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REVIEW

Development of soil microbiology methods: from respirometry to molecular approaches

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Abstract This review deals with techniques and methods used in the study of the function and development of microorganisms occurring in soil with emphasis on the contributions of Czech Academician Ivan Málek and his coworkers or fellows (Jiří Macura, František Kunc) to the development of basic techniques used in soil microbiology. Early studies, including batch cultivation and respirometric techniques, as well as later developments of percolation and continuous-flow methods of cultivation of soil microorganisms are discussed. Recent developments in the application of analytical chemistry (HPLC or GC) and of molecular biological techniques to ecological questions that have revolutionized concepts in soil microbiology and microbial ecology are also briefly mentioned, including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), phospholipid fatty acid analysis (PLFA) and others. The shift of soil microbiology from the study of individual microorganisms to entire microbial communities, including nonculturable species, is briefly discussed.

Keywords Soil microbiology · Continuous cultivation · Microbial growth · Enzymes · Biomarkers

Introduction

Soil microbiology is a discipline that describes the numbers, activity, and interactions of microorganisms present in soil, and how they are affected by their environment or human activities. Understanding the function of soil ecosystems in relation to ever changing soil conditions is a key to understanding the basic mechanisms of soil productivity. Transformation of organic soil matter, and mineralization and immobilization of soil nutrients, are considered to be two of the most important factors influencing soil fertility. Scientific studies have focused attention on a variety of ways to enhance either soil fertility or plant nutrition [52, 168]. The presence of microorganisms in soil was observed in nineteenth century. Among others, G.G. Gustavson at the VIIIth Congress of Russian scientists and physicians in St. Petersburg said, that "the soil is heavily colonized by invisible and inaudible inhabitants eagerly working to the benefit of agriculture" [58]. Stoklasa in 1911 [150] was one of the first scientists who expressed the importance of considering soil as a complex and dynamic entirety. Vinogradsky in his lecture at the International Society of Soil Microbiology in Rome in 1924 emphasized the necessity of developing methods that would be specific to soil microbiology and that would permit investigations of soil microorganisms in their natural environment. He indicated the main features of this development, which he then focused on soil microflora, nitrogen fixation, nitrification, decomposition of cellulose, and the ecology of soil microorganisms [95]. Recent decades have seen considerable progress in soil microbiology. Biochemical and ecological approaches developed significantly, followed by the development of chemical and recent molecular biology techniques. First of all, attempts were made to simulate natural conditions and to obtain more direct evidence on the biochemical and ecological activities and functions of microorganisms in soil. This research has not only brought deeper insight into soil biology and microbiology but also has contributed to a better understanding of various interactions between biotic and

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abiotic components in soil. Also, establishing contact with other scientific disciplines appeared to be necessary for further development of soil microbiology.

Batch cultivations and respirometry

Early methods of studying soil microbial processes used single enrichment of the soil with an appropriate substrate and analyses of metabolic products that led to results characteristic for a closed system. Such methods of cultivation consisted in inoculation of microorganisms into a limited amount of a more or less defined nutrient medium or soil. As the cells begin to propagate (even in mixed cultures), they utilize elements of the medium according to different needs of their growth. In this method of cultivation, individual generations of the cell population grow under different and changing conditions. However, these first experiments enabled the researchers to develop techniques for measuring respiratory activity of soil microorganisms. These methods underwent substantial changes since the first works of Stoklasa [150, 151] and were used in many studies. Respiratory activity of soil was, from the beginning, connected mainly with soil fertility [136] or with the ability of soil microorganisms to decompose organic matter [122–124]. The use of respirometry until the 1960s was exhaustively reviewed by Domsch [28] and only some examples of the techniques used will be mentioned here. In general, these techniques can be divided into three major groups: measurement of carbon dioxide production, measurement of oxygen consumption, and determination of the respiratory quotient (RQ, a ratio of the volume of carbon dioxide produced to the volume of oxygen consumed in respiration over a period of time).

The measurement of carbon dioxide production was carried out mostly by determination of CO_2 absorbed in sodium hydroxide. Classic titration methods were described by Stotzky [152] and Holm-Jensen [65], conductometric detection by Wolf et al. [178] and others [47, 48, 167]. The infra-red analysis of gas was used by Zöttl [184–189]. Gravimetric determination of barium or strontium carbonates [22] or direct Warburg method [22, 76, 135] were also widely used. The latter method was used, among others, for the study of decomposition of aromatic compounds (*p*-hydroxybenzoic, quinic acids, vanillin and coumarin) added to soil by Kunc and Macura [78].

