SOIL MICROBIAL BIOMASS AND ORGANIC MATTER DYNAMICS IN METAL-CONTAMINATED SOILS

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ABSTRACT

A main aim of this thesis was to compare and evaluate different microbiological methods (total biomass or microbial activity) to detect effects of heavy metals on the functioning of the soil ecosystem. The biomass methods, which included biomass C, biomass ninhydrin-N, biomass N, ATP, substrate induced respiration and the activity methods, which included CO_2 evolution and arginine ammonification reliably detected effects of heavy metals on the soil microbial ecosystem in metal-contaminated soils from the Woburn Market Garden Experiment which contained, due to past sludge applications, Zn, Cu or Ni at around current European Union (EU) upper limits and Cd at around three times the limit. Most microbiological indices were decreased by up to about 50% in the most metal-contaminated soils and the results were comparatively simple to interpret.

Many more problems were encountered when soils from a non-experimental site in Spain, polluted by heavy metals from past mining activity, and now containing heavy metals at up to 27 times current EU limits were examined. Huge variability, caused, for example, by site variations in soil organic C, soil texture, agricultural management and topography, were encountered. While the biomass methods and most measurements of microbial activity (except CO_2-C evolved) gave some indication of a negative response to increases soil metal concentrations, linked parameters *e.g.* biomass specific respiration or biomass as a percentage of soil organic C provided much more sensitive indicators of the effects of metals on the soil ecosystem. It was concluded that extrapolation from the field experiment to the natural environment, while difficult, is now a practical proposition.

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I dedicate this thesis to my husband

J u a n

and my sons and daughter

E r l i k

K e v i n

and

E r i n

CHAPTER 1

GENERAL INTRODUCTION

Pollution of soils by heavy metals (*e.g.* Cu, Ni, Cd, Zn) may occur either through natural processes or via anthropogenic pathways. Some soil parent materials, such as the black sedimentary sulphide-bearing shales contain high concentrations of Cd, Zn, Pb and Ni (Alloway, 1990). Anthropogenic sources of metal pollution are smelters, mining, industry and the application of metal-contaminated pesticides, fertilizer and sewage sludge to land. Sewage sludge is produced as an inevitable by-product of waste water treatment and its disposal has to face the problem of increasing quantities being produced. The UK currently produces over 1 million tonnes of sewage sludge as dry solids a year (Bruce and Davis, 1989). The common disposal practices in the UK are incineration (4%), landfill (25%), dumping at sea (30%) and application to agricultural land (42%). The latter in the UK is set to increase in the next four years as dumping at sea in the UK will be banned in 1998 (Wild and Jones, 1991; DoE, 1993). Incineration and landfill are expensive, may cause atmospheric pollution and are not suitable to accommodate the increasing quantities of sludge.

Application of sewage sludge to agricultural land is an attractive option because the sludges can be a valuable source of essential macronutrients to plants, such as N, P and K as well as micronutrients such as Fe, Cu and Zn. Sewage sludge acts also as a soil conditioner, improving the physical properties of soils, by decreasing bulk density and increasing water holding capacity (Dennis and Fresquez,

1989; Khaleel *et al.*,1981; Kladivko and Nelson, 1979). Repeated applications of sewage sludge, however, can result in elevated metal concentrations that persist in the plough layer for many years (DoE, 1981; McGrath, 1987).

Le Riche (1968) reported metal contamination of soil following the long term application of sewage sludge to the Woburn Market Garden field experiment at Woburn, UK. Since that date there have been many reports concerning metals in sludge and in crop plants grown on sludge-amended soils (Amadu *et al.*, 1989; Berrow and Webber, 1972; Chang *et al.*, 1982; Cunninham *et al.*, 1975; Mahler *et al.*, 1987; Mizuno, 1968). Therefore, limits for the safe use of sewage sludge have been proposed based on their total content of Zn, Cu and Ni, and the maximum permitted limit of heavy metals accumulated in soils have been regulated, to avoid harmful effects to plants (Commission of the European Communities, 1986). However, these guidelines do not take into account the impact of heavy metals on soil micro-organisms and their activity. The residence time of metal derived from sludge is probably of the order of 10^3 - 10^4 years (McGrath, 1987). The heavy metals entering the soil through pollution are strongly held on soil clays and organic matter, thus, metals are initially present as microscopic particles which may be sorbed, bound, chelated and/or in salt form and therefore may be less mobile and less bioavailable in soils with higher organic matter and clay contents contents (Alloway, 1990; Babich and Stotzky, 1983; Hongve *et al.*, 1980; Tyler, 1981). Their potential toxicity is controlled by several physico-chemical factors *e.g.* pH, inorganic anion concentrations, competition from other cations and chelating agent concentrations (Bache, 1979; Buckman and Brady, 1969; Gast, 1979; Hargreaves and Whitton, 1976; Stevenson, 1979). Heavy metals are more mobile in acid soils and hence,

more available for plant uptake, but become less so at higher soil pH (Chaney, 1973; Davies and Carlton-Smith, 1980; Smilde, 1981). Heavy metals released through human activities may become immediately available to micro-organisms more readily than those occurring naturally. For these reasons, the concentration of total heavy metal in a soil may not be a good indicator of its biological availability (Wilson, 1977).

Numerous studies have demonstrated that classical methods such as viable counts are highly selective and of limited value in providing information on the microbial nutrient pool size and the turnover of this pool (Jenkinson and Ladd, 1981). The concept of the microbial biomass was therefore developed (Jenkinson, 1966; Jenkinson and Powlson, 1976a) whereby the soil micro-organisms were measured as a single unit. The biomass has a multiple role in soil, affecting the decomposition and turnover of organic matter, nutrient immobilization and cycling, and soil structure. The microbial biomass is itself part of the soil organic C, comprising typically 1 to 3 % (Anderson and Domsch, 1980; Jenkinson and Powlson, 1976b; Jenkinson and Ladd, 1981; Lynch and Panting, 1980; Van Veen and Paul, 1981). The microbial biomass is defined as the living microbial component of the soil, and includes bacteria, actinomycetes, fungi, protozoa, algae and microfauna. Traditionally, soil microbial studies have focused on the isolation of specific microbial groups.

As a general criterion, a quantitative assessment of the biomass requires that the method used must take into account all the diverse components of the biomass. Several methods currently available can provide an alternative approach to estimate soils microbial biomass *in situ*. Examples are:

Microscopic counts

Fumigation-incubation

Fumigation-extraction

Substrate induced respiration

ATP content

Microcalorimetry

The use of direct microscopy to measure the total biomass of soil micro-organisms was the first method designed for this purpose. However, this method has limited application to C and nutrient cycling studies.

Since the 1970's a major advance method in estimating the soil biomass has been the introduction of the chloroform-fumigation method (Jenkinson and Powlson, 1976a). The fumigation-incubation method is based on the finding that the extra CO_2-C evolved from a $CHCl_3$ fumigated soil following fumigant removal and aerobic incubation compared to that evolved from a similar incubation of non fumigated soil, results from the decomposition of microbial cells killed by the fumigant (Jenkinson and Powlson, 1976a).

Substrate induced respiration or the Physiological Method for measuring the amount of microbial biomass present in soil is based on measurement of the maximum rate of respiration after addition of glucose to soil followed by incubation for a short time, usually one hour to avoid microbial proliferation (Anderson and Domsch, 1978). Glucose is invariably chosen as the substrate as it is rapidly metabolized and is soluble in water. The method does depend on a constant proportion of organisms in each soil responding to it in the same way. The method is calibrated against the fumigation-incubation method.

Adenosine triphosphate (ATP), as an indicator of life in soil, can be extracted and measured accurately by the fire-fly luciferin-luciferase enzyme system. The criteria for measurement of ATP in soils are: 1) effectively extraction of ATP from soil, 2) inactivation of the ATP-hydrolysing enzymes, and 3) the extractant must minimize ATP sorption onto soil colloids (Jenkinson and Ladd, 1981). The method used in this work was that of Jenkinson and Oades (1979). The extraction reagent consisted of 0.1 *M* paraquat, 0.25 M Na_2HPO_4 and 0.5 *M* trichloroacetic acid (pH 1.6). The paraquat and phosphate were added to inhibit sorption of ATP on positively and negatively charged soil sites and the trichloroacetic acid to irreversibly deactivate ATPases.

The use of microcalorimetry for measuring microbial activity in soil was first suggested by Mortensén *et al.*, (1973). This method measures the heat output from organisms, which reflects their metabolic activity. Sparling (1981) related the rate of heat output from soils to biomass C by fumigation-incubation and substrate induced respiration.

Jenkinson and Ladd (1981) described the advantages and disadvantages of most of these methods.

The fumigation-extraction method (FE) has only been developed recently, and the fumigation-incubation method has been used as the standard to test and calibrate it and the others (Anderson and Domsch, 1978; Jenkinson and Oades, 1979; Ross, 1990; Sparling and West, 1988; Vance *et al.*, 1987). The organic C extractable by 0.5 *M* K_2SO_4 after 24h $CHCl_3$ fumigation comes from the cells of the microbial biomass lysed by the fumigant and can be used to estimate soil microbial biomass C.

The method can be used in acid soils and in soils recently amended with substrate, where the fumigation-incubation method is unreliable.

Microbial biomass measurements have provided useful information on soil organic matter dynamics in terrestrial ecosystems. For example, the soil microbial biomass responds much faster to many forms of disturbance and stress caused by anthropogenic activities, *e.g.* agriculture, forestry, mining and pollution than the total soil organic C (Anderson and Domsch, 1975; Bisessar, 1982; Chander and Brookes, 1993; Goyal *et al.*, 1992; Huysman *et al.*, 1994; Luizao *et al.*, 1992; Powlson *et al.*, 1987; Wardle and Parkinson, 1992). Therefore, it has frequently been proposed as an indicator of stress and disturbance induced by these activities (Brookes and McGrath, 1984; Christie and Beattie, 1989; Insam and Domsch, 1988; Powlson *et al.*, 1987). Thus, when steady-state conditions are achieved, the biomass C as a percentage of soil organic C will, at least in agricultural ecosystems, equilibrate at a characteristic level. Deviation from this level would indicate that the soil is either losing or accumulating soil organic C. There is evidence that heavy metals at around, or a little in excess of current permitted EU limits also decreased the proportion of biomass C in total soil organic matter. The first report of significant effects of metals at levels at or below current European Union (EU) permitted limits on microbial biomass came from measurements on metal-contaminated soils from the Woburn Market Garden Experiment (Brookes and McGrath, 1984). Chander and Brookes (1993) reported that the biomass C as a percentage of soil organic C in soils contaminated with Zn (375 to 705 $\mu g\ g^{-1}$ soil) or Cu (197-690 $\mu g\ g^{-1}$ soil) or both (322-475 μg Zn g^{-1} soil, 176-262 μg Cu g^{-1} soil) was less than half (0.4 to 0.7) that in the control soil (1.5 to 1.6). Thus this parameter provides a sensitive indicator of

the effects of heavy metals on microbial biomass. Therefore, shifts in the biomass C/ organic C ratio may act as an early warning of environmentally harmful practices (Anderson and Gray, 1991; Insam *et al.*, 1989; Insam and Domsch, 1988).

Recently, results from different long-term experiments where sludges were applied at Luddington, Lee Valley and Gleadthorpe experimental farms with different soil types: sandy loam, silty loam and sandy loam, respectively, under different agricultural systems (horticultural crops in Luddington and Lee Valley and clover in Gleadthorpe) and different heavy metal concentration has been reported (Chander and Brookes, 1991a; 1993). The maximum concentrations of Cu was 2.6 times current EU limits at Luddington, Zn 2.8 times at Lee Valley, 4.9 times Cu and 2.3 times Zn and Zn and Cu in combination at 1.8 and 1.4 times in Gleathorpe soils. The values of biomass C as percentage of soil organic C were similar in all those studies (less than half that in soils which received uncontaminated sludge). These results suggested that metals at these concentrations were causing decreases in microbial biomass.

Although microbial biomass is only a small fraction of soil organic matter, it plays a prominent role in soil organic matter transformation, such as mineralization and immobilization of organic compounds. The CO_2 released from the mineralization of soil organic matter is often employed as a measure of metabolic activity (Brookes, 1985; Golebiowska and Pedziwilk, 1984; MacFadyen, 1970; Witkamp and Frank, 1969) and it reflects the availability of C for microbial maintenance and is a measure of basic turnover rate in soil (Insam and Domsch, 1988; Insam and Heselwandter, 1989). However, Brookes and McGrath (1984) reported no differences in soil respiration measured between metal-contaminated (high-metal) and uncontaminated

(low-metal) soils from the Woburn Market Garden Experiment. However, soil biomass C was only about half the amount in high-metal soils as it was in low-metal soils. Combining the two measurements to give amounts of CO_2-C evolved per unit of biomass (biomass specific respiration rate) has been shown to be a useful indicator of stress. This measurement has been applied to soil microbial biomass analyses in maintenance energy investigations (Anderson and Domsch, 1985a; 1985b), in comparison of field managements (Anderson and Domsch, 1990), soil variables such as fallow, field, meadow forest, different vegetation and crop rotation (Šandtrüčková and Straškraba, 1991) in ecosystem succession (Insam and Haselwandter, 1989) in studies on the effect of temperature (Anderson and Domsch, 1986; Joergensen *et al.*, 1990), for elucidating effects of environmental changes on microbial communities (Anderson and Gray, 1991), assessing the effects of environmental conditions on microbial biomass of forest soils (Anderson and Domsch, 1993) and in studies of heavy metal stresses (Brookes and McGrath, 1984; Killham, 1985). Brookes and McGrath (1984) reported that the biomass specific respiration rate can be a good indicator of environmental stress due to heavy metals.

So far all this work has been done using soils taken from well-managed field experiments. It is important to find out if these approaches could be used to study the effects of heavy metals in the environment where greater 'noise', due to variations in soil properties, is anticipated.

Brookes (1993) suggested that the link between biomass C and CO_2-C evolved (biomass specific respiration rate) and soil organic C (biomass as percentage of soil organic C) might provide a more sensitive indicator of soil pollution than either single measurements of microbial biomass and microbial activity. He also suggested that

these measurements by being related, might constitute some form of "internal" control that depended upon particular management, soil type and climate. Changes in the values of these linked parameters, for example biomass specific respiration, may indicate damage to the function of the soil ecosystem.

The environmental impact of mining industries has been caused not only directly from mining activity but also due to contamination caused by the spoil which remains after the extraction of minerals (Bisessar, 1982; Freedman and Hutchinson, 1980; Gingell *et al.*, 1976; Jordan and Lechavalier, 1975; Nordgren *et al.*, 1985; Olson and Thornton, 1982; Pancholy *et al.*, 1975; Rühling and Tyler, 1973). Such areas, which are polluted by heavy metals can provided an ideal opportunity to investigate the effect of metal on the natural ecosystem by measuring the amount of microbial biomass and microbial activity and their combination and the relationship between biomass and soil organic C.

This study consisted of three main parts:

The first part of this work was to study the validity of different methods for measuring microbial biomass and microbial activity in soils with metal concentrations at around current EU limits. The following methods were tested, *viz.* biomass C, biomass ninhydrin-N and biomass nitrogen measured by fumigation-extraction (FE), substrate induced respiration (SIR), ATP and microbial activities measured by arginine ammonification rate and CO_2-C evolved. A secondary aim was to investigate effects of soil storage (*i.e.* length of storage and temperature) on microbial biomass and microbial activity.

The second part was to determine at which soil concentrations of heavy metals microbial biomass and microbial activity were affected.

The third part was to investigate the application of these methods and ergosterol and dimethyl sulphoxide reduction rate to a large area in the natural environment which had been polluted by previous mining of zinc and lead. This caused a pollution gradient from the centre of the polluted site caused by past mining activity.

Ergosterol is the predominant sterol component in most fungi. Its specific absorption of uv light at 282nm enables ergosterol to be quantitatively measured by high pressure liquid chromatography (HPLC) and distinguished from other steroids present in higher plants, animals and no fungal microbes.

Ammonium, amino acids or protein contains α-amino-N which reacts with ninhydrin to give a red colour which can be determined colorimetrically. L-Leucine is used as the standard amino acid. It has advantage of giving the maximum and most reproducible color yield and appears to be generally more convenient for analyses of aqueous samples (Moore and Stein, 1954).

Chapter 2 of this thesis describes the general methodology, Chapter 3 discusses measurements of soil microbial biomass and microbial activities in low- and high-metal soils after storage and pre-incubation for different periods after sampling.

Chapter 4 reports microbial biomass and microbial activity measurements and their combinations in soils from a field experiment where different manurial treatments were applied 26 years ago causing low and high-metal concentrations in soils.

Chapter 5 investigate the validity of the methods to measure microbial biomass and microbial activities on soils from a polluted natural site, rather than a field experiment.

Chapter 6 investigates the validity of the link between microbial biomass and microbial activities, and microbial biomass and soil organic C in metal-contaminated soils from the above polluted natural site.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOIL SAMPLING AND SOIL PRETREATMENT

Soils were sampled from two sites: (1) a field experiment in UK, the Market Garden Experiment (location and description are given in Chapter 3) and (2) non-experimental grassland in Spain (full description in Chapter 5). After sampling, (0 to 23 cm soil depth) using a 5 cm diameter auger in soils sampled from U.K. and a spade in soils from Spain, all the stones, visible roots and fauna were removed. The soils were then sieved (< 2 mm) and adjusted to 40% water holding capacity by adding water if the soil was too dry or by turning the soil carefully at room temperature to avoid any part of the soil becoming air-dry. The prepared soil was stored moist at 4°C until used. Further portions of soil were air-dried and finely ground in a Tema mill (< 160 μm), for chemical analysis.

2.2 SOIL MICROBIAL BIOMASS MEASUREMENTS

Microbial biomass measurements were done by the Fumigation Extraction Method (Vance *et al.*, 1987, Wu *et al.*, 1990). Moist soil portions (25g oven-dry basis) were fumigated with ethanol-free chloroform at 25°C for 24h. The ethanol was removed (Vogel, 1965) so as not to contaminate the soils with ethanol-derived C.

The soils to be fumigated were placed in a desiccator lined with wet tissue paper. Chloroform (25ml) was added at the desiccator, then evacuated until the $CHCl_3$ boiled vigorously for 2 min. The soils were extracted immediately by shaking at 90 rpm with 0.5 *M* K_2SO_4 (4:1 solution: soil ratio) for 30 min following fumigant removal. Portions of the unfumigated soils were extracted similarly at the time fumigation commenced. The filtered soil extracts were stored at -15°C until analysis. The following measurements were all done on different aliquots the same 0.5 *M* K_2SO_4 soil extracts.

2.2.1 BIOMASS CARBON

Organic C in the soil extracts was determined by oxidation with potassium persulphate in the presence of ultraviolet light (Wu *et al.*, 1990) using a Dohrmann D.C. 80 automatic carbon analyzer.

Biomass C (Bc) was calculated from:

$$B_c = 2.22\ E_c$$

where: Ec =[(organic C extracted from fumigated soil) minus (organic C extracted from unfumigated soil)].

and the factor 2.22 is a proportionality constant to account for the fact that only about 45% of the total biomass N is extracted is extracted during fumigation extraction (*e.g.* Jenkinson, 1988; Ocio and Brookes, 1990).

2.2.2 BIOMASS NINHYDRIN-N

Ninhydrin-N was measured in the K_2SO_4 extracts of fumigated and unfumigated soils by the method of Amato and Ladd (1988), modified by Joergensen and Brookes (1990). Briefly, soil extracts or blanks (0.75ml) and citric acid buffer pH 5 (1.75ml) were put into 20ml test tubes. Ninhydrin reagent (1.25ml) was added slowly and vigorously mixed. This reagent was prepared from: ninhydrin (20g) and hydrindantin (3g) dissolved in dimethyl sulphoxide (750ml). Lithium acetate (250ml, 4 *M*) was added and the mixture flushed with oxygen-free nitrogen for 30 min. The test-tubes were heated (20 min) in a water bath at 100°C. The tubes were then cooled to room temperature in darkness, ethanol-water (4.5ml, 1:1) added, and thoroughly mixed. Finally absorbances were read at 570 nm against a water blank.

The concentration of ninhydrin-N was obtained by reference to a standard curve of 0-1000 μmM L-Leucine in 0.5 *M* K_2SO_4.

Biomass ninhydrin-N (Bnin) was calculated from:

Bnin= [(Ninhydrin-N extracted from fumigated soil) minus (ninhydrin-N extracted from unfumigated soil)]

2.2.3 BIOMASS NITROGEN

Biomass nitrogen was measured by the method of Brookes *et al.*, (1985 a, b). This method has two main steps in the nitrogen analysis of K_2SO_4 extracts of soils: 1) the reduction of soil NO_3^- to NH_4^+ and 2) the measurement of total nitrogen (i.e. inorganic and organic N) by the Kjeldhal method. Briefly, soil extracts or blanks (30ml), Zn powder (300mg) and Chromium III reagent (10ml) [25g $CrK(SO_4)_2$12 H_2O dissolved in acidified water (100ml concentrated H_2SO_4 in 500ml

water)] were mixed in a 250ml digestion tube. After 2h at room temperature, $CuSO_4$ solution (0.6ml, 0.19 *M*), concentrated sulphuric acid (10ml) and a few anti-bumping granules were added and the final mixture refluxed for 3h at 360°C. After cooling, water (20ml) was cautiously added to the digestion tube, then NaOH (25ml, 10 *M*) keeping the tubes in a cold water bath. The digestion tubes were then attached to a steam-digestion unit adding more NaOH (25ml, 10 *M*) to render the solution alkaline and the mixture steam-distilled into a titration vessel containing boric acid (5ml, 2%). The distillate (40ml) was collected and titrated to pH 4.7 with *M*/80 H_2SO_4 using an automatic pH titration unit.

Biomass nitrogen (B_N) was calculated from:

$$B_N = 2.22\ E_N$$

where, E_N = (total nitrogen extracted by 0.5 *M* K_2SO_4 from fumigated soil) minus (total nitrogen extracted by 0.5 *M* K_2SO_4 from unfumigated soil)

2.2.4 SUBSTRATE INDUCED RESPIRATION

Biomass C was measured by Substrate Induced Respiration (SIR) (Anderson and Domsch 1978) as modified and calibrated against the fumigation extraction method (Lin, 1994). Two moist portions of soil (equivalent to 30g oven-dry soil) were weighed into 320ml Quick fit flasks and pre-incubated at room temperature (30 min) after adding a mixture of glucose and talcum powder (1:4), to give a concentration of 6mg glucose g^{-1} soil. The flasks were then sealed with silicon grease and incubated for 3h at 25° C. The CO_2 evolved (10ml) was measured by gas chromatography in 10ml aliquots of air.

Biomass C measured by SIR was calculated from:

$$SIR = 16.18\ Y + 5.11$$

where Y = μl CO_2 evolved hr^{-1} g^{-1} soil.

2.2.5 ATP

Adenosine 5'-triphosphate (ATP), it was extracted from soil by the method of Jenkinson and Oades (1979). Two portions of moist soil each containing 5g soil on an oven-dry basis were weighed into 50ml centrifuge tubes and extracted with 25ml extractant A (a mixture containing 0.5 *M* trichloroacetic acid, 0.25 *M* sodium orthophosphate and 0.1 *M* paraquat) by ultrasonic dispersion for 2 min using a Branson B12 150W sonifier at full power. Two other portions were extracted with 25ml extractant B (extractant A containing 25pmol added ATP 50 μl^{-1}). After the ultrasonic dispersion, the soil extracts were cooled on ice, filtered (Whatman No. 44) and the filtrates frozen and stored at -15°C until analysis. Blanks consisted of 25ml of extractant A and B ultrasonified and filtered without soil. ATP was measured by the luciferin-luciferase enzyme assay (Tate and Jenkinson, 1982) using a TRI-CARB Liquid Scintillation counter (Model 2500TR, Parckard Instrument Company). The soil extracts (50μl) were added to 5ml arsenate buffer (0.1 *M* $Na_2HAsO_4.7H_2O$, 10 m*M* $MgSO_4$ and 2 mM EDTA, pH 7.40) in a scintillation counting vial. Luciferin-luciferase enzyme solution was prepared by adding 8ml distilled water and 10mg luciferin to each vial of Picozyme F. The enzyme solution (50μl) was then added to each scintillation vial, gently mixed, and the vial then counted for 0.1 min. ATP standards (0-100 pmol ATP 50μl^{-1} extractant A) were prepared and counted similarly.

Soil ATP contents were calculated using a computer programme based upon the equation derived by Jenkinson and Oades (1979) for conversion of counts to soil ATP content:

$$ATP\ (nmol\ g^{-1}\ soil) = \frac{(COUNT\ EX\ A - BLANK\ EX\ A)\ SF\ (25+W)}{(RECOVERY\ x\ 5\ x\ 50)}$$

$$\%\ RECOVERY\ of\ added\ ATP = 100 \left\{ \frac{(COUNT\ EX\ B - COUNT\ EX\ A)}{(BLANK\ EX\ B - BLANK\ EX\ A)} \right\}$$

where

COUNT EX B = counts from soils extracted with extractant B.

COUNT EX A = counts from soils extracted with extractant A.

BLANK EX B = counts of extractant B.

BLANK EX A = counts of from extractant A.

SF = Inverse slope of standard [ATP]/[counts] curve.

W = water content of the soil sample

25 = ml of extractant A or B

5 = g oven-dry soil

50 = μl of soil extract used for ATP analysis

2.2.6 ERGOSTEROL

Ergosterol was determined by the procedure of Seitz *et al.*, (1977) as modified by S. Higashida (personal communication). The ergosterol assay comprised several steps: extraction, saponification, partition, purification and HPLC analysis. Briefly, moist soil containing 20-30g oven-dry soil was mixed with 120ml of methanol in a 200ml centrifuge bottle. After sonification (Branson B12 150 W sonifier at full

power) for 3 min in an ice bath, then holding at 0°C for 30 min, the samples were centrifuged at 3000 rpm for 15 min (MSE Scientific Instrument, Europa 24M). Aliquots (100ml) from the supernatant were saponified with 10% (w/v) KOH and 20% (v/v) ethanol in 250ml round bottomed flasks by refluxing for 30 min in an 80°C water bath. After cooling to room temperature, distilled water (25ml) and hexane (80ml) were added to the refluxed samples in a 250ml separating funnel and vigorously shaken 30 times. This was repeated three times, with release of excess pressure each time.

Following removal of the aqueous phase, the hexane layer was transferred to a 250ml flask. Two more hexane extractions (80ml) were done and the hexane aliquots combined and evaporated to dryness in a rotatory evaporator. The dried samples were dissolved in a small amount of methanol (1.5ml) by heating the round bottom flask in an electrical mantle and transferring to a volumetric flask (5.0ml). The flask was washed again twice with methanol (1.5ml) and the washings added to the volumetric flask. After cooling at room temperature, the final volume was adjusted to 5.0ml with methanol, then the samples were mixed and stored at -15°C before HPLC analysis.

To determine the recovery of ergosterol, 1ml of methanol containing 145 μg ergosterol ml^{-1} was added to the same weight of soil 3 min before the sonification and the soil then extracted as above .

Ergosterol in the methanol solutions and ergosterol standards (10 μg ml^{-1} in HPLC grade methanol) were measured in a LDC Analyst series 7800 HPLC using methanol (HPLC grade) as the mobile phase at a flow rate of 1.5 ml min^{-1} and uv detection at 282 nm.

Ergosterol content in soil was calculated from:

$$Ergosterol\ (\mu g\ g^{-1}\ soil) = E\ V_f\ \frac{H_t}{H_d}\ \frac{(M_e + W_w)}{(M_s\ W_s\ S_k)}$$

where

E = Ergosterol in solution (μg ml^{-1})

V_f = final volume of methanol (ml)

H_t = total weight of hexane used for extracting the ergosterol (g)

H_d = sub-sample of hexane used for drying and ergosterol purification (g)

M_e = volume of methanol used for extraction (ml)

M_s = volume of methanol used for saponification (ml)

W_w = water content of the soil sample (ml)

W_s = oven-dry soil weight (g)

S_k = recovery of added ergosterol (%)/100

2.3 SOIL MICROBIAL ACTIVITY MEASUREMENTS

2.3.1 CO_2

Total CO_2-C trapped in 1 *M* NaOH (20ml) was determined by titrating 5ml aliquots of NaOH with standardized 0.5 *M* HCl from pH 8.3 to 3.7 using an automatic titrator. The CO_2-C evolved from the moist soil (containing 50g oven-dry soil) incubated for different time periods at 25°C was calculated from:

$$CO_2\text{-}C(\mu g\ C\ g^{-1}\ soil) = \frac{(V_s - V_0)\ N\ (20/5)\ x\ 12\ x\ 1000}{Ws}$$

where:

V_s = volume of HCl (ml) required to titrate the CO_2 prepared from the soil

V_0 = HCl (ml) required to titrate CO_2 from the blank.

N = the molarity of the standardized HCl

W_s = the oven-dry weight (g) of the moist soil in each measurement.

20 = the volume (ml) of the titrated NaOH solution

5 = the aliquot (ml) used for titration

12 = atomic weight of C

1000 = factor to convert the volume or HCl titrated from mg C to μg C

Biomass specific respiration rate was calculated from mg CO_2-C evolved in 10 d g^{-1} Biomass C. All measurements are expressed on an oven-dry soil basis (105°C, 24h) and are the means of three replicate determinations.

2.3.2 ARGININE AMMONIFICATION RATE

The arginine ammonification rate was determined by Alef and Kleiner's (1987) method. Briefly, moist soil samples (containing 10g oven-dry soil) were placed into 130ml plastic bottles and incubated for 3h at 25°C after arginine solution (2.5ml, 0.2%) was added (drop-wise) and then stored at -15°C until extraction. Soils and water without added arginine served as controls. After thawing the soils, KCl was added, (10ml, 2 *M*), the mixture shaken (90 rpm) for one hour and then filtered (Whatman No. 42). Ammonium was estimated by colorimetric continuous flow

analysis (Technicon AA 11 Instrument). The results were expressed as $\mu g\ NH_4^+$-N formed g^{-1} soil h^{-1}.

2.3.3 DIMETHYL SULPHOXIDE REDUCTION

Dimethyl sulphoxide reduction (DMSO) was measured by the method of Alef and Kleiner (1989). Moist soil samples (containing 10g oven-dry soil) were weighed into 307ml Quick-fit flasks and sealed with silicon grease after addition of 4ml DMSO solution (5% in water). After incubation at 25°C for 5h, a sample from the gas phase (1.0ml) was used to estimate the dimethyl sulphide (DMS) produced in a Perkin Elmer 3 gas chromatograph fitted with a flame ionization detector and a Haye Sep R column (2m). The temperature of the detector, injector and column were 220, 200 and 160°C respectively.

The time needed for each gas chromatographic DMS analysis was 4 min. The concentration of DMS in soils was obtained by a DMS calibration curve prepared as follows: 500μl liquid cold DMS (4°C) was transferred to a closed flask (about 1000ml) fitted with a gas sampling septum, and allowed to evaporate for 5 min at 25°C. Appropriate aliquots from this solution were added to other flasks to give DMS gas concentrations from 50 - 200ng ml^{-1}. Dimethyl sulphoxide reduction was calculated from:

$$DMSO\ reduction = \frac{(D\ V_a)}{(W_s\ t\ V_i)}$$

where:

D= the amount of DMS (ng) in the sample analyses and calculated from the standard curve.

V_a = (total volume Quick fit flask (307ml) minus (g wet soil + ml DMSO solution used).

W_s = the oven-dry weight (g) of the moist soil

t = the incubation time (h)

V_i = the volume (ml) of gas sample used for the gas chromatography analysis.

2.4 SOIL CHEMICAL ANALYSES

The sieved soils were air-dried and then finely ground in a Tema-mill (<160 μm). Soil pH was determined in suspensions of air-dried soil and distilled water using a 1:2.5 ratio of soil to water. Total nitrogen in soil was measured by Kjeldhal digestion (Bremner, 1965) and Technicon analysis. Total organic C was measured by dry combustion using a Europa Scientific TCD analyzer. Total metals in soils were extracted by McGrath and Cunliffe's (1985) method using Aqua Regia. The metal concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP) (A.R.L.34000 instrument).

Soil Cd concentrations at or below 5μg g^{-1} soil were determined by atomic absorption spectrometry because ICP may overestimate soil Cd concentration in this concentration range (S.P. McGrath, personal communication).

2.5 STATISTICAL ANALYSES

All measurements are the mean analyses of triplicate determinations unless otherwise stated. The statistical analyses: two-way analyses of variance, multiple

linear regression, discriminant analyses and least standard deviation were done using the Genstat 5 Program provided by the Statistics Department, Rothamsted Experimental Station.

CHAPTER 3

EFFECTS OF INCUBATION TIME AND STORAGE CONDITIONS ON MICROBIAL BIOMASS AND ACTIVITY IN SOILS OF HIGH AND LOW METAL CONCENTRATIONS

3.1 INTRODUCTION

The size of the soil microbial biomass and microbial activity are influenced by many environmental factors such as soil management, crop rotation, land use and climatic conditions (Anderson and Domsch, 1989; Biederbeck *et al.*, 1984; Carter, 1991b; Clarholm and Rosswall, 1980; Insam *et al.*,1989; Insam *et al.*, 1991; Patra *et al.*, 1990; Wu, 1991). Both the biomass and its activity are also influenced by soil treatments before analysis, such as moisture content, length of storage and incubation period after sampling (Lynch and Panting, 1980; Ross *et al.*, 1980; Ross and Tate, 1984; West *et al.*, 1986b; Zelles *et al.*, 1991).

Methods currently available to measure the soil microbial biomass and microbial activity frequently describe conditions of storage and incubation of soils prior to use. For example, commonly measurements of soil microbial biomass by the fumigation incubation method (FI), fumigation extraction method (FE) or ATP content are done on soils stored at 4°C after sampling and which are then incubated at temperatures of between 22 to 25°C for 5 to 10d before analysis (Anderson and Domsch, 1978; Brookes *et al.*, 1985a; Jenkinson and Oades, 1979; Jenkinson and Powlson, 1976b; West *et al.*, 1986a). Microbial biomass, measured by the substrate

induced respiration method (SIR) are frequently done on soils which have first been pre-incubated for 30 min, 24h or 10d at 22°C (Alef *et al.*, 1988; Anderson and Domsch, 1978; West and Sparling, 1986). In the original arginine ammonification method to measure microbial activity, the soils were first pre-incubated at room temperature (20 to 25°C) for 30 min or 1h before measurement (Alef *et al.*, 1988 and Alef and Kleiner, 1986, 1987).

It is normal procedure for soils to be given a conditioning incubation (*e.g.* Jenkinson, 1988) before analyses for biomass or microbial activity. This permits these parameters to stabilise during this period. However, it is often not clear from the literature just what effects this conditioning incubation may have on biomass or its activity. For example Ross *et al.*, (1980) gave results for soils stored at different temperatures (4, 25, -20°C) and storage periods (28 and 56d). He found that no storage temperature was entirely suitable because significant changes in some index of biomass (biomass C, N flush or ATP) could occur at any storage temperatures. Also, as in this work, a change in any one biomass index was not always accompanied by a similar proportional change in another. For example, West *et al.*, (1986a) reported a decrease in biomass C (by fumigation incubation method) by 35% and an increase in ATP by 59 to 67% after 7d incubation and further changes occurred after this.

One aim of this Chapter was to investigate this problem further and decide upon the best period of conditioning incubation, if any, in metal-contaminated and uncontaminated soils. Another main aim of this work was to investigate the validity of the methods to measure microbial biomass and activity in soils that contained low and high heavy metal concentrations and which were stored and pre-incubated for

different periods after sampling. A further aim was to assess the influence of storage period before analysis upon microbial biomass content and microbial activity in soils of high and low metal concentrations.

3.2 MATERIALS AND METHODS

3.2.1 DESCRIPTION OF THE WOBURN MARKET GARDEN EXPERIMENT

A field experiment was started in 1942 at Woburn, U.K. on a sandy loam soil (Cottenham series) on two series of plots (A and B). The aim of the experiment was to compare the value of various bulky organic manures, applied at two rates, with inorganic fertilizers (Johnston and Wedderburn, 1975). Each organic and inorganic fertilizer treatment was replicated on 4 plots, each 6.09 x 8.53m (Figure 3-1). The organic manures, the amount applied on a dry matter basis each year and the date applied are given in Table 3-1.

Sewage sludge and sludge compost were not applied after 1961 because it was found that large amounts of Zn had accumulated in these soils (Le Riche, 1968). Farmyard manure, vegetable compost and inorganic fertilizer were applied until 1967 (Table 3-1). After the applications of organic manures ceased, all plots received uniform annual applications of inorganic fertilizer only. From 1974 to 1982 the entire experiment was put down to grass. In 1982 the grass was ploughed in and then clover grown from 1983 to the present.

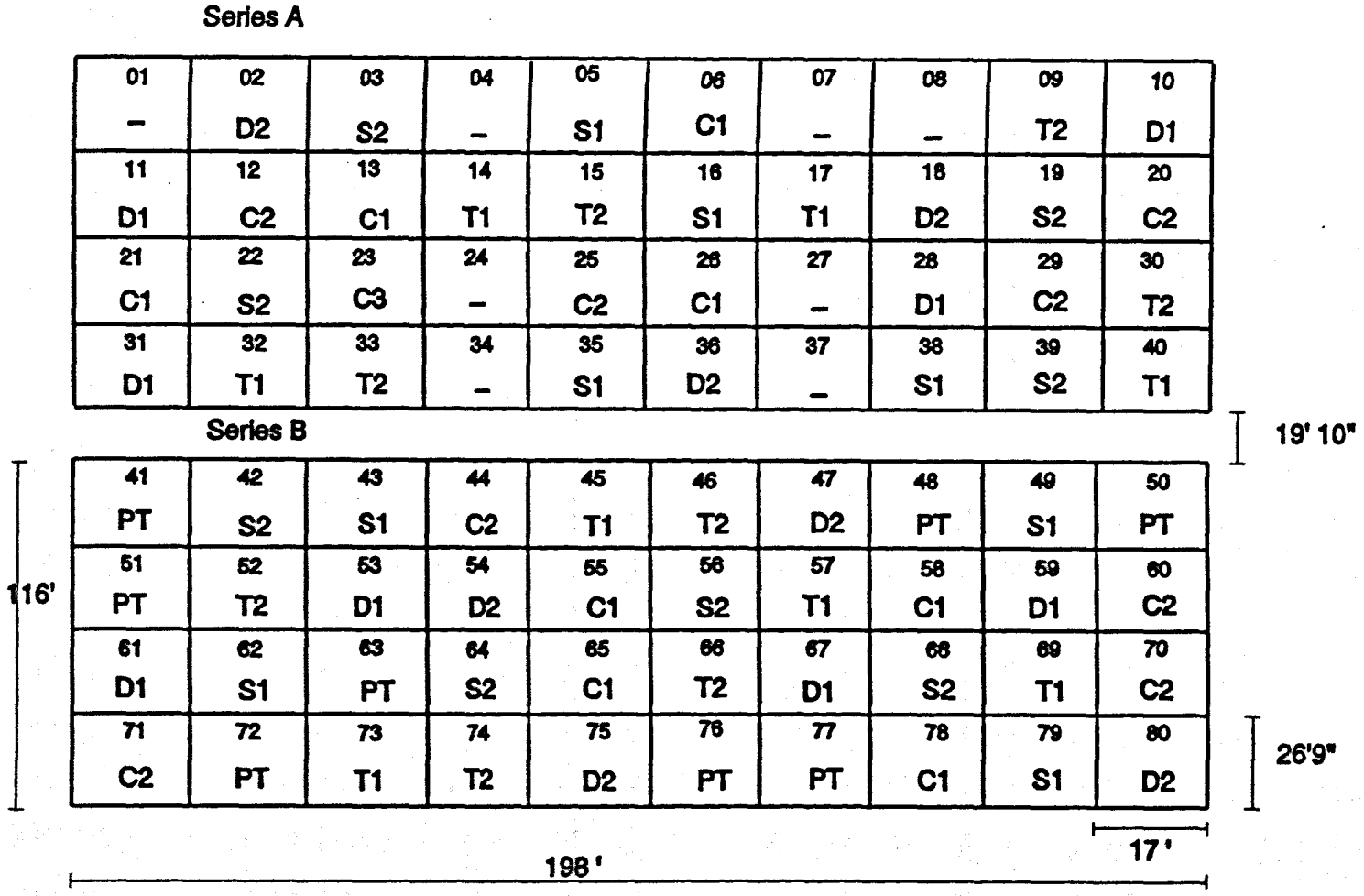

Figure 3-1: Map of the Woburn Market Garden Experiment (D = farmyard manure, C = vegetable compost, - = inorganic fertilizer, S = sewage sludge, T = sewage compost, PT = peat, 1 = rate 1, 2 = rate 2)

Table 3-1: The Woburn Market Garden Experiment manurial treatments and dates applied.

