Through the Eye of the Needle — The Story of the Soil Microbial Biomass

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Birth of the Microbial Biomass Concept

In 1966, David Jenkinson at Rothamsted Research, UK, published a paper that led to a new paradigm in soil science, despite its innocuous sounding title "Studies on the decomposition of plant material in soil. II. Partial sterilisation of soil and the soil microbial biomass." We think that this was the first time that the term *soil microbial biomass* appeared in the scientific literature; it refers to the total mass of all organisms in soil. David Jenkinson later described the microbial biomass as the "eye of the needle through which all organic matter entering the soil must pass". Ten years later, the concept was reinforced and a specific method for measuring the quantity of carbon (C) held in the soil microbial biomass was published in a series of papers by David Jenkinson, David Powlson and others that have been very widely cited (Jenkinson, 1976; Jenkinson and Powlson, 1976a, b; Jenkinson *et al.*, 1976; Powlson and Jenkinson, 1976). The

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method proposed in 1976 became known as the chloroform ($CHCl_3$) fumigation–incubation method, later abbreviated to FI to distinguish it from the $CHCl_3$ fumigation–extraction method, developed later and termed FE (Vance *et al*., 1987b). Much of the 1976 research resulted from the Ph.D. work of David Powlson carried out under the supervision of David Jenkinson. Later developments including the development of the FE method for measuring biomass C and methods for measuring N and P in the biomass were largely led by Phil Brookes.

For post-millennial soil researchers, those beginning their work after or around the year 2000, it may be difficult to understand the scientific context of soil microbiology in the 1960s. The genetics revolution had barely started — Watson and Crick had published their discovery of the structure of DNA only 13 years before the Jenkinson (1966) paper. Molecular biological concepts and methods that are now routine were unheard of. The concept of measuring the properties of an entire population, such as the quantity of C held in all living organisms in soil, was not common. At that time, the major focus of soil microbiology was the identification of organisms responsible for specific processes. Processes of interest included ammonium oxidation (nitrification), cellulose decomposition and the degradation of individual pesticides.

By far the major tool available for studying soil microorganisms was culturing under laboratory conditions using agar plates containing different nutrients to give some specificity to the species of organisms likely to grow. Population sizes were assessed by an extension of this approach based on counting the number of colonies developing on plates of nutrient agar from increasingly diluted soil extracts; the method was termed "plate counting".

While many soil microbiologists recognised the limitations of this approach for quantitative studies, little else was available. The concentration of nutrients used for plate counts was usually far greater than likely to be present in soil, thus calling into question the relevance of many findings on population sizes and trends. Despite all this, the approaches used at that time were not without success. For example, in 1952, Selman Waksman at Rutgers University, USA, was awarded the Nobel Prize for medicine in recognition of his discovery of antibiotics produced by organisms in soil. At Rothamsted, UK, Norman Walker discovered a new class of soil bacteria responsible for nitrification in soil: *Nitrospira* to be added to the better known *Nitrobacter* and *Nitrosomonas* species.

By the 1960s, it was becoming clear that the majority of bacteria and fungi in soil did not grow under the laboratory conditions normally used — the term "viable but unculturable" began to be used. This recognition arose due to the so-called direct counting of soil microbes when it was found that the number of organisms observed when soils were examined under the microscope was many

times greater than numbers based on plate counts (Skinner *et al.*, 1952). One direct counting technique, not relying on the growth of colonies on nutrient agar, was a method developed by Jones and Mollison (1948) and often called the Jones–Mollison agar film technique. In this method, a known mass of soil is dispersed in a known volume of warm liquid agar and small volumes taken and allowed to solidify as thin films in a haemocytometer slide of known depth. Agar is used not as a source of nutrients but simply as a means of converting a liquid dispersion to a solid film that can be observed under a microscope. The solidified agar film is transferred from the haemocytometer to a microscope slide, stained with an appropriate stain that is reasonably specific to living organisms as opposed to dead organic particles, and the number of stained organisms counted under a microscope. We return to this method later in the chapter.

So far as we can tell, 50 years after the event, David Jenkinson did not initially set out to develop a method for measuring the quantity of organic C held in the soil microbial population. He had entered the world of soil science from a background of a Ph.D. in organic chemistry and initially applied the methods of organic analysis to studying the chemical composition of soil organic matter (SOM). But he rapidly concluded that SOM was such a complex mixture that the chemical analytical methods available at the time were incapable of yielding much useful information, so he turned his attention to the dynamics of SOM rather than its chemical structure. He and others realised that freshly formed SOM, derived from recent inputs of plant material, behaved differently to native SOM, much of which exhibited great stability and resistance to microbial decomposition.

Jenkinson was amongst the first to apply isotopic methods to distinguish between recently derived C and native C in soil. He did this by growing ryegrass in an atmosphere labelled with $^{14}CO_2$ and then adding the ^{14}C-labelled plant material to soil under field conditions for up to 4 years. In an earlier paper Jenkinson (1965) had shown that the labelled grass initially decomposed rapidly — about two-thirds of the added C was evolved as CO_2 within 6 months in the temperate climate of Rothamsted, but thereafter decomposition was far slower with about one-fifth of the labelled C remaining in soil after 4 years. This was a strong indication that SOM could be regarded as existing in pools or fractions that differed greatly in their rates of turnover. We consider that this was one powerful strand of reasoning that led Jenkinson to the concept of the microbial biomass as a discrete and meaningful fraction within soil.

Simultaneously, there was interest in the idea of different rates of turnover of SOM in different fractions from a more practical viewpoint, namely the supply of nitrogen (N) from soil to crops. SOM in agricultural soils typically contains

several thousand kg ha^{-1} of N in organic forms, yet only a few percent of this is converted into inorganic forms and thus available for plant uptake each year, typically around 20–200 kg N ha^{-1}. During the 1960s, and still today, there is interest in better predicting the N supply from SOM during a given crop-growing season so that fertiliser applications can be adjusted accordingly. Jenkinson was involved in such work around this time and published a method for predicting N mineralisation based on the extractability of specific forms of organic C from soil using dilute barium hydroxide solution (Jenkinson, 1968).

A third strand of reasoning that certainly had a major influence on his thinking was ongoing research at the time by plant pathologists at Rothamsted and worldwide experimenting with soil fumigants to control soil-born plant fungal diseases and parasitic nematodes. The chemicals used were typically broad-spectrum biocides designed to kill virtually all soil organisms; they included formaldehyde, methyl bromide, trichloro(nitro)methane (known as chloropicrin) and other halogenated hydrocarbons. It was frequently observed in such studies that, even in soils with no apparent pest or disease problem, plant growth was stimulated; in many cases this was attributed to additional mineralisation of N compared to unfumigated soil, though other factors were also thought to be involved as discussed by Jenkinson (1966) and Powlson (1975). The source of this additional N was a matter of interest and, as a key aspect of fumigation was killing of organisms, decomposition of the killed soil microbial cells was considered a likely source. It seemed likely that a recolonising population of microorganisms in the soil would utilise the cells of killed organisms as food with the N in organic forms being mineralised and released as inorganic N. This would also account for the period of enhanced soil respiration observed following soil fumigation, measured as enhanced evolution of CO_2 and uptake of oxygen. This line of reasoning appears to have been the breakthrough in Jenkinson's thinking that led to the idea of a way of measuring the quantity of C held in organisms before being killed by the fumigant. It is interesting that this "eureka moment" came through cross-fertilisation between scientists working in different fields — SOM studies and plant pathology. It is a reminder that all scientists can benefit from hearing about ideas in other fields — something about which Jenkinson was particularly enthusiastic.

Establishing the Chloroform Fumigation–Incubation (FI) Method

^{14}C-labelled C in the soil amounted to only 1.8% of the total soil organic C, yet the CO_2-C evolved from untreated soil when incubated in the laboratory for

Table 1.1 Labelled and unlabelled CO_2 evolved by soil exposed to partial or complete sterilisation treatments, inoculated with fresh soil and incubated for 10 days at 25°C.

Treatment	CO_2-C evolved in 10 days (μg C g^{-1} soil)		Percentage of labelled C in evolved CO_2-C
	^{14}C-labelled	Unlabelled	
None	14.7	146	9.2
Air-drying	23.2	195	10.6
Irradiation (0.25 Mrad)	33.8	238	12.4
CH$_3$Br vapour	47.5	239	16.6
CHCl$_3$ vapour	49.4	259	16.0
Oven-dried (80°C)	54.6	347	13.6
Oven-dried (100°C)	56.0	493	10.2
Autoclaving (120°C)	58.6	524	10.1

Note: The soil had previously been incubated with labelled plant material for 1 year under field conditions.
Source: Adapted from Jenkinson (1966).

10 days at 25°C was more than five times as enriched (Table 1.1). This indicated that C involved in biological processes leading to CO_2 evolution was far more heavily labelled with ^{14}C than the soil organic C as a whole. All of the treatments tested increased total CO_2 evolution but, importantly, several of them greatly increased the percentage of labelled C in the CO_2 evolved. Fumigation with chloroform or methyl bromide (CH$_3$Br) almost doubled this proportion to around 16% (Table 1.1). Jenkinson reasoned that the C in cells of microorganisms (the soil microbial biomass) was likely to be the most heavily labelled organic C fraction in the soil, so a treatment giving the greatest increase in ^{14}C labelling of evolved CO_2 must be selectively acting on this fraction. Because CHCl$_3$ was easier to use than CH$_3$Br and was thought to be less toxic to humans, Jenkinson used CHCl$_3$ fumigation for further work. Although oven-drying and autoclaving increased evolution of labelled CO_2 by a similar amount to fumigation, they also increased evolution of unlabelled CO_2 considerably. Jenkinson interpreted this to mean that these treatments, in addition to killing organisms, also rendered additional non-biomass (and more lightly labelled) forms of organic C decomposable and thus were less selective.

An important conclusion from the results with labelled soil was that CHCl$_3$ had very little effect on the rate of decomposition of non-biomass organic C in

soil. Following this conclusion, and several other simplifying assumptions, Jenkinson set out the following expression for estimating the quantity of C in soil microbial biomass (B_C) as follows:

$$B_C = \frac{F_C}{k_C} \,,$$
(1.1)

where F_C is the flush of decomposition following chloroform fumigation defined as (CO_2-C evolved by fumigated soil during a 10-day incubation at 25°C) minus (CO_2-C evolved by untreated soil during the same period) and k_C is the fraction of the biomass C decomposed and evolved as CO_2-C during the incubation.

