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Review paper

# Active microorganisms in soil: Critical review of estimation criteria and approaches

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ABSTRACT

Microbial functioning refers to microbial activity because only the active microorganisms drive biogeochemical processes. Despite the importance of *active* microorganisms, most methods focus on estimating *total* microbial biomass and fail to evaluate its active fraction. At first, we have described the differences among the *active*, *potentially active*, and *dormant* microbial states in soil and suggested threshold values of parameters for their identification. Secondly, we critically reviewed the ability of a broad range of approaches to estimate and characterize the active and the potentially active microorganisms in soil. Following approaches were evaluated: plate count and microbial cultures; direct microscopy combined with cell staining; ATP, PLFA, DNA and RNA content; microarray analyses; PCR-based approaches; stable isotope probing; soil proteomics, enzymes activity; and various approaches based on respiration and substrate utilization. The "static" approaches, mainly based on the single-stage determination of cell components (ATP, DNA, RNA, and molecular biomarkers), detect well the presence of microorganisms and total biomass, but they fail to evaluate the active part and consequently the functions. In contrast, the dynamic approaches, estimating the changes of these parameters during microbial growth and based on process rates: substrate utilization and product formation, e.g., respiration, help to evaluate active microbial biomass and relate it to specific process rates. Based on a comparison of all approaches for their universality (possibility to analyze active, potentially active and dormant microorganisms), we concluded that 1) direct microscopy with complementary stains, 2) a combination of RNA-based FISH with staining of total microbial biomass, and 3) approaches based on microbial growth were the most advantageous and allowed simultaneous quantitative estimation of *active*, *potentially active*, and *dormant* microorganisms in soil.

The *active* microorganisms compose only about 0.1–2% of the *total* microbial biomass and very seldom exceed 5% in soils without input of easily available substrates. Nonetheless, the fraction of *potentially active* microorganisms (ready to start utilization of available substrates within few hours) is much higher, contributing between 10 and 40% (up to 60%) of the total microbial biomass. Therefore, we emphasize the role of *potentially active* microorganisms with quick response to fluctuating substrate input in soil microhabitats and hotspots.

The transition from the potentially active to the active state occurs in minutes to hours, but the shift from dormant to active state takes anywhere from hours to days. Despite very fast activation, the reverse process – fading to the potentially active and dormant stage – requires a much longer period and is very different for individual criteria: ATP, DNA, RNA, enzyme production, respiration rates. This leads to further difficulties in the estimation of the active part of microbial community by methods based on these parameters. Consequently, the standardization, further elaboration, and broad application of approaches focused on the portion of active microorganisms in soil and their functions are urgently needed. We conclude that because active microorganisms are the solely microbial drivers of main biogeochemical processes, analyses of the active and potentially active fractions are necessary in studies focused on soil functions.



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## 1. Introduction: why consider active microorganisms?

Studies that refer to microbial biomass are central not only in soil science but also in all biogeochemistry-related disciplines. Microbial biomass is studied not as end in itself but as a driver of biogeochemical cycles. This requires knowing which microorganisms are responsible for specific processes and, more generally, which portion of the microbial biomass is responsible for the turnover of elements.

Microbial communities in soils consist of a very broad range of organisms in different physiological states. These are frequently termed as active, viable, living, dormant, passive, dying, dead, and so on, states (Johnsen et al., 2001) and are often difficult to differentiate among (Rousk et al., 2009). These terms can be summarized as four physiological states of microorganisms. The first three are living states. The first is the *active* state of the microorganisms. The active microorganisms are involved in the ongoing utilization of substrates and associated biochemical transformations. The second is the *potentially active* microorganisms. This part is in physiological alertness (De Nobili et al., 2001; Raubuch et al., 2010) and can switch to utilization of substrates within minutes to a few hours. The last state of living microorganisms is the *dormant* state. It does not contribute to ongoing processes currently but can contribute under altered circumstances. The fourth state of microorganisms in soil is *dead* (including lysed cells and microbial residues), but also quantified by some methods and does not directly contribute to any ongoing processes. Dead microbial biomass does, however, affect turnover of C and N as a source of easily available substrates. All these parts of *total* microbial biomass are crucial for evaluating soil functions and comparing treatments, environmental conditions, land use, and management practices. However, only active microorganisms are involved in the ongoing processes and consequently, all processes should be related to the mass of *active* microorganisms driving biogeochemical elements cycling in soil.

Most methods for estimating microbial biomass (reviewed by Beck et al., 1997; Nannipieri et al., 2003; Hartmann et al., 2004; Bölter et al., 2006; Joergensen and Emmerling, 2006; Joergensen and Wichern, 2008) were developed to measure *total* microbial biomass, and these reviews are focused on methods for estimating *total* microbial biomass. However, because most processes are driven by *active* microorganisms, it is a current challenge to quantitatively distinguish active and dormant biomass and to assess ecologically relevant microorganisms actively contributing to ecosystem functions (Ellis et al., 2003).

This motivated the present review of one of the most dynamic pools and drivers in soil – the active microbial biomass. After the definition of terms, we evaluated suitable methods to estimate the active part of microbial biomass (note that this review does not focus on presenting analytical details of the methods) and then compared the approaches by their suitability to evaluate separately the three parts of living microbial biomass. Furthermore, we suggested the threshold values or parameter ranges as criteria for differentiation of the three parts of living microbial biomass by various approaches.

It was not the aim of this review to analyze various microbial activities such as respiration, decomposition rates of natural substrates or xenobiotics, transformations of biogenic elements, ATP production, or enzyme activities. However, we refer to some of these approaches if they are directly or indirectly useful to estimate or to characterize the portion of active microorganisms.

## 2. Definitions: total, dead, dormant, and active microorganisms

**The total microbial biomass** includes all living and nonliving soil organisms smaller than 150–200 μm (Swift et al., 1979; Coleman and Wall, 2007). The total amount of microbial biomass is relatively small (50–2000 μg C $g^{-1}$ soil). It averages at 2–3% (Anderson and Domsch, 2010) and usually does not exceed 4.5% of organic C content (Anderson, 2003). The **dead** microorganisms are in an irreversible state in which no growth, cell elongation, or protein synthesis can take place (Villarino et al., 2000). Dead cells, or **microbial necromass**, act as an additional pool of available substrate but do not contribute actively to any biogeochemical processes. Microbial necromass is a fraction of easily available SOM and

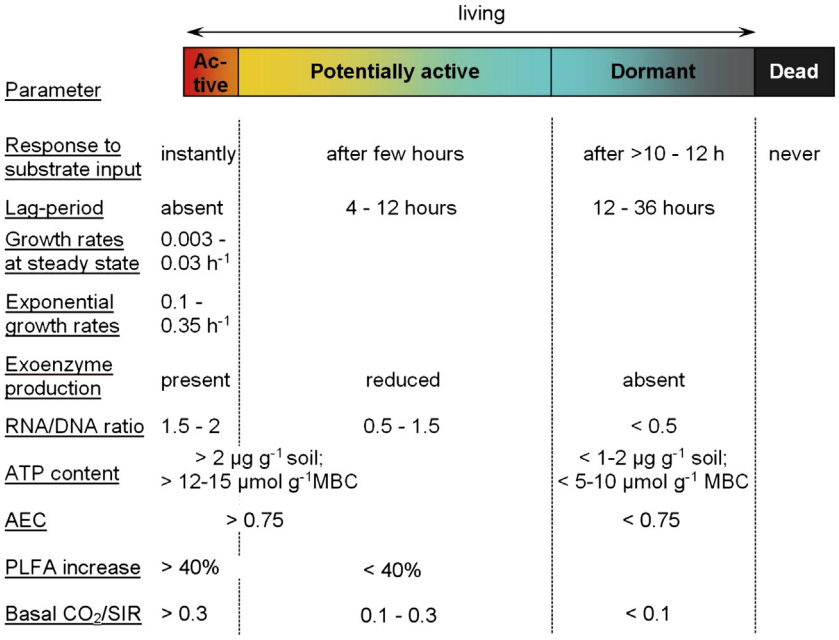



**Fig. 1.** Various physiological stages of microorganisms in soil: active, potentially active, dormant and dead. Threshold values and ranges for parameters obtained by various approaches to differentiate between the physiological stages are suggested (see text for details and references).

is very dynamic due to permanent re-utilization of microbial C, which can be 1) mineralized to $CO_2$, 2) further incorporated into microorganisms or 3) transformed to stabilized SOM (Cotrufo et al., 2012; Bradford et al., 2013). So it represents important intermediate stage of long term C stabilization in soil. The fraction of necromass must be considered, when the determination of active microorganisms is based on extracting/staining the cell components remaining in dead cells (see Section 3.2). Additionally, the intriguing pool of extracellular enzymes (Burns, 1982; Nannipieri et al., 2012; Schiemel and Schaeffer, 2012) as a "part" of non-living microbial biomass that is still "active" and can hydrolyze/oxidize substrates long after the producer is dead, should be considered as contributing to the C and N turnover.

Only a tiny portion of the total microbial biomass maintains an **active state** in soil without an input of easily available substrates, while a large proportion of living cells are inactive (Jenkinson and Ladd, 1981; Prosser et al., 2007). We define the **active microbial biomass** as the portion of total microbial biomass that 1) *is involved in current utilization of substrates*, or 2) *readily responds to substrate input* e.g., by respiration, producing enzymes, or 3) *is growing and reproducing*.

The **dormant state** of soil microorganisms has traditionally described microbial cells exhibiting *strongly reduced physiological activity*, e.g., resting cells forming spores or cysts (Roszak and Colwell, 1987). However, only some microorganisms have the ability to generate spores, e.g., it is common for Gram(+) bacteria to do so, while Gram(−) switch to a non-cultivable state under starvation. Moreover, many bacteria can persist utilizing energy reserves at a very slow rate for long periods by lowering their metabolic activity (Raubuch et al., 2002). Fungal spores are produced primarily for reproduction but survive under unfavorable conditions. Therefore, **dormant state** is defined as various resting forms with strongly reduced respiration and endogenous metabolism over an extended period. Microorganisms in the dormant state do not contribute to turnover processes.

The quickness of response to substrate addition or environmental changes (e.g. moistening) can be used to reflect the activity states (Fig. 1). At the level of individual cells, the microorganisms (also non-cultivable) increase metabolic activity within minutes (e.g., respiration) to hours in response to substrate input even if the cell division starts several hours to days later (Winding et al., 1994; Maraha et al., 2004; Konopka et al., 2011). Therefore, the *fraction of microorganisms that rapidly switch from the inactive state to activity* indicates the **potentially active** microbial population permanently existing in soil between the active and dormant physiological states. Even under long-term starvation the potentially active microorganisms maintain 'physiological alertness' to be ready to occasional substrate input (De Nobili et al., 2001). The potentially active fraction is not uniform and consists of long-term starving cells and resting forms quickly reactivated from reduced metabolism to an active state within few hours (Placella et al., 2012).

## 3. Approaches to estimate active microorganisms in soil

We refer to the reviews of Breeuwer and Abee (2000); Nannipieri et al. (2003); Hartmann et al. (2004); Bölter et al. (2006); Joergensen and Emmerling (2006); Joergensen and Wichern (2008); Musat et al. (2012) for detailed descriptions of estimation methods for microbial biomass and activity as well as their advantages and shortcomings. The present review focuses on the methods' potentials to distinguish *physiological states* of soil microorganisms. Several indirect criteria are often used to evaluate an active fraction of microbial biomass. These include cultivability or staining with specific dyes, the amount of biomarkers in microbial cells, and metabolic/respiratory activity. We also discuss the restrictions to be considered for correctly interpreting the results obtained by such indirect approaches.

