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Determination and identification of metabolites of the fungicides Iprodione and Procymidone in compost

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Abstract

The main metabolites formed from Iprodione and Procymidone during the composting process have been isolated and identified by HPLC-DAD-MSD. After addition of the fungicides to the composting pile, we monitored the reaction of the two analytes and the formation of their degradation products for eight months. We verified the nature of the metabolites by comparison with those hypothesised in the literature and by comparison with the behaviour of an abiotic process in aqueous acetonitrile pH 6 and at 35°C. After taking into account the different kinetic behaviours of the fungicides on degradation in compost and hydro-organic solution, breakdown pathways are proposed for biodegradation.

Keywords: Metabolite; Iprodione; Procymidone; Kinetic; Composting process; HPLC-DAD-MSD

1. Introduction

Application of recycled municipal sludges to agricultural land for maintaining or increasing soil fertility has become an argument of great interest for soil scientists, agronomists and environmentalists throughout the world. Although these organic additives can have a positive influence on the physical and biochemical properties of soils (Tietjen, 1975; Chen and Avnimelech, 1986), great interest arises due to the presence in the sludges of hazardously high levels of heavy metals and organic pollutants, mainly derived from industrial effluent that reach the treatment plants.

While a considerable amount of literature has been published about the negative effects caused by toxic metals in plant-soil systems (Wieteska et al., 1996; Leiter et al., 1997; Zhou and Liu, 1997), very little work has been done on the presence and fate of undesirable organic compounds.

Since compost is used for growing plants, the fate of any anthropogenic compounds in the compost must be known. In particular, pesticides are used with some regularity on turf grass, vegetables and fruit, and the fate of most of these products during the compost process is not well known (Racke and Frink, 1989; Lemmon and Pylypiw, 1992).

The two dicarboximidic fungicides Iprodione (Rovral) and Procymidone (Sialex 50 WDG) are frequently used for controlling fungal diseases such as the Botrytis Cinerea, Monilia and Sclerotinia species, and they are especially used in fruit growing, strawberry, wine, horticulture and flower and ornamental cultivations. The relative formulas are reported in Fig. 1.

The use of these pesticides is increasing as their correct application does not produce a residual content in the food products that is higher than that consented by the legal limits (Molinari et al., 1980; Cabras et al., 1982; Flori et al., 1982a,b). Nevertheless, it is possible to find crops and environments with consistent residues of these

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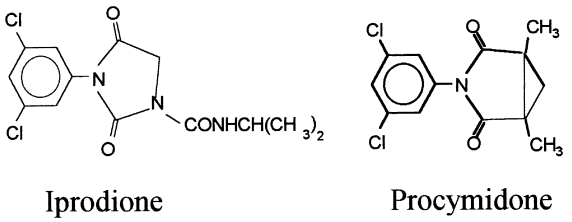

Fig. 1. Formulas of Iprodione and Procymidone.

fungicides and/or their breakdown products, as a consequence of frequent application, or because the interval between treatments (Barbero and Gaia, 1979; Flori et al., 1982a) is not respected. In a recent study (Vanni et al., in press), non-toxic amounts of dicarboximidic fungicides (Iprodione, Vinclozolin and Chlozolinate) have been found with or without their common metabolite (3,5-DCA) in commercial compost. Therefore, given the various uses of compost, the danger of bioaccumulation of the possible breakdown products with unknown toxicity cannot be excluded.

This paper reports the isolation and structural identification of the breakdown products of Iprodione and Procymidone during the composting process. After addition of the fungicides to the composting pile, the behaviour of the two fungicides and their degradation products was studied, and verifications were made by comparing the kinetic behaviour of every compound in the compost in an hydro-organic acid solution (55% acetonitrile/45% sodium phosphate buffer pH 6.5) where fungicides are completely soluble. In fact, the chemical hydrolysis of pesticides could be one of the principal mechanisms for the decomposition of organic pesticides in the environment, in soils, in plant tissues or in maintained foods. Therefore, understanding the reaction kinetics could be an important parameter for predictions on the biological activity and the persistence of the active ingredients.

Based on these data, biodegradation pathways for these fungicides are proposed.