Methods based on the determination of oxygen consumption and RQ represent another important group. Drobník [29–32] and coworkers [165, 166] proposed a method based on measuring gas pressure changes. Attention was also paid to the use of electrolytic respirometers [9, 11, 12, 57, 111, 153, 165, 179]. Microrespirometric techniques related to the original Warburg method were also frequently used and modified for the purposes of soil microbiology [18, 19, 121, 135, 148], especially when working with larger amounts of soil [33, 115, 126, 152]. The determination of RQ was mostly based on the Warburg two-vessel technique [135] or its modifications. All methods had their limitations, especially when working with a soil with pH higher than 5. Thus, a number of modifications of the above techniques were described [16, 24, 41, 53, 112, 175, 178, 184–189]. In some cases the carbon dioxide formation in situ can bring useful information. Several modifications of respirometric techniques for field measurements were also described [13, 69, 91, 99]. Respirometry is still used and represents a useful method in field experiments (e.g. [1, 2, 15, 132]).

Percolation and continuous cultivation

According to Jansson [70] a soil may be considered to be a closed system only in laboratory experiments; under natural conditions when the presence of plants and/or other organisms brings about a continual periodic removal of nutrients from the soil and an enrichment of the soil with organic compounds, the soil as a whole has the character of an open system. As Waksman [174] pointed out, human activities influencing soil properties must be taken into account as well. The disadvantage of the classic method of soil microbiology was later overcome by using percolation techniques introduced by Schloesing and Müntz [140]. The apparatus developed by Audus [3] was later modified by Lees and others [23, 71, 83–87, 127, 144, 160] and in the 1980s, among others, by Goswami and Green [55], Stevens and Cornforth [147], Weeraratne [177] and Longden and Claridge [90]. Combination of soil percolation with the measurement of produced radioactive carbon dioxide was used in the first works dealing with the effect of glucose on decomposition of (plant) organic materials added to soil [154].

Continuous cultivation developed by Ivan Málek is the most effective method of growth limitation by substrate with simultaneous preservation of balanced exponential growth [17, 25]. In this process, a fresh medium is permanently supplied at a certain rate and the fermented medium containing the culture and metabolites flows out to the reservoir for further treatment. Although, at the beginning, scientists who used this method and developed its techniques represented “an almost completely closed scientific club” (according to Málek [100]), the method soon aroused considerable interest among microbiologists as it made possible the study of topics such as microbial physiology, biochemistry and genetics from new points of view and could also be applied to industrial fermentation processes (see Symposium on continuous cultivation, [155]). The use

of continuous cultivation of microorganisms in the 1960s and 1970s has been repeatedly and exhaustively reviewed by Málek and coworkers [101–103, 105–108], including original works, reviews (and patents) dealing with the theory of continuous cultivation (e.g. [39, 100]), laboratory cultivation methods, cultivation of cells of higher organisms, growth of pathogenic microorganisms and immunological properties of the culture, physiology of microorganisms and product formation, technology and equipment, industrial application of continuous cultivation methods, decontamination of industrial effluents, production of biomass and various metabolites, study of mutations and the use of continuous cultivation in soil microbiology and agriculture. These reviews by Málek cover hundreds of studies and it is possible to mention only a part of them in this article.

The first continuous cultivation apparatus used for the study of microbial processes in soil samples was described by Ivan Málek and Jiří Macura. The apparatus consisted of six cultivation units and a feeding mechanism. The stock solution inflow was regulated by electromagnetic screws that were controlled by a time switch [156]. This mechanism ensured a constant flow and permitted regulation of its rate. The cultivation units were almost identical with the percolation apparatus described earlier by Audus [3], adapted for continuous flow. The soil samples were placed in glass tubes closed at both ends with rubber stoppers. After passing through the soil, the solution was collected in separation funnels, enabling the researchers to further analyze the culture liquid. Aeration was ensured by a side-arm tube at the upper end of the cultivation tube. The introduced air was purified by removing carbon dioxide, and after passing through the soil, it was introduced into an absorption flask with a caustic soda solution. In this manner the amount of carbon dioxide produced could be estimated [93, 94, 104]. Unlike with the percolation technique, the solution passed through the soil and was not returned to circulation. One of the first applications was a detailed investigation of nitrification conditions and of the effect of various substances on the composition of soil microflora [94]. The results showed quite clearly that the method was of great theoretical significance, so it became widely applied in soil microbiology. Perfilev [128], who studied bacterial ecology in different soil layers, described a new continuous-flow, micro-cultivation apparatus where he could form gradients of different substances. His apparatus was also used later for studying the multiplication and development of bacterial cultures [50, 129].