### 3.1. Plate count and microbial cultures

Even though plate-count techniques represent only about 1% of total soil microorganisms, the number of colony-forming units (CFU) is positively correlated with enzymes and respiratory activity (Sanchez-Peinado et al., 2009) and is still applied to characterize the relative abundance of active/potentially active microbial groups with certain functions or trophic requirements (Néble et al., 2007). Despite very low portion of microorganisms identified by plate-count techniques, their contribution to nutrient cycling in soil could be high as they represents 80–90% of bacterial biovolume in soil due to large cell size (>0.065 $\mu m^3$) of cultivable organisms (Olsen and Bakken, 1987). The majority of cells smaller than 0.065 $\mu m^3$ are viable but not cultivable (Winding et al., 1994; Oliver, 2005) because reproductive ability is not necessarily related with metabolic activity. Starving microorganisms can remain metabolically active (Maraha et al., 2004) and degrade organics (Forlani et al., 1999; Mijangos et al., 2009), although they are unable to grow on nutrient agar plates. Furthermore, cultivability of certain species is dependent on the stage of population development (e.g. the cultivability can be lost under starvation but it can be recovered under favorable conditions (Oliver, 2010; Lleo et al., 1998; De Fede and Sexstone, 2001; see Section 3.3.). Some bacterial and fungal spores, those are initially not active but are cultivable, also contribute to the CFU count and are accounted as potentially active biomass. The genetic structure of the active bacterial community represents for 30–40% (Bernard et al., 2007) or even for 80% (Roszak and Colwell, 1987) of the uncultured microorganisms. Therefore, active microbial biomass is strongly underestimated by the plate-count technique. So, isolation in liquid cultures (Chin et al., 1999) or on solid agar media (Constant et al., 2008, 2010) is mainly used presently to monitor the potential activity, morphology, and physiological characteristics of microbial groups that perform the specific functions (e.g., cellulolytic, methanogenic, or $H_2$-oxidizers) in soil (Dunfield and Conrad, 2000).

Despite these shortcomings, the *dynamics* of colony appearance was creatively interpreted to calculate both stochastic start of growth and microbial growth rate by the kinetic approach (Hashimoto and Hattori, 1989). The colony appearance, i.e. the moment when the microbial colony becomes visible, depends on 1) the period preceding microbial growth, i.e. on lag-time; 2) microbial cell size: shorter time and smaller fission number is required to visualize a colony formed by large versus small cells (at same doubling time); and 3) microbial growth rates *per se*. Lag period is shorter for microorganisms in an active physiological state. Therefore, the colony forming curves reveal which microbial group starts to grow first when growth is not limited. Because cell division begins much earlier than the colony can be detected, the *sum of active and potentially active* microorganisms is estimated by the plate count without determining the currently active microbial fraction.

Analyzing the time of colony formation revealed that "fast growing" forms (according to the definitions, the potentially active microorganisms required 11–19 h for the colony to appear) were mainly copiotrophic, Gram(+) and spore-forming (Kasahara and Hattori, 1991). Potentially active bacteria have larger cell sizes (4–7 μm), but their cell number is commonly 2.5–5 times lower than that of slow-growing or dormant bacteria (colony appearance after 33–63 h), where Gram(−) oligotrophs prevailed (Table 1).

**Table 1**
Relative contribution (in % of total) of potentially active, active, dormant and dead microorganisms to total microbial biomass in soils and sediments as determined by microbial cultures and direct microscopy with cell staining.

| Methods/staining dyes | Object | Pot. active | Active | Dormant | Dead | Reference |
|---|---|---|---|---|---|---|
| **Non-activated soils** | | | | | | |
| Acridine orange (total), CTC (active), micro-colony formation | Field soil under barley | 4–11 | 2–6 | 83–94 | | Winding et al., 1994 |
| CTC (active), propidium iodide (PI) (dead) | Microbial inoculate; initial | 5 | <1 | 90 | 4 | Maraha et al., 2004 |
| CTC (active), PI (dead) | Microbial inoculate; starvation | | <1 | 23 | 77 | Maraha et al., 2004 |
| Specific inhibitors + staining: SYBR green, PI and acridine orange | Marine sediments | 6–11 | 0.3–4 | 26–30 | 70–74 | Luna et al., 2002 |
| Frequency of dividing cells | Soil 0–10 cm | | 3.43 | | | Bloem et al., 1992a |
| | 10–25 cm | | 3.42 | | | |
| Autoradiography | Bulk soil fungi | | 0.8–0.9 | | | Bååth, 1988 |
| **Average for non-activated soils** | | **7.4%** | **1.9%** | **42%** | **56%** | |
| **Soils activated with litter or glucose** | | | | | | |
| *Fungal*: calcofluor-FB28 (total), | Bulk soil[a] | | 2.5–14 | 86 | | Busse et al., 2009 |
| FDA (active) | Mineral fraction[a] | | 10–26 | 74–90 | | Busse et al., 2009 |
| | Coarse organics[a] | | 3–6 | 94–97 | | Busse et al., 2009 |
| CTC (active), PI (dead) | Microbial inoculate activated | 30 | 4–87 | 58–73 | 25–54 | Maraha et al., 2004 |
| Autoradiography | Bulk soil bacteria[b] | | 56–72 | | | Ramsay, 1984 |
| Frequency of dividing cells | Bulk soil | | 10 | | | Bloem et al., 1992b |
| | Optim. moisture | | 16–23 | | | |
| | Drying-rewetting | | | | | |
| **Average for activated soils** | | **30%** | **25%** | **66%** | **40%** | |
| **Non-activated soils without separation of active and potentially active** | | | | | | |
| Colony forming curve | Grassland soil | 16 | | 84 | | Kasahara and Hattori, 1991 |
| Colony forming curve | Wetland rice field | 30 | | 70 | | Hashimoto and Hattori, 1989 |
| Combination of FISH with DAPI staining | Bulk soil | 5–10 | | 90 | | Christensen et al., 1999 |
| Combination of FISH with DAPI staining | Bacteria extracted from soil | 58 | | 42 | | Barra Caracciolo et al., 2005 |
| Combination of FISH with DAPI staining | Bacteria extracted from soil | 55 | | 45 | | Bertaux et al., 2007 |
| Combination of FISH with DAPI staining | Bulk soil | 41–47 | | 53–59 | | Zarda et al., 1997 |
| Direct microscopy with INT reduction | Bulk soil | 49 | | 51 | | Norton and Firestone, 1991 |
| | Bacteria | 52 | | 48 | | |
| | Fungi | 48 | | 52 | | |
| Direct microscopy with INT reduction | Rhizosphere | 55 | | 45 | | Norton and Firestone, 1991 |
| | Bacteria | 68 | | 72 | | |
| | Fungi | 51 | | 49 | | |
| Direct microscopy with CTC (active) and DTAF (total) staining | Bulk subsoils | 10–40 | | | | Bhupathiraju et al., 1999 |
| | 1.9 m | 10–30 | | | | |
| | 2.3 m | | | | | |
| Direct microscopy with CTC (active) and DTAF (total) staining | Contaminated subsoils | 45–65 | | | | Bhupathiraju et al., 1999 |
| | 1.9 m | 39–66 | | | | |
| | 2.3 m | | | | | |
| **Average for non-activated soils** | | **40%** | | **56%** | | |

[a] Soil amended with forest litter.
[b] Soil amended with glucose.

### 3.2. Direct microscopy combined with cell staining

Fluorescent microscopy allows distinguishing total and active cells by use of complementary stains. Dyes that bind to the cell components such as nucleic acids (acridine orange, SYBR Green I, 4,6-diamidino-2-phenylindole [DAPI], Europium chelate), proteins (fluorescein iso-thiocyanate – [FITC]) or polysaccharides of cell walls (phenol aniline blue, phenolic tryptophan blue, 5-4,6-dichlorotriazinyl aminofluoroscein – [DTAF]) can cross intact cell membranes. These dyes are sensitive to cells in an active, dormant, and even dead state. Another group of dyes binding to the nucleic acids (propidium iodide [PI] and ethidium bromide) are unable to penetrate membranes and cannot stain living cells. These dyes are commonly used to identify dead, membrane-destructed cells (Busse et al., 2009; Luna et al., 2002). Dead but intact cells, however, are not necessarily stained with propidium iodide (Maraha et al., 2004; Busse et al., 2009), calling for caution when interpreting unstained cells as being dormant. Such caution is necessary when the active cells are not directly stained. The application of dual staining (e.g., with DAPI and PI) to estimate active biomass by subtraction of dead cells (PI) from the total population (DAPI) is misleading because dormant microorganisms are also stained with DAPI. Neglecting the pool of dormant cells can overestimate the active biomass. Thus, an unexpectedly high fraction of active bacterial biomass comprising most of the total bacterial population (Table 1) was assessed by subtracting compromised cells stained with PI from total bacterial biomass stained with SYBR Green dye (Busse et al., 2009). In the same study, direct staining of active and total fungal biomass with fluorescein diacetate [FDA] and calcofluor-FB28 brightener, respectively, revealed a much lower biomass of active fungal hyphae (1.5–10% of total fungal biomass). Applying complementary dyes and correctly interpreting staining results (considering efficiency of staining for various dyes under soil conditions, Bölter et al., 2006) enable an estimation of the dormant pool by subtracting active and dead cell pools from the total bacterial biomass.

Living microbial cells can be directly stained with dyes that reveal microbial functioning: FDA, 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) or 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride [INT] (Nannipieri et al., 2003; Maraha et al., 2004). The fluorescence after applying such dyes corresponds to metabolic activity in cells, e.g. conversion of non-fluorescent FDA by into the green fluorescent compound, or reduction of CTC or INT by actively respiring bacteria into the red fluorescent formazan.

The frequency of cell division is determined by direct microscopy simultaneously with counting. It requires no incubation, and is applicable both for assessment of the actively growing fraction of total microbial biomass and as an index of *in situ* bacterial growth rate in soil (Bloem et al., 1992a). The fraction of active bacteria determined by the frequency of cell division was less than 3.5% of the total bacterial biomass and increased up to 10 and 23% under continuously wet or drying-rewetting conditions (Bloem et al., 1992b).

The combination of direct count with autoradiography after utilization of radioactive substrates (e.g., $^{3}$H- or $^{14}$C-glucose, $^{14}CH_4$) allows for the detection of metabolically active bacteria (Ramsay, 1984), fungi (Bååth, 1988), or microbial functional groups, e.g., methanotrophs (Stiehl-Braun et al., 2011). Less than 1% of fungi demonstrated metabolic activity after the addition of a trace amount of $^{14}$C-glucose (Table 1); while the fraction of FDA-responsive fungi was 2–4 times larger (Bååth, 1988). Certain underestimation of active fungi can occur due to internal hyphae anatomy segregated by septal pores (Heaton et al., 2012). Thus, viable fungal hyphae contain large sections that may look empty. Because empty parts of viable mycelium do not show metabolic activity they are usually considered as dormant (Waid et al., 1971; Söderström, 1979). Contrary to low fraction of active fungal biomass, more than 70% of bacteria were metabolically active after the application of $^{3}$H-glucose (Ramsay, 1984).

Applying CTC and INT staining to microbial cells extracted from soil revealed a remarkably large percentage (up to 40–55%) of active microorganisms both in the rhizosphere and in root-free soil (Table 1). Because the CTC/INT staining procedure takes 4 h, potentially active microorganisms can also contribute to the microbial fraction with active electron transport. Moreover, a physiological state of soil microorganisms may be altered dramatically during their extraction from soil. Thus, application of techniques on extracted cells may not be valid for determining microbial activity *in situ*.

The percentage of total bacteria that were CTC/INT-positive varied in top- and subsoil samples from 10 to 49%. However, in the soils with high microbial activity (e.g., in rhizosphere, Cheng, 2009), the range of potentially active microorganisms increased to 39–66% (Bhupathiraju et al., 1999; Norton and Firestone, 1991). Specifically, the contribution of INT-active fungi was similar, while the percentage of active bacteria was 30% higher in the rhizosphere than in the bulk soil (Norton and Firestone, 1991).

The application of direct microscopy to the bacterial inocula (Maraha et al., 2004) or microscopy in combination with specific inhibition (Luna et al., 2002) showed that the addition of easily available substrates to the soil increased the active microbial fraction by 11–25% of the total microbial biomass, while under starvation the fraction of active microorganisms did not exceed 1–5% (Table 1). Accordingly, most of the actively growing cultivated cells lost their activity and changed their physiological state under starvation.