Soil manurial treatment	Total organic matter[a] ($t\ ha^{-1}$)	Date applied
Inorganic fertilizer (NPK)	0	1942-1969
Farmyard manure		
Rate 1	136	1942-1969
Rate 2	272	
Vegetable compost		
Rate 1	117	1942-1969
Rate 2	234	
Sewage sludge		
Rate 1	163	1942-1961
Rate 2	326	
Sludge compost		
Rate 1	110	1942-1961
Rate 2	220	

[a] Dry matter

3.2.2 SAMPLING AND PREPARATION OF SOILS

Soils were taken only from series B of The Woburn Market Garden Experiment (Figure 3-1) that received treatments of sewage sludge, double dressing (plots: 42, 56, 64 and 68) (high-metal soil), farmyard manure, double dressing (plots: 47, 54, 75 and 80) and inorganic fertilizer (plots: 22, 25, 34 and 37) (low-metal soils). Soils were sampled (5 cm diameter cores, 0-23 cm depth) in March 1992, then the soils were treated as described in section 2.1.1. In this work, 200g sieved soil from each of the four replicate plots of each treatment were carefully mixed to provide a single bulked sample of each treatment. Each analytical measurement was the mean of triplicate determinations.

3.2.3 SOIL CHEMICAL ANALYSES

Soil pH, organic C, total N and total metal concentrations were determined in air-dried and ground portions of soil as described in section 2.4. Table 3-2 summarizes the soil analyses.

3.2.4 SOIL MICROBIAL BIOMASS MEASUREMENTS

Soils were stored at 4°C in sealed plastic bags for a short term experiment of one or eight weeks, also for a longer term of 2 months (defined as fresh soil in section 3.4) and also up to one year. The soils for the shorter term experiment were then incubated from 0, 10 and 20d and measurements of biomass C, ninhydrin-N and ATP performed as described in section 2.2.1 , 2.2.2 and 2.2.5. Substrate induced respiration (SIR) was measured after 0, 2, 4, 7, 10 and 20d of incubation as described in section 2.2.4. For longer term experiments with soil stored for one year, the soils

Table 3-2: pH, soil organic C, total N and total metals in soils from the Woburn Garden Market Experiment

Treatment	pH	Organic C	Total N	Zn	Cu	Ni	Cd	Cr	Pb
		(%)		($\mu g\ g^{-1}$ soil)					
Inorganic fertilizer NPK	7.3	0.89	0.14	109	40	17	0.3	41	43
Farmyard manure rate 2	6.9	2.03	0.18	170	52	21	0.4	60	62
Sewage sludge rate 2	6.8	1.97	0.17	308	100	30	6.0	110	109
EU recommended upper limits				300	140	75	3.0	-	300

were incubated for 10d before the microbial biomass measurements.

3.2.5 SOIL MICROBIAL ACTIVITY MEASUREMENTS

Measurements of carbon dioxide evolution and arginine ammonification rate were done at 0, 2, 4, 7, 10, 15 and 20d of incubation as described in sections 2.3.1 and 2.3.2.

3.3 RESULTS AND DISCUSSION

3.3.1 CHANGES IN SOIL BIOMASS C AND BIOMASS NINHYDRIN-N IN SOILS OF LOW AND HIGH METAL CONCENTRATIONS DURING INCUBATION

The amount of biomass C in high-metal soil (Sewage sludge) at one week or eight weeks storage at 4°C followed by a pre-incubation for 0 or 20d at 25°C was about 39 to 46% less than in low-metal soils (FYM) (Table 3-3). The decrease (40 to 47%) in high-metal soils compared to FYM was similar to biomass ninhydrin-N in soils stored and pre-incubated for the same period. Similar decreases in biomass C in high-metal soils incubated for 7d at 25°C were reported by Brookes and McGrath (1984) and Chander and Brookes (1991c).

Overall, biomass C in soils stored for eight weeks decreased compared to one week and also in soils incubated at 25°C for 20d compared to 0d. This decrease in microbial biomass was higher at one week (22 to 27%) than at eight weeks (7 to 15%) of storage at 4°C. An analysis of variance of the results showed that incubation and storage of the soils was significant at $p<0.05$ for each treatment

Table 3-3: Biomass C[a] and biomass Ninhydrin-N[a] at 0 and 20d incubation after one and eight weeks storage at 4°C in soils from the Woburn Garden Market Experiment.

	Incubation period before analysis	Storage time at 4°C prior to incubation			
		Biomass C		Biomass Ninhydrin-N	
		($\mu g\ g^{-1}$ soil)			
	days	One week	Eight weeks	One week	Eight weeks
Inorganic fertilizer NPK	0	220[b]±4.8[c]	173±5.5	8.4±0.20	8.2±0.13
	20	161±2.6(-26.8)[d]	143±3.2(-17.3)	8.0±0.08 (-4.7)	7.5±0.13 (-8.5)
Farmyard manure	0	289±3.7	242±8.0	12.0±0.22	11.3±0.18
	20	227±4.3(-21.4)	213±7.7 (-12.0)	10.6±0.23(-11.7)	10.7±0.24 (-5.3)
Sewage sludge	0	175±3.7	132±2.7	6.4±0.46	6.8±0.15
	20	131±2.5 (-25.1)	130±4.7 (-1.5)	5.8±0.20 (-9.4)	6.0±0.22 (-11.8)

[a] Measured by fumigation-extraction.
[b] Mean of four plots per treatment and three replicate determinations per plot.
[c] Standard error of the mean
[d] Values in parentheses are % change due to storage

(NPK, FYM and sewage sludge). This means that both incubation and storage affected the size of biomass C.

West *et al.*, (1986a) found a similar percentage of decrease in biomass C measured by the Fumigation Incubation Method (FI) and in soils pre-incubated at 25°C between 0 to 7d. In contrast Tate and Jenkinson (1982) obtained an increase of 23% in biomass C in soils measured by the FI method and incubated for 7d at 25°C prior to analysis. Both suggested that these changes could be an artifact of the methodology. For example the period most suitable for measuring unfumigated control soil remains problematical because the decomposition of fresh native organic matter proceed at different rate in both fumigated and unfumigated soils in soils that recently received large inputs of fresh substrate, rewetted or very recently sampled soils (Brookes *et al.*, 1985a; Jenkinson and Powlson, 1976b; Sparling *et al.*, 1985; Voroney and Paul, 1984; West *et al.*,(1986b).

Biomass ninhydrin-N decreased by very similar amounts between one and eight weeks (by 7 - 11 and 6 - 12% respectively), following incubation at 0 or 20d prior to analysis. Analysis of variance showed that incubation at 4°C for 8 weeks caused a decrease in biomass and there was no significant difference between the measurements done at 1 or 8 weeks ($p<0.05$). These results suggest that the decrease of biomass C and ninhydrin-N may be due to the decomposition of small roots which would have occurred over this period. They may have contributed to the flush of C due to fumigation. In addition, any biomass killed during soil preparation or storage which was not decomposed by week one, would also have contributed to the flush at week one but not at week eight.

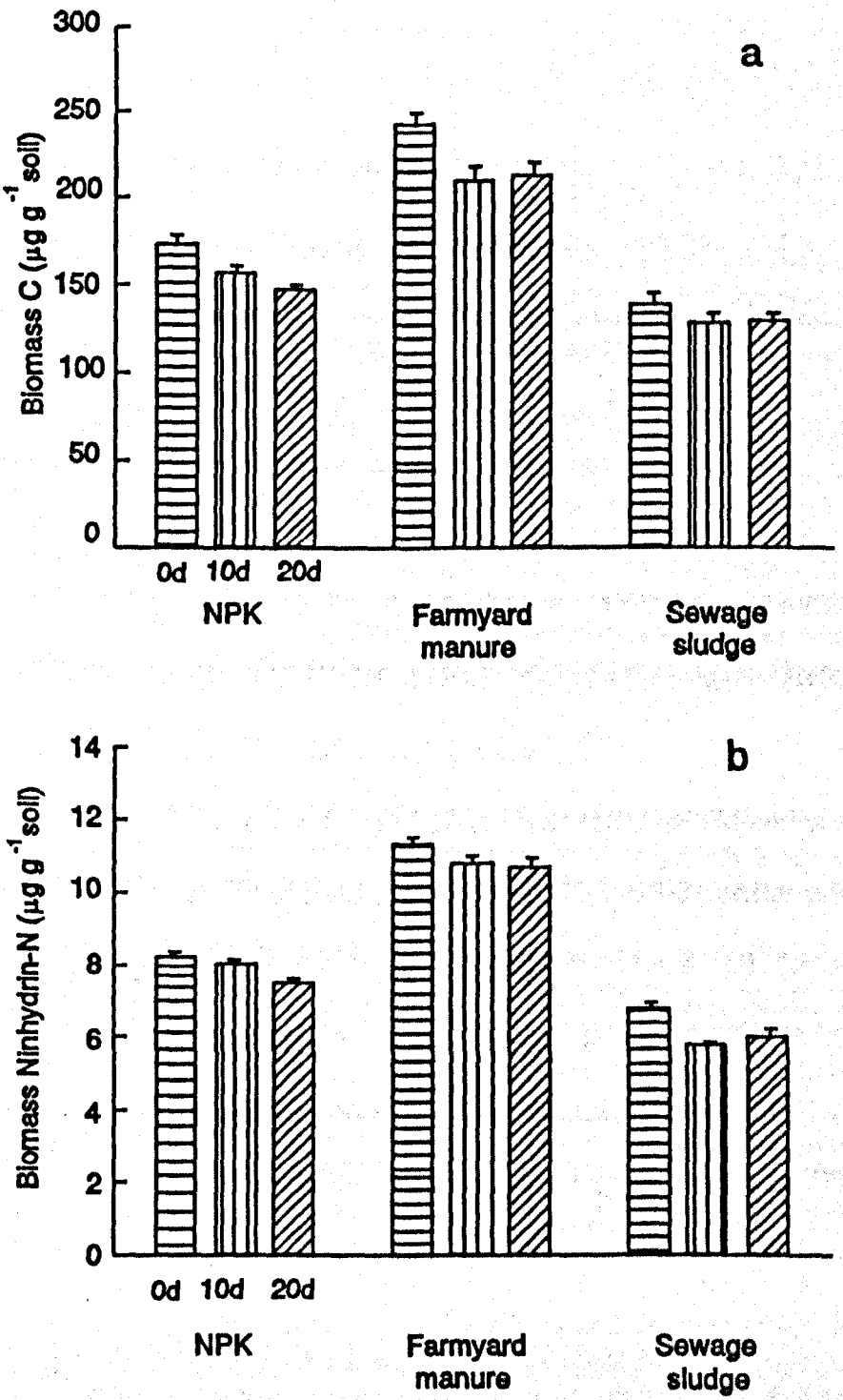

Figure 3-2: Amount of biomass C (a) and ninhydrin-N (b) at 0, 10, and 20d incubation at 25°C after eight weeks storage at 4°C (standard errors of the mean shown).

The significance of differences of biomass C and biomass ninhydrin-N between 10 and 20d of incubation after storage for eight weeks was determined by Student's t-test (data not shown) and did not change significantly at $p < 0.05$ (Figure 3-2).

These results indicate that the decrease of biomass C and ninhydrin-N because of the storage and pre-incubation, is the same in high-metal soils and low-metal soils. Thus, measurements of biomass C and biomass ninhydrin-N by FE are reliable methods to measure microbial biomass in low- and high-metal soils that were stored and pre-incubated for different periods after sampling.

3.3.2 CHANGES IN CO_2-C EVOLUTION AND BIOMASS SPECIFIC RESPIRATION RATE IN SOILS OF LOW AND HIGH METAL CONCENTRATIONS DURING INCUBATION

Carbon dioxide evolved from of all the soils (NPK, FYM and sewage sludge) declined during the first 7d of incubation at 25°C (Figure 3-3). After this period, the respiration rate proceeded at a lower and constant rate in all the soils without significant differences between low and high-metal soils.

Bottner (1985) also reported that CO_2-C released from unfumigated soil declined with increasing incubation time. Similarly, West *et al.*, (1986a) found a decrease in soil respiration when soils were incubated for 7d. Reasons for these decreases in soil respiration include; 1) the killing and decomposition of organisms by sieving, etc., 2) the exposure of previously inaccessible substrate, 3) the decomposition of root fragments detached during soil sampling and sieving and 4) complex shifts in the balance of competing sections of the soil microbial population

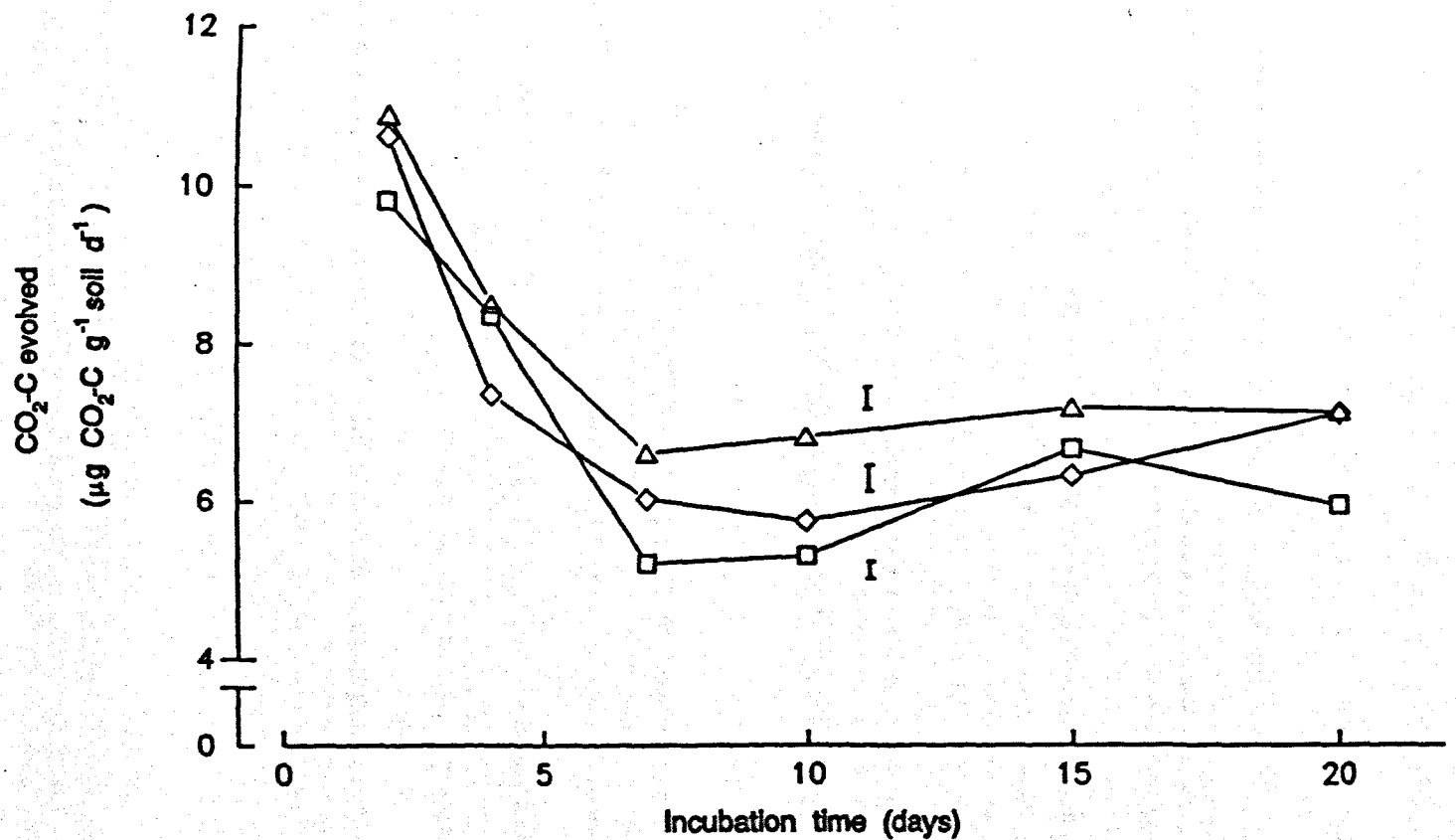

Figure 3-3: Changes in rates of carbon dioxide evolved from Woburn soils (□ NPK, ▲ FYM and ◇ Sewage sludge) during 20d incubation at 25°C after eight weeks storage at 4°C (LSD shown).

(Jenkinson and Ladd, 1981).

The biomass specific respiration rate (mg CO_2-C g^{-1} biomass carbon d^{-1}) also declined during the 0 - 7d incubation period (Figure 3-4). This was presumably for the above reasons. This agrees with the views of Insam (1990) that typically a high biomass specific respiration rate is found in soils with a recent input of easily degradable substrate. This fresh substrate induces a microflora composed of mainly r-strategy or zymogenous organisms which respire more CO_2-C per unit degradable C than k-strategy or autoctonous organisms (Jenkinson and Ladd, 1981; Pianka, 1970).

The rate of biomass specific respiration in high-metal soils was up to 1.5 times higher than in low-metal soils (*e.g.* FYM) (Figure 3-4). A similar ratio was found by Chander (1991) in unamended low- and high-metal soils incubated for 0 to 100d.

The biomass in the high-metal soil maintained a consistently faster biomass specific respiration rate which was about 1 - 2 times faster than in the low-metal soils.

The soils treated with NPK, FYM and sewage sludge maintained separate rates of biomass specific respiration in the same proportion 1.2 (NPK) and 1.5 (sewage sludge) times with respect to FYM treated soils throughout the incubation period. In contrast, CO_2-C evolution per unit weight of soil did not really separate the different treatments, unlike biomass specific respiration rate (Figure 3-3). The reason is probably because the available soil organic matter was limited in all the soils, so even the smaller biomass in high-metal soils could decompose soil organic matter at the maximum rate. . Little is known about the effect of heavy metals at these concentration upon the species composition of the soil microbial biomass. However,

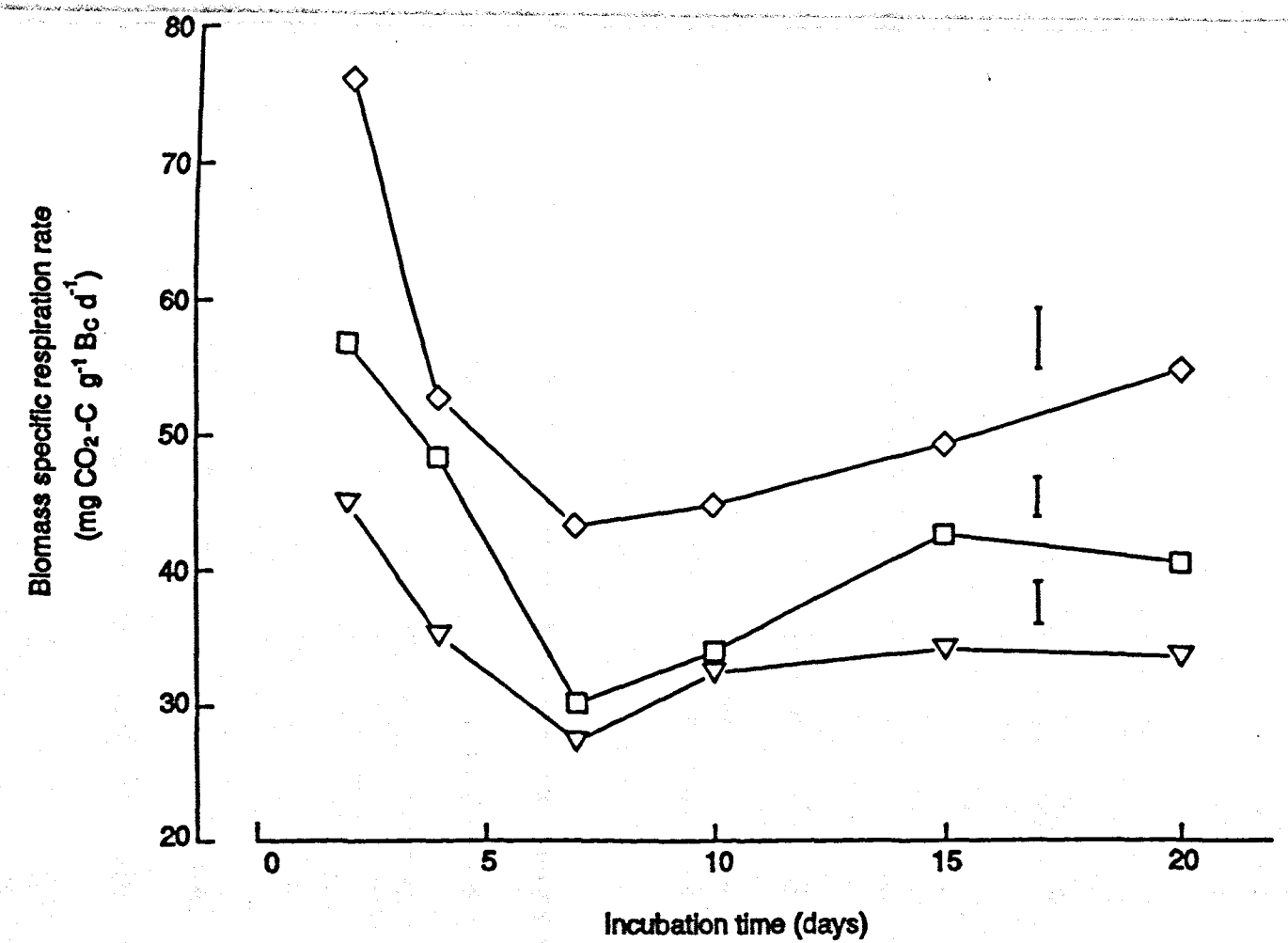

Figure 3-4: Biomass specific respiration rate of Woburn soils (□ NPK, ▽ FYM and ◇ sewage sludge) during 20d incubation at 25°C after 8 weeks storage at 4°C (LSD shown).

Brookes *et al* (1986) showed that the proportions of bacteria, larger spherical organisms (*e.g.* fungal and protozoa spores) and fungal hyphae were not affected by heavy metals in the soils of Woburn Market Garden Experiment, despite the effect of the metals on the size of total biomass. However, when a large amount of substrate was added the rate of CO_2-C evolved was proportional to substrate availability, not to the size of microbial biomass (Chander and Brookes, 1991c).

An increase in the biomass specific respiration rate has been interpreted as the effect of stress on the microbial community (Anderson and Domsch, 1990 and 1993; Chander and Brookes, 1991c; Killham, 1985; Šantrůčková and Straškraba, 1990). Thus, Chander and Brookes (1991c) recently demonstrated that much more added ^{14}C-labelled glucose or ^{14}C-labelled maize was converted to new biomass in low- than in high-metal soil. Conversely, much more of the available C from the added substrate was evolved as CO_2-C from the biomass in the high-metal than in the low-metal soil. This implies that the metals were causing decreased efficiency of substrate utilization by microbial biomass, and leading, in turn, to a smaller microbial biomass. Several mechanisms describing of the effect of heavy metals on micro-organisms have been suggested. For example the metals may affect microbial growth (Babich and Stotzky, 1977; 1979; Bhattacherjee, 1986; Smith, 1977), morphology (Barrow *et al.*, 1978; Barrow and Tornabene, 1979; Rosenzwieg and Pramer, 1980) and biochemical activities, for example by inhibiting the rate of respiration, synthesis of RNA and protein, nitrate reductive pathways and increasing the phosphorus content of the cell walls (Blundell and Wild, 1969; Cassity and Kolodziej, 1984; Siegee and Rabaee, 1986; Venkateswerlu and Stotzky, 1986).

These results suggest that biomass specific respiration rate provided a more useful parameter to detect the effect of heavy metals in pre-incubated soils than CO_2-C evolved.

3.3.3 CHANGES IN BIOMASS C MEASURED BY SUBSTRATE INDUCED RESPIRATION IN SOILS OF LOW AND HIGH METAL CONCENTRATIONS DURING INCUBATION.

Biomass C estimated by substrate induced respiration (SIR) apparently increased during the first 4d of incubation in all treatments (NPK, FYM and sewage sludge) (Figure 3-5) then, a decline started at 7d incubation. Figure 3-6 shows results of cumulative CO_2-C actually evolved from unamended soil and CO_2-C evolved due to glucose using CO_2-C measured from the SIR method (after adding glucose and incubation for 2h) in all the soils incubated at different time periods prior to analyses. The slope between 0 to 4d is higher than during the rest of the incubation time (7 to 20d) by both approaches. This means that more CO_2 was produced during this period (0 to 4d). Similar results were reported by Sparling *et al.*, (1981) in unamended soils (control) incubated for 3, 5, 7 and 14d. An increase of 30% was recorded up to 5d.

The previous data (Figure 3-2) suggest that the amount of biomass C and ninhydrin-N both measured by FE were similar when measured at 0, 10 and 20d. However, following addition of glucose, the rate of CO_2-C production increased linearly from day 0 to 10 in the three treatments (Figure 3-6). This strongly suggests that the maximum metabolic capacity of the biomass was depressed by storage and that several days of incubation at 25°C was required to produce a maximum SIR response. Similar results of depressed respiration rate caused by storage stress were

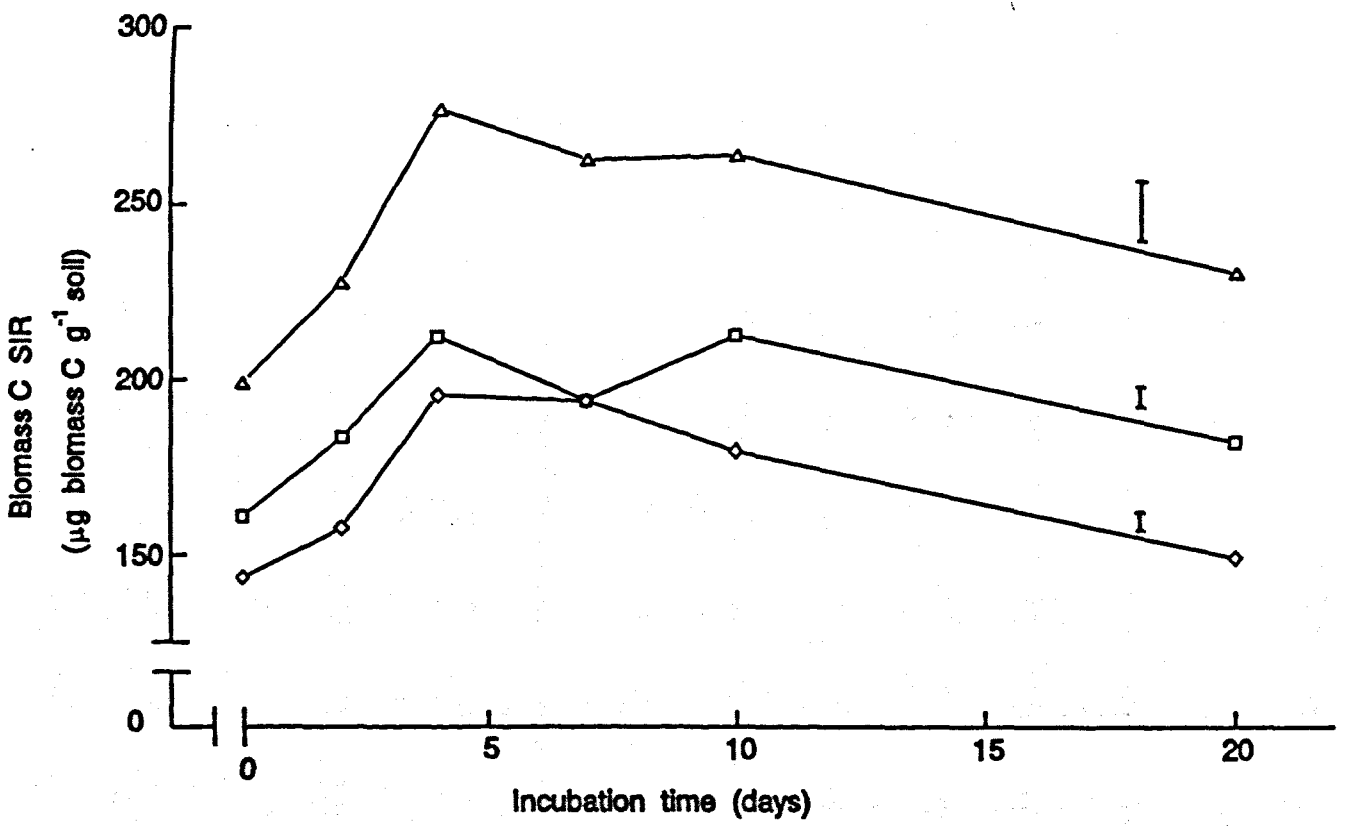

Figure 3-5: Microbial biomass (B_C) measured by substrate induced respiration (SIR) in Woburn soils (□ NPK, ▵ FYM and ◇ sewage sludge) during 20d incubation at 25°C (LSD shown).

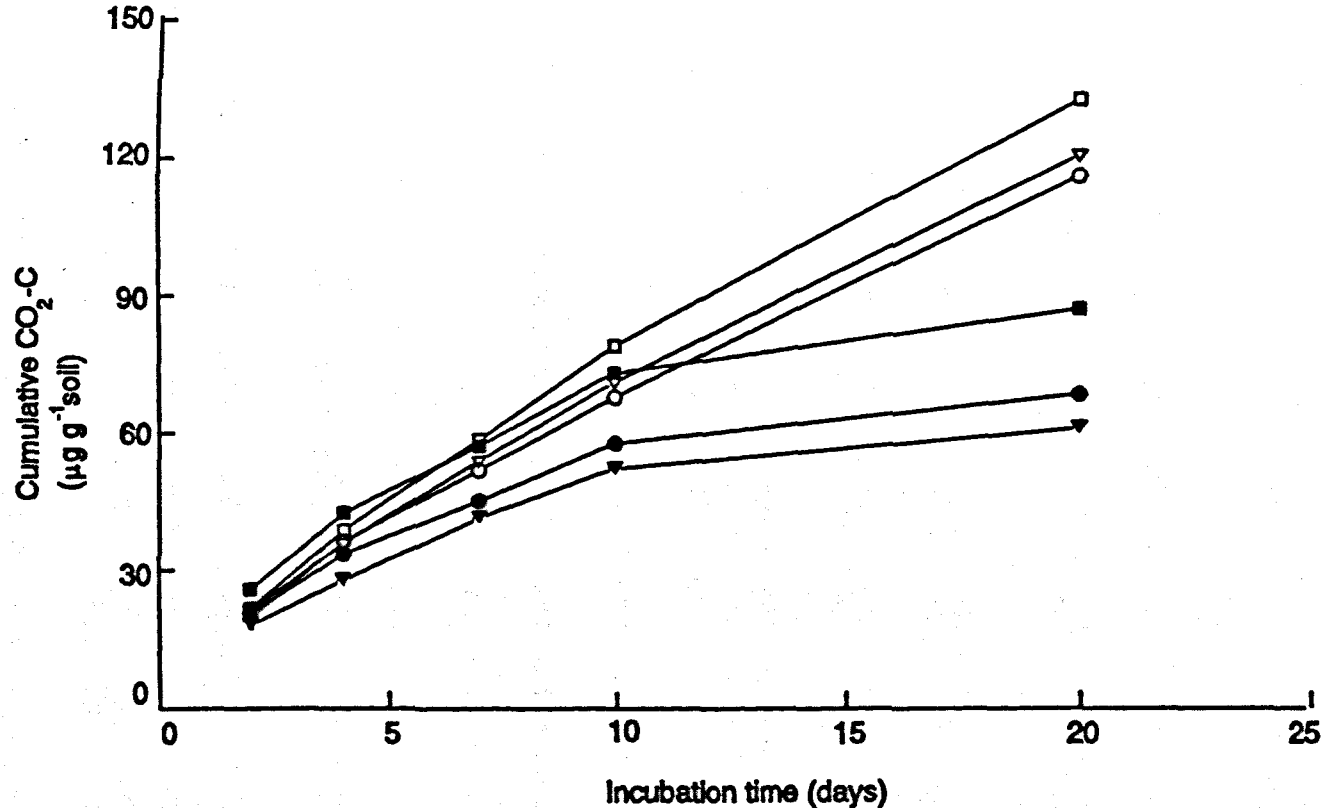

Figure 3-6: Cummulative CO_2-C in Woburn soils (O NPK, □ FYM and ▽ sewage sludge) calculated from CO_2-C measured (open symbols) and SIR (filled symbols) after eight weeks storage at 4°C.

given by Visser *et al.*, (1984) who reported that the microbial biomass of stored grassland soil responded more slowly to the addition of glucose than undisturbed soils even when no differences in amounts of biomass were detected. These results agree with Anderson and Domsch (1978) who showed that cultured micro-organisms in the early phase of growth, after harvesting and supplying with glucose, respired 2.5 times more CO_2-C per unit of biomass C than micro-organisms in log phase.

These results may suggest that during the first 4d of incubation, the SIR method was measuring the activity of the microbial biomass instead of the amount of microbial biomass, as shown by Wardle and Parkinson (1990).

The substrate induced respiration rate between 10 to 20d of incubation decreased by 14% (Figure 3-5). Similarly, Sparling *et al.*, (1981), found that SIR decreased by 12% after 12 to 14d of incubation in soils amended with different concentrations of glucose (0, 1, 5, and 10 mg glucose g^{-1} soil) and they concluded that after this time the cells would not be expected to be in the exponential phase. However, in this work, data from cumulative CO_2-C from basal respiration and from SIR (Figure 3-6) suggest that the concentration of glucose (6mg g^{-1} soil) used was not enough to obtain the maximum SIR response.

There was no correlation between CO_2-C evolved in basal respiration and biomass C (Table 3-4). In this case therefore, carbon availability from the soil organic matter itself was limiting the microbial activity. In contrast, when the large amount of glucose was added, the CO_2-C evolved was proportional to the amount of biomass present. Thus, the size of the biomass, not the nutrient availability, was limiting the microbial activity in this case.

Table 3-4: Biomass C[a], CO_2-C evolved by basal respiration and after 2h following glucose addition in soils from the Woburn Market Garden Experiment.

Treatment	Incubation time	Biomass C	CO_2-C	CO_2-C+glc[c]
	(days)	($\mu g\ g^{-1}$ soil)	($\mu g\ g^{-1}$ soil d^{-1})	($\mu l\ g^{-1}$ soil h^{-1})
Inorganic fertilizer NPK	0	173±4.5	9.91±0.338	9.63±0.119
	10	156±4.0	5.31±0.187	12.85±0.024
	20	147±2.6	5.96±0.149	10.98±0.180
Farmyard manure FYM	0	249±4.4	10.88±0.625	12.00±0.065
	10	210±6.5	6.82±0.215	16.01±0.173
	20	213±6.3	7.14±0.279	13.96±0.092
Sewage sludge	0	139±5.1	10.61±0.396	8.56±0.065
	10	128±4.8	5.75±0.393	10.81±0.273
	20	130±3.8	7.12±0.333	8.93±0.027
r[b]			0.116[ns]	0.669[**]

[a] measured by Fumigation-extraction method

[b] coefficient of linear correlation between biomass C and CO_2-C evolved by basal respiration or glucose added

[c] glucose

[ns] not significant

[**] significant at 0.05 probability level

Biomass C measured by SIR was significantly different in high-metal and low-metal soils at all times of incubation (Figure 3-5). Biomass C measured by SIR in FYM soils was 1.3 times larger and sewage sludge 0.89 times lower than similar NPK treated soils. These results suggest that biomass C by SIR can be measured in soils pre-incubated before the analysis, for short periods, *e.g.* 15d.

3.3.4 CHANGES IN ATP CONTENT IN SOILS OF LOW AND HIGH METAL CONCENTRATIONS DURING INCUBATION

The ATP contents in low and high-metal soils at one and eight weeks after sampling are shown in Table 3-5. Storage of soils for one or eight weeks at 4°C followed by analysis of soils incubated at 25°C for 20d prior to analysis after one or eight week of storage caused significant changes in ATP ($p<0.05$). Similarly ATP measured after 8 weeks storage then 20d incubation gave bigger increases in ATP than after one week of storage (33 to 35%). Figure 3-7 shows the ATP content at 0 10 and 20d of incubation before analysis. An analysis of variance of the results shown that incubation and storage were significant for all the treatments.

Biomass ATP concentrations (Table 3-5) were in the range of 6 to 16 μmol ATP g^{-1} biomass C, and overall, close to the mean value of 11.7 μmol ATP g^{-1} B_C reported by Jenkinson (1988) from data from a large number of unamended soils collected from the literature. There was an increase in ATP content in soils incubated from 0 to 20d after one or eight weeks storage of from -6 to 45%. Over this period the biomass C declined, but to a much smaller extent (about 1.5 to 27%, Table 3-3). This increase in ATP is therefore not caused by a net increase in biomass C. It is most likely to be due to an increase in the biomass ATP content in response to the

Table 3-5: Soil ATP content and biomass ATP concentration in soils from the Woburn Market Garden Experiment after 1 and 8 weeks storage at 4°C followed by incubation for 0 or 20d at 25 °C.

Treatment	ATP (nmol ATP g^{-1} soil)		% change	biomass ATP concentration (μmol ATP g^{-1} B_C[a])		% change
	0 days	20 days		0 days	20 days	
1 week after sampling						
Inorganic fertilizer NPK	1.44[b]±0.039[c]	1.74± 0.052	20.8	6.55±0.159	7.48±0.191	14.2
Farmyard manure rate 2	2.05±0.090	2.68± 0.032	30.6	7.19±0.351	12.11±0.432	68.4
Sewage sludge rate 2	1.36±0.032	1.27± 0.010	-6.6	7.68±0.179	7.78±0.153	1.3
8 weeks after sampling						
Inorganic fertilizer NPK	1.67± 0.065	2.41± 0.147	44.4	9.64±0.506	16.36±1.347	69.7
Farmyard manure rate 2	2.25± 0.116	3.30± 0.079	46.7	9.30±1.135	15.60±0.360	67.7
Sewage sludge rate 2	1.41± 0.079	1.80± 0.052	27.7	10.14±0.445	14.47±0.144	42.7

[a] biomass C

[b] Mean of four plots per treatment and three replicate determination per plot.

