

A novel pathway for mineralization of the thiocarbamate herbicide molinate by a defined bacterial mixed culture

Luísa Barreiros,^{1,2} Balbina Nogales,^{3,4}
Célia M. Manaia,² António C. Silva Ferreira,²
Dietmar H. Pieper,³ Maria A. Reis,⁵ and
Olga C. Nunes^{1*}

¹LEPAE – Departamento de Engenharia Química,
Faculdade de Engenharia, Universidade do Porto, Porto,
Portugal.

²Escola Superior de Biotecnologia, Universidade Católica
Portuguesa, 4200–072 Porto, Portugal.

³Division of Microbiology, GBF-German Research Centre
for Biotechnology, Braunschweig, Germany.

⁴Area de Microbiologia, Universitat de les Illes Balears,
Palma de Mallorca, Spain.

⁵Department of Chemistry, Faculdade de Ciências e
Tecnologia, Universidade Nova de Lisboa, Monte da
Caparica, Portugal.

Summary

A bacterial mixed culture able to mineralize molinate was established, through enrichment, using mineral medium with molinate as the only carbon, nitrogen and energy source. The combination of five cultivable isolates, purified from the enrichment culture, permitted the reconstitution of a degrading consortium. Both enrichment and defined cultures were able to mineralize molinate without accumulation of degradation products by the end of the growth. Among the five isolates constituting the defined mixed culture, an actinomycete, strain ON4, was essential for biodegradation, being involved in the cleavage of the thioester bond of molinate, the initial step of the degradation pathway. Isolate ON4 was able to grow on molinate at concentrations below 2 mM, with the accumulation of ethanethiol and diethyl disulphide. These sulphur compounds were toxic to strain ON4 when accumulating at higher concentrations. However, this inhibitory effect was avoided by the presence of other members of the mixed culture, out of which isolates ON1 and ON2 were observed to consume ethanethiol and diethyl disulphide. In this way, interactions among defined mixed culture members involve metabolic and detoxifying association.

Introduction

Molinate (S-ethyl perhydroazepine-1-carbothioate) is a systemic thiocarbamate herbicide used world-wide to control weeds in rice paddies. Molinate is applied to flooded fields, and due to the flow of water between the fields and surroundings, contamination of receiving waters has occurred in various countries at levels up to 100 µg l⁻¹ of the herbicide (Carrasco *et al.*, 1987; Julli and Krassoi, 1995; Mabury *et al.*, 1996). A review of the toxicological information of molinate indicates that this herbicide has adverse reproductive effects, is neurotoxic and possible oncogenic (Cochran *et al.*, 1997).

According to literature, dissipation of molinate in fields is mainly due to volatilization (75–85%), although photolysis (5–10%), and adsorption and biodegradation (5–10%) are also supposed to occur (Soderquist *et al.*, 1977; Rajagopal *et al.*, 1984; Mabury *et al.*, 1996).

Although molinate is considered one of the most recalcitrant thiocarbamates (Nagy *et al.*, 1995), several soil and water microorganisms, both in mixed populations or in pure cultures (fungi and bacteria), have been reported to convert molinate into partially oxidized products (Klysheva *et al.*, 1980; Golovleva *et al.*, 1981; Zyakun *et al.*, 1983; Imai and Kuwatsuka, 1986a,b,c). Three main pathways have been proposed, based on products detected in molinate transforming microbial cultures, experimental soils and environmental samples (Soderquist *et al.*, 1977; Thomas and Holt, 1980; Golovleva *et al.*, 1981; Imai and Kuwatsuka, 1982). The oxidation of the ethyl moiety leads to the production of molinate alcohol and molinate acid, whereas hydroxy- and oxomolinate are derived from azepine ring oxidation. Molinate sulphoxide and sulphone are formed as a consequence of sulphur oxidation, and it is supposed that such a chemical modification may lead to the release of hexamethyleneimine (HMI). The degradation of molinate and its oxidized derivatives, namely with regard to the enzymes/catalysts involved, is not understood. Molinate sulphoxide seems to be a widespread oxidation product of molinate, being produced by eukaryotic cells (Jewell *et al.*, 1999) or by chemical processes such as the chlorination employed in water treatment facilities (Cochran *et al.*, 1997). Molinate sulphoxide has been reported as being more persistent and, above all, more toxic to animals than molinate (Golovleva *et al.*, 1981; Ellis *et al.*, 1998; Jewell and Miller, 1998; Tjeerdema and Crosby,

1988; Jewell *et al.*, 1999). Because molinate oxidation is thought to occur extensively, the contamination of natural waters with this herbicide may have more malignant effects than previously assumed.

This report describes a mixed bacterial culture able to mineralize molinate while using it as the only source of carbon, energy and nitrogen. Under these conditions, the defined mixed culture promoted molinate degradation without accumulation of dead-end products. Among the five cultivable bacterial isolates composing the defined mixed culture, an actinomycete was the only one capable of molinate transformation, in axenic culture.

Results

Enrichment and characterization of molinate degrading cultures

An enrichment culture was obtained, using molinate as carbon source, from a sample of sediment collected from a site where a molinate containing effluent has been discharged for several years. This enrichment culture, named EC1, reduced 400 mg l⁻¹ of molinate to concentrations below the HPLC detection limit (0.9 mg l⁻¹), with a specific degradation rate of 110 mg of molinate g⁻¹ (cells dry weight) h⁻¹ and with a maximum specific growth rate of 0.04 h⁻¹. High performance liquid chromatography (HPLC) analyses gave no indication for the accumulation of intermediates or dead-end products. The analysis of the microbial composition of consortium EC1 revealed the presence of at least 45 cultivable bacterial isolates, distinguishable by their colony morphology on PCA plates. In order to obtain a culture comprising a limited number of bacteria, together capable of molinate degradation, consortium EC1 was subcultured in medium containing molinate as single carbon and nitrogen source. Furthermore, a molinate concentration of 750 mg l⁻¹, close to its maximum solubility, was used such that the selected microorganisms should be able to tolerate high concentrations of the herbicide. Plating of the thereby obtained enrichment culture (EC2) revealed the presence of five different morphological types of colonies. The respective colonies were purified and the isolates designated by isolate ON1 to ON5. None of the isolates was able to grow with 750 mg l⁻¹ molinate as a single source of carbon and nitrogen in axenic culture. However, the artificial five-membered mixed culture consisting of the mixture of equal proportions of purified cultivable isolates from enrichment culture EC2 was able to grow on and to mineralize molinate. This defined mixed culture was named DC (defined culture). It could, thus, be concluded that, enrichment culture EC2 was at least composed by the five isolates and that they had a major role in molinate degradation.

