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Anaerobic utilization of essential oils by denitrifying bacteria

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Abstract

Plant volatile organic compounds are a major carbon source in nature. We studied the degradability of these substances by anaerobic microorganisms in enrichment cultures with representative essential oils as organic substrates and nitrate as electron acceptor. Lemon and pine needle oil supported microbial growth in the presence of pure oil, whereas parsley seed, camphor, sage, fennel, and mint oil supported growth only when the essential oils were dissolved in an overlying phase of 2,2,4,4,6,8,8-heptamethylnonane. Thyme oil did not support denitrification. Analyses of the microbially degraded oils revealed the disappearance of monoterpenes, of several monoterpenoids, and of methoxy-propenyl-benzenes, including apiole and myristicin. Most-probable-number determinations for denitrifying communities in sewage sludge and forest soil yielded 10^6 to 10^7 monoterpene-utilizing cells ml^{-1}, representing 0.7 to 100% of the total cultivable nitrate-reducing microorganisms. The utilization of essential oils together with the common occurrence of this metabolic trait are indications for an environmentally important, but currently unexplored anaerobic turnover of plant volatile organic compounds in soil.

Introduction

Plants produce volatile organic compounds (VOCs) that diffuse into the atmosphere and the soil. The former receives annually 1.5×10^{15} g carbon in the form of VOCs (Günther et al. 1995), representing more than 2% of the new plant biomass (Bolin 1983). Litter is a major source for VOCs in soil, e.g., the top horizon of soil in a pine forest consists of fresh litter with 1.4 mg of monoterpenes g^{-1} and the mineral layer below the humus contains 0.6 μg g^{-1} (White 1994). The release from roots has not been studied, but larvae of the pine weevil *Hylobius abietis* move through soil towards roots by sensing sources of α-pinene (Nordenhem & Nordlander 1994).

Steam distillation of plant parts yields a major fraction of VOCs, the essential oils. These mixtures contain mainly monoterpenes and monoterpenoids together with some aromatic compounds and sequiterpenes. Primarily synthesized monoterpenes are unsaturated hydrocarbons that are also further oxidized in plants by oxygenases (Karp et al. 1987; Lupien et al. 1999). The products, monoterpene alcohols and ketones, are named monoterpenoids. Monoterpenes contribute together with isoprene to the thermal tolerance of plants (Sharkey & Singsaas 1995). Another important physiological function of monoterpenes and monoterpenoids is the defence against herbivores: plants induce the synthesis of these compounds upon insect damage (Gershenzon & Croteau 1991; Paré & Tumlinson 1997). Essential oils are used as flavours, fragrances and pharmaceutical products. The latter utilization is based on antimicrobial properties (Janssen et al. 1987; Billing & Sherman 1998; Hulin et al. 1998). The toxicology of monoterpenes and monoterpenoids, as defined by studies with aerobic organisms, has shown that monoterpenoids are more toxic than monoterpenes. This is presumably due to a higher bioavailability caused by a higher water solubility (Weidenhamer et al. 1993) and the presence of

alcohol and ketone groups which can react with proteins. Membranes have been identified as one target site where monoterpenes act (Sikkema et al. 1995). The presence of monoterpenes in forest litter may cause, according to recent studies, an ecologically important inhibition of ammonium and methane oxidation (White 1988; Ward et al. 1997; Amaral et al. 1998).

Monoterpenes and monoterpenoids are known to be anaerobically biodegradable since a few years (Harder & Probian 1995, reviewed in Hylemon & Harder 1999). In this study, we investigate, to our knowledge for the first time, the biodegradability of representative essential oils under anoxic, denitrifying conditions using sewage sludge, forest ditch mud, or *Alcaligenes defragrans* as inocula. *Alcaligenes defragrans* is the first bacterium isolated that mineralizes monoterpenes anaerobically (Harder & Probian 1995; Foß et al. 1998). Monoterpenes and monoterpenoids are also degraded by species of the genera *Thauera*, *T. linaloolentis* and *T. terpenica* (Harder & Probian 1995; Foß & Harder 1998). The contribution of bacteria related phylogenetically to *Alcaligenes defragrans* and *Thauera* to the community in enrichment cultures was determined by whole cell in situ hybridization with fluorescent 16S rRNA-targeted oligonucleotide probes. The population size of denitrifying monoterpene-utilizing bacteria was appraised to test whether the metabolic capacity is common in nature.

