Biodegradation (2009) 20:85–94
DOI 10.1007/s10532-008-9202-6

ORIGINAL PAPER

Biodegradability of 2-ethylhexyl nitrate (2-EHN), a cetane improver of diesel oil

Floriane Solano-Serena · Elodie Nicolau · Grégory Favreau · Yves Jouanneau · Rémy Marchal

Received: 23 October 2007 / Accepted: 11 June 2008 / Published online: 22 June 2008

Abstract The 2-ethyhexyl nitrate (2-EHN) is currently added to diesel oil to improve ignition and boost cetane number. The biodegradability of this widely used chemical needed to be assessed in order to evaluate the environmental impact in case of accidental release. In aerobic liquid cultures, biodegradation of 2-EHN was assessed in biphasic liquid cultures using an inert non-aqueous phase liquid such as 2,2,4,4,6,8,8-heptamethylnonane (HMN) as solvent for the hydrophobic substrate. 2-EHN was found to be biodegradable by microbial communities from refinery wastewater treatment plants, but was recalcitrant to those of urban wastewater treatment facilities. Out of eighteen hydrocarbon-polluted or non-polluted soil samples, six microbial populations were also able to degrade 2-EHN. However, strain isolation from these microbial populations was rather difficult suggesting close cooperation between members of the microbial communities. Specific axenic bacterial strains selected for their ability to catabolize recalcitrant-hydrocarbons were also tested for their capacity to degrade 2-EHN. In liquid cultures with HMN phase as non-aqueous phase liquid, some *Mycobacterium austroafricanum* strains were found to degrade and mineralize 2–EHN significantly.

Keywords 2-Ethylhexyl nitrate · Biodegradation · Mineralization · Soil microbial population · *Mycobacterium*

Abbreviations

2-EHN	2-Ethylhexyl nitrate
MTBE	Methyl *tert*-butyl ether
NOAEL	No observed adverse effect level
HMN	2,2,4,4,6,8,8-Heptamethylnonane
NAPL	Non-aqueous phase liquid
WWTP	Wastewater treatment plant

Introduction

The 2-Ethyhexyl nitrate (2-EHN), the nitric acid ester of 2-ethyl-1-hexanol (Fig. 1), is currently added in significant amounts (0.05%–0.4%) to diesel oil to improve ignition and boost cetane number (Guibet and Faure 1999; Bornemann et al. 2002). 2-EHN is a large-scale commodity, the worldwide production of

F. Solano-Serena (✉) · G. Favreau
Groupement de Recherche Eau Sol Environnement, EA 1068 Ecole Nationale d'Ingénieurs de Limoges, Université de Limoges Parc ESTER Technopole, 87068 Limoges Cedex, France
e-mail: leglise@ensil.unilim.fr

E. Nicolau · R. Marchal
Département de Biotechnologie, Institut Français du Pétrole, 92852 Rueil-Malmaison Cedex, France

Y. Jouanneau
Laboratoire de Chimie et Biologie des Métaux, iRTSV CEA, CNRS, UMR 5249, Université Joseph Fourrier, 38054 Grenoble, France

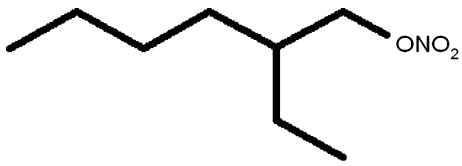

Fig. 1 Molecular structure of 2-Ethylhexyl nitrate ($C_8H_{17}NO_3$). Main physico-chemical properties (ATC 2006): vapor pressure at 20°C: 27Pa; solubility in water at 20°C: 12.6 mg l^{-1}; $\log K_{o/w}$: 5.24; liquid density: 0.96

which is estimated to be about 100,000 tons per year. It has long been considered as presenting no particular risk to human health. 2-EHN was also non-mutagenic according to the Ames test. Upon acute oral or dermal administration to mammals, it seemed relatively non-toxic (ATC 2006). Nevertheless, 2-EHN has been found to be significantly more harmful when administered in repeated doses. The No Observed Adverse Effect Level (NOAEL) in rats over a 28-day period was only 28 mg kg^{-1} d^{-1} (Someroja and Savolinen 1983). The low NOAEL level indicates that biodegradability may be a key factor in the overall 2-EHN environmental risk assessment. Indeed, in the case of accidental release biodegradability will determine the residual concentration in the environment and exposure may become chronic if no degradation occurs.

Under environmental conditions, many organic contaminants are biodegraded by local indigenous microorganisms (Roling and van Verseveld 2002). Removal of contaminants depends on the intrinsic biodegradability of molecules, on physical parameters existing on site, such as temperature and oxygen (Morgan and Watkinson 1989; Leahy and Colwell 1990) and on degradative capacity of local microbial populations. The biodegradation of contaminating molecules often involves cometabolism and cooperation between microorganisms (Marchal et al. 2003). In addition, strains with particular metabolic capacities, such as *Mycobacterium* or *Rhodococcus* bacteria (Sakai et al. 2004; Bogan et al. 2003; Ferreira et al. 2006), are frequently involved in the biodegradation of recalcitrant-hydrocarbons.