[c] Standard errors.

Table 3-6: The biomass ATP concentration in soils from the Woburn Market Garden Experiment after eight weeks storage at 4°C following incubation at 25°C.

Treatment	Incubation time	Biomass ATP concentration	%Changes[a]
	(days)	(μmol ATP g^{-1} B_C)	
Inorganic fertilizer NPK	0	9.64±0.506[b]	-
	10	13.80±0.914	43.15
	20	16.36±1.337	69.71
Farmyard manure rate 2	0	9.30±1.135	-
	10	14.94±2.513	60.65
	20	15.60±0.360	67.74
Sewage sludge rate 2	0	10.14±0.445	-
	10	14.01±0.714	38.17
	20	14.47±0.144	42.70

[a] from time 0

[b] Standard error

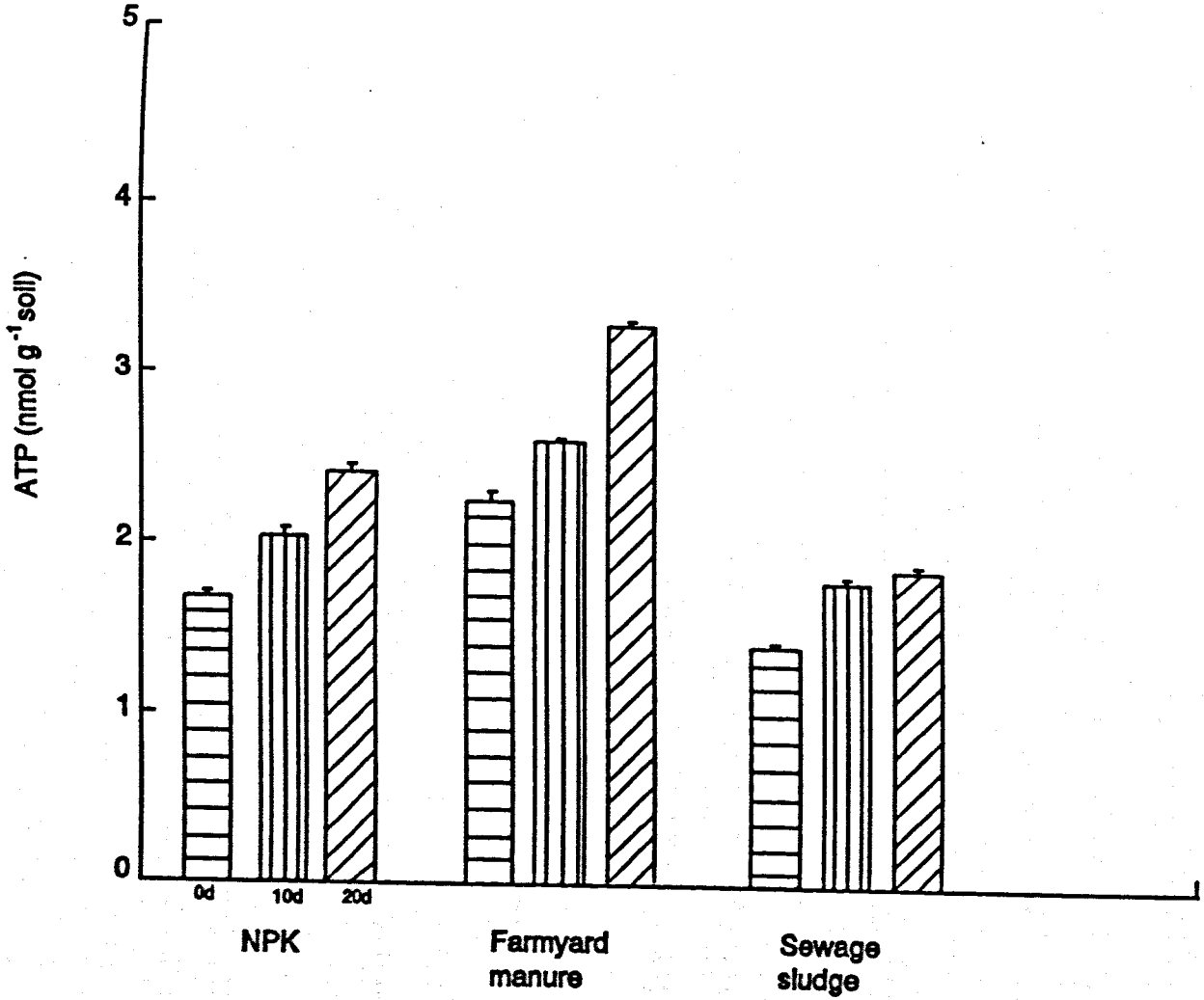

Figure 3-7: Amount of ATP in Woburn soils at 0, 10 and 20d incubation at 25°C after eight weeks storage at 4°C (standard errors of the mean shown).

increase in temperature from 4 to 25°C, increasing its metabolic rate. Similar results were reported by West *et al.*, (1986b). They found a 56% increase in ATP content in soils incubated from 0 to 7d at 25°C. Similarly, Tate and Jenkinson (1982) reported an increase of 41% during a 0 to 7d incubation at 25°C. The fumigation-extraction method measures the total microbial biomass without differences between dead and active microbial biomass. The ATP contents were changing proportionally by much larger amounts than those of the biomass (Table 3-6). This data was therefore indicative of changes of the biomass ATP concentration rather than synthesis of new biomass. These results might suggest that the energy status of the microbial community in stored soils is lower than in the incubated soils. More research is necessary to elucidate the factors which cause the increase of ATP and the connection with other measures of microbial biomass (such as SIR) and microbial activity measurements (such as CO_2-C evolved), since these measurements could be related indicators of a microbial system which is undergoing changes caused by storage and incubation conditions.

The ATP content in high-metal soil was consistently about 50% lower than low-metal soils (FYM and NPK) either at one or eight weeks storage and incubated for 0 to 20d. This means that in spite of apparent changes in soil ATP content in soils during incubation, it can separate high and low-metal soils, as can biomass C by measured FE and SIR.

3.3.5 CHANGES IN ARGININE AMMONIFICATION RATE IN SOILS OF LOW AND HIGH METAL CONCENTRATIONS DURING INCUBATION

The rate of arginine ammonification declined during the first 4d of incubation in all the soils (NPK, FYM and sewage sludge), then proceeded at a constant rate (Figure 3-8). The values of arginine ammonification rate in all the treated soils (NPK, FYM and sewage sludge) at 4d incubation decreased greatly (encircled values, Figure 3-8). I believe that these values are erroneous. This is supported by previous arginine ammonification rate measurements in soils stored for one week at 4°C after sampling (data not shown) where the values were similar compared to 7d incubation. The period of declining arginine ammonification rate coincided with decreasing CO_2-C evolved and increasing biomass C measure by SIR (Figures 3-3 and 3-5). The increasing of biomass C by SIR during the period 0 to 4d might be due to the different pathways to metabolise glucose and arginine.

The reason for the difference between arginine ammonification rate and CO_2-C evolved is because the latter one was C limited (see section 3.3.3) and the addition of an available C or N substrate such as arginine to soil, results an increase of NH_4 released, which is proportional to the size of microbial biomass (Table 3-7).

During the incubation from 0 to 4d no changes in mineralization of arginine (μg NH_4-N + NO_3-N g^{-1} soil) were observed in any of the soil treatments, (data not shown). The NO_3 measurements did not follow a defined pattern in the soil treatments during this period of incubation. The relatively large differences between replicates is probably due to other intrinsic problems of the extraction method to detect small quantities of NO_3^-. Therefore it was not possible to determine whether the decline of arginine ammonification was due to decreased mineralization or

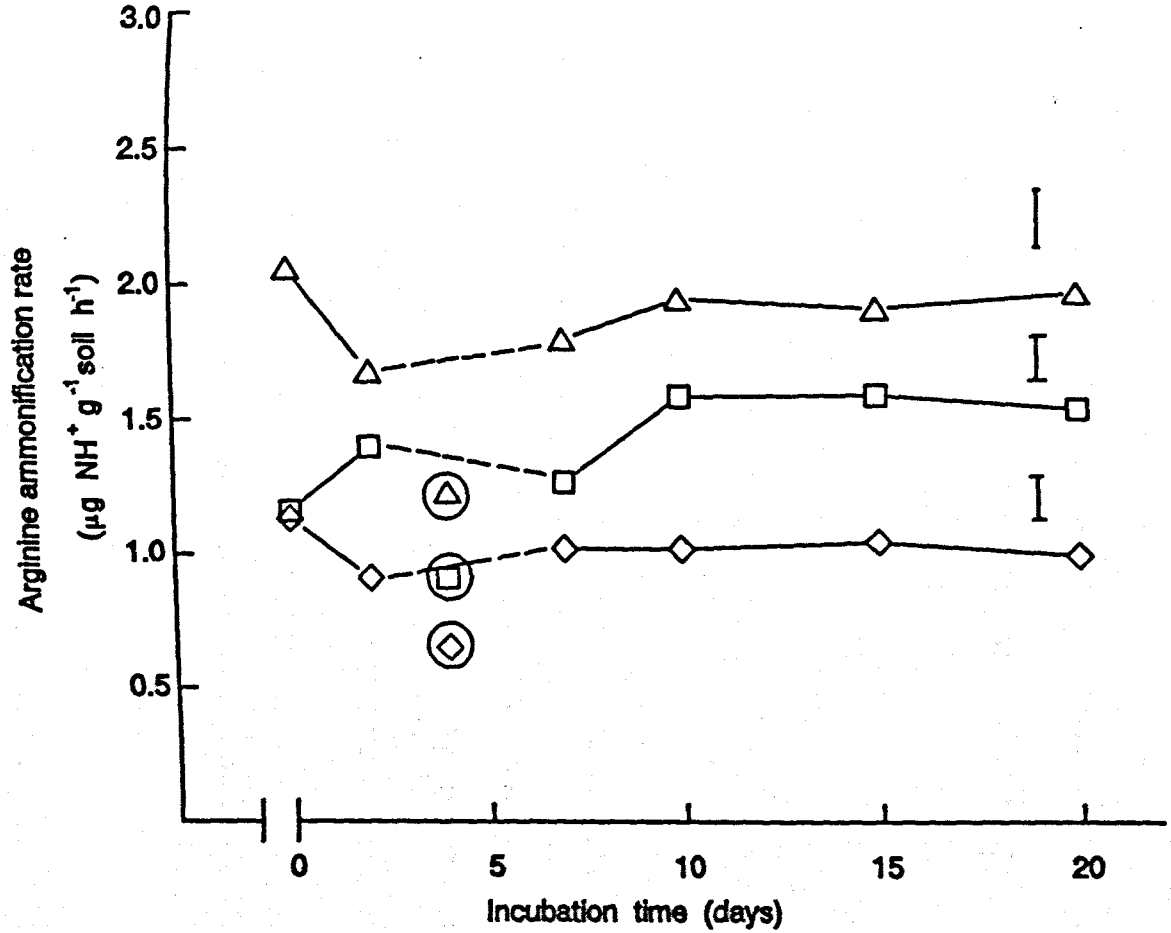

Figure 3-8: Arginine ammonification rate in Woburn soils (□ NPK, ▵ FYM and ◇ sewage sludge) during 20d incubation at 25°C after eight weeks storage at 4°C (LSD shown). For explanation of encircled values see text.

Table 3-7: Biomass C[a] and NH_4-N extracted from soil with and without added arginine followed by 3h incubation, in soils from the Woburn Market Garden Experiment.

Treatment	Incubation time	Biomass C	NH_4-N	NH_4-N +Arg[c]
	(days)		($\mu g\ g^{-1}$ soil)	
Inorganic fertilizer NPK	0	173±4.5	0.80±0.012	3.11±0.020
	10	156±4.0	0.27±0.016	3.45±0.019
	20	147±2.6	0.90±0.002	3.99±0.007
Farmyard manure FYM	0	249±4.4	0.33±0.043	4.23±0.032
	10	210±6.5	0.59±0.011	4.48±0.027
	20	213±6.2	0.91±0.012	4.82±0.028
Sewage sludge	0	139±5.1	0.60±0.022	2.86±0.062
	10	128±4.8	0.69±0.019	2.72±0.014
	20	130±3.8	1.12±0.037	3.12±0.018
r[b]			-0.378[ns]	0.795***

[a] measured by fumigation-extraction method

[b] coefficient of linear correlation between biomass C and NH_4 extracted in soils with and without arginine added

[c] arginine

[ns] not significant

*** significant at 0.001 probability level

increased immobilization.

The arginine ammonification rate (Figure 3-8) was higher in low-metal (NPK, FYM) than high-metal soils (sewage sludge) and were separated (FYM 1.35 times and sewage sludge 0.73 less than NPK) at all periods of incubation.

These results suggest that arginine ammonification rate was a useful parameter to detect the effect of heavy metal in soils that were stored and pre-incubated for different periods after sampling.

3.4 CHANGES IN SOIL MICROBIAL BIOMASS AND MICROBIAL ACTIVITIES DUE TO STORAGE OF SOILS OF LOW AND HIGH METAL CONCENTRATIONS FOR ONE YEAR

Storage of soils for one year caused a decline in all measured parameters compared to soil stored for 2 - 3 months (fresh soil). The decreases in the amount of biomass measured after storage for 2 or 3 months and after one year at 4°C were in the range of 1.6 to 12% for biomass C, from 1.1 to 7.7% for biomass ninhydrin-N and 13 to 23% for ATP. The apparent changes in ATP, although large when expressed on a percentage basis were actually very small in real terms (i.e. < 0.5nmol ATP g^{-1} soil). But for SIR the differences were larger, from 37.6 to 46.1% (Table 3-8). In contrast, CO_2-C evolved decreased from 21 - 35%, biomass specific respiration rate from 14.8 to 25.6% and arginine ammonification rate from 23.5 to 38.8% compared to initial values (Table 3-9). Analysis of variance of the data showed that differences between soil microbial biomass C and ninhydrin-N in fresh soils and soils stored for 1 year were not significant at $p<0.05$ within treatments.

Table 3-8: Effect of storage at 4°C on microbial biomass in the Woburn Garden Market Experiment soils.

Soil manurial treatment	Measured after sampling[a]	Measured 1 year after sampling	% change
	Biomass C (μg C g^{-1} soil)		
Inorganic fertilizer	149[b] ± 10.6[c]	131 ± 10.4	-12.3
Farmyard manure	196 ± 9.2	177 ± 10.8	-10.0
Sewage sludge	109 ± 8.2	107 ± 6.6	-1.6
	Biomass ninhydrin-N (μg Nin-N g^{-1} soil)		
Inorganic fertilizer	6.5 ± 1.10	6.0 ± 0.53	-7.4
Farmyard manure	8.6 ± 0.09	8.0 ± 0.34	-7.7
Sewage sludge	4.5 ± 0.32	4.5 ± 0.75	0.0
	Biomass C by SIR (μg g^{-1} soil)		
Inorganic fertilizer	289 ± 2.7	143 ± 1.8	-37.6
Farmyard manure	303 ± 1.5	173 ± 1.8	-43.0
Sewage sludge	221 ± 1.6	119 ± 1.4	-46.1
	ATP (nmol ATP g^{-1} soil)		
Inorganic fertilizer	1.7 ± 0.09	1.5 ± 0.10	-12.9
Farmyard manure	2.6 ± 0.10	2.1 ± 0.08	-35.5
Sewage sludge	1.6 ± 0.11	1.2 ± 0.04	-21.1

[a] Two months after storage at 4°C following incubation for 10d at 25°C
[b] Mean of four plots per treatment and three replicate determination per plot
[c] Standard error

Table 3-9: Effect of storage at 4°C on soil microbial activity in the Woburn Garden Market Experiment soils.

Soil manurial treatment	Measured after sampling[a]	Measured 1 year after sampling	% change
	CO_2-C evolved (μg CO_2-C g^{-1} soil)		
Inorganic fertilizer	54.0[b] ± 1.23[c]	36.8 ± 0.70	-32.0
farmyard manure	61.6 ± 0.58	40.1 ± 0.53	-35.5
Sewage sludge	57.4 ± 1.06	45.3 ± 0.63	-21.2
	Biomass specific respiration (mg CO_2-C g^{-1} B_C[d] d^{-1})		
Inorganic fertilizer	45.6 ± 4.03	30.9 ± 2.20	-32.1
Farmyard manure	38.6 ± 3.05	28.1 ± 1.22	-27.3
Sewage sludge	75.3 ± 3.61	53.2 ± 3.63	-29.3
	Arginine ammonification rate (μg NH_4 g^{-1} soil h^{-1})		
Inorganic fertilizer	1.68 ± 0.142	1.12 ± 0.291	-33.3
Farmyard manure	1.83 ± 0.180	1.40 ± 0.245	-23.5
Sewage sludge	1.21 ± 0.063	0.74 ± 0.128	-38.8

[a] Two months after storage at 4°C following incubation for 10d at 25°C

[b] Mean of four plots per treatment and three replicate determination per plot

[c] Standard error

[d] Biomass C

However the differences between ATP, SIR, CO_2-C evolved, biomass specific respiration rate and arginine ammonification rate were significant. Thus, the changes of microbial biomass measured by the fumigation extraction method were less (0 to 12%) than SIR and microbial activities (21 to 46%). The reason for these differences is unknown, probably, microbial biomass was losing its metabolic abilities as it aged physiologically during storage. Therefore, apparently reliable measurement of biomass C and ninhydrin-N can be done in soils stored for up to 1 year at 4°C and, possibly ATP (although soil ATP content did decrease, the absolute differences (nmol g^{-1} soil) were relatively small). This was not the case for CO_2-C evolved, SIR, arginine ammonification rate and biomass specific respiration rate measurements.

Ross (1991) reported, for a loam soil (33% clay), that biomass C measured by FE was unaffected by storage for 14 months at 4°C. In contrast, CO_2-C production declined by 50% and SIR by 40% over this period. He concluded that the appreciable amount of montmorillonite in the soil would be the major factor responsible for the stability of its microbial biomass. Soils from Woburn contain only 9% clay. These data suggest that other factors than soil type also influence the survival of microbial biomass, *e.g.* soil organic C content. Although nutrient reserves were enough in stored soils, clearly the microbial population aged on storage. Anderson and Domsch (1978) were the first to recognise the important of the physiological state of soil organisms in their original method. This conclusion is also supported by the measurements of biomass specific respiration rate which is higher in young populations (*e.g.* fresh soil) and lower in older ones (*e.g.* stored soils), as agrees with Anderson and Domsch (1990) and Insam and Domsch (1988).

Arginine ammonification is carried out only by metabolically active microorganisms as reported by Alef and Kleiner (1986). These data suggest that the rate of arginine ammonification is also affected by the age of the microbial biomass.

Data from this section suggest that fumigation-extraction methods are more appropriate for measuring changes in microbial biomass after long-term storage than SIR, ATP or arginine ammonification. In contrast, methods for measuring microbial biomass by SIR appears to measure microbial activity rather than microbial biomass under these conditions. It seems best to be cautious when estimating microbial biomass by SIR and ATP and microbial activity such as CO_2-C evolved, arginine ammonification and biomass specific respiration rate.

Overall, it can be concluded that both relative and absolute differences in the proportions of microbial biomass content (measured by FE, SIR and ATP) or microbial activities (biomass specific respiration and arginine ammonification rate but not CO_2-C evolved) can be detected in low- and high-metal soils when measured soon after sampling, or in the same soils after 1 year of storage.

3.5 SUMMARY

1 Biomass C, biomass ninhydrin-N and soil respiration declined during the first 7d of incubation. These results may be due to early mineralization of substrate such as microscopic roots or biomass killed during soil preparation or storage.

2 Biomass C measured by SIR increased during the first 4d of incubation. The values of cumulative CO_2-C theoretically calculated from the range where SIR increased were very close to the cumulative CO_2-C measured in NaOH absorbed. This probably means that the SIR method was measuring microbial activity during the first 4d of incubation instead of biomass C in soils.

3 Soil ATP content increased during a period of 0 to 20d incubation at one or eight weeks storage. Reasons for this anomaly awaits further investigation. Biomass ATP concentrations values were in the range 6 to 16 μmol ATP g^{-1} B_C, as reported in the literature.

4 Arginine ammonification rate decreased very little during the first 4d of incubation.

5 Biomass C from soils sampled after one week were declining faster than at eight weeks after sampling. This may be due to a small amount of biomass killed during soil preparation or storage or mineralization of micro-roots which could, erroneously, be possibly measured as biomass C at earlier stages. When soil samples have to be incubated, soil biomass carbon, biomass ninhydrin-N, CO_2-C evolved, arginine ammonification rate and SIR measurements can be measured from 7 to 10d of incubation as these measurements change little. Changes in biomass C and ninhydrin-N measured during one year of storage were small up to 12% and 7.7% respectively. Soil ATP changed from 13% to 35%. The CO_2 evolved decreased from 21 to

35% and arginine ammonification rate from 27 to 32%. The results indicate the measurements of biomass C, and ninhydrin-N can be used in soils stored for a long time at 4°C. This suggests that microbial activity is much more affected upon storage than is the total amount of biomass. Thus the biomass seems to age physiologically upon storage, losing activity, yet declining very little in population size.

6 The relative and absolute differences in the proportions of soil microbial biomass (measured by Fumigation-extraction, SIR and ATP) and microbial activity (arginine ammonification rate, biomass specific respiration rate but not CO_2-C evolved) can be detected in low and high-metal soils when measured after sampling following incubation 10d incubation, or in the same soil after one year of storage.

CHAPTER 4

MICROBIAL BIOMASS AND ORGANIC CARBON IN UNCONTAMINATED SOILS AND SOILS CONTAMINATED WITH HEAVY METALS AT AROUND CURRENT EUROPEAN UNION PERMITTED LIMITS

4.1 INTRODUCTION

Brookes and McGrath (1984) reported that agricultural soils from the Woburn Market Garden Experiment which had been contaminated with heavy metals from past applications of sewage-sludge at, or up to three times current EU permitted limits contained significantly smaller amounts of soil microbial biomass than similar, non-contaminated soils. Biomass C was measured by the fumigation incubation method and by soil ATP content. Soil microbial activity, assessed as CO_2-C evolution, was unchanged per unit of soil, by the metals. However, the biomass specific respiration rate (*i.e.* CO_2-C evolved per unit of biomass per unit time) was up to twice as high in the metal-contaminated soils as in other soils of the experiment.

Similar findings were reported by others (*e.g.* Chander and Brookes, 1991c; 1993; Werner, 1988). Since 1984, a range of new methods to measure microbial biomass and microbial activity have been developed. These include fumigation-extraction, substrate induced respiration, arginine ammonification and dimethyl sulphoxide reduction. These have not yet been systematically tested together to see

how suitable they are to detect effects of heavy metals on the soil microbial biomass and its activity.

The aims of this work were to 1) systematically measure the microbial biomass of the full treatments of part B of the Woburn Market Garden Experiment. Brookes and McGrath (1984) used only selected plots of part A. A full description of the experiment is given by Johnston and Wedderburn (1975) and in Chapter 3 in this thesis. A second aim was to systematically and rigorously test the effects of the different methods to measure both microbial biomass and microbial activity in these soils to determine their suitability for measuring the effects of heavy metals on the soil ecosystem.

4.2 MATERIALS AND METHODS

4.2.1 DESCRIPTION OF THE WOBURN MARKET GARDEN EXPERIMENT.

The Woburn Garden Market Experiment was described in section 3.2.1. Table 3-1 summarizes the forms of organic manures, total amounts of organic manures and the dates of application.

4.2.2 SAMPLING AND PREPARATION OF SOILS.

Soils were sampled from Cottenham series B of the experiment in February 1991. Each treatment was sampled (0-23 cm soil depth with a 5 cm diameter auger) from four different plots in the field experiment, then the soils were treated as described in section 2.1.1.

4.2.3 SOIL CHEMICAL ANALYSIS.

Soil pH, organic C, total N and total metals were determined in air-dried and ground (160μm) portions of each soil, as described in section 2.4. Table 4-2 summarizes the soil analyses.

4.2.4 SOIL MICROBIAL BIOMASS AND ACTIVITY MEASUREMENTS.

Soil microbial biomass C, biomass ninhydrin-N, biomass N and ATP were measured after 10d of incubation at 25°C, as described in sections 2.2.1-3 and 2.2.5. The rates of CO_2-C evolved and arginine ammonification were assessed at 10d of incubation, again as described in section 2.3.1 and 2.3.2. Each measurement was done in duplicate and the results are the mean of the four replicate plots of each treatment.

4.3 RESULTS AND DISCUSSION

4.3.1 SOIL pH, ORGANIC C, TOTAL N AND TOTAL SOIL METAL CONCENTRATIONS.

Soil pH, organic C, and total N in all treatments are shown in Table 4-1. The soil pH was about 7.0 and did not differ between treatments. The amounts of organic C and total N in soils that received inorganic fertilizer were lower than those in soils which received organic manure. There was no obvious difference in organic matter between soils that received uncontaminated (*i.e.* inorganic fertilizer, farmyard manure and vegetable compost) or metal-contaminated (*i.e.* sewage sludge and sludge-compost) manures.

Table 4-1: Soil pH, soil organic C, total nitrogen and total metals in soils of Series B of the Woburn Market Garden Experiment.

Soil manurial treatment	pH	Organic C	Total N	Cu	Ni	Cd	Zn	Cr	Pb
		%		($\mu g\ g^{-1}$ soil)					
Inorganic fertilizer (NPK)	7.24	1.43	0.142	50	21	2.3	163	68	50
Farmyard manure									
Rate 1	7.10	1.72	0.161	57	18	1.8	197	79	64
Rate 2	7.02	2.28	0.183	50	21	1.7	185	70	58
Vegetable compost									
Rate 1	6.98	1.88	0.158	47	19	3.2	159	68	52
Rate 2	7.02	1.68	0.156	42	17	1.8	147	62	43
Sewage sludge									
Rate 1	6.95	1.88	0.154	77	25	6.4	240	99	78
Rate 2	7.01	1.78	0.173	110	30	8.5	347	132	110
Sludge compost									
Rate 1	7.03	1.62	0.160	72	27	4.7	253	93	88
Rate 2	7.05	1.87	0.177	97	26	6.7	335	113	118
EU[a] recommended upper limit				140	75	3	300	-	300

[a] European Union

Sludged soils contained Zn at around the current EU permitted upper limit and Cd at about 3 times respectively (Table 4-1). The concentration of Cd and Zn in the sludge treated soils were 3 and 1.7 times greater than those soils that received NPK, FYM and vegetable compost (low-metal).

The amount of total metals (Zn, Cu, Ni and Cd) in farmyard manure treated soils (series B) were slightly greater than in the soils from series A with the same treatment as reported by Brookes *et al.*, (1986) and Chander and Brookes (1991c). However, in soils which had received sewage sludge, the soil metal concentrations were similar in Cu, Cr and Pb, but slightly higher for Zn and Cd, for series A and B. I can not offer a satisfactory explanation for these differences. This unexplained differences may be due to sampling error.

4.3.2 EFFECTS OF HEAVY METALS ON SOIL MICROBIAL BIOMASS AND MICROBIAL ACTIVITY.

The amount of biomass C in soils containing increased concentrations of heavy metals from past applications of metal-contaminated manures (high-metal soils) was consistently less (from 52 - 72 % less) that in soils which received non-contaminated manures such as farmyard manure, vegetable compost and inorganic fertilizer (low-metal soils) (Figure 4-1 and Table 4-1). This finding is similar to Brookes and McGrath (1984) and Chander and Brookes (1991c) for series A. Table 4-2 shows similar decreases in biomass ninhydrin-N, biomass N and ATP in the high-metal soils. These results suggest that soil microbial biomass measurements by FE (biomass C, ninhydrin-N and N) and ATP content are suitable methods to determine the effect of heavy metal on microbial biomass in soils from long-term field experiments.

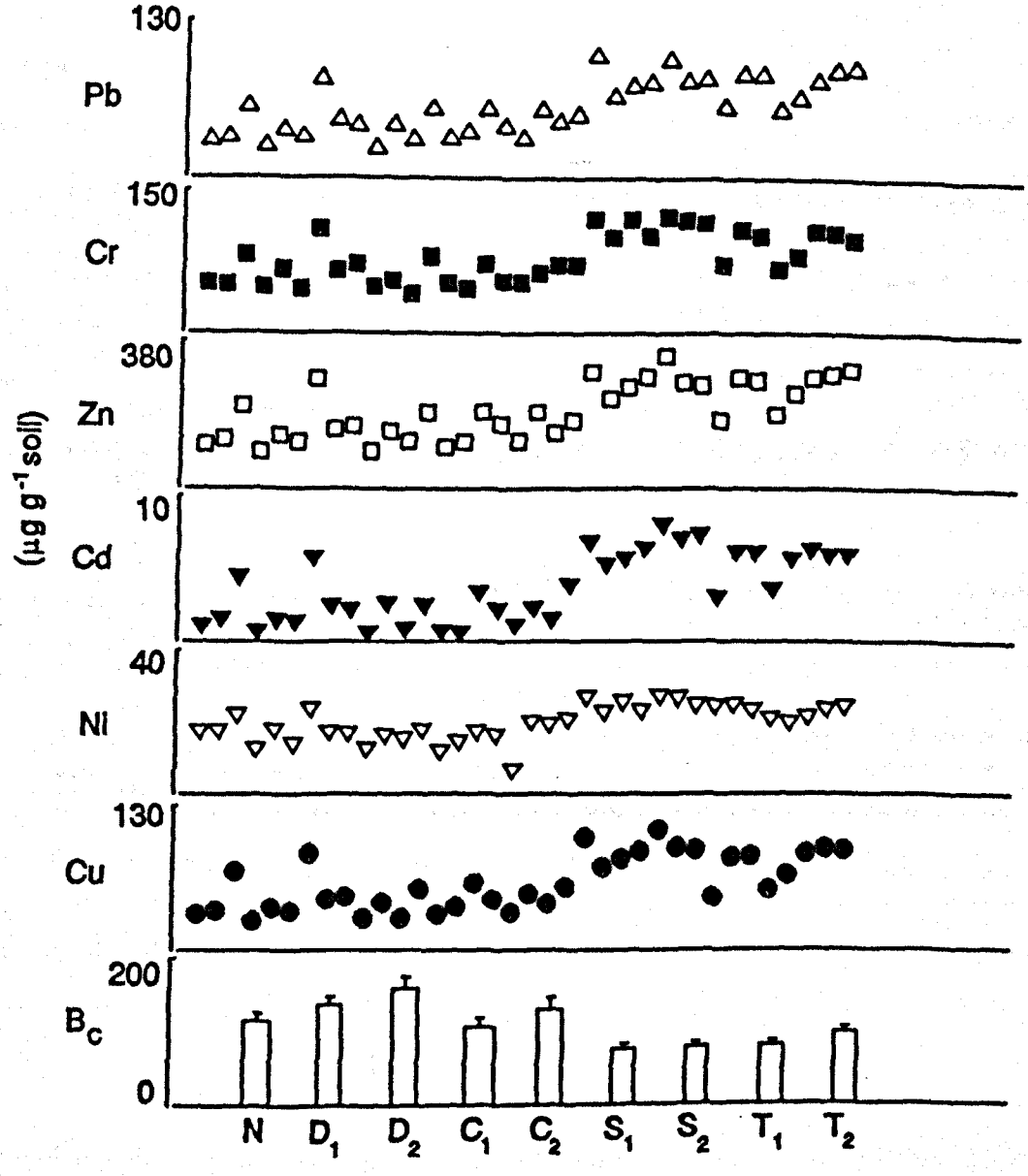

Figure 4-1: Total concentration of heavy metal and biomass C in soils from the Woburn Market Garden Experiment (N=NPK, D=FYM, C=vegetable compost, S=sewage sludge and T=sludge compost, 1=rate 1, 2=rate 2)

Table 4-2: Microbial biomass in soils from the Woburn Market Garden Experiment

Soil manurial treatment	Biomass Ninhydrin-N	Biomass N	ATP
	(μg g^{-1} soil)		(nmol g^{-1} soil)
Inorganic fertilizer (NPK)	6.52[a] ± 0.600[b]	25.2 ± 0.30	1.72 ± 0.092
Farmyard manure			
Rate 1	7.66 ± 0.609	27.3 ± 3.41	2.01 ± 0.189
Rate 2	8.61 ± 0.118	31.6 ± 2.21	2.62 ± 0.330
Vegetable compost			
Rate 1	6.67 ± 0.756	25.4 ± 3.26	2.15 ± 0.359
Rate 2	7.74 ± 0.861	29.7 ± 2.47	2.29 ± 0.226
Sewage sludge			
Rate 1	5.13 ± 0.268	19.2 ± 3.96	1.54 ± 0.086
Rate 2	4.50 ± 0.194	18.7 ± 1.10	1.63 ± 0.083
Sludge compost			
Rate 1	5.14 ± 0.468	21.7 ± 1.85	1.57 ± 0.269
Rate 2	5.62 ± 0.126	21.7 ± 2.20	1.98 ± 0.117
L.S.D. (P = 0.05)	1.498	7.40	0.636

[a] Mean of four plots per treatment and two replicate determinations per plot

[b] ± Standard error of the mean

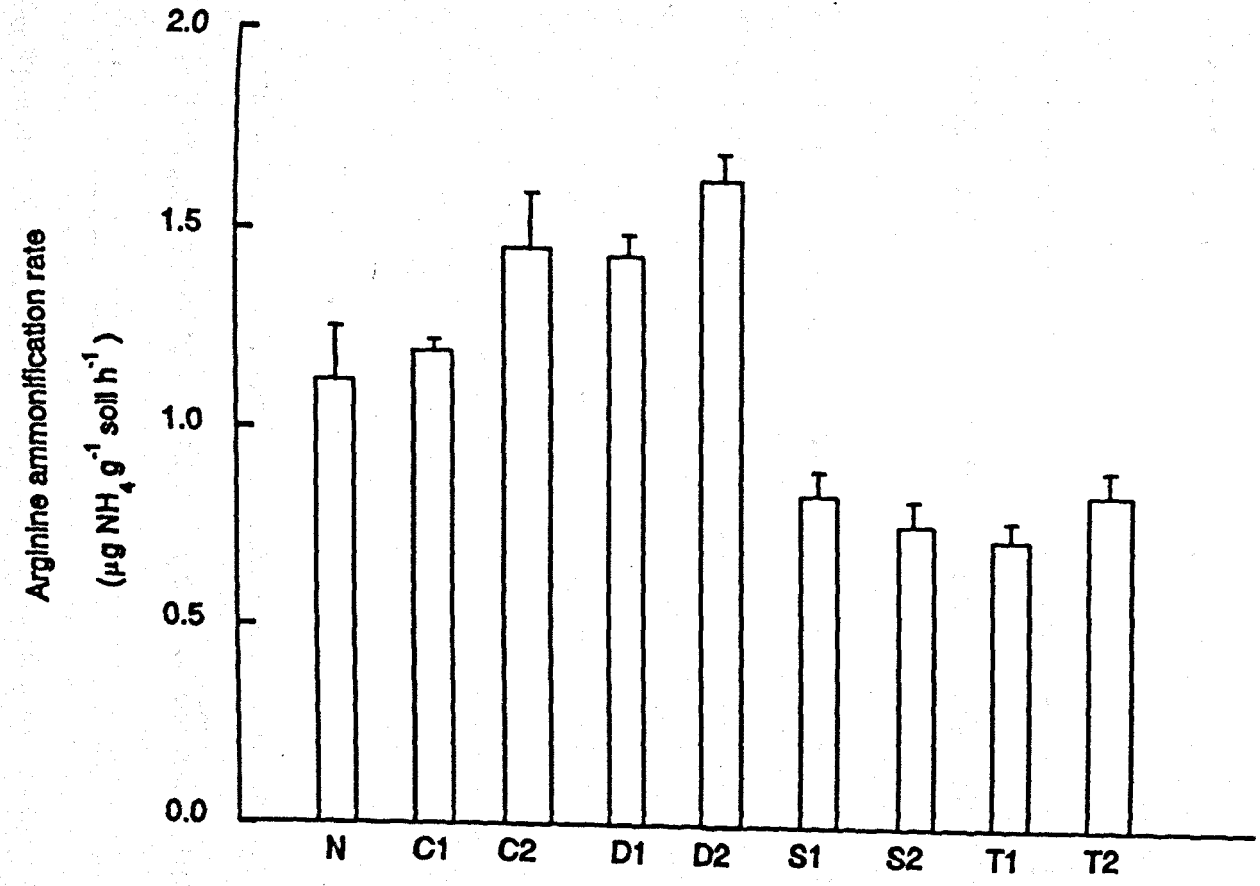

Figure 4-2: Arginine ammonification rate in soils from the Woburn Market Garden Experiment (N=NPK, D=FYM, C=vegetable compost, S=sewage sludge, T=sludge compost, 1=rate 1, 2=rate 2). Standard errors shown.

The arginine ammonification rate was about 58% less in the high-metal than the low-metal soils (Figure 4-2). Thus, changes in arginine ammonification rate may also reflect the effects of heavy metals at around these concentrations on microbial activity in soils. In contrast, the amount of CO_2-C evolved was very similar between soils and there were not statistically significant differences between high and low-metal soils (Table 4-3). Similar results of CO_2-C evolved were reported by Brookes and McGrath (1984). Reasons for these differences are discussed in section 4.3.3.

4.3.3 CORRELATION BETWEEN MICROBIAL BIOMASS AND MICROBIAL ACTIVITY MEASURED BY DIFFERENT METHODS IN UNCONTAMINATED AND METAL-CONTAMINATED SOILS

Soil microbial biomass C (B_C) and biomass ninhydrin-N (B_{NIN}) were closely correlated in all the treatments (Figure 4-3) irrespective of soil metal concentration. The overall regression equation was $B_c = (17.0 \pm 1.93)\ B_{NIN}$ and the linear correlation coefficient between biomass C and ninhydrin-N was $r = 0.95$. The proportionality factor (17) was within the range of 15 to 34 reported by Joergensen and Brookes (1990)(mean 20.6), Carter (1991a) (24.3), but smaller than that reported by Ocio and Brookes (1990) (31.2). Amato and Ladd (1988) reported a linear relationship between biomass C and biomass ninhydrin-N in unamended soils ($B_C = 21\ B_{NIN}$) after a 10d $CHCl_3$ fumigation of the soils. Chander and Brookes (1991a) reported a proportionality factor of 28.2 by combining data from Luddington (sandy loam soil) and Lee Valley (silty loam soil) for all the treatments (low- and high-metal soils), 28.1 from Gleathorpe soils (1993) and a factor 25.9 in low- and high-metal unamended soils and soil amended with glucose from the Woburn Market

Table 4-3: The CO_2-C evolved and biomass specific respiration rate during a 10d incubation period in soils from the Woburn Market Garden Experiment.