A preliminary value for k_C was derived by growing a ^{14}C-labelled culture of a single species of soil bacteria, *Nitrosomonas europaea*, adding this to soil and measuring the amount of $^{14}CO_2$ evolved after $CHCl_3$ fumigation and incubation under the standard conditions. It was found that 28% of the bacterial C was evolved (Jenkinson, 1966) giving a k_C value of 0.28, but to avoid an appearance of spurious precision, this was rounded to 0.3.

The Jenkinson (1966) paper also contains descriptions of several experiments designed to test other hypotheses that had been put forward to explain the flush of CO_2 evolution and N mineralisation observed after the so-called partial sterilisation treatments. The results strongly argued against the other hypotheses and supported the idea that the C came from decomposition of organisms killed by the treatments. This paper is elegantly written and warrants re-reading today as an example of a clear but closely argued account of quite complex experiments. Ten years later, David Jenkinson and David Powlson revisited the issues. They examined in detail the impacts of $CHCl_3$ fumigation on soil metabolism (Jenkinson and Powlson, 1976a) and measured the flushes of decomposition caused by fumigation, gamma irradiation, air-drying or autoclaving when applied to a range of soils differing in organic matter content, soil type, pH and management (Figure 1.1). These experiments confirmed the earlier conclusions that chloroform fumigation killed soil organisms, rendering them decomposable and giving rise to the flush of decomposition, but had little effect on the decomposability of non-biomass soil organic C. The flush of N mineralisation following fumigation was also taken as strong indication of an impact on soil organisms followed by their decomposition as organisms are rich in N compared with SOM as a whole: they typically have a C/N ratio of around 5 or less compared with 10 or more in total SOM. Powlson and Jenkinson (1976) found that in a range of soils the ratio (CO_2-C evolved)/(N mineralised) after $CHCl_3$ fumigation was typically about 6 compared to a range of about 12–20 in unfumigated soil.
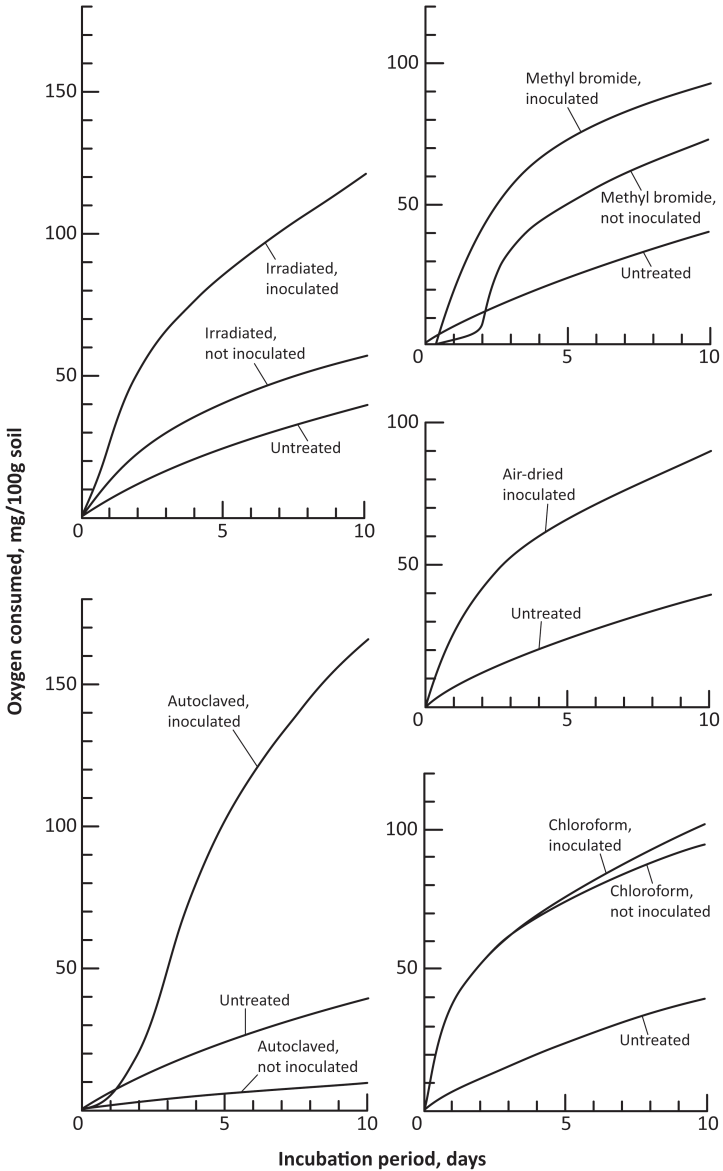
Figure 1.1   Oxygen uptake by soil from the farmyard manure-treated plot of the Broadbalk experiment when incubated for 10 days at 25°C after different partially or completely sterilising treatments, with or without an inoculum of fresh soil. (Adapted from Powlson and Jenkinson, 1976.)

Applying the range of treatments to soils differing in total organic C content demonstrated the complexity of the impacts of the treatments. For example, autoclaving was very effective at killing organisms as shown by the almost complete absence of CO_2 evolution or oxygen uptake if the autoclaved soils were not inoculated with fresh soil (Figure 1.1). It was also clear that this treatment rendered large amounts of non-biomass soil C decomposable in addition to killing organisms. Gamma irradiation at the high dose used, 2.5 Mrad, was thought to be sterilising the soil but considerable CO_2 evolution was observed after treatment, even in uninoculated soil. It was thought that this was either due to the chemical action of free radicals formed during irradiation or to continued activity of enzymes in or released from cells that had been killed in the sense that they were incapable of division but still retained enzymes capable of at least some activity (Jenkinson and Powlson, 1976a). In contrast, air-drying, a treatment to which soils are frequently subjected in the field, had a limited killing effect but had a significant impact on the decomposability of non-biomass organic matter. This is consistent with the observation that the C/N ratio of the organic matter mineralised during the flush following air-drying and rewetting was wider than for the fumigation treatments: generally 8–12, closer to that of SOM as a whole (Powlson and Jenkinson, 1976).

In another of the 1976 series of papers (Jenkinson, 1976), 11 different laboratory-grown organisms were added to soil, fumigated with chloroform and the amount of C evolved as CO_2 measured. Averaged over all the organisms (yeasts, fungi and bacteria), 50% of the added C was evolved giving a value of 0.5 for k_C in Eq. (1.1); this value was recommended in the fifth paper of the series (Jenkinson and Powlson, 1976b) setting out a practical method for measuring microbial biomass C in soil. On the basis of later work, the value of k_C was modified to 0.45 (Jenkinson and Ladd, 1981). In the method proposed in 1976, the CO_2 evolved by fumigated and unfumigated soil is absorbed in sodium hydroxide solution and the quantity determined by titration; in some later developments the quantity of CO_2 evolved is determined by taking gas samples from the incubation vessel and determining CO_2 concentration using gas chromatography. In the detailed studies leading to the final FI method much was learned by monitoring oxygen uptake during incubation, as shown in Figure 1.1, using a manometer connected to the incubation vessel (Jenkinson and Powlson, 1976a; Powlson and Jenkinson, 1976).

In a completely independent test of the FI method, Jenkinson *et al*. (1976) reported, for a set of eight soils, a comparison of microbial biomass C as estimated by this method and as derived from direct microscopy. A modification of the Jones and Mollison (1948) direct counting method was used. Organisms in

the agar films were stained using phenolic aniline blue, as originally used by Jones and Mollison: several fluorescent stains were tested but proved unsatisfactory because they did not stain such a wide range of organisms as aniline blue. The main modification to the Jones–Mollison method was to allocate stained organisms to size classes rather than simply count total numbers. Organisms classed as roughly spherical, such as bacteria, were divided into 13 size classes with diameters ranging from 0.3 to 19 μm, and the numbers in each size class counted. Hyphae, regarded as cylinders, were divided into seven diameter classes ranging from 1 to 11 μm, and the lengths in each class measured. In this way a total biovolume for each soil was derived and a biomass C value calculated by making assumptions about dry matter content and C concentration of organisms. This work was incredibly tedious and time-consuming and also subject to a fair degree of subjectivity; three people took part in the work and considerable efforts were necessary to ensure that their results were consistent with each other due to the difficulty of being certain of whether some blue marks were truly stained organisms or artefacts. For seven of the eight soils studied there was a close relationship between biovolume and biomass C as measured by FI. Quite remarkably there was also a close correspondence between actual values for biomass C derived from the two methods (Jenkinson *et al.*, 1976). This numerical correspondence was not taken too seriously in view of assumptions made in converting biovolume to biomass C but the close correlation between the two totally different approaches was taken as strong independent evidence for the validity of the FI method. The conclusion was later strengthened by Vance *et al.* (1987b) using a wider range of soils.

The soil that was an exception to the relationship was acidic (pH 3.9): direct counting of organisms showed a large population but the flush of decomposition following chloroform fumigation, and other treatments, showed a long lag phase and a very small increase in CO_2 evolution compared to untreated soil as shown in Figure 1.2 (Powlson and Jenkinson, 1976). Without fully understanding the reasons, it was concluded that the FI method was not applicable to acid soils (Jenkinson *et al.*, 1976; Jenkinson and Powlson, 1976b). Unfortunately, in subsequent years, it was not uncommon for scientists to ignore this and apply the method to acid soils — then complain that the method did not work!

