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Transformation of the herbicide 2,6-dichlorobenzonitrile to the persistent metabolite 2,6-dichlorobenzamide (BAM) by soil bacteria known to harbour nitrile hydratase or nitrilase

Maria Sommer Holtze*, Jan Sørensen, Hans Christian B. Hansen & Jens Aamand
*Department of Geochemistry, Geological Survey of Denmark and Greenland, 1350, Copenhagen, Denmark (*author for correspondence: e-mail: msh@geus.dk)*

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Abstract

In soil the herbicide 2,6-dichlorobenzonitrile (dichlobenil) is degraded to the persistent metabolite 2,6-dichlorobenzamide (BAM) which has been detected in 19% of samples taken from Danish groundwater. We tested if common soil bacteria harbouring nitrile-degrading enzymes, nitrile hydratases or nitrilases, were able to degrade dichlobenil *in vitro*. We showed that several strains degraded dichlobenil stoichiometrically to BAM in 1.5–6.0 days; formation of the amide intermediate thus showed nitrile hydratase rather than nitrilase activity, which would result in formation of 2,6-dichlorobenzoic acid. The non-halogenated analogue benzonitrile was also degraded, but here the benzamide intermediate accumulated only transiently showing nitrile hydratase followed by amidase activity. We conclude that a potential for dichlobenil degradation to BAM is found commonly in soil bacteria, whereas further degradation of the BAM intermediate could not be demonstrated.

Introduction

The aromatic nitrile 2,6-dichlorobenzonitrile (dichlobenil) is the active component of e.g. the herbicides Prefix G and Casoron G. The sale of these herbicides have been prohibited in Denmark since 1997 but they are still used in many other countries world wide. In soil dichlobenil is mainly degraded to the persistent metabolite 2,6-dichlorobenzamide (BAM) which, in contrast to dichlobenil, is very mobile and hence easily leached to groundwater (Clausen et al. 2004). The acute toxicity of BAM is considered to be low to moderate with LD_{50} ranging from 1144 to 2330 mg kg^{-1} body weight in mice and rats; though, the carcinogenic effect of BAM and toxicity towards reproduction has not been fully investigated (Cohr & Simonsen 2004). BAM has been detected in 19% of 5000 groundwater samples tested in Denmark; in 7.5% of the samples the concentration exceeded the EU threshold concentration of 0.1 μg l^{-1} for pesticides in drinking water (Brüsch & Juhler 2003). BAM is also detectable in groundwater in other countries; in 15% of 359 samples from German groundwater the concentration exceeded the EU limit and in Sweden BAM has been detected in 10 out of 24 groundwater samples although below the EU threshold (Kreuger et al. 2003; Wolter et al. 2001).

Two different pathways for enzymatic hydrolysis of nitriles are known: (i) a direct hydrolysis of nitrile to the corresponding acid catalysed by nitrilases and (ii) a two-step reaction where the nitrile is transformed to an amide by nitrile hydratases followed by amide transformation to the corresponding acid by amidases (Figure 1) (Banerjee et al. 2002; Kato et al. 2000). Hence, in the following it is assumed that cells harbour nitrilase when, during the hydrolysis of nitriles no

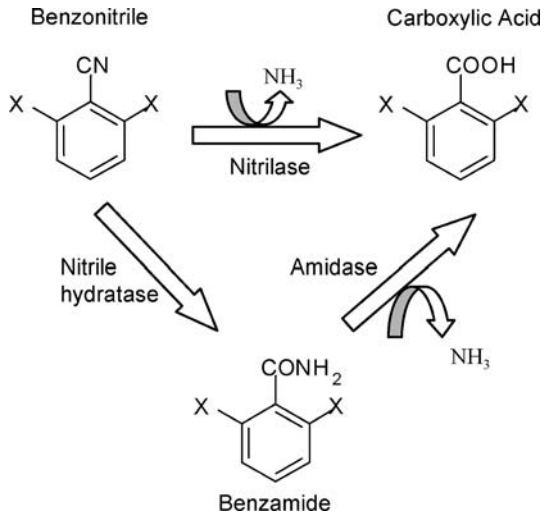

Figure 1. Initial reactions of the pathways of aromatic nitrile degradation. X represents either H in non-chlorinated compounds (benzonitrile, benzamide and benzoic acid) or Cl in chlorinated compounds (dichlobenil, BAM and 2,6-dichlorobenzoic acid).

