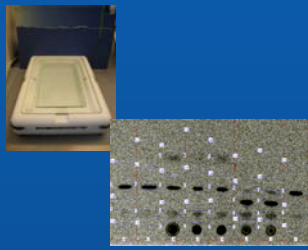

TABLE 1. Distribution of ^{14}C residues from [^{14}C]diuron in agricultural soil inoculated with *Variovorax* sp. strain SRS16 or *A. globiformis* D47 or with an SRS16-D47 coculture as estimated by thin-layer chromatography and autoradiographic quantification, including detection of the demethylated diuron metabolite DCPMU, the didemethylated metabolite DCPU, and 3,4-DCA

Prepn	^{14}C recovery (%)[a]						
	[^{14}C]diuron	[^{14}C]DCPMU	[^{14}C]DCPU	[^{14}C]3,4-DCA	$^{14}CO_2$	^{14}C-labeled humic fraction	Nonextractable ^{14}C
Soil + SRS16	23.1 (5.5)	1.8 (0.5)	1.5 (0.1)	0.0	36.6 (4.1)	5.4 (0.4)	31.5 (1.5)
Soil + D47	16.6 (3.2)	1.6 (0.1)	1.7 (0.3)	3.9 (1.5)	5.9 (0.5)	14.9 (0.8)	55.4 (3.2)
Soil + SRS16 + D47	1.1 (0.6)	0.2 (0.1)	0.6 (0.2)	0.0	59.9 (2.3)	4.4 (0.5)	33.7 (0.9)
Soil	78.8 (0.9)	3.0 (0.5)	1.7 (0.2)	0.0	1.2 (0.0)	0.0	15.3 (1.2)

[a] The values are means and standard deviations (in parentheses) for triplicate experiments expressed as percentages of the initial amount of [^{14}C]diuron.

Unexpected mineralization activity

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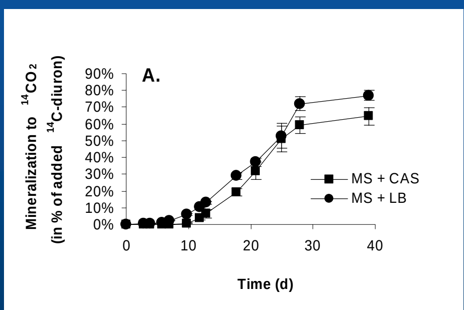
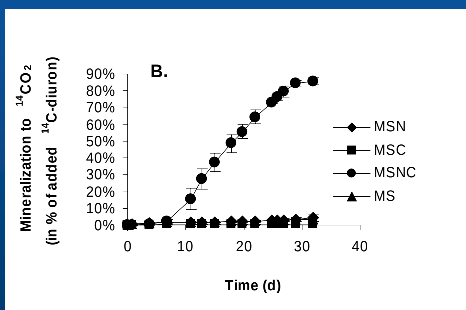
Sørensen *et al*. AEM. 2008

Unexpected finding: Enhanced diuron mineralization by strain SRS16 in pure culture





Mineralization of ^{14}C-ring-labeled diuron to $^{14}CO_2$ by *Variovorax* sp. SRS16 in MS supplemented with either Casamino acids (CAS) or LB or in MS, MSC or MSCN (succinate-based). Initial diuron concentration was 10 mg l^{-1}. The data are means (n=3).

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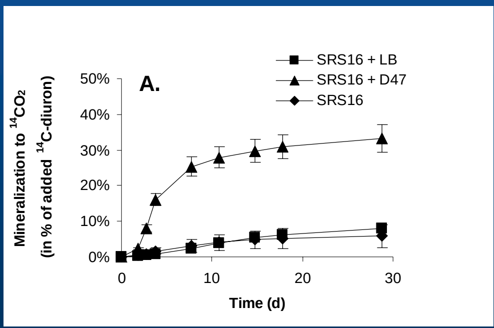
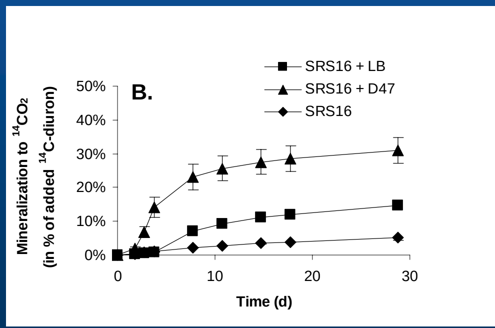
Mineralization of ecologically relevant concentrations of diuron by SRS16 or coculture D47-SRS16

16 µg l^{-1}



39 µg l^{-1}



Mineralization of ^{14}C-ring-labeled diuron to $^{14}CO_2$ in MS.
The data are means (n=3).

