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ORIGINAL PAPER

The most-probable-number enumeration of dichlobenil and 2,6-dichlorobenzamide (BAM) degrading microbes in Finnish aquifers

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Abstract In groundwater subsurface deposits and a topsoil from five aquifers having 2,6-dichlorobenzamide (BAM) in water, we determined the most-probable-number (MPN) of 2,6-dichlorobenzonitrile (dichlobenil) and metabolite BAM degrading microorganisms. Dichlobenil and BAM were combined nitrogen sources in the MPN tubes, which were scored positive at concentrations <75% after 1 month incubation. Aerobic and anaerobic microbes degrading dichlobenil and BAM were common in samples in low numbers of 3.6–210 MPN g dw^{-1}. Additional degradation occurred in high MPN dilutions of some samples, the microbial numbers being 0.11–120 × 10^5 MPN g dw^{-1}. The strains were isolated from low and high dilutions of one deposit, and degradation in pure cultures was confirmed by HPLC. According to the 16S rDNA sequencing, strains were from genera *Zoogloea*, *Pseudomonas*, *Xanthomonas*, *Rhodococcus*, *Nocardioides*, *Sphingomonas*, and *Ralstonia*. Dichlobenil (45.5 ± 18.3%) and BAM (37.6 ± 14%) degradation was low in the MPN tubes. Despite of microbial BAM degradation activity in subsurface deposits, BAM was measured from groundwater.

Keywords 2,6-Dichlorobenzonitrile · 2,6-Dichlorobenzamide · Most-probable-number enumeration · Biotic degradation · Groundwater environment · Topsoil

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Introduction

Dichlobenil (2,6-dichlorobenzonitrile) is a herbicide used against germinating seeds and the growth of young plants. In the topsoil dichlobenil is rather quickly metabolized to 2,6-dichlorobenzamide (BAM) (Beynon and Wright 1972; Holtze et al. 2007a; Koopman and Daams 1960; Miyazaki et al. 1975; Montgomery et al. 1972; Verloop and Nimmo 1970; Vosáhlová et al. 1997). With a high water solubility and low sorption affinity of 0.10–0.93 l kg^{-1} BAM easily leaches to groundwater, where it is persistent (Albrechtsen et al. 2001; Broholm et al. 2001; Clausen et al. 2004, 2007; Tuxen et al. 2000, 2002). BAM is a problem in groundwater world widely, including Denmark, Italy, Germany, and Finland (Brüsch 2004; Porazzi et al. 2005; Wolter et al. 2001).

The information on the microbiological BAM degradation in the groundwater environment is

lacking. Microbes able to degrade BAM or dichlobenil have been isolated from soil surface, where BAM has been persistent, slowly or fast degraded by microbes after the exposure to dichlobenil (Beynon and Wright 1968; Clausen et al. 2007; Holtze et al. 2007a, b; Miyazaki et al. 1975; Montgomery et al. 1972; Verloop 1972; Vosáhlová et al. 1997). The long-term exposure to herbicides generally improves the ability of soil microbes to degrade these compounds. The microbial degradation is affected by several different environmental conditions, such as the amount of oxygen, nutrients and organic matter, temperature, carbon to nitrogen ratio, redox potential, inhibiting chemicals, and bioavailability of the herbicide (Häggblom 1992). We studied the most-probable-number (MPN) of dichlobenil and BAM degrading microbes in groundwater well and sampling pipe deposits, and a surface soil from five aquifer areas with BAM in groundwater in Southern Finland. The MPN counts were compared to the chemical compositions of the samples.

Materials and methods

Chemicals

Dichlobenil, 2,6-dichlorobenzamide and simazine were from Ehrenstorfer GmbH (Augsburg, Germany), and the HPLC grade acetonitrile and methanol from Merck (Darmstadt, Germany).

