JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Electrochemical Monitoring of the Biodegradation of 2,4-Dimethylaniline

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2,4-Dimethylaniline is a recalcitrant degradant of the pesticide amitraz and is also an industrial pollutant which is genotoxic, teratogenic and carcinogenic. The biological degradation of 2,4-dimethylaniline was examined and monitored by cyclic voltammetry. *Pseudomonas* species isolated from cattle dip tanks initially metabolized 2,4-dimethylaniline by oxidative deamination, following a degradation pathway via a 3-methylcatechol intermediate. The bacteria were capable of utilizing 2,4-dimethylaniline as a nitrogen source and, following deamination, as a carbon source. The formation of the metabolite, 3-methylcatechol, was monitored and confirmed by voltammetric monitoring.

KEYWORDS: Amitraz; pesticide; degradation; voltammetry; *Pseudomonas*

INTRODUCTION

The environmental risk of pesticide application is often compounded by the degradation of parent compounds to toxicologically stable degradants. The pesticide amitraz, (*N*-(2,4-dimethylphenyl)- *N* ′-[dimethylphenyl)-imino]methyl-*N*-methyline ethanimidamide), has been shown to degrade to the environmentally stable aniline derivative, 2,4-dimethylaniline (2,4-DMA), **Figure 1** (*1*−*3*), which has been shown to be teratogenic (*4*), genotoxic (*5*, *6*), and carcinogenic. Given that 2,4-DMA is also found in high levels in fuels (*7*), and is used as a precursor in dye, chemical, and pharmaceutical manufacture, studies aimed at the degradation of this environmental pollutant have been conducted, largely through abiotic methods, such as the use of pyrilium-containing zeolite photocatalysts (*8*).

Studies on the biological degradation of 2,4-dimethylaniline have been more limited, with work by Rozkov et al. (*7*) reporting on the feasibility of bacterial metabolism of dimethylanilines. While no proposed degradation pathway was presented in that study, a significant body of research into the biodegradation pathway of anilines, aniline derivatives (such as azo dyes), and, to a lesser extent, methylanilines, have proposed degradation routes via catechol intermediates. *Pseudomonas* strains have been shown to be capable of metabolizing aniline-based derivatives both through cometabolism and direct utilization as a source of energy. *Ps. putida* has been shown to metabolize recalcitrant *m*- and *p*-methylanilines as both carbon and nitrogen sources via oxidative deamination to a 4-methylcatechol intermediate followed by meta cleavage of the ring (*9*), while studies propose the degradation of azo dyes via catechol to β-ketoadipic acid, which is completely mineralized in the tricarboxylic acid (TCA) cycle (*10*).

The mechanism and pathway of biodegradation of 2,4-DMA by *Pseudomonas* spp. has thus not yet been fully shown. In this study, the feasibility of the biodegradation of 2,4-DMA by *Pseudomonas* spp. isolated from cattle vats (to which the pesticide amitraz has been applied) is examined. Using electrochemical methods for the analysis of 2,4-DMA as described earlier (*11*), we also present the use of electroanalysis as a tool for monitoring the biodegradation of 2,4-DMA as well as the identification of the main metabolites formed.

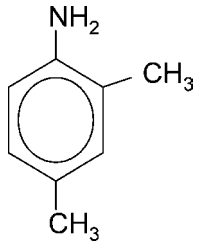

Figure 1. 2,4-dimethylaniline

MATERIALS AND METHODS

Isolation and Enrichment of *Pseudomonas* spp. from Spent Cattle Dip. *Pseudomonas* spp. confirmed by a Gram stain, where Gram− negative, rod-shaped bacteria were observed, was isolated from spent cattle dip by plating dilutions onto MacConkey purple agar (Difco) plates to which 0.1 μM 2,4-DMA (Sigma Aldrich) solubilized with 0.2 M NaOH was added. The plates were incubated at 37 °C overnight.

