

Soil microbial biomass and its activity estimated by kinetic respiration analysis – Statistical guidelines

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Abstract The use of kinetic respiration analysis to determine soil microbial biomass its active part and maximum specific growth rate has recently increased. With this method, the increase in soil respiration rate initiated by application of carbon growth substrate, e.g. glucose, and mineral nutrients is used to estimate parameters describing microbial growth in soil. This study refines the method by developing statistical guidelines for the data analysis and processing. The kinetic respiration analysis assumes that microbial growth is not limited by substrate and energy. That is why it is critically important to identify the time period corresponding to the unlimited growth. In this work, we studied how the unlimited growth phase can be identified in less subjective ways by examining 121 data sets of respiration time series of 44 different soil samples taken from field plots. Deflection of the respiratory curve from the exponential pattern indicates growth limitation. Subjective selection of the part of respiratory curve which fits to the exponential pattern resulted in a 30% bias in specific microbial growth rates. We propose rules that are based on inspecting the patterns in a series of plots of residuals of fitted respiration rate. By comparing those rules with a set of statistical criteria we find that the weighted-coefficient of determination (r^2) can be used to objectively constrain the unlimited growth phase in those cases where double-limitation does not occur. Furthermore, we discuss how the uncertainty of estimated microbial parameters is influenced by a) measurement uncertainty, b) biased measurement at the beginning of the experiment, and c) the number and timing of respiration measurements. We recommend checking plots of fits and residuals as well as reporting uncertainty bounds together with the estimated microbial parameters. A free statistical package is provided to easily deal with those aspects.

Keywords Microbial growth rate, Microbial activity, Microbial biomass, Respiration kinetics, Uncertainty analysis, Non-linear regression

Introduction

The determination of the active microbial soil biomass is important to characterize soil sustainability and to understand the microbial control on the decomposition of soil organic matter (SOM) (Stenström et al. 2001). The most widely applied methods to estimate soil microbial biomass are chloroform fumigation extraction (Vance et al. 1987) and substrate induced respiration (SIR) (Anderson and Domsch 1978). However, only kinetic respiration analysis is able to distinguish between sustaining and growing biomass and is able to quantify the initial activity state of the soil microbial biomass (Panikov and Sizova 1996,

Blagodatsky et al.)(details in section 2.1). Neglecting the activity state and relating rates of soil SOM decomposition solely to maximum specific microbial growth rates, will lead to approximations of the conditions in some soil microzones, e.g. rhizosphere, but will not explicitly describe the important dynamics in bulk soil (Stenström et al. 2001) and the dynamics of the huge amounts of slowly decomposing SOM. Hence, the determination of the activity state is important to constrain different model structures about the role of soil microbial biomass in soil carbon decomposition (Wutzler and Reichstein 2008), which in turn will strongly affect our understanding of feedbacks between soil carbon cycling and global warming (Heimann and Reichstein 2008). Furthermore, quantifying the fraction of actively growing biomass, which is capable for immediate growth on added substrate, is extremely important for the estimation of all microbially mediated process rates, as only active microorganisms drive the processes. Application of this approach is helpful for the exact assessment of microbial community reaction on pollution by heavy metals (Blagodatsky et al. 2006), quantification of rhizosphere effect (Blagodatskaya et al. 2009), impact of elevated CO₂ (Blagodatskaya et al. 2010) or soil tillage (Blagodatskii et al. 2008). With the kinetic respiration analysis a soil sample is incubated together with a carbon substrate and substrate consumption or respiration is monitored over several hours. Microbial properties are inferred by fitting a model of microbial growth to the observed respiration time series. The usage of the kinetic respiration analysis

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has increased over the last several years (Blagodatskaya et al. 2010, Blagodatskaya et al. 2009, Dorodnikov et al. 2009, Esberg et al. 2010, Gnankambary et al. 2008, Ilstedt et al. 2006, Ilstedt and Singh 2005, Ilstedt et al. 2007, Lipson et al. 2009, Lipson et al. 1999). However, a comparison or synthesis of results of this method is hampered because of differences in statistical and mathematical treatment of respiration data. Hence, it is desirable to advance the methodology of the kinetic respiration analysis and study how to resolve problems associated with this treatment of the data. These problems include issues of the statistical analysis of the respiration curves, such as:

1. the selection of a subset of the respiration dataset that represents the initial unlimited growth phase (i.e. the time of the exponential growth of respiration rate)
2. prior information about the uncertainty of respiration measurements
3. determination of quantity and timing of respiration measurements
4. biasing of the measurements at the beginning of the experiment

This study focuses on problem 1 while discussing 2-4.

One of the assumptions of kinetic respiration analysis is that only those observations of the respiration rate are used where soil microbes are being activated and grow without limitation. However, limitation will occur as soon as supplied growth substrates are consumed in the course of the experiment. Hence, the observed dynamic of respiration rate must be confined to the data subset with no significant limitation effects. Applying the model fit to different subsets results in different estimates of the specific growth rate as shown for the example in Figure 1. Limitation of growth occurs at the late stage, so it would seem that the last 3 to 6 records should be discarded. Ignoring the growth limitation and using the

full dataset would have led to an underestimation of specific growth rate by 28% and to differing conclusions. On the other hand, discarding too many records leads to increasing uncertainty of the estimated parameters. In order to compare results between different studies, it is necessary to agree on criteria of confining the experimental growth phase.

As can be seen from Figure 1, three standard statistical criteria r^2 , r^2w and Q (detailed explanation below) suggest different number of records (i.e. 24, 25, or 26) to be chosen for estimating the maximum specific growth rate. So, the agreement on criteria is needed for the successful application of kinetic respiration analysis.

This study addresses all potential users of the kinetic respiration method as well as researchers estimating microbial specific growth rates using CLPP method (Lindstrom et al. 1998, Mondini and Insam 2003). Users do not need a strong statistical background; they are only required to operate a computer program (e.g. SAS, R, or ModelMaker) that is able to fit a nonlinear model to a given dataset. The program should allow specifying uncertainty of the respiration data and should provide uncertainty estimates together with estimated coefficients. In this study, we use the freely available statistical software R, specifically the function `gnls` from package `nlme`, used by the `twKinresp` package (Wutzler 2010).