### 3.3. Methods based on cell size of active microorganisms

There are various attempts to relate the metabolism and cell size of the active fraction of the soil microbial community: combination of culture-independent approaches—direct microscopy with viability stains (Norton and Firestone, 1991), flow cytometry (Maraha et al., 2004), estimation of ribosome-rich bacteria (Christensen et al., 1999) by the FISH technique (see below), extraction of viable cell compartments (ATP, PLFA, RNA), and assessment of metabolic activity (respiration, and enzyme activity). They revealed that a decrease in active microbial biomass does not necessarily indicate a decrease in a number of active cells (Elliott et al., 1983). A decrease in the size of active cells to smaller than 0.5 μm in diameter (Christensen et al., 1999) and subsequent increase in population heterogeneity (Maraha et al., 2004) illustrates the ability of microorganisms to change cell morphology but keep an active state under nutrient limitation. Furthermore, the size of active cells in a liquid culture—cell volume >0.18 μm$^3$ (Christensen et al., 1995); >0.3 μm$^3$ (Norton and Firestone, 1991) can strongly differ from that *in situ* due to the fraction of bacterial and archaeal cells of ultra-micro size comprising up to 75% of the total cell population in soil (as reviewed by Panikov, 2005). The small cell size of nanobacteria indicates either a starvation with reduced metabolic activity and slower growth rates (De Fede and Sexstone, 2001), which increased when the organisms grew at high substrate concentrations (Lleo et al., 1998), or the category of intrinsic dwarf bacteria of a permanently small size (Rutz and Kieft, 2004) able to grow fast—generation time of 6 h (Iizuka et al., 1998). The relative domination of one of these physiological categories of nanoforms determines their contribution to total microbial activity. In a community with 15% of small cells the ultrasmall bacteria contributed only 0.1% of the total soil DNA pool, and only 2% to total respiration (Panikov, 2005). Thus, physiology of nanoforms needs to be considered for estimation of their contribution to active microbial biomass.

Fungal hyphae usually vary between 2 and 20 μm in diameter (Thorn and Lynch, 2007) with only few exceptions (<10% of total fungal biomass) thinner than 0.4 μm (Panikov, 2005). Therefore, there are no fragments of fungal mycelium in fraction of nanosized cells. About 20 times decrease in metabolically-active fungal length was observed between topsoil (20–120 m g$^{-1}$) and subsoil- (0.6–1.6 m g$^{-1}$) (Bååth, 1988).

### 3.4. Molecular approaches and estimations of cell components

#### 3.4.1. DNA and RNA content

DNA is universally present in both active and inactive microorganisms (Levy-Booth et al., 2007). Therefore, quantitative extraction of microbial DNA from soil (Marstorp et al., 2000; Blagodatskaya et al., 2003) is a measure of total microbial biomass (Joergensen and Emmerling, 2006; Renella et al., 2006). An active microbial state can be revealed: 1) by the DNA increase during cell growth (at the DNA replication level) or 2) by the increase in RNA content during protein synthesis (at the gene-expression level). In DNA and RNA studies it is important to obtain quantitative DNA/RNA extraction. Both quality and quantity of NA are strongly dependent on the extraction procedure, in which a combination of mechanical, chemical and enzymatic disruption of microbial cell walls is usually enclosed (Bakken and Frostegård, 2006). Therefore, a unifying procedure of cell lysis and NA extraction is required to compare the results of different studies (Nannipieri et al., 2012). Most efficient mechanical cell destruction was attained by multi-size beads beating at 6 m s$^{-1}$ for 45 s although inevitable DNA shearing occurred under such treatment (Bakken and Frostegård, 2006). As high molecular weight is not obligatory necessary for quantitative NA determination the standardized beads beating can be suggested for efficient destruction of both fragile and robust cells to improve NA recovery from soil.

The DNA content increased simultaneously with the growth-related microbial respiration (Marstorp and Witter, 1999) or after few hours of delay (Blagodatskaya et al., 2003). During transition from active growth to starvation, however, the high DNA level was maintained for about one week longer than the decrease of microbial respiration (Anderson and Martens, 2013). This indicates that the reduced metabolism during the switch from the active to the potentially active state is not accompanied by an immediate decrease in DNA. Thus, *DNA dynamics* mirror the changes in

microbial biomass during growth. Considered alone however, the DNA content failed to distinguish the active from the potentially active states.

At the level of gene expression, the tracking of the active state of microorganisms by the *increase* in mRNA content during transcription, or by the high level of rRNA content during translation is hampered by their fast decomposition during extraction from soil (Musat et al., 2012). Therefore, techniques that do not require nucleic acid extraction (e.g. fluorescence *in situ* hybridization, FISH) are preferable for distinguishing active microorganisms within the microbial community. Combining FISH with staining of total microbial biomass with dyes having a high affinity to DNA (DAPI or acridine orange) facilitates the recognition of active microorganisms.

The number of active bacteria ($3.8–4.8 \cdot 10^8$ cells $g^{-1}$ soil, FISH) in soil activated by rewetting or by glucose addition composed 5–10% of the total bacterial counts ($5–9 \cdot 10^9$ cells $g^{-1}$ soil) determined by DAPI staining (Christensen et al., 1999). Very large (40–75%) contribution of active to total amount of microorganisms detected in several studies (Table 1) needs to be interpreted with caution. Overestimation of active microbial fraction can occur due to high sensitivity of FISH which enables detection of rRNA in resting cells despite rRNA content is 2–3 times lower in resting than in growing cells (Zarda et al., 1997). Another artifact common in soils is the unspecific binding of the oligonucleotide probes to soil organic matter causing unspecific fluorescence and resulting in overestimation of active biomass by FISH. Accordingly, not the absolute fluorescence signal but the *relative increase* in ribosomal RNA content indicated directly the active microorganisms (Christensen et al., 1999).

The FISH approach is often used to improve the resolution of other techniques. Coupling FISH with microautoradiography (FISH-MAR) enables the phylogenetic classification of single active cells (Nielsen and Nielsen, 2005; Rogers et al., 2007; Wagner, 2010). Combining FISH with Raman spectroscopy or with secondary ion mass spectrometry (nanoSIMS) enables the observation of single-cell metabolic activity (Chandra et al., 2008; Tourna et al., 2011) and the distribution of microorganisms in micro-habitats (Herrmann et al., 2007). It also helps to monitor the incorporation of isotope-labeled ($^{14}C$, $^{3}H$) substrates in biomolecules such as nucleic acids, proteins, carbohydrates, and lipids within living microbial cells (Huang et al., 2007). In most studies, however, the modern single-cell techniques were applied to analyze metabolic functions of cells in pure cultures (reviewed by Musat et al., 2012).

The combination of FISH with $^{14}C$-labeled substrates and analysis of $^{14}C$ incorporation by microautoradiography identifies active consumers and simultaneously quantifies their contribution to total microbial community. Thus, 5% of the bacteria (associated with the phyla *Betaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria)* were able to degrade $^{14}C$ labeled naphthalene, whereas phenanthrene-degrading microorganisms did not exceed 1% of the total microbial community (Rogers et al., 2007). Similarly, the bacteria degrading simazine represented approximately 5% of the total population (Martínez-Inigo et al., 2010). Application of FISH allowed relating the fraction of active microorganisms to certain functions that revealed that half of the detected archaea were able to oxidize ammonia, while the ammonia-oxidizing bacteria made up only 4% of the total bacterial community (Pratscher et al., 2011). In some cases the intensity of certain processes (e.g., methane uptake) could be directly related to the amount of active bacteria able to perform the specific process. Thus, the 14.5% reduction in $CH_4$ uptake rates under elevated $CO_2$ was explained by the 54% decrease of methanotrophic bacteria as revealed by FISH (Kolb et al., 2005). The application of the FISH technique to polyethylene terephtalate films buried in soil was employed to directly monitor the changes in microbial communities developing in soil microhabitats (Moshynets et al., 2011). Such an approach is very promising for *in situ* investigations of the spatial distribution of active microorganisms, including their growth, competition, and plant–microbial interactions.

### 3.4.2. Microarray analysis

The active taxa of the microbial community (with high level of mRNA and rRNA) can be distinguished and quantified simultaneously at the level of gene expression by microarrays after RNA extraction from soil (Poulsen et al., 1993).

The fluorescence signal in the microarray assay is sensitive to distinguish the target genes, e.g., the fluorescence intensity differed between the tested probes in the order of magnitude (Pathak et al., 2011; Urbanova et al., 2011). Despite this sensitivity, quantitative application of the microarray technique is restricted by the low RNA yield from soil, by nonspecific fluorescence, by uncertainties in fluorescence calibration, and by humic acid coextraction (Wang et al., 2011). Due to such difficulties, the microarray analyses primarily focused on the qualitative characteristics of interactions of active phylotypes within microbial community (He et al., 2012). Microarray-based study detected a 25-fold increase in the number of active bacterial taxa, corresponding to a five-fold increase in the richness of active bacterial phyla as a result of switch from the dormant to active state induced by sugars and organic acids (Shi et al., 2011). Linkage between size of active microbial taxa and variation in abundances of functional and phylogenetic genes remains a challenge (Wakelin et al., 2013). Therefore, the microarray applications have still not reached their potential in soil microbial ecology and remain to be developed for evaluation of active microorganisms. Further studies coupling microarray (e.g., PhyloChip) with 16s rRNA pyrosequencing can be used for quantitative estimation of microbial groups and their contribution to the total microbial community, revealing active members. A high-density phylogenetic microarray PhyloChip analysis (Brodie et al., 2006) revealed phylogenically distinct groups with rapid (15 min–1 h), intermediate (1–3 h), and delayed (3–72 h) activation strategies in rewetted soils (Placella et al., 2012). This corresponds to our estimation of the response of active, potentially active, and dormant microorganisms to the substrate addition (Fig. 1).

Microarray assay represents a group of techniques based on known gene sequences. An application of such techniques is benefiting for tracing interactions between certain microbial groups (see e.g. Section 3.4.3.3. for qPCR). However, most soil microorganisms are unknown (Anderson, 2003) and often less than 50% of the amino acid sequences can be identified by databases (reviewed by Nannipieri et al., 2012). Thus, we still have an open question: do the techniques based on known genes representatively reflect the activity of a whole soil genomics? (see also Section 3.5).

### 3.4.3. PCR-based approaches

Another group of methods is based on DNA/RNA extraction from soil, RNA reverse transcription to cDNA, amplification by PCR and subsequent fingerprinting or profiling of the microbial community by denaturing gradient gel electrophoresis (DGGE/TGGE), or by terminal restriction fragment length polymorphism (T-RFLP) analyses.

*3.4.3.1. Comparison of DNA and RNA sequences.* Total community composition determined by DNA-based fingerprint does not correspond to its functional activity even if it was applied to 16S/18S rRNA gene sequences. There was no link between rRNA phylogeny (gene sequence) and microbial metabolic activities (Jaspers and Overmann, 2004; Enwall et al., 2007), illustrating that gene existence does not indicate its activity (Nannipieri, 2006).

Community profiling based on direct RNA extraction reflects the metabolically active microorganisms (Anderson and Parkin, 2007). Sequences of bands from 18S rDNA and 18S rRNA T-RFLP/DGGE profiles exhibited significant differences in the total (DNA) and active (RNA) fungal community composition (Bastias et al., 2007). The dominance of certain fungal species (related to *Blastocladiomycota*, *Catenomyces*, *Basidiomycota*, and *Agaricomycetes* phylum) and the reduction of other fungal species (related to *Ascomycota*) caused by long-term devegetation were revealed by 18S rDNA/rRNA DGGE profile analyses (Ros et al., 2009). Nonetheless, the similarity often observed between DNA and RNA profiles (e.g. Pennanen et al., 2004; Anderson et al., 2008; Ros et al., 2009) indicates that the slow metabolism of potentially active microorganisms and even RNA co-extracted from dormant cells also contribute to the RNA profiles. Because DNA/RNA profiling allows only phylogenetic characteristics of active microorganisms (LeBaron et al., 2001), its application along with complementary methods to distinguish the quantities of active groups remain a challenge (Mijangos et al., 2009).

*3.4.3.2. Estimation of total microbial biomass by real-time PCR analysis: uncertainties for estimation of active microorganisms.* Evaluating the active microbial pool requires quantifying total microbial biomass in the same units. This prerequisite is also valid for the application of nucleic-acids-based technologies. Quantification of total fungal and bacterial/archaeal communities by taxon-specific 18S rRNA and 16S rRNA primers, respectively, are based on real-time PCR analysis (qPCR and RT-PCR). This approach produces the gene copy numbers in relation to the size of the targeted microbial group.