Environmental samples

The environmental samples were collected from five aquifers in Southern Finland (the northernmost aquifer 61°29′N), all having BAM in groundwater. The areas of the aquifers were as follows: aquifer A, 221 ha; aquifer B, 98 ha; aquifer C, 284 ha; aquifer D, 266 ha; and aquifer E, 96 ha. The surface soil above the aquifer B, and groundwater well and sampling pipe deposits from aquifers A and B were collected in May 2005. The groundwater well and sampling pipe deposits from aquifers C, D, and E were collected in June 2005. The deposits were accumulated for several years to the groundwater sampling pipes through sieves with the pore size of 0.3 mm. The deposit in the newest sampling pipe D was the smallest in volume. The pump Waterra HL 21507 (Waterra, Ontario, Canada) equipped with the aggregate Poweri 2601 BV (2.5 kW) (Hollola, Finland) was used to collect the deposits from the groundwater sampling pipes. The groundwater well deposits were taken using an Ekman grab sampler (Duncan and Associates, Cumbria, United Kingdom). The aquifer B surface soil was collected from the depth of 0–20 cm in a dichlobenil-exposed garden, and sieved (pore size 5 × 5 mm). The groundwater well and sampling pipe deposits were allowed to settle overnight, and water was removed and filtered with Whatman filter paper 40 (Maidstone, UK) for dichlobenil and BAM analyses. All samples were stored at 4°C until MPN enumeration (3–7 days) and then at −20°C.

Chemical and physical analyses

The water pH was measured with the pH paper (Macherey–Nagel, Düren, Germany). The dry weight was determined from the weight loss of 1–2 g of sample after drying at 105°C for 24 h in duplicate (SFS-EN 13040 2000). To determine the organic matter content the sample was heated at 550°C for 4 h, and then weighed (SFS-EN 13039 2000).

Total carbon, nitrogen and sulfur were determined with the LECO Model 2000 CNS analyzer (St. Joseph, MI, USA) using helium as a carrier gas. Three parallel samples were weighed and analyzed according to the instructions of LECO. The water soluble NH_4^+, NO_3^-, and NO_2^- were analyzed according to standards SFS 5505 (1988), SFS-EN ISO 10304-1 (1995) and SFS-EN ISO 10304-2 (1997). The elements Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn were analyzed according to the standards ISO 11885 (1996) and ISO/CD 22036 (2005). The NH_4^+, NO_3^-, NO_2^-, and element analyses were done in Ramboll Analytics Ltd. (Lahti, Finland) using accredited methods (FINAS, Finnish Accreditation Service T039; SFS-EN ISO/IEC 17025 2005).

MPN enumeration

To enumerate dichlobenil and BAM degrading microbes, the most-probable-number (MPN) method in three replicates was done using medium modified from those of Alvey and Crowley (1996), and Ostrofsky et al. (2002). The medium contained per litre: 75 mg dichlobenil, 75 mg BAM, 1.6 g K_2HPO_4, 0.4 g

$NaH_2PO_4 \cdot H_2O$, 1 mg thiamine, 0.4 mg biotin, 1.0 g sodium citrate, 5.5 mg $FeSO_4 \cdot 7H_2O$, 9 mg $CaSO_4$, 10 mg $MnSO_4 \cdot H_2O$, 12 mg $MgSO_4 \cdot 7H_2O$, 0.1 mg $NaVO_3 \cdot H_2O$, 0.05 mg H_3BO_3, 0.2 mg $NiCl_2 \cdot 6H_2O$, 0.1 mg $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg Na_2SeO_3, 0.2 mg Na_2WO_4, 0.2 mg $CuSO_4$, 2 mg $ZnSO_4 \cdot 7H_2O$, and 2 mg $CoSO_4 \cdot 7H_2O$. The initial dilutions were made by adding 1 g of deposit or soil on the dry weight basis (1 ml of water from aquifer D pipe) into 9 ml of the MPN medium. After thorough mixing additional 10-fold dilutions from 10^{-2} to 10^{-8} were prepared. From each dilution, 1 ml was transferred to three parallel test tubes containing 5 ml of the MPN medium. The MPN tubes were protected from light and incubated in a shaker at 120 rpm and 16°C (the groundwater well and sampling pipe deposits) or 21°C (the surface soil) for 30 days. The experiment was carried out both in aerobic and anaerobic conditions, the anaerobic atmosphere being created with Mikrobiologie Anaerocult A and Anaerotest (Merck, Germany). After 30 days, 100 µl samples were taken for the HPLC analysis. The MPN results were scored positive when at least 25% of dichlobenil or BAM was degraded, and the MPN scores were converted to cell numbers and the 95% confidence limits were calculated as has been presented (de Man 1983).

