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Catabolic profiles as an indicator of soil microbial functional diversity

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Executive Summary

This project assessed the potential of a multivariate profiling approach to quantify the functional diversity of soil microbial communities, and its potential application in Ecological Risk Assessment scenarios.

The basic concept is that of functional profiling by a multiple substrate-induced respiration (MSIR) assay. This involves the high-resolution measurement of the time-courses of soil respiration that arise following the addition of a prescribed suite of carbonaceous substrates to sub-samples of a test soil. The resultant profiles reflect the ability of the soil community to degrade the added substrates.

A series of 62 soils from different ecological origins that had been contaminated to various extents by heavy metals or organics were profiled. Multivariate analysis of the profiles demonstrated that they were clearly and consistently discriminatory on basis of broad soil type and ecological background, and were sensitive to factors likely to influence soil communities and their function. The method was sensitive to increasing levels of toxin stress, as indicated by increasing inhibition of catabolic response across a range of substrates. A simple impact framework was developed that provides a graphical representation of changes in functional diversity as a result of soil management or contamination.

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1. Introduction

1.1 Background

The Environment Agency is charged with reporting on the state of the environment, including soils. As such, it is responsible, through a number of regulatory regimes, for protecting soil quality. The European Commission and the UK government have adopted a functional approach to soil protection, which involves identifying the functions soil performs (for us and for the wider environment), characterising the mechanisms that support those functions and developing methodologies to determine whether those functions are performing satisfactorily.

Environmental interactions (soil has a key role linking the atmosphere, geology, water resources and land use) have been identified as a key function of soils. It underlines the role of soil in the environment as a processor of organic material, thereby protecting surface and groundwater and recycling nutrients. The soil microbial community (bacteria and fungi) is the main agent of this processing. It is the ability of soil biomass to degrade a wide range of organic substrates that underpins the soil's filtering, buffering and nutrient recycling activities.

The soil community is remarkably complex, generally comprising the highest levels of biodiversity on the planet. The origins and consequences of this are manifold (Ritz 2005), not least the continued development of a plethora of ways of characterising and measuring its composition and function (Leckie 2005, Kirk *et al*. 2004, Bloem *et al*. 2005). These can be grouped into three basic approaches: genetic profiling, which relates to the base structure of the community (the 'library' of fundamental information that is present); phenotypic profiling, which relates to the prevailing expression of the genetic background (the 'living form' of the community); and functional profiling, which relates to the various processes the community is engaged in or are potentially capable of carrying out. The latter approach is a particularly powerful concept for assessing the status of soils in relation to environmental interactions, because it is directly associated with ecological processes as they occur.

Despite the importance of the carbon-transformation function, there are, as yet, no robust and validated methodologies for assessing the ability of the soil microbial community to metabolise a wide range of substrates. One potential approach is the so-called community-level physiological profiling (CLPP) profiling technique, pioneered by Garland and Mills (1994). The basis of this concept is to measure simultaneously the extent to which the soil community can metabolise a range of carbonaceous substrates supplied separately but in tandem. A popular version of the technique utilises Biolog™ plates, which allow researchers to measure simultaneously the metabolic utilisation of a suite of 95 (or more) compounds.

Many studies have demonstrated how such Biolog response profiles discriminate between soils, communities and environmental factors (such as, Garland 1996, Insam and Rangger 1997, Grayston *et al*. 2004). However, there are significant flaws in this method that compromise its applicability and interpretability (Preston-Mafham *et al*.

2002). Fundamentally, the method relies upon the growth and expression of soil organisms in small volumes of enriched culture media in vitro. It is clear that the majority of soil micro-organisms do not grow or metabolise under such circumstances. Typically, only a few percent of micro-organisms at most are represented and these are heavily biased in favour of fast-growing gram negative bacteria. The Biolog method therefore provides a very limited perspective on the likely substrate-utilisation capability of soil communities, although it does demonstrate that the fundamental concept of catabolic (involving the metabolic breakdown of large molecules into smaller ones with a release of energy) profiling is sound. Indeed, ecological interpretation of Biolog data can be fraught (for instance, Haack *et al*. 1995,Campbell *et al*. 1997, Preston-Mafham *et al*. 2002).

A logical extension to the concept was pioneered by Degens and Harris (1997). They developed a catabolic community profiling technique, in which substrate suites are applied directly to whole soil and the respiratory response then measured. This technique is essentially an amplification of the substrate-induced respiration (SIR) method developed by Anderson and Domsch (1978), now adopted as an ISO standard for soils (ISO 2002). Degens and Harris measured SIR responses of soils to a large range of substrates over the first four hours. This was before any cell division was likely to have occurred and hence was related to the response of the in situ community. The resultant activity profiles ('functional profiles') showed a clear discrimination between different soils and soil management regimes, and recent work has reinforced the potential utility of the technique (for instance, Degens 1998, Schipper *et al*. 2001, Stevenson *et al*. 2004). This 'multiple substrate-induced respiration' (MSIR) technique shows promise, but there are significant technical constraints since measuring catabolic rates in a large number of discrete soil samples is labour-intensive and technically demanding. In addition, MSIR responses have generally only been measured at two time-points (the minimum required to establish a rate), and it is likely that considerably more information could be gleaned if more detailed response dynamics were available.

Contemporary developments in automated respirometry, such as the MicroResp® micro-scale system (Campbell *et al*. 2003), open up the possibility of performing such measurements at reasonable replication using a wide range of substrates on a range of soils. However, the MicroResp® system assays units of a few hundred mg of soil and with limited temporal resolution. Larger-scale multi-channel respirometers offer the potential to derive respiration curves with high temporal resolution.

1.1.1 Objectives

The overall aim of this project was to measure detailed time-courses of MSIR responses from a range of soils, in order to explore the potential of such data in functional profiling.

The specific objectives of this proof-of-concept study are detailed below.

1. To refine the methodology of the MSIR functional profiling method using an automated respirometer for arable, grassland and woodland soils, and to produce a Standard Operating Procedure (SOP) for this instrument.

2. To apply this refined methodology to a range of soils contaminated with heavy metals and organics.

3. To analyse the MSIR responses to assess the discriminatory power of the approach, in terms of its ability to separate soils on the basis of land-use or level of contamination.

4. To review the potential suitability of the approach as a generally applicable diagnostic test for soil quality or as a biological test in Tier 2 of the Environment Agency's Ecological Risk Assessment Framework.

2 Methods

2.1 Approaches

2.1.1 Soil respiration

Carbon dioxide (CO_2) emitted from soils can originate via a number of mechanisms, including microbial and faunal respiration, free enzyme activity, and from abiotic sources such as acid reaction with carbonates. In soils that contain low concentrations of carbonate and with pH <= 7, it is conventionally accepted that the majority of such CO_2 is biotic in nature, reflecting catabolic activity, and is termed soil respiration. In this report, we simply use the term soil respiration to denote gross CO_2 evolution from soils and do not distinguish between the different potential origins. The rate at which CO_2 is evolved from soils in an unamended state is termed 'basal' respiration and reflects the underlying gross metabolic activity of the soil community.

2.1.2 Soil

If MSIR profiling is to be useful, it must be sufficiently discriminating to allow the identification of baseline functional profiles for principal land uses and to detect deviations from those baselines as a result of contamination or environmental pressures. To this end, soils derived from a range of sites were studied and grouped into five sets.

- Set 1: 'Baseline' soils from arable, grassland and woodland sites, designed to give a wide ecological range. These were used to develop the SOP.

- Set 2: Soils variously contaminated and derived from contrasting sites.

  - Set 2a: Soils derived from a landscape-scale gradient of heavy metal contamination from the zinc (Zn) smelter at Avonmouth.

  - Set 2b: Soils subjected to biocide application (Dursban®) and associated controls from the NERC Soil Biodiversity field plots at Sourhope.

- Set 3: Soils from the UKWIR/Defra/Environment Agency Sewage Sludge Network. These formed the majority of the samples since the design of this extensive network of field sites carries an appropriate level of replication to robustly field-test the MSIR functional profiling concept.

  - Set 3a: Inter-site comparison – control and high copper (Cu) concentrations at Gleadthorpe, Woburn and Watlington sites.

  - Set 3B: Intra-site study; three levels of control, high and low Cu and Zn, and high cadmium (Cd) at Watlington site.

Details of the origins and basic properties of the soils are given in Table 2.1.

For Sets 1 and 2 at each site or plot, core samples (10 cm in diameter) were taken from each of nine nodes on a W-shaped transect across the area to be sampled. The cores were pooled and the soil passed through a 2-mm sieve, providing one composite soil

sample per site or plot. Set 3 soils were sampled from the prescribed field plots by the Agricultural Development and Advisory Service (ADAS) and passed through a two-mm sieve following delivery. All soils were stored at 4°C prior to use and pre-incubated at 25°C at c. 45% of water-holding capacity for seven days. Preliminary work established that this period was adequate to ensure that post-sampling disturbance effects on microbial activity had subsided.

2.1.3 Respirometry

The principle of the MSIR technique is to add a suite of carbonaceous substrates separately, but simultaneously, to a test soil (each to its own sub-sample) and measure the evolution of CO_2 from each sub-sample. In this study, the aim was to establish detailed time-courses for the respiratory responses. To this end, soil respiration was measured using a Rapid Automated Bacterial Impedance Technique (RABIT®) instrument (Don Whitley, Bradford U.K.; http://www.dwscientific.co.uk/; Figure 2.1a), which was originally designed to measure the electrical impedance of microbial broth cultures. The system is based on arrays of polypropylene cells, each with a pair of electrodes located in the base (Figure 2.1b). The impedance between these electrodes is measured and recorded with high precision at prescribed intervals by a data-logging computer. In the configuration used here, the electrodes are embedded in an agar gel containing an alkali, and soil samples are introduced into the tubes and sealed (Figure 2.1c). As CO_2 is evolved from the soil, it reacts with the alkali and causes a decline in conductivity within the gel. There is a well-established and direct relationship between the change in impedance and the quantity of dissolved CO_2. The system used in this study had a capacity of 96 tubes.

2.1.4 Substrates

The principle of the MSIR method allows for any number of substrates to be used in profiling, and these can be prescribed according to the particular needs of the study. In practice, limitations are set by the capacity for respirometry and the time required to prepare samples. In this study, a set of 31 substrates was used, plus an unamended control (Table 2.2). They were chosen based upon the substrates adopted in the range of Biolog plates and previous MSIR work (Degens and Harris 1997, Campbell *et al*. 2003).

Table 2.1 Locations and basic properties of soils

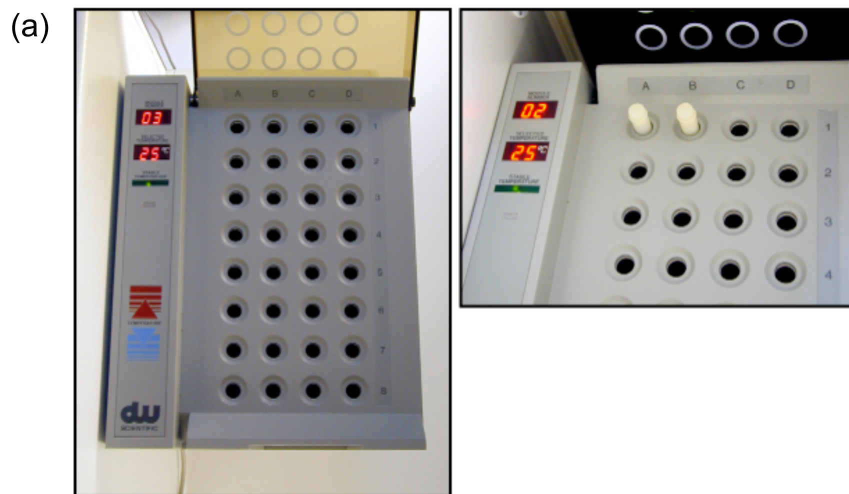
Ref	Set and Location	Description	Abbreviation	NS Grid Ref	pH	WHC (ml/g)	N (%)	C (%)	C/N
Set 1									
1	Silsoe Farm	Arable	ST1_ARB	TL 075358	6.2	0.35	0.12	1.1	9.2
2	Silsoe Farm	Grassland	ST1_GRS	TL 071358	7.0	0.78	0.18	1.9	10.3
3	Chicksands Wood	Woodland (deciduous)	ST1_DCW	TL 095398	6.6	0.89	0.39	5.2	13.3
4	Chicksands Wood	Woodland (coniferous)	ST1_CFW	TL 096396	4.8	0.74	0.34	4.6	13.6
5	Maulden Wood	Sandy heath	ST1_SDY	TL 066382	5.4	0.33	0.09	1.0	12.0
Set 2a: Avonmouth									
6	Over	8.1 km from Zn smelter	AVN_TS_1	ST 595827	5.8	0.78	0.45	4.9	10.7
7	Easter Compton	5.6 km from smelter	AVN_TS_2	ST 568826	7.0	0.91	0.56	7.2	12.8
8	Hallen Hill	3.2 km from smelter	AVN_TS_3	ST 554802	6.4	1.45	0.75	9.8	13.0
9	Near Avonmouth	1.5 km from smelter	AVN_TS_4	ST 535797	5.8	0.83	0.36	3.7	10.5
10	Avonmouth	0.85 km from smelter	AVN_TS_5	ST 532789	6.1	0.99	0.40	4.3	10.5
Set 2b: NERC Field Experiment									
11	Sourhope	Control (Plot 1D)	SHP_Con_1	NT 844206	3.1	2.58	1.28	18.2	14.1
12	Sourhope	Control (2F)	SHP_Con_2	NT 844206	3.8	3.31	1.40	19.4	13.9
13	Sourhope	Control (3E)	SHP_Con_3	NT 844206	3.3	2.72	1.19	15.0	12.6
14	Sourhope	Control (4A)	SHP_Con_4	NT 844206	3.4	2.77	1.16	15.8	13.6
15	Sourhope	Control (5F)	SHP_Con_5	NT 844206	3.4	2.59	1.08	15.0	14.0
16	Sourhope	Biocide (Plot 1E)	SHP_Bio_1	NT 844206	3.1	3.17	1.24	17.6	14.2
17	Sourhope	Biocide (2D)	SHP_Bio_2	NT 844206	3.5	2.56	1.12	14.6	13.1
18	Sourhope	Biocide (3C)	SHP_Bio_3	NT 844206	3.8	2.37	0.94	12.3	13.1
19	Sourhope	Biocide (4B)	SHP_Bio_4	NT 844206	3.3	3.77	1.49	20.1	13.5
20	Sourhope	Biocide (5E)	SHP_Bio_5	NT 844206	3.5	2.47	1.12	14.9	13.3

Table 2.1 continued

Ref	Set and Location	Description	Abbreviation	NS Grid Ref	pH	WHC (ml/g)	N (%)	C (%)	C/N
Set 3a: Sewage Sludge Trial									
21	Gleadthorpe	Control B1	GLD_Con_1	SK 593702	5.6	0.44	0.16	1.7	10.7
22	Gleadthorpe	Control B2	GLD_Con_2	SK 593702	5.7	0.45	0.14	1.5	10.9
23	Gleadthorpe	Control B3	GLD_Con_3	SK 593702	6.2	0.40	0.14	1.5	10.8
24	Gleadthorpe	Cu high B1	GLD_Cu_1	SK 593702	6.0	0.46	0.24	2.5	10.5
25	Gleadthorpe	Cu high B2	GLD_Cu_2	SK 593702	5.6	0.49	0.29	3.0	10.4
26	Gleadthorpe	Cu high B3	GLD_Cu_3	SK 593702	5.6	0.45	0.22	2.3	10.2
27	Woburn	Control B1	WOB_Con_1	SP 948332	6.0	0.34	0.11	1.3	11.7
28	Woburn	Control B2	WOB_Con_2	SP 948332	6.3	0.30	0.11	1.3	11.5
29	Woburn	Control B3	WOB_Con_3	SP 948332	6.6	0.32	0.11	1.6	14.8
30	Woburn	Cu high B1	WOB_Cu_1	SP 948332	6.3	0.43	0.24	2.7	11.0
31	Woburn	Cu high B2	WOB_Cu_2	SP 948332	6.3	0.34	0.22	2.4	10.9
32	Woburn	Cu high B3	WOB_Cu_3	SP 948332	6.4	0.42	0.26	2.8	10.7
33	Watlington	Control B1	WAT_Con_1	SU 685945	6.1	0.58	0.15	1.4	9.2
34	Watlington	Control B2	WAT_Con_2	SU 685945	6.3	0.41	0.14	1.4	9.6
35	Watlington	Control B3	WAT_Con_3	SU 685945	6.1	0.51	0.16	1.5	9.8
36	Watlington	Cu high B1	WAT_Cu_1	SU 685945	5.9	0.51	0.25	2.3	9.4
37	Watlington	Cu high B2	WAT_Cu_2	SU 685945	5.9	0.59	0.24	2.4	9.9
38	Watlington	Cu high B3	WAT_Cu_3	SU 685945	6.4	0.53	0.27	2.6	9.6

Table 2.1 continued

Ref	Set and Location	Description	Abbreviation	NS Grid Ref	pH	WHC (ml/g)	N (%)	C (%)	C/N
Set 3B									
39	Watlington	Control (TR1) B1	WTL_Con_1	SU 685945	5.9	0.45	0.14	1.3	9.2
40	Watlington	Control (TR1) B2	WTL_Con_2	SU 685945	5.9	0.43	0.14	1.4	9.9
41	Watlington	Control (TR1) B3	WTL_Con_3	SU 685945	5.8	0.48	0.17	1.7	10.2
42	Watlington	Digested sludge (TR2) B1	WTL_Dig_1	SU 685945	5.7	0.62	0.32	2.9	9.1
43	Watlington	Digested sludge (TR2) B2	WTL_Dig_2	SU 685945	5.6	0.54	0.30	2.8	9.3
44	Watlington	Digested sludge (TR2) B3	WTL_Dig_3	SU 685945	5.7	0.55	0.31	2.8	9.3
45	Watlington	Raw cake (TR3) B1	WTL_Raw_1	SU 685945	6.3	0.65	0.30	2.8	9.3
46	Watlington	Raw cake (TR3) B2	WTL_Raw_2	SU 685945	6.2	0.59	0.28	2.5	8.7
47	Watlington	Raw cake (TR3) B3	WTL_Raw_3	SU 685945	6.0	0.54	0.25	2.3	9.2
48	Watlington	Zn low (TR4) B1	WTL_ZnL_1	SU 685945	5.9	0.66	0.36	3.4	9.4
49	Watlington	Zn low (TR4) B2	WTL_ZnL_2	SU 685945	5.9	0.64	0.30	2.9	9.6
50	Watlington	Zn low (TR4) B3	WTL_ZnL_3	SU 685945	6.0	0.56	0.30	2.9	9.6
51	Watlington	Zn high (TR7) B1	WTL_ZnH_1	SU 685945	6.0	0.63	0.28	3.0	10.4
52	Watlington	Zn high (TR7) B2	WTL_ZnH_2	SU 685945	6.0	0.58	0.28	2.9	10.5
53	Watlington	Zn high (TR7) B3	WTL_ZnH_3	SU 685945	6.0	0.56	0.29	3.1	10.6
54	Watlington	Cu low (TR8) B1	WTL_CuL_1	SU 685945	6.1	0.58	0.28	2.6	9.1
55	Watlington	Cu low (TR8) B2	WTL_CuL_2	SU 685945	6.2	0.58	0.27	2.5	9.2
56	Watlington	Cu low (TR8) B3	WTL_CuL_3	SU 685945	6.3	0.59	0.27	2.5	9.3
57	Watlington	Cu high (TR11) B1	WTL_CuH_1	SU 685945	5.9	0.60	0.31	3.0	9.6
58	Watlington	Cu high (TR11) B2	WTL_CuH_2	SU 685945	6.0	0.57	0.26	2.6	9.8
59	Watlington	Cu high (TR11) B3	WTL_CuH_3	SU 685945	6.0	0.57	0.28	2.7	9.7
60	Watlington	Cd high (TR15) B1	WTL_CdH_1	SU 685945	5.9	0.64	0.30	2.9	9.6
61	Watlington	Cd high (TR15) B2	WTL_CdH_2	SU 685945	6.0	0.60	0.31	3.0	9.6
62	Watlington	Cd high (TR15) B3	WTL_CdH_3	SU 685945	5.9	0.61	0.31	2.9	9.4



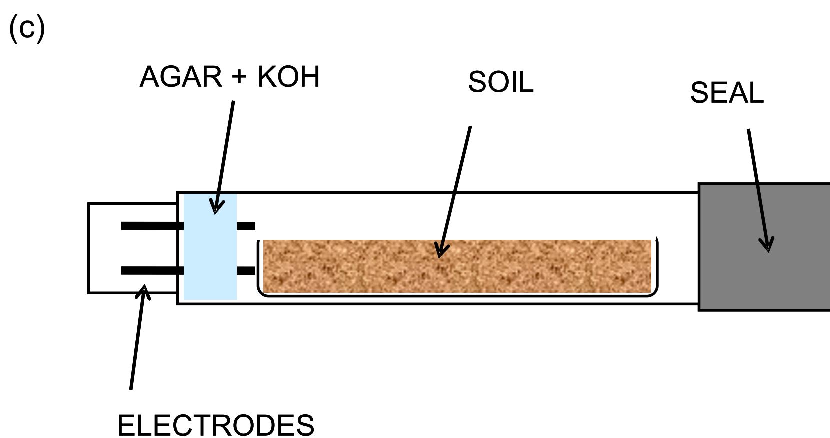


Figure 2.1 Rapid Automated Bacterial Impedance System (RABIT) used to measure soil respiration. (a) Overview of system; each hole in constant-temperature incubation block holds one cell. (b) Detail of cells and glass troughs used to hold soil (length of tube = 12 cm). (c) Configuration of cells.

Table 2.2 Substrate suite used in all assays (final concentration is that manifest in incubation solution at start of incubation).

Abbreviation	Substrate	Final concentration
MGL	B methyl D glucoside	75 mM
BSA	Bovine serum albumen	3.6%
CLB	Cellobiose	75 mM
CTA	Citric acid	100 mM
CDX	Cyclodextrin	0.5%
GLC	D-glucose	200 mM
MLA	DL-malic acid	100 mM
MNL	D-mannitol	75 mM
MNS	D-mannose	75 mM
GLA	Gluconic acid	100 mM
GLY	Glycogen	100 mM
ERY	I-erythritol	75 mM
ARG	L-arginine	15 mM
ASC	L-ascorbic acid	100 mM
ASP	L-asparagine	15 mM
GLA	L-glutamic acid	15 mM
GLM	L-glutamine	15 mM
HST	L-histidine	15 mM
LYS	L-lysine	15 mM
PHN	L-phenylalanine	15 mM
SER	L-serine	15 mM
MAL	Malonic acid	100 mM
PNT	Pantothenic acid	100 mM
QNA	Quinic acid	100 mM
STC	Starch	0.5%
SNC	Succinic acid	100 mM
TWN	Tween 80	1%
URE	Urea	100 mM
WAT	Water	
XYL	Xylose	75 mM
KBA	α-ketobutyric acid	100 mM
KGA	α-ketoglutaric acid	100 mM

2.2 Development of basic method

The non-standard application of the RABIT system for MSIR functional profiling of soils was developed as part of this project. Soil Sets 1 and 2a were used in the development process, in order to optimise procedures and test the reproducibility of the system. The laboratory SOP relating to application of the RABIT system for MSIR is given in Appendix II. Profiling 96 samples (aliquots) takes one fully experienced technician between one and two days to complete all operations, if starting with sieved soil. Weighing aliquots of soils into assay boats is the most laborious step, and the friability of the soil has a large impact on this procedure. Hence, heavy-textured soils tend to be problematic in this respect.