Assessing microbial C based on gene copy numbers remains problematic because of the absence of studies comparing the amount of microbial C (determined by basic methods such as FE or SIR) to the bacterial and fungal 16S/18S rRNA gene copy numbers. Because at least one gene copy represents either a bacterial or fungal cell, the quantity of the microbial biomass can be roughly estimated by the number of gene copies using the conversion factor for microbial C. Such estimations are valid for certain microbial groups, e.g., for the nitrate-reducing and denitrifying bacteria, ammonia-oxidizing archaea, which have a relatively small variation: one to three targeted gene copies (e.g., narG, nirK, nirS, nosZ and amoA) per cell (Hallin et al., 2009). However, total bacterial biomass can be strongly overestimated by calculations based on 16S rRNA gene-copy numbers, because the amount of ribosomal gene copies per prokaryotic cell commonly varies from 1 to 15 (Hallin et al., 2009). Nonetheless, the qPCR results are consistent with alternative biomass measurements (Rousk et al., 2010–a). Thus, the maximal bacterial biomass calculated by qPCR assays assuming one gene copy per cell and 20 fg C per cell (Bååth, 1994) varied from 62 to 700 μg C $g^{-1}$ in similar soils (Table 2). The first three examples in Table 2 show realistic total microbial C corresponding to 1–3% of $C_{org}$ content considering that the contribution of fungal biomass is equal to or even exceeds the bacterial biomass. Bacterial biomass content >6% of $C_{org}$ (last example in Table 2) indicates the occurrence of taxa, in which more than one gene copy represents one bacterial cell. Thus, for partitioning active and dormant microbial pools, the estimation of total microbial biomass based on gene copy numbers can be refined by simultaneously determining microbial C, e.g. by fumigation–extraction method and applying the qPCR to the large set of soils.

*3.4.3.3. Shift in phylogenic domination as indicator of active microbial taxa.* The qPCR is based on the DNA extraction from soil or from microbial cultures and on quantifying the targeted rRNA genes. It therefore estimates total microbial biomass. Even though qPCR does not reflect microbial activity directly, the qPCR-detected changes in phylogenic or functional groups allow for comparison of their relative abundance. As active biomass is a part of total biomass a strong shift in phylogenic domination of total microbial community is indirect indication of corresponding phylogenic shift in its active fraction. Due to lack of experimental confirmations, however, it remains still not clear whether strong increase in the contribution of certain phyla to bacterial community (e.g. two- to three-fold increase of *Bacteriodetes* and *Actinobacteria* after land use change) indicates their larger activity (Philippot et al., 2009; Wessén et al., 2010). The unexpectedly large number of gene copies of acidophilic *Acidithiobacillus ferrooxidans* revealed the occurrence of acidic microniches in neutral postmining soils (Urbanova et al., 2011) but an activity state of *A. ferrooxidans* needs experimental proof. The qPCR-based comparison of fungal and bacterial abundance along a soil pH gradient revealed that three major groups (*Ascomycetes*, *Basidiomycetes* and *Chytridiomycetes*) governed fungal community independently of pH (Rousk et al., 2010–a). In contrast, the bacterial community composition was strongly altered by pH: the *α-Proteobacteria* and the *Acidobacteria* dominated in the high- and low-pH soils, respectively (Rousk et al., 2010-a). However, it remains unclear whether the PCR-revealed phylogenic domination is directly related to the functionally active portion of microbial community. Thus, the contribution of functional groups responsible for the activity at the community level (e.g., ammonia oxidation, nitrate reduction, and denitrification) composed from 0.03–4–14% of total bacterial biomass. This was considerably lower than the contribution of dominating phyla varying from 12 to 40% (Hallin et al., 2009). The quantitative PCR of the 16S rRNA genes did not reveal the changes in the bacterial community caused by reduced N deposition despite the increase in substrate-induced respiration and the decrease in nitrate reductase activity (Kandeler et al., 2009). The dynamics of active fungal and bacterial communities were revealed using growth-based approaches, but the estimations based on qPCR assays did not detect changes in bacterial or fungal gene copy numbers (cf. Rousk et al., 2009; Rousk et al., 2010-a). The next challenge in assessing active microorganisms is therefore to estimate the physiologically active microbial cells along with the qPCR analyses. Indeed, combining the PCR-based T-RFLP approach with FISH representing active microorganisms (see Section 3.4.1.) revealed that the contribution of archaea exceeded 10% of the active anaerobic cellulolytics (Chin et al., 1999). This is considerably larger than the proportion of archaea in the total prokaryotic community (0.3–3%) estimated by specific primers (Wessén et al., 2010).

Another method to distinguish between the active and dormant microorganisms is the application of qPCR simultaneously to the DNA and RNA extracted from soil. The qPCR assay based on DNA extraction estimates the amount of genes responsible for ribosome production (i.e. rDNA copy numbers = rRNA gene copy numbers).

**Table 2**
Example calculations of total bacterial biomass based on qPCR assays of the 16S rRNA (assuming one gene copy and 20 fg C per cell; see explanations in Section 3.4.3.2.) in comparison with the range of total microbial C content in soil (calculated as 1–3% of organic C content).

| Soil | pH | C content | Range of total microbial C | Bacterial biomass | Reference |
|---|---|---|---|---|---|
| | | mg C $g^{-1}$ soil | μg C $g^{-1}$ soil | μg C $g^{-1}$ soil | |
| Eutric Arenosol | 7.2 | 12.7 | 127–381 | 62 | Djigal et al., 2010 |
| Eutric Cambisol | 5.6 | 12.2 | 122–366 | 200 | Hallin et al., 2009 |
| Stagnic Luvisol | 6.8 | 15.5 | 155–465 | 160 | Marhan et al., 2011 |
| Eutric Cambisol | 6.2 | 11.0 | 110–330 | 700 | Wessén et al., 2010 |

Abundant genes mean abundant ribosomes that can be potentially produced per time unit. Nonetheless, the actual number of ribosomes (which can be estimated by qPCR of RNA, i.e. rRNA copy numbers) also depends on transcription activity. Thus, larger amounts of rRNA- versus rDNA-copy numbers indicate ribosome production by active microorganisms. Low amounts of rRNA- at large rDNA-copy numbers correspond to the dormant state (no ribosome production). However, the thresholds for the rRNA- versus rDNA-copy numbers are not quantified yet. This calls for studies on DNA/RNA qPCR assays to quantitatively assess the portion of active microorganisms in soil.

*3.4.4. ATP*

ATP is a component of living cells that is decomposed by microorganisms within several hours after its release into the soil (Conklin and Macgregor, 1972). The ATP content in microbial cells depends on the growth phase, cell size, species and activity. For example, in the senescent phase of *Escherichia coli*, ATP amounts to $3.5 \cdot 10^{-10}$ μg per cell (Wildish et al., 1979), and it varies in bacterial cells between 0.2 and $7 \cdot 10^{-10}$ μg per cell (Velten et al., 2007). This gives ATP potential as an index of microbial activity. Unexpectedly, most studies failed to distinguish an activity state of soil microorganisms. This provoked us to discuss methodological uncertainties of ATP determination as a possible cause of low sensitivity of ATP to microbial activity state in soil.

*3.4.4.1. Why ATP content does not reflect active biomass?* Although the ATP content in microbial cells varies by more than one order of magnitude, surprisingly stable mean values (10–12 μM $g^{-1}$ MBC) were obtained for a broad range of soils independently of soil type, geography, and management (Jenkinson, 1988; Contin et al., 2001). This reflects the ATP determination procedure, which was originally developed as a measure of *total* microbial biomass, considering that most microorganisms in soil are dormant (Jenkinson and Ladd, 1981). Soil preconditioning for 1–2 weeks at optimal temperature and moisture was recommended before ATP determination to avoid microbial activation during sample preparation (Oades and Jenkinson, 1979; Ciardi and Nannipieri, 1990). As

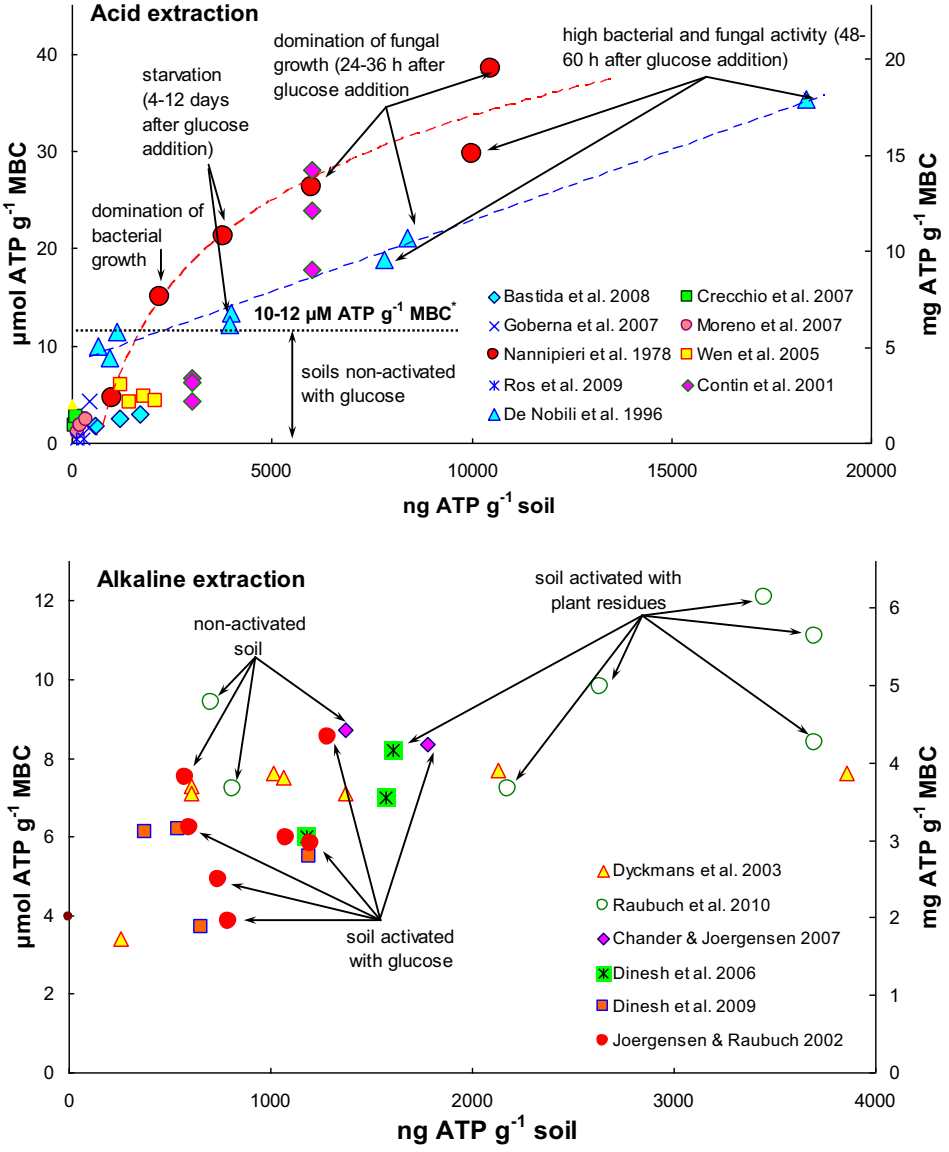



**Fig. 2.** Changes in ATP content determined by acid (top) and alkaline (bottom) extraction in activated and non-activated soils. Individual points present the values from the studies referred in the legend.

available substrates are quickly consumed during such pre-conditioning, the microorganisms are mainly inactive, and consequently the ATP content does not truly reflect the active part.

Another uncertainty of ATP-based estimations of the active microorganisms is the absence of a unified procedure for ATP extraction from soil. Both acid and alkaline extractions have been used. Higher ATP contents and higher sensitivity to disturbances were always observed for acid (Jenkinson, 1988; Nannipieri et al., 1978; De Nobili et al., 1996; Contin et al., 2001) versus alkaline (Dyckmans et al., 2003; Chander and Joergensen, 2007; Raubuch et al., 2010) extractions (Fig. 2, compare top and bottom). High recovery of added ATP was usually obtained both in acid and alkaline extractions (Martens, 1992; Joergensen and Raubuch, 2002). The possible cause for discrepancies in the ATP content between studies could be the incomplete cell lysis during the disruption procedure. Adding cell lysing agents such as paraquat dichloride (Oades and Jenkinson, 1979; De Nobili et al., 1996) or chloroform (Nannipieri et al., 1978) increased ATP extraction for 2–5 times as compared with the phosphoric acid and dimethylsulphoxide DMSO (Webster et al., 1984; Ciardi and Nannipieri, 1990).

