EFFECTS OF PESTICIDES ON THE SOIL MICROBIAL BIOMASS AND MICROBIAL ACTIVITY.

by

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ABSTRACT

This thesis describes research investigating the side-effects of pesticides on soil microbial biomass and microbial activity, with particular reference to two recently developed pesticides, a fungicide, epoxiconazole, and a herbicide, quinmerac.

In a dose-response experiment, application of these pesticides to a sandy loam soil, at up to 10 and 20 times field rate, had no significant effect on soil microbial biomass C or ninhydrin-reactive N, over 84 days incubation. There was also no effect on soil respiration, except for the higher rate quinmerac-treated soil, which evolved 13% less CO_2-C than the control.

The rate of mineralisation of epoxiconazole and quinmerac, and their long-term effect on soil respiration, were measured in three contrasting soils: a sandy loam, a silty clay loam, and a clay soil, using ^{14}C-labelled active ingredients. The kinetics of the pesticides' mineralisation were quite different, epoxiconazole being hyperbolic, while quinmerac was sigmoidal. The maximum amount of mineralisation of both pesticides occurred in the silty clay loam soil, which had the lowest microbial biomass content. The mineralisation of the pesticides was increased by the addition of ryegrass, with the greatest effect in the silty clay loam soil, probably because of the large ryegrass C : biomass C ratio. The mineralisation of epoxiconazole was affected by the ryegrass amendment much more than quinmerac. Further additions of the pesticides had no significant effect on soil respiration or pesticide mineralisation.

The mineralisation of epoxiconazole and quinmerac was further investigated in the silty clay loam soil, using samples with different crop management histories, and the effects of ryegrass and glucose amendment. Pesticide mineralisation was shown to be related to the amount of soil microbial biomass, indicating that the difference in mineralisation rate between the three soil types above was not due to differences in their crop management, but innate differences in soil chemistry and microbiology. Ryegrass addition stimulated the mineralisation of epoxiconazole more than quinmerac, while the reverse was true for glucose, indicating that the pesticides were being degraded by two distinct fractions of the microbial biomass.

The effects of long-term cumulative field application of the pesticides benomyl, chlorfenvinphos, aldicarb, triadimefon and glyphosate, on soil microbial biomass and mineralisation of soil organic matter were investigated. The addition of aldicarb consistently increased the microbial biomass, due to its beneficial effect on crop growth, but this effect was not reflected in the rate of organic matter mineralisation. However, in general, the continued application of these pesticides for up to 19 years, at slightly higher than the recommended rates, had very little effect on the soil microbial population.

The effects of epoxiconazole and triadimefon on soil ergosterol content and microbial biomass C were compared in a sandy loam soil. Both pesticides temporarily reduced soil ergosterol by about 30%, while biomass C remained largely unaffected. However, when straw was added to the soils, the inhibition of ergosterol was still evident, as was an inhibitory effect on biomass C. The measurement of soil ergosterol was more sensitive to the pesticide effects than biomass C, and could be a useful test in determining changes in fungal populations.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
CEC	cation exchange capacity
HPLC	high performance liquid chromatography
L.S.D.	least significant difference
me	milliequivalents
o.d.	oven dry
ppm	parts per million
s.d.	standard deviation
s.e.d.	standard error of difference
SIR	substrate-induced respiration
WHC	water holding capacity

To my mother and father

CHAPTER 1

INTRODUCTION

Agriculture in many ways represents the antithesis of natural ecosystems. The essential requirements for economically viable systems of crop production, *e.g.* monoculture of a single cultivar, addition of fertilizers, climate control in glasshouses, remove most natural checks on pathogens, and also create the problem of weeds (*i.e.* plants in the wrong place). The selective breeding of wild plants to produce crops giving greater yields and more palatable food, benefitted not only humans, but also every other species capable of utilising them, which then became pests.

Records of pests date back thousands of years. However, although early writers mentioned pests, they suggested no means of overcoming them. Later classical writers on agriculture did record remedies, many based on "magic", but some which were more practical. For example, Virgil recorded seeing seed dressed with soda and olive oil lees, while Cato mentions the use of banding grease made of olive oil lees, bitumen and sulphur, against vine leaf roller (Ordish, 1976). Over the ensuing centuries, many other chemical methods of pest control were used, including bitumen fumes, rue water, vinegar and tobacco water (refer to Ordish (1976) for an excellent short history on pests and their control).

The widespread use of pesticides did not become really established until the 19th century, and this took many decades to occur. For instance,

a recipe containing sulphur for spray application to fruit was reported in 1803, and sulphur was described by the London Horticultural Society in 1821 as the only specific remedy for treating mildew on peaches. However, the use of sulphur as a pesticide did not become established until 1848, when it was used to combat vine powdery mildew in France. Similarly, copper sulphate was being used by a few farmers as a seed dressing in 1815, but its fungicidal properties were not widely recognised until 1882, when Millardet noticed the effects of Bordeaux mixture, as it became known, on downy mildew on vines (Ordish, 1976; Martin and Woodcock, 1983; Lang and Clutterbuck, 1991).

Around 1890, copper sulphate was discovered to be a selective herbicide as well, killing charlock in cereals. It was later replaced in this role by ferrous sulphate (Lang and Clutterbuck, 1991). The 19th century also saw the widespread introduction of botanicals *i.e.* pesticides derived from plants. These include pyrethrum and nicotine, in 1828, rotenoids, commonly as derris dust, in 1848, and quassia, in 1884 (Martin and Woodcock, 1983). As with sulphur and copper compounds, these also took time to become established, due in part to lack of communication and also a reluctance on the part of farmers to change their ways (Ordish, 1976). However, the three main groups of pesticides; herbicides, insecticides and fungicides, were all well established by the end of the 19th century.

During the early 20th century, mainly inorganic pesticides were introduced, including sulphur derivatives, arsenicals, and compounds of lead, copper and mercury. The first synthetic pesticide, however, the

insecticide potassium dinitro-2-cresylate, was marketed in Germany in 1892 (Lang and Clutterbuck, 1991). Synthetic pesticides began to come on the market in force in the 1930's, for example nitrophenols in 1932, and dithiocarbamates in 1934, but it was the advent of World War II that caused pesticide research to take off, stimulating three major discoveries - DDT, organo-phosphorus insecticides and phenoxyacetic herbicides. Many of the early generations of pesticides were discovered by accident, or by the systematic examination of the properties of compounds originally synthesized for other purposes. For example, Bordeaux mixture was originally used to deter grape thieves, organo-phosphorus insecticides were developed from nerve gas compounds, and dithiocarbamates were developed for use as vulcanizing agents for the rubber industry (Dickinson and Lucas, 1982; Lang and Clutterbuck, 1991).

Following World War II, the pesticide industry grew and prospered. Between 1945 and 1950, 20 companies entered the market and 28 new pesticides were produced. By 1960, 50 companies had joined the field, and 145 new compounds, and by 1970, 500 pesticides had been introduced and 75 companies were screening chemicals for pesticidal activity. As more pesticides were introduced, however, the rate of discovery of new compounds fell markedly. For example, in 1956 it was estimated that it was necessary to screen about 1,800 compounds to find one new product, whereas 25 years later the ratio was estimated to be around 15,000 to 1 (Dickinson and Lucas, 1982; Lang and Clutterbuck, 1991). Currently there are over 400 active ingredients approved for use in the UK, and a similar

number in the EC, while in the USA there are over 1,200 basic active ingredients, formulated into more than 30,000 products and brands (Hurst *et al.*, 1991).

During the 1970s demand for agrochemicals was strong, with the world market averaging around 7.6% real growth per annum. During the 1980s the real growth of the market fell to about 3.1% per annum, and is now in decline. The world end-user agrochemical market value peaked in 1990 at about $27.35 billion, while in 1992 it was valued at $25.2 billion. This fall is mostly due to a decline in the West European and ex-Soviet Union markets (McDougall and Phillips, 1993). West Europe remains the second largest market (Table 1-1), while the split of the market between product types has remained fairly constant (Table 1-2).

The decline in sales in the West European market during the 1990s was mainly due to poor weather and latterly to reforms in the Common Agricultural Policy, such as the introduction of set aside schemes and cuts in the cereal intervention price (McDougall and Phillips, 1993). Patterns of pesticide use are also changing. In the UK between 1982 and 1988, the volume of pesticide use in arable crops declined, while the area treated increased. Usage, measured by area treated, increased by 17% from 19.7 million ha in 1982 to 23.1 million ha in 1988. By weight, pesticide usage on cereals decreased by 6% during the same period (Hurst *et al.*, 1991).

The current extent of the damage caused by pests is very great. The International Group of National Associations of Pesticide Manufacturers (GIFAP) has stated that, "even with modern cultural

Table 1-1: 1992 world pesticide sales by region

North America	29.2%
West Europe	26.7%
Far East	24.4%
Latin America	9.4%
East Europe	4.6%
Rest of world	5.6%

Source: McDougall and Phillips (1993)

Table 1-2: World agrochemical sales by product type ($ million)

	1990	1991	1992
Herbicides	12034	11792	11340
Insecticides	7932	7772	7560
Fungicides	5743	5628	4788
Others	1641	1608	1512
Total	27350	26800	25200

Source: Watkins (1993)

techniques, at least 30% of the world's potential crop production is lost each year. Crop losses would be doubled if existing pesticide uses were abandoned". The British Agrochemical Association estimates that as much as 45% of world food potential is lost to pests – 30% pre- and 15% post-harvest (Hurst *et al.*, 1991). With such large losses, the massive growth of the pesticide manufacturing industry and market is hardly surprising, and although it has currently stagnated, renewed growth in the longer term is expected (McDougall and Phillips, 1993).

As the pesticide industry grew in the post-war period, there was also increasing concern over the possible impact of pesticides on non-target organisms, most famously expressed in 1962 by Rachel Carson in her book *Silent Spring*. This was by no means the first publication addressing this problem, and there were already many papers and reviews in the literature at this time, solely concerned with side-effects on soil micro-organisms *e.g.* Smith *et al.* (1945), Fletcher and Bollen (1954), Fletcher (1960), and Bollen (1961).

Some pesticides act on biochemical processes that are common to many animals, plants and micro-organisms, and thus are a greater hazard to non-target organisms (Moorman, 1989). Many pesticides are applied directly to soil, while of those applied to crop foliage, a large percentage will also enter the soil. For example, in a field experiment where the fungicide propiconazole was sprayed onto winter wheat, 15–45% of the chemical was directly deposited on the soil surface, depending on the time of application (Elmholt, 1992). It has been estimated that, often, less than

0.1% of pesticides applied to crops actually reaches the target organisms (Pimental and Levitan, 1986). Further additions of pesticides to soil may occur following rainfall or incorporation of crop residues, so that most of the pesticide will eventually enter the soil environment. As soil micro-organisms are of prime importance in the cycling of nutrients and hence to soil fertility, the concern over possible side-effects of pesticides on them is obviously well-founded.

This has led to a profusion of papers on various aspects of this subject, of at least 1,540 between 1938 and 1983 (Domsch, 1984), and numerous review articles *e.g.* Parr (1974), Anderson (1978), Wainwright (1978), Simon-Sylvestre and Fournier (1979), Goring and Laskowski (1982) and Moorman (1989). These have covered the effects of many, but by no means all, available pesticides on many parameters, including microbial activities such as soil respiration, ammonification, nitrification, nitrogen fixation and enzyme activities, and microbial populations such as cellulolytic organisms, thermophiles, acidophiles, actinomycetes and algae.

Despite the enormous amount of data produced in these reports, there is still much uncertainty about the potential risk of pesticide side-effects (Domsch, 1984). There are a number of reasons for this, for example, many responses were only achieved through the application of unrealistically high rates of pesticide (Parr, 1974), while conflicting results have been reported of the effects of the same pesticide on the same parameter (Parr, 1974; Anderson, 1978; Greaves, 1987). Many older, and some not so old, papers include the use of *in vitro* culturing methods to

enumerate microbial populations, where such methods are now generally considered to be severely limited in their usefulness. There are also areas of research that have not been adequately explored, such as chronic effects of relatively low levels of pesticide application (Parr, 1974; Biederbeck *et al.*, 1987), and possible effects of pesticide degradation products (Camper, 1991). It is somewhat disquieting that, according to Domsch (1991), of 577 registered pesticides only 24% had been "sufficiently" investigated with regard to side-effects on soil micro-organisms.

There is a further problem, which is that many of the published reports fail to evaluate or interpret their results with regard to the relationship of soil microbial activity and soil fertility. Given the difficulties involved, this is understandable. It is also crucial that any artificially-induced effects are considered in the context of effects induced by natural stresses, such as extremes of water potential, pH, temperature and physical disturbance of the soil (Greaves and Malkomes, 1980; Cook and Greaves, 1987).

It was with this in mind that Domsch *et al.* (1983) proposed a scheme for ecologically assessing pesticide side-effects on soil micro-organisms. According to this scheme, almost all microbial responses to biocidal chemicals could be described by four different, reversible or irreversible reactions. The effects are considered in two ways, by magnitude and by duration of the response, with the latter being of greater importance from an ecological point of view.

In assessing ecological data from many publications, Domsch *et al.*

(1983) concluded that depressions of up to 99% are likely to occur under natural conditions. After considering doubling times of microbial populations observed in soil environments, they concluded that for reversible responses, a delay in recovery less than 30 days was negligible (*i.e.* of no ecological significance), between 31 and 60 days was tolerable, and over 60 days was critical. For irreversible effects, the same three categories are used, but which category a response should fall into is a function of the size of the depression and the length of the monitoring period. Hence, after 30 days only a depression of 99% or greater would be considered critical, while after 90 days a depression of 70% would fall into this category.

By applying these criteria to data on pesticide side-effects in the literature, Domsch *et al.* (1983) concluded that, for reversible effects, 89% of all cases were negligible, 9% tolerable and only 2% were critical. Similarly, in a partial examination of irreversible effects, 38% were negligible, 55% tolerable and 7% critical. While this scheme goes a long way in improving the objective assessment of pesticide side-effects, the authors admit that there is room for refinement.

The difficulties of selecting adequate criteria for measuring pesticide effects on soil micro-organisms and their activities result mainly from fundamental problems of soil microbiology (Greaves and Malkomes, 1980). Thus, improving our understanding of the significance of pesticide side-effects largely depends on improving our understanding of soil microbiology, and therefore partly on the development and improvement

of new methods and techniques to measure the effects.

One set of techniques which has mostly been developed relatively recently are measurements of the total soil microbial biomass. This has been defined as the living part of the soil organic matter, excluding organisms larger than about 5×10^3 μm^3 and plant roots (Jenkinson and Ladd, 1981). Although it accounts for only around 1-3% of the soil organic carbon in most soils, the soil microbial biomass is both the agent of biochemical change in soil and a repository of plant nutrients that is more labile than the bulk of the soil organic matter (Jenkinson, 1988b).

The composition of the soil microbial biomass varies depending on soil characteristics, particularly pH and moisture content, but fungi and bacteria are usually the dominating groups (Fig. 1-1). The biomass can also be divided into zymogenous and autochthonous populations. The former, also known as 'K' strategists, represent opportunist micro-organisms which, upon exposure to fresh substrate, undergo rapid growth and division, followed by a period of dormancy until the next input of substrate is encountered. The autochthonous population (or 'r' strategists) consists of micro-organisms with a low but steady level of activity, probably utilising humified organic matter (Harris, 1988a; Jenkinson, 1988b).

The size of the soil microbial biomass is actually quite large. For example, the plough layer of the unmanured plot of the Broadbalk Continuous Wheat Experiment at Rothamsted contains about 500 kg microbial carbon ha^{-1}, equivalent to approximately 5 tonnes of living cells (Jenkinson and Ladd, 1981). This large population (predominantly

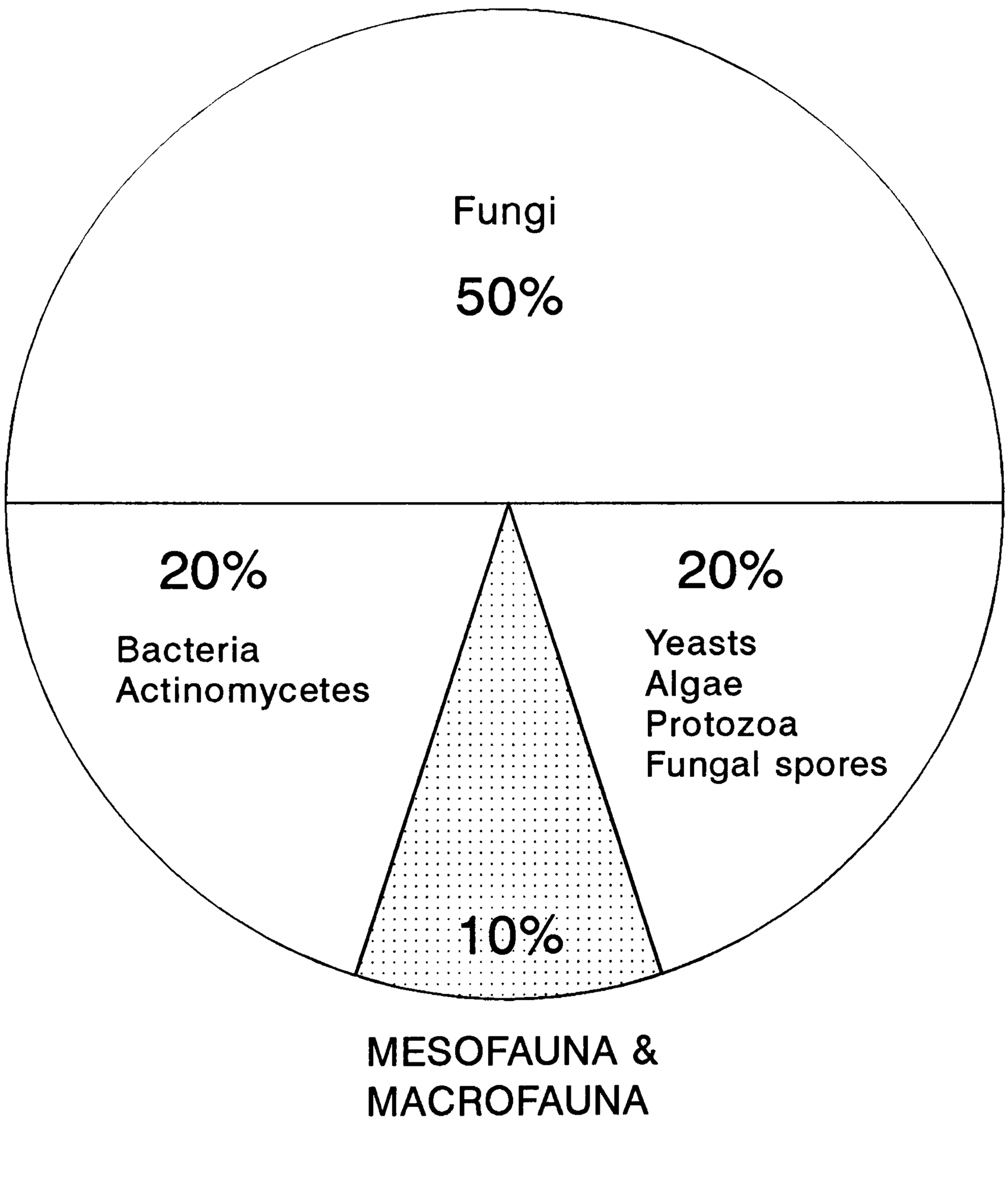


Figure 1-1: The approximate composition of the soil microbial biomass (source: *New Scientist* **96** No. 1334).

chemoheterotrophic) is maintained by a very small annual carbon input of about 1.2 t total C ha^{-1}, little more than twice the total weight of biomass present. The soil microbial biomass therefore has many features typical of a dormant, or resting population, including a very slow respiration rate, long mean cell division time and a long turnover time (Brookes *et al.*, 1990).

However, paradoxically, the biomass maintains, in moist soils, an ATP concentration of about 10–11 μmol ATP g^{-1} biomass C and an adenylate energy charge (AEC), the proportion of ATP to ADP and AMP, of 0.8–0.95, both of which are characteristic of exponentially-growing micro-organisms *in vitro*. This high biomass ATP concentration has been found to be remarkably constant in moist soils varying widely in texture, organic matter content and pH, and in soil amended and unamended with straw (Brookes *et al.*, 1990; Ocio and Brookes, 1990a). The means by which soil micro-organisms maintain such a high ATP concentration and AEC under starvation conditions are not yet understood, but this helps to explain their ability to respond immediately to additions of readily available substrates (Lethbridge and Lynch, 1987).

A range of methods for measuring the size of the microbial biomass have been developed over the last 15 years or so, most of which have been reviewed by Jenkinson and Ladd (1981) and Jenkinson (1988b). In brief, the main methods are:

(i) direct microscopy (*e.g.* Jenkinson *et al.*, 1976; Williamson and Johnson, 1990);

(ii) the fumigation-incubation method (Jenkinson and Powlson, 1976);

(iii) the fumigation-extraction method (Vance *et al.*, 1987; Wu *et al.*, 1990)

(iv) the substrate-induced respiration method (Anderson and Domsch, 1978);

(v) measurement of microbial ATP (*e.g.* Jenkinson and Oades, 1979; Tate and Jenkinson, 1982).

All these methods have been subject to modification and improvement, but nevertheless still have disadvantages as well as advantages.

Of the above methods, the fumigation-extraction method is the most versatile, as it can be used to measure several microbial elements, including biomass carbon, nitrogen and phosphorus, and can be used in a number of situations where fumigation-incubation does not work *e.g.* in the presence of decomposing substrates, in acidic soils and in waterlogged soils (Vance *et al.*, 1987; Inubushi *et al.*, 1991). Also, with the introduction of automated procedures for measuring soluble organic carbon (Chaussod *et al.*, 1988; Wu *et al.*, 1990), large numbers of extraction samples can be processed in a short space of time.

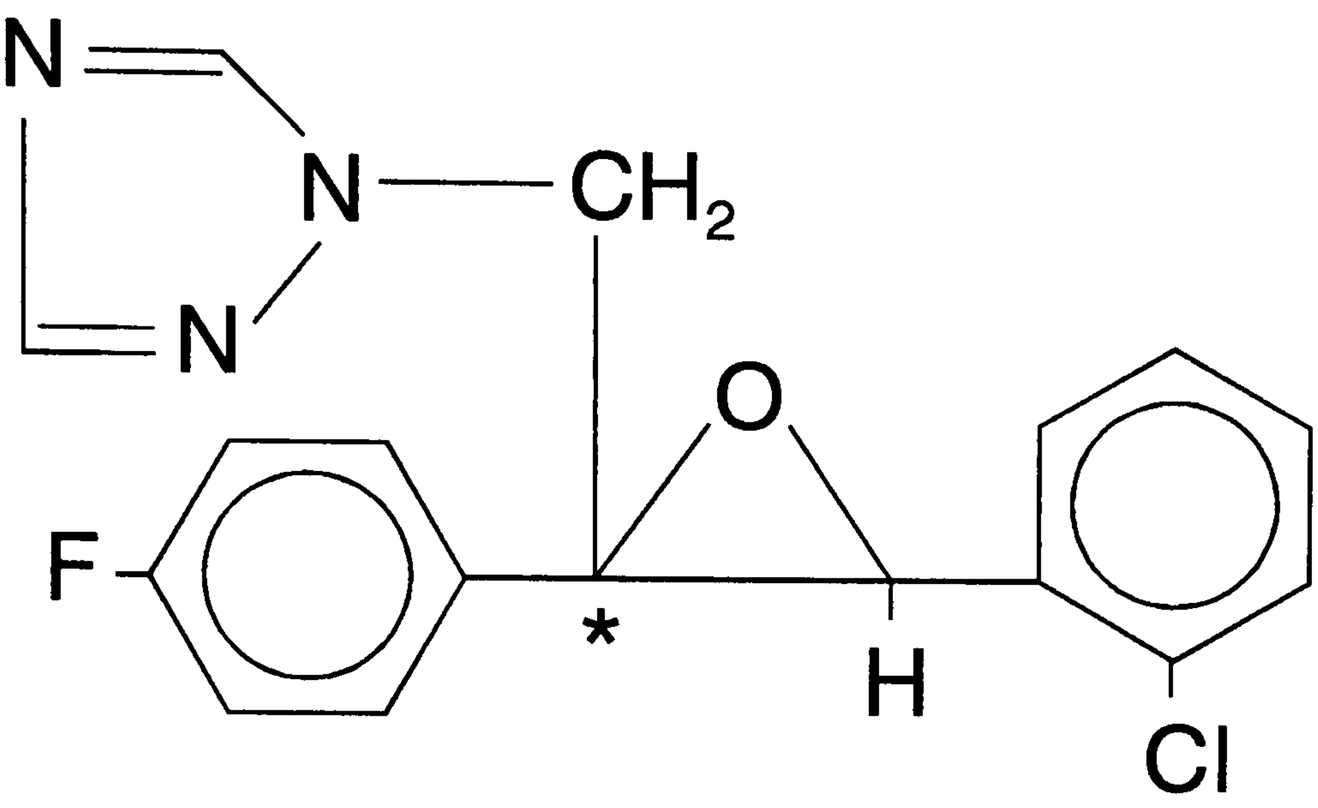
In most soils that are at or near steady-state conditions (*i.e.* inputs are equal to outputs), there is a reasonably close linear relationship between amounts of microbial biomass C and amounts of soil organic C (Anderson and Domsch, 1989). The soil microbial biomass increases or decreases in response to changes in soil management far more rapidly than soil organic matter as a whole. Biomass measurements can therefore

provide an early indication of changes in total soil organic matter following, for example, long-term straw incorporation (Powlson *et al.*, 1987). It has been shown that the linear relationship between biomass C and soil organic C does not hold in soils which have been contaminated with heavy metals. Thus, the ratio between these two parameters may be used as an indicator of changes in soil conditions caused by chemical pollution (Brookes and McGrath, 1984; Chander, 1991). As yet, there have been relatively few publications on pesticide side-effects which have included the use of this method, but they are gradually becoming more frequent (*e.g.* Fournier *et al.*, 1992; Harden *et al.*, 1993).

With the competitiveness of the global pesticide market, and problems with pesticide-resistance in pathogens and weeds, particularly with systemic compounds, new chemicals with pesticidal activity are continually being sought. Modern pesticides tend to be applied at much lower doses than older compounds, but this does not mean they will be less harmful to non-target organisms, as environmental risk arises from dose and activity, not dose alone (Greaves, 1987). There is therefore no room for complacency in testing new compounds as they are developed. Also, if the assertion of Domsch (1991) above is correct, there are many compounds currently in use which remain to be adequately assessed.

The main aim of this study was to investigate some aspects of the potential side-effects on the soil microbial biomass of two new pesticides developed by BASF Aktiengesellschaft, a fungicide, epoxiconazole, and a herbicide, quinmerac (Fig. 1-2a and b). Epoxiconazole is a triazole

(a)



(b)

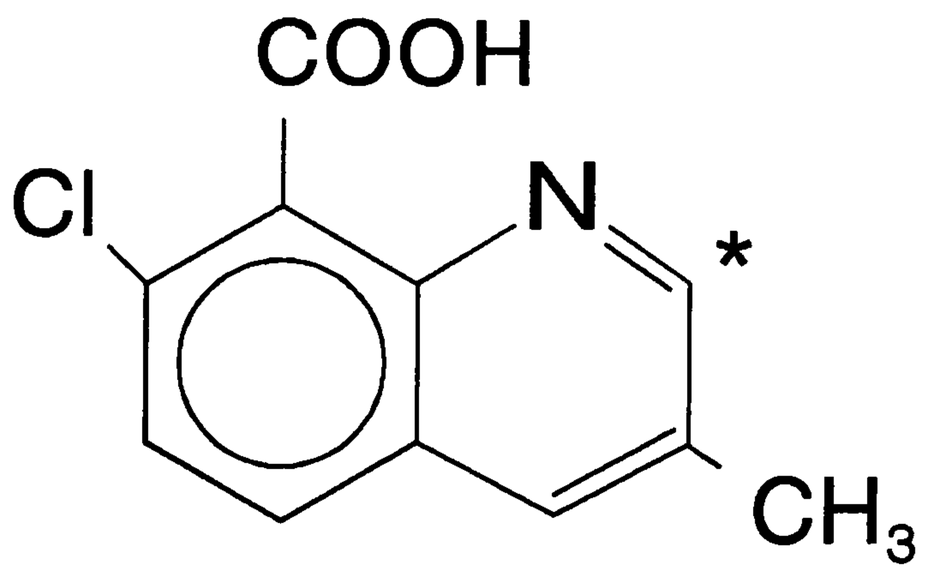


Figure 1-2: The structural formulae of (a) epoxiconazole (rel-(2R,3S)-3-(2-chlorophenyl)-2-(4-fluorophenyl)-2-[(1H-1,2,4-triazol-1-yl)methyl]-2-oxirane), and (b) quinmerac (7-chloro-3-methylquinoline-8-carboxylic acid). * denotes position of ^{14}C label, when applicable.

systemic and contact fungicide to be used mainly on cereals, to control rusts and several other pathogens (*e.g. Septoria nodorum, Rhynchosporium* spp., *Fusarium* spp.), while quinmerac is a quinolinecarboxylic acid herbicide, active against *Galium aparine*, *Veronica* spp. and other weeds, to be used on cereals, oilseed rape and sugar beet. Quinmerac has an acid pK_a of 4.31, and a K_{ow} (pH 7) of 0.9, and is degraded to two main metabolites, a hydroxylated and a dicarboxylic acid form. Information concerning epoxiconazole is currently confidential, neither is information on effects of either pesticide on specific non-target organisms available.

In Chapter 2, the general methodology employed in this thesis is described. Chapter 3 describes a simple dose-response experiment on epoxiconazole and quinmerac in a sandy loam soil. Chapter 4 investigates the long-term effects and mineralisation of these two chemicals in three contrasting soils. In Chapter 5 the mineralisation of the two pesticides is further investigated, following the addition of substrates to a soil to enhance microbial activity. Chapter 6 describes results of laboratory experiments conducted on soil taken from a long-term field experiment, where five different pesticides, unrelated to the BASF chemicals, had been applied annually in all combinations for up to 19 years. In Chapter 7 the side-effects of the mode of action of epoxiconazole on soil micro-organisms are examined in detail, and compared with those of an established triazole fungicide, triadimefon. In Chapter 8 the significance of pesticide side-effects in the light of the research presented in this thesis is discussed, and likely areas for further work indicated.

CHAPTER 2

GENERAL MATERIALS AND METHODOLOGY

2.1 ROUTINE EXPERIMENTAL METHODS

2.1.1 SOIL SAMPLING AND SOIL PRETREATMENT

Soils were sampled from 0–10 cm depth using a 5 cm diameter Dutch auger. After collection, the soils were stored at 5°C overnight, in loosely-tied polythene bags. Within 24 hours of collection, the soils were spread out on plastic sheeting and discrete pieces of plant material, stones and visible soil fauna (earthworms, spiders, larvae *etc.*) removed. The soils were then sieved (<2 mm), or if necessary, first allowed to dry partially, the soil being turned frequently to ensure that no part of it became air-dry. After sieving, any further discrete pieces of non-soil material still present were removed. If not used immediately, the soils were stored in polythene bags loosely-tied with an elastic band, at 5°C in the dark. Soils were usually used within 2 months of collection.

Before use, the sieved soils were adjusted to 40% of full water holding capacity (WHC), determined volumetrically using glass funnels, by slowly applying distilled water with an ordinary hand sprayer, and pre-incubated for 7–10 days, over soda lime and water, in air-tight steel slip-lid drums (dia. 35.5 cm, height 61 cm – Percy Metal Co. Ltd., Wembley, Middlesex) at 25°C. This period of pre-incubation is necessary to allow uniform wetting of the soil, and to give time for the effects of disturbance on the microbial biomass to subside (Jenkinson, 1988). A portion of the

soils was then air-dried for chemical analyses, at either <2 mm or finely ground (<160 μm) in an agate ball-mill.

Further details on the collection and preparation of soil, adapted from the laboratory manual of the microbial biomass group of the Soil Science Department, Rothamsted Experimental Station, are given in Appendix 1.

2.1.2 SOIL INCUBATION

After the pre-incubation period, sub-samples of soil (50 g on oven-dry (o.d.) basis – 105°C, 24 h) were weighed into 100 ml glass jars of known weight. After the appropriate treatment was applied, these were then placed in 1 l brown-glass jars, stoppered with rubber bungs, together with a Trident vial containing 20 ml 1.0 M NaOH solution and 10 ml free distilled water. The soil samples were incubated under these conditions for the duration of the experiment, with the vials of NaOH being periodically replaced as necessary.

N.B. All results are expressed on a soil oven-dry weight basis, as the mean of three replicates, unless specifically stated otherwise.

2.1.3 PESTICIDE TREATMENT

The pesticides epoxiconazole and quinmerac were added to the soils as the formulated products, being an emulsifiable concentrate (125 g l^{-1}) and a wettable powder (50% w/w), respectively. The carrier materials were of unknown composition. The formulated products were diluted with

distilled water, and were adjusted so that solutions with different concentrations of active ingredient all contained approximately the same amount of carrier material. The solutions were prepared so that the required pesticide doses were contained in a small volume, typically 1–2.25 ml. The recommended field rate application of epoxiconazole was 0.25 kg ha^{-1}, while that of quinmerac varies from 0.25–1.0 kg ha^{-1} depending on the crop. The pesticides were therefore applied at concentrations reflecting these rates, usually at 0, 1 and 10 times field rate (approximately), as μg active ingredient g^{-1} soil. However, the recommended application rate of epoxiconazole was subsequently halved (R. Gerhardt, pers. com.), and so this fungicide was actually applied at 0, 2 and 20× field rate in this work. The higher field rate was adopted for quinmerac.

The pesticide solutions were added to the soils using glass syringes, with 51 mm hypodermic needles. The needle was slowly inserted into the soil to the bottom of the jar while the plunger was depressed very gradually. The needle was withdrawn and the procedure repeated at several points around the soil, until the full volume had been added. This procedure was adopted in an attempt to distribute the pesticides as evenly as possible throughout the soil sample, in a consistent manner. Separate syringes were used for the various pesticide solutions, and each was washed out twice with the particular solution between soil samples. The hypodermic needles were changed periodically, as they tended to become blocked after a while. Distilled water, of varying volume, was similarly

added to the soils, to bring them to 50% WHC.

2.2 ANALYTICAL METHODS

2.2.1 SOIL MICROBIAL BIOMASS C

Soil microbial biomass carbon was measured by the fumigation-extraction method (Vance *et al.*, 1987). In this technique, moist soil portions, each equivalent to 25 g dry soil, were fumigated in a desiccator lined with wet tissue paper to maintain humidity, containing about 40 ml ethanol-free chloroform ($CHCl_3$) (Jenkinson and Powlson, 1976) in a 50 ml beaker with a few anti-bumping granules. A small vial of soda lime was also placed in the desiccator. Latterly, $CHCl_3$ stabilised with 25 ppm amylene (2-methyl-2-butene) (Fisons, Loughborough, UK) was used, which has been shown to give identical results to other forms of ethanol-free $CHCl_3$ (Mueller *et al.*, 1992). The desiccator was evacuated using a water-pump until the $CHCl_3$ boiled vigorously for 2 min., the valve was then closed and the desiccator placed in the dark at 25°C. After 24 h, the soils were placed in a clean, empty desiccator and the $CHCl_3$ removed by repeated evacuation, firstly with a water pump and then with an electrical vacuum pump. The soils were extracted after being transferred to 350 ml plastic bottles, 100 ml 0.5 M K_2SO_4 added, and the bottles shaken on a reciprocal shaker (200 strokes $min.^{-1}$) for 30 min. The suspensions were then filtered (Whatman No. 42) into 125 ml plastic bottles, and stored at -15°C until analysis. A set of non-fumigated soils were similarly extracted at the beginning of the 24 h fumigation period. Soils taken from

incubation experiments were removed from their 100 ml jars and mixed thoroughly before being split into halves of 25 g (o.d. equivalent) and treated as above.

Organic C in the soil extracts was measured by an automated procedure, using a Dohrmann DC 80 carbon analyser (Wu *et al.*, 1990). In this procedure, 10 ml soil extract (or blank solution – 100 ml 0.5 M K_2SO_4 filtered as above) was mixed with 10 ml sodium hexametaphosphate solution (SLR grade, 5% w/v, adjusted to pH 2 with conc. H_3PO_4). The mixture was sparged with O_2 to remove dissolved CO_2, then 1 ml of the solution automatically injected into the machine, and the organic C oxidized to CO_2 by potassium persulphate solution (2% w/v in HPLC water, acidified as above) in the presence of ultra-violet light. The CO_2 produced was then measured by infra-red analysis, and the organic C content of the solutions automatically printed out in ppm. The analyses were calibrated against a standard curve produced from potassium phthalate solutions of 0–80 ppm C in 0.5 M K_2SO_4, diluted 1:1 as above. Samples with C contents >40 ppm were suitably diluted with acidified HPLC water to fall within the calibration range. When this was necessary, a set of blank solutions were similarly diluted also.

Soil microbial biomass carbon (B_C) was calculated from:

$$B_C = 2.22\ E_C \qquad \text{(Wu } \textit{et al.}\text{, 1990)}$$

where E_C = [(organic carbon extracted from fumigated soil) – (organic carbon extracted from non-fumigated soil)]

expressed as μg C g^{-1} soil.

2.2.2 SOIL MICROBIAL BIOMASS NINHYDRIN-REACTIVE N

Potassium sulphate-extractable ninhydrin-reactive N (essentially ammonium-N and α-amino N) was measured using the method of Amato and Ladd (1988), as modified by Joergensen and Brookes (1990). Briefly, 0.5 M K_2SO_4 extracts or blanks (0.75 ml) and 0.2 M citric acid buffer, pH 5.0 (1.75 ml) were placed in 20 ml test tubes. Ninhydrin reagent [ninhydrin (4 g) and hydrindantin (0.6 g), dissolved in 150 ml dimethylsulphoxide to which was then added 50 ml 4 M lithium acetate buffer, pH 5.2 (Pierce, Rockford, Ill.)] (1.25 ml) was slowly added and the contents thoroughly mixed using a vortexer, and the tubes then heated in a vigorously boiling water bath for 25 min. After cooling to room temperature in the dark, 1:1 ethanol-water (4.5 ml) was added, the contents mixed as before and the absorbance at 570 nm measured against a distilled water blank, with a Lambda 3 UV/VIS spectrophotometer (Perkin Elmer, Beaconsfield, UK). Absorbances were converted to μg ninhydrin-N g^{-1} soil by reference to a standard curve of 0.5 M K_2SO_4 solutions of D-leucine within the range 0–500 μM N. Biomass ninhydrin-N (B_{NIN}) was calculated from:

B_{NIN} = [(ninhydrin-N extracted from fumigated soil) − (ninhydrin-N extracted from non-fumigated soil)].

2.2.3 CO_2 EVOLUTION

Carbon dioxide C trapped in 1 M NaOH was determined by titration of 5 ml aliquots from pH 8.3 to 3.7 with standardised 0.5 M HCl

(after Tinsley *et al.*, 1951), using an autotitration system (Radiometer Copenhagen, Denmark). The CO_2-C evolved from the soil was calculated from :

$$CO_2\text{-C } (\mu g\ g^{-1}\ soil) = \frac{(V_1 - V_2) \times M \times (20/5) \times 12 \times 1000)}{W}$$

where V_1 and V_2 are the volumes of HCl (ml) used to titrate the 5 ml aliquots of NaOH from the soil sample and blank (1 L jar with no soil), respectively, *M* is the molarity of the standardised HCl, and W is the weight of oven dry soil.

2.2.4 ^{14}C ANALYSES

2.2.4.1 *Microbial biomass ^{14}C and $^{14}CO_2$ evolution*

The amount of ^{14}C-labelled organic C in the 0.5 M K_2SO_4 soil extracts was determined by mixing 0.75 ml extract with 2 ml distilled water and 15 ml Ultima Gold scintillation cocktail (Canberra Packard, Pangbourne, UK), in duplicate. Each sample was then counted for 10 min. in a Canberra Packard Tri-carb 2500TR scintillation counter. Measurements were corrected for background radiation by subtracting the mean count of the blank 0.5 M K_2SO_4 solutions, and for luminescence automatically by the counter. The counts per min. (CPM) detected were converted to disintegrations per min. (DPM) using an internal ^{14}C-sucrose quench curve (LKB Wallac, Turku, Finland). The DPM counts were converted to Bq g^{-1} soil, and the amounts of ^{14}C-labelled biomass C calculated from:

$$B_C\text{-}^{14}C = \frac{(F - NF)}{SA} \times 2.22$$

where F and NF are the counts of the fumigated and non-fumigated soils and SA the specific activity of the relevant pesticide molecule (Bq μg^{-1}).

The amount of ^{14}C-labelled CO_2 derived from the pesticides, trapped in 1 M NaOH, was measured by mixing 2 ml NaOH with 10 ml Ultima Gold scintillation cocktail, and counted for 10 min. as described above. The amount of $^{14}CO_2$ evolved from the soils was expressed in terms of % of total radioactivity applied, and was calculated from:

$$^{14}CO_2 = \frac{(C-B)}{60} \times \frac{(20/2)}{A} \times 100$$

where C is the count of the NaOH (DPM), B is the mean count of 4 blank NaOH solutions and A is the activity of the ^{14}C-labelled pesticide solutions (Bq ml^{-1}).

2.2.4.2 *Soil ^{14}C*

Total ^{14}C content of soils at the end of experiments was determined using a Canberra Packard Tri-carb 306 sample oxidiser. Samples were mixed with cellulose powder and combusted in pure O_2 at 1000°C for 1.5 min., and the $^{14}CO_2$ generated trapped in 15 ml Carbo-Sorb (Canberra Packard) to which was automatically added 10 ml Permafluor V scintillation cocktail (Canberra Packard). The system's accuracy was checked by combusting 5000 DPM ^{14}C paper strip standards (Amersham International, Amersham, UK). A known amount of ^{14}C, as ^{14}C-labelled pesticide solution, was added to previously untreated soil samples which were combusted as above, to correct for the efficiency of combustion and ^{14}C recovery. Samples were counted for 20 min. as described in section

2.2.4.1, except that a factory-produced ^{14}C-quench curve was employed (Canberra Packard), with automatic subtraction of background radiation.

2.2.5 SOIL CHEMICAL ANALYSES

Soil pH was determined in suspensions of air-dried soil in both distilled water and 0.01 M $CaCl_2$, with a 1:2.5 soil:liquid ratio, using a glass electrode with a calomel reference electrode (Radiometer Copenhagen). Total organic C in the soils was measured by the dichromate digestion method of Kalembasa and Jenkinson (1973), and total soil N by Kjehldahl digestion (Bremner, 1965). Soil cation exchange capacity (CEC) was determined by ICP measurement of exchangeable cations leached into neutral ammonium acetate, while soil texture was determined using the pipette method.

Ammonium-nitrogen (NH_4-N) and nitrate-nitrogen (NO_3-N) in 0.5 M K_2SO_4 soil extracts were determined by microcontinuous flow analysis using an Alpkem Rapid Flow Analyser (Alpkem, Oregon, USA), based on the methods of Crooke and Simpson (1971) and Henriksen and Selmer-Olsen (1970), for NH_4^+ and NO_3^-, respectively.

All chemicals and reagents used in these studies were of analytical (AR) grade, except where specifically stated otherwise.

2.2.6 STATISTICAL ANALYSES

All measurements are the mean of triplicate treatments, unless specifically stated otherwise. Statistical analyses (t-test, analysis of

variance) were carried out using Genstat 5, Release 3.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 1992), except for linear regressions, which were produced using SigmaPlot for Windows, 1.01 (Jandel Scientific).

Notes on the analysis of a 2^n factorial experiment.

A factorial experiment is one where the treatments comprise all combinations of levels of 2 or more factors. When there are n factors, each at 2 levels, this is called a 2^n factorial. For example, in a 2^3 experiment with three factors A, B, C, which each have 2 levels, say presence and absence, there are eight treatment combinations, for which the conventional notation is: (1) a b c ab ac bc abc. Treatment ab indicates that A and B are at their higher level (present) while C is at its lower level (absent), and the symbol (1) indicates the treatment where all factors are at their lower level.

In a single-replicate 2^2 experiment, 2 estimates of the effect of A are supplied, at each level of B. The average observed effect of A over the 2 levels of B is called the *main effect* of A. In a single-replicate 2^5 experiment, such as in Chapter 6, there will be 5 main effects A, B, C, D, E, 10 "first-order" interactions *e.g.* AB, AC *etc.* and 10 "second-order" interactions *e.g.* ABC, ABD *etc.* It is not usual to consider interactions higher than second-order.

The analysis of variance of a factorial experiment must contain contributions to the total sum of squares from each main effect and interaction, as well as from blocks, if present. These items are subtracted from the total sum of squares to leave the residual sum of squares. As each of the main effects and interactions in a 2^n factorial has only 1 degree of freedom, the mean square of each of these is equal to the sum of squares. Each of these sums of squares may be tested against the residual mean square (equal to the pooled sample variance - s^2) to give the variance ratio, F.

Where the level of replication is constant, as in Chapter 6, the standard error of the difference (SED) of any two means is also constant, and may be calculated from $SED = \sqrt{(2s^2/r)}$ (where r = no. of reps.). If preferred, the least significant difference (LSD), the smallest difference between two treatment means which a t-test would report as significant, may be calculated from $LSD = t^*_p \times SED$ (where t^*_p = critical value of t at a given level of probability, on p degrees of freedom, *i.e.* that of the residual mean square). The LSD allows significant differences between means to be easily seen, but either method may be appropriately used.

CHAPTER 3

EFFECTS OF EPOXICONAZOLE AND QUINMERAC ON SOIL MICROBIAL BIOMASS AND MICROBIAL ACTIVITY IN A SANDY LOAM SOIL

3.1 INTRODUCTION

There has been increasing concern about the potential side-effects of pesticides on soil micro-organisms since around 1950 (Domsch, 1984), and thus the effects of many established chemicals, and others which are now obsolete, are relatively well documented (see reviews referred to in Chapter 1). However, new pesticides are continually being developed, so the need for testing for possible effects on non-target organisms is an on-going one. Tests to determine the extent of such effects on soil micro-organisms have been many and varied, including plate counts (Schreven *et al.*, 1970; Voets *et al.*, 1974), direct counts (Duah-Yentumi and Johnson, 1986; Jones *et al.*, 1991) soil enzyme activity (Atlas *et al.*, 1978; Biederbeck *et al.*, 1987), carbon cycling (Johnen and Drew, 1977; Lewis *et al.*, 1978; Harden *et al.*, 1993), nitrogen cycling (Tu, 1978; Marsh and Greaves, 1979; Mårtensson, 1992) and substrate-induced respiration (Anderson *et al.*, 1981; Wardle and Parkinson, 1990). Many of these methods have severe limitations, *e.g.* the selectivity of plate counts, the lack of correlation between soil enzymes and other microbial parameters, and yield little relevant information.

Greaves (1987) noted that the most commonly recommended

parameters for assessing pesticide side-effects; soil respiration, ammonification and nitrification, were not necessarily the best ones to measure. He suggested that measurement of changes in the microbial biomass itself might be a useful approach, although pointing out that there would be disagreement as to the choice of the exact method. Since the publication of this paper, there have been a number of significant improvements in the methodology for measuring microbial biomass in soil, specifically in methods based on chloroform-fumigation and extraction (usually termed fumigation-extraction) (Vance *et al.*, 1987; Wu *et al.*, 1990). However, there have been relatively few reports on pesticide side-effects in the literature that have included the use of fumigation-extraction (*e.g.* Jones *et al.*, 1991; Jones *et al.*, 1992; Harden *et al.*, 1993). Although the use of soil respiration has had some criticism, when coupled with measurements of total microbial biomass, it can be a sensitive indicator of soil pollution (Brookes, 1993).