2. Experimental

2.1. Materials

Iprodione [3-[(3,5-dichlorophenyl)-*N*-(1-methylethyl) -2,4-dioxo-1-imidazolidinecarboxyamide] was furnished by Supelco. Procymidone [*N*-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide] and 3,5-dichloroaniline were supplied from Dr Ehrenstorfer GmbH. The commercial products Rovral (Iprodione) and Sialex 50 WDG (Procymidone) were furnished by RHÔNE-POULENC and SIAPA, respectively.

Solvents employed included acetic acid (glacial (100%); GR, Merck), ortho-phosphoric acid 99% (extra pure, Riedel-de Haën), di-sodium hydrogen phosphate dihydrate 99.0% (Fluka), sodium dihydrogen phosphate dihydrate 99.0% (Fluka), ammonium acetate (analytical reagent, Riedel-de Haën), acetonitrile gradient-grade (Chromasolv, Merk). Ultra pure water was obtained from a water purification Elgastat system.

2.2. Procedure

2.2.1. Addition on composting pile for biotic degradation

Fig. 2, shows a diagram of a generic composting pile. The analyte additions have been made in the sketched zones (about 10 m) of two extremes, while the central one (about 10 m) has been considered as blank.

The medium quantity of technical product (50% analyte + coformulates) to be applied on crops is about 1.5–2 kg/ha, therefore 450 $g_{product}$/10 m_{pile} has been applied in order to obtain detectable quantities of analyte. Taking into consideration the parameters reported in Table 1, the added quantity is therefore 20 μg of analyte versus g dried compost (DC).

The pile was turned regularly and sampled at compost initiation and at 4, 11, 18, 25, 32, 43, 60, 94, 119, 151, 186 and 241 days after treatment. During the composting process, pH variations were evaluated using the official method reported in DIVAPRA (1998) from pH 5.5 at acid phase to pH 7.5 before grinding until pH 8.7 after maturation in the farmyard (eight months).

The compost samples were frozen at −18°C and preserved in plastic bags.

2.2.2. Extraction from compost samples

The analytical technique using sonication disruption and acetonitrile extraction have been reported elsewhere (Vanni et al., in press). The obtained extracts have been preserved at 4°C in screw-capped vials.

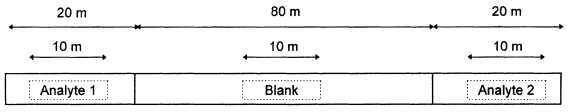

Fig. 2. Diagram of the composting pile: sketched areas show sampling zones.

Table 1
Characteristics of composting pile

Pile width (m)	2
Pile length (m)	120
Pile height (m)	1.5
Compost density (t/m^3)	0.6
Compost humidity (%)	50

Table 2
Operative conditions for the analysis of fungicides, metabolites and hydrolyses

	Biotic process		Abiotic process	
	Fungicides	Metabolites	Hydrolyses	
Column (Lichrospher, HP)	RP-C18, 5 μm, 250 mm×2 mm 1 i.d.	RP-C18, 5 μm, 250 mm×2 mm 1 i.d.	RP-C18, 5 μm, 250 mm×3 mm 1 i.d.	RP-C18, 5 μm, 250 mm×2 mm 1 i.d.
Flow (ml/min)	0.3	0.3	1	0.3
Eluent	H_2O/CH_3CN	H_2O/CH_3CN CH_3COOH 0.05 M pH 3/CH_3CN	H_2O/CH_3CN Phosphate buffer 0.05 M pH 3/CH_3CN	H_2O/CH_3CN
Elution	Gradient[a]	Gradient[a]	Isocratic[b]	Gradient[a]
Detector	DAD/APCI	DAD/API-ES	DAD	DAD/API-ES

[a] See the solvent programming diagram below.
[b] 70% organic solvent/30% phosphate buffer.

2.2.3. Abiotic hydrolysis: operative conditions

The pH (6 and 8.7) values have been chosen following evaluations made during the composting process. The temperatures used (35°C and 55°C) refer to the values of the mesophilic and thermophilic steps of the T (°C) curves versus time, as reported in Golueke (1977).

Aliquots of 0.2 ml of a single standard solution at 500 μg/ml in acetonitrile were mixed with 0.01 M phosphate buffer at two different levels of pH 6 and 8.7. The final concentration of fungicide in the buffered solutions was 20 μg/ml. Aliquots of 1 ml of the buffered standard solutions were transferred into vials and incubated at 35°C and 55°C in a water bath and in darkness. After incubation, the samples were acidified with H_3PO_4 to pH 3 to stop the reaction.