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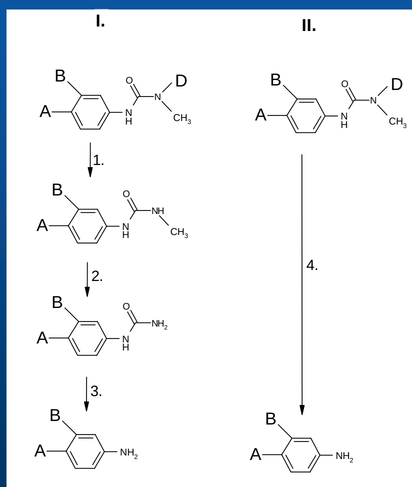
New questions raised

- Is there an overlooked capacity for diuron degradation among previously isolated bacterial strains described recently in the literature?

- Will it be possible to find isolates harbouring an initial diuron degradation pathway resembling that previously described for structurally related herbicides such as isoproturon and linuron?



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Strains tested for degradative activity towards diuron

Strain:	Source:	Known degradation capacity:
Variovorax sp. PBS H4	Agricultural soil, Belgium	Linuron$^+$, 3,4-DCA$^-$, Diuron$^-$
Variovorax sp. WDL1	Orchard soil, Belgium	Linuron$^+$, 3,4-DCA$^+$, Diuron$^-$
Variovorax sp. PBL H6	Agricultural soil, Belgium	Linuron$^+$, 3,4-DCA$^+$, Diuron$^-$
Variovorax sp. PBL E5	Agricultural soil, Denmark	Linuron$^+$, 3,4-DCA$^+$, Diuron$^-$
Variovorax sp. SRS16	Agricultural soil, Denmark	Linuron$^+$, 3,4-DCA$^+$, Diuron$^+$
Hydrogenophaga sp. PBL-H3	Agricultural soil, Belgium	Linuron$^+$, 3,4-DCA$^+$
Sphingomonas sp. SRS2	Agricultural soil, UK	Linuron$^-$, Isoproturon$^+$, Diuron$^+$,
Arthrobacter globiformis D47	Agricultural soil, UK	Linuron$^+$, Diuron$^+$, 3,4-DCA$^-$

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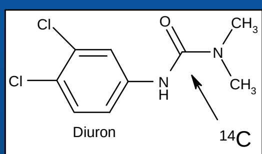
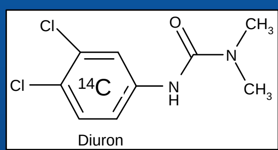
Assays used for determining degradative activity

^{14}C-mineralisation assay



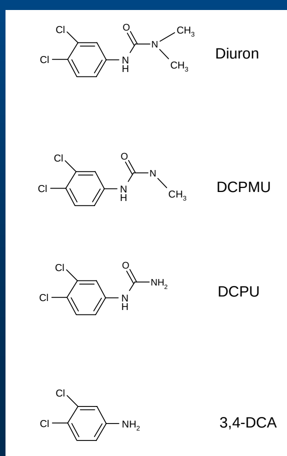
^{14}C-carbonyl-labeled diuron



^{14}C-U-ring-labeled diuron

UPLC assay

Waters ACQUITY UPLC photodiode array detector
ACQUITY UPLC BEH C18 column.



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Partial or complete diuron mineralization by known phenylurea degraders

Functional categories:
- Side chain mineralisers
- Side and ring mineralisers

	Cumulative $^{14}CO_2$ production (in % of added ^{14}C) [a]			
	^{14}C-*carbonyl*-diuron		^{14}C-*ring*-diuron	
Strain	**MS**	**R2B**	**MS**	**R2B**
Arthrobacter globiformis D47	ND [b]	77.6 (±3.1)	ND	1.8 (±0.5)
Hydrogenophaga sp. PBL-H3	1.8 (±0.0)	27.3 (±5.2)	0.8 (±0.2)	6.2 (±3.6)
Sphingomonas sp. SRS2	6.4 (±4.2)	86.7 (±1.9)	0.1 (±0.1)	2.7 (±0.5)
Variovorax sp. PBL-H6	2.0 (±0.5)	78.0 (±11.5)	2.2 (±0.1)	18.8 (±0.4)
Variovorax sp. PBL-E5	1.1 (±0.0)	43.8 (±1.8)	1.1 (±0.5)	46.8 (±12.7)
Variovorax sp. WDL1	5.7 (±1.3)	1.2 (±0.5)	3.3 (±0.4)	4.1 (±0.3)
Variovorax sp. PBS-H4	0.9 (±0.2)	2.0 (±0.2)	0.2 (±0.0)	0.4 (±0.1)
Variovorax sp. SRS16	1.3 (±0.2)	57.6 (±12.3)	2.1 (±0.1)	43.5 (±2.0)
Uninoculated control	0.1 (±0.0)	0.1 (±0.0)	0.1 (±0.1)	0.1 (±0.0)