In this chapter, the potential side-effects of two newly developed pesticides; a fungicide, epoxiconazole, and a herbicide, quinmerac, described in Chapter 1, were assessed in a simple dose-response experiment. The aim of this experiment was to determine whether these new pesticides would affect the total soil microbial biomass, measured by the fumigation-extraction method, and microbial activity, measured by soil respiration, in a sandy loam soil.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLING AND PREPARATION OF SOIL

The soil was sampled from plots 75 and 76 of the Arable and Ley Rotation field experiment in Stackyard D, Woburn Experimental Farm, Bedfordshire (Johnston, 1973). Both plots were in the last year of an 8-year grass-clover ley, and differed only in that plot 75 received 38 t ha^{-1} farmyard manure in 1966, 25 years before being used here, compared to none for plot 76.

The soils were bulked, then sieved as described in section 2.1.1, mixed thoroughly, and then stored at 5°C for 2 weeks before use. Six kg soil (*ca.* one third the total) was then removed and pre-incubated, as described in section 2.1.1.

3.2.2 SOIL CHEMICAL ANALYSES

Soil pH, CEC, organic C, total N and texture were measured as described in section 2.2.5, on dried subsamples of the bulked soil.

3.2.3 PESTICIDE TREATMENTS

Epoxiconazole and quinmerac were added as described in section 2.1.3, to a total of 15 replicate soil samples (50g on an oven-dry basis) per pesticide treatment. Epoxiconazole was added at 0, 0.25 and 2.5 $\mu g\ g^{-1}$ soil, and quinmerac at 0, 1.0 and 10.0 $\mu g\ g^{-1}$ soil, and the soil samples then incubated as described in section 2.1.2.

3.2.4 SOIL MICROBIAL BIOMASS AND CO_2 EVOLUTION MEASUREMENTS

Soil microbial biomass C and ninhydrin-reactive N were measured as described in section 2.2.1 and 2.2.2, on soil samples removed after 0, 7, 14, 28, 56 and 84 days incubation. Evolution of CO_2 was measured as described in section 2.2.3, from NaOH samples removed after 3, 7, 14, 28, 42, 56 and 84 days incubation.

3.3 RESULTS AND DISCUSSION

3.3.1 SOIL CHARACTERISTICS

The soil was a sandy loam (Cottenham series) containing 72% sand, 12% silt and 16% clay (refer to Hodge *et al.* (1984) for description of soil series named in this thesis). The pH was 6.6 in distilled water, 6.0 in 0.01 M $CaCl_2$, with a CEC of 11.7 me 100 g^{-1}. Soil organic C and total N were 1.49 and 0.142%, respectively.

3.3.2 EFFECT OF PESTICIDES ON MICROBIAL BIOMASS

The initial soil microbial biomass C content was about 300 μg g^{-1} soil. During the incubation period, the biomass C contents of both epoxiconazole and quinmerac-treated soil were almost identical. After 7 days incubation the biomass C increased to about 335 μg g^{-1} soil, then gradually declined to about 200 μg g^{-1} soil after 84 days incubation (Figs. 3-1 and 3-2). The initial increase in biomass seen at day 7 may have been due to a residual effect of the soil's disturbance during it's preparation.

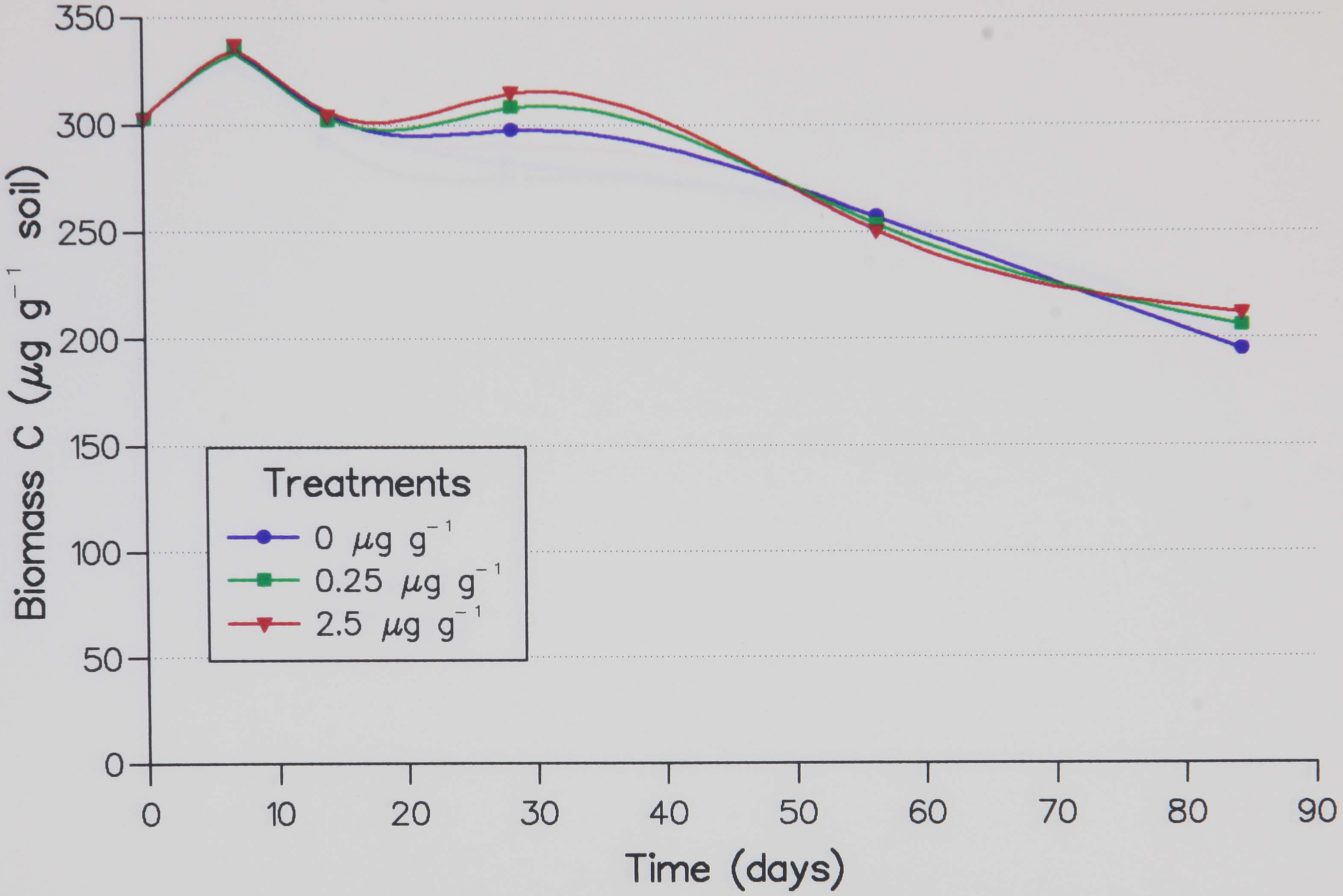


Figure 3-1: Microbial biomass C contents of epoxiconazole-treated soil (see Appendix 2 for s.d.).

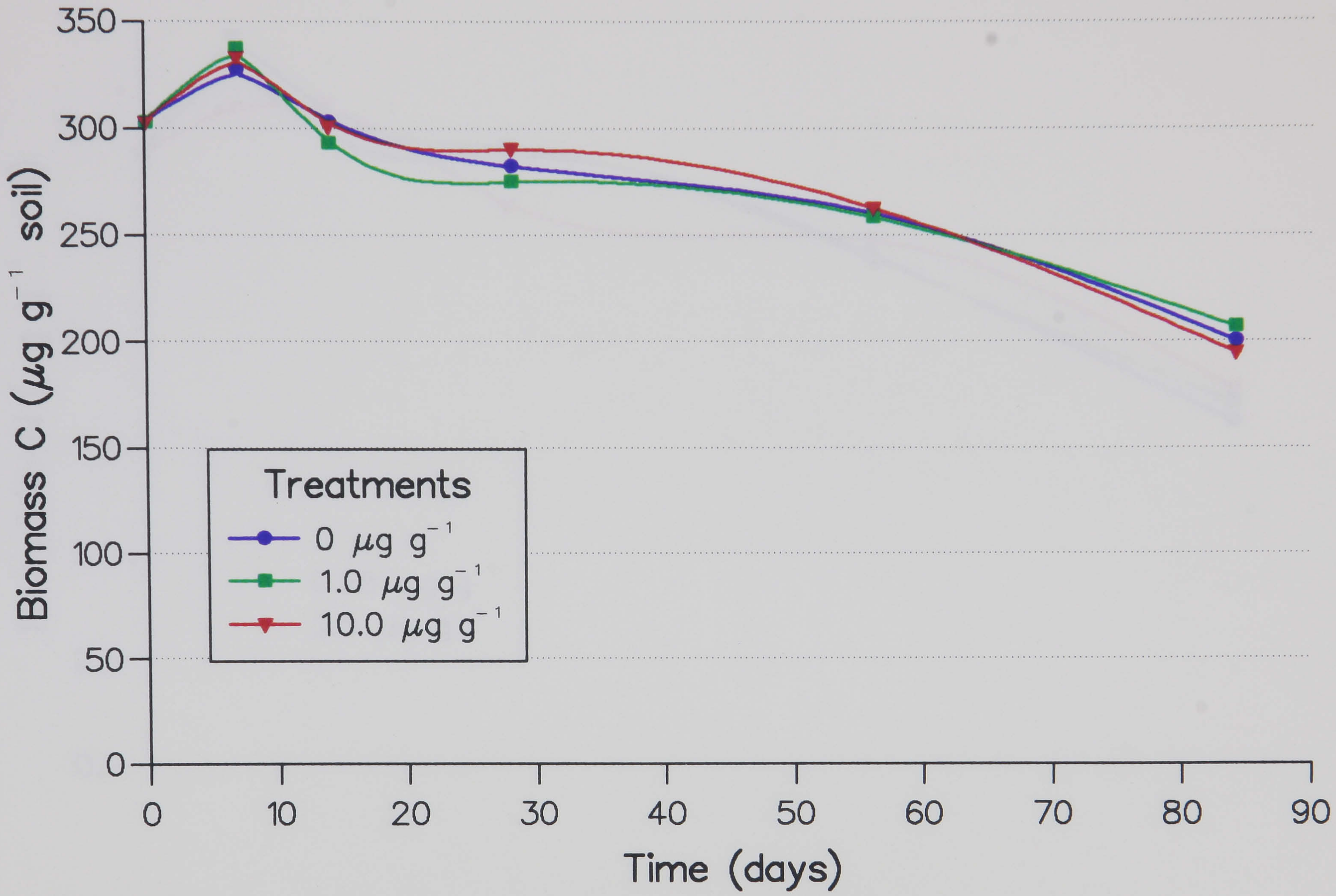


Figure 3-2: Microbial biomass C contents of quinmerac-treated soil (see Appendix 2 for s.d.).

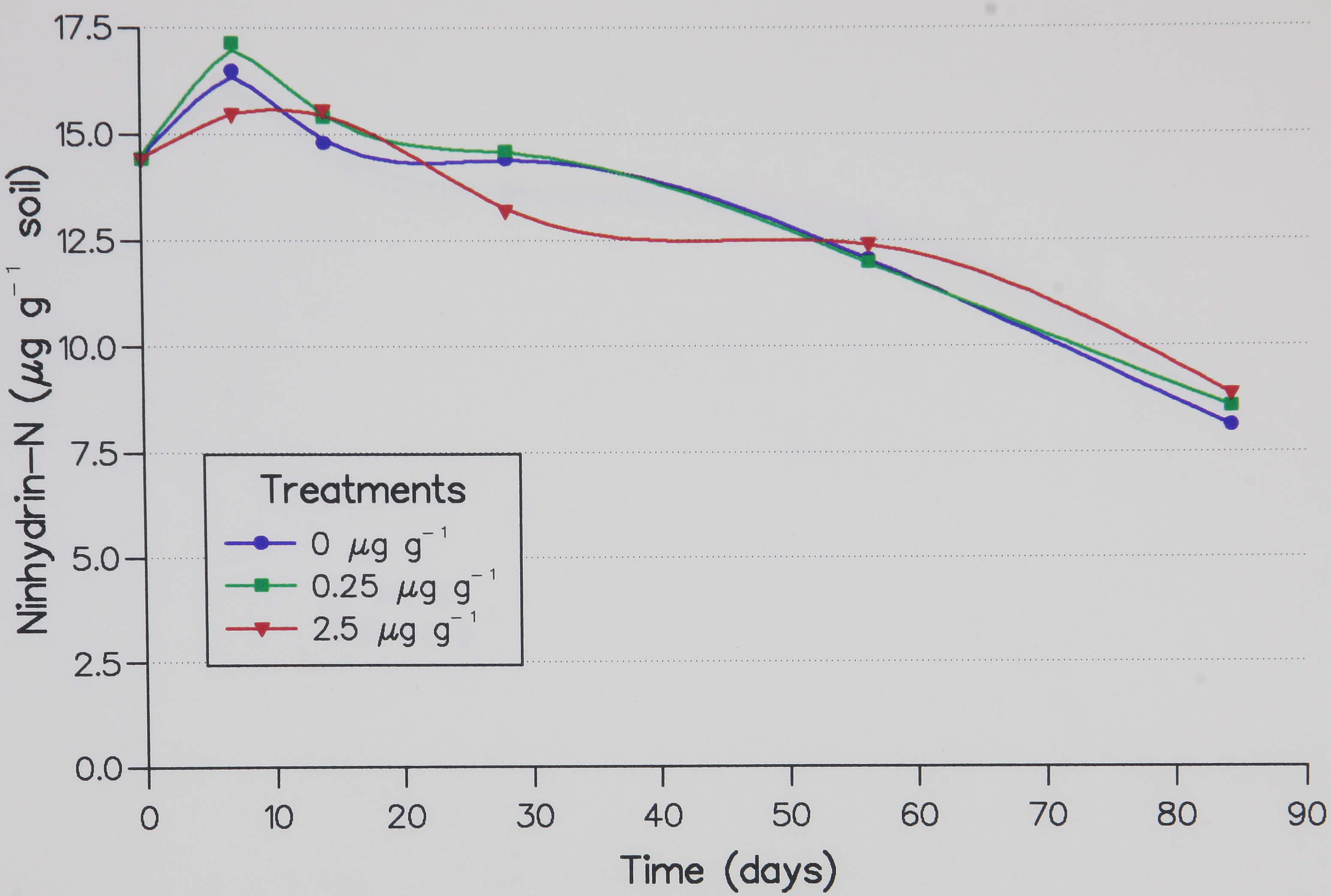


Figure 3-3: Microbial biomass ninhydrin-N contents of epoxiconazole-treated soil (see Appendix 2 for s.d.).

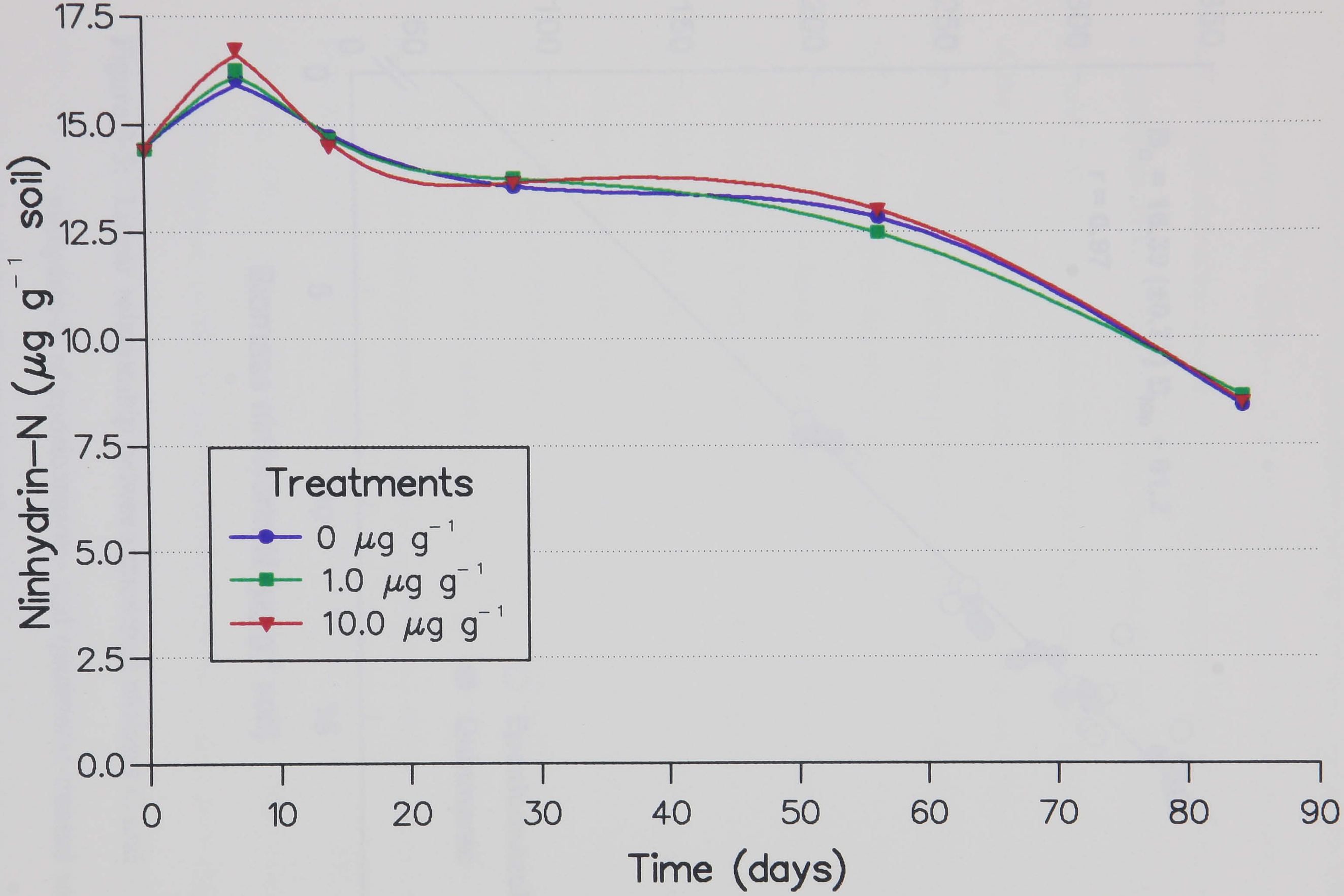


Figure 3-4: Microbial biomass ninhydrin-N contents of quinmerac-treated soil (see Appendix 2 for s.d.).

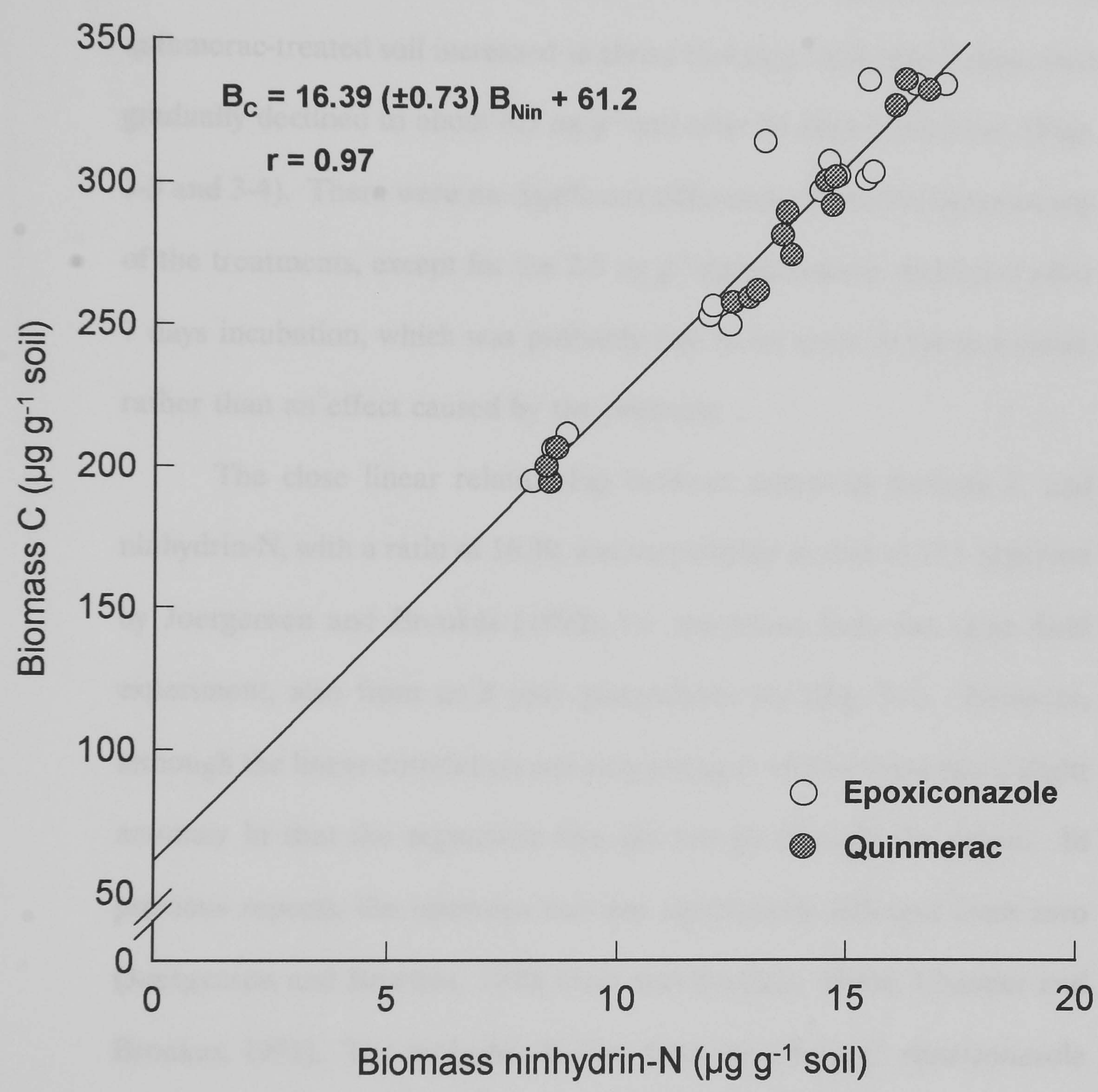


Figure 3-5: Linear relationship between microbial biomass C and ninhydrin-N of epoxiconazole- and quinmerac-treated soil (including 0× treatments).

There were no significant differences (P=0.05) between any of the treatments.

The initial biomass ninhydrin-N of the soil was 14.4 μg g^{-1} soil. The pattern of decline over the incubation period was very similar to that of the biomass C. Biomass ninhydrin-N of both epoxiconazole- and quinmerac-treated soil increased to about 16.4 μg g^{-1} soil after 7 days, then gradually declined to about 8.5 μg g^{-1} soil after 84 days incubation (Figs. 3-3 and 3-4). There were no significant differences (P=0.05) between any of the treatments, except for the 2.5 μg g^{-1} epoxiconazole treatment after 7 days incubation, which was probably due to an error in measurement, rather than an effect caused by the pesticide.

The close linear relationship between microbial biomass C and ninhydrin-N, with a ratio of 16.39, was very similar to that of 17.1 reported by Joergensen and Brookes (1990), for soil taken from the same field experiment, also from an 8 year grass/clover ley (Fig. 3-5). However, although the linear correlation was very strong (r=0.97), there was a slight anomaly in that the regression line did not go through the origin. In previous reports, the intercept was not significantly different from zero (Joergensen and Brookes, 1990; Ocio and Brookes, 1990a; Chander and Brookes, 1991). The ninhydrin-N data from the 2.5 μg g^{-1} epoxiconazole treatment did not match the corresponding biomass C data very closely, but omitting these data did not affect the ratio, and only shifted the intercept towards zero by a negligible amount (data not shown). The shift away from the origin was caused largely by the data from day 84, where

the ninhydrin-N values were slightly low (or biomass C slightly high). This was most probably due to experimental error.

The Woburn soil was chosen for this experiment largely because of its texture. As a sandy loam soil with a low clay content and C.E.C., and a fairly low organic C content, its sorptive properties were also low, and hence if the chemicals were to exhibit any adverse effects on the soil microbial biomass, they would theoretically be greatest in a soil of this type, as a relatively large proportion of the applied chemicals would be biologically available. From these initial biomass measurements, it would appear that neither epoxiconazole nor quinmerac had any effects on the size of the total soil microbial population, at the concentrations applied.

3.3.3 EFFECTS OF PESTICIDES ON SOIL CO_2 EVOLUTION

Addition of epoxiconazole to the soil had no significant effect on CO_2 evolution at either concentration (Fig. 3-6). Addition of quinmerac at 1.0 μg g^{-1} soil also had no effect on CO_2 evolution compared to the control. However, the amount of CO_2-C evolved from the 10.0 μg g^{-1} quinmerac-treated soil was about 13% less than from the control, after 84 days incubation (Fig. 3-7). The respiration rates at day 42 and 56 were significantly different from the control ($P<0.001$), but that at day 84 was not ($P=0.05$), due to the large variance of the CO_2 measurements (see Appendix 2).

The respiration rates of all soils were generally similar throughout the incubation. The rate was greatest over the first 3 days incubation, at

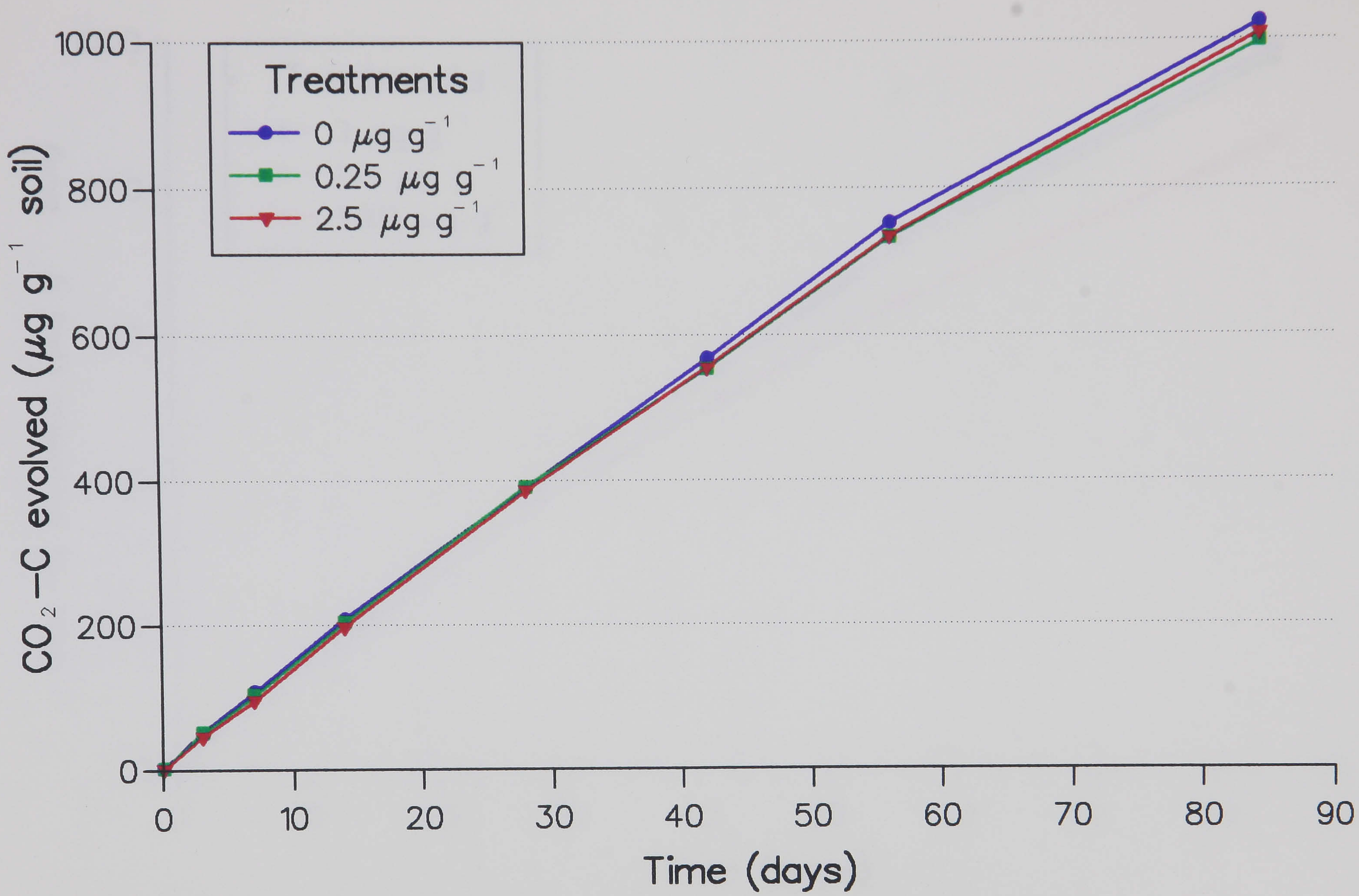


Figure 3-6: Cumulative CO_2 evolution in epoxiconazole-treated soil (see Appendix 2 for s.d.).

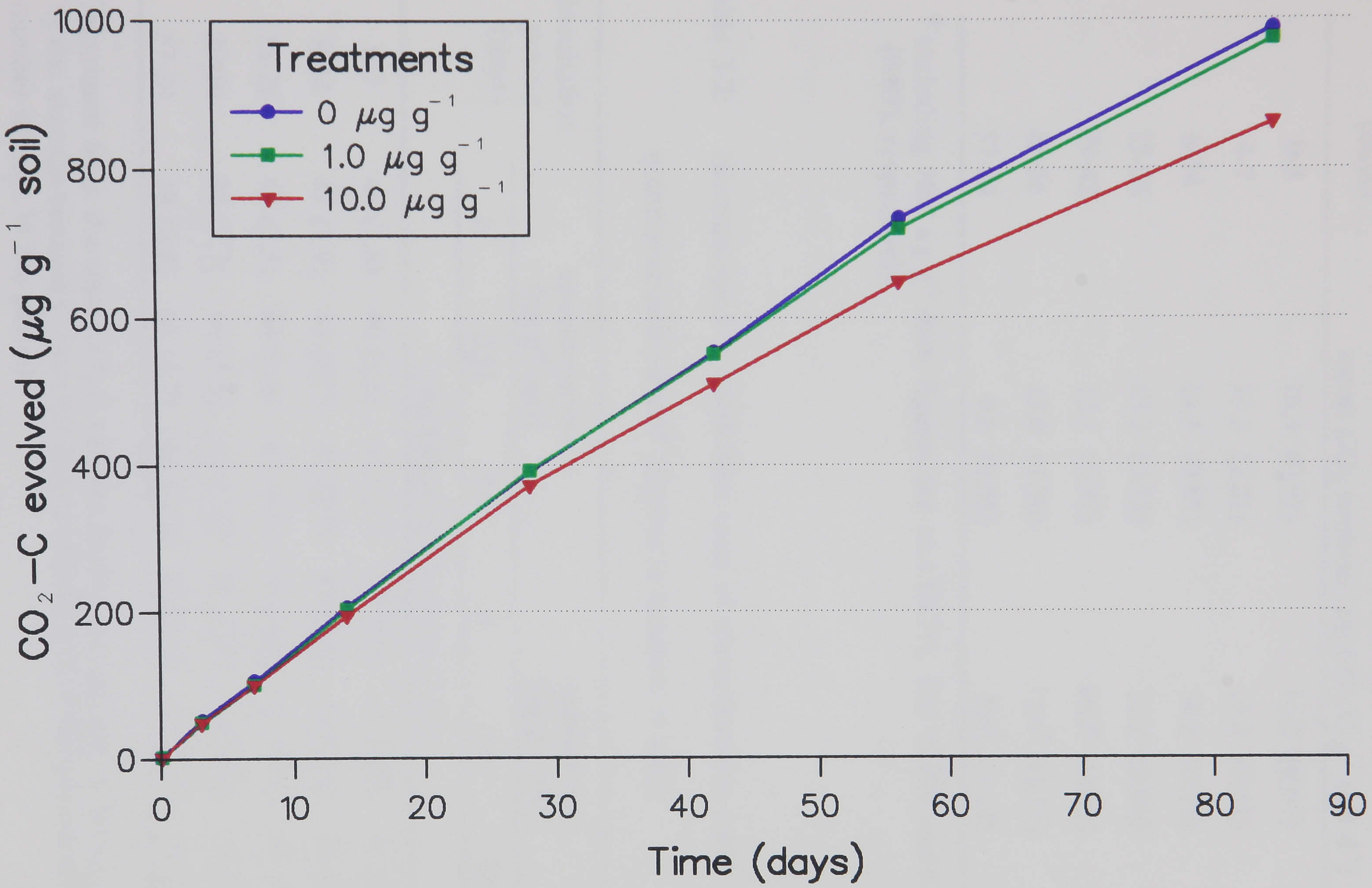


Figure 3-7: Cumulative CO_2 evolution in quinmerac-treated soil (see Appendix 2 for s.d.).

Table 3-1: Respiration rates of epoxiconazole- and quinmerac-treated soil (mean of all treatments) (figures in brackets = s.d.).

Incubation period (days)	epoxiconazole	quinmerac
	mean CO_2 evolved (μg CO_2-C g^{-1} soil d^{-1})	
0-3	16.4 (0.99)	16.2 (0.67)
4-7	13.2 (1.21)	13.2 (0.41)
8-14	14.5 (0.55)	14.2 (0.51)
15-28	13.2 (0.22)	13.2 (0.66)
29-42	12.1 (0.92)	10.2† (1.01)
43-56	13.0 (1.30)	11.6† (1.51)
57-84	9.6 (0.55)	8.6† (1.22)

† excluding 10.0 μg g^{-1} data, figures are 11.4 (0.71), 12.5 (0.87) and 9.1 (0.89), respectively

Table 3-2: Biomass-specific respiration rates of epoxiconazole- and quinmerac-treated soil‡ (figures in brackets = s.d.).

Incubation period (days)	epoxiconazole (μg g^{-1} soil)			quinmerac (μg g^{-1} soil)		
	0	0.25	2.5	0	1.0	10.0
	(mg CO_2-C g^{-1} biomass C d^{-1})					
0-7	48 (1.8)	46 (1.5)	43 (2.0)	47 (0.6)	45 (2.0)	45 (0.8)
8-14	47 (1.9)	46 (0.7)	44 (2.7)	47 (3.0)	46 (1.6)	44 (0.8)
15-28	43 (2.3)	44 (1.4)	42 (0.8)	45 (4.1)	47 (2.7)	42 (1.7)
29-56	43 (7.7)	44 (5.6)	42 (1.0)	46 (5.5)	44 (3.9)	36 (2.3)
57-84	43 (2.8)	41 (1.7)	42 (3.0)	40 (4.1)	39 (4.7)	34 (5.7)

‡ Calculated from the total CO_2-C evolved during the incubation period divided by the average biomass C from the beginning and end of the period, and by the number of days in the period.

about 16 μg CO_2-C g^{-1} soil d^{-1}. Over the following 53 days the soil respiration rates were fairly constant, at around 13 ($\pm$ 0.9) μg CO_2-C g^{-1} soil d^{-1}, but declined over the last 28 days incubation to about 9 μg CO_2-C g^{-1} soil d^{-1} (Table 3-1). This corresponds quite closely to the initial increase, then gradual decline in soil microbial biomass (Figs. 3-1 – 3-4).

The possible inhibition in the 10.0 μg g^{-1} quinmerac-treated soil was also evident in the biomass-specific respiration rates (*i.e.* CO_2-C evolved per unit of biomass C) (Table 3-2). The rates tended to decrease slightly towards the end of the experiment, but the decreases in the 10.0 μg g^{-1} quinmerac treatment in the last two incubation periods were about 18% greater than the average of the other treatments. The variation between replicate measurements of CO_2 evolution became increasingly large towards the end of the experiment, particularly in the quinmerac treatments, with standard deviations of up to 17%. Because of this large variation, it is possible that this inhibition of CO_2 evolution caused by the 10 μg g^{-1} herbicide treatment was not a genuine effect, but an artefact of some kind.

Measurements of the microbial biomass as a whole can mask shifts between the component populations (Greaves and Malkomes, 1980). That this did not occur in this instance, at least to any significant degree, is indicated by there being no increase in CO_2 evolved from the pesticide-treated soil compared to the control. Over 10 days incubation, about 45% of the C of any organisms killed by the pesticides would have been evolved as CO_2 (Jenkinson and Ladd, 1981).

Excepting the decreased CO_2 evolution from the 10.0 μg g^{-1} quinmerac-treated soil, neither pesticide had any significant effect on soil microbial activity, as measured by soil respiration. Given its mode of action and fairly wide range of target organisms, epoxiconazole was considered the more likely of the two pesticides to exhibit side-effects. That it did not may be due to a number of reasons, including its strong adsorption to soil colloids, even in a sandy loam soil (R. Bromilow, pers. com.), relatively low application rate and possibly a low proportion of susceptible micro-organisms.

3.4 SUMMARY

The effects of two new pesticides, epoxiconazole and quinmerac, on microbial biomass contents and respiration in a sandy loam soil were investigated.

Microbial biomass C and ninhydrin-N declined steadily over the course of the 84 days incubation, with no significant effects caused by the chemicals. Similarly, there was no significant effect on soil CO_2 evolution, with the exception of quinmerac at 10.0 μg g^{-1}, about which there was some doubt as to the veracity of the results. Hence, both epoxiconazole and quinmerac, at or above field application rates, appeared to have no harmful side-effects on the soil microbial biomass.

CHAPTER 4

THE MINERALISATION OF EPOXICONAZOLE AND QUINMERAC IN THREE CONTRASTING SOILS

4.1 INTRODUCTION

There are numerous chemical, physical and biological factors known to affect the degradation of pesticides in soil, such as clay and organic matter content, temperature, pH, redox potential *etc*. The interactions of these various factors determine the overall persistence of a chemical under any given set of conditions. Degradation products include compounds which have undergone only slight molecular changes, and inorganic molecules or ions, *e.g.* carbon dioxide, water, ammonium and nitrate, which represent complete mineralisation (Kaufman *et al.*, 1985; Camper, 1991). Abiotic chemical reactions rarely lead to appreciable changes in pesticide molecular structure; thus it is enzymatic reactions that are responsible for major structural changes (Alexander, 1981). Soil micro-organisms are clearly the major source of such degradative enzymes, and microbial degradation is an important factor affecting the persistence of most pesticides in soil (Bollag, 1982; Kaufman *et al.*, 1985).

In investigations of pesticide side-effects in soil, often no attempt is made to measure the degradation of the pesticide under examination. It is very important that the fate of the pesticide under the experimental conditions is known, particularly in long-term experiments, otherwise side-effects may be found and ascribed to a pesticide long after it has been

effectively degraded (Greaves and Somerville, 1987). Using pesticides labelled with ^{14}C is a simple method often employed to measure their breakdown, *e.g.* Anderson, 1984; Soulas *et al.*, 1984; Soulas, 1993; Levanon, 1993. Mineralisation, as represented by $^{14}CO_2$ evolution, can be easily monitored, and also intermediate breakdown products, if desired.

When repeated applications of a pesticide are given to a soil, the selection and growth of degradative microbial populations can lead to succeeding applications having progressively shorter residence times. Continued application of a pesticide to susceptible soils may therefore eventually lead to a reduced or complete loss of that pesticide's efficacy (Kaufman *et al.*, 1985). The accelerated degradation of pesticides following repeated applications in the field has been demonstrated for a number of compounds, including, for example, carbofuran (Read, 1986) and aldicarb (Suett and Jukes, 1988).

The aims of this experiment were, firstly, to measure the rate of mineralisation of the two pesticides epoxiconazole and quinmerac, in three contrasting soils, and also the long-term effects of the pesticides on soil respiration. The formation of organic pesticide metabolites was not measured, as this was the subject of another, separate study. Secondly, to determine the effects of repeated applications of the pesticides on the rate of mineralisation and soil respiration, and thirdly to compare the treatment effects at two different temperatures. This experiment also afforded the opportunity of confirming the possible inhibition of soil respiration found in the 10.0 $\mu g\ g^{-1}$ quinmerac-treated soil, described in Chapter 3.

4.2 MATERIALS AND METHODS

4.2.1 SAMPLING AND PREPARATION OF SOIL

Soil was collected from three different sites: Woburn Experimental Farm (a subsample of the bulk soil collected previously - see section 3.2.1); plot 09, section 3 of the Broadbalk Continuous Wheat Experiment, Rothamsted Experimental Station - a 1 year fallow after wheat; and Boot Field, Silsoe Agricultural College Farm - stubble after wheat.

The Broadbalk soil was sieved while field moist, as described in section 2.1.1, and stored at 5°C with the Woburn soil. The Silsoe soil, however, was treated quite differently. Because of its high clay content, the soil could not be sieved while at field moisture content. Instead, the soil was spread out and partially air-dried, as described in section 2.1.1. As the soil dried it was carefully broken up by hand into smaller and smaller lumps, and that part of it <2 mm periodically sieved out. This soil was slightly re-moistened with distilled water and stored at 5°C. At the higher moisture contents, the soil smeared when sieving was attempted. However, as the soil dried it became harder and harder, until it could no longer be broken up by hand, and yet still would not pass through the sieve. It was therefore decided to fully air-dry the remaining soil, approximately 75% of the total, which was then ground with a mortar and pestle to pass through a 2 mm sieve. This soil was then thoroughly mixed with the partially air-dried soil, about 1 L of distilled water gradually added with a hand sprayer, and the soil then incubated at 25°C, as described in section 2.1.1, for seven days. Particulate organic matter was

then removed, and the soil stored at 5°C for 2 weeks until use. Subsamples of each soil were then removed, adjusted to 40% WHC and incubated as described in section 2.1.1.

4.2.2 SOIL CHEMICAL ANALYSES

Soil pH, CEC, organic C, total N and texture were determined on dried subsamples of each soil, after the 7 days pre-incubation period, as described in section 2.2.5. Soil ^{14}C content at the end of the experiment was determined as described in section 2.2.4.2.

4.2.3 PESTICIDE TREATMENTS

Pesticide solutions of 0, 12.5 and 125 $\mu g\ ml^{-1}$ epoxiconazole and 0, 50 and 500 $\mu g\ ml^{-1}$ quinmerac were prepared, with part of the active ingredient of each solution being labelled with ^{14}C at a specific point on the molecule (Fig. 1a, 1b). The labelled chemicals were supplied dissolved in toluene (epoxiconazole) and acetone (quinmerac). The appropriate volume of labelled pesticide solution was placed in 25 ml volumetric flasks, and the solvent allowed to evaporate off. The labelled active ingredients were redissolved in aliquots of stock solutions, made up manually from pure active ingredient and carrier material, and the final solutions were then made up with distilled water. The solutions were prepared to contain approximately 16.67 kBq (10^6 DPM) ml^{-1}. The final radioactivity was checked by scintillation counting of 0.1 ml aliquots in 10 ml Ultima Gold cocktail, in duplicate.

A total of 36 samples of each soil were weighed out as described in section 2.1.2. One ml aliquots of the pesticide solutions were added to triplicate soil samples, as described in section 2.1.3, giving 6 samples per treatment. Thus, epoxiconazole was added at 0, 0.25 and 2.5 μg g^{-1} soil, and quinmerac at 0, 1.0 and 10.0 μg g^{-1} soil. An appropriate volume of distilled water was also added, to adjust the soil moisture content to 50% WHC. The soil samples were then incubated as described in section 2.1.2, one half at 25°C, the other at 15°C, for a total of 266 days. The replicates of each treatment were arranged on shelves in randomised blocks.

Further pesticide treatments, *without* ^{14}C-labelled active ingredient, were given to the soils after 84 and 172 days incubation. These pesticide solutions were prepared from the formulated products provided by BASF.

4.2.4 SUBSTRATE AMENDMENTS

As this experiment involved a long-term incubation, 0.125 g air-dry, ground ryegrass was added to each soil sample after 0, 56, 112 and 172 days incubation, to maintain microbial activity. The ryegrass contained 45.5% C, as determined by dichromate digestion (Kalembasa and Jenkinson, 1973); thus each amendment was equivalent to about 1140 μg C g^{-1} soil.

4.2.5 TOTAL AND ^{14}C-LABELLED MICROBIAL BIOMASS C MEASUREMENTS

Total and ^{14}C-labelled soil microbial biomass was determined as

Table 4-1: Characteristics of the Woburn, Broadbalk and Silsoe soils.

	Woburn	Broadbalk	Silsoe
pH (H_2O)	6.6	8.2	7.8
pH (0.01M $CaCl_2$)	6.0	7.6	7.5
CEC (meq 100 g^{-1})	11.7	34.6	69.7
Organic C (%)	1.49	0.83	3.17
Total N (%)	0.142	0.101	0.326
Sand (%)	72	20	14
Silt (%)	12	51	14
Clay (%)	16	28	72
Texture	Sandy loam	Silty clay loam	Clay
Soil Series	Cottenham	Batcombe	Evesham

described in sections 2.2.1 and 2.2.4.1, respectively, at day 0 (total biomass C only) and at the end of the experiment, after 266 days incubation.

4.2.6 TOTAL CO_2 AND $^{14}CO_2$ EVOLUTION MEASUREMENTS

Total CO_2 and $^{14}CO_2$ evolution were determined as described in sections 2.2.3 and 2.2.4.1, respectively, after 7, 14, 28, 42, 56, 70, 84, 98, 112, 140, 172, 199, 224 and 266 days incubation.

4.3 RESULTS AND DISCUSSION

4.3.1 SOIL CHARACTERISTICS

The characteristics of the three soils are shown in Table 4-1.

4.3.2 TOTAL AND ^{14}C-LABELLED MICROBIAL BIOMASS C

It is impossible to say how great an effect the air-drying treatment had on the microbial population of the Silsoe soil (section 4.2.1). However, at day 0 the Silsoe soil contained roughly three and five times as much microbial biomass C as the Woburn and Broadbalk soils, respectively (Figs. 4-1 and 4-2). As there was no significant increase in the initial respiration rate, or the biomass C content of the Silsoe soil over the course of the experiment, it is reasonable to assume that the microbial biomass had largely recovered, or at least stabilised, by the time the experiment began.

Addition of epoxiconazole to the three soils had no significant effect on microbial biomass C at either concentration or either temperature, at

the end of the experiment (Fig. 4-1). This was also the case with the quinmerac-treated soils, except that at 15°C the biomass C content of the 1.0 μg g^{-1}-treated Broadbalk soil (325 μg g^{-1}) was significantly greater ($P<0.05$) than both the 0 (289 μg g^{-1}) and the 10.0 μg g^{-1}-treated Broadbalk soil (304 μg g^{-1}) (Fig. 4-2). These differences are not great, and are probably of statistical significance only. There was little overall difference between the microbial biomass C contents of the epoxiconazole and quinmerac-treated soils.

The amount of ryegrass added to each soil sample over the course of the experiment was approximately equivalent to 4560 μg C g^{-1} soil. The ratios of total ryegrass C added to the initial soil microbial biomass C contents were 4.63 (0.1), 13.29 (0.25) and 24.4 (1.55):1, for the Silsoe, Woburn and Broadbalk soils, respectively (figures in brackets are standard deviations). Thus, roughly 5 and 2× as much ryegrass C μg^{-1} biomass C was added to the Broadbalk soil than to the Silsoe and Woburn soils, respectively.

