Sélection de souches dégradant le 2-EHN

Au début de ce projet de thèse, la question de la biodégradabilité du 2-EHN était en suspens car aucune étude n'avait été menée pour déterminer quels pouvaient être les critères importants présidant à biodégradation de ce composé. Nous étions en lieu de craindre que la biodégradabilité du 2-EHN serait difficile pour les raisons suivantes :

- la rareté dans l'environnement des microorganismes dotés de l'équipement enzymatique pour dégrader les composés branchés;
- la difficulté d'accession des microorganismes à un substrat quelque peu volatil (tension de vapeur de 27 Pa à 20°C),
- et la toxicité d'un substrat, légèrement soluble dans les milieux de culture mais cependant largement hydrophobe (log $k_{O/W} = 5,24$).

L'ensemble de ces éléments devait être pris en compte pour définir les conditions de sélection de micro-organismes appropriés.

Pour sélectionner des souches capables de dégrader du 2-EHN, il convenait de disposer d'un test de dégradation adapté aux particularités du substrat. Le test de Sturm a été considéré à l'origine car il est utilisé couramment pour mesurer la biodégradabilité des produits commerciaux (OCDE). Il s'agit d'un test où le composé à tester est soumis à une biomasse cellulaire constituée par une boue de station d'épuration (STEP) d'eaux urbaines. La dégradation est alors mesurée par la production totale de CO_2 piégé en milieu alcalin. Comme l'a noté justement Battersby *et al.* (1999), le test de Sturm n'est applicable qu'aux produits organiques solubles dans l'eau et ayant une faible volatilité. Ce test ne convenait donc pas pour le 2-EHN qui est doté d'une solubilité restreinte (12, 6 $mg.L^{-1}$) et d'une tension de vapeur appréciable (27 Pa à 20°C).

Un autre test, celui défini par la norme NF ISO 14593 d'octobre 2005 sur "l'évaluation en milieu aqueux de la biodégradabilité aérobie ultime des composés organiques par analyse du carbone inorganique dans des récipients hermétiquement clos" pouvait être également envisagé, puisque le 2-EHN est un composé volatil. Ce test dit "du CO_2 dans l'espace de tête" se déroule dans une fiole fermée, avec un faible volume de liquide, contenant des microflores de boues de station d'épuration urbaine (STEP), et un espace de tête suffisamment grand pour contenir

l'oxygène nécessaire à la dégradation du substrat. Le temps du test de dégradation est de 28 jours et la dégradation du substrat résiduel est évaluée par la mesure du CO_2 produit dans l'espace de têtede la fiole et du COT (carbone organique total) résiduel dans le milieu. La quantité de substrat est de 2 à 40 $mg.L^{-1}$. Pour appliquer le test du "CO_2 dans l'espace de tête" au cas du 2-EHN, il eût fallu que le 2-EHN soit d'avantage soluble. Compte tenu de la pression de vapeur saturante et d'après la loi des gaz parfaits, 2 $mg.L^{-1}$ de 2-EHN environ sont contenus à saturation dans la phase vapeur à 20°C et de l'ordre de 3 $mg.L^{-1}$ à 30°C, température à laquelle s'effectuent les tests. A saturation, peu de 2-EHN est donc solubilisé et accessible au micro-organisme dans la phase aqueuse (12,6 $mg.L^{-1}$ à 20°C). Dans les conditions du test "CO_2 dans l'espace de tête", les quantités de substrat était alors trop faibles pour conduire à des mesures précises du substrat.

Nous avons donc préféré nous inspirer du test mis au point pour la biodégradabilité des gazoles (Penet *et al.* 2004), celui-ci autorisant de surcroît l'examen des cas où la biodégradation du substrat est incomplète et pour lesquels s'accumulent certains métabolites. Afin de prendre en compte le caractère inhibiteur probable du substrat, le 2-EHN a été introduit dans le réacteur après dissolution dans une phase solvant inerte non métabolisable (non aqueous phase liquid ou NAPL, dans la terminologie anglo-saxonne) telle que 2,2,4,4,6,8,8-heptamethylnonane (HMN). La phase NAPL sert alors de réservoir pour le substrat hydrophobe et atténue la concentration solubilisée dans le milieu. Au fur et à mesure de sa consommation, le substrat est transféré de la phase solvant vers le milieu de culture. De la sorte, l'inhibition par excès de concentration de substrat a toutes chances d'être levée.

A l'aide du test défini pour le 2-EHN, les premiers essais de biodégradation ont été effectués en utilisant des microflores de STEP urbaines, puis en utilisant des microflores de raffineries. Les tests ont été étendus à des souches pures de collection qui étaient connues pour leur capacité de dégradation des hydrocarbures. Comme nous le verrons, certains micro-organismes présentent une capacité de dégradation intéressante.

ARTICLE 1

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

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ORIGINAL PAPER

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

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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*

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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

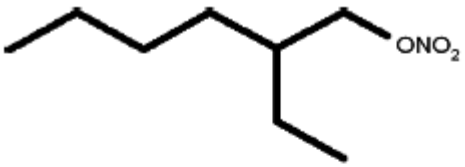

Fig. 1 Molecular structure of 2-Ethylhexyl nitrate ($C_8H_{17}NO_3$). Main physico-chemical properties (ATC 2006): vapor pressure at 20°C: 27Pa; solubility in water at 20°C: 12.6 mg l^{-1}; $logK_{o/w}$: 5.24; liquid density: 0.96

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)

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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

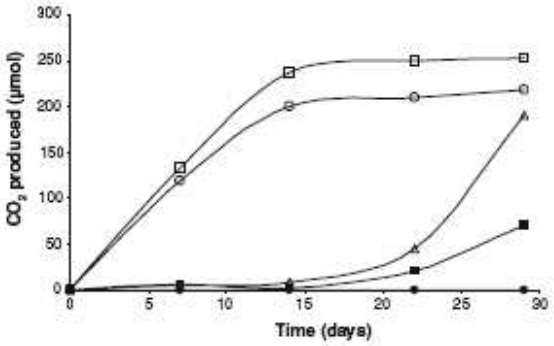

Fig. 2 Kinetics of CO_2 production for 2-EHN-free (●), first (■), second (Δ), third (O) and fourth (□) cultures from soil sample 14 Cultures were incubated at 30°C, with HMN. Flasks contained 8 µl of 2-EHN, i.e. 351 µmol C flask^{-1}

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

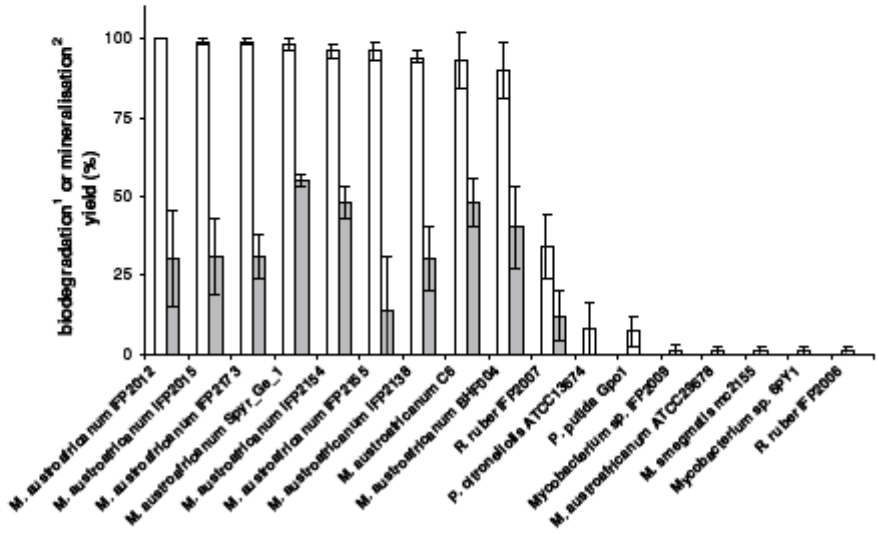

Fig. 3 Biodegradation (open bar) and mineralization (grey bar) yields for 17 specific axenic bacterial strains The biodegradation test was performed at 30°C for 28 days, with 5 µl of 2-EHN, i.e. 219 µmol C $flask^{-1}$. Calculated as the ratio of the amount of substrate biodegraded in test flasks to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

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2 Dégradation du 2-EHN par *M. austroafricanum* IFP 2173

Comme le montrent les précédents résultats, il est difficile d'isoler des souches capables de dégrader le 2-EHN. Parmi les 18 souches pures hydrocarbonoclastes seules certaines souches de *M. austroafricanum* testées sont capables de dégrader le 2-EHN sans toutefois le minéraliser totalement. *M. austroafricanum* IFP 2173 a pour particularité de dégrader le 2-EHN sans phase solvant (HMN). C'est la raison pour laquelle nous avons entrepris l'étude cinétique de la dégradation du 2-EHN avec cette souche.