amide is detected and, furthermore, the amide is not used as a substrate. When an intermediate accumulation of the corresponding amide during nitrile hydrolysis is observed we assume the bacteria to harbour nitrile hydratase whereas a further hydrolysis of the amide shows that the bacteria harbour amidase. The degradation pathway of dichlobenil in soil is not fully known but BAM has been shown to be the main metabolite of dichlobenil degradation in soils and in three bacterial cultures belonging to the genera *Arthrobacter*, *Rhizobium* and *Rhodococcus* (Beynon & Wright 1968; Blakey et al. 1995; Briggs & Dawson 1970; Meth-Cohn & Wang 1997; Miyazaki et al. 1975; Montgomery et al. 1972; Verloop & Nimmo 1970; Vosáhlová et al. 1997). This indicates the involvement of nitrile hydratases although it cannot be excluded that nitrilases are involved as well since other nitriles are degraded by this enzyme. If BAM is further degraded or if dichlobenil is degraded by nitrilase excluding BAM formation the risk of contaminating the groundwater with BAM would obviously be lower. The activity of both enzyme systems results in nitrogen being released from the nitrile. In accordance, the nitrile hydratase–amidase system can be regulated by the availability of N (Takashima et al. 2000).

We hypothesise that dichlobenil may be transformed either to (i) BAM due to catalysis by nitrile hydratase and further on to 2,6-dichlorobenzoic acid by amidase, or (ii) directly to 2,6-dichlorobenzoic acid by nitrilase. The objective of the present study was to test whether dichlobenil is degraded by both nitrile hydratases and nitrilases. This was tested using common soil bacteria known to degrade non-halogenated nitriles in N-limited cultures by either nitrile hydratases or nitrilases. The degradation of dichlobenil was compared to degradation of benzonitrile, which is a well-studied non-halogenated analogue to dichlobenil; the benzonitrile degradation was taken to represent a positive control of enzyme activity. Furthermore, the effect of inorganic N (ammonia) on the degradation of dichlobenil was studied to reveal if the enzymes were controlled by availability of N.

Materials and methods

Chemicals

Analytical-grade 2,6-dichlorobenzonitrile (99.5%, CAS RN 001194-65-6), 2,6-dichlorobenzamide (99.5%, CAS RN 002008-58-4) and benzonitrile (99.5%, CAS RN 000100-47-0) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Analytical-grade benzamide (99.3%, CAS RN 000065-85-0) was purchased from MP Biomedicals, Inc. (Aurora, Ohio, USA).

Bacteria

The nitrile hydratase- and amidase-producing bacteria *Rhizobium radiobacter* 9674, *Rhodococcus erythropolis* 9675 and *Rhodococcus erythropolis* 9685, and the nitrilase-producing bacteria *Rhizobium* sp. 11401 (re-classified from *Agrobacterium* to *Rhizobium* in accordance with (Young et al. 2001)), *Pseudomonas fluorescens* 11387 and *Pseudomonas putida* 11388 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). All strains were originally isolated from soil (Layh et al. 1997).

Growth media

R2A plates and R2A-based broth (R2B) were prepared according to the manufacturer (Difco, Detroit, Michigan, USA) and (Reasoner & Geldreich 1985), respectively. A mineral salt (MS)

medium without carbon and nitrogen was prepared as described previously (Sørensen & Aamand 2003).

Incubations

Freeze stocks of the bacteria were streaked on R2A plates and grown for 2 days before inoculation in R2B. The cultures were grown in R2B (30 °C, 24 h) under orbital shaking (100 rpm, Certomat® R, Sartorius BBI Systems GmbH, Melsungen, Germany) and were harvested by centrifugation (5000×*g*, 10 min). The pellet was washed and resuspended in 10 mM phosphate buffer (5.7 ml 1.0 M $NaH_2PO_4 \cdot H_2O$ and 8.4 ml of 0.5 M $Na_2HPO_4 \cdot 2H_2O$ l^{-1}) to obtain an optical density ($OD_{600\ nm}$) of 0.5 as measured in a Perkin Elmer lambda 12 UV/VIS spectrophotometer (Perkin Elmer Inc., Boston, USA). One millilitre was transferred to 20 ml autoclaved glass test tubes with 9 ml MS medium containing 5.9 mM sodium succinate l^{-1} (Sigma–Aldrich, St. Louis, Missouri, USA) as the primary C source (23.7 mM C) and 50 μM dichlobenil or benzonitrile [added from stock solutions of 8.6 and 5.2 g l^{-1}, respectively, in dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany)]. The nitriles represented the only N source in the tubes, except in one experiment with *R. erythropolis* 9675 where additional 10 mM NH_4Cl was included. All test tubes including sterile and unamended controls (without dichlobenil or benzonitrile) were incubated in triplicate under orbital shaking (100 rpm) at 30 °C in the dark.