2.2.1 Data analysis and visualisation

The raw data accrued when a soil is profiled using the SOP comprise a series of conductivity values (µS) taken every six minutes between 0.5 hours and 16 hours for each sample. Since the initial conductivity in each cell is different, values were first normalised by subtracting the initial value from each successive reading. Each soil/substrate combination was replicated three times within each incubation run. Arithmetic means of these replicates were calculated and plotted as cumulative respiration curves (Figure 2.2a). Conductivity values for all substrates were then corrected for basal respiration by subtracting the mean signal for unamended controls (for instance, water [WAT] treatment) from the mean signal relating to each substrate. These values were subsequently converted to µg CO_2-C using a conversion factor derived empirically (Appendix III). Respiration rates were calculated for each six-minute time interval, corrected for dry mass of soil to normalised units of µg CO_2-C g^{-1} h^{-1} and plotted as rate curves (Figure 2.2b). Rates attributed to each substrate are therefore the mean of three internally-consistent determinations. It is not appropriate to view the three determinations made for each soil/substrate combination as replicates since the association with individual instances of control rates is arbitrary. Cumulative data was further expressed as carbon (C) evolved as a proportion of that added in the substrate, assuming no priming effect (such as enhanced mineralisation of native soil C in substrate-induced soils).

The resultant data sets can be analysed in a variety of ways. In principle, respiration rate curves follow a universal sigmoidal pattern (Anderson and Domsch 1978, ISO 2002). However, in this study a wide variety of curves were manifest (Figure 2.3), severely confounding attempts to summarise them by means of standard curve-fitting methods. Although a variety of methods were explored, the fundamental requirement of independence of shape meant that curve-fitting proved untenable. Hence, the approach adopted was to calculate mean respiration rates over a series of time intervals (Table 2.3). A factor based on the integral of the cumulative curves was also calculated. This total area under the curve (TAUC) approach has been adopted in previous work using Biolog profiling (Hackett and Griffiths 1997). An evenness parameter was calculated using the rates between two hours and six hours based on the Simpson-Yule index – $E=1 / \Sigma p^2_i$ where pi is the respiration response to individual substrates as a proportion of total respiration activity induced by all substrates (Magurran 1988). This parameter ranges from zero (no utilisation) to the maximum number of substrates involved (equitable utilisation of all substrates) and is widely used in a variety of studies (such as Degens *et al*. 2000). Examples of the summarised data output are given in Figures 2.2c–e. The full set of such outputs relating to all profiled soils is presented in Appendix 1.

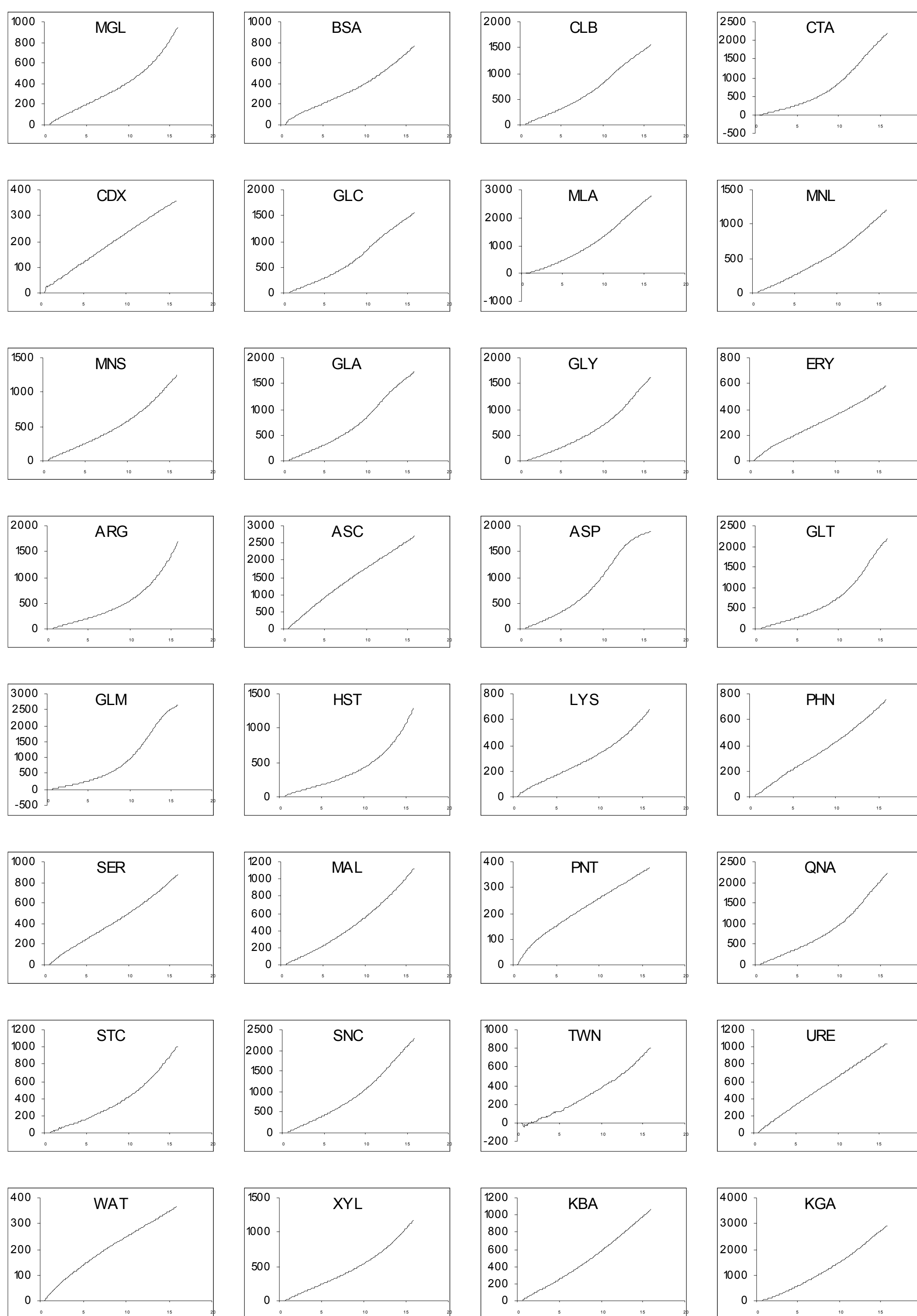


Figure 2.2 Example output and derived parameters from respirometry. Notes: Time-courses over 16 hours of cumulative respiration from arable soil (Set 1). Three-letter abbreviations relate to substrates as listed in Table 2.2. All ordinates are scaled according to their respective maxima: units are µS in respiration cell.

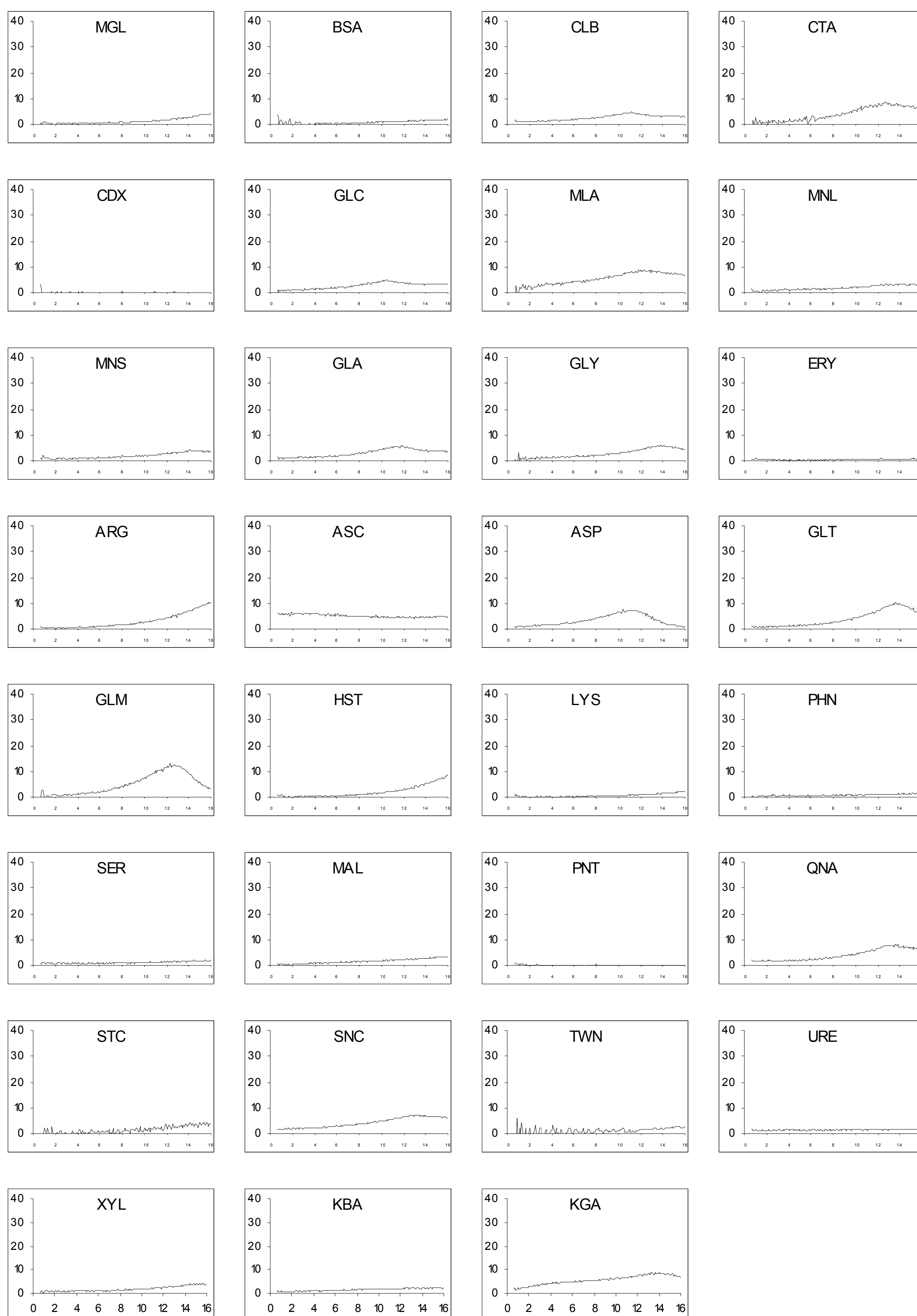


Figure 2.2 continued.
Notes: Time-courses over 16 hours of respiration rates from arable soil (Set 1). Three-letter abbreviations relate to substrates as listed in Table 2.2. All ordinates have the same scale: units are µg CO_2-C g^{-1} h^{-1}.

Sample: ST1_ARB

BSL	0.7	**D**	15.5
SIR	1.9		

	MGL	BSA	CLB	CTA	CDX	GLC	MLA	MNL	MNS	GLA	GLY	ERY	ARG	ASC	ASP	GLT
Rate T_{2-6}	0.4	0.3	1.4	1.3	-0.1	1.4	3.1	1.1	0.9	1.5	1.3	0.3	0.6	5.7	1.8	1.2
Rate $T_{0.5-4}$	0.4	0.5	1.2	0.7	-0.2	1.0	2.1	0.8	0.8	1.2	0.8	0.4	0.5	5.9	1.3	0.9
Rate T_{4-8}	0.6	0.4	1.9	2.1	0.0	2.0	4.0	1.4	1.1	2.0	1.7	0.3	1.0	5.3	2.7	1.7
Rate T_{8-12}	1.1	0.9	3.7	5.4	0.1	3.9	6.7	2.0	2.1	4.3	3.1	0.5	2.7	4.7	6.0	4.4
Rate T_{12-16}	2.9	1.6	3.3	7.3	0.0	3.3	7.7	3.0	3.5	4.1	5.2	0.7	7.1	4.6	3.0	8.3
Util% T_4	0.1	0.0	0.1	0.1	-0.1	0.1	0.4	0.1	0.1	0.1	0.1	0.1	0.3	0.6	1.4	0.8
Util% T_8	0.1	0.1	0.3	0.3	-0.1	0.2	1.1	0.3	0.3	0.4	0.3	0.2	1.2	1.3	4.8	2.5
Util% T_{12}	0.3	0.1	0.6	1.0	0.0	0.4	2.4	0.7	0.7	0.9	0.7	0.3	3.5	1.9	12.3	7.0
Util% T_{16}	0.7	0.2	0.8	1.9	0.0	0.6	3.8	1.2	1.2	1.4	1.3	0.5	9.4	2.4	15.9	15.2
TAUC	0.8	0.7	2.2	2.7	-0.1	2.2	4.3	1.5	1.4	2.5	1.9	0.4	1.6	5.7	3.0	2.5

	GLM	HST	LYS	PHN	SER	MAL	PNT	QNA	STC	SNC	TWN	URE	XYL	KBA	KGA
Rate T_{2-6}	1.3	0.3	0.2	0.6	0.8	0.8	0.0	1.9	0.3	2.3	0.4	1.4	0.9	1.0	4.0
Rate $T_{0.5-4}$	0.8	0.3	0.2	0.5	0.8	0.5	0.2	1.8	0.1	2.0	-0.3	1.4	0.7	0.8	2.9
Rate T_{4-8}	2.3	0.5	0.2	0.6	0.8	1.2	-0.1	2.4	0.6	2.8	0.7	1.4	1.1	1.2	4.9
Rate T_{8-12}	7.5	1.8	0.7	0.9	1.1	1.9	0.0	4.6	1.6	4.8	1.2	1.5	1.8	1.8	6.2
Rate T_{12-16}	8.6	5.4	1.6	1.3	1.6	2.8	0.0	7.1	3.1	6.7	2.1	1.6	3.3	2.1	7.9
Util% T_4	0.7	0.2	0.1	0.8	1.2	0.1	0.0	0.2	0.0	0.3	0.0	0.9	0.1	0.1	0.4
Util% T_8	3.0	0.7	0.3	1.8	2.6	0.4	0.0	0.5	0.3	0.9	0.1	2.0	0.3	0.4	1.1
Util% T_{12}	10.6	2.2	0.6	3.3	4.5	0.9	0.0	1.1	0.9	1.8	0.3	3.1	0.7	0.7	2.0
Util% T_{16}	19.0	6.6	1.5	5.5	7.2	1.6	0.0	1.9	2.2	3.0	0.6	4.3	1.4	1.1	3.2
TAUC	3.4	1.0	0.4	0.7	1.0	1.2	0.1	3.1	0.8	3.4	0.5	1.5	1.3	1.3	4.9

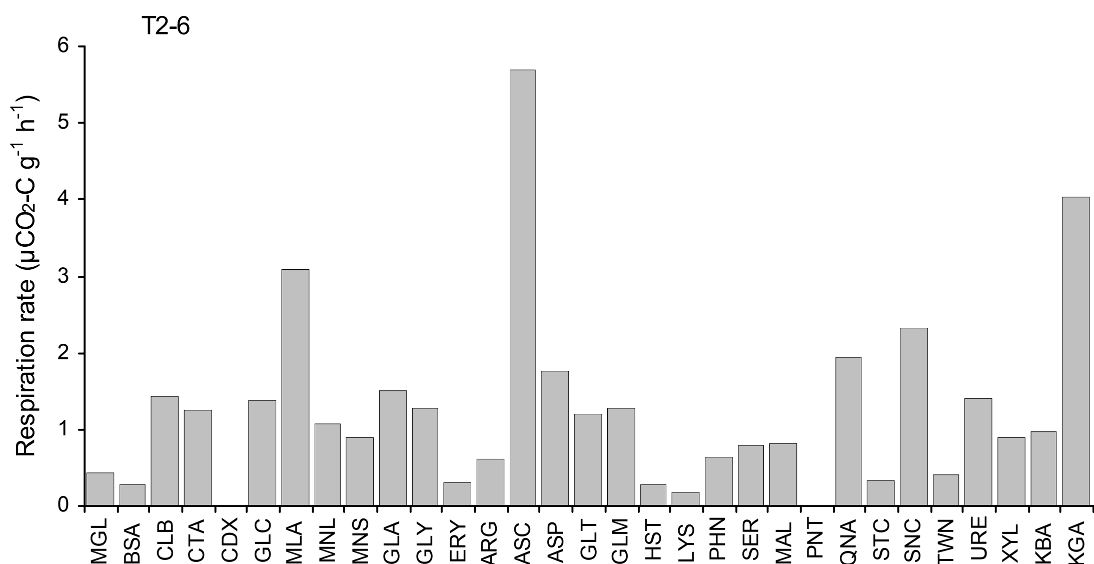


Figure 2.2 continued.
Notes: Derived data from arable soil (Set 1). Three-letter abbreviations relate to substrates as listed in Table 2.2; parameter codes as in Table 2.3.

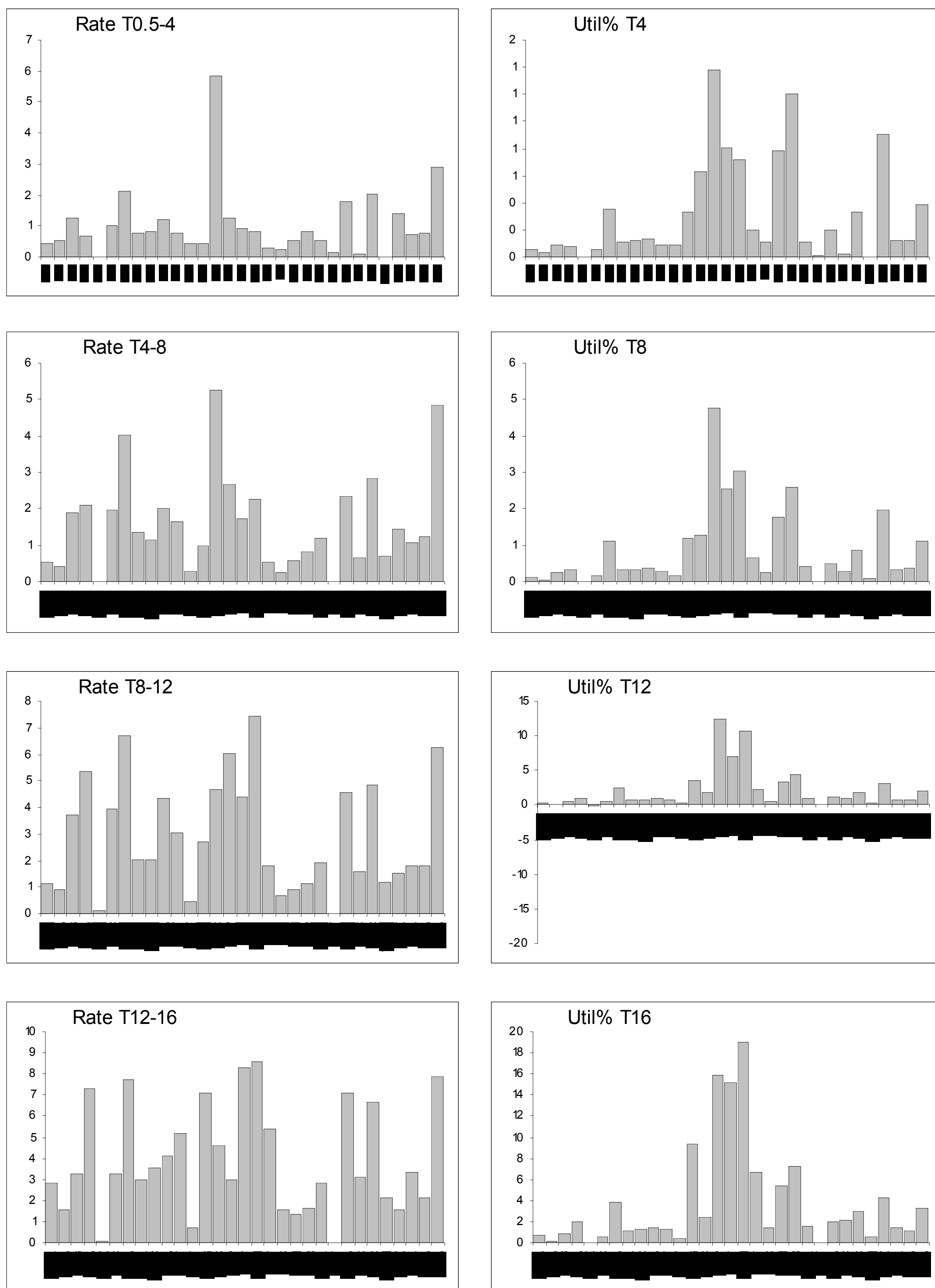


Figure 2.2 continued.
Notes: Derived data from arable soil (Set 1). Three-letter abbreviations relate to substrates as listed in Table 2.2; parameter codes as in Table 2.3. Ordinates scaled according to maxima; units are µg CO_2-C g^{-1} h^{-1}.

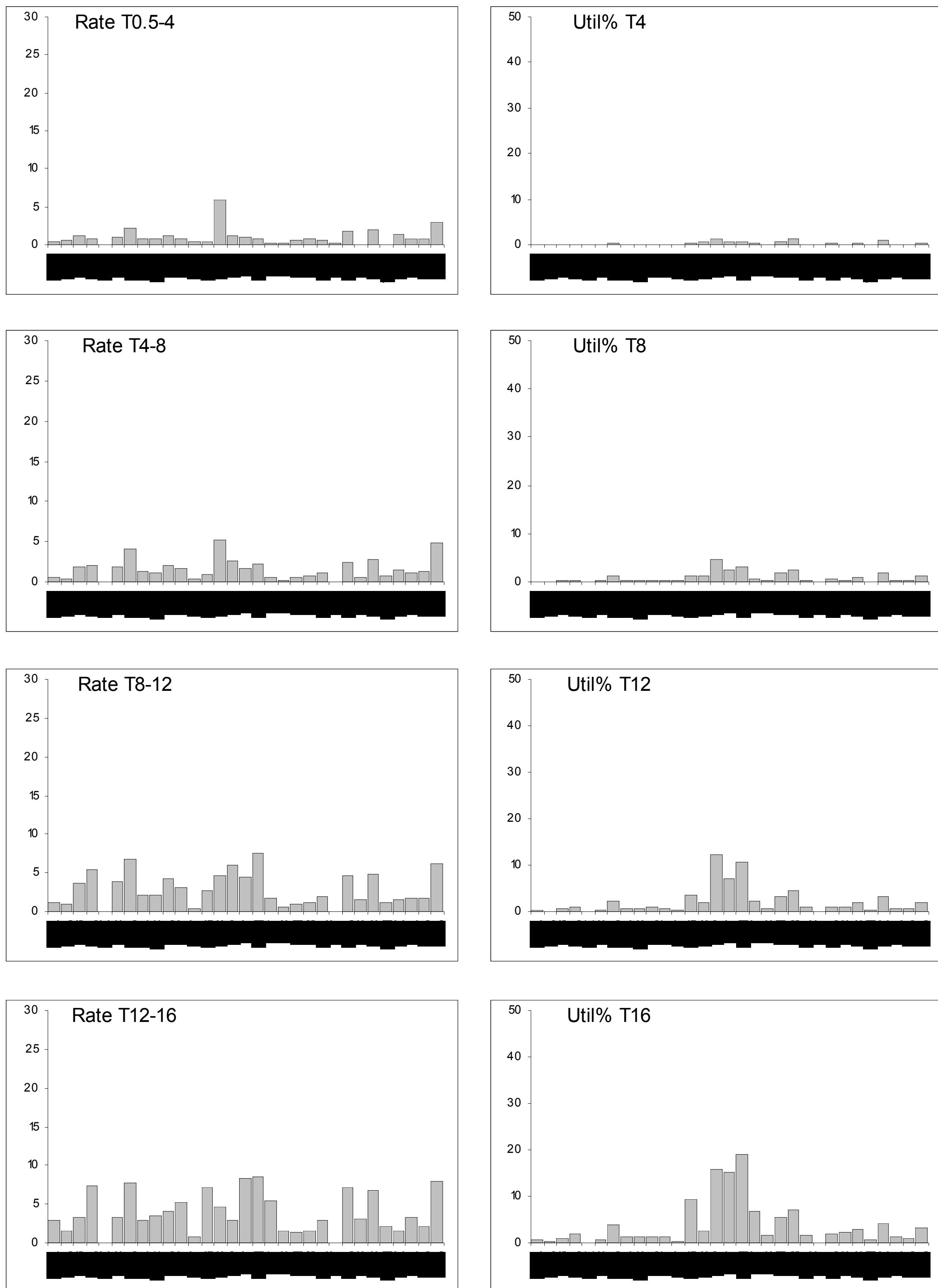


Figure 2.2 continued.
Notes: Derived data from arable soil (Set 1). Three-letter abbreviations relate to substrates as listed in Table 2.2; parameter codes as in Table 2.3. Ordinates within columns have the same scale; units for respiration are µg CO_2-C g^{-1} h^{-1}.