Materials and methods

Materials and culture conditions

Natural oils (Italian lemon oil, pine needle oil, parsley seed oil, camphor oil, Dalmatian sage oil, fennel oil, mint oil (China), and thyme oil) were obtained from Roth, Karlsruhe, Germany. GC-MS analyses of the essential oils confirmed the analyses published by Formácek & Kubeczka (1982). Activated sewage sludge was sampled from a local wastewater treatment plant (Lintel, Osterholz-Scharmbeck), and a mud-ditch water slurry was collected in a forest near Bremen. *Alcaligenes defragrans* strain 54Pin^T was from our laboratory culture collection. Experiments performed with the species included a chemical reductant, 4 mM of ascorbate, that is not metabolized by the bacteria (Foß et al. 1998). Due to the low solubility, all presented monoterpene concentrations are calculated values based on the monoterpene present in the culture flask and the size of the aqueous phase in the experiment.

Anoxic media and cultivation techniques were used as described (Widdel & Bak 1992; Foß et al. 1998). Enrichment cultures on essential oils were attempted with 5 ml of activated sludge or 20 ml of a mud-ditch water slurry from a forest ditch as inoculum in 250 ml-round bottle flasks that contained 96 or 240 μl of essential oil and 150 ml of the ascorbate-free freshwater medium described in Foß et al. (1998). The amount of 2,2,4,4,6,8,8-heptamethylnonane (HMN) was 0 or 5 ml. Control samples with 240 μl of oil, 5 ml of HMN and 150 ml of medium were performed in parallel to account for physical and chemical losses. The cultures were incubated for four months at 28 °C in the dark. They were not shaken to allow initially the transient formation of a gradient of oil components from the lighter organic phase to the mud on the bottom of the culture bottle. Overpressure due to gas formation was determined regularly with a gas-tight syringe and depletion of nitrate (initially 10 mM) was observed with a semi-quantitative indicator strip (Merck, Darmstadt, Germany). Upon electron acceptor depletion, 10 mM of nitrate were re-established in the enrichment culture by addition of nitrate from an anoxic stock solution (5 M).

Alcaligenes defragrans 54Pin^T was cultured in 21 ml-tubes containing 15 ml of medium, 10 μl of essential oil, and 0 or 1 ml of HMN. Inoculation occurred with 400 μl of a culture recently grown on α-pinene.

The size of denitrifying populations in activated sewage sludge and in the upper soil layer (0-5 cm) of a local forest near Bremen was estimated with most-probable-number dilutions in liquid medium (APHA 1969). MPN counts were performed in steps of a ten-fold dilution with three replicates per dilution. Each portion contained 10 ml of freshwater medium including 10 mM of nitrate (Foß et al. 1998) and (i) a mixture of acetate, butyrate, succinate, lactate and ethanol (1 mM of each compound), (ii) a mixture of eucalyptol, 2-carene, α-pinene, (+)-sabinene and α-terpinene (0.3 mM of each compound in totally 0.4 ml of HMN), or (iii) 1.5 mM of α-terpinene in 0.4 ml of HMN. The MPN culture tubes were incubated for eight weeks at 20 °C in the dark. They were placed on a rotary shaker (60 rpm) in an inverted position with an angle of fifteen degrees above the horizontal to improve the bioavailability of the monoterpene.