2.1 Cinétique de la dégradation du 2-EHN par M. austroafricanum IFP 2173

Afin de caractériser la dégradation du 2-EHN par *M. austroafricanum* IFP 2173, diverses études cinétiques de dégradation ont été mises en œuvre. Pour ce faire, des mesures de la dégradation, rapides globales et indirectes, ont été effectuées. Elles concernent la production de CO_2 ou de la consommation de dioxygène.

2.1.1 Influence du substrat de préculture

Avant chaque test de biodégradation, chaque souche est cultivée sur MMSYE avec 2.5 $g.L^{-1}$ de Tween 80 (Polysorbate 80 CAS: 9005-65-6). Cette source de carbone amphiphile évite la formation de flocks au cours de la préculture. A titre de comparaison, d'autres sources de carbone ont été testées comme substrat de préculture. Il s'agit du succinate de disodium (4 $g.L^{-1}$) choisi car il est directement dégradé en l'absence de voie anaplérotique et ne nécessite théoriquement pas d'induction enzymatique particulière pour son utilisation en tant que source de carbone. L'acétate de sodium (4 $g.L^{-1}$) est aussi une source de carbone facilement métabolisable pour les bactéries du groupe CMN. L'iso-octane ou 2,2,4-triméthylpentane (500 $mg.L^{-1}$) est un substrat particulier que la souche *M. austroafricanum* IFP 2173 est capable de dégrader en induisant à cette occasion un cytochrome P450. Le glycérol (500$mg.L^{-1}$) est aussi une source de carbone simple mais nécessitant la présence de certaines enzymes (déshydrogénases). A l'issue de ces précultures contenant différentes sources de carbone, des tests de biodégradation du 2-EHN ont été mis en place et la cinétique de production de CO_2 a été mesurée (**Figure 4.1**).

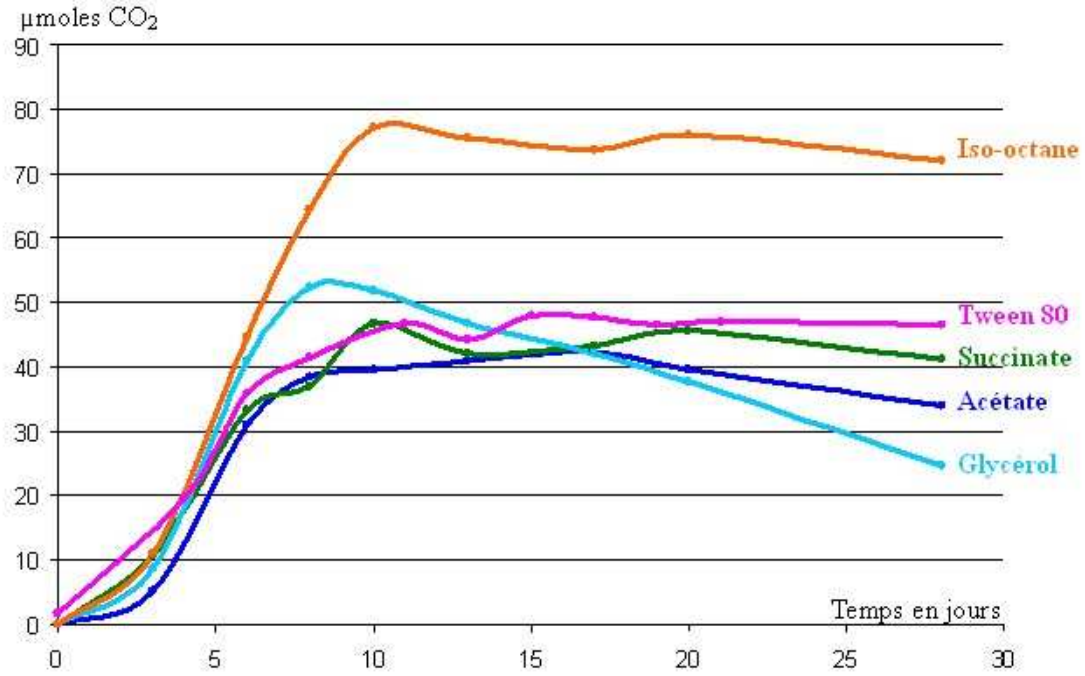


Figure 4.1: *Cinétique dégradation du 2-EHN par M. austroafricanum IFP 2173 en présence de HMN : Influence du substrat de préculture*

Le succinate, l'acétate et le Tween80 sont des sources de carbone adéquates pour les précultures de *M. austroafricanum* IFP 2173 et assurent une dégradation convenable du 2-EHN. Le **tableau 4.1** rassemble les performances finales des tests de biodégradation effectués avec ces précultures.

Tableau 4.1: *Influence du substrat de préculture sur les performances finales de biodégradation du 2-EHN par M. austroafricanum IFP 2173*

Substrat de préculture	Vitesse maximale de dégradation (µmole CO_2/jour)	Taux de recouvrement abiotique (%)	Taux de dégradation (%)	Rendement de minéralisation (%)
Tween 80	8.9	72	99 (+/- 1)	31 (+/- 7)
Acétate de sodium	10.1	71	94 (+/- 4)	37 (+/- 6)
Succinate de di-sodium	8.6	71	93 (+/- 2)	37 (+/- 16)
Glycérol	12.4	70	92 (+/- 2)	50 (+/- 1)
Iso-octane	12.2	70	97 (+/- 3)	51 (+/- 3)

Les taux de dégradation avoisinent les 100% après 28 jours avec les précultures sur iso-octane et Tween 80. Avec préculture sur glycérol, la croissance est plus lente. Le taux de minéralisation du 2-EHN est de 50% pour les précultures sur glycérol et isooctane. Il est

d'environ 35% sur Tween80, acétate et succinate. Lorsque *M. austroafricanum* IFP 2173 est précultivée sur iso-octane, l'induction du cytochrome P450 ou du système Alk (Solano-Serena *et al.* 2004) pourrait être à l'origine de l'amélioration d'activité qui est constatée pour la dégradation du 2-EHN.

2.1.2 Influence du mode d'apport du 2-EHN

L'influence du mode d'apport du substrat a été testée par respirométrie. Dans les essais standards, le milieu de culture contient 0,05% (v/v) de 2-EHN, additionné de 5% de HMN (v/v). Une autre phase réservoir inerte a été testée, il s'agit de l'huile de silicone (47V20, Prolabo). Par ailleurs, la souche *M. austroafricanum* IFP 2173 est capable de croissance en l'absence de HMN. Nous avons suivi la dégradation du 2-EHN à 30°C par mesure de l'O_2 consommé en présence de 5% d'HMN, en présence de 5% d'huile de silicone et sans phase solvant (**Figure 4.2**).

La dégradation du 2-EHN est légèrement améliorée par le remplacement du HMN par l'huile de silicone. La consommation finale d'O_2 est légèrement augmentée. Cependant, la vitesse maximale de biodégradation n'est pas modifiée. L'huile de silicone semble assurer une meilleure disponibilité du substrat pour le micro-organisme.

En l'absence de phase solvant, la croissance de *M. austroafricanum* IFP 2173 est possible à 30°C. La croissance est cependant plus lente (μ = 0,3 jour^{-1}) et la quantité finale d'O_2 consommé est aussi moins importante.