Estimation of bacterial growth

Cell densities (CFU) of *R. erythropolis* 9675 amended with dichlobenil and NH_4Cl was estimated at day 0 and 6. Serial dilutions were made in the 10 mM phosphate buffer and five drops of 10 μl were pipetted on R2A plates; incubations were performed at 20 °C and colonies were counted after 1–7 days of incubation.

Measurements of degradation

Aliquots of 750 μl taken from the incubated test tubes were filtered through a 0.2-μm-mesh PTFE membrane syringe filter (Titan Filtration Systems, Wilmington, North Carolina, USA) and the last 250 μl of the filtrate was collected and stored at –20 °C until further analysis. Concentrations of dichlobenil, benzonitrile, BAM and benzamide were determined in a Hewlett-Packard Series 1050 HPLC equipped a 250×2 mm i.d. Hypersil 5 μm-C_{18} column (Phenomenex, Cheshire, UK) and a UV detector. The mobile phase was acetonitrile in Milli-Q water (2:3 by volume), injection volume was 4 μl and flow rate was 0.7 ml min^{-1}. Detection and subsequent quantification were made by measuring the absorbance at 200 nm (Juhler et al. 2001) including a comparison with pure standards.

Statistical analyses

Statistical analyses were performed to test whether the concentrations of dichlobenil and benzonitrile were significantly lowered in the bacterial cultures compared to the sterile control. Furthermore, it was tested if the number of CFU increased over time in the N-amended culture of *R. erythropolis* 9675. All data were tested for normality and equal variance and subjected to a two-way ANOVA in SigmaStat™ for Windows® version 2.03 (SPSS Inc., 1997) with a significance level of 0.05.

Results and discussion

Degradation by Rhodococcus *strains*

In cultures of *R. erythropolis* 9675 and *R. erythropolis* 9685 dichlobenil was rapidly and efficiently transformed to the corresponding amide, BAM, showing nitrile hydratase activity. No amidase activity was seen and BAM accumulated over time. Benzonitrile was even more rapidly degraded to the corresponding amide, benzamide, by the two bacterial strains. In contrast to BAM, however, benzamide was further degraded demonstrating both nitrile hydratase and amidase activity when the cultures were amended with benzonitrile (Figure 2).

Dichlobenil has previously been shown to be transformed to BAM by bacterial cultures due to nitrile hydratase activity (Meth-Cohn & Wang 1997; Miyazaki et al. 1975; Vosáhlová et al. 1997). Meth-Cohn & Wang (1997) showed nitrilase activity as benzonitrile was degraded to benzoic acid, but the bacterium was not tested for amidase activity. It has been shown that nitrilases