The enumeration of both dichlobenil and BAM degrading microbes was carried out in the same MPN tubes. Therefore, the percentage of BAM degraded was calculated by comparing the analyzed BAM amount in the MPN tubes to the theoretical total amount of BAM, that is the sum of the initially added BAM and that metabolized from dichlobenil. The analyzed BAM amount was always $\geq$100% of the initial concentration. In most studies dichlobenil has been shown to be degraded to BAM, but the formation of 2,6-dichlorobenzoic acid from dichlobenil is also possible (Beynon and Wright 1972; Holtze et al. 2007a; Montgomery et al. 1972; Verloop 1972; Verloop and Nimmo 1970).

Microbial isolation and 16S rRNA gene sequencing

To confirm the microbial dichlobenil and BAM degradation, the MPN dilutions 10^{-1}, and the dilution 10^{-6} from well E deposit were cultivated at 21°C under aerobic conditions on the MPN medium supplemented with 1.5% agar, and either dichlobenil or BAM as the sole nitrogen source. The isolates were re-plated until pure microbial cultures were obtained. The strains were grown aerobically in 10 ml of the liquid MPN medium at 21°C for approximately 20 days in duplicate, and the ability of the microbes to degrade dichlobenil or BAM as the N-source was determined by the HPLC analysis. The degradation percentage was calculated in relation to the non-inoculated controls with dichlobenil or BAM.

For the 16S rRNA gene sequencing, the strains isolated from the well E MPN dilutions 10^{-1} and 10^{-6} were cultivated at 21°C on a medium having per litre 5 g tryptone, 2.5 g yeast extract, 1 g glucose, and 15 g agar. The cells were collected, and the DNA was purified with the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The 1,500 bp fragment of the 16S rRNA gene was amplified using the polymerase chain reaction (PCR) with the DNA Engine DYAD Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The 50 µl reaction mixture contained approximately 25 ng of template DNA, 50 pmol of primers pA and pH (Oligomer, Helsinki, Finland) (Edwards et al. 1989), 4 µmol of each deoxynucleoside triphosphate (Finnzymes, Espoo, Finland), 5 µl of 10× buffer (10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), and 2.0 U of DyNAzyme II DNA Polymerase (Finnzymes, Espoo, Finland). After heat denaturation at 94°C for 5 min, 25 amplification cycles of denaturation at 95°C for 20 s, primer annealing at 55°C for 20 s, and primer extension at 72°C for 30 s, were performed. Finally, 72°C was maintained for 5 min, followed by cooling to 11°C for 5 min and then to 4°C. The 16S rRNA gene was sequenced in duplicate using the universal primers pA, pD, or pE (Edwards et al. 1989), 1 µl of template DNA, and ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v.3.1 (Perkin Elmer, Applied Biosystems, Foster City, USA). The sequencing conditions were: an initial denaturation at 95°C for 2 min, 36 cycles of denaturation at 95°C for 10 s, annealing and elongation at 55°C for 2 min 10 s, and the final extension at 72°C for 5 min. The sequencing products were detected with the ABI PRISM® 3700 DNA analyzer (Perkin Elmer, Applied Biosystems, Foster City, USA). The sequencing was performed in the DNA Sequencing Laboratory (Institute of Biotechnology, University of Helsinki,

Helsinki, Finland). The obtained sequences were compared to the 16S rRNA gene sequences in the EMBL database using the Fasta program. The 16S rDNA sequences of isolated strains have been deposited in the EMBL Nucleotide Sequence Database under accession numbers FM999989-FM999997.