2.2.4. Fungicides and metabolites analysis

The analysis of the extracts was executed by HPLC, injecting 20 μl of filtered solutions into a liquid chromatograph (HP 1100) equipped with a 1100 Binary Pump and with UV–Vis/diode array detector (HP 1100 DAD) and a mass detector (HP 1100 MS).

The operative conditions are reported in Table 2.

Every solvent was previously filtered and degassed. The solvent programming was as follows:

1 min		5 min				
40%	→	70%	→	100%	→	40%
	34 min		30 min		10 min	

Changes in the percentage of organic solvent in the mobile phase through the gradient program occurred linearly. The absorption of analytes was monitored at 210 nm and the entire absorbance data were recorded from 190 to 400 nm during every analysis.

The working conditions for APCI and API-ES are reported in Table 3.

The quantification of peaks was carried out by an external standard method, using measurements of peak areas and a calibration curve for each pesticide. The limits of detection (LOD) are reported in Table 4.

Table 3
Working conditions for the MS detector

Operative parameters	APCI	API-ES
$T_{\text{drying gas}}$ (°C)	350	350
$T_{\text{vaporiser}}$ (°C)	325	/
Flow-rate$_{\text{drying gas}}$ (ml/min)	7	10
Capillary voltage (V)	5000	5000
Corona current (μA)	6	/
Fragmentor (V)	70	70
Polarity	Positive	Negative

Table 4
Detection limits of tested compounds from compost samples

	DAD	MSD
Compounds	LOD (μg/kg) ± RSD (%)	LOD (μg/kg) ± RSD (%)
Iprodione	35 ± 8.9	50 ± 7.1
Procymidone	35 ± 7.4	200 ± 6.5

The quantitative determination of the degradation products was made using external standards, if commercially available, or in any case, was determined approximately with the assumptions that follow.

In Cabras et al. (1983) and Pirisi et al. (1986), 3,5-DCA is given as the final product of degradation of two analytes. By comparing absorbance spectra (see Fig. 3), it is evident that the absorbance at 210 nm due to the aromatic ring is slightly influenced by the chromophor groups (C═O) adjacent to –N<. Therefore, supposing that at a chosen wavelength the ε (molar absorbivity) of several formed metabolites remains practically equal to that of 3,5-DCA and analyte, we can assume that the Lambert–Beer equation could be applied to other breakdown products, C_{Met} is thereby calculated by the following equation:

$$C_{\text{Met}} = (C_{\text{Analyte/3,5-DCA}} * A_{\text{Met}})/A_{\text{Analyte/3,5-DCA}}, \quad (1)$$

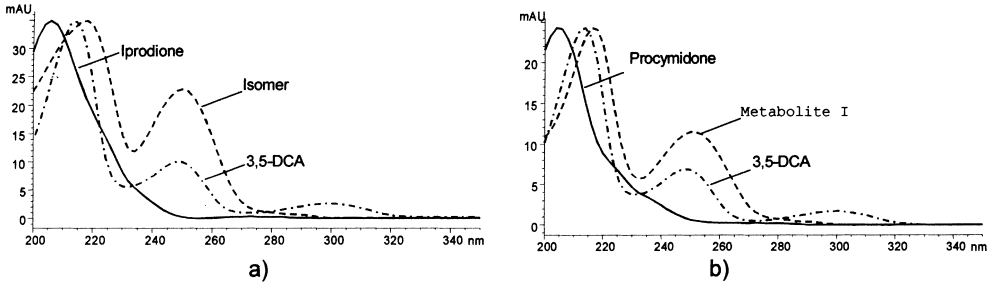

Fig. 3. Comparison with absorbance spectra: (a) Iprodione and its metabolites, and (b) Procymidone and its metabolites.

where C_{Met} and $C_{\text{Analyte/3,5-DCA}}$ are the concentrations of metabolite and of analyte (Procymidone or Iprodione) or 3,5-DCA, respectively.

After semi-quantitative evaluations of breakdown products, the kinetic behaviour for each compound was studied and the relative constants rate and pattern by mathematical expressions was determined using the literature method based on parallel-consecutive reaction (Rodiguin and Rodiguina, 1964), which is well suited for this kind of kinetics.

3. Results and discussion

3.1. Biodegradation of analytes

In the present study, we previously studied the degradation, said 'primary', of two analytes during the composting process, then our attention focused on compounds formed during the process, in order to identify them through MSD and to verify that no accumulation of toxic reaction products is produced in the reaction media.