Soil manurial treatment	Biomass C	Soil respiration	Biomass specific respiration rate
	(mg CO_2-C g^{-1} soil)		(mg CO_2-C g^{-1} B_C[c] d^{-1})
Inorganic fertilizer (NPK)	137.6±18.10	54.0[a]± 3.03[b]	45.7 ± 4.05
Farmyard manure			
Rate 1	163.9±14.17	58.8 ± 2.46	41.3 ± 5.78
Rate 2	188.7±13.51	61.6 ± 0.66	38.6 ± 3.00
Vegetable compost			
Rate 1	132.9±13.94	60.0 ± 1.71	53.1 ± 3.14
Rate 2	155.0±15.31	57.4 ± 1.56	41.3 ± 3.85
Sewage sludge			
Rate 1	91.5±10.47	58.0 ± 0.63	62.8 ± 11.05
Rate 2	95.1±15.31	57.4 ± 2.26	75.3 ± 10.56
Sludge compost			
Rate 1	102.6±9.58	58.8 ± 1.37	57.5 ± 2.38
Rate 2	116.7±10.19	64.0 ± 2.95	52.2 ± 1.63
L.S.D. (P=0.05)	43.70	5.83	16.98

[a] Mean of four plots per treatment and two replicates per plot

[b] ± standard errors

[c] B_C = biomass C

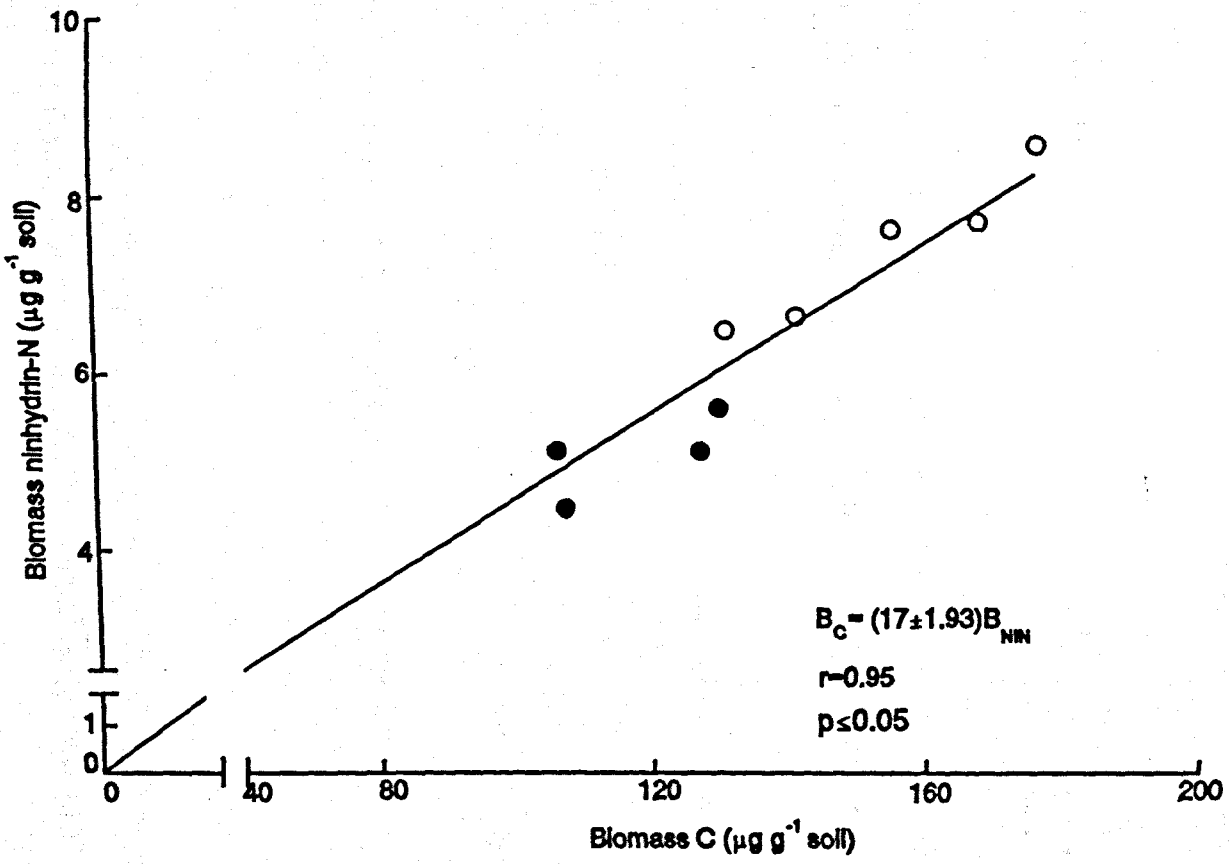

Figure 4-3: The correlation between biomass C and biomass ninhydrin-N in soils (O low-metal, ● high-metal) from the Woburn Market Garden Experiment.

Garden Experiment (1991c). Although, there are differences between proportionality factors for the relationship between biomass C and biomass ninhydrin-N in these different studies, their correlations were all significant. Thus, these results suggest that ninhydrin-N measurement could provide a very rapid method to measure microbial biomass in uncontaminated and metal-contaminated soils, and that the metals do not affect the ratio of C to ninhydrin-N in the microbial biomass.

Similarly, the amount of biomass C was closely correlated with biomass N and ATP in all the soils, irrespective of metal content (Figures 4-4 and 4-5). The linear correlations between biomass C and biomass N were statistically significant ($p<0.05$ - $p<0.01$). The biomass C/biomass N ratio ($B_C = (5.10 \pm 0.43)\ B_N$) was between the range 4 - 6 reported by Jenkinson (1988). The ratio C/N (5.10) is very close to that measured in pure cultures of bacteria (3 - 5) Jenkinson (1976) and 4.5 Bowen (1966). These results therefore suggest that the biomass in uncontaminated and metal-contaminated soils had the same N concentration. Thus, these data indicate that biomass N is a suitable method to estimate microbial biomass in metal contaminated soils and the biomass C/N ratio is not affected by the heavy metals at these soil concentrations.

The regression equation between ATP and biomass C was $B_C = (67.4 \pm 10.5)$ ATP ($r=0.90$). The mean biomass ATP concentration, averaged over both the low- and high-metal soils was 12.4 μmol ATP g^{-1} biomass C, close to the value of 11.7 μmol ATP g^{-1} biomass C obtained by Jenkinson (1988) who collected literature values from a large number of unamended soils. Chander and Brookes (1991c) reported an average value of 12.3 μmol ATP g^{-1} biomass C, from high and low-metal soils with and without added substrate from the Woburn Market Garden Experiment.

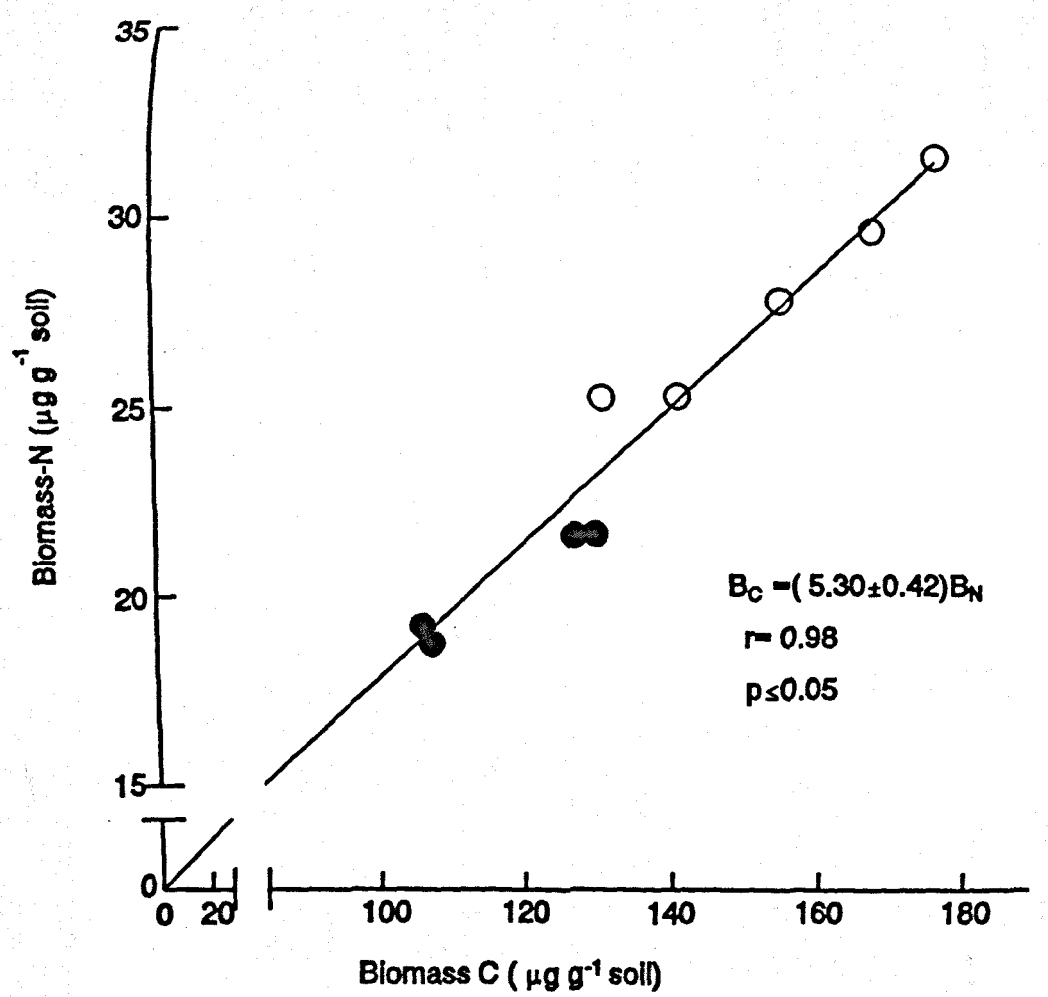

Figure 4-4: The correlation between microbial biomass C and microbial biomass N in soils (O low-metal, ● high-metal) from the Woburn Market Garden Experimant.

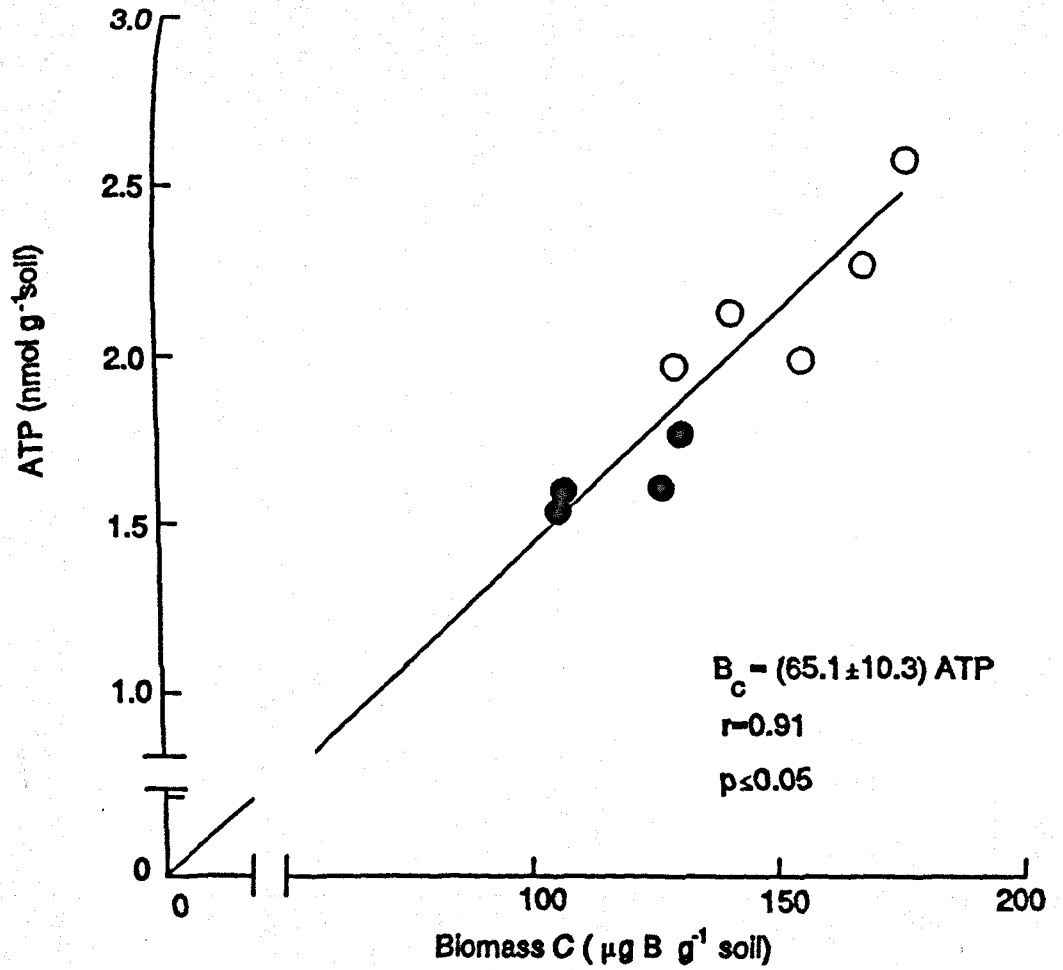

Figure 4-5: The correlation between microbial biomass C and ATP in soils (○ low-metal, ● high-metal) from the Woburn Market Garden Experiment.

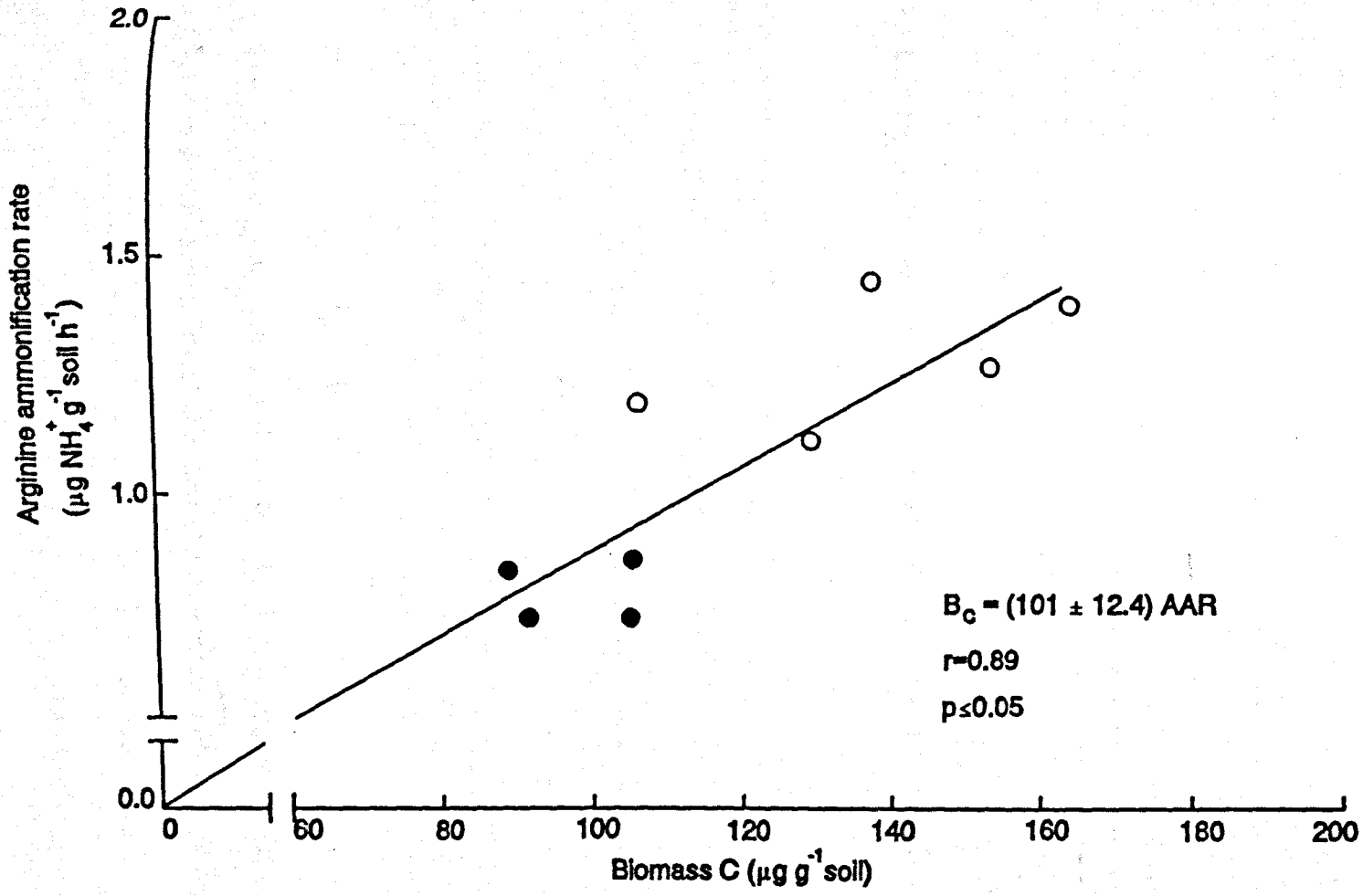

Figure 4-6: The correlation between microbial biomass C and arginine ammonification rate in soils (O low-metal , ● high-metal) from the Woburn Market Garden Experiment.

Biomass C was also linearly correlated with arginine ammonification rate (AAR), B_C = (101 ± 12.4) AAR (r=0.89, $p<0.05$), (Figure 4-6). In contrast, Kaiser *et al.*, (1992) reported a poor correlation between biomass C by FE and arginine ammonification rate. He used soils over a wide range of texture (3.0 to 42.8% clay) and organic matter content (0.69 - 2.61 % organic C), which may account for their lower coefficient of correlation (B_C = 152.6 AAR, r=0.54). The results in this section show that both ATP and arginine ammonification rate may reliably measure soil microbial biomass and microbial activity in soils with low- and high-metal content. Also, the ratio biomass C/ arginine ammonification and the biomass ATP concentration are not affected by the soil metal concentration.

The similar rates of CO_2-C evolved from low- and high-metal soils suggests that the metals did not affect the rate of mineralization of soil organic matter. In contrast, the rate of mineralization of added arginine was proportional to the size of the initial soil microbial biomass, being significantly lower in the high-metal soils (Figures 4-2 and 4-6). This difference is probably because, in the first case, the availability of decomposable soil organic matter was very low at any time, so that both the larger biomass in the low-metal soil and the smaller biomass in the high-metal soil could metabolise it completely. However, when arginine was added in excess, although the rate of mineralization per microbial cell may have been similar, the measured rates of mineralization in high-metals soil were less than in the low-metal soils, simply because of the smaller microbial population in the former case.

4.3.4 BIOMASS SPECIFIC RESPIRATION RATE.

The biomass specific respiration rate (mg CO_2-C g^{-1} biomass C d^{-1}) in the high-metal soils was about 1.6 times that in low-metal soils, averaged over all treatments (Table 4-3). In contrast soil respiration was similar in all soils, irrespective of fertilizer treatment or soil metal concentration. This means that there was more CO_2-C evolved per gram of biomass C per day in high-metal soils than in low-metal soils. These results therefore show that the smaller biomass in the high-metal soil had a faster specific respiration rate than the larger biomass in the low-metal soil. This increase in the biomass specific respiration rate is interpreted as the effect of stress on the microbial community as described in Chapter 3 section 3.3.2. Thus, high concentrations of heavy metals causes the soil microbial biomass to divert more energy from growth to maintenance as discussed by Chander and Brookes, (1991c); Killham, (1985) and Killham and Firestone, (1984). Recently, Chander and Brookes (1991c) reported that more total and ^{14}C-labelled CO_2-C was evolved from the high-metal than from low-metal soil after addition of ^{14}C-labelled glucose and maize. In contrast, about 30% less ^{14}C-labelled biomass was synthesised per unit of added substrate in high-metal than in low-metal soils. This suggests that the biomass in the metal-contaminated soil was less efficient in the utilization of substrate for synthesis of biomass C.

4.3.5 RELATIONSHIP BETWEEN BIOMASS C AND SOIL ORGANIC C.

There was an approximate relationship between the amount of soil microbial biomass and soil organic C in soils containing low concentrations of metal (Figure 4-7). Similar results were reported by Brookes and McGrath (1984) in Woburn soils,

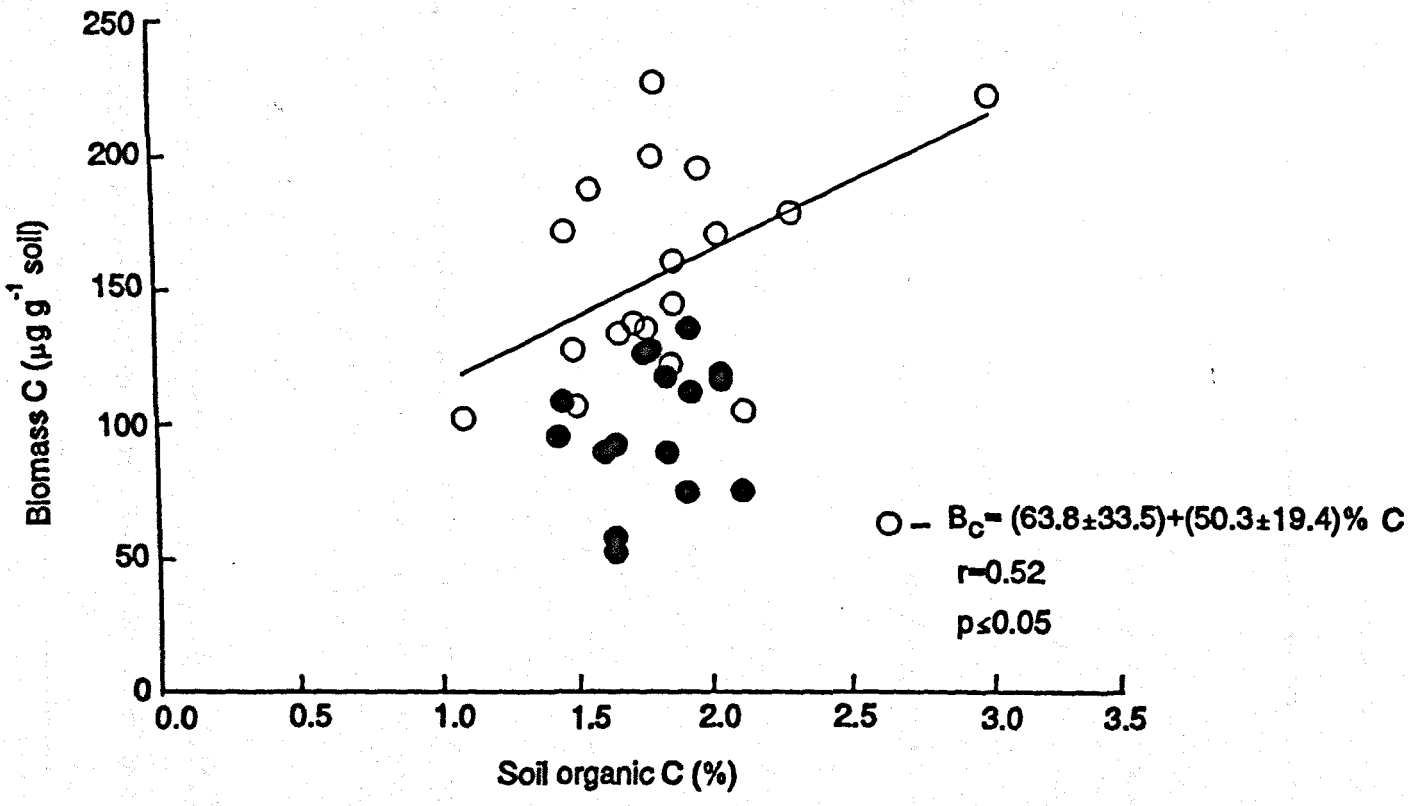

Figure 4-7: Biomass C and soil organic C in soils (O low-metal, ● high-metal) from the Woburn Market Garden Experiment.

Chander and Brookes (1993) in low-metal soils from Gleathorpe. The correlations were similar between biomass ninhydrin-N, ATP and soil organic C (Figures 4-8 and 4-9). The regression coefficients were statistically significant ($p < 0.05$) in each case. The data were also analyzed by Discriminant Analysis, the results are shown in Figure 4-10. The purpose of this statistical method is to examine separations among groups by estimation of the boundaries (likelihood functions) of each population (Kendal, 1980). In this study, two population were assigned, one of these groups was assigned to low-metal and the second to high-metal soil. Points following the side of negative score are allocated to the low- metal group, those on the other side, positive score, to the high-metal soil group. Discriminant scores of each plot showed that 16 out of 20 low-metal plots analyzed had negative scores and 15 out of the 16 high-metal plots had positive scores. The anomalous plots were mainly in low-metal soils which were contaminated with heavy metals because of boundary contamination from adjacent plots which received metal contaminated sludge. The analysis indicated that separate communities exist in the low and high-metal soils. The biomass developed under soils with high concentrations of metals (*i.e.* soils that received sludge and sludge compost) were not significantly correlated with soil organic C content.

Several authors (*e.g.* Anderson and Domsch, 1989; Insam and Domsch, 1988; Powlson and Jenkinson, 1981; Ross *et al.*,1980; Wu, 1991) have also reported that there is an approximate relationship between microbial biomass and total soil organic C. These results suggest that soil organic C is an important factor affecting the amount of soil biomass C. However, the relationship between these parameters also varies according to different physical characteristics (*e.g.* pH, clay content etc.) or under different management, cropping history and climate. For example, clay soils

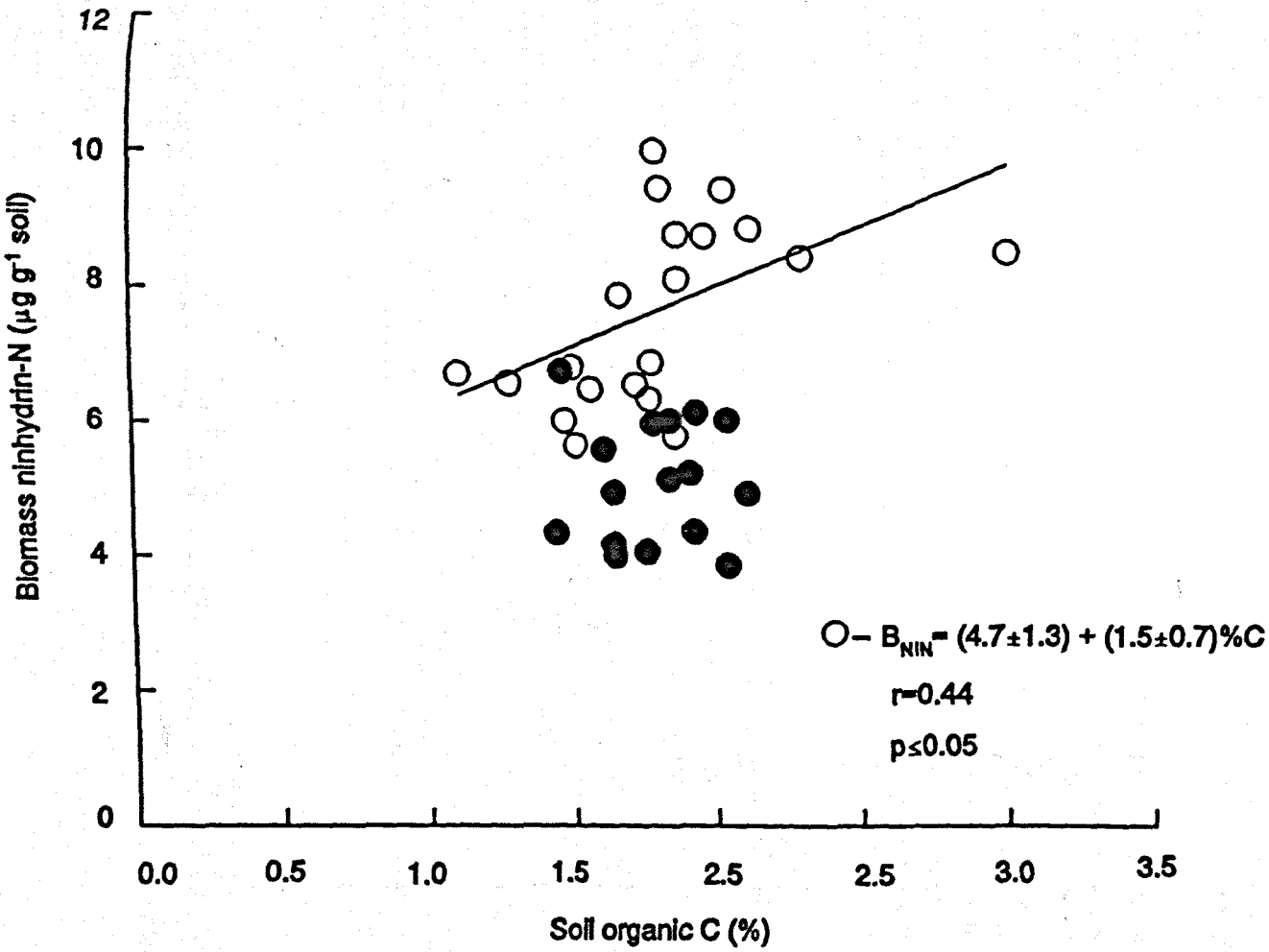

Figure 4-8: Biomass ninhydrin-N and soil organic C in soils (O low-metal, ● high-metal) from the Woburn Market Garden Experiment.

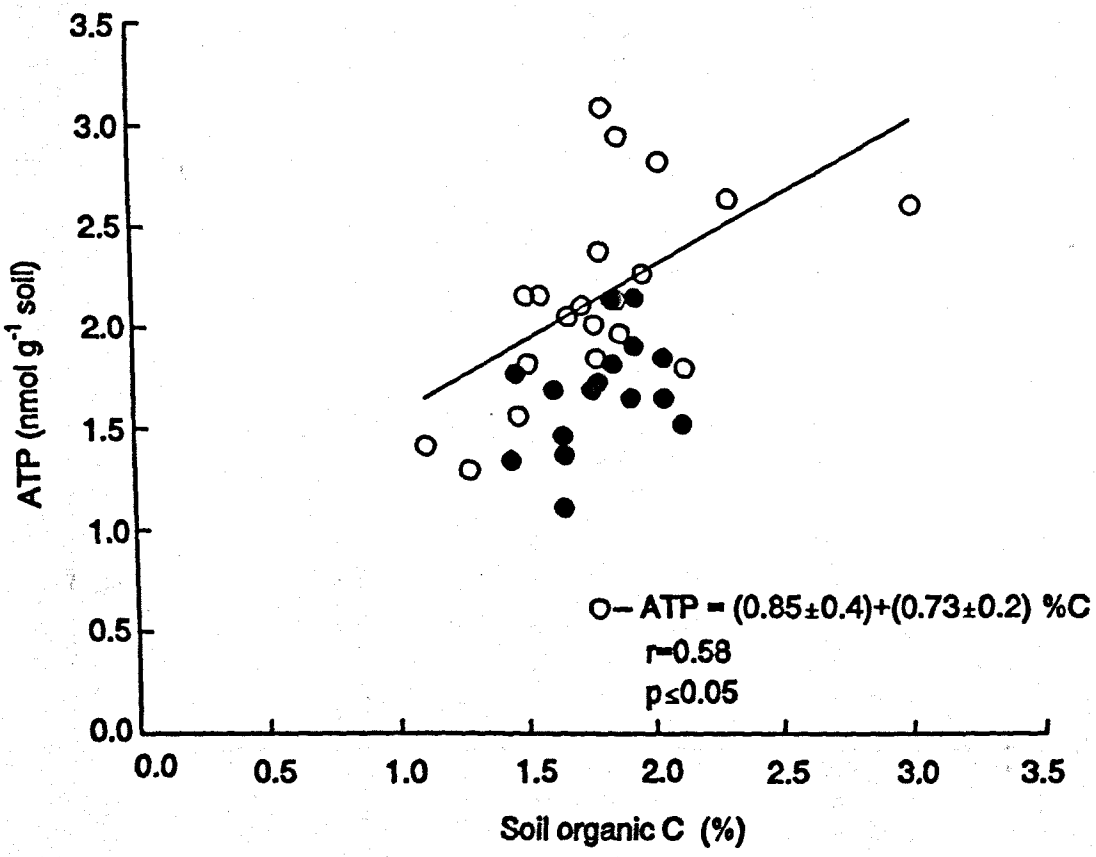

Figure 4-9: ATP and soil organic C in soils (O low-metal, ● high-metal) from the Woburn Market Garden Experiment.

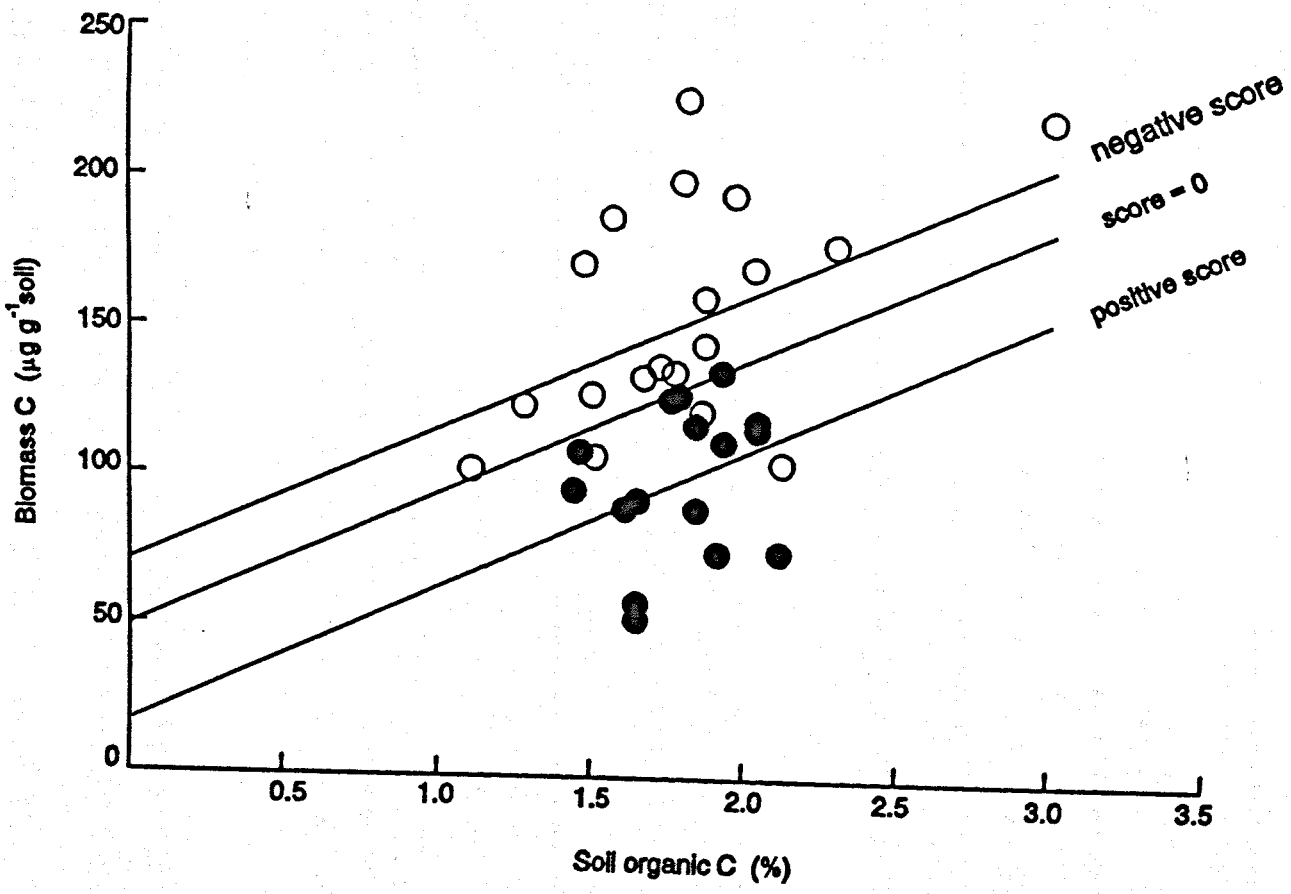

Figure 4-10: Discriminant analysis of low-metal (○) and high-metal (●) soils from the Woburn Market Garden Experiment.

contain several times more biomass C than sandy soils under similar management and climate (Alef *et al.*, 1988; Ayanaba *et al.*, 1976; Badalucco *et al.*, 1990; Gregorich *et al.*, 1990; Insam *et al.*, 1989). Also, biomass C is larger in grassland and forest than arable soils (Insam and Domsch, 1988; Jenkinson and Ladd, 1981; Lynch and Panting, 1980; Van Veen *et al.*, 1985; Wu, 1991). Biomass C responds to changes in soil management, changing in the same direction as total organic C changes (Carter, 1991a and 1986; Insam *et al.*, 1989; Wu, 1991). However the changes in biomass C have been suggested to be much faster and proportionally greater than the changes in total soil organic C (Jenkinson and Ladd, 1981; Wu, 1991). This is supported by the observation that biomass C as proportion of total soil organic matter decreased as the heavy metals increased. This link between biomass C and total soil organic C may constitute a form of "internal control" within soils of similar type and under similar management. Thus, if the ratio [biomass C]/[soil organic C] changes under these conditions, this could indicate damage to the soil ecosystem long before such changes could be detected by classical analysis of, for example, total soil organic C. If generally applicable this could enable more work to be done under non-experimental condition where, usually, the lack of suitable "control" soils which interpretation of environmental damage, due for example, to heavy metals extremely difficult.

