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REVIEW ARTICLE

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# Current methods for measuring microbial biomass C in soil: Potentials and limitations

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**Abstract** Methods for measuring soil microbial biomass C were reviewed. The basic ideas behind the fumigation–incubation method, the fumigation–extraction method, the substrate-induced respiration method, and the ATP method were examined together with the advantages, disadvantages, and limitations as reported in the literature and those found by our own recent investigations.

The fumigation–incubation method is the basic technique which is also used for calibration of the three other methods. It is characterized by simple performance without the need of expensive equipment. Its application is limited to soils with a pH above 5 and to soils that do not contain easily degradable C sources. If these limitations are not considered, too low or even negative biomass values will be obtained. These restrictions are largely overcome by the fumigation–extraction method. However, the $k_{EC}$ factor applied to calculate microbial biomass C from the C additionally made extractable by the fumigation is still controversial. The substrate-induced respiration requires expensive equipment for the hourly measurement of soil respiration. This method is also susceptible to amendment of soils with C sources, leading to an overestimate of biomass C. Although a few authors disagree with some basic assumptions behind the methods described, they are widely used and accepted. The use of ATP to measure biomass C in soil is far more uncertain. A high diversity of applied techniques for the extraction and measurement of ATP has led to biomass C : ATP ratios which vary between about 150 and 1000. Our own current investigations are expected to shed more light on the problems of ATP extraction. Preliminary results indicate that a constant biomass C : ATP ratio of about 200 may be more realistic.

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**Key words** Microbial biomass C · Estimation methods Fumigation–incubation method · Fumigation extraction method · Substrate induced respiration method

## Introduction

The microbial biomass accounts for only 1–3% of soil organic C but it is the eye of the needle through which all organic material that enters the soil must pass (Jenkinson 1977). During this process these materials are converted by microorganisms in order to generate energy and to produce new cellular metabolites to support their maintenance and growth. In the C-limited soil system available C in organic materials entering the soil is the driving force behind these processes but other essential nutrient elements (particularly N, P, K) are also involved. Under suitable environmental conditions the extent of the turnover will mainly be controlled by the size and activity of the microbial biomass. In order to elucidate the intricate interrelationships and controlling mechanisms of the input/output fluxes of nutrients and energy in the soil ecosystem a reliable quantification of the microbial biomass is required. Valuable information on biomass growth, turnover time, death rates, and the efficiency of C use can be derived from reliable biomass C data.

The microbial biomass itself may represent a labile pool of C and nutrient elements. In agricultural soils 200–1000 μg biomass C $g^{-1}$ soil is often found. This cell mass fixes 100–600 kg N and 50–300 kg P per hectare in the upper 30 cm of soil. These amounts often exceed the annual application of nutrients supplied as fertilizer to soils in agricultural practice. The liberation or fixation of these nutrients depends on the life dynamics of the microorganisms. Growth of biomass and fixation of nutrients is promoted by rhizodeposits and plant debris and the liberation of nutrients is the consequence of microbial death.

These processes provide the incentive for a reliable quantification of the microbial biomass as a whole and

for the inclusion of its life dynamics in considerations about nutrient cycling in soil.

This review was written to give the inexperienced reader a guide to the application of current biomass C estimation methods in order to decide which method can best answer a particular scientific question and whether reliable biomass C data can be expected. This paper reviews the most widely applied techniques which have been developed over the last 15 years. The latest results from our own investigations are also integrated and new information on the application of these techniques is discussed. The methods reviewed include (1) the fumigation–incubation method (Jenkinson and Powlson 1976b); (2) the fumigation–extraction method (Vance et al. 1987b); (3) the substrate-induced respiration method (Anderson and Domsch 1978b); and (4) the ATP extraction method (Tate and Jenkinson 1982; Bai et al. 1988).

## The fumigation–incubation method

### Origin of method

As early as 1908, under the title "Über die Wirkungen des Schwefelkohlenstoffs und ähnlicher Stoffe auf den Boden" (Effects of carbon disulfide and related compounds on soil) K. Störmer described and interpreted the effects of biocidal fumigants on soils. He postulated that (1) the observed effect of improved plant growth after a transient treatment of soils with toxic fumigants is caused by a liberation of additional N; (2) this N originates from the bodies of the organisms killed by the toxicant; and (3) after treatment of the soils an increased proliferation of bacteria can be observed, which degrade the killed organisms and liberate the N fixed in the cell mass.

This explanation for the observed phenomena, now accepted as correct, did not find the general acceptance it deserved and was overlain by other explanations. Jenkinson (1966) summarized the most important early theories. One hypothesis assumed that microbial activity and development is restrained in unsterilized soil by unknown toxic compounds, reduced microbial vigour, or by inhibiting, antagonistic effects between different sections of the microbial populations. Partial sterilization suspends these effects for a transient time. A second theory postulated a physical or chemical protection of otherwise-unavailable substrates in unsterilized soil. This protecting barrier may consist of waxes which are dissolved by exposure to lipophilic solvents, such as $CHCl_3$, $CS_2$, or $CCl_4$. The third possible explanation was based on the observation that most ways of partial sterilization (heating, air drying, irradiation) increase the amount of water-soluble organic matter. This can be explained by chemical alteration of the non-living parts of the soil organic matter but also by killing of microorganisms and the ensuing lysis.

In order to examine these different theories, Jenkinson (1966) investigated the $CO_2$ and $^{14}CO_2$ liberation from soil samples subjected to different treatments. These samples were collected from agricultural fields 1–4 years after they had been amended with tops or roots of $^{14}C$-labelled ryegrass under field conditions. The first theory of inhibited microbial activity in unsterilized soil was evaluated by observing the respiration of $^{14}CO_2$ from partially sterilized (oven dried) soil after mixing it with different amounts of unsterilized soil. If one of the above-mentioned inhibiting processes were to be valid for the postulated reduced microbial activity in unsterilized soil, the respiration from partially sterilized soil would be diminished in the presence of unsterilized soil. However, the results did not confirm this assumption and strongly contradicted these theories. In a second set of experiments, Jenkinson (1966) compared the liberation of labelled and unlabelled $CO_2$ from soil samples which had been subjected to irradiation, $CH_3Br$ vapour, $CHCl_3$ vapour (24-h exposure) and oven drying at 80° C. All four treatments caused a roughly comparable evolution of $CO_2$ with the same percentage of labelled C from non-uniformly labelled soil.