3.2. Procymidone

From a kinetic point of view, the results of primary degradation point out a pseudo-first-order law, described by the equation

$$-\mathrm{d}C/\mathrm{d}t = k_{\text{obs}}C, \tag{2}$$

where C is the substrate concentration and k_{obs} is the observed first-order rate constant. According to Eq. (2), linear plot of $\ln(C/C_0)$ versus time is obtained (see insert in Fig. 4), from which slopes k_{obs} can be estimated. A value of 0.073×10^{-4} min^{-1} was determined working under the conditions reported in Fig. 4. Each point represented three replicate experiments.

As shown in Fig. 4, the degradation curve of fungicide seemed to present the same biphasic degradation shown by other pesticides reported in Vandervoort et al. (1997). There is an initial rapid dissipation rate followed by a slower process. Vandervoort and others hypothesised that the first step could be related to volatilisation and photolysis, while the second step to microbial degradation.

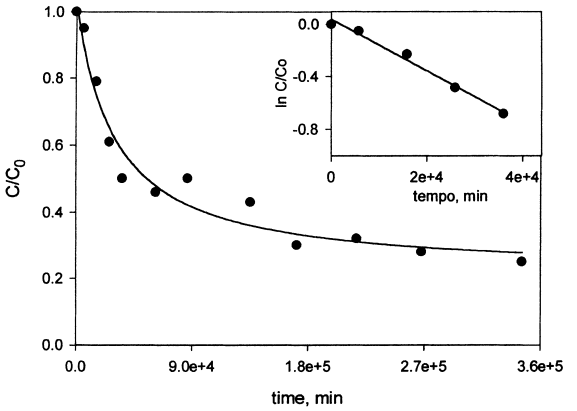

Fig. 4. Degradation kinetics of Procymidone during composting process. Initial substrate concentration, C_0: 1.23×10^{-4} $\text{mol}_{\text{Analyte.}}/\text{g}_{\text{dry}}$.

In the examined time range (0–241 days), the total conversion of Procymidone did not occur: 30% remained unchanged in the compost.

As shown in Fig. 5, the partial primary biodegradation led up to two breakdown products which were identified with MSD, 3,5-dichloronaline and 2-(3,5-dichlorophenylcarbamoil)-1,2-dimethylpropane carboxylic acid (`metabolite I`) (Pirisi et al., 1986). Their mass spectra are reported in Fig. 6.

3.3. Iprodione

Fig. 7 shows that a rapid transformation to only a breakdown product happens in approximately 30 days for this fungicide.

Following characterising with MSD, the metabolite formed during the composting process was identified: the parent compound was quantitatively converted to its `Isomer`, 3-isopropyl-*N*-(3,5-dichlorophenyl)-2,4-dioxo-

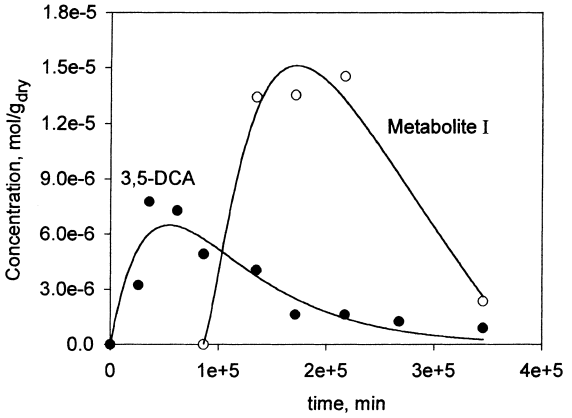

Fig. 5. Formation kinetics of Procymidone metabolites during the composting process.

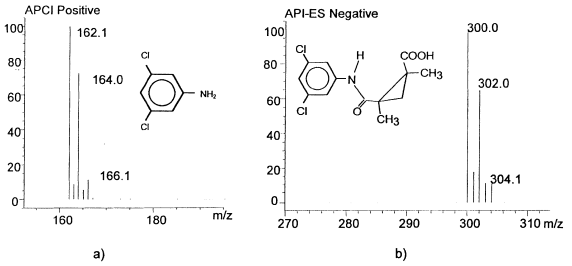

Fig. 6. Mass spectra of 3,5-DCA (a) and `metaboliteI` (b). Operative conditions are reported in Table 3.