Chemical analyses

Nitrate and nitrite were determined quantitatively by HPLC (Harder & Probian 1995). Essential oils and monoterpenes were extracted with hexane and were analysed by capillary-column GC with a Perkin-Elmer Autosystem gas chromatograph (Überlingen, Germany) equipped with flame ionisation detectors, a CTC A200S autosampler and a Turbochrom digital data-analysing system. Compounds were separated by using an Optima-5 column (0.32 mm by 50 m, 0.25 μm film thickness, Macherey-Nagel, Germany), H_2 at a flow rate of 2 ml min^{-1}, and the following temperature program: injection port temperature, 270 °C; column temperature, 60 °C for 2 min, increasing to 140 °C at a rate of 4 °C min^{-1}, increasing to 320 °C at a rate of 40 °C min^{-1}, 320 °C for 5 min; flame ionization detector temperature 350 °C. GC-MS was performed as described by Harder & Probian (1995). Compound identification was based on relative retention times, mass spectra and, in several cases, comparison with authentic standards.

In situ hybridization with oligodeoxynucleotide probes

The presence of *Alcaligenes defragrans* in enrichment cultures was detected with 16S rRNA-targeted oligodeoxynucleotide probes that were labelled with the fluorescent dye Cy3.18 (Biological Detection Systems, Pittsburgh, PA). In a nested approach (Amann et al. 1995), probes EUB338 for *Bacteria*, BET42a with a non-labelled competitor probe, GAM42a, for *Proteobacteria* of the beta subclass, AT1458 for the genera *Thauera* and *Azoarcus* plus *Alcaligenes defragrans*, and the species-specific probe Ade441 for *Alcaligenes defragrans* were applied as described (Manz et al. 1992; Rabus et al. 1999).

Results

Enrichments of denitrifying bacteria on essential oils

Essential oils vary in their chemical composition from oils containing only monoterpenes to oils with a large amount of monoterpenoids (Formácek & Kubeczka 1982). We selected lemon, pine needle, and parsley seed oil as examples of the former group, and camphor, sage, fennel, mint, and thyme oil as representatives of the latter group. The essential oils served as the only electron donors in attempts to enrich denitrifying bacteria from sewage sludge or a forest ditch mud sample. We enriched also on essential oils diluted in 2,2,4,4,6,8,8-heptamethylnonane that is nearly water-insoluble, not biodegradable, and not poisonous, in order to reduce detrimental effects of the essential oils (Alexander 1994).

Several enrichment cultures started denitrification within a few days: six of eight cultures with sewage sludge utilizing pine needle or lemon oil consumed 10 mm of nitrate within four days. Forest ditch mud was an inferior inoculum in comparison to sewage sludge. The slowest enrichment rate was observed with the mud: 53 days were required to consume 10 mm of nitrate on camphor or sage oil. The incubation was continued for totally four months, but further enrichment culture did not grow. Lemon and pine needle oil enabled growth of enrichment cultures from sewage sludge in the absence of an organic carrier phase. To the opposite, thyme oil did not support denitrification even in the presence of HMN. Other essential oils containing monoterpenoids were not toxic when diluted into HMN, e.g., sage and mint oil were excellent carbon sources for enrichment cultures (Table 1).

GC and GC-MS analyses of the essential oils, and GC analyses of the residual essential oil after microbial growth enabled the identification of compounds that were biologically consumed or formed. The analyses for samples with sewage sludge as inoculum and HMN are summarised in Table 2. Myrcene was the least degradable monoterpene in lemon oil, whereas camphene and α-thujene were recalcitrant in pine needle oil. Camphene was also fractious in camphor and sage oil. Parsley seed oil is characterised by the aromatic insecticides apiole and myristicin. These antibiotics and two related compounds, elemicin and 1,2,3,4-tetramethoxy-5-(2-propenyl)-benzene (1,2,3,4-TMPB), were depleted in the enrichment cultures. The organic phase recovered from enrichment cultures on camphor oil corresponding to 10 mM of monoterpene contained slightly increased amounts of myrcene, α-phellandrene, α-terpinene, cymene, *trans*-ocimene, and γ-terpinene. Monoterpenoid consumption from mint oil resembled the substrate range of *Thauera terpenica* strain 21Mol (Foß & Harder 1998). Other major monoterpenoids degraded were bornyl acetate, eucalyptol, α-terpineol, 4-terpineol, α-thujone, β-thujone, camphor, and borneol. Methoxy-propenyl-benzenes, estragol, *cis*- and *trans*-anethole were partially depleted from