As discussed by Jenkinson (1966), this result is difficult to explain by theories which postulate that partial sterilization breaks down physical or chemical barriers protecting otherwise-available substrates. For instance, $CHCl_3$ may dissolve waxy films protecting decomposable substrates, but it is very difficult to see how irradiation could act in the same way. Similarly, the result is difficult to explain if it is assumed that partial sterilization makes other non-living parts of the soil organic matter decomposable. If so, all four different treatments should have the same, unlikely property of acting on the same fraction of the soil organic matter to roughly the same extent. The only common action of all treatments is that they kill microorganisms and that the flush of N and $CO_2$ can only be caused by their degradation. By subjecting soil samples to a repeated partial sterilization by oven drying (80° C) or $CHCl_3$ fumigation, Jenkinson (1966) found that the amount of labelled $CO_2$ was increased only slightly but not significantly by a second treatment with heat or $CHCl_3$ vapour. The amount of unlabelled $CO_2$ increased significantly with oven drying, but the repeated $CHCl_3$ fumigation did not show this effect. This suggests that the additional exposure to $CHCl_3$ vapour kills few more organisms and causes little, if any, alteration to the decomposability of the non-living parts of the soil organic matter. By these results Jenkinson (1966) confirmed the explanation postulated by Störmer (1908) and concluded that the size of the $CO_2$ flush should provide a measure of the original biomass.

These results and conclusions faded almost without trace in soil biology (Jenkinson 1987). It took another 10 years for Jenkinson and Powlson to present the chloroform fumigation–incubation method for quantifying soil microbial biomass C. The method was carefully developed in a series of five papers (Jenkinson and Powlson 1976a, b; Powlson and Jenkinson 1976; Jenkinson et al. 1976; Jenkinson 1976) and it replaced former tedious and inaccurate microscopic counting techniques. Other methods of measuring soil microbial biomass have

since been developed and have been calibrated against this method.

## Requirements of the method and problems arising

In Fig. 1 the experimental procedure and background is illustrated. It shows that two basic requirements must be fulfilled to obtain accurate results. (1) $b' \gg b$: The amount of $CO_2$ generated by the decomposition of killed organisms must be very much greater in the fumigated sample than that which develops from organisms that die in the non-fumigated sample during the 10 days of incubation. (2) $h' = h$: The amount of $CO_2$ that originates from the degradation of the non-living parts of the soil organic matter must be equal in the fumigated and non-fumigated samples.

From these requirements the main restrictions for the practical application of the CFI-method can be derived. The requirement $b' \gg b$ means that the control should not contain greater amounts of dead organisms. This will be the case when dry soils are investigated. Under these circumstances a pre-incubation of the rewetted soil for at least 10 days is recommended. A similar treatment is advisable for soils which have been kept frozen. The second requirement, $h' = h$, is of special importance because many interesting scientific questions concern the development of the microbial biomass under practical agricultural conditions. These often include soils which are amended with organic materials, e.g., root and other plant materials or organic fertilizers. In these cases the application of the fumigation–incubation method can lead to considerable faults, which means that too low or even negative biomass values are calculated. We have illustrated this problem by amending soils with chopped $^{14}C$-labelled wheat roots (Martens 1985). Different times after the addition of the root material, biomass $^{14}C$ was estimated by the fumigation–incubation method with a daily measurement of $^{14}CO_2$ liberation during the 10-day incubation of the fumigated and control samples. The results are presented in Fig. 2. It shows that within the first 2 weeks the controls were better able to degrade the added root material than the fumigated samples. This is feasible if the mass of the newly developed microbial population in the re-inoculated fumigated samples corresponds to only 10–20% of the original biomass and consists mainly of bacteria (Martens 1985). Twenty-eight days after the addition of the roots the $^{14}CO_2$ respiration curves indicated that the requirement $h = h'$ was fulfilled. On each day of the 10-day incubation time the fumigated samples liberated more $^{14}CO_2$ than the controls. Depending on the amount of easily degradable organic material the fumigation–incubation method will give more or less incorrect biomass C results. These can only be avoided by a careful removal of the amendment (e.g., roots) or a sufficient pre-incubation of at least 3 weeks.

Some users of this method have tried to solve the problem of different degradation capacities in fumigated and control samples by choosing a different control. In some investigations the $CO_2$ respired from days 10–20 (Jenkinson and Powlson 1976b) or the $CO_2$ liberated from the fumigated sample between days 10 and 20 (Chaussod and Nicolardot 1982) was used to calculate biomass C. From tracer experiments Voroney and Paul (1984) concluded that biomass C can be calculated without subtraction of an unfumigated control. Shen et al. (1987) examined the consequences for biomass C values if these different controls are applied. From his experiments he concluded that only a sufficient pre-incubation of the soil plus the use of the unfumigated sample from days 0–10 as a control gives reliable biomass C values.

We tried to overcome the problems with amended soils by increasing the amount of the inoculum that is added to the fumigated soil. This procedure was supposed to support the development of a new population in the fumigated samples with a qualitative and quantitative composition similar to that of the controls. However, these efforts did not give plausible results (Martens 1985).

A further important limitation of the fumigation–incubation method occurs with soils at pH values below 5. This is explained by the impeded development of the mainly bacterial population in the fumigated and re-inoculated soil samples under conditions of low pH. This causes a reduced mineralization of the killed microorganisms which makes the usual $k_c$ factor invalid.

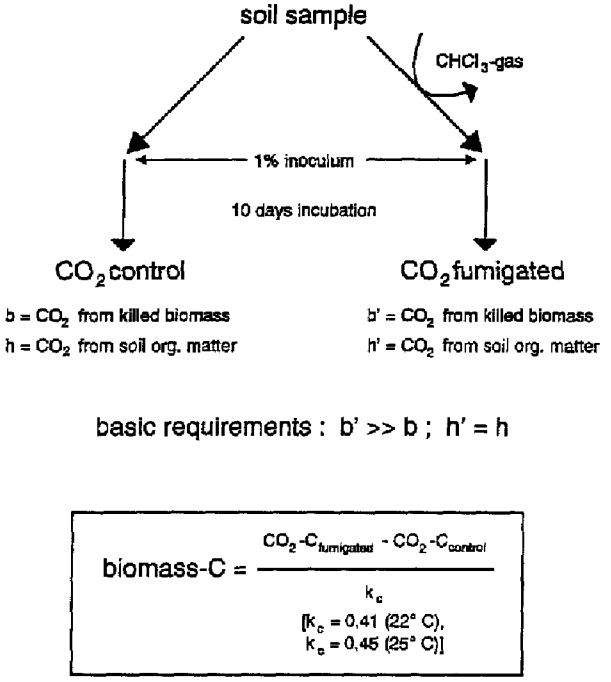


**Fig. 1** Experimental procedure and calculation for the estimation of microbial biomass C by the $CHCl_3$ fumigation–incubation method (*org.* organic)