Material and Methods

The background of kinetic respiration analysis

With kinetic respiration analysis, a soil sample is amended with a carbon substrate, e.g. glucose and mineral nutrients to avoid limitation by substrates other than carbon. The soil sample is incubated with constant temperature and moisture level of 60% water holding capacity and the respiration rate is monitored during the

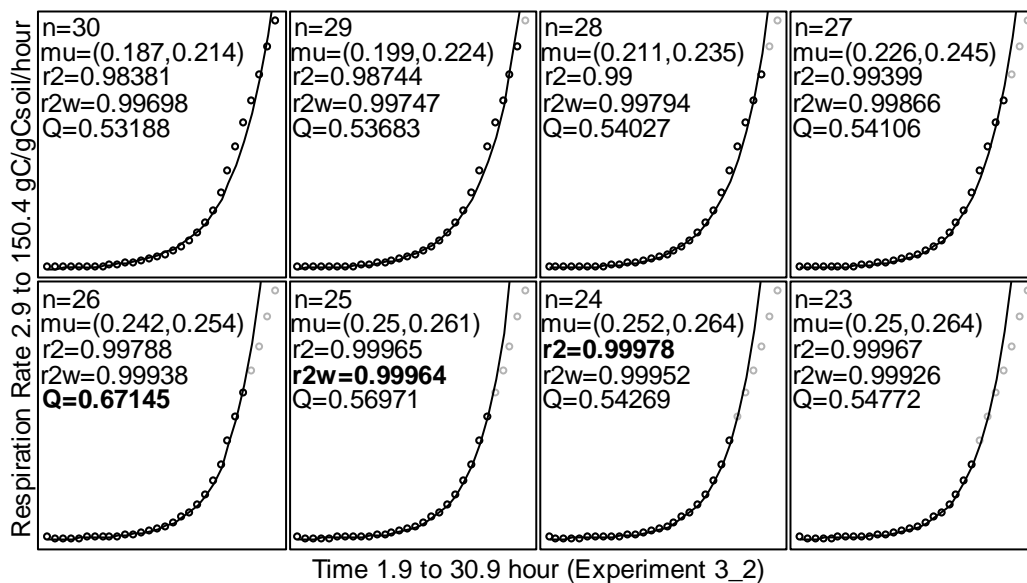


Figure 1: A typical case: plot series of fits of model equation 1 to different subsets of the time series of respiration data set 3_2. Black points: n records that are included in the model fit, grey points: excluded records, mu: 95% confidence interval of maximum growth rate, r²: coefficient of determination, r²w: Q: alternative goodness of fit measure. Bold text indicates the maximum of the measure.

following 24-48 hours (experimental details are described below in the following section). Under such conditions of initially unlimited microbial growth an exponential increase of the respiration rate is observed. The increase in respiration often cannot be explained solely by growth of microbial biomass. One explanation is that many microbial cells in soils are usually in an energy-saving sustaining state and are initially not a full potential to metabolize the substrate but must adapt their molecular equipment (Panikov 1995). Hence the population or microbial community will change from a physiological state of low activity towards their full potential metabolic activity. The physiological state is the fraction of actual to potential substrate turnover of the microbial community that is responding to the substrate addition. In chemostat this is interpreted as the state of most of the cells (Appendix A), however, in heterogeneous soil it can also be interpreted as the fraction of sustaining cells to fully active cells (Blagodatsky et al.). With kinetic respiration analysis the respiration curve (eq. 1) is fitted to the respiration rates and used to calculate microbial parameters.

$$p(t) = \beta_o + \beta_1 e^{\beta_2 t} \quad (1)$$

where $p(t)$ is the measured respiration rate at time t , expressed as respired CO_2 -C per time, β_i are fitted coefficients. This respiration curve and the coefficients are interpreted in slightly different ways (Colores et al. 1996, Marstorp and Witter 1999, Stenström et al.). Here, we concentrate on the interpretation where the growth associated respiration is allowed to change with changing activity of microbial biomass (eq. 2 to 4) (Panikov 1995, Panikov and Sizova 1996). In the beginning the exponential curve is often dominated by adapting the physiological state of the cells, called the lag-phase. During this phase often only weak increase in microbial biomass is observed. Later on during the so called exponential growth phase, the

respiration curve is dominated by growth of the active microbial biomass.

$$\mu_{\max} = \beta_2 \quad (2)$$

$$r_o = \frac{\beta_1(1-\lambda)}{\beta_o + \beta_1(1-\lambda)} \quad (3)$$

$$x_o = \frac{\beta_1 \lambda Y_{\text{CO}_2}}{r_o \mu_{\max}} = \frac{\beta_o + \beta_1(1-\lambda)}{(\frac{1}{\lambda} - 1) \beta_2} Y_{\text{CO}_2} \quad (4)$$

where μ_{\max} is maximum specific growth rate, i.e. potential maximum of fully active cells, r_o is the initial physiological state, x_o is the initial biomass. Those three are the microbial parameters of interest. Parameter λ is the ratio between productive (growth associated) part and total of the specific respiration activity and Y_{CO_2} is biomass yield per unit of respired CO_2 . If the kinetic respiration analysis is performed shortly after soil sampling, the initial physiological state ($0 < r_o < 1$) characterizes relative microbial activity in situ.

For numerical reasons it historically has been easier to fit Eq. 1 to the data and calculate microbial parameters in a second step. Current statistical software, however, can easily and directly fit models of complex formulations. Hence, we recommend representing the model in the form of eq. 5 and fit this equation to the data. In this way the estimated coefficients and also the estimated uncertainties directly refer to the microbial parameters of interest.

$$p(t) = x_o (1 - r_o) \left(\frac{1}{\lambda} - 1 \right) \frac{\mu_{\max}}{Y_{\text{CO}_2}} + x_o r_o \frac{1}{\lambda} \frac{\mu_{\max}}{Y_{\text{CO}_2}} e^{\mu_{\max} t} \quad (5)$$

Equation 5 is effectively a three parameter equation when accepting the following assumptions. First, λ may be accepted as a basic stoichiometric constant of 0.9 during unlimited growth (Akimenko et al. 1983). And second $Y_{\text{CO}_2} = Y/(1-Y)$ can be assumed to be the constant 1.5 during the experiment (

Blagodatsky et al.). A more advanced statistical treatment that accounts for the uncertainty of those two parameters would slightly increase the confidence range of the microbial parameters of interest. The derivation of Eq. 5 is summarized in Appendix A.

Soils samples and respiration measurement

We used 121 data sets of respiration time series of 44 different soil samples taken from field plots (upper 10 cm) to test several methods of confining the experimental growth phase. The first part of the soil sample data consisted of 16 samples of the same soil (Cambisol, sandy loam, from FAL, Braunschweig, Germany field experiment, (Blagodatsky et al. 2006)), which differed in the rate of soil fertilization, the type of grown agricultural crop, and

Table 1: Soil characteristics and field descriptions for subset FACE.

Soil type, texture, location	Crop	Sampling site	N, kg N ha ⁻¹ year ⁻¹	CO ₂ in atmosphere ppm	Sample No
Cambisol, Sandy loam FAL	Sugar beet	rhizosphere	126	350	1
				550	2
		root-free soil	63	350	3
				550	4
		rhizosphere	63	350	5
				550	6
	Winter wheat	root-free soil	126	350	7
				550	8
		rhizosphere	126	350	9
				350	10
		rhizosphere	63	350	11
				350	12
		rhizosphere	126	550	13
				550	14
		rhizosphere	63	550	15
				550	16

Table 2: Soil characteristics and description of applied treatments for sample subsets FAL and Pushchino. Standard deviations are in parentheses.

