An appraisal of five methods for the measurement of the fungal population in soil treated with chlorothalonil

Rosana F Vieira,[1]* Célia MMS Silva,[1] Aline HN Maia,[1] Elisabeth F Fay[1] and Kátia C Coelho[2]

[1]*Embrapa Environment, CP 69, CEP 13820-000, Jaguariúna, SP, Brazil*
[2]*National Center of Research, Brazil*

Abstract: An experiment was carried out in a greenhouse with the aim of determining an acceptable method to quantify the fungal population in a soil treated with the fungicide chlorothalonil. Doses of the fungicide ranging from 12 to 96 μg AI g^{-1} soil were applied and microbial biomass carbon (C), soil ergosterol content, living hyphal length, as well as counts of total and cellulolytic fungi colonies, were measured 1, 2, 3, 4 and 5 weeks after application. At the end of 16 weeks a new evaluation was done using the three first methods. The microbial biomass C was the least sensitive parameter and, on balance, the living hyphal length was the most sensitive parameter for demonstrating effects of chlorothalonil on the fungal population. Some problems related to the efficiency of the ergosterol content and living hyphal length measurements in the evaluation of the effect of the fungicide on the fungal microflora are discussed, as is the need to compare short-term (0–5 weeks after treatment) with longer-term (16 weeks) results.

Keywords: fungicide; chlorothalonil; ergosterol; living hyphal length; microbial biomass C; fungi population

1 INTRODUCTION

Chlorothalonil (tetrachloroisophthalonitrile), a fungicide widely used in Brazil,[1] is a non-systemic, contact material which protects plant surfaces from fungal pathogens; the amount applied varyies with the crop to be treated. It is a broad-spectrum material which conjugates with, and thus depletes, the thiols (particularly glutathione) present in the target fungi.[2]

The fungi are an important part of the terrestrial ecosystem. They usually dominate the microbial biomass in most soils[3] and are the heterotrophs primarily responsible for the decomposition of organic residues.[4] Furthermore, fungi represent a large nutrient pool, contribute to soil aggregation[5] and participate in mycorrhizal symbiosis.[6] In spite of their importance in the terrestrial ecosystem, the fungi are vulnerable to damage from fungicides which may accumulate in soil. These pesticides, like others, could affect biochemical and physiological processes that are common to target and non-target organisms.

Several techniques have been used to quantify fungi in soil, but serious sources of error exist in all of these.[7] The method most used for measuring fungal populations in soil is the direct microscopic counting of hyphae. This method, along with the quantification of fluorescein diacetate-stained hyphal length, tends to underestimate the fungal population and may be influenced by a high degree of observer's subjectivity.[8] The soil ergosterol content is a biochemical method which has been cited as an indirect and sensitive indicator of changes in fungal populations[9] and also as a possible indicator of pesticide side-effects.[10] Ergosterol is the major sterol in the majority of eucomycotic fungi.[11] An important disadvantage of this fungal marker compound is the absence of its production by oomycetes and by a number of yeasts, both of which may be important in some soils.[8] Another problem associated with this method is that the ergosterol content of fungal tissue varies with their physiological state.[12,13]

Considering that, on a mass basis, fungi represent the most abundant group of micro-organisms in soils,[3] the microbial biomass carbon (C) could be also an adequate parameter to measure the toxic effects of fungicides in soil. Although that premise has been verified with some fungicides, other authors have shown the ergosterol content to be a more sensitive measure of pesticide side-effects than microbial biomass carbon.[10]

The aim of this work was to determine the most appropriate and sensitive method for measuring possible deleterious effects of the fungicide chlorotha-

* Correspondence to: Rosana F Vieira, Embrapa Environment, CP 69, CEP 13820-000, Jaguariúna, SP, Brazil
E-mail: rosana@cnpma.embrapa.br
(*Received 29 November 1999; accepted 5 January 2000*)

lonil on the soil fungal population. The following methods were evaluated: microbial biomass C, soil ergosterol content, living hyphal length, and counts of total and cellulolytic fungal colonies on specific culture media.

2 MATERIALS AND METHODS

2.1 Materials

The experiment was conducted in a greenhouse, in Embrapa Environment, Jaguariúna, SP, Brazil, using a soil classified as Red Yellow Oxisol, with the following chemical and physical characteristics: pH(H_2O) 4.8; cationic exchange capacity 73.6 $mmol_C\,dm^{-3}$; organic matter 30.5 $g\,dm^{-3}$; clay 387 $g\,kg^{-1}$; sand 485 $g\,kg^{-1}$ and silt 128 $g\,kg^{-1}$.

The soil was collected at 0–15 cm depth in an area occupied by molasse grass (*Melinis minutiflora* Beauv), which had not been cultivated in the last 10 years. It was contained in white plastic boxes without drainage (20 kg soil per box). The commercial product Vanox® was used at doses of 12, 24, 48 and 96 μg AI g^{-1} soil (doses 1–4), with untreated soil (dose 0) included for comparison; dose 4 represented twice the greatest amount normally affected in the field. Before the addition of the fungicide to the experimental units, the soil remained in the boxes for seven days, with humidity corrected to 50–70% water holding capacity (WHC). At the end of this period the initial evaluations of the microbiological parameters were performed and the pesticide was then added. In each case, the chlorothalonil was affected in a sufficient volume of water to bring the soil to 60–70% WHC. Soils were assessed at weekly intervals for five weeks (short-term evaluation) and at the end of 16 weeks (long-term evaluation) after fungicide addition. The soil was sampled periodically for humidity evaluation in the laboratory, and the humidity was maintained at the standard level. Soil temperature was also monitored periodically; it remained around 19 °–23 °C in the morning, and around 27–28 °C, in the afternoon, during the whole experimental period, these temperatures being typical of ambient conditions in the area.

For all the methods described below, calculations were based on the weight of a sample dried at 105 °C, for 24 h

2.2 Analytical methods

2.2.1 *Soil microbial biomass C*

Soil microbial biomass C was measured by the fumigation-extraction method.[14] Soil (50 g) was divided into two equal parts, one of which was exposed to chloroform for 24 h and the soil was then extracted with potassium sulfate solution (0.5 M; 100 ml); the other portion was not fumigated but was extracted, under the same conditions, at the time fumigation began. Organic C in the extracts was determined by dichromate digestion. The soil microbial biomass C content (C_{mic}) was calculated using the relation:

$$C_{mic} = 2.64 \,.\, E_c \qquad (1)$$

where E_c is the extracted organic C of the fumigated soil less the extracted organic C of the non-fumigated soil.