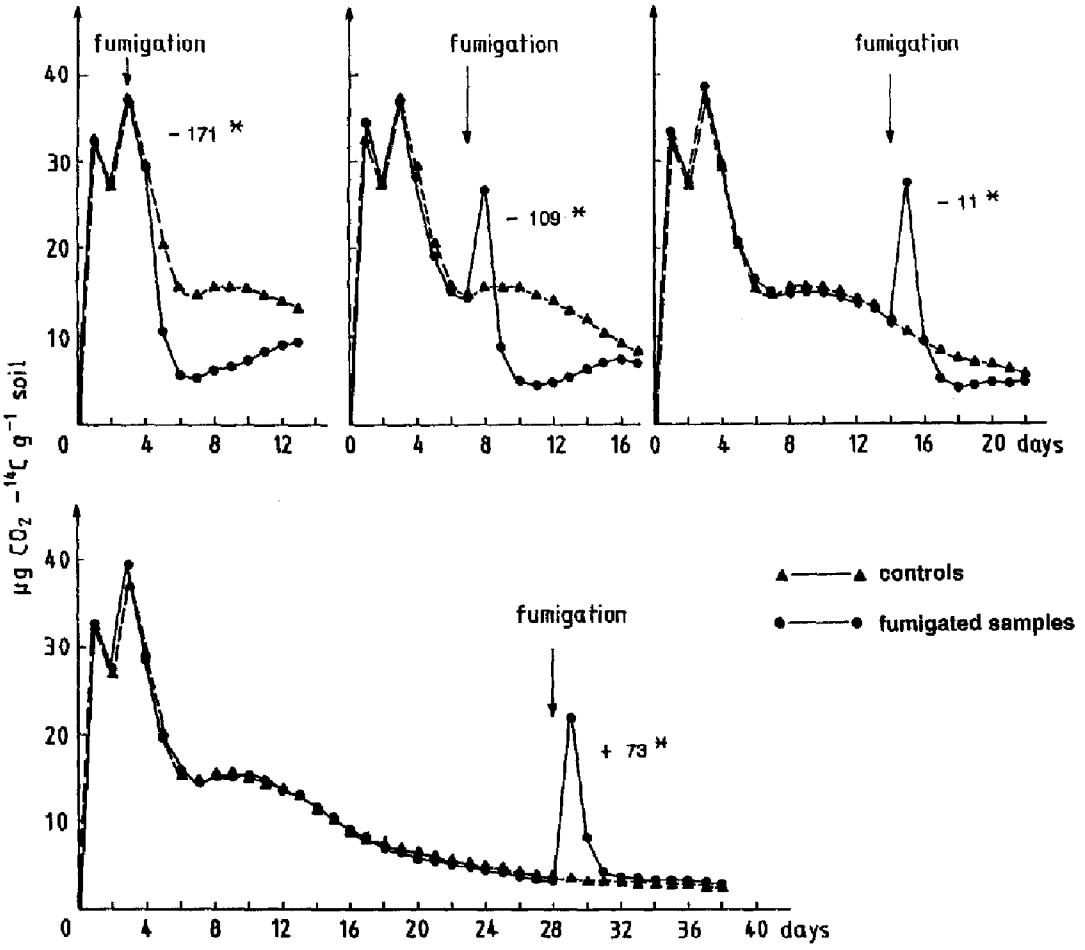


**Fig. 2** Evolution of $CO_2$ from control and fumigated samples of a chernozem soil after an amendment with 1000 μg wheat root $^{14}C\,g^{-1}$. Fumigation was carried out 3, 7, 14, or 28 days after addition of roots. * Calculated increase in biomass $^{14}C$

## Criticism of the method

Some authors argue about the application of the fumigation–incubation technique because they doubt some fundamentals of the method. Alef (1993) summarized most of the critical points as (1) chloroform does not kill all microorganisms in soil; (2) the direct quantification of soil microorganisms by other techniques does not correspond to the results of the fumigation–incubation method, (3) the fumigation with $CHCl_3$ makes some of the humic fraction of soils more available for degradation; and (4) the $k_c$ factor depends on the composition of the microbial populations in soils and the soil pH.

The first objection was proposed by Ingham and Horten (1987) who found that bacterial and fungal populations in a prairie soil were reduced by only 37–79%. This result contradicts several other investigations (Shields et al. 1974; Lynch and Panting 1980; McGill et al. 1986) which indicated that viable populations of soil fungi, bacteria and actinomycetes were reduced by 90–100% after a fumigation with $CHCl_3$. From the result that a second treatment with $CHCl_3$ did not significantly increase the evolution of $^{14}CO_2$ and $CO_2$, Jenkinson (1966) concluded that exposure to $CHCl_3$ vapour kills almost all soil organisms. In view of these results it seems possible that an incomplete killing by $CHCl_3$ may be caused by an incorrect performance of the fumigation procedure.

The lack of agreement sometimes observed between the fumigation–incubation method and the results of direct microscopy must take into account the fact that the results of this technique depends very much on the technique applied staining, its ability to distinguish between dead and living microbial cells, and the factor used for the conversion of observed cell shapes into corresponding biomass C values. The particular skill of an investigator will also influence the results of counting. Under these conditions a high degree of variation and uncertainty in the results from direct microscopic counting must be expected. This means that all comparisons between the fumigation–incubation method and microscopic counting must be considered with scepticism, regardless of whether the results indicated a close (Jenkinson et al. 1976; Vance et al. 1987a; Martikainen and Palojärvi 1990) or no (Schnürer et al. 1985; Ingham and Horten 1987; Ingham et al. 1991) correlation. The question of whether fumiga-

tion also liberates degradable C from the non-living parts of the organic matter (Tate et al. 1988; Coûteaux et al. 1989, 1990) has already been discussed by Jenkinson (1966). Investigating the decomposition of fumigated organisms in soil, Jenkinson (1976) later confirmed his former conclusion that the $CO_2$ flush after fumigation is mainly due to the decomposition of killed organisms, but he could not entirely exclude a small contribution from non-biomass material. Because of this uncertainty he assumed that the calculated biomass data are probably not better than ±20%.

Another point of dissension is the high variability in $k_c$ factors (0.2–0.6) found for different microorganisms in soils of different pH (Vance et al. 1987a). If the recommendation not to use the fumigation–incubation method in soils with a pH below 5 is followed, the high variability is appreciably reduced. This was demonstrated by Jenkinson (1988), who calculated an average $k_c$ factor of 0.46±0.046 from several data in the literature, obtained with soils of a pH between 5 and 8 and an incubation temperature of 25° C. However, some degree of uncertainty in the $k_c$ factor must be expected if the composition of the soil populations differs, because dead fungi are degraded in soils at a higher rate than bacteria within a 10-day incubation (Vance et al. 1987a; Anderson and Domsch 1978a). Anderson and Domsch (1978a) estimated $k_c$ factors for 27 $^{14}$C-labelled species of soil fungi and bacteria and calculated the possible errors in case of different population compositions. On the basis of a frequent average fungi:bacteria ratio of 75:25 in arable soils, they calculated a $k_c$ factor of 0.411 for 22° C. An overall variation of 5% in the $k_c$ factor must be expected if the biomass of bacteria differs by 80% or the biomass of fungi differs by 25%. The same variation must be expected if the mineralization rate of bacteria differs from the average by 25% (i.e., 41.6% instead of 33.3%) or that of fungi by 6% (i.e., 46.5% instead of 43.7%). This indicates that the mineralization of fungi is the sensitive factor.