The intrinsic biodegradability of organic pollutants such as 2-EHN has to be determined since it is a key element in risk assessment. It is usually assessed using laboratory tests derived from those designed for commercial products (Strotmann et al. 2004). However, as indicated by Battersby et al. (1999), current test guidelines designed for water-soluble, organic compounds with low volatility are unsuitable for most oil products. Tests have therefore been specifically designed for oil products (Battersby et al. 1999); they are performed using a pre-exposed inoculum with a duration of three months. Specific tests involving an oil-degrading microbial population and biological additives to enhance biodegradation have also been designed for particular products, such as bioremediation additives (Haines et al. 2005).

The intrinsic biodegradability of 2-EHN has received little investigation. 2-EHN was not found to be readily biodegradable in the standard CO_2 headspace test since the yield of biodegradation failed to attain 60% within 28 days (ATC 2006). As little information was available on aerobic biodegradation of 2-EHN, our study aimed at evaluating the intrinsic biodegradability of 2-EHN under different laboratory conditions. For this purpose, microbial populations from wastewater treatment plants and from soil were used. 2-EHN biodegradation by some *Actinobacteria* species was also investigated because of the recognized ability of these microorganisms to attack recalcitrant molecules.

Materials and methods

Culture medium

The vitamin-supplemented mineral salts medium described by Bouchez et al. (1995) was used as nutrient solution. The solution was autoclaved at 121°C for 20 min without vitamins, which were sterilized by filtration at 0.22 µm and added to the medium after cooling. The carbon source was 2-EHN at 768 mg l^{-1} of culture medium (equivalent to 421 mg C l^{-1} or 351 µmol C $flask^{-1}$) for microbial populations and 480 mg l^{-1} (equivalent to 263 mg Cl^{-1} or 219 µmol C $flask^{-1}$) for pure strains.

The equation for complete mineralization of 2-EHN under aerobic conditions is:

$$C_8H_{17}NO_3 + 12O_2 \rightarrow 8CO_2 + 8\ H_2O + HNO_3$$

The conditions used allowed aerobic biodegradation to occur under non limiting conditions, in particular with respect to oxygen, which was in excess in flask headspace.

Microorganisms

A variety of sources were used for 2-EHN biodegradation tests:

- Samples from five distinct urban wastewater treatment plants (WWTP) and from two refinery WWTP treating water previously used along refinery treatment processes. Sludge was centrifuged at 3,000 g for 20 min and re-suspended in the nutrient solution at a final concentration of 1 g l^{-1} (dry weight). After centrifugation (3,000 g for 20 min), the supernatant was discarded and sludge pellets were stored at −20°C for up to six months without significant loss of biodegradation capacity.
- Samples from ten non-polluted soils (samples 1 to 10) and from eight polluted soils (samples 11–18). Microbial suspensions were directly obtained by dispersing 5 g l^{-1} of soil sample into the nutrient solution. The characteristics of the soil samples which had been used for diesel biodegradation (Penet et al. 2006) are indicated in Table 3.

Several pure strains (Table 1) were also used for 2-EHN biodegradation tests. These strains are mainly branched-hydrocarbon-degrading bacteria. Precultures were performed in liquid medium containing Tween 80 (2.5 g l^{-1}). 2-EHN biodegradation tests were inoculated with centrifuged precultures at an optical density (580 nm) of 0.2.

Culture conditions

The biodegradation tests were performed in 120-ml flasks closed with Teflon-coated stoppers and sealed with aluminum caps. 2-EHN was added to 10 ml of inoculated culture medium in presence of 500 µl of 2,2,4,4,6,8,8-heptamethylnonane (HMN). HMN was used as a non-aqueous phase liquid (NAPL) to reduce the toxicity and volatility of 2-EHN. Cultures were incubated at 30°C with shaking (150 rpm). The overall aerobic biodegradation kinetics were regularly monitored by GC analysis of CO_2 in the flask headspace. CO_2 production was similarly monitored in control flasks without 2-EHN addition.

Experiments were carried out in duplicate. Abiotic controls supplemented with sodium azide (1 g l^{-1}) were performed under similar conditions. At the end of the incubation period, 2 ml of dichloromethane were introduced into the flasks and abiotic controls which were shaken for 1 h and then stored overnight at 4°C before analysis. The residual 2-EHN was determined by GC-FID-analysis of dichloromethane phase.

The abiotic recovery ratio of the substrate was calculated as 2-EHN recovered in the abiotic flasks

Table 1 Strains used in the study

Strain	Reference or sources
Mycobacterium austroafricanum IFP2012	Francois et al. (2002)
Mycobacterium austroafricanum IFP2015	Lopes Ferreira et al. (2006)
Mycobacterium austroafricanum IFP2138	IFP collection
Mycobacterium austroafricanum IFP2154	IFP collection
Mycobacterium austroafricanum IFP2155	IFP collection
Mycobacterium austroafricanum IFP2173	Solano-Serena et al. (2004)
Mycobacterium austroafricanum Spyr_Ge_1	J. C. Willison [a]
Mycobacterium austroafricanum BHF004	J. C. Willison [a]
Mycobacterium austroafricanum C6	Jouanneau et al. (2005)
Mycobacterium austroafricanum ATCC 29678	Johnson et al. (2004)
Mycobacterium smegmatis mc2155	Poupin et al. (1999)
Mycobacterium sp. IFP2009	Béguin et al. (2003)
Mycobacterium sp. 6PY1	Krivobok et al. (2003)
Rhodococcus ruber IFP2006	Chauvaux et al. (2001)
Rhodococcus ruber IFP2007	Chauvaux et al. (2001)
Pseudomonas citronellolis ATCC 13674	Fall et al. (1979)
Pseudomonas putida Gpo1	Smith and Hyman (2004)

[a] Laboratoire de Chimie et Biologie des Métaux, iRTSV—CEA, CNRS, Université Joseph Fourrier UMR 5249, 38054 Grenoble, France

with respect to initial 2-EHN supplied. The 2-EHN biodegradation yield was the ratio of 2-EHN biodegraded in test flasks to substrate recovered in abiotic controls.