2.2.2 *Living hyphal length*

The living hyphal length was evaluated according to method proposed by Melloni and Cardoso[15] with some adaptations reported by Nogueira and Cardoso.[16] To soil (20 g) in a 500-ml beaker, water (*c*300 ml) was added under pressure and the resulting suspension was passed through 0.71- and 0.25-mm sieves, mounted above a funnel placed over a 2-litre beaker. The sieves were then washed with a strong jet of water (*c*1.4 litres) and the resulting suspension was transferred to a blender and the volume made up to 1.5 litres. The blender was operated for 10 s at a low speed to ensure aggregate dispersal and the mixture was then allowed to stand for 2 min, after which a sample (500 ml) was passed slowly through a 44-mesh sieve. The retained material was then suspended in phosphate buffer solution (pH 7.4; KH_2PO_4 0.1 M + NaOH 0.1 M; 10 ml) For the determination of living hyphal length, 5 ml of this suspension was mixed with fluorescein diacetate solution (FDA; 5 ml), as in Reference 17, except that the FDA (5 mg) was dissolved in acetone (2 ml).[16] After incubation at room temperature for 5 min., hyphae were collected by filtration onto a gridded Millipore filter (0.45 μm). The length of fluorescing hyphae was determined using an epifluorescent microscope equipped with a blue filter (60 × magnification). Hyphal length was enumerated in 64 squares delineated on the centre of the filter. For the calculation of the living hyphal length, Newman's equation[18] was used:

$$R = (\pi An)/2H \qquad (2)$$

where: R is the length of extra-radicular mycelium evaluated on the 64 fields of the Millipore filter (mm); A is the filter area; n is the number of hyphal intersections on the horizontal lines of the grid of the reticulated ocular; H is the total length of the horizontal lines of the grid.

2.2.3 *Soil ergosterol content*

Soil ergosterol content was evaluated using the simplified method proposed by Eash *et al*[19] with a few modifications. Briefly, soil (5 g) in a centrifuge tube was mixed with methanol (15 ml) and potassium hydroxide solution (40 g $litre^{-1}$ in 95% ethanol; 5 ml), homogenized in a vortex mixer for a few seconds, and sonicated for 1 min. The supernatant was removed and placed in a water bath (80–83 °C) for 15 min, homogenized in a vortex and returned to the water bath for a further 15 min. Tubes were then cooled. The extraction mixture was filtered through Whatman No 1 paper, which was rinsed with water (5 ml) and

methanol (10 ml). The filtrate was transferred to a separatory funnel and extracted with hexane (3×10 ml). The hexane extracts were combined and concentrated to *c*2–3 ml in a rotary vacuum evaporator at 42–43 °C, transferred to a graduated flask, made up to *c*6 ml and then dried using a stream of nitrogen. The residue was dissolved in methanol (4 ml), homogenized, and filtered through a HV Millex 0.45-μm pore size membrane filter. Determination of ergosterol was performed in four replicates for each dose and in four replicate samples spiked with standard ergosterol at 0.2 μg g^{-1} soil. Extracted ergosterol was analysed by HPLC using a Shimadzu LC-10AD pump and a SPD-10AV UV-VIS detector, with a CR7A integrator chromatopac. The wavelength was set to 282 nm. The column was a C18 reversed phase Shim-Pack CLC-ODS (250×4.6 mm $\times 5.0$ μm) operated at room temperature. The mobile phase was methanol. The flow rate was 0.8 ml min^{-1}. A 20-μl injection loop was used. The retention time for ergosterol was approximately 20 min. The HPLC data were quantified by measuring appropriate liquid chromatographic peak heights in the sample chromatograms and then referring to calibration curves prepared with ergosterol standards.

2.2.4 Plate counting of total and cellulolytic fungal colonies

For estimating the number of soil fungi, the dilution plate technique was employed. The fungi were assessed on Martin's medium.[20] The cellulolytic fungi content was determined using cellulose-asparagine-agar.[20] The cellulolytic fungi formed cellulose degradation halos around their colonies. Both fungal counts were performed only at the fifth week after treatment.

2.3 Statistical methods

A complete randomized block design with four replications was used. For all techniques, the absolute measures at each time were converted to relative measures, dividing the observed value by the corresponding observed value for time zero, before the fungicide addition. Expressing all the measurements using the same scale allowed us to compare their variability patterns, among the different chlorothalonil doses, over time. The time variability patterns in the short-term evaluation for microbial biomass C, soil ergosterol content and living hyphal length were evaluated using quadratic or linear models, fitted for each chlorothalonil dose. Key parameters such as intercept, slope or vertex coordinates were estimated for each dose. After that, the effect of dose on such key parameters was evaluated, using regression analysis. For plate counts of total and cellulolytic fungi colonies, no model adequately described variability pattern over time. The effect of chlorothalonil doses on the relative counts at the fifth week was analysed, using non-linear models. The significance of the parameters in these models was evaluated by means of unilateral *t*-tests for contrasts. In the long-term evaluation, non-linear models were also fitted to describe decreases in relative measurements as function of the applied chlorothalonil doses. For the non-linear models, the parameters were estimated by the non-linear least squares method. The modified Gauss–Newton iterative technique,[21] was employed using the SAS® procedure PROCNLIN. The linear and quadratic models were adjusted by maximum likelihood using the SAS procedure PROCMIXED.[22]

2.3.1 Short-term evaluation

2.3.1.1. Microbial biomass C. For microbial biomass C, quadratic models with intercept equal to 100% were adjusted for each dose, in order to describe changes in relative microbial biomass C across time. Some additional parameters, useful to describe changes in relative microbial biomass C, were estimated: minimal microbial biomass C achieved (MB), time to achieve the minimal microbial biomass C (TM) and recuperation rate of the minimal microbial C (RR). MB and TM correspond to the vertex ordinate and abscissa, respectively, from the adjusted model. RR is the velocity of recuperation in relative microbial biomass C after the MB has been achieved. The MB, TM and RR were estimated for each block and fungicide dose. In order to obtain a mean value for each treatment, the four block values were averaged. To quantify effects of fungicide dose on the parameters of the relative microbial biomass C model (MB, TM and RR), linear regressions were adjusted using the MIXED procedure from SAS®.