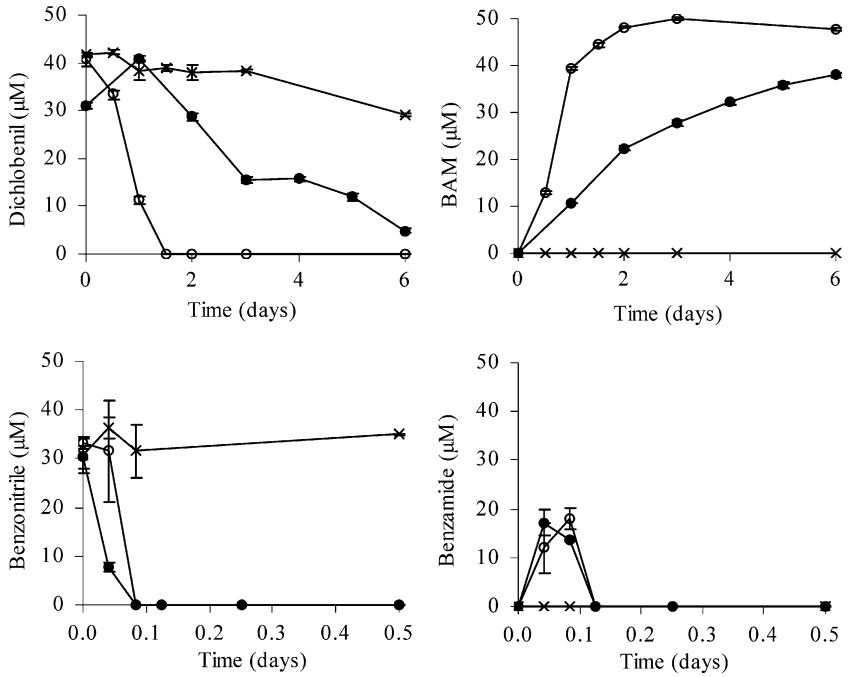

Figure 2. Degradation of nitriles by *Rhodococcus* sp. strains. Concentration versus incubation time for dichlobenil and benzonitrile to the upper and lower left, respectively, and for BAM and benzamide to the upper and lower right, respectively. Open circles represent *R. erythropolis* 9675, closed circles *R. erythropolis* 9685 and crosses the sterile control. All data represent mean ± SE of triplicate samples.

sometimes fail to degrade nitriles all the way to the corresponding acids resulting in production of the intermediate amide (Stevenson et al. 1992). Since the non-chlorinated analogue benzonitrile was also degraded to the corresponding amide in the present study the production of BAM was not due to a failure of a nitrilase but instead nitrile hydratase is the enzyme involved in degradation of dichlobenil to BAM. This is in accordance with *R. erythropolis* 9675 and 9685 being originally enriched on the aliphatic nitrile naproxen nitrile, which they also degrade to the corresponding amide by nitrile hydratases and further on to the corresponding acid by amidases (Layh et al. 1997).

Even though the non-halogenated analogue benzamide was degraded no enzyme activity was seen towards BAM in the present study; hence, the two *ortho*-positioned chlorides apparently hindered the degradation of BAM. It is not known if amidases were indeed produced by the dichlobenil-amended cultures, but if they were produced they were unable to catalyse degradation of the BAM intermediate. A steric hindrance exerted by the chloride atom(s) in the *ortho*-position(s) was also seen in a former study where 13 *Arthrobacter* sp. strains isolated from soil were tested for their ability to degrade benzamide, *ortho*-chlorobenzamide and BAM (Heinonen-Tanski 1981). All strains degrade benzamide but not *ortho*-chlorobenzamide or BAM. Others showed that *para*- and *meta*-substituted benzonitriles are hydrolysed efficiently to the corresponding benzoic acids by *R. rhodochrous* AJ270 whereas *ortho*-substituted benzonitriles are rapidly and efficiently transformed into amides; further degradation of the amides proceeds slowly if at all (Meth-Cohn & Wang 1997). It was proposed that while initial hydration of the linear nitrile group is not significantly affected by steric factors, the subsequent amidase-mediated step is very sensitive. In the present study, the amidase-mediated step of BAM degradation was completely hindered by the chloro substituents.

Regulation of nitrile hydratase activity by N

To test if degradation of dichlobenil was regulated by the availability of N we performed an

experiment with *R. erythropolis* 9675 supplemented with both dichlobenil and NH_4Cl. When the culture was amended with NH_4Cl as a supplementary N source the concentration of dichlobenil during time was not significantly different from the concentration in the sterile control presented in Figure 2. The N amendment resulted in a significantly higher number of CFU ($3.2\times10^8 \pm 1.2\times10^8$) at day 6 compared to day 0 ($9.6\times10^6 \pm 1.9\times10^6$) showing that the culture was growing and not inhibited by the high amendments of nutrients.

The fact that no degradation of dichlobenil was observed in the culture amended with NH_4Cl demonstrates that activity of the nitrile hydratase of *R. erythropolis* 9675 was down-regulated by high N availability. A former study showed that production of nitrile hydratase by another bacterium, *Bacillus smithii* SC-J05-1, ceased when excess N as $(NH_4)_2SO_4$ was available (Takashima et al. 2000). The apparent N regulation of the nitrile hydratase in *B. smithii* SC-J05-1 seems explicable since activity of nitrile hydratase was proceeded by activity of amidase thus making the nitrile-N available by degradation of the amide (Takashima et al. 1998).