The net CO_2 production was calculated as the difference between the final quantities of CO_2 in the test flasks and in the hydrocarbon-free flask. The mineralization extent was the carbon ratio between the net CO_2 produced and the carbon content of the substrate at the beginning of the experiment. Mineralization yield was taken as the carbon ratio between the net CO_2 produced and the substrate consumed.

Strain isolation

Microbial populations enriched in 2-EHN biodegraders were obtained from 4-week-old subcultures. Strain isolation was performed on solid medium composed of the mineral salts solution (Bouchez et al. 1995) and 15 g l^{-1} agar. Petri dishes were incubated at 30°C in sealed jars saturated with 2-EHN vapor as only carbon and energy source. Isolated strains were then tested in liquid cultures for their biodegradation capacity, 2-EHN being provided either through a liquid HMN phase (8 µl of 2-EHN in 0.5 ml of HMN) or a saturated headspace. Microbial growth was determined by optical density at 580 nm.

Chromatographic analyses

Carbon dioxide was measured with a Girdel Serie 30 chromatograph equipped with a catharometric detector and a CTRI column (Alltech). The carrier gas was helium and the column temperature was 60°C. The injector and detector temperatures were 100°C. Samples (250 µl) of the headspace gas in culture flasks were withdrawn with a gas-tight syringe and injected into the chromatograph for CO_2 analysis.

Residual 2-EHN was quantified with a Varian model 3400 gas chromatograph equipped with a flame ionization detector and a CP SIL 5CB capillary column (Chrompack) (30 m × 0.25 mm × 0.25 µm). The carrier gas was hydrogen. The injector temperature was 250°C. The detector temperature was 280°C. The column temperature was first set at 100°C and increased to 125°C at 4°C min^{-1}. Decaline was used as an internal standard.

Nucleic acid extraction and strain identification

For each strain, genomic DNA was extracted from the pellet from a 5 ml Tween 80-grown culture (Solano-Serena et al. 2004).

The 16S rRNA genes were cloned in pCR2.1 TOPO vector (Invitrogen) using TOPO TA cloning, according to manufacturer's instructions. The sequence of the forward primer F8 was 5′-AGAGTTTGATYMTG GCTCAG-3′, and the sequence of the reverse primer 1492R was 5′-CGGTTACCTTGTTACGACCT-3′ (Grabowski et al. 2005). The 16S rRNA gene was sequenced by Genome Express. Strains were identified using Blast on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences have been deposited in the GenBank database under accession numbers EU544515 for strain IFP 14.1 and EU544516 for strain S5.

Chemicals

The 2-EHN (CAS Number 27247-96-7) and HMN, were obtained from Sigma Aldrich Chimie (France). Mineral salts were from VWR (France).

Results and discussion

Because of its hydrophobic nature (log $K_{o/w}$ of 5.24), and slight solubility in water (12.6 mg l^{-1} at 20°C), 2-EHN biodegradability could not be adequately estimated by the standard Sturm test which has been designed for freely-soluble substrates of low volatility (Battersby et al. 1999). As currently performed for hydrophobic compounds, a NAPL system involving HMN was used (Bouchez et al. 1995; Garcia-Junco et al. 2003; Muñoz et al. 2008; Kirkwood et al. 2008). Our preliminary experiments confirmed that HMN had no toxic effect on microbial cells (Wodzinski and Johnson 1968), that it was not biodegradable (Schaeffer et al 1979) and that it could prevent 2-EHN toxicity (data not shown).

Biodegradation of 2-EHN by WWTP microbial populations

As mainly recommended by the current norms on chemicals (OECD 1992a, 1992b), biodegradability was determined using activated-sludge as inoculum.

Table 2 CO_2 productions from 2-EHN by WWTP microbial populations

Microbial population	Incubation period (days)	CO_2 produced[a] (μmol)	Mineralization extent[b] (%)
Urban WWTP 1	35	0	0
Urban WWTP 2	35	37	11
Urban WWTP 3	28	8	2
Urban WWTP 4	28	0	0
Urban WWTP 5	28	−3	0
Refinery WWTP 1	49	192	55
Refinery WWTP 2	35	235	67

WWTP: Waste Water Treatment Plant

[a] Amount of CO_2 produced per flask. CO_2 production of substrate-free flasks has been subtracted. Tests were performed in duplicate for 28 days with 8 μl of 2-EHN, i.e. 351 μmol C $flask^{-1}$. Mean value are indicated

[b] Carbon molar ratio of production CO_2 to initial carbon introduced

Five samples from distinct urban WWTPs on the one hand and two samples from oil refinery WWTPs on the other were used. Biodegradation tests were performed in HMN-containing medium. CO_2 released in the flask headspaces was monitored during incubation until no additional CO_2 was produced. Final CO_2 productions by WWTP microbial populations are indicated in Table 2.