### Value of the method

Balancing the advantages and disadvantages of the fumigation–incubation method, I conclude that it allows a reliable measurement of microbial biomass C if the known limitations are taken into account. I feel it is not justifiable to reject the method because some of the basic assumptions are possibly not completely fulfilled. This may lead to some degree of uncertainty, but if we can accept a variability of ±20%, as assumed by Jenkinson (1976), the method can be used to investigate aspects of microbial life in soil.

This view has obviously been generally accepted and the fumigation–incubation method has become the most widely applied technique for estimating microbial biomass C in soils since details were published in 1976. The importance of the method was documented (Jenkinson 1987) when it was identified by the *Science Citation Index* as a so-called citation classic. This indicated that the paper by Jenkinson and Powlson (1976b) had been cited in over 205 publications up to 1987.

## The fumigation–extraction method

### Estimating the $k_{EC}$ factor

In his paper of 1966 Jenkinson mentioned that fumigation of soils with $CHCl_3$ increases the amount of C extractable with 0.5 $M$ $K_2SO_4$. Voroney (1983) pointed out that there is obviously a close correlation between $K_2SO_4$-extractable C additionally liberated by fumigation and the microbial biomass C content of soils. This was confirmed by Vance et al. (1987b), who proposed the following calculation to measure biomass C by means of an extraction with 0.5 $M$ $K_2SO_4$:

$$\text{Biomass C} = k_{EC} \times E_C$$

$E_C$ = (organic C extracted from fumigated soil) – (organic C extracted from non-fumigated soil)

A $k_{EC}$ factor of 2.64 was calculated from a regression between $E_C$ and biomass C as estimated by the fumigation–incubation method. Analogous to the $k_C$ factor of this method, the factor 2.64 or its reciprocal 0.38 is named the $k_{EC}$ factor.

As the $k_{EC}$ factor 2.64 was derived from a relatively small data base, other research groups made attempts to check or to establish a new $k_{EC}$ factor. These investigations included experiments with in situ labelling of the microbial biomass with $^{14}$C-labelled substrates and different techniques to calibrate the C additionally made extractable by fumigation. Table 1 gives information about the different $k_{EC}$ factors, as reported in the literature.

Besides different methods to calibrate the extractable C from microorganisms after fumigation, two techniques were applied to estimate the C content in the extractant $K_2SO_4$. Some of the research groups quantified the C by an oxidation with $K_2Cr_2O_7$ and a subsequent back-titration of the unreduced dichromate. An alternative method is the use of automatic total organic C (TOC) analysers, which oxidize the C by ultraviolet radiation in the presence of $K_2S_2O_8$.

Table 1 shows that most investigators estimated a $k_{EC}$ factor of between 0.30 and 0.35 with an average of about 0.32 (value of 0.20 not included) when the dichromate method was applied. Wu et al. (1990) demonstrated that the oxidation of C with dichromate is obviously not complete. They found an average of about 20% more C with TOC analyser. This is consistent with general chemical knowledge that complex organic molecules cannot be completely oxidized by dichromate. In addition, organic compounds that are already partly oxidized consume less oxidation equivalents, leading to an underestimate of C. This explains the higher $k_{EC}$ factors found with TOC analysers.

**Table 1** $k_{EC}$ Factors published in the literature. Soil details comprise number of soils, organic C status (min. = < 10%, org. = > 10%), land use, range of pH values, and pre-incubation of soils in days at 20 – 25 °C after sampling. *CFI* fumigation – incubation, *SIR* substrate-induced respiration

| Method and authors | Details about soils | Method of calibration | Range of $k_{EC}$ values | Average $k_{EC}$ value |
|---|---|---|---|---|
| *C estimate by dichromate consumption* | | | | |
| Vance et al. (1987b) | 10 min. soils, arable and wilderness, pH 3.9 – 8.0, pre-incubated 10 days | CFI | 0.27 – 0.46 | 0.34[a] |
| Sparling and West (1988) | 26 min. soils, pasture, pH 4.4 – 6.4, pre-incubated 4 – 7 days | SIR | 0.20 – 0.56 | 0.35 |
| | 6 org. soils, pasture a forest, pH 4.1 – 5.3, pre-incubated 4 – 7 days | SIR | 0.10 – 0.25 | 0.20 |
| | 4 min. and 2 org. soils, pH 4.1 – 5.3 pre-incubated 70 days and 2 days after addition of $^{14}$C-glucose | $^{14}$C biomass | 0.25 – 0.38 | 0.33 |
| | 6 min. soils pasture, pH 5.3 – 6.4, pre-incubated 4 – 7 days | CFI | 0.22 – 0.38 | 0.30[a] |
| Ross (1990) | 7 min. soils, grassland, pH 5.2 – 7.4, fresh and pre-incubated 7 days | CFI (variations) | 0.30 – 0.73 | 0.33 |
| Martens (unpublished data) | 25 min. soils, arable, pH 6.0 – 7.5, pre-incubated 14 – 28 days | CFI | 0.20 – 0.47 | 0.28[a] |
| *C estimate by $CO_2$ measurement* | | | | |
| Bremer and van Kessel (1990) | 1 min soil, arable, pH 7.4, pre-incubated 105 and 7 days after addition of $^{14}$C-glucose | $^{14}$C biomass | 0.32 – 0.38 | 0.34 |
| Martikainen and Palojärvi (1990) | 2 min. soils, arable, pH 5.2 and 6.1, no pre-incubation | Microscopic counting | 0.16 and 0.24 | 0.20 |
| | 8 org. soils, forest, pH 3.9 – 6.8, no pre-incubation | Microscopic counting | 0.25 – 0.66 | 0.47 |
| Wu et al. (1990) | 9 min. soils, arable and grassland, pH 5.2 – 7.9, pre-incubated 10 days | CFI | Not given | 0.45[a] |
| Kaiser et al. (1992) | 24 min. soils, arable, pH 5.5 – 7.3, pre-incubated 7 days | CFI | 0.31 – 0.47 | 0.40[a] |
| Zagal (1993) | 7 min. soils, arable, pH 5.7 – 7.1, pre-incubated 28 days | CFI | 0.34 – 0.63 | 0.47 |
| | 2 min. soils, arable, pH 5.7 and 6.7, pre-incubated 7 and 2 days after addition of $^{14}$C-glucose | $^{14}$C biomass | 0.23 and 0.23 | 0.23 |

[a] Used to estimate the frequency of distribution of the $k_{EC}$ factor

In considering the different efficiencies of C oxidation and a uniform $k_C$ factor for the applied fumigation – incubation method, Joergensen (personal communication 1993) recalculated the single $k_{EC}$ data in the different publications marked *a* in Table 1. These data, plotted as a frequency distribution, show that most $k_{EC}$ values were between 0.36 and 0.48 when calculated with TOC analysers (Fig. 3). Under these conditions an average $k_{EC}$ factor of 0.43 should be chosen. For an application of the dichromate method this factor has to be divided by 1.19 (Wu et al. 1990), giving a recommended $k_{EC}$ factor of 0.36.