The FI method immediately attracted controversy. Shields *et al.* (1974) claimed that the value obtained for biomass C was erroneously large, their argument being based on experiments in which ^{14}C-labelled glucose was incubated with soil for 14 days and the flush of ^{14}C-labelled CO_2 after $CHCl_3$ fumigation determined. From their data, they concluded that the calculated size of the newly formed ^{14}C-labelled biomass was unrealistically large, meaning that $CHCl_3$ was
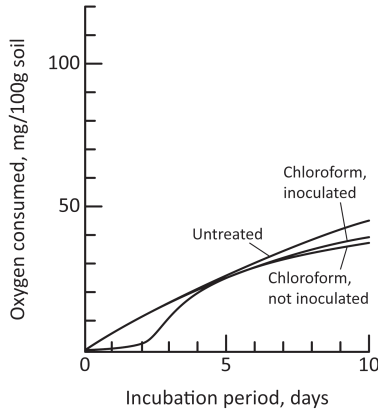
Figure 1.2   Oxygen uptake by an acid soil (pH 3.9) following fumigation with $CHCl_3$ and incubation for 10 days at 25°C with and without an inoculum of fresh soil. (Adapted from Powlson and Jenkinson, 1976.)

rendering non-biomass C fractions decomposable. Jenkinson and Powlson (1976a) conducted several experiments designed to test whether this was true, in part repeating some of the experiments conducted by Shields *et al.* (1974) but also using combinations of $CHCl_3$ fumigation and gamma irradiation. Because of the complexities of the effects of the treatments, the general complexity of SOM and our continuing poor understanding of many aspects of its dynamics, it proved difficult to obtain entirely unambiguous evidence. Similar difficulties arose in arguments over the use of a control in calculating biomass C, as discussed later. However, a crucial issue was that Shields *et al.* (1974) used a k_C value of 0.3, the preliminary value proposed by Jenkinson (1966). In refuting the arguments of Shields *et al.* (1974), Jenkinson and Powlson (1976a) pointed out that using the larger k_C value of 0.5 (Jenkinson, 1976), later revised to 0.45, gave an entirely realistic value for biomass C.

The FI Method: Benefits, Drawbacks and Conundrums Raised

A great advantage of the FI method as proposed and thoroughly tested by Jenkinson (1966) and Jenkinson and Powlson (1976b) was that it made soil microbiology accessible to a far wider range of soil researchers than was previously the case. In fact, Jenkinson once said "now everyone can be a soil microbiologist

within 24 hours". Perhaps of broader significance is that the methodology opened a new way of thinking about soil processes that linked chemistry and elemental cycling to the biology driving some of the key processes in soil. In this way, the development of the FI method, and subsequent developments discussed later in this chapter, can truly be regarded as a paradigm shift in soil science. It might be regarded as a bridge between classical soil microbiology, with an emphasis on identifying organisms responsible for specific processes, and modern concepts of biogeochemical cycling. However, the FI method did have some practical disadvantages. Firstly, the method required a 10-day aerobic incubation, often preceded by a pre-incubation to allow effects of disruption due to sampling, sieving and handling of soil to subside — necessary if the CO_2 evolution by the control unfumigated soil is to be truly representative of "untreated" soil. Secondly, it could not be used in acid soils or those containing actively decomposing substrates. Nevertheless, it soon became accepted as a standard method and transformed our understanding of SOM dynamics.

A drawback was the need to purify the $CHCl_3$ to be used for soil fumigation. For many years, $CHCl_3$ purchased from chemical suppliers contained 1% ethanol as a stabiliser to prevent the formation of free radicals. It is essential that all traces of ethanol are removed prior to fumigation otherwise some is retained by the soil and mineralised to CO_2 during the 10-day incubation, giving erroneously inflated biomass C measurements. For many years we used a tedious acid pretreatment of the ethanol-containing $CHCl_3$ followed by washing, distillation and storage over anhydrous Na_2SO_4 at 4°C. However, on storage, even at 4°C, a fraction of the distilled $CHCl_3$ rapidly decomposed to phosgene gas. Not only does this gas have a most unpleasant smell, but it is also extremely toxic, accounting for its use as a chemical weapon in World War I. Fortunately, this tedious purification procedure is no longer necessary as ethanol-free chloroform can be readily purchased containing traces of an alternative stabiliser, usually amylene.

In both the FI and FE methods (described later), it is essential that ethanol-free $CHCl_3$ is used or the ethanol will give inflated biomass measurements: in the FI method $CHCl_3$ will be mineralised to CO_2 and in FE it will be measured as organic C. Table 1.2 illustrates the magnitude of error introduced if $CHCl_3$ containing ethanol is used.

Controversy over the Control in the FI Method

A remarkable feature of the FI method is that, after fumigation and the dissipation of the initial flush of CO_2 due to the mineralisation of the fumigation-killed

Table 1.2   Biomass C (μg g^{-1} soil) measured following fumigation without and with alcohol-free $CHCl_3$.

Soil number	Pure chloroform	Chloroform + ethanol
1	476 ± 11	884 ± 21
2	1238 ± 38	1658 ± 28
3	291 ± 8	502 ± 10
4	433 ± 16	838 ± 48
5	372 ± 20	677 ± 24

biomass, the rates of evolution of CO_2 in the fumigated and non-fumigated soils are approximately equal for periods of weeks or even months (e.g. Powlson and Jenkinson, 1976a; Kemmitt *et al.*, 2008). The implications of this were largely overlooked during the method development and for a long time after. It implies that the small recolonising microbial population that develops in the fumigated soil after fumigation (initially ca. 10% of that in the corresponding non-fumigated soil) and the large, intact, population in the non-fumigated soil are both mineralising recalcitrant, humified SOM at the same rate. This does seem an unlikely occurrence. Surely the larger population would be expected to mineralise SOM faster than the fumigant-damaged one? Reber (1967) reported that the recolonising population, as well as being smaller, was mainly composed of bacteria, particularly *Pseudomonas* species, which largely utilise labile substrates. Most of the fungal population was eliminated and remained low for many weeks. Other work, using modern molecular methods (Dominguez-Mendoza *et al.*, 2014; Chen *et al.*, 2015), also showed that there are distinctly different microbial community structures in fumigated and non-fumigated soils.

Empirically, it therefore seems highly unlikely that this small recolonising population in the fumigated soil, having limited diversity, could possibly mineralise the large pool of humified and recalcitrant SOM to the same extent as the large, structurally diverse native population. Yet, this is precisely what was proposed in the 1976 papers.

The rates of CO_2-C evolution from the fumigated and non-fumigated soils have repeatedly been shown to be remarkably similar after 4–5 days of aerobic incubation, i.e. once the flush of decomposition of the fumigant-killed biomass is over (e.g. Powlson and Jenkinson, 1976; Wu *et al.*, 1996; Kemmitt *et al.*, 2008). Similarly, Jenkinson (1966), using soil previously incubated with ^{14}C-labelled plant material for several years, found that the ^{14}C enrichments of the CO_2 evolved by fumigated and unfumigated soils were approximately equal after the initial

$CHCl_3$-induced flush of decomposition had subsided. This indicated that the same pool of non-biomass SOM was being mineralised in both cases. However, Voroney and Paul (1984) suggested that the two different microbial populations were incapable of the same rates of SOM mineralisation. They considered that the small recolonising population was incapable of significant organic matter mineralisation so that biomass C (B'_C) was best calculated from

$$B'_C = \frac{F'_C}{k'_C},\qquad(1.2)$$

where B'_C and k'_C are as in Eq. (1.1) but $F'_c = CO_2$-C evolved from $CHCl_3$-fumigated soil in 0–10 days, i.e. CO_2-C evolved by the unfumigated control soil is not subtracted to derive the value for the flush of decomposition because the quantity of CO_2 derived from non-biomass SOM during the incubation is taken as zero.

Clearly, this is much more than a philosophical debating point. Both Eqs. (1.1) and (1.2) cannot be correct. If a control is not subtracted (Eq. (1.2)), the calculated biomass will be considerably larger than if it is (Eq. (1.1)).

The original data from Jenkinson and Powlson (1976b) is given in Table 1.3. It clearly shows a very consistent increase in calculated biomass C of around 30% if a control is not used. It should be noted that they used the control value obtained over 10–20 days, to overcome initial increases in respiration caused by sampling or plant residues. Nowadays, the standard procedure is to give the soil a conditioning incubation at 25°C and 50% water-holding capacity over soda lime for 5–7 days to trap CO_2 and to permit respiration and microbial activity to stabilise before analysis. With the advent of the FE method described later this procedure is theoretically unnecessary. However, it is still usually adopted by us to minimise possible artefacts arising from soil sampling and disturbance. The approach of Voroney and Paul (1984) would seem to have advantages in that the measurements of biomass without the need to measure a control would be much simplified. In fairness, they argued against this. However, if their approach is used, biomass measurements would be much larger than if the original approach of Jenkinson and Powlson (1976b) is adopted. Indeed, the difference is often much larger than those in Table 1.3, sometimes as much as twice as large (e.g. Schnürer *et al.*, 1985). The modification of Voroney and Paul (1984) was, unfortunately, adopted by many workers, particularly in North America. While some (e.g. Schnürer *et al.*, 1985) wisely published actual CO_2 measurements and biomass C values calculated using both approaches, others did not.

Table 1.3   Biomass C (μg g^{-1} soil) determined by the FI method calculated with and without a 10–20-day control.

Soil number	Control subtracted (BC1)	Control not subtracted (BC2)	Ratio of BC1/BC2
1	227	356	0.63
2	184	287	0.64
3	524	773	0.68
4	431	637	0.68
5	246	347	0.71
6	242	351	0.71
7	1178	1733	0.68
8	1308	1920	0.69
9	1033	1555	0.66

Source: Data modified from Jenkinson and Powlson (1976b).

After considering all the evidence, we are able to state, with a high degree of certainty, that the original method of Jenkinson and Powlson (1976b) provides the best estimate of biomass C by FI and that of Voroney and Paul (1984) is not valid. Our evidence is as follows:

(1) As stated above, Jenkinson *et al.* (1976) found that there was a remarkably close relationship between biomass C measured by direct microscopy and FI in seven forest, arable and grassland soils having near-neutral pHs. Only in a single acidic soil did this not hold. Vance *et al.* (1987a) found similar results.

(2) The finding that fumigated soil evolves CO_2 at roughly the same rate over long periods as the comparable non-fumigated soil cannot be accounted for by the Voroney and Paul (1984) method. Using their approach means that the magnitude of biomass C is determined by the length of incubation, increasing indefinitely — an improbable event. Surely the respiration rate in the fumigated soil should approach zero after the fumigant-killed biomass has been mineralised? In fact, the observed result is that it returns to almost the same rate as in the non-fumigated soil after 4–5 days. This is very much in line with what would be expected if mineralisation of native SOM proceeded at the same rate in both fumigated and unfumigated soils.