Microbial communities from urban WWTPs 1, 4 and 5 exhibited no biodegradation capacity since the CO_2 evolved in test flasks after about four weeks did not significantly differ from that of 2-EHN-free flasks. The CO_2 productions from the urban WWTPs 2 and 3 showed only a slight mineralization of 2-EHN. Urban WWTP microbial populations were therefore not competent for complete 2-EHN-biodegradation although they had been previously shown to be able to degrade both diesel oil (Penet et al. 2006) and gasoline (Solano-Serena et al. 1999). In contrast to urban WWTP microbial populations, activated-sludge samples from refinery WWTPs mineralized 2-EHN significantly, since up to 67% of carbon in the initial substrate was transformed into CO_2. Their efficiency in 2-EHN biodegradation suggested that they might be composed of microorganisms having the specific catabolic ability to biodegrade branched hydrocarbons. Such a peculiar metabolic trait had already been observed for branched-hydrocarbon biodegradation (Solano-Serena et al. 2000b).

Biodegradation of 2-EHN by environmental microbial populations

Various soil samples were used as inocula in order to assess the distribution of 2-EHN biodegradation activity in environmental microbial populations. Soil samples were taken either from uncontaminated (microbial populations 1–10) or contaminated (microbial populations 11–18) soils. The tests were performed in HMN-containing liquid cultures (Table 3).

The amount of recovered 2-EHN in abiotic flasks was at least 71% of the amount initially supplied. Considering the volatility of 2-EHN, such an abiotic recovery value was quite satisfactory. Actually, abiotic losses were in the same order of magnitude as those determined for hydrocarbons of low molecular weight (Solano-Serena et al. 2000a) or for diesel oil (Penet et al. 2006). 2-EHN biodegradation yield was found to be variable depending on the soil. Five microbial populations (no. 6, 10, 11, 12, 13) degraded 2-EHN by less than 10%, confirming the partial recalcitrance of the molecule. A biodegradation yield higher than 90% was obtained with six microbial communities (no. 4, 7, 9, 14, 15, 16) which had previously been shown to extensively degrade diesel oil (Penet et al. 2006). Among microbial populations from polluted soils, the most efficient ones were from diesel-polluted soils. Although the lag phase was sometimes long, these results confirm the huge and varied biodegradation capacity of soil microbial populations toward xenobiotics. The microbial populations origin had no influence on the biodegradation capacity, since competent populations were found both in polluted and unpolluted soils. In contrast to gasoline or diesel oil biodegradation (Marchal et al. 2003), soil-exposure to hydrocarbon contaminants was not compulsory for the microbial population to acquire 2-EHN-biodegradation capacity.

Selection of 2-EHN biodegraders

In order to enrich microbial populations in 2-EHN degraders, microbial populations exhibiting biodegradation capacities were sub-cultured in liquid HMN-containing medium. Because growth of biomass could not be measured in soil suspensions, subcultures were monitored by following CO_2 production in the headspace (Solano-Serena et al. 2000c). The lag period decreased with sub-culturing and disappeared

Table 3 2-EHN biodegradation by soil microbial populations

Soil	Micro-organisms source[a]	Incubation period (days)	Lag time(days)	Abiotic recovery[b] (%)	Biodegradation yield[c] (%)	Mineralization yield[d] (%)
1	Garden	60	18	82	17[e]	0[e]
2	Field	75	0	102	43	34
3	Garden	60	n.d.	96	41	5
4	Pine forest	75	10	71	97[e]	67[e]
5	Garden	60	22	78	11	41
6	Garden	60	n.d.	74	0	0
7	Vineyard	86	23	99	100	81
8	Forest	60	18	97	27[e]	86[e]
9	Garden	71	23	91	102	62
10	Garden	60	n.d.	84	0	0
11	Polluted site (jet fuel, 2.0)	60	8	89	7	75
12	Polluted site (jet fuel, 2.4)	60	29	107	3	100
13	Polluted site (jet fuel, 2.2)	60	n.d.	84	4	0
14	Polluted site (diesel oil, 10.0)	60	17	89	100	74
15	Polluted site (diesel oil, 2.7)	60	35	89	100	59
16	Polluted site (diesel oil, 4.4)	60	8	107	100	68
17	Polluted site (crude oil, 9.0)	47	10	87	17	25
18	Polluted site (crude oil, 10.0)	43	0	108	59	44

Tests were performed in duplicate unless otherwise stated, at 30°C with 8 µl of 2-EHN, i.e. 7.68 mg flask^{-1}

[a] Pollution type and amounts of contaminating hydrocarbons in g per kg of soil dry weight are indicated between parentheses

[b] 2-EHN recovered in the abiotic flasks with respect to initial 2-EHN supplied

[c] Calculated as the ratio of the amount of substrate biodegraded in test flasks to the amounts of recovered substrate in the abiotic controls

[d] Carbon molar ratio of production CO_2 to biodegraded substrate

[e] Only one test was performed

n.d., Not determined

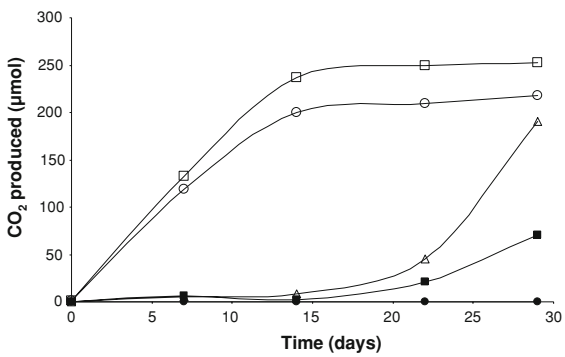

Fig. 2 Kinetics of CO_2 production for 2-EHN-free (●), first (■), second (Δ), third (O) and fourth (□) cultures from soil sample 14 Cultures were incubated at 30°C, with HMN. Flasks contained 8 µl of 2-EHN, i.e. 351 µmol C flask^{-1}

at the third culture (Fig. 2). Similarly, the time required for maximal CO_2 production to occur decreased from 30 days in the first culture to 12 days in the fourth. Total CO_2 production was also higher in the fourth culture than in the third indicating a clear adaptation of the bacterial community.