Assuming an efficiency of biosynthesis of 20% (Jenkinson and Ladd, 1981), each 1140 μg addition of ryegrass C would have produced about 228 μg new biomass C g^{-1} soil (this assumes complete mineralisation, which would not have actually occurred in the time period (Jenkinson, 1988); the actual amount of new biomass would have been less). At 15°C, the synthesis of this new biomass would take longer than at 25°C, as would its subsequent decline once the substrate was used up. The last ryegrass amendment was given 94 days before the experiment ended. At 15°C, the

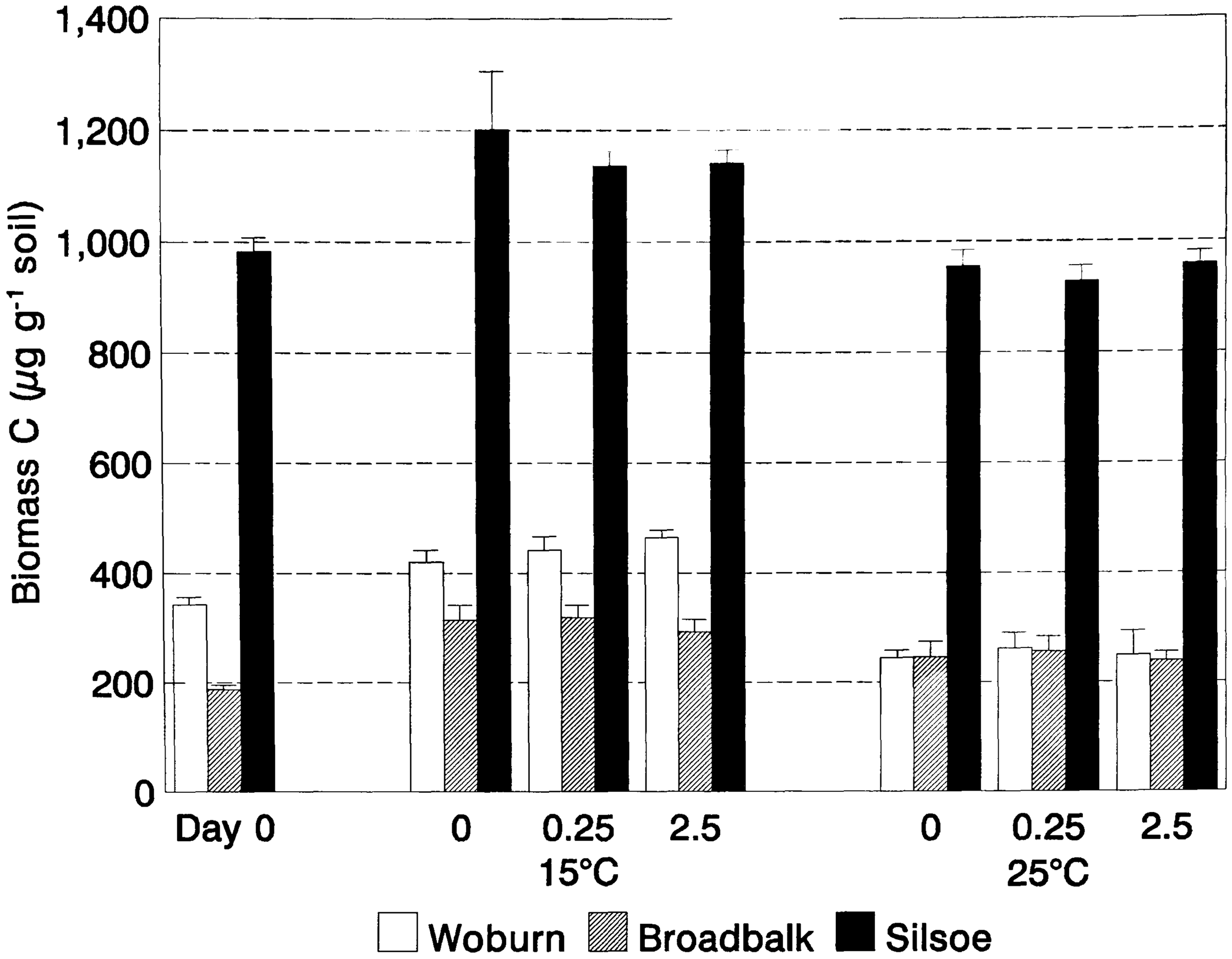


Figure 4-1: Microbial biomass C of soils at day 0, and of epoxiconazole-treated soils (0, 0.25 and 2.5 μg g^{-1} soil) at day 266 (bars = s.d.).

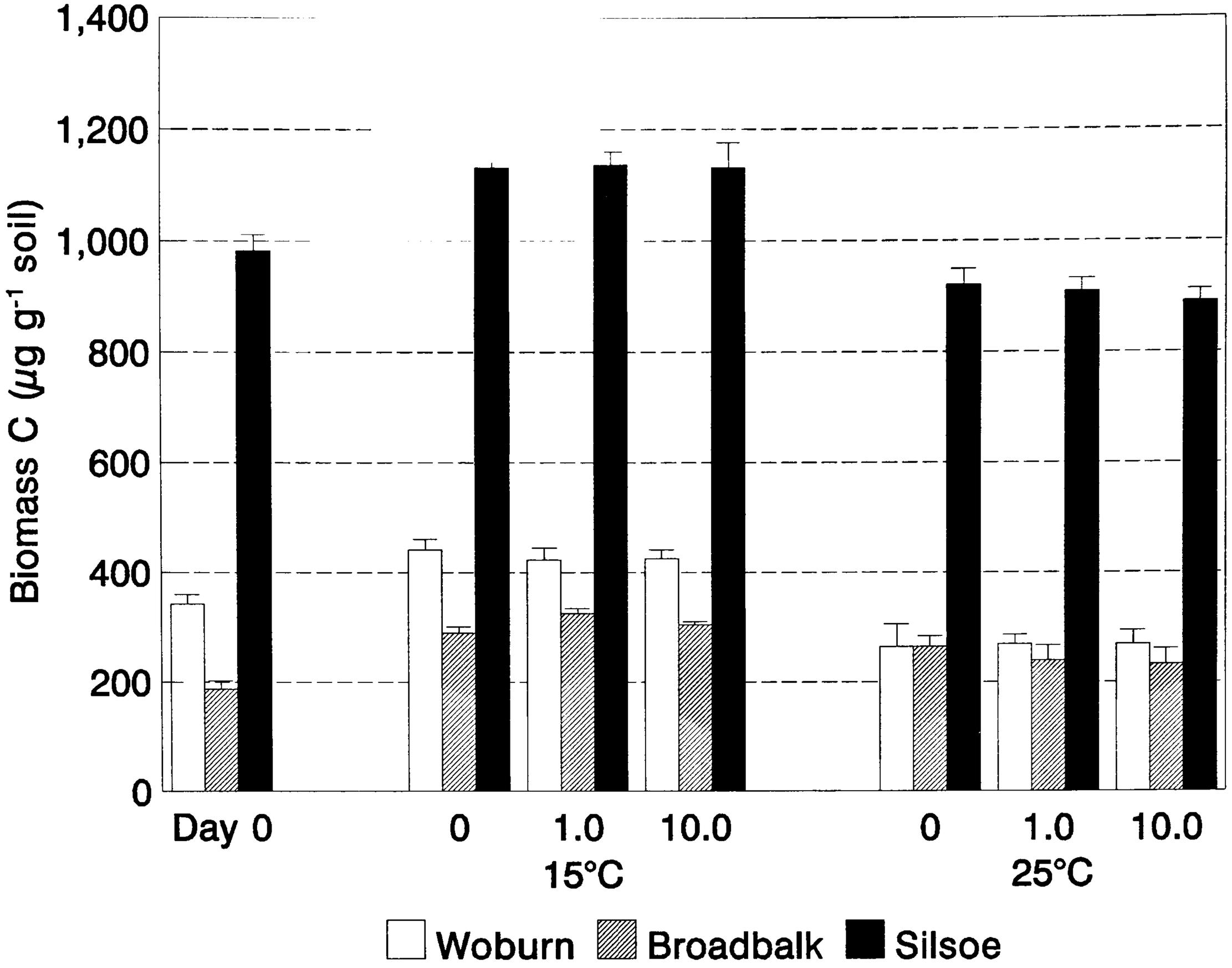


Figure 4-2: Microbial biomass C of soils at day 0, and of quinmerac-treated soils (0, 1.0 and 10.0 μg g^{-1} soil) at day 266 (bars = s.d.).

Table 4-2: Mean net change in soil microbial biomass C from day 0 to day 266 (figures in brackets = s.d.).

Incubation Temp.	Soil	epoxiconazole		quinmerac	
		Mean change in biomass C ($\mu g\ g^{-1}$)	% change	Mean change in biomass C ($\mu g\ g^{-1}$)	% change
15°C	Woburn	+100 (27)	+29.2	+84 (19)	+24.5
	Broadbalk	+121 (24)	+64.6	+121 (17)	+64.6
	Silsoe	+172 (56)	+17.5	+151 (24)	+15.4
25°C	Woburn	-91 (35)	-26.5	-76 (33)	-22.2
	Broadbalk	+60 (19)	+32.0	+58 (29)	+31.0
	Silsoe	-36 (28)	-3.7	-76 (28)	-7.3

Table 4-3: Amount of microbial biomass ^{14}C at day 266, as % of initial amount of radioactivity applied (figures in brackets = s.d.).

Incubation Temp.	Soil	Microbial biomass ^{14}C (%)			
		epoxiconazole ($\mu g\ g^{-1}$ soil)		quinmerac ($\mu g\ g^{-1}$ soil)	
		0.25	2.5	1.0	10.0
15°C	Woburn	1.2 (0.44)	0.3 (0.05)	4.5 (0.05)	5.4 (1.09)
	Broadbalk	2.8 (0.63)	0.5 (0.65)	4.4 (0.57)	1.5 (2.13)
	Silsoe	2.2 (0.07)	0.8 (0.22)	9.5 (0.14)	14.4 (1.42)
	L.S.D. (P=0.05)	0.89	0.81	0.68	3.20
25°C	Woburn	0.8 (0.25)	0.6 (0.26)	2.9 (0.14)	3.5 (0.54)
	Broadbalk	2.6 (0.24)	0.9 (0.42)	3.9 (0.26)	3.3 (2.77)
	Silsoe	2.3 (0.13)	1.4 (0.45)	4.4 (0.73)	3.6 (1.30)
	L.S.D. (P=0.05)	0.43	0.77	0.91	3.59

decline in microbial biomass content was slow enough so that there was a net increase at day 266, ranging from 84 – 172 μg g^{-1} (Table 4-2). The decline was slowest in the Silsoe soil, which, with its high clay content, is as would be expected (van Veen *et al.*, 1985). However, at 25°C the decline in biomass C was slowest in the Broadbalk soil, and this was the only soil with a net increase in microbial biomass at day 266, while those of the Woburn and Silsoe soils had decreased by 36 – 91 μg g^{-1} (Table 4-2).

This may be because the increased rate of metabolism at 25°C caused the microbial biomass of the Woburn and Silsoe soils to use up the readily-available ryegrass C well before the end of the incubation period, but the amount of ryegrass C added to the Broadbalk soil was so much in excess of the microbial biomass C that, even with the increased microbial metabolic rate, the ryegrass sustained the biomass for significantly longer than in the other two soils.

The amounts of ^{14}C-labelled microbial biomass present at day 266 were measurable, but the levels were on the picogram g^{-1} soil scale, and so in terms of total biomass C, were completely negligible. This is not unsurprising, however, as the amount of ^{14}C added to the soils at day 0 was very small: roughly 3 and 8 ng g^{-1} soil for epoxiconazole and quinmerac, respectively. When expressed as a percentage of the initial amount of ^{14}C applied, however, the amounts of ^{14}C-labelled biomass ranged from 0.3 – 2.8% and 1.5 – 14.4% for the epoxiconazole and quinmerac-treated soils, respectively (Table 4-3).

The increase in concentration of epoxiconazole led to a decrease

in the percentage of pesticide-derived biomass C in all three soils, which would be expected, from increased competition from unlabelled pesticide. However, the increase in concentration of quinmerac actually caused an increase in pesticide-derived biomass C in the Woburn soil, and also in the Silsoe soil, although only at 15°C. The increase in concentration of epoxiconazole also affected the relative amounts of biomass ^{14}C between soils. At 0.25 $\mu g\ g^{-1}$ soil, the Broadbalk soil contained the greatest amount of pesticide-derived biomass, the Silsoe slightly less and the Woburn soil least of all. But at the higher application rate, the Silsoe soil contained more pesticide-derived biomass than the Broadbalk soil. In the quinmerac-treated soils, the relative amounts of pesticide-derived biomass were also inconsistent. However, no definite conclusions can be drawn from any of these data, because of the large variation between the replicates.

It was noticeable that the treatment with the greatest amount of pesticide-derived microbial biomass, the 10.0 $\mu g\ g^{-1}$ quinmerac-treated Silsoe soil incubated at 15°C, evolved the least amount of $^{14}CO_2$ (Fig. 4-6). However, as the relationship between the biomass ^{14}C and $^{14}CO_2$ evolved in the corresponding 1.0 $\mu g\ g^{-1}$ treatment was disproportionate to that of the higher rate, this result should be treated with caution.

4.3.3 EFFECT OF PESTICIDES ON SOIL RESPIRATION

Neither pesticide had a permanent effect on CO_2 evolution at either concentration (Tables 4-4 – 4-7). The higher rate treatments tended to

cause more and greater inhibition than the lower rate ones, although the addition of quinmerac to the Broadbalk soil stimulated respiration (Tables 4-5 and 4-7). Significant differences between treatments were sporadic, with no apparent pattern, in part due to occasional large standard deviations. Further pesticide additions during the experiment had no discernable effect on the rate of respiration compared to the control treatments. The total CO_2-C evolved from the 10× quinmerac treatments of the Woburn soil was slightly less, over the whole of the incubation period, than the other treatments, as was found in the previous experiment (section 3.3.3), although to a lesser extent (about 1.5 and 4.5% at 15 and 25°C, respectively, compared to the 0× treatments). However, the differences at each sample time were rarely significant ($P=0.05$), and so the effect observed in the previous chapter was not confirmed. Thus, the higher-rate quinmerac treatment did not significantly inhibit soil respiration. The effect of epoxiconazole on the Woburn soil incubated at 25°C was similar to that reported in section 3.3.3, and there was also little effect on the respiration rate of the Broadbalk soil. Epoxiconazole did appear to have a slight inhibitory effect in the Silsoe soil, however (Tables 4-4 and 4-6), but this was only temporary.

The effect of temperature on the respiration rates of each soil was largely quantitative, as would be expected. However, some differences in CO_2 evolution between soil types could be seen. The mean amount of CO_2-C evolved from the epoxiconazole-treated Woburn soil at 15°C, after 7 days incubation, was about 12 and 15% less than that from the

Table 4-4: Cumulative CO_2 evolution from epoxiconazole-treated soils at 15°C (figures in brackets = s.d.).

Sample Time (Days)	μg CO_2-C evolved g^{-1} soil								
	Woburn			Broadbalk			Silsoe		
	0×	1×	10×	0×	1×	10×	0×	1×	10×
7	266 (5.2)	269 (5.3)	267 (13.5)	307 (7.5)	304 (3.3)	299 (9.9)	321 (2.6)	314 (2.3)	312** (1.9)
14	414 (1.6)	420 (3.7)	416 (1.4)	435 (1.5)	430* (1.5)	422* (1.9)	468 (0.1)	459* (1.1)	457 (2.8)
28	619 (3.9)	625 (5.2)	616 (10.8)	578 (2.9)	573 (3.3)	563 (2.0)	637 (0.4)	620 (11.8)	612 (10.0)
42	774 (5.3)	782 (2.5)	764 (9.6)	672 (2.0)	670 (4.4)	656 (2.2)	752 (1.5)	730 (13.0)	714 (13.8)
56	901 (3.1)	910 (1.2)	884* (2.6)	747 (0.8)	740 (4.4)	725** (1.3)	845 (2.4)	816 (11.9)	793 (12.8)
70	1421 (10.9)	1440 (7.2)	1401 (9.7)	1237 (14.5)	1219 (4.6)	1205 (8.0)	1277 (0.5)	1232 (23.0)	1191 (17.7)
84	1643 (1.2)	1653 (7.4)	1610** (2.6)	1385 (8.1)	1375 (6.0)	1359 (1.3)	1425 (1.5)	1374 (5.7)	1328 (8.7)
98	1791 (9.6)	1785 (6.4)	1739* (6.0)	1483 (9.3)	1475 (1.5)	1459 (1.2)	1534 (1.6)	1478 (7.9)	1430 (7.3)
112	1883 (7.2)	1877 (4.6)	1828 (3.4)	1550 (6.4)	1543 (4.8)	1527 (3.7)	1621 (1.0)	1556 (13.2)	1504 (11.3)
140	2592 (33.0)	2568 (32.5)	2527 (9.0)	2230 (20.7)	2225 (4.0)	2207 (7.0)	2224 (10.4)	2138 (44.4)	2076 (34.7)
172	2809 (9.0)	2786 (7.4)	2740 (7.8)	2406 (1.0)	2409 (16.6)	2389 (9.4)	2463 (38.1)	2348 (10.7)	2298 (12.8)
199	3509 (31.5)	3487 (27.6)	3416 (4.4)	3065 (8.9)	3070 (12.3)	3054 (10.5)	3117 (61.8)	2970 (26.1)	2935 (18.6)
224	3692 (6.1)	3677 (9.1)	3596 (12.5)	3227 (1.7)	3233 (6.8)	3218 (3.0)	3340 (32.4)	3181 (18.1)	3166 (8.8)
266	3879 (9.5)	3860 (19.4)	3784 (2.5)	3391 (8.7)	3384 (2.9)	3379 (2.2)	3598 (13.4)	3456 (36.6)	3467 (43.6)

Values significantly different from 0× treatment indicated by * ($P<0.05$) and ** ($P<0.01$)

Table 4-5: Cumulative CO_2 evolution from quinmerac-treated soils at 15°C (figures in brackets = s.d.).

Sample Time (Days)	μg CO_2-C evolved g^{-1} soil								
	Woburn			Broadbalk			Silsoe		
	0×	1×	10×	0×	1×	10×	0×	1×	10×
7	270 (1.0)	295** (5.1)	287* (7.1)	293 (1.0)	327** (2.5)	311** (1.7)	300 (5.0)	320* (4.0)	311 (11.9)
14	422 (1.1)	440 (5.5)	440 (3.7)	414 (1.9)	461** (2.0)	440 (5.8)	446 (0.5)	470 (4.0)	461* (2.5)
28	625 (1.9)	647* (1.8)	653** (2.2)	554 (0.4)	607 (3.6)	581 (10.4)	597 (12.7)	638 (3.3)	611 (12.2)
42	780 (2.0)	811** (1.4)	814 (3.2)	646 (2.6)	704 (2.5)	672 (5.9)	699 (14.0)	753 (1.7)	709 (3.5)
56	906 (3.2)	943* (0.2)	939* (6.0)	714 (0.2)	773 (2.3)	741 (6.1)	776 (13.5)	844 (0.9)	7829 (0.5)
70	1443 (6.5)	1478 (11.0)	1475 (11.9)	1184 (1.4)	1244 (9.9)	1226 (7.2)	1177 (16.0)	1256 (11.6)	1173 (1.7)
84	1658 (4.1)	1697 (3.5)	1688 (3.9)	1329 (3.4)	1386 (3.3)	1369 (6.1)	1311 (12.6)	1400 (3.3)	1303 (1.3)
98	1792 (4.7)	1834 (4.0)	1811 (19.9)	1424 (7.5)	1477 (2.6)	1460 (2.2)	1409 (11.0)	1507 (1.6)	1397 (2.6)
112	1883 (1.6)	1924 (3.5)	1897 (3.5)	1487 (4.2)	1541 (1.1)	1522 (2.2)	1480 (12.7)	1590 (1.5)	1471 (8.1)
140	2570 (6.5)	2621 (26.4)	2564 (13.2)	2159 (0.3)	2192* (5.9)	2174** (2.5)	2043 (29.7)	2166 (7.0)	2024 (2.2)
172	2781 (4.5)	2834 (3.5)	2755 (21.3)	2337 (0.7)	2365 (4.8)	2349 (5.9)	2254 (10.8)	2354* (6.8)	2242 (6.1)
199	3477 (23.0)	3524 (12.5)	3427 (12.1)	2923 (90.2)	3025 (14.9)	3011 (9.2)	2869 (19.1)	2954 (6.0)	2891 (7.4)
224	3672 (10.6)	3704 (18.2)	3611 (2.9)	3061 (8.0)	3177 (3.4)	3170* (2.8)	3059 (22.4)	3133 (9.0)	3115 (9.0)
266	3874 (10.4)	3887 (26.3)	3792* (8.5)	3211 (1.4)	3337 (7.4)	3332* (4.0)	3308 (41.0)	3360 (11.4)	3423 (24.7)

Values significantly different from 0× treatment indicated by * ($P<0.05$) and ** ($P<0.01$)

Table 4-6: Cumulative CO_2 evolution from epoxiconazole-treated soils at 25°C (figures in brackets = s.d.).

Sample Time (Days)	μg CO_2-C evolved g^{-1} soil								
	Woburn			Broadbalk			Silsoe		
	0×	1×	10×	0×	1×	10×	0×	1×	10×
7	467 (11.0)	446 (13.1)	447 (5.5)	467 (23.6)	448 (15.4)	428 (12.5)	526 (7.2)	499 (16.2)	490** (9.1)
14	703 (3.0)	685 (2.6)	686 (2.6)	622 (1.8)	612* (4.1)	588* (0.6)	730 (7.4)	701 (5.3)	692 (3.1)
28	972 (3.2)	970* (3.9)	979* (13.3)	775 (2.0)	769 (5.7)	740 (2.7)	958 (3.2)	930 (4.3)	915 (1.0)
42	1188 (4.3)	1195 (0.3)	1199 (10.4)	884 (3.5)	880 (4.8)	850 (2.1)	1137 (9.0)	1108 (1.0)	1089 (5.5)
56	1388 (5.5)	1401 (23.8)	1396 (25.6)	960 (2.8)	962 (3.6)	931* (1.3)	1289 (4.9)	1261 (1.8)	1238 (2.1)
70	2083 (16.1)	2107 (11.5)	2090 (8.4)	1576 (9.6)	1579 (12.4)	1538 (1.2)	1863 (2.9)	1829 (4.3)	1806 (8.8)
84	2272 (14.5)	2287 (6.4)	2270 (0.5)	1695 (9.8)	1697 (6.3)	1658 (1.0)	2035 (1.8)	2004 (1.4)	1970* (4.2)
98	2387 (11.5)	2388 (4.9)	2377 (12.0)	1778 (2.8)	1785 (2.3)	1744 (1.4)	2174 (2.2)	2148 (2.5)	2101* (2.8)
112	2479 (3.1)	2468 (7.8)	2461 (11.6)	1852 (1.0)	1865 (12.3)	1823 (9.0)	2318 (2.9)	2305** (2.0)	2229** (4.4)
140	3268 (8.9)	3247 (13.0)	3252 (19.8)	2610 (19.3)	2627 (12.8)	2575 (4.7)	3087 (17.7)	3072 (15.0)	2983 (8.4)
172	3449 (33.0)	3413 (17.2)	3436 (11.4)	2766 (4.7)	2782 (6.8)	2740 (5.7)	3357 (8.8)	3345 (10.4)	3237 (6.2)
199	4176 (29.2)	4142 (34.5)	4165 (27.2)	3516 (13.1)	3525 (21.8)	3468 (7.1)	4108 (19.4)	4086 (6.7)	3966 (13.0)
224	4321 (13.8)	4292 (13.5)	4330 (17.3)	3645 (14.7)	3666 (21.9)	3603 (8.2)	4349 (18.1)	4317 (12.2)	4192 (9.7)
266	4481 (24.6)	4466 (14.8)	4509 (28.0)	3785 (8.8)	3819 (9.4)	3744 (8.5)	4628 (12.3)	4591 (6.7)	4471 (19.6)

Values significantly different from 0× treatment indicated by * ($P<0.05$) and ** ($P<0.01$)

Table 4-7: Cumulative CO_2 evolution from quinmerac-treated soils at 25°C (figures in brackets = s.d.).

Sample Time (Days)	μg CO_2-C evolved g^{-1} soil								
	Woburn			Broadbalk			Silsoe		
	0×	1×	10×	0×	1×	10×	0×	1×	10×
7	464 (14.7)	464 (15.7)	471 (7.0)	457 (30.6)	454 (16.3)	460 (11.1)	509 (4.6)	499 (10.1)	500 (9.4)
14	704 (0.7)	710** (1.5)	713 (5.4)	615 (5.6)	620 (3.5)	627 (5.5)	714 (2.9)	705 (4.6)	708 (5.0)
28	977 (9.9)	986 (5.5)	976 (8.5)	761 (1.3)	781** (2.0)	786** (4.8)	938 (2.1)	934 (3.0)	939 (5.2)
42	1198 (14.0)	1207 (7.7)	1180 (9.3)	871 (1.6)	901** (0.5)	895 (6.9)	1113 (5.0)	1113 (4.5)	1117 (4.8)
56	1401 (14.6)	1406 (11.2)	1357* (7.2)	948 (2.9)	978 (10.5)	973 (4.6)	1261 (2.2)	1268* (3.2)	1272 (6.1)
70	2092 (10.5)	2108 (13.9)	2055 (15.4)	1548 (11.4)	1583 (18.7)	1587 (3.9)	1840 (5.3)	1845 (5.5)	1842 (8.8)
84	2297 (26.7)	2302 (23.8)	2265 (21.2)	1660 (3.1)	1701 (11.7)	1706 (3.6)	2003 (2.2)	2013 (2.5)	2009 (1.9)
98	2422 (18.9)	2414 (12.3)	2372 (2.7)	1743 (4.0)	1790 (11.5)	1799* (0.4)	2135 (4.0)	2150 (3.3)	2145 (2.3)
112	2518 (11.1)	2493 (2.2)	2447 (10.4)	1812 (3.5)	1863 (7.1)	1882* (4.6)	2269 (5.2)	2285 (2.6)	2279 (4.8)
140	3302 (18.5)	3257 (23.2)	3203 (66.3)	2556 (19.4)	2641 (2.0)	2657 (10.3)	3032 (14.6)	3068 (4.0)	3037 (1.2)
172	3483 (28.6)	3417 (12.0)	3363 (30.7)	2705 (9.3)	2809 (1.8)	2821 (6.8)	3270 (8.5)	3340* (12.7)	3282 (10.3)
199	4191 (20.0)	4095 (20.7)	4016* (20.2)	3418 (10.2)	3544 (34.6)	3538 (36.3)	3972 (16.9)	4089* (6.5)	3976 (19.8)
224	4333 (22.1)	4224 (15.9)	4141 (13.2)	3538 (11.6)	3685 (14.6)	3679 (14.4)	4191 (14.7)	4305 (12.0)	4178 (12.0)
266	4485 (58.3)	4361 (20.3)	4282 (12.8)	3674 (20.9)	3837 (12.4)	3839 (29.3)	4447 (6.2)	4559 (11.8)	4420 (3.6)

Values significantly different from 0× treatment indicated by * ($P<0.05$) and ** ($P<0.01$)

Broadbalk and Silsoe soils, respectively ($P<0.001$). By day 266, however, the Woburn soil had evolved about 13 and 10% more CO_2-C than the Broadbalk ($P<0.01$) and Silsoe ($P<0.05$) soils, respectively. At 25°C, the mean amount of CO_2-C evolved from the Woburn soil was approximately the same as that from the Broadbalk soil, and about 10% less than from the Silsoe soil ($P<0.001$), after 7 days incubation. By day 266, the total amount of CO_2-C evolved from the Woburn soil was about 19% greater than that from the Broadbalk soil ($P<0.001$), but was not significantly different from the Silsoe soil.

The situation with the quinmerac-treated soils was quite similar. At 15°C the mean amount of CO_2-C evolved from the Woburn soil after 7 days incubation was about 8% less than the other two soils ($P<0.001$), and by day 266, the total amount evolved was about 17 and 14% greater than that from the Broadbalk and Silsoe soils, respectively ($P<0.05$). At 25°C, the mean amount of CO_2-C evolved from the Woburn soil was about 2% greater than that from the Broadbalk soil ($P<0.05$), and about 7% less than from the Silsoe soil ($P<0.001$), after 7 days incubation. By day 266, the total amount of CO_2-C evolved from the Woburn soil was about 16% greater than that from the Broadbalk soil ($P<0.05$), but was not significantly different from the Silsoe soil.

The main differences caused by the 10°C increase in temperature therefore, were that at 25°C the Woburn soil evolved more CO_2 relative to the Broadbalk soil than at 15°C, and that the Silsoe soil evolved more CO_2 relative to the Woburn soil at 25°C than at 15°C, for both

epoxiconazole- and quinmerac-treated soils. This could possibly be due to differences in the mesophilic and thermophilic components of the microbial population of each soil.

4.3.4 EFFECT OF SOIL TYPE ON PESTICIDE MINERALISATION

4.3.4.1 *Epoxiconazole*

The general pattern of $^{14}CO_2$-C evolution over time from the ^{14}C-labelled epoxiconazole in all three soils was hyperbolic (Figs. 4-3 and 4-4). The mineralisation rate was initially fastest in the sandy loam Woburn soil, slowest in the clay Silsoe soil, and intermediate in the silty clay loam Broadbalk soil. This was as might be expected, even though the Woburn soil did not have the largest microbial biomass (Fig. 4-1), as clay particles are known to increase the stability of organic substrates in soil (van Veen *et al.*, 1985). However, sorption of most uncharged, low-solubility pesticides (as is epoxiconazole) in surface soils is generally dominated by interaction with hydrophobic sites on soil organic matter (Green and Karickhoff, 1990). Thus, the availability of epoxiconazole, and hence its mineralisation, might be expected to be less in the Woburn than the Broadbalk soil, as the former's organic C content was nearly twice that of the latter (Table 4-1).

At 15°C the amount of $^{14}CO_2$ evolved from the Broadbalk soil exceeded that evolved from the Woburn soil after only 14 days incubation, and continued to do so from then onwards. The Broadbalk soil had the smallest microbial biomass (Fig. 4-1), and also contained nearly twice as

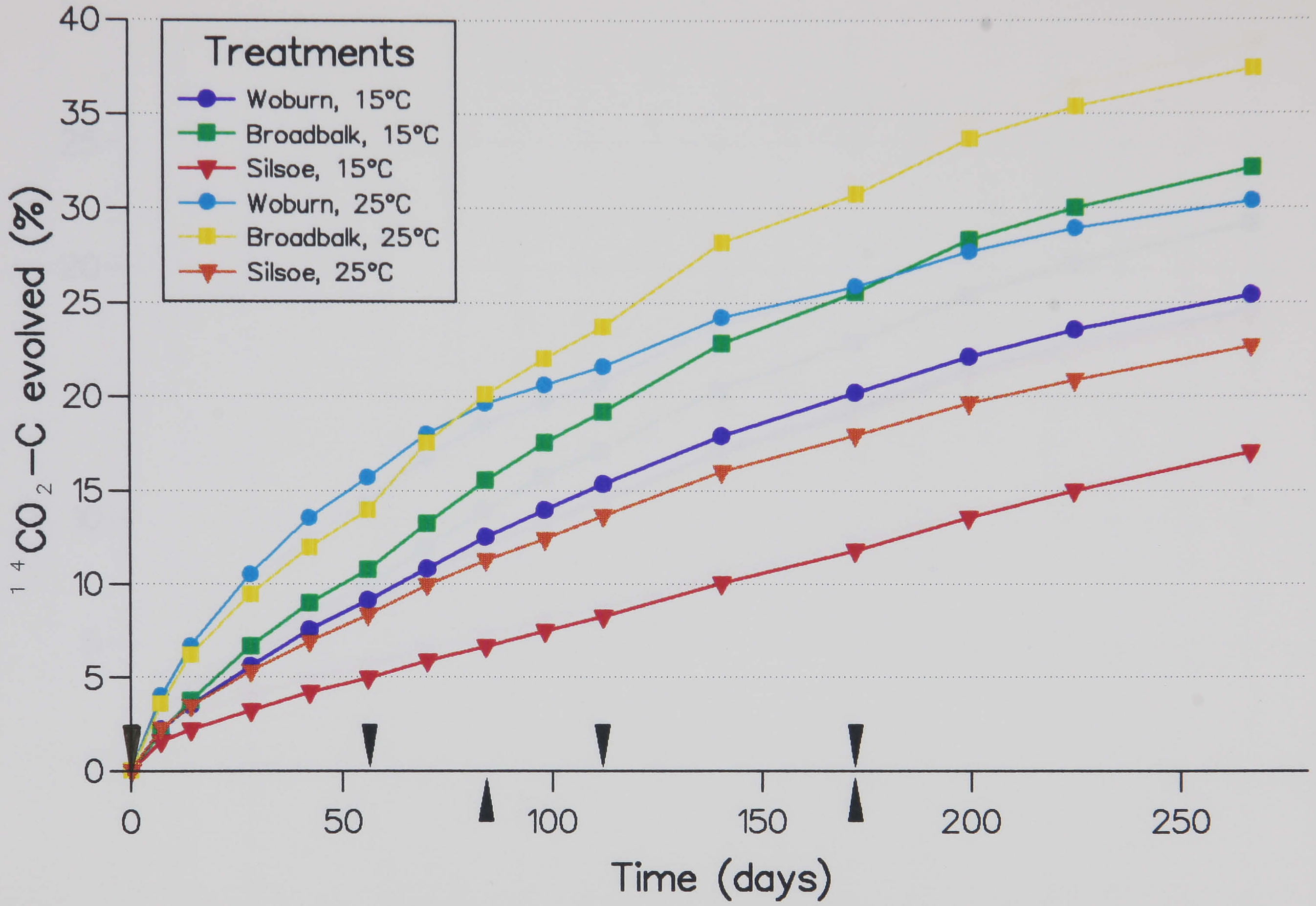


Figure 4-3: Cumulative $^{14}CO_2$-C evolution of 0.25 μg g^{-1} epoxiconazole-treated soils, expressed as % of the original amount of ^{14}C added. Down arrows indicate addition of ryegrass, up arrows indicate further additions of fungicide (see Appendix 2 for s.d.).

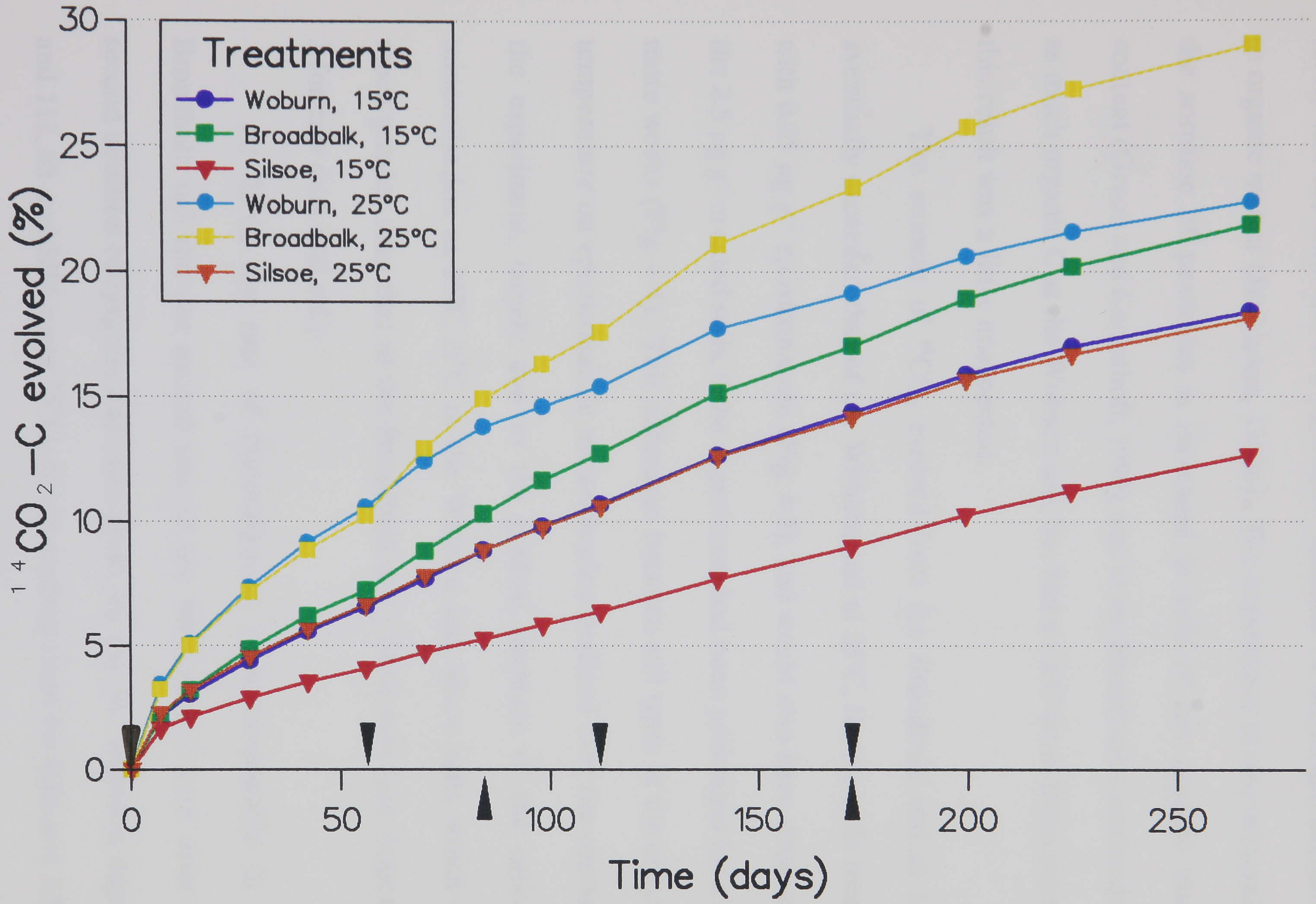


Figure 4-4: Cumulative $^{14}CO_2$-C evolution of 2.5 μg g^{-1} epoxiconazole-treated soils, expressed as % of the original amount of ^{14}C added. Down arrows indicate addition of ryegrass, up arrows indicate further additions of fungicide (see Appendix 2 for s.d.).

much clay as the Woburn soil (Table 4-1), but the difference in soil organic matter content may account for this. However, both organic matter and clay contribute to the colloidal properties of a soil, and only rarely can the sorption of a particular pesticide be attributed exclusively to organic matter (Jenkinson, 1988a). The importance of clay minerals in the sorption of pesticides is increased in soils of low organic matter content (Green and Karickhoff, 1990), and as the Broadbalk contained half as much organic C as the Woburn soil, but had a CEC three times greater, this result was a little unexpected.

The amount of $^{14}CO_2$ evolved from the Broadbalk soil at 15°C eventually exceeded that of the Woburn soil at 25°C, for the soils treated with 0.25 μg g^{-1} epoxiconazole (Fig. 4-3), and would also have done so in the 2.5 μg g^{-1}-treated soils, if the experiment had been prolonged for a few more weeks (Fig. 4-4). This difference between soil types of the effect of temperature on epoxiconazole mineralisation declined over the course of the experiment, largely due to the gradual decrease of the ratio of mineralisation at 25°C:15°C in the Woburn and Silsoe soils, which was much greater than that of the Broadbalk soil (Table 4-8) (see Appendix 2 for raw data and s.d.).

At 25°C, the rate of mineralisation of epoxiconazole in the Broadbalk soil did not exceed that of the Woburn soil until after the second addition of ryegrass had been given, on day 56. Between days 56 and 112, 65 and 52% more $^{14}CO_2$-C was evolved from the 0.25 and 2.5 μg g^{-1} epoxiconazole-treated Broadbalk soil than from the correspondingly

Table 4-8: Ratios of total $^{14}CO_2$-C evolved at 25°C:15°C in epoxiconazole-treated soils

Soil/Pesticide rate (μg g^{-1} soil)	Time (days)		
	56	112	266
Woburn – 0.25	1.72:1	1.41:1	1.20:1
Woburn – 2.5	1.61:1	1.44:1	1.24:1
Broadbalk – 0.25	1.29:1	1.23:1	1.16:1
Broadbalk – 2.5	1.42:1	1.38:1	1.33:1
Silsoe – 0.25	1.68:1	1.65:1	1.33:1
Silsoe – 2.5	1.64:1	1.66:1	1.43:1

Table 4-9: Ratios of total $^{14}CO_2$-C evolved at 25°C:15°C in quinmerac-treated soils

Soil/Pesticide rate (μg g^{-1} soil)	Time (days)		
	56	112	266
Woburn – 1.0	1.78:1	1.36:1	1.20:1
Woburn – 10.0	2.04:1	1.66:1	1.34:1
Broadbalk – 1.0	2.77:1	2.22:1	1.47:1
Broadbalk – 10.0	2.69:1	2.23:1	1.67:1
Silsoe – 1.0	3.08:1	1.79:1	1.18:1
Silsoe – 10.0	7.74:1	6.34:1	3.39:1

treated Woburn soil, respectively. Thus, the mineralisation rate of epoxiconazole in these two soils did not follow the same relative pattern at the two incubation temperatures, possibly due to differences in the composition of the soil microbial populations.

The increase in concentration of epoxiconazole caused quite similar decreases in the mineralisation rates in the three different soils, which were slightly greater at 15°C than at 25°C. In the Woburn soil, 27.5 and 25.1% less $^{14}CO_2$-C was evolved from the 2.5 μg g^{-1}-treated soil, compared to the 0.25 μg g^{-1}-treated soil, 32 and 22.4% less in the Broadbalk soil, and 25.6 and 20.1% less in the Silsoe soil, at 15 and 25°C, respectively. Of course, the total amount of pesticide mineralised in the 2.5 μg g^{-1}-treated soils was much greater than in the 0.25 μg g^{-1}-treated soils; it was the relative rate of mineralisation that was decreased. This effect may have been partly due to competition from the increased amount of unlabelled pesticide.

As with total CO_2 evolution, further additions of epoxiconazole had no discernable effect on the mineralisation of the initial fungicide added to the soils. This indicates that the microbial biomass was not becoming adapted to the fungicide, at least in the experimental time period. However, this is not absolute proof, which would require additions of ^{14}C-labelled chemical after an increasing number of non-labelled pesticide additions. The later pesticide additions in this experiment were not ^{14}C-labelled, as it would have been impossible to distinguish between $^{14}CO_2$ evolved from the different pesticide additions.

The increase in $^{14}CO_2$ evolution caused by ryegrass addition varied considerably between the three soils, with the greatest effect occurring in the Broadbalk soil, the least in the Silsoe soil, and the effect in the Woburn soil intermediate between the other two. This was probably related to the relative sizes of the initial soil microbial biomasses, and thus the relative amount of ryegrass added per unit of biomass, and also means that within a given soil type, the rate of mineralisation of epoxiconazole was directly related to the amount of biomass present, although this does not hold between different soils. The successive ryegrass amendments produced smaller and smaller increases in $^{14}CO_2$ evolution. Epoxiconazole is strongly adsorbed by soil colloids (R. Bromilow, pers. com.), but not all the pesticide would be adsorbed to the same degree. It is possible that as the fungicide was mineralised, the fraction that was most strongly bound, *i.e.* least available to the microbial biomass, became proportionately greater and greater. Thus, as the incubation progressed, the remaining fungicide became relatively more resistant to microbial degradation, and the increases in microbial biomass caused by the ryegrass amendments had progressively less and less effect on the rate of fungicide mineralisation.

4.3.4.2 *Quinmerac*

The general pattern of $^{14}CO_2$-C evolution over time from the ^{14}C-labelled quinmerac in all three soils was sigmoidal (Figs. 4-5 and 4-6). As with epoxiconazole, the mineralisation rate of quinmerac was initially fastest in the Woburn soil (16% clay), slowest in the Silsoe soil (72% clay),

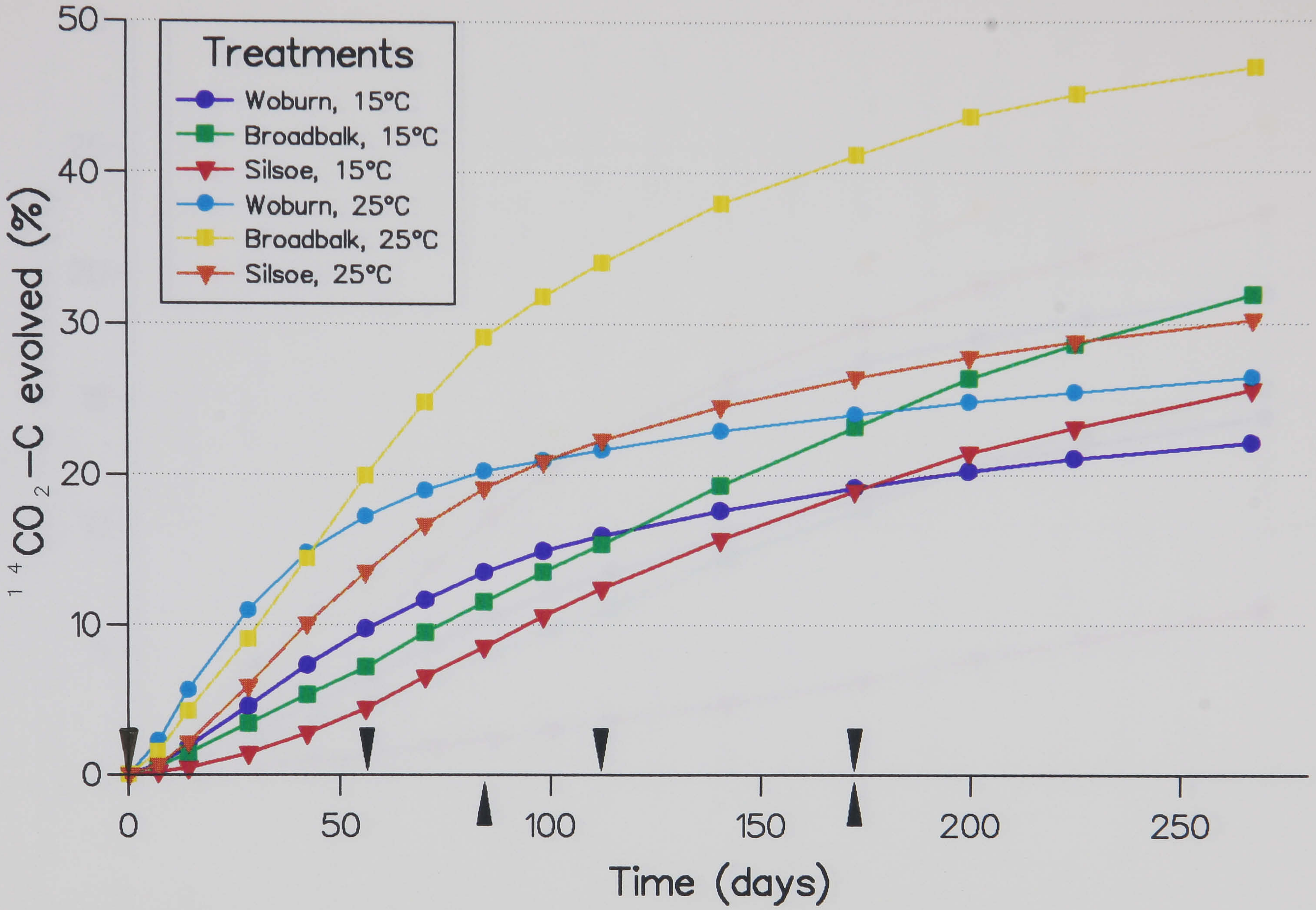


Figure 4-5: Cumulative $^{14}CO_2$-C evolution of 1.0 μg g^{-1} quinmerac-treated soils, expressed as % of the original amount of ^{14}C added. Down arrows indicate addition of ryegrass, up arrows indicate further additions of herbicide (see Appendix 2 for s.d.).