Characterization of members of the defined mixed culture DC

Isolates ON1 to ON5 were characterized using FAMES profiling, Biolog assays and 16S rDNA sequencing analysis. Out of the five isolates, only ON4 was Gram-positive. The combination of the three employed methodologies permitted the identification of the Gram-negative isolates (ON1, ON2, ON3 and ON5), at least to the genus level. Their 16S rDNA sequences presented high percentage of identity (above 99%) with reference 16S rDNA sequences in the EMBL database. Biolog and GC-FAME coefficients for these isolates were above the minimal matches values referred in guidelines of these systems (0.5 and 0.3 respectively). Isolates ON1 and ON3 were identified as members of the genus *Pseudomonas*, with similarities of 99.84% with the 16S rRNA sequence of *P. chlororaphis* IFO 3904^T and of 99.77% with *P. nitroreducens* IAM 1439^T respectively. The 16S rRNA sequence of isolate ON5 had 99.60% similarity with that of *Achromobacter xylosoxidans* ssp. *denitrificans* ATCC 15173^T. Isolate ON2 was identified as *Stenotrophomonas maltophilia* showing a similarity of 99.32% with *S. maltophilia* ATCC 15173^T. The fifth isolate, ON4 was a Gram-positive bacterium, affiliated by 16S rDNA sequence analysis to the actinomycetes with peptidoglycan Group B (Rainey *et al.*, 1994), being *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* LMG 3645^T the closest validly described species (95.30% similarity).

Degradation of molinate by enrichment culture EC2 and defined mixed culture DC

Both, the enrichment culture EC2 and the defined mixed culture DC were able to grow and mineralize molinate when grown with concentrations up to 750 mg l⁻¹ of the herbicide. For both mixed cultures, the association between cell growth and molinate depletion was evidenced by the fact that the exponential growth phase was coincident with molinate consumption, and growth ceased after molinate had been consumed (Fig. 1A and B). There was a direct association between the total organic carbon of the culture supernatants (referred as dissolved organic carbon, DOC) and molinate depletion by both cultures; DOC contents decreased by more than 91% when cultures were grown with molinate as the sole source of carbon and nitrogen (Table 1). No degradation of molinate was observed in uninoculated controls. Both cultures EC2 and DC were able to degrade molinate to concentrations below the detection limit, and the maximum specific growth and molinate degradation rates of defined mixed culture DC [0.081 ± 0.01 h⁻¹ and 189.4 ± 18.9 mg of molinate g⁻¹ (cells dry weight) h⁻¹, respectively] were slightly higher than those of enrichment culture EC2 [0.073 ± 0.01 h⁻¹

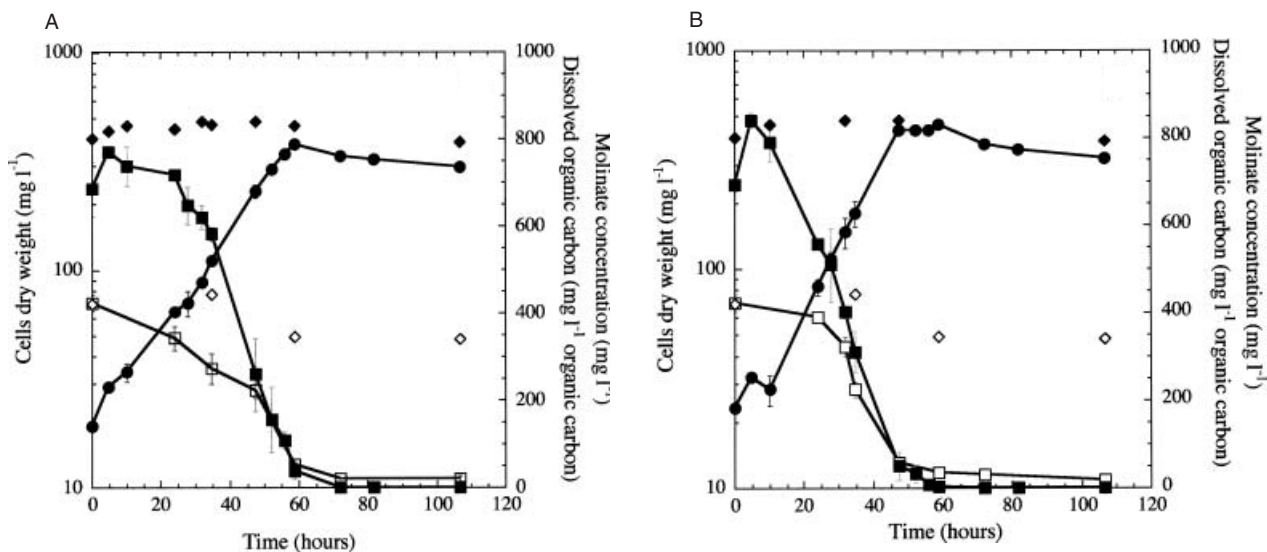


Fig. 1. Enrichment culture EC2 (A) and defined mixed culture DC (B) grown in medium B with 750 mg l⁻¹ molinate. Symbols: ■, Molinate concentration (mg l⁻¹) in culture supernatant. ◆, Molinate concentration in uninoculated control. ●, Growth curve. □, Dissolved organic carbon (mg l⁻¹ organic carbon) in culture supernatant. ◇, Dissolved organic carbon in uninoculated control. The values are the means of three independent experiments, and the error bars show standard deviations.

and 133.8 ± 9.8 mg of molinate g⁻¹ (cells dry weight) h⁻¹, respectively]. Resting cells analyses confirmed that both cultures, EC2 and DC, exhibited similar molinate degradation rates of 119 and 122 mg of molinate g⁻¹ (cells dry weight) h⁻¹ respectively.

Solid-phase micro extraction followed by gas chromatography analysis (SPME/GC) methodology revealed that molinate solutions (Herbex) contained impurities (Fig. 2A). Two of the contaminating compounds were identified as ethanethiol, and diethyl disulphide by comparison of retention time and MS spectra with those of authentic standards, and each of these compounds represented less than 5% of the compound mixture. Two further compounds were tentatively identified, by comparing the Kovats indices and the mass spectra present in the NIST 98 MS Library Database, as diethyl trisulphide and S,S-diethyl ester carbonodithioic acid respectively. No changes were observed when uninoculated medium with molinate was incubated. As is shown in Fig. 2D, defined mixed culture DC was not only capable to degrade molinate but also to degrade impurities present in the medium, as evidenced by the disappearance of the respective GC signals during growth.