4.3.5.1 BIOMASS C AS A PERCENTAGE OF SOIL ORGANIC C.

Microbial biomass C expressed as a percentage of soil organic C= 100 x (biomass C/organic C) was between the range 1.2 - 1.9 times higher in low-metal soils than in high-metal soils (Figure 4-11). In soil treated with vegetable

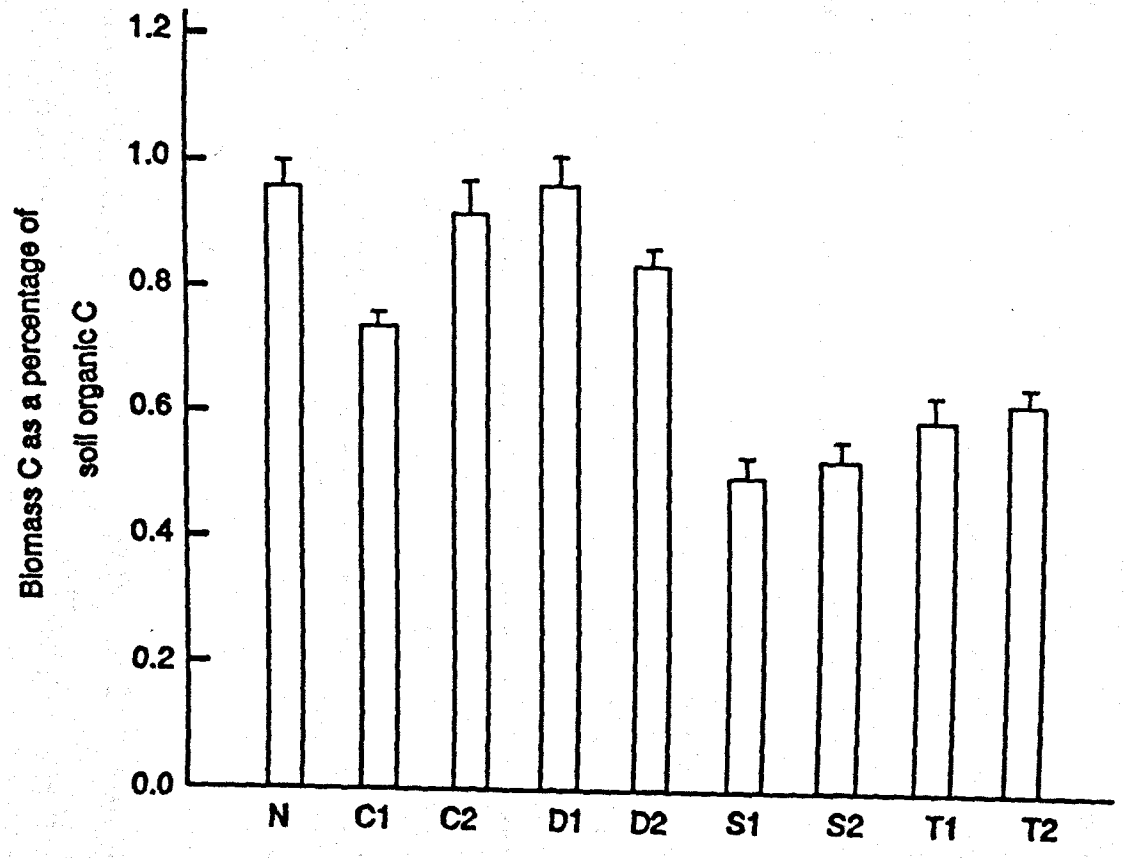

Figure 4-11: Biomass C as a percentage of soil organic C in soils from the Woburn Market Garden Experiment (N=NPK, D=FYM, C=vegetable compost, S=sewage sludge, T=sludge compost, 1=rate 1, 2=rate 2). Standard errors shown.

compost, rate 1 had the smallest biomass C as a percentage of total soil organic C (0.76) within the low-metal soil group. Plot 65 of this treatment contained unexpectedly high concentrations of heavy metals because of boundary contamination from adjacent high-metal plots, as mentioned previously (Figure 4-1). The biomass C as a percentage of soil organic C in low-metal soils (0.90 to 0.84%) were at the bottom of the range reported by Jenkinson and Ladd (1981) (0.8 - 4.0%), Anderson and Domsch (1989) (2.3-2.9%), Wu (1991) (0.5 to 2.6%) and lower than those of Jenkinson and Powlson (1976b) (1.7 - 3.7%) using soils with different texture, land use and crop rotation.

However the biomass C as a percentage of soil organic C in the high-metal soils (0.5 - 0.62%) were much lower than in low-metal soils. Similar results were reported by Chander and Brookes (1991a) between low-metal soils (1.5 - 2%) and in high-metal soils which received contaminated sewage sludge in UK in Luddington and Lee Valley soils (1.3 - 0.6%) and Chander and Brookes (1993) in low-metal (1.5 to 1.6) and Zn, Cu or both contaminated soils (0.4 to 0.7) in Gleadthorpe soils.

Different values of biomass C as percentage of soil organic C have been observed by others and related to different managements, crop rotation, land use, and climatic conditions (Anderson and Domsch, 1989; Carter, 1991b; Insam *et al.*, 1989; Insam *et al.*, 1991; Wu, 1991). Thus biomass as percentage of soil organic carbon directly reflects the quantity and quality of the fresh input of organic C and biological availability of the total organic matter in soil.

The changes in the biomass C as percentage of soil organic C in the present work indicate that the metals are affecting the size of biomass C. The soil organic C contents in the manurial treatments with high and low-metal soils were very similar

and within the range 1.4 to 1.9%, except the soils which received vegetable compost, rate 2, where it was 2.3% (Table 4-1).

Linking soil microbial biomass with soil organic matter in this way permits detection of the effects of the metals on the soil ecosystem while measurement of, for example, total soil organic C does not. It must be remembered that these data were all obtained from a well-managed field experiment, where variation in crop yield, other than due to treatment effects, was negligible. This also applies to other parameters e.g. soil pH, clay content, draining etc., all of which may have significant effects upon soil organic matter or biomass content and relations between them. However, it does seem from this, and other work, that these simple relationships between for example CO_2-C evolution and biomass C (biomass specific respiration rate) or biomass as a percentage of total soil organic C may constitute some form of "internal control", which might permit investigation of soil from the natural environment which may also be under stress due to pollution by heavy metals or other agents. This is examined later in the thesis (Chapter 6) on soils from a naturally occurring heavy metal gradient in Northern Spain.

4.4 SUMMARY

1 Microbial biomass C, ninhydrin-N, nitrogen and ATP decreased by up to about half in high-metal soils compared to low-metal soils. Similar decrease were found in the arginine ammonification rate in the high-metal soils.

2 The amounts of CO_2-C evolved from all the treatments were very similar, with no statistical differences. In contrast, biomass specific respiration rate

and the ratios CO_2/B_{Nin}, CO_2/B_N and CO_2/ATP in high-metal soils were about 1.5 times higher than in low-metal soils.

3 Biomass carbon as percentage of soil organic carbon was 0.60 times lower in the high-metal than low-metal soils.

4 Correlations between B_C, B_{Nin}, B_N and ATP were statistically significant ($p<0.05$), indicating that the heavy metals contained in the soils which received sewage sludge and sludge compost did not affect the ratios C/Nin, C/N and C/ATP of the native microbial biomass.

5 There was an approximate correlation between the amount of soil microbial biomass and soil organic carbon in low-metal soils. The same results were obtained with biomass ninhydrin-N and ATP where the regression coefficients were statistically significant at $p<0.05$ in each case.

6 Discriminant analysis of the data indicated that two separate communities exist in low and high-metal soils. The biomass developed under soils with high concentrations of heavy metal was not significantly correlated with soil organic C content. The correlation between soil organic C and microbial biomass constitutes an 'internal control' of the ecosystem in study. Thus, the link between biomass C and total soil organic C may permit detections of the effects of the metals on the soil ecosystem.

CHAPTER 5

RELATIONSHIPS BETWEEN MICROBIAL BIOMASS AND MICROBIAL ACTIVITIES IN SOILS FROM A NATURAL ENVIRONMENT CONTAINING LOW AND HIGH CONCENTRATIONS OF METALS

5.1 INTRODUCTION

The main sources of heavy metal pollution in soils are from mining and industrial wastes, sewage sludge, fertilizers and pesticides. Past research on the potential problems of heavy metals pollution has mainly been focused on the risk of contamination of crops (Marks *et al.*, 1977; Webber, 1980). More attention is now being given to effects on soil biological processes (Brookes *et al.*, 1986; Lighthart *et al.*,1983; Linares *et al.*,1989). There is widespread interest in the quantitative estimation of soil microbial biomass as an ecological indicator in determining the degree of disturbance in contaminated habitats. However, soil microbial biomass and microbial activity are influenced by several factors such as soil organic carbon, clay content, management practice, cropping history and climate (Anderson and Domsch, 1989; Insam and Domsch, 1988; Jenkinson and Ladd, 1981; Lynch and Panting, 1980; Paul and Voroney, 1980; Powlson and Jenkinson, 1981; Schnürer *et al.*, 1985; Van Veen *et al.*, 1985). To assess the effect of heavy metals on soil microbial biomass and microbial activities it is important to take into account all such factors. So far studies have mainly been done in soils from well managed field experiments. For example, Brookes and McGrath (1984) and Brookes *et al.*, (1986), found

decreased amounts of microbial biomass and some decreased microbial activities (*e.g.* non-symbiotic N_2 fixation) in a sandy loam soil (9 %clay) which had last received metal-contaminated sewage sludge 20 years before the biological measurements. The soil concentration of Zn, Cu and Ni were slightly less, but Cd was about 3 times more than the maximum current permitted European Union (EU) soil metal limits. Chander and Brookes (1991a) reported decreasing toxicity of metals to the microbial biomass in the following order Cu>Zn>Ni>>Cd. The toxic effects of the metals on the biomass were greater in sandy soils than in those of higher clay content (Chander and Brookes, 1991a). For example, Zn and Cu when present separately at about 1.4 times current permitted EU limits each decreased the biomass by about 20% whereas the decrease by Zn and Cu in combination, was about 57% compared to soil given uncontaminated sludge. Thus, the combination of Zn and Cu decreased the microbial biomass much more than individual metals at comparable concentrations. Correlations between biomass C measured by the Fumigation Incubation and Fumigation Extraction methods, ATP and biomass ninhydrin-N in the above work, were determined in soils with the same texture and management in each long-term experiment. These parameters provided information about the effects of different concentrations of heavy metals (single or combined) on microbial biomass or activities in soils from the field experiment. The toxic effects of Zn concentration at about 2.3 times EU permitted limits was greater (40%) in sandy loam soil (Luddington, 15% clay) than in silt loam soils (Lee Valley 21% clay) where it was about 30%.

However, there is currently little similar information about microbial biomass and activities in polluted soils from natural environments (Bååth *et al.*, 1991; Ohya

et al., 1988; Fritze *et al.*, 1989). Therefore, the specific objectives in this part of the work were: 1) To measure and compare a) the soil microbial biomass by different methods *e.g.* FE, SIR, ATP, ergosterol and b) microbial activities by *e.g.* CO_2-C evolved, arginine ammonification rate and dimethyl sulphoxide reduction rate in field soils of different texture and soil management contaminated with Zn at different concentrations. 2) To determine the relationship between microbial biomass and microbial activities, measured by different techniques. The site selected (from Gipuzkoa, Spain) had a natural gradient of heavy metals due to contamination from mine-spoil waste.

5.2 MATERIALS AND METHODS

5.2.1 DESCRIPTION OF THE SAMPLING SITE

Soils were collected from the agricultural valley of Oyarzun in Gipuzkoa, Spain (Figure 5-1). The soil of the valley is contaminated mainly by Zn and Pb, which were the two metals obtained from the mine of Arditurri at the top of the river Oyarzun. The mine was started in Roman times, and has now been finally abandoned. Mine wastes (e.g. fine material from ground rocks) have been transported by the river stream and rain and deposited in the estuaries and in the valley.

The slope of the land where the transects were collected gradually increased with increasing distance from the river. The slope steepened from 90 to 200m in transect 1 and from 180 to 240m in transect 2. The area where transect 1 was collected is a grassland and some ornamental and vegetable crops are grown on the

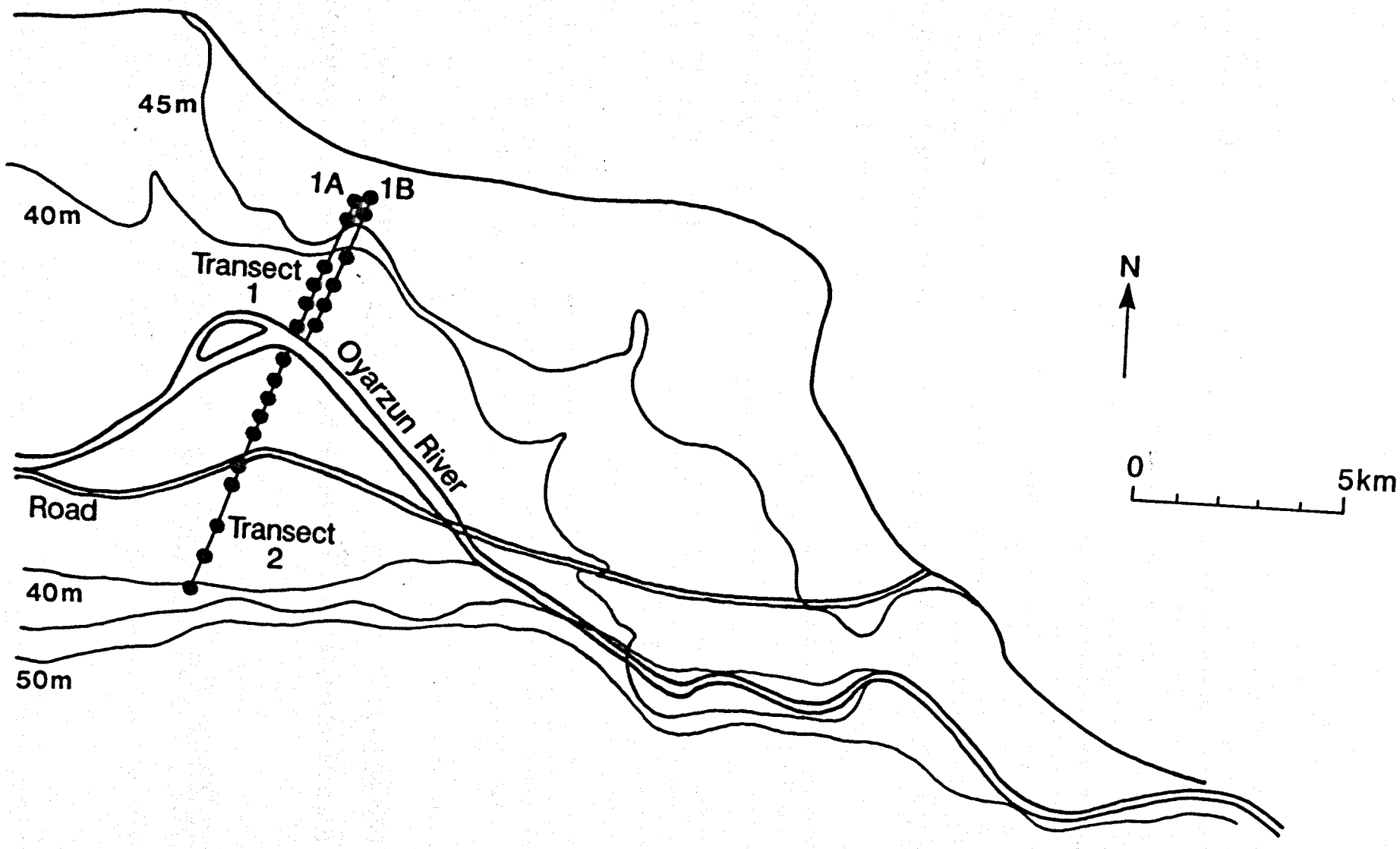

Figure 5-1: Localization of study site in Gipuzkoa, Spain.

hill. Fruit trees, maize, ornamental plants and protected crops under glasshouses are grown on the land where transect 2 was collected.

5.2.2 SAMPLING AND PREPARATION OF SOILS

Soil samples were collected with a spade (0-23cm soil depth) along a pollution gradient perpendicular to River Oyarzun. The two transects (number 1 and 2) were sampled in November 1992, one was a grassland soil and the other was agricultural land in the other side of the river. In order to obtain more information, more samples from transect 1 were obtained in March 1993. Generally, the results from transect 1 are pooled from both sampling occasions (November 1992 and March 1993). However, they are treated separately in section 5.3.6 when ergosterol measurements are discussed. In this case the soils sampled in November 1992 are referred to as sample 1A and those from March 1993 as transect 1B. The soils were treated as described in section 2.1.1 prior to analysis.

5.2.3 SOIL CHEMICAL ANALYSIS

Soil pH, organic C, total nitrogen and total metal concentrations were determined in air-dried and ground (160μm) portions of each soil, as described in section 2.4. The texture of the soils was analysed in the air-dried soil at Laboratorio Agrario de Fraisoro, Diputación Foral de Gipuzkoa, Spain. Tables 5-1, 5-2 and 5-3 summarize the soil analysis.

5.2.4 SOIL MICROBIAL BIOMASS AND ACTIVITIES MEASUREMENTS

Soil microbial biomass C, ninhydrin-N, substrate induced respiration, ATP and ergosterol were measured after 10d of incubation at 25°C , as described in sections 2.2.1 - 2 and 2.2.4 - 6. The rates of CO_2-C evolved, arginine ammonification rate and dimethyl sulphoxide reduction rate were assessed at 10d of incubation as described in sections 2.3.1 to 3. Each analytical measurement was the mean of triplicate determinations.

5.3 RESULTS

5.3.1 TOTAL METAL CONCENTRATIONS IN THE SOILS

The amounts of total metals (Zn, Cu, Ni, Cd and Pb) in the soils of the two transects are shown in Table 5-1, together with the maximum currently permitted EU soil metal limits. The Zn , Cd and Pb concentrations of the soils close to the edge of the river (5 to 15 m) were very high, with averages of about 6320 μg Zn, 10.8 μg Cd and 1030 μg Pb g^{-1} soil, compared to that in soils far from the river (120 to 240 m) where the averages were 278 μg Zn and 0.44 μg Cd g^{-1} soil. However, the soil Cu, Ni and Pb did not vary systematically along the river (Table 5-1). The concentration of Zn was significantly correlated to Cd concentration ($r=0.90$, $p<0.05$), less to Pb ($r=0.50$) but not to Cu and Ni.

5.3.2 SOIL pH, ORGANIC C, TOTAL N AND SOIL TEXTURE

Soil pH, organic C, total N and texture of the soils of the two transects are shown in Tables 5-2 and 5-3. The soil pH for all the transects ranged from 7.7

Table 5-1: Total metal concentration in soils from Gipuzkoa, Spain.

Sample	Distance from river (m)	Zn	Cu	Ni	Cd	Pb
		(ug g^{-1} soil)				
Transect 1						
1	5	6488	87.3	35.2	10.0	1543
2	15	6208	176.0	37.1	11.9	483
3	20	6551	85.2	38.6	11.7	719
4	30	3132	98.3	37.5	6.4	495
5	40	3237	63.1	28.6	5.6	439
6	50	3150	99.3	33.2	6.8	518
7	60	2390	54.4	29.8	4.2	383
8	70	3709	100.0	34.3	7.6	378
9	90	859	64.3	49.0	0.7	244
10	105	188	60.8	39.7	0.3	140
11	120	332	75.9	41.9	0.3	170
12	200	286	92.8	72.2	0.6	128
Transect 2						
1	8	6265	94.2	32.9	10.8	1052
2	20	1570	65.1	30.4	0.9	2777
3	40	823	51.4	23.9	0.3	1458
4	80	908	57.8	32.7	0.3	1121
5	110	437	49.6	29.8	0.3	2484
6	130	334	46.4	32.4	0.3	1862
7	180	243	43.6	28.4	0.3	432
8	200	258	37.9	31.4	0.3	282
9	240	217	33.9	35.2	0.3	139
EU upper limits		300	140	75	3.0	300

Table 5-2: Soil organic carbon, total nitrogen and pH in soils from Gipuzkoa, Spain.

Sample	Distance from river	pH	Soil Organic Carbon	Total Nitrogen
	(m)		%	
Transect 1				
1	5	7.7	3.42	0.14
2	15	7.6	1.67	0.16
3	20	6.9	4.67	0.33
4	30	5.8	2.40	0.24
5	40	6.1	2.73	0.22
6	50	5.8	1.78	0.17
7	60	6.0	2.70	0.24
8	70	6.0	1.67	0.17
9	90	6.2	4.28	0.37
10	105	5.5	2.81	0.31
11	120	6.1	4.06	0.32
12	200	6.0	2.44	0.26
Transect 2				
1	8	7.4	4.00	0.23
2	20	5.9	2.94	0.26
3	40	5.9	2.68	0.25
4	80	5.8	2.80	0.25
5	110	6.2	3.52	0.24
6	130	5.9	3.65	0.21
7	180	5.9	2.98	0.15
8	200	5.5	2.49	0.22
9	240	5.6	3.24	0.25

to 5.5. Soils collected between 5 to 20m from the edge of the river had the highest pH (about 7.0), changing very little along the rest of the transect. Soil organic C ranged from 1.67 to 4.67% and soil organic N from 0.14 to 0.37% respectively.

Generally, the soil texture was sandy in soils collected near the river and changed through sandy loam, sandy clay loam to clay with increasing distance from the edge of the river.

Figure 5-2 shows the soil organic matter content in soils where the main contaminant was Zn for all the transects. The sandy soils with the highest concentrations of Zn had the highest % soil organic C (average 3.45 %C) and it was similar in the low-metal soils with clay texture (3.096 %C). A 5 cm layer of litter accumulation was observed in areas with high concentrations of Zn (personal observation).

5.3.3 SOIL MICROBIAL BIOMASS C AND BIOMASS NINHYDRIN-N

The amount of biomass C decreased as soil Zn concentration increased (Figure 5-3). However the amount of biomass was affected by factors other than high concentrations of heavy metals. For example, the amounts of biomass C in low-metal soils ranged from 400 to 800 $\mu g\ g^{-1}$ soil, and in high-metal soils (1000 μg Zn g^{-1} soil) ranged from 200 to 800 $\mu g\ g^{-1}$ soil. Figure 5-4 show that the amount of microbial biomass was also related to clay content and/or total soil organic C content in transect 1 and 2. The amount of biomass C in transect 2 (horticultural land) in soil at 80m from the river was 50% less than that of soil at 40m (Figure 5-4). The soil at 80m contain similar Zn concentrations, % organic C and the same texture as the soil at 40m (823, 908 μg Zn g^{-1} soil; 2.68, 2.80 % soil organic C respectively and both with

Table 5-3: Soil texture in soils from Gipuzkoa Spain.

Sample	Distance from river (m)	Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)	Classification
Transect 1						
1	5	39.4	47.5	7.7	5.4	Sand
2	15	33.2	52.2	6.6	8.1	Sand
3	20	3.5	67.7	14.1	14.7	Sandy loam
4	30	34.0	34.8	15.2	16.0	Sandy clay loam
5	40	48.9	33.3	6.5	11.2	Sandy loam
6	50	33.7	42.8	10.2	13.3	Sandy loam
7	60	30.9	41.7	10.4	17.0	Sandy clay loam
8	70	37.7	39.5	9.7	13.1	Sandy loam
9	90	11.1	28.2	25.5	35.3	Clay
10	105	11.8	28.0	25.1	35.2	Clay
11	120	12.2	30.8	25.5	31.5	Clay
12	200	11.7	32.0	24.8	31.5	Clay
Transect 2						
1	8	21.2	62.9	7.9	8.0	Sandy loam
2	20	28.5	34.7	16.2	20.6	Sandy clay loam
3	40	31.6	32.5	13.5	22.4	Sandy clay loam
4	80	21.6	38.6	14.7	25.1	Sandy clay loam
5	110	22.9	27.7	21.7	27.7	Clay
6	130	19.0	26.7	23.2	31.1	Clay
7	180	23.3	26.1	25.8	24.8	Clay loam
8	200	17.4	27.1	26.6	28.9	Clay
9	240	14.1	22.8	26.9	36.2	Clay

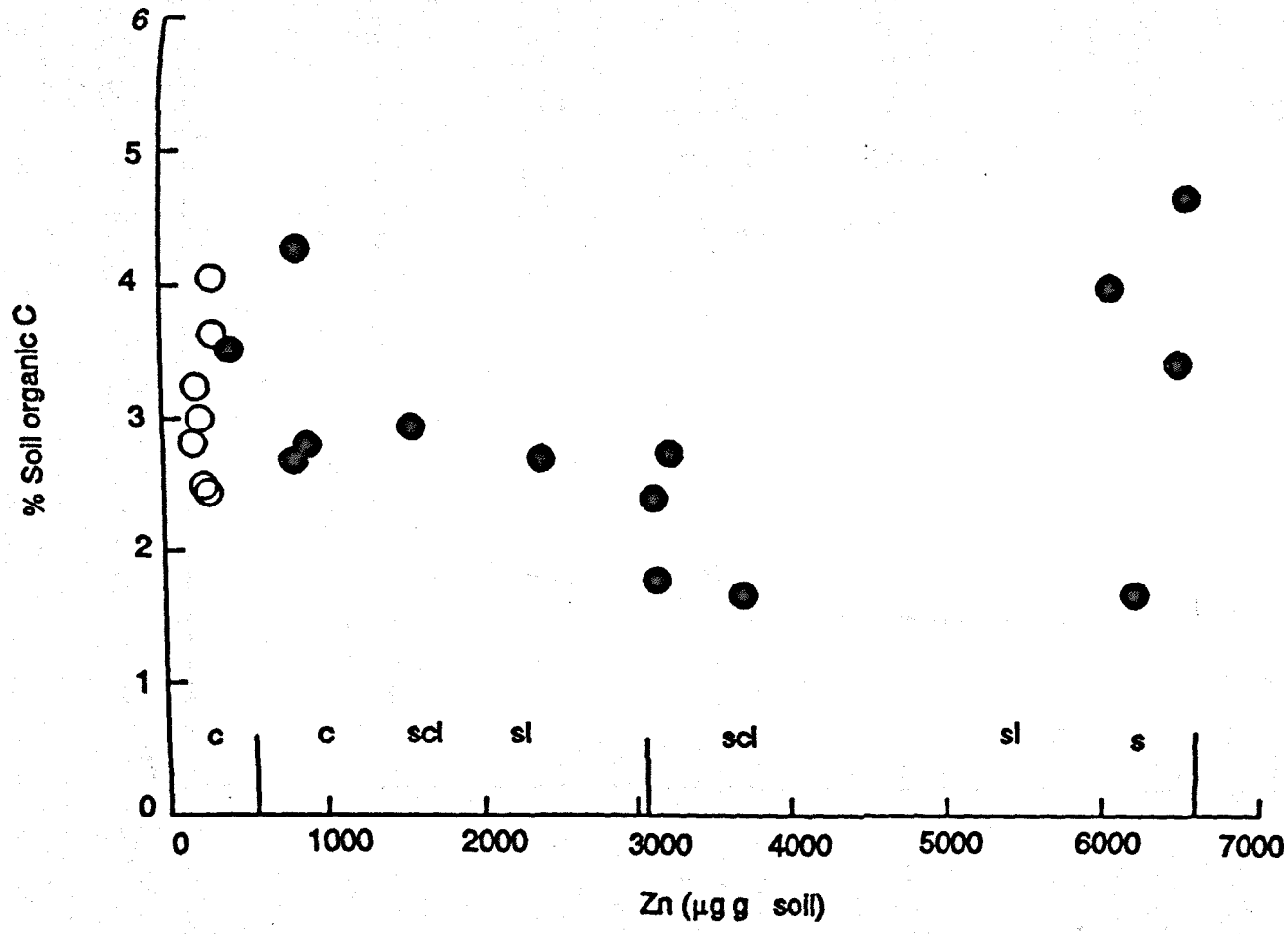

Figure 5-2: Total soil organic C and soil texture (s=sandy, sl=sandy loam, scl=sandy clay loam and c=clay) in low- (○) and high-metal (●) soils from Gipuzkoa, Spain.

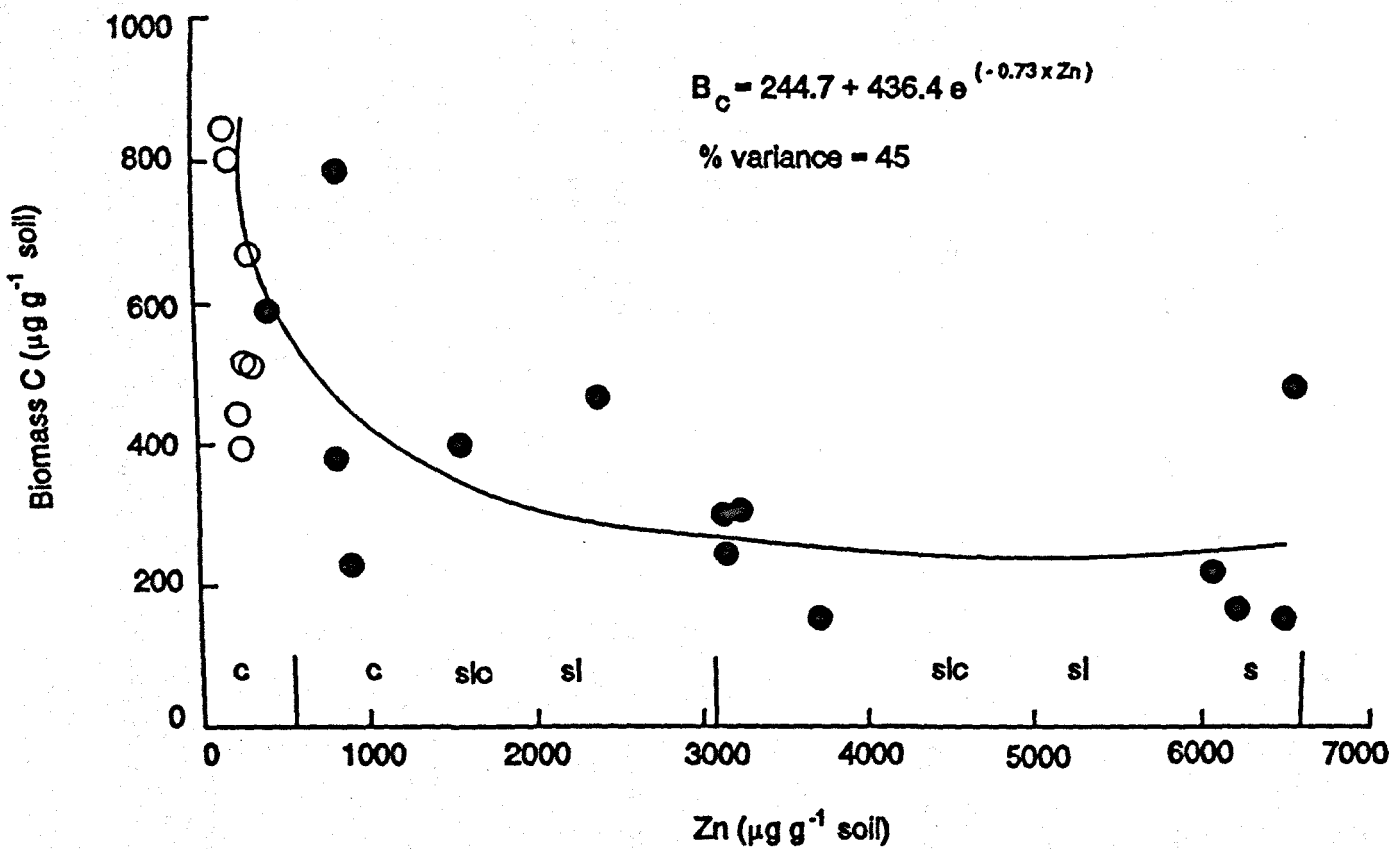

Figure 5-3: Soil microbial biomass C and soil texture (s=sandy, sl=sandy loam, slc=sandy clay loam, c=clay) in low- (O) and high-metal (●) soils from Gipuzkoa, Spain.

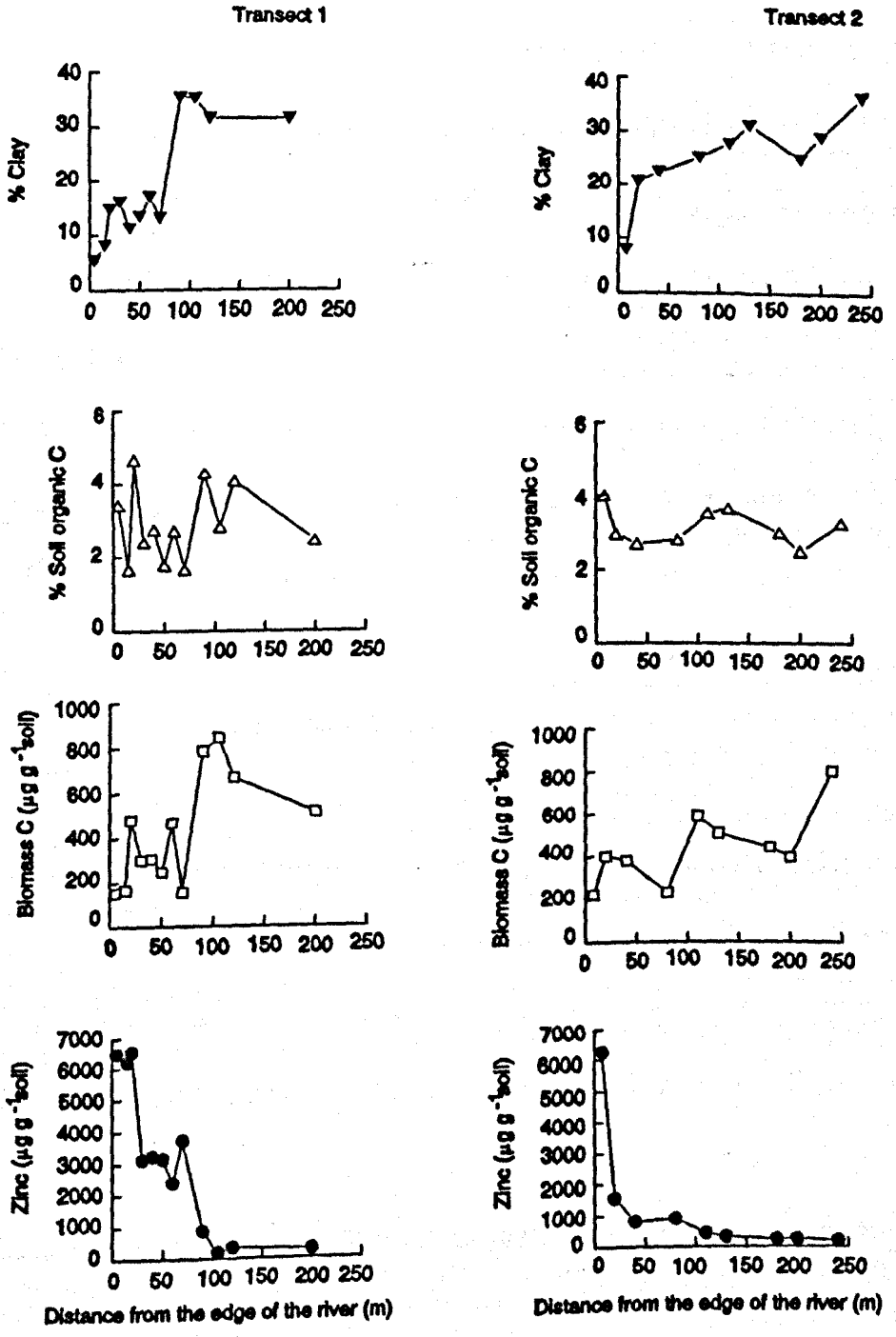

Figure 5-4: Biomass C, soil organic C, clay content and Zn concentrations in soils from transect 1 and 2 along a distance from the edge from the river in Gipuzkoa, Spain.

sandy clay loam textures), but at 80m there was grassland soil near to a road.

Multiple regression analysis show that biomass C was most significantly correlated to clay content (r=0.854) rather than soil organic C (r=0.475) or Zn concentration (r=-0.638) (Table 5-4). Paired correlations between biomass C and soil parameters such as clay + Zn or Clay + soil organic C accounted for correlations of 0.88 which were very close to the correlation between biomass C and clay (0.85) (Table 5-5).

The amounts of biomass C (B_C) and biomass ninhydrin-N (B_{NIN}) were significantly linearly correlated (r=0.97, $p<0.05$), combining the data from all the transects (Figure 5-5). The overall regression equation was $B_C=(21.7 \pm 1.3)B_{NIN}$.

5.3.4 SOIL MICROBIAL BIOMASS C AND ATP

Soil ATP, as soil microbial biomass C, was also positively correlated with soil clay content (r=0.719), soil organic C (r=0.590) and negatively correlated with Zn concentration (r=-0.452) (Table 5-4) and the trend along a gradient of Zn concentration was similar to biomass C.

The correlation between ATP and biomass C was significant (r=0.88, $p<0.05$). The regression equation between biomass C and soil ATP was: $B_C = (83.1 \pm 9.7)$ ATP (Figure 5-6). Thus the biomass in the three transects in the low and high-metal soils had the same ATP concentration (Table 5-6). The biomass ATP concentration, averaged over the soils from both transects was 10.7 μmol ATP g^{-1} B_C.

Table 5-4: Correlation matrix for soil parameters compared by linear regression.

	% Soil organic C	% clay	Zn
% Soil organic C	-	0.261[ns]	0.016[ns]
% Clay	-	-	-0.887***
Biomass C	0.475**[a]	0.854***	-0.638***
Biomass nin-N	0.452**	0.784***	-0.538**
SIR[b]	0.270[ns]	0.873***	-0.622**
ATP	0.590**	0.719***	-0.452**
Ergosterol	0.789***	0.230[ns]	0.300[ns]
AAR[c]	0.538**	0.698***	-0.409*
CO_2-C evolved	0.519**	-0.349[ns]	0.691***
DMSO[d]	0.687***	0.746***	-0.540**

[ns] = not significant

[a] = values marked with ***, **, * are significant at 0.001, 0.05 and 0.1 probability levels respectively

[b] = Substrate induced respiration

[c] = Arginine ammonification rate

[d] = Dimethyl sulphoxide reduction rate

Table 5-5: The best pair of multiple linear regression between microbial biomass and microbial activity with soil parameters.