In order to isolate 2-EHN biodegraders from the last subcultures, samples were diluted and spread on solid mineral medium with 2-EHN provided as vapor. After three transfers on solid medium, thirteen bacterial colonies were isolated and tested for their growth capacity on 2-EHN in the HMN-containing liquid medium. Only one of them (strain IFP14.1) was able to grow under these conditions. Using this strain, total 2-EHN biodegradation was observed, the mineralization yield reaching 40%. Strain isolation from 2-EHN-

Table 4 Mineralization of 2-EHN by two isolated strains

Strain	CO_2 produced[a] (μmol)		Growth ($O.D._{580\ nm}$)	
	2-EHN free flask	Test flask	2-EHN free flask	Test flask
Mycobacterium sp. S5	0.23	4.67	0.010	0.230
Aurantimonas altamirensis IFP14.1	0.00	1.16	0.001	0.217

[a] Amount of CO_2 produced per flask after 4 days

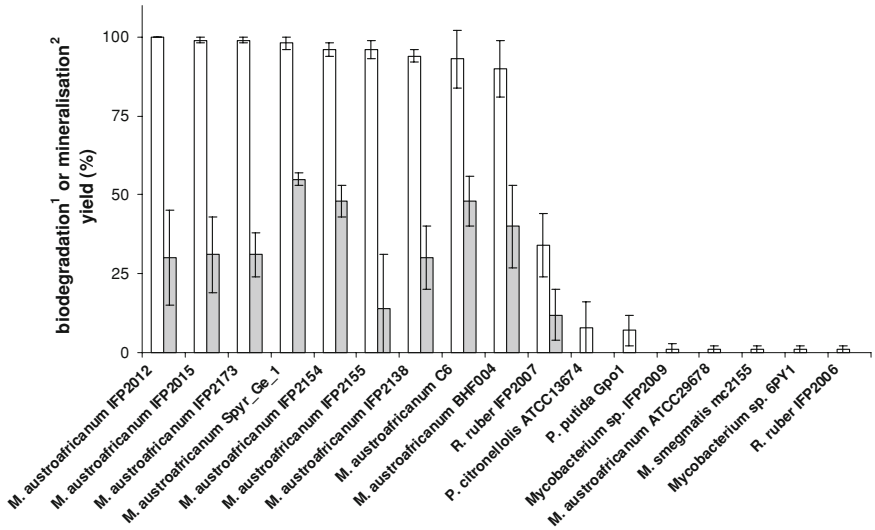

Fig. 3 Biodegradation (open bar) and mineralization (grey bar) yields for 17 specific axenic bacterial strains The biodegradation test was performed at 30°C for 28 days, with 5 μl of 2-EHN, i.e. 219 μmol C $flask^{-1}$. Calculated as the ratio of the amount of substrate biodegraded in test flasks to the amount of recovered substrate in the abiotic controls. Test was performed in triplicate. Carbon molar ratio of CO_2 production to biodegraded substrate. Test was performed in triplicate

degrading microbial populations was therefore rather complex, probably because of the cooperative processes prevailing in the microbial communities. A similar difficulty in strain isolation was encountered for cyclohexane and complete biodegradation could only be obtained using strain association (Beam and Perry 1974; Solano-Serena et al. 2000a).

The thirteen strains isolated on solid medium were able to form colonies, which however indicates that they could use 2-EHN as a growth substrate when supplied as vapor phase. Thus, liquid cultures with 2-EHN provided in the gas phase were performed. Only two isolates were able to produce minor CO_2 amounts in these conditions (Table 4), which were identified as *Aurantimonas altamirensis* and *Mycobacterium* sp., respectively, by 16S rRNA gene analysis.

Biodegradation of 2-EHN by pure strains

Since strain isolation was not really successful, various hydrocarbon-degrading-microorganisms from strain collections were tested for their 2-EHN biodegradation capacity in liquid cultures. As 2-EHN is an ethyl-substituted ester, strains with capacities in branched-alkane biodegradation were chosen. These mainly belonged to the *Corynebacterium-Mycobacterium-Nocardia* (CMN) group. Other strains tested included *P. putida* Gpo1, harboring a non hemic system of alkane hydroxylation (van Beilen et al. 2003), and *P. citronellolis* ATCC 13674, a strain capable of branched-alkane biodegradation (Fall et al. 1979; Bhattacharya et al. 2003). The kinetics of 2-EHN mineralization was monitored over 28 days. As already noted for soil microbial populations, values of abiotic recovery were satisfactory and reached at least 70% of the initial substrate (data not shown). From the CO_2 evolved and the substrate consumed in tests, biodegradation and mineralization yields were calculated (Fig. 3). Mineralization was not calculated when the biodegradation yield was below 10%, i.e. for seven strains. Only one strain not belonging to *M. austroafricanum* was able to significantly degrade and mineralize 2-EHN *(R. ruber* IFP2007). For nine strains of *M. austroafricanum*, the biodegradation yield was over 90%. With *M. austroafricanum* C6, IFP2154 and Spyr_Ge_1, mineralization yields were close to 50%.