(3) The finding that similar rates of lightly labelled $^{14}CO_2$ were evolved from a long-term ^{14}C-labelled soil with and without fumigation, after the initial flush

was over at 4–5 days (Jenkinson, 1966), strongly suggests that the same pool of pre-existing non-biomass SOM was being mineralised at the same rate in both soils.

(4) Wu *et al.* (1990) heavily labelled a soil with ^{14}C-labelled glucose, so producing a heavily labelled biomass. Following fumigation and incubation, after the initial flush had subsided, fumigated and unfumigated soils both evolved $^{14}CO_2$ at the same rate for many weeks. This should not have occurred according to Voroney and Paul (1984).

(5) The FE method will be discussed in more detail later. It is based on extraction and chemical analysis of biomass components (C, N, P, etc.), unlike the FI method which is based on microbial activity. In the FE method the control is not controversial and biomass C is simply measured from the difference between C extracted from a fumigated minus unfumigated soil. Therefore, apart from the fumigation step, it is a fundamentally different method to FI. Wu *et al.* (1990), using nine soils containing between 0.9 and 4.2% C, reported a linear 1:1 relationship ($y = 0.99x$; $r = 0.99$) between biomass C measured by FI and FE. Clearly, these practically identical biomass measurements, using two very different approaches, could not have been measured from the approach of Voroney and Paul (1984).

(6) Wu *et al.* (1996) incubated a grassland soil with ^{14}C glucose for 27 days and then fumigated it with $CHCl_3$. Biomass C was measured by both FI and FE. Both methods gave extremely similar results. The rates of ^{14}C-labelled C and unlabelled CO_2-C evolved were each near-identical and the CO_2-C evolution curves were approximately parallel. The specific activities of the CO_2-C evolved from both the fumigated and non-fumigated soils were extremely similar after the flush. The specific activity of F_C (measured in the FI method) and E_C (measured by FE) were not significantly different.

These results all concur in suggesting that the original proposal of Jenkinson and Powlson (1976b) is approximately correct and the use of a non-fumigated soil as a control incurs a trivial error in biomass C calculations compared to the approach advocated by Voroney and Paul (1984).

Microbial Biomass C Measurements by the Chloroform Fumigation–Extraction (FE) Method

Following $CHCl_3$ fumigation there is an increase in the C extractable with aqueous solutions from the fumigated compared to the non-fumigated soil. This was

first described by Jenkinson and Powlson (1976a) and Powlson and Jenkinson (1976) but, quite remarkably, its significance was not recognised at that time. Probably they were preoccupied with the issues raised in developing the FI method. Brookes *et al.* (1982) published a method for measuring the quantity of phosphorus (P) in the soil microbial biomass based on soil fumigation followed by extraction with 0.5 M $NaHCO_3$. This was the first FE method and stimulated thinking that led towards an extraction method for biomass C.

In 1985 an American Ph.D. student, Eric Vance, joined David Jenkinson's group as a Fulbright Scholar. His aim was to investigate, with Phil Brookes, the use of the FI method in acidic forest soils. Hitherto, the FI method had proved to be invalid in low-pH soils. Eric showed that the reason for the failure of FI under such conditions was that mineralisation of non-biomass SOM was largely inhibited in fumigated acidic soils (Vance *et al*., 1987a); so it transpired that, in the special case of strongly acidic soils, the contention of Voroney and Paul (1984) regarding the invalidity of the control, as discussed above, was correct. Eric Vance also showed that the extra C released by $CHCl_3$ was closely related to soil adenosine 5′ triphosphate (ATP) content and so could be used as an estimate of biomass C (Vance *et al*., 1987b). This gave rise to the FE method for biomass C, and the paper describing the method (Vance *et al*., 1987c) remains the most widely cited paper in the journal *Soil Biology & Biochemistry*. Indeed, some of the original data from Powlson and Jenkinson (1976) was used to calibrate the FE method in Vance *et al.* (1987c). So,

$$B_C = 2.22E_C, \tag{1.3}$$

where E_C = (organic C extracted after $CHCl_3$ fumigation) minus (organic C extracted from non-fumigated soil).

The factor 2.22 represents the proportion of biomass C extracted by 0.5 M K_2SO_4 solution after $CHCl_3$ fumigation and is analogous to k_C in the FI method; it is based on 45% of the C in the fumigated biomass being extracted after $CHCl_3$ fumigation. The same value is used for biomass N, as discussed later. The factor was not directly derived experimentally. Rather it was obtained from regressions between E_C and F_C (Vance *et al*., 1987c) and, for biomass N, between F_N and E_N (Brookes *et al*., 1987). This is why the control issue remains of key importance. If the use of a control in the FI method is wrong, then it follows that the factor 2.22 (equivalent to K_{eC} and K_{eN}) is also wrong and the estimation of biomass C and N by the FE method is flawed. The organic C in the extraction solution (normally 0.5 M K_2SO_4) was originally measured by digestion with potassium dichromate but later a far more convenient method was developed using an automated C analyser (Wu *et al.*, 1990).

The FE method overcame many of the limitations of FI and has now super-seded it. Measurements can be made immediately after fumigation rather than having to wait for 10 days as with FI. It may be used in acid soils (Vance *et al.*, 1987a), soils containing actively decomposing substrates under laboratory and field conditions (Ocio and Brookes, 1990; Ocio *et al.*, 1991) and in waterlogged soils including those used for growing paddy rice (Inubushi *et al.*, 1991; Qiu *et al.*, 2015). Neither method can be used in dry soils, at least only after the soil has received a conditioning incubation for 5–7 days, to allow the C released on air-drying to be mineralised (De Nobili *et al.*, 2006). The FE method is ideal for ^{14}C tracer studies involving microbial C dynamics, as the ^{14}C in the soil extracts can be measured directly by scintillation counting.

Biomass N

In the early work developing the original FI method, the flush of N mineralisation following $CHCl_3$ fumigation and incubation was measured and ascribed to N being released from killed biomass. Thus, the flush of N in the FI method was implicitly regarded as an indicator of the quantity of N held in the microbial biomass but it was not until considerably later that an explicit method for measuring biomass N was developed. This only occurred as interest in the FE method for biomass C (Vance *et al.*, 1987c) and P (Brookes *et al.*, 1985) progressed. Biomass N can be measured in the same extracts as biomass C, usually following Kjeldahl digestion, a rather tedious approach. Biomass N is calculated exactly as for biomass C, from

$$B_N = 2.22E_N, \tag{1.4}$$

where E_N = (organic N extracted after $CHCl_3$ fumigation) minus (organic N extracted from unfumigated soil).

Biomass N measurements derived in this way are very closely correlated with biomass C measured by FI and FE (Brookes *et al.*, 1985). Again, biomass N measured by FE overcomes the limitations of biomass C, as discussed above. FE measurements using ^{15}N are quite easily made. The main problem with biomass N is measuring very small concentrations of N in the extraction solutions.

Amato and Ladd (1988) introduced the ninhydrin-N method to measure biomass N. The ninhydrin reagent reacts with ammonium ions and the α-amino N groups in amino acids and proteins to produce an intense purple colour which can easily be measured spectrophotometrically. Ninhydrin-N and biomass N (B_N) measurements are strongly correlated and biomass N can be fairly accurately calculated from

$$B_N = 5.1E_{Nin}, \tag{1.5}$$

where E_{Nin} is (ninhydrin-N extracted from fumigated soil) minus (ninhydrin-N extracted from unfumigated soil).

The ninhydrin method has all the advantages of the other FE methods over FI and is rapid and accurate. It largely superseded the method for biomass N described above that ended with the rather cumbersome measurement of organic N in the extracting solution following FE. However, in studies where ^{15}N labelling of N in the biomass is to be measured, it is necessary to follow the original procedure or something similar as it is essential to measure ^{15}N enrichment in the entire biomass: Ninhydrin-N only represents a small sample of total biomass N.

Biomass P and S

Brookes *et al.* (1982) published a method for measuring biomass P in which the increase in P extractable by 0.5 M $NaHCO_3$ following $CHCl_3$ fumigation is measured. This was the first example of an FE method and laid the foundations for the FE methods for biomass C and N. In the biomass P method a correction is made for released P that is fixed on soil surfaces. Several analogous methods for measuring biomass S have been developed, based on extraction with various reagents, following 24 h $CHCl_3$ fumigation. The extraction reagents include 10 mM $CaCl_2$, 100 mM $NaHCO_3$, 16 mM NaH_2PO_4 (e.g. Saggar *et al.*, 1981; Chapman, 1987; Wu *et al.*, 1994).

Why is SOM so Stable? The Regulatory Gate Hypothesis

The FI method was more than a new approach to measure the soil microbial biomass. It also gave us a novel insight into the mechanisms controlling mineralisation of SOM. The global stock of soil organic carbon (SOC) is around 1500 Pg C, twice that in the atmosphere and about three times the amount in global vegetation (Lal, 2004). This C enters soil by photosynthesis and returns to the atmosphere by mineralisation. This exchange is about 10 times that entering the atmosphere from fossil fuels. Direct measurement of soil C stocks is an insensitive means of detecting changes, while the soil processes governing mineralisation and stabilisation of C are poorly understood. The mineralisation of SOC is a fundamental process upon which all life on the planet ultimately depends. It is therefore vital that we have a correct understanding of the key processes involved if we are to better manage this huge C pool. Furthermore, it is remarkable that no one has yet satisfactorily explained the paradox of SOC mineralisation, as outlined above. The paradox is that even if 90% of the soil microorganisms are destroyed by $CHCl_3$ fumigation, following fumigant removal, SOC mineralisation continues at the

same rate as in the undamaged soil once the initial decomposition flush is over, usually within about 4 days (Jenkinson and Powlson, 1976a; Wu *et al.*, 1996). This phenomenon has been known for nearly 60 years but has never been satisfactorily explained, even though $CHCl_3$ fumigation forms the basis for all the widely used methods of measuring soil microbial biomass, as discussed above. It is important to note that this mechanism only operates in soils above pH 5.5. Below this pH the C dynamics after fumigation are totally different, decomposition being inhibited by increased Al availability (Vance *et al.*, 1987a).