[a] Data are mean values (n =3) with the standard deviation after 30 days of incubation
[b]. ND, not determined in this study

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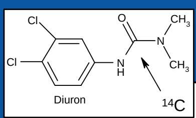
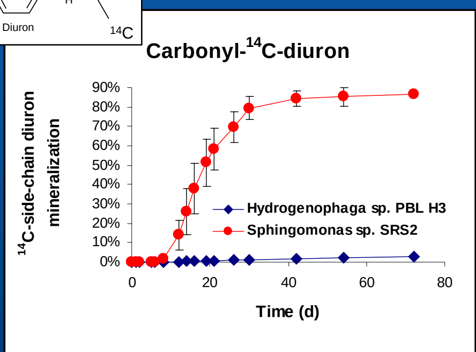
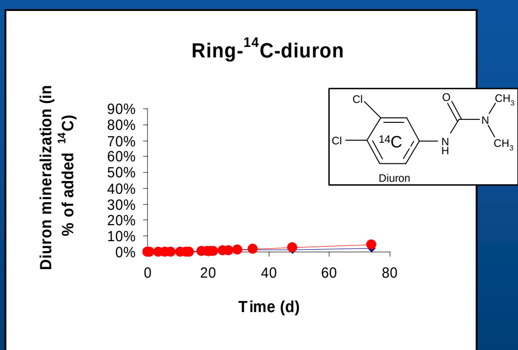
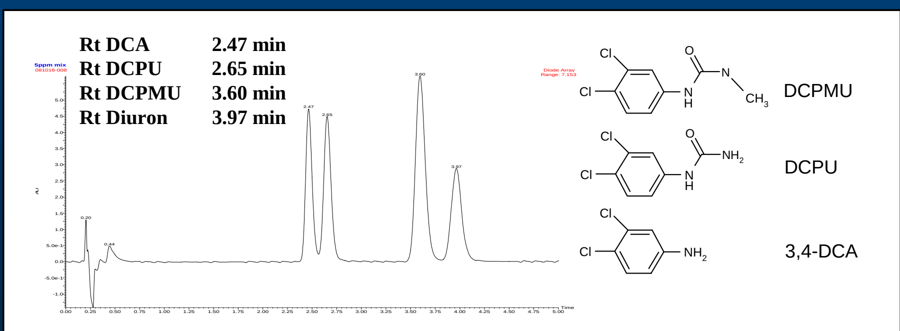
Enhanced diuron degradation when supplemented with growth substrates

Elucidating diuron degradation pathway harboured by *Sphingomonas* sp. SRS2









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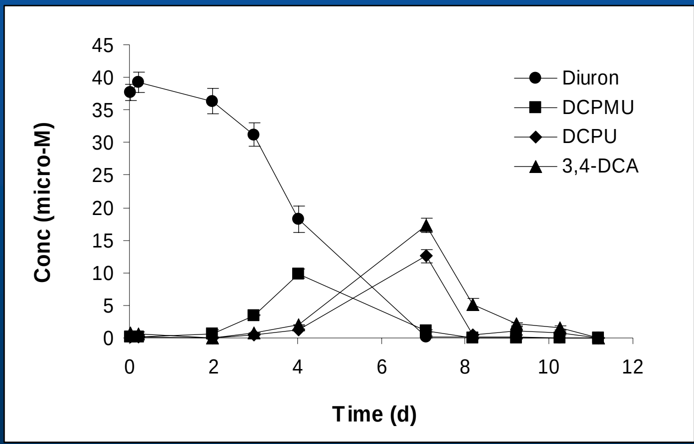
Partial diuron degradation by *Sphingomonas* sp. SRS2



Degradation of diuron by SRS2 in MS + Casamino Acids and occurrence of the metabolites DCPMU, DCPU and 3,4-DCA. The initial diuron concentration was 10 mg l^{-1} and the initial cell densities were approximately 10^7 ml^{-1}. The data is mean values (n = 3). The bars indicate the standard deviation.