Soil type, texture, location	pH (KCl)	Total C (%)	Total N (%)	Management	Amendments (mg·g ⁻¹ soil)	Sample No
Cambisol, Sandy loam, FAL	5.8	1.11 (0.05)	0.102 (0.006)	Arable, since 33 yrs no N; since 12 yrs no fertiliser	No nutrients	17
					0.4 N	18
					0.4 P	19
					0.8 N; 0.4 P	20
					0.4 N; 0.4 P	21
					0.2 N; 0.4 P	22
					0.133 N; 0.4 P	23
Cambisol, Sandy loam, FAL	5.6	2.54 (0.05)	0.221 (0.002)	Grassland, 300 m3 ha ⁻¹ sewage sludge (1980- 1990), until 1992 45 kg min N twice per yr since 10 yrs no treatment; 2x mowing per yr; cuttings not removed	No nutrients	24
					0.4 N	25
					0.4 P	26
					0.8 N; 0.4 P	27
					0.4 N; 0.4 P	28
					0.2 N; 0.4 P	29
					0.133 N; 0.4 P	30
Luvisol, Loamy silt, Pushchino	4.6	1.5 (0.035)	0.126 (0.001)	Arable, no fertiliser since 7 yrs	No nutrients	31
					0.4 N	32
					0.4 P	33
					0.8 N; 0.4 P	34
					0.4 N; 0.4 P	35
					0.2 N; 0.4 P	36
					0.133 N; 0.4 P	37
Luvisol, Loamy silt, Pushchino	4.2	1.67 (0.01)	0.151 (0.002)	Grassland, turned from arable to meadow 22 yrs ago; no fertiliser since 22 yrs; 2x mowing per yr and cuttings harvested	No nutrients	38
					0.4 N	39
					0.4 P	40
					0.8 N; 0.4 P	41
					0.4 N; 0.4 P	42
					0.2 N; 0.4 P	43
					0.133 N; 0.4 P	44

the concentration of atmospheric CO₂ at which these crops were grown (Table 1).

The second part of the soil sample data consisted of 28 samples from FAL and Pushchino. It originated from laboratory experiments on four different soils with varying levels of mineral N and P applied to the soil during determination of the respiration response (Table 2). All soil samples were sieved (<5 mm) at day of sampling and stored field-fresh in aerated polyethylene bags usually for 1-3 days at 4 °C (maximum 2 weeks). Before measurements of the substrate induced respiration kinetics the soil moisture content was adjusted to 50% of the water holding capacity (WHC), and then the soil was preincubated at 22 °C for 24 h.

Ten grams (dry weight) of soil were amended with a mixture containing glucose (10 mg g⁻¹), talcum (20 mg g⁻¹), and mineral salts (standard mixture consists of (NH₄)₂SO₄, 1.9 mg g⁻¹; K₂HPO₄, 2.25 mg g⁻¹; MgSO₄*7H₂O, 3.8 mg g⁻¹). For the second part of data, some soil samples were amended with smaller amount of N or P as shown in Table 2. After substrate addition, the soil samples were placed in triplicate into an ADC2250 24- Multichannel Soil Respiration System (ADC Bioscientific Ltd, Great Amwell, UK), which

consisted of 24 plastic tubes, to measure CO₂ production rate at 22°C. Each sample was continuously aerated (300 ml min⁻¹), and the evolved CO₂ was measured every hour using an infrared detector and a mass flow meter (Heinemeyer et al. 1989). Air-flow rate, CO₂ concentration and standard error of CO₂ measurements were continuously monitored.

Model fitting

Each of the 121 respiration rate time series has been first truncated to exclude the records after the maximum of the initial growth phase. Next, for each truncated time series, a series of subsets of data were constructed where the last 0 to n records were omitted. For each of these subsets a slightly modified version of (eq. 1) was fitted by non-linear regression (eq. 6).

$$v(t_i) = e^{\beta_{0l}} + \beta_1 e^{e^{\beta_{2l} t_i}} + \varepsilon_i \quad (6)$$

Variables β_0 and β_2 were replaced by $\exp(\beta_{0l})$ and $\exp(\beta_{2l})$ respectively, where β_{0l} and β_{2l} are the natural logarithm of the corresponding variable. By this variable transformation, we ensured that the parameters at original scale and their 95% confidence intervals are

always positive. ε_i is the standard deviation of the measurement error. In order to account for the fact that higher respiration rates were measured with a higher uncertainty, we used the following variance model (7).

$$sd(\varepsilon_i) = \sigma |v(t_i)|^\delta \quad (7)$$

The expected standard deviation $sd(\varepsilon_i)$ increases with the model prediction $v(t_i)$. The coefficient σ was estimated by the model fit in the gnls function (Pinheiro and Bates 2000), and the coefficient δ has been set to 0.5, 0.6, and 0.5 for the FACE, FAL, and Pushchino subsets (Table 1) of the data, respectively. A value of 0 corresponds to unchanging absolute errors, 1 to unchanging relative errors, and intermediate values correspond to increasing absolute errors or decreasing relative errors. We will elaborate on the choice of δ in more detail in the discussion section.

Model fitting was done using the gnls function of the nlme package version 3.1-96 of the free statistical software R version 2.10.1. The starting values for each fit were determined by a linear regression of $\ln(p(t) - 0.99 \cdot \min(p(t)))$ against time t , where $p(t)$ is the respiration rate measured at time t .

Standard errors and confidence bounds of the microbial parameters were obtained by fitting a slight modification of equation 5 directly to the respiration using the gnls function. The modification was a variable transformation to represent our prior knowledge that initial biomass, specific growth rate are strictly positive and the initial activity ratio is bounded between zero and one (eq. 8).

$$p(t) = e^{x_{ol}} (1 - s(r_{ol})) \left(\frac{1}{\lambda} - 1 \right) \frac{e^{\mu_{max} t}}{Y_{CO2}} + e^{x_{ol}} s(r_{ol}) \frac{1}{\lambda} \frac{e^{\mu_{max} t}}{Y_{CO2}} \quad (8)$$

We replaced x_0 and μ_{max} by $\exp(x_{ol})$ and $\exp(\mu_{max,l})$ respectively, and we replaced r_0 by $s(r_{ol})$, where s is the inverse of the logit function $\log(r_0 / (1 - r_0))$. The transformed parameters x_{ol} , $\mu_{max,l}$, and r_{ol} are estimated within an unbounded domain. The back-transformations to the original scale ensured

proper bounds and proper confidence intervals for the microbial parameters.

The necessary starting values were obtained by applying equation 2 to 4 to the estimates of fitting equation 6 to the respiration data.

Rules of comparing residual plots to confine the unlimited growth phase

In order to identify a subset of the data within the unlimited growth phase, we compared several fits on subsets of the original data that differ by the number of included records (n).

If the subset of data included records where growth conditions were actually limited, the time course of the respiration rate diverged from simulated exponential increase (see Figure 1, $n=30$). This effect is often identified more clearly by plotting the log of the respiration minus the estimated respiration at time zero, i.e. β_0 (Fig. 2, $n=30$).

Plotting the standardized residuals (differences between model fits and measurements divided by the corresponding standard errors) revealed a distinct shape resembling the letter V in the subsets that include records out of the unlimited growth phase (Fig. 3). This deviation from a random distribution indicated a significant positive autocorrelation in the residuals.