2.3.1.2 Soil ergosterol content and living hyphal length. Linear models were fitted to describe changes in relative soil ergosterol content and in relative living hyphal length between the first and the fifth week, for each chlorothalonil dose. The slope corresponds to the rate of decrease in soil ergosterol content over time (ERG_VD). The intercept of these models should not be interpreted as ergosterol level at time zero because models were adjusted to take into account only the data from first to fifth week. The soil ergosterol content at the first week (ERG_W1) was estimated instead from the intercept parameter. Regression methods were used to investigate the influence of chlorothalonil doses on ERG_VD and ERG_W1. The statistical procedures used for ergosterol content were also utilized for living hyphal length. The code LHL_VD was used to designate the decrease rate in the living hyphal length and LHL_W1 was utilized to indicate the living hyphal length on the first week.

2.1.1.3 Plate counting of total and cellulolytic fungal colonies. The effect of chlorothalonil concentration on the relative counts of total fungi (RTF) and relative counts of cellulolytic fungi (RCF) at the fifth week was investigated using non-linear regression analysis. Non-linear models were fitted to describe decreases in RTF

or RCF as the applied chlorothalonil doses increases:

$$Y_{ij} = \alpha - \beta[(1 - \exp(-y \,.\, x_{ij})] + \epsilon_{ij} \qquad (3)$$

where Y_{ij} is the observed RTF or RCF corresponding to dose i and block j at five weeks after the fungicide application; α is the mean of the RTF or RCF at dose zero, β is the decrease in those relative measurement between dose zero and the doses for which the curve flattens off, γ is a parameter related to rate of decrease in RTF or RCF as the chlorothalonil dose increases and ϵ_{ij} is the random disturbance associated with each observation Y_{ij}. The difference (α–β) corresponds to the RTF or RCF achieved when the model flattens off. Plots of standardized residuals and coefficients of determination (R^2) were employed to evaluate the adequacy of the negative exponential models.[23]

2.3.2 Long-term evaluation

The effect of chlorothalonil doses in the long-term evaluation of the relative measurements were described by a non-linear model similar to that represented in eqn (3), where Y_{ij} is the relative measurement corresponding to dose i and block j at 16 weeks after chlorothalonil application; α is the mean of the relative measurement corresponding to dose zero. The parameters β, γ, (α–β), as well as the error term ϵ_{ij} have the same interpretation as in eqn (3). R^2 and plots of standarized residuals were also employed to evaluate the model adequacy.

Based on the fitted models, chlorothalonil concentrations corresponding to reductions of 50, 75 and 90% in relative microbial biomass, relative living hyphal length, and relative soil ergosterol content at 16 weeks after fungicide application were estimated.

Using the results of the five-week experiment, the variation in the relative measurements over the period (0–5) and 5–16 weeks was evaluated. This is important information for assessing the influence of the period of evaluation on the comparison between different methods.

3 RESULTS AND DISCUSSION

3.1 Evaluation of short-term (1–5 weeks) tests

The values for soil ergosterol content in the control ranged from 1.28 to 2.00 $\mu g\, g^{-1}$ soil over the period of the experiment, while those in the chorothalonil treatments varied from 0.21 to 0.74 $\mu g\, g^{-1}$ soil. These values, particularly those for the control soil, are very close to values obtained by other authors in ploughed soils.[10,24] The extraction efficiency, based on analysis of soil samples spiked with ergosterol standard, was 86%. On the other hand, the values obtained for living hyphal length, ranging from 1.39 to 6.41 $m\, g^{-1}$ soil in the control, and from 0.61 to 1.90 $m\, g^{-1}$ soil in the chlorothalonil treatments, were lower than those found in other soil types.[25] The microbial biomass C ranged from 215.5 to 333.1 $\mu g\, g^{-1}$ soil in the control and from 126.8 to 247.8 $\mu g\, g^{-1}$ soil in the chlorothalonil treatments (data not shown).

3.1.1 Microbial biomass C

The variability of the microbial biomass C over five weeks showed a clear quadratic pattern (t-test, $P < 0.0001$) for all pesticide doses (Fig 1A). The key parameters for the models, MB, TM and RR, were not affected over the 12–96 $\mu g\, g^{-1}$ dose range (F-test, $P > 0.38$) (Table 1). The overall mean MB value for those doses was 43% smaller than that obtained in the treatment without the fungicide. The TM and RR values were 23% and 67% higher, respectively, than those for the control (t-test, $P < 0.0001$). Although the recuperation rate of the minimal microbial C (RR) was greater in the chlorothalonil treatments, than that in the untreated soil, the achieved relative microbial biomass C at five weeks in those treatments was still 28% smaller than that observed in the control.

Although the soil microbial biomass C values apparently demonstrate that the effect of chlorothalonil treatment is transitory, other factors must be taken into consideration when interpreting these data. The observed initial decline can be attributed to chlorothalonil activity as well as to the micro-organisms adapting to the new growth conditions, as shown also by the decline of the microbial biomass C in the control. Therefore, the recuperation of this biomass C, from the second to the third week after treatment, may be caused, at least partially, by the presence of dead micro-organisms over that period and not due to the transitory effect of the fungicide. The fungitoxic effect of chlorothalonil on the microbial biomass C is demonstrated by the minimum value of the microbial biomass C (MB). The MB of the treatments which received the fungicide was reached about one week after the MB was achieved in the control. The highest recovery rate (RR) of the microbial biomass C in the chlorothalonil-treatmented soils could have been a result of a higher availability of carbon due not only to micro-organisms which died due to non-adaptation to the new environmental conditions, but also to the micro-organisms that were killed as a result of the fungicide application.

3.1.2. Soil ergosterol content

There was a decline in relative soil ergosterol content over the five-week duration of the experiment for all treatments (Fig 1B). During the first week, there was

Table 1. Minimum relative microbial biomass C (MB), relative time to achieve the minimum (TM) and relative recuperation rate (RR) corresponding to each chlorothalonil dose

Dose ($\mu g\, AI\, g^{-1}$)	*MB[a] (%)*	*TM (weeks)*	*RR (%)*
0	70.34	2.21	13.25
12	38.09	2.71	23.07
24	41.21	2.73	21.63
48	44.60	2.69	20.58
96	37.92	2.70	23.03

[a] Expressed as percentage of the respective values before chlorothalonil application.