In accord with Fig. 3, a relatively large variation in the $k_{EC}$ factor must be expected. Jenkinson (1988) suggested that the liberated, soluble organic matter will partition between soil constituents and the extractant. This may depend on the soil type, especially on its clay content. However, I found no correlation ($r = 0.016$) between the $k_{EC}$ factor and the clay content when the corresponding data given by Kaiser et al. (1992) were used. Further research is required to elucidate this aspect in order to reach a more consistent $k_{EC}$ value.

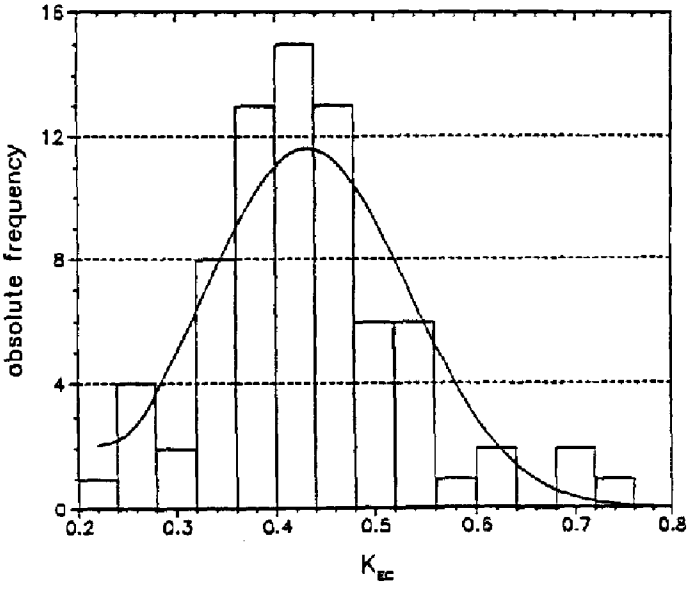


**Fig. 3** Frequency distribution of the $k_{EC}$ factor obtained by a calibration with the fumigation–incubation method as reported in the literature (R. G. Joergensen, personal communication 1993). C contents in $K_2SO_4$ extracts estimated by the dichromate method were recalculated with a factor of 1.19 according to Wu et al. (1990)

### Value of the fumigation–extraction method

In spite of these uncertainties the fumigation–extraction method offers new opportunities for estimates of biomass C in soils which are not suitable for the fumigation–incubation technique, especially, as described above, in soils with a low pH or with degradable organic materials. Since the fumigation–extraction method can also be used for waterlogged soil (Inubushi et al. 1991), Mueller et al. (1992) developed a technique to estimate biomass C in soils with a high root content, which often occur in agricultural fields. By wet sieving soil samples with 0.05 *M* $K_2SO_4$, and subsequent centrifugation of the resulting soil suspension, roots can be removed. In root-free and $K_2SO_4$-saturated soil an effective $CHCl_3$ fumigation can be performed and the usual fumigation–extraction method (Vance et al. 1987b) applied. As opposed to the fumigation–incubation method this variation of the fumigation–extraction technique by Mueller et al. (1992) allows biomass C estimates in field samples without adjustment of the soil samples to a suitable water content, without time-consuming removal of roots by hand, and without pre-incubation.

## The substrate-induced respiration method

### Application and equipment

Investigating the contribution by fungal and bacterial biomass to total soil respiration, Anderson and Domsch (1973) observed that after the addition of glucose to soil samples, respiration was raised to a new elevated level for a few hours (2–8 h) before the liberation of $CO_2$ increased due to the proliferation of the soil populations. This new respiration level was called "maximum initial response" and is induced by an amount of glucose which depends on the particular soil under examination. A further increase in the glucose concentration did not lead to a higher maximum initial response. By adding selective inhibitors with the glucose, Anderson and Domsch (1975) developed a method to measure the relative bacterial and fungal contributions to soil respiration without obtaining information on the weight of the biomass. The fumigation–incubation method of Jenkinson and Powlson (1976b) gave them the opportunity to check the maximum initial response values against the corresponding microbial biomass C contents of agricultural soils. The regression thus calculated by Anderson and Domsch (1978b) indicated that 40 mg biomass C respires 1 ml $CO_2\,h^{-1}$ at the stage of the maximum initial response. The feasibility of this ratio was confirmed by corresponding measurements carried out with pure cultures of soil fungi.

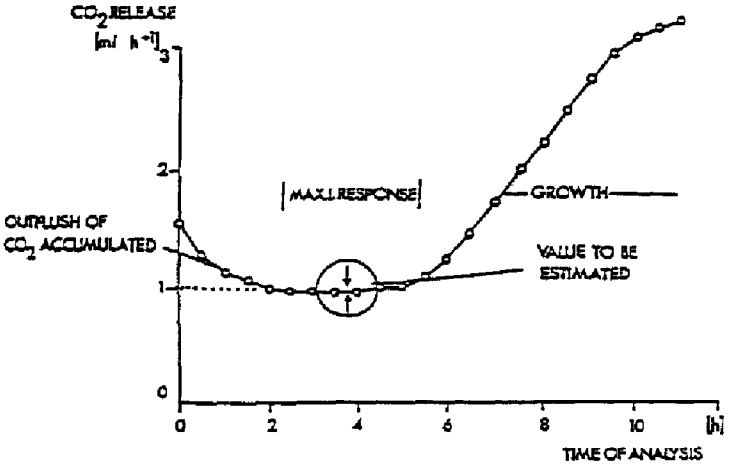


**Fig. 4** Evolution of $CO_2$ after addition of an appropriate amount of glucose to induce the maximum initial response (*MAX. I. RESPONSE*) for estimation of microbial biomass C by the substrate-induced respiration method

Figure 4 shows the typical respiration curve obtained with the substrate-induced respiration method. After the addition of glucose as a glucose/talc mixture an immediate increase in respiration will be recorded with a subsequent slight decrease within in the next 2–3 h. The latter is caused by the diminishing effect of the mixing process. After this a constant respiration rate will be observed for 2–8 h, followed by a rapid increase in $CO_2$ liberation due to the proliferation of the soil organisms. One value within the period of constant respiration is used to calculate the biomass C.