Considering that a significant fraction of the carbon source was used to produce cell biomass, it could be inferred that little or no intermediary metabolites accumulated during growth. In contrast, *M. austroafricanum* IFP2012, IFP2015, IFP2173 and IFP2138 displayed mineralization yields close to 30%, suggesting that metabolite accumulation occurred. Only one strain of *M. austroafricanum* displayed no biodegradation capacity (*M. austroafricanum* ATCC29678, *formerly M. vaccae* JOB5).

In fact, *M. austroafricanum* belongs to the fast-growing Mycobacteria group, which forms a coherent phylogenetic branch clearly separated from the slow-growing Mycobacteria. The fast-growing Mycobacteria can be readily isolated from environmental samples and their biodegradation capacities for various hydrocarbons such as mono- (Tay et al. 1998) and poly- (Leys et al. 2005) aromatic hydrocarbons, linear, branched (Solano-Serena et al. 2000a) and cyclic (Beam and Perry 1973; Bogan et al. 2003) alkanes, methyl *tert*-butyl ether (MTBE) and ethyl *tert*-butyl ether (Francois et al. 2002; Lopes Ferreira et al. 2006) have clearly been demonstrated. Hydrocarbon uptake is favored by the high hydrophobicity of the cell wall composed of large amounts of C60–C90 mycolic acids, contributing up to 60% of the cell wall (Brennan and Nikaido 1995). The mycolic acid profile in the CMNs depends on the carbon source and influences the selectivity of uptake and transport of alkanes (Sokolovska et al. 2003). Among Gram-positive bacteria, the huge flexibility of Mycobacteria in terms of both metabolism and cell wall structure certainly explains their capacity for 2-EHN biodegradation.

Conclusion

Although 2-EHN is a large-scale commodity that can be considered as a potential pollutant for soils and aquifers, little information on its biodegradability is available. According to the standard procedure of CO_2 Headspace Test (ATC 2006), 2-EHN was considered as being not readily biodegradable. In the present study, the biodegradability of 2-EHN was demonstrated. This was achieved using a NAPL system involving HMN or silicone oil (data not shown) and microbial communities derived from refinery WWTPs. HMN addition to culture system could remove the inhibition resulting from substrate excess by gradually releasing 2-EHN into aqueous phase as it was consumed.

Biodegradation capacities of 2-EHN were not extensively distributed among microbial populations, since only refinery WWTP microbial populations and some of the soil microbial populations were able to degrade 2-EHN. This molecule has a branched structure, the biodegradation of which probably requires particular microorganisms harbouring specific pathways such as the so-called citronellol pathway which removes *anteiso* methyl groups of branched alkanes (Fall et al. 1979). The hydrocarbon contaminations might enrich microbial populations with degraders, but exposure duration to contaminant had to be long enough for giving the opportunity for the microbial population to adapt to degrade xenobiotic, as already suggested for MTBE (Moreels et al. 2004).

Several microbial populations were able to degrade 2-EHN. Strains isolation from microbial populations was, however, quite difficult, as it was for the biodegradation of gasoline additives such as MTBE (Francois et al. 2002, Rohwerder et al. 2006). Only two isolates utilized 2-EHN when provided in the vapor phase and only one isolate grew slightly under the conditions of our standard liquid test. In fact, complex microbial communities appeared to be efficient for 2-EHN biodegradation, probably because of the commensalisms phenomenon existing between microorganisms of the population. Some intermediary metabolites might be produced by biodegraders with limited biodegradation capacities. Accumulated metabolites can then serve as carbon sources for other organisms, allowing extensive mineralization of the substrate by complex microbial population.

Most strains of *M. austroafricanum* were found capable of extensive 2-EHN biodegradation. These findings actually highlight the great versatility of those strains for the biodegradation of hydrophobic compounds to which 2-EHN belongs.

Acknowledgments We thank Dr. John C. Willison for the gift of *M. austroafricanum* strains BHF004, Spyr_Ge_1, C6 and for critical reading of the manuscript.

References

ATC (2006) High production volume: challenge program for nitric acid, 2-ethylhexylester. http://www.epa.gov/hpv/pubs/summaries/nitracd2/c14932rt.pdf

Battersby NS, Ciccognani D, Evans MR, King D, Painter HA, Peterson DR, Starkey M, CONCAWE Biodegradation Task Force (1999) An 'inherent' biodegradability test for oil products: description and results of an international ring test. Chemosphere 38:3219–3225. doi:10.1016/S0045-6535(98)00552-9

Beam HW, Perry JJ (1973) Co-metabolism as a factor in microbial degradation of cycloparaffinic hydrocarbons. Arch Mikrobiol 91:87–90. doi:10.1007/BF00409542

Beam HW, Perry JJ (1974) Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. J Gen Microbiol 82:163–169

Béguin P, Chauvaux S, Miras I, Francois A, Fayolle F, Monot F (2003) Genes involved in the degradation of ether fuels by bacteria of the *Mycobacterium/Rhodococcus* group. Oil Gas Sci Technol Rev IFP 58:489–495