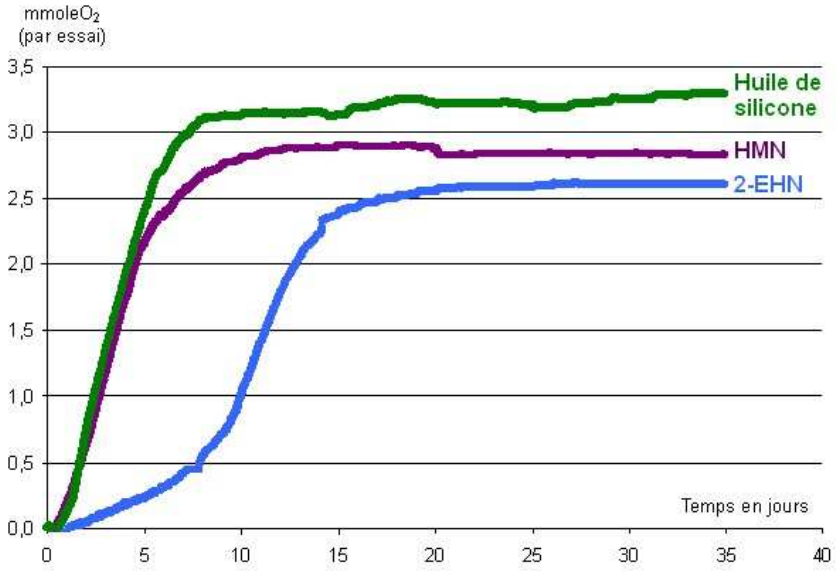


Figure 4.2: *Influence de la nature de la phase solvant sur la dégradation du 2-EHN par M. austroafricanum IFP 2173.*

2.1.3 Influence de la température

La cinétique de consommation d'O_2 par *M. austroafricanum* IFP 2173 a été testée à 20°C et à 30°C en présence de HMN, en conditions standard (**Figure 4.3**).

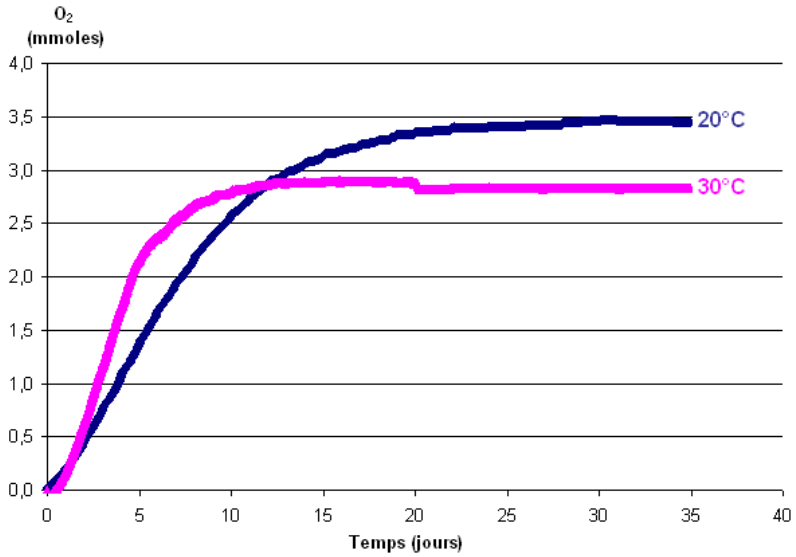


Figure 4.3: *Cinétiques de consommation d'O_2 par M. austroafricanum IFP 2173 : Influence de la température*

La consommation d'O_2 à 30°C est plus rapide qu'à 20°C. En revanche, la quantité finale d'O_2 consommée est plus forte à 20°C qu'à 30°C. A 30°C, la tension de vapeur du 2-EHN est plus élevée (27 Pa à 20°C contre 42 Pa environ à 30°C). Pour une température plus forte, il y a plus de 2-EHN dans la phase vapeur, qui n'est pas immédiatement disponible pour le microorganisme.

Le **Tableau 4.2** représente les paramètres de croissance calculés pour les différents systèmes.

Tableau 4.2 : *Variations des conditions de culture sur 2-EHN de M. austroafricanum IFP 2173*

Systèmes testés	Vitesse max de consommation d'O_2 (mmole/jour)	Consommation d'O_2 maximale expérimentale (mmoles)	Quantité de 2-EHN résiduel ($mg.L^{-1}$)
avec HMN à 20°C	3.1	3.5	1
avec huile de silicone à 20°C	3.5	3	0
sans solvant à 20°C	NC*	NC*	270‡
avec HMN à 30°C	5.3	4.5	0
avec huile de silicone à 30°C	5.8	5	0
sans solvant à 30°C	0,3	2.6	0*

* NC: absence de croissance

‡ : L'extraction du 2-EHN est partielle en l'absence de HMN.

Quelle que soit la température du test, les cinétiques de consommation d'O_2 sont légèrement plus rapides en présence d'huile de silicone qu'en présence de HMN. Quel que soit le solvant utilisé la consommation d'O_2 est plus rapide à 30°C qu'à 20°C. Alors que la croissance est possible à 30°C en l'absence de solvant, elle ne l'est plus à 20°C.

Ces résultats soulignent le fait que le 2-EHN est biodégradable dans des conditions particulières de température et de mode d'apport du substrat.

2.2 Étude préliminaire de la voie de dégradation

2.2.1 Étude du système d'attaque du substrat

Afin de caractériser l'étape initiale de dégradation du 2-EHN, nous avons examiné l'action des inhibiteurs des mono-oxygénases. Les tests de dégradation du 2-EHN ont été réalisés en présence de pentyne ou d'azoles (**Tableau 4.3**).

Tableau 4.3*: Action des inhibiteurs de mono-oxygénases sur la dégradation du 2-EHN*

Inhibiteur	Consommation du substrat[a] (%)	Croissance[b] (DO_{600})	Rendement de minéralisation (%)	Présence d'intermédiaire[c]
Pentyne	0	0	-	-
Clotrimazole	94	0,45	94	++
Methimazole	93	0,36	93	++
Metronidazole	85	0,40	85	++
Econazole	94	0,10	94	++
Miconazole	80	0,14	79	++
Ketoconazole	73	0,20	73	++

[a] : par rapport au témoin abiotique
[b]: en tenant compte de l'absorption du témoin endogène.
[c]: abscence (-) ou présence (+) de la 4-éthyl dihydrofuran-2(3H)-one.

La pentyne est un alcyne en C_5. C'est un substrat suicide des mono-oxygénases. Le 2-EHN n'est pas dégradé lorsque de la pentyne est introduite dans le milieu (400 $mg.L^{-1}$). L'inhibition de la dégradation du 2-EHN par la pentyne montre donc l'intervention d'une mono-oxygénase, vraisemblablement au niveau de l'attaque initiale du substrat.

Les azoles sont des inhibiteurs possibles des cytochromes P450. Leur utilisation à raison de 10 $mg.L^{-1}$ n'empêche pas la dégradation du 2-EHN par *M. austroafricanum* IFP 2173. La dégradation du 2-EHN pourrait donc impliquer une mono-oxygénase de type non hémique. Les fortes valeurs de rendement de minéralisation s'expliquent par le fait que la souche IFP 2173 a utilisé le DMSO, qui a servi à diluer les azoles, comme source de carbone.

2.2.2 Utilisation du 2-EHN comme source d'azote

Un milieu MMSYE sans azote ni ammoniaque a été testé afin de savoir si le 2-EHN pouvait servir de source d'azote pour *M. austroafricanum* IFP 2173 (**Tableau 4.4**).

Tableau 4.4: *Utilisation du 2-EHN comme source d'azote*

Conditions de culture	Taux de dégradation (%)	Rendement de minéralisation (%)	Recouvrement abiotique (%)
avec HMN, avec NH_4^+	99 (+/-1)	20 (+/-4)	72
avec NH_4^+	99 (+/-1)	20 (+/-6)	ND*
avec HMN sans NH_4^+	98 (+/-1)	21 (+/-6)	80
sans HMN, sans NH_4^+	98 (+/-1)	13 (+/-1)	ND*

ND* : Le calcul du taux de recouvrement abiotique en l'absence de HMN n'est pas possible en raison de la forte tension de vapeur du 2-EHN.

Même en l'absence de NH_4^+, le 2-EHN est dégradé totalement dans conditions testées. Ainsi, le groupement nitrate du 2-EHN semble pouvoir servir de source d'azote pour le micro-organisme.