Colonies were picked and inoculated into conical flasks containing nutrient broth. The flasks were placed on a gyrator shaker (Labcon) (120 RPM) and incubated at 37 °C. The growth of the *Pseudomonas* spp. was monitored spectroscopically at OD_{600nm}. The *Pseudomonas* spp. were harvested by centrifugation using a Beckman JA14 rotor (10 000*g*) when the cells were in exponential growth phase, resuspended in saline solution, and stored at 4 °C.

Toxicity of 2,4-DMA to *Pseudomonas* spp. The degree of toxicity of 2,4-DMA to the isolated *Pseudomonas* spp. was assessed. This was achieved by growing *Pseudomonas* spp. in the presence of different concentrations of 2,4-DMA. Batch flasks of nutrient broth inoculated

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10.1021/jf061951k CCC: $33.50
Published on Web 10/18/2006

Table 1. Experimental Setup Used for the Assessment of Whether the Isolated *Pseudomonas* spp. Can Utilize 2,4-DMA as an Alternate Carbon or Nitrogen Source, or Both

flask set	carbon source	nitrogen source		
1	no glucose	2,4-DMA	NH_4Cl	2,4-DMA
2	glucose	2,4-DMA	no NH_4Cl	2,4-DMA
3	no glucose	2,4-DMA	no NH_4Cl	2,4-DMA
4	glucose	2,4-DMA	NH_4Cl	2,4-DMA

with harvested *Pseudomonas* spp. cells were prepared, each containing a different concentration of 2,4-DMA. The concentrations of 2,4-DMA used ranged between 0.1 μM and 5 μM. The flasks were incubated at 37 °C on a gyrator shaker (120 RPM), and samples were aliquoted aseptically on an hourly basis. The effect of 2,4-DMA on the growth of the *Pseudomonas* spp. cells was monitored using UV−vis spectroscopy at OD_{600nm}. All experiments were performed in triplicate.

Assessment of 2,4-DMA as a Carbon or Nitrogen Source. Fifty milliliters of autoclaved nitrogen- and carbon-free minimal salts medium was added to 250 mL conical flasks. The minimal salts medium consisted of (per liter of distilled water) 1.0 g H_2PO_4, 0.6 g NaH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 2 mg yeast extract (Difco), and 1 mL of trace element solution containing 0.05 mg H_3BO_3, 0.2 mg $CaSO_4$, 0.1 mg $CoSO_4$, 0.2 mg $CuSO_4$, 3 mg $FeSO_4$, 0.02 mg $MnCl_2$, and 0.03 mg $ZnSO_4 \cdot 7H_2O$ (*12*). Once added to the batch flasks, 2,4-DMA was aliquoted into each to yield a final concentration of 1 μM 2,4-DMA. Four batch flask studies were prepared in triplicate. **Table 1** summarizes the experimental setup.

Pseudomonas spp. was inoculated into flasks containing the carbon and nitrogen sources outlined in **Table 1**. *Pseudomonas* spp. cell viability and growth state was assessed by UV−vis spectroscopy at OD_{600nm}. Cell viability was confirmed by serial dilution and plating of broth containing *Pseudomonas* cells onto autoclaved minimal salts agar containing 1 μM 2,4-DMA solubilized by the addition of 0.1 M NaOH and incubated at 37 °C.

The decrease in 2,4-DMA concentration due to its biodegradation was monitored electrochemically by cyclic voltammetry at a Autolab Potentiostat/ Galvanostat 30 (PGSTAT 30) (Eco Chemie, Netherlands) coupled to a Voltammetric Analytical stand (VA 663), Metrohm, Netherlands. A glassy carbon working electrode referenced against a Ag/AgCl reference electrode, with a platinum wire as counter electrode, was obtained from Bioanalytical Systems (BAS). Aliquots of each sample analyzed voltammetrically was added to 0.2 M BR buffer pH 7.0 and degassed with nitrogen prior to analysis.