Role of isolates in the degradation of molinate

As mentioned above, none of the five isolates (ON1-ON5) was able to grow in pure cultures in medium B with 750 mg l⁻¹ molinate as the only source of carbon, nitrogen and energy or even in the presence of ammonia as additional nitrogen source. All isolates were further tested for their capability to transform molinate as single source of

carbon and nitrogen and in the presence of other carbon sources (yeast extract). None of the Gram-negative isolates was able to grow or degrade the herbicide in axenic culture at any molinate concentration tested, as the only source of carbon and nitrogen or when the medium was supplemented with 0.1% (wt/vol) yeast extract. Resting cells assays with individual Gram-negative isolates confirmed that they were unable of molinate transformation. In contrast, isolate ON4 was able, in pure culture, to grow with molinate as the sole nutrient, producing biomass when the substrate was supplied at low concentrations (≤2 mM) and reducing the herbicide to undetectable levels (Table 1). However, growth ceased after successive transfers in medium B with 187 mg l⁻¹ molinate, suggesting that reserve materials might be used as growth factors. In fact, good growth was observed in medium B with 187 mg l⁻¹ molinate supplemented with 0.02% (wt/vol) of yeast extract, suggesting that unknown growth requirements may be involved. These results also may indicate that growth requirements can be fulfilled by other isolates when isolate ON4 is growing in defined mixed culture DC. The molinate degradation rate by resting cells of isolate ON4 [111 mg of molinate g⁻¹ (cells dry weight) h⁻¹] was similar to those obtained for cultures EC2 and DC [119 and 122 mg of molinate g⁻¹ (cells dry weight) h⁻¹, respectively], which suggests that isolate ON4 might be important for the whole degradation process.

No growth occurred when isolate ON4 was inoculated in medium with higher concentrations of molinate (Table 1), neither when this medium was supplemented with 0.1% (wt/vol) of yeast extract, in sealed vials. How-

Table 1. Molinate degradation by enrichment culture EC2 and defined mixed culture DC and isolate ON4 with different herbicide concentrations.

	Cultures in medium B with 187 mg l ⁻¹ molinate						Cultures in medium B with 750 mg l ⁻¹ molinate					
	Cells dry weight (mg l ⁻¹)		Molinate (mg l ⁻¹)		DOC (mg l ⁻¹)		Cells dry weight (mg l ⁻¹)		Molinate (mg l ⁻¹)		DOC (mg l ⁻¹)	
	0 d	5 d	0 d	5 d	0 d	5 d	0 d	5 d	0 d	5 d	0 d	5 d
Consortium EC2	50	100	170	<0.9	90	10	20	300	690	<0.9	420	20
Consortium DC	50	130	180	<0.9	100	10	20	320	690	<0.9	420	20
Isolate ON4	30	120	190	<0.9	105	20	30	25	700	260	450	180
Gram-negative isolates	40	20	190	190	100	105	–	–	–	–	–	–
Abiotic control	–	–	200	210	100	110	–	–	800	790	420	340

Values are means of at least two independent experiments. HPLC detection limit of 0.9 mg l⁻¹. d, days.

ever, significant molinate transformation was observed. These results suggest that an inhibitory effect due to the presence of high concentrations of molinate degradation products was taking place (see below).

Previous studies on molinate (bio)degradation have detected molinate derivatives, such as sulphoxide and sulphone, oxo- and hydroxy-, and alcohol and acid (Soderquist *et al.*, 1977; Klysheva *et al.*, 1980; Golovleva

et al., 1981; Imai and Kuwatsuka, 1982; 1986a,b,c; Zyakun *et al.*, 1983; Cochran *et al.*, 1997) as dead-end metabolites. The occurrence of some of these products during biological degradation of molinate by defined mixed culture DC and isolate ON4 was investigated using organic extracts of supernatants obtained from growing cultures as described by Imai and Kuwatsuka (1982). The extracts were analysed by HPLC and GC/FID and com-

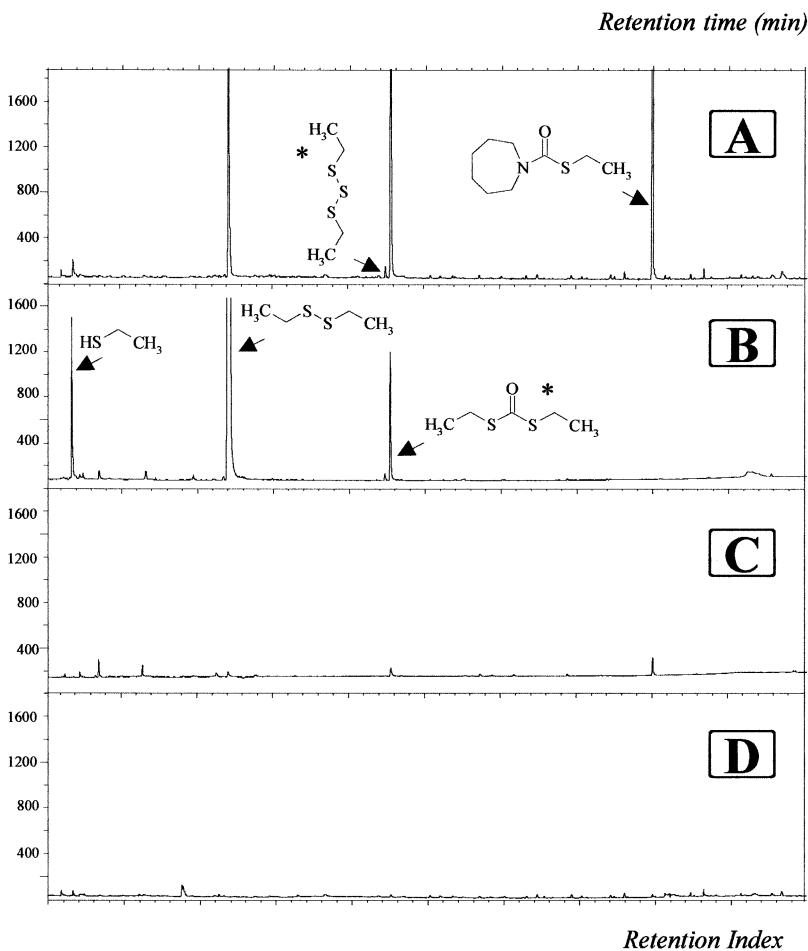


Fig. 2. Chromatograms obtained by SPME/GC/FID analysis of headspace of different cultures in medium B with 187 mg l⁻¹ molinate. Uninoculated medium after 7 days of incubation (A). Culture of isolate ON4 after 7 days of incubation (B). Culture of isolate ON4 with a mixed culture of the Gram-negative isolates incubated in a vial placed in the headspace, after 7 days of incubation (C). Culture of defined mixed culture DC after 7 days of incubation (D). (*) Tentatively identified by comparing the Kovats indices and the mass spectra present in the NIST 98 MS Library Database. Retention time of the compounds: ethanethiol, 3.2 min; diethyl disulphide, 24 min; diethyl trisulphide, 44 min; S,S-diethyl ester carbonodithioic acid, 45 min; and molinate, 80 min.

pared with authentic standards of molinate sulphoxide, molinate sulphone, 2-oxo-molinate, molinate acid and molinate alcohol. None of these compounds were detected after molinate degradation by cultures of defined mixed culture DC or of isolate ON4.