Table 1. Essential oils as growth substrates for enrichment cultures and *Alcaligenes defragrans*.

Essential oil	Activated sludge as inoculum				Forest ditch mud as inoculum				*A. defragrans* 54Pin^T	
	− HMN[a]	− HMN[b]	+ HMN[a]	+ HMN[b]	− HMN[c]	− HMN[d]	+ HMN[c]	+ HMN[d]	− HMN[e]	+ HMN[e]
Lemon oil	+	++	++	+++	–	–	+	+++	+++	+++
Pine needle oil (*Pinus*)	+	++	++	++	–	–	+	++	++	+++
Parsley seed oil	–	–	–	+++	–	–	–	–	+	+++
Camphor oil	–	–	+	++	–	–	+	–	+	+
Sage oil	–	–	++	+++	–	–	–	+	±	±
Fennel oil	–	–	–	+	–	–	–	–	–	±
Mint oil	–	–	++	++++	–	–	–	–	–	±
Thyme oil	–	–	–	–	–	–	–	–	–	±

[a] 96 μl essential oil, corresponding to 0.62‰ vol essential oil/vol aqueous phase and to 4 mM monoterpene; 3.2% vol HMN/vol aqueous phase.
[b] 240 μl essential oil, corresponding to 1.55‰ vol essential oil/vol aqueous phase and to 10 mM monoterpene; 3.2% vol HMN/vol aqueous phase.
[c] 96 μl essential oil, corresponding to 0.56‰ vol essential oil/vol aqueous phase and to 4 mM monoterpene; 2.9% vol HMN/vol aqueous phase.
[d] 240 μl essential oil, corresponding to 1.41‰ vol essential oil/vol aqueous phase and to 10 mM monoterpene; 2.9% vol HMN/vol aqueous phase.
[a–d] Nitrate concentrations were monitored. Upon electron acceptor depletion, 10 mM nitrate were added. Each (+) indicates the consumption of 10 mM nitrate by the culture. Endogeneous electron donors present in the inoculum caused according to control experiments the consumption of less than 10 mM nitrate. Heptamethylnonane did not serve as electron donor.
[e] 0.65‰ vol essential oil/vol aqueous phase, corresponding to 4 mM monoterpene; 6.5% vol HMN/vol aqueous phase. Consumption of nitrate (10 mM) occurred not at all (−), up to 20% (±), up to 50% (+), more than 50% (++) or completely (+++).

fennel oil. Traces of limonene disappeared from thyme oil.

Denitrification of 10 mM of nitrate is coupled to the mineralization of 1.2 to 1.5 mM of monoterpene, according to pure culture studies (Foß & Harder 1997; Foß et al. 1998; Foß & Harder 1998). We tested two amounts of essential oil, 96 and 240 μl. The lower amount, corresponding to 4 mM of monoterpene, was expected to limit nitrate reduction closely to 30 mM of nitrate and to select for consumption of all substances present in the oil. The higher amount, corresponding to 10 mM of monoterpene, was anticipated to reveal the compounds preferentially consumed. Nitrate reduction of the enrichment cultures correlated with the amount of oil supplied; the consumption of 40 mM of nitrate occurred only in the presence of 240 μl of oil (Table 1). A large nitrate consumption (Table 1) correlated with a low recovery of oil compounds (Table 2).