Humified SOC is thought to comprise, at least in large part, random condensation products of microbial metabolites and has no clearly defined chemical structure. It is highly resistant to microbial degradation, so that it is only mineralised at a very slow rate by the soil microbial biomass. It therefore seems incredible that when the soil microbial biomass is reduced to 10% of its original size by $CHCl_3$ fumigation, it can mineralise the same SOC pool at the same rate as the large undamaged population for many months without any fresh substrate inputs (e.g. Joergensen *et al*., 1990). It has recently been proposed that the description of SOC given above is incorrect and that it is largely composed of smaller molecules given protection from decomposition through association with minerals and metal oxides (Lehmann and Kleber, 2015) but this remains controversial. However, even if the recently proposed model of SOC is correct, the paradox remains of a small recovery of biomass following fumigation achieving the same rate of SOC decomposition as the far larger population in the unfumigated soil.

It is clear that only an extremely small microbial population survives $CHCl_3$ fumigation as shown by the negligibly small concentration of ATP in fumigated soil before $CHCl_3$ removal (Kemmitt *et al*., 2008). The population developing during a subsequent incubation must therefore develop from the very small number of surviving organisms, or from a small added inoculum. It is theoretically possible that the community structure of the small recolonising microbial population is the same as that in the intact non-fumigated soil. However, Chen *et al.* (2015) showed that the much diminished soil microbial biomass in the fumigated soil has a much smaller diversity (survivors mainly belong to the classes *Bacilli*, *Eurotiomycetes* and *Sordariomycetes*). Others have also shown vast differences in the community structure of pre- and post-fumigation populations (Reber, 1967; Dominguez-Mendoza *et al*., 2014). This suggests that the basal (or underlying) rate of SOC mineralisation is unrelated to the size, community structure or activity of the soil microbial biomass. Since SOC is mineralised, although very slowly, by the soil microbial biomass, it seems very strange that a group, or groups, of soil microorganisms have not evolved the ability to mineralise SOC more rapidly, thereby gaining more energy and achieving a competitive advantage over other less efficient microorganisms. If not, why
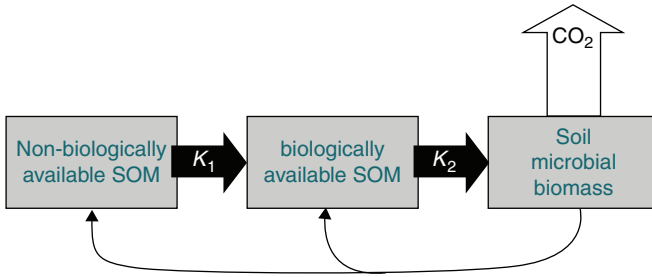
Figure 1.3   Diagrammatic representation of the 'Regulatory Gate Hypothesis'. (Adapted from Kemmitt *et al*., 2008.)

not? This surely suggests that the mineralisation rate of SOC is not under microbial control. If so, the question is, what is the mechanism?

Kemmitt *et al.*(2008) proposed a new theory to explain this phenomenon. They considered that SOC had to first undergo an abiotic process, or processes, before it could be mineralised by the microbial biomass. They termed this the Regulatory Gate Hypothesis (Figure 1.3).

In Figure 1.3, K_1 is the abiological transformation of non-bioavailable SOC. K_2 is the biological mineralisation of (now) bioavailable SOC. Arrows indicate that the soil microbial biomass may create both non-biologically available and biologically available SOC but is not able to directly influence the rate of K_1. The precise nature of K_1 is not known. It may include processes such as desorption, chemical oxidation, free radical activity, cleavage of phenolic rings (Majcher, 2000) or stabilised exocellular enzymes (thought unlikely). Probably, more than one mechanism operates simultaneously. Kuzyakov *et al.* (2008) suggested that enzymes released from the $CHCl_3$-induced lysis of microbial cells remained active and could mineralise soil organic C for several months. We discount this: exocellular non-stabilised enzymes would be readily utilised as substrate and have been shown to have a short half-life in soil (Burns, 1982; Nannipieri *et al*., 1983).

We are currently investigating the underlying mechanisms of the Regulatory Gate Hypothesis. In one experiment, we attempted to see whether we could measure abiotic release of mineralisable soil organic C. Six soils were fumigated for 24 hours, the $CHCl_3$ removed, then incubated four times for 4 days to destroy most of the microbial biomass. The soils were then washed carefully with 0.01 M $CaCl_2$ to remove as much $CHCl_3$-released microbial C as possible. They were then very slowly perfused for 63 days with water saturated with $CHCl_3$ (Kemmitt *et al*., 2008), the $CHCl_3$ being added to inhibit microbial activity. The perfusates
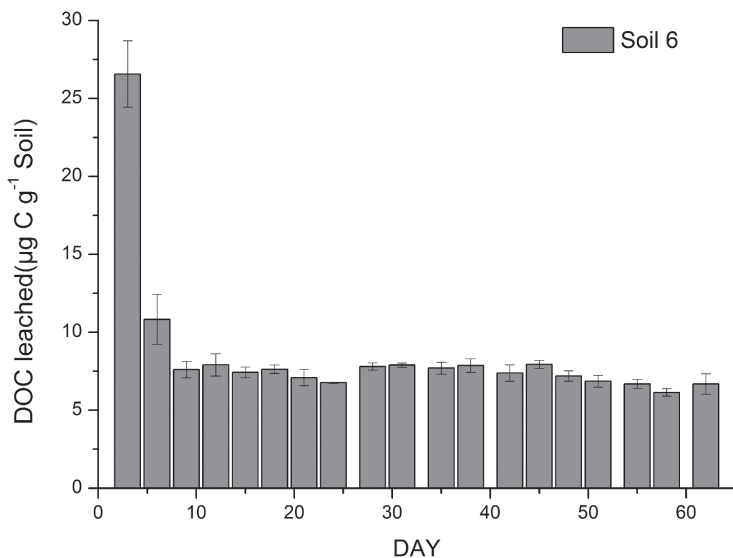
Figure 1.4   Soil organic C leached during a 63-day perfusion of a grassland soil.

were collected at approximately 5-day intervals and analysed for solubilised organic C. In newer work (Yang Feng Chen, unpublished data), the soil in Figure 1.4 was taken from a non-experimental grassland site near Hangzhou, China, that contained 7.02 g kg^{-1} organic C and had a pH of 8.7. After the initial flush of soluble C, the quantity released declined to a low level but the rate of release was remarkably constant for the duration of the experiment. This shows that organic C can be released abiotically into soluble forms at a slow but constant rate, which is in line with the Regulatory Gate Hypothesis. In another experiment (Yan Feng Chen, unpublished data), the perfusates were bulked, freeze-dried and added back to their parent soil. Soil-derived organic C in the freeze-dried extracts was mineralised rapidly, showing that the C released during perfusion was highly decomposable and readily available to the soil population, unlike total soil organic C.

The Regulatory Gate Hypothesis explains why mineralisation of SOC proceeds at the same rate, even if the microbial population size has been decimated, with both activity and diversity drastically changed. It suggests a mechanism whereby SOC is decomposed at the same rate, irrespective of the size of the population in the soil. The results are consistent with the idea that the soil population, whether large or small, damaged or intact, is acting on the same small but constant supply of SOC. This SOC has been transformed abiotically from forms that are not biologically available to forms that are available, and which have been

brought into contact with the microbes through diffusion into the soil solution. However, the mechanism(s) responsible for this first and abiotic step remain unidentified. We believe that the other possibility, that the SOC is mainly being mineralised directly by microbial activity, cannot be justified in the light of the above evidence.

Soil Adenosine 5′ Triphosphate Measurements

Cellular adenosine 5′ triphosphate (ATP) has been termed the energy currency of the cell. It can be readily measured in soil, following extraction with a suitable reagent, by the firefly luciferin–luciferase enzyme. The question here is, what is a suitable reagent? A good example of the problem is demonstrated by the long-running debate between the late Professor David Jenkinson and Professor Eldor Paul in the 1980s. Jenkinson and Oades (1979) proposed a method to measure biomass ATP based on ultrasonic dispersion of soil to disrupt microbial cells, followed by extraction of ATP with a reagent containing paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride), phosphate and trichloroacetic acid (TCA). The paraquat (a planar molecule, as is ATP) and phosphate inhibit ATP fixation on the soil colloids, and TCA is a very powerful inhibitor of enzyme activity. The phosphatases and ATPases, once liberated from the ultrasonified cells, would, if not deactivated, almost instantaneously dephosphorylate adenosine 5′ triphosphate (ATP) to adenosine 5′ diphosphate (ADP) and adenosine 5′ monophosphate (AMP) — it was for this reason that Jenkinson and Oades (1979) selected TCA as the extracting reagent. Paul and Johnston (1977) published an alternative ATP method based on extraction with a $NaHCO_3$–$CHCl_3$ reagent, which was later modified by Martens (1985) to also contain P and adenosine (the $NaHCO_3$ reagent). Both this, and the method of Paul and Johnston (1977), consistently measured very much smaller soil ATP concentrations than the TCA reagent. Brookes *et al.* (1983) measured the total soil adenylates ATP, ADP and AMP extracted with both reagents. They found that both reagents extracted similar amounts of total adenylates (i.e. $\sum A_T = (ATP + ADP + AMP)$). However, ATP comprised 80–84% of A_T with the TCA reagent but only 32–55% with the $NaHCO_3$ reagent: clearly a large discrepancy. Brookes *et al.* (1987) considered that it was due to phosphatases and ATPases retaining activity in the $NaHCO_3$ reagent and denaturing ATP. In contrast, they were immediately and completely deactivated in the TCA reagent. In the TCA method, one set of soils is extracted with a "spike" of ATP to correct for incomplete extraction as some of the ATP released from microbes will be sorbed on soil particles, thus giving a low ATP recovery in the extractant. Martens (2001) considered that the spike

approach considerably underestimated the true soil ATP content. He found that when soils were extracted six times, each time with fresh extractant, the sum of the ATP extracted greatly exceeded (by 2–9 times) the ATP measured using the spike approach. In contrast, Contin *et al.* (2002) found no significant difference in the ATP contents of four soils when extracted by the conventional "spike" method and by six separate extractions. Clearly there is an unbridgeable gap between the two sets of data and we still remain uncertain of the reasons. We continue to use the spike and consider it to be approximately correct.