Excluding additional records from the end time of the dataset, the V-shape gradually disappeared. With the example of Fig. 3 with $n=25$ records, residuals were scattered around without a distinct pattern. Hence, the assumption of independence of the residuals was valid for this subset and the applied model (eq. 6) successfully described the time course of the respiration. We selected the subset that includes the most records out of the sets where the assumption of independent residuals has not been violated. This can be formalized as selecting the number of records n of the first plot in the sequence where the pattern in residuals disappeared. The influence of limitations during the growth cycle starts gradually. Hence the choice of the dataset when this pattern disappeared is not strict and still a bit subjective. Therefore we strived to find

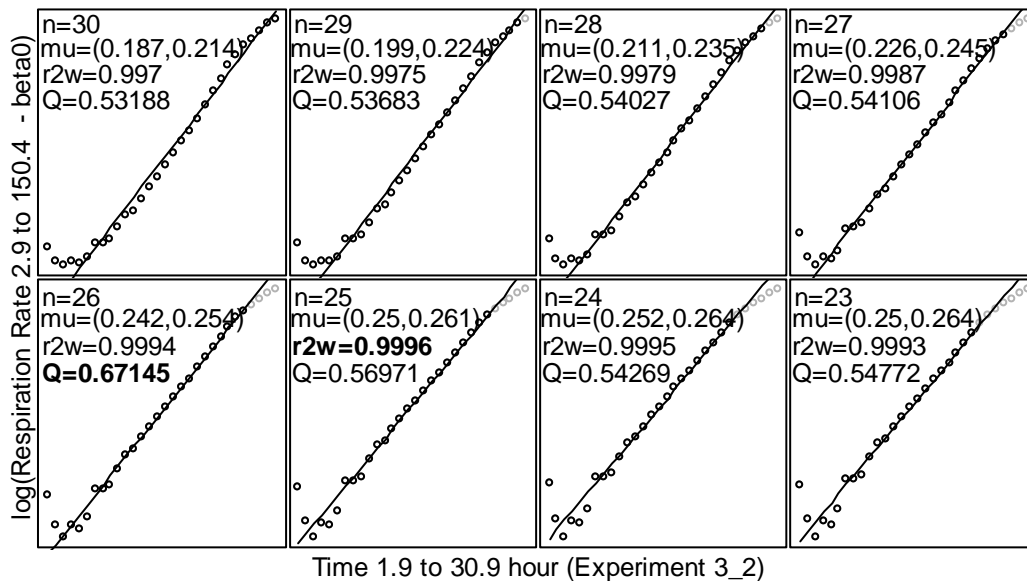


Figure 2: Same as Fig. 1 with y-axis is at log-scale. Coefficient β_0 is the respiration rate at the time zero directly after the amendment. β_0 is estimated during model fit and slightly changes between figure panels.

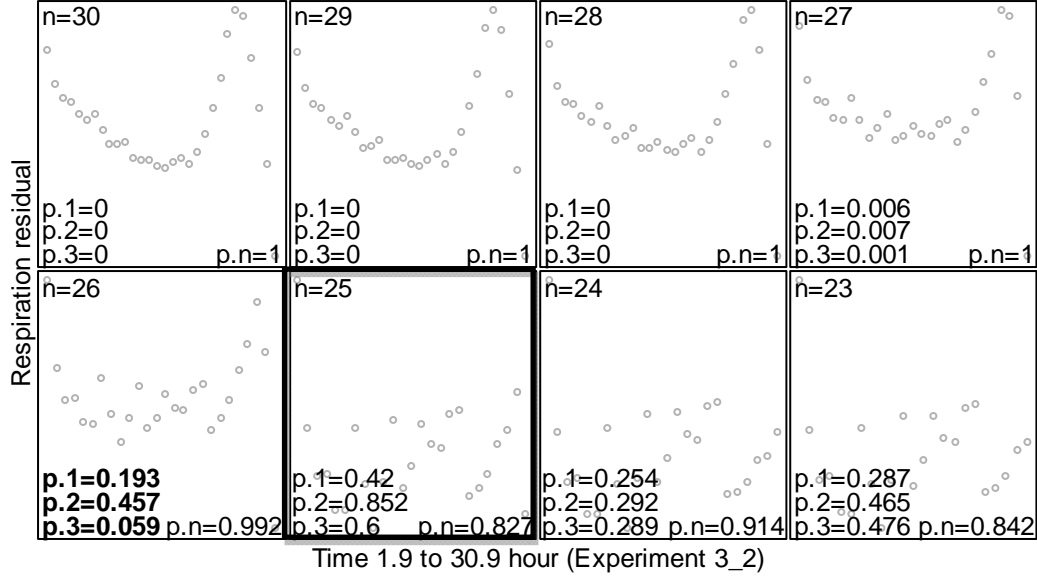


Figure 3: Plot series of the residuals of Figure 1. n : number of records in the subset of the data. For this example the statistical criteria tell that there is no significant auto-correlation starting from panel $n=26$. From visual inspection of the V-shaped patterns, however, we decide that unlimited growth phase extends only until $n=25$ records. $p.1$: probability of autocorrelation according to the Breusch-Godfrey test for the entire series of residuals, $p.2$: same as $p.1$ but applying the test only for the subset of residuals excluding the records before the minimum of respiration., $p.3$: same as $p.2$: but additionally excluding the last residual in the test statistics, $p.n$: probability of the Durbin Watson test for negative autocorrelation.

statistical criteria to identify the unlimited growth phase.

Statistical criteria to confine the unlimited growth phase

The model fits to subsets of the respiration time series differing by the number of included records n can be also compared by statistical criteria. We benchmarked several criteria by comparing them to the result of applying the rules of comparing residual plots to the 121 respiration time series.

I. Maximum value of r^2 and Q-value

The first measure that we tested was the coefficient of determination, denoted r^2 (eq. 9) (Quinn and Keough 2002).

$$r^2 = 1 - \frac{SS_{err}}{SS_{tot}} = \frac{SS_{reg}}{SS_{tot}} = \frac{\sum_i (v(t_i) - \bar{v})^2}{\sum_i (v_i - \bar{v})^2} \quad (9)$$

where r^2 is the coefficient of determination, SS_{err} is the residual sum of squares, SS_{tot} is the total sum of squares, SS_{reg} is the regression sum of squares, $v(t)$ is the respiration rate predicted by the fitted model for time t , \bar{v} is the mean respiration rate across all i , and v_i is the observed respiration rate.

The coefficient of determination has two useful properties: it decreases with the exclusion of records from the data set, and it decreases with inclusion of records that are far apart from the model prediction, i.e. outside the exponential growth phase. Hence, we expected the dataset with the maximum number of

points in the exponential growth phase to result in the maximum r^2 .

In order to account for the varying measurement uncertainties, we additionally calculated a weighted version of the coefficient of determination denoted r^2w according to eq. 10.

$$r^2w = \frac{\sum_i (v(t_i) - \bar{v})^2 sd(\varepsilon_i)^{-2}}{\sum_i (v_i - \bar{v})^2 sd(\varepsilon_i)^{-2}} \quad (10)$$

where $sd(\varepsilon_i)$ is the expected standard deviation according to equation 7.