Pesticide analyses

Dichlobenil and BAM concentrations in water from the groundwater sampling pipes and wells were analyzed in Ramboll Analytics Ltd. (Lahti, Finland) using a method based on the solid phase extraction of pesticides, followed by the high resolution gas-liquid chromatography—mass spectrometry. The method was accredited according to the guidelines of FINAS, Finnish Accreditation Service T039 (SFS-EN ISO/IEC 17025).

To analyze dichlobenil and BAM from the MPN medium, HPLC standards were prepared in methanol:water (3:1 v/v) containing dichlobenil and BAM at six concentrations ranging from 2.6 to 174 μM, and 69.4 μM of simazine as an internal standard. The obtained calibration curves were linear with the correlation coefficients ($r^2 \pm$ standard deviation) of 0.983 ± 0.026 for dichlobenil and 0.970 ± 0.025 for BAM. The internal standard, 69.4 μM simazine, was added to 100 μl of the MPN sample, and the volume was filled to 600 μl with methanol:water (3:1 v/v). Standards and samples were filtered through a 0.45 μm GHP membrane (Acrodisc®, Gelman, Pall Corporation Ltd., NY, USA), and 20 μl was analyzed with the HPLC.

The HPLC was equipped with the sample processor Waters 712 WISP (MA, USA), two Waters Associates Inc model 6000A pumps (Milford, Massachusetts, USA) with the flow rate of 1.0 ml min^{-1}, SunFire Column (C_{18}, 3.5 μm, 3.0 × 150 mm, Waters, Ireland), and the UV detector (Hewlett Packard HP 1050, MI, USA) set at 225 nm. The Maxima 820 chromatography workstation (Millipore, Ventura, California, USA) was used to control the HPLC system and to collect data from the UV detector. Acetonitrile and 10 mM phosphate buffer pH 7.0 were used as the mobile phase with the following gradient profile: acetonitrile concentration increased from 30 to 70% in 12 min, then held at 70% for 1 min, after which back to 30% in 5 min, and held for 5 min.

Statistical analyses

The Pearson two-tailed correlation analyses were performed using the SPSS Statistical package for Windows (SPSS Inc., Chicago, IL, USA).

Results and discussion

Dichlobenil degradation

According to the MPN enumeration, the microbial dichlobenil degradation was common in the surface soil and all the subsurface deposits from below the groundwater table, independent of the amounts of organic and inorganic compounds in the samples (Tables 1, 2). Microbes metabolizing dichlobenil under the aerobic conditions were present in all samples, and under anaerobic conditions in all the subsurface deposits but not in the surface soil B (Table 1). However, the numbers of dichlobenil degrading microbes remained unclear, due to the detection of dichlobenil degradation in both low and high MPN dilutions, but not in dilutions lying between. The dichlobenil degradation exceeded the limit of positive degradation (>25%) in the MPN dilutions of 10^{-1} to 10^{-3}, the microbial numbers being low, 3.6–210 MPN g^{-1} on the dry weight (gdw) basis. In addition, dichlobenil degradation was observed in the high 10^{-4} to 10^{-8} MPN dilutions under the aerobic conditions in the surface soil B, and pipe C and well E deposits, and under the anaerobic atmosphere in well E deposit. The microbial numbers were 1.1×10^4 to 1.2×10^7 MPN g dw^{-1} (Table 1). Although two different numbers could be calculated for dichlobenil degrading microbes, the amount of dichlobenil degraded in one month was within 45.5 ± 18.3% (mean ± standard deviation) in all the positive tubes. The low MPN numbers for dichlobenil degrading microbes correlated with chromium ($P < 0.01$, $r = 0.949$) and copper ($P < 0.01$, $r = 0.963$) contents in samples under aerobic conditions, and with iron ($P < 0.001$, $r = 0.999$) under the anaerobic atmosphere (Tables 1, 2).