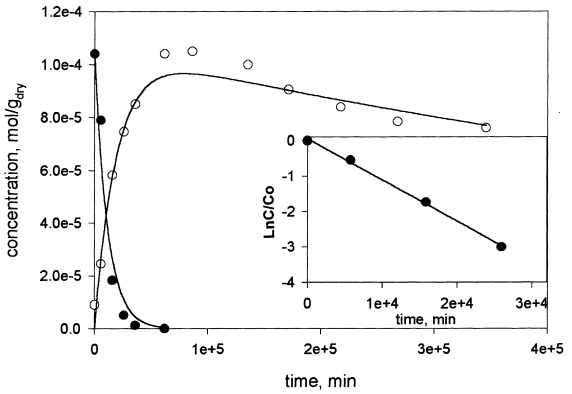

Fig. 7. Degradation kinetics of Iprodione (●) and its metabolite (○) during composting process. Initial substrate concentration, C_0: 1.04×10^{-4} $mol_{Analyte}/g_{dry}$.

1-imidazolidine-carboxamide (for the formula see Fig. 8) in accordance with the formula reported in a study on spiked vegetables samples (Newsome and Collins, 1990).

Also shown in Fig. 7, after 70 days, 20% of the `Isomer` undergoes microbial degradation to other metabolites which is probably not seen due to the fact that the metabolites are under the detection limits.

In addition, for Iprodione, according to Eq. (2), slope k_{obs} has been estimated (see insert in Fig. 7) in a value of 0.83×10^{-4} min^{-1}.

3.4. Abiotic degradation of fungicides

In the literature, several studies have reported (Cabras et al., 1984; Melkebeke et al., 1986; Pirisi et al., 1986; Newsome and Collins, 1990) on analytes hydrolysis with precise reference to their breakdown products. To confirm the nature of biotic metabolites, and to establish the kind of reactions that take part during the composting process, abiotic degradation has been carried out. Hydrolysis has been taken with standard solutions at several pH and temperatures. Kinetics of fungicides degradation and metabolites formation have been evaluated.

In the following figures, degradation kinetics and the relative metabolite formations are reported for both analytes.

3.5. Procymidone

In the degradation rate and time range, the hydrolysis conditions which approach the biotic conditions (see Fig. 4) are pH 6 and 35°C. The identification of the two breakdown products derived with MSD, and whose formation kinetics are reported in Fig. 10, result to be the same as those obtained during the composting process (see Fig. 6).

The data plotted in Fig. 9 show that the fungicide behaved very differently in several analysed conditions. At pH 6.0 and at a 35°C temperature, Procymidone degraded to only 15% in 40 days. With the increase of pH it was completely degraded (100%) within 35 days, and with the increase of temperature it was completely degraded within 10 days.

The linear lines of the pseudo-first-order kinetics, obtained at two different pH values (6 and 8.7), are shown in the insert of Fig. 9. The k_{obs} values of 2.4×10^{-4} min^{-1} and 41.5×10^{-4} min^{-1} were determined for pH 6 and 55°C and for pH 8.7 and 35°C, respectively, while at pH 6 and 35°C linearisation was not possible because $t_{1/2}$ has not been reached.

3.6. Iprodione

As shown in Fig. 11, the data show different behaviour at different conditions for this fungicide. The hydrolysis conditions which approach those of the biotic process, such as degradation rate and time (see Fig. 7), are again pH 6 and 35°C. Nevertheless, from the formation kinetics of Iprodione metabolites reported in Fig. 12, the identification of the three formed breakdown products have confirmed only the `Isomer`-like compound also formed during the composting process.

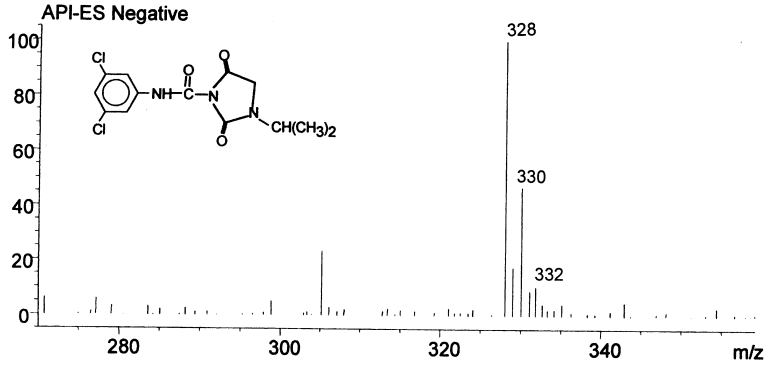

Fig. 8. Mass spectrum of `Isomer`. Operative conditions are reported in Table 3.