Spiking of Carbon- and Nitrogen-Free Flasks with 2,4-DMA. In a further batch flask experiment where both the carbon and nitrogen source were replaced by 1 μM 2,4-DMA, the breakdown of 2,4-DMA was assessed and monitored by cyclic voltammetry for 120 h (at 2 h intervals for the first 20 h and then at 10 h intervals). After 120 h, the batch flasks were spiked with 2,4-DMA to once again obtain a final concentration of 1 μM 2,4-DMA. The breakdown of 2,4-DMA was monitored initially at 2 h intervals for 20 h and then at 10 h intervals. The rate of 2,4-DMA breakdown was assessed by cyclic voltammetry and the growth of the *Pseudomonas* spp. cells analyzed by UV−vis spectroscopy at OD_{600nm}.

RESULTS AND DISCUSSION

***Toxicity of 2,4-DMA to Pseudomonas* spp. Figure 2** shows the absorbance readings for the growth of *Pseudomonas* spp. in minimal salts batch flask culture, in concentrations of 2,4-DMA between 1 μM and 5 μM. The control study shows growth of *Pseudomonas* spp. in absence of 2,4-DMA, as illustrated by an increase in optical density. While growth of the bacteria was observed for all concentrations of 2,4-DMA studied, the decrease in optical density in the presence of increasing concentrations of 2,4-DMA (compared to the control) indicates that this toxin does inhibit the growth of *Pseudomonas* spp. When compared to the control, 1 μM 2,4-DMA yielded the lowest degree of inhibition in the study, and this concentration was used for all further studies of 2,4-DMA metabolism by *Pseudomonas* spp.

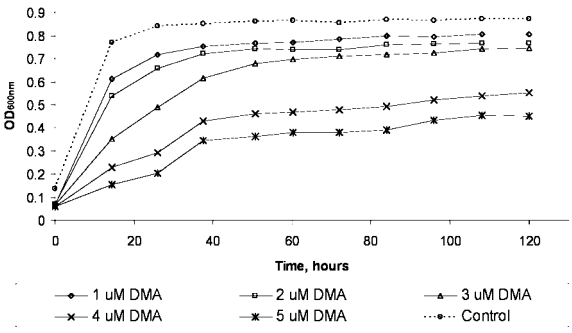

Figure 2. Line graphs showing the toxicity inhibition on the growth of *Pseudomonas* spp. in the presence of 1 to 5 μM 2,4-DMA. Error bars were removed for graphical simplicity.

Assessment of 2,4-DMA as a Carbon or Nitrogen Source. Figure 3a−d presents results from the assessment of 2,4-DMA utilization as a carbon or nitrogen source, or a combination thereof, by *Pseudomonas* spp. For ease of visual comparison, the scale for **Figure 3a−d** are the same. The study shown in Figure 3 (a) assessed 2,4-DMA as a carbon source for *Pseudomonas* spp. with NH_4Cl present as the readily available nitrogen source. **Figure 3a** shows a minimal decrease in the concentration of 2,4-DMA over 180 h, indicating that 2,4-DMA was not readily degraded by *Pseudomonas* spp. and, thus not readily utilized as a carbon source. Low OD_{600nm} absorbance values for *Pseudomonas* spp., **Figure 3a**, suggest lysis of the *Pseudomonas* spp. cells, indicating cell death.

The utilization of 2,4-DMA as a potential nitrogen source is presented in **Figure 3b**, in the presence of glucose and absence of NH_4Cl. After 48 h, a decrease in the concentration of 2,4-DMA was observed, with a 60% decrease in the concentration of 2,4-DMA after 180 h. OD_{600nm} of 0.58 indicates the utilization of the amine group in 2,4-DMA as a nitrogen source for growth. This ready utilization of 2,4-DMA as a nitrogen source suggests that the initial step in the breakdown of 2,4-DMA by *Pseudomonas* spp. could be deamination under aerobic conditions.

In the study shown in **Figure 3c**, the readily available carbon and nitrogen sources were substituted with 2,4-DMA. From the line curve it is evident that 2,4-DMA decreased in concentration in the presence of *Pseudomonas* spp. while the OD increased, indicating that the microorganisms utilize 2,4-DMA as both a carbon and nitrogen source for growth.