Table 2.3. Summary parameters derived from respiration rate curves.

Abbreviation[1]	Parameter
SIR	Mean respiration rate at 2–5 hours after addition of glucose to soil. This equates to the 'standard' substrate-induced respiration (SIR) rate as defined in ISO17155 (2002). This measure, as adopted in conventional soil microbiology, is deemed to be directly proportional to the microbial biomass.
$Rate{\cdot}T_{2–6}$	Mean respiration rate at 2–6 hours, corrected for basal respiration from unamended controls. This was adopted as a standard unitary SIR rate for the purposes of this study, in order to contrast with the analyses where respiration over 16 hours was incorporated.
	Mean respiration rates over four intervals at 0.5–16 hours incubation, duly corrected for basal respiration over concomitant periods, such as:
$Rate{\cdot}T_{0.5–4}$	… between 0.5 and 4 h
$Rate{\cdot}T_{4–8}$	… between 4.1 and 8 h
$Rate{\cdot}T_{8–12}$	… between 8.1 and 12 h
$Rate{\cdot}T_{12–16}$	… between 12.1 and 16 h
	The amount of C evolved as CO_2 from the soils as a proportion (%) of the amount of C added to the soils as substrate, as manifest at four intervals.
$Util\%{\cdot}T_{4}$	… after 4 h
$Util\%{\cdot}T_{8}$	… after 8 h
$Util\%{\cdot}T_{12}$	… after 12 h
$Util\%{\cdot}T_{16}$	… after 16 h
TAUC	The integral of the cumulative respiration curves ('total area under curve').

[1] as used in data output tables in text and Annex 1

Data reduction was carried out by principal component analysis (PCA), using logical combinations of the parameters. PCA was chosen because it is a robust test of the discriminatory power of the method, carrying no a priori information about the samples' relationships. The method is essentially a data-reduction technique producing a series of values (principal components, PCs) that reduce multivariate data to a few values that capture the essence of the original data (specifically in relation to the samples being compared). If samples have the same multivariate properties, the PCs will be the same; the more divergent the profiles, the less similar the PCs. Loadings provide information about which substrates contribute to such discrimination.

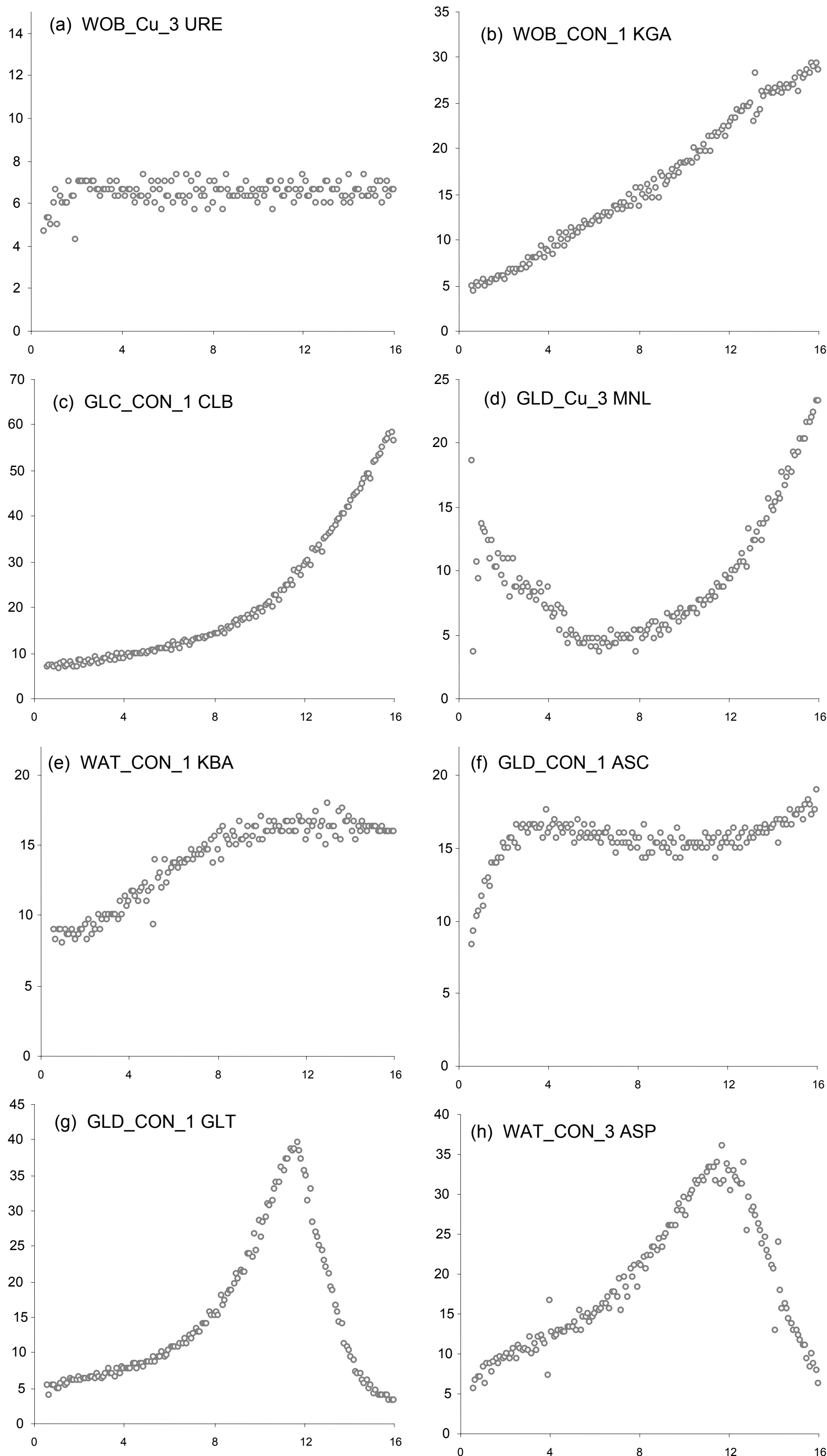


Figure 2.3 Examples of the variety of respiration rate curves obtained for different substrates over 16 hours incubation from soils within sample Set 3a. Notes: Sample codes as in Tables 2.1 and 2.2. Points show rate at six-minute intervals. Ordinates show rates in µg CO_2-C g^{-1} h^{-1}, not to same scale.

3 Results and discussion

3.1 Set 1 Baseline soils

Since one composite soil sample per site was profiled, soils from Set 1 were used primarily for SOP development. The precision of the MSIR assay was assessed by considering the three internal assay replicates of the Rate·T2–4 data, including the unamended control (32 substrates). The mean coefficient of variation (CV) across all substrates was 20% for arable and 10% for grassland; these were the first two soils profiled using the draft SOP. For the remaining three Set 1 soils, profiled using the finalised SOP, the mean CV was 6%.

The profiles can be regarded as pseudo-replicates (not capturing the true variance relating to the environmental factors affecting the soils), but do allow preliminary visualisation of the degree of similarity in MSIR profiles between the different soils. The assay replicates clustered strongly (as the CVs would suggest). There was a distinct separation of soils by both PC1 and PC2, which together accounted for 97% of the variation (Figure 3.1a). PC3 [1% variation] further separated grassland soil from the others.

The loadings demonstrated that many substrates contributed to the separation of samples on PC1. However, PC2, which predominately separated coniferous woodland soils from the others, was dominated by citric acid and ketoglutaric acid (Figure 3.1b). The ranking of these soils in terms of basal and SIR rates followed an ecologically intuitive pattern, with deciduous woodland and grassland soils showing the greatest evenness values (Table 3.1).

Table 3.1 Summary statistics for basal respiration (BSL), substrate induced respiration (SIR), evenness (E) for Set 1a samples

Sample	BSL	SIR	E
	μg CO_2-C g^{-1} h^{-1}		
Arable	0.67	1.94	15.5
Grassland	1.26	5.81	22.2
Deciduous woodland	2.27	9.94	24.2
Coniferous woodland	1.32	3.77	16.5
Sandy heath	0.69	2.38	18.8

3.2 Set 2a heavy metal contaminated soils

Respiration and evenness parameters did not follow a particular trend with distance from the smelter, but Zn concentration in the soil increased with proximity to the smelter (Table 3.2). Metal concentrations were apparently greatest at 3.2 km from the smelter, but this was not statistically verifiable due to the sampling design. The mean CV across all substrates for the three internal assay replicates of the Rate·T2–4 data was 5%, confirming that the SOP was reliable for routine application. PCA analysis of this data demonstrated a distinct separation of soils based on their distance from the smelter (Figure 3.2a), and a notably strong association between location in PC space and the Zn concentration measured in the soils (Figure 3.2a). Loadings were complex in relation to PC1, but high for urea and ascorbic acid separating Location 3 soils from the remainder in PC2, and KGA separating Location 1 soils in PC3 (Figure 3.2b).

Table 3.2 Summary statistics for basal respiration (BSL), substrate induced respiration (SIR), evenness (E) and Zn concentration for Avonmouth samples (Set 2a)

Sample	BSL	SIR	E	Zn
	μg CO_2-C g^{-1} h^{-1}			μg g^{-1}
8.1 km from Zn smelter	1.12	6.76	20.6	529
5.6 km from smelter	1.87	12.0	23.1	849
3.2 km from smelter	2.12	9.75	24.9	3465
1.5 km from smelter	1.07	6.14	23.2	2386
0.85 km from smelter	1.27	7.17	22.1	2635

3.3 Set 2b Biocide contaminated soils

There was no effect of biocide on basal respiration and SIR or other soil properties (Table 3.3) in the Sourhope soils. The respiration profiles of these upland grassland soils were markedly different to those measured in all other samples within this study, showing considerably greater respiration rates for many substrates and contrasting rate curves in the MSIR assay. These properties were manifest across all samples. The sampling design of these soils was such that true field-level replication was attained, such that the effect of biocide could be tested. There was no significant effect of biocide treatment upon MSIR profiles, using either Rate·T2–6 data or the four-interval data for PC1 or PC2 (Figure 3.3) or the remaining PCs (data not shown). The reason for the particular respiratory behaviour of these soils could not be established within the confines of this project. It was notable that the respiration curves for the GLC (standard SIR) substrate were unusual and that production of CO_2 from urea-amended soils was extremely high. This may be attributable to the soil being grazed pasture and historically subject to repeated urine inputs.

Table 3.3 Summary statistics for basal respiration (BSL), substrate induced respiration (SIR), evenness (E) and chemical soil properties for Set 2b samples

Treatment	BSL	SIR	E	pH	%C	%N
	μg CO_2-C g^{-1} h^{-1}					
Control	1.66	12.6	19.8	3.43	15.9	1.18
Biocide	1.99	12.7	19.5	3.40	16.7	1.22
Pooled s.e.	0.29	1.2	0.23	0.11	1.15	0.08
P_t	ns	ns	ns	ns	ns	ns

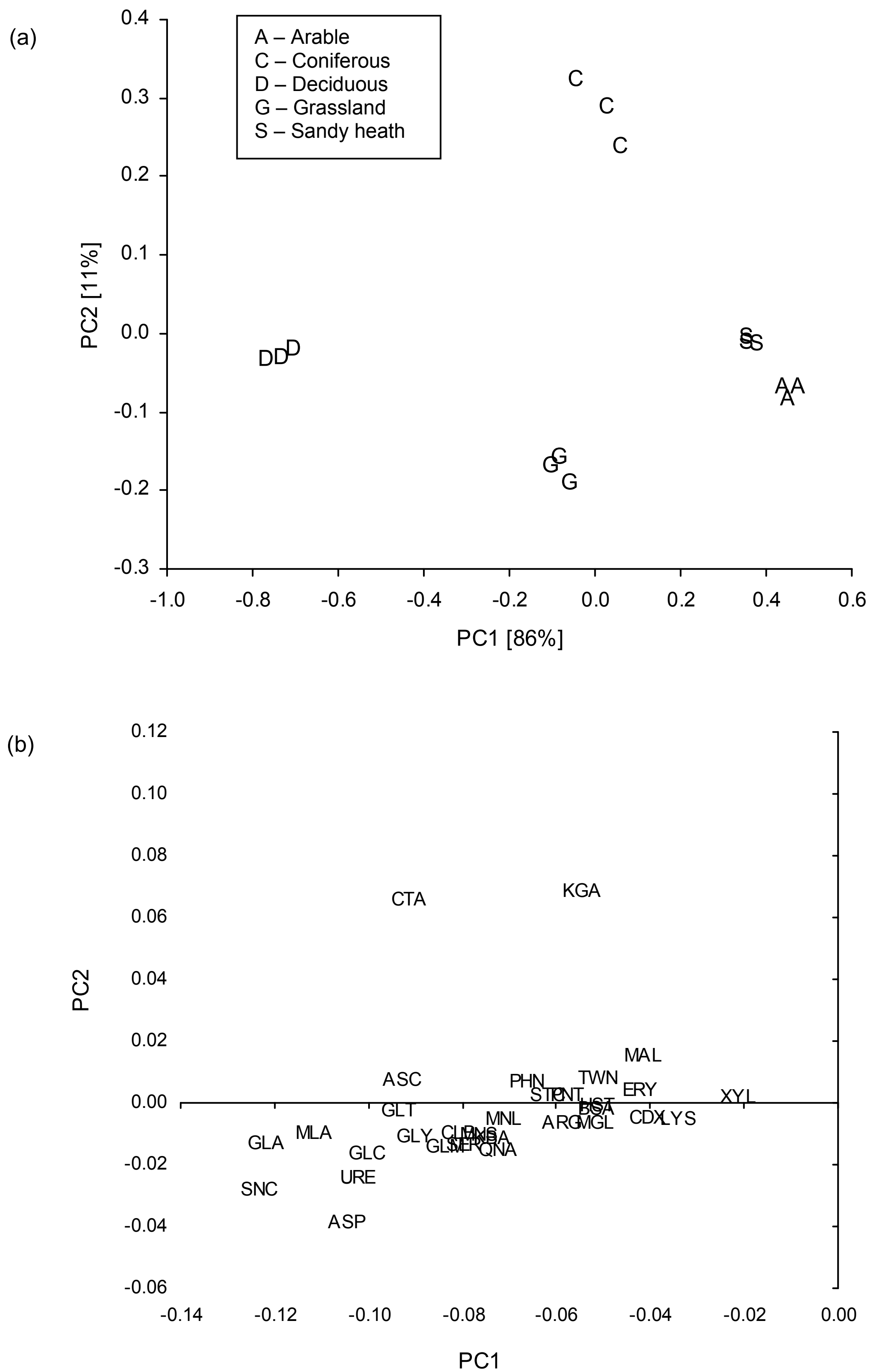
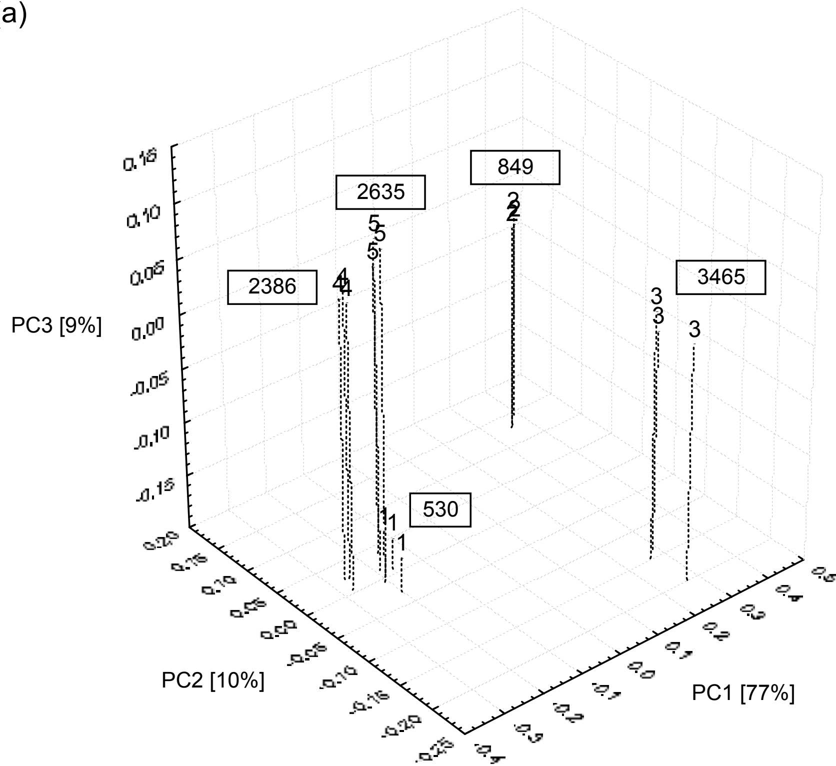


Figure 3.1 Principal component plot for Set 1 soils. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.



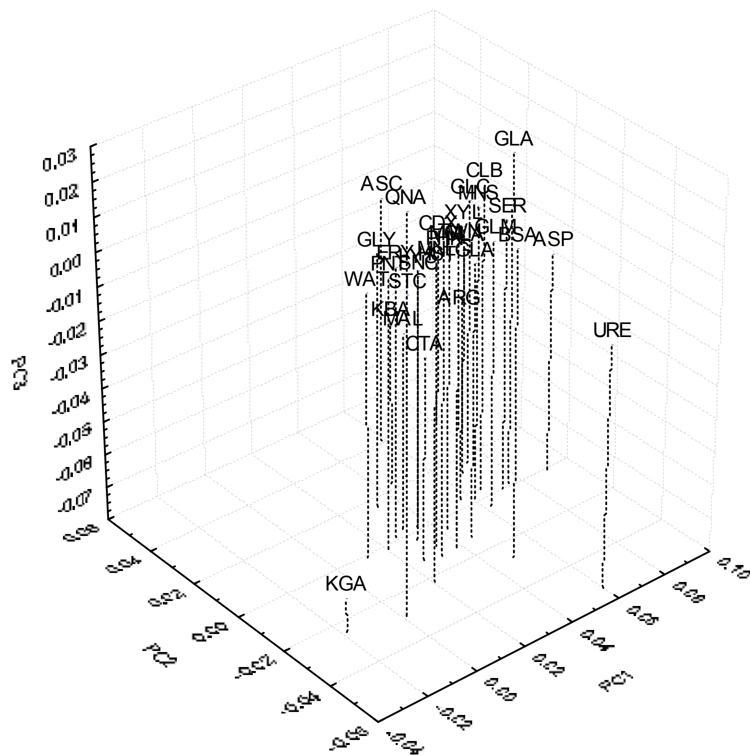


Figure 3.2 Principal component plot for Set 2A soils. (a) Principal components, 1=distal, 5 = proximal to smelter; values in boxes are soil Zn concentrations [µg g^{-1}]. (b) Loadings; abbreviations for substrates as in Table 2.2.

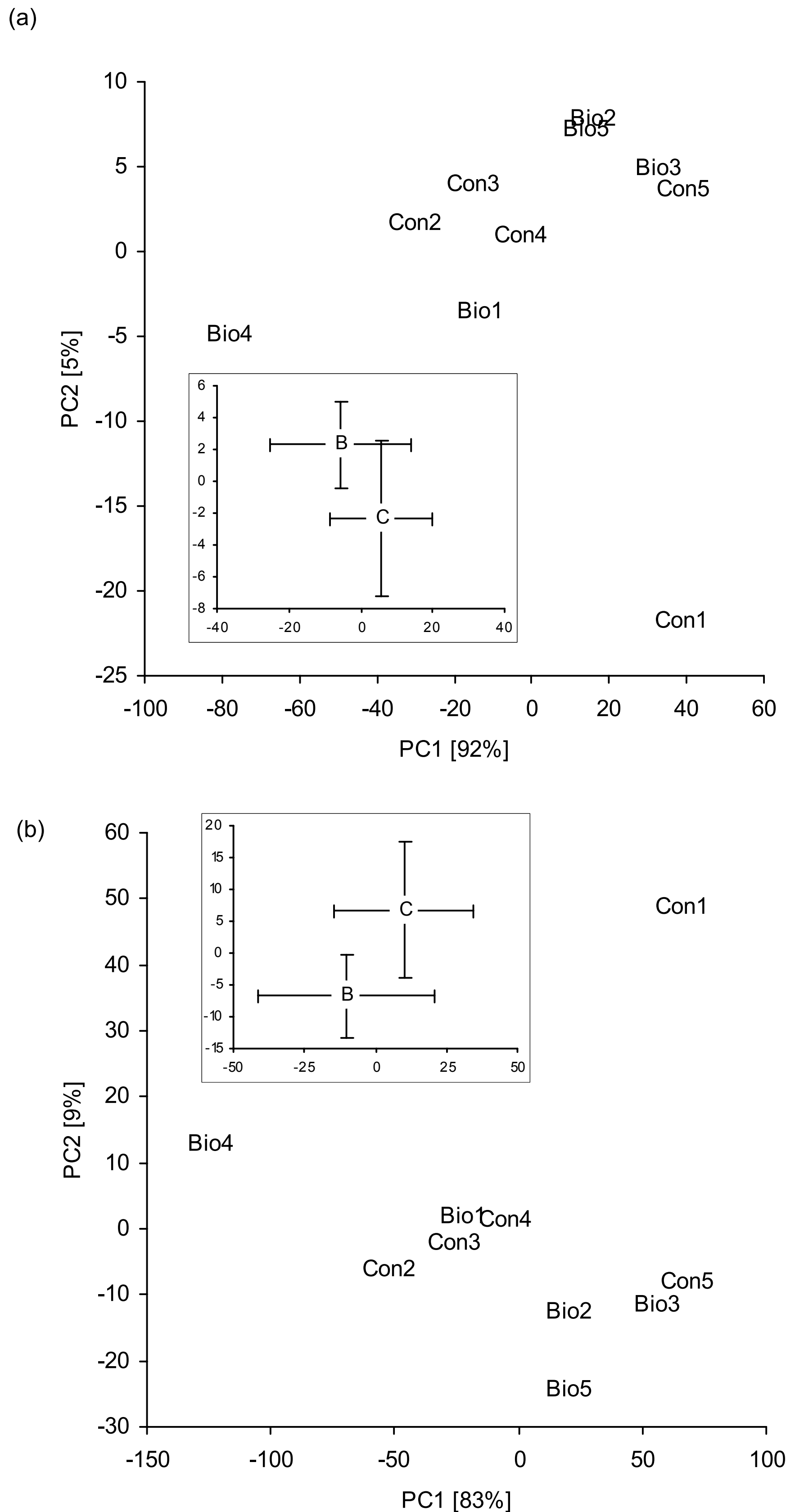


Figure 3.3 Principal component plot for Sourhope (Set 2B) soils. (a) Based on respiration rates $T_{2–6}$. (b) Based on rates at four intervals across $T_{0.5–16}$. Notes: Con = control plots; Bio = biocide treated plots. Insets show means with associated standard errors.