A similar measure of goodness of fit is the sum of weighted residuals (eq. 11).

$$X^2 = \sum_i \frac{(v(t_i) - v_i)^2}{sd(\varepsilon_i)^2} \quad (11)$$

where $v(t_i)$ and v_i are the model prediction and the observed respiration respectively at time t_i and $sd(\varepsilon_i)$ the estimated standard deviation of observation i (eq. 7). The measure Q is the probability of obtaining the statistics X^2 (eq. 11) in a Chi-square distribution with the number of degrees of freedom equal to the number of data values minus the number of adjusted parameters, i.e. three in this case. Q corresponds to the probability that the differences between the model and the data has occurred by chance. Similar to r^2 , Q is expected to have maximum values at the dataset with the maximum number of points in the exponential growth phase.

II. Absence of autocorrelation between residuals

Deviations from unlimited growth will result in patterns in the residuals. If there was a distinctive pattern in the residuals, then testing for autocorrelation in residuals is probably significant. If there was no distinctive pattern, the tests for autocorrelation have higher probability of obtaining non-significant results.

The Durbin-Watson statistic tests for first-order autocorrelation in the residuals from a regression analysis (Durbin and Watson 1951). The function `dwtest` in the free R software provides probabilities for positive or negative autocorrelation based on this statistics.

The Breusch-Godfrey test (Breusch 1978) also tests for autocorrelation in the residuals but is considered to be more general and more powerful than the Durbin-Watson test. The function `bgtest` of the free R software provides probabilities for serial autocorrelation for the Breusch-Godfrey test.

We used these probabilities of the tests to choose a subset of the data with the following rules.

- 1) The fit to the data set has no significant autocorrelation on the 5% rejection level.
- 2) The fit to the dataset including one more point has significant autocorrelation at the 5% rejection level.
- 3) If there are several cases that fulfill rules 1 and 2, select the case that includes the most records.

We found that these tests detected more autocorrelations in the respiration data than that was obvious in the residual plots when applied to all the residuals. Hence, we present the results of applying the tests to subsets of the residuals. The first subset includes all residuals (denoted **dwtestfull** and **bgtestfull**). The second subset omits all the residuals before the minimum of respiration and excludes the very first records that are prone to measurement errors (denoted **dwtest** and **bgtest**). Further we found that the last observation had a large influence on the test statistics. Hence, the third subset additionally excluded the last observation (denoted **dwtest1** and **bgtest1**).

III. Combined criteria

Because each of the basic criteria identified incorrect number of records in unlimited growth phase results for a few respiration series, we further refined and experimented with several combinations of the basic criteria. Here we present one combined criterion that was quite successful and at the same time not too complicated. We denote it **cortest**. The combined criterion works on the following rules

- 1) Choose the dataset identified by `bgtest1` rules
- 2) If there is a dataset with negative autocorrelation according to a `dwtest` that includes more records then the dataset identified by 1 then choose this one.

This combined criterion improved the `bgtest1` decision in the cases where there was a negative autocorrelations by chance.

The second combined criterion, here denoted as **r2wComb**, took account for deviations between several criteria. When both the `r2` criterion and the `cortest`

criterion identified a dataset that deviated by more than one point from the dataset identified by the `r2w` criterion, the criterion `r2wComb` did not give a number of points but was undecided (NA, not a value).

Results and discussion

Confining the experimental growth phase

Series of plots of model fits at original and log scale (Figs. 1, 2) and the series of model residuals plots (Fig. 3) greatly aided the identification of the records within the unlimited growth phase. We applied the rules of comparing residual plots (section 2.4) to the 121 respiration datasets. In 8 datasets, there were inherent autocorrelations that may have arisen from measurement errors that were correlated with time, making the rules of comparing residual plots unsuccessful. In those cases, the V-pattern did not disappear with exclusion of additional records (Fig. 4). So we selected a subset of unlimited growth by inspecting the series of fits at original and log scale.

The importance of properly confining the unlimited growth phase as demonstrated in Fig. 1 was confirmed by a sensitivity analysis across the 121 time series of microbial growth respiration (Appendix B). The specific growth rate μ_{\max} was significantly biased downwards and the initial activity r_0 was highly sensitive to including records after the unlimited growth phase.

When benchmarking several statistical criteria to confine the growth phase, the `r2w` criterion performed best among the non-combined criteria. It successfully identified the unlimited growth phase in all time series where nutrients were not limiting (104 of 121) (Table 3).

Table 3: Frequency of matches and mismatches between the number of records in subsets of unlimited growth data identified by several statistical criteria compared to the number of records identified by rules of comparing residual plots across $n=121$ datasets.

Crit.	Undecided	< -1	> +1	Matches	Failure rate
Correlation measures					
r2	0	31	3	87	28%
r2w	0	1	6	114	6%
Q	0	56	35	30	75%
Patterns in residuals					
dwtestfull	23	30	1	67	26%
dwtest	11	29	1	80	25%
dwtest1	10	28	10	73	31%
bgtestfull	6	13	15	87	23%
bgtest	4	13	17	87	25%
bgtest1	3	14	17	87	26%
Combined measures					
cortest	3	14	17	87	26%
r2wComb	15	0	2	104	2%

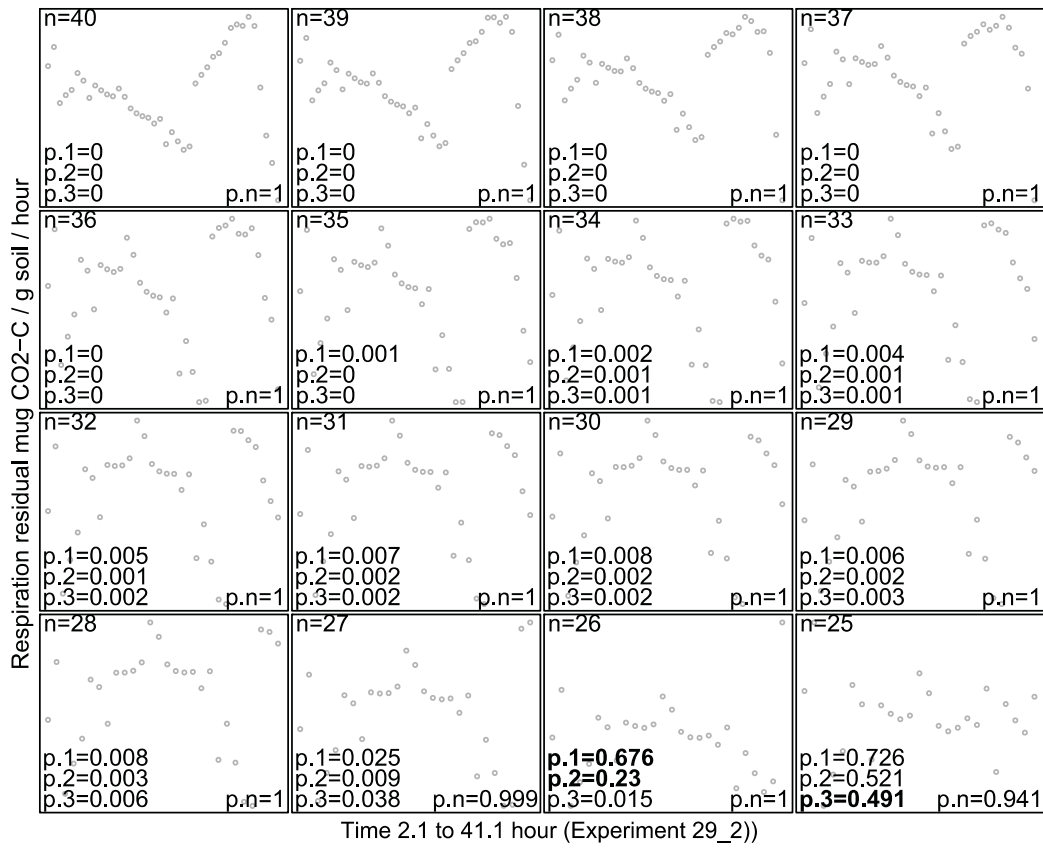


Figure 4: Example plot series of standardized residuals where the inspection of residual pattern was not applicable.