In **Figure 3d**, readily accessible carbon and nitrogen sources were available to *Pseudomonas* spp. in the form of glucose and ammonia, in the presence of 2,4-DMA. For 60 h no decrease in the concentration of 2,4-DMA was noted while increase in the OD_{600nm} indicated growth of the *Pseudomonas* spp. owing to utilization of glucose and ammonia. After 60 h, the readily available carbon and nitrogen sources were exhausted as indicated by the lag in the growth phase. Beyond this lag phase, the concentration of 2,4-DMA began decreasing, indicating its use as a carbon and nitrogen source by the *Pseudomonas* spp. followed by the associated increase in growth of the *Pseudomonas* spp.

Spiking of Carbon- and Nitrogen-Free Flasks with 2,4-DMA. The following study assessed the rate of 2,4-DMA degradation by *Pseudomonas* spp. that have acclimatized to utilizing 2,4-DMA as a carbon and nitrogen source. **Figure 4** shows the decrease in 2,4−DMA concentration in the presence of *Pseudomonas* spp. with time in the absence of alternative sources of carbon and nitrogen. Growth of the *Pseudomonas*

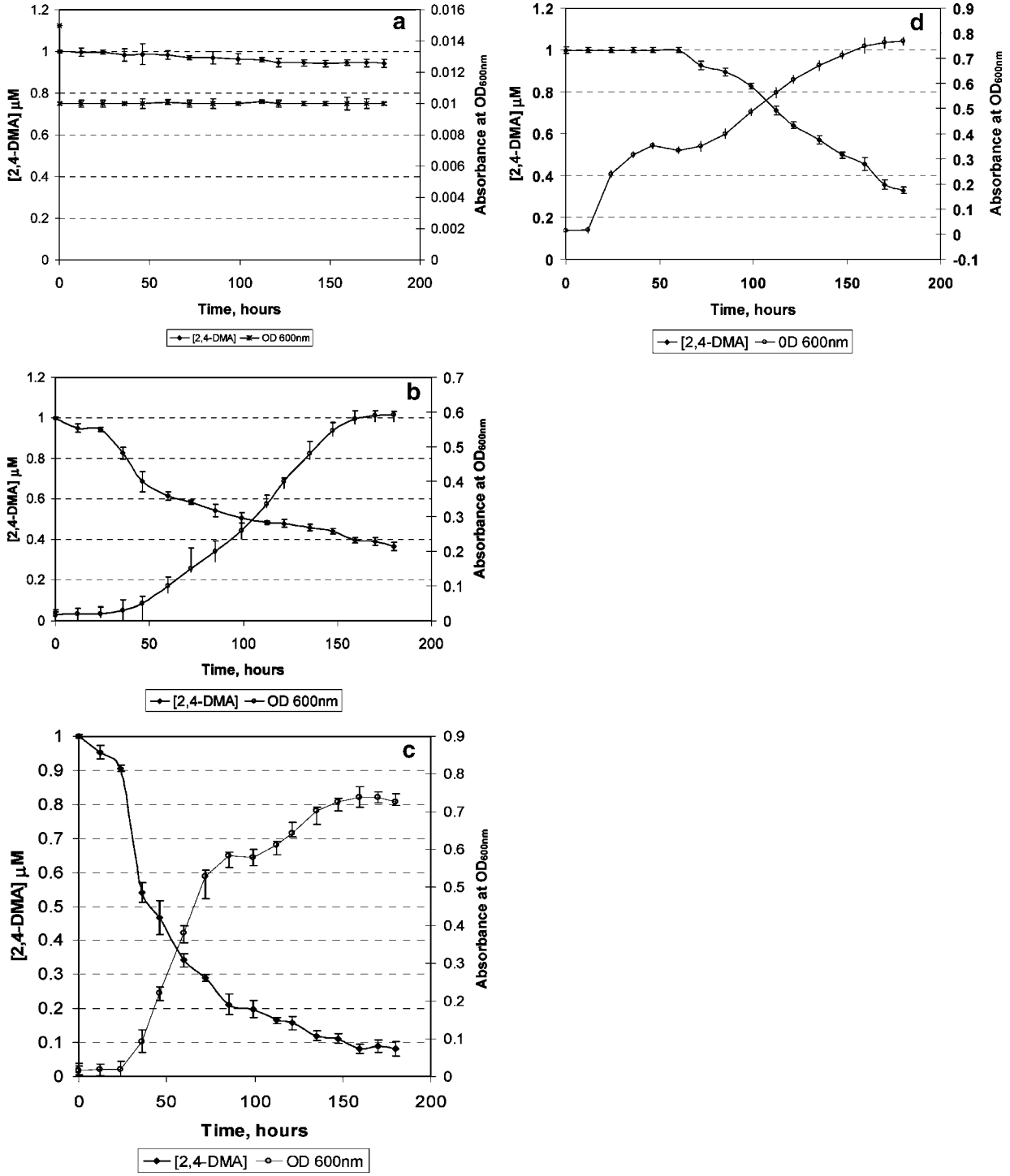

Figure 3. (a). Examination of 2,4-DMA utilization as a carbon source by *Pseudomonas* spp. in the presence of 1 μM 2,4-DMA and NH_4Cl. (b). Examination of 2,4-DMA utilization as a nitrogen source by *Pseudomonas* spp. in the presence of 1 μM 2,4-DMA and glucose. (c). Examination