Later, Macura and Kunc [96] studied the metabolism of glucose in soil. They found that continuous feeding with glucose affected the composition of the microbial population. Experiments with starch-containing medium led to findings showing that microbial population in the soil also contained a considerable amount of protozoa [59]; the ratio between protozoa and bacteria varied in dependence on the dilution rate. Worth mentioning, also, is the paper by Stewart et al. [149] on the use of continuous cultivation for studies of rumen microflora. Anaerobic decomposition of glucose continuously added to soil was described by Takai et al. [159]. In contrast to the conditions in waterlogged soil, in these experiments a nitrogen atmosphere was used. In this study, carbon dioxide, formic, acetic, butyric, and lactic acids were detected as the main products of anaerobic decomposition of glucose. In the case of glycine continuously added to the soil samples, the extent of glycine mineralization was related to the weight of soil. Glycine was nitrified most effectively in a soil sample, having a mass of 30 g where ca. 66% of the added glycine nitrogen was oxidized to nitrites and nitrates [98]. Biological immobilization of mineral forms of nitrogen and phosphorus in soil was studied by Macura and Kunc [97]. The amount of immobilized nitrogen and phosphorus was closely associated with glucose decomposition and carbon dioxide evolution. The ratio of the amount of glucose carbon assimilated by the soil microflora to the amount of nitrogen immobilized depended on the C:N ratio in the added solution. Heterocontinuous flow cultivation was also used to study degradation of xenobiotics. Vokounová et al. [172] studied degradation of the herbicide Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile [118, 170, 173], in soil in the presence of glucose or ribose. In chernozem soil inoculated with *Pseudomonas putida* 90 and 47% of the herbicide was degraded, respectively. In another work, degradation of 2-chlorobenzoic and 2,5-dichlorobenzoic acids by *Pseudomonas stutzeri* [77] was described. Other applications of heterocontinuous flow cultivation method in soil microbiology are reviewed in the work of Kunc [79]. The apparatus for both the percolation and continuous techniques used in the Institute of Microbiology ASCR, Prague are shown in Figs. 1 and 2 (Kuncostroj, according to Vokounová [171]). Other photos can be found, for instance, in works of Málek and Macura [104] or Longden and Claridge [90].

Measuring of soil enzyme activities

Instrumental tools that allowed rapid determination of enzyme activities contributed to the further development of soil microbiology. The techniques were based mostly on spectrophotometry, fluorescence, radiolabelling and gas- or high-pressure liquid chromatography. The sources of enzymes in soil may be microorganisms, plants or animals, both living and dead, from which enzymes can be released due to changes in membrane permeability or after cell decomposition. Soil enzymes are mostly of microbial origin and are closely related to microbial abundance and/or

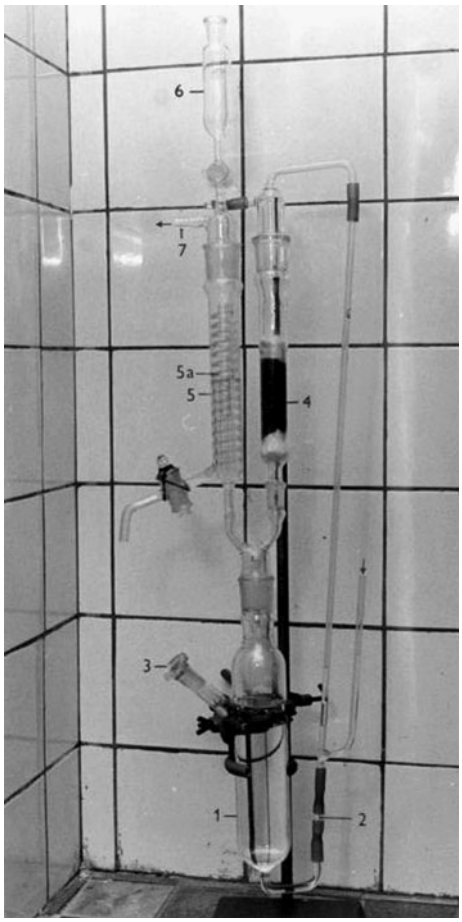

Fig. 1 Cultivation unit of a percolation apparatus. The apparatus contains: *1* reservoir with percolation solution *2* capillary tube *3* outlet for sampling of the percolation solution *4* soil sample *5* adsorption column with NaOH solution and (*5a*) glass spiral tube ensuring CO_2 retention *6* vessel for dosing NaOH and *7* vacuum pump outlet