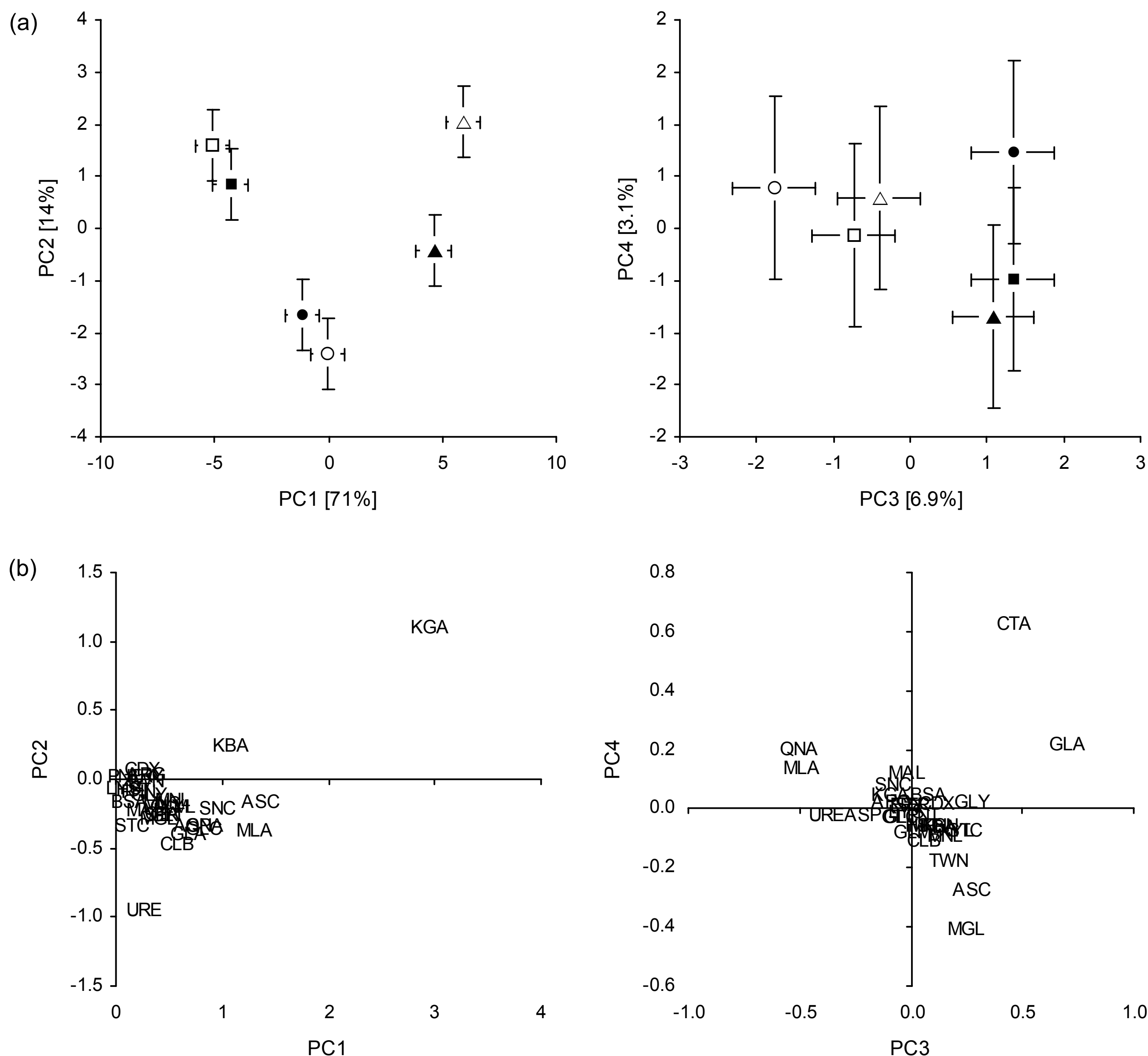
3.4 Set 3a sewage sludge network soils (inter-site)

The sampling design for this set of soils was such that a factorial analysis of parameters could be attained. For basal respiration and SIR, and other soil properties, there were a variety of site and metal effects but no significant interactions (Table 3.4).

Table 3.4 Summary statistics for respiration, evenness and soil parameters for Sewage Sludge samples (Set 3a)

	Gleadthorpe	Watlington	Woburn	Metal mean	ems	P_F		
						Site	Metal	Site x Metal
(a) Basal respiration (µg CO_2-C g^{-1} h^{-1})					0.002	***	***	ns
Control	0.55	0.54	0.51	0.53				
Cu	0.66	0.66	0.63	0.65				
Site mean	0.6	0.6	0.57					
(b) SIR (µg CO_2-C g^{-1} h^{-1})					0.15	***	ns	ns
Control	3.6	3.97	3.3	3.62				
Cu	3.5	3.94	3.28	3.57				
Site mean	3.55	3.95	3.29					
(c) Evenness					0.87	ns	**	ns
Control	20.5	19	19.9	19.8				
Cu	21.5	21.2	21.5	21.4				
Site mean	21	20.1	20.7					
(d) pH					0.05	**	ns	ns
Control	5.84	6.32	6.10	6.09				
Cu	5.74	6.33	6.05	6.04				
Site mean	5.79	6.32	6.08					
(e) %C					0.04	ns	***	ns
Control	1.57	1.39	1.46	1.48				
Cu	2.57	2.62	2.54	2.58				
Site mean	2.07	2.01	2.00					
(f) %N					0.000 30	ns	***	ns
Control	0.15	0.11	0.13	0.13				
Cu	0.25	0.24	0.25	0.25				
Site mean	0.20	0.18	0.19					

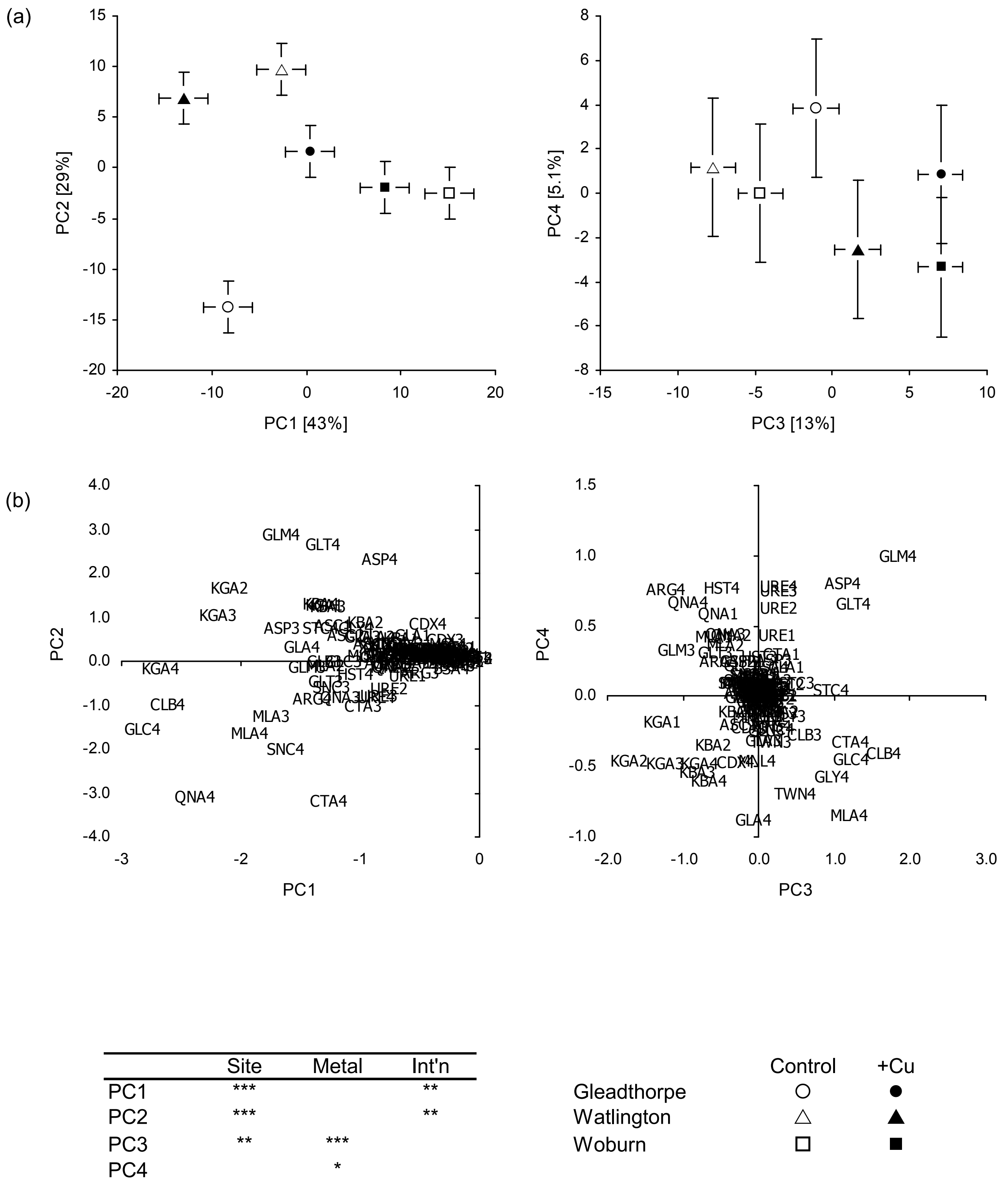
The full suite of parameters listed in Table 2.3 was investigated for discriminatory power of MSIR. PCA based on Rate·T2–6 x discriminated by site with PC1 and PC2, and metal in PC3 (Figure 3.4a). When based on the four-interval data, there was significant site x metal interactions for PC1 and PC2, distinct separation of site and metal for PC3, and metal for PC4 (Figure 3.5a).



	Site	Metal	Int'n
PC1	***		
PC2	***		
PC3		***	
PC4			

	Control	+Cu
Gleadthorpe	○	●
Watlington	△	▲
Woburn	□	■

Figure 3.4 Principal component plot for Sewage Sludge trial (Set 3A) soils, based on Rates·$T_{2–6}$. (a) Principal components. (b) Loadings; abbreviations as in Table 2.2.



	Site	Metal	Int'n
PC1	***		**
PC2	***		**
PC3	**	***	
PC4		*	

Figure 3.5 Principal component plot for Sewage Sludge trial (Set 3A) soils, based on respiration rates at four intervals across $T_{0.5–16}$. (a) Principal components. (b) Loadings; three-letter abbreviations for substrates as in Table 2.2; numbers following these denote time intervals for respiration rates where 1 = $T_{0.5–4}$, 2 = $T_{4–8}$, 3 = $T_{8–12}$, 4 = $T_{12–16}$.

When rates were expressed as Util%, the nature and extent of discrimination varied according to the incubation time being considered. Effects tended to strengthen after 4 hours, with interaction terms becoming significant in relation to the 12 hour and 16 hour data (Figures 3.6–3.9). PC4 did not significantly discriminate Util% data in any case. TAUC data significantly discriminated between sites in PC1, giving a site x metal interaction in PC2 and a metals effect in PC3 (Figure 3.10). This method of summarising the respiration curves appeared to give notably ‘clean’ discrimination in the PC plots. In general, Util% data gave more complex responses in relation to the pattering within PC plots.

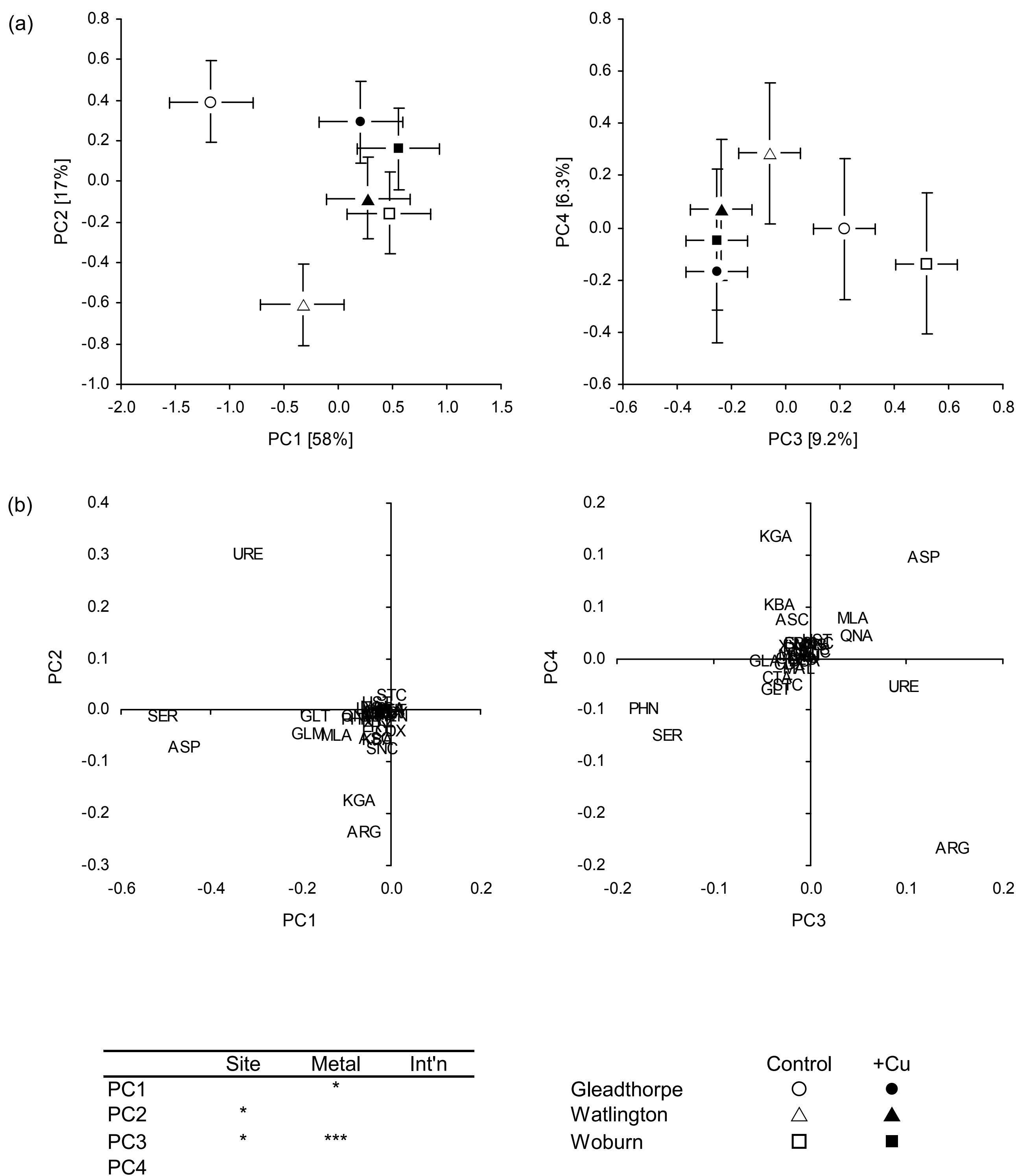
Substrates that contributed to the greatest extent in loadings are summarised in Table 3.5. Substrates that featured most frequently were ketoglutaric acid and asparagine (with 15 and nine instances in Table 3.5 respectively), followed by arginine, glutamine, urea and serine.

3.5 Set 3b sewage sludge network soils (intra-site)

There was no difference in basal respiration between any of the Watlington soils, but SIR and other soil properties all showed highly significant treatment effects (Table 3.6). The carbon content of soils was significantly greater where sludge had been applied, and double where metal-containing sludges were applied (Table 3.6). Evenness was significantly lower in control soils than all others (Table 3.6). PCA analysis revealed a wide variety of patterns of distinct and significant discrimination between many of the soils, but the nature of the sampling regime and the large series of samples resulted in quite complex patterns (Figures 3.11–3.14). Significant differences between samples were apparent in all cases for PCs1–3, and for PCs1–4 from the four-interval data (Figure 3.12). Generally, no-sludge control soils were distinctly separated from all others, but not always by the same PC (Figures 3.11–3.14). There was also a general trend that metal-treated soils separated from the controls and that low metal rates separated from high metal rates (Figures 3.11-3.14). However, formal testing of this trend is difficult because ad hoc aggregation of the treatment factors is statistically unsound.

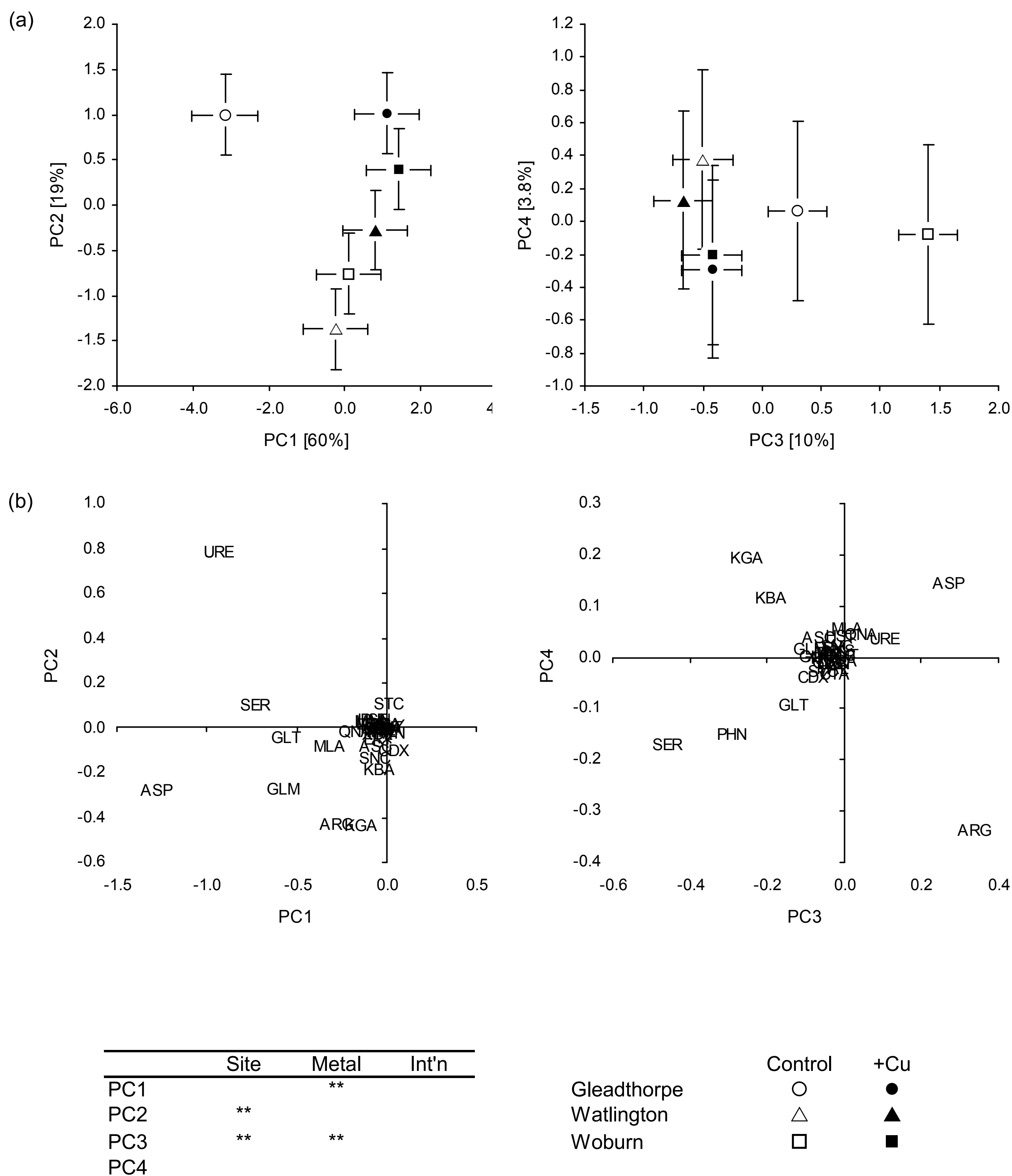
3.6 All samples

As a further test of the discriminatory power of the MSIR assay, all samples were combined in one PCA using Rate·T2–6 data. The samples were separated distinctly and highly significantly according to the Set, with Sourhope samples being particularly distinct (Figure 3.15). This is to be expected on the basis of the ‘unusual’ respiration profiles generated by the upland grassland soil. The standard errors for the Sewage Sludge soils were particularly small, as would be expected given their coherent location. However, the variance of both Set 1 and Set 2a samples was also notably small given the regional spread of their origins. Soils derived from Watlington in both Set 3a and Set 3b were most similar within the Sewage Sludge series in both PC1 and PC2 (Figure 3.15). A range of substrates carried high loadings for both PC1 and PC2, but these all feature in the dominant substrates recorded for Set 3a profiling (Table 3.5).