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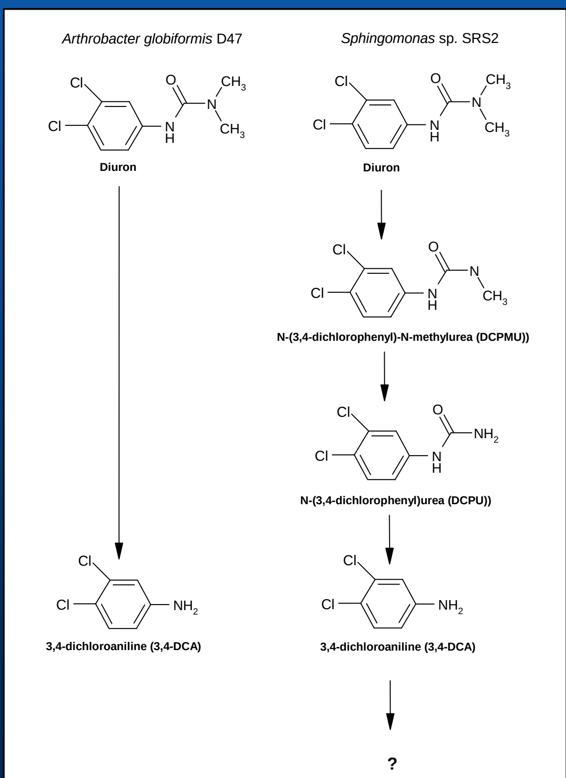
Proposed initial degradation steps for diuron by strains D47 and SRS2



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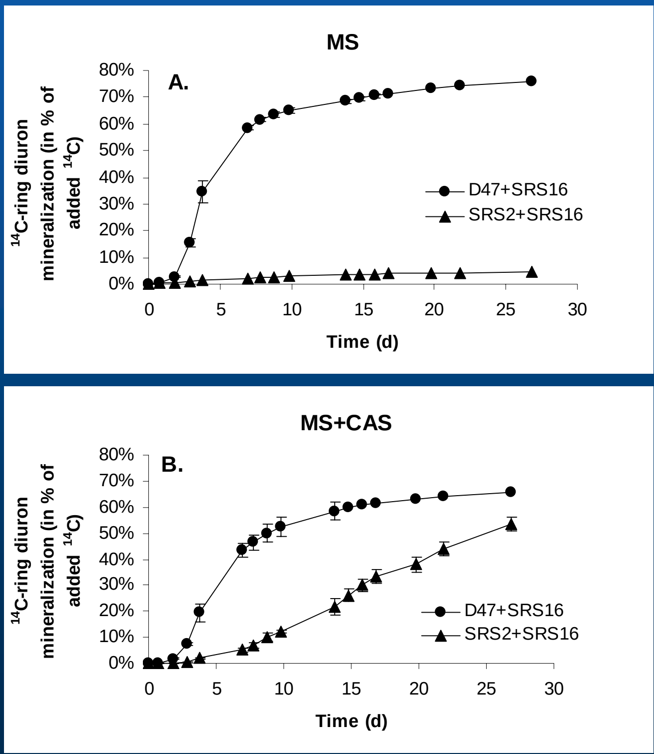
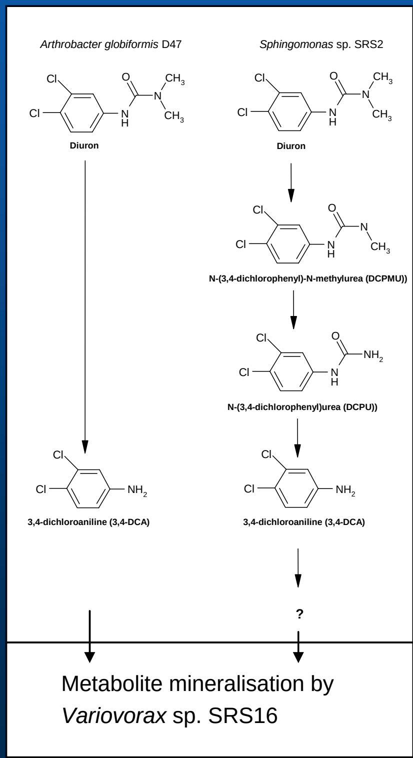
Mineralisation of ^{14}C-ring-labeled diuron by cocultures D47-SRS16 and SRS2-SRS16