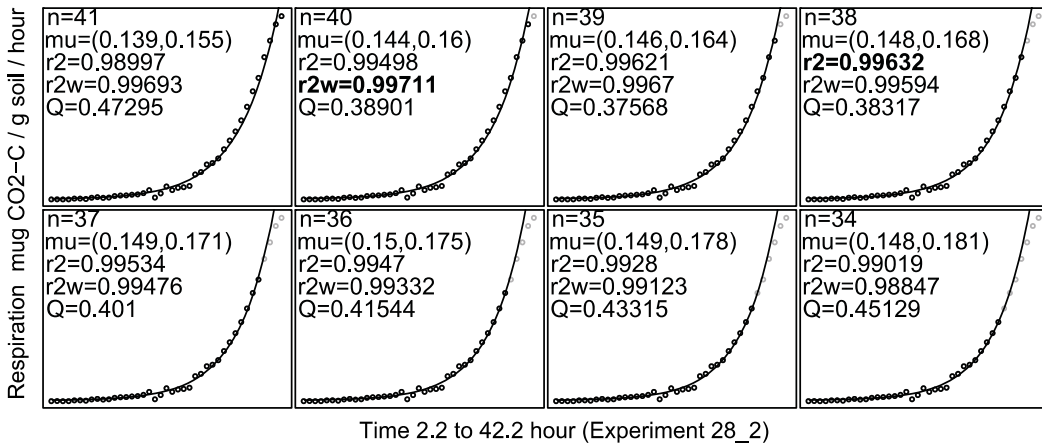


Figure 5: Example plot series of model fits where the $r2wComb$ criterion was not successful. The rules of comparing residual patterns suggested the series of 36 records, the $r2wComb$ suggested 40 records. The best estimates of microbial parameters, though, did not significantly differ between the two subsets of the data.

Robust criteria confining the experimental growth phase

The weighted $r2$ is a simple statistical criterion to constrain the unlimited growth phase. However, we further studied whether the criteria can distinguish the situations where the restrictions for unlimited growth are violated. If a criterion does not give unreasonable results in those cases we call it robust. An example test case was the FAL arable sandy soil that was incubated with incomplete nutrient amendment. As a result the $r2w$ criterion sometimes provided incorrect conclusions (time series of samples 24, 26, and 29). This was because of to deviations from unlimited growth due to shortage of nutrients instead of carbon substrate. We

experimented with several methods of automated detection of these problematic cases. In most of the unsuccessful cases, the $r2w$ criterion diverged from both the $r2$ and the cortest criterion by more than one record. The check on this divergence was formalized with the combined $r2wComb$ criterion, which identified almost all problematic cases. There were only two of the 121 series (time series 28_2 and 37_2) where the $r2wComb$ criterion provided approximation instead of indentifying the problems of double limitation.

In both series, consistent deviations of the respiration from the growth model in the middle of the growth phase hampered the test for correlations (Fig. 5). Such consistent deviations can arise due to

equipment error. However, the microbial parameters based on an improperly defined growth phase did not significantly differ from the ones based on visually confining the growth phase.

In summary, the r2w criterion was applicable for confining the experimental growth phase in experiments with complete nutrient amendment. For low nutrient studies, the more complex combined r2wComb criterion was able to detect the problematic cases where further inspection is necessary. We conclude that the r2wComp criterion represents a robust way, how to statistically constrain the unlimited growth phase.

Uncertainty of respiration measurements

In addition to the experimental growth phase, the estimates of microbial parameters depend on the prior information about measurement uncertainty. Ordinary least squares parameter estimation assumes that measurement errors are independent and identically distributed. However, this assumption is often not met with respiration measurements. The uncertainty of the respiration measurements usually increases with the magnitude of the respiration. The usage of generalized

linear models allows the magnitude of the variance of each measurement to be a function of its predicted value (Quinn and Keough 2002). Hence, we modelled the variation of the residuals by equation 7. The parameter δ was assumed to be dependent only on the measurement setup and was assumed not to change between datasets measured with the same device and setup.

The number of records in the unlimited growth phase and the estimated microbial parameters was sensitive to the choice of the magnitude of increase of measurement uncertainty with the magnitude of the respiration. For example, in Fig. 6, the 95% confidence interval of specific growth rate estimated at $\delta=0.5$ does not include the best estimate at $\delta=0.3$. However, assuming δ within the range of 0.4 to 0.8 does not introduce significant bias.

One might be tempted to estimate the uncertainty of the measurements by the distribution of measurement errors across replicates. However, the differences between replicates are only in part caused by measurement errors but are in a big part caused by variation in initial biomass x_0 and physiological state r_0 . Even with totally precise measurements of respiration, the observed respiration would differ because of differences of microbial biomass across replicates. Hence, estimating the measurement uncertainty from differences across respiration observations of several replicates would lead to an overestimation of measurement uncertainty, especially in the later stages of growth.

In the best case, measurement uncertainty has been determined by a calibration of the measurement setup against a known flux of carbon dioxide. However, such information is rarely available. The identification of δ from the data is an unsolved problem. In this study we eventually included parameter δ as a free parameter in model fits to each experiment and used the median of the results across all the experiments.

For modern Model-data synthesis reporting of uncertainties of estimated parameters is as essential as reporting the best estimates (Raupach et al. 2005). The modifications of the model fitting process suggested in section 2.1 proved very useful for estimating parameter uncertainty. In accordance with (Hess and Schmidt 1995) we suggest to use respiration rates instead of cumulated respiration. Next, by directly fitting eq. 5 that includes microbial parameters allowed the numerical algorithms to infer parameter uncertainty. Further, the parameter transformations of eq. 8 yielded asymmetric confidence bounds for microbial parameters, ensuring valid ranges. This procedure corresponds to assuming a log-normal distribution for x_0 , and μ_{\max} , which is reasonable for strictly positive variables (Jaynes and Bretthorst 2003, Tarantola 2005).