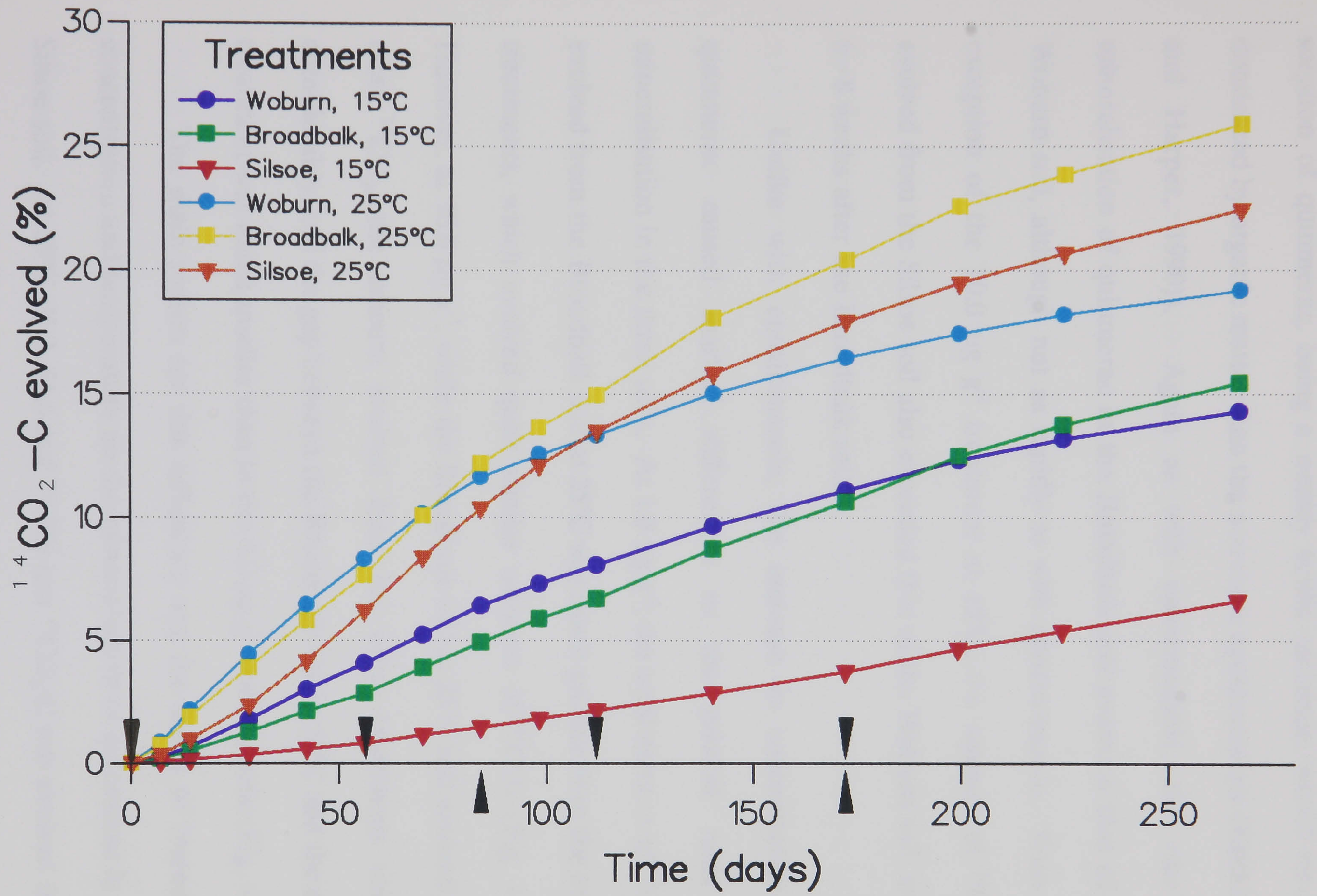


Figure 4-6: Cumulative $^{14}CO_2$-C evolution of 10.0 μg g^{-1} quinmerac-treated soils, expressed as % of the original amount of ^{14}C added. Down arrows indicate addition of ryegrass, up arrows indicate further additions of herbicide (see Appendix 2 for s.d.).

and intermediate in the Broadbalk soil (28% clay) (up to day 14 for all treatments). This was again as might be expected, and was probably directly related to the different clay contents and CEC of the soils (the sorption of quinmerac, being a weakly acidic molecule, would be less dominated by organic matter than the non-ionic epoxiconazole (Koskinen and Harper, 1990)). Again as with epoxiconazole, the rate of mineralisation of quinmerac in the Broadbalk soil overtook that of the Woburn soil, although not as rapidly as with epoxiconazole. With the exception of the 10.0 μg g^{-1} treatment at 15°C, the amount of $^{14}CO_2$ evolved from the Silsoe soil also exceeded that of the Woburn soil, some 6–8 weeks after the Broadbalk soil.

Unlike with epoxiconazole, the increase in concentration of quinmerac caused marked differences in the relative rates of mineralisation in the three soils. At 1.0 μg g^{-1}, the total amount of $^{14}CO_2$ evolved from the Broadbalk soil at 25°C was much greater than the other treatments, which evolved quite similar amounts of $^{14}CO_2$ (Fig. 4-5). However, at 10.0 μg g^{-1}, while the Broadbalk soil at 25°C still evolved the most $^{14}CO_2$, the amount evolved from the other treatments varied considerably, and the gap between the Broadbalk soil at 25°C and the next treatment was much smaller than in the 1.0 μg g^{-1} treated soils (Fig. 4-6).

The main reason for this difference was the effect of increased concentration and temperature on the mineralisation of quinmerac in the Silsoe soil. At 15°C, 35.3, 51.6 and 74.2% less $^{14}CO_2$-C was evolved from the 10.0 μg g^{-1} compared to the 1.0 μg g^{-1} treatments, for the Woburn,

Broadbalk and Silsoe soils, respectively ($P<0.001$). But at 25°C, the corresponding figures were 27.4, 44.8 and 25.8% ($P<0.001$). The difference in the effect of temperature on the mineralisation of quinmerac is shown in Table 4-9, and was particularly marked in the 10.0 μg g^{-1} treatment. Thus it seems that the increase in concentration of quinmerac had the least effect on its mineralisation at 25°C, but the greatest effect at 15°C. As with epoxiconazole, further additions of quinmerac had no effect on the mineralisation of the initial herbicide added to the soils.

After the fact that the pattern of quinmerac mineralisation was quite different from that of epoxiconazole, the most noticible difference between the two pesticides was that the addition of ryegrass had much less effect on the evolution of $^{14}CO_2$ from the quinmerac-treated soils. In all soils treated with 1.0 μg g^{-1} quinmerac, the increase in the rate of herbicide mineralisation was negligible, while in the 10.0 μg g^{-1}-treated soils, the increase was greatest in the Broadbalk soil, less, but still noticeable in the Woburn soil, and remained negligible in the Silsoe soil.

A possible explanation for this is that, at the 1.0 μg g^{-1} rate of application, the amount of herbicide present was less than the maximum amount that the microbial biomass was capable of mineralising. However, at 10.0 μg g^{-1} soil, the amount of microbial biomass in the Broadbalk and Woburn soils was rate limiting, and thus the increase in biomass caused by the ryegrass amendments had a noticeable effect on the rate of quinmerac mineralisation. In general, the mineralisation of quinmerac appeared to have little or no direct relationship to the size of the total microbial

biomass, within or between soil types. Both epoxiconazole and quinmerac underwent the greatest amount of mineralisation in the Broadbalk soil, which had the lowest microbial biomass content. The effect of the microbial biomass on the degradation of these chemicals must therefore have been qualitative as well as quantitative.

One possible explanation for this may be the different management of the three soils. The Woburn and Silsoe soils had both supported growing plant populations prior to being sampled, while the Broadbalk soil was taken from a fallow strip, and had received virtually no plant inputs for at least 12 months. It is possible that this lack of organic C input into the Broadbalk soil put selective pressure on the microbial population, which favoured micro-organisms with the ability to metabolise recalcitrant organic material such as humus and lignin, and also the two pesticides. If the Broadbalk soil contained proportionately more of such micro-organisms than the other two soils, this might explain the increased rates of pesticide mineralisation. The following chapter describes an experiment designed to test this hypothesis.

4.3.5 ^{14}C BALANCE SHEET

The total amount of ^{14}C accounted for, from measurements of residual soil ^{14}C and $^{14}CO_2$ evolved over the incubation period, ranged from around 83 to 102% of the original amounts of ^{14}C added to the soils, with an average of 90.8% (±4.22) (Table 4-10). Some of the ^{14}C unaccounted for would have been lost to the atmosphere as $^{14}CO_2$ when

Table 4-10: Balance sheet of residual soil ^{14}C and $^{14}CO_2$-C evolved from Woburn (W), Broadbalk (B) and Silsoe (S) soil.

Temperature	Soil/ Treatment	Residual soil ^{14}C (Bq g^{-1})	$^{14}CO_2$-C evolved (Bq g^{-1})	Total (Bq g^{-1})	% ^{14}C accounted for
15°C	WF×1	222.72	89.63	312.35	88.6
	WF×10	237.20	59.84	297.04	91.4
	WH×1	200.35	67.42	267.77	88.0
	WH×10	204.95	39.98	244.93	87.8
	BF×1	211.84	113.23	325.07	92.2
	BF×10	226.03	70.99	297.02	91.4
	BH×1	155.59	97.16	252.75	83.0
	BH×10	225.91	43.11	269.02	96.4
	SF×1	300.01	60.00	360.01	102.1
	SF×10	254.24	41.12	295.36	90.9
	SH×1	184.20	78.13	262.33	86.2
	SH×10	230.73	18.47	249.20	89.3
25°C	WF×1	210.57	107.24	317.81	90.1
	WF×10	206.54	74.05	280.59	86.3
	WH×1	196.76	80.63	277.39	91.1
	WH×10	199.98	53.62	253.60	90.9
	BF×1	211.50	131.86	343.36	97.3
	BF×10	192.27	94.26	286.53	88.2
	BH×1	142.86	142.66	285.52	93.8
	BH×10	175.79	72.12	247.91	88.9
	SF×1	246.55	79.97	326.52	92.6
	SF×10	257.28	58.87	316.15	97.3
	SH×1	174.82	92.11	266.93	87.7
	SH×10	184.33	62.64	246.97	88.5

Amounts of ^{14}C added at day 0 (Bq g^{-1} soil): epoxiconazole (F) ×1 - 352.75; epoxiconazole ×10 - 325.05; quinmerac (H) ×1 - 304.38; quinmerac ×10 - 279.00.

the NaOH vials were changed on each sample date. The distilled water in the bottom of a few incubation jars also contained a small amount of ^{14}C (data not shown). Given that a number of corrections had to made for the efficiency of the combustion system (see section 2.2.4.2), analytical error presumably accounted for the remainder.

4.4 SUMMARY

The mineralisation rates of the pesticides epoxiconazole and quinmerac at two concentrations in three contrasting soils: Woburn (16% clay); Broadbalk (28% clay); and Silsoe (72% clay), and also the pesticides' effect on long-term soil respiration were measured.

The major findings were:

1) The kinetics of the mineralisation of the two pesticides were quite different: the rate of mineralisation of epoxiconazole was fastest over the first few days following its addition to the soil, while there was a delay before the maximum rate of mineralisation of quinmerac was seen. The length of the delay depended on the soil type, pesticide concentration and temperature.

2) Both pesticides underwent the greatest amount of mineralisation in the Broadbalk soil, which had the lowest microbial biomass content of the three soils. This may be related to the low organic matter input into this soil, which was taken from a 1 year fallow.

3) The mineralisation of the pesticides was facilitated to varying degrees by the addition of ground ryegrass to the soils. This effect

was most marked in the Broadbalk soil, probably because it had the largest ratio of ryegrass C to biomass C. The mineralisation of epoxiconazole was affected to a much greater extent than quinmerac by this treatment.

4) The pesticides had only a small temporary effect on soil respiration rates, usually inhibitory, which was generally more marked at the higher concentrations. The slight inhibition caused by quinmerac at 10.0 $\mu g\ g^{-1}$ in the Woburn soil, reported in the previous chapter, was not confirmed, and it was concluded that this treatment had no significant effect. Further additions of the pesticides during the experiment had no significant effect on soil respiration or on the rate of mineralisation of the original ^{14}C-labelled pesticide.

5) Neither pesticide had any really significant effects on the soil microbial biomass C contents at the end of the experiment. A small amount of the biomass C was derived from the pesticides, ranging from 0.3 to 14.4% of the initial amount of ^{14}C-labelled pesticide applied.

CHAPTER 5

MINERALISATION OF EPOXICONAZOLE AND QUINMERAC IN BROADBALK SOILS UNDER DIFFERENT MANAGEMENT AND THE EFFECTS OF RYEGRASS AND GLUCOSE ADDITIONS

5.1 INTRODUCTION

It has been suggested that the rate of microbial degradation of pesticides in soil is a function of three key variables:

1) the availability of the chemicals to the soil micro-organisms or enzyme systems which can degrade them;

2) the quantity of these micro-organisms or enzyme systems; and

3) the level of activity of these micro-organisms or enzyme systems

(Frehse and Anderson, 1983). The availability of a pesticide is mainly determined by its adsorption to and desorption from soil colloids, while the quantity and level of activity of soil micro-organisms is related to a number of factors, such as recent plant inputs, soil organic matter content, soil moisture content, temperature, *etc.* (Anderson, 1984; Harris, 1988b).

Since micro-organisms and microbial activity have such a major role in the degradation of most pesticides in soil, the addition of a readily available carbon source, which would result in a rapid increase in microbial growth and activity, would be expected to result in enhanced pesticide degradation. However, this is not the case with all pesticides. Indeed, different workers have reported contradictory results with the same pesticide. Thus, Soulas and Fournier (1987) reported that the addition of

glucose increased the mineralisation of ^{14}C-labelled 2,4-D, while Kunc (1992) reported the precise opposite. Gunalan and Fournier (1992) found that glucose could either increase or decrease the mineralisation of 2,4-D, the difference apparently depending on the time between the addition of glucose and of 2,4-D to the soil. There are also reports of other substrates, such as sewage sludge and farmyard manure, having no or negative effects on pesticide degradation (Yaron *et al.*, 1985).

In Chapter 4, results showed that the mineralisation of the pesticides epoxiconazole and quinmerac in three soils of contrasting clay content did not proceed quite as expected. The mineralisation of the two pesticides was greatest in soil from the Broadbalk Continuous Wheat Experiment at Rothamsted, which contained the least amount of microbial biomass of the three soils. It was postulated that this may have been related to the low C inputs into the Broadbalk soil, which was from a 1 year fallow, selecting for micro-organisms with the ability to metabolise recalcitrant organic substances.

To test this hypothesis, the mineralisation of epoxiconazole and quinmerac in soil taken from three distinct areas of Broadbalk, of differing crop management, was measured. These soils received very different organic C inputs over the course of the experiment's 150 year history, and so may have developed quite distinct microbial populations. Thus, if the above hypothesis is correct, the rate of mineralisation of the two pesticides should be reflected by the soils' cropping history.

In Chapter 4, ground ryegrass was added simply to prevent excessive

decline in the microbial population during the incubation. It was not intended as an experimental treatment, and an appropriate control (*i.e.* soils with no ryegrass amendment) was not included. However, the addition of ryegrass to the soils also had an effect on the mineralisation of the two pesticides, with epoxiconazole affected much more than quinmerac. In the work reported below, a nil treatment is included, and the effects of ryegrass amendment on the rate of pesticide mineralisation are also compared with a more readily available C substrate, glucose.

5.2 MATERIALS AND METHODS

5.2.1 SAMPLING AND PREPARATION OF SOIL

Soil was collected from three distinct areas of the Broadbalk Continuous Wheat Experiment at Rothamsted: plot 09, Section 9 - 150 years continuous wheat (35 years since last fallow); plot 09, Section 2 -1.5 years fallow after wheat; and from the centre of the path between plots 08 and 09, Section 9 - 150 years "fallow" (Johnston, 1969). Soil from these samples are subsequently referred to as "Wheat", "Fallow" and "Path", respectively. The soils were sieved, and pre-incubated for 10 days, as described in section 2.1.1.

5.2.2 SOIL CHEMICAL ANALYSES

Soil pH, CEC, organic C, total N and texture were determined for each soil sample, as described in section 2.2.5. Soil ^{14}C at the end of the experiment was determined as described in section 2.2.4.2.

5.2.3 PESTICIDE TREATMENTS

Each pesticide was added at a single rate: epoxiconazole at 0.25 μg g^{-1} soil, quinmerac at 1.0 μg g^{-1} soil. The pesticide solutions were prepared in the same manner as described in section 4.2.3, with a proportion of the active ingredients labelled with ^{14}C, to give approximately 0.333 kBq g^{-1} soil. The solutions were prepared such that the appropriate amount of pesticide was contained in the volume of solution required to bring the soil to 50% WHC, and were added to the soil as described in section 2.1.3. The final ^{14}C content of the solutions was checked by scintillation counting of duplicate 0.1 ml aliquots in 10 ml Ultima Gold cocktail.

A total of 18 samples of each soil type were weighed out as described in section 2.1.2. Aliquots (2.25 ml) of the epoxiconazole solution were added to half of these samples, and 2.25 ml aliquots of the quinmerac solution to the other half. The soil samples were incubated for 84 days at 25°C as described in section 2.1.2, arranged in randomized blocks.

5.2.4 SUBSTRATE AMENDMENTS

After 28 days incubation, 1 ml 0.69 M glucose solution was added to three samples of each set of pesticide-treated soils, using a syringe and hypodermic needle. A further three samples were taken, and 125.6 mg air-dry, ground ryegrass carefully mixed into each soil sample. Each of these amendments added 1000 μg C g^{-1} soil. Ammonium sulphate was added to the glucose solution, to give a C:N ratio of 15:1. Ammonium sulphate solution (1 ml, 0.69 mM) was added as above to the soils receiving

ryegrass, which, including the N content of the ryegrass, also gave a C:N ratio of 15:1. Distilled water (1 ml) was added to the remaining three samples of each set of soils, which served as controls. This procedure was repeated on day 56.

5.2.5 TOTAL AND ^{14}C-LABELLED MICROBIAL BIOMASS C MEASUREMENTS

Total and ^{14}C-labelled microbial biomass was determined as described in sections 2.2.1 and 2.2.4.1, respectively, at day 0 (total microbial biomass C only) and at day 84 (total and ^{14}C-labelled microbial biomass C).

5.2.6 TOTAL CO_2 AND $^{14}CO_2$ EVOLUTION MEASUREMENTS

Total CO_2 and $^{14}CO_2$ evolution was measured as described in sections 2.2.3 and 2.2.4.1, respectively, after 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77 and 84 days incubation. Measurements on days 3 to 28 were the mean of nine replicates, while those after day 28 were meaned from three replicates.

5.3 RESULTS AND DISCUSSION

5.3.1 SOIL CHARACTERISTICS

The characteristics of the three soils are shown in Table 5-1.

Table 5-1: Characteristics of the three Broadbalk soils.

	Wheat	Path	Fallow
pH (H_2O)	7.9	8.0	8.1
pH (0.01M $CaCl_2$)	7.4	7.4	7.7
CEC (meq 100 g^{-1})	21.8	17.9	29.7
Organic C (%)	1.10	0.80	0.74
Total N (%)	0.130	0.105	0.100
Texture	Silty clay loam (Batcombe series)		

5.3.2 TOTAL AND ^{14}C-LABELLED MICROBIAL BIOMASS C

At day 0, the total microbial biomass C contents of the three soils were about 284, 117 and 107 $\mu g\ g^{-1}$ soil, for the Wheat, Path and Fallow soils, respectively (Fig. 5-1). Thus, the soil from the Continuous Wheat plot contained more than twice as much microbial biomass as the other two soils. The difference between the Path and Fallow soil was statistically significant ($P < 0.001$). This result, and the organic C and total N contents of the two soils, indicate that the Path soil received a greater input of organic material than the Fallow soil, contrary to expectations. This was probably due to lateral root growth from the wheat of the plots on either side of the path.

At day 84, the microbial biomass C in the epoxiconazole-treated, unamended soils had declined slightly, to about 259, 103 and 96 $\mu g\ g^{-1}$ soil, for the Wheat, Path and Fallow soils, respectively (Fig. 5-1). Only the decrease from the day 0 value in the Wheat soil was statistically significant ($P < 0.05$). The addition of glucose caused a greater increase in biomass C than ryegrass in the Wheat soil, but the difference was not significant at the 5% level because of the high standard error. There was no difference between the increase in biomass C content caused by the substrate amendments in the other two soils.

Biomass C contents in the quinmerac-treated, unamended soils at day 84 had increased slightly compared to that at day 0, to about 315, 140 and 141 $\mu g\ g^{-1}$ soil, for the Wheat, Path and Fallow soils, respectively (Fig. 5-2). Only the increase in the Wheat soil was statistically significant

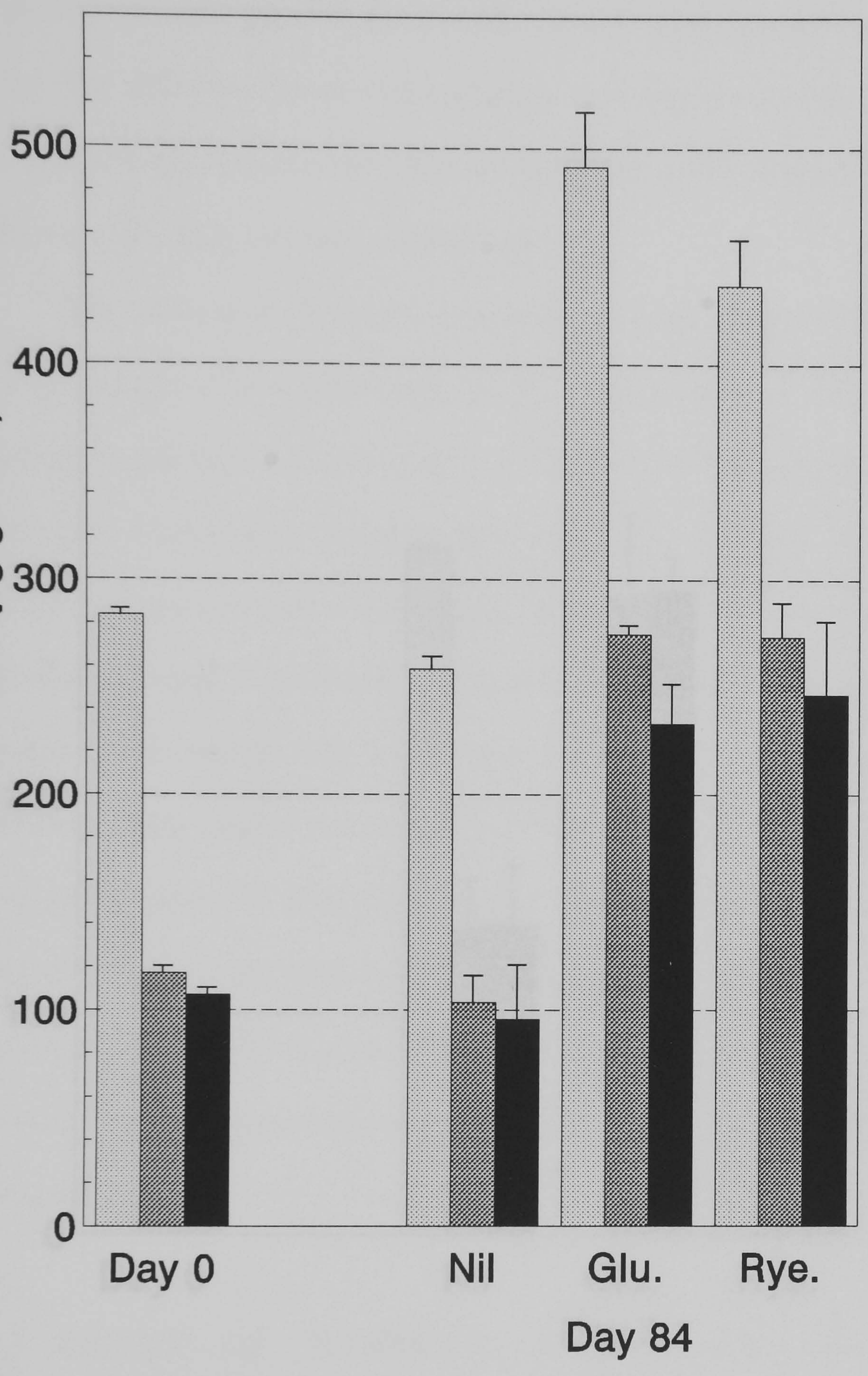


Figure 5-1: Microbial biomass C contents of the Wheat, Path and Fallow soils at day 0, and of unamended (Nil), glucose- (Glu.) and ryegrass-amended (Rye.) epoxiconazole-treated soils at day 84 (bars = s.d.).

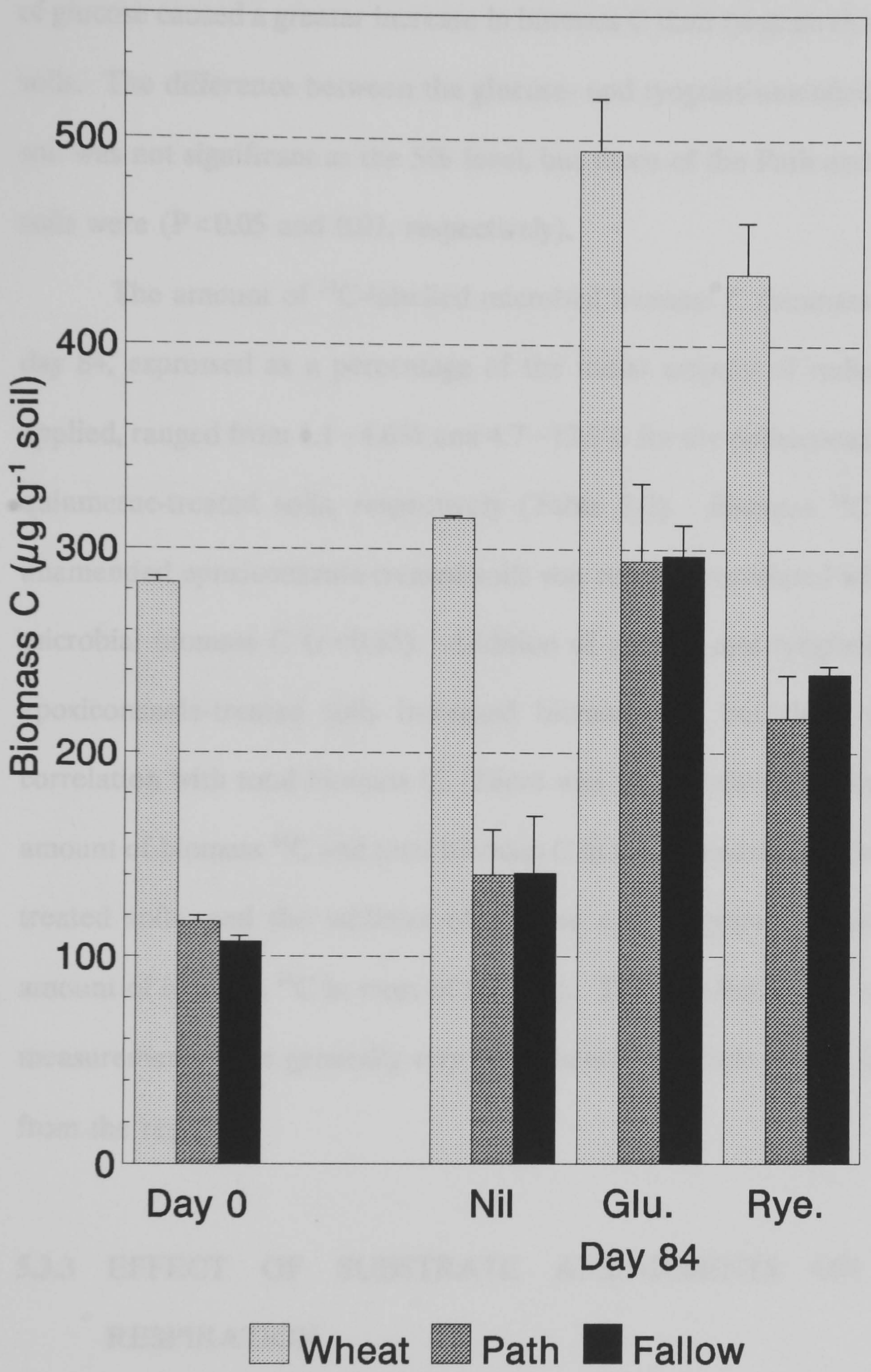


Figure 5-2: Microbial biomass C contents of the Wheat, Path and Fallow soils at day 0, and of unamended (Nil), glucose- (Glu.) and ryegrass-amended (Rye.) quinmerac-treated soils at day 84 (bars = s.d.).

($P<0.001$), the other two soils having high standard errors. The addition of glucose caused a greater increase in biomass C than ryegrass in all three soils. The difference between the glucose- and ryegrass-amended Wheat soil was not significant at the 5% level, but those of the Path and Fallow soils were ($P<0.05$ and 0.01, respectively).

The amount of ^{14}C-labelled microbial biomass C (biomass ^{14}C) at day 84, expressed as a percentage of the initial amount of radioactivity applied, ranged from 1.1 – 4.6% and 4.7 – 12.3% for the epoxiconazole and quinmerac-treated soils, respectively (Table 5-2). Biomass ^{14}C in the unamended epoxiconazole-treated soils was roughly correlated with total microbial biomass C ($r=0.85$). Addition of glucose and ryegrass to the epoxiconazole-treated soils increased biomass ^{14}C, but there was no correlation with total biomass C. There was no correlation between the amount of biomass ^{14}C and total biomass C in the unamended quinmerac-treated soils, and the addition of glucose and ryegrass decreased the amount of biomass ^{14}C in most of the soils. The standard errors of these measurements were generally very large, hence very little can be inferred from the results.

5.3.3 EFFECT OF SUBSTRATE AMENDMENTS ON SOIL RESPIRATION

The initial amounts of CO_2-C evolved from the epoxiconazole-treated soils were in the order Wheat > Fallow > Path (Fig. 5-3), each soil evolving significantly different amounts of CO_2 over the first 28 days

Table 5-2: Soil microbial biomass ^{14}C contents at day 84, as % of the initial amount of radioactivity applied (figures in brackets = s.d.).

Pesticide	Soil	Microbial biomass ^{14}C (%) substrate amendment		
		Nil	Glucose	Ryegrass
Epoxiconazole	Wheat	2.4 (0.24)	3.6 (0.13)	3.7 (0.17)
	Path	1.8 (0.92)	3.6 (0.70)	3.8 (1.86)
	Fallow	1.1 (0.65)	2.3 (1.12)	4.6 (0.67)
L.S.D. (P=0.05)		1.23	1.80	2.14
Quinmerac	Wheat	8.3 (0.19)	7.3 (0.59)	9.3 (0.72)
	Path	10.1 (2.29)	7.3 (2.00)	12.3 (6.86)
	Fallow	10.3 (2.99)	4.7 (4.10)	7.5 (4.21)
L.S.D. (P=0.05)		5.38	5.92	10.11

of incubation ($P<0.001$). Over the next 56 days incubation, the unamended Wheat soil continued to evolve significantly more CO_2 than the other two unamended soils. This was as expected, as the Wheat soil had a much greater microbial biomass and organic matter content than the other two soils. The Path soil tended to evolve less CO_2-C than the Fallow soil, but there were no significant differences between these two soils. This was most likely due to the reduction in the number of sample replicates after day 28, when there was a concomitant increase in standard error. The initial difference in CO_2 evolution between the Path and Fallow soil was not as expected, as the Path soil had a slightly greater biomass and organic matter content than the Fallow soil, and is difficult to account for.

Addition of ryegrass and glucose caused substantial increases in soil respiration (Fig. 5-3). However, although the amount of C added was the same, the glucose-amended soils evolved significantly more CO_2-C than the ryegrass-amended soils. It is likely that this was mostly because the glucose was more readily available than the ryegrass, and there may also have been a small priming effect (*i.e.* increased mineralisation of soil organic matter) caused by the glucose additions (Wu *et al.*, 1993).

The amounts of CO_2-C evolved from the epoxiconazole-treated soils immediately following glucose addition were not significantly different from one another, on both day 35 and 63. This was partly because there was a marked increase in the standard errors of the measurements (with a mean L.S.D. of 13.08, compared to a mean of 6.25 for the remaining

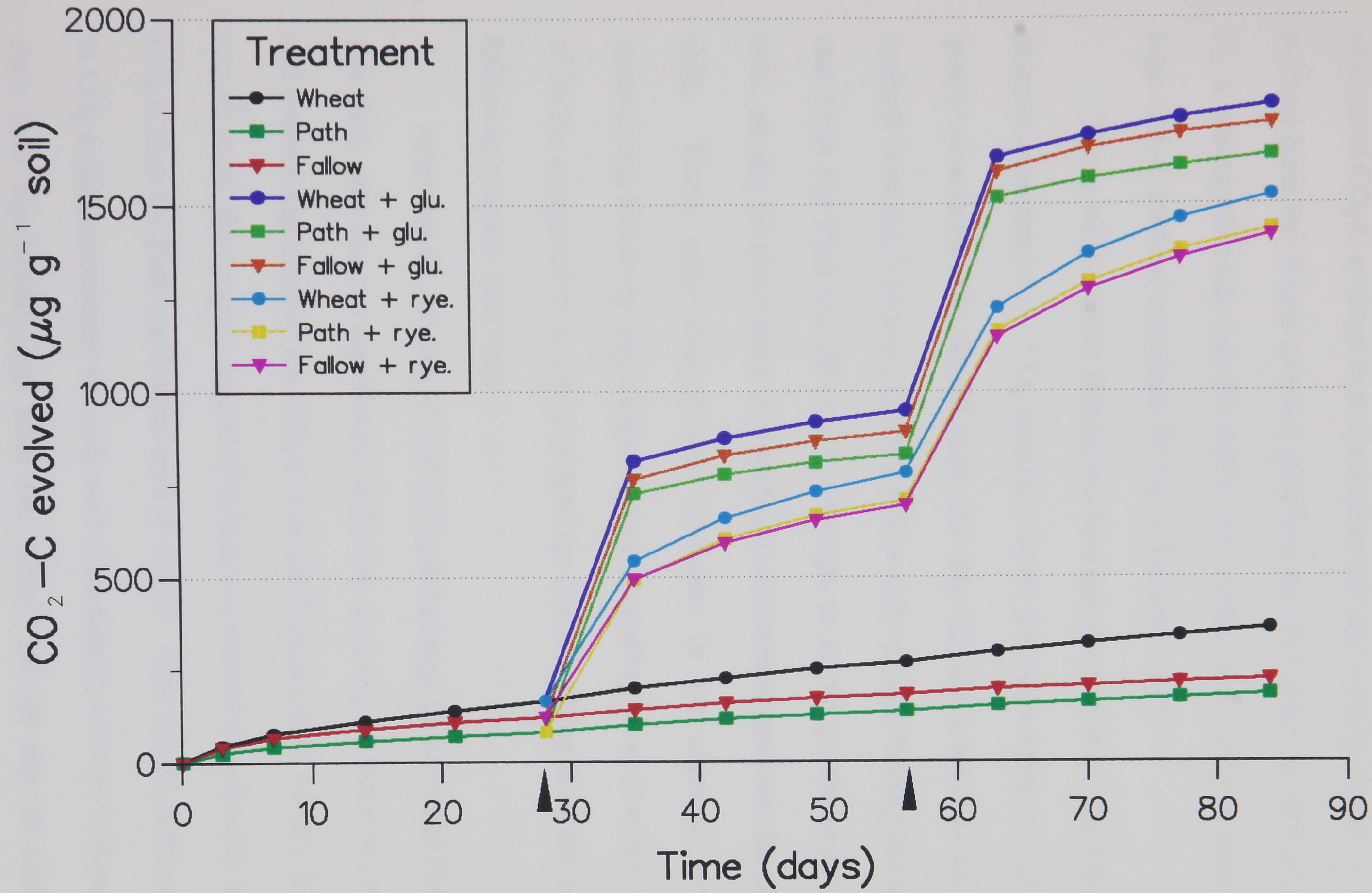


Figure 5-3: Cumulative total CO_2-C evolution in unamended, glucose-amended and ryegrass-amended epoxiconazole-treated Broadbalk soils. Up arrows indicate substrate addition (see Appendix 2 for s.d.).

measurements following glucose addition). From day 35 to 56 and 70 to 84, the CO_2-C evolved from the Path soil was significantly less than that from the Wheat soil, and initially the Fallow soil as well ($P<0.05$). The amount of CO_2-C evolved from the Fallow soil was initially similar to that evolved from the Wheat soil, but gradually declined until, on days 56 and 84, it was significantly less ($P<0.05$). The order of the soils in terms of total CO_2-C evolved remained Wheat > Fallow > Path.

There were relative differences between the effects of ryegrass and glucose amendment on CO_2 evolution from the three soils, as well as quantitative ones. On day 35 (and also day 42), the amount of CO_2-C evolved from the ryegrass-amended Fallow soil was significantly less than that from the Path soil ($P<0.01$), although, as in the glucose-amended soils, on day 63 there were no significant differences between the three soils. There were even greater increases in the standard errors immediately following ryegrass amendment than with glucose (mean L.S.D. of 36.20, compared to a mean of 8.44 for the remaining measurements following ryegrass addition).

After day 42, the CO_2-C evolved from the ryegrass-amended Fallow and Path soils was not significantly different, until day 84, when the Path soil evolved significantly less CO_2-C than the Fallow soil ($P<0.05$). From day 42 to 56, the ryegrass-amended Wheat soil evolved significantly more CO_2-C than the Path and Fallow soils ($P<0.05$). After day 63, differences in CO_2 evolution between the soils were inconsistent, with the Fallow and Path soils significantly less than the Wheat soil on day 70 and 84,

respectively, and no significant differences on day 77. In contrast to the unamended and glucose-amended soils, while the Wheat soil still evolved the greatest amount of CO_2-C at day 84, the order of the Fallow and Path soils was reversed, with the Path soil evolving slightly more CO_2-C than the Fallow soil (Fig. 5-3). There is no obvious explanation for this occurrence.

The initial CO_2-C evolution from the quinmerac-treated soils was in the same order as the epoxiconazole-treated soils, *i.e.* Wheat > Fallow > Path (Fig. 5-4). As before, the amount of CO_2-C evolved from the three soils over the first 28 days incubation were all significantly different from each other ($P<0.001$). The total CO_2-C initially evolved from the quinmerac-treated soils was greater than that from the epoxiconazole-treated soils; approximately 12, 37 and 17% more, for the Wheat, Path and Fallow soils, respectively, at day 28. By day 84, however, there was no difference between the quinmerac- and epoxiconazole-treated, unamended Wheat soil, while the differences between the other two unamended soils remained approximately constant over the latter 56 days incubation. As with the epoxiconazole-treated unamended soils, there were no significant differences between the CO_2 evolution of the Path and Fallow soils from days 35 to 84.

The effect of glucose addition to the soils was much less consistent in the quinmerac-treated soils. There were no significant differences between the CO_2 evolution of the three soils on days 35, 49 or 63 to 84. On day 42, the amount of CO_2-C evolved from the Wheat and Path soils was significantly less than that from the Fallow soil ($P<0.05$), while the

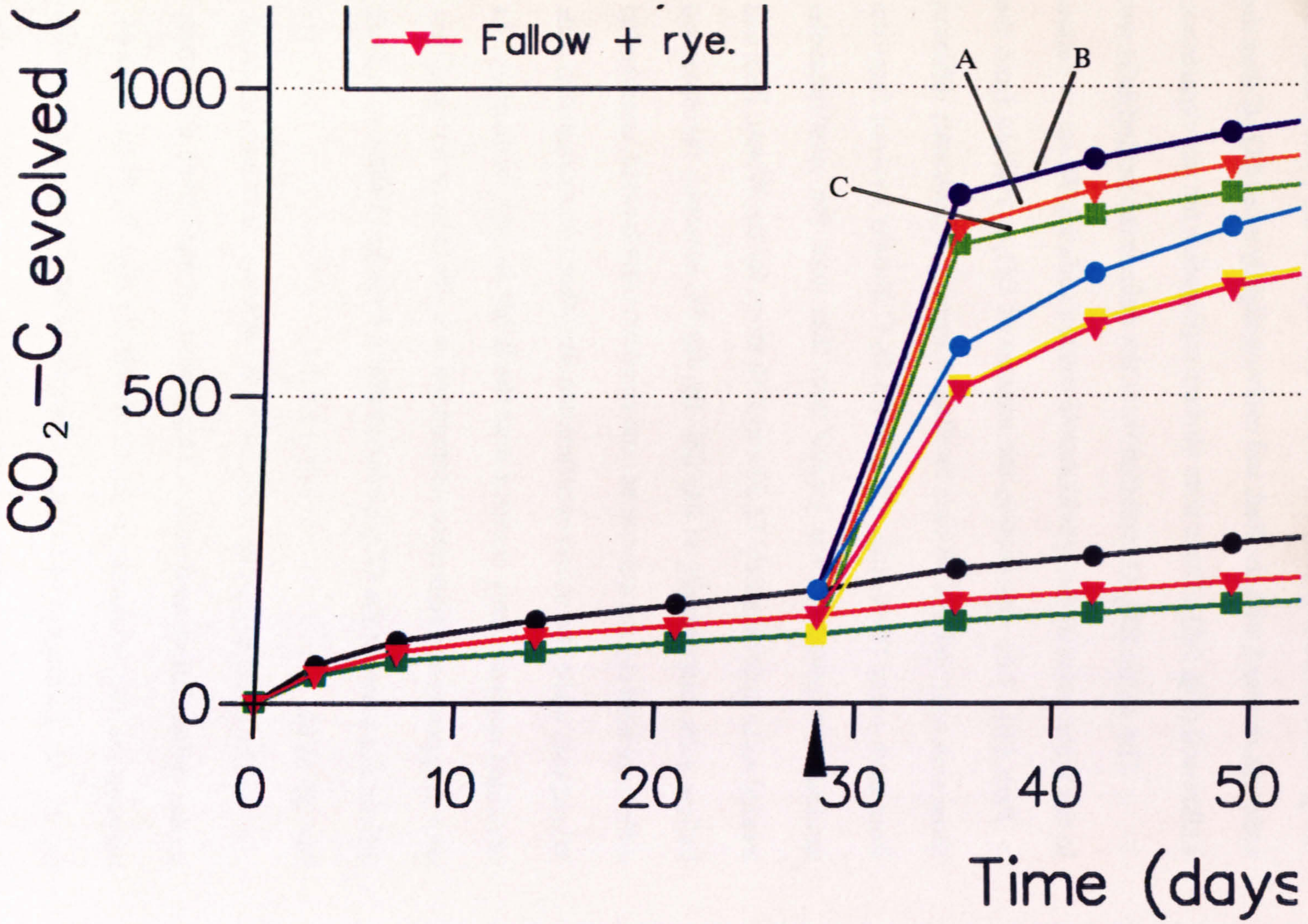


Figure 5-4a: Enlargement of Fig. 5-4 to show the slight difference in respiration rates from days 35-42, between the glucose-amended Fallow soil (A) and the glucose-amended Wheat (B) and Path (C) soils (see p. 91).

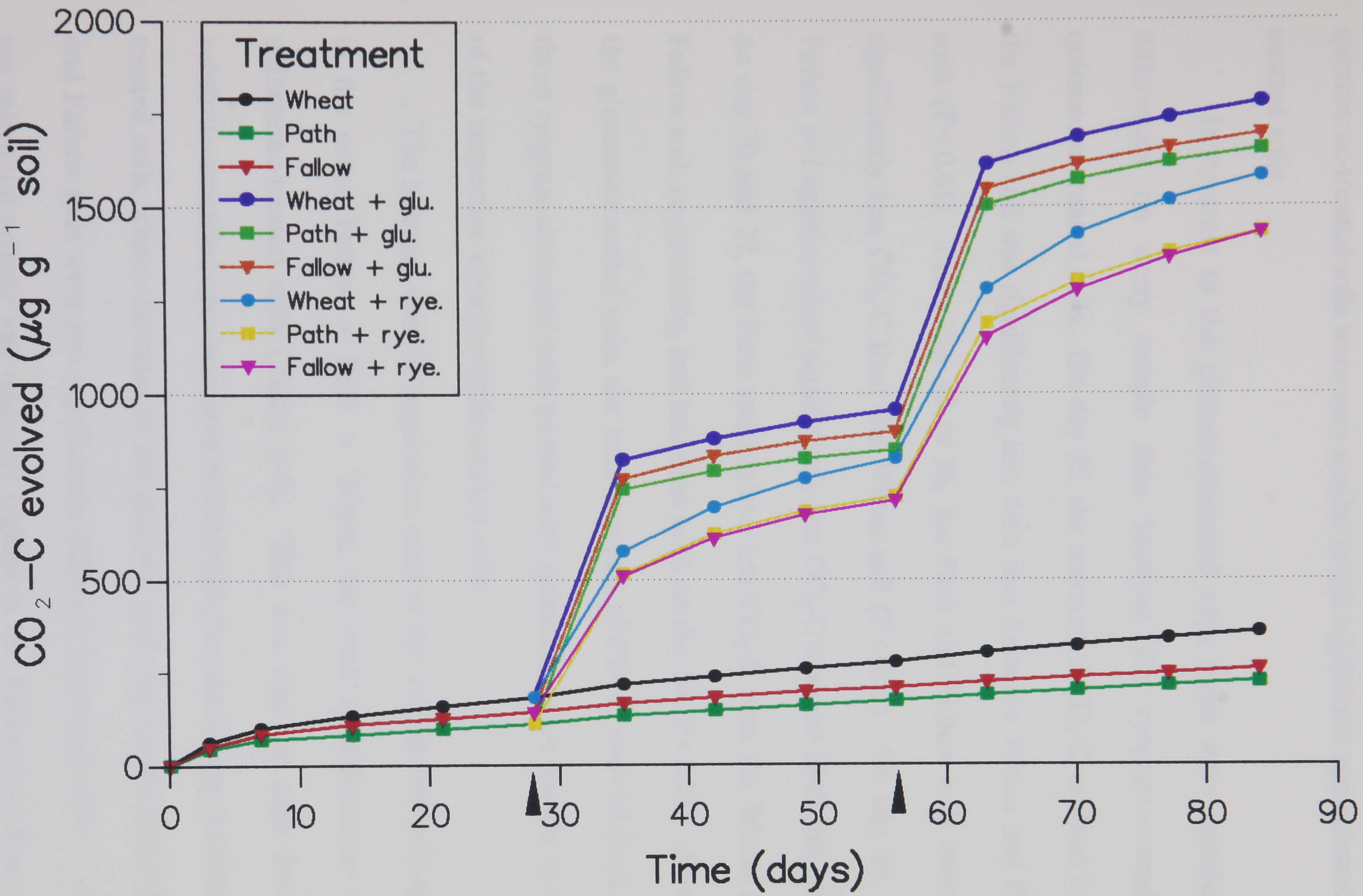


Figure 5-4: Cumulative total CO_2-C evolution in unamended, glucose-amended and ryegrass-amended quinmerac-treated Broadbalk soils. Up arrows indicate substrate addition (see Appendix 2 for s.d.).

only other significant differences were on day 56, where the Path and Fallow soils evolved significantly less CO_2-C than the Wheat soil ($P<0.05$). The total amounts of CO_2-C evolved from the three glucose-amended, quinmerac-treated soils were very similar to the equivalent epoxiconazole-treated soils.