The original TCA method of Jenkinson and Oades (1979) suffered from the disadvantage that paraquat was included to inhibit ATP fixation by soil during ultrasonic extraction. Paraquat, which was used as a herbicide until it was banned in Europe in around 2007, is highly toxic to both plants and animals, including humans. For ATP measurements, the paraquat was purified from Gramoxone, the commercial form in which it was purchased, by alcohol extractions to remove various unwanted components followed by re-extraction with ethanol–charcoal and crystallisation. This is a potentially dangerous operation, as Phil Brookes found when he attempted to hasten the process by boiling the ethanol–paraquat mixture over a Bunsen burner. Shortly after catching fire, a white cloud of ethanol and paraquat vapour poured out of the fume cupboard, out of the door, travelled a few metres down the corridor then dissipated. Unfortunately, the Rothamsted Safety Officer was walking down the corridor, saw what had happened and nearly had a fit. Any fool can follow the instructions in a Methods manual. This fool nearly poisoned a large number of his colleagues and burnt the building down!

Paraquat's dangerous properties always made the TCA method unattractive to many soil scientists. Its banning in Europe meant that a substitute was needed and Redmile-Gordon and Brookes (2011) tested a number of possible alternatives. Imidazole was shown to give the same ATP results as paraquat at concentrations of 1.10 M TCA, 0.25 M P and 0.6 M imidazole (termed the TIP reagent). Imidazole is also toxic, as are many laboratory chemicals, so great care must be taken. However, it is readily available, cheap, avoids the need for purification as with paraquat and no cumbersome conversion factors are required to compare ATP extracted by the two reagents. Apart from the above careless incident, we have experienced no health problems associated with either the TCA or TIP reagent despite frequent use.

The essential features of an ATP extracting reagent are that it must have the property of preventing the almost instant deactivation of ATP to ADP and AMP, as the firefly luciferin–luciferase system only detects ATP. It must also be able to prevent the fixation of ATP to soil via the negatively charged P groups or positively charged adenine groups. Finally, it must not inhibit the

luciferin–luciferase enzyme system during the quantitative measurement of ATP. For example, the Paul and Johnston (1977) method for measuring soil ATP, based on extraction with $NaHCO_3$–$CHCl_3$, gave higher recoveries of added ATP but soil ATP concentrations 2–3 times lower than with the TCA reagent. Brookes *et al.* (1987) showed that this reagent failed to inhibit phosphatases and ATPases, which explained their low ATP measurements. They considered that strongly acidic reagents were essential because they inhibit enzyme activity virtually instantaneously and that near-neutral reagents, such as $NaHCO_3$, should be avoided. Other reagents which have been tested include the H_2SO_4–P reagent of Eiland (1983), the H_3PO_4–DMSO–adenosine–urea–Zwittergent reagent (Webster *et al.*, 1984) and the dimethylsulfoxide–Na_3PO_4–NRB reagent (Bai *et al*., 1998). These, together with the TCA (Jenkinson and Oades, 1979) and TIP reagents (Redmile-Gordon and Brookes, 2011), give comparable results. In waterlogged, that is, paddy soils, ATP can be measured in conjunction with biomass C measurements by FE (Inubushi *et al.*, 1989, 1991, see Chapter 4).

The ATP Concentration of the Soil Microbial Biomass

All living organisms contain ATP, so it must have been selected for its role in cellular energy dynamics very early in the evolution of life. It has an exocellular half-life of less than 1 h (Conklin and McGregor, 1972) and is therefore a very reliable indicator of life in soil. The soil microbial biomass is generally considered as a largely resting population, with a mean cell division time of about 6 months and a much longer turnover time. Jenkinson and Ladd (1981) estimated a turnover time of 2.5 years for the biomass in soil of the Broadbalk continuous wheat experiment at Rothamsted. This long turnover time is a reflection of the very low substrate availability in most soils. Oades and Jenkinson (1979) measured a mean microbial ATP concentration of 10.6 μmol ATP g^{-1} biomass C in a group of Australian and UK soils. Jenkinson (1988) later reported a mean value of 11.2 ± 2.74 μmol ATP g^{-1} biomass C for a much wider range of ATP and biomass C measurements from Australian, Danish, English and New Zealand soils collated from the literature. He laid down strict criteria for including soils in his correlation. These were:

(1) The soils had all received a conditioning incubation of at least several days at 25°C before analysis.
(2) Soil moisture was below 60% water holding capacity.
(3) Soil pH was not below 4.8.

(4) All measurements were done by FI.

(5) Only results obtained with acidic extractants were included.

Since Jenkinson (1988) published the above correlations, there have been some significant changes in biomass methodology. The FE method permits biomass C measurements in acid soils, soils containing actively decomposing substrates and waterlogged soils (not included by Jenkinson, 1988). There is now a much larger literature base than previously. Contin *et al.* (2001) combined all the available literature, including soils below pH 4.8, those containing actively decomposing substrates and soils without a conditioning incubation. The mean biomass ATP concentration was 11.0 μmol ATP g^{-1} biomass C, based on 207 separate analyses, remarkably close to the earlier values.

This analysis demonstrates that, for all practical purposes, the microbial biomass has a constant ATP concentration of around 10–12 μmol ATP g^{-1} biomass C. This is close to the mean biomass ATP concentration reported by Knowles (1977) for a range of prokaryotic and eukaryotic microorganisms undergoing exponential growth *in vitro*. It is because of the observed constant concentration of ATP in biomass that ATP can be used as a proxy for biomass measurements and for testing or calibrating biomass methods, as discussed earlier in relation to FI and FE methods. The data in Figure 1.5(a) is an example of this close relationship.

The relationship is not particularly sensitive to temperature, with only a slow increase in biomass ATP concentrations with increasing temperature. Contin *et al.* (2000) showed that biomass C changed little in grassland and arable soils incubated between 0 and 25°C for 23 days. Soil microbial biomass ATP concentrations did gradually increase with increasing incubation temperature, from about 7 to 10 μmol ATP g^{-1} biomass C. Both values are well within the range of ATP values of exponentially growing microorganisms.

Biomass C can be measured in waterlogged soils by FE and by ATP using either the TCA or TIP extractants (Inubushi *et al.*, 1989, 1991; Qiu *et al.*, 2016). Both biomass C and ATP decline considerably during anaerobic incubation: ATP levels are rapidly restored, but not to the original levels, upon aeration. Biomass C and ATP relationships are shown in a number of aerobically (Figure 1.5(a)) and anaerobically (Figure 1.5(b)) incubated soils, incubated with and without ryegrass as a substrate. The aerobic soils had a mean biomass ATP concentration of 7.9 but in the anaerobic soils it was very much lower, at 2.83 μmol g^{-1} biomass C. Also, in the anaerobic soils there was no observable relationship between biomass C and ATP concentrations. We do not know the reasons for this difference between aerobic and anaerobic soils.
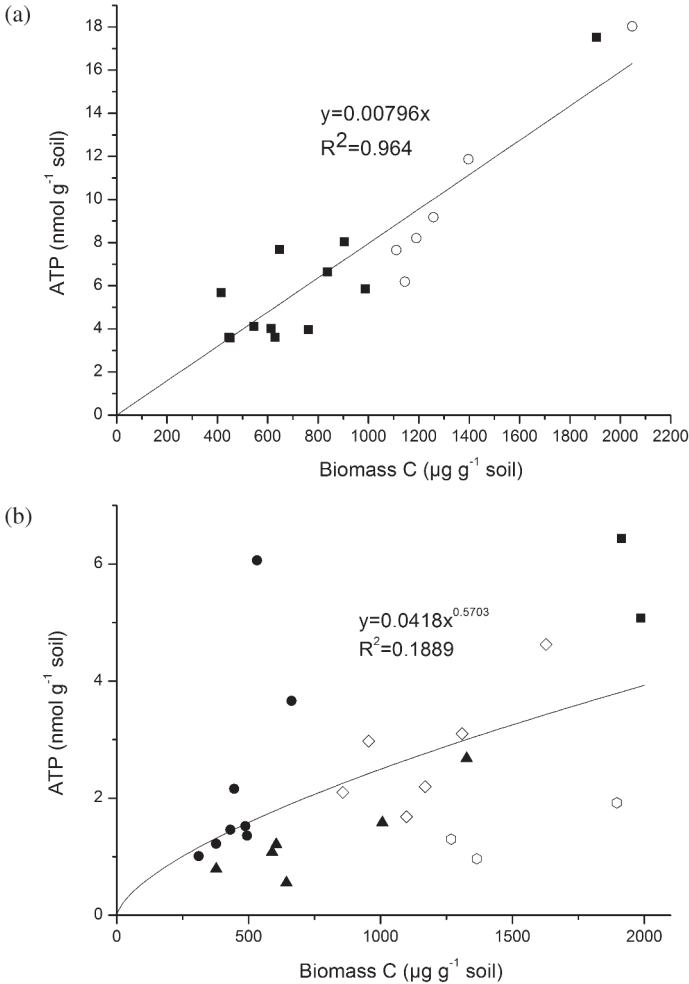
(a)

(b)

Figure 1.5   Biomass C and ATP measurements in (a) aerobically and (b) anaerobically incubated soils. (Adapted from Qiu *et al*., 2016.)

The Adenylate Energy Charge of the Soil Microbial Biomass

The adenylate energy charge (AEC) is defined as a linear measure of the metabolic energy stored in the adenylate energy pool (Atkinson, 1977). AEC = [(ATP) + 0.5(ADP)]/[(ATP) + (ADP) + (AMP)]. In actively growing cells it is stabilised between about 0.8 and 0.95, but it can theoretically range from 0 (all AMP) to

1.0 (all ATP). Lower values indicate a population that is stressed, senescent or incapable of cellular biosynthesis (Atkinson, 1977; Karl, 1980). Brookes *et al.* (1983) measured AEC by an enzymatic procedure following extraction with the TCA reagent. The aim was to determine whether the high values for ATP concentration in the soil microbial biomass resulted from (a) a small part of the population having an exceedingly high ATP concentration but with the majority being low in ATP (i.e. low AEC) or (b) a population as a whole that maintained a relatively high and constant ATP concentration (high AEC) independent of whether or not it is in active growth. A grassland soil at a field temperature of 8.4°C at the time of sampling, when extracted moist as soon as possible at 10°C, had an AEC of 0.85. In the same soil, air-dried for 5 days, AEC had decreased to 0.45, and 2.5 hours after rewetting it had increased to 0.76. Very comparable AEC values to those of Brookes *et al.* (1987) were reported by Joergensen and Raubuch (2002) in a moist, sandy soil during an 8-day incubation whether unamended (range 0.81–0.84) or glucose-amended (range 0.76–0.82). Anaerobically incubated soils behave differently: Inubushi *et al.* (1989) showed that AEC decreased rapidly upon anaerobic incubation, falling from a value of 0.76 to values in the range 0.54 to 0.34. Aeration increased AEC, but not to its pre-anaerobic level.