of 2,4-DMA utilization as both a carbon and nitrogen source by *Pseudomonas* spp. in the presence of 1 μM 2,4-DMA. (d). Examination of 2,4-DMA utilization as an alternate carbon and nitrogen source by *Pseudomonas* spp. in the presence of 1 μM 2,4-DMA, NH_4Cl, and glucose.

spp. is shown by an increase in OD_{600nm}. When 70% of the 2,4-DMA was utilized, spiking this culture with additional 2,4-DMA after 120 h induced a second phase of 2,4-DMA breakdown (phase B in the aforementioned figure).

At time = 132 h, 12 h, after spiking with 2,4-DMA, a more pronounced decrease in the concentration of 2,4-DMA was noted, owing to the acclimation of the *Pseudomonas* spp. to 2,4-DMA as a growth source. At time = 24 h (after spiking with 2,4-DMA), a 40% decrease in the 2,4-DMA concentration was observed whereas in the first phase (A), a 40% decrease was only observed after 50 h. The OD_{600nm} readings confirm the utilization of 2,4-DMA as a carbon and nitrogen source by *Pseudomonas* spp. as the organism enters a second exponential growth phase, as shown by the diauxic curve, A and B, shown in **Figure 4**.

On the basis of this evidence in which *Pseudomonas* spp. readily accessed 2,4-DMA as a nitrogen source, in the absence of a more readily available nitrogen source, and the lack of 2,4-DMA utilization as a carbon source in the presence of a more readily available carbon source, it is evident that a pathway in