2.2.3 Dégradation du 2-éthylhexanol et de l'acide 2-éthylhexanoïque

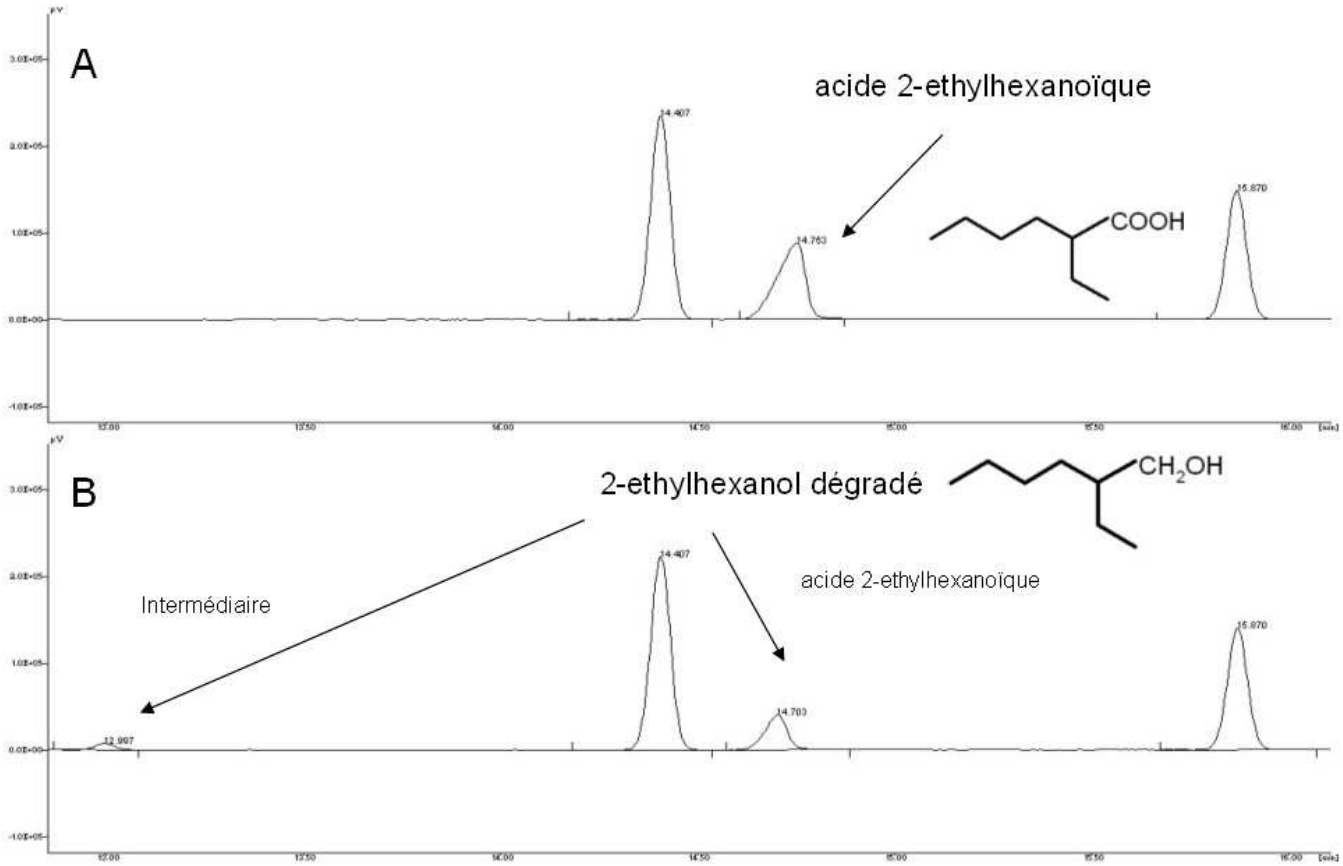
Afin de comprendre si la dégradation du 2-EHNest susceptible de passer par le 2-éthylhexanol puis à l'acide 2-éthylhexanoïque, la dégradation de ces deux intermédiaires potentiels a été testée (**Tableau 4.5**).

Tableau4.5: *Dégradation du 2-éthylhexanol et de l'acide 2 éthylhexanoïque par M. austroafricanum IFP 2173*

Substrat	Taux de dégradation (%)*	Rendement de minéralisation (%)*
2-éthylhexanol	100 (+/-0)	10,6 (+/-11)
acide 2-éthylhexanoïque	40 (+/-0)	23 (+/- 20)
2-EHN	99 (+/- 1)	31 (+/- 7)

* : Les essais ont été réalisés en triplicates pendant 28 jours.

Le 2-éthylhexanol est complètement dégradé alors que l'acide 2-éthylhexanoïque ne l'est qu'incomplètement. Pour le 2-éthylhexanol, le rendement de minéralisation est de 10,6 % seulement, ce qui signifie que des métabolites se sont probablement accumulés. Si l'on examine le chromatogramme final de dégradation du 2-éthylhexanol, on repère un intermédiaire de dégradation (dénommé provisoirement "intermédiaire X") ainsi que l'acide 2-éthylhexanoïque (**Figure 4.4 B**). En tant que substrat, l'acide 2-éthylhexanoïque est dégradé à 40% et minéralisé à 23%, ce qui laisse supposer que ce dernier est dégradé plus extensivement mais très lentement. La **figure 4.4 A** met en évidence que l'intermédiaire X ne s'accumule pas. Signalons que dans la littérature, l'acide 2-éthylhexanoïque est parfois considéré comme toxique pour les micro-organismes (Nalli *et al.,* 2002).



Figures 4.4: *Chromatogrammes obtenus après la dégradation par M. austroafricanum IFP 2173 de: A: L'acide 2-éthylhexanoïque, B: Le 2-éthylhexanol*
Les pics non identifiés sont la cis- et trans-décaline utilisée comme standard interns pour le dosage du 2-EHN résiduel.
D'après ces résultats, la dégradation du 2-EHN est susceptible passer par le 2-éthylhexanol. On assiste à l'accumulation de l'intermédiaire X, mais pas à celle de celle de l'acide 2-éthylhexanoïque.

2.2.4 Essais de dérivatisation de l'intermédiaire accumulé

Afin de déterminer sa structure, l'intermédiaire X a été estérifié puis silylé. L'estérification permet de savoir s'il y a un acide carboxylique dans la molécule inconnue. Un groupement méthyle se fixe sur le groupement R–COOH libre pour former un groupement R-COO-CH_3 qui se traduit par un déplacement de pic en CPG.. Ensuite, on effectue une silylation qui permet de fixer un groupement Si-$(CH_3)_3$ sur les groupements R-OH formant ainsi un groupement R-O-Si-$(CH_3)_3$. Ces transformations chimiques ont été effectuées sur le milieu de culture où le 2-EHN a été dégradé, c'est-à-dire soit sur le milieu lyophilisé soit sur l'intermédiaire extrait par un solvant. Les conditions d'analyse testées et leurs résultats figurent dans le **Tableau 4.6**.

L'intermédiaire X n'a pas pu être dérivé dans ces conditions. Les méthodes d'estérification et de silylation ont été validées avec des produits standards (alcools et acides carboxyliques). Aucune dérivation n'a été réussie sur l'intermédiaire X

Tableau 4.6: *Essais de synthèses de dérivés sur l'intermédiaire de dégaradtion.*

Traitement de l'intermédiaire	Estérification*	Silylation*	Résultats
Filtré puis lyophilisé	-	Selon Matériel et Méthodes	Pas de dérivé
Filtré puis lyophilisé	En milieu acide	Selon Matériel et Méthodes	Pas de dérivé
Saponifié puis Lyophilisé	En milieu acide	Selon Matériel et Méthodes	Pas de dérivé
MTBE évaporé sous N_2	En milieu acide	-	Pas de dérivé
MTBE	En milieu acide	Selon Matériel et Méthodes	Pas de dérivé
Cyclohexane	En milieu alcalin	-	Pas de dérivé
Cyclohexane	-	Selon Matériel et Méthodes	Pas de dérivé
Cyclohexane	Par du diazométhane	-	Pas de dérivé

*Le protocole expérimental est détaillé dans la partie matériel et méthodes.

L'ensemble de ces résultats font partie des essais préliminaires qui ont pour objectif de comprendre la voie de dégradation du 2-EHN par *M. austroafricanum* IFP 2173. Ils ne sont pas présentés dans l'article n°2 "Catabolism of 2-ethyhexyl nitrate by *M. austroafricanum* IFP 2173", même si certaines observations y sont citées.

L'article présente certains aspects cinétiques de la voie de dégradation du 2-EHN ainsi que l'identification de l'intermédiaire X (qui n'aurait pu aboutir sans le travail d'analyse de Lucien Kerhoas et la synthèse de la molécule par Martine Lettere à l'INRA de Versailles).

2.3 Voie catabolique du 2-EHN

ARTICLE 2 ACCEPTE DANS APPLIED ENVIRONMENTAL MICROBIOLOGY, LE 12 AOUT 2008.