Significance of ATP and AEC Concentrations in the Soil Microbial Biomass

Because the energy inputs to soil from plant material are generally small compared to the size of the population, the soil microbial biomass must be considered as a largely resting, or dormant population, with a very low metabolic rate overall. Blagodatsky *et al.* (2000) and Blagodatskaya and Kuzyakov (2013) estimated that the active pool of soil microorganisms in soil that has not recently received labile substrate addition is very small: generally 1–2% of the total biomass and up to a maximum of 5%. However, the potential is much higher, with organisms able to begin substrate utilisation within a few hours when the active fraction may comprise up to 60% of the total population. The question is: why does the soil microbial biomass maintain an ATP concentration and AEC at levels found in microorganisms in exponential growth, when only a small fraction is undergoing active growth for most of the time? Surely a strategy based on spores or resting cells with low ATP and AEC would be more energy efficient in the normally substrate-poor environment of soil?

De Nobili *et al.* (2001) added small amounts of root extract or glucose (11 or 34 μg C g^{-1} soil), termed "trigger molecules", to three soils. Up to at least seven

times more CO_2-C was evolved from the soils than was added in the substrate. The largest response was with the root extract, suggesting that this substrate contained a wider diversity of "trigger molecules" than did glucose. More work is required to characterise this response and to determine the exact molecules that stimulate the biomass and how they are detected. De Nobili *et al.* (2001) hypothesised that the survival strategy of the microbial biomass is based upon the population of "resting cells" using energy to maintain a state of "metabolic alertness" and that it is ready to expend more energy if some cells detect early signals from the substrate, indicating that a more substantial "food event" is to come. The "trigger response" may therefore be an evolutionary response to the fact that although easily available substrate is a rare event in soil, it is never an impossibility. It is hypothesised that a survival strategy based on spores or other resting states may be less advantageous because the process of transforming from spore to a state capable of utilising fresh substrate may be too slow a response. In contrast, investing energy in maintaining both a high ATP concentration and AEC may give an evolutionary advantage.

Some Applications of Microbial Biomass Measurements

Long-lasting damage to soil microbes following soil fumigation for pest control

Although less widely used than in the past, volatile broad-spectrum biocides are still used in some situations for fumigating soils to control soil-borne pests and fungal diseases. Soil fumigation is mainly used for high-value crops in the horticultural industry and especially, but not exclusively, in greenhouses. Methyl bromide was used as a nematocide in various horticultural situations until it was phased out in 2005 under the Montreal Protocol because of its damaging impact on the Earth's ozone layer. As mentioned at the beginning of this chapter, in the 1960s there were many experiments worldwide testing the effectiveness of various fumigants in controlling crop pests and diseases and seeking to elucidate the mechanisms through which they influenced crop yields. Jenkinson and Powlson (1970) used the FI method (though it had not yet been fully developed) to examine the residual impact of past soil fumigation treatments in the field on the microbial biomass. Table 1.4 shows some results, recalculated from this work. In this example, soils had been fumigated with formaldehyde in the field either 6 months or 3 years before they were sampled and analysed in the laboratory.

In the soil that had received three treatments with formaldehyde, the last being 6 months before sampling, biomass C was less than half that in the untreated soil.

Table 1.4   Impact of field fumigation with formaldehyde on the size of the soil microbial biomass as measured using FI, and its rate of recovery.

	Period since last fumigation		
Field treatment with formaldehyde	**Unfumigated control**	**6 months**[a]	**3 years**
Soil microbial biomass C (μg g^{-1} soil)	322	147	242
Microbial biomass as a percentage of that in soil not fumigated in the field (%)	—	47	75

[a] Soil had received a total of three formaldehyde treatments during the previous 2 years.
Source: Adapted from Jenkinson and Powlson (1970).

After 3 years, it had recovered to some extent but was still significantly smaller than in soil that had never been fumigated, showing a slow rate of recovery. Measurements of this type clearly demonstrated the drastic impact of soil fumigation on the soil population, indicating that great caution was required if such biocidal treatments were to be used. Biomass N was affected similarly; at the time there was much interest in the role of N released from killed soil organisms contributing to observed increases in crop growth. If the biomass was as slow to recover as indicated here, less N would be released on subsequent field fumigations compared with the first: this was thought to be one factor contributing to observed declines in crop yield responses to repeated fumigations applied over several years.

Damage to Soil Microbes from Heavy Metal Pollution

All scientists like to believe that their research flows effortlessly from one step to the next, based on their previously carefully planned, and logically based, experiments. If only if it were true! Most of us, if we are really honest, would have to admit that, more than occasionally, we owe a fair deal to luck. The discovery of a negative impact on soil microbes of heavy metals, even at relatively low concentrations, is such an example. One of the less well-known long-term experiments run by Rothamsted Research is the Woburn Market Garden Experiment. It was started in the 1940s to test the value of farmyard manure and sewage sludge compared to inorganic fertiliser on the yields of vegetable crops including carrot and red beet. The produce was often served for lunch in the Rothamsted canteen until a famous Rothamsted scientist decided to analyse the heavy metals in the vegetables. Although what happened next is anecdotal, it comes from a reliable source. Apparently, he rushed into the canteen one lunch time, arms waving and

demanded that any vegetables were immediately removed from the menu. He had detected high cadmium (Cd) concentrations in some of them, at levels possibly injurious to human health. Hopefully everyone survived. However, his findings triggered considerable research into the effects of organic fertilisers, especially sewage sludge, which chelates and concentrates heavy metals, and thus influences their dynamics in soil and uptake by crops.

In the 1980s Phil Brookes decided to investigate whether heavy metal availability was increased due to enhanced microbial activity in organically manured soils, including those receiving sewage sludge (now often termed "biosolids"), compared to those given inorganic fertilisers. He did a very simple preliminary experiment which included measuring biomass C in soils from the Woburn Market Garden Experiment that had received inorganic fertiliser, sewage sludge or farmyard manure. At this time, it was believed that heavy metals in soils were at around the (then) European Union permitted concentrations and had no deleterious effects on plants, animals or soil microorganisms. He was amazed to find that soils treated with sewage sludge contained as little as half the biomass C concentrations as those which received farmyard manure or inorganic fertiliser. These were very preliminary results but then with Steve McGrath, an expert on heavy metals in soil and crops, the soils from the entire Woburn experiment were rigorously sampled and analysed for biomass, ATP and heavy metals. The results were conclusive. Heavy metals at quite low concentrations were damaging the soil microbial population (Figure 1.6; Brookes and McGrath, 1984). The biomass concentrations in the metal-contaminated soils were much smaller than in soils given farmyard manure or inorganic fertiliser. Furthermore, in these soils, there was no relationship between biomass C and total soil organic C, in contrast to the usual situation and as shown in the low-metal soils in this experiment (Figure 1.6). As the last applications of metal-contaminated sludge were added around 30 years before this work was done, it was clear that the metals had a long-lasting impact on the soil microbial population.

Shortly after this work was published (Brookes and McGrath, 1984) all hell broke loose. The then UK Prime Minister, Margaret Thatcher, and her Government, were about to privatise the organisations responsible for water supply and sewage treatment. Sewage sludge was normally disposed of by the water treatment plants by providing it free of charge to farmers as an organic fertiliser. Anything that stopped this, even partially, could have serious economic effects and damage water privatisation. The work of Brookes and McGrath (1984) had been noticed by the national and farming press, and this was the last thing the water authorities or the Government wanted prior to privatisation. The work was heavily criticised as irrelevant by the water authority chiefs, as the soil Cd levels in the sewage
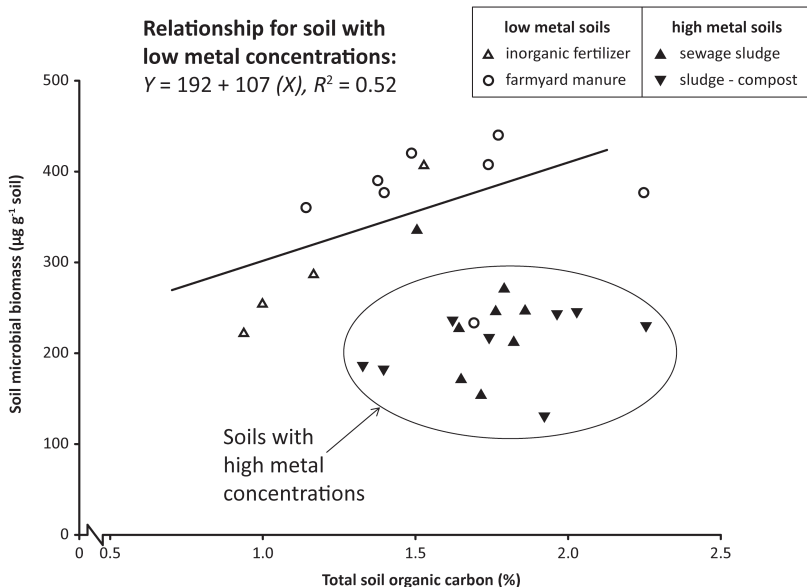
Figure 1.6   Soil microbial biomass and organic C concentrations in soils from the Woburn Market Garden Experiment that had previously received various treatments, leading to either high or low metal concentrations in soil. (Adapted from Brookes and McGrath, 1984.)

sludge-treated soils in the Woburn experiment exceeded European Union permitted limits. This was because it was an old experiment, started long before these limits were set and at a time when sewage sludge was often higher in metal concentrations that was currently the case. Brookes and McGrath clashed with these people at several acrimonious public meetings. However, other work (Chander and Brookes, 1994) showed that in the absence of Cd, zinc–copper interactions could produce similar results, so the effect was not specific to Cd. It was also found that autotrophic N_2 fixation by cyanobacteria was severely inhibited in the metal-contaminated soils (Brookes *et al*., 1986). This led to other research (McGrath *et al.*, 1988) showing a significant decrease in N_2-fixation by the Rhizobium–clover symbiosis in the metal-contaminated soils, with strong implications for the long-term sustainability of applying metal-contaminated sewage sludge to soil. This body of work led to a review of sewage sludge applications in the UK and a decrease in the permitted metal loadings. All this arose from a few chance biomass C measurements made for entirely different reasons!