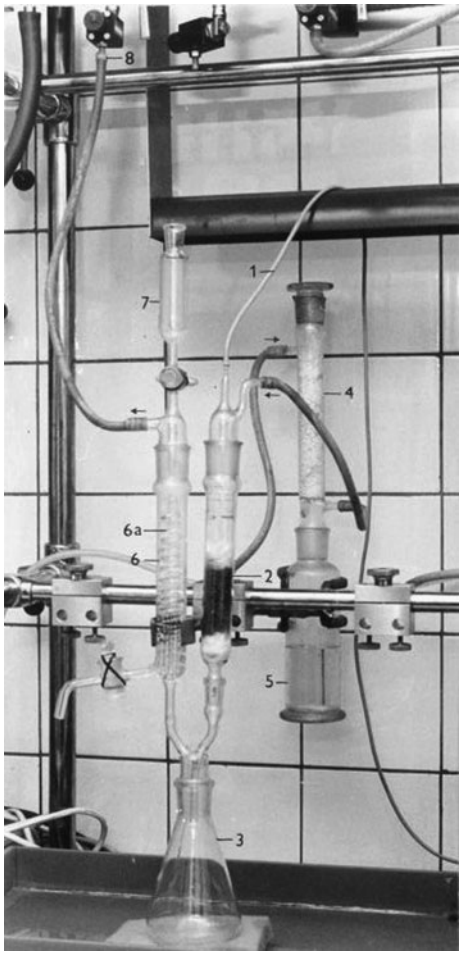

Fig. 2 Heterocontinuous cultivation. The apparatus contains: *1* inlet for sterile substrate solution *2* soil sample *3* vessel for eluate collection *4* column with caustic soda *5* bubble-through vessel with NaOH solution *6* adsorption column with NaOH solution and (*6a*) glass spiral tube ensuring CO_2 retention *7* vessel for dosing NaOH and *8* vacuum pump outlet

activity [68]. Enzymes were detected in soils stored for 60 years and even in geologically preserved soils. According to Macura [95], urease and phosphatase activities were found in permafrost samples of 9,000-year-old peat. Primary attention was originally paid to urease [14, 26, 63, 74, 110, 158], phosphatases [38, 62], and dehydrogenases [163]. Methods for the determination of proteases [80, 89, 119, 131], L-asparaginase [42, 43, 180], L-glutaminase [44, 45, 67], L-histidine ammonia lyase [40, 60], and amidase [8, 20, 41, 66, 73] were also described. However, the enzymes degrading cellulose [61], hemicelluloses and other polysaccharides and the enzymes involved in lignin transformation are considered to be the most important in soil. While the enzymes involved in N, P and S acquisition are produced by a wide variety of soil microorganisms, and some of them are also secreted by plant roots, the production of several polymer-degrading enzymes is often ascribed to fungi [6, 114, 145, 157]. These enzymes comprise endoglucanase, cellobiohydrolase, *β*-glucosidase [7, 92], endoxylanase, endomannanase, *β*-glycosidases [10, 21], esterases [10], endochitinase, *N*-acetylglucosaminidase, and α-glucosidase [141]. From the lignin-degrading enzymes, mostly produced by white-rot fungi, the main attention has been paid to Mn-peroxidase [64], lignin peroxidase [109], phenoloxidase (laccase) [5], and H_2O_2-producing enzymes [109]. A major weakness of the enzyme tests is that the actual microbial activity of a soil is not necessarily well reflected. Enzyme–clay and enzyme–organic polymer complexes show a remarkable resistance to proteolytic and thermal denaturation (e.g. [120, 138, 139, 162]) and the measured activities thus can represent the maximum potential of the soil sample rather than the actual enzyme activities.