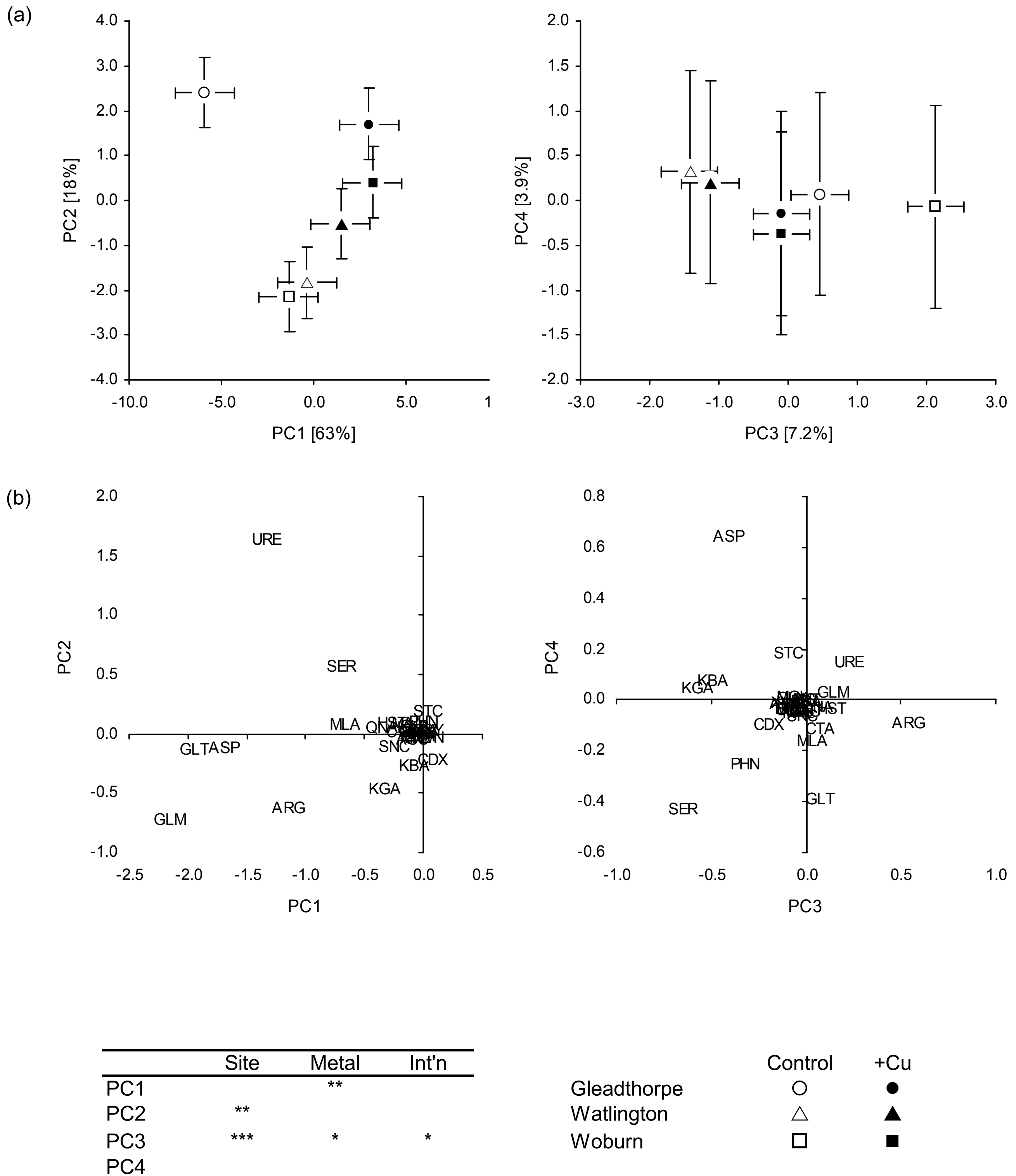
	Site	Metal	Int'n
PC1		*	
PC2	*		
PC3	*	***	
PC4			

Figure 3.6 Principal component plot for Sewage Sludge trial (Set 3A) soils, based on C evolved after 4 hours, expressed as a proportion (%) of that added as substrate. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.



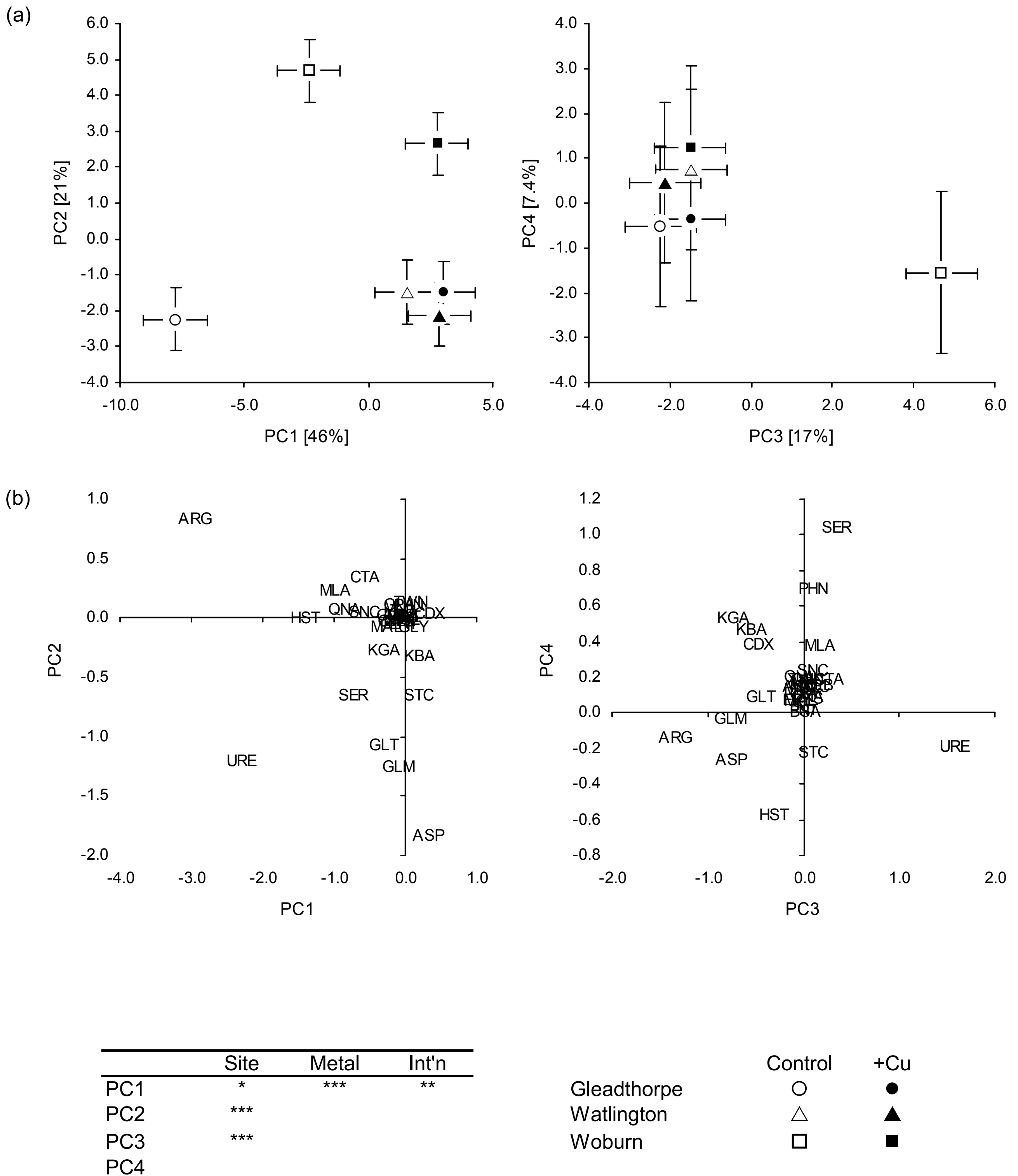
	Site	Metal	Int'n
PC1		**	
PC2	**		
PC3	**	**	
PC4			

Figure 3.7 Principal component plot for Sewage Sludge trial (Set 3A) soils, based on C evolved after 8 hours, expressed as a proportion (%) of that added as substrate. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.



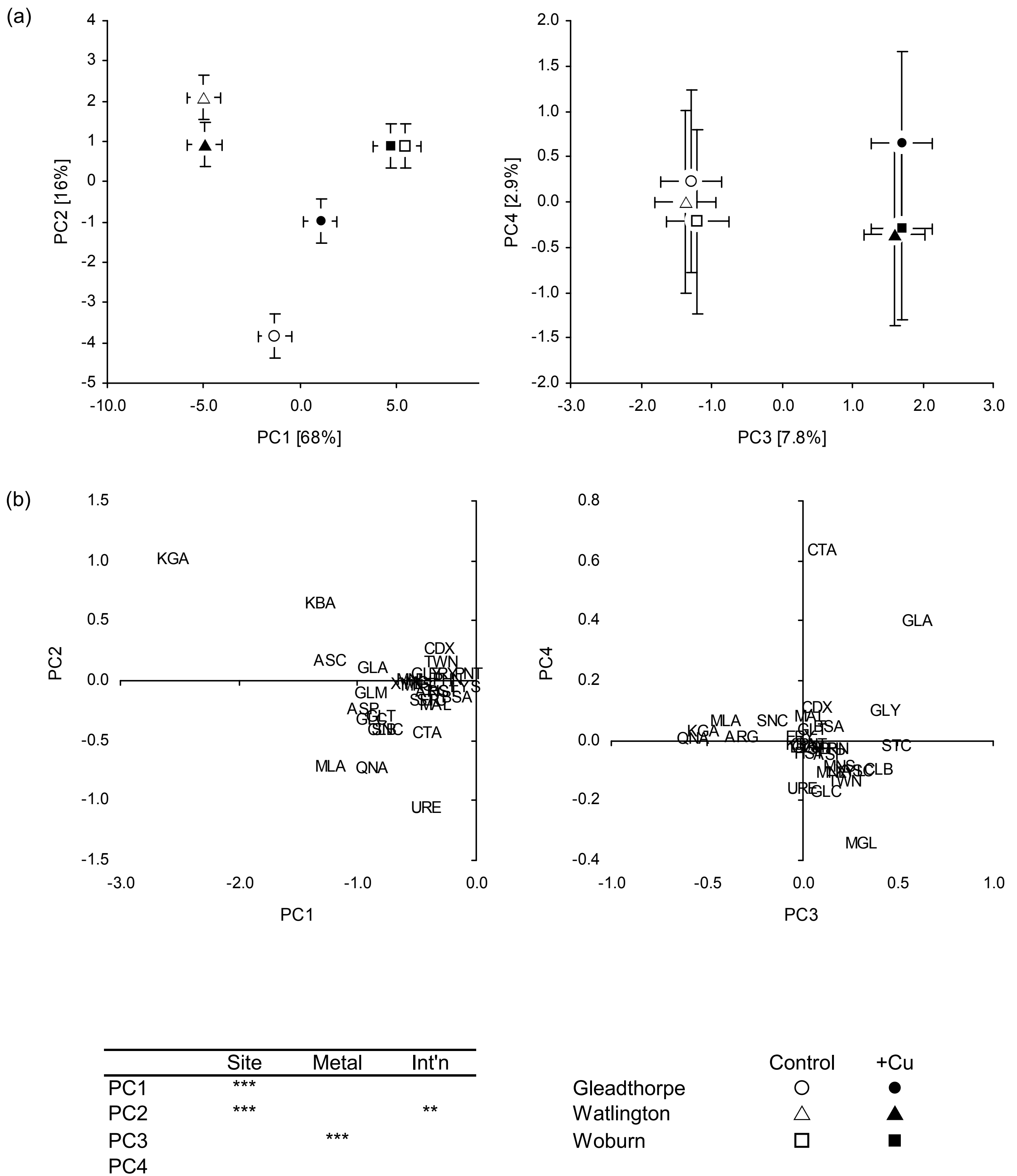
	Site	Metal	Int'n
PC1		**	
PC2	**		
PC3	***	*	*
PC4			

Figure 3.8 Principal component plot for Sewage Sludge trial (Set 3A) soils, based on C evolved after 12 hours, expressed as a proportion (%) of that added as substrate. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.



	Site	Metal	Int'n
PC1	*	***	**
PC2	***		
PC3	***		
PC4			

Figure 3.9 Principal component plot for Sewage Sludge trial (Set 3A) soils, based on C evolved after 16 hours, expressed as a proportion (%) of that added as substrate. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.



	Site	Metal	Int'n
PC1	***		
PC2	***		**
PC3		***	
PC4			

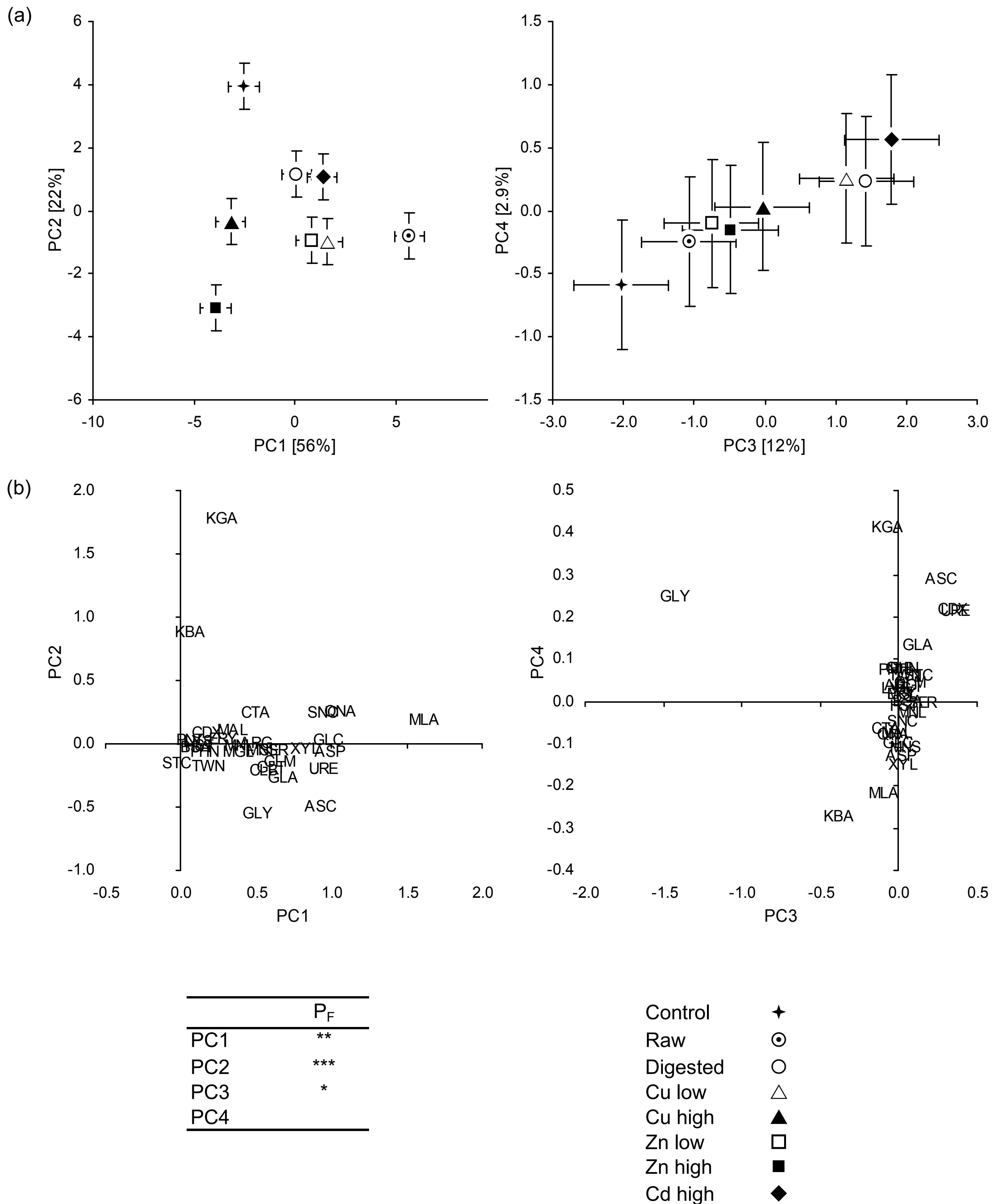
Figure 3.10 Principal component plot for Sewage Sludge trial (Set 3A) soils, based on total area under cumulative respiration curve (TAUC) over 16 hours. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.

Table 3.5 Substrates that contributed high loading values within principal component analyses (abbreviations for substrates as in Table 2.2)

	PC1	**PC2**	**PC3**	**PC4**
Rate·$T_{2–6}$	KGA	KGA	CTA	CTA
		URE	GLA	MGL
			MLA	
			QNA	
Four-interval	CLB4	ASP4	ARG4	GLA4
	GLC4	CTA4	CLB4	GLM4
	KGA2	GLM4	KGA1	MLA4
	KGA3	GLT4	KGA2	
	KGA4	QNA4	KGA3	
	QNA4		GLM3	
			GLM4	
			QNA4	
TAUC	KGA	KGA	GLA	CTA
		URE	GLY	GLA
			KGA	MGL
			MLA	
			STC	
			QNA	
Util% frequent across all time intervals	ASP, SER, URE, KGA, ARG			
Util% infrequent across all time intervals	GLM, PHN, KBA, GLT			

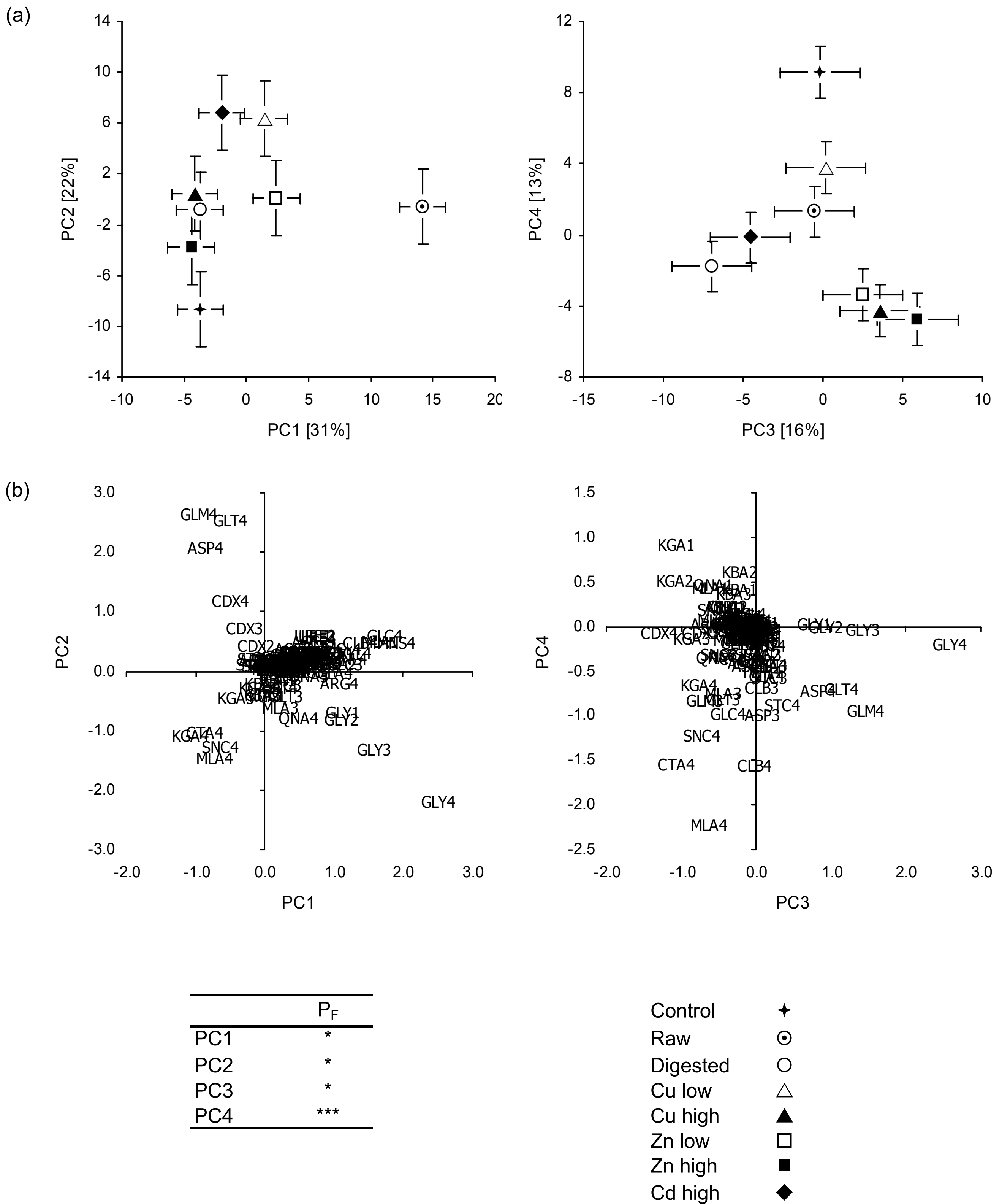
Table 3.6 Summary statistics for basal respiration (BSL), substrate induced respiration (SIR), evenness (E) and chemical soil properties for Watlington (Set 3b) samples

Treatment	BSL	SIR	E	pH	%C	%N
	$\mu g\ CO_2\text{-}C\ g^{-1}\ h^{-1}$					
Control	0.61	4.73	19.3	5.86	1.46	0.15
Raw	0.67	6.58	21.0	6.17	2.52	0.28
Digested	0.68	5.22	20.6	5.64	2.82	0.31
Cu low	0.61	5.37	20.7	6.17	2.52	0.27
Cu high	0.66	4.39	21.0	5.97	2.77	0.29
Zn low	0.68	5.35	21.4	5.94	3.04	0.32
Zn high	0.70	3.99	20.3	5.97	2.97	0.28
Cd high	0.64	5.35	20.9	5.93	2.93	0.31
Pooled s.e.	0.044	0.28	0.26	0.041	0.102	0.011
P_F	ns	***	***	***	***	***



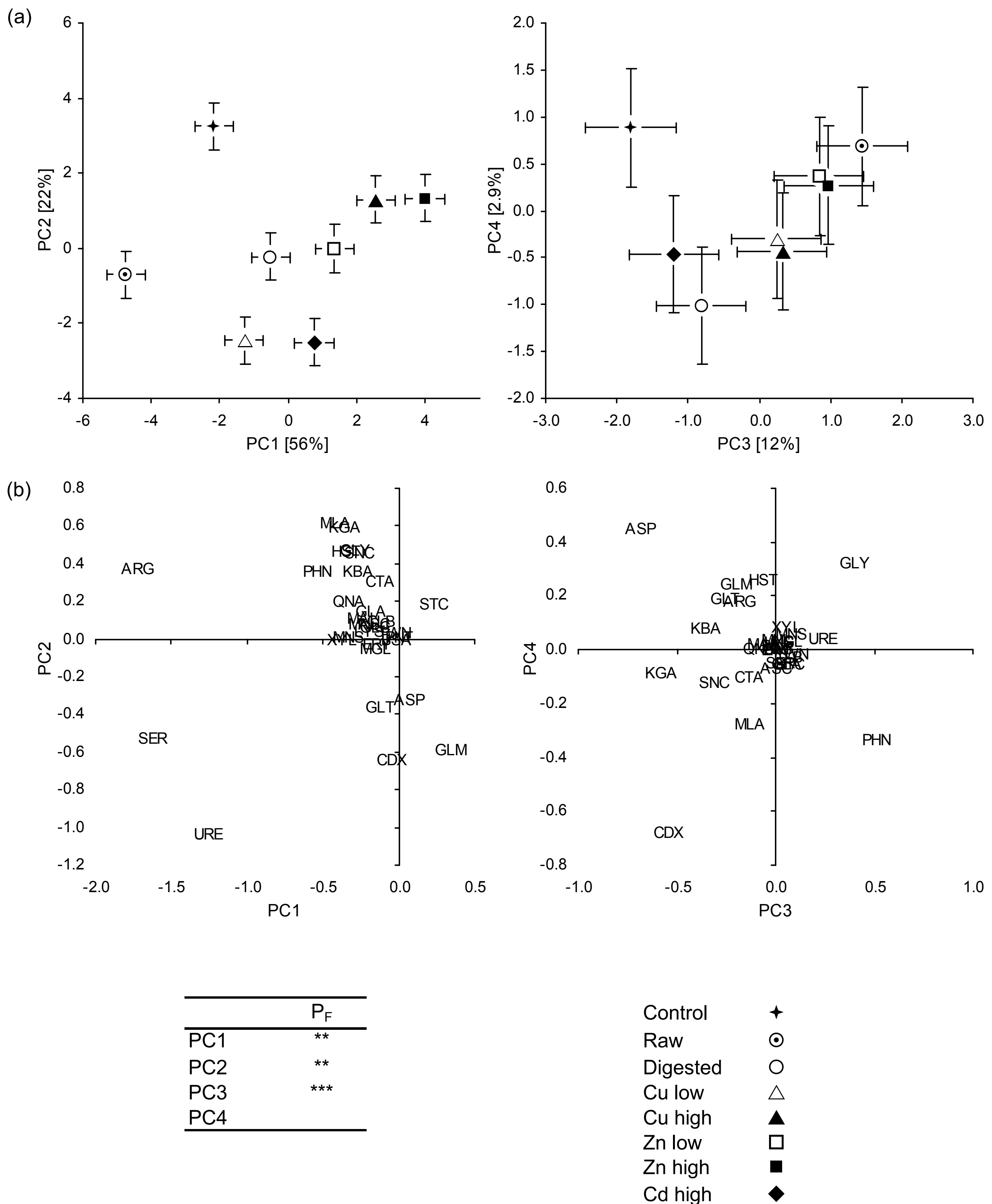
	P_F
PC1	**
PC2	***
PC3	*
PC4	

Figure 3.11 Principal component plot for Watlington Sewage Sludge trial (Set 3B) soils, based on Rates·T_{2-6}. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.