Semiquantitative SPME/GC analysis of cultures and resting cell suspensions of isolate ON4, revealed that the disappearance of molinate was concomitant with the increase in concentration of ethanethiol and diethyl disulphide. Although ethanethiol and diethyl disulphide were present in molinate solutions, the levels of these compounds clearly increased due to the activity of isolate ON4 (Fig. 2B). In fact, during molinate (1 mM) degradation, concentrations of ethanethiol and diethyl disulphide consistently increased 20 to 30-fold (corresponding to approximately 1 mM) whereas for abiotic controls no significant changes were observed. These results suggested that isolate ON4 was able to cleave the thioester bond of molinate, releasing ethanethiol that would be further spontaneously oxidized to diethyl disulphide (see below).

The DOC values found after degradation of 1 mM molinate by isolate ON4 were low (average of 20 mg l⁻¹) and comparable to those determined for a 1 mM solution of ethanethiol (average of 22 mg l⁻¹). These results suggested that isolate ON4 was able to mineralize the azepine moiety of molinate, but not the ethyl moiety.

As ethanethiol and diethyl disulphide were not present after growth of the defined mixed culture DC, which contained all the five isolates (see Fig. 2D for a comparison), the role of the Gram-negative isolates (ON1-ON3, and ON5) was analysed. The reconstitution of defined mixed culture DC by the addition of a mixture of resting cells of the Gram-negative members to cultures or resting cells of isolate ON4 preincubated with molinate, resulted in the degradation of these sulphur compounds (results not shown). In the same way, when a mixed culture of the Gram-negative isolates was incubated in a separated compartment of the flask containing a culture of isolate

ON4, the Gram-negative isolates were capable to use volatile hydrocarbons from the headspace and no products were accumulated during growth (Fig. 2C). Thus, these results indicate that defined mixed culture members other than isolate ON4 were responsible for degrading ethanethiol released from molinate by isolate ON4. In accordance with the assumption that high concentrations of ethanethiol might be toxic to isolate ON4, this organism was able to grow on 4 mM molinate, when a mixed culture of the Gram-negative isolates was present in a separate compartment of the same flask (Table 2). These results prove the ability of the Gram-negative isolates to grow while consuming the metabolites produced by isolate ON4, therefore indicating the co-operative nature of the degradation of molinate between isolate ON4 and the Gram-negative isolates.

In order to evaluate which of the Gram-negative isolates were involved in the depletion of sulphur compounds, each individual isolate was incubated in medium B supplemented with 2.5 mM ethanethiol and ammonium sulphate. According to SPME/GC analysis of the headspace of axenic cultures, only isolates ON1 and ON2 could degrade that sulphur compound among the individual members of defined mixed culture DC. However, ethanethiol was spontaneously converted to diethyl disulphide during the incubation period. In fact, in uninoculated medium with ethanethiol, half of its initial concentration was converted to diethyl disulphide within 4 days. Because the spontaneous conversion of ethanethiol to diethyl disulphide was not quantitative, but quantitative depletion was observed in the presence of isolates ON1 or ON2, it can be concluded that ethanethiol is transformed by those organisms. Moreover, both organisms are also capable of transforming diethyl disulphide.

The analytical methods utilised for liquid phase analysis (HPLC and CG/FID) of cultures or resting cells of isolate ON4 did not permit the detection of the azepine ring of molinate or its by-products. Hexamethyleneimine (HMI),

Table 2. Growth of isolate ON4 on 750 mg l⁻¹ molinate and of a mixed culture of Gram-negative isolates incubated in a vial placed in the headspace of the culture of isolate ON4.

Incubation conditions	Cells dry weight of isolate ON4 (mg l ⁻¹)		Cells dry weight of Gram-negative isolates (mg l ⁻¹)	
	0 days	10 days	0 days	10 days
Bottom: Culture of isolate ON4 in medium B with 750 mg l ⁻¹ molinate. Headspace: Culture of Gram-negative isolates in medium B with (NH ₄) ₂ SO ₄ .	40	140	40	110
Bottom: Medium B with 750 mg l ⁻¹ molinate. Headspace: Culture of Gram-negative isolates in medium B with (NH ₄) ₂ SO ₄ .	Not applicable	Not applicable	40	50
Bottom: Culture of isolate ON4 in medium B without molinate. Headspace: Culture of Gram-negative isolates in medium B with (NH ₄) ₂ SO ₄ .	40	20	40	60
Bottom: Culture of isolate ON4 in medium B with 750 mg l ⁻¹ molinate. Headspace: Medium with (NH ₄) ₂ SO ₄ .	40	50	Not applicable	Not applicable

Values are means of two independent experiments.

an analogue compound of molinate, has been referred in the literature as a possible product of molinate degradation (Thomas and Holt, 1980; Imai and Kuwatsuka, 1982; 1986c). In this way, it was tested if the individual members of the defined mixed culture DC could grow in medium B with 2.5 mM HMI. Under these conditions, only the *Pseudomonas* spp. isolates ON1 and ON3 were able to grow in axenic culture on HMI as carbon and nitrogen source. Because HMI could not support growth of isolate ON4, whereas this organism could grow on 187 mg l⁻¹ molinate with the accumulation of sulphur compounds (see above), it seems improbable that HMI is an intermediate of molinate degradation by this organism.

Discussion

The majority of published studies on molinate biodegradation show that the transformation of this herbicide takes place only in the presence of other carbon sources (Klysheva *et al.*, 1980; Golovleva *et al.*, 1981; Imai and Kuwatsuka, 1982; 1986a,b; Molinari *et al.*, 1992; Daffonchio *et al.*, 1996; Daffonchio *et al.*, 1999), leading to the accumulation of partially oxidized products (Klysheva *et al.*, 1980; Golovleva *et al.*, 1981; Zyakun *et al.*, 1983; Imai and Kuwatsuka, 1986a,b,c). The only exception is the work reported by Carrasco *et al.* (1992) with a mixed microbial culture that was able to degrade 50 mg l⁻¹ molinate, as the only carbon source, in a continuous flow reactor fed with mineral medium with ammonia as nitrogen source. Carrasco *et al.* (1992) reported a molinate depletion of about 97% for a residence time of 23 days, and accumulation of metabolic products derived from molinate degradation were detected, although in trace amounts (1–2 p.p.b.). In this respect, the present work is innovative as the microbial cultures isolated were able to use molinate as the single source of carbon, nitrogen and energy. Moreover, when compared to other published reports (Klysheva *et al.*, 1980; Golovleva *et al.*, 1981; Imai and Kuwatsuka, 1982; 1986a,b,c; Carrasco *et al.*, 1992; Daffonchio *et al.*, 1996; Daffonchio *et al.*, 1999), enrichment culture EC2 and defined mixed culture DC revealed an increased tolerance to molinate (c.a. seven times), and were able to reduce molinate to undetectable levels, accompanied by DOC depletion when grown with 750 mg l⁻¹ molinate.