Multiple linear regression	
Biomass C = -146.20 + 69.75(%soil organic C)+16.96(%clay)	r=0.88
Biomass C = 189.83 + 125.43(%soil organic C) - 0.591(Zn)	r=0.77
Biomass C = -322.37 + 29.21(%clay) + 0.0515(Zn)	r=0.88
Biomass nin-N = - 8.27 + 2.54(%soil organic C)+0.69(%clay)	r=-0.84
Biomass nin-N = 5.66 + 5.82(%soil organic C) - 0.02(Zn)	r=0.73
Biomass nin-N = - 17.39 + 1.33(%clay) + 0.026(Zn)	r=-0.82
SIR[a] = -36.69 + 21.36(%soil organic C) + 12.22(%clay)	r=-0.88
SIR = 199.34 + 61.37(%soil organic C) - 0.397(Zn)	r=0.70
SIR = -247 + 20.83(%clay) + 0.387(Zn)	r=-0.92
ATP = -2.52 + 1.26(%soil organic C) + 0.143(%clay)	r=-0.84
ATP = 0.310 + 1.73(%soil organic C) - 0.005(Zn)	r=0.77
ATP = -4.34 + 0.324(%clay) + 0.007(Zn)	r=-0.79
Ergosterol = - 6.14 + 0.26(%clay) + 0.011(Zn)	r=0.78
AAR[b] = -2.46 + 1.08(%soil organic C) + 0.13(%clay)	r=-0.80
AAR = 0.155 + 1.51(%soil organic C) - 0.004(Zn)	r=0.70
AAR = -5.06 + 0.32(%clay) + 0.008(Zn)	r=-0.79
CO_2-C evolved = 67.70+77.31(%soil organic C) - 5.41(%clay)	r=0.77
CO_2-C evolved = -57.03 + 59.14(%soil organic C) + 0.27(Zn)	r=-0.88
CO_2-C evolved = -170.95 + 9.60(%clay) + 0.63(Zn)	r=-0.84
DMSO[c] = -101.73 + 77.00(%soil organic C)+8.40(%clay)	r=-0.88
DMSO = 89.21 + 105.74(%soil organic C) - 0.37(Zn)	r=0.89
DMSO = -91.35 + 15.64(%clay) + 0.24(Zn)	r=0.74

[a] Substrate induced respiration

[b] Arginine ammonification rate

[c] Dimethyl sulphoxide reduction rate

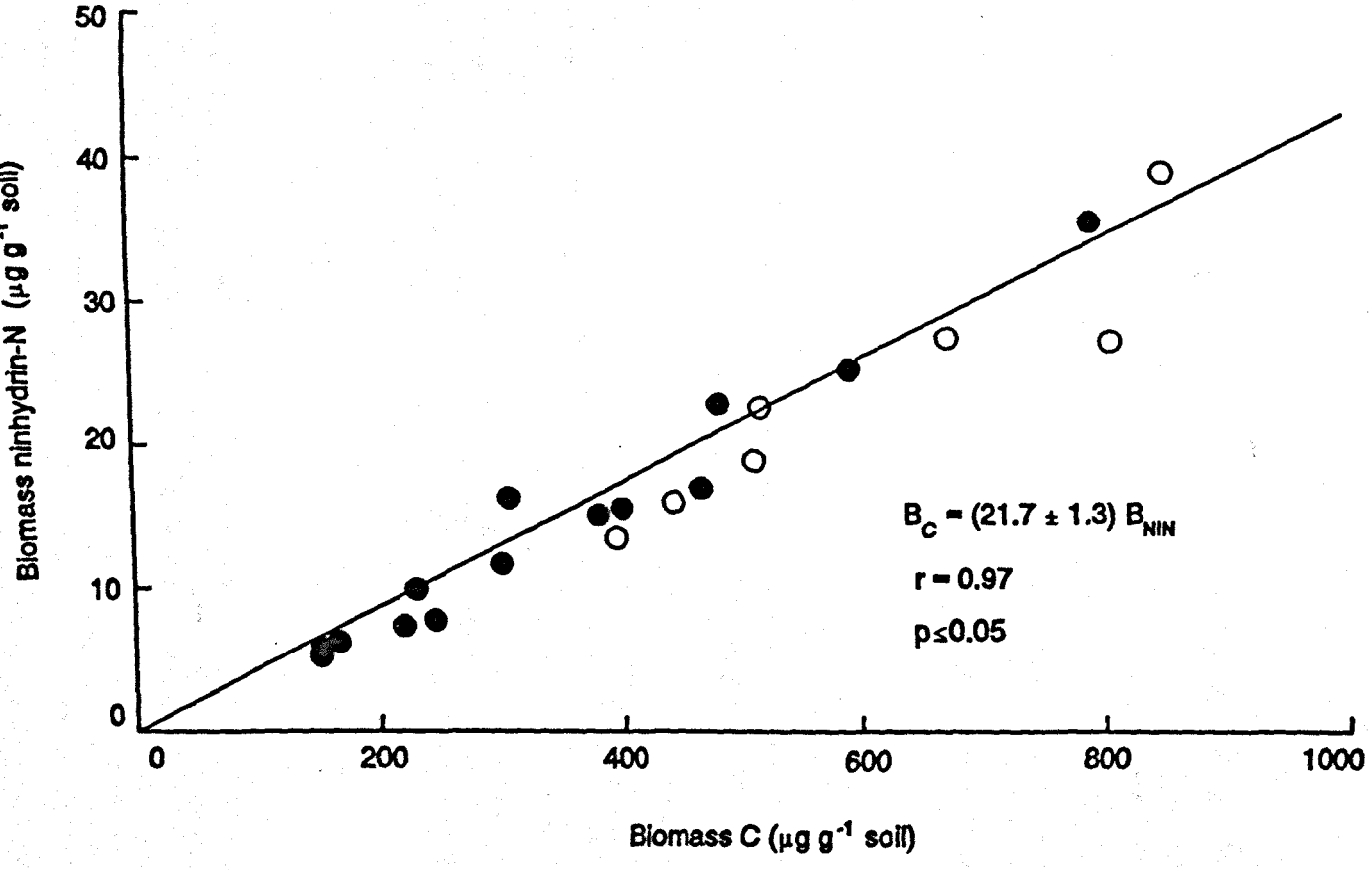

Figure 5-5: The correlation between microbial biomass C and biomass ninhydrin-N in low- (○) and high-metal(●) soils from Gipuzkoa, Spain

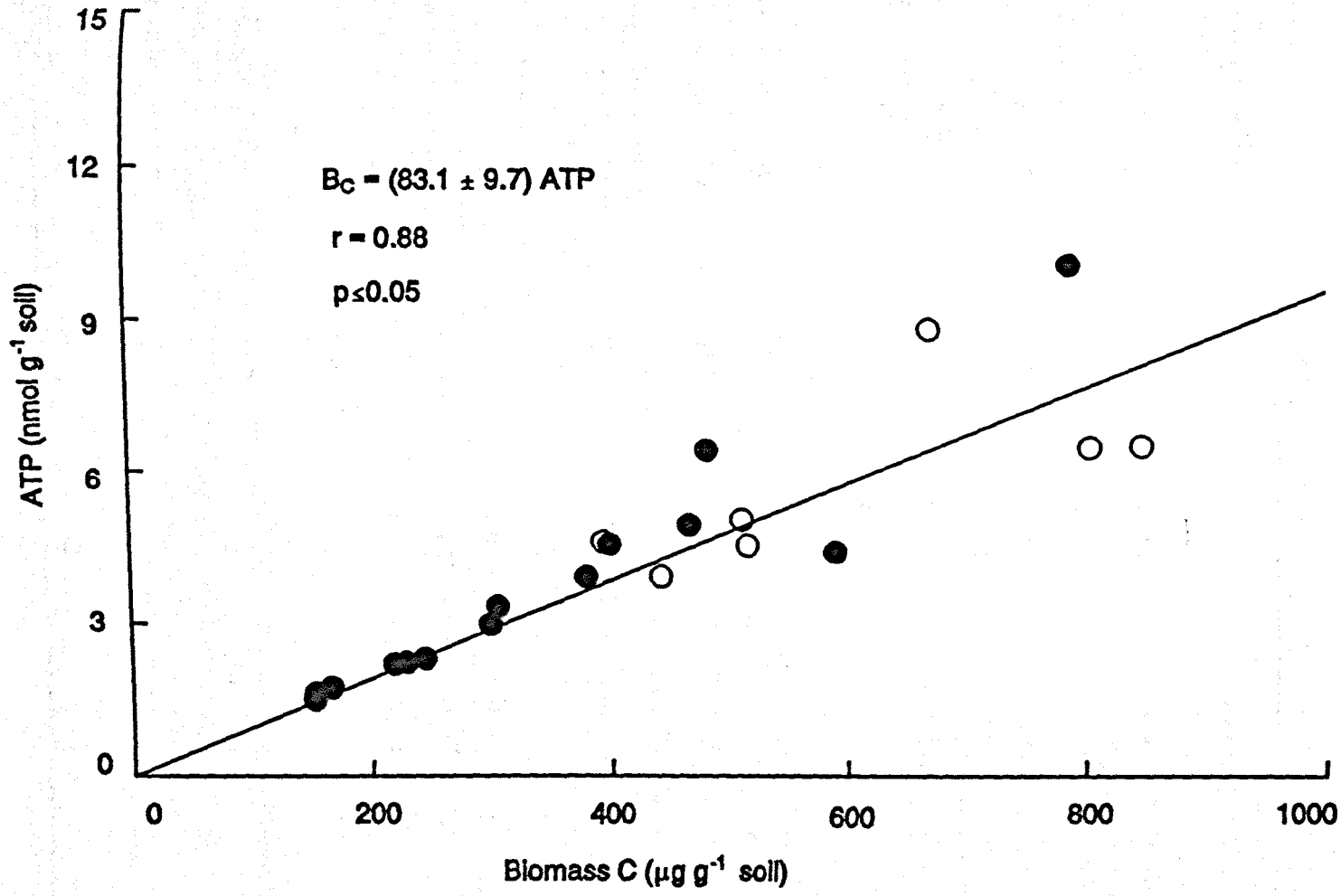

Figure 5-6: The correlation between microbial biomass C and ATP in low- (O) and high-metal (●) soils from Gipuzkoa, Spain.

Table 5-6: Soil ATP content, biomass ATP concentration and Zn in soil along the transect from the river in soils from Gipuzkoa, Spain.

Sample	Distance	Zn	ATP	ATP/B_C[a]
	(m)	($\mu g\ g^{-1}$ soil)	(nmol g^{-1} soil)	($\mu mol\ g^{-1}\ B_C$)
Transect 1				
1	5	6488	1.51	10.12
2	15	6208	1.74	10.43
3	20	6551	6.42	11.00
4	30	3132	2.98	9.90
5	40	3237	3.33	14.60
6	50	3150	2.31	9.40
7	60	2390	4.94	12.55
8	70	3709	1.64	10.64
9	90	859	10.08	11.64
10	105	188	6.50	7.68
11	120	332	8.81	12.31
12	200	286	4.53	8.78
Transect 2				
1	8	6265	2.20	9.57
2	20	1570	4.55	10.48
3	40	823	3.91	12.20
4	80	908	2.23	9.12
5	110	437	4.40	10.63
6	130	334	5.04	12.14
7	180	243	3.92	10.81
8	200	258	4.60	9.93
9	240	217	6.47	11.30

[a] Biomass ATP concentration

5.3.5 SOIL MICROBIAL BIOMASS C AND SUBSTRATE INDUCED RESPIRATION

Soil microbial biomass by SIR was affected as was biomass C by clay content ($r=0.873$) and Zn concentration ($r=-0.622$) but not by soil organic C (Table 5-4). The trend of SIR along a gradient of Zn concentration was similar to that of biomass C, as SIR declined with increasing Zn content.

Biomass C measured by fumigation-extraction and substrate induced respiration (SIR) were significantly correlated ($r=0.93$, $p<0.05$). The linear regression equation was $B_C=(1.41\pm0.12)$SIR and the intercept was not significantly different from zero (Figure 5-7).

5.3.6 SOIL MICROBIAL BIOMASS C AND ERGOSTEROL

Soil ergosterol content was measured in only transect 1. However, it was measured from this transect on two occasions, in November 1992 (transect 1A) and March 1993 (transect 1B) as discussed in section 5.2.2. Because soil ergosterol was so different on these two occasions the results have not been pooled, but treated separately. There was statistically significant correlation between ergosterol and soil organic C ($p<0.05$) but not with biomass C, %clay and Zn content (Figure 5-10, Table 5-4). Figure 5-8 shows ergosterol content and Zn concentration along the transect from the edge of the river. The amounts of ergosterol in soils from transect 1A and 1B were very different, while the biomass C content was similar (Figure 5-9). The biomass C ratio (biomass C in transect 1A/biomass C in transect 1B) in every soil sample from transect 1A and 1B with similar concentration of Zn and soil texture ranged between 0.9 - 1.6 with a mean of 1.3, while ergosterol ratios were more

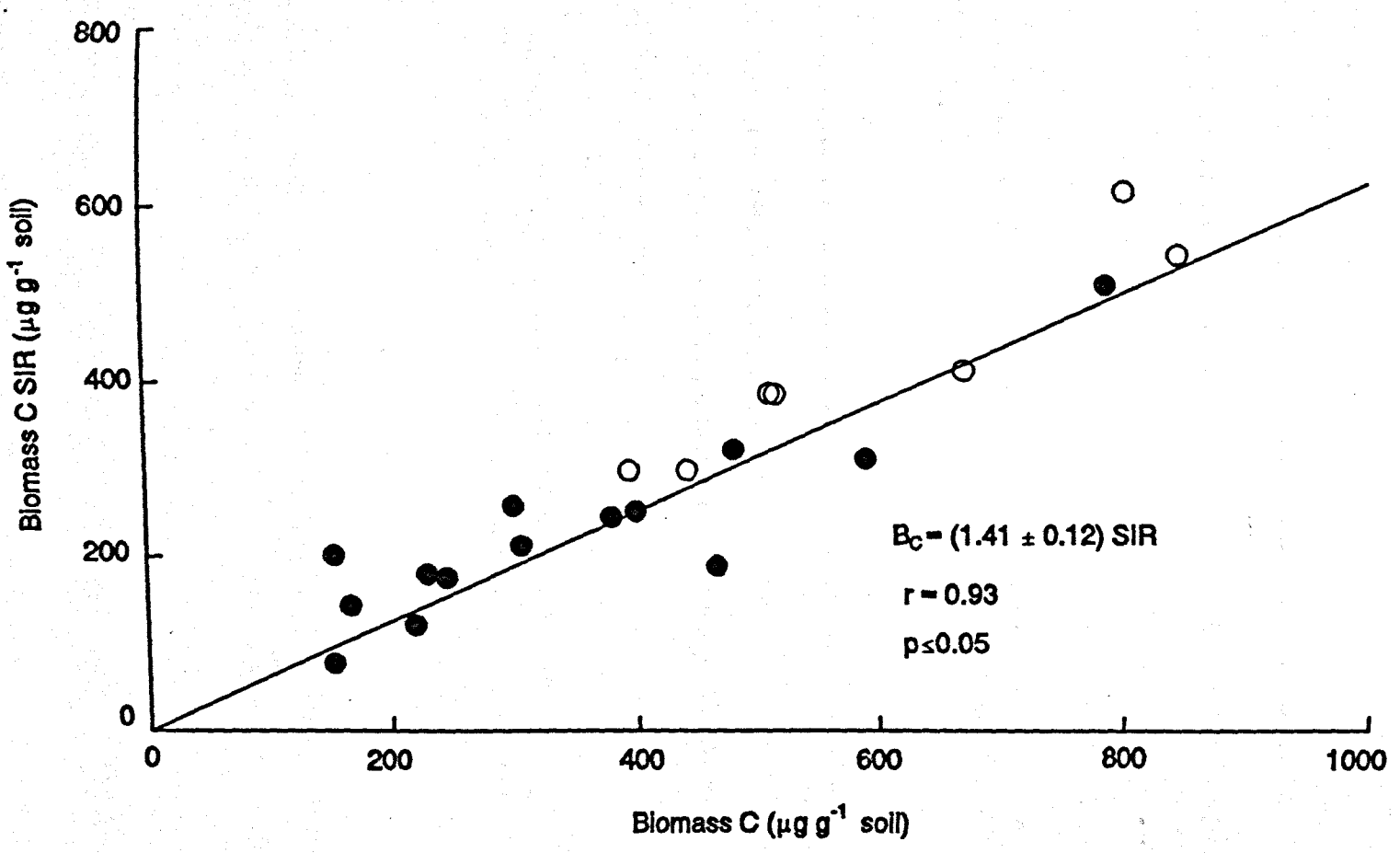

Figure 5-7: The correlation between microbial biomass C measured by fumigation-extraction and SIR in low- (○) and high- metal (●) soils from Gipuzkoa, Spain.

Table 5-7: Ergosterol and biomass C ratios in soils from transect 1A and 1B with similar texture and Zn concentration in soils from Gipuzkoa, Spain.

Zn[a] ($\mu g\ g^{-1}$ soil)	Texture[b]	Ergosterol ratio[c]	Biomass C ratio[d]
6488 - 6208	s , s	5.36	0.92
6551 - 3132	sl , s	5.60	1.60
3237 - 3150	sl , sl	6.45	1.25
2390 - 3707	scl , sl	3.13	1.55
859 - 188	c , c	2.54	0.93
332 - 286	c , c	4.02	1.30

[a] Zn concentration transect 1A - transect 1B

[b] Soil texture transect 1A , transect 1B : s=sandy, sl= sandy loam, scl= sandy clay loam, c= clay

[c] [Ergosterol] transect 1A/[ergosterol] transect 1B

[d] [Biomass C] transect 1A/[biomass C] transect 1B

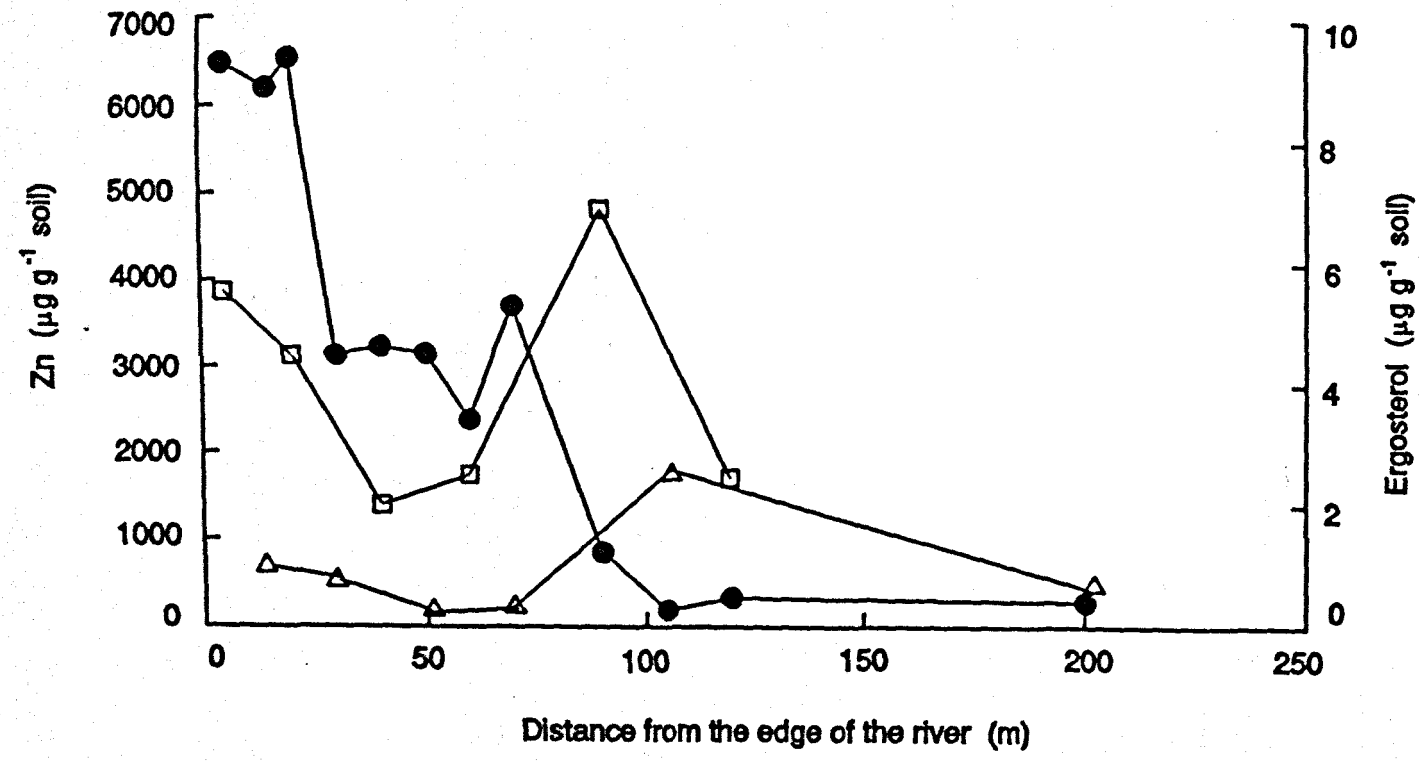

Figure 5-8: Soil ergosterol content in transect 1A (□) and transect 1B (▵) and Zn concentration (●) along a distance from the edge of the river in Gipuzkoa, Spain.

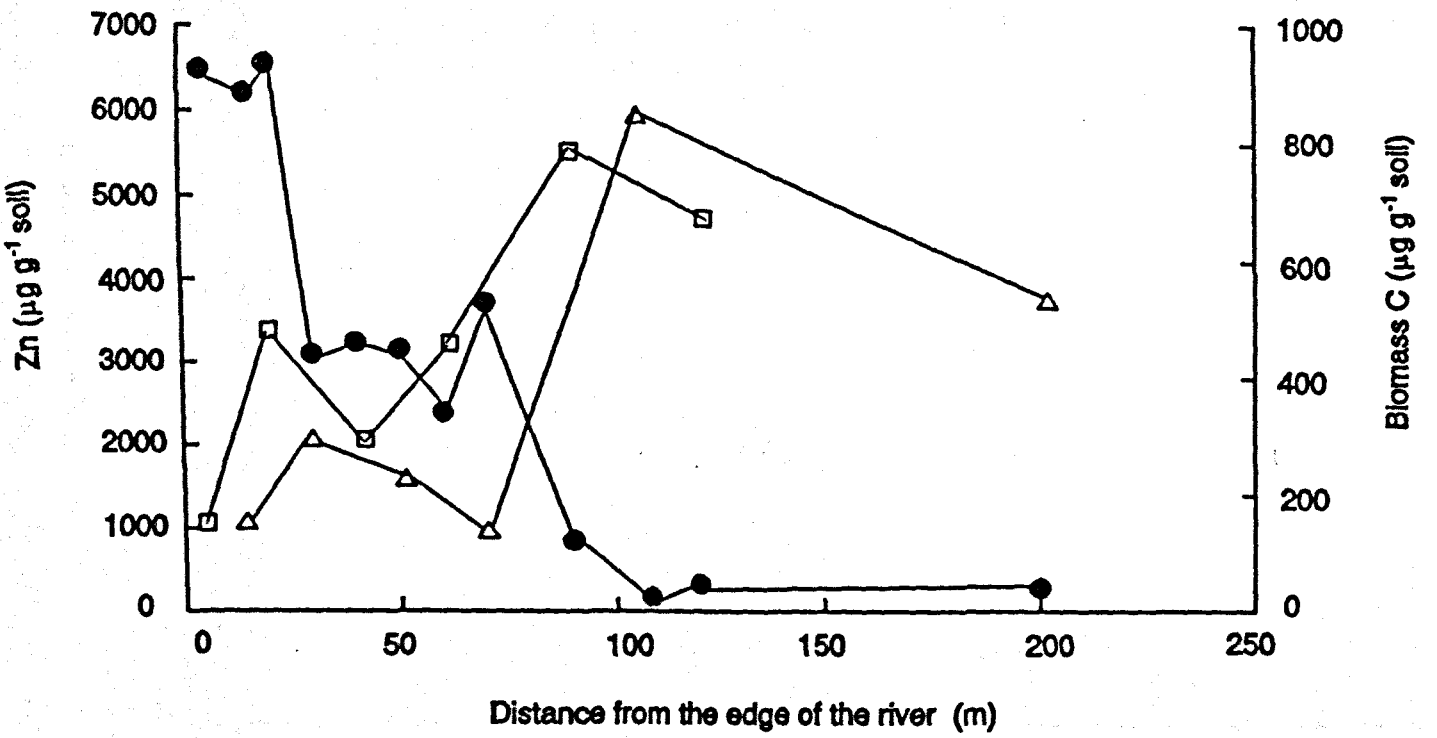

Figure 5-9: Soil biomass C in transect 1A (□) and 1B (▵) and Zn concentration (●) along a distance from the edge of the river in Gipuzkoa, Spain.

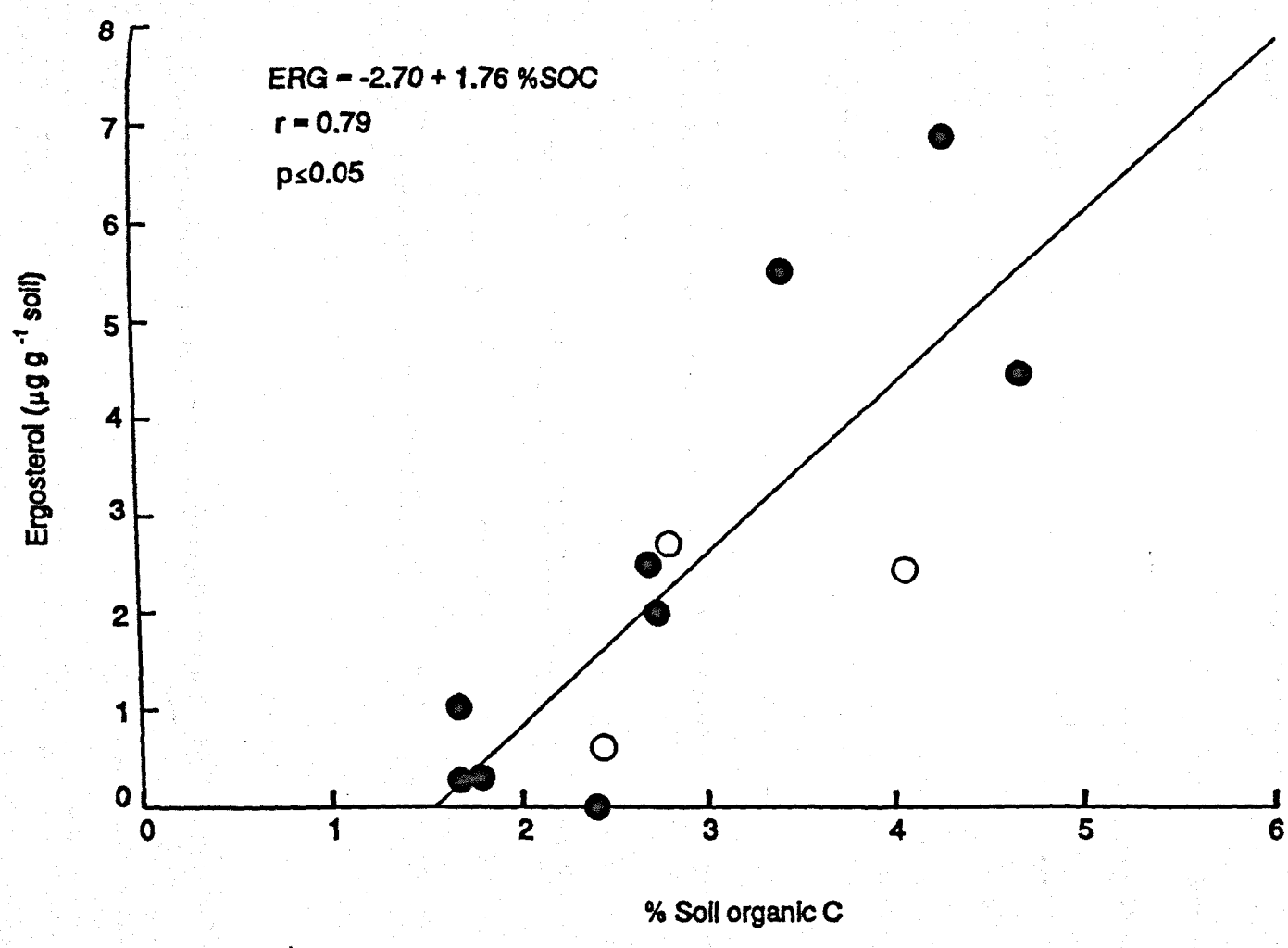

Figure 5-10: The correlation between ergosterol (ERG) and % soil organic C in low- (○) and high-metal (●) soils from Gipuzkoa, Spain.

variable, range 2.5 - 6.5, mean 4.5 (Table 5-7).

Transect 1A was sampled in November 1992, following heavy rain. Samples were kept at room temperature for 24h after sampling, sieved (<2mm) and then stored at 4°C for 18 weeks before the ergosterol analysis. Soils from transect 1B were collected at the beginning of March 1993, the temperature (10°C) was similar to that in November 1992 (10°C) but the weather was drier. After sampling the soils were stored for 8d at room temperature, then sieved and stored during 4 weeks at 4°C before the ergosterol analysis.

5.3.7 SOIL MICROBIAL BIOMASS C AND CO_2-C EVOLVED

Carbon dioxide evolved and biomass C were not significantly correlated (Figure 5-11). About twice as much CO_2-C evolved was measured from the very high-metal soils (Zn concentration ranged from 6208 - 6551 $\mu g\ g^{-1}$ soil), than in low-metal soils (188 - 334 $\mu g\ g^{-1}$ soil) (Figure 5-12). Although the mean of % soil organic C (3.44%) in the very high-metal soils was similar to that in low-metal soils (3.10 % organic C) as in section 5.3.2 (Figure 5-2); biomass C was 57% lower in the very high-metal soils compared to low-metal soils (Figure 5-4). These results may suggest a higher rate of utilization of organic C, and thus a faster rate of mineralization, produced by a smaller size of biomass C. Figure 5-13 shows that the basal respiration is derived from the available organic material in soil and it is not related to the size of microbial biomass. Multiple regression analysis showed that CO_2-C evolved was significantly correlated to Zn at $p<0.05$ ($r=0.691$) and soil organic C ($r=0.519$) and not significantly correlated to clay content or biomass C (Table 5-4).

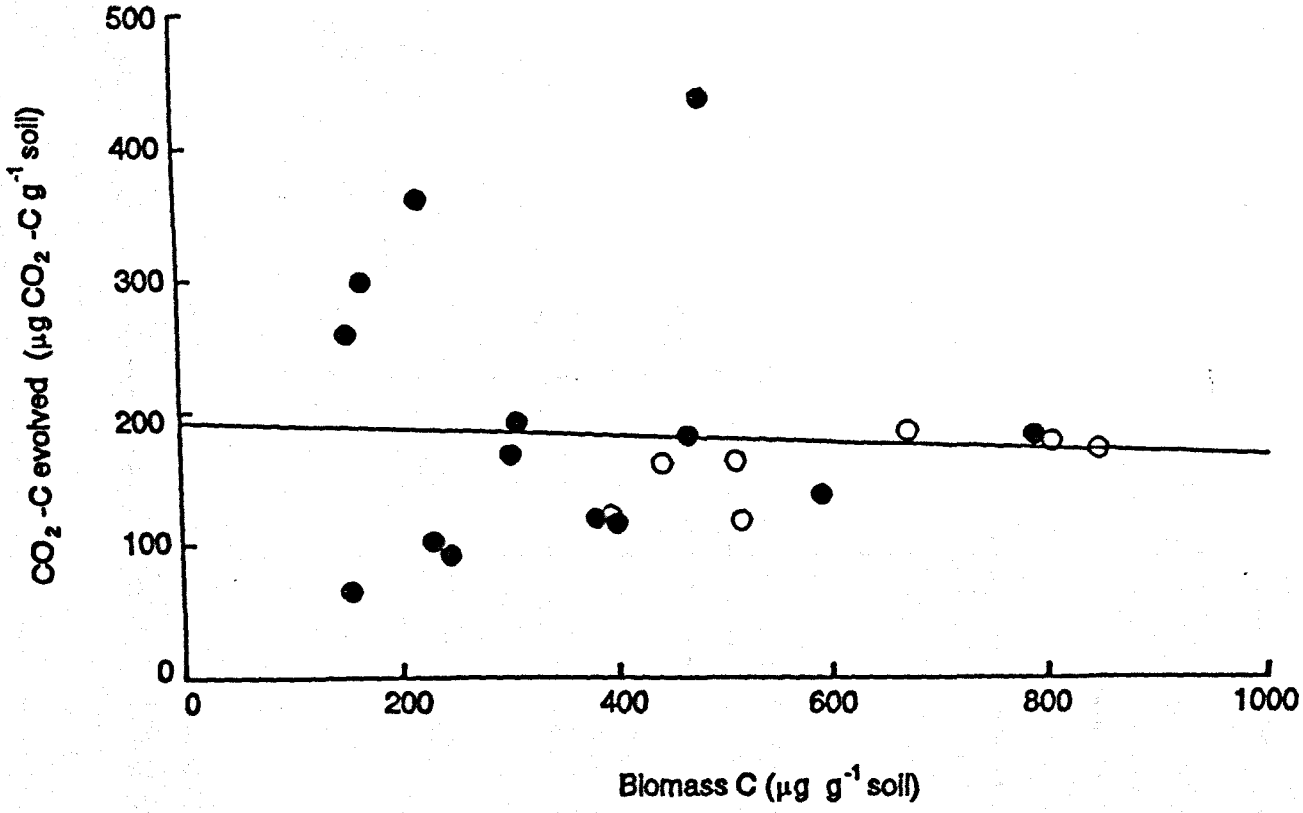

Figure 5-11: The correlation between microbial biomass C and CO_2-C evolved in low- (○) and high-metal (●) soils from Gipuzkoa, Spain.

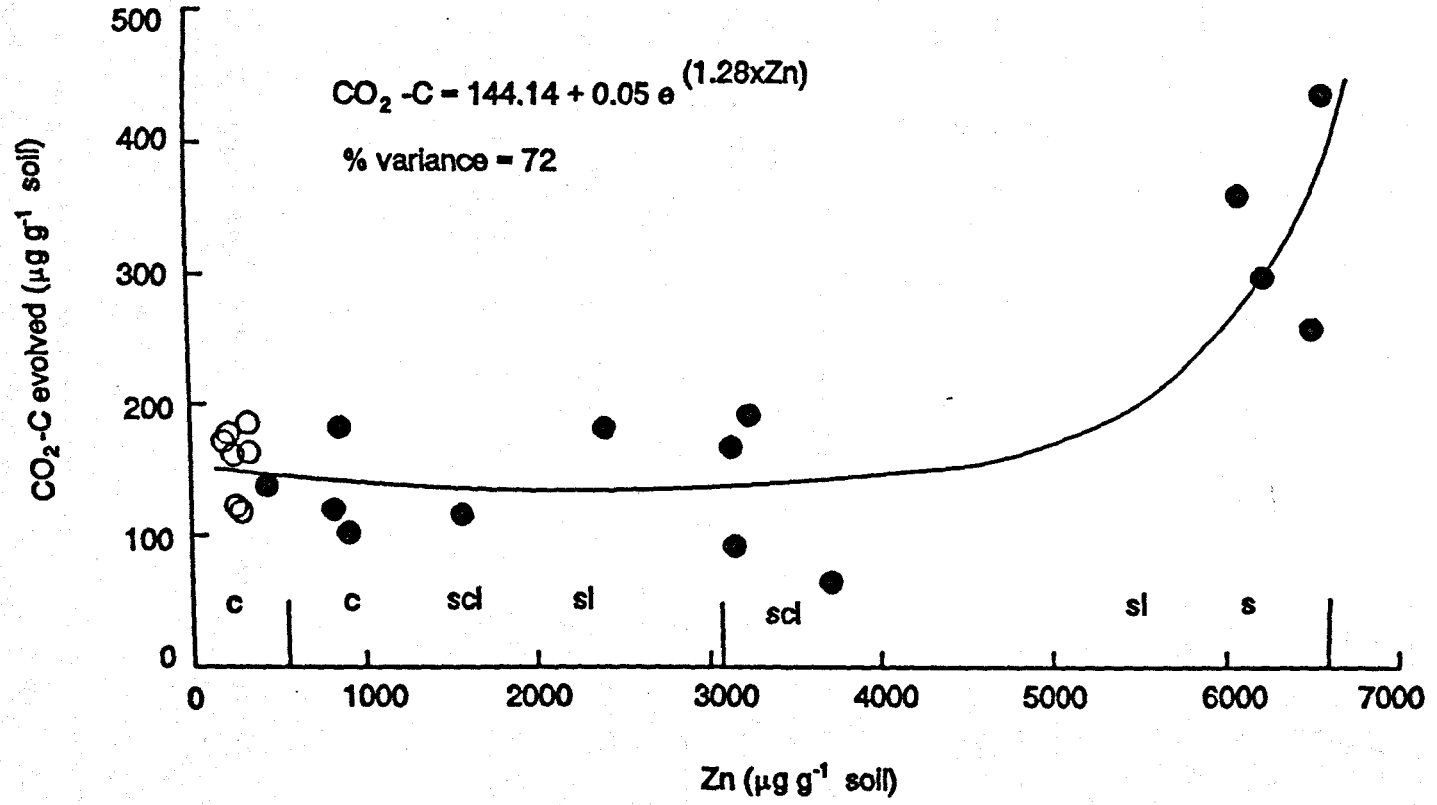

Figure 5-12: Carbon dioxide evolved, soil texture (s=sandy, sl=sandy loam, scl=sandy clay loam and c=clay) and low (○) and high (●) Zn concentration in soils from Gipuzkoa, Spain.

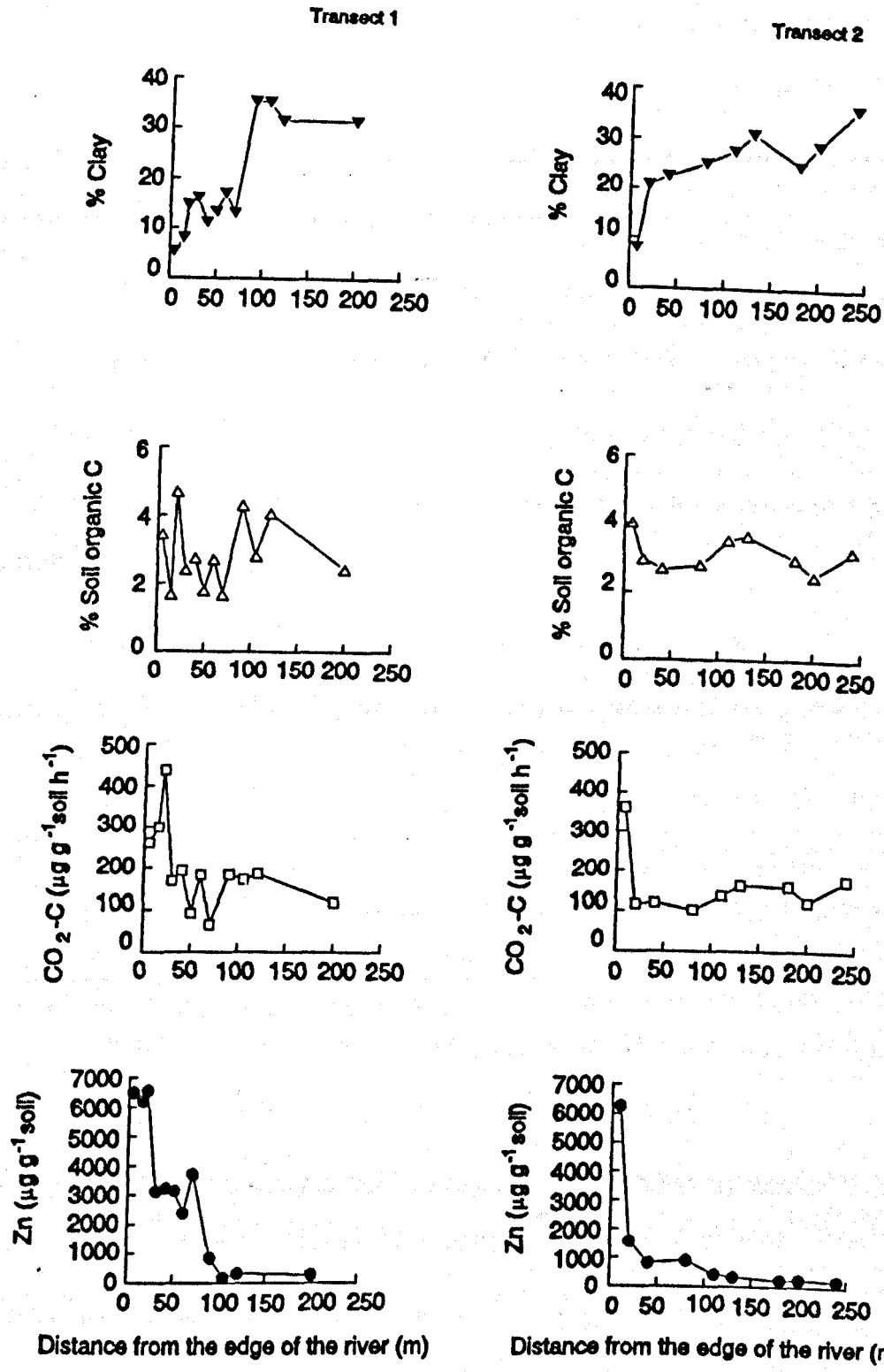

Figure 5-13: Soil respiration, soil organic C, clay and Zn content in soils from transect 1 and 2 along a distance from the edge of the river in Gipuzkoa, Spain.

5.3.8 SOIL MICROBIAL BIOMASS C AND ARGININE AMMONIFICATION RATE

Microbial biomass C, arginine ammonification rate, %clay content and Zn concentration (Table 5-8) were analysed by multiple linear regression. The rates of arginine ammonification and soil biomass C contents were both significantly correlated at $p<0.05$ to clay content ($r=0.698$) and soil organic C ($r=0.538$) and less strongly but significantly correlated to Zn concentration ($r=-0.409$) (Table 5-4). Arginine ammonification rate decreased with increasing Zn concentration and the trend was similar to biomass C.