Because of 2 weeks pre-incubation the significant effect of rhizosphere soil (Crecchio et al., 2007), vegetation removal (Ros et al., 2009), plant canopy (Goberna et al., 2007), and atrazine applications (Moreno et al., 2007) on ATP content can be interpreted as changes in *total* microbial biomass (Fig. 2, top). The relatively low absolute ATP content found in these studies prohibits linking it with the active portion of microorganisms because of pre-incubation. In the absence of pre-incubation, distinct effects of sewage sludge and compost on the specific ATP content was observed despite low ATP extraction (Bastida et al., 2008). The application of quick, deep freezing and thawing, and beads beating, commonly used for microbial cell destruction (Section 4.1; Bakken and Frostegård, 2006), can increase the yield and sensitivity of ATP determination as an index of active microbial part.

*3.4.4.2. ATP content in dormant and active microorganisms.* ATP amounts below 1–2 μg $g^{-1}$ soil and 5–10 μmol $g^{-1}$ MBC correspond to the non-activated soil microbial community (Fig. 2, top). An ATP content exceeding the threshold of 2 μg $g^{-1}$ soil and 12–15 μmol $g^{-1}$ MBC indicates active microorganisms. According to the detailed study of Nannipieri et al. (1978), the ATP content in microbial cells strongly increased after activation with glucose: from 4 to 5 μmol $g^{-1}$ MBC in the dormant cells to 11–20 μmol $g^{-1}$ MBC during domination of bacterial growth (12–24 h), and further up to 20–40 μmol $g^{-1}$ MBC when fungal growth contributed to substrate decomposition (Fig. 2, top). Activation of heterotrophic microorganisms with glucose caused a much larger increase in ATP content, by a factor of 3–8 (De Nobili et al., 1996; Nannipieri et al., 1978), compared with the 25–50% increase in the ATP-to-biomass ratio observed during decomposition of lower available plant residues (Fig. 2, bottom). After substrate exhaustion, the very high ATP content in microbial cells (16–30 μmol $g^{-1}$ MBC) was maintained in the starving (~5 days after glucose addition) and even in the resting microbial community (12–22 days after glucose addition). This indicates the ability of microorganisms to maintain the potentially active state over long periods.

Changes in microbial physiology caused by a transition from dormancy to growth are much stronger than the changes in the microbial biomass content (Table 3). The biomass increased by a factor of 1.6 in the soil amended with glucose, whereas an 8.4-fold increase in ATP content was observed (Nannipieri et al., 1978). This corresponds to the 15-fold higher ATP content in newly formed (active) cells than in the initial (mostly dormant) microbial community (Table 3). In contrast, the reverse shift from activity to dormancy caused a much stronger reduction of microbial biomass (2.6 times) compared with ATP (1.3 times) (Table 3). Thus, the ATP increase could serve as an appropriate indicator of active microbial biomass, but it is less sensitive to trace the transition of microorganisms from growth to dormancy.

**Table 3**
Microbial biomass and ATP content expressed on a soil dry weight and MB basis. Data extracted from Nannipieri et al. (1978).

| Physiological state of microorganisms | Mainly dormant (after prolonged storage) | Activated (30–40 h after glucose, N and P addition) | Active (biomass increment) | 3 weeks starvation after activation |
|---|---|---|---|---|
| Microbial biomass, mg DW $g^{-1}$ soil | 0.8 | 1.3 | 0.5 | 0.5 |
| ATP, μg ATP $g^{-1}$ soil | 1 | 10 | 9 | 4 |
| ATP/MB, μM ATP $g^{-1}$ MB | 2.3 | 19.2 | 35.5 | 14.7 |

In summary, the ATP content in soils determined after pre-incubation indicates mainly dormant microorganisms. Direct estimations of ATP in fresh soil samples and comparative studies on changes in the ATP and biomass contents involving a) inhibition of ATP-degrading enzymes; b) complete cells lysis; c) avoiding the adsorption of released ATP molecules on the surface reactive particles (Nannipieri et al., 2003) by addition of inorganic phosphate (Contin et al., 2002) are necessary to distinguish the active and potentially active portions of microorganisms.

*3.4.4.3. Adenylate energy charge.* The energetic status of soil microorganisms determined by adenylate energy charge (AEC): $(ATP + 0.5 \cdot ADP)/(AMP + ADP + ATP)$ was suggested as a promising indicator of active microbial biomass (Chapman et al., 1971; Brookes et al., 1983). For pure microbial cultures, AEC values of 0.80–0.95 corresponded to microbial growth, whereas values of 0.5–0.75 represented the microbial stationary phase, which could be interpreted as the potentially active stage of *in situ* microorganisms (Atkinson, 1977; Martens, 1985). The dormant, senescent and dying microbial cells *in vitro* cultures were characterized by AEC values below 0.5.

The AEC values determined directly in soil (Brookes et al., 1983; Joergensen and Raubuch, 2002; Raubuch et al., 2002, 2010) were always unexpectedly high (0.78–0.85). This underlines that even non-growing and potentially active microorganisms maintain a high energetic status in terms of being able to react rapidly to a 'food event' (De Nobili et al., 2001). The high *in situ* AEC values independently confirm the intensive metabolism of the 25–30% potentially active fraction of the microbial community observed by direct counts (Maraha et al., 2004). They also correspond to the strong respiratory increase observed after substrate input. Unfavorable climatic conditions (e.g. drought) markedly reduced the AEC value (Ciardi et al., 1993) despite the AEC was insensitive to amendments with glucose (Joergensen and Raubuch, 2002) or with plant residues (Raubuch et al., 2010). This makes the applicability of AEC ambiguous as a quantitative indicator of the active microbial state in soil. However, a high cumulative potentially active + active state of soil microorganisms can be distinguished from the dormant state if the AEC value exceeds 0.75.

*3.4.5. PLFA content*

*3.4.5.1. Do the differences in PLFA content indicate the active microorganisms?* Phospholipid fatty acids (PLFA) are found only in living cells and thus are characteristic biomarkers for living microorganisms (Zelles, 1999; Kramer and Gleixner, 2006; Denef et al., 2009). PLFA composition reflects the ecophysiological groups of microbial community but does not indicate microbial activity.

However, comparison of the PLFA content in non-activated and in growing microorganisms is an option to get a link with the microbial activity state. Assuming the PLFA content of 65 μmol $g^{-1}$ microbial biomass (Bååth and Anderson, 2003), the PLFA compose in average 1.74% of total biomass. This content is not uniform however. The contribution of fungal versus bacterial PLFA to total PLFA is smaller because of the higher surface-to-volume ratio of smaller bacterial cells compared to fungal hyphae. So, the PLFA content does not exceed 0.5% of fungal biomass (assuming the conversion factors of 42 nmol PLFA $\mu g^{-1}$ fungal C [Joergensen and Wichern, 2008] and of 15 μmol PLFA $g^{-1}$ fungal biomass [Frostegård and Bååth, 1996]).

The PLFA content is two times larger in G(−) versus G(+) bacteria considering additional phospholipic bilayers in the outer membrane of G(−) bacteria cells. Evidence of larger PLFA content (two versus one phospholipid layer) in the cell membranes of Gram(−) than of Gram(+) bacteria is usually ignored by data interpretation. Thus, the larger $^{13}C$ enrichment in Gram(−) versus Gram(+) PLFA biomarkers after $^{13}CO_2$ pulse-labeling (Denef et al., 2009) does not necessarily indicate higher biomass of active Gram(−) bacteria.

Furthermore, the PLFA content in laboratory cultures is not suitable for extrapolation to soil conditions: the PLFA content in a growing culture of Gram(−) bacteria *Phyllobacterium myrsinacearum* composed <0.1% of cell DW, i.e., was 50–90 times smaller (!) than in bacterial cells extracted from soil (Table 4). This can be due to acclimation to harsh environmental conditions such as varying substrate availability, temperature, pH, mechanical pressure and drought (Rousk et al., 2010b). Consequently, microbial cells in soil are smaller than in pure cultures (Kieft, 2000); their higher surface-to-volume ratio leads to a higher cell membrane content and higher PLFA content per cell. Thus, PLFA content *per se* is not indicative of activity state; however, the changes in PLFA content after soil disturbance has a potential to distinguish dormant, activated, and growing microorganisms (discussed in the section 3.4.5.2).

*3.4.5.2. Increase in PLFA content: application for estimation of active microbial biomass.* Increase in PLFA content as a response to soil treatment has a potential to indicate changes in active microbial biomass. Similar to higher ATP content in active versus dormant cells (see Section 3.4.4.2.), the PLFA increase in activated cells should be considered to avoid overestimating the amount of active biomass using common conversion factors. Assuming the up to 40% differences in PLFA content between active and dormant bacterial cells (Table 4) (Ehlers et al., 2010), the 2.5-fold increase in PLFA (from 30 to 75 nmol PLFA $g^{-1}$ soil) corresponded to only a 1.75-fold increase in active biomass. Consequently, the increase in absolute PLFA content (<40%) in activated soil reflects the switch of dormant microorganisms to active state and not necessarily microbial growth. This was confirmed by adding small amounts of $^{13}C$-labeled glucose (15–83 μg C $g^{-1}$ soil) resulting in a 35% increase in PLFA content, independent on the added glucose amount (Dungait et al., 2011). This indicates activation (a switch from dormancy to activity) of the microbial community rather than exponential microbial growth (cell multiplication). The PLFA increase indicates an increase in cell-membrane content and is also related to an increase in mitochondrion number in active cells. Thus, the conversion factors for absolute PLFA content in active microbial biomass, still require experimental proof.

The sensitivity of PLFA as an indicator of the active microbial biomass is hampered by the PLFAs originating from non-active cells or humic-acid-derived fatty acids (Nielsen and Petersen, 2000). A relatively high, about 30–40% background of such 'non-indicative' PLFA (Fig. 1 in Rousk et al., 2010-b) explains the very small and often insignificant differences in PLFA content between activated and non-activated soils. So, the dynamics of bacterial PLFA in soil after plant residue addition did not correspond to the leucine uptake or to the dynamics of the leucine-to-thymidine incorporation ratio (see below), which reflects metabolically active microorganisms (Rousk and Bååth, 2007). A 10- and 30-fold increase in substrate-induced respiration and in the fungal:bacterial growth ratio corresponded only to a 2- to 3-fold increase in total and in bacterial/fungal PLFAs (Rousk et al., 2009, 2010-b). Thus, relative changes in PLFA may be useful in comparing the dynamics of soil microbial communities in terrestrial ecosystems. However, extrapolation of such observations for total microbial biomass changes remains problematic.

We conclude that an *increase* in PLFA content of <40% indicates potential microbial activity, whereas the PLFA increment >40% is necessary to confirm an actively growing state. Such a rough threshold needs to be further approved and refined experimentally.

Although microbial activation can be revealed by an increase of some cell compounds (ATP, PLFA), this does not necessarily indicate microbial growth. However, with such a "small" increase (below threshold, see e.g. Fig. 1), it is useful to trace the switch from "dormant" to "potentially active" or to "active" state, especially in combination with molecular techniques.