In contrast to the glucose-amended soils, there were significant differences on every sample date between the ryegrass-amended, quinmerac-treated soils. On day 35, the amount of CO_2-C evolved from the Fallow soil was significantly less than that from the Wheat and Path soils ($P<0.01$). From day 42 to 56, the Path and Fallow soils evolved significantly less CO_2-C than the Wheat soil ($P<0.05$). On day 63, the Fallow soil again evolved significantly less CO_2-C than the Path soil, while on day 70 and 77, the Path soil evolved less CO_2-C than the Wheat and Fallow soil, respectively, and less than both on day 84 ($P<0.05$). As with the glucose-amended soils, the total amounts of CO_2-C evolved from the three ryegrass-amended soils treated with quinmerac were similar to that of the respective epoxiconazole-treated soils.

The biomass specific respiration rates of the unamended soils were in the order Fallow > Path > Wheat, for both epoxiconazole- and quinmerac-treated soils (Table 5-3). This was also the case for the substrate-amended epoxiconazole-treated soils, but not for the quinmerac-treated soils, where the rates of the glucose- and ryegrass-amended Path and Fallow soils were not significantly different from each other. Thus, the microbial activity was relatively highest in the Fallow and Path soils

Table 5-3: Biomass-specific respiration rates of epoxiconazole- and quinmerac-treated Broadbalk soils * (figures in brackets = s.d.).

Pesticide	Soil	substrate amendment		
		Nil	Glucose	Ryegrass
		(mg CO_2-C g^{-1} biomass C d^{-1})		
Epoxiconazole	Wheat	16 (1.1)	54 (2.2)	51 (3.3)
	Path	20 (3.0)	99 (2.4)	88 (3.6)
	Fallow	27 (4.8)	120 (7.4)	103 (21.3)
L.S.D. (P=0.05)		6.6	9.3	25.2
Quinmerac	Wheat	14 (0.3)	54 (2.4)	53 (3.5)
	Path	22 (3.6)	96 (9.4)	102 (6.7)
	Fallow	26 (3.4)	100 (5.1)	98 (4.3)
L.S.D. (P=0.05)		5.7	12.6	10.0

* Calculated from the total CO_2-C evolved over the experiment divided by the mean microbial biomass C from days 0 and 84, and by the number of days' incubation.

and lowest in the Wheat soil. This is presumably related to the different amounts of organic C input into each soil.

5.3.4 PESTICIDE MINERALISATION

The rates of $^{14}CO_2$ evolution in the unamended epoxiconazole-treated soil were in the order Wheat > Path > Fallow (Fig. 5-5), and were correlated with the amount of soil microbial biomass ($r=0.98$). The differences were all statistically significant at day 3 ($P<0.001$), but thereafter the amounts of $^{14}CO_2$ evolved from the Path and Fallow soils were not statistically different. The Wheat soil consistently evolved more $^{14}CO_2$ than the other two soils, and the differences remained statistically significant throughout the experiment.

The pattern of $^{14}CO_2$ evolution was hyperbolic, as was found before (see section 4.3.4.1). However, the initial rate of $^{14}CO_2$ evolution was significantly greater than in the Broadbalk soil in the previous experiment (see Fig. 4-3). This was due to deterioration of the ^{14}C-labelled epoxiconazole during the period of storage between the two experiments (R. Bromilow, pers. com.).

The addition of ryegrass led to a greater increase in the evolution of $^{14}CO_2$ than did addition of glucose to the epoxiconazole-treated Wheat and Fallow soils, despite the fact that the ryegrass-amended soils had similar or smaller microbial biomass contents than the glucose-amended ones. This effect did not occur in the Path soil, where the amounts of $^{14}CO_2$ evolved from the two amended soils were almost identical (Fig. 5-5).

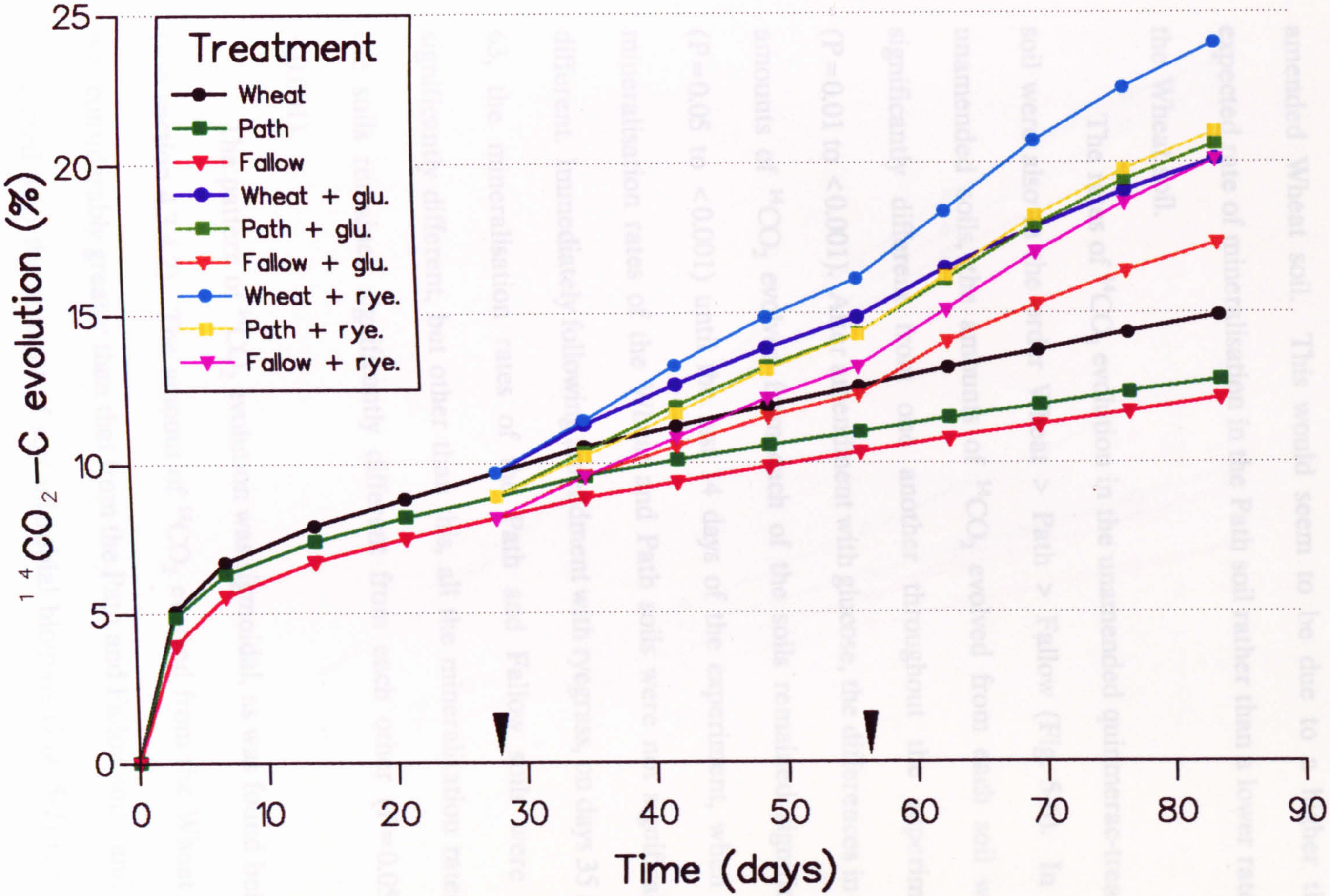


Figure 5-5: Cumulative $^{14}CO_2$-C evolution in unamended, glucose-amended and ryegrass-amended epoxiconazole-treated Broadbalk soils. Down arrows indicate substrate addition (see Appendix 2 for s.d.).

The order of mineralisation rate between the amended soils remained Wheat > Path > Fallow, except that the amount of $^{14}CO_2$ evolved from the glucose-amended Path soil gradually surpassed that from the glucose-amended Wheat soil. This would seem to be due to a higher than expected rate of mineralisation in the Path soil rather than a lower rate in the Wheat soil.

The rates of $^{14}CO_2$ evolution in the unamended quinmerac-treated soil were also in the order Wheat > Path > Fallow (Fig. 5-6). In the unamended soils, the amounts of $^{14}CO_2$ evolved from each soil were significantly different from one another throughout the experiment ($P = 0.01$ to < 0.001). After amendment with glucose, the differences in the amounts of $^{14}CO_2$ evolved from each of the soils remained significant ($P = 0.05$ to < 0.001) until the last 14 days of the experiment, when the mineralisation rates of the Wheat and Path soils were not significantly different. Immediately following amendment with ryegrass, on days 35 and 63, the mineralisation rates of the Path and Fallow soils were not significantly different, but other than this, all the mineralisation rates of the soils remained significantly different from each other ($P = 0.05$ to < 0.001).

The pattern of $^{14}CO_2$ evolution was sigmoidal, as was found before (see section 4.3.4.2). The amount of $^{14}CO_2$ evolved from the Wheat soil was considerably greater than that from the Path and Fallow soils, and was correlated with the amount of soil microbial biomass (Fig. 5-2) ($r = 1.0$). This appears to be in direct contrast to the findings of the previous

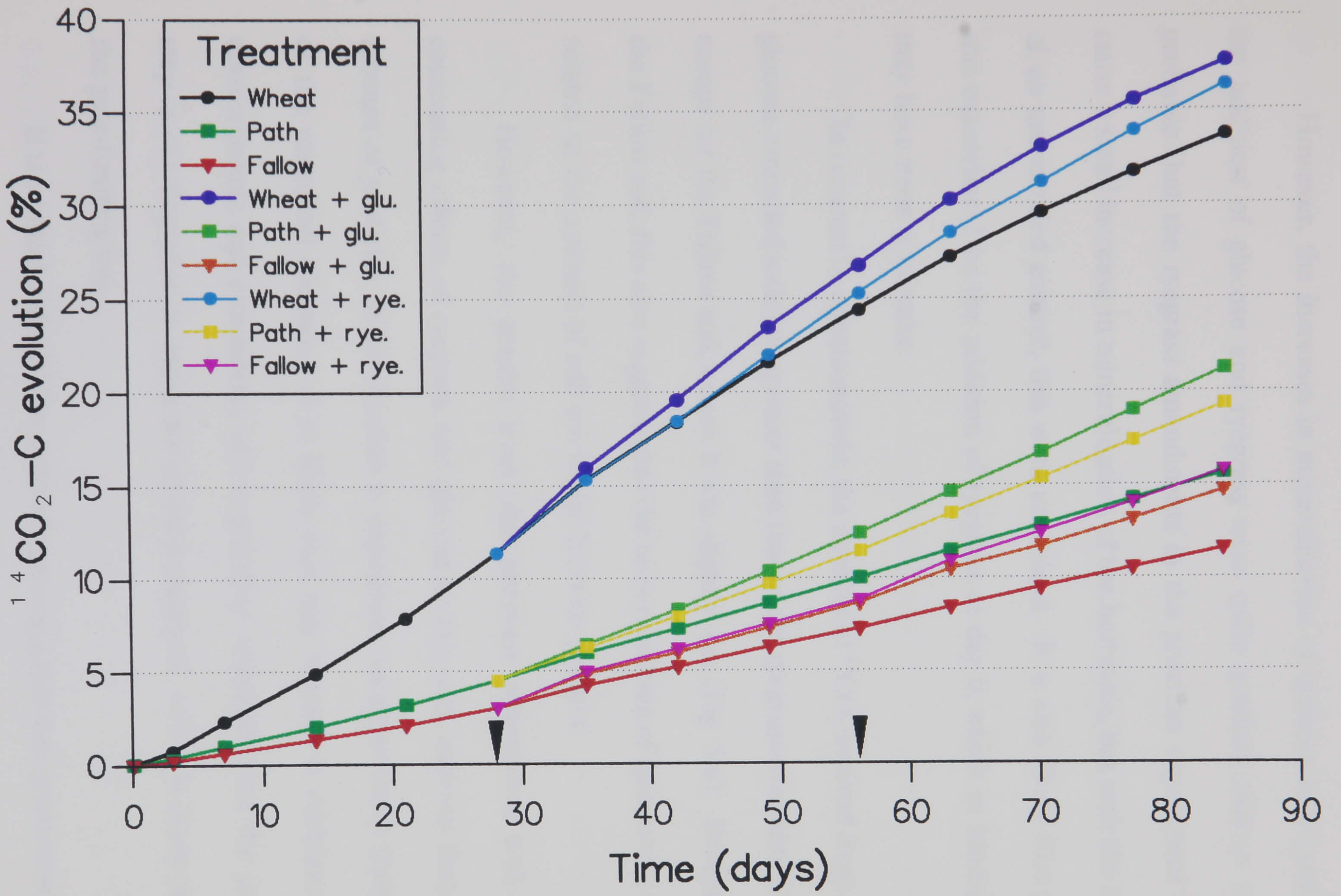


Figure 5-6: Cumulative $^{14}CO_2$-C evolution in unamended, glucose-amended and ryegrass-amended quinmerac-treated Broadbalk soils. Down arrows indicate substrate addition (see Appendix 2 for s.d.).

experiment (see section 4.3.4.2). Also in contrast to the earlier results was the significant increase in $^{14}CO_2$ evolution caused by the substrate amendments.

However, the increases in mineralisation of quinmerac caused by the addition of glucose and ryegrass were quite gradual, and so it is probable that the ryegrass amendments in the previous experiment did cause a small increase in mineralisation of the herbicide, but with the lack of an unamended control, this went unnoticed. It is also likely that this was exacerbated by the addition of ryegrass at day 0, which in hindsight may have been a mistake.

In contrast to epoxiconazole, the amount of $^{14}CO_2$ evolved from the glucose-amended soils was greater than that of the ryegrass-amended soils, except for the Fallow soil, where it was slightly less (Fig. 5-6). Excepting the Fallow soil, this also suggests that the mineralisation of quinmerac was related to the amount of soil microbial biomass present.

However, the results from the previous experiment and the contrasting effects of ryegrass and glucose in this one, indicate that the amount of pesticide mineralisation is dependent on a particular fraction of the microbial biomass. It is likely that this fraction is different for epoxiconazole and quinmerac, and is probably responsible for the initial step in the degradation sequence of each chemical, which is likely to be the rate-limiting one.

If the initial degradation steps of epoxiconazole and quinmerac are carried out by different fractions of the microbial biomass, this may explain

the different effects of the ryegrass and glucose amendments on $^{14}CO_2$ evolution. It is likely that the substrate amendments stimulated the growth of the various fractions of the microbial biomass by differing degrees. The ryegrass additions probably stimulated the growth of fungi to a greater extent than bacteria, while glucose probably stimulated bacteria slightly more than the ryegrass did.

Amendment of the soils with ryegrass generally increased the mineralisation of epoxiconazole more than amendment with glucose, while the opposite was the case with quinmerac. One could hypothesize, therefore, that epoxiconazole was predominantly decomposed by fungi, while quinmerac was predominantly decomposed by bacteria. There is no direct evidence to support this of course, but further investigation of this issue is really outside the scope of this thesis.

The biomass-specific epoxiconazole mineralisation rates of the three soils were inversely proportional to the amount of microbial biomass ($r = -0.99$), and in the same order as the biomass-specific respiration rates, although there was no significant difference ($P = 0.05$) between the Path and Fallow soils (Table 5-4). There appeared to be little pattern to the biomass-specific quinmerac mineralisation rates.

Viewed in isolation, the biomass-specific epoxiconazole mineralisation results appear to support the hypothesis that the Path and Fallow soils contained a higher proportion of pesticide-degrading micro-organisms than the Wheat soil. However, as the overall biomass-specific respiration rates were similar, it is likely that the increase in the amount

Table 5-4: Biomass-specific pesticide mineralisation rates of epoxiconazole- and quinmerac-treated Broadbalk soils * (figures in brackets = s.d.).

Pesticide	Soil	substrate amendment		
		Nil	Glucose	Ryegrass
		(%$^{14}CO_2$-C evolved μg^{-1} biomass C d^{-1})		
Epoxiconazole	Wheat	33 (0.9)	31 (1.8)	40 (1.4)
	Path	69 (5.4)	63 (1.4)	64 (5.5)
	Fallow	72 (10.3)	61 (5.9)	68 (8.0)
L.S.D. (P=0.05)		13.5	7.2	11.3
Quinmerac	Wheat	67 (0.4)	57 (1.9)	60 (2.3)
	Path	73 (6.0)	62 (7.1)	69 (6.4)
	Fallow	57 (12.0)	43 (3.5)	54 (3.7)
L.S.D. (P=0.05)		15.4	9.4	8.9

* Calculated from the total $^{14}CO_2$-C evolved over the experiment, as % of the amount initially applied, divided by the mean microbial biomass C from days 0 and 84, and by the number of days' incubation (results multiplied by 10^3).

of pesticide mineralisation per unit of microbial biomass was simply a reflection of the general increase in metabolic activity, caused by carbon limitation, and not a specific adaptation to the pesticides. The rate of pesticide mineralisation in the previous experiment being highest in the Broadbalk soil, which had the lowest microbial biomass content, was therefore probably due to innate differences in the composition of the soil microbial biomasses, and not to the fact that the soil was taken from a one-year fallow. It is also possible that the chemicals were most available in the Broadbalk soil, due to its low organic carbon content.

5.3.5 ^{14}C BALANCE SHEET

The total amount of ^{14}C accounted for, from measurements of residual soil ^{14}C, and $^{14}CO_2$ evolved over the experiment, ranged from about 89 to 104% of the initial amounts of ^{14}C added to the soils, with a mean of 96.5% (±4.7) (Table 5-5). This indicates that the analyses were of acceptable accuracy and precision.

5.4 SUMMARY

The rate of mineralisation of the pesticides epoxiconazole and quinmerac in three soils from the Broadbalk Continuous Wheat field experiment, with different crop management histories: "Wheat", "Path" and "Fallow", and the effects of soil amendment with ryegrass and glucose, were investigated.

The amount of pesticide mineralised was related to the amount of

Table 5-5: Balance sheet of residual soil ^{14}C and $^{14}CO_2$-C evolved from the epoxiconazole- and quinmerac-treated Wheat (W), Path (P) and Fallow (F) soils.

Pesticide	Soil/ Amendment	Residual soil ^{14}C (Bq g^{-1})	$^{14}CO_2$-C evolved (Bq g^{-1})	Total (Bq g^{-1})	% ^{14}C accounted for
Epoxiconazole	W(nil)	229.66	46.40	276.06	88.7
	W(glu.)	242.71	62.65	305.36	98.1
	W(rye.)	230.70	74.58	305.28	98.0
	P(nil)	272.96	39.76	312.72	100.4
	P(glu.)	239.39	64.21	303.60	97.5
	P(rye.)	223.06	65.42	288.48	88.6
	F(nil)	285.98	37.80	323.78	104.0
	F(glu.)	237.33	53.93	291.26	93.5
	F(rye.)	213.43	62.59	276.02	88.6
Quinmerac	W(nil)	226.91	114.16	341.07	100.6
	W(glu.)	204.74	127.34	332.08	98.0
	W(rye.)	219.44	123.07	342.51	101.1
	P(nil)	276.29	52.91	329.20	97.1
	P(glu.)	233.98	71.79	305.77	90.2
	P(rye.)	248.01	65.35	313.36	92.5
	F(nil)	305.14	39.18	344.32	101.6
	F(glu.)	290.91	49.76	340.67	100.5
	F(rye.)	262.33	53.45	315.78	93.2

Amounts of ^{14}C added at day 0 (Bq g^{-1} soil): epoxiconazole - 311.39; quinmerac - 338.95.

soil microbial biomass, in the unamended soils. Thus it was greatest in the Wheat soil, which contained around twice as much microbial biomass as the other two soils. The amendment of the soils with the substrates significantly increased the soil microbial biomass contents, but the mineralisation rates of the pesticides did not increase to the same degree. Ryegrass addition stimulated the mineralisation of epoxiconazole more than glucose, while the reverse was true for quinmerac, with the unexplained exception of the Fallow soil. This was possibly due to the mineralisation of the two pesticides being carried out by distinct fractions of the soil microbial biomass, whose growth was also differentially stimulated by the two different C substrates.

It was concluded that the different crop management of the soils in this and the previous experiment, although this would have affected the composition of the soil microflora, had only an indirect effect on the rate of pesticide mineralisation, and that the differences in mineralisation rates between the soil types was probably due to ecological and soil chemical differences caused by other factors.

CHAPTER 6

EFFECTS OF LONG-TERM CUMULATIVE APPLICATIONS OF PESTICIDES IN THE FIELD ON SOIL MICROBIAL BIOMASS AND MINERALISATION OF SOIL ORGANIC MATTER

6.1 INTRODUCTION

Most studies investigating side-effects of pesticides on soil micro-organisms have involved laboratory-based experiments, often concerned with short-term effects following application of a single pesticide. However, in the field, one or more pesticides may be repeatedly applied to the same soil for many years, which may lead to a build-up of pesticide residues or metabolites, whereby the possibility of damaging effects upon the soil microbial biomass or its activity is much greater (Grossbard, 1971; Greaves, 1979).

Of the relatively few reports that have addressed long-term effects, most have been concerned with field experiments, as it is difficult to maintain laboratory incubations for more than a few months (Greaves, 1979). There have been some papers on the repeated application of a single pesticide to a soil over many years (*e.g.* Voets *et al.*, 1974; Duah-Yentumi and Johnson, 1986; Biederbeck *et al.*, 1987), and on the application of several pesticides to a soil over a few years (*e.g.* Schuster and Schröder, 1990; Jones *et al.*, 1991), but very few on the repeated application of several pesticides over many years (*e.g.* Grossbard, 1971; Heinonen-Tanski *et al.*, 1985; 1986). Given that the latter is common in

agriculture, it is surprising that there have been few such studies. The continuing introduction of new chemicals, and the use of mixtures or sequences of different chemicals, demands a continued, and perhaps increased, research effort to ensure that harmful effects on the soil microflora will be avoided (Greaves, 1979).

In 1973, a field experiment, known as the Chemical Reference Plots, was begun on Long Hoos field at Rothamsted Experimental Station, in which the effects of the long-term application of three pesticides on the yield of barley was to be investigated. The pesticides were benomyl (methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate) – a benzimidazole fungicide, chlorfenvinphos (2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate) – an organophosphorus insecticide, and aldicarb (2-methyl-2-(methylthio)propionaldehyde *O*-methylcarbamoyloxime) – a carbamoyloxime insecticide/nematicide. Two other pesticides were later incorporated into the experiment: glyphosate (*N*-(phosphonomethyl)glycine) – a phosphonic acid herbicide, in 1980; and triadimefon (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)butanone) – a conazole fungicide, in 1982. The chemicals were added in the following formulations in 1992: benomyl – Benlate wettable powder (50% a.i.); chlorfenvinphos – Birlane granules (10% a.i.); aldicarb – Temik granules (10% a.i.); triadimefon – Bayleton wettable powder (25% a.i.); glyphosate – Gallup soluble concentrate (36% a.i.). The pesticides were applied annually, at slightly greater than normal field application rates (see Fig. 6-1).

All the plots also received other pesticides as standard farm practice, consisting of late spring applications of herbicides, *e.g.* mecoprop and bromoxynil (the compound(s) differed between years), to the growing crop to control broadleaved weeds. The experiment was terminated after 20 years, with the last pesticide applications being made in September 1992 and March 1993.

The Chemical Reference Plots were designed as a fully randomised, single-replicate 2^5 factorial experiment. Each of the 32 plots measured 15′×12′10″, separated by 3′6″ and 8′ paths along the long and short edges, respectively. Pesticide application was split over the year – benomyl, chlorfenvinphos and aldicarb were applied to the seed bed each spring immediately prior to sowing, while triadimefon and glyphosate were applied in the autumn, usually both on the same day, after the barley had been harvested. The experimental design is shown in Fig. 6-1, and the chemical structures of the five pesticides are given in Fig. 6-2.

This experiment affords a unique opportunity to assess the effects on soil micro-organisms of long-term repeated applications of several pesticides in the field, in a statistically balanced experimental design. In this chapter, the effects of 19 years of pesticide application on the size of the microbial biomass in soil taken directly from the field are measured, and also the rate of mineralisation of soil organic matter, measured by basal soil respiration, ammonification and nitrification, in laboratory-based incubations.

01	02	03	04	05	06	07	08
–	BE	–	BE	BE	BE	BE	–
CS	CS	CS	CS	–	–	–	–
–	–	–	AL	AL	–	–	–
TR	–	–	TR	TR	TR	–	TR
GL	–	GL	GL	–	GL	–	–
09	10	11	12	13	14	15	16
BE	–	BE	BE	–	BE	BE	–
CS	–	–	–	CS	–	–	CS
–	AL	–	AL	AL	AL	–	–
TR	–	–	–	–	TR	TR	–
GL	–	GL	–	–	GL	–	–
17	18	19	20	21	22	23	24
BE	–	–	–	–	–	–	–
CS	CS	CS	–	–	–	–	–
–	AL	–	AL	AL	AL	–	–
TR	TR	TR	TR	TR	–	–	–
–	GL	–	–	GL	GL	GL	–
25	26	27	28	29	30	31	32
–	BE	BE	BE	BE	–	–	BE
–	–	CS	CS	CS	CS	CS	CS
–	AL	AL	AL	AL	AL	AL	–
TR	–	TR	–	–	–	TR	–
GL	GL	–	–	GL	GL	–	GL

TREATMENTS (cumulative annually)

Fungicide to seedbed
– , BE None, benomyl at 4 kg ha^{-1}

Insecticide to seedbed
– , CS None, chlorfenvinphos at 2 kg ha^{-1}

Insecticide to seedbed
– , AL None, aldicarb at 6 kg ha^{-1}

Fungicide in autumn
– , TR None, triadimefon at 0.25 kg ha^{-1} (since 1982 only)

Herbicide to stubble
– , GL None, glyphosate at 1.5 kg ha^{-1} (since 1980 only)

BASAL MANURING: 'Nitram' *ca.* 435 kg ha^{-1}

CROP: Spring barley

Figure 6-1: Plan of the Chemical Reference Plots field experiment, Long Hoos V, Rothamsted Experimental Station.

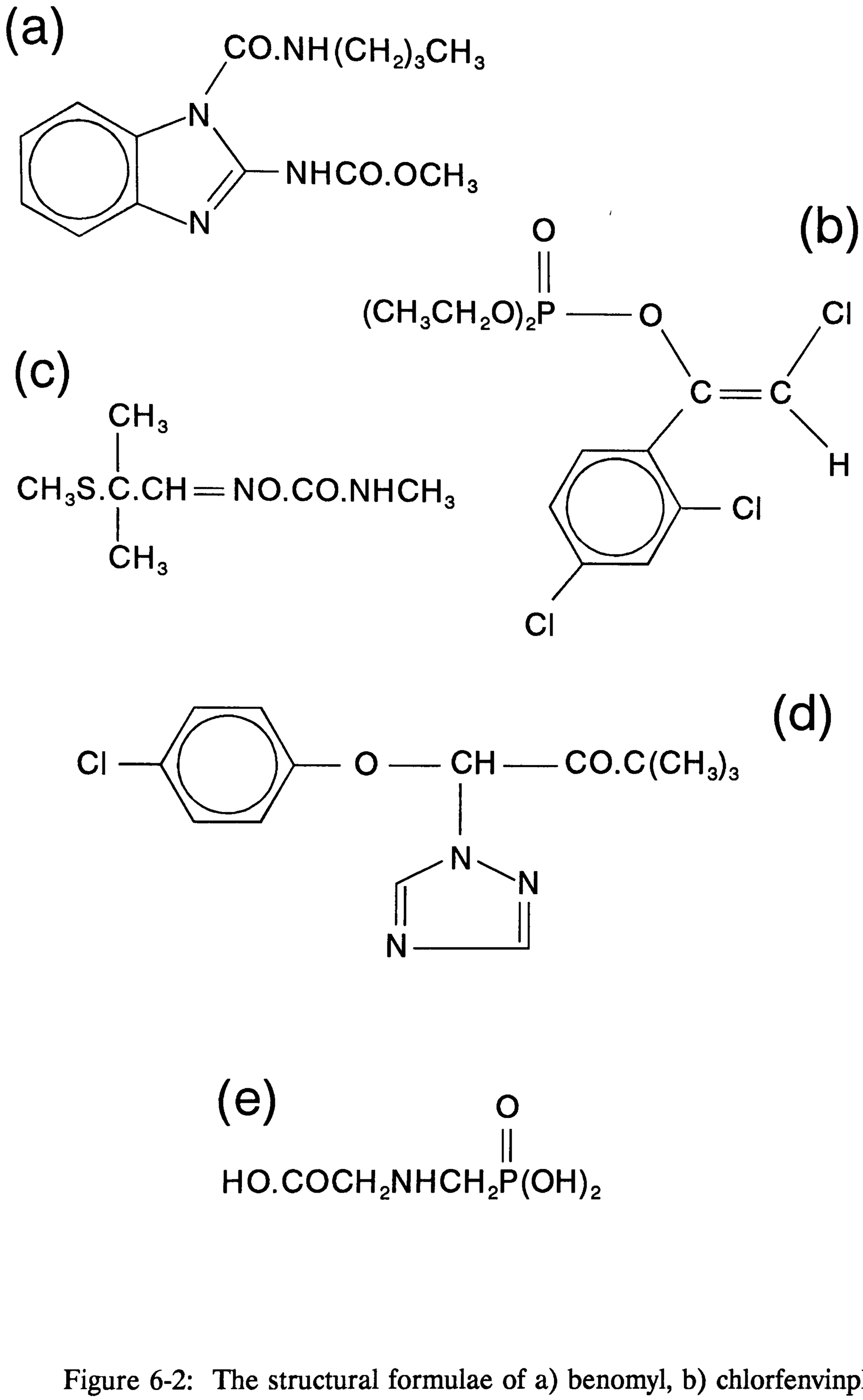


Figure 6-2: The structural formulae of a) benomyl, b) chlorfenvinphos, c) aldicarb, d) triadimefon and e) glyphosate.

6.2 MATERIALS AND METHODS

6.2.1 SAMPLING AND PREPARATION OF SOIL

About 3.5 kg soil was collected from each plot, from within *ca.* 8 – 18″ from the long edges, in the discard strips of the plots, in April 1992 and again in October 1992. Both sampling times were 4 weeks after the appropriate pesticides had been applied. The soil samples were sieved and prepared as described in section 2.1.1, over a period of about 5 weeks, during which time the soils not undergoing processing were stored at 5°C. Care was taken at all times to ensure that there was no cross-contamination of soil between plots. Once prepared, the soil samples were stored at 5°C until use.

The microbial biomass contents of the spring-collected soil were first determined on sub-samples removed after about 6 weeks storage at 5°C, at field moisture content (*ca.* 43% WHC). For the incubation experiment using the spring samples, 500 g soil was removed from each plot sample, after a further 4 weeks storage at 5°C, adjusted to 50% WHC, and pre-incubated for 7 days as described in section 2.1.1. Samples were then removed for microbial biomass analyses, and also weighed out for the incubation experiment.

For the incubation experiment using the autumn samples, the soils were adjusted to 50% WHC immediately after the sieving was completed, and pre-incubated for 11 days. Samples were then removed for microbial biomass analyses and the incubation experiment, as before. No prior biomass measurements were made with the autumn soil samples.

In both incubation experiments, the triplicate samples from each plot were stored in randomised blocks, and incubated at 25°C in a constant environment room as described in section 2.1.2, for a total of 56 days.

6.2.2 SOIL CHEMICAL ANALYSES

Soil pH, CEC, organic C, total N and texture were determined on soil taken from each plot and bulked, as described in section 2.2.5, for both sets of samples.

6.2.3 MICROBIAL BIOMASS MEASUREMENTS

Soil microbial biomass C and ninhydrin-N were measured as described in sections 2.2.1 and 2.2.2, respectively. Biomass ninhydrin-N was measured in the spring soil samples, at field moisture content, only.

Microbial biomass was also measured by the substrate-induced respiration (SIR) method of Anderson and Domsch (1978), as modified by Lin (1994), at the beginning of the incubations of the spring and autumn-sampled soils. Briefly, 30 g moist soil was amended with a 4:1 mixture of talc and glucose, to give 6 mg glucose g^{-1} soil. The soil was then incubated at 25°C for 2 hours, and the CO_2 concentration of the flask headspace determined by gas chromatography.

6.2.4 CO_2 EVOLUTION MEASUREMENTS

Soil CO_2 evolution was determined as described in section 2.2.3, after 3, 7, 14, 21, 28, 35, 42, 49 and 56 days incubation.

Table 6-1: Characteristics of the spring and autumn-sampled soil

	Spring	Autumn
pH (H_2O)	6.9	6.9
pH (0.01M $CaCl_2$)	6.4	6.3
CEC (meq 100 g^{-1})	14.9	15.1
Organic C (%)	1.36	1.36
Total N (%)	0.143	0.145
Sand (%)	20	
Silt (%)	61	
Clay (%)	19	
Texture	Silty clay loam (Batcombe series)	

6.2.5 SOIL NH_4^+ AND NO_3^- MEASUREMENTS

Soil NH_4^+ and NO_3^- were determined as described in section 2.2.5, in 0.5 M K_2SO_4 extracts taken after 0 and 56 days incubation.

6.3 RESULTS AND DISCUSSION

6.3.1 SOIL CHARACTERISTICS

The spring- and autumn-sampled soil characteristics are shown in Table 6-1.

6.3.2 INITIAL MICROBIAL BIOMASS CONTENT

The microbial biomass C content of the soil from each plot is shown in Fig. 6-3. There was a gradual decrease in biomass C across the plots, from west to east, presumably due to changes in soil texture or some other characteristic. The numbers on the X and Y axes refer to the adjacent plots (*cf.* Fig. 6-1). These data cannot be used to interpret the effects of the pesticide treatments, and all subsequent results will refer to statistically processed data.

The effects of the individual pesticide treatments (*i.e.* the main effects in statistical terminology) on microbial biomass C in the spring-sampled soil are shown in Fig. 6-4. The addition of aldicarb had the greatest effect, increasing biomass C by about 21.5%, although this increase was not quite significant at the 5% level. Jones *et al.* (1991) also found increased microbial biomass in aldicarb-treated soil in the field, although at lower doses than were applied in the Chemical Reference

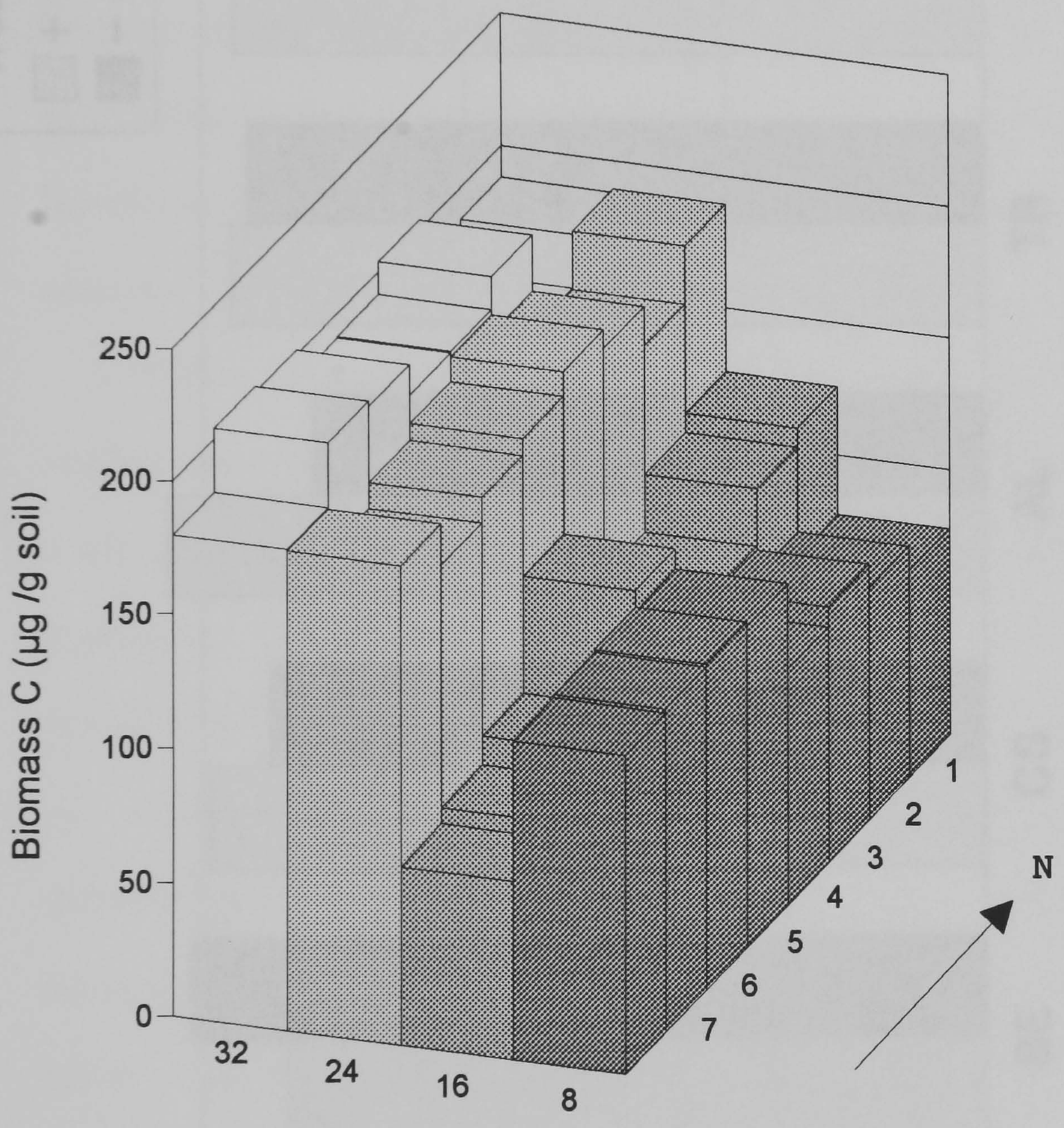


Figure 6-3: Initial soil microbial biomass C contents of the Chemical Reference Plots field experiment (mean s.d. = 11.1 μg g^{-1}).

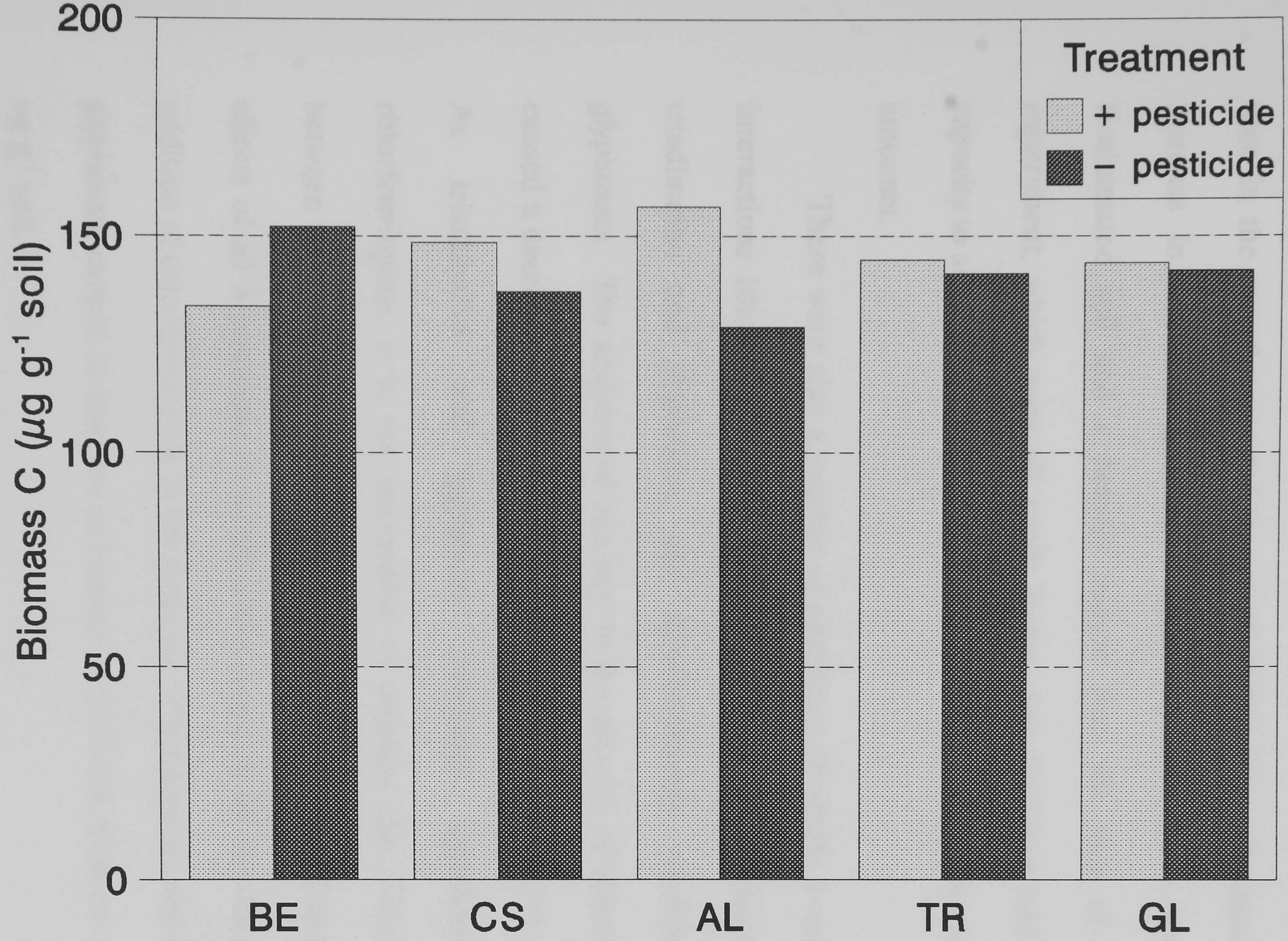


Figure 6-4: Initial mean biomass C contents of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated spring-sampled soil. L.S.D. (P=0.05) = 30.1.

Plots. The maximum increase was caused by applications of 1.4 kg ha^{-1}, while at the maximum rate of aldicarb, 4.0 kg ha^{-1}, the microbial biomass was not significantly different from the control soil. The rate of aldicarb applied to the Chemical Reference Plots, 6.0 kg ha^{-1}, was much greater than in the experiment of Jones *et al.* (1991), but still caused a large increase in microbial biomass. This may have been because the Rothamsted soil had a heavier texture than the soils of the other experiment, which were all sandy loams, and therefore had a greater capacity to adsorb the chemical and protect the soil microflora from excess amounts.

There were also a number of significant (P=0.05) 2- and 3-factor interactions (data not shown), between benomyl and chlorfenvinphos, triadimefon and glyphosate, and chlorfenvinphos, triadimefon and glyphosate. The addition of benomyl in the absence of chlorfenvinphos caused a decrease in biomass C of 32.5%, from *ca.* 164 to 111 μg g^{-1} soil. As triadimefon and glyphosate interacted significantly with chlorfenvinphos, it is not reasonable to consider the 2-factor effects between these two (*i.e.* as the 3-factor interaction is significant, only the effects of all 3 pesticides in combination ought to be considered). The addition of chlorfenvinphos in the presence of triadimefon and absence of glyphosate caused an increase in biomass C of 55.4%, from *ca.* 125 to 195 μg g^{-1} soil.

The main effects of pesticide application on microbial biomass ninhydrin-N are shown in Fig. 6-5. The pattern of significant differences

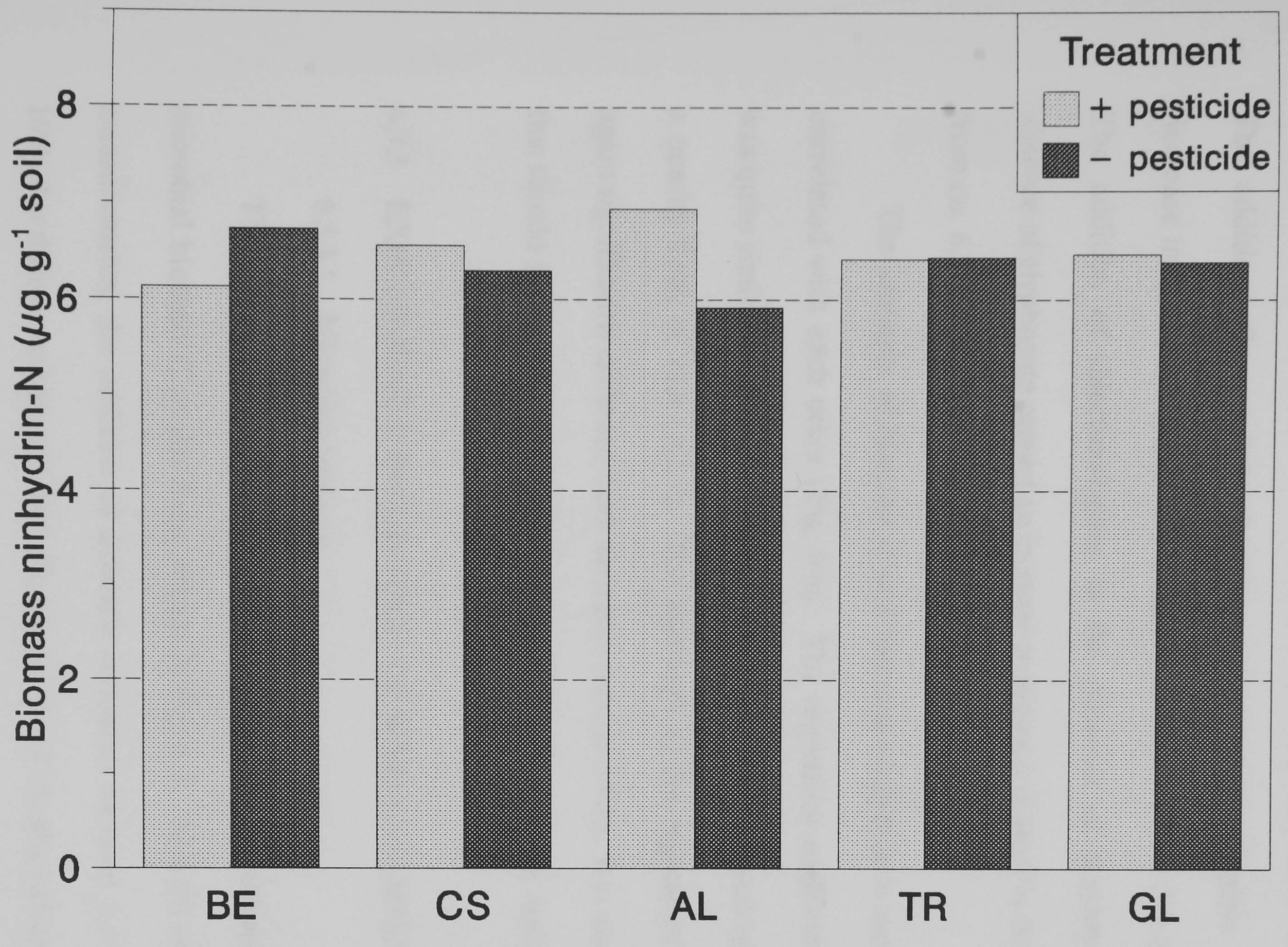


Figure 6-5: Initial mean biomass ninhydrin-N contents of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated spring-sampled soil. L.S.D. (P=0.05) = 1.2.

between treatment effects was exactly the same as for biomass C (data not shown), but with slightly lower levels of significance (all greater than 90% probability, however), indicating that this method gave less precise measurements of microbial biomass than the determination of biomass C. The addition ofbenomyl in the absence of chlorfenvinphos caused a decrease in biomass ninhydrin-N of 33.6%, from *ca.* 7.2 to 5.4 μg g^{-1} soil. The addition of chlorfenvinphos in the presence of triadimefon and absence of glyphosate caused an increase in biomass ninhydrin-N of 27.9%, from *ca.* 6.2 to 7.9 μg g^{-1} soil.