Bhattacharya D, Sarma PM, Krishnan S, Mishra S, Lal B (2003) Evaluation of genetic diversity among *Pseudomonas citronellolis* strains isolated from oily sludge-contaminated sites. Appl Environ Microbiol 69:1435–1441. doi:10.1128/AEM.69.3.1435-1441.2003

Bogan BW, Lahner LM, Sullivan WR, Paterek JR (2003) Degradation of straight-chain aliphatic and high-molecular-weight polycyclic aromatic hydrocarbons by a strain of *Mycobacterium austroafricanum*. J Appl Microbiol 94:230–239. doi:10.1046/j.1365-2672.2003.01824.x

Bornemann H, Scheidt F, Sander W (2002) Thermal decomposition of 2-ethylhexyl nitrate (2-EHN). J Chem Kinet 34:34–38. doi:10.1002/kin.10017

Bouchez M, Blanchet D, Vandecasteele JP (1995) Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. Appl Microbiol Biotechnol 43:156–164. doi:10.1007/BF00170638

Brennan PJ, Nikaido H (1995) The envelope of mycobacteria. Annu Rev Biochem 64:29–63. doi:10.1146/annurev.bi.64.070195.000333

Chauvaux S, Chevalier F, Le Dantec C, Fayolle F, Miras I, Kunst F et al (2001) Cloning of a genetically unstable cytochrome P-450 gene cluster involved in degradation of the pollutant ethyl tert-butyl ether by *Rhodococcus ruber*. J Bacteriol 183:6551–6557. doi:10.1128/JB.183.22.6551-6557.2001

Fall RR, Brown JL, Schaeffer TL (1979) Enzyme recruitment allows the biodegradation of recalcitrant branched hydrocarbons by *Pseudomonas citronellolis*. Appl Environ Microbiol 38:715–722

Francois A, Mathis H, Godefroy D, Piveteau P, Fayolle F, Monot F (2002) Biodegradation of methyl tert-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. Appl Environ Microbiol 68:2754–2762. doi:10.1128/AEM.68.6.2754-2762.2002

Garcia-Junco M, Gomez-Lahoz C, Niqui-Arroyo JL, Ortega-Calvo JJ (2003) Biosurfactant- and Biodegradation-Enhanced Partitioning of Polycyclic Aromatic Hydrocarbons from Nonaqueous-Phase Liquids. Environ Sci Technol 37:2988–2996. doi:10.1021/es020197q

Guibet JC, Faure BE (1999) Fuels and Engines: technology, energy, environment. Editions Technip, Paris

Haines JR, Kleiner EJ, McClellan KA, Koran KM, Holder EL, King DW et al (2005) Laboratory evaluation of oil spill bioremediation products in salt and freshwater systems. J Ind Microbiol Biotechnol 32:171–185. doi:10.1007/s10295-005-0218-1

Johnson EL, Smith CA, O'Reilly KT, Hyman MR (2004) Induction of methyl tertiary butyl ether (MTBE)-oxidizing activity in *Mycobacterium vaccae* JOB5 by MTBE. Appl Environ Microbiol 70:1023–1030. doi:10.1128/AEM.70.2.1023-1030.2004

Jouanneau Y, Willison JC, Meyer C, Krivobok S, Chevron N, Besombes JL et al (2005) Stimulation of pyrene mineralization in freshwater sediments by bacterial and plant bioaugmentation. Environ Sci Technol 39:5729–5735. doi:10.1021/es050412d

Kirkwood KM, Chernik P, Foght JM, Gray MR (2008) Aerobic biotransformation of decalin (decahydronaphthalene) by *Rhodococcus* spp. Biodegradation. doi:10.1007/s10532-008-9181-7

Krivobok S, Kuony S, Meyer C, Louwagie M, Willison JC, Jouanneau Y (2003) Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: evidence for two ring-hydroxylating dioxygenases. J Bacteriol 185:3828–3841. doi:10.1128/JB.185.13.3828-3841.2003

Leahy JG, Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315

Leys NM, Ryngaert A, Bastiaens L, Wattiau P, Top EM, Verstraete W et al (2005) Occurrence and community composition of fast-growing *Mycobacterium* in soils contaminated with polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol 51:375–388. doi:10.1016/j.femsec.2004.09.015

Lopes Ferreira NL, Maciel H, Mathis H, Monot F, Fayolle-Guichard F, Greer CW (2006) Isolation and characterization of a new *Mycobacterium austroafricanum* strain, IFP 2015, growing on MTBE. Appl Microbiol Biotechnol 70:358–365. doi:10.1007/s00253-005-0074-y

Marchal R, Penet S, Solano-Serena F, Vandecasteele JP (2003) Gasoline and diesel oil biodegradation. Oil Gas Science and Technol–Rev IFP 58:441–448

Moreels D, Bastiaens L, Ollevier F, Merckx R, Diels L, Springael D (2004) Evaluation of the intrinsic methyl tert-butyl ether (MTBE) biodegradation potential of hydrocarbon contaminated subsurface soils in batch microcosm systems. FEMS Microbiol Ecol 49:121–128. doi:10.1016/j.femsec.2004.02.016

Morgan P, Watkinson RJ (1989) Microbiological methods for the cleanup of soil and ground water contaminated with halogenated organic compounds. FEMS Microbiol Rev 5:277–299. doi:10.1016/0168-6445(89)90025-9