The cells grown in these enrichment cultures were characterised by fluorescent in situ hybridization in a nested approach, in order to identify the growth success of bacteria that are phylogenetically closely related to *Alcaligenes defragrans* (Amann et al. 1995). Bacteria detectable with the oligonucleotide probes AT1458 and Ade441 that are specific for the genera *Azoarcus* and *Thauera*, and for *Alcaligenes defragrans* (Rabus et al. 1999) were found in enriched communities, but did dominate only a few communities (Table 3).

Growth of Alcaligenes defragrans *on essential oils*

The aforementioned essential oils were tested as growth substrates for *Alcaligenes defragrans*. Nitrate reduction of *Alcaligenes defragrans* strain 54Pin^T occurred on all essential oils in the presence of an organic carrier phase (Table 1). Monoterpenes that were previously identified as growth substrates for the strain 54Pin^T were depleted in all oils except mint and thyme oil (data not shown). Compounds present in these oils apparently inhibited the consumption of monoterpenes. The antimicrobial properties of thyme, mint, fennel, and parsley seed oil are also evident from reduced nitrate consumption in cultures containing no HMN (Table 1).

Population size of monoterpene-mineralising denitrifying microorganisms

Most-probable-number estimations with a mixture of primary fermentation products, a mixture of monoterpenes, and α-terpinene were performed to quantify the cultivable denitrifying bacteria and the monoterpene-utilizing subpopulation (Table 4). The physiological

Table 2. Biological consumption of essential oils by denitrifying enrichment cultures.

Essential oil/ compound	Content[a] (%)	Recovery after incubation (%)[b] 4 mM	10 mM
Lemon oil			
α-pinene	1.3	0	n.d.[c]
sabinene	1.3	1	n.d.
β-pinene	11.5	0	n.d.
myrcene	0.7	18	n.d.
limonene	71.7	0	n.d.
γ-terpinene	9.0	0	n.d.
neral	0.6	0	n.d.
geranial	0.3	0	n.d.
geranyl formate	0.3	3	n.d.
geranyl acetate	0.3	5	n.d.
Σ	97.0	0	
Pine needle oil			
α-thujene	0.2	68	112
α-pinene	35.7	0	60
camphene	4.5	28	109
β-pinene	28.4	0	47
myrcene	2.4	0	83
3-carene	4.2	1	86
cymene	2.6	0	82
limonene	11.5	0	34
γ-terpinene	0.2	1	88
terpinolene	1.2	0	16
α-terpineol	0.2	0	0
bornyl acetate	1.9	1	34
Σ	93.0	1	53
Parsley seed oil			
α-pinene	22.4	5	84
β-pinene	15.4	4	81
cymene	1.3	21	57
eucalyptol	3.3	44	47
cresol	2.4	6	132
α-terpineol	2.4	0	1
camphor	0.4	0	0
myristicin	29.8	7	0
elemicin	4.7	1	1
1,2,3,4-TMPB	3.1	0	0
apiol	9.3	0	0
Σ	94.5	6	37

Table 2. Continued.

Essential oil/ compound	Content[a] (%)	Recovery after incubation (%)[b] 4 mM	10 mM
Camphor oil			
α-pinene	1.2	3	92
camphene	0.4	58	110
sabinene	0.6	5	76
β-pinene	0.9	3	96
myrcene	1.0	12	154
α-phellandrene	0.3	11	157
α-terpinene	0.6	0	297
cymene	2.0	1	125
eucalyptol	33.5	0	21
trans-ocimene	0.3	74	277
γ-terpinene	1.2	10	131
terpinolene	2.2	0	87
endo-fenchol	11.4	0	0
camphor	32.1	0	107
borneol	0.6	0	11
4-terpineol	5.6	2	0
α-terpineol	2.9	0	0
trans-isosafrole	1.5	0	0
Σ	98.3	1	51
Sage oil			
α-thujene	0.6	3	31
α-pinene	2.8	1	14
camphene	6.3	38	108
sabinene	0.8	0	2
β-pinene	0.7	0	19
myrcene	0.6	13	62
α-terpinene	0.7	6	151
cymene	6.3	0	6
eucalyptol	9.8	0	0
terpinolene	0.8	0	10
α-thujone	21.8	0	0
β-thujone	4.9	0	1
camphor	27.5	0	57
borneol	1.8	0	0
4-terpineol	0.5	31	7
bornyl acetate	1.8	0	13
sabinyl acetate	1.0	0	4
Σ	88.7	3	25