The amounts of biomass C and biomass ninhydrin-N were closely correlated with each other (Fig. 6-6). The regression coefficient, 26.67, was quite similar to that obtained by Ocio *et al.* (1991) for soil taken from a nearby field, of 21.4 (±2.7). The intercept of the regression line was again significantly different from zero (see section 3.3.2). It is unclear why this should be so, but experimental error is the most likely explanation.

6.3.3 EXPERIMENT 1: INCUBATION OF SPRING-SAMPLED SOIL

6.3.3.1 *Microbial biomass*

The effects of the statistical main treatments on the amounts of soil microbial biomass C at day 0 are shown in Fig. 6-7. As with the earlier measurements, the addition of aldicarb increased biomass C, by about 16%, this difference being statistically significant ($P < 0.05$), while the other pesticides had no significant effect. The higher order interactions between benomyl and chlorfenvinphos, and triadimefon and glyphosate, were still

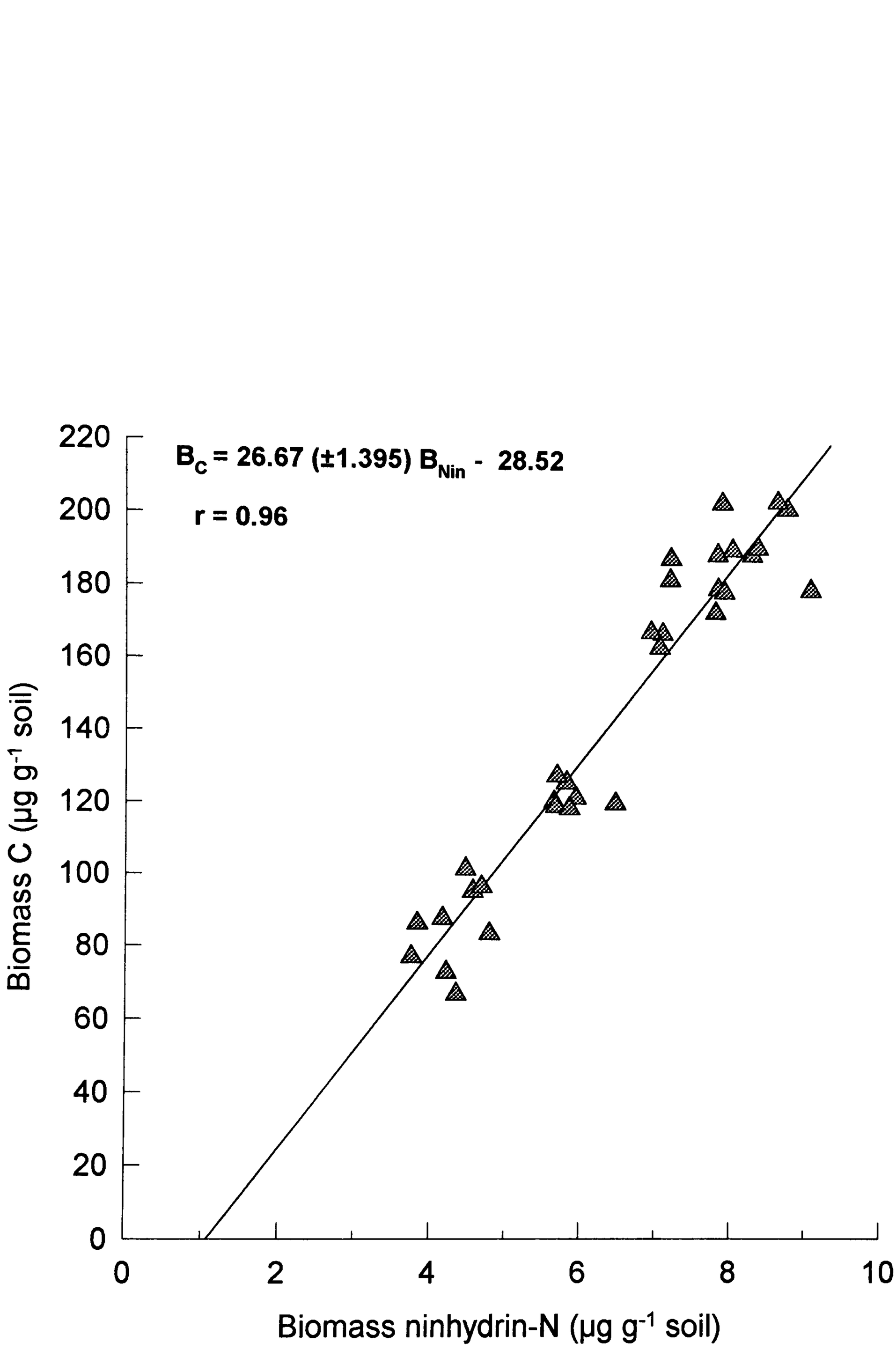


Figure 6-6: The linear relationship between microbial biomass C and ninhydrin-N in the spring-sampled soil.

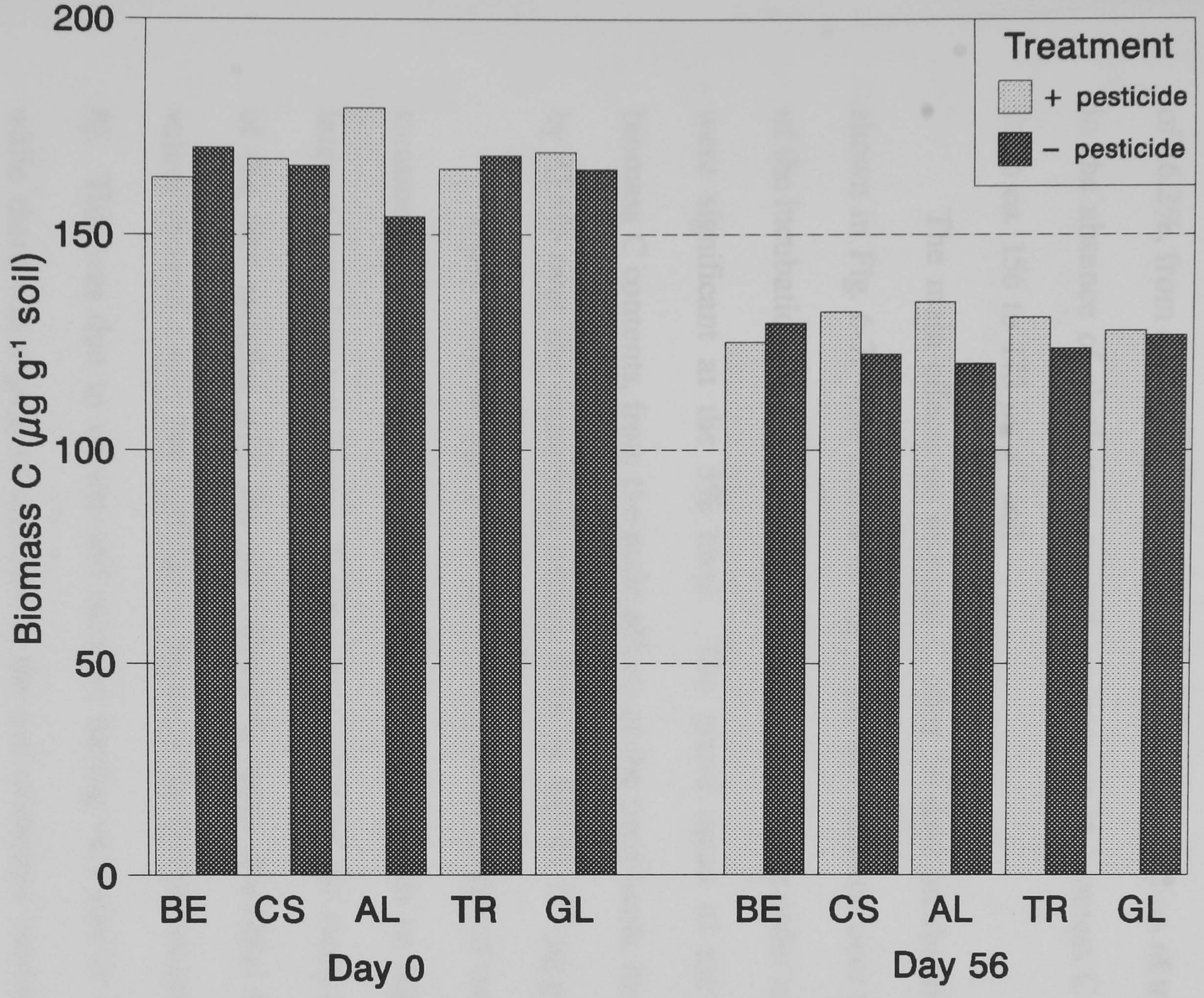


Figure 6-7: Microbial biomass C contents of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated spring-sampled soil after 0 and 56 days incubation. L.S.D. (P=0.05) = 20.6 (Day 0), 18.9 (Day 56).

present (P=0.05). However, the interaction between chlorfenvinphos, triadimefon and glyphosate was not significant at the 5% level. The addition of benomyl in the absence of chlorfenvinphos caused a decrease in biomass C of 15.2%, from *ca.* 180 to 152 μg g^{-1} soil. The addition of glyphosate in the absence of triadimefon caused an increase in biomass C of 16.2%, from *ca.* 156 to 181 μg g^{-1} soil, while the addition of triadimefon in the absence of glyphosate caused an increase in biomass C of 11.3%, from *ca.* 156 to 173 μg g^{-1} soil.

The main effects on biomass C after 56 days incubation are also shown in Fig. 6-7. The effects of the pesticides lessened over the course of the incubation, and none of the main effects or higher order interactions were significant at the 5% level. The grand mean of the microbial biomass C contents, from the main effects of the treatments, had declined by 23.6% over the incubation period, from *ca.* 167 to 127 μg g^{-1} soil.

There were no significant differences between any of the pesticide treatments as measured by SIR, either as main effects or higher order interactions (data not shown). Regression analysis of the mean SIR values of the plot samples with the corresponding mean microbial biomass C values showed a very low correlation between the two parameters (Fig. 6-8). This was due to all the soil samples having very similar SIR rates, while there was a gradual increase in the soil microbial biomass content from plots 1–8 and 25–32 (Fig. 6-3). No attempt was made to convert the SIR rates to microbial biomass C, as calibration of the SIR method is problematic (Wardle and Parkinson, 1990).

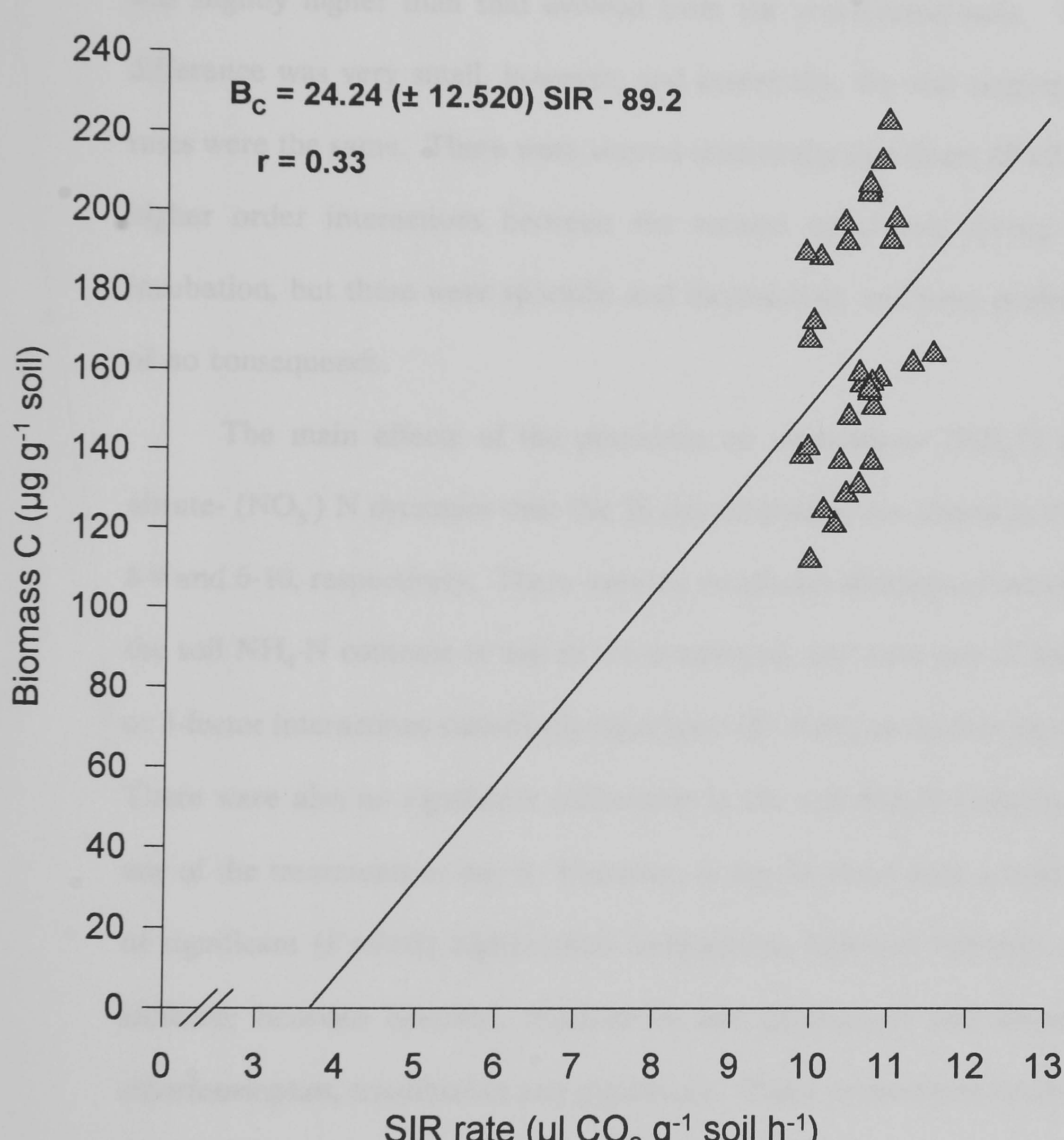


Figure 6-8: The relationship between microbial biomass C and SIR in the spring-sampled soil.

6.3.3.2 *Mineralisation of soil organic matter*

The main effects of the pesticides on CO_2 evolution over the 56 day incubation are shown in Table 6-2. There were no significant differences ($P = 0.05$) between the main effects over the incubation, with one exception on day 42, when the mean CO_2 evolved from the triadimefon-treated soils was slightly higher than that evolved from the non-treated soils. This difference was very small, however, and essentially, the soil respiration rates were the same. There were several statistically significant ($P < 0.05$) higher order interactions between the various treatments during the incubation, but these were sporadic and inconsistent, and were probably of no consequence.

The main effects of the pesticides on ammonium- (NH_4^+) and nitrate- (NO_3^-) N dynamics over the 56 day incubation are shown in Figs. 6-9 and 6-10, respectively. There were no significant differences between the soil NH_4-N contents of any of the treatments, nor were any of the 2- or 3-factor interactions statistically significant ($P < 0.05$), at day 0 or day 56. There were also no significant differences in the soil NO_3-N contents of any of the treatments at day 0. However, at day 56 there were a number of significant ($P < 0.05$) higher-order interactions, between benomyl and aldicarb; between benomyl, triadimefon and glyphosate; and between chlorfenvinphos, triadimefon and glyphosate. These interactions involved differences of around 10%, but were probably not of any consequence.

Thus, none of the pesticide treatments affected the rates of ammonification or nitrification of soil N. The grand mean NH_4-N content

Table 6-2: Cumulative CO_2 evolution from the benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated spring-sampled soil.

Day	CO_2-C evolved from soil (pesticide main effects) ($\mu g\ g^{-1}$ soil)										L.S.D. (P=0.05)
	+BE	−BE	+CS	−CS	+AL	−AL	+TR	−TR	+GL	−GL	
3	15	15	15	15	16	15	15	15	15	15	1.4
7	30	30	31	29	31	30	31	30	30	30	1.0
14	52	53	53	51	52	52	53	51	52	53	2.0
21	70	72	72	71	71	71	72	70	70	72	1.7
28	86	88	87	87	87	88	89	86	86	89	1.5
35	103	105	103	104	103	105	105	102	102	106	1.6
42	118	120	118	120	118	120	121	117	117	121	1.2
49	132	135	133	134	133	135	136	131	131	136	1.7
56	144	148	145	147	145	147	149	144	144	148	1.1

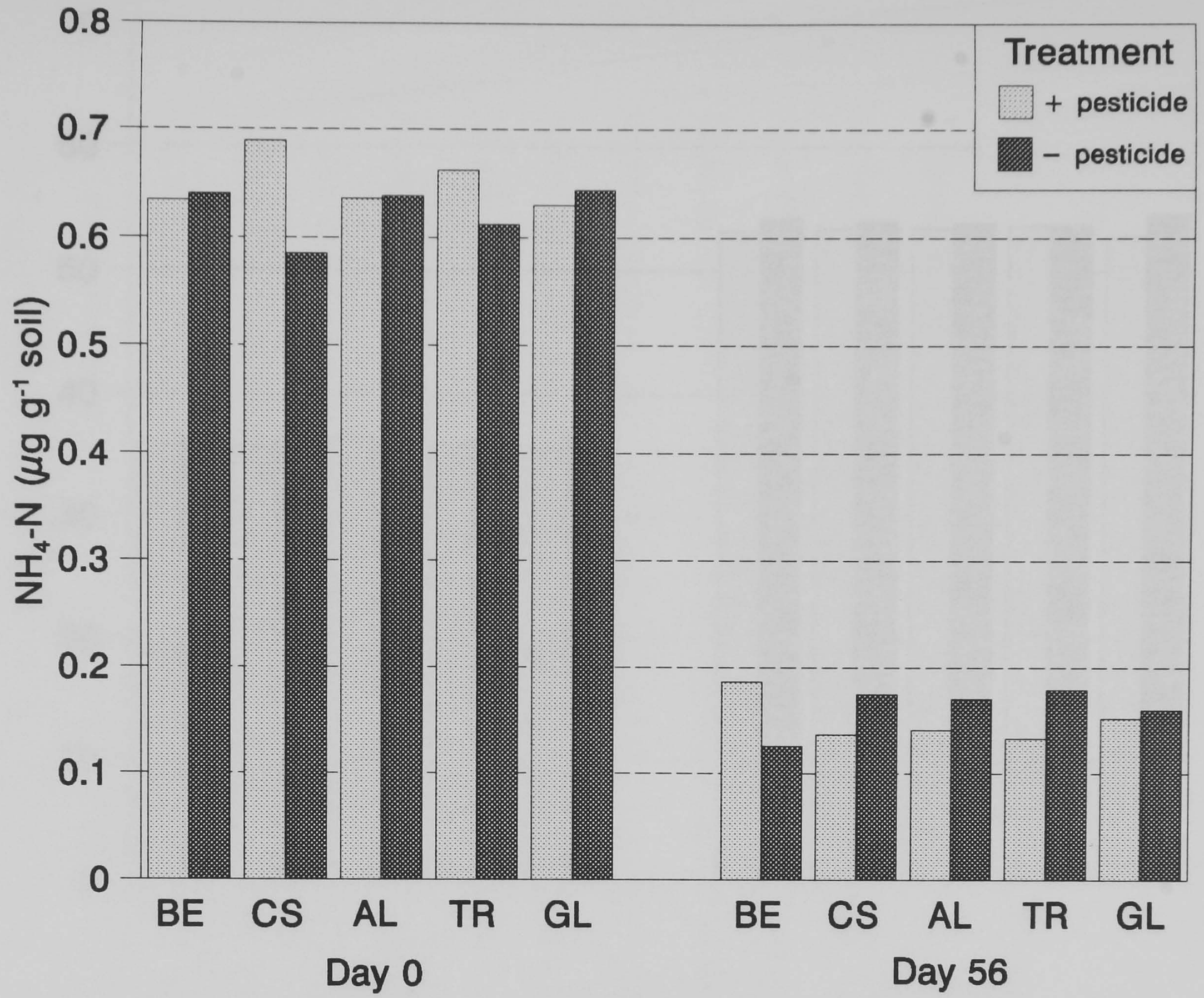


Figure 6-9: Ammonium-N contents of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated spring-sampled soil after 0 and 56 days incubation. L.S.D. (P=0.05) = 0.169 (Day 0), 0.125 (Day 56).

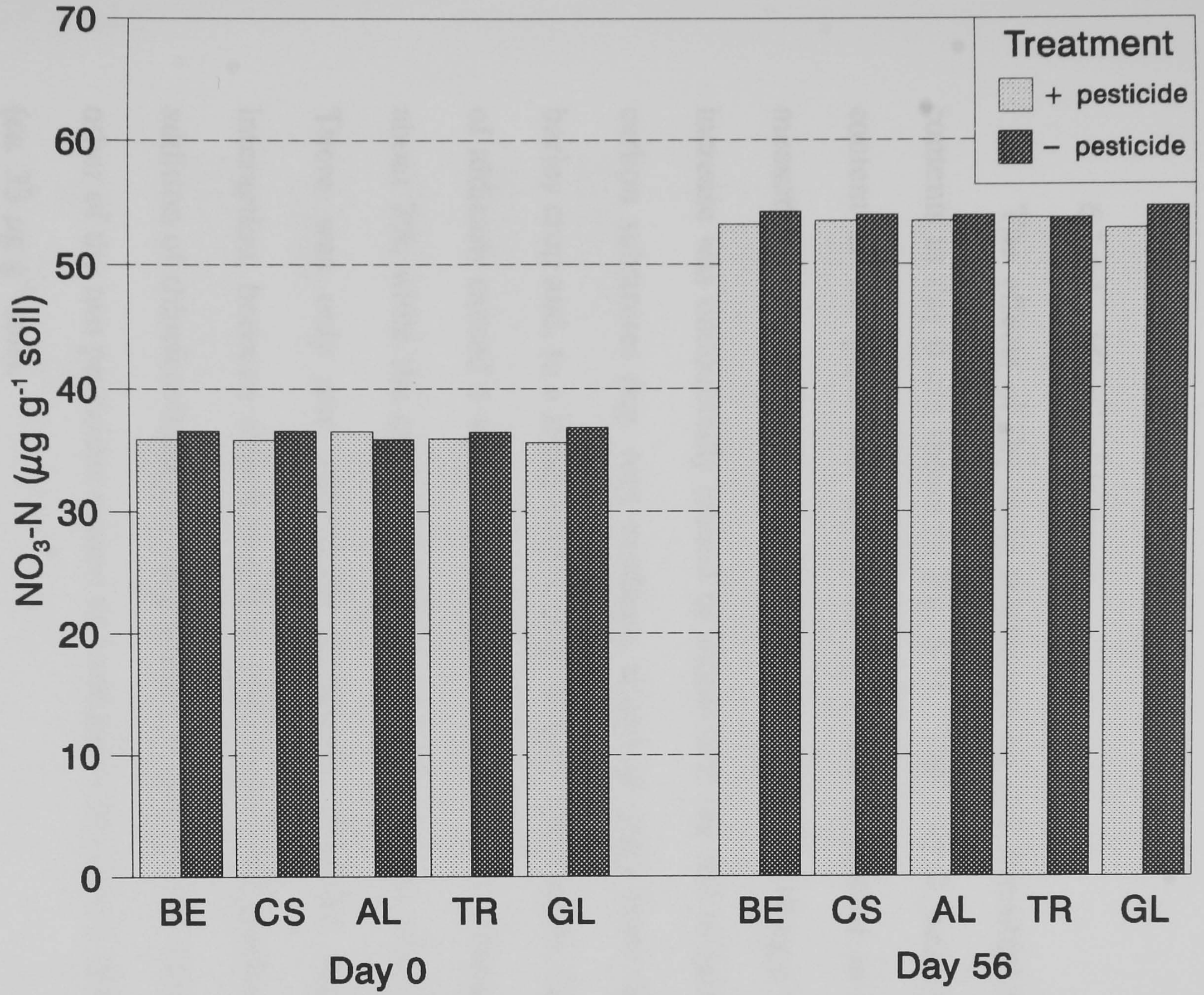


Figure 6-10: Nitrate-N contents of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated spring-sampled soil after 0 and 56 days incubation. L.S.D. (P=0.05) = 3.771 (Day 0), 2.677 (Day 56).

of the soils decreased by 75.5% over the 56 days incubation, from *ca.* 0.64 to 0.16 μg g^{-1} soil, while the NO_3-N content increased by 48.4%, from *ca.* 36.19 to 53.72 μg g^{-1} soil, over the same period.

6.3.4 EXPERIMENT 2: INCUBATION OF AUTUMN-SAMPLED SOIL

6.3.4.1 *Microbial biomass*

The effects of the main treatments on soil microbial biomass C contents at day 0 are shown in Fig. 6-11. The overall mean biomass C content of the plots had increased by 47.1% compared to the initial measurements of the spring-sampled soil, from 143 to 210 μg g^{-1} soil. This increase was undoubtedly caused by inputs into the soil of labile organic carbon substrates (*e.g.* root exudates, sloughed cells) from the growing barley crop and, to a lesser extent, from weeds. Once again, the addition of aldicarb caused a significant increase in biomass C, although only of about 7%, while the other pesticides had no significant effect ($P=0.05$). There was only one statistically significant ($P<0.05$) higher order interaction, between chlorfenvinphos, triadimefon and glyphosate. The addition of chlorfenvinphos in the presence of one and the absence of the other of the two pesticides caused an increase in biomass C of about 16% (*ca.* 33 μg g^{-1} soil).

The main effects on biomass C after 56 days incubation are also shown in Fig. 6-11. Unlike the spring-sampled soil, the effect of aldicarb was still significant at the end of the incubation ($P<0.05$), the biomass C

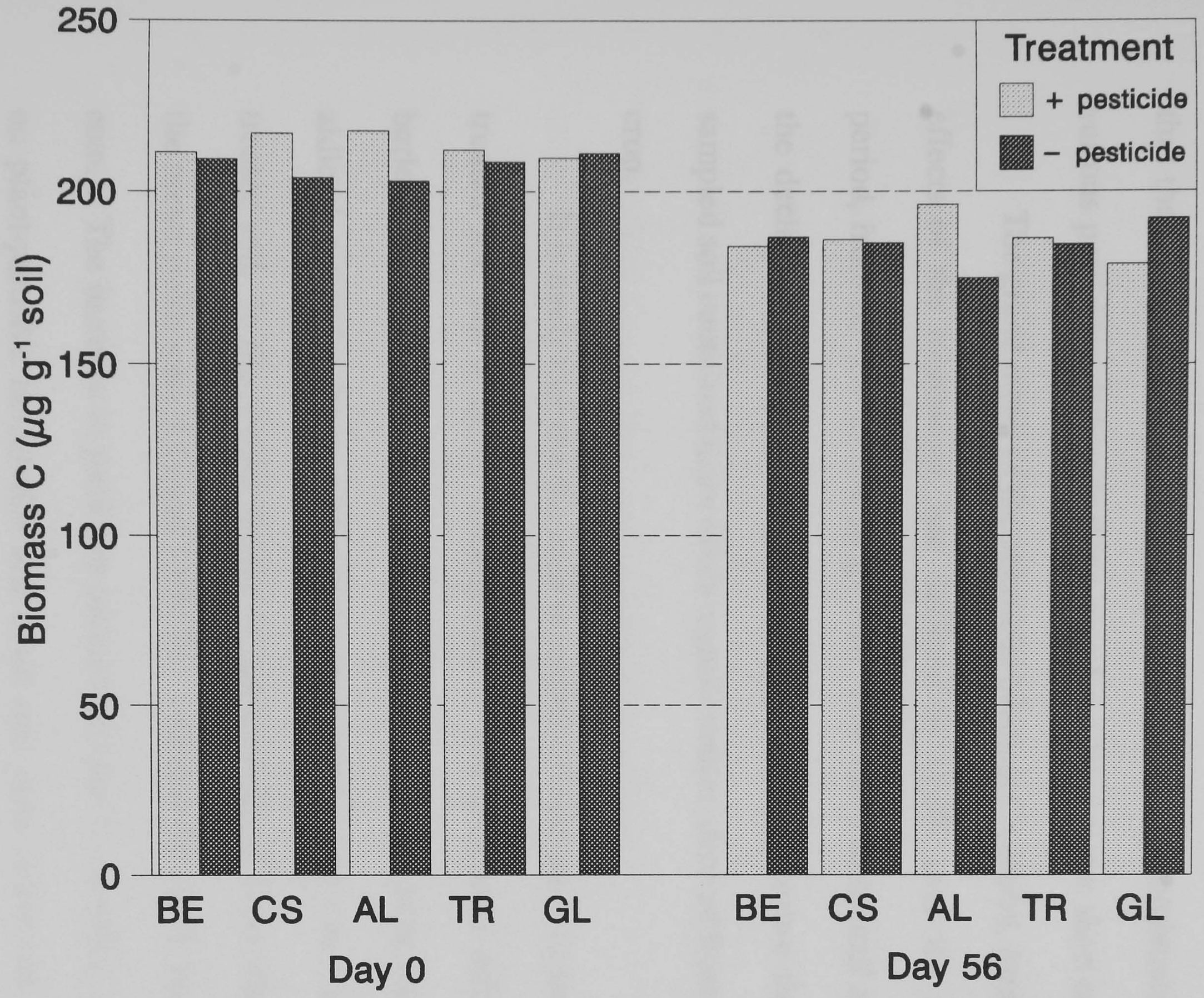


Figure 6-11: Microbial biomass C of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated autumn-sampled soil after 0 and 56 days incubation. L.S.D. (P=0.05) = 14.6 (Day 0), 20.0 (Day 56).

content of the treated soil being about 12% greater than that of the untreated soil. However, the 3-factor interaction between chlorfenvinphos, triadimefon and glyphosate was no longer significant at the 5% level at day 56. The gradual lessening in the number of higher order interactions in the biomass C measurements over the course of the experiments, indicates that there were probably no long-term effects of the combinations of the various pesticides, although there may have been some short-term ones.

The grand mean of the microbial biomass C content, from the main effects of the treatments, had declined by 11.8% over the incubation period, from *ca.* 210 to 186 μg g^{-1} soil. This was exactly half as much as the decline in the spring-sampled soil, presumably because the autumn-sampled soil contained more labile organic matter, derived from the barley crop.

It is likely that the increase in microbial biomass C in the aldicarb-treated soil was a direct consequence of the chemical's effect on the barley. Between 1974 and 1983, the mean yield of spring barley in the aldicarb-treated soil was 5.17 t ha^{-1}, compared to 4.86 t ha^{-1} in the non-treated soil. In the second decade of the experiment, from 1984 to 1993, the mean yields were 5.21 and 5.09 t ha^{-1}, respectively (R. Bromilow, pers. com.). The increase in yield was presumably due to the effect of aldicarb on plant-parasitic nematodes and other soil pests, while the difference between the first and second decades was probably caused by increasing adaptation to and mineralisation of the chemical by the soil microflora (Suett and Jukes, 1988). Although the increases in barley yield were not

particularly large, it is probable that, over the 20 year period of the experiment, significantly more plant material entered the soil in the aldicarb-treated plots than in the non-treated plots as a result of more vigorous growth, thereby causing an increase in soil microbial biomass.

In contrast to the spring-sampled soil, statistical analysis of the SIR data gave similar results to biomass C, although only the chlorfenvinphos, triadimefon and glyphosate interaction was significant at the 5% level (data not shown). The linear correlation between SIR and biomass C was also much stronger than in the spring-sampled soil ($r=0.83$) (Fig. 6-12). The autumn-sampled soil was pre-incubated for 11 days compared to 7 for the spring-sampled soil. Presumably, the latter was not long enough for the soil microflora to stabilise for the purposes of the SIR method, while the fumigation-extraction method was more robust. While the SIR results from the autumn-sampled soil were a marked improvement on the earlier incubation, the fumigation-extraction method still gave more reliable results.

6.3.4.2 *Mineralisation of soil organic matter*

The main effects of the pesticides on CO_2 evolution over the 56 day incubation are shown in Table 6-3. Approximately 76% more CO_2-C was evolved overall from the autumn-sampled soil compared to that from the spring-sampled soil, reflecting the larger microbial biomass and readily decomposable organic matter inputs from the crop. There were no significant differences ($P=0.05$) between the main effects over the

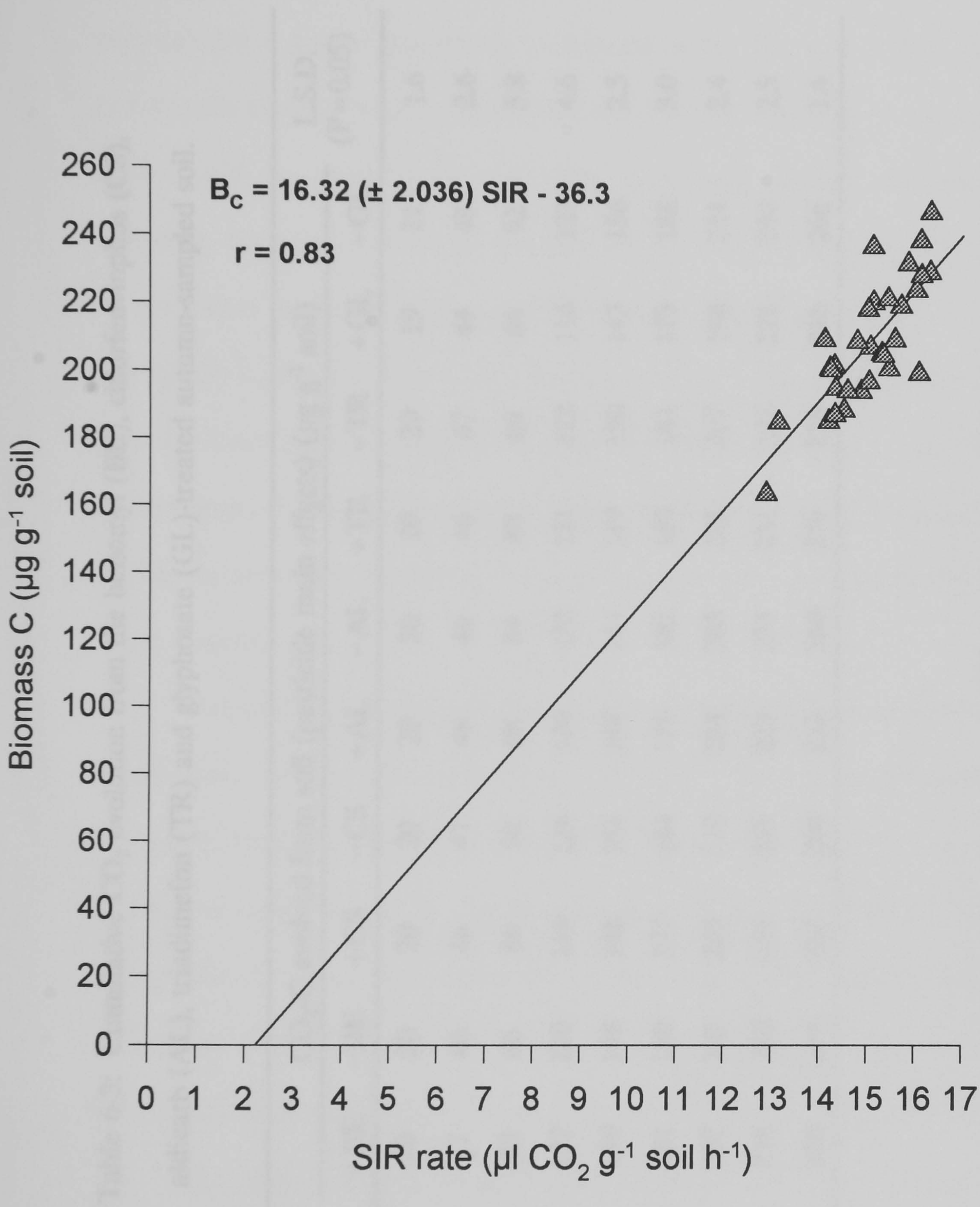


Figure 6-12: The relationship between microbial biomass C and SIR in the autumn-sampled soil.

Table 6-3: Cumulative CO_2 evolution from the benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated autumn-sampled soil.

Day	CO_2-C evolved from soil (pesticide main effects) ($\mu g\ g^{-1}$ soil)										L.S.D. (P=0.05)
	+BE	−BE	+CS	−CS	+AL	−AL	+TR	−TR	+GL	−GL	
3	20	20	20	20	20	20	20	20	19	21	1.6
7	47	46	46	47	46	46	46	47	44	49	2.6
14	90	86	86	90	88	89	88	89	84	92	5.8
21	122	120	119	124	120	122	121	122	116	127	4.6
28	150	148	146	152	148	151	149	150	143	156	2.5
35	181	180	177	184	179	182	180	181	173	188	3.0
42	207	205	203	210	204	208	205	207	198	214	2.4
49	234	230	229	235	229	235	231	233	223	240	2.5
56	259	254	253	260	253	260	256	258	248	266	1.4

incubation, except for a small decrease caused by glyphosate at day 3. The decreases on day 7 and day 28 caused by glyphosate were not quite significant at this level ($P<0.06$), but help to account for the difference between the + and − glyphosate treatments at day 56. Less CO_2-C was consistently evolved from the glyphosate-treated soil compared to the non-treated soil, by about 2−3 $\mu g\ g^{-1}$ soil over most of the incubation. However, the somewhat large standard errors of the differences of the means meant that most of the apparent inhibitions by glyphosate were not statistically significant. There were again a number of significant ($P<0.05$) 2- and 3-factor interactions over the incubation, although fewer than in the spring-sampled soil. These were also inconsistent and bore no relation to the higher order interactions in the earlier incubation, and were probably of no consequence.

One slightly puzzling aspect of the CO_2 evolution in both incubation experiments, is that the statistically significant increase in microbial biomass C caused by the addition of aldicarb was not reflected in the soil respiration rates. It may be that the measurement of CO_2 evolution was not sufficiently sensitive to detect what would have been relatively small differences between treatments, probably due to the larger errors in the CO_2 measurements compared to those of the soil microbial biomass C.

The main effects of the pesticides on NH_4- and NO_3-N dynamics over the 56 day incubation are shown in Figs. 6-13 and 6-14, respectively. In contrast to the previous incubation, there was a significant decrease in the NH_4-N contents of the aldicarb-treated soil compared to the non-

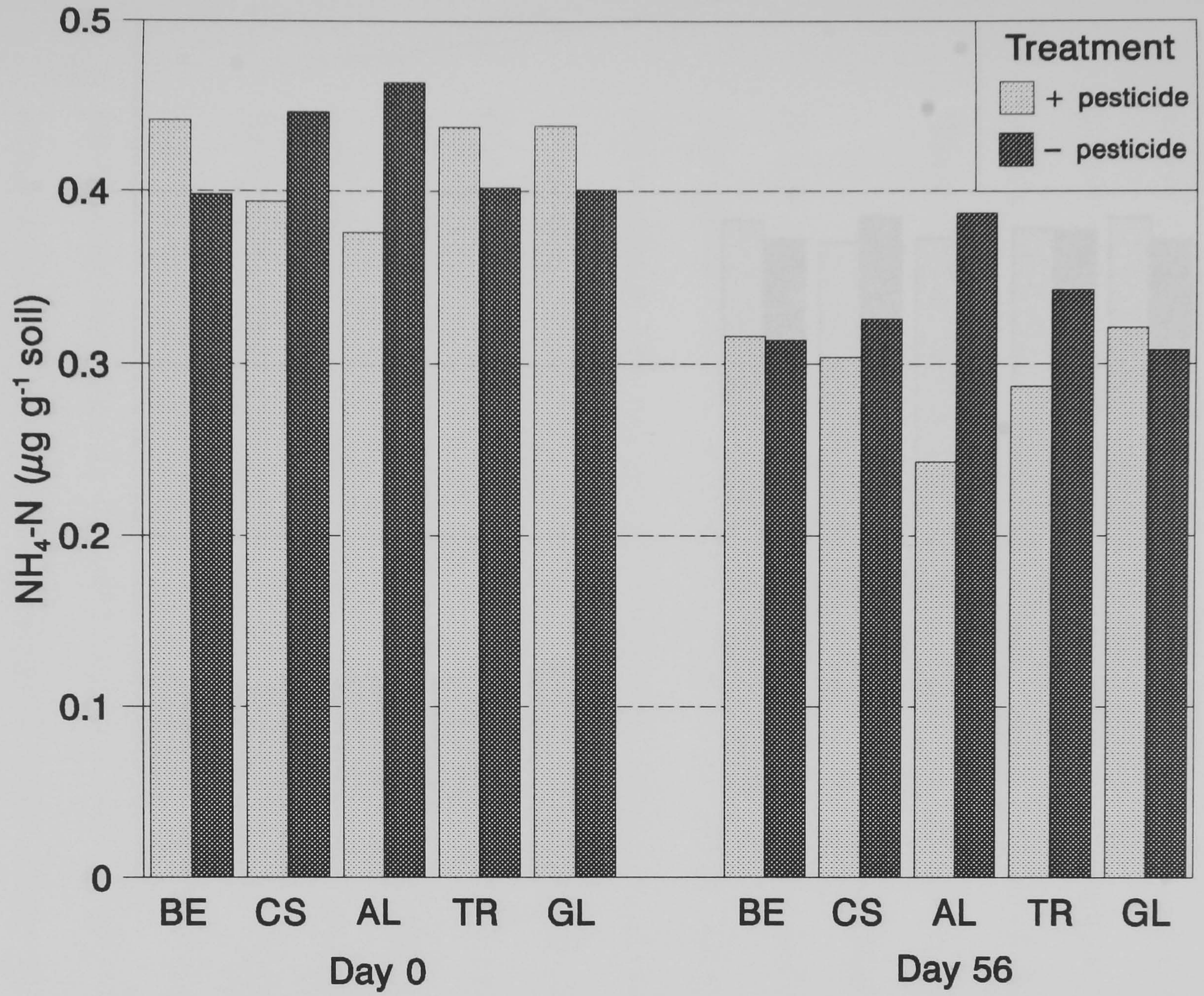


Figure 6-13: Ammonium-N contents of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated autumn-sampled soil after 0 and 56 days incubation. L.S.D. (P=0.05) = 0.099 (Day 0), 0.139 (Day 56).

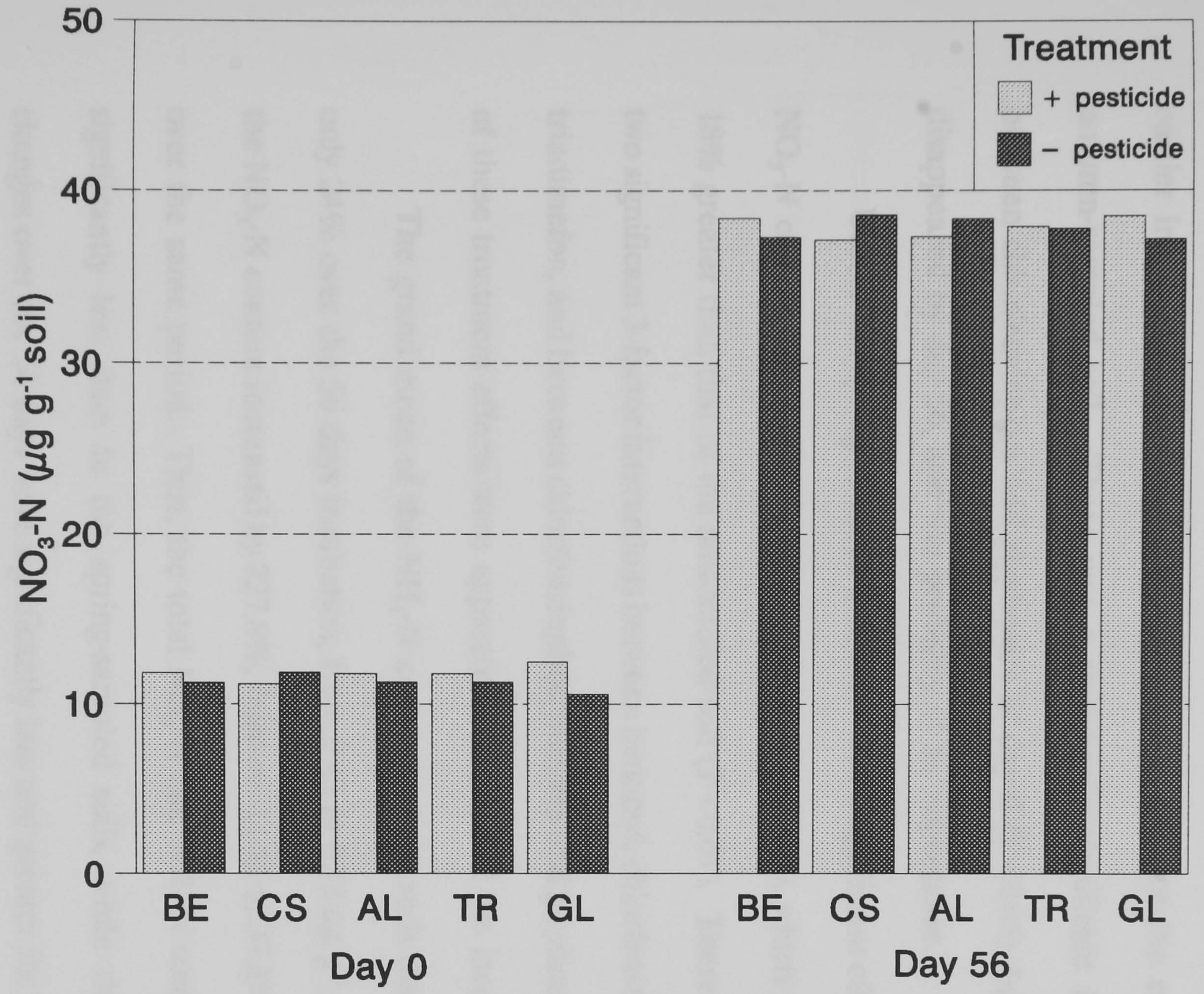


Figure 6-14: Nitrate-N contents of benomyl (BE), chlorfenvinphos (CS), aldicarb (AL), triadimefon (TR) and glyphosate (GL)-treated autumn-sampled soil after 0 and 56 days incubation. L.S.D. (P=0.05) = 1.125 (Day 0), 2.973 (Day 56).

treated soil at day 56 ($P<0.05$), of about 37%. There was also a similar effect at day 0, although it was less marked in size and statistical significance ($P=0.075$). This may be due to the uptake of NH_4^+ by the larger microbial biomass in the aldicarb-treated soil. This effect was not noticeable in the spring-sampled soil, but the effect of aldicarb in the earlier incubation decreased over time, which was not the case in the autumn-sampled soil. There was a statistically significant interaction between chlorfenvinphos and glyphosate at day 0 ($P<0.05$), but this had disappeared by day 56, and was probably of no importance.