Catabolism of 2-Ethylhexyl nitrate (2-EHN) by *Mycobacterium austroafricanum* IFP 2173

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Running title: Catabolism of 2-Ethylhexyl nitrate

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Biodegradation of 2-Ethylhexyl Nitrate by *Mycobacterium austroafricanum* IFP 2173▽

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2-Ethyhexyl nitrate (2-EHN) is a major additive of fuel that is used to increase the cetane number of diesel. Because of its wide use and possible accidental release, 2-EHN is a potential pollutant of the environment. In this study, *Mycobacterium austroafricanum* IFP 2173 was selected from among several strains as the best 2-EHN degrader. The 2-EHN biodegradation rate was increased in biphasic cultures where the hydrocarbon was dissolved in an inert non-aqueous-phase liquid, suggesting that the transfer of the hydrophobic substrate to the cells was a growth-limiting factor. Carbon balance calculation, as well as organic-carbon measurement, indicated a release of metabolites in the culture medium. Further analysis by gas chromatography revealed that a single metabolite accumulated during growth. This metabolite had a molecular mass of 114 Da as determined by gas chromatography/mass spectrometry and was provisionally identified as 4-ethyldihydrofuran-2(3H)-one by liquid chromatography-tandem mass spectrometry analysis. Identification was confirmed by analysis of the chemically synthesized lactone. Based on these results, a plausible catabolic pathway is proposed whereby 2-EHN is converted to 4-ethyldihydrofuran-2(3H)-one, which cannot be metabolized further by strain IFP 2173. This putative pathway provides an explanation for the low energetic efficiency of 2-EHN degradation and its poor biodegradability.

2-Ethyhexyl nitrate (2-EHN) is the nitric ester of 2-ethyl-1-hexanol. It is added at 0.05% to 0.4% to diesel formulations in order to increase the cetane number. As a result of the extensive use of diesel worldwide, the 2-EHN market is about 100,000 tons per year.

Although biodegradability has for a long time been regarded as a relevant characteristic of chemicals, it was only recently incorporated into safety assessments. In the case of fuel oils, large volumes of oxygenates, such as methyl-*tert*-butyl ether (MTBE), have been added to gasoline since 1992 (19). Because of a lack of knowledge about their biodegradability and insufficient safety regulation, pollution cases resulting from accidental releases occurred in many countries. In the United States, for example, as many as 250,000 sites may have been polluted from leaking underground fuel tanks (35). Poor knowledge of the biodegradation of widely used chemicals may also hide unforeseen concerns relating to the toxicity of metabolic products. For example, the degradation of chlorinated aromatics, such as 4-chlorocatechol, in soil gave rise to the formation of an antibiotic, protoanemonin, which is detrimental to soil microcosms (6).

In case of accidental release of 2-EHN into the environment, the fate and impact of the pollution are unpredictable because of the scarcity of data on 2-EHN biodegradation. Screening tests have been recommended by both the U.S. Environmental Protection Agency (34) and the Organization for Economic Cooperation and Development (24) to evaluate the biodegradability of commercial substances. In this context, the so-called criterion of "ready biodegradability" requires that the tested substance be biodegraded to a level of 60% within 28 days (5). Standard degradation experiments showed that 2-EHN could not be considered readily biodegradable (American Chemistry Council Petroleum Additives Panel High Production Volume Challenge Program final submission for nitric acid, 2-ethylhexyl ester, 2006). It was assumed in this study that 2-EHN was poorly available to microbial communities because of its low water solubility and its high volatility.

In fact, 2-EHN displays both a low vapor pressure, corresponding to about 1.9 mg/liter at 20°C, and a moderate solubility in water (12.6 mg/liter at 20°C). Therefore, 2-EHN is expected to form a separate organic phase in aqueous solution even when present in small amounts. 2-EHN is also a rather hydrophobic molecule, as indicated by a log $K_{o/w}$ (octonal-water partition coefficient) value of 5.24. Hydrophobic compounds with log $K_{o/w}$ values in the range of 1 to 5 are often toxic to cells because they insert into the lipid bilayer of the cell membrane, disturbing its integrity and causing cell permeabilization (13, 22).

The backbone of 2-EHN is a branched alkane, a type of molecule that is more resistant to biodegradation than linear alkanes. The metabolism of both linear and branched hydrocarbons by bacteria involves enzymes of the β-oxidation pathway (3). In the case of branched alkanes, their degradation may lead to the formation of β-substituted acyl-coenzyme A intermediates that block β-oxidation (27). Such a metabolic blockage has been encountered during the degradation of terpenoids, such as citronellol, geraniol, and nerol (10, 28). If a

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quaternary carbon atom occurs at the end of an alkane chain, the result is a molecule quite resistant to microbial attack (18).

In a recent study, microbial communities endowed with the ability to degrade 2-EHN were obtained by enrichment from activated sludge or soil samples (33). The isolation of pure strains able to utilize 2-EHN as a sole source of carbon and energy proved rather difficult. Nevertheless, among several strains of fast-growing mycobacteria previously isolated on other hydrocarbons, some strains, all identified as *Mycobacterium austroafricanum*, were found to degrade 2-EHN.

In the present study, the kinetics of 2-EHN degradation by selected strains was investigated. *M. austroafricanum* IFP 2173, which showed the highest rate of degradation, was chosen for further investigation of 2-EHN catabolism. As a means to reduce the expected toxic effect of 2-EHN on bacterial cells and increase its bioavailability in aqueous media, bacterial cultures were mostly carried out in biphasic media. Such biphasic cultures, including a non-aqueous-phase liquid (NAPL) that serves as a solvent for the hydrophobic substrate, have already been implemented to facilitate the degradation of various toxic or recalcitrant compounds (2, 4, 8, 12, 25, 26). A metabolite that accumulated during growth was detected in the culture medium and identified by liquid chromatography-tandem mass spectrometry (LC/MS-MS). Based on our data, a plausible pathway for 2-EHN catabolism by *M. austroafricanum* IFP 2173 is proposed.

MATERIALS AND METHODS

Microorganisms and culture conditions. The strains used in this study were *M. austroafricanum* IFP 2173 (30), isolated on iso-octane; *M. austroafricanum* IFP 2012 (11) and *M. austroafricanum* IFP 2015 (15), both isolated on MTBE; and *M. austroafricanum* C6 (14), *M. austroafricanum* Spyr_Ge_1, and *M. austroafricanum* BHF 004 (J. C. Willison, unpublished data), all isolated on pyrene.

The culture medium consisted of a mineral salts solution (7) supplemented with 0.1 g/liter of yeast extract. The carbon source was added after medium sterilization (120°C for 20 min). All cultures were incubated at 30°C with shaking (150 rpm).

Chemicals. 2-EHN (Chemical Abstract Service [CAS] registry number 27247-96-7), 2-ethylhexanol, 2-ethylhexanoic acid, MTBE, decahydronaphthalene, 3-methyldihydrofuran-2(3H)-one, diethyl zinc, and heptamethylnonane (HMN) were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Mineral salts were from VWR (Fontenay-sous-Bois, France).

Biodegradation experiments. Biodegradation tests were performed in 120-ml flasks closed with Teflon-coated stoppers and sealed with aluminum caps. Unless otherwise indicated, 4.8 mg of 2-EHN (or 2-ethylhexanol or 2-ethylhexanoic acid) was added to 10 ml of the medium supplemented with 500 μl of 2,2,4,4,6,8,8-HMN. Cultures were adjusted to an optical density at 600 nm of 0.2 using washed pellets of centrifuged precultures grown on Tween 80 (2.5 g/liter) as the sole source of carbon. The degradation rate was monitored by measuring at regular intervals by gas chromatography (GC) the CO_2 evolved in the headspace. Residual 2-EHN was estimated as described below in triplicate. Abiotic controls were supplemented with mercuric chloride (0.2 mg/liter), and endogenous controls, lacking a carbon source but containing HMN, were performed under similar conditions.

Analyses of substrate and products. Cultures grown on 2-EHN were filtered on a polytetrafluoroethylene membrane (0.45 μm), and the cell biomass was determined as dry weight after lyophilization of the cell pellet. When HMN was omitted from the growth medium, the total organic carbon (TOC) was measured on the filtrates using a TOC-5050 carbon analyzer (Shimadzu) according to the European norm NF EN 1484. Residual 2-EHN in the culture filtrate, as well as derived metabolites, was extracted with 10 ml of MTBE containing 0.05% (vol/vol) decahydronaphthalene as an internal standard. After 30 min of shaking and static overnight incubation at 4°C, the solvent extracts were analyzed by GC with flame ionization detection (FID). A Varian 3400 chromatograph (Sugarland) equipped with a CP-Sil Pona CB column (0.25 mm by 50 m) obtained from Chrompack (Raritan, NJ) was used. The carrier gas was helium. The temperatures of the injector and the detector were set at 250 and 280°C, respectively. The column temperature was varied from 100°C to 200°C at 4°C/min and then from 200°C to 259°C at 20°C/min.