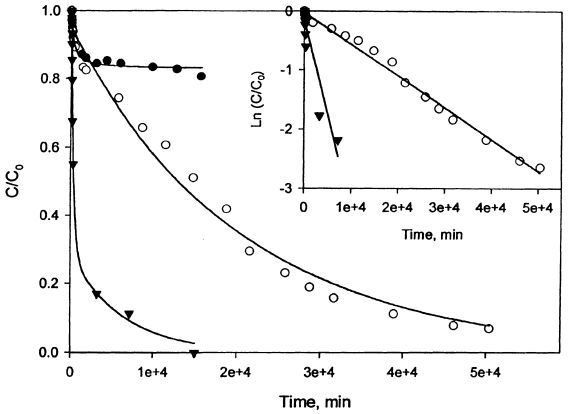

Fig. 9. Degradation kinetics of Procymidone during the hydrolysis process. Initial substrate concentration: 3.6×10^{-5} M. Operative conditions: (●) pH 6, 35°C; (○) pH 6, 55°C; (▼) pH 8.7, 35°C.

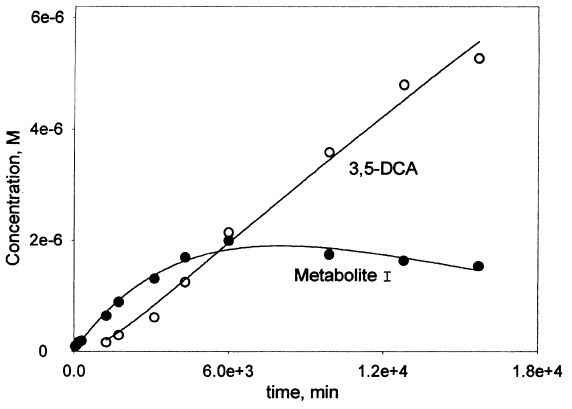

Fig. 10. Formation of Procymidone metabolites during hydrolysis (pH 6, 35°C).

The relative mass spectra is reported in Fig. 8. The two new metabolites have been identified by MSD as 3-isopropil carbamoil-5-(3,5-dichlorophenyl)hydantoic acid (`met. II`), in accordance with Newsome and Collins (1990), and `metabolite III` whose mass spectra are reported in Fig. 13.

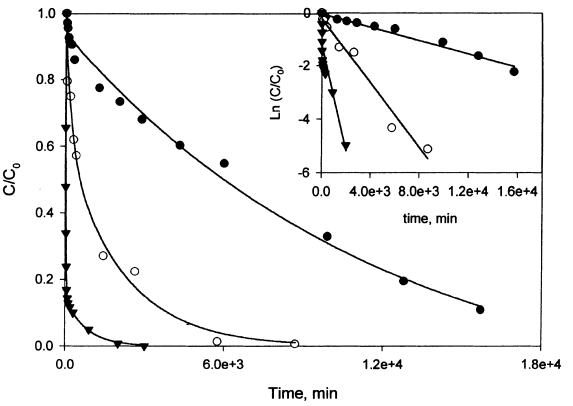

Fig. 11. Degradation kinetics of Iprodione during the hydrolysis process. Initial substrate concentration: 4.0×10^{-5} M. Operative conditions: (●) pH 6, 35°C; (○) pH 6, 55°C; (▼) pH 8.7, 35°C.

On the contrary, compared to the biotic process, generally the isomerisation has not been quantitatively (a low concentration of `Isomer` was formed) and at the highest temperature (55°C) the presence of 3,5-DCA has been determined.

In the insert in Fig. 11, the linear lines of the pseudo-first-order kinetics at the three different analysed pH values are shown. The k_{obs} values of 1.4×10^{-4} min^{-1}, 10.7×10^{-4} min^{-1} and 268×10^{-4} min^{-1} were estimated for pH 6 at 35°C and 55°C and for pH 8.7 at 35°C, respectively. These data show that Iprodione rate constants are also influenced by pH and temperature variations, but more by pH rather than by temperature.

Table 5 summarises a qualitative comparison between the biotic and abiotic processes. All metabolites obtained are reported for both analysed fungicides.