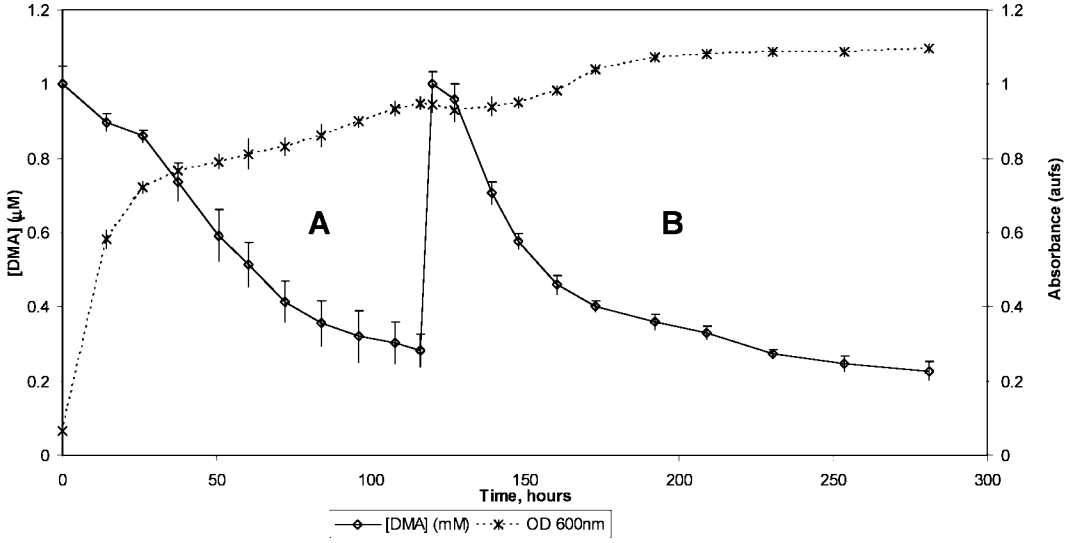

Figure 4. The breakdown of 1 μM 2,4-DMA by *Pseudomonas* spp. and spiking of the batch culture with 2,4-DMA at 120 h to yield a final concentration of 1 μM 2,4-DMA. The diauxic growth curve for *Pseudomonas* spp. is shown by the broken line.

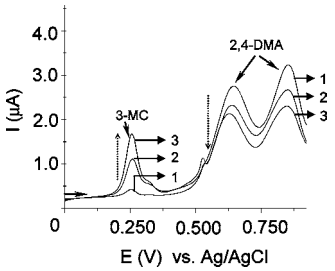

Figure 5. CVs showing the breakdown of 2,4-DMA by *Pseudomonas* spp. and the formation of 3-MC between 48 h and 60 h. Legend: 1 = 48 h, 2 = 54 h, 3 = 60 h.

which the amine group of 2,4-DMA is degraded as the initial step is plausible. Given however that the *Pseudomonas* spp. was capable of utilizing 2,4-DMA as both a carbon and nitrogen source, it follows that oxidative deamination yields an intermediate which can readily be accessed as a carbon source. On the basis of studies by earlier authors on anilines (*13*) and methylanilines (*9*, *10*), it is likely then that metabolism of 2,4-dimethylanilines proceeds via oxidative deamination of the amine through a series of intermediates which represent an accessible carbon source, such as the catechols.

To test this theory, cyclic voltammograms of a solution in which 2,4-DMA was utilized as the sole carbon and nitrogen source by *Pseudomonas* spp. was performed over time (**Figure 5**). The anodic couple between 0.6 and 0.8 V is attributed to 2,4-DMA and the oxidation peak at 0.25 V to formation of 3-methylcatechol, (3-MC), as confirmed by CV of pure 3-MC. With time, the oxidation peaks attributed to 2,4-DMA decrease as that of the 3-MC increases, indicative of metabolism of 2,4-DMA to 3-MC by *Pseudomonas* spp. While it is evident that the concentration of 2,4-DMA decreases as the concentration of 3-MC increases, a direct correlation between the decrease in 2,4-DMA concentration and 3-MC increase in concentration is difficult, given that 3-MC is further mineralized.

In conclusion, these observations provide evidence for the proposed oxidative deamination of 2,4-DMA via a series of intermediates to a catechol metabolite before further metabolism by *Pseudomonas* spp., showing the feasibility of using electrochemical studies for direct monitoring of the biodegradation. Furthermore, as a consortium of mixed bacterial cultures including *Pseudomonas* spp. isolated from cattle dip tanks has been shown to degrade amitraz to 2,4-DMA, (*1*, *14*), it is likely then that complete mineralization of amitraz is possible in dipping tanks in the presence of bacteria capable of degrading this pesticide and its degradants.

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Received for review July 12, 2006. Revised manuscript received September 11, 2006. Accepted September 19, 2006. This material is based on work supported by the National Research Foundation (NRF) of South Africa and Rhodes University. Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

JF061951K