The method requires a measuring system which allows an hourly $CO_2$ analysis. Anderson and Domsch (1978b) used an Ultragas 3 $CO_2$ analyzer (Wöstoff Company, Bochum, Germany) which operates in a non-continuously flushing mode. The soil samples are incubated for 40 min without aeration followed by preflushing for 10 min to remove accumulated $CO_2$. Within in the next 10 min respired $CO_2$ is flushed into a 0.04 *N* NaOH and the change in its conductivity is a quantitative measure of absorbed $CO_2$. Ten years with this equipment has shown up deficiencies in its operation and handling. A comparison of biomass estimations by the substrate-induced respiration method and the fumigation–incubation method indicated that in neutral and alkaline soils the Ultragas 3 overestimates biomass (Martens 1987). This investigation showed that the respiration of the microorganisms steadily increases the partial pressure of the $CO_2$ in the air of the sample tubes during the 40 min of non-flushing. Depending on the soil pH, an increasing amount of $CO_2$ will be dissolved in the soil solution as $HCO_3^-$. The following 10 min of preflushing is not sufficient to remove all dissolved $CO_2$. Hence, during the subsequent 10 min of aeration for $CO_2$ quantification, $CO_2$ from the soil solution will be liberated and will be additionally detected.

## Further techniques

The disadvantages of the Ultragas 3 encouraged Heinemeyer et al. (1989) to develop a new technique. Based on a continuous flushing of soil samples and a quantification of the $CO_2$ by an infrared detector, the system is controlled by computer, which also calculates the biomass C data. Up to 48 samples can be processed per day. About 15 laboratories use this commercially available system, mainly in Germany.

The high costs of $CO_2$-measuring systems have led to attempts to use the substrate-induced respiration method with simpler laboratory techniques. Sparling (1981) incubated soil samples after the addition of glucose in glass bottles, sealed with a septum. The respired $CO_2$ in the headspace of the bottles was quantified each hour by gas chromatography analysis. However, this technique is restricted to soils with a pH below 6.5. At higher pH values the accumulated $CO_2$ in the headspace partly dissolves in the soil water as $HCO_3^-$, and $CO_2$ is underestimated. Martens (1987) applied the substrate-induced respiration method in a self-constructed aeration system in which the respired $CO_2$ was absorbed by 4 ml 2 *N* NaOH. Each hour the alkaline was replaced by hand. For analysis of its small $CO_2$ content the conventional titration technique proved to be not sufficiently accurate. Therefore aliquots of the NaOH in sealed medical flasks were acidified (pH 1) with 5 *N* $H_2O_4$ which was injected into the flasks via a septum. After vigorously shaking the flasks the $CO_2$ content in the headspace was assayed by gas chromatography. Although a reasonable estimate of biomass C is possible by these simpler techniques, they are very time-consuming and not suitable for routine analysis.

## Soil treatment

Like the fumigation–incubation method, the substrate-induced respiration method was established with soils which had not recently been subjected to special treatments like drying, freezing, or amendments. Anderson and Domsch (1978b) pointed out that their proposed correlation between the maximum initial response and biomass C may only be valid for an "average" situation in which living cells of all physiological ages, including resting stages, are present. From their pure culture studies they assumed that soils with a population of predominantly young cells will give a higher than "average" $CO_2$ production per unit biomass C compared with aged cells, which will give lower values. It is likely that special treatments, especially the addition of C amendments, will change the composition of the soil population and will lead to an overestimate of biomass C. This was confirmed by Sparling et al. (1981), who estimated biomass C in glucose-amended soils. To fulfil the requirement of an "average" population it is recommended that soils be sampled from fields in early spring when the influences of agricultural practices have subsided. A pre-incubation of soil samples of at least 1 week after sieving will correspond to the conditions used by Anderson and Domsch (1978b) in establishing the substrate-induced respiration method.

## Correlation with other methods

The close correlation between the maximal initial response and biomass C was confirmed when the abovementioned conditions were considered and a greater number of soils were investigated. Martens (1987) found a relationship based on 22 soils which does not differ significantly (Wardle and Parkinson 1991) from that of Anderson and Domsch (1978b). Kaiser et al. (1992) also confirmed the close relationship when they investigated 27 soils which had been sampled in spring and pre-incubated for 1 week before use. The $CO_2$ production was measured hourly by the method described by Heinemeyer et al. (1989). However, they calculated that the substrate-induced respiration and the fumigation–incubation method gave corresponding biomass C values only when the conversion factor of ml $CO_2$ $h^{-1}$ to biomass C is 30, in-

stead of 40 as found by Anderson and Domsch (1978b). The basic difference between the Anderson and Domsch (1978b) investigation and that of Kaiser et al. (1992) is the use of different apparatus with different flushing modes and detectors for the measurement of $CO_2$ and, in addition, the different performance of the fumigation–incubation method used to calibrate the maximum initial response. Anderson and Domsch (1978b) also used the Ultragas 3 $CO_2$ analyzer for the latter method. This means that the soil samples were flushed each hour for 20 min. Kaiser et al. (1992) applied the technique of Jenkinson and Powlson (1976b) who incubated the soil samples in closed glass vessels with absorption of the liberated $CO_2$ in NaOH without any aeration. These differences may explain the different conversion factors, although no further comparable investigations have been done to prove this further. Since Kaiser et al. (1992) used the fumigation–incubation method as reported in the original literature and the measurement of the maximum initial response was performed in a more appropriate continuous flushing system (Heinemeyer et al. 1989), I recommend a factor of 30 to convert the $CO_2\,h^{-1}$ into biomass C. Kaiser et al. (1992) also included the fumigation–extraction method in their investigation and found close linear relationships between all three methods. The correlation coefficients between the three methods ranged between 0.96 and 0.99, excluding one acidic and two peat soils.

Biomass estimate in amended soils are of special interest when one is concerned with the fixation and liberation of nutrients by microbial cells in soil under conditions of agriculture practice. In order to test the reliability and limitations of the fumigation–incubation, fumigation–extraction, and substrate-induced respiration methods, we amended agricultural soils with 1% dried sewage sludge. Biomass C estimates were carried out 3, 7, 14, 21, and 28 days after the addition of the organic material as described by the original methods. Table 2 gives the results. They show that the three methods analyzed similar but not identical biomass C contents in the non-amended soils. Three days after the amendment the fumigation–incubation and the substrate-induced respiration methods gave undoubtedly erratic results. The fumigation–incubation method showed the typical underestimate caused by the control problem discussed above. Biomass values comparable with the fumigation–extraction method were found after 2–3 weeks. The results of the substrate-induced respiration method confirmed the expected overestimate, indicating a shift in the physiological ages of the microbial cells, i.e., the populations consisted of younger cells than usually found under "average" conditions (see above). With the substrate-induced respiration method the amendment affected the biomass C estimates even after 4 weeks. These results show that the substrate-induced respiration technique requires long pre-incubation times before reliable estimates can be expected after the addition of a C source. Compared with the results obtained at later sampling dates, the fumigation–extraction method obviously gave overestimates in two soils 3 days after the amendment. After further incubation of the amended soil samples this method was obviously no longer essentially influenced by the sewage sludge, indicated by fairly constant biomass values from day 7 on.