Although among the five individual isolates composing defined mixed culture DC only isolate ON4 was able to degrade the herbicide, it was not responsible for molinate mineralization. SPME/GC analysis revealed that the activity of isolate ON4 leads to the accumulation of ethanethiol and mainly diethyl disulphide in headspace of its cultures or resting cells. Although ethanethiol, along with other sulphur compounds, seems to be a contaminant of molinate itself, the increase in the levels of this thiol could be

clearly attributed to the activity of isolate ON4. The production of ethanethiol was accompanied by the accumulation of diethyl disulphide, which might be the result of the spontaneous oxidation of ethanethiol. A similar description is made by Sipma *et al.* (2002) who reported the accumulation of dimethyl disulphide in an upflow anaerobic sludge-digester during methanethiol degradation, due to the presence of minor amounts of oxygen. On the other hand, the inability of isolate ON4 to grow in axenic culture in the presence of 750 mg l⁻¹ of molinate, was due to the accumulation of high concentrations of sulphur degradation products. It is reported in literature that polysulphide bounds, as di- or tri-sulphides, may retard or inhibit microbial growth (Kyung and Fleming, 1997; Tsao and Yin, 2001). In this respect, the intervention of Gram-negative isolates may be very important as detoxifying agents, as isolate ON4 was able to grow with 750 mg l⁻¹ molinate when the remaining defined mixed culture members removed those compounds from headspace of its culture. In fact, it was observed that at least isolates ON1 and ON2 had the capability to consume such degradation products. These results suggests that a mixture of isolate ON4 with one of these isolates (ON1 or ON2) are enough to perform the herbicide mineralization. However, it was not possible, until now, to establish which mixed culture composition presents the maximal efficiency of molinate mineralization.

The requirement of a consortium to achieve molinate mineralization is not surprising, as combined metabolic activity of mixed bacterial communities seems to be rather common in degradation of pollutants. In general, three distinct types of co-operation may occur among bacterial consortia members during degradation of organic pollutants – metabolic deficiency, metabolic association and metabolite detoxification. In metabolic deficiency the degrading organism has specific nutritional requirements, necessary to promote or increase degradation, which are provided by secondary strains. Examples of this co-operative association are described by Hay *et al.* (2001), reporting the degradation of the antimicrobial triclosan or by Sorensen *et al.* (2002), on a study of degradation of the herbicide isoproturon. Metabolic association is characterized by the cross-feeding of metabolites from the degradation pathway within the consortium members. Eventually, this is the commonest form of co-operation, and numerous examples can be found (e.g. Arfmann *et al.*, 1997; Chapalamadugu and Chaudhry, 1991; De Souza *et al.*, 1998; Carvalho *et al.*, 2002). Metabolite detoxification, which can be considered a particular case of metabolic association, involves the production of a toxic and/or inhibitory metabolite by the degrading organism and its consumption by secondary strains. Examples of this kind of co-operative association are described by Feigel and Knackmuss (1993), reporting the mineraliza-

tion of 4-aminobenzenesulphonic acid, or Pelz *et al.* (1999) on a study about chlorosalicylate degradation. Although the interspecies interactions in defined mixed culture DC are not completely understood yet, it is clear that metabolic association and detoxification impose the interdependence between consortium DC members. However, the similarity of molinate degradation rates observed for isolate ON4 and for defined mixed culture DC suggests that isolate ON4, in mixed culture, is the first to attack molinate and probably the rate limiting member.

Although some authors have proposed that thiocarbamate oxides are precursors for biodegradation (Rajagopal *et al.*, 1984; Imai and Kuwatsuka, 1982; 1986c), it was Nagy *et al.* (1995) who presented the first evidence for an oxidative metabolism of the herbicide EPTC (S-ethyl dipropylcarbamothioate), involving an inducible cytochrome P450. These authors found EPTC-sulphoxide as a degradation product, although did not conclude whether this compound constituted a dead-end metabolite. Because several thiocarbamates, but not molinate, could interact with that cytochrome P450, Nagy *et al.* (1995) concluded that this herbicide could not be degraded by such process. An alternative degradation process is described for EPTC, involving the hydrolysis of the thioester bond, with the release of ethanethiol, dipropylamine and CO₂ (McClung *et al.*, 1994).

The mechanism and catalysts used by isolate ON4 in molinate transformation are not characterized yet. However, attending to the results obtained throughout this study and considering the hypothesis of McClung *et al.* (1994) it is possible to propose a metabolic route for molinate degradation involving the hydrolysis of the thioester bond by isolate ON4. Through this hydrolysis, isolate ON4 would originate ethanethiol and N-carboxy hexamethyleneimine, supplying carbon and nitrogen sources for its own growth and for other consortium members. Future studies will focus on the characterization of this enzyme system of isolate ON4, which is responsible for the beginning of molinate mineralization by the defined mixed culture.

Defined mixed culture DC is robust and highly efficient in molinate mineralization and represents a valuable tool for decontamination of molinate polluted waters under field conditions. Further studies will contribute to better understand the contribution of each mixed culture member to promote and maximise the efficiency of molinate mineralization.

Experimental procedures

Chemicals

Molinate (S-ethyl perhydroazepine-1-carbothioate) of 99% purity was obtained from Riedel-de Haën (Seelze, Germany) and molinate of 97% purity from Herbex, Produtos Químicos (Estoril, Portugal). Molinate sulphoxide, molinate-sulphone,

molinate-acid, molinate-alcohol and 2-oxo-molinate were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). Ethanethiol, diethyl disulphide and hexamethyleneimine (HMI) were obtained from Sigma-Aldrich (Steinheim, Germany).

Mineral media

Two kinds of mineral media were used during this study. Medium A contained Na₂HPO₄ (6.0 mM), KH₂PO₄ (4.0 mM), CaCl₂·H₂O (0.47 mM), NaCl (0.14 mM), MgSO₄·7H₂O (0.41 mM), nitritotriacetate (0.52 mM), FeSO₄·7H₂O (2 mg l⁻¹), ZnSO₄·7H₂O (0.1 mg l⁻¹), MnSO₄·H₂O (0.03 mg l⁻¹), H₃BO₃ (0.3 mg l⁻¹), CoSO₄·7H₂O (0.24 mg l⁻¹), CuSO₄·5H₂O (0.01 mg l⁻¹), NiSO₄·7H₂O (0.02 mg l⁻¹), NaMoO₄·2H₂O (0.03 mg l⁻¹), Ca(OH)₂ (0.5 mg l⁻¹), and EDTA (5 mg l⁻¹).