Degradation by Rhizobium *strains*

To test if other soil bacteria known to degrade nitriles also degraded dichlobenil we included *R. radiobacter* 9674 known to harbour nitrile hydratase and amidase and *Rhizobium* sp. 11401 known to harbour nitrilase (Layh et al. 1997); we expected the former to produce BAM but the latter to degrade dichlobenil directly to 2,6-dichlorobenzoic acid. As shown in Figure 3, *Rhizobium radiobacter* 9674 and *Rhizobium* sp. 11401 significantly lowered the concentrations of both dichlobenil and benzonitrile compared to the sterile control. In both cultures, BAM and benzamide were produced but while BAM accumulated over time benzamide was rapidly degraded. This shows that both cultures harboured nitrile hydratase and amidase, which has been reported for *R. radiobacter* 9674 but not for *Rhizobium* sp. 11401. Since the latter strain was previously shown

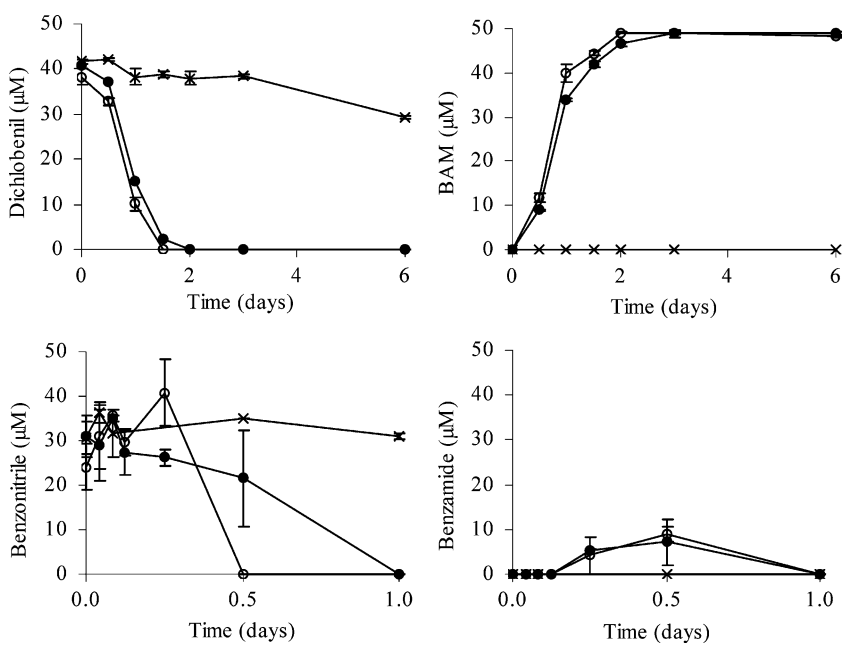

Figure 3. Degradation of nitriles by *Rhizobium* sp. strains. Concentration versus incubation time for dichlobenil and benzonitrile to the upper and lower left, respectively, and for BAM and benzamide to the upper and lower right, respectively. Open circles represent *Rhizobium radiobacter* 9674, closed circles *Rhizobium* sp. 11401 and crosses the sterile control. All data represent mean ± SE of triplicate samples.

to harbour nitrilase, the strain was capable of producing both types of enzyme systems.

Degradation by Pseudomonas *strains*

To test whether nitrile hydratase activity could be observed in other bacteria only known to harbour nitrilases we finally tested *P. fluorescens* 11387 and *P. putida* 11388 (Layh et al. 1997). As shown in Figure 4, the degradation patterns observed for the other bacteria were also seen in *P. fluorescens* 11387, while *P. putida* 11388 did not degrade the nitriles within the experimental time allocated. An earlier experiment with larger inoculum size and longer incubation time did show that *P. putida* 11388 was indeed capable of degrading 29 μM dichlobenil within 16 days while benzonitrile was still not degraded (data not shown). The lack of benzonitrile degradation in this strain is ascribed to a relatively high enzyme specificity as shown in *Klebsiella pneumoniae* ssp. *ozaenae* where nitrilase catalyses transformation of 3,5-dibromo-4-hydroxybenzonitrile to 3,5-dibromo-4-hydroxybenzoic acid but not benzonitrile *per se* (McBride et al. 1986). Both *Pseudomonas* strains are known to produce nitrilase but our study shows that they are also capable of producing nitrile hydratase and *P. fluorescens* 11387 produce amidase as well.

Nitrile hydratase versus nitrilase activity

We showed that degradation of dichlobenil to BAM (or benzonitrile to benzamide) is not confined to bacteria known to produce nitrile hydratases; degradation was also observed in *Rhizobium* sp. 11401, *P. fluorescens* 11387 and *P. putida* 11388 known to degrade aliphatic nitriles by nitrilases (Layh et al. 1997). Nitrilases could not be demonstrated to be active in degradation of the two nitriles tested in the present study. We therefore suggest that *Rhizobium* sp. and *Pseudomonas* sp. strains, shown to harbour nitrilases by Layh et al. (1997), were able to produce both nitrile hydratase and nitrilase as observed previously for *Rhodococcus* sp. strains only (Kato et al. 2000; Nagasawa et al. 1988, 1991).