**Table 2** Comparison of the fumigation–extraction (*CFE*), fumigation-incubation (*CFI*), and substrate induced respiration (*SIR*) methods applied to soils with an amendment of 1% sewage sludge (2350 μg C $g^{-1}$ soil)

| Soil | Days after amendment | Biomass C (μg C $g^{-1}$ soil) | | |
|---|---|---|---|---|
| | | CFE ($k_{EC} = 0.36$) | CFI ($k_C = 0.41$) | SIR ($F = 30$) |
| Völkenrode | Before | 235 | 222 | 251 |
| | 3 | 684 | 113 | 1169 |
| | 7 | 440 | 301 | 1154 |
| | 14 | 377 | 359 | 940 |
| | 21 | 390 | 390 | 806 |
| | 28 | 342 | 415 | 691 |
| Jerxheim | Before | 323 | 442 | 413 |
| | 3 | 585 | 389 | 917 |
| | 7 | 551 | 467 | 1077 |
| | 14 | 507 | 521 | 1151 |
| | 21 | 526 | 663 | 998 |
| | 28 | 512 | 655 | 947 |
| Timmerlah | Before | 245 | 286 | 350 |
| | 3 | 615 | 51 | 971 |
| | 7 | 416 | 299 | 663 |
| | 14 | 392 | 380 | 753 |
| | 21 | 383 | 451 | 668 |
| | 28 | 408 | 452 | 874 |

In other investigations the biomass methods were also tested on a small number of soils which had been subjected to special treatments like different moisture and storage conditions, amendments with organic C sources, or contamination with heavy metals (West et al. 1986; Dumontet and Mathur 1989; Ocio and Brookes 1990; Wardle and Parkinson 1990; Ross 1991). In most cases relationships between pairs of methods were weak when conversion factors cited in the literature were used. However, these conversion factors are only average values from a greater number of investigated soils, and single soils can differ markedly from the general relationship. In addition, the limitations given above regarding the applications of the biomass methods have to be taken into account.

West et al. (1986) pointed out that conversion factors obtained with a relatively narrow range of soils may not be universally applicable. In studies on New Zealand grassland soils they found out wide range of soil-dependent conversion factors between some of these methods. This was supported by Wardle and Parkinson (1991). They evaluated and recalculated equations and common conversion factors given in the literature by an application of statistical methods. For the $k_C$ factor of the fumigation–incubation method they reviewed the published data and calculated a high degree of variability. The relationship between this method and the substrate-induced respiration method was found to be uncertain. They ex-

plain this result by the fact that the latter measures only the glucose responsive microbial biomass while fumigation–incubation measures the $CHCl_3$-susceptible biomass. It remains an open question whether the glucose responsive microbial biomass is a constant fraction of the $CHCl_3$-susceptible biomass, a prerequisite for a constant conversion factor. The calibration of the fumigation–extraction method against the fumigation–incubation method appears to these authors to be also uncertain, probably because $k_C$ and $k_{EC}$ values vary differently across soil types.

## The ATP extraction method

### Difficulties in the use of ATP

The amount of biomass in soil can, in theory, be assessed by the quantification of a particular cell constituent of the microorganisms. Jenkinson and Ladd (1981) mentioned the basic requirements for this approach: The substance must be present in all organisms in the same known concentration at all times. It must be present only in living organisms from which the compound can be extracted quantitatively. An accurate and sensitive method must be available to quantify it in the obtained soil extract. ATP is a universal cell constituent which meets these requirements best, although not in a perfect way. It is present in all living cells and can be estimated with great sensitivity by the luciferin–luciferase system. Both intracellular ATP in dead organisms and free ATP in soil are degraded very rapidly. The main difficulties in using ATP as a quantitative measure of soil biomass are (1) the extraction of ATP from the cells is not complete; (2) ATP is decomposed by enzymatic or chemical hydrolysis during the extraction process; and (3) after its extraction ATP is strongly adsorbed by soil constituents.

Since early investigations on the ATP content of soils, these problems have been taken into account. Some research groups have tried to overcome the difficulties by the addition of in vitro cultivated microorganisms as an internal standard. It was supposed that this technique can monitor all ATP losses. However, this approach is open to criticism. In relation to the extractability of ATP from cells, it is not sensible to assume that in vitro cultivated organisms can represent the complex native soil flora. The ATP pool of microorganisms reacts very rapidly with changes in their environment, which means that the addition of the organisms to a soil can lead to an immediate, uncontrolled change in their ATP content. In addition, the production and maintenance of such an internal standard is laborious. For these reasons, it is now usual to apply the ATP standard as a chemical compound. However, the losses monitored with this standard can only be referred to the ATP which has already been extracted from the organisms. This concerns the losses by adsorption and hydrolysis. No information can be obtained about the efficiency of ATP extraction from the cells. By the application of a suitable extractant and the simultaneous sonication of the soil suspension it is assumed that a complete extraction will be reached and that hydrolysis is also avoided. It has been found that this aim can best be reached with acid extractants like trichloroacetic acid or $H_2SO_4$. The tendency of ATP to be strongly adsorbed by soil constituents is reduced by the addition of organic and inorganic compounds which compete with ATP for the binding sites. The molecular constituents of the ATP, phosphate and adenosine, have been proved to reduce adsorption. The same effect has been found for a synthetic zwitterionic detergent (Webster et al. 1984) or the cationic organic compound paraquat (Jenkinson and Oades 1979).

### ATP extractants

During the last 20 years numerous extractants have been proposed but there has been no extensive comparison of these reagents, tested on a greater number of different soils. The available investigations compared only two or three different extractants with a very limited number of soils and so they do not answer the question about the most effective extractant. A suitable evaluation of a given procedure can be made by an estimate of the biomass C : ATP ratio. Highly efficient ATP estimation methods are characterized by a low value. These were found by Oades and Jenkinson (1979) and Tate and Jenkinson (1982), who estimated ratios between 120 and 210 with an average of 171 by using their trichloroacetic acid/phosphate/paraquat extractant. Eiland (1983), with his sulphuric acid/phosphate/NRB extractant, obtained biomass C : ATP ratios of between 124 and 240, which increased to values between 171 and 477 when soils were stored for about 7 months. A high variability in this ratio was also found by Ross et al. (1980) and Sparling (1981), with values of between 163 and 423 or 201 and 858, respectively. In a review Jenkinson (1988) selected those ATP data from the literature which were obtained with an acid extractant. In addition, data were only included when the soils under investigation were pre-incubated for at least a few days and when biomass C estimates were carried out with the fumigation–incubation method. Under these conditions the relationship between biomass C and ATP was found to be $169 \pm 5$. This low value is somewhat surprising for two reasons: (1) Pure cultures of growing microorganisms have biomass C : ATP ratios of about 200–250 (Knowles 1977; Karl 1980). (2) The soil population is supposed to be predominantly in a dormant state with low metabolic and turnover rates (Jenkinson and Ladd 1981). Brookes et al. (1987) discussed this phenomenon of high ATP levels in the soil biomass without giving a convincing explanation for the observed results.