Table 2. Continued.

Essential oil/ compound	Content[a] (%)	Recovery after incubation (%)[b] 4 mM	10 mM
Fennel oil			
α-pinene	2.3	57	26
myrcene	0.3	78	90
limonene	4.0	41	32
γ-terpinene	0.4	84	104
fenchone	4.8	101	111
fenchyl acetate	0.5	0	0
α-terpineol	1.7	0	0
estragol	5.1	40	119
cis-anethole	1.0	41	107
trans-anethole	74.3	65	91
Σ	94.4	58	83
Mint oil			
α-pinene	0.9	5	108
β-pinene	1.1	3	104
myrcene	0.5	4	110
3-octanol	0.6	0	0
limonene	2.3	0	93
isopulegol	0.9	0	0
menthone	20.4	0	67
isomenthone	11.7	0	37
neomenthol	4.1	0	11
menthol	46.1	0	3
isomenthol	1.4	0	0
α-terpineol	0.5	0	15
pulegone	0.3	0	2
peritone	1.0	0	0
menthyl acetate	0.6	1	68
Σ	92.4	1	25

Table 2. Continued.

Essential oil/ compound	Content[a] (%)	Recovery after incubation (%)[b] 4 mM	10 mM
Thyme oil			
α-pinene	0.2	94	105
camphene	0.3	93	104
myrcene	0.3	92	108
3-carene	0.4	94	107
cymene	42.6	86	110
limonene	0.9	15	18
γ-terpinene	3.7	91	115
terpinolene	1.4	89	114
endo-fenchol	5.1	77	115
borneol	1.5	72	107
α-terpineol	2.3	81	107
thymol	33.5	86	109
carvacrol	4.6	81	108
Σ	96.8	82	106

[a] Essential oil composition was analysed by GC with a flame ionization detector and individual contents were calculated from peak areas. Sequiterpenes present in several oils could not unambiguously be identified and were not included in the table.
[b] Enrichment cultures containing 96 μl or 240 μl of essential oil – correspondingly 4 or 10 mM monoterpene, respectively – were incubated together with chemical control experiments that included 240 μl of oil, 5 ml of HMN and 150 ml of anoxic fresh water medium. After incubation, all cultures were extracted with 6 ml of hexane and the organic phase was analysed by GC-FID. Peak areas of individual compounds recovered from the control experiments were set to 100%. The standard deviation of GC determinations was $\pm$ 5%.
[c] n.d. not determined. A hexane phase was not recovered due to a high surfactant activity of the culture.

capacity was widespread: one of one-hundred-and-fifty nitrate-reducing bacteria present in sewage sludge and each denitrifying microorganism in coniferous soil grew anaerobically on the natural unsaturated hydrocarbons.