To confirm the microbial dichlobenil degradation, the microbes were isolated from the MPN dilutions 10^{-1} of aquifers B and E, and MPN dilution 10^{-6} of aquifer E under aerobic conditions. The strains were cultivated in the MPN medium, and the dichlobenil

Table 1 The MPN enumeration of dichlobenil and BAM degrading microbes in groundwater well and sampling pipe deposits, and surface soil from aquifers A, B, C, D, and E

	Aquifer A		Aquifer B		Aquifer C	Aquifer D[b]	Aquifer E
	Pipe	Well	Well	Surface soil	Pipe	Pipe	Well
Dichlobenil degradation							
Aerobic (MPN g^{-1} dry wt)	9.2	210	23.0	21/11000[a]	93/11000[a]	7.2	210/12000000[a]
Low confidence limit (95%)	1.4	40	4.6	4.5/3600[a]	18/3600[a]	1.3	40/3700000[a]
High confidence limit (95%)	38	430	94	42/38000[a]	420/38000[a]	18	430/42000000[a]
Anaerobic (MPN g^{-1} dry wt)	7.2	3.6	3.6	0	150	11	27/920000[a]
Low confidence limit (95%)	1.3	0.17	0.17		37	3.6	8.7/140000[a]
High confidence limit (95%)	18	18	18		420	38	94/3800000[a]
BAM degradation							
Aerobic (MPN g^{-1} dry wt)	9.2	120	23.0	3.6	93/36000[a]	7.2	210/6400000[a]
Low confidence limit (95%)	1.4	37	4.6	0.17	18/1700[a]	1.3	40/1700000[a]
High confidence limit (95%)	38	420	94	18	420/180000[a]	18	430/18000000[a]
Anaerobic (MPN g^{-1} dry wt)	3.6	3.6	3.6	0	23.0	0	27
Low confidence limit (95%)	0.17	0.17	0.17		4.6		8.7
High confidence limit (95%)	18	18	18		94		94

[a] MPN numbers from high dilutions of 10^{-4} to 10^{-6} (soil B and pipe C, dichlobenil), 10^{-5} to 10^{-7} (pipe C, BAM), or 10^{-6} to 10^{-8} (well E, dichlobenil and BAM)

[b] MPN ml^{-1}

degradation as the nitrogen source of 28.4 ± 9.0% in comparison to non-inoculated controls was verified by the HPLC analysis. In aquifer E, according to the 16S rRNA gene sequencing, the bacterial strains degrading dichlobenil in the 10^{-1} dilution were from the genera *Zoogloea*, *Pseudomonas* and *Xanthomonas*. They differed from the strains degrading dichlobenil in the 10^{-6} dilution, which were from the genera *Rhodococcus*, *Nocardioides* and *Sphingomonas* (Table 3). The dichlobenil degraders reported in previous studies have been isolated from surface soil, or pond water and sediment. They have been from the genera *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Actinomyces*, *Rhodotorula*, *Penicillium*, *Trichoderma*, *Rhizobium*, *Rhodococcus*, *Pseudomonas*, *Aminobacter*, or *Agrobacterium* (Heinonen-Tanski 1981; Holtze et al. 2006; Miyazaki et al. 1975; Sørensen et al. 2007; Vosáhlová et al. 1997).

BAM degradation

The BAM degrading microbes were also common in the surface soil, and all the groundwater well and pipe deposits with the organic and inorganic compound compositions presented in Table 2. Under the aerobic and anaerobic conditions, microbes degrading BAM were found in all samples, except for the surface soil B and the newly built groundwater sampling pipe D with the small deposit volume under the anaerobic atmosphere (Table 1). Due to the BAM degradation in the low MPN dilutions between 10^{-1} and 10^{-3}, the calculated MPN numbers were low, 3.6–210 MPN g dw^{-1} (Table 1). However, the MPN numbers of BAM degrading microbes also were inaccurate, due to the additional BAM degradation in the high dilutions of 10^{-5} to 10^{-7} and 10^{-6} to 10^{-7} in the aerobic pipe C and well E deposits, the MPN values being 3.6×10^4 and 6.4×10^6 MPN gdw^{-1}, respectively (Table 1). When positive, the BAM degradation was within 37.6 ± 14% in both the low and high MPN dilutions. The low MPN numbers for BAM degrading microbes under aerobic conditions correlated with chromium ($P < 0.01$, $r = 0.940$) and copper ($P < 0.05$, $r = 0.844$) in the deposits and soil (Tables 1, 2).