By the low biomass C : ATP ratios that have been measured, the acid extractants and especially the trichloroacetic acid/phosphate/paraquat reagent have proved to be effective reagents for ATP extraction from soil. Therefore it is rather unlikely that the $H_3PO_4$/dimethylsul-

**Table 3** ATP contents (nmol $g^{-1}$ soil) estimated by the standard addition technique or by a sixfold extraction. Extractant: trichloroacetic acid/$HPO_4^{2-}$/paraquat

| Soil | ATP extract 1 | Recovery of standard (%) | ATP calculated | ATP extracts 2–6 | ATP extracts 1–6 |
|---|---|---|---|---|---|
| Edemissen | 3.33 | 88.1 | 3.78 | 1.99 | 5.32 |
| Ensmad | 4.02 | 83.1 | 4.79 | 5.30 | 9.32 |
| Rot am See | 5.45 | 78.9 | 6.91 | 5.41 | 10.86 |
| Göttingen | 3.43 | 82.2 | 4.18 | 1.92 | 5.35 |

phoxide/Zwittergent Detergent 3–10 (Calbiochem)-based reagent introduced by Webster et al. (1984) extracts 1.7–3 times more ATP than the trichloroacetic acid/phosphate/paraquat extractant, as found by Ciardi and Nannipieri (1990). This would mean that the biomass C: ATP ratio would fall far below 100.

As mentioned above, the addition of pure ATP as a standard does not give any information about the efficiency of ATP extraction from microbial cells. This makes ATP estimates uncertain. In order to elucidate this problem we started an investigation to find out how much ATP a soil sample really contains. For this purpose repeated extractions were carried out with the trichloroacetic acid/phosphate/paraquat extractant, each time with the fresh reagent. In general, six extractions of the same sample were necessary to obtain an extract with an ATP content at the limit of detection in the luciferin–luciferase system. The amounts of ATP found in the six extracts were added and compared with the amount of ATP as estimated by a single extraction and corrected for recovery of the added ATP standard (Table 3). The latter technique indicated losses of the standard of between 12 and 21%. The amounts of ATP found in the extracts 2–6 (Table 3) should correspond to these losses. However, far more ATP was detected by repeated extraction. This result shows that the usual standard addition technique does not give the correct information about the amount of non-extracted ATP.

Single extractant

For a practical application repeated extractions are too time- and labour-consuming. A more convenient method with a single extraction step is required, which gives ATP values very similar to those obtained by a repeated extraction. Besides other methods, we also tested the extractant used by Bai et al. (1988) with this in mind. This method is based on an extraction with a $Na_3PO_4$/dimethyl sulphoxide (pH 11.7) reagent and a subsequent treatment with NRB (Lumac). This quaternary detergent is supposed to support the release of ATP from microbial cells. During the current investigation we estimated the ATP contents of four soils and compared these with the ATP values obtained after a sixfold extraction with trichloroacetic acid/phosphate/paraquat. Table 4 shows that both methods gave nearly identical results, considering that parallel ATP estimates in the same soil generally differ by

**Table 4** ATP contents (nmol $g^{-1}$ soil) of soils as estimated by a sixfold extraction with TCA/$HPO_4^{2-}$/paraquat or a single extraction with $Na_3PO_4$/DMSO/NRB (*TCA* trichloroacetic acid, *DMSO* dimethylsulphoxide)

| Soil | Biomass C: ATP[a] | TCA/$HPO_4^{2-}$/paraquat | $Na_3PO_4$/DMSO/NRB |
|---|---|---|---|
| Edemissen | 179 | 5.32 | 5.08 |
| Ensmad | 233 | 9.32 | 9.61 |
| Rot am See | 231 | 10.86 | 11.77 |
| Göttingen | 234 | 5.35 | 5.01 |

[a] Estimated by the fumigation-incubation method (Jenkinson and Powlson 1976b)

5–10%. From these results I draw the preliminary conclusion that with the $Na_3PO_4$/dimethylsulphoxide/NRB extractant a convenient reagent is available which extracts all ATP from soils. The most important aspect of this investigation is to test whether rather constant and low biomass C: ATP ratios can be confirmed as reported by Jenkinson (1988). The results obtained so far with four soils are insufficient to draw firm conclusions. Therefore experiments are under way to investigate more soils of different texture and different pretreatments.

## Conclusions

When selecting a biomass C method for a special application we must be aware of two points: (1) The methods were developed and calibrated with soils where the biomass was in a more or less steady state. (2) Good correlations between the different techniques obviously exist, but only for a greater number of soils. This means that for a single soil, the usual conversion factors may differ from the calculated average value. If we wish to quantify microbial biomass C by fumigation–incubation or substrate-induced respiration methods, we have to take care that the biomass is not in a stage of rapid change or that conditions exist (organic substrates, more favourable climatic conditions) which will support this in the near future. If a soil under investigation does not fulfil this requirement a suitable pretreatment (removal of visible plant residues, pre-incubation) is necessary. However, the initial biomass C content may change during a pre-incubation. A possible solution for this dilemma in many cases will be an ap-

plication of the $CHCl_3$ fumigation–extraction method, which does not depend on the physiological state of the soil microflora and which does not present the control problem of the fumigation–incubation method in amended soils. However, the relatively high variability of the $k_{EC}$ factor means that special care is required in applying the average value as reported in the literature. An individual estimate of the $k_{EC}$ factor for a special soil will be advisable in many cases. I recommend calibration of the additional extractable C after fumigation (EC) by the fumigation–incubation method, considering the limitations of this technique and including a rough assessment of the bacteria : fungi ratio for a better evaluation of the estimated $k_C$ factor. This procedure will give information on whether or not the $k_{EC}$ factor obtained is close to the average.

No general conclusion can be drawn concerning the reliability and acceptability of the four biomass C methods discussed. It remains the responsibility of the scientist to decide which one of the techniques described is suitable for a particular problem.

Under the circumstances, we have to concede that an exact determination of the conversion factors is not an attainable goal. I therefore agree with Sparling et al. (1990) who stated that in many cases "a greater precision is not needed when soil microbial C is being used to reveal relative differences between soils, the effects of agronomic practices or seasonal fluctuations".

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