Time courses of 2-EHN degradation and metabolite excretion were performed in flasks that were sacrificed at regular time intervals. CO_2 in the flask headspace was measured with a Varian 3400 gas chromatograph (Sugarland) equipped with a catharometric detector and a PorapackQ (80/100 mesh, 2 m; Chrompack, Raritan, NJ). The net amount of CO_2 produced was determined as the difference between the final quantities found in the test flasks and that found in hydrocarbon-free flasks.

Kinetics of O_2 consumption. Continuous monitoring of substrate oxidation was carried out through measurement of O_2 consumption using a respirometer (Sapromat D12-S; Voith, Germany). Flasks containing 250 ml of culture medium and 125 μl of 2-EHN as a carbon source were inoculated with *M. austroafricanum* IFP 2173 to an optical density at 600 nm of 0.1. Incubation was carried out at 30°C with shaking in the presence or absence of HMN (12.5 ml). Cultures and substrate-free controls were performed in triplicate.

Chemical synthesis of 4-EDF. 4-Ethyldihydrofuran-2(3H)-one (4-EDF) was synthesized according to a published procedure (1). To a three-necked flask containing dry toluene (5 ml), copper(II) trifluoromethanesulfonic acid (0.025 mmol) and triethylphosphite (0.05 mmol) were successively added. The mixture was stirred for 30 min at room temperature to obtain a colorless solution. After it cooled to −20°C, zinc diethyl (5 mmol previously dissolved in hexane) was added, followed by furan-2(5H)-one (5 mmol). The reaction mixture was allowed to warm to 0°C for 6 h and was then incubated at room temperature and monitored by GC. After completion of the reaction, the mixture was hydrolyzed with aqueous 5 N HCl and then extracted with diethyl ether (2 × 15 ml); the organic phase was dried over $MgSO_4$ and concentrated in vacuo. The crude product was purified by column chromatography on SiO_2 using a mixture of diethyl ether/pentane [80/20]) as the eluent.

Coupled MS analyses. GC/MS-MS analysis was carried out under chromatographic conditions identical to those described above for GC-FID. Mass spectra were acquired in the split mode with a time-of-flight mass spectrometer (Tempus TOF MS; Thermo Finnigan).

LC/MS-MS was performed using a high-performance LC system (Alliance 2695; Waters, Guyancourt, France) coupled to a Quattro LC triple-quadrupole mass spectrometer (Micromass, Manchester, United Kingdom) with an electrospray interface. Data were acquired in the positive or negative ionization mode and processed with the MassLynx NT 4.0 system. The electrospray source voltages were as follows: capillary, 3.2 kV; extractor, 2 V; and cone voltage, 22 and 17 V under positive mode. The source block and desolvation gas stream were heated at 120°C and 350°C, respectively. Nitrogen was used as the nebulization and desolvation gas (75 and 350 liters h^{-1}, respectively). For MS-MS, collisional induced dissociation was performed under argon (2.5×10^{-3} mbar) at a collision energy set between 10 and 40 eV.

RESULTS

Time course of 2-EHN biodegradation by selected strains. The kinetics of 2-EHN biodegradation was studied using a few bacterial strains previously selected from among environmental isolates and collection strains for the ability to attack the compound (33). Most of these strains were identified as members of the genus *Mycobacterium*. In order to avoid growth inhibition due to 2-EHN toxicity, HMN was added as NAPL to the bacterial cultures, and biodegradation time courses were monitored by measuring the CO_2 production in the culture headspace. The biodegradation kinetics were found to vary widely depending on the bacterial strains (data not shown). *M. austroafricanum* IFP 2173 was the fastest and most efficient of the microorganisms tested, since it produced the largest amount of CO_2 (37 μmol per flask) after 13 days of incubation. *M. austroafricanum* IFP 2173 was also the only strain able to grow on 2-EHN in the absence of HMN (data not shown).

Effect of the 2-EHN supply mode on the biodegradation rate. The impact of NALP addition on 2-EHN biodegradation by

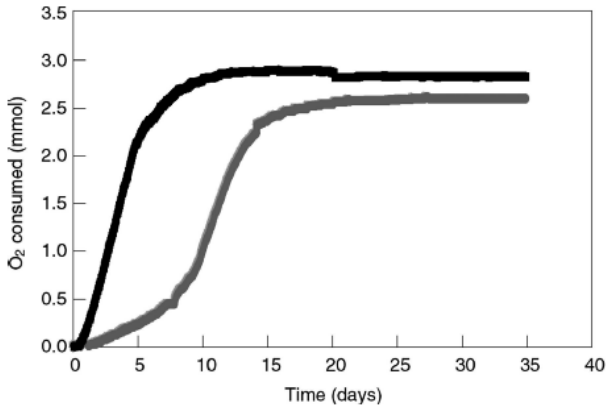

FIG. 1. Effect of a NALP (HMN) on the rate of oxygen consumption by *M. austroafricanum* IFP 2173. Cultures (250 ml) were grown in the flasks of a respirometer and contained 125 μl of 2-EHN as a carbon source. The cultures were incubated in the presence (black line) or absence (gray line) of HMN (12.5 ml).

strain IFP 2173 was studied through continuous monitoring of substrate-dependent oxygen consumption by respirometry.

In the culture lacking HMN, O_2 uptake started after a lag phase of about 1 day and then increased with time according to a sigmoidal curve (Fig. 1). The maximal growth rate (μ_{max}) could be deduced from the oxygen uptake rate, assuming that the biomass yield remained constant during growth. Over a 9-day period of growth, μ_{max} was calculated to be 0.29 day^{-1} on average, corresponding to a generation time of 2.4 days. In the HMN-containing culture, the lag phase was shorter, and the O_2 uptake became linear after a very short exponential phase (μ_{max} = 0.29 day^{-1}). The maximal rate of O_2 uptake was 5.3 mmol/day, and the overall O_2 consumption reached a maximum of 2.9 mmol, compared to 2.6 mmol for cells grown without HMN.

The effect of the 2-EHN concentration on growth was studied in HMN-containing cultures (Fig. 2). The concentration of 2-EHN had little effect on the specific growth rate. During the linear phase of growth, the O_2 uptake rate increased proportionally to the 2-EHN concentration in the culture medium up to 3 g/liter. This indicated that the 2-EHN diffusion rate from

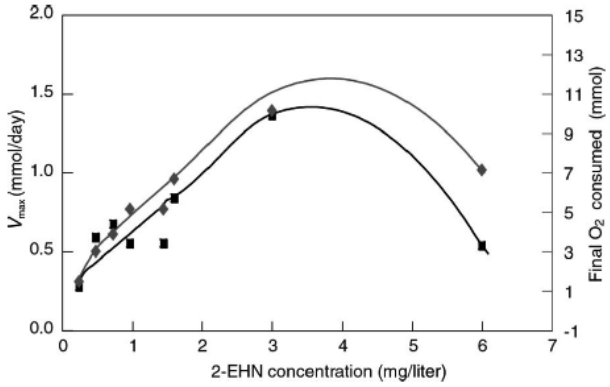

FIG. 2. Effect of the 2-EHN concentration on oxygen consumption by *M. austroafricanum* IFP 2173. Biphasic cultures contained a variable concentration of 2-EHN and 12.5 ml of HMN. The maximal rates of O_2 uptake or metabolism (V_{max}) (■) and overall O_2 consumption (◆) were determined.

TABLE 1. Carbon balance of 2-EHN biodegradation by *M. austroafricanum* IFP 2173[a]

Substrate or product	Mass change[b] (mg/liter)	Carbon balance	
		Carbon change[b] (mg/liter)	Carbon recovery (%)
2-EHN	482	269	0
Cell biomass	94	50[c]	19
CO_2[d]	115	31	12
TOC[e]	165	165	61
Total products			92

[a] Cultures (10 ml) were performed at 30°C in 120-ml flasks.
[b] Considering the whole content of the culture flasks.
[c] The carbon/dry-biomass ratio was assumed to be 52% (17). The dry biomass was determined from 100-ml cultures grown in 1-liter flasks.
[d] CO_2 was determined after acidification of the culture.
[e] TOC was measured in the culture fluid after filtration through a 0.22-μm membrane.

HMN to the water phase was a limiting factor for bacterial growth. At 2-EHN concentrations higher than 3 g/liter, bacterial growth was inhibited, as indicated by both lower oxygen uptake rates and lower overall O_2 consumption. For 2-EHN concentrations lower than 3 g/liter, no residual substrate was detected in the culture medium by the end of growth, and the O_2 consumption was roughly proportional to the amount of substrate supplied.