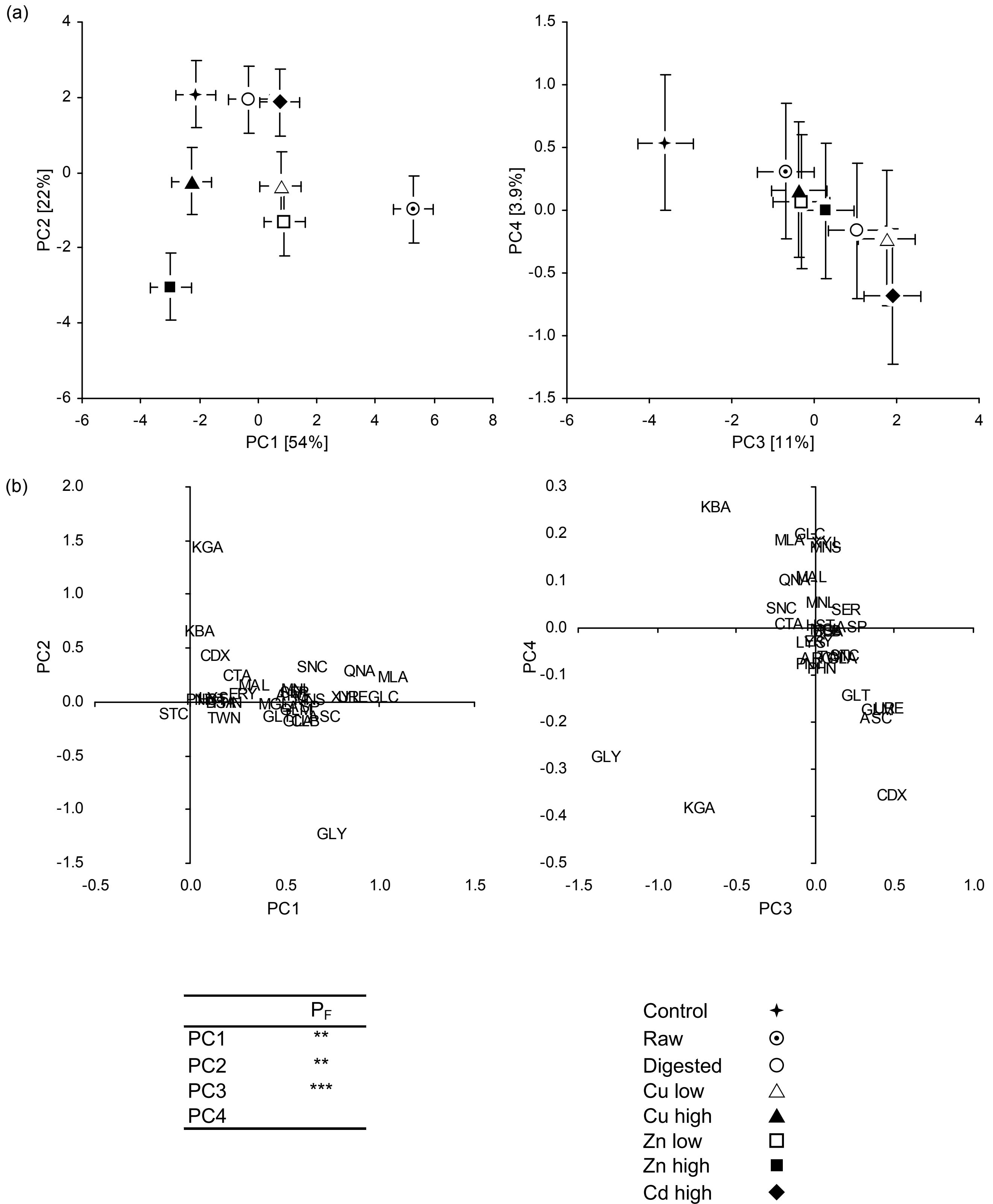
	P_F
PC1	*
PC2	*
PC3	*
PC4	***

Figure 3.12 Principal component plot for Watlington Sewage Sludge trial (Set 3B) soils, based on respiration rates at four intervals across $T_{0.5–16}$. (a) Principal components. (b) Loadings; three-letter abbreviations for substrates as in Table 2.2; numbers following these denote time intervals for respiration rates where 1 = $T_{0.5–4}$, 2 = $T_{4–8}$, 3 = $T_{8–12}$, 4 = $T_{12–16}$.



	P_F
PC1	**
PC2	**
PC3	***
PC4	

Figure 3.13 Principal component plot for Watlington Sewage Sludge trial (Set 3B) soils, based on C evolved after 16 hours, expressed as a proportion (%) of that added as substrate. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.



	P_F
PC1	**
PC2	**
PC3	***
PC4	

Figure 3.14 Principal component plot for Watlington Sewage Sludge trial (Set 3B) soils, based on total area under cumulative respiration curve (TAUC) over 16 hours. (a) Principal components. (b) Loadings; abbreviations for substrates as in Table 2.2.

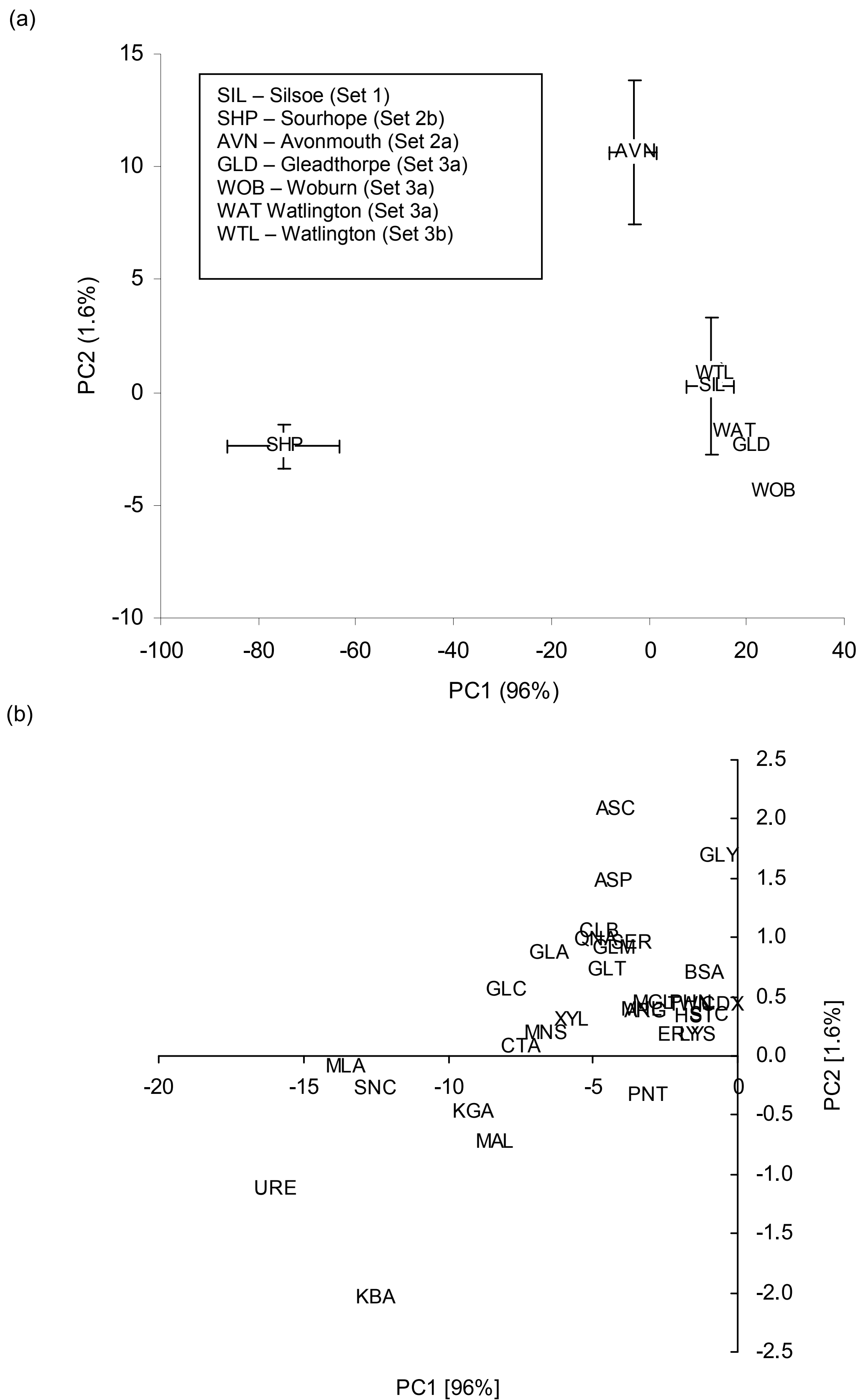


Figure 3.15 Principal component plot for main groups of soils in this study. (a) Principal components; points show means (n = variable), bars show standard errors; where bars not visible they fall within scope of marker. (b) Loadings; abbreviations for substrates as in Table 2.2.

3.7 Towards application of MSIR profiles in impact assessment analysis

Data from the Watlington intra-site samples (Set 3b) were used to explore a potential method for impact assessment. The sample set was suitable for this approach since it contained a range of ‘control’ samples against which to compare the range of metal ‘treatment’ samples, with appropriate replication. The basis of the proposed method is to calculate the nature of and extent to which utilisation of each substrate is affected by the treatment relative to a control. The nature of the effect is defined as inhibition (reduction in respiration rate relative to control), stimulation (significant increase in rate) or neutral (no significant effect), and the extent can be quantified due to the nature of the MSIR assay. Hence, ‘control’ data were subtracted from ‘treatment’ data for each substrate, and expressed both as an absolute change and as a proportional change (%) relative to the control. Resultant data were then plotted as a profile, with an associated frequency count of inhibition, stimulation and neutral effects. This principle could be applied to any of the MSIR parameters, but in this evaluation it was applied to the Rate·T2–6 data. The approach revealed a very clear stimulatory impact of sludge to the substrate utilisation capability of the Watlington soil, as well as an apparently greater effect for raw compared to digested sludge. This result is supported by the significant increase in the evenness factor (Table 3.6), but the profiling technique affords a much more detailed perspective on the nature of the effect.

Table 3.6 Summary statistics for basal respiration (BSL), substrate-induced respiration (SIR), evenness (E) and chemical soil properties for Watlington (Set 3b) samples

Treatment	BSL	SIR	E	pH	%C	%N
	$\mu g\ CO_2\text{-}C\ g^{-1}\ h^{-1}$					
Control	0.61	4.73	19.3	5.86	1.46	0.15
Raw	0.67	6.58	21.0	6.17	2.52	0.28
Digested	0.68	5.22	20.6	5.64	2.82	0.31
Cu low	0.61	5.37	20.7	6.17	2.52	0.27
Cu high	0.66	4.39	21.0	5.97	2.77	0.29
Zn low	0.68	5.35	21.4	5.94	3.04	0.32
Zn high	0.70	3.99	20.3	5.97	2.97	0.28
Cd high	0.64	5.35	20.9	5.93	2.93	0.31
Pooled s.e.	0.044	0.28	0.26	0.041	0.102	0.011
P_F	ns	***	***	***	***	***

Following sludge addition, there was a very restricted inhibition of ketobutyric acid and glycine utilisation (Figure 3.16). When compared to the no-sludge control treatment, Cu

effects were generally stimulatory at low Cu and neutral at high Cu (Figure 3.17). However, when compared to raw sludge, inhibition was more frequent and high Cu was inhibitory to the relative utilisation of many substrates (Figure 3.18).

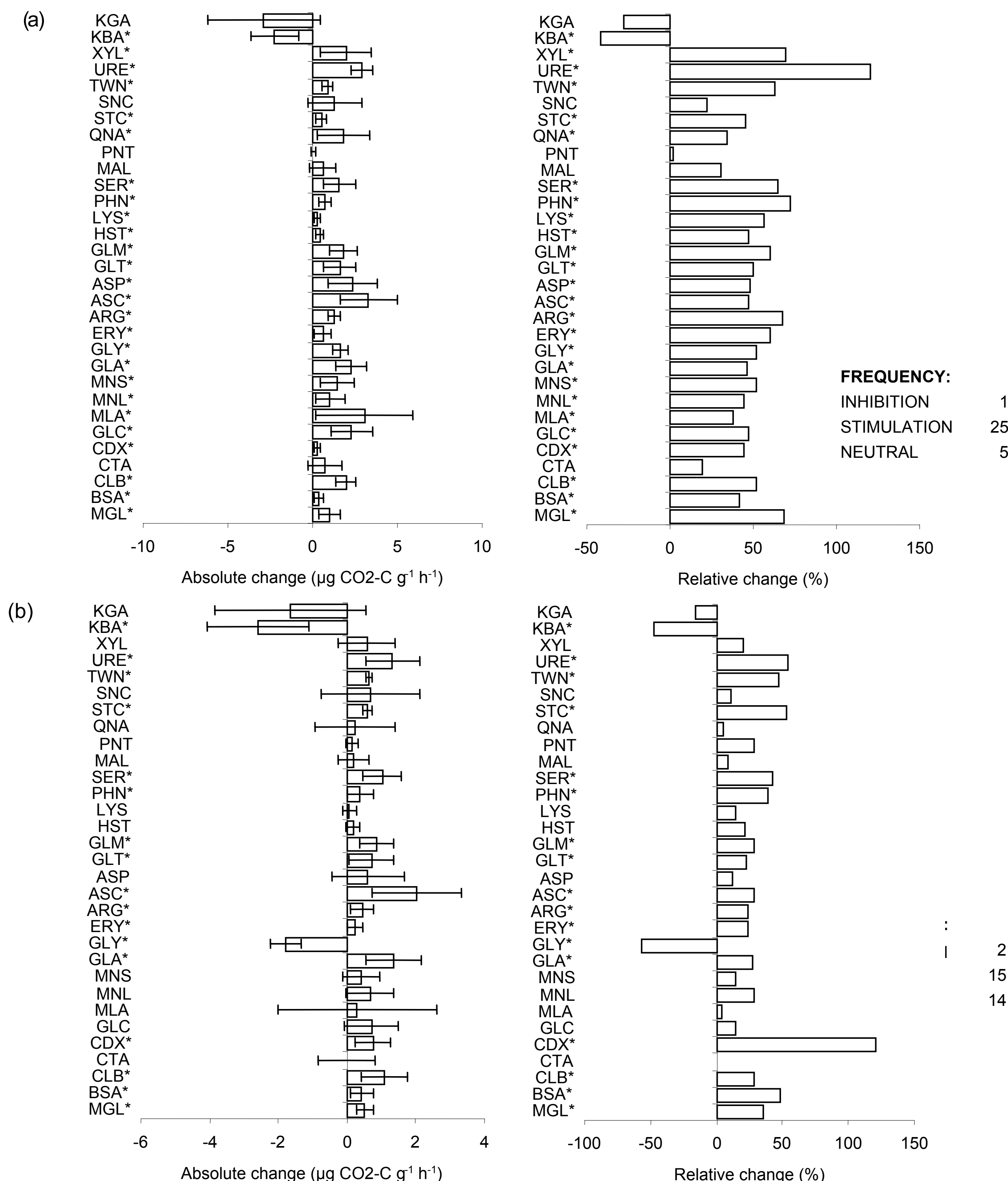
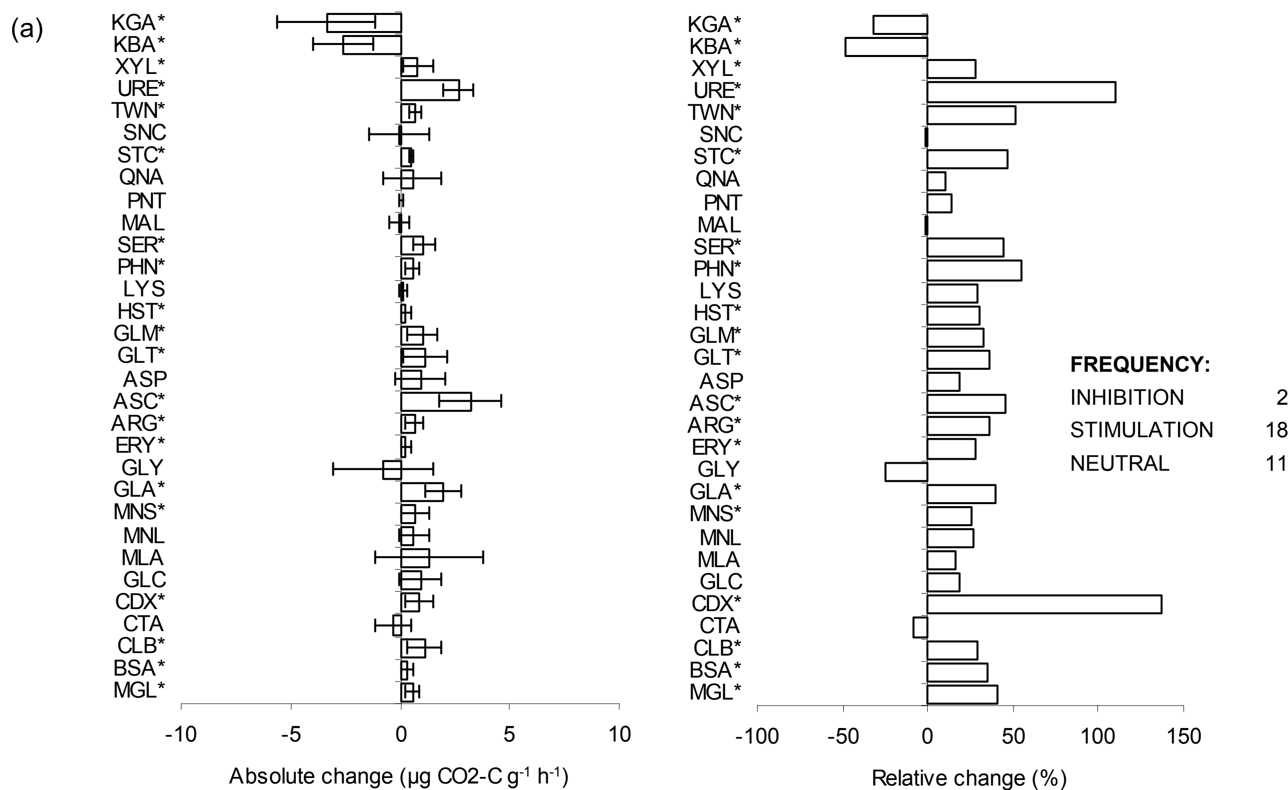


Figure 3.16 Effect of sludge treatment on MSIR profiles in Watlington soils (Set 3b), expressed as the change in Rate·$T_{2–6}$ relative to the no sludge control treatment. (a) Raw sludge. (b) Digested sludge. Notes: Bars show means, whiskers show LSD. Abbreviations for substrates as in Table 2.2; where these are followed by asterisk, the value is significantly different from zero. Inset shows number of significant effects.

(a)



(b)

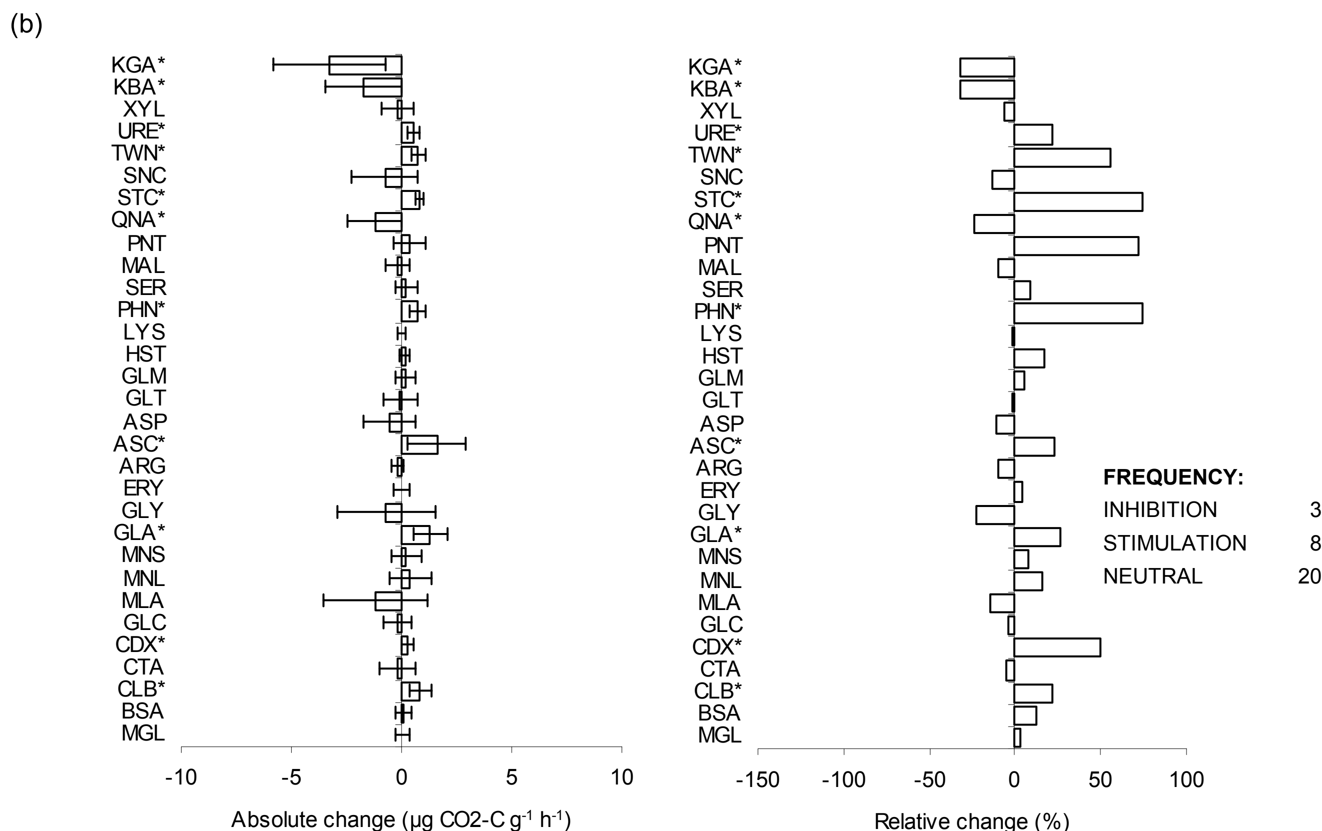


Figure 3.17 Effect of Cu sludge treatment on MSIR profiles in Watlington soils (Set 3b), expressed as the change in Rate·T_{2-6} relative to the no sludge control treatment. (a) Low Cu. (b) High Cu. Notes: Bars show means, whiskers show LSD. Abbreviations for substrates as in Table 2.2; where these are followed by asterisk, the value is significantly different from zero. Inset shows number of significant effects.