Unlike the spring-sampled soil, there was a significant effect on the NO_3-N content of the glyphosate-treated soil at day 0, which was about 18% greater than that of the non-treated soil ($P<0.01$). There were also two significant 3-factor interactions between benomyl, chlorfenvinphos and triadimefon, and between chlorfenvinphos, aldicarb and glyphosate. None of these treatment effects were apparent at the end of the incubation.

The grand mean of the NH_4-N contents of the soils decreased by only 24% over the 56 days incubation, from 0.42 to 0.32 $\mu g\ g^{-1}$ soil, while the NO_3-N content increased by 227.8%, from *ca.* 11.55 to 37.85 $\mu g\ g^{-1}$ soil over the same period. Thus, the total NH_4-N and NO_3-N contents were significantly less than in the spring-sampled soils, while the relative changes over the 56 days were significantly less and greater for NH_4^+ and NO_3^-, respectively, compared to the earlier incubation. The former presumably reflects the flush of mineral N that begins in the spring, while the latter is probably due to the increased microbial biomass and readily

decomposable organic matter of the autumn-sampled soil.

6.4 SUMMARY

The effects of 19 years of cumulative annual field application of five pesticides (benomyl, chlorfenvinphos, aldicarb, triadimefon and glyphosate), in 2^5 combinations, on soil microbial biomass and the mineralisation of soil organic matter were investigated. Soil samples were taken one month after the application of benomyl, chlorfenvinphos and aldicarb in April 1992, and again in October 1992, one month after the application of triadimefon and glyphosate.

The addition of aldicarb caused a significant increase in soil microbial biomass, an effect which appeared to be more or less permanent. This increase was probably an indirect effect, caused by aldicarb's beneficial effect on crop growth, which would have led to increased organic inputs into the soil.

This effect of aldicarb was not reflected in the mineralisation rate of soil organic C, possibly because the measurements of CO_2 evolution showed a greater variation those of biomass C. Measurement of microbial biomass activity by the substrate-induced respiration method also gave much less precise results than measurements of biomass C.

The mineralisation of soil organic N to ammonium and nitrate was mostly unaffected by the pesticide treatments. In the autumn-sampled soil, there was significantly less NH_4^+ in the aldicarb-treated soil. It is possible that this was due to immobilisation by the increased microbial biomass in

these treatments, and did not represent a loss to the soil system.

Overall, the application of these pesticides to the same sites, for up to 19 years, had very little effect on the microbial parameters measured. The one consistent significant side-effect on soil microbial biomass, caused by the addition of aldicarb, was actually of a beneficial nature. Thus, it would seem that the continuous use of these pesticides at or slightly above the recommended rates, singly or in combination, had no long-term harmful effects on the soil microbial biomass or its activity.

CHAPTER 7

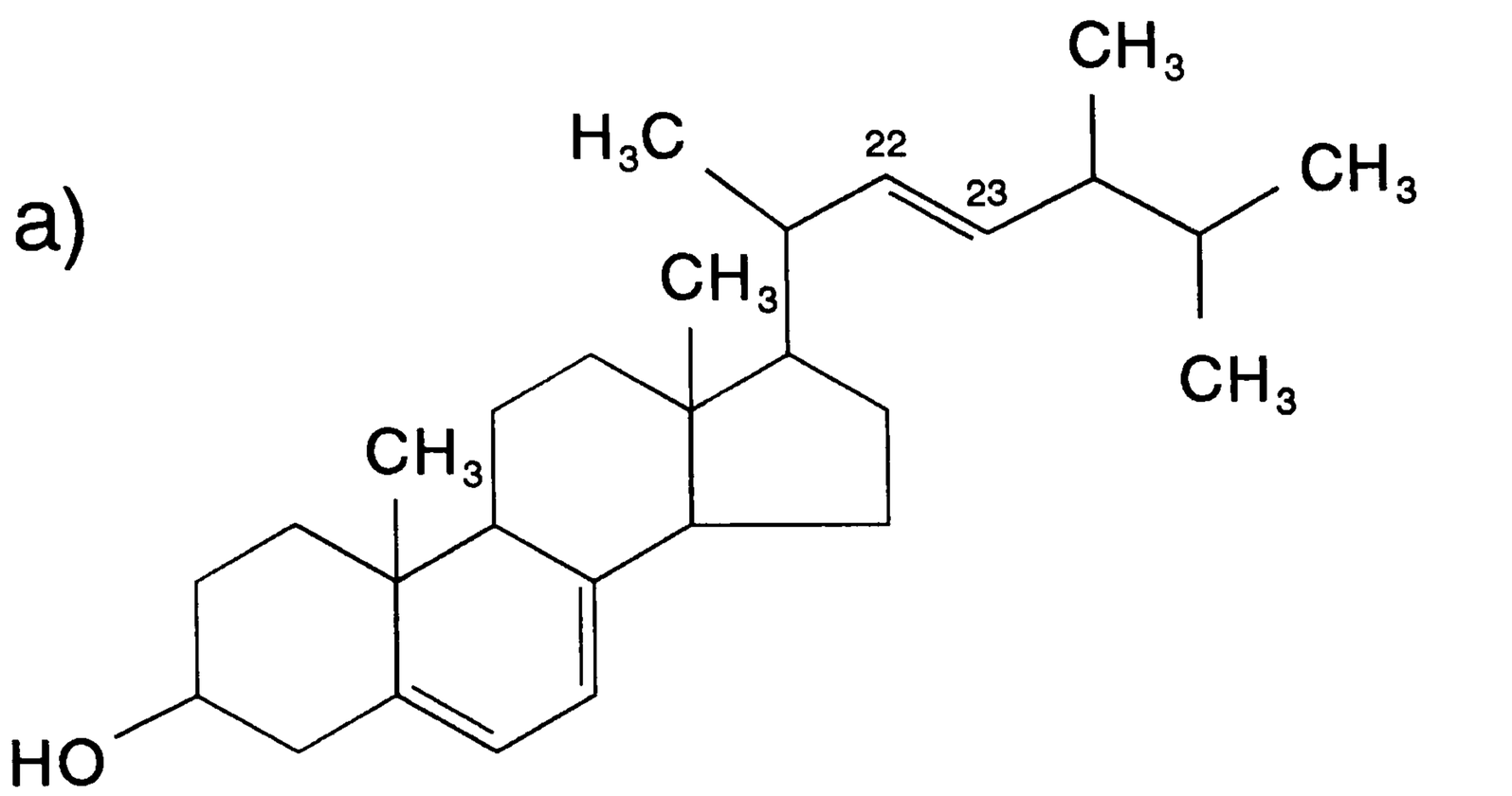
COMPARISON OF THE INFLUENCE OF EPOXICONAZOLE AND TRIADIMEFON ON SOIL ERGOSTEROL AND MICROBIAL BIOMASS

7.1 INTRODUCTION

Ergosterol [(3β,22E)-ergosta-5,7,22-trien-3-ol] (Fig. 7-1a) was first discovered in the ergot fungus *Claviceps purpurea*, and has subsequently been found to be the major sterol of many fungal species. It is not found in all fungi, being rare or absent in the Mastigomycotina, Hymenoascomycetidae I, Pyrenomycetes I and Uredinales (Weete, 1989).

Ergosterol predominantly occurs in the phospholipid bilayer of the fungal cell membrane, as the free alcohol, ester and/or glycoside (Weete, 1989). It is thought to increase the membrane microviscosity, and thus may influence a number of processes, including cell wall synthesis, by modulating chitin synthetase, and the activity of other membrane-bound enzymes, such as cytochrome c peroxidase and ATPases (Peacock and Goosey, 1989; Buchenauer, 1990).

Ergosterol is endogenous almost exclusively to fungi, with certain green microalgae and protozoa being the only non-fungal sources, and so may be a useful index of fungal presence (Newell, 1992). The $\delta^{5,7}$-diene double bonding of ergosterol gives it a unique pattern of ultra-violet absorption, with a maximum at 282 nm, which makes it readily distinguishable from the major sterols of animals, vascular plants and non-



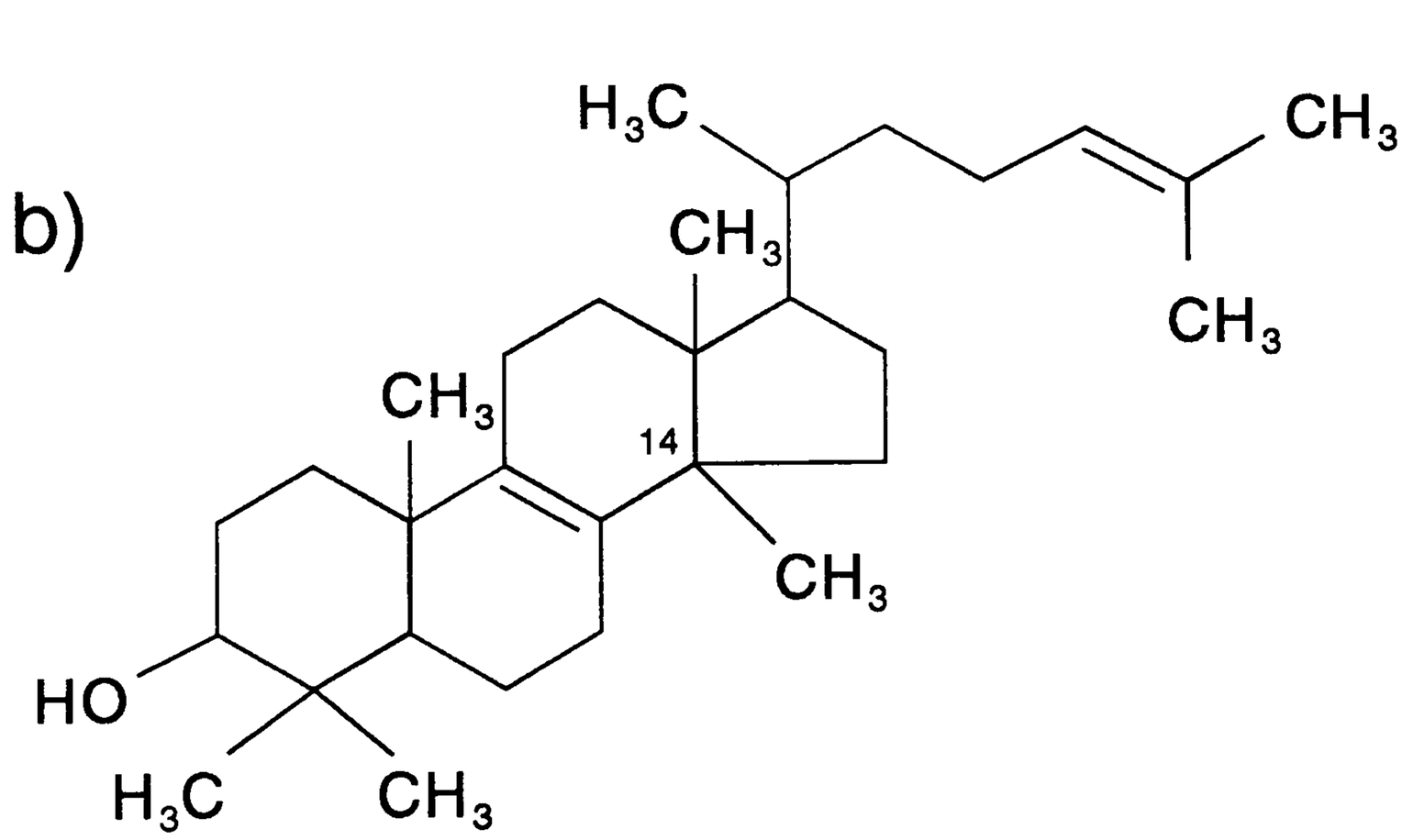


Figure 7-1: Molecular structure of a) ergosterol, and its precursor b) lanosterol.

fungal micro-organisms (Newell, 1992; Davis and Lamar, 1992).

Ergosterol was first used as an indicator of fungal growth by Seitz *et al.* (1977, 1979) in cereal grains. It has subsequently been used to estimate fungal biomass in decomposing plant material (Lee *et al.*, 1980; Newell *et al.*, 1987, 1988; Gessner and Chauvet, 1993), soil (Grant and West, 1986; West *et al.*, 1987; Zelles *et al.*, 1987; Davis and Lamar, 1992) and ecto- and VA mycorrhizae (Salmanowicz and Nylund, 1988; Johnson and McGill, 1990a,b; Schmitz *et al.*, 1991; Frey *et al.*, 1992).

The discovery that the blocking of sterol biosynthesis suppressed fungal growth led to the development, begun in the late 1960s, of a wide range of fungicides with this mode of action (Cremlyn, 1991). These target a number of biochemical processes in sterol biosynthesis, the most common of which is the C-14 demethylation step during the conversion of lanosterol (Fig. 7-1b) to ergosterol, or a functionally equivalent sterol (Weete, 1989). These demethylation-inhibitor fungicides are now extremely important in crop protection, due to their wide spectrum of target organisms and low application rate (Elmholt, 1992; Kwok and Loeffler, 1993). They include a variety of azole, pyridine, pyrimidine and piperazine derivatives, the most important group of which are those derived from triazole and imidazole.

The removal of the 14α-methyl group is a stage in the conversion of 24-methylene-dihydrolanosterol, and is achieved in three $NADPH_2$-dependent oxygenase reactions. The first step is catalysed by a microsomal cytochrome-P450 enzyme, and it is this C-14 demethylase ($P450_{14DM}$) that

is inhibited by the DMI fungicides (Cremlyn, 1991). The mode of action of azole DMIs appears to be twofold: first, the relatively unhindered N_3 (imidazole) or N_4 (triazole) of the azole ring binds to the ferric iron at the haem centre of the $P450_{14DM}$, and second, the N_1 substituent of the azoles interacts with the apoprotein of the enzyme. There is evidence that the N_1 substituent also effects the interaction of the azole moiety with the haem iron (Kapteyn *et al.*, 1992).

The two fungicides used in this experiment, epoxiconazole and triadimefon, are both triazole derivatives, with modes of action as described above. Epoxiconazole is a new fungicide recently developed, while triadimefon is a well established fungicide which has been on the market for many years. With the increasing use and wide spectrum of activity of this type of fungicide, it is important that they be fully assessed for side-effects on soil micro-organisms. With the development of methods to measure ergosterol, and this being the main target of these fungicides, determining the effects of demethylation inhibitors on soil ergosterol contents would seem an ideal method to carry out such assessments.

The aim of this experiment was to determine the suitability of using ergosterol as an indicator of the side-effects of pesticides on soil fungi. To date there have only been reports of studies done *in vitro* (e.g. Peacock and Goosey, 1989; Guan *et al.*, 1992; Kwok and Loeffler, 1993). The work described here appears to be the first report to investigate effects of pesticides on ergosterol contents in soil.

7.2 MATERIALS AND METHODS

7.2.1 SAMPLING AND PREPARATION OF SOIL

The soil (approx. 4 kg $plot^{-1}$) was collected from plots 21 and 22 of the Arable and Ley Rotation field experiment at Stackyard D, Woburn Experimental Farm. Both plots were in the final year of an 8 year grass-clover ley with no added inorganic N fertiliser, and differed only in that plot 21 received 38 t ha^{-1} of farm-yard manure, compared to none for plot 22, in 1963, 30 years prior to the time of sampling. The soil samples were stored unprocessed in separate polythene bags at 5°C for 16 weeks before use.

The soils were sieved and pre-incubated as described in section 2.1.1. The two samples were mixed together thoroughly and incubated in a single polythene bag for 7 days, at field moisture content (33.5% WHC). The soil sample was then split into 5 equal parts, which were stored at 5°C until pesticide addition (see below).

7.2.2 SOIL CHEMICAL ANALYSES

Soil pH, CEC, organic C, total N and texture were determined on the combined soil, as described in section 2.2.5.

7.2.3 PESTICIDE TREATMENTS

Pesticide solutions were all prepared to contain the same amount of epoxiconazole carrier material (*ca.* 160 μg C ml^{-1}, adding *ca.* 5 μg C g^{-1} soil), as described in section 2.1.3. Epoxiconazole was added to soil at 0.25

and 2.5 μg g^{-1}, and triadimefon at 0.125 and 1.25 μg g^{-1} soil. The latter concentrations are roughly equivalent to 1× and 10× field rate application. The solutions were prepared such that the appropriate amount of pesticide was added in the volume required to bring the soils to 40% WHC. The pesticide solutions were added using a hand-held sprayer to soil spread in a thin layer on polythene sheeting, and not as described in section 2.1.3. The soil was then mixed thoroughly and transferred to a large polythene bag, loosely tied with a rubber band, and incubated in steel drums, as described in section 2.1.1. Due to the amount of work involved in the ergosterol analyses, the pesticide treatments were staggered: the epoxiconazole solutions were added 2 days after the control treatment (epoxiconazole carrier only - added immediately after the pre-incubation period), and the triadimefon solutions 3 days after this.

7.2.4 STRAW ADDITION

Finely chopped wheat straw (41.3% C, 0.52% N - determined as for soil, described in section 2.2.5) was added to one half of each soil, at 1000 μg C g^{-1} soil, after 7 (or 6 - see below) days incubation. The unamended soils were adjusted to 50% WHC with distilled water, while the straw-amended soils were adjusted to the same moisture content with ammonium sulphate solution, at a concentration to give a C:N ratio with the straw of 15:1 (including the N content of the straw). Each soil was then placed in a fresh polythene bag and returned to the steel drums.

7.2.5 SOIL ERGOSTEROL MEASUREMENTS

The method employed was based on that of Grant and West (1986), but with a number of modifications, the main ones being that ^{14}C-labelled ergosterol was not used to calculate losses during extraction, and that soil-methanol extracts were not filtered.

Moist soil (25g o.d. basis, in triplicate) was weighed into 250 ml centrifuge bottles (MSE, Crawley, UK). 120 ml methanol, at 5°C, was added and the soil ultrasonified for 3 min in an ice-water bath, using a 20 kHz 140 W MSE sonifier with a 12.5 mm probe, set at high power with amplitude = 5, giving an inter-peak distance of 18 μm. The bottles were swirled vigorously for 20 s at the start, and half way through the sonication period. The bottles were kept in ice for 15 min, then centrifuged at 6000 rpm for 15 min, at 0°C (MSE Europa 24M).

Supernatant (100 ml) was transferred to 250 ml round-bottom Quickfit flasks, and 20 ml absolute ethanol and 10 g potassium hydroxide added. The methanol extracts were then saponified under reflux in a water bath at *ca*. 90°C for 1 hour. The flasks were then removed from the water and allowed to cool for 2 min whilst still attached to the condenser. The flasks were then detached, capped with aluminium foil and cooled to 5°C.

Once cooled, the extracts were transferred to 250 ml separating funnels. HPLC-grade water (25 ml) was used to rinse the round-bottom flasks and to dissolve any remaining KOH. This was then added to the separating funnels, and the round-bottom flasks rinsed thoroughly with

methanol and left to drain. Hexane (60 ml) was added to the funnels, which were then shaken vigorously with 100 inversions. The two phases were allowed to separate, and the lower methanol layers transferred to 250 ml conical flasks, while the upper hexane layers were carefully decanted into the, now clean and dry, round-bottom flasks. Care was taken to ensure that none of the grease formed during the partitioning was transferred with the hexane layers. The methanol layers were returned to the separating funnels and the hexane partitioning step repeated twice.

The combined hexane phases were then evaporated to dryness on a rotary evaporator (Büchi Rotorvapor R) at *ca.* 28°C. The extracted ergosterol was immediately redissolved in *ca.* 1 ml HPLC-grade methanol, which was transferred to a 5 ml volumetric flask. A second aliquot of methanol was added to the flask, warmed gently on an electrothermal mantle for 15 s, swirled round the flask and then added to the 5 ml flask. The round-bottom flask was rinsed out with 2 further lots of methanol (not warmed), added to the 5 ml flask as before, which was then made up to volume. The sample was then transferred to a cork-stoppered 75 × 12 mm flat-bottomed sample tube (Scientific Furnishings, Chichester, UK), and stored at −20°C until analysis.

In order to calculate the recovery of ergosterol by the extraction procedure, 1 ml of a 150 mg l^{-1} solution of re-crystallised ergosterol (Aldrich, Gillingham, UK) in HPLC-grade methanol, was added to an additional portion of soil (25 g o.d. weight) at the same time as the 120 ml methanol, and extracted exactly as described above. One soil sample only,

taken from a different treatment each time, was so spiked on each sample day. The percentage recovery was calculated from the difference in peak height of the spiked soil and the corresponding mean of the unspiked soil.

The ergosterol extracts were analysed by reverse-phase high performance liquid chromatography, using an LDC Analyst 7800 HPLC, with wavelength set at 282 nm. The column was a LiChroCART 250 × 4 mm stainless steel Merck cartridge packed with 10 μm LiChrospher 100RP-18 end-capped sorbent, preceded by a Merck guard column (4 × 4 mm) packed with 5 μm LiChrospher sorbent, as above. The mobile phase was 98:2 methanol:water (HPLC grade), with a flow rate of 2 ml min^{-1}, giving a pressure of 1500 p.s.i. The retention time of the ergosterol under these conditions was 6.5 to 7 min. Samples were injected using a 100 μl Hamilton micro-syringe, via a 20 μl loop, and were analysed in duplicate at least.

The analyses were calibrated against a standard curve produced from ergosterol solutions of 2.5 – 40 mg l^{-1}. Results were then calculated from:

$$\mu g \text{ ergosterol } g^{-1} \text{ soil} = \frac{P \times V_1 \times V_2 \times R}{W \times C}$$

where P is the sample peak height (mm), V_1 is the volume of the ergosterol sample, V_2 is the volume of extractant (methanol + soil water) divided by the volume saponified, R is 100 divided by the % recovery, W is the dry weight of soil, and C is the regression coefficient of the standard curve.

7.2.6 MICROBIAL BIOMASS C MEASUREMENTS

Soil microbial biomass C was measured as described in section 2.2.1. Due to shortage of soil towards the end of the experiment, measurements on day 28 were done in duplicate.

Sub-samples for analysis of both parameters were removed after 7, 14 and 28 days incubation. However, due to electrical shutdowns, the day 7 and 14 samples of the triadimefon-treated soils had to be taken 1 day earlier, *i.e.* on days 6 and 13. Due to an accident during the extraction procedure, the ergosterol measurement at day 0 was from a single soil sample only, which had been stored at −15°C for 28 days.

7.3 RESULTS

7.3.1 SOIL CHARACTERISTICS

The soil was a sandy loam (Cottenham series), containing 70% sand, 15% silt and 15% clay. Soil pH was 6.7 in distilled water, 6.1 in 0.01 M $CaCl_2$, with a CEC of 12.0 me 100 g^{-1}. Organic C and total N were 1.46 and 0.139%, respectively.

7.3.2 EFFECTS OF PESTICIDES ON SOIL ERGOSTEROL CONTENTS

7.3.2.1 *Unamended soil*

After 7 days incubation, the ergosterol content of the control soil had increased by about 30%, from 1.58 to 2.05 μg g^{-1} soil (Fig. 7-2). The ergosterol content of the epoxiconazole-treated soil, at both 2× and 20×

rates, after 7 days incubation was not significantly different from that at day 0, but, at 1.49 μg g^{-1}, was about 27% lower than the control soil at 7 days. After 6 days incubation, the ergosterol content of the triadimefon-treated soil, at both rates, had declined to 1.45 μg g^{-1}, and was 30% lower than the control soil after 7 days incubation (Fig. 7-3). Thus there was no significant difference between the epoxiconazole and triadimefon treatments at either of their two respective concentrations, at this stage.

After 14 days incubation, the ergosterol content of the control soil had declined to 1.28 μg g^{-1} soil. The ergosterol content of the 20× epoxiconazole-treated soil, at 1.25 μg g^{-1}, was not significantly different from the control. However, that of the 2× treatment at 14 days, 1.39 μg g^{-1}, was slightly greater than both the control and the 20×-treated soils ($P<0.01$) at this time (Fig. 7-2). After 13 days incubation, the ergosterol content of the 1× and 10× triadimefon-treated soils had declined to 1.36 and 1.32 μg g^{-1} soil, respectively. Neither of these figures were significantly different from the control soil (Fig. 7-3).

After 28 days incubation, the ergosterol contents of the 2× and 20× epoxiconazole-treated soils were significantly less than the control ($P<0.001$), being 1.10, 1.18 and 1.39 μg g^{-1} soil, respectively. Thus, the ergosterol contents of the treated soils had declined by about 21 and 15%, respectively, although there was no significant difference between the two pesticide concentrations (Fig. 7-2). By contrast, the ergosterol contents of the 1× (1.40 μg g^{-1}) and 10× (1.36 μg g^{-1}) triadimefon-treated soils were not significantly different from the control or each other (Fig. 7-3).

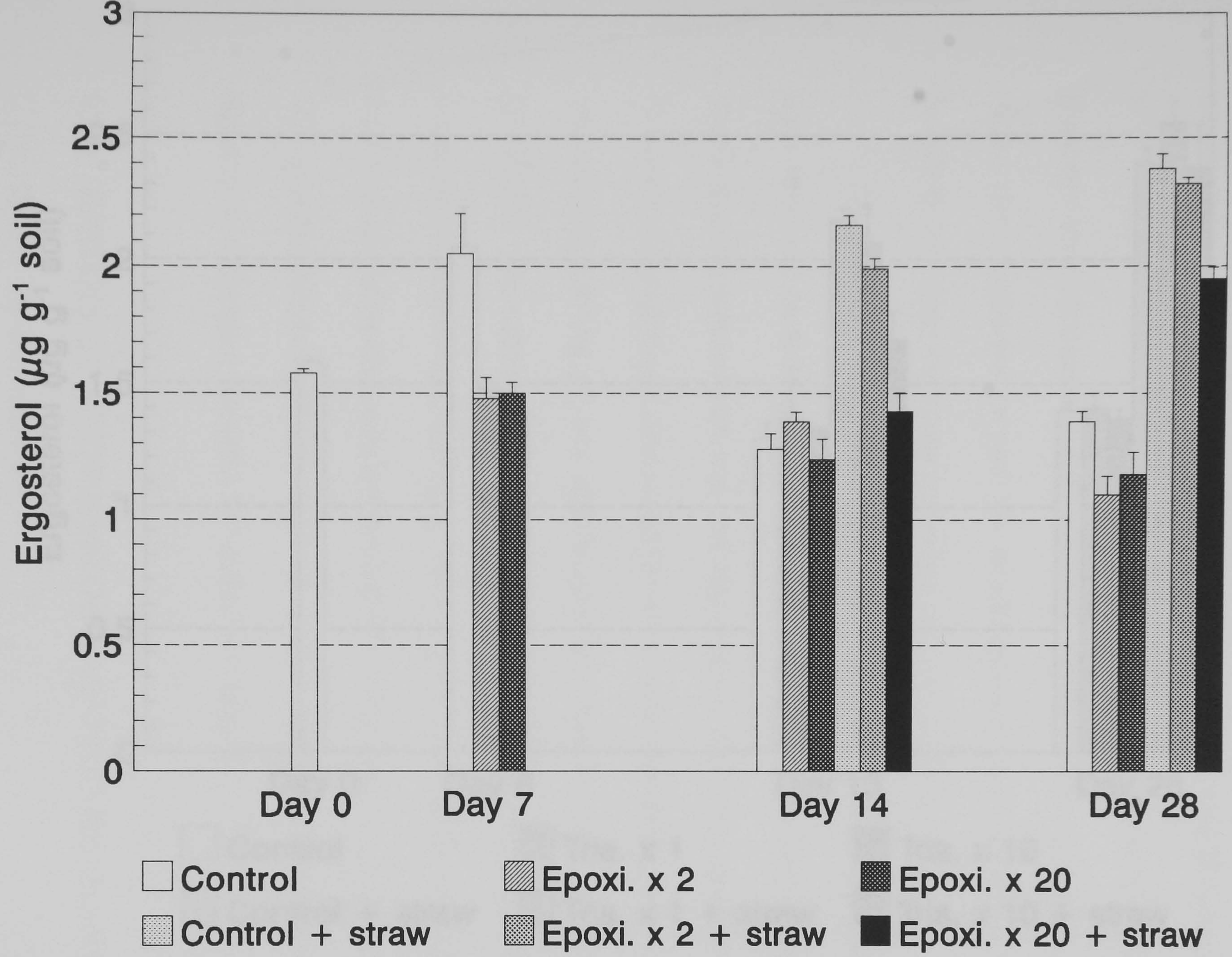


Figure 7-2: The ergosterol content of epoxiconazole-treated soil (bars = s.d.).

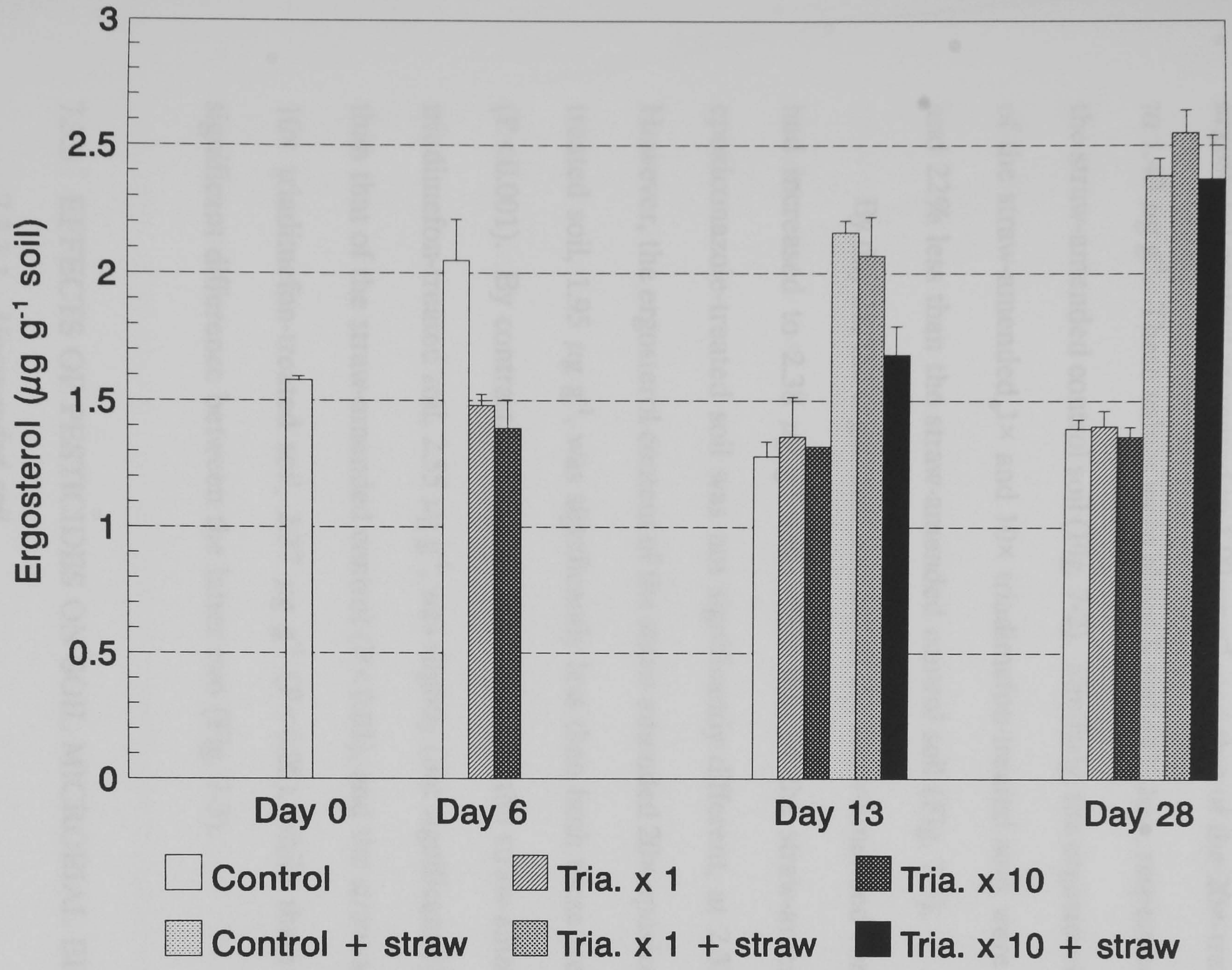


Figure 7-3: The ergosterol content of triadimefon-treated soil (bars = s.d.).

7.3.2.2 *Straw-amended soil*

Addition of straw caused a 69% increase in soil ergosterol content, compared to the unamended control, on day 14, from 1.28 to 2.16 $\mu g\ g^{-1}$ soil. However, in the straw-amended 2× epoxiconazole-treated soil the ergosterol content increased to 1.99 $\mu g\ g^{-1}$, and that of the 20×-treated soil to 1.43 $\mu g\ g^{-1}$. These values were about 8 and 44% less, respectively, than the straw-amended control soil (Fig. 7-2). Similarly, the ergosterol content of the straw-amended 1× and 10× triadimefon-treated soils were about 4 and 22% less than the straw-amended control soil (Fig. 7-3).

By day 28, the ergosterol content of the straw-amended control soil had increased to 2.38 $\mu g\ g^{-1}$ soil, while that of the straw-amended 2× epoxiconazole-treated soil was not significantly different, at 2.32 $\mu g\ g^{-1}$. However, the ergosterol content of the straw-amended 20× epoxiconazole-treated soil, 1.95 $\mu g\ g^{-1}$, was significantly less than both these treatments ($P<0.001$). By contrast, the ergosterol content of the straw-amended 1× triadimefon-treated soil, 2.55 $\mu g\ g^{-1}$, was slightly (but significantly) greater than that of the straw-amended control ($P<0.01$), and the straw-amended 10× triadimefon-treated soil, 2.37 $\mu g\ g^{-1}$ ($P<0.05$), while there was no significant difference between the latter two (Fig. 7-3).

7.3.3 EFFECTS OF PESTICIDES ON SOIL MICROBIAL BIOMASS

7.3.3.1 *Unamended soil*

Addition of epoxiconazole caused a very slight, but statistically significant ($P<0.001$), increase in biomass C of about 11 $\mu g\ g^{-1}$ soil, after

7 days incubation, compared to the control soil (Fig. 7-4). This was an increase of about 3% on average, there being no significant difference between the two pesticide concentrations. After 6 days incubation, the biomass C of the 1× triadimefon-treated soil was not significantly different from the control soil. The differences between the biomass C in the 10×, and the control and 1× triadimefon soils, although statistically significant ($P<0.01$ and 0.05, respectively), were only of about 2 and 1%, respectively (Fig. 7-5).

After 14 days incubation, the biomass C of the 2× epoxiconazole-treated soil was not significantly different from that of the control. The biomass C content of the 20× epoxiconazole soil was about 12 $\mu g\ g^{-1}$ greater than the control and 2× epoxiconazole treatments, on average, a statistically significant difference ($P<0.001$) of about 3.5%. By contrast, after 13 days incubation, the biomass C of both triadimefon-treated soils was significantly lower than the control, declining by about 12% on average, from about 328 to 289 $\mu g\ g^{-1}$ (Fig. 7-5). There was no significant difference between the two pesticide concentrations.

After 28 days incubation there were no significant differences between the epoxiconazole treatments and the control soil. By contrast, both rates of triadimefon caused small but significant increases compared to the control soil of 2.5 and 8%, for the 1× ($P<0.05$) and 10× ($P<0.001$) treated soils, respectively.

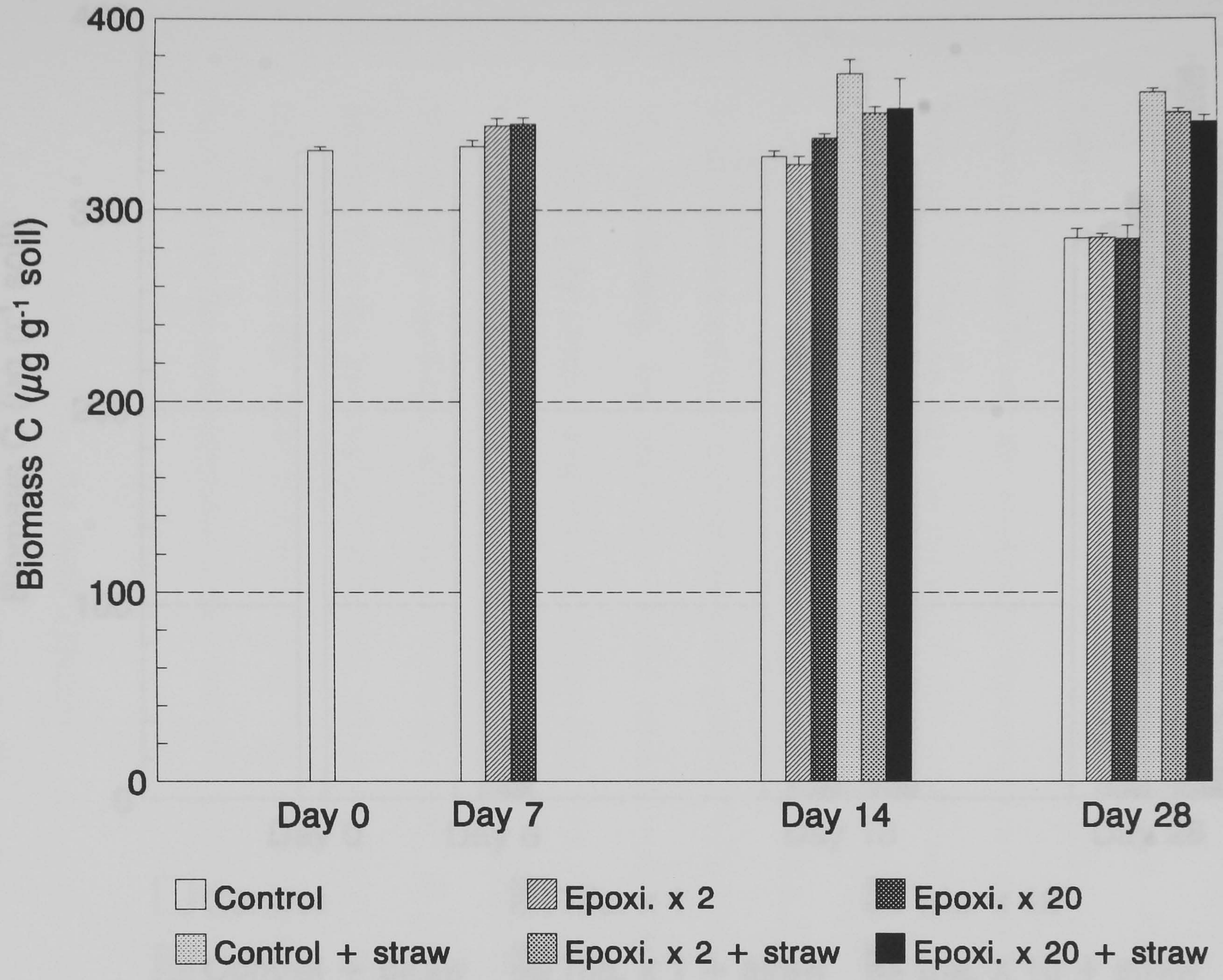


Figure 7-4: Biomass C content of epoxiconazole-treated soil (bars = s.d.).

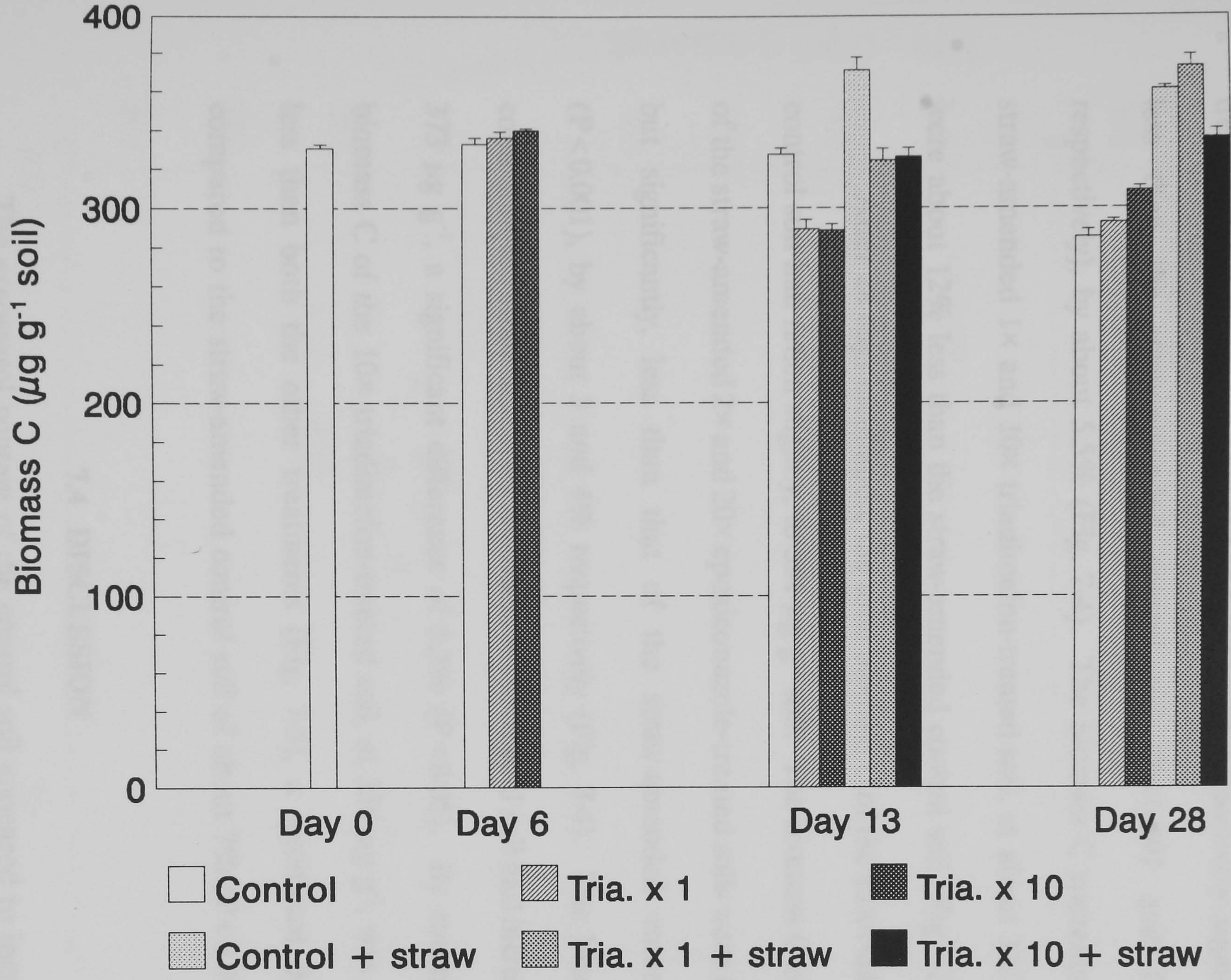


Figure 7-5: Biomass C content of triadimefon-treated soil (bars = s.d.).

7.3.3.2 *Straw-amended soil*

Addition of straw caused an increase in biomass C of 13% in the control soil, from 328 to 371 μg g^{-1} soil, on day 14. However, in the epoxiconazole-treated soils, the biomass C contents of the 2× and 20×-treated soils increased to only 350 and 352 μg g^{-1}, respectively; significantly less than the straw-amended control soil ($P<0.001$ and $P<0.05$, respectively), by about 5.5% (Fig. 7-4). The biomass C contents of the straw-amended 1× and 10× triadimefon-treated soil, at about 325 μg g^{-1}, were about 12% less than the straw-amended control soil (Fig 7-5).

After 28 days incubation, the total biomass C of the straw-amended control soil had fallen slightly, to 361 μg g^{-1} soil. The biomass C contents of the straw-amended 2× and 20× epoxiconazole-treated soils were slightly, but significantly, less than that of the straw-amended control soil ($P<0.001$), by about 3 and 4% respectively (Fig. 7-4). The biomass C content of the straw-amended 1× triadimefon-treated soil had increased to 373 μg g^{-1}, a significant difference of 3.3% ($P<0.05$). By contrast, the biomass C of the 10× triadimefon-treated soil, at 336 μg g^{-1}, was slightly less than both the other treatments (Fig. 7-5), a significant decrease compared to the straw-amended control soil of about 7% ($P<0.001$).

7.4 DISCUSSION

The ergosterol content of the control soil appeared to increase substantially over the first 7 days incubation, by about 30%. However, there was no concurrent increase in soil microbial biomass C over this

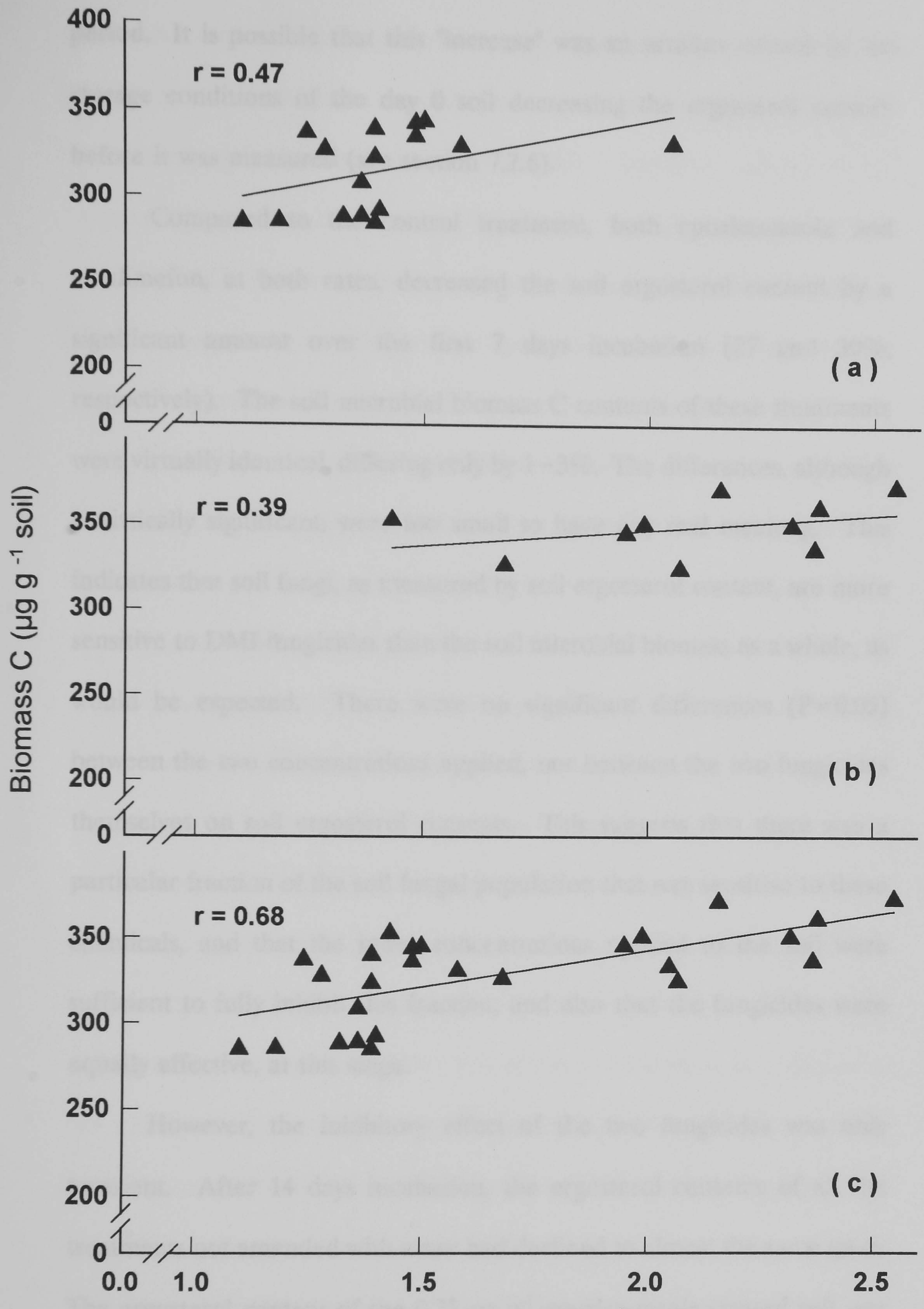


Figure 7-6: Linear correlation of ergosterol and microbial biomass C in a) unamended soils; b) straw-amended soils; c) all soils.

period. It is possible that this "increase" was an artefact caused by the storage conditions of the day 0 soil decreasing the ergosterol content before it was measured (see section 7.2.6).