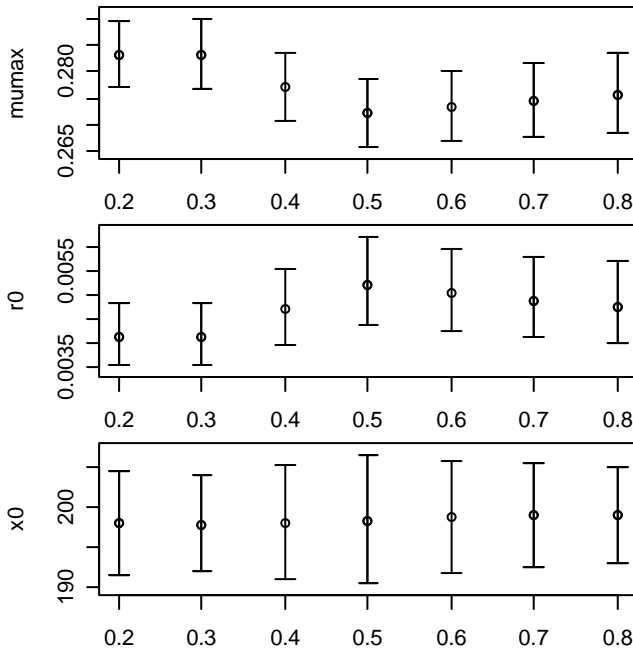


Figure 6: Comparison of microbial parameter estimates across varying assumption of increasing measurement uncertainty for respiration time series 2.1. X-labels denote δ parameter for equation 7. Points denote best estimates and arrows denote 95% confidence interval after identifying the unlimited growth dataset and fitting microbial parameters.

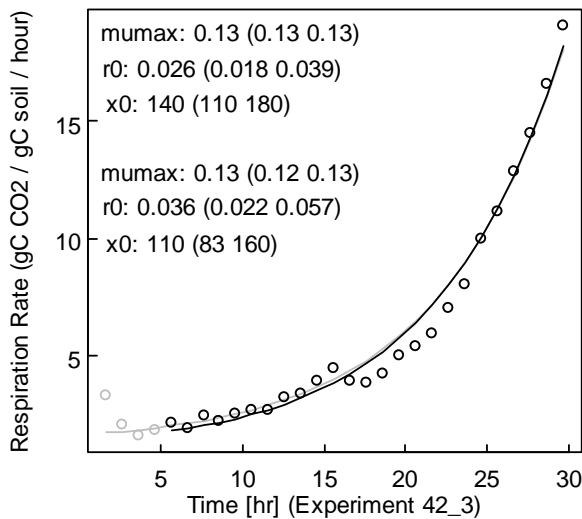


Figure 7: . Effect of biased measurements at the beginning of the time series. The gray line and the first set of estimated parameters are inferred from the entire time series. The black line and the second set of estimated parameters are inferred from a time series where the first 5 hours (gray points) have been excluded. Values in brackets denote the 95% confidence interval.

Biased measurements at the beginning of the growth phase

Several methods of measuring respiration from soil give imprecise measurements after disturbing the soil sample at the beginning of the experiment. With gas analyzers (Heinemeyer et al. 1989), the flow has to equilibrate throughout the tubing system. With the Respicond method (Nordgren 1988), temperature has to be restored. Hence, it is necessary to discard the imprecise measurements at the beginning before the fitting procedure. In the example of figure 7 the decline of respiration rates within the first 4 hours does not correspond to the assumption of unlimited growth of microbes. Omitting the first 4 observations from the kinetic respiration analysis changed the estimates of initial (time=0) microbial biomass and initial activity ratio.

Number and spacing of respiration measurements

The required precision of microbial parameters eventually determines the required number of measurements. From a statistical perspective, the fitting method requires at least 6 records. This is because there are 6 parameters to estimate, the three microbial parameters, the two parameter of residual uncertainty, and the time (or number of records) within the unlimited growth phase. More important than the number of measurements, however, is that the measurements are well dispersed across the time of unlimited growth. We demonstrate this issue using the example from the introduction (Fig. 8). We repeated the kinetic analysis for several subsets of the time series. Using only 6 records equally spaced across the growth phase, the confidence intervals of microbial parameters

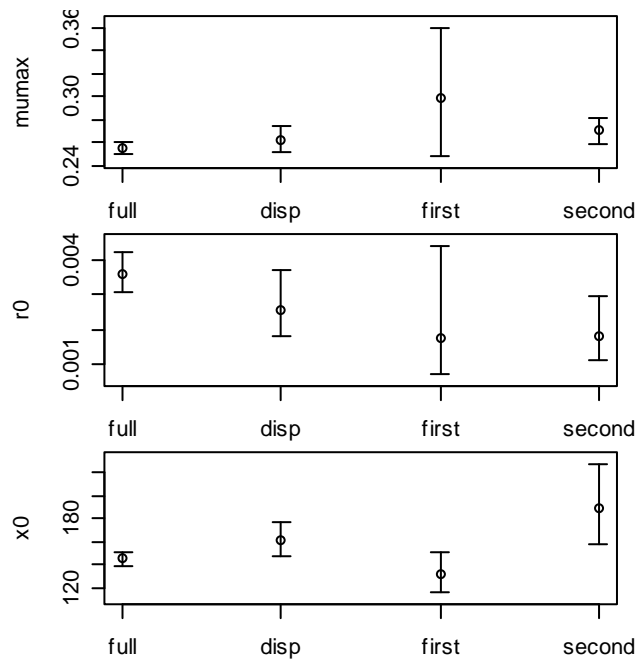


Figure 8: Comparison of microbial parameter estimates across varying scenarios of subsets of the respiration time series 3_2. full: dataset with 30 records (Fig. 1), disp: 6 dispersed records (1 7 13 19 25 30), first: first 15 records, second: last 15 records. Points denote best estimates and arrows denote 95% confidence interval.

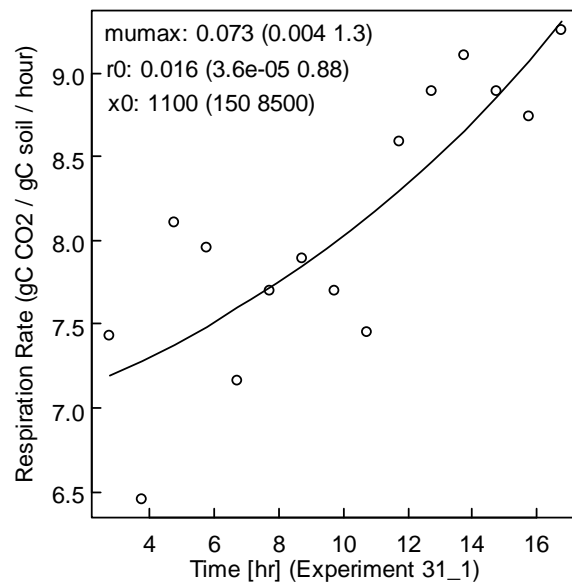


Figure 9: Example of wide 95% confidence intervals (given in brackets) of estimated microbial parameters.

were only inflated by a factor of about 2 and included the best estimate that was based on all 30 records. In contrast, when using all 15 measurements of the first or second half respectively, the confidence intervals were inflated much more.