Carbon balance of 2-EHN biodegradation by *M. austroafricanum* IFP 2173. In order to determine the carbon balance of 2-EHN biodegradation, *M. austroafricanum* IFP 2173 was cultivated in mineral medium lacking HMN to avoid perturbation of TOC measurements by HMN. The culture was stopped when no more CO_2 was released, which coincided with the total consumption of 2-EHN (see Fig. 4). The biomass formed, the TOC in the filtered culture medium, and the amount of CO_2 released were measured. The carbon recovery as metabolites and cell biomass was calculated by taking into account the elementary compositions of the substrate and products (Table 1). A carbon recovery rate of 92% was obtained for the 2-EHN bioconversion. Carbon converted into biomass (94 mg/liter) and CO_2 (165 mg/liter) amounted together to only 33% of the total carbon produced. Accordingly, a high proportion of the substrate-derived carbon was recovered in the clarified culture medium (67%), possibly reflecting metabolite accumulation.

Identification of a metabolite excreted in the culture. GC-FID analysis of culture fluid extracts performed during 2-EHN degradation experiments revealed a gradual increase in the concentration of an unknown compound with a retention time shorter than that of 2-EHN. This finding suggested that a metabolite might have accumulated during growth and accounted for the substantial level of TOC previously detected in the supernatants of 2-EHN-grown cultures. High-resolution mass spectral analysis of this compound (Fig. 3a) showed that it had a molecular mass of 114.07 Da and the chemical formula $C_6H_{10}O_2$. The mass spectrum of this compound did not match any of the spectra currently available in the databases. Nevertheless, a comparison of the LC/MS-MS data of the excreted product with those of 3-methyldihydrofuran-2(3H)-one, a commercially available product, revealed several common fragment ions. The analysis also indicated that the molecule

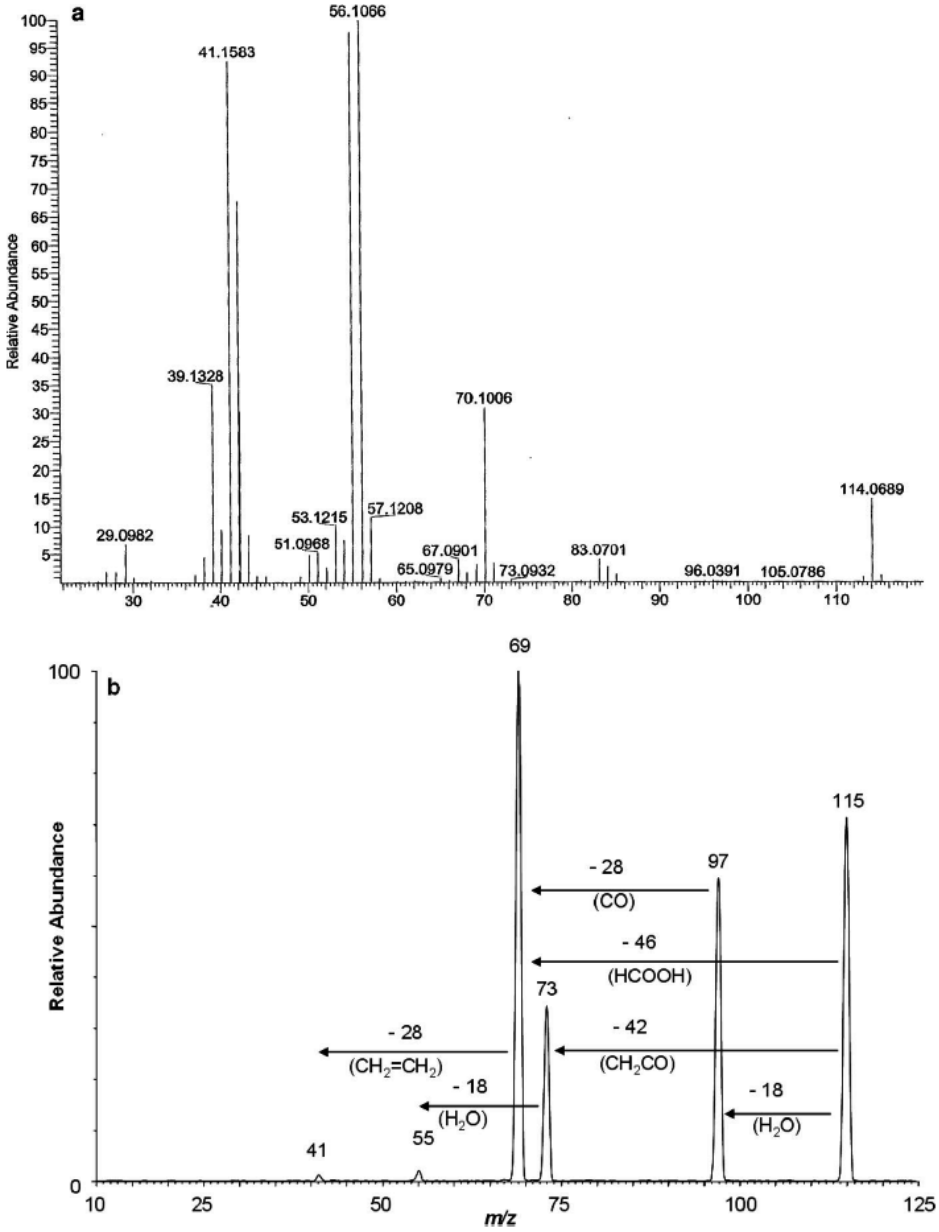

FIG. 3. MS characterization of the metabolite produced by strain IFP 2173 upon degradation of 2-EHN. (a) High-resolution electron impact mass spectrum of the accumulated metabolite as obtained by GC-MS analysis. (b) Collisional induced dissociation/MS-MS product ion spectrum of the protonated molecule (MH^+) obtained by LC/MS-MS analysis at a collision energy of 10 eV.

did not contain any carboxylic or hydroxyl groups (Fig. 3b). Taken together, our data indicated that the product of interest might be 4-EDF, which can also be designated β-ethyl-γ-butyrolactone. In order to confirm the structure of the metabolite, the chemical synthesis of 4-EDF was undertaken as described in Materials and Methods (1). The LC/MS-MS characteristics of the synthesized lactone were identical to those of the metabolite, confirming that the product that accumulated in cultures of *M. austroafricanum* IFP 2173 grown on 2-EHN was 4-EDF.

The rate of 4-EDF accumulation was assessed by GC-FID analysis of the culture fluid during growth. Figure 4 shows that 4-EDF formation and CO_2 release were concurrent with 2-EHN degradation.

Biodegradation of 2-EHN-derived compounds. As a means to elucidate the pathway of 2-EHN biodegradation by *M. austroafricanum* IFP 2173, we tested compounds with structures derived from 2-EHN as possible substrates. 2-Ethylhexanol, the primary alcohol resulting from 2-EHN hydrolysis, was biodegraded, yielding 2-ethylhexanoic acid and 4-EDF. 2-Ethylhexanoic acid, the product resulting from 2-ethylhexanol oxidation, was not biodegraded, even in the presence of HMN. This compound is considered to be toxic for most bacteria (21). It should be noted that 2-EHN can be used as a sole nitrogen source by strain IFP 2173, indicating that nitrate is formed, probably as a result of an initial attack on 2-EHN by an esterase (data not shown). 2-EHN biodegradation was also tested in the presence of isooctane, the compound on which *M. austroaf-*

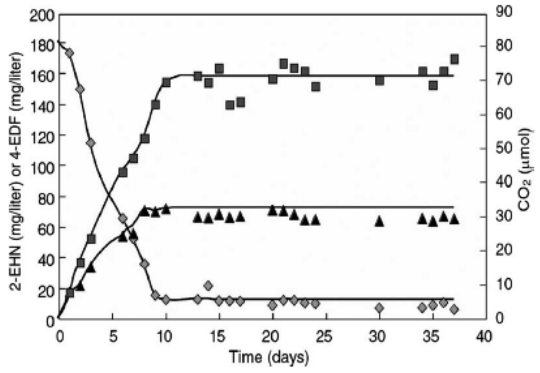

FIG. 4. Accumulation of 4-EDF during 2-EHN biodegradation. Parallel cultures were carried out in 120-ml flasks and removed at the times indicated for extraction and measurements of 2-EHN (♦) and 4-EDF (▲). CO_2 (■) was measured in a separate culture flask. Residual 2-EHN is the fraction of hydrocarbon that stayed bound to the flask wall and stopper and remained inaccessible to bacteria.

ricanum IFP 2173 was selected. Diauxic growth was observed, with the strain degrading isooctane first and then 2-EHN into 4-EDF (data not shown).