To confirm the microbial BAM degradation, the microbes were isolated from the MPN dilutions 10^{-1} of aquifers A, B, C, and E, and MPN dilution 10^{-6} of aquifer E under aerobic conditions. The strains were

Table 2 The characteristics of the groundwater well and sampling pipe deposits, and surface soil from aquifers A, B, C, D, and E

	Aquifer A		Aquifer B		Aquifer C	Aquifer D	Aquifer E
	Pipe	Well	Well	Surface soil	Pipe	Pipe	Well
Water							
Dichlobenil (μg l^{-1})	<0.02	<0.02	<0.02	ND	<0.02	<0.02	<0.02
BAM (μg l^{-1})	0.02 ± 0.01	<0.02	1.60 ± 0.40	ND	0.04 ± 0.01	0.15 ± 0.04	0.12 ± 0.03
pH	7.0	5.5	7.0	ND	6.0	6.0	6.0
Sediment							
Organic matter (mg g^{-1} dry wt)	18.3 ± 6.5	250.0 ± 64.5	7.8 ± 3.2	18.5 ± 1.9	47.5	ND	58.4 ± 2.3
Total-C (mg g^{-1} dry wt)	4 ± 0.7	100 ± 13	7 ± 0.2	0.4 ± 0.08	4 ± 0.09	ND	6 ± 0.7
Total-S (mg g^{-1} dry wt)	3 ± 0.3	3 ± 0.3	0.1 ± 0.002	0.03 ± 0.02	0.5 ± 0.7	ND	5 ± 0.6
Total-N (mg g^{-1} dry wt)	1 ± 0.07	7 ± 0.1	1.6 ± 0.05	1 ± 0.05	1 ± 0.06	ND	0.8 ± 0.03
NH_4-N (μg g^{-1} dry wt)	0	263 ± 7	0	1 ± 0.3	2 ± 0.6	ND	3 ± 0.9
NO_3-N (μg g^{-1} dry wt)	0	0	0	24 ± 6	0	ND	0
NO_2-N (μg g^{-1} dry wt)	0	0	0	0	0	ND	0
Iron (mg g^{-1} dry wt)	16 ± 4	21 ± 5.3	4.2 ± 1	8.5 ± 2.1	385 ± 96	ND	80 ± 20
Manganese (μg g^{-1} dry wt)	795 ± 199	120 ± 30	38 ± 10	115 ± 29	410 ± 103	ND	770 ± 193
Zinc (μg g^{-1} dry wt)	710 ± 249	125 ± 44	17 ± 6	41 ± 14	93 ± 33	ND	205 ± 72
Cadmium (μg g^{-1} dry wt)	0	9 ± 4.1	0	0	0	ND	1 ± 0.5
Cobalt (μg g^{-1} dry wt)	21 ± 7.4	7 ± 2.5	2 ± 0.7	4 ± 1.4	9 ± 3.2	ND	34 ± 11.9
Chrome (μg g^{-1} dry wt)	8 ± 2.4	64 ± 19.2	6 ± 1.8	13 ± 3.9	19 ± 5.7	ND	90 ± 27.0
Copper (μg g^{-1} dry wt)	45 ± 13.5	205 ± 61.5	0	13 ± 3.9	62 ± 18.6	ND	175 ± 52.5
Lead (μg g^{-1} dry wt)	195 ± 68.3	23 ± 8.1	0	12 ± 4.2	66 ± 23.1	ND	46 ± 16.1
Nickel (μg g^{-1} dry wt)	35 ± 11	16 ± 5	0	0	26 ± 8	ND	78 ± 23