Discussion

In nature, a concentration gradient of plant volatile organic compounds is present from the releasing plant part to its environment. Now we tested similar conditions with essential oils as substitutes for plant volatiles in denitrifying enrichment cultures. Microbial communities grew within one to eight weeks of incubation on several essential oils, with the exception of thyme oil. The biological consumption of substances of the essential oils was determined by GC analyses in comparison with non-inoculated, incubated control experiments. These controls cover the diffusion into the butyl stopper of the flask. In the absence of an organic carrier phase, Dolfing et al. (1990) observed after an incubation of two weeks a recovery of 69% of [^{14}C]-toluene in the stopper. The presence of an organic phase reduces the loss, e.g., we observed after an incubation time of 105 days a monoterpene recovery of 90% in non-inoculated samples and of 75% in experiments with autoclaved inocula (Harder & Foß 1999). In this study we observed in the absence of HMN, but not in the presence of HMN, low recovery rates of monoterpenes in samples with a negligible biological activity (data not shown). Hence we selected the samples with HMN in order to identify sub-

Table 3. Detection of *Alcaligenes defragrans* in growing[a] denitrifying enrichment cultures on essential oils by fluorescent in situ hybridization.

Essential oil	Amount of oil	Presence of HMN	Probe-positive cells/DAPI-stained cells (%)			
			EUB338	BET42a	AT1458	Ade441
Activated sludge as inocululum						
Lemon oil	96 μl	–	67	33	11	12
	240 μl	–	91	74	44	77
	96 μl	+	75	25	27	24
	240 μl	+	82	59	10	9
Pine needle oil (*Pinus*)	96 μl	–	66	3	0	0
	240 μl	–	68	46	20	43
	96 μl	+	67	26	22	17
	240 μl	+	92	47	5	25
Camphor oil	96 μl	+	56	34	32	26
	240 μl	+	62	22	11	6
Parsley seed oil	240 μl	+	72	50	48	44
Mint oil	96 μl	+	85	20	9	0
	240 μl	+	85	37	36	11
Sage oil	240 μl	+	28	3	0	2
Forest ditch mud as inocululum						
Lemon oil	96 μl	+	89	35	4	0
	240 μl	+	69	51	0	5
Pine needle oil	96 μl	+	52	51	5	11
	240 μl	+	66	50	11	24
Camphor oil	96 μl	+	63	40	14	12
Sage oil	240 μl	+	28	3	0	2

[a] All cultures had consumed more than 10 mM nitrate (see Table 1). Ten cultures with lower nitrate consumption were tested by FISH. Nine of these enrichments showed a low hybridization with AT1458 and Ade441: $\leq$ 72% for EUB338, $\leq$ 30% for BET42a $\leq$ 12% for AT1458, and $\leq$ 12% for Ade441.The exception was the enrichment on 96 μl parsley seed oil containing activated sludge and HMN: 100% cells hybridized with EUB338, 85% with BET42a, 65% with AT1458, and 87% with Ade441.

stances utilized (Table 2). The compounds included growth substrates for pure strains (*Alcaligenes defragrans*, *Thauera terpenica*, and *Thauera linaloolentis* (Foß et al. 1998; Foß & Harder 1998)) and enrichment cultures already established (reviewed by Hylemon & Harder 1999) as well as substances that have not been shown to be biodegradable under anoxic conditions, notably mono- and poly-methoxy-propenyl-benzenes. Small absolute increases of some monoterpenes were only observed in a culture on camphor oil, similar to earlier observations in enrichment cultures (Harder & Probian 1995). Quantitative formation of cymene, as observed under methanogenic enrichment conditions (Harder & Foß 1999), or of dead-end metabolites, e.g., isoterpinolene (Heyen & Harder 1998), did not occur.

The toxicology of monoterpenes and monoterpenoids towards anaerobic bacteria seems to resemble that towards aerobic microorganisms. Growth on lemon and pine needle oil in the absence of HMN strongly suggests that monoterpenes are less toxic than monoterpenoids. Enrichment cultures on essential oils with monoterpenoids required reduced concentrations of the compounds. This was achieved by dilution into HMN. The toxic compounds may be polymethoxy-propenyl-benzenes in parsley seed oil, *p*-methoxy-propenyl-benzenes and fenchone in fennel oil, and different monoterpenoids in camphor, sage and mint oil. Thyme oil that was not attacked contains as major constituents the aromatic cymene and the phenolic thymol. Recently, *Azoarcus* sp. strain pCyN1 and *Thauera* sp. strain pCyN2 were isolated on cymene under denitrifying conditions (Harms et al. 1999). These strains tolerated a HMN phase with 10% v/v cymene. In our experiments, cymene represented up to 2.4% v/v of the organic phase. In addition, Knobloch et al. (1986) identified thymol as one of the most toxic