Muñoz R, Chambaud M, Sergio Bordel S, Villaverde S (2008) A systematic selection of the non-aqueous phase in a bacterial two liquid phase bioreactor treating α-pinene. Appl Microbiol Biotechnol 79:33–41. doi:10.1007/s00253-008-1400-y

OECD (1992a) 301 Ready Biodegradability, OECD guidelines for testing of chemicals. Organisation for Economic Co-operation and development, Paris

OECD (1992b) 302 B Zahn-Wellens/EMPA Test, OECD guidelines for testing of chemicals. Organisation for Economic Co-operation and development, Paris

Penet S, Vendeuvre C, Bertoncini F, Marchal R, Monot F (2006) Characterisation of biodegradation capacities of

environmental microflorae for diesel oil by comprehensive two-dimensional gas chromatography. Biodegradation 17:577–585. doi:10.1007/s10532-005-9028-4

Poupin P, Ducrocq V, Hallier-Soulier S, Truffaut N (1999) Cloning and characterization of the genes encoding a cytochrome P450 (PipA) involved in piperidine and pyrrolidine utilization and its regulatory protein (PipR) in *Mycobacterium smegmatis* mc2155. J Bacteriol 181:3419–3426

Rohwerder T, Breuer U, Benndorf D, Lechner U, Müller RH (2006) The alkyl tert-butyl ether intermediate 2-hydroxyisobutyrate Is degraded via a novel cobalamin-dependent mutase pathway. Appl Environ Microbiol 72:4128–4135. doi:10.1128/AEM.00080-06

Roling WF, van Verseveld HW (2002) Natural attenuation: what does the subsurface have in store? Biodegradation 13:53–64. doi:10.1023/A:1016310519957

Sakai Y, Takahashi H, Wakasa Y, Kotani T, Yurimoto H, Miyachi N (2004) Role of alpha-methylacyl coenzyme A racemase in the degradation of methyl-branched alkanes by *Mycobacterium* sp. strain P101. J Bacteriol 186:7214–7220. doi:10.1128/JB.186.21.7214-7220.2004

Schaeffer TL, Cantwell SG, Brown JL, Watt DWS, Fall RR (1979) Microbial growth on hydrocarbons: terminal branching inhibits biodegradation. Appl Environ Microbiol 38:742–746

Smith CA, Hyman MR (2004) Oxidation of methyl tert-butyl ether by alkane hydroxylase in dicyclopropylketone-induced and n-octane-grown *Pseudomonas putida* GPo1. Appl Environ Microbiol 70:4544–4550. doi:10.1128/AEM.70.8.4544-4550.2004

Sokolovska I, Rozenberg R, Riez C, Rouxhet PG, Agathos SN, Wattiau P (2003) Carbon source-induced modifications in the mycolic acid content and cell wall permeability of *Rhodococcus erythropolis* E1. Appl Environ Microbiol 69:7019–7027. doi:10.1128/AEM.69.12.7019-7027.2003

Solano-Serena F, Marchal R, Ropars M, Lebeault JM, Vandecasteele JP (1999) Biodegradation of gasoline: kinetics, mass balance and fate of individual hydrocarbons. J Appl Microbiol 86:1008–1016. doi:10.1046/j.1365-2672.1999.00782.x

Solano-Serena F, Marchal R, Casaregola S, Vasnier C, Lebeault JM, Vandecasteele JP (2000a) A *Mycobacterium* strain with extended capacities for degradation of gasoline hydrocarbons. Appl Environ Microbiol 66:2392–2399. doi:10.1128/AEM.66.6.2392-2399.2000

Solano-Serena F, Marchal R, Lebeault JM, Vandecasteele JP (2000b) Distribution in the environment of degradative capacities for gasoline attenuation. Biodegradation 11:29–35. doi:10.1023/A:1026594717754

Solano-Serena F, Marchal R, Lebeault JM, Vandecasteele JP (2000c) Selection of microbial populations degrading recalcitrant hydrocarbons of gasoline by monitoring of culture-headspace composition. Lett Appl Microbiol 30:19–22. doi:10.1046/j.1472-765x.2000.00631.x

Solano-Serena F, Marchal R, Heiss S, Vandecasteele JP (2004) Degradation of isooctane by *Mycobacterium austroafricanum* IFP 2173: growth and catabolic pathway. J Appl Microbiol 97:629–639. doi:10.1111/j.1365-2672.2004.02344.x

Someroja S, Savolinen H (1983) Neurochemical effects of ethylhexyl nitrate in rats. Toxicol Lett 19:189–193. doi:10.1016/0378-4274(83)90281-3

Strotmann U, Reuschenbach P, Schwarz H, Pagga U (2004) Development and evaluation of an online CO(2) evolution test and a multicomponent biodegradation test system. Appl Environ Microbiol 70:4621–4628. doi:10.1128/AEM.70.8.4621-4628.2004

Tay ST, Hemond HF, Polz MF, Cavanaugh CM, Dejesus I, Krumholz LR (1998) Two new *Mycobacterium* strains and their role in toluene degradation in a contaminated stream. Appl Environ Microbiol 64:1715–1720

van Beilen JB, Li Z, Duetz WA, Smits TH, Witholt B (2003) Diversity of alkane hydroxylase systems in the environment. Oil & Gas Science and Technology - Rev. IFP 58:427–440

Wodzinski RS, Johnson MJ (1968) Yields of bacterial cells from hydrocarbons. Appl Microbiol 16:1886–1891