ND, not determined

Table 3 Dichlobenil or BAM degrading strains isolated from well E deposit MPN dilutions of 10^{-1} and 10^{-6}, and their partial 16S rRNA gene sequence similarities with those in the EMBL database

Strain	*N*-source	MPN dilution	Position 5′ → 3′	Reference accession number and strain	DNA sequence similarity (%)
Zoogloea VPMK8	Dichlobenil	10^{-1}	8-908	X74914 *Zoogloea ramigera*	99.5
Pseudomonas VPMK9	Dichlobenil	10^{-1}	8-908	AB021401 *Pseudomonas marginalis*	99.8
Xanthomonas VPMK10	Dichlobenil	10^{-1}	8-908	AE012540 *Xanthomonas campestris*	99.9
Zoogloea VPMK12	BAM	10^{-1}	8-908	X74914 *Zoogloea ramigera*	99.6
Ralstonia VPMK13	BAM	10^{-1}	8-908	AF312022 *Ralstonia basilensis*	99.7
Rhodococcus VPMK15	Dichlobenil	10^{-6}	8-908	AF532870 *Rhodococcus erythropolis*	100.0
Nocardioides VPMK18	Dichlobenil	10^{-6}	8-908	U82666 *Nocardioides simplex*	100.0
Sphingomonas VPMK20	Dichlobenil	10^{-6}	8-518	AF131295 *Sphingomonas* sp.	100.0
Rhodococcus VPMK17	BAM	10^{-6}	8-908	AF532870 *Rhodococcus erythropolis*	100.0

The positions (*Escherichia coli* 16S-numbering) of the 16S rRNA gene sequences are presented

cultivated in the MPN medium, and the BAM degradation as the nitrogen source of 13.5 ± 4.0% in comparison to the controls was verified by the HPLC analysis. In aquifer E, according to the 16S rRNA gene sequencing, the bacteria degrading BAM in the 10^{-1} dilution were from the genera *Zoogloea* and

Ralstonia, while a strain from the genus *Rhodococcus* was isolated from the 10^{-6} dilution (Table 3). Interestingly, the bacterium from the genus *Zoogloea* degrading both dichlobenil and BAM was isolated from the 10^{-1} MPN dilution, and the bacterium from the genus *Rhodococcus* degrading both compounds was isolated from the 10^{-6} dilution. The two previously reported BAM degraders from the genus *Aminobacter* have been isolated from topsoil (Simonsen et al. 2006; Sørensen et al. 2007).

Conclusions

Based on the MPN enumeration, microbes able to degrade dichlobenil and BAM were commonly present in the surface soil, and all the sub surface deposits collected from five different aquifers in the northern boreal region, South Finland (Tables 1, 2). The obtained MPN values were low, 3.6–210 MPN gdw^{-1}, although in some of the MPN dilution series an interesting pattern was observed: dichlobenil and BAM were also degraded in the high dilutions, the MPN numbers being 1.1×10^4–1.2×10^7 MPN gdw^{-1}. However, the remarkable differences in the microbial MPN numbers did not affect the amount of pesticide or its metabolite degraded. When positive degradation was observed, it was always in the range of $45.5 \pm 18.3\%$ for dichlobenil and $37.6 \pm 14\%$ for BAM.

The dichlobenil and BAM degrading bacteria in the 10^{-1} MPN dilution differed from those isolated from the 10^{-6} dilution in the groundwater well E deposit (Table 3). In the 10^{-1} dilution, *Zoogloea*, *Pseudomonas* and *Xanthomonas* degraded dichlobenil, and *Zoogloea* and *Ralstonia* degraded BAM, while in the 10^{-6} dilution *Rhodococcus*, *Nocardioides*, and *Sphingomonas* degraded dichlobenil, and *Rhodococcus* degraded BAM. The microbial strains present in the low MPN dilutions could prevent the activity of dichlobenil or BAM degrading strains until diluted out, leading to two different MPN values. Similarly, the conditions in the groundwater environment (Table 2) likely prevent the BAM degradation, even though the samples clearly contained microbial BAM metabolizing activity.

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