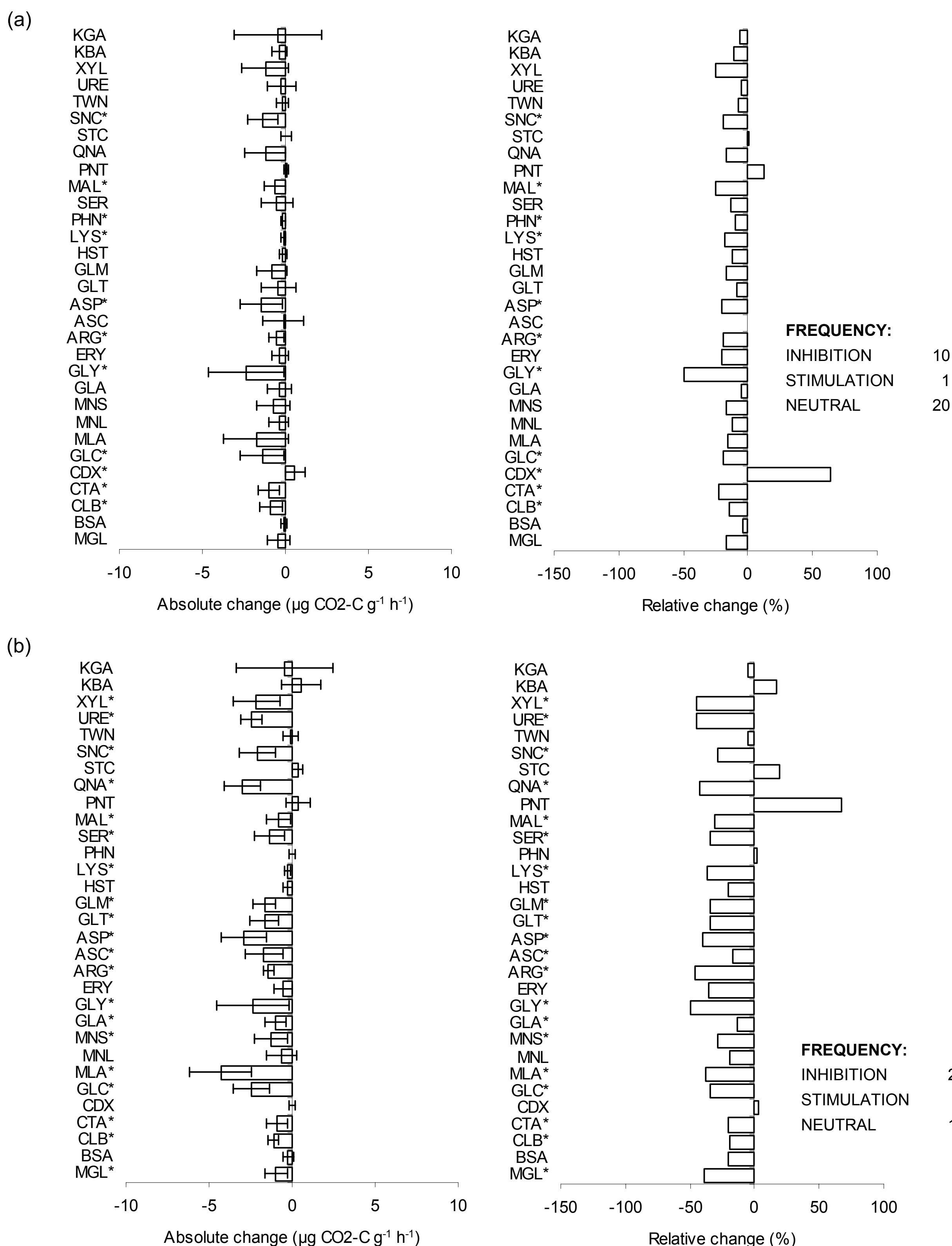


Figure 3.18 Effect of Cu sludge treatments on MSIR profiles, expressed as change in Rate·T2–6 relative to raw sludge control treatment. (a) Low Cu. (b) High Cu. Notes: Bars show means, whiskers show LSD. Abbreviations for substrates as in Table 2.2; where these are followed by asterisk, the value is significantly different from zero.

This demonstrates the importance of the definition for the control. Where sludge is applied, even carrying a metal, the stimulatory effect of the organic material may offset toxic effects arising from the metal. A similar situation occurred with respect to Zn (Figures 3.19 and 3.20). Cadmium apparently had relatively small effects (Figure 3.21). For the purposes of this exercise, a t-test was used to test for significance, but to avoid Type I errors a more rigorous approach would produce superior results. Increased (true) replication would also markedly increase the sensitivity of the approach.

3.8 Phospholipid fatty acid analysis (PLFA)

Soil communities can be described and analysed in terms of their genetic, phenotypic and functional properties (Ritz *et al*. 2004). A small supplementary study was carried out, using soils from Sets 2b and 3b, to investigate whether phenotypic profiling based on phospholipid fatty acid (PLFA) analysis of soils discriminated between samples to a similar extent to MSIR profiling. For Sourhope soils, PCs 1, 2 and 4 did not significantly discriminate control from biocide soils, but PC3 did (P=0.053; Figure 3.22a). This discrimination was attributable to three PLFAs: 18:1w9, 19:0c and an 18-length acid (Figure 3.22b). For Watlington soils, PCs 1, 2 and 4 showed significant discrimination (Figure 3.23). Control and raw sludge soils were separated from all others by PC1, and high Cu was strongly discriminating, with the PLFA 16:0 having a major influence (Figure 3.23). There was a general trend for soils receiving high metal rates to separate from those with low metal rates. Control soils were strongly separated by PC4, which related to the PLFAs 19:2 and 18:2w6c.

The two profiling methods therefore showed some consistency in the manner in which the samples were discriminated, such as the minor effect of biocide treatment on Sourhope soil, and the strong sludge and metal effects in the ADAS Watlington trial. There is little ecological reason to propose any particular relationship between PLFA and MSIR profiles, since the relationships between community structure and function in soil systems are rarely direct and generally very complex.

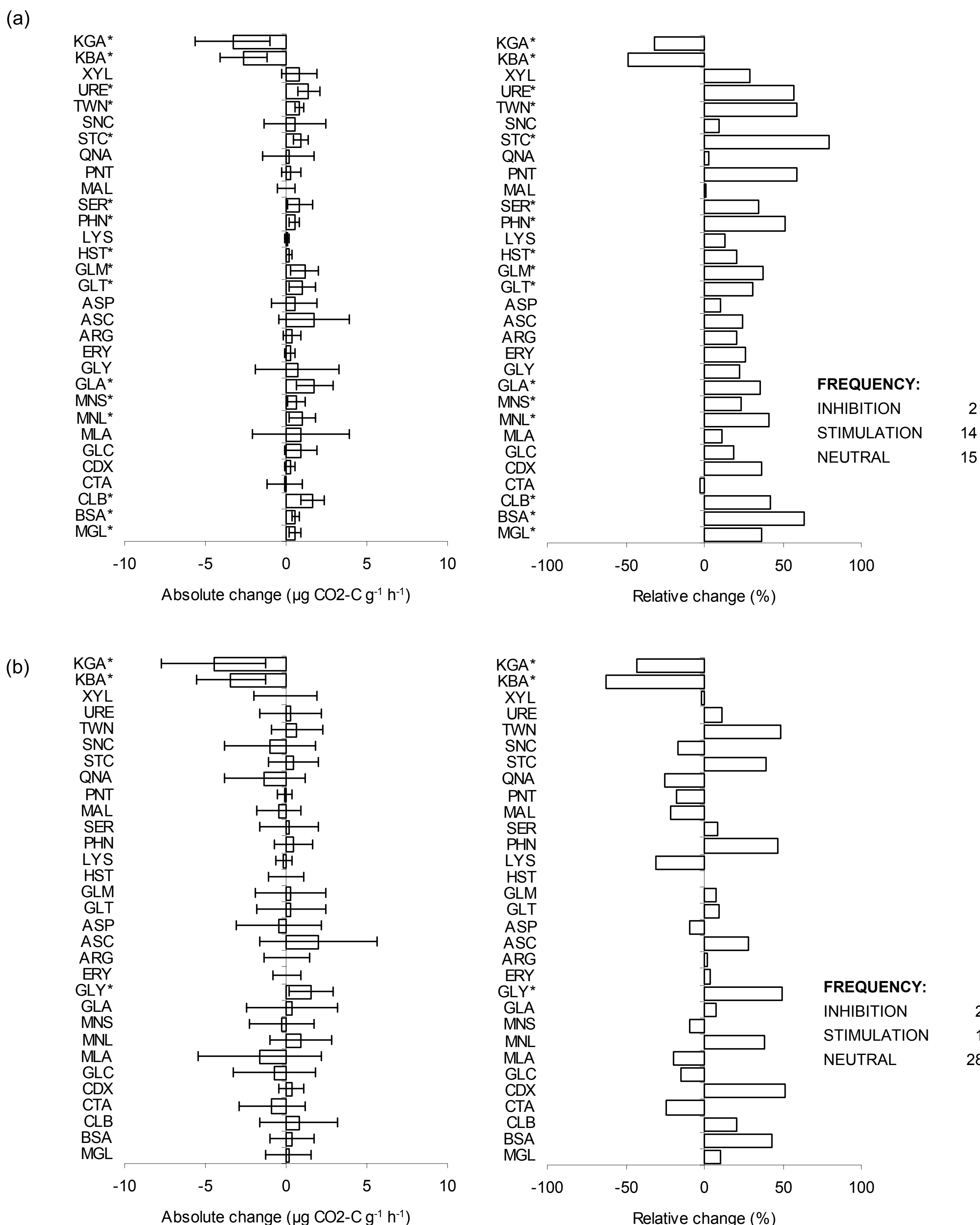


Figure 3.19 Effect of Zn sludge treatment on MSIR profiles in Watlington soils (Set 3b), expressed as the change in Rate·$T_{2–6}$ relative to the no sludge control treatment. (a) Low Zn. (b) High Zn. Notes: Bars show means, whiskers show LSD. Abbreviations for substrates as in Table 2.2; where these are followed by asterisk, the value is significantly different from zero. Inset shows number of significant effects.

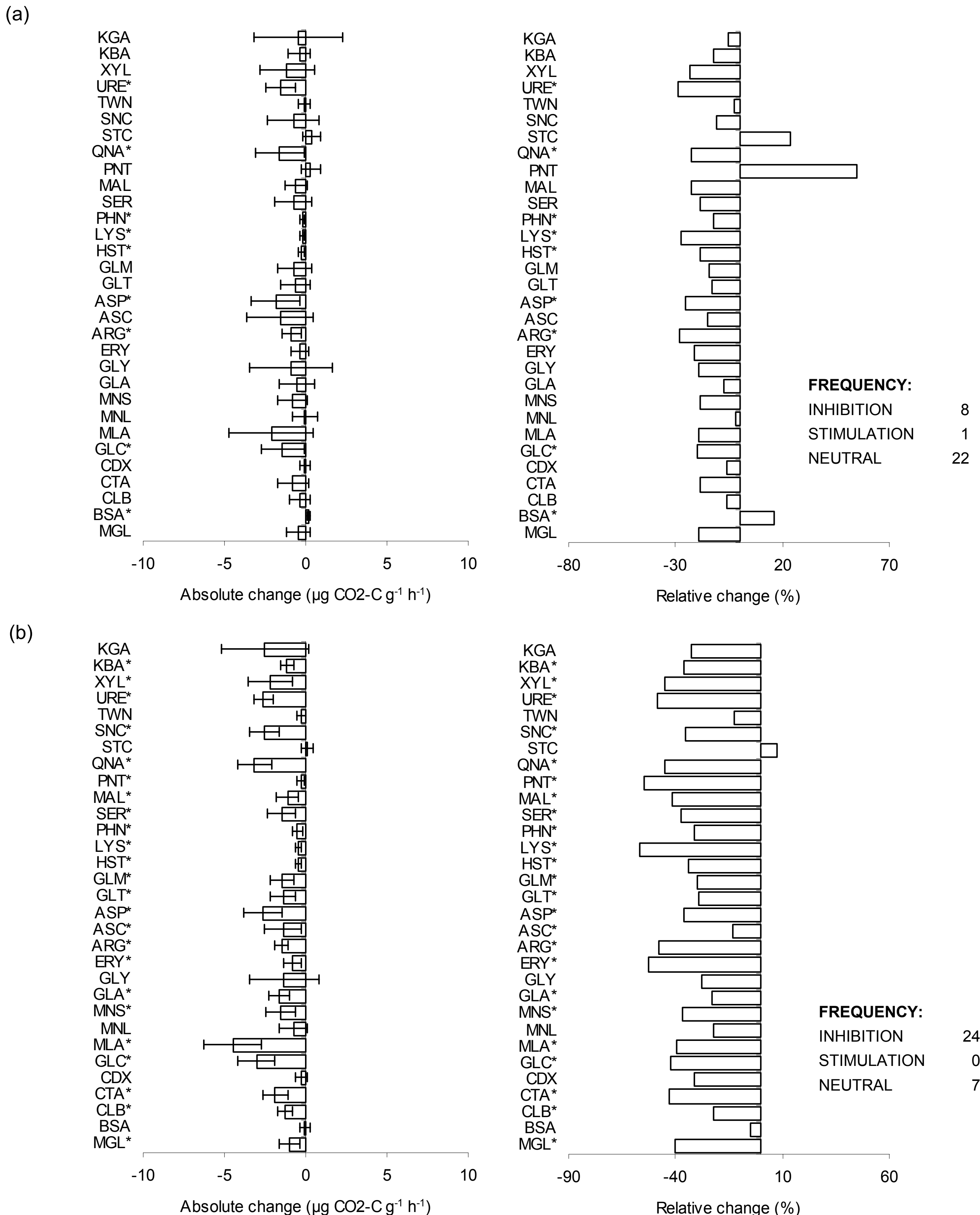


Figure 3.20 Effect of Zn sludge treatment on MSIR profiles in Watlington soils (Set 3b), expressed as the change in Rate·$T_{2–6}$ relative to the raw sludge control treatment. (a) Low Zn. (b) High Zn. Notes: Bars show means, whiskers show LSD. Abbreviations for substrates as in Table 2.2; where these are followed by asterisk, the value is significantly different from zero. Inset shows number of significant effects.

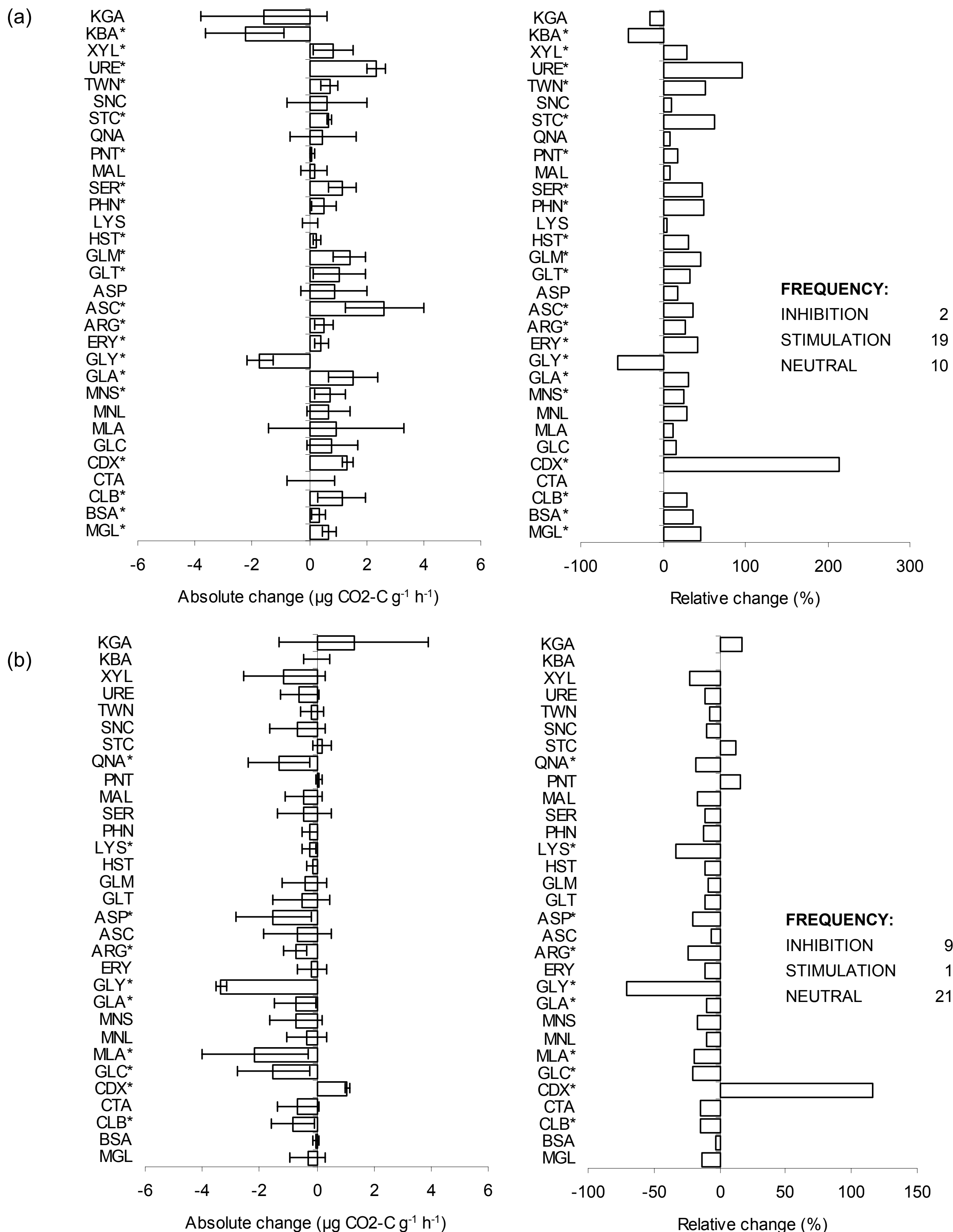


Figure 3.21 Effect of Cd sludge treatment on MSIR profiles in Watlington soils (Set 3b), expressed as the change in Rate·$T_{2–6}$ relative to control treatments. (a) No sludge control. (b) Raw sludge control. Notes: Bars show means, whiskers show LSD. Abbreviations for substrates as in Table 2.2; where these are followed by asterisk, the value is significantly different from zero. Inset shows number of significant effects.

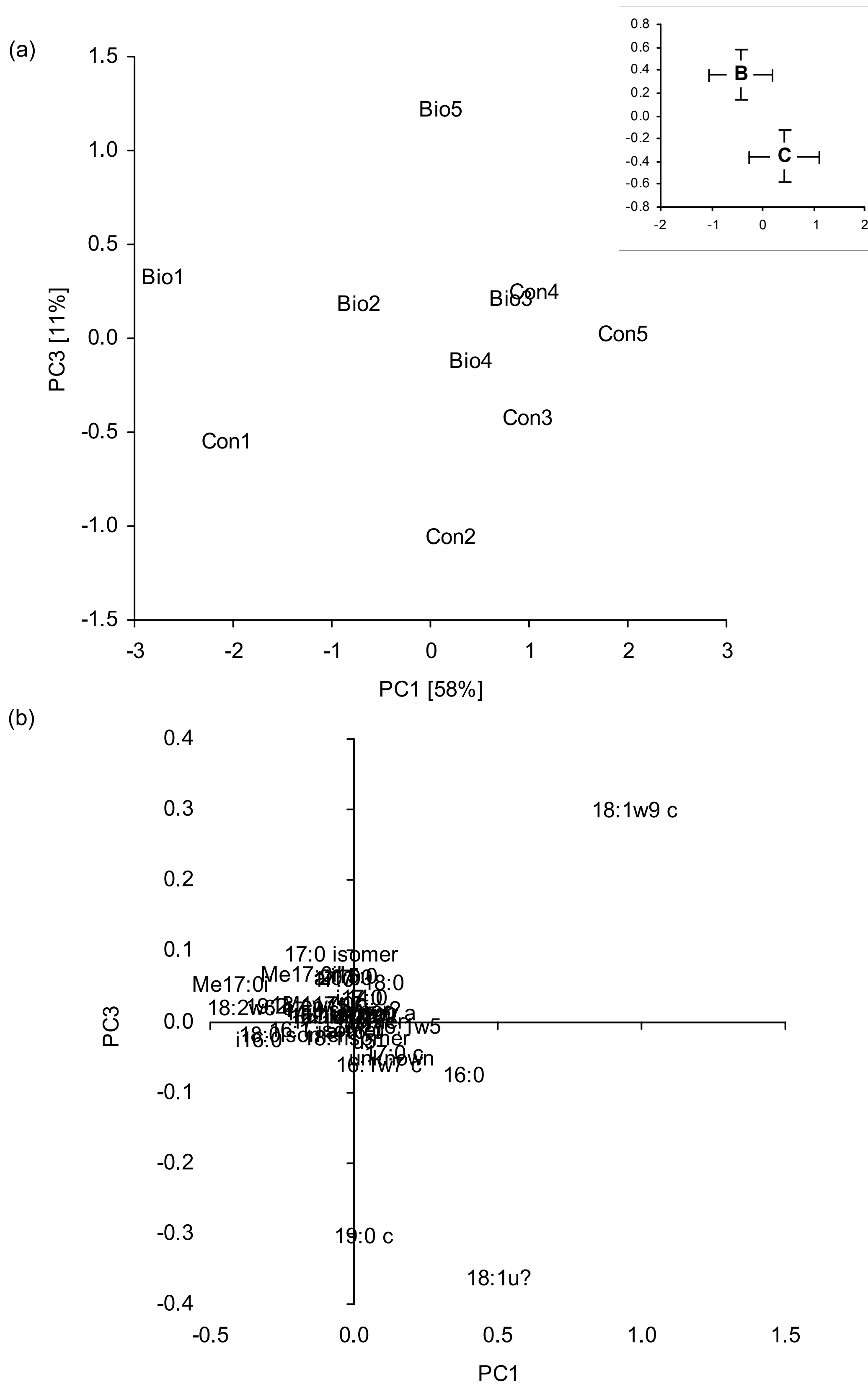
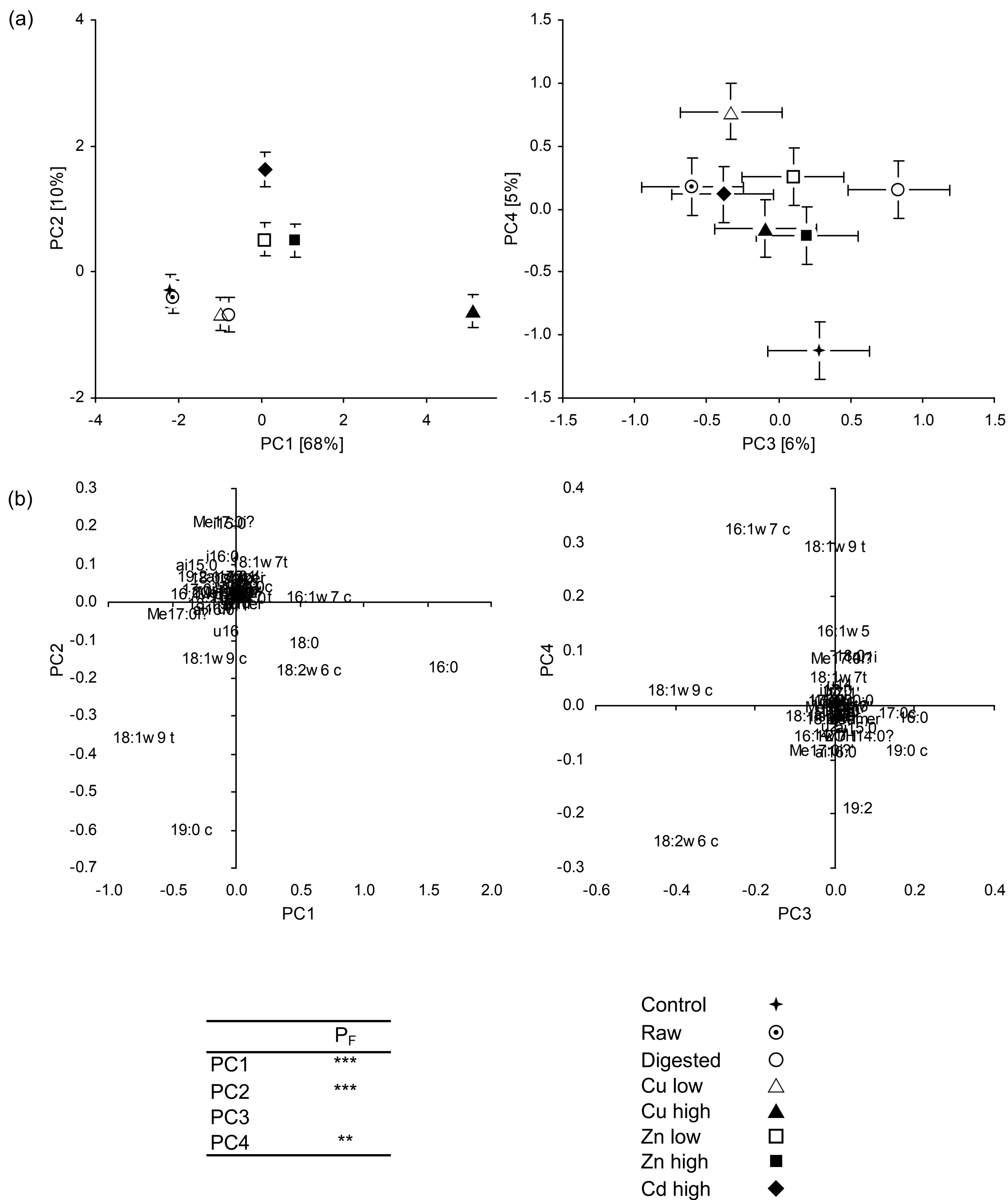


Figure 3.22 Principal component plot for Sourhope (Set 2B) soils based on PLFA profiles. (a) Principal components; inset show means with associated standard errors. (b) Loadings.