*3.4.6. Stable isotope probing and compound-specific $^{13}C$ analysis*

Microbial activation and substrate utilization can be revealed by combining microbial biomarkers with the stable isotope probing (SIP) approach and with compound specific isotopic analysis (CSIA). Both approaches refer to microbial activity only if they are coupled with $^{13}C$ (or $^{15}N$) labeling (Paterson et al., 2009). The fate of $^{13}C$ from labeled plant residues, rhizodeposits or specific organic compounds added to soil can be traced in DNA/RNA, PLFA, and other biomarkers to identify the microorganisms metabolizing the added substrates (Glaser, 2005; Amelung et al., 2008). By DNA/RNA-SIP, the active

**Table 4**
PLFA content in microbial cells in a pure culture of *Phyllobacterium myrsinacearum* and in microbial cells extracted from soil by centrifugation with a nonionic density gradient medium in comparison to the bacterial PLFA content in soil.

| PLFA/microbial cells content | *P. myrsin-acearum* | Bacterial PLFA extracted from 15 soils | Bacterial cells extracted from soil | | Relative change by activation |
|---|---|---|---|---|---|
| | | | Non-activated | Activated soil | |
| μmol PLFA $g^{-1}$ cell mass | 3.7 | 521–537 | 155 | 222 | 1.43 |
| amol PLFA $cell^{-1}$ | 0.17 | 14 | 7 | 9–11 | 1.43 |
| PLFA, of cell mass | 0.09 | 8.3 | 4.2 | 5.9 | 1.4 |
| nmol PLFA $g^{-1}$ soil | | 16.4–174 | 30 | 75 | 2.5 |
| Cells $g^{-1}$ soil | | $0.7–7.2 \cdot 10^9$ | $4.3 \cdot 10^9$ | $7.5 \cdot 10^9$ | 1.74 |
| Source | Zelles, 1999 | Frostegård and Bååth, 1996 | Ehlers et al., 2010 | | |

Calculations are based on data from Zelles (1999) and Ehlers et al. (2010), assuming a C content in bacterial cells of 20 fg C $cell^{-1}$ (Bååth, 1994), 48 C in bacterial biomass (Christensen et al., 1995) and a molar mass of PLFA between 267 and 298 g $mol^{-1}$.

microorganisms utilizing the $^{13}C$-labeled substrate are separated physically in density gradient (Radajewski et al., 2000). Further identification of active microorganisms incorporated the substrate is possible by PCR and gene sequencing. The sampling by stable isotope probing should be done in short periods to avoid cross metabolism and sequential utilization of labeled metabolites. Heavy $^{13}C$ enrichment of DNA/RNA for at least several $^{13}C$ atom-percent excess necessary for the DNA/RNA-SIP approach can be reached only by artificial labeling. Thus, decomposition of $^{13}C$-labeled wheat residues was revealed by $^{13}C$ incorporated in DNA and RNA (Bernard et al., 2007). The RNA $^{13}C$ enrichment as well as the different diversity of sequences of light and heavy RNA indicated that, even during intensive decomposition, not all active microorganisms were involved in wheat residue utilization.

In contrast to DNA/RNA-SIP, the $^{13}C$ enrichment in PLFA may remain on a very low level (few per mil ‰). Despite small changes in total PLFA content, the higher contribution of $^{13}C$ in specific PLFAs indicated the presence of microbial groups active in the rhizosphere (Denef et al., 2009; Tian et al., 2013). The $^{13}C$-PLFA showed that Gram(+) bacteria assimilated $^{13}C$ more actively in the rhizosphere than in root-free soil (Denef et al., 2009). In the rhizosphere, however, Gram(−) bacteria most actively assimilated root-derived C, while Gram(+) bacteria were less successful in such assimilation (Butler et al., 2003; Treonis et al., 2004; Lu et al., 2007; Tian et al., 2013). Furthermore, the high activity of both saprophytic and AMF fungi revealed by PLFA in competition for root-derived C (Denef et al., 2009) indicates the importance of fungal functional diversity in C turnover in the rhizosphere. Similarly to other molecular biomarkers, the PLFA serve as qualitative rather than quantitative indicators of microbial activity specific to the $^{13}C$-labeled substrate.

### 3.5. Enzymes as indicators of physiological state of soil microorganisms

The active state of soil microorganisms can be recognized by their ability for enzyme production. Enzymes can be studied in soil at the level of specific genes (genomics), by protein production (proteomics), or by decomposition products (metabolomics) (Burns et al., 2013). The enzymes newly produced by active microorganisms are not the sole source of the extracellular enzymes because immobilized enzymes also contribute to total enzyme activity in soil and hamper determination of enzyme production (Nannipieri, 2006). Therefore, there are intensive discussions as to what extent soil ***genomics***, i.e., diversity and expression of coding genes, reflect the actual metabolic diversity and the corresponding enzyme production (Burns and Dick, 2002; Burns et al., 2013). Again, as a gene's presence *per se* does not necessarily reflect its activity (Nannipieri, 2006), there is no positive relationship between gene diversity and enzyme production. Lower gene diversity due to the domination of certain microbial groups can even boost enzyme activity (Metcalfe et al., 2002). The functioning of a single gene can result in the simultaneous syntheses of isoenzymes catalyzing the same reactions but with different rates (Graves and Haystead, 2002; Khalili et al., 2011). Characterization of proteins synthesized during gene expression – analyzed by two-dimensional gel electrophoresis in combination with mass-spectrometry – is very promising in environmental ***proteomics*** (Nannipieri and Paul, 2009). Nonetheless, the quantitative characterization of enzymes by soil proteomics is limited due to the poor efficiency of protein extraction from soil (Nannipieri et al., 2012). Accordingly, small amounts of extracted proteins do not necessarily indicate low enzyme activity (Masciandaro et al., 2008; Giagnoni et al., 2011). Thus, the active state of soil microorganisms can be revealed by enzymes production at the level of gene expression (e.g., by mRNA) but it cannot be directly related to the rates of enzyme-mediated reactions (Nannipieri, 2006; Nannipieri and Paul, 2009), which are dependent on the C availability in soil microhabitats (Acosta-Martinez et al., 2003; Geisseler and Horwath, 2009). At high C availability enzyme activity depends on the maximal reaction rate ($V_{max}$). Under substrate limitation common for soils, the apparent $K_m$ (as a result of simultaneous activity of multiple enzymes from both active and "stabilized" pools) is a preferable indicator of enzymes properties (German et al., 2011).

It is still not clear whether the pools of stabilized and newly produced enzymes can be distinguished by their reaction rates. Furthermore, how the short delay in enzyme activity as a response to substrate addition indicates the switch of microorganisms from a dormant to an active state remains to be studied.

The increase of hydrolytic enzymes' production usually begins only within 24–48 h after substrate input (Allison and Vitousek, 2005) or even later (e.g., casein-hydrolyzing protease, Nannipieri et al., 1983) and remains high during 2–14 days (Renella et al., 2007) and up to 2–4 months (Nannipieri et al., 1983). In certain cases, however, a long-term delay in enzyme production indicates either 1) dormant physiological state of microorganisms responsible for enzymes production and temporal sequence in microbial succession (Nannipieri et al., 1983) or 2) the catabolic repression of enzyme synthesis by product excess. Thus, the addition of inorganic P repressed phosphatase activity (Nannipieri et al., 1978); the activity of β-glucosidase (produced glucose monomer units breaking cellulose chains) was repressed during the 3–5 days after glucose addition (Renella et al., 2007). After substrate exhaustion, however, the β-glucosidase activity increased dramatically (up to three- to six-fold) and remained stable up to 40 days (Renella et al., 2007), demonstrating an active state of starving microorganisms.

Despite enzyme activity reflects the general microbial activity, it is not possible to estimate the portion of active microorganisms in soil based on this proxy either at the level of genomics, proteomics, or metabolomics.

### 3.6. Approaches based on respiration and substrate utilization

#### 3.6.1. The ratio basal-to-substrate induced respiration as indicator of physiological state of soil microorganisms

Microbial respiration is commonly used to quantify metabolic activity (Cederlund and Stenström, 2004; McIntyre et al., 2009; Chodak et al., 2009). Basal respiration, i.e., respiration without substrate addition is mainly driven by substrate availability, but it also depends on the physiological state and on microbial maintenance requirements (Anderson and Domsch, 1985; Insam and Haselwandter, 1989). Therefore, basal respiration can be considered as indication of integrated metabolic activity (Panikov, 2005) but not of active microbial biomass.

Addition of available C to soil at saturation concentrations causes a strong (five- to ten-fold) increase in the respiration rate (substrate induced respiration, SIR) and so, reveals the group of microorganisms utilizing the substrate (Anderson and Domsch, 1978). This group, however, is apparently not uniform and consists of two parts: 1) growing, or capable for immediate growth and 2) potentially active copiotrophic and oligotrophic microorganisms, which can switch from a dormant to active stage within a few hours (Placella et al., 2012). The ratio between basal and SIR respiration ($Q_R = V_{basal}/V_{SIR}$) is restricted to the interval between 0 and 1 and indicates the respiratory ratio between growing and potentially active microorganisms. $Q_R$ values near zero correspond to the very low basal respiration, i.e., to the reduced actual heterotrophic activity (Blagodatskaya et al., 1996; Anan'eva et al., 2002) (Table 5). Such a situation can occur in soil after the long-term absence of available substrates, e.g. long-term fallow soil (Hirsch et al. 2009;

**Table 5**
Effect of substrate availability on respiration parameters and contribution of actual and potential respiratory activity to $Q_R$ values (see explanations in text) reflecting the physiological state of soil microorganisms.

| Substrate availability | $V_{basal}$ | $V_{SIR}$ | $Q_R$ | Physiological state of microorganisms |
|---|---|---|---|---|
| Extremely low | ≈ 0 | High | < 0.1 | A \| Potentially active \| Dormant \| Dead |
| Low | Low | High | 0.1-0.2 | Active \| Pot.active \| Dormant \| Dead |
| High | High | ≈ $V_{basal}$ | 0.6-0.8 | Active \| Dormant \| Dead |
| Stress conditions | Low | ≈ $V_{basal}$ | 0.8-1 | A \| Dormant \| Dead |

The data are extracted from Hund and Schenk (1994); Wardle and Ghani (1995); Blagodatskaya et al. (1996, 2006, 2008); Blagodatskaya and Anderson (1999); Anan'eva et al. (2002).

Barre et al., 2010) or under extreme drought or prolonged freezing when microorganisms exhaust a particular substrate pool, and thus, strongly reduce the respiration. Because most soils have some available C (Allison, 2006) the $Q_R$ –values near zero are seldom and they indicate environmental stress.

In contrast, a $Q_R$ approaching 1 reflecting the absence of an increase in respiratory response to substrate addition, i.e., the absence of potentially active microorganisms, can occur in two contrasting situations. First, in soils enriched with plant residues, manures, or in the rhizosphere, i.e., with an ample amount of energy-rich substrate (Table 5) most of the microbial community is active rather than dormant (Hund and Schenk, 1994). Such cases were observed in a few studies in which a large fraction of the active microbial cells was detected by FISH or by direct counts (see Sections 3.2 and 3.4.1. above). Second, it can occur in soils in which potentially active microorganisms are strongly suppressed by some stress factors, e.g., acidification (Blagodatskaya and Anderson, 1999), heavy metal contamination (Blagodatskaya et al., 2006, 2008), soil heating (Wardle and Ghani, 1995) or for soils under permanent long-term starvation/conservation, e.g., buried soils (Blagodatskaya et al., 2003). In the second situation, most of the microbial community is dormant (Table 5). Most common under natural conditions are the $Q_R$ values 0.1–0.2 revealing the contribution of active and potentially active microorganisms to soil respiration (Hund and Schenk, 1994; Wardle et al., 1999). Note, however, that the respiratory activity and not the fraction of active biomass can be evaluated by the $Q_R$ values because of maintenance respiration which contributes disproportionally to SIR and basal respiration.

### 3.6.2. *Substrate induced respiration – measure of active or of total biomass?*

SIR is the respiration of active glucose-responsive microorganisms. However, the rate of substrate-induced $CO_2$ production is converted to total microbial biomass by the conversion factor 40.04 calculated by Anderson and Domsch (1978) from the correlation between SIR and fumigation techniques. So, SIR represents the total microbial biomass (fumigation is a measure of total microbial biomass) (Jenkinson, 1988). To avoid misinterpretations, the SIR rates without conversion to SIR biomass are used to characterize the active fraction (Fierer and Schimel, 2003; Kandeler et al., 2009). Note here that application of SIR along with the selective inhibitors of bacterial and fungal protein synthesis enable the determination of the fungal-to-bacterial respiratory ratio (Busse et al., 2009) but cannot be interpreted as a measure of the glucose-active fungal and bacterial biomass.