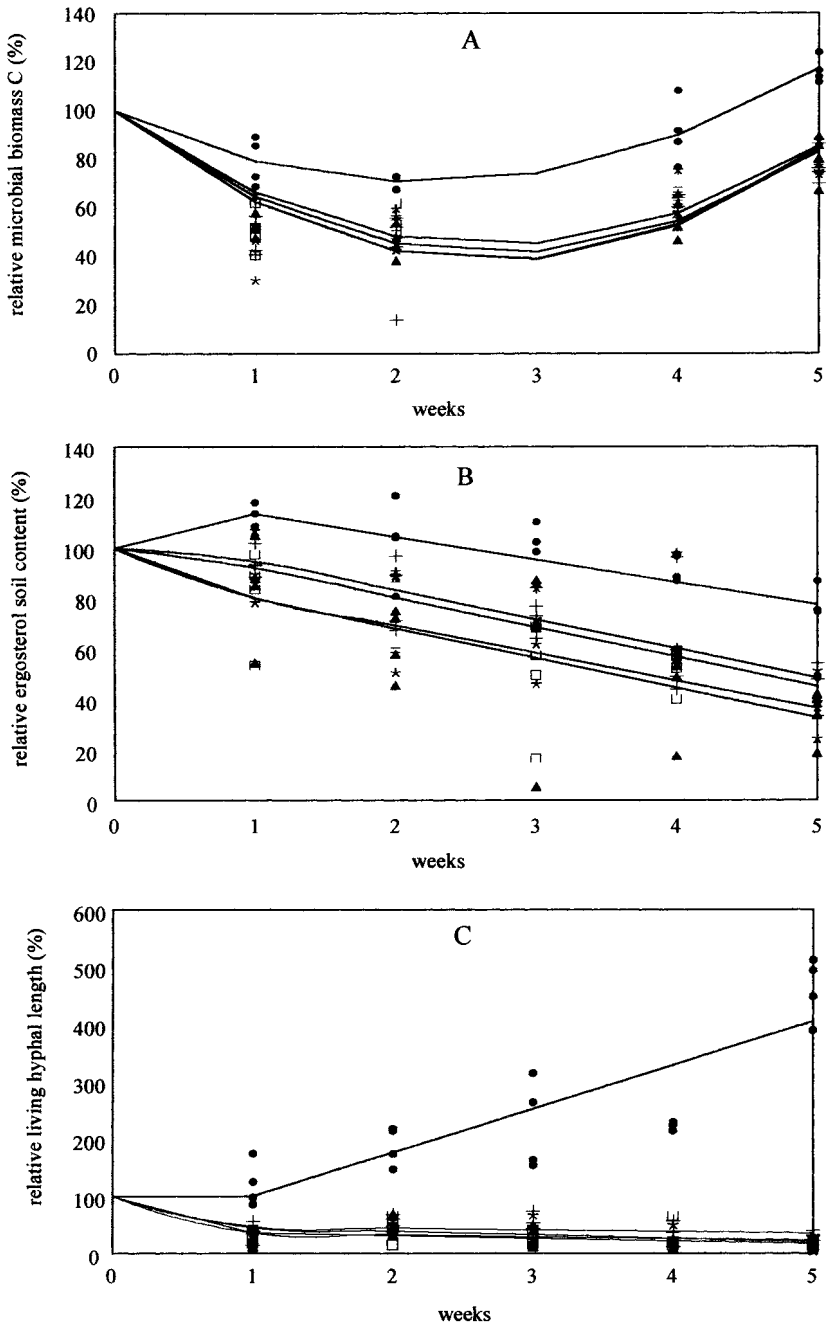

Figure 1. Time variability patterns of relative fungal measurements over five weeks: (A) microbial biomass C, (B) living hyphal length and (C) soil ergosterol content. Doses (●) 0, (+) 12, (*) 24, (□) 48, (▲) 96 μg chlorothalonil g^{-1} soil.

an increase in the relative soil ergosterol content (13.3%) in the untreated soil, but an average decrease of 13.3% with fungicide treatments (Table 2). The ERG_W1 decreased linearly with increasing chlorothalonil dose (*t*-test, $P = 0.073$). No significance in the ERG_VD with time was observed over the range of doses tested (*t*-test, $P = 0.500$). The overall mean ERG_VD for the chlorothalonil treatments was around 28% greater than in the untreated control soil.

Although the soil ergosterol content has been recommended recently as a means of quantifying changes in soil fungal populations,[9] as an indicator of living fungal biomass[24] or as a useful method for assessing pesticide side-effects,[10] this method presents some problems. The ergosterol content, besides varying with the phase of fungal growth,[13] also varies among fungal species.[12] Several studies have demonstrated that soil ergosterol content is significantly greater in older and stationary-phase fungal mycelia than in young or growth-phase mycelia.[13] The decline in the ergosterol content in all treatments in this work could lead to mistaken interpretations with regard to the effect of chlorothalonil on the soil fungal population, if used in isolation from other data. The decrease in the soil ergosterol content in the control suggests that other factors as well as the fungicide were affecting the soil fungal population. It would be difficult to evaluate the contribution of such factors to variations in the soil fungi microflora. The recuperation of the microbial biomass C after two to three weeks, however, could indicate that a large proportion of the soil fungi population was in the initial phase of growth

Dose (μg AI g^{-1})	ERG_W1 (%)	SE[a] (%)	ERG_VD (%)	SE[a] (%)	P-value[b]
0	113.3	7.2	−9.0	2.2	< 0.001
12	94.7	7.2	−11.5	2.2	< 0.001
24	92.0	7.2	−11.7	2.2	< 0.001
48	80.2	7.2	−10.9	2.2	< 0.001
96	79.8	7.2	−11.8	2.2	< 0.001

[a] The standard error was estimated under the assumption of homogeneous variances.
[b] *P*-value corresponding to unilateral *t*-test for the null hypothesis ERG_VD = 0.

Table 2. Relative soil ergosterol contents at the first week (ERG_W1), rate of decrease in relative ergosterol content over time (ERG_VD) and their respective standard errors (SE), for each chlorothalonil dose

and, therefore, with low ergosterol content. The living hyphal length, in the same way, indicated increasing fungal growth in the control with time.

3.1.3 *Living hyphal length*

In contrast to observations with microbial biomass C and soil ergosterol content, the variability pattern of the living hyphal length over five weeks was different in the control soil from those that received fungicide (Fig 1C). In the first week the living hyphal length (LHL-W1) remained constant in the control soil, while in the other treatments an average decrease of 58% was observed (Table 3). Over the five-week period there was an increase of 76% per week in the living hyphal length (*t*-test, $P < 0.001$) in the treatment without chlorothalonil; in the treated soils the LH_VD values were not significantly different (*t*-test, $P > 0.131$), indicating no influence of chlorothalonil dose on the LH_VD.