Conclusions – part 1

- We constructed a bacterial coculture capable of rapid diuron mineralization and efficient in degrading realistic diuron concentrations
- Mineralization of both diuron and linuron suggests that this coculture could be relevant for remediation of soil and water affected by these herbicides, as well as their shared metabolite 3,4-DCA
- Unexpectedly, *Variovorax* sp. SRS16 performed diuron mineralization alone making it the first bacterium capable of mineralizing both a N,N-dimethyl-substituted phenylurea herbicide and an N-methoxy-N-methyl-substituted phenylurea herbicide

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Conclusions – part 2

- There is an overlooked potential for degradation of diuron based on the fact that previous studies have screened for diuron degradative activity in MS-based media
- Diuron degradative activity by known phenylurea strains can be induced by supplementing the strains with appropriate growth substrates
- The ability to degrade the dimethylurea-side chain of diuron was more common among the tested strains compared to complete aromatic ring mineralisation
- Among the tested strains, only *Variovorax* spp strains had the ability to mineralize the ring structure of diuron
- *Sphingomonas* sp. SRS2 (a known isoproturon-degrading soil bacterium) harbours the “classical” initial phenylurea degradation pathway and is capable of partially degrading diuron. Endproduct (s) unknown

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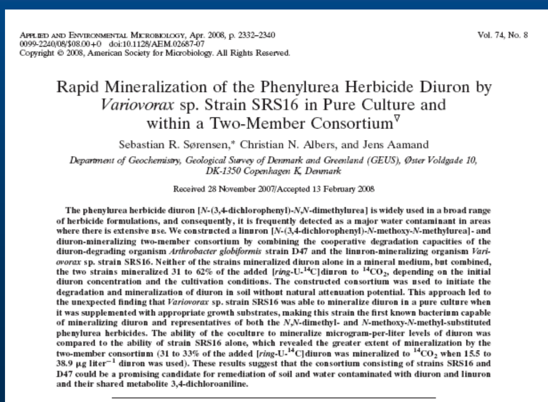
Publications summarising the diuron data

- **S. R. Sørensen, C. N. Albers and J. Aamand. 2008. Rapid mineralization of the phenylurea herbicide diuron by *Variovorax* sp. strain SRS16 in pure culture and within a two-member consortium. Appl. Environ. Microbiol. 74:2332-2340**
- **S. R. Sørensen, R.K. Juhler and J. Aamand. Partial and complete mineralization of the herbicide diuron by known phenylurea-degrading bacteria isolated from agricultural soils. *In-prep***



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0099-2240/08/$08.00+0 doi:10.1128/AEM.02687-07

Rapid Mineralization of the Phenylurea Herbicide Diuron by *Variovorax* sp. Strain SRS16 in Pure Culture and within a Two-Member Consortium∇

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The phenylurea herbicide diuron [*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea] is widely used in a broad range of herbicide formulations, and consequently, it is frequently detected as a major water contaminant in areas where there is extensive use. We constructed a linuron [*N*-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea]- and diuron-mineralizing two-member consortium by combining the cooperative degradation capacities of the diuron-degrading organism *Arthrobacter globiformis* strain D47 and the linuron-mineralizing organism *Variovorax* sp. strain SRS16. Neither of the strains mineralized diuron alone in a mineral medium, but combined, the two strains mineralized 31 to 62% of the added [*ring*-U-^{14}C]diuron to $^{14}CO_2$, depending on the initial diuron concentration and the cultivation conditions. The constructed consortium was used to initiate the degradation and mineralization of diuron in soil without natural attenuation potential. This approach led to the unexpected finding that *Variovorax* sp. strain SRS16 was able to mineralize diuron in a pure culture when it was supplemented with appropriate growth substrates, making this strain the first known bacterium capable of mineralizing diuron and representatives of both the *N*,*N*-dimethyl- and *N*-methoxy-*N*-methyl-substituted phenylurea herbicides. The ability of the coculture to mineralize microgram-per-liter levels of diuron was compared to the ability of strain SRS16 alone, which revealed the greater extent of mineralization by the two-member consortium (31 to 33% of the added [*ring*-U-^{14}C]diuron was mineralized to $^{14}CO_2$ when 15.5 to 38.9 μg liter^{-1} diuron was used). These results suggest that the consortium consisting of strains SRS16 and D47 could be a promising candidate for remediation of soil and water contaminated with diuron and linuron and their shared metabolite 3,4-dichloroaniline.

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- A. Simonsen
- Erkin Gödzdereliler

HRI-Warwick, United Kingdom

- Gary D. Bending

Catholic University of Leuven, Belgium

- Philip Breugelmans
- Dirk Springael

See also posters:
A8 (Sand filter inoculation)
B7 (Linuron degradation)

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