Our findings are in contrast with the former hypothesis that degradation of aromatic nitriles is catalysed mainly by nitrilases (Nagasawa & Yamada 1990; Nawaz et al. 1992; Stevenson et al.

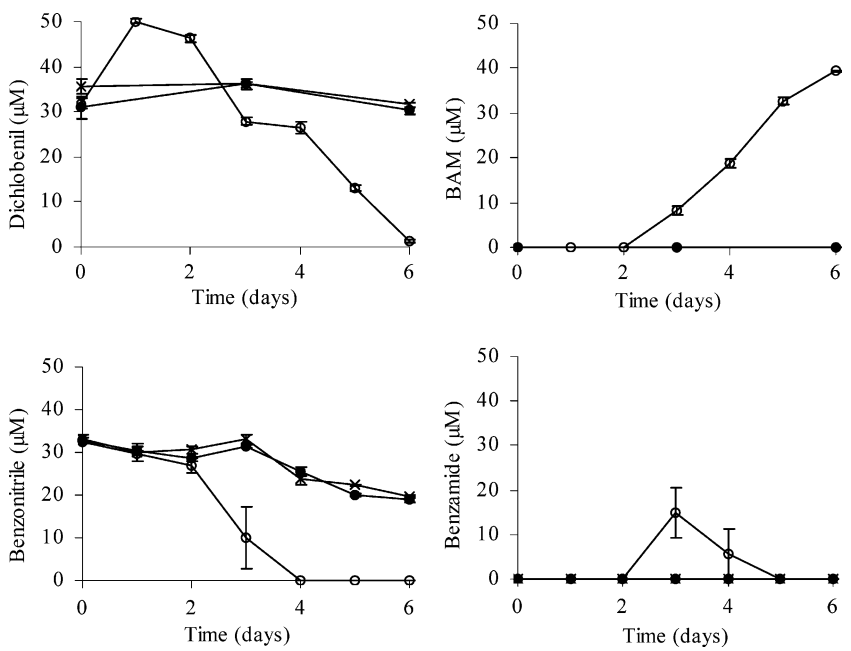

Figure 4. Degradation of nitriles by *Pseudomonas* sp. strains. Concentration versus incubation time for dichlobenil and benzonitrile to the upper and lower left, respectively, and for BAM and benzamide to the upper and lower right, respectively. Open circles represent *P. fluorescens* 11387, closed circles *P. putida* 11388 and crosses the sterile control. All data represent mean ± SE of triplicate samples.

1992; Wyatt & Linton 1988). The validity of this hypotheses has been challenged previously by others but by performing experiments with benzonitrile only (Blakey et al. 1995; Hjort et al. 1990; Hoyle et al. 1998; Kobayashi et al. 1990; Layh et al. 1997). Layh et al. (1997) further proposed that nitrilase is the main nitrile-hydrolysing enzyme in Gram-negative bacteria, while the nitrile hydratase/amidase system dominates in Gram-positive bacteria such as the *Rhodococcus* spp. known for commercial synthesis of acrylamide from acrylonitrile (Bunch 1998; Warhurst & Fewson 1994). We have shown here that all four Gram-negative bacteria belonging to *Rhizobium* and *Pseudomonas* spp. hydrolysed dichlobenil or benzonitrile using nitrile hydratases. Hence, it cannot be predicted which enzyme system will be active simply by looking at the type of nitrile or bacteria.

Conclusions

Dichlobenil was degraded by nitrile hydratase to BAM by all of the common soil bacteria tested; even by the bacteria known to harbour nitrilase. Hence, it was not possible to demonstrate degradation of dichlobenil due to the activity of nitrilases. Apparently, the nitrilase-producing bacteria were able to produce both enzyme systems. Furthermore, the activity of the nitrile hydratase of *R. erythropolis* 9675 was down-regulated by high N availability. The subsequent BAM degradation was in all of the bacteria hindered by the two chloro substituents. If the degradation pattern observed among the common soil bacteria in the present study represents the pattern in soils, dichlobenil will be transformed to BAM, which can be transported to the groundwater. A potential for BAM degradation may still occur in dichlobenil-polluted soils, however, if adaptation over time can alter the specificity of the amidase thereby enabling the enzyme to overcome the steric hindrance.

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