The living hyphal length, however, was the parameter which supplied the most useful information in relation to the effects of chlorothalonil on the soil fungal population. Effects could be observed visually, but the high data variability, possibly due to deficiencies in methodology, made it difficult to obtain significant differences between doses over the five-week period of the experiment.

3.1.4 *Plate counting of total and cellulolytic fungal colonies*

In untreated soil, counts of total fungi and cellulolytic fungi increased by 51 and 58%, respectively, over the five-week period (Table 4). By contrast, counts in treated soils decreased with increasing dose indicating a negative exponential pattern (Fig 2), with the counts of total and cellulolytic fungi evening out at 62 and 52% of the initial counts, corresponding to the (α–β) parameter of the respective models (Table 4; see eqn (3)).

In comparison to the other methods utilized in this work, the two methods of plate counting were not sufficiently efficient to demonstrate the effect of chlorothalonil, except at the higher doses and then only at 35 days after treatment: for shorter times after treatment the variability in fungal counts was so large that appropriate data interpretation was not possible. These results agree with the general consensus that dilution plating, as commonly used to isolate bacteria, has severe drawbacks in ecological studies of fungi.[26]

The results demonstrated that, at least during five weeks, the use of the microbial biomass C as an isolated factor is not adapted to verify possible adverse effects of chlorothalonil on the soil fungal population, in comparison to soil ergosterol content and living hyphal length. Hart and Brookes[10] also demonstrated, in an experiment conducted over 28 days, that the soil fungal ergosterol content is a more sensitive indicator of side-effects of ergosterol-inhibiting fungicides than measurements of the soil microbial biomass C. However, for the better interpretation of data on ergosterol content, it should be associated with a parameter that indicates growth of the microbial microflora, since its content in soil is closely linked to the fungal growth stage. Although all the data demonstrated that the lowest dose of the fungicide applied to the soil in this work was sufficient to fully inhibit the fungal population, the soil ergosterol content seems to be the more useful parameter for obtaining differentiated results in the fungal population, when different doses are considered, in experiments conducted over a short period.

3.2 Evaluation of long-term tests

All methods used were effective for the determination of the effects of chlorothalonil on soil microflora 16

Concentration (μg AI g^{-1})	LHL_W1 (%)	S.E.[a] (%)	LHL_VD (%)	S.E.[a] (%)	P value[b]
0	100.7	18.5	76.1	5.5	<0.001
12	47.2	18.6	−2.7	5.4	0.619
24	45.4	18.6	−6.1	5.4	0.263
48	36.4	18.6	−3.3	5.4	0.542
96	36.4	18.6	−4.7	5.4	0.385

[a] The standard error was estimated under the assumption of homogeneous variances.
[b] *P*-value corresponding to unilateral *t*-test for the null hypothesis LHL_VD = 0.

Table 3. Relative living hyphal length at the first week (LHL_W1), the rate of increase (dose 0) or decrease (treated soils) (LHL_VD) and their respective standard errors (SE)

Evaluated measurements	Parameter[a]	Calculated Value	Asymptotic P-Value	Asymptotic 95% confidence limits (%) Lower	Upper
Relative total fungi	α	150.6	0.004[b]	126.0	175.1
	β	88.7	<0.0001[c]	55.6	121.9
	γ	4.5	0.017[d]	0.4	8.6
Relative cellulolytic fungi	α	157.8	0.0001[b]	132.9	182.8
	β	105.8	0.001[c]	41.4	170.2
	γ	2.1	0.082[d]	−0.9	5.1

Table 4. Values, derived from eqn (3), for the relative total fungal content and relative cellulolytic fungal content, over increasing doses of fungicide

[a] Derived from eqn (3) in section 2.1.1.3 of the text.
[b] *P*-value corresponding to asymptotic two-tailed *t*-test for the hypothesis $\alpha = 1$.
[c] *P*-value corresponding to asymptotic upper-tailed *t*-test for the hypothesis $\beta = 0$.
[d] *P*-value corresponding to asymptotic upper-tailed *t*-test for the hypothesis $\gamma = 0$.

weeks after the fungicide application. Increasing the dose brought about a decrease of the evaluated parameters, which followed a negative exponential pattern, ie a rapid reduction was caused by increase of the pesticide concentration, followed by a slow reduction, with a tendency to stabilization (Fig 3). The relative decreases of microbial biomass C, caused by fungicide doses 1 to 4, were 38, 50,62 and 70%, respectively (Table 5). The values for soil ergosterol were decreased by 78, 87, 94 and 96%, respectively. However, the decreases were not dependent only on the fungicide addition to the soil, since the average data obtained with the dose zero, α value, (Table 5) showed that, regardless of the chlorothalonil application, the microbial biomass C and soil ergosterol content decreased by around 20 and 61%, respectively, during the 16 weeks of experimentation. Some other factors, therefore, must be implicated in soil microflora growth limitation.

Taking into consideration that such factors also affected the soil micro-organisms in the treatments with the fungicide, the decreases of the microbial biomass C and soil ergosterol content caused by the doses 1 to 4 of the pesticide were, respectively, 18, 30, 42 and 50% and 17, 26, 33 and 35%. The marked decline of ergosterol content, even in the untreated soil, could be explained by the fact that the ergosterol content varies with growth conditions,[9] and with other factors as discussed previously. During the whole experimental period no external sources of carbon were added to the soil, and that might have affected the soil ergosterol content more markedly than the microbial biomass C as a whole. West and Grant[9] have observed that substrate glucose amendment increased or decreased the ergosterol content depending on soil type. Nevertheless, the marked decline of ergosterol content during the experimental period did not limit its capacity to demonstrate the toxic effect of chlorothalonil on the soil fungal population.

In relation to the living hyphal length, the α value, in the untreated soil, remained unaltered from the first to the last evaluation, 16 weeks later. Decreases caused by fungicide doses 1 to 4 were 47, 73, 91 and 96%, respectively (Table 5). Considering the percentages of decrease of the microbial biomass C and soil ergosterol content, as a consequence of fungicide application, those results might demonstrate a high sensitivity of the living hyphal length measurement to the toxic effects of chlorothalonil. However, values of hyphal length obtained at short-term evaluation showed an increase of 360% in relation to the evaluation performed at time zero (Fig 4B). Therefore, living hyphal length also presented decreases not associated with fungicide addition, which demonstrated the importance of the adequate establishment of the evaluation periods in order to permit an efficient data interpretation.