	P_F
PC1	***
PC2	***
PC3	
PC4	**

Figure 3.23 Principal component plot for Watlington Sewage Sludge trial (Set 3B) soils, based on PLFA profiles. (a) Principal components. (b) Loadings.

4 Conclusions

1. MSIR functional profiling has been shown to be discriminatory on the basis of broad soil type and ecological background, and is sensitive to a wide variety of factors likely to influence soil communities and function.

2. The method is sensitive to increasing levels of environmental stress caused by the presence of toxins, as indicated by increasing inhibition of catabolic response across a range of substrates.

3. The method is data-rich. Optimal methods of data analysis have not been conclusively established, but by applying a range of approaches it has become apparent that the patterns of discrimination are sensitive to the analysis method. Nonetheless, fundamental relationships between samples and impacts appear to prevail whichever method is used. More detailed analyses appear to increase sensitivity, but also increase complexity and make interpretations more difficult.

4. Certain substrates appear to be consistently implicated in discrimination. This may mean that reduced substrate suites are adequate for gross discriminatory purposes, such as initial screening, and that particular suites will be discriminatory for particular environmental pressures.

5. The simple impact framework that was developed shows some promise, and provides results that are consistent with intuitive relationships between soils and the impacts of sludges and metals.

6. MSIR functional profiling has proved to be complementary to phenotypic profiling.

7. The MSIR approach, using the RABIT methodology developed in this proof-of-concept project, has been shown to satisfy many of the requirements of Tier II of the Ecological Risk Assessment Framework. Its relation to the ‘five R’ framework is detailed below.

   i) Responsive: certainly – the method has been shown to provide a measurable response after exposure to pollutants, when compared to the results of assays conducted in uncontaminated soils.

   ii) Robust: apparently – whether the assay responds to environmental factors unrelated to pollution or environmental degradation requires further elucidation.

   iii) Reproducible: apparently (within a laboratory context, certainly) – the number of samples studied here was not adequate to rigorously confirm the method’s reproducibility, but there were strong indications of reproducibility associated with sites (for instance, Woburn site clustering across two time-points in Set 3).

   iv) Representative: certainly – the assay is demonstrably applicable across ranges of potentially contaminated sites. In addition, the ecological compartment (the soil community and it’s attendant C-utilisation function) is certainly present at each site, allowing comparisons to be made between separate locations.

v) Relevant: certainly – MSIR profiles are ecologically meaningful and show potential to be related directly to ecosystem-level effects. The mechanistic interpretation of such data requires further study.

8. The MSIR concept is clearly one that shows great promise for assessing the functional status of soils, particularly in the context of environmental interactions and impact assessment. It offers real potential as an effective tool for use in ecological risk assessment that is reliable and ecologically interpretable.

5 Recommendations

Recommendations for further development of the MSIR concept are detailed below.

1. More extensive studies are needed to confirm the utility of this approach in relation to the ‘five R’ criteria of a Tier II protocol. This would best be realised via a two-phase approach.

    a. In the scoping study, field-based samples were used. These were taken from sites with varying degrees of prescription and control of environmental factors that were expected to impact upon soil function. Interpretation of such data is confounded by other factors that will also have varied across these sites. More precise calibration of controlled laboratory microcosm manipulations would allow the precise nature of measured changes in functional profiles and their associated interpretation to be determined.

    b. A wider range of samples need to be taken from environmental contexts, including ‘reference’ sites against which to assess impact. Such references would provide the end-points against which to measure impacts.

2. Some key technical/analytical developments are needed.

    a. More considered study of the substrate suites used and their respective concentrations, mapped to the context of particular environmental circumstances.

    b. Further testing to determine the appropriateness of using reduced numbers of substrates for gross discrimination.

    c. More rigorous consideration of statistical analytical approaches for analysing the complex data sets produced by the method.

3. To develop the approach as a tool for impact assessment.

    a. The continued development of the risk-assessment methodology to make it suitable for widespread adoption.

    b. Study of how MSIR profiles or inhibition profiles map against other soil functions.

    c. Calibration of a simplified technique for routine deployment. The RABIT system is a ‘research grade’ instrument and staff-intensive, which has implications for throughput.

In conclusion, the approach offers objective quantification and allows direct comparison across different stressors.

It is anticipated that a substantive three-year programme of research into the areas detailed above would produce an effective tool for assessing the risk to ecological function across a wide spectrum of environmental challenges.

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We sincerely thank land owners for permission to sample from their respective sites, David Spurgeon for assistance relating to the Avonmouth site, Paul Gibbs for assistance and liaison with ADAS sites and Gordon Common for assistance at Sourhope.

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List of abbreviations

ARG	L-arginine
ASC	L-ascorbic acid
ASP	L-asparagine
BSA	Bovine serum albumen
BSL	Basal respiration
CDX	Cyclodextrin
CLB	Cellobiose
CLPP	Community-level physiological profiling
CTA	Citric acid
CV	Coefficient of variation
E	Evenness
ERY	I-erythritol
GLA	Gluconic acid
GLA	L-glutamic acid
GLC	D-glucose
GLM	L-glutamine
GLY	Glycogen
HST	L-histidine
KBA	α-ketobutyric acid
KGA	α-ketoglutaric acid
LYS	L-lysine
MAL	Malonic acid
MGL	B methyl D glucoside
MLA	DL-malic acid
MNL	D-mannitol
MNS	D-mannose
MSIR	Multiple substrate-induced respiration
PCA	Principle component analysis
PHN	L-phenylalanine
PLFA	Phospholipid fatty acid analysis
PNT	Pantothenic acid
QNA	Quinic acid
RABIT	Rapid automated bacterial impedance technique
SER	L-serine
SIR	Substrate-induced respiration
SNC	Succinic acid
SOP	Standard operating procedure
STC	Starch
TAUC	Total area under the curve
TWN	Tween 80
URE	Urea
WAT	Water
XYL	Xylose

Appendix I

Checklist of objectives as listed in the project specification.

Objective	Notes
1. To refine the methodology of the functional profiling method (FPM) using arable, grassland and woodland soils.	Completed.
2. To apply the refined methodology to soils contaminated with heavy metals and organics.	Completed.
3. To analyse the respiration curves in terms of lag time, initial rate and cumulative CO_2 evolution, as set out in the BSI-ISO standard 2.	This approach proved to be untenable given the nature of the respiration profiles that were measured.
4. To use the results to assess the discrimination of the approach in terms of its ability to separate soils on the basis of land use or level of contamination.	Completed.
5. To produce a Standard Operating Procedure (SOP) setting out the steps employed in the final methodology.	Completed, as Appendix II.
6. To report the findings of the work, with the SOP as an appendix, in a Technical Report.	Completed.
7. To advise the Environment Agency on the suitability of the approach for the three uses set out above and, where appropriate, to make recommendations for further work.	Given in report.
8. To make the raw respiration results, and any ancillary data required to reproduce the results presented in the report, available to the Environment Agency as an Excel spreadsheet.	Provided.
9. To deliver the draft report by the end of December 2005.	Completed 13th February 2006, with agreement of submission date from EA Project Manager
10. To deliver the finalised reports by March 2006.	Report finalised March 2006.

Appendix II

Standard Operating Procedure for use of RABIT system for measuring soil respiration

1. Introduction

1.1. Soil Respiration

The greater proportion of soil micro-organisms are normally dormant (Jenkinson and Ladd, 1981) and so their rate of respiration is low (basal metabolic rate). Adding an easily degradable substrate, such as glucose, causes an immediate increase in microbial respiration. The increase in respiration before cellular growth is related to substrate-responsive soil microbial biomass (Anderson and Domsch, 1978). This technique is known as Substrate Induced Respiration (SIR).

Glucose is used for SIR measurements because virtually all soil micro-organisms can readily utilise it. The SIR method was further developed by Degens and Harris (1997), who assessed the catabolic diversity of soil microbial communities by measuring short-term (four-hour) evolution of CO_2 following the addition of a range of substrates, in addition to glucose. This approach has now been used in various soil functional studies, such as Degens and Harris 1997, Degens *et al* 2000, Degens *et al* 2000, Schipper *et al* 2001 and Stevenson *et al*. 2004. This technique will be refereed to hereafter as multiple substrate-induced respiration (MSIR).

Applying impedance technology to soil functional profile studies allows respiration to be continually monitored over a much greater time frame. In addition, readings taken every six minutes allow observations of respiration kinetics, unlike taking one single reading after four hours. This method uses the Rapid Automated Bacterial Impedance Technique (RABIT) developed by Don Whitley Scientific Ltd (Bradford, UK).

1.2. Impedance Theory

Impedance is the resistance to flow of an alternating current through a conducting material. As such it can be defined as:

$$\text{Impedance } (Z) = R^2 + (1/2\pi FC)\ 2$$

Where: R = resistance, C = Capacitance (electrode polarisation, the ability of an object or surface to store a charge), F = frequency of data collection.

Impedance technology is widely used as a microbiology tool and has been used to study bacteria, yeasts and moulds, and is also used in the food, beverage, pharmaceutical, petrochemical, public health and dairy industries. The RABIT system can be used to assess microbial change using either direct or indirect measurements.

1. **The direct** method is based on the monitoring of microbial metabolites, which can affect the impedance of a culture medium by releasing ionised molecules. Uncharged or weakly-charged substances such as polysaccharides, fats and proteins are metabolised by micro-organisms into highly charged end-products, such as organic acids, fatty acids and amino acids. The resultant changes in the electrical properties (conductivity and

resistance) of the culture medium can be measured by metal electrodes placed in the container of the inoculated growth medium.

2. **The indirect** impedance technique monitors the electrical change of alkaline agar due to ionisation of CO_2 to carbonate. In this case, the electrodes are immersed into a CO_2 absorbing trap containing potassium hydroxide, rather than the microbial cultures associated with the direct method. CO_2 produced from the soil microbial community is trapped in an alkaline absorbent solution and causes a decrease in the conductance of the alkaline agar. This decrease is plotted against time.

The indirect method is used in this study to measure soil respiration parameters. Unlike standard soil respiration methods that measure CO_2 emission at a single point in time, impedance technology monitors real-time activity and therefore offers the following advantages.

- Results are determined in a shorter time frame.
- The time-course of respiratory activity is determined and available for kinetic analysis.

2. Health and Safety

Ensure that the laboratory COSHH form has been read and signed before proceeding with this method.

Before using the autoclave ensure that you have been trained by a suitable member of staff. In particular: ensure that the autoclave cycle is complete before opening the autoclave; handle hot equipment with care using dry, heat-resistant gloves to remove items; Pyrex bottles (whether full or empty) should have their caps placed on loosely to prevent explosion due to expansion.

3. RABIT operational conditions

RABIT operational conditions are set up as follows:

- Test Duration = 16 hours
- Time Resolution = 6 minutes
- Temperature = 25°C
- Detection Criteria = 10 µSiemens.

The operational conditions are saved as a Test Code file called 'MP16hr.rtc'. The laboratory air conditioning unit is set at 20°C.

The electrical signal is temperature dependent and so temperature control is critical. A 1°C rise in temperature will result in an average increase of 0.9% in capacitance and 1.8% in conductance (Eden and Eden, 1984). This means that a 5 millidegree temperature drift would cause a resultant 1 µS change. Temperature stability within the RABIT block is greater than ±2 millidegrees.

Individual incubator modules can be run at different temperatures from 4°C above ambient temperature to a maximum of 50°C. For the purposes of this method each block will be set at 25°C.

4. Preparation of conductance cells

NB. Care in the preparation of indirect cells will ensure maximal shelf life and consistent sensitivity.

4.1. Reagents

1. 10% potassium hydroxide stock solution. Dissolve 25g of KOH in 250 ml of deionised water.

2. 1% potassium hydroxide working solution. Dilute the 10% solution by adding 10ml to a 100ml volumetric and make to the mark (prepare on the day of use).

3. Bacteriological Agar No.1

4.2. Method

1. Weigh 1.0 g of Bacteriological Agar No.1 into a 100 ml screw-capped bottle and disperse in 50 ml of deionised water.

2. Dissolve the agar by autoclaving.

3. While the molten agar is still hot (approx. 70 ºC) add 50 ml of the cold 1% potassium hydroxide solution and mix thoroughly.

4. Dispense 1.0 ml of the agar/KOH mixture into the base of each clean, dry RABIT cell. Take care to minimise the quantity of agar that runs down the inside of the cell.

5. Allow the agar to cool and solidify for 15–30 minutes (maximum), then tightly seal each cell using rubber stoppers.

6. After the agar has solidified allow the cells to stabilise for a minimum of three hours (preferably overnight) prior to use.

7. If tightly stoppered and protected from strong light, RABIT cells may be stored at room temperature for one month. Do not refrigerate the cells.

1. To check the condition of cells before use: place a representative sample in an incubator module, allow to warm through for 15–20 minutes, and observe their conductivity readings using the Global > Conductivity Table function. These values should typically be 15000–16000 µS at 25 ºC. Do not use a cell with a conductivity reading below 14000 µS at 25 ºC.

4.3. Cleaning the conductance cells after use

1. Remove the used cells from the RABIT, remove the bung and the cells' contents.

2. Autoclave the cell for 15 minuets at 121°C.

3. Remove the cell from the autoclave and discard the hot agar/potassium hydroxide mix.

4. Ensure that the cells are visually clean, scrub the inside if necessary.

5. Wash the cell in deionised water.

6. Dry the cell in the drying cupboard.

Note: Periodic cleaning of the electrode pins that contact the socket with Scotchbrite® or something similar will ensure good electrical connections. If necessary, detach the tube from the cell base assembly and place the cell base and/or tube in a beaker containing 0.1M HCl and then ultrasonicate for 30 minutes. After sonication, wash the cells/tubes in deionised water, dry the cells and then re-attach the tube to the cell.

5. Procedure

5.1. Reagents

200 mM glucose (13.51 g l^{-1})
Other substrates as needed for MSIR assay

5.2. Method

1. Pre-condition the soil (2 mm sieved) at 250 ºC for seven days. It should be in a friable state (typically at c. 45% of water-holding capacity, but such that it readily passes a 2-mm mesh. Determine proportion of its water holding capacity in this state by procedure of Schipper *et al*. 2001).

2. Ensure that enough conductance cells have been prepared for the analysis.

3. Turn on the computer. The RABIT software will load automatically.

4. Prepare a RABIT worksheet for the analysis. Refer to Table AI.1 for an example of a worksheet. This is done by selecting 'Start' from the menu bar. Ensure samples are sequenced to provide a randomised block design within RABIT cells. These can be prescribed by application of the RABIT randomiser Excel macro.

Table AI.1: Worksheet example

w/s no.	Sample Code	Test Code	Temp.	Delay	Location	Run
Sample number.	Sample details.	Refers to the test parameters.	Set at 25°C.	Set at 30 minutes.	Location in the RABIT block.	A tick appears here when the test cell has been accepted.
0345	+glucose	MP16hr	25	00.30	02 A8	√
0345	+water	MP16hr	25	00.30	02 A8	√

5. On completion of the worksheet select ‘Allocate’. The cells location will be automatically allocated. The location will be listed in the ‘Location’ column of Table AI.1.

6. When cells have been allocated a position, select ‘Fit Cells’.

7. For each soil sample, weigh 1.0g of soil (2-mm sieved and pre-incubated) into a glass boat in duplicate. One will be the control and the other will be the glucose amended soil.

8. Add water to the control soil and 200mM glucose (or appropriate substrate) to the amended soil to raise the moisture status of the soil to 100% of its water holding capacity.

9. Insert the conductance cell into the allocated position (cells accepted are indicated by a green tick in the ‘Run’ column of the worksheet).

10. Place the soil sample into the RABIT cell, taking care not to spill the sample.

11. Stopper the RABIT cell.

12. When all cells have been accepted click ‘Done’.

13. To terminate tests, firstly ensure that the analysis is complete by selecting the RABIT module containing the cells (select the module using a left mouse click) then select an impedance cell (left mouse click onto individual cells) and ensure that the ‘cell status’ window indicates ‘test completed’.

14. If the test is complete, remove conductance cells from the module.

15. Select the module containing the analysis to be terminated (select the module using a left mouse click) and then select ‘Terminate’ from the menu bar. Mark the cells to be terminated by clicking each one using the left mouse button. To select all the cells in one module click the right mouse button.

5.3. Exporting Data

1. Insert a USB stick into the ‘F’ drive. Create a folder on the USB stick and identify it with the date.

2. Go to ‘Reports’ on the tool bar. Select the reports that you want from the drop-down menus available and then click on ‘make report’.

3. Select File on the tool bar then export all. Select the file location (file created above). File name is 001, file type is ‘comma separated variable’.

6. RABIT collator

This is an Excel macro that collates any defined number of .csv files from the RABIT output and places copies on one worksheet to assist further analysis.

Path: File Location written as drive:\date\ (for instance, g:\080205\)
Source prefix: 0 (i.e. first digit of the file)
Cells: 1 to n (where n is number of cells to be collated)
Collated filename: saves file as this name

7. Data analysis

The raw data accrued when a soil is profiled using the SOP comprises a series of conductivity values (µS) taken every six minutes between 0.5 hours and 16 hours for each sample. Since the initial conductivity in each cell is different, values are first normalised by subtracting the initial value from each successive reading. Where each soil/substrate combination is replicated within each incubation run, arithmetic means of these replicates are calculated. Conductivity values for all substrates are then corrected for basal respiration by subtracting the mean signal for unamended controls (such as water [WAT] treatment) from the mean signal relating to each substrate. The values were subsequently converted to µg CO_2-C using a conversion factor derived empirically. For the Silsoe instrument, this factor is 1 µS change in conductivity = 5.82 mg CO_2 l^{-1} evolved into headspace. Respiration rates are calculated for each six-minute time interval, corrected for dry mass of soil to normalised units of µg CO_2-C g^{-1} h^{-1}.

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Appendix III

Calibration of RABIT respirometer

A range of soils were selected to give diverse respiration rates. Respiration rates from triplicate samples were determined using the RABIT impedance technique in one set of cells, and by a reference method using headspace gas chromatography in a paired set of RABIT cells. The calibration curve obtained is shown in Figure AII.1. From this curve, a factor was derived to convert RABIT µS readings into units of µg CO_2-C g^{-1} dry soil equivalent, and thence to rates and proportions of C present in substrates.

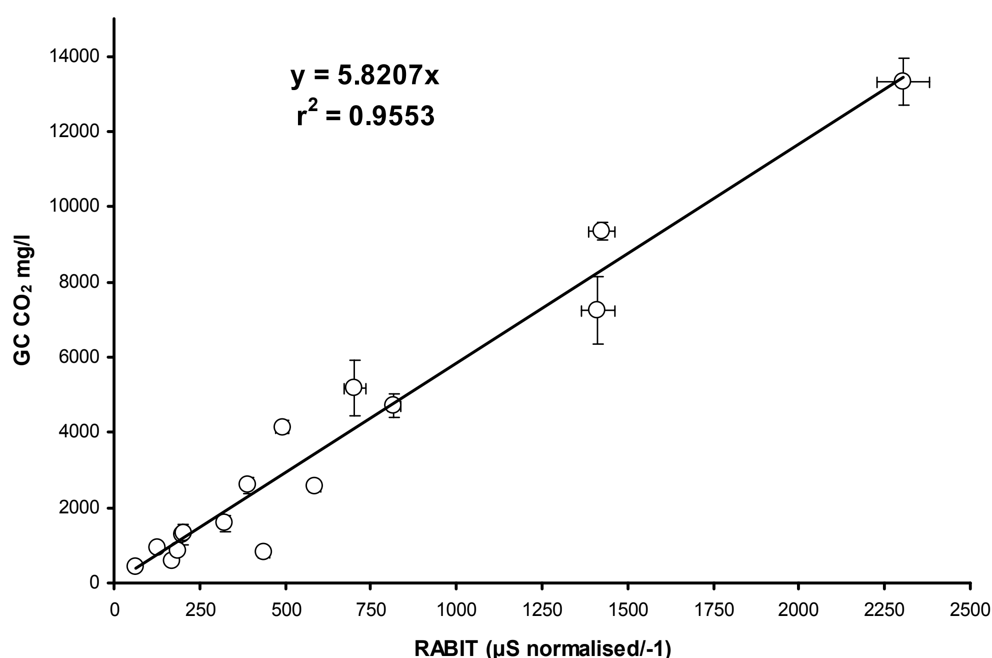

Figure AII.1 Calibration curve for change in conductivity in RABIT cells to headspace concentration in equivalent cells measured by gas chromatography

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