Analysis of biomarkers

Fungi play an important role in the decomposition of organic matter such as cellulose, hemicelluloses and

lignin. The quantification of fungal biomass was originally restricted to staining and microscopical methods [4, 125]. Later, ergosterol content was shown to be a reliable indicator of fungal growth [72, 81, 137, 142, 143]. Ergosterol is the main sterol of most Ascomycetes, Basidiomycetes and Fungi Imperfecti. There are also fungi such as Mucorales, which possess ergosterol but not as the principal sterol. In general, some lower fungi also produce sterols other than ergosterol [75, 183] and its content may vary among individual species. So the interpretation of ergosterol-content measurements should be conducted carefully. Besides the ergosterol method, other methods based on detection of low molecular compounds include the analysis of muramic acid [113, 164, 181], teichoic acid and components [51, 161, 176], glucosamine [46, 181], and diaminopimelic acid [34–37, 56, 146]. Muramic acid, in contrast to ergosterol, is found only in prokaryotes and has been used as an indicator of bacterial and cyanophyte biomass [113]. Teichoic acid makes up 20–40% of dry weight of the cell wall of gram-positive bacteria and can thus be used as a biomarker for the estimation of biomass of gram-positive bacteria in soil and sediments [51]. In the case of glucosamine, a relatively specific compound found in the prokaryotic wall, there is a significant problem that occurs when using glucosamine analysis to estimate fungal biomass—the contribution of glucosamine from chitin of invertebrates. *m*-Diaminopimelic acid has been found in peptidoglycan of most bacterial cell walls and can also be a precursor in the biosynthesis of lysine. Little is known about the decomposition of diaminopimelic acid in soils. It has received little attention as an indicator of bacterial biomass [34–37]. A breakthrough in the use of biomarkers for community characterisation was the introduction of phospholipid fatty acid analysis (PLFA). These compounds are found in membranes of all living cells and can be used for the determination of community composition [68, 130, 134, 182]. The method is still being developed and undergoing interesting improvements, for example those reported by Gomez-Brandón et al. [54]. Its main advantage is the ability to discriminate between individual microbial groups. For archaebacteria, fatty acid residues ether-linked to glycerol are typical. Bacteria in general contain saturated or monosaturated fatty acids ester-linked to glycerol. Anaerobes contain sphingolipids that are largely absent in aerobes. While Gram-negative bacteria contain more hydroxylated fatty acids, Gram-positive ones contain more branched fatty acids. Cyanobacteria and eucaryotes usually possess lipids containing unsaturated fatty acids and, for fungi, 18:2ω6 PLFA is typical [49, 68]. Using this method, bacterial groups and fungi can be reliably characterised.

Modern DNA and RNA-based methods and approaches

Recent developments in the application of analytical chemistry and molecular biological techniques to ecological questions have revolutionized concepts in soil microbiology and microbial ecology. In particular, soil DNA and RNA extraction, nucleic acid reassociation, determination of G/C content of the whole microbial population, specific hybridization and PCR techniques, cloning and sequencing, denaturing gradient gel electrophoresis (DGGE), and many others have proven to be powerful tools for the assessment of the ecology of microorganisms in their natural environment. Techniques based on the detection of other compounds, such as fatty acid lipids or ergosterol mentioned above, have also provided new insights into the nonculturable fraction of microorganisms present in soil. Advantages and disadvantages of the individual methods are described in the recent work of Valášková and Baldrian [169] and the papers cited therein. Both the denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) represent tools for obtaining full sequences that can be subjected to further analysis. In the case of TGGE, only short sequences (<400 bp) can be analyzed [116, 117, 133]. Single-strand conformation polymorphism analysis (SSCP) leads to separation of single-stranded DNA molecules based on differences in their secondary structures. Similarly to TGGE, only short sequences can be analyzed [27, 82]. Terminal restriction length polymorphism (T-RFLP) found differences in the localization of restriction sites in DNA sequences. The method is simple and reproducible but reveals low phylogenetic specificity of terminal restriction sites [88]. PLFA enables us to analyse the diversity of both bacterial and fungal communities simultaneously, however with a very low level of taxonomic discrimination.

These methods that are used to describe the diversity of microbial communities in soil represent an important shift from the earlier cultivation-based approaches to more comprehensive culture-independent methods. This paper, dealing mostly with the contribution of I. Málek to the development of soil microbiology, cannot, of course, cover all the past, present and future aspects of the topic. Many other important original works describing different approaches can be found in excellent reviews of Macura and Insam that cover periods of 1924–1974 [95] and mid 1960s–2000 [68] as well as in recent works of Baldrian and coworkers [6, 7, 169].

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