Medium B contained phosphate buffer (27 mM, pH 7.2), CaCl₂·2H₂O (0.2 mM), NaCl (7.56 mM), MgCl₂·6H₂O (0.81 mM), FeCl₂·4H₂O (5.19 μM), HCl (1.3 μl, 25%), ZnCl₂ (0.07 mg l⁻¹), MnCl₂·4H₂O (0.1 mg l⁻¹), H₃BO₃ (0.062 mg l⁻¹), CoCl₂·6H₂O (0.19 mg l⁻¹), CuCl₂·2H₂O (0.017 mg l⁻¹), NiCl₂·6H₂O (0.024 mg l⁻¹) and NaMoO₄·2H₂O (0.036 mg l⁻¹). These mineral media were supplemented with different carbon and nitrogen sources (see below).

Culture enrichment

A mixed water and soil sample was collected from the runoff of a Portuguese molinate producing industry. The sample (10%, v/v) was incubated in mineral medium A with 400 mg l⁻¹ molinate as carbon and energy source and (NH₄)₂SO₄ (3.8 mM), as well as KNO₃ (1.02 mM) and NaNO₃ (8.21 mM) as additional nitrogen sources. Ten successive transfers at 8-day intervals were made to fresh medium A with the same composition (5%, v/v of inoculum). This culture (named EC1) was further enriched in medium B using molinate (750 mg l⁻¹) as sole source of carbon and nitrogen, as no additional nitrogen sources were added. Three more successive transfers at 5-day intervals were made to fresh medium B (5%, v/v of inoculum). The final enrichment culture obtained has been named EC2.

Isolation, purification and identification of isolates from the enrichment cultures

In order to identify members of the consortia important for molinate degradation, cultures were serially diluted in sterile saline solution (NaCl 0.85%, wt/vol), spread on plate count agar (PCA) and incubated at 30°C for 2 days. Individual colonies with distinct morphologies were purified by subculturing on the same medium. Cultivable organisms isolated from enrichment culture EC2 (named ON1 to ON5) were characterized subsequently. The nutritional pattern of the isolates was tested using the Biolog Microplate System, according to manufacturer indications, and identification was done by comparison with the Microlog Gram-negative and Gram-positive databases (version 3.0) using the Microlog Distance Coefficient Method (Biolog™). Fatty acid methyl esters (FAMES) were extracted and analysed as described previously (Kuykendall *et al.*, 1988; Moreira *et al.*, 2000). Identification and quantification of the FAMES, as well as the

numerical analysis of the fatty acid profiles, was performed by using the standard MIS Library Generation Software (Microbial ID, Newark, Delaware, USA). Sequencing of the 16S rRNA genes (16S rDNA) of the isolates was determined after PCR amplification using primers 27f and 1492r or 1525r (Lane, 1991). The PCR reactions were performed as described previously (Nogales *et al.*, 2001). The nucleotide sequence of purified PCR products was determined by using the BigDye Terminator Cycle Sequencing Kit and ABI 373 and 377 sequencers (Applied Biosystems), according to the manufacturer's instructions. The sequence data have been submitted to the EMBL database under accession numbers AJ306832 to AJ306836, for isolates ON1 to ON5, respectively. Sequences were aligned and evolutionary distances determined as described by Nogales *et al.* (2001).

A defined mixed culture, named DC, was constituted by the mixture of equal proportions of the five cultivable isolates (ON1-ON5) purified from enrichment culture EC2.

Culture conditions

The capability of each individual isolate to grow on molinate or hexamethylenimine (HMI) as carbon and nitrogen sources was tested in medium B containing 187 mg l⁻¹ (1 mM) to 750 mg l⁻¹ (4 mM) of molinate or 1 mM to 2.5 mM HMI respectively. The ability of these organisms to use molinate, HMI, ethanethiol (2.5 mM) and diethyl disulphide (2.5 mM) as carbon sources was tested using the same medium supplemented with ammonium sulphate (3.8 mM). Whenever necessary, the media were supplemented with yeast extract (0.2 g l⁻¹ or 1 g l⁻¹).

Cultures used for kinetic determinations were grown in screw-capped Erlenmeyer flasks with Teflon-lined caps. Cultures used for headspace analysis were grown in 100 ml PTFE sealed flasks containing 20 ml of medium B. Incubations were made at 30°C, 150 r.p.m.

The ability of isolates ON1, ON2, ON3 and ON5 to grow on sulphur compounds produced by isolate ON4 was tested. A mixture of isolates ON1, ON2, ON3 and ON5 were grown in medium B supplemented with ammonium sulphate, in a 10-ml vial containing 2.5 ml of medium, placed in the headspace of a culture of isolate ON4 in medium B with 750 mg l⁻¹ molinate. The system was tightly sealed in order to avoid any loss of volatile compounds.

Cell dry weight

Calibration curves of optical density (OD_{610 nm}) versus dry weight were obtained by filtering cell suspensions with optical densities ranging from 0.1 to 0.7 through previously weighted glass fibre membranes (Whatman) and drying at 90°C until constant weight.

Resting cell assays

Resting cell assays were performed in phosphate buffer 54 mM, pH 7.2 (PB) with 187 mg l⁻¹ molinate and cell densities corresponding to 1 g l⁻¹ cells (dry weight). Enrichment culture EC2 and defined mixed culture DC were grown in medium B with 750 mg l⁻¹ molinate and isolates ON1 to ON5

were grown in medium B with 187 mg l⁻¹ molinate supplemented with yeast extract (1 g l⁻¹) in axenic culture. The late exponential phase cultures were centrifuged, washed twice with PB, and suspended in 10 ml of PB containing molinate. The suspensions were incubated at 30°C and shaken at 150 r.p.m.

Analysis of molinate and degradation products

Molinate was quantified using a High Performance Liquid Chromatograph (Merck) equipped with an UV-VIS detector (Merck) operating at 210 nm and a Lichrosphere 5 µm RP-18 column (Merck). A mixture of methanol:water (80:20, v/v) was used as mobile phase at a flow rate of 0.8 ml min⁻¹. Cell free supernatants or dilutions thereof were directly injected in the HPLC.

The presence of molinate sulphoxide, molinate-sulphone, molinate-acid, molinate-alcohol, and 2-oxo-molinate in organic extracts of culture supernatants or liquid phase of resting cell suspensions of isolate ON4 was evaluated using the same mobile phase in a proportion of 50:50 (v/v). The organic extracts were obtained as described previously (Imai and Kuwatsuka, 1982). Briefly, each sample (5 ml) was acidified with concentrated HCl (pH 3) and extracted twice with the same volume of *n*-hexane. The pH of the remaining aqueous phase was adjusted to 10 with 10 N NaOH, and extracted twice with ethyl ether. The remaining aqueous phase was acidified again with HCl (pH 2), and extracted twice with ethyl ether. Each organic extract (acidic hexane, basic ether and acidic ether) was analysed independently.