Table 4. MPN determination of denitrifying bacteria

Carbon source and electron donor	Population size of denitrifying bacteria	
	Activated sludge (cells ml^{-1})[a]	Coniferous soil (cells ml^{-1})[a]
Acetate, butyrate, succinate, lactate and ethanol	4.7 (0.7–14) $\times 10^8$	1.0 (0.2–2.9) $\times 10^7$
α-Terpinene, sabinene, 2-carene, α-pinene and eucalyptol	3.1 (0.5–16) $\times 10^6$	1.6 (0.2–8.7) $\times 10^6$
α-Terpinene	3.1 (0.5–16) $\times 10^6$	1.6 (0.2–8.7) $\times 10^7$

[a] Values in parentheses describe 95% confidence limits.

monoterpenoids. Hence, the failure to enrich on thyme oil may be caused mainly by the biocidal property of thymol. However, the degradation of thymol by aerobic bacteria has been reported involving oxygenation reactions (Dagley 1971). The mud-ditch mixture from a forest was an inferior inoculum. Enrichment cultures on lemon and pine needle oils developed only in the presence of an HMN phase. This provides evidence for the presence of toxic compounds present in the mud. An alternative explanation, the absence of denitrifying bacteria that are resistant to a pure monoterpene phase seems unlikely in a presumably monoterpene-rich habitat.

The growth of denitrifying communities on essential oils suggests that plant volatiles released from roots or litter in the soil can serve as carbon sources for anoxic soil bacteria. To support this opinion, we determined the microbial population able to grow on monoterpenes with the most-probable-number technique. Earlier we demonstrated that primary fermentation products as substrate support the whole cultivable denitrifying population (Kniemeyer et al. 1999). Menthadienes together with an organic carrier phase were used to minimize toxic effects. The large populations of monoterpene-degrading denitrifying bacteria that were found in sewage sludge and forest soil may represent a biological response to the huge participation of monoterpenes in the global carbon cycle. Additional evidence for a wide distribution of the metabolic capability was provided by FISH analyses of the enrichment cultures: the denitrifying cultures were not dominated by β-proteobacteria related to the isolated denitrifying species *Alcaligenes defragrans*, *Thauera terpenica*, and *Thauera linaloolentis*.

A large population of monoterpene-degrading microorganisms is expected to support a short lag phase in the first enrichment culture. In this study, the development of enrichment cultures required less than ten days (activated sludge and lemon or pine needle oil) to six to eight weeks (forest mud and pine needle or sage oil). Enrichment on pure compounds diluted in HMN were reported to require ten days to three weeks for several monoterpenes and monoterpenoids (Harder & Probian 1995), four weeks with cymene (Harms et al. 1999), three to 14 weeks for alkylated benzenes (Rabus & Widdel 1995, Harms et al. 1999) and six weeks for hexane, heptane or octane (Ehrenreich et al. 2000). Although different inocula were used in these studies, the fastest growth on monoterpenes and monoterpenoids agrees with a large degrading community in nature.

Conclusion

Research in chemical ecology of plant volatiles, notably the monoterpenes, was in the past focused on plant-eucaryal herbivore interactions. Now our study indicates that even anaerobic bacteria are capable of utilizing plant volatiles and that this metabolic ability is common. It seems desirable to study in more detail the ecological interactions between plants, soil microbes, monoterpenes and other volatiles, and the regulation of biological processes that are inhibited by the volatiles, e.g., aerobic methane and ammonia oxidation.

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