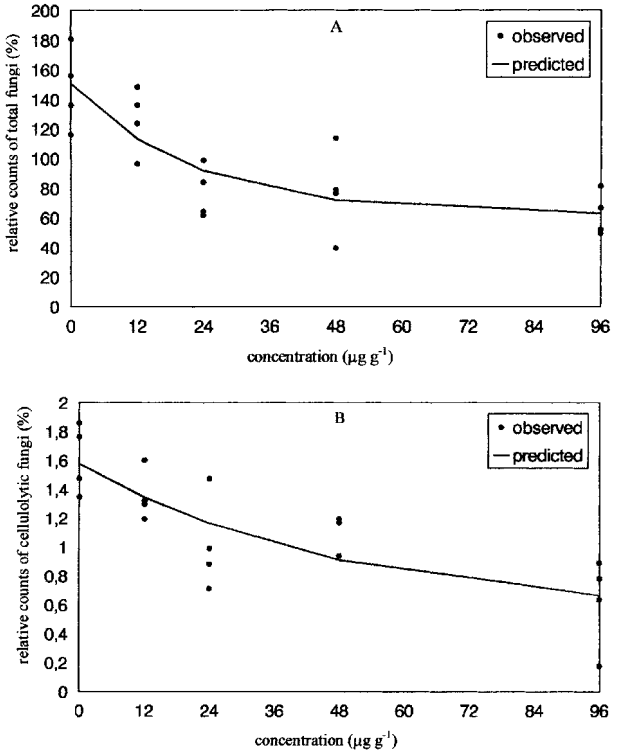

Figure 2. Negative exponential models describing changes in (A) relative counts of total fungi and (B) relative counts of cellulolytic fungi for increasing chlorothalonil concentrations.

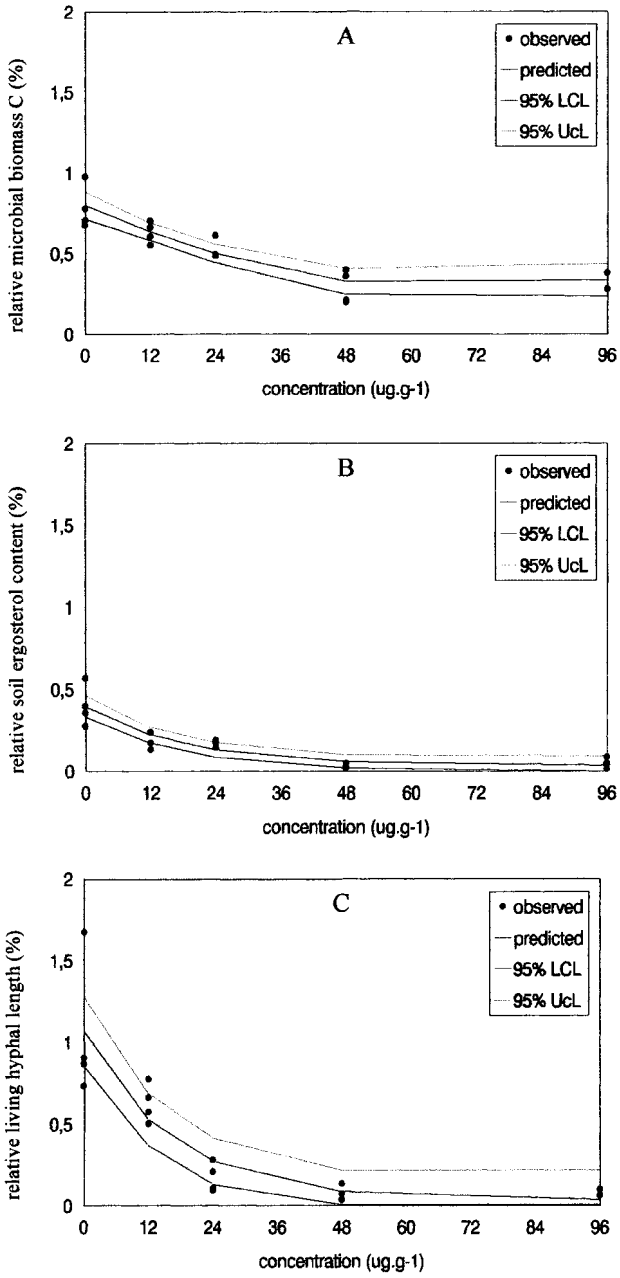

Figure 3. Non-linear models describing changes in (A) relative microbial biomass C, (B) living hyphal length and (C) relative soil ergosterol content for increasing chlorothalonil concentrations. LCL: 95% lower confidence limit; UCL: 95% upper confidence limit.

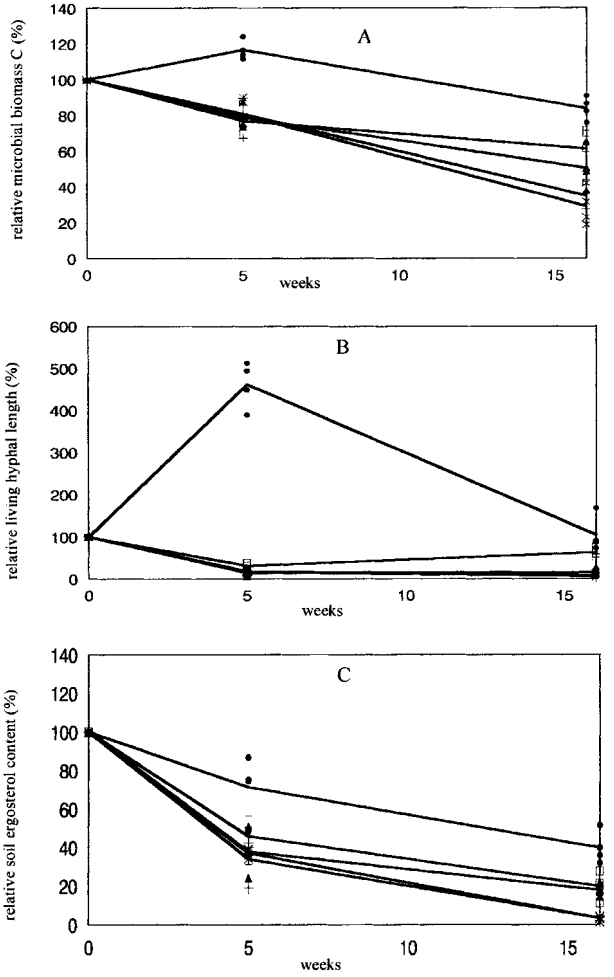

Figure 4. Variation in relative microbiological measurements over the periods 0–5 weeks and 5–16 weeks: (A) microbial biomass C, (B) living hyphal length and (C) soil ergosterol content. Doses: (●) 0, (□) 12, (▲) 24, (×) 48, (+) 96 g chlorothalonil g^{-1} soil.