Biomass C and arginine ammonification rate (AAR) were significantly correlated ($r=0.90$, $p<0.05$) (Figure 5-14). The intercept was significantly different from zero ($B_C = (104 \pm 41.2) + (89.2 \pm 9.7)$ AAR).

5.3.9 SOIL MICROBIAL BIOMASS C AND DIMETHYL SULPHOXIDE REDUCTION

The reduction rate of dimethyl sulphoxide (DMSO) was also affected by the clay content ($r=0.746$), soil organic C ($r=0.687$) and Zn concentration ($r=0.540$) (Table 5-4), and the trend along a gradient of Zn concentration was similar to that of biomass C.

The amount of biomass C (B_C) and dimethyl sulphoxide reduction rate (DMSO) in all the soils from transect 1A and 1B (DMSO was not determined in transect 2) were significantly correlated ($r=0.92$, $p<0.05$). The regression analyses gave the linear relationship: $B_C=(1.45\pm0.19)$DMSO (Figure 5-15). The linear

Table 5-8: Microbial biomass C and arginine ammonification rate along a gradient concentration of Zn in soils from Gipuzkoa, Spain.

Sample	Distance	Zn	Biomass C	Arginine[a]	Clay
	(m)	($\mu g\ g^{-1}$ soil)		($\mu g\ g^{-1}$ soil h^{-1})	%
Transect 1					
1	5	6488	153	1.1	5.4
2	15	6208	167	1.4	8.1
3	20	6551	483	5.5	14.7
4	30	3132	301	2.7	16.0
5	40	3237	307	3.3	11.2
6	50	3150	245	1.5	13.3
7	60	2390	467	4.5	17.0
8	70	3709	154	1.4	13.1
9	90	859	789	9.8	35.3
10	105	188	846	6.0	35.2
11	120	332	670	6.0	31.5
12	200	286	517	3.7	31.5
Transect 2					
1	8	6265	220	1.5	8.0
2	20	1570	400	2.7	20.6
3	40	823	380	2.5	22.4
4	80	908	230	1.9	25.1
5	110	437	591	4.7	27.7
6	130	334	512	3.6	31.1
7	180	243	444	3.1	24.8
8	200	258	395	3.9	29.0
9	240	217	803	6.5	36.2

[a] Arginine ammonification rate

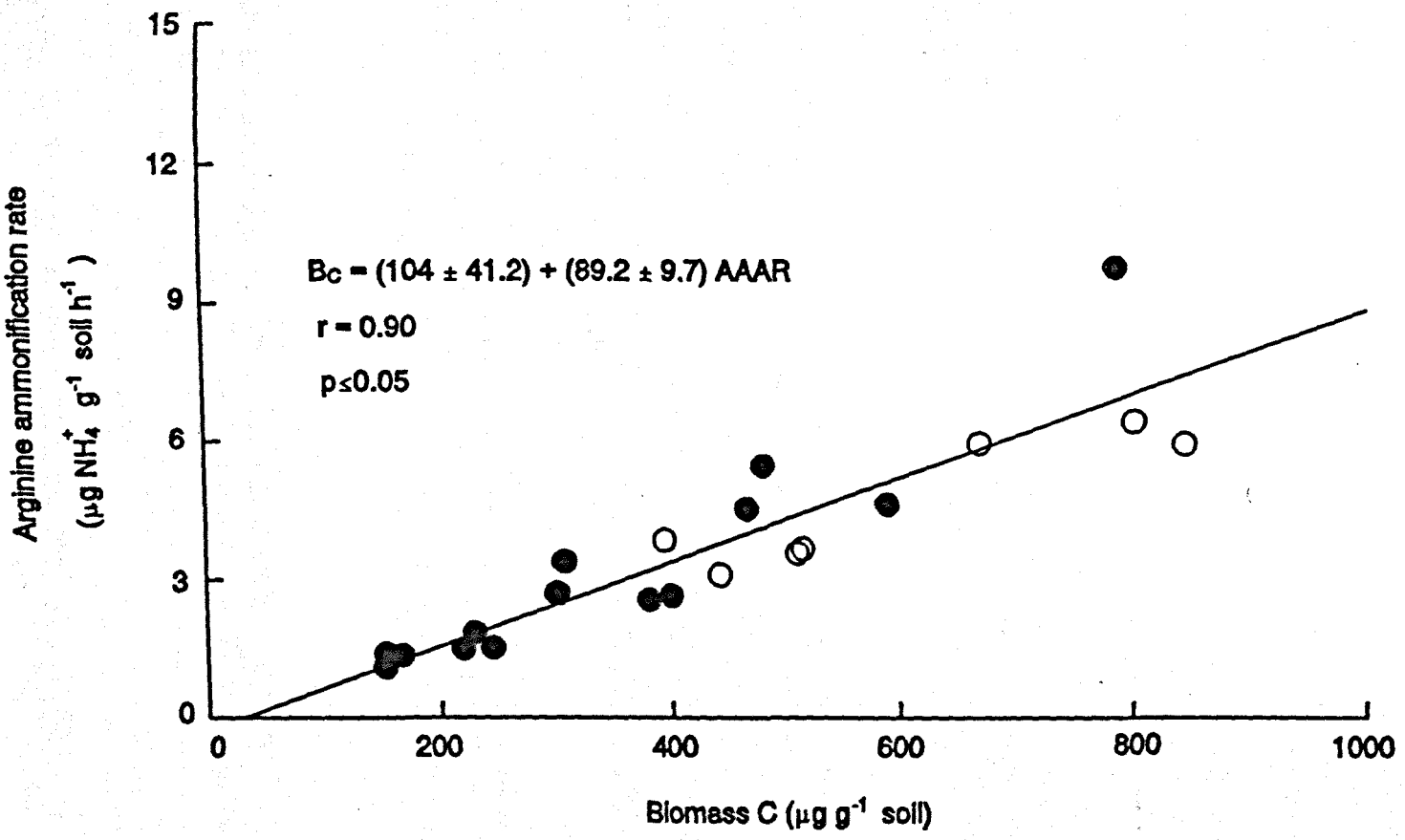

Figure 5-14: The correlation between microbial biomass C and arginine ammonification rate (AAR) in low- (○) and high-metal (●) soils from Gipuzkoa, Spain.

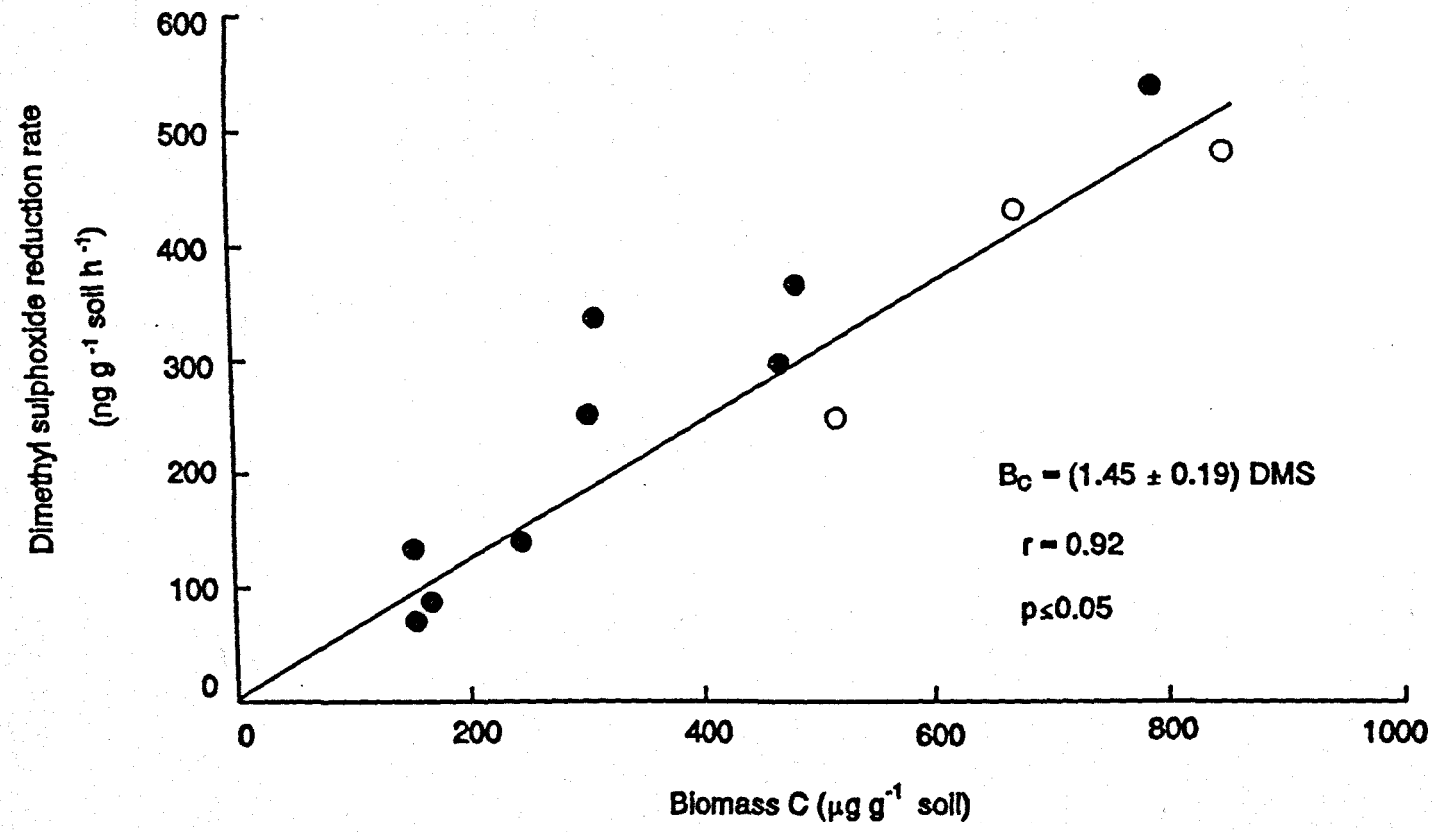

Figure 5-15: The correlation between microbial biomass C and dimethyl sulphoxide reduction rate (DMSO) in low- (○) and high-metal (●) soils from Gipuzkoa, Spain.

Table 5-9: Soil microbial biomass measured by SIR and dimethyl sulphoxide reduction rate along a gradient of Zn in soils from Gipuzkoa, Spain.

Sample	Distance	Zn	SIR[a]	DMSO[b]
	(m)	($\mu g\ g^{-1}$ soil)		($ng\ g^{-1}$ soil h^{-1})
Transect 1				
1	5	6488	78	134
2	15	6208	144	88
3	20	6551	323	367
4	30	3132	258	253
5	40	3237	212	337
6	50	3150	176	141
7	60	3390	190	298
8	70	3709	202	71
9	90	859	512	542
10	105	188	547	485
11	120	332	414	433
12	200	286	387	250

[a] Substrate induced respiration

[b] Dimethyl suphoxide reduction rate

correlation between SIR and DMSO was also significant (SIR=0.814 DMSO + 56.49, r=0.85, $p<0.05$) (Table 5-9).

5.4 DISCUSSION

Generally, the Ni and Cu (except one soil sample: transect 1, sample 2) concentrations in the soils were lower than the maximum currently permitted EU limits and Zn, Cd and Pb were up to 21, 3 and 9 times EU upper limits respectively. Similar heavy metal concentrations of soils sampled in this area were reported by Ansorena and Marino (1990). In their work, concentrations of heavy metals such as Zn, Pb and Cd were determined in several agricultural and non-agricultural soils collected from a gradient going through areas near the mine until the urban zone in the deep valley of River Oyarzun. Soil metal concentrations ranged from 140 to 9000 μg Zn, 70 to 11000 μg Pb and 1.1 to 20 μg Cd g^{-1} soil.

The high % soil organic C and litter accumulation in the sandy soils in the zones with the highest concentration of heavy metal suggest the litter and organic matter decomposition were affected. Similar reports of organic matter accumulation in sites close to smelter operations, in woodlands and in forest have been published (Coughtrey *et al.*, 1979; Freedman and Hutchinson, 1980; Strojan, 1978; Tyler, 1981). Coughtrey *et al.*, (1979) showed that Zn and Cd exerted a strong influence on litter decomposition and there appeared to be little influence of Pb, Cu or pH on litter decomposition in woodland contaminated by Pb, Zn, Cd and Cu.

In this work, soil Zn and Cd concentrations were significantly correlated (section 5.3.1). Thus it is not possible to analyse statistically which of the metals

could have separate influences on the accumulation of soil organic C in sandy soils that had the highest concentration of Cd and Zn (Table 5-1, 5-2).

5.4.1 SOIL MICROBIAL BIOMASS AND MICROBIAL ACTIVITY

The multiple regression analysis results suggest that clay content is an important factor in the stabilization of soil organic C and biomass C. This suggestion is in agreement with Jenkinson (1977), Sørensen (1983), Van Veen *et al.*, (1985, 1987).

Paired correlations demonstrate that a range between 78.9 to 91.9% of the total variation of biomass C can be accounted for by including clay + Zn (79.6 to 87%) or clay + soil organic C (70 to 87%) (Table 5-5), which was very close to the correlation between microbial biomass and clay (range from r=0.70 to 0.87). These results show that clay is a more important factor in the variation of biomass C than Zn concentration (r=0.41 to 0.64). This probably also means that clay content in high-metal soils affects the toxicity of Zn to the biomass C. For example, the amount of biomass C in the soil with 6551 μg Zn g^{-1} soil and 14.7 % clay is higher than the other soils with similar Zn concentration (6208 - 6488 μg g^{-1} soil) but lower clay content (5.4 to 8.1%) (Figure 5-3, Tables 5-1, 5-3). Kiekens (1990), reported that clay and soil organic C are known to adsorb Zn quite strongly especially at alkaline soil pH values as discussed in Chapter 2.

The proportional factor of the regression equation between biomass C and ninhydrin-N ($B_c = (21.7 \pm 1.3)\ B_{NIN}$) was within the range reported by other authors as discussed in Chapter 4.

The proportional factor between biomass C and SIR (1.41) was similar to those reported by Ocio and Brookes (1990) (1.35) in soils amended and unamended with straw and incubated for 13 and 35d at 25°C. Sparling and West (1988) also reported a similar relationship between the flush of organic C extracted by K_2SO_4 and SIR from 26 mineral soils from arable and pasture sites (SIR = 1.85 E_C + 242). Martens (1987) reported a very close correlation (r=0.98) between biomass C measured by FI and SIR (B_C=1.81 SIR-141.63) in 22 different soils taken from arable, grassland and forest. All these proportionality factors were very similar to the proportionality factor reported in this work, but the intercepts were significantly different from zero and very different from each other.

The ATP concentration in the biomass (10.7 μmol ATP g^{-1} B_C) was similar to those values (11.7 μmol ATP g^{-1} B_C) reported by Jenkinson (1988), who collected literature data from a large number of unamended soils. Chander and Brookes (1991c) reported a value of 12.3 μmol ATP g^{-1} B_C meaned over high- and low-metal soils with and without added substrate. In Chapter 4 (this thesis) in low- and high-metal soils from the Woburn Market Garden Experiment a value of 12.4 μmol ATP g^{-1} B_C was reported. This suggests that the biomass ATP concentration was not decreased in the high-metal soils, although, microbial biomass (biomass C, ninhydrin-N, SIR) and microbial activity (arginine ammonification rate) were considerable reduced as discussed in Chapter 4 and in this Chapter. The energy status of micro-organisms is principally indicated by the ratio between ATP and the other nucleotides (ADP and AMP). The ratio ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]) was defined as the adenylate energy charge (AEC) (Atkinson and Walton, 1967). Brookes and McGrath (1987) found high AEC's (0.8 - 0.9) in both low- and high-metal soil,

again with smaller biomass in the latter. The soil micro-organisms in the high-metal as in low-metal soils may possibly maintain this high ATP concentration and AEC in order to respond rapidly to added substrate or to substrate which becomes available (Brookes *et. al.*, 1983; 1987; Tateno, 1985). Generally, however, the available substrate is in very low concentrations in soils (Lynch, 1982; Paul and Voroney, 1980; Wagner, 1975) so the growth and activity of the biomass is restricted, and the small amount of substrate available is used for maintenance. This suggests that a high AEC and biomass ATP concentration is an advantage in both low- and high-metal soils for the survival of the biomass over long periods (Brookes *et. al.*, 1983).

The proportionality factor between arginine ammonification rate (AAR) and biomass C (104) was close to that (101) obtained from low- and high-metal soils from the Woburn Market Garden Experiment reported in Chapter 4 (section 4.3.3). In contrast, Kaiser *et al.*, (1992), reported a poor correlation between biomass C by FE and arginine ammonification in 25 arable soils with a wide range of textural classes (3 - 42.8 %clay), the soil organic matter content (0.69 - 2.61) and total N (.05 - 0.41). In the soils of this study, the clay content (5.4 - 36.2) and % total N (0.128 - 0.39), were between the range of those soils. Data in this section showed that low- and high-metal soils did not affect the ratio B_C/AAR in soils from the natural environment, as was also the case in the experimental soils from the Woburn experiment.

The correlation between biomass C by SIR and arginine ammonification rate was also significant ($r=0.83$, $p<0.05$) (SIR = 54.45 AAR + 91.92). The correlation coefficient (54.45) was close to that reported by Alef *et al.*, (1988) (SIR = 59.20 AAR + 120.07) using 22 soils of different origins and management

histories. In their work the clay content ranged from 5 - 45 and the % organic matter from 0.88 - 12.79. The %clay content and % organic matter were between those ranges in this work (as mentioned above).

The linear correlation between SIR and dimethyl sulphoxide reduction rate (DMSO) was very similar to that reported by Sparling and Searle (1993) (SIR = 0.72 DMSO + 220, r=0.81) obtained from 45 soils representing a variety of soil types and land uses. They reported that DMSO was more sensitive than SIR to As and Cr. They suggested that the mechanisms of this response may not be entirely biological, as DMSO reduction can be affected by the presence of other oxidizing agents (*e.g.* NO_3^-, SO_3^{2-}, $S_2O_3^{2-}$) (Zinder and Brock, 1978). In this work, the correlation between DMSO and B_C by FE or the SIR methods was consistent and linear over different Zn concentrations and soil types. The extremely close correlations between biomass C and biomass ninhydrin-N, SIR, ATP and microbial activities such as arginine ammonification rate and dimethyl suphoxide reduction rate strongly suggest that all these methods provided a rapid measure of biomass and activities in low- and high-metal soils from this natural environment and that the microbial biomass in metal contaminated soils had the same biomass ninhydrin-N, SIR, ATP, arginine ammonification and DMSO as the biomass C in the uncontaminated soils. However, it was not possible to compare the amount of microbial biomass and microbial activities in high-metal soils from this natural environment with a control soil (low-metal soil), since unfortunately the polluted soils had different properties to the unpolluted or control soils, such as clay contents. This illustrates well the problem of monitoring soil pollution in the environment, rather than under the carefully control conditions of the laboratory or field experiment.

5.4.2 SOIL ERGOSTEROL CONTENT

The significant difference in soil ergosterol content in soils from transect 1A and 1B with similar Zn concentration might be due to the rain increasing the fungal population in transect 1A. This suggestion agrees with Söderström (1979) who reported that the amount of metabolically-active fungal biomass determined with fluorescein diacetate (FDA) was correlated with soil moisture content. Further experiments are necessary to test this hypothesis.

Because of concern that the slightly different storage conditions and storage times may themselves have caused changes in soil ergosterol contents, sample number 5 of transect 1B was also analyzed with the soils of transect 1A. The ergosterol content of soil number 5 was 2.9 $\mu g\ g^{-1}$ soil at the first analysis and 2.7 $\mu g\ g^{-1}$ soil at the second. These similar results suggest that soil ergosterol was not affected by the different storage conditions and also that between batch variation was also acceptable. According to West *et al.*,(1987), ergosterol content in sandy loam and silt loam soils declined slightly during storage for one week at 25°C, from 8.5 to 1.2%. Factors other than a high concentration of Zn affected the amount of ergosterol in soils. Further work is required to determine if soil ergosterol content correlates with biomass C in long-term experimental soils contaminated with heavy metal, where soil heterogeneity to less than in soils from natural environment.

There is currently little information in the relationships between soil ergosterol and microbial biomass contents and whether the ratio [ergosterol]/[biomass] is constant enough for ergosterol to be a useful biomarker. There is some evidence (Lin, PhD Thesis, University of Nottingham, 1994) that ergosterol decomposes quite slowly in soil. For example, the soils in that study were amended with ergosterol in

unfumigated or fumigated soils and then incubated for 10d. The added ergosterol was not degraded in either the unfumigated or fumigated soils during 10d incubation. Similarly, the soils were incubated with and without pesticides. The ergosterol of the fungi killed by pesticides was not decomposed in 25d incubation. In contrast, fungal biomass measured by direct microscopy decreased markedly in pesticide amended soil, which might suggest either that significant quantities of exocellular ergosterol accumulated or there was a change in the [ergosterol]/[biomass C] ratio. It is currently unknown which of these factors operated. Certainly my results, obtained from soils sampled from a gradient of increasing heavy metals from the natural environment, rather than from a field experiment, are difficult to interpret, presumably for previously stated reasons.

5.4.3 THE CO_2-C EVOLVED

The lack of significant correlation between CO_2-C evolved and biomass C agree with Kaiser *et al.*, (1992), Shen *et al.*, (1987), Sparling and Ross, (1993) but the data in this work did not agree with the negative correlation between CO_2-C evolved and heavy metal concentration reported either in different types of soils amended with heavy metal (Cornfield, 1977; Doelman and Haanstra, 1979a; 1979b; 1984) or polluted with heavy metal (Ohya *et al.*, 1988; Rühling and Tyler, 1973; Tyler, 1974). Possible reasons could be that available soil organic C and nutrients are not limited in this soil. The microbial biomass may also have adapted in this contaminated soil from the environment. It may therefore not be comparable to soils which have received heavy metals in a short-term experiment (Ohya *et al.*, 1985; 1986).

The results from Figure 5-2 and 5-12 and the high correlation between CO_2-C and Zn concentration with % soil organic C (Table 5-4) show that more available soil organic C accumulated in sandy soil of high metal concentrations (Zn concentration ranged: 6208 - 6551 $\mu g\ g^{-1}$ soil) and more of it was evolved as CO_2-C. Chander and Brookes (1991c) showed that much more of the available C from added glucose or ryegrass was evolved as CO_2-C from the biomass in the high-metal than in low-metal soil. This suggests that soil microbial biomass in metal contaminated soil is under stress due to the high concentration of metals, as suggested by Killham (1985). Furthermore, the organic C turnover is faster in sandy soils than in clay soils (Jenkinson and Rayner, 1977; Merckx *et al.*, 1985; Van Veen and Paul, 1981).

Carbon dioxide evolved might be used as a indicator of the effects of heavy metals on microbial activity in these soils where the available C was not a limitation. However, a proper control, with other polluted soils is still required in order to compare its variations due to pollution by heavy metals. However, it might be possible, by combining both biomass and activity measurements, to overcome this problem as this approach may provide an internal control, so avoiding the need for more conventional control soils. This is discussed in the next Chapter.

5.5 SUMMARY

1 Transects collected at increasing distances from the river had different Zn concentration, % soil organic C content, texture, agricultural management and topography. Soil microbial biomass and microbial activities were measured

by different methods in soils collected along the transect from a natural environment.

2 Soil organic C accumulated in soils heavily contaminated with Zn (6208 - 6551 $\mu g\ g^{-1}$ soil). The amount of microbial biomass (measured as biomass C, ninhydrin-N, SIR and ATP) and microbial activities (arginine ammonification rate and dimethyl sulphoxide reduction rate) decreased as Zn concentration increased, but CO_2-C evolved increased with increasing Zn concentration. This demonstrates that more mineralization occurs when the soil microbial biomass is under stress, caused by high metal concentrations. For example Zn ranged from 6208 - 6551 μg Zn g^{-1} soil, Cu from 85.2 - 176 μg Cu g^{-1} soil, Cd from 10.0 - 11.9 μg Cd g^{-1} soil and Pb from 483 - 1543 μg Cu g^{-1} soil.

3 The proportionality factors defining the linear relationship between microbial biomass and activities were not affected by heavy metal concentrations. Thus, biomass measured by FE, SIR and ATP and microbial activities such as arginine ammonification rate and dimethyl sulphoxide reduction rate can both be reliably determined in soils contaminated with metals from a natural environment. The size of microbial biomass and rates of activities were also affected by clay content and less by soil organic C content. These parameters were unable to be used as single indicators of heavy metal toxicity because it was not possible to compare them with results from proper control soils.

4 Soil ergosterol content was not correlated with biomass C. The relationship between the amount of ergosterol and Zn concentrations was also not clear. Further work is necessary to determine the correlation between ergosterol and biomass C in these soils.

CHAPTER 6

SOIL MICROBIAL BIOMASS AND SOIL ORGANIC C IN A GRADIENT OF Zn CONCENTRATIONS IN SOILS FROM A NATURAL ENVIRONMENT

6.1 INTRODUCTION

Microbial biomass and microbial activity measurements have been used to evaluate the effects of heavy metals on soil micro-organisms (Brookes and McGrath, 1984; Chander and Brookes 1991a; 1991b; 1993). In long-term experimental studies of metal-contaminated soils these parameters have often been conventionally compared to control soils, in order to obtain valuable information about the soil metal concentrations at which soil microbial biomass or microbial activity is affected (Brookes and McGrath, 1984; Chander and Brookes 1991a; 1993). However, in polluted soils from natural environments, variable factors may include different pH's, soil texture, plant C-input, etc. Therefore, to obtain a valid control soil might be difficult. Furthermore, the complexity of the natural environment may introduce variations not only in the size of microbial biomass and microbial activity (Babich and Stotzky, 1978; Jenkinson and Powlson, 1976a; Sørensen, 1981; Van Veen *et al.*, 1985), but also the relative toxicity of heavy metals. For example, it is known that metal toxicity decreases with increasing clay content (Stotzky, 1986; Tyler, 1981). Organic matter can influence the mobility and bioavailability of heavy metals and, thereby, their toxicity (Babich and Stotzky, 1983; Hongve *et al.*, 1980; Liang and

Tabatabai, 1977). Effects of pH on the toxicity of Zn to micro-organisms appear to vary with the type of organisms, *e.g.* fungi appear more sensitive to Zn toxicity as the pH increases from 5.5 to 7.5 (Babich and Stotzky, 1983; Hargreaves and Whitton, 1976). Therefore it seems unlikely that any single microbial parameter may be used as an indicator of heavy metal toxicity in a naturally polluted environment.

Brookes (1993) suggested that combining microbial biomass and microbial activity (*e.g.* biomass specific respiration rate) or biomass as a percentage of soil organic C, might be more sensitive measurements of soil pollution than either activity or biomass measurement alone. Such linked parameters might be possibly used to determine how much a natural ecosystem is being altered by pollutants without recourse to long field experiments. He further suggested that these related measurements might also serve as independent controls, so obviating the need for tedious and expensive field experiment.

The main aim of this work was to test if the use of such ratios might indeed be more useful measurements than single parameters. Therefore, biomass C as a percentage of soil organic C, the correlation between biomass C and soil organic C and biomass specific respiration rate, where the biomass was measured by different methods such as FE, SIR, ATP and ergosterol, were considered. A secondary aim was to determine which of these parameters were most suitable as indicators of environmental stress caused by heavy metals.

6.2 MATERIALS AND METHODS

6.2.1 DESCRIPTION OF THE SAMPLING SITES

The area selected for this study is located near Alditurri Mine, in the Valley of Oyarzun in Gipuzkoa, Spain. A description of the site is given in Chapter 5.

6.2.2 SAMPLING AND PREPARATION OF SOILS

Transect 1 and 2 were sampled in November 1992. Six samples more were collected from transect 1 in March 1993 (treated as transect 1A (November 1992) and 1B (March 1993) when ergosterol measurements are discussed). Twelve samples in total from transect 1 and 9 samples for transect 2 were collected. All the transects were collected along a pollution gradient of Zn and other heavy metals, *e.g.* Cu, Cd and Pb, perpendicular to the River Oyarzun. The soils were treated as described in section 2.1.1.

6.2.3 SOIL CHEMICAL ANALYSIS

Soil pH, organic C, total N and total metal concentrations were determined in the air-dried and ground (160μm) portions of each soil, as described in section 2.4. The textures of the soils were determined at Laboratory Agrario de Fraisoro, Diputación Foral de Gipuzkoa, Spain. Table 5-1, 5-2 and 5-3 (Chapter 5) summarizes the soil analysis.

6.2.4 SOIL MICROBIAL BIOMASS AND ACTIVITIES MEASUREMENTS

Soil microbial biomass C, ninhydrin-N, substrate induced respiration, ATP and ergosterol were measured after 10d of incubation at 25°C , as described in sections 2.2.1 to 2 and 2.2.4 to 6. The rates of CO_2-C evolved, arginine ammonification and dimethyl sulphoxide reduction (DMSO) were assessed after 10d incubation as described in sections 2.3.1 to 3. Each analytical measurement is the mean of triplicate determinations.

6.3 RESULTS AND DISCUSSION

6.3.1 RELATIONSHIP BETWEEN BIOMASS C AND SOIL ORGANIC C

There was a significant linear relationship between biomass C and soil organic C in high-metal soils (437 - 6551 μg Zn g^{-1} soil) (B_C= (116.4 $\pm$ 45.4) %C , r=0.55, n=14, $p<0.05$), but not in low-metal soils (188 - 334 μg Zn g^{-1} soil) (Figure 6-1). Brookes and McGrath (1984) and data in this work (Chapter 4) reported that in soils from the Woburn Market Garden Experiment, that a similar linear relationship was obtained in low-metal soils but not in high-metal soils. Those soils contained 9 %clay and 1.2 to 1.8 % soil organic carbon. In the Woburn Market Garden Experiment, soil Zn ranged up to about 300 μg g^{-1} soil, considerably lower than in this work.

Several reports have been published showing that organic C content in mineral soils usually correlates with biomass C (Anderson and Domsch, 1989; Insam and Domsch, 1988; Powlson and Jenkinson, 1981; Wu, 1991). The relationship is generally most strong in soils with less than 2.5 % organic C, as demonstrated by Anderson and Domsch (1989). In this work, the main reason for a non correlation

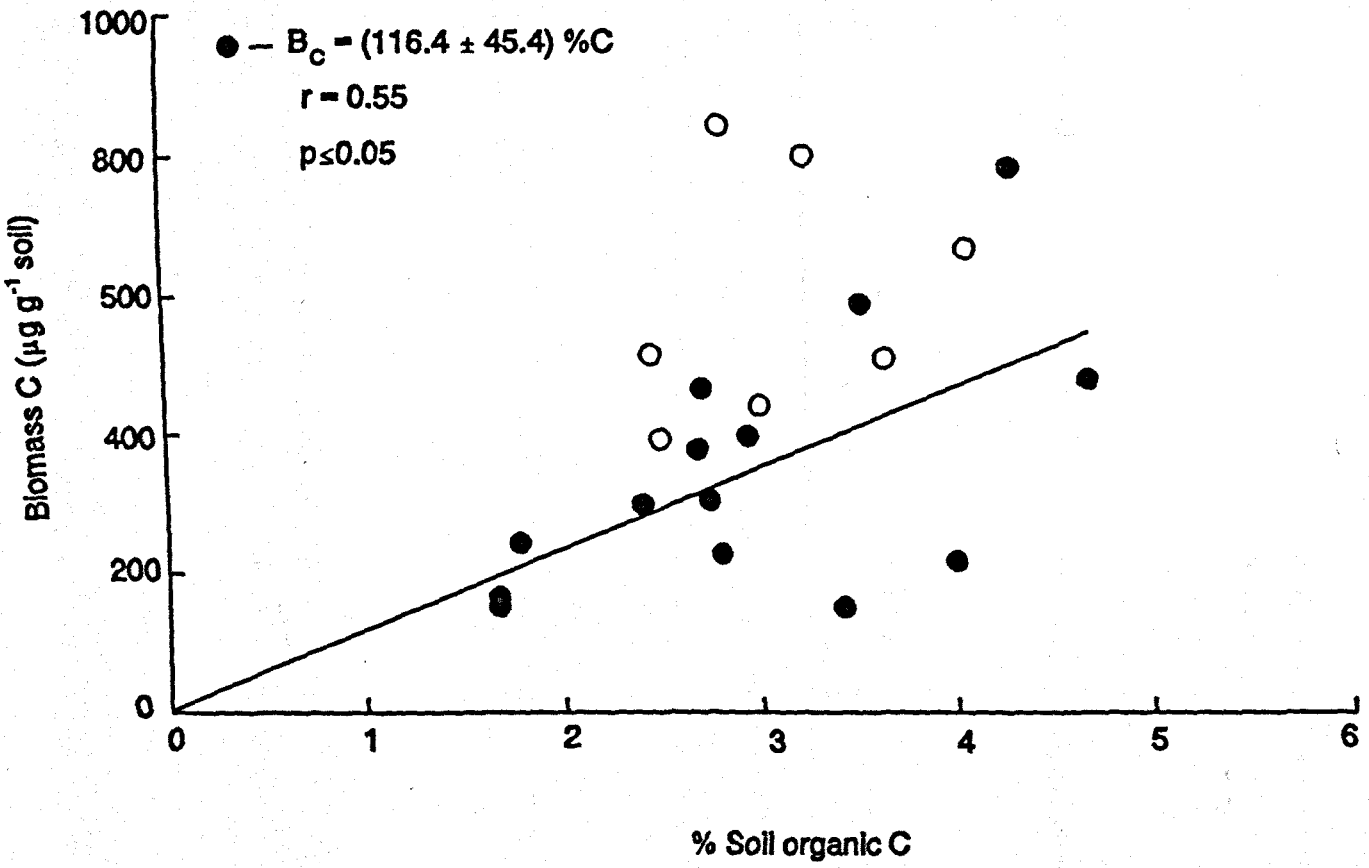

Figure 6-1: Biomass C and soil organic C in low-metal(○) and high-metal(●) soils from Gipuzkoa, Spain.

Table 6-1: Biomass C, % soil organic C, % clay content, pH and Zn concentration ranges in low- and high-metal soils from Gipuzkoa, Spain.

Soil treatment		range	ratio[a]
Low-metal	Biomass C[b]	444 - 846	1.91
	% soil organic C	2.44 - 4.06	1.67
	% clay	24.8 - 36.2	1.46
	pH	5.5 - 6.1	1.12
	Zn[b]	188 - 334	1.78
High-metal	Biomass C[b]	153 - 788	5.15
	% soil organic C	1.67 - 4.67	2.80
	% clay	5.4 - 35.3	6.54
	pH	5.8 - 7.7	1.33
	Zn[b]	437 - 6551	15.00

[a] [maximum value]/[minimum value]

[b] $\mu g\ g^{-1}$ soil

between biomass C and soil organic C in low-metal soil may be due to the few samples (n=7) with similar clay content (24.8 to 36.2), soil organic C (2.44 to 4.06 % soil organic C), and pH (5.5 to 6.13) that caused similar effects on the size of microbial biomass (Table 6-1). Thus, a high clay content in soil is known to favour the accumulation of organic C (Jenkinson, 1977; Sørensen, 1975; 1981; 1983; Van Veen *et al.*, 1984, 1985) by causing slower turnover of organic matter and enhanced survival of microbial biomass (Jenkinson and Rayner, 1977; Van Veen *et al.*, 1987; Van Veen and Paul, 1981).

In contrast, the high-metal soils had large variations in texture (sandy, sandy loam, sandy clay loam and clay), and also in the % organic C, which ranged from 1.67 to 4.67 (Table 5-2 and 5-3). The ratio between the maximum value and minimum value of biomass C in high-metal soil was similar to that in low-metal soils (Table 6-1). The significant correlation between biomass C and % clay over all the soils in this study (Chapter 5) and these data suggest that the reason why the linear correlation between biomass C and % organic C can hold in high-metal soils is because the organic matter and clay content in mineral soils acts as a buffer against toxification by the metal as shown by Mathur (1983), Dumontet and Mathur (1989), Collins and Stotzky (1989), Doelman and Haanstra (1986). They suggested that organic matter is the more important factor as a buffer against toxification by metals than clay content.

Similarly, a linear correlation of soil biomass C and % organic C calculated from data reported by Chander and Brookes (1991a) can be obtained in low-metal soils with 15 to 21 % clay and 1.5 to 4.6 % organic C from two long-term experiments: Luddington and Lee Valley and, in low- and high-metal soils with 9 %

clay and 1.04 to 1.99 % organic C in soils from the long-experiment: Gleathorpe (Chander and Brookes, 1993).

Discriminant analysis of low- and high-metal soils in Figure 6-2 show the allocation to the population of low- and high-metal soils (Chapter 4) where 6 out of 7 low-metal soils had a negative score, and 10 out of 14 high-metal soils had positive score. The texture of soils that were the anomalous points in low- and high-metal soils were clay, sandy loam and sandy clay soils. This analysis indicates that two separated communities exist in the low- and the high-metal soils.

The linear correlation between biomass C and % organic C cannot be used as an indicator of toxicity of heavy-metal in these soils, from non-experimental sites because the interference of other factors such as soil texture, organic C content and management also affected the correlation between biomass C and soil organic C in both the low-metal soils or high-metal soils. Therefore, the correlation between biomass C and soil organic C gave information about the two separated communities, but did not provide a sensitive indicator of the effects of high-metal concentrations on the size of soil microbial biomass.