We conclude that SIR reflects the respiration of active and potentially active microorganisms in soil if conversion factor is not considered. For that matter the soil should be not pre-incubated (see comments to pre-incubation for ATP analysis in Section 3.4.4.1). Further studies on the conversion factor from SIR to the portion of active biomass based on modern approaches (e.g., RNA + FISH + DAPI – see Fig. 5) can help to make the SIR-method into a suitable tool for estimating the active part of the microbial community.

### 3.6.3. *Microbial growth rates as characteristics of active microorganisms in soil*

Growth rates of heterotrophic microorganisms are estimated by two main approaches. First, the addition of trace amounts of labeled biomolecules corresponding to their content in soil ($^{14}$C-sugars, $^{14}$C-($^{15}$N)-amino acids, $^{14}$C-acetate, $^{14}$C-leucine or $^{3}$H-thymidine) and tracing their direct incorporation in cell components, which does not alter microbial growth rates. Second, is the addition of large amounts of substrates that induce unlimited exponential growth. These two approaches are complementary and simulate 1) steady-state conditions for microbial biomass at equilibrium between growth and death, or 2) non steady-state conditions when microbial growth exceeds death, causing an increase in microbial biomass. An incorporation of trace substrate amounts reflects real microbial growth under limitation but is usually applied either to soil slurry (Rousk and Bååth, 2007) or to the suspension of bacterial cells extracted from soils (Bååth, 2001). The second approach (substrate-induced growth) estimates the universal microbial parameter—maximal specific growth rate ($\mu_m$) *in situ* after applying substrate directly to the soil. Note, that its modifications allow determination of growth parameters of various physiological groups, e.g. autotrophic microorganisms as well (Panikov, 1995). The differences in $\mu_m$ between soils correspond to the physiology of active microorganisms indicating dominating growth strategies and intensities of microbial turnover.

*3.6.3.1. Microbial growth under substrate limitation.* The range of bacterial growth rates from 0.003 to 0.02 $h^{-1}$was determined by $^{14}$C-leucine incorporation (calculations based on Bååth, 1998). Large bacteria (>1 μm) grew faster ($\mu = 0.01\ h^{-1}$) than did the small cells <0.4 μm ($\mu = 0.001\ h^{-1}$; as determined by $^{3}$H-thymidine incorporation, calculations based on Bååth, 1994). The fungal growth rates calculated by $^{14}$C-acetate incorporation in ergosterol (which is the measure of total fungal biomass) were much slower, 0.0003 and 0.0014 $h^{-1}$for unamended and substrate-amended soil, respectively (Rousk and Bååth, 2007). Fungal growth can be underestimated, because of indirect calculations: the $^{14}$C incorporation was related to the total amount of fungal biomass determined by the ergosterol content. Considering that the actively growing fungal biomass was about 1% of the total biomass in soil (Bååth, 1988, Table 1), the value 0.028 $h^{-1}$ equals fungal growth (Rousk and Bååth, 2007). These fungal growth rates correspond with those of

bacteria under substrate limitation (Rousk and Bååth, 2007; Rousk and Nadkarni, 2009).

*3.6.3.2. Substrate induced growth respiration (SIGR)—estimation of active biomass, growth rates and microbial turnover.* Despite the heterogeneity of soil microbial community, the pattern of respiratory response to glucose addition used for SIGR estimation is uniform and is similar to the exponential increase of biomass in pure batch cultures. The microbial growth on added substrate allows for estimates of the: 1) period before growth (lag-time), 2) exponential growth phase, and 3) the decrease of respiration after substrate exhaustion. The models describing the kinetics of substrate-induced respiration consider either the exponential part of the respiratory curve only (Schmidt, 1992) or both the exponential part and the part corresponding to the lag-period (Panikov, 1995; Stenström et al., 1998). It is considered that the lag-period is a function of the physiological state of soil microorganisms and is inversely proportional to the maximum specific growth rate (Baranyi and Pin, 1999; Blagodatskaya et al., 2007). The latter model allows estimation of *the growing fraction of microorganisms in native soil* (before glucose addition), whereas the fraction of total biomass can be interpreted as the portion of potentially active microorganisms able to grow on the added substrate (Wutzler et al., 2012). As total SIGR-biomass is usually for 30–50% lower than SIR-biomass (Blagodatsky et al., 2000) the fraction of glucose-utilizing microorganisms amounts to 50–70% of a whole microbial community. However, the interpretations should be done with caution for activated microbial communities considering that actively growing microorganisms respire more $CO_2$–C per mass unit than sustaining microorganisms (Anderson and Domsch, 1978). Thus, instead of theoretical value the direct determination of yield factor, $Y_{CO_2}$, is required for correct estimation the fraction of glucose-utilizing microbial biomass in soil (Blagodatsky et al., 2000). Estimations based on the SIGR approach showed that 0.2–0.6% of the total microbial biomass in un-amended soils are growing (Blagodatsky et al., 2000, 2010; Blagodatskaya et al., 2009). This corresponds to the estimations based on direct counting (Maraha et al., 2004; Luna et al., 2002) and on autoradiography (Bååth, 1988) (Table 1).

The absolute values of microbial growth rates depend strongly on the calculation method: they increase from the linear approaches (e.g., the log-transformed respiration rate plotted against time during exponential increase) through simple exponential models to the model considering both the lag and exponential growth phases (Panikov, 1995). Thus, applying Panikov's model to the data set of Ehlers et al. (2010) yielded a $\mu_m$ value of 0.15 $h^{-1}$, which was twice as high as $\mu = 0.072$ $h^{-1}$ calculated by the linear approach in the original study. The maximal specific growth rates on glucose at 22 °C usually vary in soil between 0.10 and 0.35 $h^{-1}$ (Blagodatskaya et al., 2009); they exceed the growth rates under various limitations: by a factor of 2–7 at limitation by low temperature ($\mu = 0.03$–$0.05$ $h^{-1}$) (Lipson et al., 2009); by the factor of 2–4 under drying-rewetting ($\mu = 0.04$–$0.08$ $h^{-1}$ as determined by the frequency of dividing cells (Bloem et al., 1992-a, 1992-b, see Section 3.2) or by a factor of 10–50 at trace amounts of substrate (Bååth, 1998; Rousk and Bååth, 2007, see Section 3.4.3.1.). Correspondingly, depending on growth conditions, the generation (doubling) time of active microorganisms can vary from several hours (no limitations) to 10 days for non-cultivable forms (Winding et al., 1994) or up to 6–22 days for growth at temperatures below zero (Panikov and Sizova, 2007).

*3.6.3.3. How to increase the fraction of active biomass?.* As substrate is a main driver of microbial activity in soil, it is reasonable to know how the portion of active microorganisms depends on the amount

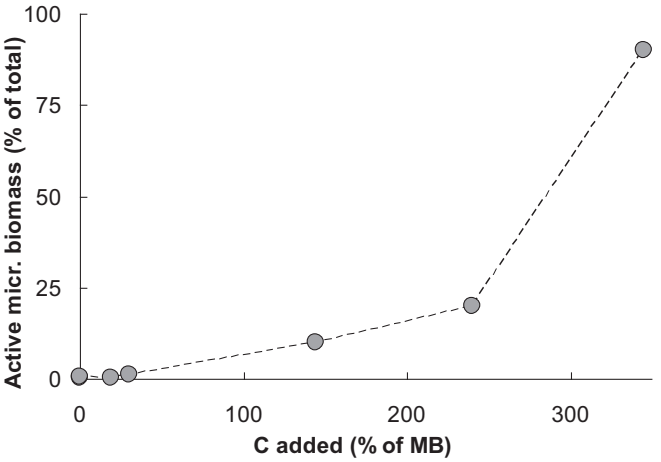



**Fig. 3.** Percentage of microbial biomass able to utilize glucose within 24–48 h after adding increasing glucose amounts (from Blagodatsky et al., 2000; Blagodatskaya et al., 2007, 2009).

of available substrate. The activation of microbial biomass with a glucose amount 5 times lower than MBC did not increase the active fraction at all (Fig. 3). A strong increase in active biomass up to 10% of the total was observed after adding a glucose amount comparable (*ca.* 1.5 times higher) with MBC. When the added glucose was enough for unlimited exponential growth (exceeded MBC by a factor of 2.5–3.5), almost the entire microbial community (90–100%) able to utilize glucose was active within two days (Blagodatsky et al., 2000; Blagodatskaya et al., 2007, 2009). Growth on substrates of low availability requires longer time for activation of large fraction of microorganisms. Thus, increase of fraction of active fungi to 97% was detected by autoradiography during 21 days of litter decomposition (Waid et al., 1973). So, adding available C in amounts higher than 2.5-fold of microbial C causes transfer of most soil microorganisms to transfer from potentially active and dormant stages to an active stage within a few days (in the absence of other limitations). This highlights the role of potentially active microorganisms in utilization of fluctuating amounts of available soil organics. Despite the transition from the potentially active to the active state occurs within minutes to hours, such transition occurs only under favorable conditions and as a response to substrate input (Marschner et al., 2008; Garcia-Pausas and Paterson, 2010). Under common nutrients limitation only small pool of active microorganisms participates in the C turnover. Therefore, the calculation of real turnover rates should be based on active biomass.

*3.6.4. Biolog and MicroResp*

Growth-related (instead of biomass-based) measurements referred to the community-level physiological profile (CLPP) determined by the Biolog Ecoplates system (Insam, 1997). The Biolog enables comparison of relative contribution of microorganisms decomposing various substrates to cumulative metabolic ability of the microbial community (Gomez et al., 2006; Mijangos et al., 2009). Despite its high potential to measure the absolute microbial growth rates (Insam, 1997), this approach remains mainly qualitative and yields only relative estimations of metabolic diversity in soil extracts (Degens et al., 2001; Girvan et al., 2004). The rates of substrate utilization correspond to the activity only of the selected microbial groups that are fast growing in the plates; they do not reflect the microbial growth *in situ* (Smalla et al., 1998; Ros et al., 2008; Chodak et al., 2009).

The shortcomings of Biolog are eliminated when dynamics of the substrate utilization are traced by microbial respiration directly

in soil without extraction (Degens and Harris, 1997), e.g. in the MicroResp system (Campbell et al., 2003, 2008). Thus, combination of the SIGR approach in Biolog and $^{14}C$-labeled substrates in the MicroResp system has potential for estimation of active microorganisms and their growth rates on various substrates (Williamson and Wardle, 2007).

## 4. Dynamics of parameters indicating changes in the physiological activity stage and microbial growth

Most of the approaches presented above showed that the portion of active microorganisms in soil is very small and is strongly dependent on the amount of easily available substrates (Fig. 3). Because the amount of available substrates in soil varies temporally and spatially by orders of magnitude, the changes of the physiological state of microorganisms are common for soil conditions. The transition of potentially active and dormant microorganisms to active state is accompanied by the sequence of processes: respiration increase, ATP and enzymes production, DNA, RNA and PLFA synthesis, and growth *per se*. However, these processes start at different transition stages and consequently the lag-phase described above for microbial growth (see SIGR approach) is not identical to the lag-phase of other activity parameters (Fig. 4). Furthermore, the duration of these transition stages varies for members of microbial community representing various activation strategies (Placella et al., 2012). Additionally, the increase of these parameters is different. There are very few studies reflecting the change of metabolic parameters by transition from dormant to active state (or back) under soil conditions. Most of the studies were focused on the changes during microbial growth. Therefore, the dynamics of the activity parameters described below reflect the transition not only from dormant to active stage but mainly during the growth.

The first indication of switch from dormant or potentially active to active state is an increase in respiration. The exponential increase in respiration reflects microbial growth. It starts usually within 4–12 h after substrate input (transition from potentially active to active stage) and its maximal intensity depends on the amount and availability of the substrate Respiration slows down very sharply after substrate exhaustion (Fig. 4).

An increase in the DNA content directly indicates cell propagation following accelerated respiration with a 2–5 h delay. After reaching the maximum of about three times higher than that in dormant cells, the high DNA content is maintained for several days or weeks despite the respiration decrease. In contrast, the RNA content quickly decreases up to 60% within few hours under starvation conditions (Poulsen et al., 1993).

The maximal ATP production (8–10 times higher than in dormant cells) is delayed for 1–3 days after the respiratory peak (Nannipieri et al., 1978; Renella et al., 2007). Then the ATP production decreases more slowly than does the respiration and levels off at a several-fold higher content compared with the soil before substrate addition. The increased activity of most hydrolytic enzymes corresponds to the period of maximal microbial respiration. A decrease in microbial respiration, however, does not mean reduced hydrolytic enzyme activity. Most of the enzymes maintain high activity during one month after substrate input (Renella et al., 2007).