By analysing the Procymidone results first, as both processes present the same metabolites, we could

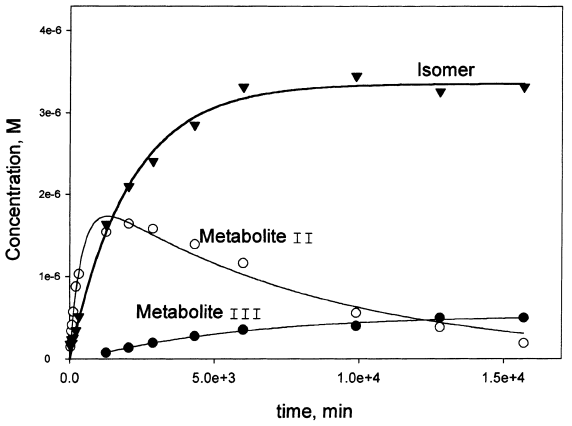

Fig. 12. Formation kinetics of Iprodione metabolites during hydrolysis (pH 6, 35°C).

hypothesise that during the composting process hydrolytic degradation prevails. This hypothesis could be supported by the analysis of the semi-quantitative data reported in Table 6. During the biotic process the predominance of one of the two reaction steps is not present: k_1 and k_2 values have the same order. In accordance with the more similar abiotic condition (pH 6 and T 35°C), the biotic degradation of Procymidone is characterised by a slow breakdown as shown by the higher value of $t_{1/2}$.

In the abiotic process, the reaction rate of step 1, determining the formation of metabolite I, the results are influenced more by the increase of pH than by the increase of temperature. For the second reaction step, determining the formation of 3,5-DCA, the rate constant (k_2) is affected by the opposite influence.

For Iprodione, chemical hydrolysis is probably of limited importance to the fate of this chemical during

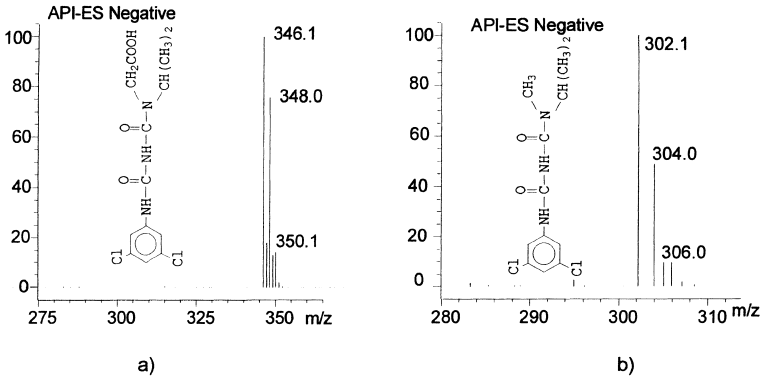

Fig. 13. Mass spectra of met. II (a) and met. III (b). Operative conditions are reported in Table 3.

Table 5
Comparison between biotic and abiotic metabolites

Fungicides, (*m/z*) t_r	Procymidone, (283) 21.2		Iprodione, (329) 20.5			
Metabolites	I (*m/z*) t_r	3,5-DCA (*m/z*) t_r	II (*m/z*) t_r	III (*m/z*) t_r	3,5-DCA (*m/z*) t_r	Is. (*m/z*) t_r
Biotic process	(301) 6	(161) 15.5	/	/	/	(329) 29
Abiotic process	(301) 6	(161) 15.5	(347) 2.5	(303) 9.6	(161) 15.5	(329) 29

Table 6
Biotic and abiotic kinetic constants for Procymidone

	Biotic process	Abiotic process		
		pH = 6, T = 35°C	pH = 6, T = 55°C	pH = 8.7, T = 35°C
$10^4 \times k_{obs}$ min^{-1}	$k_1 = 0.032$	$k_1 = 0.18$	$k_1 = 2.26$	$k_1 = 41.4$
	$k_2 = 0.041$	$k_2 = 1.58$	$k_2 = 0.14$	$k_2 = 0.096$
	/	$k_3 = 0.84$	$k_3 = 0.53$	/
$t_{1/2}$ (days)	39.9	/[a]	12.06	0.43

[a] $t_{1/2}$ has not been reached.