6.3.2 BIOMASS C AS A PERCENTAGE OF SOIL ORGANIC C

Biomass C as a percentage of organic C in low-metal soils (188 - 334 μg Zn g^{-1} soil) ranged from 3.24 to 1.41% (Figure 6-3). These values were within the range of those reported for other soils (Adams and Laughlin, 1981; Jenkinson and Ladd, 1981; Robertson *et al.*, 1988; Sparling *et al.*, 1990). The proportion of biomass C as a percentage of organic C was markedly decreased (average 0.771) in soils with Zn concentrations between 3709 to 6551 μg g^{-1} soil (Table 6-2). However, in high-

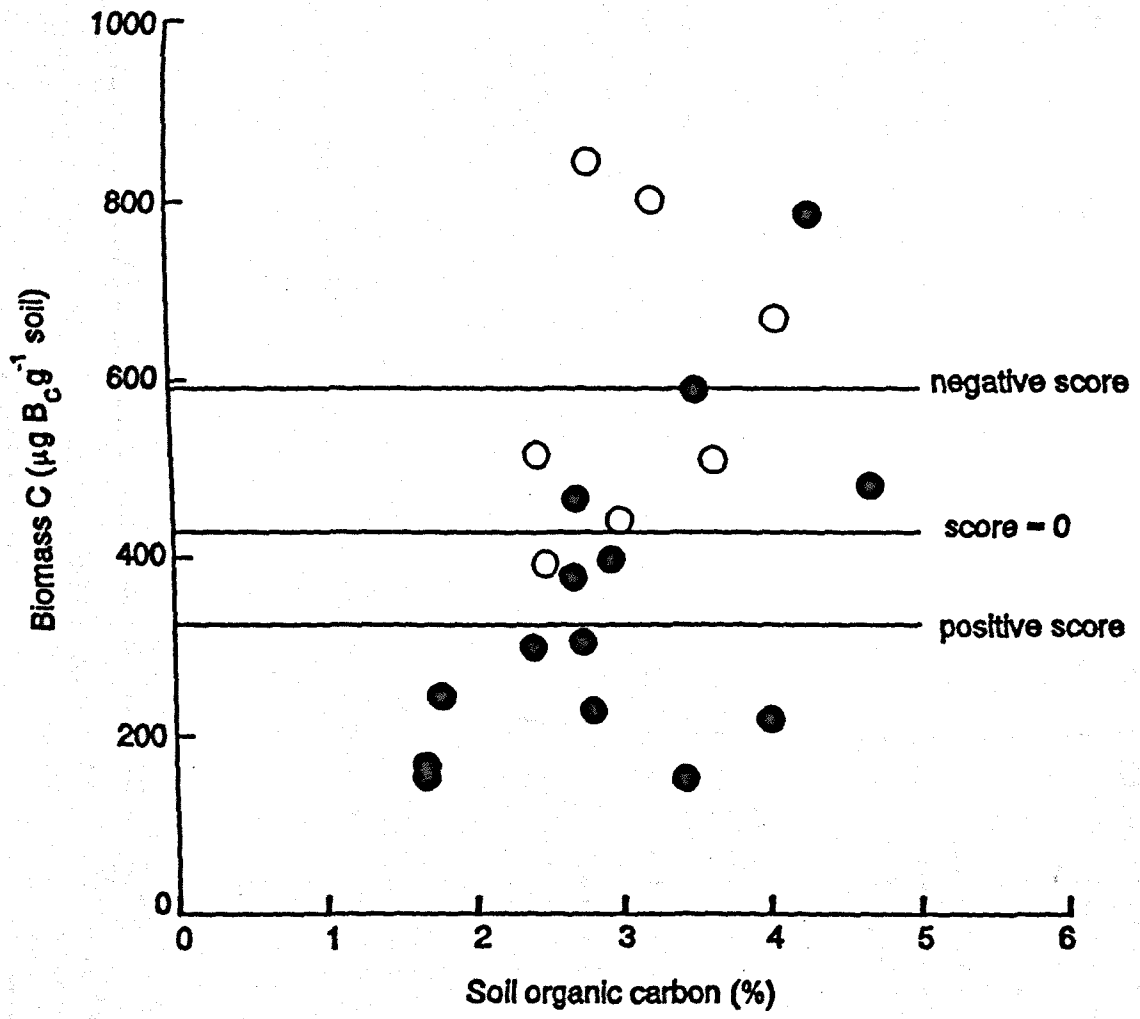

Figure 6-2: Discriminant analysis of low-metal (○) and high-metal (●) soils from Gipuzkoa, Spain.

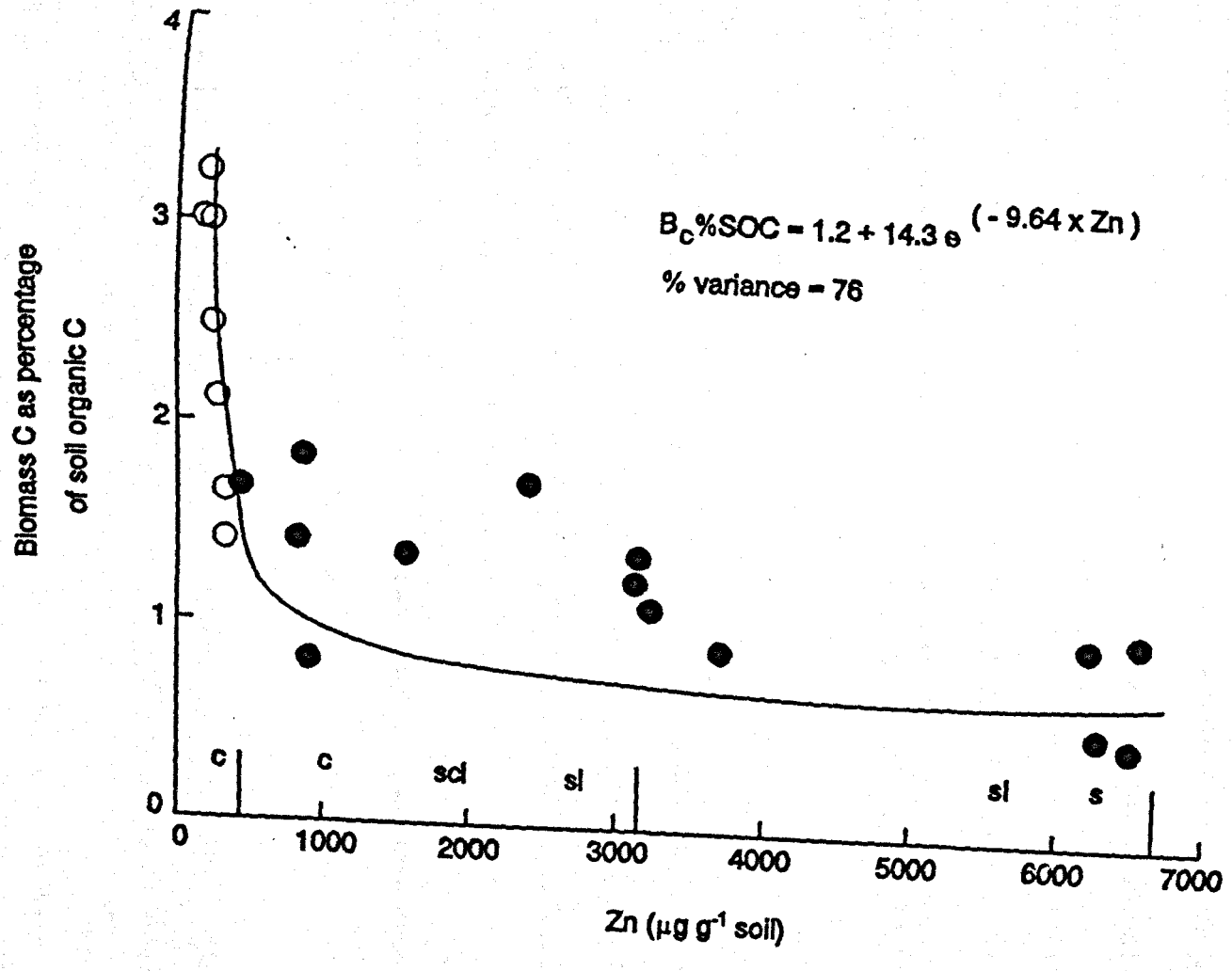

Figure 6-3: Biomass C as percentage of soil organic C, soil texture (s=sandy, sl=sandy loam, scl=sandy clay loam and c=clay) and low (○) and high (●) Zn concentration in soils from Gipuzkoa, Spain.

metal soils in which Zn concentrations ranged from 437 to 3237 $\mu g\ g^{-1}$ soil, the biomass C as a percentage of organic C was in the same range as low-metal soils (average 1.44). These values in high-metal soils (3709 to 6551 μg Zn g^{-1} soil) were less than half (0.5 to 1.03) those in low-metal soils (68% less). In soils where Zn ranged between 437 to 3237 $\mu g\ g^{-1}$ soil, the percentage was 40.5 % less than those in low-metal soils. There was a significant exponential relationship between biomass C as a percentage of soil organic C and Zn concentration (BC%SOC = $1.2 + 14.3\ e^{(-9.64 \times Zn)}$, percent of variance accounted for = 76)

The smaller amounts of biomass carbon as a percentage of soil organic C in Zn contaminated soils and the significant exponential relationship between biomass C as percentage of soil organic C and Zn (Figure 6-3) strongly indicates that metals are causing decreased biomass as reported in high-metal soils from long-term experiments (Brookes *et al.*,1984; 1986; Brookes and McGrath, 1984; Chander and Brookes, 1991a; 1993 and data in Chapter 4 in this work). Therefore, the ratio biomass C as percentage of soil organic C provides a sensitive indicator of the effects of heavy-metal on microbial biomass in this ecosystem. The link between these two parameters can itself constitute an 'internal control' which involves a particular management, soil type and climate, and it may indicate damage to the function of the soil ecosystem as in agreement with the suggestion of Brookes (1993) and Chander and Brookes (1993). However, it is important to point out that the values of biomass C as percentage of soil organic C are very similar in sandy loam (ranged Zn concentrations ranged between 437 - 1570 $\mu g\ g^{-1}$ soil) and clay loam soils (Zn ranged between 2390 - 3709 $\mu g\ g^{-1}$ soil) (Table 6-2). Again, this may be due to soil heterogeneity such as differences in the available organic matter content, soil texture

Table 6-2: Biomass C as percentage of soil organic C, % soil organic C, biomass C and soil texture in low- and high-metal soils from Gipuzkoa, Spain

Zn[a]	%C[b]	BC%SOC[c]	B_c[d]	Type soil[e]	Class[f]
range		average			
188-334	3.10	2.42	598	c	low
437-1570	3.24	1.42	478	scl, c	medium
2390-3709	2.28	1.28	295	sl, scl	high
6208-6551	3.44	0.77	256	s, sl	very high

[a] (μg Zn g^{-1} soil)

[b] % Soil organic C

[c] Biomass C as percentage of soil organic C

[d] Biomass C = (μg B_c g^{-1} soil)

[e] c=clay, slc=sandy loam clay, sl=sandy loam, s=sandy

[f] class= classification according to Zn concentration

and crop management (Anderson and Domsch, 1989), having larger effects than Zn concentration between these metal ranges. It is therefore not possible to provide from this work, 'normal' relationships within different soil types and managements for biomass C as a percentage of soil organic C and to use this as a predictor of the effects of heavy metals in the soil ecosystem.

6.3.3 BIOMASS SPECIFIC RESPIRATION RATE

The mean biomass specific respiration rate (measured as mg CO_2-C produced g^{-1} B_C d^{-1}) in high-metal soils where Zn concentration ranged from 6208 to 6551 μg g^{-1} soil was 151, in soils where Zn ranged between 437 to 3709, it was 39 and in low-metal soils (188 to 332 μg Zn g^{-1} soil) it was 27.5 mg CO_2-C g^{-1} B_C d^{-1} (Figure 6-4). There was a significant exponential relationship between biomass specific rate and Zn concentration (BSRR = $11.9+12.7\ e^{(0.37 \times Zn)}$, percent of variance accounted 1for = 83).

Table 6-3 shows the mean of CO_2-C evolved, biomass C values (the general values are given in Chapter 5, Figures 5-8 and 5-3), the range of Zn concentration, texture of soils and the Zn concentrations classes in the soils of transects 1 and 2. The total amount of CO_2-C evolved from the very high-metal soil was 2.38 times more than the average for medium, high- and low-metal soil. In contrast, biomass C was least at this soil Zn concentration (Table 6-3). The biomass specific respiration rate in the very high-metal soil was about 5.3, 5 and 3.2 times more than the low-metal, medium and high-metal soils, respectively. These results indicate that more CO_2 was evolved per g^{-1} biomass C in sandy soils with 6208 - 6551 μg Zn g^{-1} soil due to the stress caused by the heavy metal. The mechanisms of heavy metal

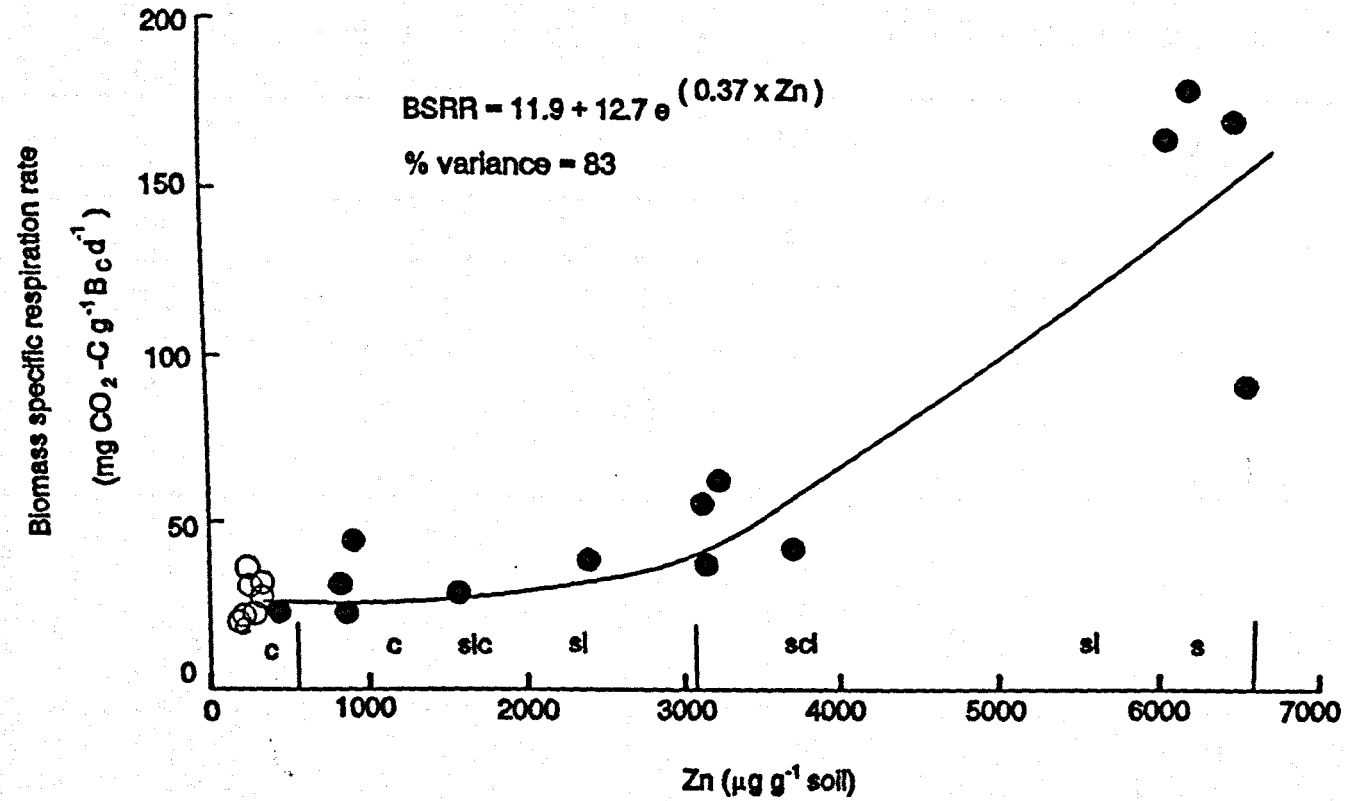

Figure 6-4: Biomass specific respiration rate, soil texture (s=sandy, sl=sandy loam, scl=sandy clay loam and c=clay) and low (○) and high (●) Zn concentration in soils from Gipuzkoa, Spain.

Table 6-3: Biomass specific respiration rate, % soil organic C, $C0_2$-C evolved, biomass C and soil texture in low- and high-metal soils from Gipuzkoa, Spain.

Zn[a]	%C[b]	BSRR[c]	CO_2-C[d]	B_C[e]	Type soil[f]	class[g]
range	average					
188-334	3.10	27.5	157	598	c	low
437-1570	3.24	30.3	132	478	slc, c	medium
2390-3709	2.28	47.4	140	295	sl, scl	high
6208-6551	3.44	151.0	339	256	s, sl	very high

[a] (μg Zn g^{-1} soil)

[b] % Soil organic C

[c] Biomass Specific respiration rate = (mg CO_2-C g^{-1} B_C d^{-1}), B_C = biomass C

[d] CO_2-C evolved= (μg CO_2-C g^{-1} soil d^{-1})

[e] Biomass C = (μg B_C g^{-1} soil)

[f] c=clay, slc=sandy loam clay, sl= sandy loam, s= sandy

[g] class= classification according to Zn concentration

effects on microorganisms are described in Chapter 3.

Slightly more soil organic C accumulated in very high-metal soil (6208 to 6551 μg Zn g^{-1} soil) while amounts were similar in low- and medium-metal soils (Table 6-3). In contrast, more CO_2-C was evolved from the very high-metal soils. These results indicate that more organic C was available in very high-metal soil while the efficiency of microbial biosynthesis was less efficient, therefore more C was lost via respiration. These results therefore suggest again that the microbial biomass was under stress due to heavy metals in these very highly contaminated soils. Although the values of the biomass specific respiration rate in medium- and high-metal soil indicate that biomass was also under stress, CO_2-C evolved was not as great it was in very high-metal soils. This was probably due to limitations in availably of organic C. Furthermore, although the heavy-metals had influence on microbial biomass content in these soils, other factors also had effects on soil microbial biomass and activity, such as clay content (see section 5.3.2, Chapter 5). These results are supported by studies done in unpolluted soils with high clay content, where the biomass turnover rate was slower than in sandy soil (Gregorich *et al.*,1991; Ladd *et al.*,1981; Van Veen *et al.*, 1985). In addition, the basal respiration was related to the amount of available C but not to the size of microbial biomass (as section 5.3.7, Chapter 5) (Shen *et al.*,1987) or clay content (Verstraete and Voets, 1977).

Measurements of biomass specific respiration rate therefore seems to be a useful and sensitive indicator of environmental stress in heavy-metal polluted soils than either biomass C or CO_2-C evolution alone.

6.3.4 BIOMASS SPECIFIC RESPIRATION RATE MEASURE BY NINHYDRIN-N, SIR, ATP AND ERGOSTEROL

Trends of biomass specific respiration rates calculated using ninhydrin-N, SIR and ATP (mg CO_2-C g^{-1} B_{NIN-N}, SIR or ATP) along a gradient of a Zn concentration were similar to those measured with biomass specific respiration rate when biomass C was measured by fumigation-extraction (Figures 6-4 to 6-7). There were significant exponential correlations between biomass specific respiration measured by ninhydrin-N, SIR and ATP and soil Zn (0.874, 0.872, and 0.866 respectively). The interpretation of these data is the same as biomass C specific respiration rate, thus, more CO_2-C was produced per g of biomass ninhydrin-N, SIR or ATP due to the effect of stress caused by the high-metal soils. Maire (1983, 1987) showed that combining biomass and activity as CO_2/ATP to study the recolonization of a sterilized peat on pots and in soils following air-drying then rewetting of agricultural soils was a good approach to measure changing microbial dynamics in soils following perturbation. The changes in biomass specific respiration, calculated from by biomass ninhydrin-N, SIR and ATP also similarly reflected effects of heavy metals on the soil ecosystem in soils from this non-experimental gradient. Thus, any of the methods could be used to measure biomass specific respiration.

In contrast the biomass specific respiration rate calculated from soil ergosterol content did not reflect effects of Zn on the soil ecosystem (Table 6-4). This is because the ratio [ergosterol]/[biomass] was itself variable, as discussed in Chapter 5.

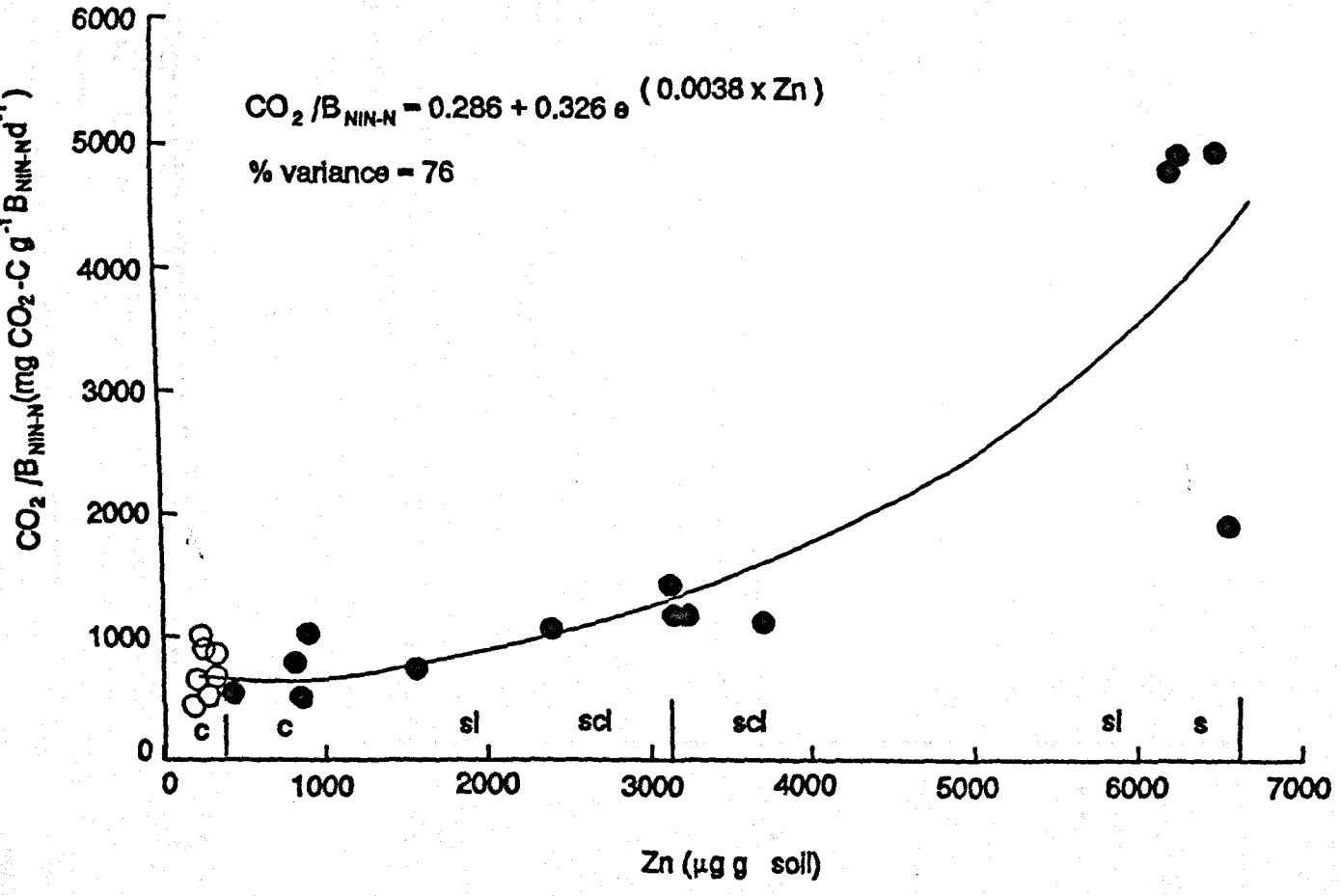

Figure 6-5: Biomass specific respiration rate calculated from biomass ninhydrin-N, soil texture (s=sandy, sl=sandy loam, scl= sandy clay loam and c=clay) and low- (○) and high-metal (●) soils from Gipuzkoa, Spain.

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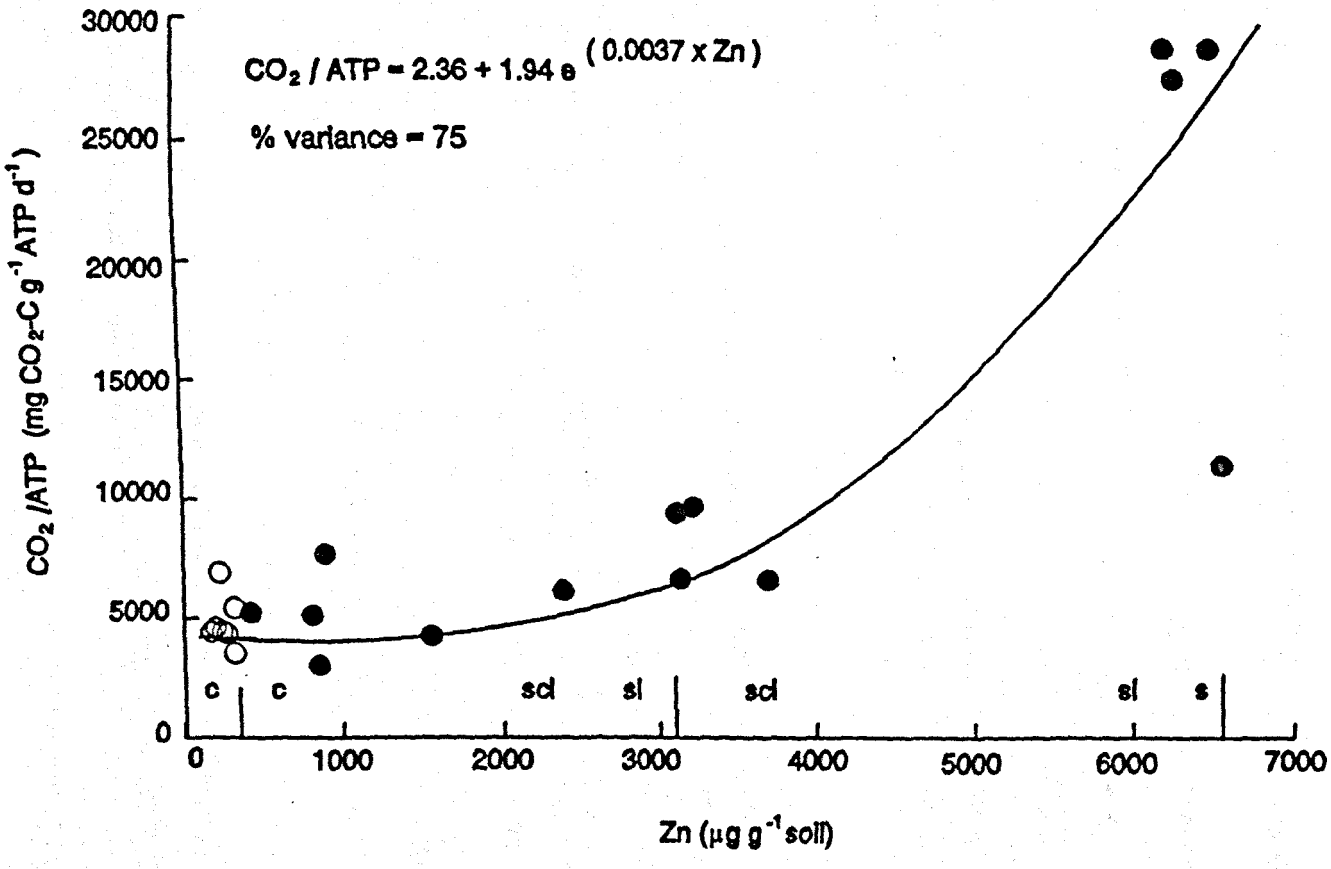

Figure 6-7: Biomass specific respiration rate calculated from ATP, soil texture (s=sandy, sl=sandy loam, scl=sandy clay loam and c=clay) and low- (○) and high-metal (●) soils from Gipuzkoa, Spain.

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6.4 SUMMARY

Amounts of total soil organic C, total metal concentrations, soil microbial biomass (measured by FE, SIR, ATP and ergosterol) and microbial CO_2-C evolved were measured in soils sampled along a gradient of Zn concentration from a natural polluted area from Gipuzkoa, Spain.

The important finding are:

1 Amounts of microbial biomass were linearly correlated with soil organic C content in high-metal soils, but not in low-metal soils. The data may indicate that clay content and soil organic C act as buffers against toxic effects of metals in soils with high metal content. Discriminant analysis showed that two microbial communities exist, the community in the low-metal soils was separated by 96% (6 out of 7 low-metal soils had a negative score) from the high-metal soil and in the high-metal soils 71% (10 out of 14 high-metal soils had positive score) of the community was separated from the low-metal soil.

2 The amount of biomass C as a percentage of soil organic C was negatively related with Zn concentrations. The values of this ratio in low-metal soils were within the range of values already published, but in high-metal soils the ratio drastically decreased (average 0.77%). However this was due not only to the effects of heavy metals on the microbial biomass but also to the changes in soil organic C, clay content and soil texture (*e.g.* in soils with Zn concentration between 1000 and 4000 $\mu g\ g^{-1}$ soil). Therefore it is not possible to compare the values with appropriate control soil in this Zn concentration

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CHAPTER 7

GENERAL DISCUSSION

7.1 HEAVY METALS IN SITES CONTAMINATED BY SEWAGE SLUDGE AND MINE SPOIL.

Heavy metal pollution of soils, irrespective of their source, is causing increasing concern because heavy metals tend to persist in the soil for a very long time. In extreme cases the effects on the surrounding ecosystems from mining and smelting areas are obvious in the poor growth of vegetation due to phytotoxicity and accumulation of organic matter in the soil. Areas affected by this severe pollution are frequently restricted in extent. However, contaminated sites containing quite high soil metal concentrations are some times used as agriculture land (for example in Chapter 5). Most of the concern about the accumulation of heavy metals in agricultural soils relates to the possibility of uptake by crops and consequent adverse effects on the yield of crops and on livestock and human diets. When assessing the potential hazard of heavy metals in ecosystems with mine spoil and sewage sludge at high concentrations there is a particular need to decide whether the land is to be used for agricultural purposes or requires remedial treatment. In the latter case, removing the metals from contaminated soils by growing plants that hyper-accumulate metals has been suggested as a possibility (*e.g.* Baker and Brooks, 1989; McGrath *et al.*, 1993). However, this is still in the research stage. Liming and a combination of liming and the addition of clay are currently the most important and practical means

of decreasing heavy metal availability in soils and plant uptake (Dijkshoorn *et al.*, 1981; John *et al.*, 1972; King and Morris, 1972; Williams, 1975). However, the availability of some elements are more sensitive to changes in soil pH than others. For example Cu availability is more sensitive to soil pH than Pb availability (Alloway, 1990). Several measures could be taken to restrict metal-contaminated sludge application to agricultural soils

a) If the metal concentration in the already polluted soils is known, then any further sludge additions can be limited to reduce the risk of exceeding the limits of recommended metal concentrations. By knowing the metal content of the sludge, the application rates can be calculated.

b) Cleaning the sludge sources *i.e.* in the factories and industries where metals originate. For example, removing the metals from the sludge by chemical or physical processes which involve mobilization, separation of the sludge particles and removal of the heavy metals from the water phase. The most successful mobilization process is with inorganic acids such as HCl, H_2SO_4, HNO_3. The removal of sludge particles and mobilized heavy metals by chelating agents (EDTA, nitrilotriacetic acid and citrate) has also been attempted. Several methods can be used to remove the heavy metals from the extraction phase: microfiltration, ultrafiltration, reverse osmosis, neutralization followed by sedimentation or flotation, precipitation with hydrogen sulphide or sodium sulphide, electrodialysis or solvent extraction (Scott *et al.*, 1980; Stoveland *et al.*, 1979; Wozniak and Huang, 1982).

In both situations (cleaning up and restriction of sewage sludge application), the risk of exceeding set limits can only be enforced through legislation. Current guidelines control metal-contamination of soil through sewage sludge addition,

specific loading rates and maximum permitted total metal-concentration in soils. However the guidelines do not take into account the effects of heavy metals on soil microbial biomass and microbial activity.

Therefore, practical guidelines are desirable based on microbiological criteria for assessing the significance of hazards either in polluted sites from the natural environment or in sewage-sludge treated soils.

7.2 BACKGROUND TO PROJECT

Previous work reported by Brookes and McGrath (1984), Chander and Brookes (1991a; 1991b; 1993) showed that microbial biomass and microbial activity measurements may be used as biological indicators of the effect of heavy metals at low metal concentration (1-3 times current EU limits). At these concentrations, individual metal or combinations of metals were shown to decrease the amount of microbial biomass and microbial activity. This work was done using soils from well-managed field experiments where these measurements can be easily compared with similar measurements from appropriate control soils. It was not known if these approaches could be applied to polluted non-experimental soils from the natural environment with different textures, soil management and metal concentrations.

The first aim of this project was to thoroughly test current methods to measure microbial biomass and microbial activity using metal-contaminated and uncontaminated soils from a field experiment where natural variability was minimised by the application of standard and well-replicated treatments. The second aim was to determine if storage conditions prior to analysis caused changes in amounts of

microbial biomass and microbial activity. If so, how could these effects be minimised. This work led to the third aim of the project which was to use the methodologies and procedures developed above to determine the effects of heavy metals on microbial biomass and microbial activity in natural-polluted soils sampled from a natural gradient of decreasing soil metal concentrations, caused by past mine-spoil workings.

7.3 RESEARCH ACHIEVEMENTS

7.3.1 EVALUATION OF METHODS TO MEASURE MICROBIAL BIOMASS AND MICROBIAL ACTIVITY IN METAL-CONTAMINATED AND NON-CONTAMINATED SOILS.

A wide range of methods to measure microbial biomass and microbial activity were examined. Indeed the work in this thesis probably represents one of the most exhaustive examinations of the suitability of these procedures to determine effects of metals on the soil microbial ecosystem yet attempted. Total biomass measurements included biomass C, N, ninhydrin-N, ATP and biomass measured by substrate induced respiration. Ergosterol was also used to estimate the fungal biomass. Microbial activity measurements included soil CO_2 evolution, arginine ammonification and dimethyl sulphoxide reduction. Very close linear correlations were found between all the biomass methods, irrespective of whether the soils were contaminated, or not, with heavy metals. This was also true at all concentrations of heavy metals in soil, which ranged from 42 - 110 μg Cu g^{-1} soil, 17 - 30 μg Ni g^{-1} soil, 1.7 - 8.5 μg Cd g^{-1} soil, 147 - 335 μg Zn g^{-1} soil, 62 - 113 μg Cr g^{-1} soil and 43 - 118 μg Pb

g^{-1} soil in the soils of the Woburn Market Garden field experiment, the site chosen for this work. Evidence that 'linked' parameters *e.g.* biomass specific respiration rate or biomass as percentage of soil organic C were more sensitive indicators of the effects of heavy metals on soil microbial processes than single measurements were obtained.

7.3.2 EFFECTS OF STORAGE OF SOIL ON MICROBIAL BIOMASS AND MICROBIAL ACTIVITY.

The effects of storage of soils upon changes in different biomass indexes (*e.g.* biomass C, biomass ninhydrin-N, SIR, ATP) and biomass activity (CO_2-C evolved, arginine ammonification and biomass specific respiration rate) in soils of high- and low- metal concentration were investigated.

A bigger decrease in biomass C, biomass ninhydrin-N and soil respiration were found during the first 7d of incubation than after eight weeks storage. This mean that probably the FE method was influenced by the decomposition of biomass killed during soil preparation and small plant roots which may have contributed to the flush of C due to fumigation at one week but not at eight weeks of storage at 4°C. Soil microbial biomass measured by the SIR method increased during the first 4d of incubation. This suggest that the SIR method measured microbial activity during the first days of incubation instead of biomass C in soil. Soil ATP content increased during a period of 0 to 20d incubation at 25°C after one or eight weeks storage.

Biomass C and biomass ninhydrin-N changed very little after one year of storage. However, biomass C measured by SIR and microbial activity such as CO_2-C evolved and arginine ammonification rate were affected by storage period. Thus, the

biomass seems to age physiologically upon storage, losing activity and yet declining only very little in population size.

Overall, soil microbial biomass (measured by FE, SIR and ATP) and microbial activity measurements (arginine ammonification rate and biomass specific respiration rate, but not CO_2-C evolved) were able to detect relative and absolute differences between low- and high-metal soils when measured immediately after sampling following 10d incubation, or after one year of storage.

7.3.3 MICROBIAL BIOMASS AND ACTIVITY MEASUREMENTS IN SOILS FROM A NON-EXPERIMENTAL METAL-POLLUTED SITE IN SPAIN.

Transects collected along a natural gradient of heavy metals due to contamination from mine-spoil waste had different Zn concentrations, % soil organic C content, texture, agricultural activities and topography.

The amount of microbial biomass (measured as biomass C, biomass ninhydrin-N, SIR, ATP) and microbial activity (arginine ammonification rate and dimethyl sulphoxide reduction rate) were negatively significantly correlated with Zn concentration in exponential relationships. All these parameters (microbial biomass and microbial activity) were also affected by soil clay and organic C content. However, CO_2-C evolved was not correlated with biomass C and clay content but it was correlated with soil Zn and soil organic C content.

Soil ergosterol content was not correlated with biomass C or Zn concentration but it was with soil organic C content. Further work is necessary to determine the reasons for the lack of the correlation between ergosterol and biomass C in these soils.

Two separate microbial communities exist in the low-metal and in the high-metal soils. The linear correlation between biomass C and soil organic C in high-metal soils (which had a high clay content) but not in low-metal soils (which had a low clay content), suggest that soil organic C and clay content acts as a buffer against the toxic effects of metal in soils with high concentration of heavy metals.

Biomass C as a percentage of soil organic C was negatively and biomass specific respiration rate was positively correlated with soil Zn concentration. Both parameters provided sensitive indicators of the effect of heavy metals on soil microbial biomass in the ecosystem.

As a general conclusion, the measurements of soil microbial biomass (FE, SIR and ATP) and microbial activity (arginine ammonification and dimethyl sulphoxide reduction rate) by the different methods described in this thesis show that the methods are generally suitable to estimate the effect of heavy metal in soils both from field experiments and from a natural environment.

It is not possible to judge, on the basis of my own data of the soils from the natural environment whether the amount of microbial biomass or microbial activity as single parameters have been altered by heavy metals because of the complex interactions between physico-chemical (soil texture, Zn concentration) and natural environment (soil management) parameters. Only by knowing the background values of heavy metal concentration from uncontaminated soils from the same area under similar climatic conditions and with the same soil texture and soil management, can this information be obtained. However, the link between biomass C and soil organic C (biomass as percentage of soil organic C) and biomass C and CO_2-C evolved (biomass specific respiration rate) provided a more sensitive indicator of the effect of

heavy metal on soil microbial biomass (as was shown by statistical analysis) not only for small scale field experiment but for the whole ecosystem from the natural environment.

7.4 FUTURE WORK

1) Future work should be directed towards determining the community structure of the microbial community in heavy-metals polluted soils. This information will be valuable in understanding the soil organic matter dynamics and the factors which cause toxicity of heavy metals to the soil microbial biomass and microbial activity. However, it has been difficult to determine the community structure in soils receiving different treatments such as pesticides, substrate amendments and fumigation (Q. Lin, *Ph.D Thesis*, University of Nottingham, 1994). Possibly new techniques such as analysis of fatty acid and/or DNA can supply valuable information. In this way, the research effort can be directed to increase our knowledge of the effects of heavy metals in the environmental, to help with environmental protection.

2) The principal aim of soil protection is conservation or restoration of desirable soil quality. The behaviour of heavy metals in the soil system are important in the evaluation of soil quality. The behaviour of heavy metals is controlled by a large number of variables, the most important are: buffering capacity of soil, heavy metal speciation, heterogeneity of the soil system (spatial variation) and bioavailability of heavy metals. These variables must be linked with soil microbial biomass and microbial activity measurements in order to obtain sufficient reliability for

interpretation of results about the soil quality and the risk evaluation of soil pollution for contamination of crops in soil ecosystems from the environment.

3) Usually the bioavailability of heavy metals in soil is studied in relation to crop protection. Furthermore, no simple relationship exists between the total heavy metal content and the amount of microbial biomass and microbial activity (Chapter 5). The reason is because only part of the total amount of heavy metal is available in soils. Only a small fraction of the total soil metal concentration is extractable by currently available chemical methods representing different forms of the heavy metals such as soluble, absorbed, exchangeable and precipitated forms. Thus, an extraction solution for all these fractions of the total soil metal concentration would be a major advance. However such an extractant has yet to be developed.

4) The smaller microbial biomass in metal-contaminated soils, even at metal concentrations only 1-3 times current EU limits, have faster metabolic rates than the larger biomass in similar, non-contaminated soils. This seems a direct response to the toxic effects of the metals. The mechanisms of resistance of the soil microbial community to heavy metals also requires investigation.

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