When the parameters of the negative exponential models (Table 6) were analysed it was shown that the soil ergosterol β value, although 29% and 65% lower than the microbial biomass C and living hyphal length β values, respectively, had a γ value 20% higher than that observed for microbial biomass C, and only 17% lower than that for living hyphal length. This demonstrates that, in spite of the marked decreases in ergosterol content, for non-determined reasons, this method still shows a rapid decrease as a result of increasing the chlorothalonil dose. Comparing the living hyphal length β and γ values, however, it is observed that they were, respectively, on the average, 58% and 25% greater than those obtained for soil ergosterol content and microbial biomass C, respec-

Table 5. Decreases in relative microbial biomass C, relative soil ergosterol content and relative living hyphal length, over increasing fungicide concentrations

Concentration ($\mu g\ g^{-1}$)	Relative microbial biomass C	Asymptotic 95% confidence interval		Relative soil ergosterol content	Asymptotic 95% confidence interval		Relative living hyphal length	Asymptotic 95% confidence interval	
		Lower	Upper		Lower	Upper		Lower	Upper
0	0.80	0.69	0.90	0.39	0.33	0.46	1.07	0.86	1.28
12	0.62	0.55	0.69	0.22	0.17	0.27	0.53	0.37	0.70
24	0.50	0.42	0.58	0.13	0.09	0.18	0.27	0.13	0.41
48	0.38	0.31	0.44	0.06	0.02	0.10	0.09	0.00	0.22
96	0.30	0.20	0.40	0.04	0.00	0.01	0.04	0.00	0.21

Measurement		Estimated parameters values	Asymptotic P-value	Asymptotic 95% confidence limits	
				Lower	Upper
Microbial biomass C	α	0.80	0.0009[b]	0.69	0.90
	β	0.51	<0.0001[c]	0.36	0.67
	γ	0.04	0.4920[d]	0.009	0.06
Soil ergosterol content	α	0.39	<0.0001[b]	0.33	0.46
	β	0.36	<0.0001[c]	0.27	0.44
	γ	0.05	<0.0001[d]	0.02	0.09
Living hyphal length	α	1.07	0.0065[b]	0.06	1.20
	β	1.04	0.0010[c]	0.77	1.30
	γ	0.06	0.0018[d]	0.02	0.01

Table 6. Values for parameters derived from negative exponential models[a] to describe changes in relative microbial biomass C, relative living hyphal length and relative soil ergosterol content as a result of increasing fungicide dose

[a] See Eqn (3) in text.
[b] *P*-value corresponding to asymptotic two-tailed *t*-test for the hypothesis $\alpha = 1$.
[c] *P*-value corresponding to asymptotic upper-tailed *t*-test for the hypothesis $\beta = 0$.
[d] *P*-value corresponding to asymptotic upper-tailed *t*-test for the hypothesis $\gamma = 0$.

tively. These data demonstrate that, despite the sensitivity demonstrated by the soil ergosterol method, the living hyphal length method presents a greater number of parameters which lead to its being considered as the most sensitive of the three methods. The smaller response of the microbial biomass C to chlorothalonil, in relation to the other two measurements, can also be observed by the greater value of the α–β parameter and by the data in Table 7. In this method, in spite of a 20% decrease of the α value, the relative microbial biomass C stabilized before reaching a reduction of 75%, which did not occur with the living hyphal length and soil ergosterol determinations. Doses necessary for 75% and 90% reduction of the soil ergosterol content were lower than those for living hyphal length; it is likely that this was a consequence of the low soil ergosterol content at the end of the sixteenth week. On the other hand, the data under discussion were obtained at time zero and at the end of 16 weeks, which for the case of living hyphal length might not be the most adequate, since its increase at 35 days was not considered (Fig 4B).

The stabilization of fungicide effect, within the studied doses, occurred near 48 μg AI g^{-1} soil for all evaluated measurements. For microbial biomass C, ergosterol content and living hyphal length, differences in effect between the doses of 48 and 96 μg AI g^{-1} soil were 8.6, 5.6 and 0.44%, respectively.

The living hyphal length determination, although providing excellent results in short-duration experiments, does not allow, under those conditions, determination of the minimum dose needed to fully inhibit the soil fungal population. The soil ergosterol content, on the other hand, in contrast with the observations obtained with the microbial biomass C and living hyphal length, showed a similar behaviour in both experimental periods, although the data only showed a tendency to differentiation among doses in the short-term evaluation (*t*-test, $P = 0.07$). Consequently, the soil ergosterol content seems to be the more adequate method for short-duration evaluations (about 30 days) when one wishes to assess the effects of change in fungicide dose, although a larger response amplitude would also demand longer incubation of soil with the pesticide. The absence of a differentiated response of ergosterol content to two doses of two ergosterol-inhibiting fungicides, as obtained by Hart and Brookes,[10] might also be associated with the short duration of soil incubation (28 days) in their work.

Therefore, the results of the present study demonstrate that, to obtain significantly different results between doses, it is necessary to use experiments involving longer than five and up to 16 weeks incubation. On the other hand, it is important to emphasize that some sort of stress must have occurred in the soil during the experimental period. Stresses associated with the presence of chlorothalonil in the soil could intensify its fungitoxicity, thus making the higher doses more prejudicial. Additional studies to examine the influence of different types of stress on the overall effect of chlorothalonil on the fungal population are needed. It was also well established that, in short- or long-duration evaluations, and in comparison

Table 7. Chlorothalonil concentrations corresponding to reductions of 50, 70 and 90% in microbial biomass C, soil ergosterol content and living hyphal length at 16 weeks after chlorothalonil application

Microbial variable	Dose (μg g^{-1} soil) required to give% reduction		
	50%	75%	90%
Microbial biomass C[a]	24.1	–	–
Soil ergosterol content[b]	–	9.5	31.7
Living hyphal length	13.0	25.6	45.1

[a] Relative microbial biomass stabilized before reaching reduction of 75%.
[b] Relative ergosterol reached reduction of 60% even at concentration zero.

to soil ergosterol content and living hyphal length, the microbial biomass C was the least sensitive method for the measurement of chlorothalonil effects, although this parameter has been cited as a indicator of pesticide side-effects.[27,28] The soil ergosterol content, although offering excellent results, still requires further studies relating ergosterol production to environmental conditions to permit more conclusive interpretations. Therefore, the living hyphal length technique has to be regarded as currently the most sensitive method for the determination of the effect of chlorothalonil on soil fungi. However, it is important to point out that, as a consequence of the various problems associated with the living hyphal length technique, this method could be utilized to compare treatments without concern for obtaining actual values for soil fungi population.