Solid-phase microextraction (SPME) and gas chromatography was used to analyse molinate degradation products present in the headspace of cultures or resting cells suspensions. The SPME fibre used, 50/30 µm Divinylbenzene/carboxen/PDMS, was obtained from Supelco (Bellefonte, PA, USA). For each SPME analysis, the fibre was exposed to the headspace of a culture or a resting cells suspension (in 100 ml PTFE sealed vials containing 20 ml of liquid phase, as described above) for 30 min, removed from the vial and inserted into the injection port of the gas chromatograph for 10 min. The fibre was cleaned between each extraction by inserting the fibre in the auxiliary injection port, during 30 min, at 220°C.

GC analysis were performed on a Hewlett-Packard 5890 (Palo Alto, CA, USA) chromatograph equipped with a flame ionization detector (FID) and CP-WAX 58 (FFAP)-CB (50 m × 0.32 mm, 1.2 µm) column (Chrompack). The splitless time was 0.5 min. The carrier gas was H₂ at a flow rate of 1.2 ml min⁻¹. The FID used hydrogen at 35 ml min⁻¹ and a mixture of nitrogen/oxygen (80/20) at 350 ml min⁻¹. The make-up gas was nitrogen at 20 ml min⁻¹. The initial temperature was 40°C. After 1 min the temperature was increased to 220°C at a rate of 2°C min⁻¹. The final temperature was held for 30 min. The operating temperatures of injector and detector were 220°C. Identification of compounds derived from molinate degradation was done by comparison of retention times with those of authentic standards. Degradation products extracted by SPME were also analysed by GC-MS analysis, using a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 2000 mass selective detector (USA) and a Saturn GC/MS workstation software version

5.51, as described previously (Silva Ferreira *et al.*, 2003). Identification of chromatographic peaks was achieved by comparisons of mass spectra obtained from the sample with those from authentic standards analysed under the same conditions and by comparing the Kovats indices and the mass spectra present in the NIST 98 MS Library Database.

Analysis of dissolved organic carbon

The total organic carbon present in culture supernatants (DOC) or dilutions thereof was directly analysed using a Shimadzu 5000 A Total Organic Carbon Analyser (Kyoto, Japan), with high temperature catalytic combustion and Pt alumina catalyst. For calibration, potassium diphthalate and anhydrous sodium carbonate were used as organic and inorganic standards respectively.

Determination of specific growth rate and molinate specific degradation rate

Specific growth rates were calculated during the exponential phase of growth. Molinate consumption rate can be expressed as $-\frac{dS}{dt} = \frac{X_0\mu}{Y}e^{\mu t}$, where S is the molinate concentration, μ is the specific biomass growth rate, Y is the observed biological yield and X_0 is the biomass concentration at $t=0$. Integrating this equation between $t=0$ and t ($S = S_0$ for $t=0$) we get $S = S_0 - \frac{X_0}{Y}(e^{\mu t} - 1)$. This expression was fitted to the experimental data and the derivative of S with respect to t was computed to obtain the molinate consumption rate $-\frac{dS}{dt}$. The specific molinate degradation rate was calculated as $\frac{1}{X}\left(-\frac{dS}{dt}\right)$ where X is the biomass concentration at time t .

Acknowledgements

We gratefully acknowledge Herbex, Produtos Químicos, S.A. for supplying molinate, Alexandra Diogo and Milton Costa from Departamento de Bioquímica da Faculdade de Ciências e Tecnologia da Universidade de Coimbra for the FAMES analysis by MIDI system, Hans-Adolf Arfmann from Division of Microbiology, GBF-German Research Centre for Biotechnology, Braunschweig for valuable discussions, and Rute Oliveira and Patrícia Correia from LEPAE for technical assistance.

This work was financially supported by Fundação para a Ciência e Tecnologia from Ministério da Ciência e Tecnologia (project PRAXIS/P/BIO/12193/1998 and grants Praxis XXI/BPD/9936/96, and SFRH/BD/4558/2001).

References

Arfmann, H.A., Timmis, K.N., and Wittich, R.M. (1997) Mineralization of 4-chlorodibenzofuran by a consortium consisting of *Sphingomonas* sp. strain RW1 and *Burkholderia* sp. strain JWS. *Appl Environ Microbiol* **63**: 3458–3462.

Carrasco, J.M., Planta, M., Gomez-Casals, V., and Moraes, V. (1987) Pesticide residues in lake Albufera, Valencia, Spain. *J Assoc Off Anal Chem* **70**: 752–753.

Carrasco, J.M., Sabater, C., Alonso, J.L., Gonzalez, J., Botella, S., Amoros, I., *et al.* (1992) Molinate decontamination processes in effluent water from rice fields. *Sci Total Environ* **123/124**: 219–232.

Carvalho, M.F., Alves, C.C.T., Ferreira, M.I.M., de Marco, P., and Castro, P.M.L. (2002) Isolation and initial characterization of a bacterial consortium able to mineralize fluorobenzene. *Appl Environ Microbiol* **68**: 102–105.

Chapalamadugu, S., and Chaudhry, G.R. (1991) Hydrolysis of carbaryl by a *Pseudomonas* sp. and construction of a microbial consortium that completely metabolizes carbaryl. *Appl Environ Microbiol* **57**: 744–750.

Cochran, R.C., Formoli, T.A., Pfeifer, K.F., and Aldous, C.N. (1997) Characterization of risks associated with the use of molinate. *Regul Toxicol Pharmacol* **25**: 146–157.

Daffonchio, D., Baggi, G., Molinari, G.P., Ranalli, G., and Sorlini, C. (1996) Effects of the herbicide molinate on the metabolic activities of a degradative *Streptomyces griseus* strain. *J Environ Sci Health* **B31**: 257–268.

Daffonchio, D., Zanardini, E., Vatta, P., and Sorlini, C. (1999) Cometabolic degradation of thiocarbamate herbicides by *Streptomyces* sp. strain M2 and effects on the cell metabolism. *Annali Di Microbiologia Ed Enzimologia* **49**: 13–22.

De Souza, M., Newcombe, D., Alvey, S., Crowley, D., Hay, A., Sadowsky, M., and Wackett, L. (1998) Molecular basis of a bacterial consortium: interspecies catabolism of atrazine. *Appl Environ Microbiol* **64**: 178–184.

Ellis, M.K., Richardson, A.G., Foster, J.R., Smith, F.M., Widowson, P.S., Farnworth, M.J., *et al.* (1998) The reproductive toxicity of molinate and metabolites to the male rat: effects on testosterone and sperm morphology. *Toxicol Appl Pharmacol* **151**: 22–32.

Feigel, B.J., and Knackmuss, H.-J. (1993) Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. *Arch Microbiol* **159**: 124–130.