We conclude that the switch from dormancy to activity at substrate level sufficient to growth is relatively fast (several hours to several days), whereas the switch from activity to dormancy is very slow (several weeks to several months). Activated microorganisms maintain the potentially active state (high ATP production, PLFA content, and enzyme activity) despite the exhaustion of available substrates. This situation indicates that starvation after activation and/or substrate-induced growth increases the contribution of potentially active microorganisms to the total microbial community. Consequently, the portion of active microorganisms in soil is a function not only of the amount of available substrates (as presented in Fig. 3) but also of the period after the substrate exhaustion (Fig. 4). Surely, environmental and climatic conditions (e.g., soil moisture and temperature), as well as specific requirements of microbial populations (e.g. sulfur oxidizers or autotrophic nitrifies), etc. affect both the response of microorganisms

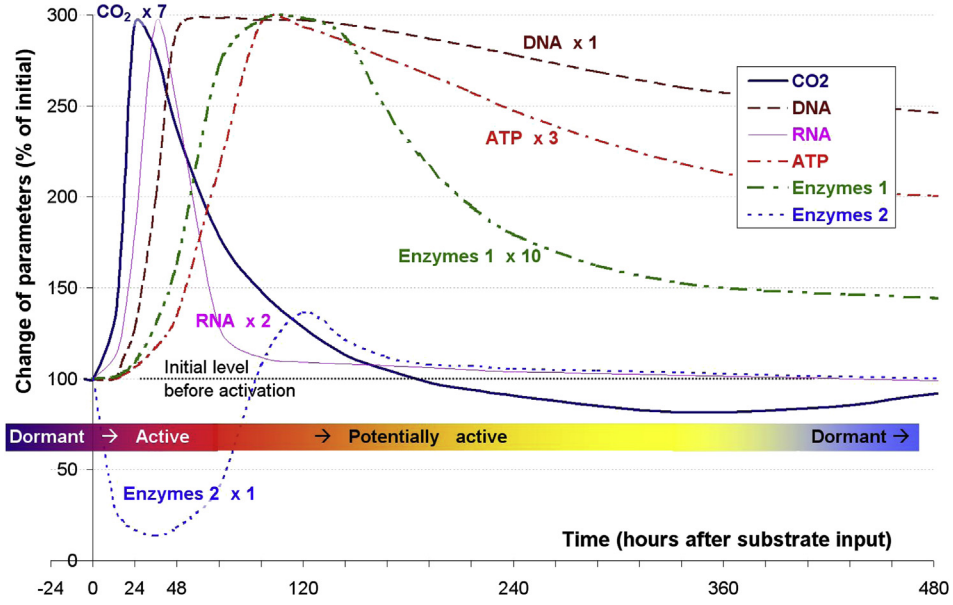
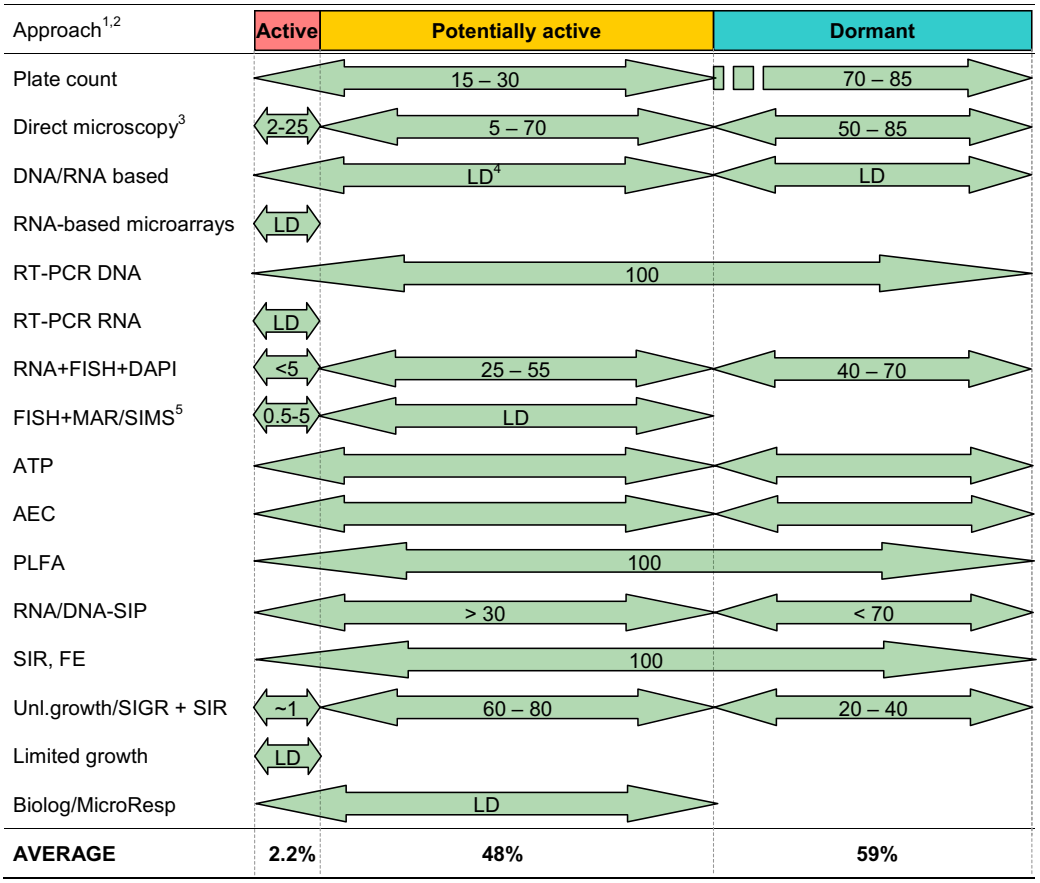



**Fig. 4.** Dynamics of microbial activity parameters during the transition of microorganisms from a dormant to active state after adding easily available substrate to soil. This conceptual diagram is based on the summary of results of studies included in the review (De Nobili et al., 1996; Blagodatsky et al., 2000, 2010; Blagodatskaya et al., 2009; Nannipieri et al., 1978; Poulsen et al., 1993; Marstorp and Witter, 1999; Blagodatskaya et al., 2003; Anderson and Martens, 2013; Allison and Vitousek, 2005; Nannipieri et al., 1983; Renella et al., 2007). Note the strongly different parameter scaling level shown by the multiplication number after the parameter label. Enzymes 1 represent the group of hydrolytic enzymes. Enzymes 2 represent catabolic repression of enzyme synthesis in case of product excess (e.g. glucose for β-glycosidase).

on the substrate input and the decline rate of parameters after substrate exhaustion. Because various parameters change with different rates, the methods analyzing these activity parameters show different portions of active microbial biomass in soil. Thus, the suggested concept of transition between dormant to active state and back through potentially active to dormant state (Fig. 4) needs further extended experimental analysis and generalization for spatial–temporal variation in the response times of parameters reflecting activity of specific microbial populations (aerobic vs. anaerobic, copiotrophic vs. oligotrophic, autotrophs vs. heterotrophs, etc.) with a diverse range of activation/growth rates.

## 5. Conclusions on method comparison and outlook

The importance of active microorganisms for all biogeochemical processes motivated us to prepare this review of approaches to




[1] Not all approaches described in the review are presented here, as some approaches (e.g. estimation of enzyme activities) cannot be directly related to the portion of active, potentially active and dormant microorganisms.

[2] The numbers on arrows reflect average estimations of percentage (%) of the portions of active, potentially active and dormant state of soil microbial biomass. These numbers are presented only for methods allowing quantitative estimation of the portions of active, potentially active and dormant state. The estimations by other approaches cannot be recalculated for the percentage of microorganisms in the three physiological stages. The percentage ranges are rounded up from Table 1, as well as from Rogers et al. (2007), Bernard et al. (2007).

[3] Direct microscopy in combination with various stains allows estimation of dead microbial biomass (not shown in the Fig. 5).

[4] These methods works in combination with application of substrates labeled by radioactive (MAR) or stable isotopes (SIMS).

Abbreviations: DNA: deoxyribonucleic acids, RNA: ribonucleic acids, RT-PCR: Real-time polymerase chain reaction, FISH: fluorescent in situ hybridization, DAPI: 4,6-diamidino-2-phenylindole, MAR: microautoradiography, SIMS: Secondary Ion Mass Spectrometry, ATP: adenosine triphosphate, AEC: adenylate energy charge, PLFA: phospholipids fatty acids, SIP: stable isotope probing, SIR: substrate induced respiration, FE: fumigation extraction, SIGR: substrate induced growth respiration. LD: limited data

**Fig. 5.** Suitability of various approaches for quantitative and qualitative analysis of the active, potentially active and dormant state of soil microbial biomass (modified from Blagodatsky et al., 2000).

estimate the active and potentially active parts of microbial communities. We show that despite the broad range of approaches developed to estimate the *content* of microbial biomass in soil, only a few have focused on its *active fraction*. Some of the approaches initially based on flux- or growth-related measurements (and not the content-related analyses of specific cell components) would be suitable for analyses of active microbial fraction. However, they were standardized to reflect total microbial biomass and consequently lost their focus on the activity state.

This review and the comparison of approaches underline the importance of *potentially active* microorganisms able to quickly switch to active state and exist in alertness for easily available substrates. Despite a very low portion of active microorganisms in soil (merely 0.1–2%), the potentially active microorganisms contribute to the extremely high spatial and temporal variation of microbial activity. This is a prerequisite for the major differences in process rates in such an extremely heterogeneous environment as soil. Furthermore, we do not really know whether these 0.1–2% of active microorganisms are common for the steady state of microbial community or whether they reflect the very small soil volume with high local content of available substrates—the hotspots, where an important part of potentially active and partly dormant microorganisms switched to the active state?

We evaluated the contributions of active, potentially active, and dormant microbial biomass in bulk soil using the data from studies referred to in corresponding chapters about individual approaches. The active microorganisms vary in bulk soil between 0.1 and 2% and do not exceed 5% of total microbial biomass (Fig. 5). The potentially active microorganisms compose between 10 and 60%.

We showed that none of the static methods—analyzing the content of C, N in microbial biomass or specific cell components and biomarkers—can indicate the active state of soil microorganisms. In contrast, approaches based on *changes* in the state of microorganisms after their activation by substrate addition (these approaches are termed here as dynamic approaches) do provide information about the active fraction. These approaches are based on *changes* in respiration or in the content of specific cell components (ATP, DNA, RNA, PLFA, etc.) after substrate addition.

To evaluate which of the approaches hold the most advantages and potential for further development, we compared their universality, i.e., an ability for quantitative and separate analyses of all three states of microorganisms: active, potentially active, and dormant (Fig. 5). This comparison clearly showed strong differences in the universality of the approaches. Three approaches allow separate estimation of active, potentially active, and dormant fractions: 1) direct microscopy combined with complementary stains, 2) a combination of RNA-based FISH with staining of total microbial biomass, and 3) approaches based on unlimited microbial growth (SIGR + SIR). Importantly, these approaches applied in dynamics allow quantitative estimation of the portions of microorganisms in the three physiological states.

Many approaches allow the estimation of two physiological states of microorganisms in soil (Fig. 5). They are suitable to evaluate the dormant state, but integrate the active and potentially active microorganisms. This urgently calls for further developing new approaches and standardizing the existing approaches (e.g. RNA-based microarrays, FISH + MAR/SIMS, RT-PCR RNA, see Fig. 5) for evaluating the portion of active microorganisms and their specifics for various functions. We assume that the major progress in the approaches allowing estimation of the three physiological stages should be based on combining methods. This effort should also be oriented toward dynamic approaches that are based on changes of microbial parameters in response to utilization of suitable substrates.

In conclusion, the evaluation of active microbial biomass and specific functional activities is an extremely important step in our understanding of biogeochemical processes and their biotic drivers. The total microbial biomass content is a static parameter that is insufficient to describe dynamic processes. Making the step from static to dynamic properties of soil and to evaluating process drivers requires estimating active and potentially active microbial biomass.

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