Compared to the control treatment, both epoxiconazole and triadimefon, at both rates, decreased the soil ergosterol content by a significant amount over the first 7 days incubation (27 and 30%, respectively). The soil microbial biomass C contents of these treatments were virtually identical, differing only by 1−3%. The differences, although statistically significant, were too small to have any real meaning. This indicates that soil fungi, as measured by soil ergosterol content, are more sensitive to DMI fungicides than the soil microbial biomass as a whole, as would be expected. There were no significant differences ($P=0.05$) between the two concentrations applied, nor between the two fungicides themselves on soil ergosterol contents. This suggests that there was a particular fraction of the soil fungal population that was sensitive to these chemicals, and that the lower concentrations applied to the soil were sufficient to fully inhibit this fraction, and also that the fungicides were equally effective, at this stage.

However, the inhibitory effect of the two fungicides was only transient. After 14 days incubation, the ergosterol contents of all the treatments not amended with straw had declined to almost the same level. The ergosterol content of the 0.25 $\mu g\ g^{-1}$ epoxiconazole-treated soil was statistically greater than the control and the 2.5 $\mu g\ g^{-1}$ treatments, but the differences amounted to only about 0.11 and 0.13 $\mu g\ g^{-1}$ soil, respectively,

and were not really meaningful. The biomass C contents of the epoxiconazole-treated and control soil were essentially the same after 14 days incubation, at about 329 μg g^{-1} soil. However, those of the triadimefon-treated soil were significantly less than the control, at about 289 μg g^{-1} soil. This might be caused by side-effects of triadimefon on the bacterial population of the soil, or possibly the fungal population, as ergosterol concentration may not be constant during fungal growth (Newell *et al.*, 1987; Marfleet *et al.*, 1991; Schnürer, 1993), although others have reported the contrary (Newell, 1992; Nylund and Wallander, 1992; Gessner and Chauvet, 1993).

At the end of the experiment, the ergosterol contents of the unamended triadimefon-treated soil were about the same as that of the control soil (*ca.* 1.38 μg g^{-1} soil), as on day 13. However, the ergosterol contents of the epoxiconazole-treated soil were significantly less than the control soil (both *ca.* 1.14 μg g^{-1} soil). It seems implausible that epoxiconazole would cause the soil ergosterol content to fluctuate in this way, so the most likely explanation is that these relatively small decreases were due to experimental error. The soil microbial biomass contents of the epoxiconazole-treated soil were, as earlier, no different from the control soil. Those of the triadimefon-treated soil had increased by 2.5–8% relative to the control, the inhibition shown on day 13 only being temporary. The increase in microbial biomass may have been in part due to mineralisation of the microbes apparently killed at day 13 (*ca.* 8 μg C g^{-1}, assuming 20% efficiency of biosynthesis (Jenkinson and Ladd, 1981)).

As would be expected, straw addition increased the ergosterol content of the control soil by a significant amount, as fungal growth is generally assumed to be stimulated following straw addition (Allison and Killham, 1988). However, while there were no major differences between the unamended treatments after 14 days incubation, a renewed inhibitory effect was shown in the straw-amended treatments. This suggests that the fungicides were more active against an actively growing fungal population than against a stationary one. Also, unlike the inhibition shown on day 7, the higher rate of each fungicide caused significantly greater inhibition than the lower rate (22–34% and 4–8%, respectively, compared to the straw-amended control soil).

Interestingly, epoxiconazole was a more potent inhibitor of ergosterol biosynthesis than triadimefon, again in contrast to the unamended treatments (Figs. 7-2 and 7-3). Indeed, the inhibitory effect of triadimefon in the straw-amended treatments had disappeared after a further 2 weeks incubation, while the 20× epoxiconazole-treated soil still had a significantly decreased ergosterol content. This may be because epoxiconazole was applied at a higher concentration, or that the newer fungicide was more effective than the older triadimefon, or a combination of the two.

Addition of straw caused proportionately much smaller increases in microbial biomass C than in ergosterol (in the ranges 4.6–26.6% and 15.3–110.9%, respectively). The increase in total microbial biomass caused by straw-amendment was inhibited by the two fungicides. Unlike

with soil ergosterol, there was no significant difference between the inhibition caused by the two pesticide concentrations at 7 days following straw addition. As with the unamended soil, triadimefon inhibited microbial biomass to a greater extent than epoxiconazole (12 and 5%, respectively, compared to the straw-amended control soil). There was a similar recovery of microbial biomass C in the 1× triadimefon-treated soil as with soil ergosterol, but the higher rate still caused some inhibition at day 28, of about 9% compared to the straw-amended control soil. Both levels of epoxiconazole also caused some inhibition of soil microbial biomass C at day 28, of about 4% compared to the amended control soil. If it is assumed that most of the new biomass formed due to the straw amendment was fungal (Allison and Killham, 1988), this also indicates that the fungicides were more active against growing fungi.

When all treatments were included, there was a measure of linear correlation between ergosterol and microbial biomass C (Fig. 7-6c). However, when the treatments were divided into unamended and straw-amended soil, the correlation decreased markedly (Figs. 7-6a, 7-6b). Overall, only about 46% of the variation was accounted for by the linear relationship in Fig. 7-6c (*i.e.* $r^2 < 0.5$), most of which was due to gross differences between the unamended and straw-amended soil. The overall linear correlation between ergosterol and microbial biomass of 0.68 was similar to that reported by West *et al.* (1987) of 0.65. However, the results shown in Figs. 7-6a and 7-6b indicate that there was only a weak correlation between the two parameters. The relationship was probably

distorted somewhat by the inhibiting action of the fungicides, hence no firm conclusions should be drawn.

Losses of ergosterol during the extraction process ranged between 3 – 12%, with an average of 8% (± 3). These losses are very similar to those reported by Grant and West (1986), but larger than those reported later by West *et al.* (1987), of an average of 2%. Davis and Lamar (1992) criticised the method of Grant and West (1986), where pure ergosterol was added with the extractant to determine the recoveries. Instead, they proposed that determinations of extraction efficiencies should be based upon recoveries from fungal tissue added to soils. Davis and Lamar (1992) correctly pointed out that addition of ergosterol with extractants measured potential losses during extraction, but not extraction efficiencies from soil. They reported that extraction efficiencies of pure ergosterol from soil and inoculated wood chips failed to model extraction efficiencies of fungal ergosterol. They hypothesised that the losses of pure ergosterol may have been due to adsorption to soil components or to soil-catalysed oxidative degradation.

However, as the extraction procedure of Grant and West (1986) was much more rapid and vigorous than that of Davis and Lamar (1992), which involved extracting soil samples for 16 h. on a rotating tumbler shaker, it is likely that these problems would have been insignificant in the procedure described in section 7.2.5. Nevertheless, the use of fungi to determine extraction efficiency is worthy of further study. Davis and Lamar (1992) used only a single fungal species, *Phanerochaete*

chrysosporium, which had been cultured *in vitro* several times. Because of the possibility of variation between different species, a wider range would have been better. The ideal approach might be to use fungal hyphae extracted directly from soil (*e.g.* Vilariño *et al.*, 1993).

At the present time, the use of ergosterol as a fungal biomarker is limited. There is room for refinement in the methodology, and the question of variability of ergosterol content with growth stage, nutritional status and environment needs to be resolved. The recommendation of West *et al.* (1987) that ergosterol can be used "to quantify *changes* in the fungal populations of soils" cannot yet be improved upon. The results presented in this chapter do indicate, however, that this method could be very useful as a test in assessing pesticide side-effects, particularly in pesticides which specifically inhibit ergosterol biosynthesis.

7.5 SUMMARY

The effects of straw-amendment and two ergosterol biosynthesis-inhibiting fungicides, epoxiconazole and triadimefon, on ergosterol content and microbial biomass C in a sandy loam soil were investigated.

Both fungicides reduced the soil ergosterol contents of the unamended soil by about 30% after 7 days incubation, after which the contents remained similar to that of the control soil. Microbial biomass C remained largely unaffected, except for triadimefon-treated soil after 13 days incubation, where it fell by about 12%, though this inhibition was only temporary.

Following straw-amendment, the inhibition of ergosterol biosynthesis reappeared, ranging from 4–34%. This effect was also transient, but was longer-lasting than in the unamended soil. Inhibition of microbial biomass C also occurred in the straw-amended soil, ranging from 5–12%. The higher fungicide concentrations generally caused greater inhibition in soil amended with straw, in both ergosterol and microbial biomass C, than occurred in unamended soil.

Measurement of soil ergosterol content was a much more sensitive assay to measure pesticide side-effects than soil microbial C. With further research, it could be a useful method in determining changes in fungal populations, although it should not be used in isolation.

CHAPTER 8

GENERAL DISCUSSION

8.1 THE SIGNIFICANCE OF PESTICIDE SIDE-EFFECTS ON SOIL MICRO-ORGANISMS

There seems little doubt that the use of pesticides in agriculture will continue to increase, particularly in developing countries. Also, as all types of pests tend to develop tolerance or resistance to many pesticides, new chemicals will continue to be developed. Thus, there will be a need for the testing of pesticides for side-effects on soil micro-organisms for the foreseeable future.

However, although concern has been expressed over the potential side-effects of pesticides for many years, they should be considered with a sense of perspective. According to Domsch *et al.* (1983), only a small percentage of papers have reported effects attributable to pesticides greater than those caused by natural stresses, such as changes in temperature, water potential, pH *etc.* Most of these papers related to a small group of very toxic chemicals such as soil fumigants and non-selective fungicides. Most published results were considered to involve negligible effects, compared to the natural fluctuations that microbial populations and activities undergo.

Furthermore, most pesticides which enter the soil will eventually be either partially or wholly degraded, either chemically or by soil micro-organisms. Their residence times in soil are therefore usually relatively

short. Those pesticides that do persist in the environment, such as the organochlorine insecticides DDT, dieldrin and γ-HCH, have been banned or restricted in their use, or, like the cationic herbicides diquat and paraquat, are so strongly bound to soil colloids that their availability to soil micro-organisms is negligible (Alexander, 1981; Kaufman *et al.*, 1985; Simon-Sylvestre and Fournier, 1979).

When compared to heavy metals, for example, which persist in soil almost indefinitely, and whose toxic effects are more-or-less permanent (*e.g.* Brookes and McGrath, 1984), the side-effects of most pesticides are negligible, and are certainly outweighed by their beneficial effects on crop yield. Although this does not mean that there should be any laxity in assessing current and future pesticides for side-effects on soil micro-organisms, it is likely that, for most chemicals, the effects of soil microbes on the pesticides will be of greater overall concern.

8.2 RESEARCH ACHIEVEMENTS

The pesticides epoxiconazole and quinmerac were shown to have no detectable side-effects on total soil microbial biomass in several different soils, nor on microbial activity as measured by soil respiration, except for a minor inhibition caused by quinmerac at 10 times the recommended application rate in a sandy loam soil. Repeated application of the pesticides also had no noticeable effects on these parameters, and thus these two chemicals appear to be harmless to the total soil microbial population.

The mineralisation of epoxiconazole proceeded quite differently to quinmerac. Epoxiconazole was mineralised most rapidly immediately following its addition to soil, while there was a delay of varying length before the maximum mineralisation rate of quinmerac was observed. The mineralisation of the pesticides seemed to be related qualitatively and quantitatively to the soil microbial biomass content, within a given soil type, and the two chemicals were probably mineralised by distinct fractions of the microbial biomass.

The long-term cumulative annual field application of 5 pesticides, singly and in combination, for up to 19 years was shown to have no adverse effects on soil microbial biomass content or the mineralisation of soil organic matter. Application of one pesticide (aldicarb) corresponded with increases in soil microbial biomass by up to 21.5%, probably as an indirect effect stemming from the chemical's beneficial effect on crop growth.

An assay for soil ergosterol content was used, for the first time, to show that the fungicides epoxiconazole and triadimefon caused a minor, temporary inhibition of the soil fungal population, and that this inhibition reappeared following stimulation of fungal growth by the addition of straw. The ergosterol assay was more sensitive than measurements of soil microbial biomass, and could be a useful method to assess pesticide side-effects.

8.3 RECOMMENDATIONS FOR FUTURE RESEARCH

1) Results from side-effect tests should be evaluated in the light of the degradation and adsorption of the pesticide concerned. The effects of stable degradation products themselves remain relatively unexplored, and more needs to be known.

2) More effort should be made to include the effects of plant exudates and detritus in laboratory experiments. This would facilitate extrapolation of results to the field situation, which at present is not really feasible.

3) So far, very little work has been conducted on the effects of interactions between pesticides and fertilisers (*e.g.* Marsh, 1985), even though they are almost always used in conjunction with each other.

4) There needs to be an improvement in the criteria for the interpretation of the significance of pesticide side-effects, which requires an increase in our knowledge and understanding of soil microbiology. This may be achieved through the development of new methods and the improvements of current ones, such as the use of biomarkers such as ergosterol and phospholipid fatty acids, and molecular biology techniques, such as the use of monoclonal antibodies and gene probes, which may allow the identification and isolation of specific micro-organisms very accurately. More basic research is essential if substantial progress is to be made.

APPENDIX 1

COLLECTION AND PREPARATION OF SOIL

Soil for microbiological studies was collected from the top 10 to 23 cm depth for arable soils and 0-10 cm depth for grassland, usually sampled with an auger or soil corer. When a large area of land was sampled, soil samples were taken at intervals along the lines of a series of imaginary zig-zags or "W"s across it. If the sampling area of a plot was restricted in some way, *e.g.* perhaps only the edges were permitted to be sampled, every effort was made to ensure that the pooled soil samples gave as representative a bulk soil sample as possible. *N.B.* At least 20% more soil was collected than was actually required; more if the soil was very stony.

It has been recommended that soils for use in experiments involving pesticide testing should not be collected when there has been no rainfall for 30 days (Anderson, 1987). Given that this is a fairly rare occurrence in the UK, this was not be a common problem. If a period of drought and a sampling date coincided, the researcher may probably use his/her discretion as to whether or not to go ahead with the soil collection, depending on the nature of the experiment for which it was required. Once sieved and *carefully* adjusted to about 40% WHC the biomass can be reliably measured after about 7 days conditioning incubation. Of course the measurements would be of biomass content in the conditioned soil, rather than in the soil as sampled. However, there are theoretical

reasons and empirical observations which strongly indicate that the measurements may not be in serious error (Jenkinson, 1988b).

Once the soil was collected and brought back to the station, it was spread out on a piece of plastic sheeting, and large pieces of plant material, animals and stones removed by hand. If the soil was not to be processed immediately, it was stored at 4°C until preparation.

Most soils at field moisture content are usually too wet to be sieved immediately after collection, without smearing or rolling the soil. The soil must therefore be dried to a moisture content where sieving is possible without these problems occurring. The soil was spread out as thinly as possible, while remaining as a continuous and even layer. This is particularly important around the edges of the soil. Larger lumps of soil were carefully broken up by hand, so that the pieces of soil were more or less of the same size. If the soil did not break apart along natural fracture lines, but simply stretched or smeared, then it was left to dry intact, until the moisture content was such that the lumps could be broken apart in this way. The ideal moisture content of a soil is that which allows it to crumble easily when sieved, whilst still being moist enough to support the whole of its original microbial biomass.

While the soil was drying it was turned (*i.e.* mixed) regularly, so that no part of the soil became too dry. The soil at the edges dries the quickest, and can become air-dry while the soil in the centre is still too wet to sieve. A piece of soil that is not in contact with others will dry quicker still, hence the importance of keeping the soil as a continuous layer at all

times. If a few soil aggregates did become air-dry, they were discarded. If, by accident, a significant proportion of the soil becomes air-dry, then it will be useless for experimental purposes, and a fresh sample of soil will have to be collected. If the soil was being dried in a glasshouse, it was checked very frequently on sunny days, and kept out of direct sunlight by the use of blinds. If the temperature of the glasshouse became very high (> 25°C or so), the soil was removed to a cooler place, or returned to storage. If the soil took longer than one day to reach the proper moisture content (as was usually the case), it was returned to storage overnight, or, if it was very wet or in a *permanently* shady and *cool* position, it was simply covered by another sheet of plastic.

Once the soil had reached the necessary moisture content, it was then passed through a 2 mm mesh sieve. This was best achieved with the fingers or the back of a scrubbing brush. The entire soil sample was sieved, soil which would not pass readily through the sieve was not discarded. It was sometimes necessary to carry out the drying and sieving process several times. *N.B.* For soils of a very high clay content, it may not in fact be possible to pass more than a small proportion of the soil through the sieve, whatever its moisture content. If this was the case, then the remaining soil was air-dried, then sieved using a mechanical roller mill. This soil was then re-moistened with distilled water using a hand-held sprayer and then mixed thoroughly with the soil that was sieved by hand. The soil was then incubated at 25°C for at least 7 days before continuing with the procedure, as described below. Alternatively, the soil could be

broken up by hand as much as possible, and used in an unsieved state (Ocio and Brookes, 1990b).

Once the soil was sieved, as much of the particulate plant debris, animals, stones, *etc.*, was removed as possible. This was best done with a pair of forceps, on a small sub-sample of soil at a time. It was found advisable to keep a hand-held sprayer of distilled water to hand, to prevent the soil from drying out any further. When the soil had been prepared, sub-samples were taken for determination of moisture content and water holding capacity, and the soil stored at 4°C until use. It is advisable to use the soil as quickly as possible, but if storage is unavoidable, it should not normally exceed three months.

When the soil was to be used, it was spread out thinly on some plastic sheeting, and using a hand-held sprayer, enough water was added to adjust the soil to 40% WHC (the moisture content of soils previously stored at 4°C was redetermined). This was done gradually, by wetting the surface of the soil, mixing it gently but thoroughly, then applying more water, mixing, and so on until the required amount of water had been added. This was best determined by measuring the weight loss of the sprayer, rather than by filling the sprayer with the exact volume of water.

The soil was then covered with a plastic sheet and left for 1–2 hours, before being put in a polythene bag, tied loosely with a rubber band. The bag was then placed in an air-tight metal drum, together with a 100 ml jar of soda lime and several bottles containing distilled water, and the soil incubated at 25°C for 7-10 days, before experimental use.

APPENDIX 2

RAW DATA AND STATISTICAL ANALYSES

CHAPTER 3

Figure 3-1.

Time (days)	Treatment ($\mu g\ g^{-1}$)	Biomass C ($\mu g\ g^{-1}$)	s.d.
0	-	303	6.8
7	0 0.25 2.5	336 335 337	3.4 6.6 13.1
14	0 0.25 2.5	303 302 305	6.4 23.0 11.5
28	0 0.25 2.5	298 308 315	14.2 3.3 6.9
56	0 0.25 2.5	257 254 251	3.8 12.4 13.1
84	0 0.25 2.5	195 206 212	0.8 10.3 2.1

Figure 3-2.

Time (days)	Treatment ($\mu g\ g^{-1}$)	Biomass C ($\mu g\ g^{-1}$)	s.d.
0	-	303	6.8
7	0 1.0 10.0	328 337 333	7.9 2.4 2.0
14	0 1.0 10.0	303 293 302	12.4 16.9 6.3

28	0 1.0 10.0	282 275 290	11.8 7.6 8.0
56	0 1.0 10.0	260 259 263	4.1 5.6 3.7
84	0 1.0 10.0	200 207 195	3.5 3.2 5.4

Figure 3-3.

Time (days)	Treatment ($\mu g\ g^{-1}$)	Ninhydrin-N ($\mu g\ g^{-1}$)	s.d.
0	-	14.4	0.44
7	0 0.25 2.5	16.5 17.1 15.5	0.32 1.06 0.29
14	0 0.25 2.5	14.8 15.4 15.6	0.65 2.73 2.54
28	0 0.25 2.5	14.4 14.6 13.2	2.00 0.65 1.19
56	0 0.25 2.5	12.1 12.0 12.4	0.72 1.83 0.32
84	0 0.25 2.5	8.1 8.6 8.9	0.26 0.82 1.05

Figure 3-4.

Time (days)	Treatment ($\mu g\ g^{-1}$)	Ninhydrin-N ($\mu g\ g^{-1}$)	s.d.
0	-	14.4	0.44
7	0 1.0 10.0	16.1 16.3 16.8	0.58 0.67 0.27

14	0 1.0 10.0	14.7 14.7 14.5	0.22 0.19 0.59
28	0 1.0 10.0	13.6 13.8 13.7	0.57 0.11 0.96
56	0 1.0 10.0	12.9 12.5 13.1	0.56 0.08 0.35
84	0 1.0 10.0	8.4 8.7 8.5	0.45 0.31 0.30

Figure 3-6.

Time (days)	Treatment ($\mu g\ g^{-1}$)	CO_2-C evolved ($\mu g\ g^{-1}$)	s.d.
3	0 0.25 2.5	51 50 46	1.2 2.6 1.2
7	0 0.25 2.5	107 103 95	4.2 1.8 2.0
14	0 0.25 2.5	208 204 198	6.6 3.0 10.3
28	0 0.25 2.5	389 390 386	3.9 1.0 0.9
42	0 0.25 2.5	567 554 555	19.9 7.9 8.7
56	0 0.25 2.5	753 733 734	26.0 16.6 11.8
84	0 0.25 2.5	1023 997 1008	17.7 17.4 16.3

Figure 3-7.

Time (days)	Treatment ($\mu g\ g^{-1}$)	CO_2-C evolved ($\mu g\ g^{-1}$)	s.d.
3	0	51	1.7
	1.0	48	2.0
	10.0	48	1.0
7	0	105	0.8
	1.0	100	2.4
	10.0	100	1.0
14	0	206	8.0
	1.0	203	1.8
	10.0	194	5.5
28	0	391	10.1
	1.0	392	8.1
	10.0	372	5.1
42	0	553	11.9
	1.0	550	8.4
	10.0	510	4.5
56	0	733	13.0
	1.0	719	9.0
	10.0	648	10.7
84	0	988	27.8
	1.0	972	27.7
	10.0	862	36.6

CHAPTER 4

Figure 4-3.

Time (days)	Soil (15°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.	Soil (25°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.
7	Woburn	2.19	0.049	Woburn	3.98	0.121
	Broadbalk	2.11	0.022	Broadbalk	3.58	0.053
	Silsoe	1.58	0.004	Silsoe	2.24	0.058
14	Woburn	3.47	0.050	Woburn	6.68	0.070
	Broadbalk	3.74	0.039	Broadbalk	6.21	0.057
	Silsoe	2.17	0.072	Silsoe	3.46	0.083

28	Woburn Broadbalk Silsoe	5.60 6.66 3.24	0.033 0.012 0.130	Woburn Broadbalk Silsoe	10.52 9.44 5.32	0.174 0.048 0.159
42	Woburn Broadbalk Silsoe	7.52 8.99 4.18	0.059 0.084 0.111	Woburn Broadbalk Silsoe	13.53 11.94 6.90	0.115 0.004 0.086
56	Woburn Broadbalk Silsoe	9.13 10.79 4.97	0.051 0.044 0.086	Woburn Broadbalk Silsoe	15.71 13.95 8.35	0.108 0.023 0.077
70	Woburn Broadbalk Silsoe	10.81 13.21 5.90	0.067 0.176 0.058	Woburn Broadbalk Silsoe	18.01 17.53 9.92	0.289 0.193 0.030
84	Woburn Broadbalk Silsoe	12.53 15.54 6.66	0.075 0.270 0.039	Woburn Broadbalk Silsoe	19.63 20.12 11.27	0.204 0.008 0.052
98	Woburn Broadbalk Silsoe	13.96 17.56 7.48	0.022 0.168 0.063	Woburn Broadbalk Silsoe	20.60 22.02 12.42	0.121 0.100 0.043
112	Woburn Broadbalk Silsoe	15.35 19.20 8.27	0.017 0.055 0.071	Woburn Broadbalk Silsoe	21.61 23.70 13.65	0.116 0.082 0.027
140	Woburn Broadbalk Silsoe	17.91 22.84 10.04	0.034 0.237 0.056	Woburn Broadbalk Silsoe	24.20 28.17 16.00	0.223 0.340 0.081
172	Woburn Broadbalk Silsoe	20.21 25.57 11.79	0.110 0.178 0.153	Woburn Broadbalk Silsoe	25.88 30.74 17.93	0.148 0.188 0.076
199	Woburn Broadbalk Silsoe	22.09 28.31 13.56	0.138 0.058 0.361	Woburn Broadbalk Silsoe	27.72 33.66 19.64	0.037 0.264 0.043
224	Woburn Broadbalk Silsoe	23.57 30.04 14.99	0.102 0.019 0.438	Woburn Broadbalk Silsoe	28.93 35.35 20.90	0.077 0.066 0.031
266	Woburn Broadbalk Silsoe	25.41 32.10 17.01	0.113 0.069 0.215	Woburn Broadbalk Silsoe	30.40 37.38 22.67	0.070 0.145 0.042

Figure 4-4.

Time (days)	Soil (15°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.	Soil (25°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.
7	Woburn	2.16	0.032	Woburn	3.43	0.089
	Broadbalk	2.14	0.022	Broadbalk	3.21	0.080
	Silsoe	1.63	0.134	Silsoe	2.26	0.071
14	Woburn	3.04	0.019	Woburn	5.07	0.033
	Broadbalk	3.17	0.008	Broadbalk	4.98	0.122
	Silsoe	2.11	0.042	Silsoe	3.17	0.029
28	Woburn	4.37	0.088	Woburn	7.34	0.101
	Broadbalk	4.84	0.046	Broadbalk	7.15	0.148
	Silsoe	2.88	0.094	Silsoe	4.53	0.011
42	Woburn	5.57	0.112	Woburn	9.16	0.055
	Broadbalk	6.18	0.024	Broadbalk	8.84	0.131
	Silsoe	3.53	0.066	Silsoe	5.63	0.094
56	Woburn	6.57	0.092	Woburn	10.58	0.108
	Broadbalk	7.23	0.037	Broadbalk	10.24	0.099
	Silsoe	4.06	0.045	Silsoe	6.64	0.086
70	Woburn	7.69	0.130	Woburn	12.44	0.144
	Broadbalk	8.80	0.047	Broadbalk	12.92	0.138
	Silsoe	4.72	0.032	Silsoe	7.82	0.157
84	Woburn	8.83	0.127	Woburn	13.82	0.088
	Broadbalk	10.32	0.040	Broadbalk	14.94	0.081
	Silsoe	5.27	0.071	Silsoe	8.86	0.137
98	Woburn	9.79	0.082	Woburn	14.62	0.045
	Broadbalk	11.64	0.011	Broadbalk	16.32	0.039
	Silsoe	5.85	0.064	Silsoe	9.75	0.122
112	Woburn	10.71	0.078	Woburn	15.43	0.118
	Broadbalk	12.73	0.032	Broadbalk	17.60	0.050
	Silsoe	6.39	0.057	Silsoe	10.62	0.111
140	Woburn	12.66	0.184	Woburn	17.72	0.152
	Broadbalk	15.15	0.115	Broadbalk	21.09	0.142
	Silsoe	7.69	0.079	Silsoe	12.63	0.177
172	Woburn	14.39	0.190	Woburn	19.15	0.084
	Broadbalk	17.06	0.106	Broadbalk	23.34	0.171
	Silsoe	9.00	0.063	Silsoe	14.19	0.010

199	Woburn Broadbalk Silsoe	15.89 18.94 10.28	0.177 0.104 0.089	Woburn Broadbalk Silsoe	20.64 25.75 15.70	0.129 0.222 0.153
224	Woburn Broadbalk Silsoe	16.99 20.22 11.25	0.133 0.082 0.118	Woburn Broadbalk Silsoe	21.59 27.23 16.71	0.050 0.164 0.101
266	Woburn Broadbalk Silsoe	18.41 21.84 12.65	0.142 0.066 0.224	Woburn Broadbalk Silsoe	22.78 29.00 18.11	0.039 0.123 0.143

Figure 4-5.

Time (days)	Soil (15°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.	Soil (25°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.
7	Woburn Broadbalk Silsoe	0.69 0.57 0.19	0.026 0.167 0.033	Woburn Broadbalk Silsoe	2.25 1.55 0.62	0.031 0.138 0.069
14	Woburn Broadbalk Silsoe	1.86 1.41 0.46	0.029 0.043 0.021	Woburn Broadbalk Silsoe	5.60 4.20 2.09	0.071 0.046 0.143
28	Woburn Broadbalk Silsoe	4.51 3.36 1.38	0.012 0.094 0.068	Woburn Broadbalk Silsoe	10.97 9.04 5.90	0.159 0.062 0.003
42	Woburn Broadbalk Silsoe	7.30 5.33 2.75	0.019 0.074 0.060	Woburn Broadbalk Silsoe	14.81 14.39 10.06	0.202 0.133 0.095
56	Woburn Broadbalk Silsoe	9.72 7.19 4.38	0.061 0.082 0.027	Woburn Broadbalk Silsoe	17.26 19.95 13.51	0.093 0.128 0.110
70	Woburn Broadbalk Silsoe	11.69 9.46 6.52	0.046 0.083 0.037	Woburn Broadbalk Silsoe	18.98 24.82 16.65	0.022 0.071 0.294
84	Woburn Broadbalk Silsoe	13.54 11.51 8.52	0.080 0.058 0.023	Woburn Broadbalk Silsoe	20.23 29.10 19.13	0.018 0.132 0.221
98	Woburn Broadbalk Silsoe	14.91 13.51 10.59	0.063 0.026 0.035	Woburn Broadbalk Silsoe	20.99 31.82 20.90	0.012 0.041 0.102

112	Woburn Broadbalk Silsoe	15.95 15.38 12.44	0.044 0.038 0.031	Woburn Broadbalk Silsoe	21.64 34.07 22.29	0.043 0.030 0.025
140	Woburn Broadbalk Silsoe	17.63 19.32 15.72	0.059 0.092 0.088	Woburn Broadbalk Silsoe	22.95 37.97 24.58	0.122 0.024 0.029
172	Woburn Broadbalk Silsoe	19.19 23.21 18.97	0.069 0.062 0.073	Woburn Broadbalk Silsoe	24.05 41.15 26.49	0.089 0.130 0.045
199	Woburn Broadbalk Silsoe	20.28 26.42 21.49	0.063 0.053 0.017	Woburn Broadbalk Silsoe	24.86 43.65 27.82	0.057 0.861 0.042
224	Woburn Broadbalk Silsoe	21.07 28.69 23.17	0.025 0.051 0.033	Woburn Broadbalk Silsoe	25.52 45.15 28.83	0.027 0.094 0.068
266	Woburn Broadbalk Silsoe	22.15 31.92 25.67	0.047 0.032 0.014	Woburn Broadbalk Silsoe	26.49 46.87 30.26	0.079 0.121 0.092

Figure 4-6.

Time (days)	Soil (15°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.	Soil (25°C)	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.
7	Woburn Broadbalk Silsoe	0.22 0.20 0.07	0.011 0.022 0.018	Woburn Broadbalk Silsoe	0.82 0.71 0.34	0.109 0.067 0.008
14	Woburn Broadbalk Silsoe	0.66 0.50 0.14	0.103 0.042 0.012	Woburn Broadbalk Silsoe	2.13 1.87 0.94	0.112 0.134 0.019
28	Woburn Broadbalk Silsoe	1.77 1.25 0.32	0.111 0.092 0.051	Woburn Broadbalk Silsoe	4.41 3.89 2.34	0.145 0.178 0.032
42	Woburn Broadbalk Silsoe	2.99 2.09 0.56	0.106 0.084 0.037	Woburn Broadbalk Silsoe	6.45 5.81 4.13	0.121 0.124 0.038
56	Woburn Broadbalk Silsoe	4.07 2.84 0.80	0.090 0.073 0.055	Woburn Broadbalk Silsoe	8.30 7.64 6.19	0.082 0.118 0.041

70	Woburn Broadbalk Silsoe	5.22 3.89 1.15	0.133 0.079 0.083	Woburn Broadbalk Silsoe	10.16 10.07 8.38	0.077 0.129 0.110
84	Woburn Broadbalk Silsoe	6.41 4.92 1.45	0.091 0.088 0.083	Woburn Broadbalk Silsoe	11.67 12.18 10.39	0.086 0.124 0.099
98	Woburn Broadbalk Silsoe	7.30 5.88 1.80	0.202 0.075 0.092	Woburn Broadbalk Silsoe	12.56 13.65 12.10	0.100 0.096 0.031
112	Woburn Broadbalk Silsoe	8.06 6.70 2.13	0.090 0.066 0.097	Woburn Broadbalk Silsoe	13.34 14.96 13.51	0.127 0.056 0.114
140	Woburn Broadbalk Silsoe	9.65 8.74 2.84	0.097 0.121 0.214	Woburn Broadbalk Silsoe	15.06 18.08 15.85	0.313 0.202 0.277
172	Woburn Broadbalk Silsoe	11.10 10.67 3.74	0.254 0.105 0.243	Woburn Broadbalk Silsoe	16.50 20.45 17.96	0.387 0.170 0.251
199	Woburn Broadbalk Silsoe	12.34 12.50 4.64	0.041 0.118 0.230	Woburn Broadbalk Silsoe	17.48 22.59 19.51	0.082 0.224 0.122
224	Woburn Broadbalk Silsoe	13.21 13.78 5.37	0.068 0.046 0.158	Woburn Broadbalk Silsoe	18.22 23.85 20.69	0.079 0.233 0.057
266	Woburn Broadbalk Silsoe	14.33 15.45 6.62	0.092 0.082 0.314	Woburn Broadbalk Silsoe	19.22 25.85 22.45	0.041 0.383 0.060

Figure 5-3.

Time (days)	Soil/Treatment	CO_2-C evolved ($\mu g\ g^{-1}$)	s.d.
3	Wheat	42	1.9
	Path	24	1.3
	Fallow	40	2.9
7	Wheat	77	2.6
	Path	41	1.5
	Fallow	66	1.0
14	Wheat	110	2.2
	Path	57	2.1
	Fallow	88	2.0
21	Wheat	138	3.4
	Path	70	3.4
	Fallow	106	2.3
28	Wheat	163	3.1
	Path	80	1.2
	Fallow	121	2.2
35	Wheat	199	4.0
	Path	101	0.2
	Fallow	142	3.4
	Wheat + glucose	813	5.1
	Path + glucose	726	9.7
	Fallow + glucose	765	1.9
	Wheat + ryegrass	545	16.8
	Path + ryegrass	492	2.1
	Fallow + ryegrass	495	6.1
42	Wheat	224	3.7
	Path	115	1.3
	Fallow	157	2.5
	Wheat + glucose	874	3.0
	Path + glucose	776	3.1
	Fallow + glucose	828	1.0
	Wheat + ryegrass	659	1.2
	Path + ryegrass	603	2.0
	Fallow + ryegrass	593	1.1

49	Wheat	249	4.0
	Path	126	2.7
	Fallow	171	2.2
	Wheat + glucose	917	3.9
	Path + glucose	809	0.6
	Fallow + glucose	867	3.0
	Wheat + ryegrass	730	2.2
	Path + ryegrass	666	3.9
	Fallow + ryegrass	655	0.5
56	Wheat	269	4.4
	Path	135	2.7
	Fallow	182	2.8
	Wheat + glucose	949	1.9
	Path + glucose	832	3.7
	Fallow + glucose	892	2.5
	Wheat + ryegrass	783	3.3
	Path + ryegrass	709	1.8
	Fallow + ryegrass	695	2.9
63	Wheat	297	2.2
	Path	150	0.6
	Fallow	196	3.1
	Wheat + glucose	1626	8.1
	Path + glucose	1520	6.0
	Fallow + glucose	1589	0.3
	Wheat + ryegrass	1223	28.3
	Path + ryegrass	1162	19.1
	Fallow + ryegrass	1147	24.6
70	Wheat	319	2.9
	Path	161	3.6
	Fallow	206	1.2
	Wheat + glucose	1687	3.4
	Path + glucose	1570	1.3
	Fallow + glucose	1652	0.0
	Wheat + ryegrass	1370	10.4
	Path + ryegrass	1293	13.6
	Fallow + ryegrass	1275	3.8
	Wheat	341	3.3
	Path	171	2.0
	Fallow	216	1.4

77	Wheat + glucose	1731	3.6
	Path + glucose	1604	4.2
	Fallow + glucose	1690	2.4
	Wheat + ryegrass	1462	8.2
	Path + ryegrass	1379	5.1
	Fallow + ryegrass	1357	2.4
84	Wheat	361	3.1
	Path	182	1.1
	Fallow	223	1.4
	Wheat + glucose	1766	2.3
	Path + glucose	1632	7.0
	Fallow + glucose	1717	2.1
	Wheat + ryegrass	1524	3.0
	Path + ryegrass	1434	0.0
	Fallow + ryegrass	1419	2.4

Figure 5-4.

Time (days)	Soil/Treatment	CO_2-C evolved ($\mu g\ g^{-1}$)	s.d.
3	Wheat	61	3.0
	Path	44	4.9
	Fallow	48	3.6
7	Wheat	100	1.9
	Path	68	2.7
	Fallow	83	1.9
14	Wheat	134	3.2
	Path	82	3.0
	Fallow	109	2.2
21	Wheat	159	2.5
	Path	97	5.0
	Fallow	125	1.4
28	Wheat	182	2.3
	Path	110	2.9
	Fallow	142	2.4
35	Wheat	217	0.4
	Path	132	2.5
	Fallow	165	3.6
	Wheat + glucose	824	16.9
	Path + glucose	743	19.7
	Fallow + glucose	771	2.2

	Wheat + ryegrass	578	5.0
	Path + ryegrass	515	3.6
	Fallow + ryegrass	510	1.8
42	Wheat	237	1.6
	Path	146	2.4
	Fallow	181	3.1
	Wheat + glucose	880	2.4
	Path + glucose	792	5.5
	Fallow + glucose	833	2.0
	Wheat + ryegrass	696	1.9
	Path + ryegrass	623	1.3
	Fallow + ryegrass	612	3.9
49	Wheat	259	1.6
	Path	159	4.7
	Fallow	197	3.7
	Wheat + glucose	923	2.4
	Path + glucose	827	4.6
	Fallow + glucose	872	2.0
	Wheat + ryegrass	771	7.4
	Path + ryegrass	683	0.2
	Fallow + ryegrass	674	1.3
56	Wheat	276	2.5
	Path	171	3.4
	Fallow	207	0.5
	Wheat + glucose	956	1.9
	Path + glucose	849	4.4
	Fallow + glucose	897	1.7
	Wheat + ryegrass	826	7.6
	Path + ryegrass	722	1.9
	Fallow + ryegrass	712	1.2
63	Wheat	302	2.6
	Path	186	4.4
	Fallow	223	2.6
	Wheat + glucose	1614	7.6
	Path + glucose	1505	8.6
	Fallow + glucose	1548	16.5
	Wheat + ryegrass	1281	25.4
	Path + ryegrass	1188	3.5
	Fallow + ryegrass	1151	15.8

70	Wheat	322	3.2
	Path	200	4.1
	Fallow	235	4.4
	Wheat + glucose	1686	5.9
	Path + glucose	1573	3.8
	Fallow + glucose	1614	0.7
	Wheat + ryegrass	1428	19.7
	Path + ryegrass	1302	2.7
	Fallow + ryegrass	1277	7.4
77	Wheat	340	0.6
	Path	213	1.1
	Fallow	247	2.4
	Wheat + glucose	1737	6.2
	Path + glucose	1619	2.2
	Fallow + glucose	1658	2.5
	Wheat + ryegrass	1518	11.2
	Path + ryegrass	1378	4.8
	Fallow + ryegrass	1364	2.6
84	Wheat	358	1.4
	Path	224	3.0
	Fallow	258	2.4
	Wheat + glucose	1778	6.8
	Path + glucose	1652	0.9
	Fallow + glucose	1694	0.8
	Wheat + ryegrass	1582	3.8
	Path + ryegrass	1431	1.4
	Fallow + ryegrass	1431	2.8

Figure 5-5.

Time (days)	Soil/Treatment	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.
3	Wheat	5.05	0.081
	Path	4.86	0.038
	Fallow	3.95	0.010
7	Wheat	6.70	0.032
	Path	6.32	0.043
	Fallow	5.58	0.019

14	Wheat Path Fallow	7.91 7.39 6.72	0.045 0.031 0.007
21	Wheat Path Fallow	8.83 8.20 7.49	0.018 0.029 0.016
28	Wheat Path Fallow	9.68 8.89 8.17	0.020 0.082 0.017
35	Wheat Path Fallow	10.54 9.57 8.84	0.009 0.011 0.012
	Wheat + glucose Path + glucose Fallow + glucose	11.30 10.38 9.59	0.052 0.091 0.056
	Wheat + ryegrass Path + ryegrass Fallow + ryegrass	11.41 10.23 9.56	0.088 0.057 0.052
42	Wheat Path Fallow	11.23 10.09 9.36	0.011 0.023 0.031
	Wheat + glucose Path + glucose Fallow + glucose	12.61 11.88 10.54	0.112 0.102 0.064
	Wheat + ryegrass Path + ryegrass Fallow + ryegrass	13.24 11.65 10.83	0.037 0.048 0.073
49	Wheat Path Fallow	11.91 10.59 9.88	0.007 0.020 0.033
	Wheat + glucose Path + glucose Fallow + glucose	13.83 13.21 11.54	0.084 0.099 0.104
	Wheat + ryegrass Path + ryegrass Fallow + ryegrass	14.86 13.10 12.14	0.045 0.252 0.110
	Wheat Path Fallow	12.53 11.04 10.35	0.021 0.023 0.039

56	Wheat + glucose	14.84	0.062
	Path + glucose	14.31	0.057
	Fallow + glucose	12.29	0.069
	Wheat + ryegrass	16.14	0.046
	Path + ryegrass	14.26	0.096
	Fallow + ryegrass	13.22	0.072
63	Wheat	13.18	0.011
	Path	11.51	0.014
	Fallow	10.82	0.066
	Wheat + glucose	16.48	0.102
	Path + glucose	16.10	0.133
	Fallow + glucose	14.06	0.184
	Wheat + ryegrass	18.39	0.052
	Path + ryegrass	16.24	0.181
	Fallow + ryegrass	15.10	0.120
70	Wheat	13.77	0.013
	Path	11.94	0.022
	Fallow	11.27	0.067
	Wheat + glucose	17.86	0.154
	Path + glucose	17.92	0.130
	Fallow + glucose	15.29	0.238
	Wheat + ryegrass	20.72	0.027
	Path + ryegrass	18.22	0.201
	Fallow + ryegrass	17.03	0.098
77	Wheat	14.35	0.008
	Path	12.36	0.014
	Fallow	11.71	0.072
	Wheat + glucose	19.06	0.108
	Path + glucose	19.40	0.017
	Fallow + glucose	16.38	0.243
	Wheat + ryegrass	22.48	0.009
	Path + ryegrass	19.75	0.182
	Fallow + ryegrass	18.68	0.055
84	Wheat	14.90	0.013
	Path	12.77	0.010
	Fallow	12.14	0.082
	Wheat + glucose	20.12	0.056
	Path + glucose	20.62	0.027
	Fallow + glucose	17.32	0.173

	Wheat + ryegrass Path + ryegrass Fallow + ryegrass	23.95 21.01 20.10	0.104 0.203 0.059

Figure 5-6.

Time (days)	Soil/Treatment	$^{14}CO_2$-C evolved (% of ^{14}C applied)	s.d.
3	Wheat Path Fallow	0.71 0.32 0.17	0.020 0.008 0.007
7	Wheat Path Fallow	2.32 0.97 0.60	0.029 0.015 0.023
14	Wheat Path Fallow	4.88 2.00 1.30	0.058 0.037 0.034
21	Wheat Path Fallow	7.87 3.16 2.08	0.069 0.021 0.064
28	Wheat Path Fallow	11.32 4.48 3.04	0.092 0.050 0.063
35	Wheat Path Fallow	15.27 6.00 4.23	0.038 0.036 0.101
	Wheat + glucose Path + glucose Fallow + glucose	15.89 6.43 4.85	0.176 0.151 0.074
	Wheat + ryegrass Path + ryegrass Fallow + ryegrass	15.28 6.26 4.96	0.097 0.081 0.092
42	Wheat Path Fallow	18.39 7.28 5.21	0.040 0.037 0.078
	Wheat + glucose Path + glucose Fallow + glucose	19.57 8.29 6.00	0.143 0.072 0.046

	Wheat + ryegrass	18.44	0.089
	Path + ryegrass	7.92	0.041
	Fallow + ryegrass	6.20	0.132
49	Wheat	21.60	0.027
	Path	8.68	0.044
	Fallow	6.29	0.087
	Wheat + glucose	23.47	0.006
	Path + glucose	10.38	0.073
	Fallow + glucose	7.33	0.059
	Wheat + ryegrass	21.94	0.031
	Path + ryegrass	9.69	0.070
	Fallow + ryegrass	7.52	0.152
56	Wheat	24.40	0.031
	Path	10.01	0.056
	Fallow	7.30	0.066
	Wheat + glucose	26.76	0.082
	Path + glucose	12.41	0.045
	Fallow + glucose	8.64	0.060
	Wheat + ryegrass	25.27	0.064
	Path + ryegrass	11.42	0.038
	Fallow + ryegrass	8.82	0.173
63	Wheat	27.18	0.019
	Path	11.46	0.048
	Fallow	8.41	0.091
	Wheat + glucose	30.24	0.068
	Path + glucose	14.63	0.182
	Fallow + glucose	10.45	0.113
	Wheat + ryegrass	28.50	0.052
	Path + ryegrass	13.44	0.078
	Fallow + ryegrass	10.91	0.081
70	Wheat	29.54	0.002
	Path	12.83	0.080
	Fallow	9.46	0.104
	Wheat + glucose	33.04	0.029
	Path + glucose	16.74	0.112
	Fallow + glucose	11.69	0.093
	Wheat + ryegrass	31.14	0.056
	Path + ryegrass	15.35	0.061
	Fallow + ryegrass	12.45	0.143

77	Wheat	31.73	0.018
	Path	14.23	0.083
	Fallow	10.52	0.084
	Wheat + glucose	35.53	0.062
	Path + glucose	18.98	0.134
	Fallow + glucose	13.14	0.103
	Wheat + ryegrass	33.87	0.008
	Path + ryegrass	17.33	0.081
	Fallow + ryegrass	14.07	0.184
84	Wheat	33.68	0.032
	Path	15.61	0.084
	Fallow	11.56	0.066
	Wheat + glucose	37.57	0.025
	Path + glucose	21.18	0.081
	Fallow + glucose	14.68	0.103
	Wheat + ryegrass	36.31	0.054
	Path + ryegrass	19.28	0.078
	Fallow + ryegrass	15.77	0.162

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