Golovleva, L.A., Finkelstein, Z.I., Popovich, N.A., and Skryabin, G.K. (1981) Transformation of ordram by microorganisms. *Izv Akad Nauk SSSR Series Biol* **3**: 348–358.

Hay, A., Dees, P., and Sayler, G. (2001) Growth of a bacterial consortium on triclosan. *FEMS Microbiol Ecol* **36**: 105–112.

Imai, Y., and Kuwatsuka, S. (1982) Degradation of the herbicide molinate in soils. *J Pesticide Sci* **7**: 487–497.

Imai, Y., and Kuwatsuka, S. (1986a) Characteristics of microflora degrading the herbicide molinate in soil. *J Pesticide Sci* **11**: 57–63.

Imai, Y., and Kuwatsuka, S. (1986b) The mode of metabolism of the herbicide molinate by four strains of microorganisms isolated from soil. *J Pesticide Sci* **11**: 111–117.

Imai, Y., and Kuwatsuka, S. (1986c) Metabolic pathways of the herbicide molinate in four strains of isolated soil microorganisms. *J Pesticide Sci* **11**: 245–251.

Jewell, W.T., and Miller, M.G. (1998) Testicular toxicity in the rat: metabolic activation via sulfoxidation. *Toxicol Appl Pharmacol* **149**: 159–166.

Jewell, W.T., Hess, R.A., and Miller, M.G. (1999) Comparison of human and rat metabolism of molinate in liver

- microsomes and slices. *Drug Metab Dispos* **27**: 842–847.
- Julli, M., and Krassoi, F.R. (1995) Acute and chronic toxicity of the thiocarbamate herbicide, molinate, to the Cladocean *Moina australiensis* Sars. *Bull Environ Contam Toxicol* **54**: 690–694.
- Klysheva, A.L., Golovleva, L.A., and Ilyaletdinov, A.N. (1980) Transformation of ordram by microorganisms isolated from soils of Kazakhstan rice paddies. *Izv Akad Nauk SSSR Series Biol* **4**: 29–34.
- Kuykendall, L.D., Roy, M.A., O'Neill, J.J., and Devine, T.E. (1988) Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* **38**: 358–361.
- Kyung, K.H., and Fleming, H.P. (1997) Antimicrobial activity of sulfur compounds derived from cabbage. *J Food Prot* **60**: 67–71.
- Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*. Stackebrandt, E., and Goodfellow, M. (eds). Chichester: John Wiley & Sons, pp. 115–175.
- Mabury, S.A., Cox, J.S., and Crosby, D.G. (1996) Environmental fate of rice pesticides in California. *Rev Environ Contam Toxicol* **147**: 71–117.
- McClung, G., Dick, W.A., and Karns, J. (1994) EPTC degradation by isolated soil microorganisms. *J Agric Food Chem* **42**: 2926–2931.
- Molinari, G.P., Sorlini, C., Daffonchio, D., Baggi, G., and Ruffo, L. (1992) Activity and evolution of mixed microbial culture degrading molinate. *Sci Total Environ* **123/124**: 309–323.
- Moreira, C., Rainey, F.A., Nobre, M.F., Silva, M.T., and da Costa, M.S. (2000) *Tepidimonas ignava* gen nov., sp. nov., a new chemolithoheterotrophic and slightly thermophilic member of the β -*Proteobacteria*. *Int J Syst Evol Microbiol* **50**: 735–742.
- Nagy, I., Schoofs, G., Compennolle, F., Proost, P., Vanderleyden, J., and De Mot, R. (1995) Degradation of the thiocarbamate herbicide EPTC (S-Ethylidiproylcarbamoithioate) and biosafening by *Rhodococcus* sp. strain NI86/21 involve an inducible cytochrome P-450 system and aldehyde dehydrogenase. *J Bacteriol* **177**: 676–687.
- Nogales, B., Moore, E.R.B., Llobet-Brossa, E., Rossello-Mora, R., Amann, R., and Timmis, K.N. (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microbiol* **67**: 1874–1884.
- Pelz, O., Tesar, M., Wittich, R.M., Moore, E.R.B., Timmis, K.N., and Abraham, W.R. (1999) Towards elucidation of microbial community metabolic pathways: unravelling the network of carbon sharing in a pollutant-degrading bacterial consortium by immunocapture and isotopic ratio mass spectrometry. *Environ Microbiol* **1**: 167–174.
- Rainey, F., Weiss, N., Prauser, H., and Stackebrandt, E. (1994) Further evidence for the phylogenetic coherence of actinomycetes with group B-peptidoglycan and evidence for the phylogenetic intermixing of the genera *Microbacterium* and *Aureobacterium* as determined by 16S rDNA analysis. *FEMS Microbiol Lett* **118**: 135–140.
- Rajagopal, B.S., Brahma Prakash, G.P., Reddy, B.R., Singh, U.D., and Sethunathan, N. (1984) Effect and persistence of selected carbamate pesticides in soil. *Residue Rev* **93**: 1–199.
- Silva Ferreira, A.C., Hogg, T., and Guedes de Pinho, P. (2003) Key odorants of oxidative spoiled white wines. *J Agric Food Chem* **51**: 1377–1381.
- Sipma, J., van Bree, R., Janssen, A.J., Arena, B., Hulshoff, P.L., and Lettinga, G. (2002) Degradation of methanethiol in a continuously operated upflow anaerobic sludge-blanket reactor. *Water Environ Res* **74**: 264–271.
- Soderquist, C.J., Bowers, J.B., and Crosby, D.G. (1977) Dissipation of molinate in a rice field. *J Agric Food Chem* **25**: 940–945.
- Sorensen, R.S., Ronen, Z., and Aamand, J. (2002) Growth in coculture stimulates metabolism of the phenylurea herbicide isoproturon by *Sphingomonas* sp. strain SRS2. *Appl Environ Microbiol* **68**: 3478–3485.
- Thomas, V.M., and Holt, C.L. (1980) The degradation of [¹⁴C]molinate in soil under flooded and nonflooded conditions. *J Environ Sci Health* **B15**: 475–484.
- Tjeerdema, R.S., and Crosby, D.G. (1988) Disposition, biotransformation, and detoxification of molinate (ordram) in whole blood of the common carp (*Cyprinus carpio*). *Pestic Biochem Physiol* **31**: 24–35.
- Tsao, S.M., and Yin, M.C. (2001) In vitro antimicrobial activity of four diallyl sulphides occurring naturally in garlic and Chinese leek oils. *J Med Microbiol* **50**: 646–649.
- Zyakun, A.M., Nefedova, M.Y., Baskunov, B.P., and Finkelstein, Z.I. (1983) The new products of ordram microbial degradation. *Izv Akad Nauk SSSR Series Biol* **1**: 126–130.