Detecting long-term changes in soil organic C content from organic additions

When a soil undergoes a change in management, it can often take a period of years or even decades before a change in total SOC content can be reliably measured (e.g. Poeplau *et al.*, 2011), unless impracticably large numbers of samples are taken (Smith, 2004): this is the case even in replicated field experiments and it is even more difficult to detect changes in soils under practical management in farmers' fields. However, it has been found that the microbial biomass changes more rapidly than SOC as a whole. Thus, although microbial biomass C typically only constitutes 2–4% of total SOC, it can be used as a sensitive indicator of change. This is illustrated in Table 1.5 for impacts of land-use changes for soils in temperate and humid tropical climates.

In the UK temperate climate example, an area of old grassland was ploughed and converted into arable use 22 years before the total SOC and biomass C measurements were made. Total SOC had decreased to 61% of that in the soil that continued under grass but microbial C was only 42% of the grassland soil value. In the area of arable land that was converted from grassland into a bare fallow,

Table 1.5   Total SOC and microbial biomass C in two situations following land-use changes.

Land management	SOC (%)[a]	Soil microbial biomass C (μg g^{-1} soil)[a]
UK, temperate climate		
Old grassland	3.84 (100)	960 (100)
Arable (converted from grassland 22 years previously)	2.34 (61)	400 (42)
Bare fallow (converted from grassland 11 years previously)	1.89 (49)	127 (13)
Nigeria, humid tropical climate		
Secondary forest	1.46 (100)	542 (100)
Arable (cleared from forest 2 years previously)	1.23 (84)	280 (52)

[a] Values in parentheses are SOC or biomass C contents expressed as a percentage of those in grassland soil (UK) or secondary forest (Nigeria).

Source: Adapted from Powlson and Jenkinson (1976).

with virtually no inputs of plant material for 11 years (Barre *et al.*, 2010), SOC declined to 49% of the grassland value but biomass had declined much more to only 13%, clearly showing the sensitivity of biomass C to management changes that influence organic C input. In the tropical example, total SOC in the soil cleared from secondary forest and converted into arable cropping was 1.23% compared to 1.46% in soil still under forest, or 84% of its starting value. Although a change in SOC of this magnitude has major implications for soil properties and fertility, it is not easy to reliably detect given the considerable spatial variability in a situation such as this. However, microbial biomass had decreased to almost half that in the forest soil, a change that was easy to detect and gave a more practical and sensitive means of gauging the direction and rate of change in SOM content following the change in land use.

The modelling study in Figure 1.7 illustrates the movement of total SOC and biomass C to new equilibrium values following a change in management for a
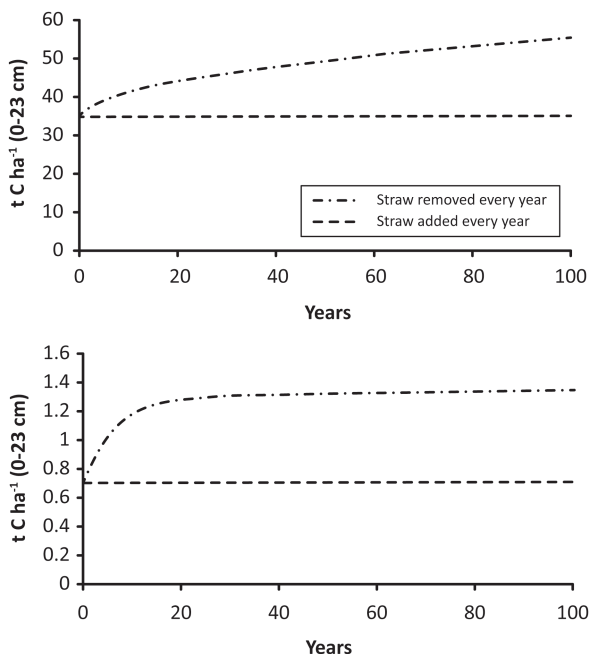

Figure 1.7   Modelled changes in total SOC and microbial biomass in a silty clay loam soil in south-east UK following annual wheat straw incorporation as compared to straw removal. (Adapted from Powlson *et al*., 2011.)

silty clay loam soil under arable cropping in a temperate climate. It was assumed that total SOC and biomass C were both at equilibrium values when the model run was started and that wheat straw had been removed every year. The RothC model (Coleman and Jenkinson, 1996) was then used to assess the changes resulting from annual incorporation of straw. Figure 1.7(a) shows that the total SOC increased slowly after straw incorporation began and was still increasing after 100 years. In contrast, biomass C (Figure 1.7(b)) increased rapidly during the first 10 years, and by 20 years had virtually reached an equilibrium level with no further increase.

This effect was illustrated by results published in 1987 from two experimental sites in Denmark where spring barley was grown and straw either removed (by burning, which was still allowed at the time) or incorporated every year. At both sites, even after 18 years of continuous straw incorporation, total SOC was only marginally greater than in soil where straw had been burned every year: the small increase in the "incorporated" treatment (Figure 1.8) was not statistically significant. In contrast, microbial biomass C was about 40% greater where straw had been incorporated and easily measurable (Figure 1.8). This was the first time that
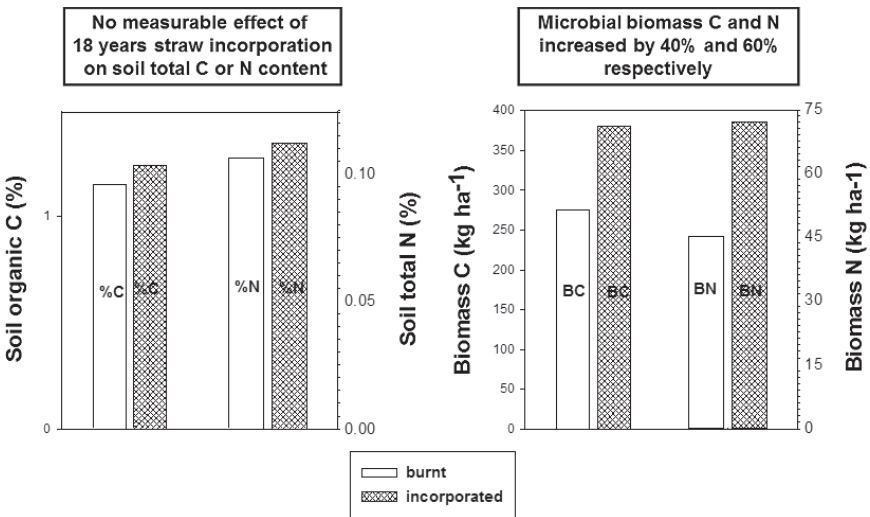

Figure 1.8   The effect of incorporating spring barley straw compared to straw removal by burning on total soil organic C and N and biomass C and N content at a sandy soil site in Denmark after 18 years. The small increases in total soil C or N are not statistically significant but microbial biomass C and N were increased by straw incorporation by 40 and 60%, respectively. (Adapted from Powlson *et al.*, 1987.)

microbial biomass measurements were specifically proposed as an "early warning system" for detecting changes in soil organic C before they are detectable by direct measurements of total SOC. The idea was demonstrated again in a vertisol in a subtropical environment where sorghum residues were either retained or removed in combination with conventional or zero tillage (Saffigna *et al.*, 1989). After 6 years, the treatments that tend to cause an increase in SOM content in the surface soil (residue retentions and zero tillage) caused increases in biomass C and N that were proportionately much greater than those in total SOC or N and much easier to detect.

Concluding Remarks

We now have a suite of methods available to measure the quantities of C, N, P and S held in the cells of living microorganisms in soil, all based on the principle of chloroform fumigation followed by extraction. All of these fumigation–extraction (FE) methods are suitable for measuring biomass in soils containing actively decomposing substrates in both laboratory (Ocio and Brookes, 1989) and field conditions (Ocio *et al.*, 1991), and in acidic soils (Vance *et al*., 1987c). Biomass measurements can also be made in anaerobic (e.g. paddy) soils following a simple modification (Inubushi *et al*., 1991; Qiu *et al.*, 2016). We also have the possibility of measuring the turnover rate of C in the biomass following substrate addition (Kouno *et al*., 2002).

Building on the Jenkinson (1966) paper, we now have the concept of the soil microbial biomass as a meaningful fraction within SOM that can provide considerable insights into soil biology and elemental transformations. The biomass has emerged as a surprisingly large population in view of the small quantities of substrate and energy generally available in soils: in a UK arable soil, the biomass in the plough layer is estimated to have a fresh weight equivalent to about 100 sheep per hectare! But it has a very low metabolic rate, orders of magnitude lower than that of microorganisms growing exponentially under laboratory conditions and with a very slow turnover rate of about 1.2 years. Yet, it has an ATP concentration and AEC comparable to microorganisms growing exponentially *in vitro*. We are still uncertain of the significance of these contradictory characteristics.

Microbial biomass measurements can act as an early indicator of trends in organic matter content resulting from management changes and in some situations a means of detecting damage to soil organisms caused by pollutants. In addition, incubation studies derived from the early work on the chloroform FI method have led to new understanding of the mechanisms influencing the surprising stability of

organic matter in soil. Although other approaches are also proving extremely valuable, especially molecular methods and the use of phospholipid fatty acid (PLFA), we owe a huge debt to the insights of David Jenkinson: much of our current understanding of SOM can be traced back to his innovative thinking and experimentation starting with the Jenkinson (1966) paper that was published 50 years ago.

Acknowledgments

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