In the case, when there are too few measurements, the uncertainty bounds will indicate this. For example Fig. 9 displays an example of strong N limitation where the kinetic respiration analysis formally works, however, essentially does not add much information

about microbial parameters. In this case it would be misleading to just report best estimates without reporting the uncertainty bounds.

Conclusions

The kinetic respiration analysis is currently the only method of soil microbial biomass estimation that allows the estimation of the active part of total biomass. The synthesis of results of the method, however, has been hampered by differences in statistical treatment of the resulting data, which is discussed in this study.

One precondition of the method is unlimited growth and the dataset has to be confined to this initial phase, i.e. the lag phase and the exponential growth phase. The estimates of the microbial kinetic parameters are sensitive to differences in this process of confining the unlimited growth phase. The process is inherently vague because the onset of deviation from unlimited growth due to the influence of a limitation appears to be gradual. Still, the scientific community should aim to establish a common procedure of confining the unlimited growth phase to allow comparison and synthesis of the estimates between studies. We propose rules of comparing residual plots that are based on the disappearance of a pattern in the residuals in a series of fits. Inclusion of records outside the unlimited growth phase introduces larger deviations than exclusion of records inside the unlimited growth phase. Hence, excluding records is the more conservative choice.

From the tested statistical criteria, the simple r2w criterion (the weighted coefficient of determination) is applicable for confining the experimental growth phase in experiments with complete nutrient amendment. The more complex r2wComb criterion can in addition detect problematic cases. It is only necessary, if the researcher wants to automate the analysis of many time series and a wants to have a guide for finding those series, e.g. from low nutrient studies, where further inspection is necessary. This study confirms the importance of experimental precautions. Nitrogen and Phosphorus limitation should be avoided in using kinetic respiration analysis for the purpose estimating microbial biomass and its activity state.

The estimates of microbial parameters are sensitive to the specification of measurement uncertainty. This uncertainty is overestimated when inspecting the variance in respiration across replicates and neglecting the differences in initial conditions between replicates. The robust estimation of uncertainty parameters from the data itself is subject to further research.

The estimates of microbial parameters are also sensitive to the number and timing of the respiration measurements across the phase of unlimited growth and introduce a bias in the initial measurements due to experimental conditions. It is important that the measurements are well dispersed across the time period of unlimited growth. The case of only too few measurements will be reflected in the uncertainty bounds of microbial estimates. We strongly recommend

always reporting those bounds together with the estimates.

We provide the tools of using the freely available R software to fit the kinetic respiration analysis model to the data and to constrain the unlimited growth phase as an R-package. The most critical statistical issues are under control and they should not hamper the application of the kinetic respiration analysis.

Appendix A: Derivation of the kinetic model

Here we present the derivation of model equation 5. The kinetic respiration analysis is based on the dynamic synthetic chemostat model (SCM) (Panikov 1995). This model describes the activity of microbial biomass by the physiological state r . Variable r is defined as the ratio between the instantaneous quantity of cell constituents absolutely necessary for growth (P-components) and its total changeable part. The SCM assumes that a change in P-components is complemented by a reverse change in components needed for survival under restricted growth (U-components), and that hence the amount of U-components should be proportional to $1-r$. It can be said that r indicates the ratio between active and sustaining biomass.

When integrating the dynamic SCM model over a time t of unlimited growth the respiration rate $p(t)$ follows equation 12 (Panikov and Sizova 1996,

Blagodatsky et al.).

$$\begin{aligned} p(t) &= [(1-Y)r(t)Q + Q']x(t) \\ &= x_0(1-r_0)Q + x_0r_0[(1-Y)Q + Q']e^{\mu_{\max}t} \\ &= x_0(1-r_0)Q + x_0r_0Q_T e^{\mu_{\max}t} \end{aligned} \quad (12)$$

where $p(t)$ is the respiration rate at time t , expressed as respired carbon per time, μ_{\max} is maximum specific growth rate, r_0 is the initial physiological state, x_0 is the initial biomass, Q and Q' are maximum substrate uptake rates for coupled and uncoupled respiration respectively. The part Y , termed biomass yield, of the uptake for coupled respiration is incorporated into biomass, i.e., is associated with growth, and part $(1-Y)$ is respired. The uncoupled part Q' is not coupled with growth and is completely respired. It can be approximated by measuring cyanide-resistant respiration. Variable $Q_T = (1-Y)Q + Q'$ is the total specific respiration. With $Y_{CO_2} = Y/(1-Y)$ and $\lambda = (Q_T - Q')/Q_T$, Equation 5 above is a direct reformulation of eq. 12.

Appendix B: Sensitivity of growth parameters to biases in confining the unlimited growth phase

Do differences in confining the unlimited growth phase cause improper estimates of microbial parameters? This question can be answered by a sensitivity analysis. For each of the 121 datasets where we identified an

unlimited growth phase, we compared resulting microbial parameters using different subsets 7 of the respiration data. The first subset corresponded to the subset defined by the rules of comparing residual plots to microbial parameters. The other six subsets included either up to three more records or omitted up to three records at the end of the unlimited growth phase.

As a measure of the expected absolute and relative magnitude of the deviation, we calculated the mean, standard deviation, and mean of the absolute (i.e. positive) of the relative error. As a measure of expected bias, we calculated the mean of the relative differences. Significance of the bias was tested by a paired t-test.

The sensitivity analysis showed significant bias of microbial parameters when constraining the unlimited growth phase in different ways. The specific growth rate μ_{\max} was significantly biased upwards when excluding records of the end of the unlimited growth phase and significantly biased downwards when including records after the unlimited growth phase

Table 4: Statistics of difference of parameter estimates with adding records to or excluding records from to the unlimited growth phase. Numbers in the heading correspond to deviations in number of records compared to the subset identified by the rules of comparing residual plots. sd: standard deviation of the difference, rel.Err: mean of the absolute of relative errors, bias: mean of the difference, p: probability of the bias by a two-sided t-test (n=121).

Parameter	Statistics	-3	-2	-1	1	2	3
μ_{\max}	sd	0.061	0.039	0.016	0.026	0.043	0.042
	rel.Err	18%	11%	5%	9%	15%	19%
	bias	0.027	0.018	0.0065	-0.018	-0.033	-0.04
	p	3.10E-06	1.60E-06	1.00E-05	7.00E-12	2.80E-13	2.20E-16
r_0	sd	0.1	0.046	0.037	0.099	0.15	0.28
	rel.Err	205%	49%	18%	103%	511%	1699%
	bias	-0.0013	-0.0075	-0.0062	0.019	0.048	0.12
	p	0.89	0.074	0.065	0.036	0.0012	1.70E-05
x_0	sd	84	140	38	70	97	51
	rel.Err	9%	9%	4%	8%	14%	17%
	bias	-19	-4	-0.35	13	1.3	-31
	p	0.014	0.75	0.92	0.04	0.89	7.00E-09

(Table 4). The initial activity r_0 was highly sensitive to inclusion of more records, as indicated by the relative errors. There was a strong significant overestimation when including records after the unlimited growth phase. For initial biomass x_0 there was no clear direction of the deviations. Based on the magnitude of the relative errors we recommend that if one is in doubt about including a record within the unlimited growth phase, one should rather exclude it.

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