The soil physical characteristics (texture, organic matter content) will have also a major influence on the efficiency of hyphal extraction from the soil. This implies that this technique is not suitable for comparative studies of fungicide effects in different soil types, when a suitable control without the fungicide is not available. In this experiment only one soil type was examined, so that consistent comparison between treatments was possible. In comparison to the other techniques utilized, the living hyphal length method was cheaper and faster.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Geraldo Stachetti Rodrigues of Embrapa Environment for supporting this work and for his motivation.

REFERENCES

1 Andrei E, *Compêndio de defensivos agrícolas: guia prático de produtos fitossanitários para uso agrícola*. Andrei, São Paulo, 492 pp 1987.
2 Vincent PG and Sisler HD, Antifungal action of 2,4,5,6-tetrachloroisophthalonitrile. *Physiol Plant* **21**:1249–1251 (1968).
3 Morton JB, Fungi, in *Principles and Applications of Soil Microbiology*, ed by Sylvia DM, Fuhrmann JJ, Hartel PG and Zuberer DA. Prentice-Hall, New Jersey, pp 72–93 (1998).
4 Paul EA and Clark FE, Carbon cycling and soil organic matter, in *Soil Microbiology and Biochemistry*, ed by Paul EA and Clark FE, Academic Press, San Diego, pp 130–155 (1996).
5 Tisdall JM, Fungal hyphae and structural stability of soils *Austral J Soil Res* **29**:729–743 (1991).
6 Habte M, Aziz T and Yuen JE, Residual toxicity of soil-applied chlorothalonil on mycorrhizal symbiosis in *Leucaena leucocephala*. *Plant and Soil* **140**:263–268 (1992).
7 Frankland JC, Dighton J and Boddy I, Methods for studying fungi in soil and forest litter. *Method Microb* **22**:343–404 (1990).
8 Stahl PD, Parkin TB and Eash NS, Sources of error in direct microscopic methods for estimation of fungal biomass in soil. *Soil Biol Biochem* **27**:1091–1097 (1995).
9 West AW and Grant WD, Use of ergosterol, diaminopimelic acid and glucosamine contents of soils to monitor changes in microbial populations. *Soil Biol Biochem* **19**:607–612 (1987).
10 Hart MR and Brookes PC, Effects of two ergosterol-inhibiting fungicides on soil ergosterol and microbial biomass. *Soil Biol Biochem* **28**:885–892 (1996).
11 Gessner MO and Newell SY, Bulk quantitative methods for the examination of eukaryotic organoosmotrophs in plant litter, in *Manual of Environmental Microbiology* ed by Hurst C. ASM Press, Washington. pp 295–308 (1997).
12 Hung BH, Yung KH and Chang ST, The sterol composition of *Volvariella volvacea* and other edible mushrooms. *Mycologia* **77**:959–963 (1985).
13 Newell SY, Miller JD and Fallon RD, Ergosterol content of salt marsh fungi: effect of growth conditions and mycelial age. *Mycologia* **79**:688–695 (1987).
14 Vance ED, Brookes PC and Jenkinson DS, An extraction method for measuring soil microbial biomass C. *Soil Biol Biochem* **19**:703–707 (1987).
15 Melloni R and Cardoso EJBN, Quantificação de micélio extrarradicular de fungos micorrízicos arbusculares em plantas cítricas. I. Método empregado. *Rev Bras Ci Solo*, **23**:53–58 (1999).
16 Nogueira MA and Cardoso FJBN, Produção de micélio externo por fungos micorrízicos arbusculares e crescimento de soja em funcão de doses de fósforo. *Rev Bras Ci. Solo* (1999, in press).
17 Bloem J, Bolhuis PR, Veninga MR and Wieringa J, Microscopic methods for counting bacteria and fungi in soil, in *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press., San Diego, USA. pp 162–173 (1995).
18 Newman EI, A method for estimating the total length of root in a sample. *J. Appl. Ecol* **3**:139–145 (1966).
19 Eash NS, Stahl PD, Parkin TB and Karlen DI, A simplified method for extraction of ergosterol from soil. *Soil Sci Soc Am J* **60**:468–471 (1996).
20 Martin JD, Use of acid rose bengal and streptomycin in the plate method for estimating soil fungi. *Soil Sci* **69**:215–232 (1950).
21 Hartley HO, The modified Gauss–Newton method for the fitting of nonlinear regression functions by least squares. *Technomet* **3**:269–288 (1961).
22 SAS Institute Inc. SAS/STAT® *User's Guide*. Cary NC. pp 1136–1193 (1989).
23 Montgomery DC and Peck EA, Measures of model adequacy, in *Introduction to linear regression analysis*, John Wiley and Sons. New York. pp 57–104 (1992).
24 Stahl PD and Parkin TB, Relationship of soil ergosterol concentration and fungal biomass. *Soil Biol Biochem* **28**:847–955 (1996).
25 Wick B, Kuhne RF and Ulek PLG, Soil microbiological parameters as indicators of soil quality under improved fallow management systems in south-western Nigeria. *Plant and Soil* **202**:97–107 (1998).
26 Tuite J, *Plant Pathological methods: fungi and bacteria*. Burgess Publishing Company, Minneapolis, USA. 239 pp (1969).
27 Vieira RF, Parametros microbiológicos indicadores do efeito do diuron sobre a microflora do solo. *Pesq Agropec Bras* **34**:897–902 (1999).
28 Van der Werf H and Verstraete W, Estimation of microbial biomass by stimulation of respiration, in *Pesticide Effects on Soil Microflora*, ed by Somerville L and Greaves MP, Taylor & Francis, London, UK. pp 147–170 (1987).