Table 7
Biotic and abiotic kinetic constants for Iprodione

	Biotic process	Abiotic process		
		pH = 6, T = 35°C	pH = 6, T = 55°C	pH = 8.7, T = 35°C
$10^4 \times k_{obs}$ min^{-1}	/	$k_1 = 1.08$	$k_1 = 7.9$	$k_1 = 252$
	$k_2 = 0.83$	$k_2 = 0.36$	$k_2 = 2.78$	$k_2 = 16$
	/	$k_3 = 1.8$	$k_3 = 4.93$	$k_3 = 337$
	/	/	$k_4 = 1.36$/	/
$t_{1/2}$ (days)	5.54	4.78	0.45	0.013

the composting process, while other transformations could prevail due to enzymatic activity of a particular micro-organism. As a matter of fact, the analyte is completely converted to a more stable isomer compound that has an identical ratio *m*/*z* to that of the parent compound (Iprodione) but has a different retention time (see Table 5).

As shown in Table 7, in the analysis of semi-quantitative data of the abiotic process, the Isomer is formed with the rate constant (k_2) in the same order to that of the biotic process, even if halved ($k_2 = 0.36$) in accordance with the decrease of transformation percent, from the biotic 100% to the abiotic 10% (see Fig. 12).

Nevertheless, in the abiotic conditions there is also the contemporary formation of metabolite II (reaction step 1) which is transformed successively into metabolite III (reaction step 3). Moreover, at pH 6 and at the highest temperature (55°C) within 24 h, another reaction (step 4), relative to the transformation of metabolite II to the 3,5-DCA, final metabolite, results to be very interesting.

As the Procymidone data have already plotted out, the first and the third reaction steps are quite influenced by the increase of pH and temperature while the second step, also present in the biotic process, shows a minor effect.

4. Conclusions

From the analysis of the curve-fitting data and from previous considerations, the degradation of Procymidone and Iprodione could occur in accordance with hypothesised pathways reported in Figs. 14 and 15 for the biotic and abiotic (pH 6 and T 35°C) processes.

On this basis, Fig. 14 shows the course of the abiotic process for Procymidone could proceed through parallel-consecutive reactions: parallel chemical reactions in which the initial substance produces two different substances simultaneously (reaction steps 1 and 2), and a consecutive reaction in which one of the intermediate products can react again contributing to the formation of the other metabolite (reaction step 3). On the contrary, as seen in the verified slowness of the biotic process, such a process could proceed only through parallel reactions.

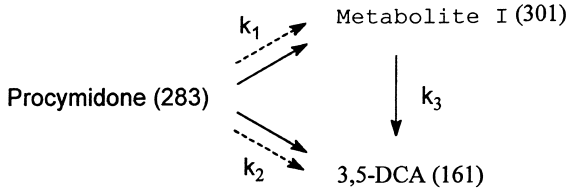

Fig. 14. Biotic (⇢) and(→) abiotic pathways for Procymidone.

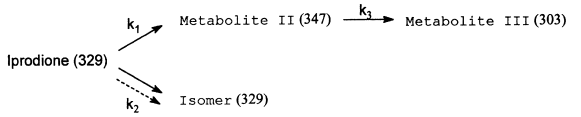

Fig. 15. Biotic (⇢) and(→) abiotic pathways for Iprodione.

As shown in Fig. 15, the abiotic process for Iprodione probably also proceeds through parallel-consecutive reactions which comprise the two parallel chains, one composed of two simple reactions (reaction steps 1 and 3) and the other composed of one simple reaction (reaction step 2). Instead, the biotic process could be described kinetically as a system of only one consecutive reaction: Iprodione is completely converted to the Isomer.

By also taking into consideration the various possible detoxification mechanisms (absorption, leaching, biodegradation, etc.) that occur in soils (Ellwardt, 1975; Herbes, 1981; Kaufman and Horwick, 1983), these results suggest that the accumulation phenomenon cannot be excluded in the alimentary chain. The presence of 30% residue of an intact pesticide (Procymidone) after 241 days, or of a toxic pesticide metabolite (Isomer of Iprodione, 3,5-DCA and Met. I for Procymidone), is a real concern because of the potential damaging effects. Furthermore, the possibility of the presence of the active product or breakdown products formation during the composting process has an importance for public health researchers because these substances, such as chlorinated aromatic amine, result to be highly toxic to living

organisms. Therefore, the presence of metabolites could also be used analytically as a marker to test the hoarding danger with the use of compost, to introduce toxic substances into the alimentary chain.

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