DISCUSSION

2-EHN is a recalcitrant compound that was considered not readily biodegradable according to standard procedures (American Chemistry Council Petroleum Additives Panel High Production Volume Challenge Program final submission for nitric acid, 2-ethylhexyl ester, 2006). However, we demonstrated in the present study that selected strains of mycobacteria were able to slowly utilize 2-EHN as a sole source of carbon under defined culture conditions. The poor biodegradability of 2-EHN might be the consequence of two factors: first, the rare occurrence of microorganisms able to use it as a carbon source, and second, its inhibitory effect on bacterial growth even at a low concentration. 2-EHN inhibition was illustrated by the experiment shown in Fig. 2 and by the lack of growth of all strains tested in HMN-free cultures, except *M. austroafricanum* IFP 2173. This strain, isolated for its ability to degrade isooctane, a branched alkane (31), demonstrated broad capabilities for hydrocarbon biodegradation (16, 32). Like many members of the *Corynebacterium-Mycobacterium-Nocardia* group of gram-positive bacteria, it may be resistant to toxic hydrocarbons, thanks to the properties of its cell envelope, which is highly rigid and contains mycolic acids (29). In mycobacteria, mycolic acids are very long fatty acids (C_{60} to C_{90}) that contribute up to 60% of the cell wall (9). The specific cell wall composition of the *M. austroafricanum* strains studied here probably accounts for their resistance to 2-EHN.

However, it is unclear whether the unique ability of strain IFP 2173 to grow on 2-EHN without NAPL is due to a cell wall composition slightly different from that of other strains or to some other strain-specific trait.

Biphasic cultures, involving the addition of an inert NAPL like HMN, were found to be critical for 2-EHN biodegradation and bacterial growth. In the HMN-free cultures, the dissolved fraction of 2-EHN represented only a minor part of the substrate supplied, since it partitioned into three distinct phases, i.e., the gas phase, the aqueous phase, and the bulk of insoluble 2-EHN. During the biodegradation process, the uptake of dissolved substrate was counterbalanced by the equilibrium transfer of 2-EHN from the bulk of the substrate ($S_{subNAPL}$) to the aqueous substrate ($S_{sub/aq}$) according to the following scheme:

$$S_{sub/NAPL} \underset{}{\overset{T_{NAPL/aq}}{\rightleftharpoons}} S_{sub/aq} \xrightarrow{\text{Growth phase}} x,$$

where $S_{subNAPL}$ and $S_{sub/aq}$ represent the amounts of substrate in the bulk and in the aqueous phases, respectively; x is the cell biomass; and $T_{NAPL/aq}$ is the substrate transfer rate of 2-EHN to the culture medium. In HMN-containing cultures, the dissolved 2-EHN was mainly confined to NAPL. Because of the high hydrophobicity of their cell walls, microbial cells tightly adhered to NAPL, and direct contact was thus the most probable mode of substrate uptake (8, 12). Accordingly, the large NAPL volume (500 μl of HMN versus 5 μl of 2-EHN in the case of the NAPL-free culture), which increased substrate bioavailability, probably accounted for its higher efficiency of assimilation by the microorganisms. Such conditions of substrate delivery were apparently required to promote the growth on 2-EHN of *M. austroafricanum* strains other than strain 2173.

The biodegradation of 2-EHN by *M. austroafricanum* IFP 2173 illustrates the remarkable metabolic capabilities of this strain for recalcitrant hydrocarbons. Indeed, it can degrade another methyl branched alkane, 2,2,4-trimethylpentane (31), suggesting that it produces enzymes specific for the degradation of anteiso-alkanes. Nevertheless, our results indicate that the degradation of 2-EHN by strain IFP 2173 is partial and gives rise to the release of an acyl with an ethyl substituent in the beta position. At least two reasons might explain the accumulation of this metabolite: (i) strain IFP 2173 lacks enzymes able to degrade it, and (ii) because of the ethyl group in the beta position, the metabolite might block the enzyme catalyzing the next step in the degradation of branched alkanes.

Considering the high biodegradation potential of strain IFP 2173, it was recently observed that this strain can degrade other xenobiotic compounds structurally related to 2-EHN, such as bis(2-ethylhexyl)phthalate (data not shown), used as a plasticizer (21, 23). The biodegradation of this compound by *Mycobacterium* sp. strain NK0301 has been reported (20). This bacterium utilized phthalate as a carbon and energy source and left the carbon skeleton of the 2-ethylhexyl moiety intact, releasing it as 2-ethylhexanol or 2-ethylhexanoic acid. In comparison, strain IFP 2173 degraded bis(2-ethylhexyl) phthalate and utilized the 2-ethylhexyl moiety, achieving a higher degree of degradation (data not shown).

The biodegradation of 2-EHN by strain IFP 2173 gave rise to the accumulation of a lactone, which was identified as 4-EDF. The lactone formed by cyclization of a breakdown product, a branched pentanoic acid, which was not metabolized further by the bacteria. The partial degradation of 2-EHN certainly explains the observed slow growth (μ_{max} = 0.29 day^{-1}) and poor growth yield of cultures utilizing this compound as a sole C source.

Considering the structure of the intermediate metabolite and the known degradation pathway of *n*-alkanes (18), we propose for the first time a plausible metabolic pathway for

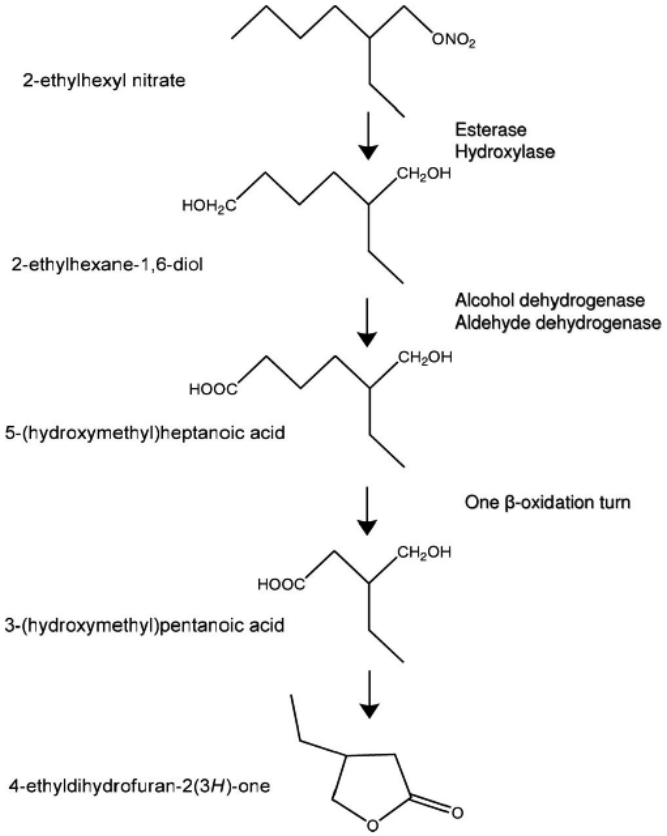

FIG. 5. Proposed pathway for 2-EHN biodegradation by *M. austroafricanum* IFP 2173.

2-EHN degradation (Fig. 5). The pathway would start by a simultaneous or sequential attack of the molecule on both extremities, with an esterase activity hydrolyzing the nitric ester bond and an oxygenase catalyzing the hydroxylation of the distal methyl group. The involvement of an esterase that would release nitrate was inferred from the observation that strain IFP 2173 utilized 2-EHN as a nitrogen source. The existence in this strain of a hydroxylase active on branched alkane is expected, since it grows on isooctane (31). The intermediate metabolite that would form, 2-ethylpentan-1,6-diol, is proposed to be oxidized to a carboxylic acid in two steps involving successively an alcohol and an aldehyde dehydrogenase. After activation by coenzyme A, the resulting 5-(hydroxymethyl)heptanoic acid would undergo one cycle of classical β-oxidation to give 3-(hydroxymethyl)pentanoic acid, which would spontaneously convert to 4-EDF by cyclization. Since the substrate underwent a single turn of β-oxidation, only two carbon atoms (out of eight in 2-EHN) could reach the tricarboxylic acid cycle, accounting for the low percentage of carbon released as CO_2 (12%).

The proposed pathway now needs to be assessed experimentally by identifying enzymes involved in 2-EHN degradation. To this end, we have undertaken a proteomic analysis to discover the proteins that are induced upon incubation of strain IFP 2173 with 2-EHN.

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