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POSITION AND OPINION PAPER

Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis

Paolo Nannipieri[1] · **Carmen Trasar-Cepeda**[2] · **Richard P. Dick**[3]

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Abstract Enzyme activity as a method for soil biochemistry and microbiology research has a long history of more than 100 years that is not widely acknowledged in terms of adherence to strict assay protocols and the interpretation of results. However, in the recent past, there is a growing lack of recognition of the historic advancements among researchers that use soil enzymology. Today, many papers are being published that use methods that either do not follow exact protocols as originally vetted in the research literature or individual labs use their own method that has not been optimized for pH, co-factors, substrate concentrations, or other conditions. This is of particular concern for fluorogenic substrates and microplate methods. Furthermore, there is a lack of understanding of the origin and location of a given enzyme being studied. Notably, regardless of the enzyme, it is too often assumed that enzyme activity equals microbial activity—which is not the case for most hydrolytic enzyme assays. Because as established by Douglas McLaren in the 1950s, a considerable amount of activity can come from catalytic enzymes stabilized in the soil matrix but that are no longer associated with viable cells (known as abiontic enzymes). In summary, today, many papers are using imperfect methods and/or misinterpret enzyme activity data that at a minimum confounds cross paper studies and meta-analysis. However, most importantly, lack of historical perspectives and ignoring strict protocols cause redundancy and fundamentally undermine the discipline and understanding of soil microbiology/biochemistry when enzymology methods are used.

Keywords Soil enzyme activity · Enzyme protocols · Extracellular enzymes · Abiontic enzymes · Fluorogenic substrates · Microplate · Microbial activity

✉ Carmen Trasar-Cepeda
ctrasar@iiag.csic.es

[1] Department of Agrifood Production and Environmental Sciences, University of Firenze, P.le delle Cascine 28, 50144 Florence, Italy

[2] Soil Biochemistry Department, Instituto de Investigaciones Agrobiológicas de Galicia-Consejo Superior de Investigaciones Científicas (IIAG-CSIC), Apartado 122, 15780 Santiago de Compostela, Spain

[3] School of Environment and Natural Resources, Ohio State University, Columbus, OH 43210, USA

Introduction

The literature on soil enzymology is extensive, as evidenced by the number of books (Burns 1978a; Kiss et al. 1998; Burns and Dick 2002; García et al. 2003; Dick 2011a), some of them also available online (Rao et al. 2014), and reviews (among the most recent Dick and Burns 2011; Nannipieri et al. 2012) and by the international conference Enzymes in the Environment held every 4 years since 1998 (Granada, Spain; Praha, The Czech Republic; Viterbo, Italy; Nauheim, Germany; and Bangor, UK).

Soil enzyme activities are of interest to soil scientists because they provide information on the ability of soils to perform biogeochemical reactions, they can be used as an index to detect impacts of anthropogenic management (agriculture and forestry) or pollution on soils, and they are generally simple, rapid, accurate, and inexpensive. This paper addresses the following aspects of soil enzyme activity assays: (1) interpretation, (2) origins and distribution of enzymes in the soil matrix, (3) underlying mechanisms of extracellular enzymes, (4) contributions of extracellular and intracellular enzymes to total enzymatic activity, (5) contributions of enzyme-like reactions to actual enzyme activity, and (6) methodological

problems and the development of new methods for measuring diverse soil enzyme activities, as reviewed by Tabatabai (1994).

Many if not the entirety of each of these topics have been investigated and discussed in publications going back more than 100 years, which in many instances are not available in electronic databases. In fact, nearly all of the major milestones and our fundamental knowledge of soil enzymology were established before the 1990s (Table 1). Yet despite the lack of major breakthroughs in the last 30 years, many soil researchers are not considering the early research which too often leads to misinterpretation of soil enzymology results. Many papers today only cite publications from the last 5–10 years, without utilizing or considering past publications. This has led to the lack of innovative research or is redundant of past research and, even worse, research being done based on imperfect methods and misleading interpretations of enzyme activity data. The objectives of this position paper are to summarize the principles that have allowed the development of soil enzymology, some of the main outcomes of the past research in soil enzymology, and ultimately to guide and suggest future research on soil enzymology. For brevity and due to the vast literature on this topic, in general, we have relied heavily upon books and some review chapters as citation. Therefore, we apologize for the many papers and some reviews that we have not cited that normally would be in a full review paper but are beyond what is possible in an opinion paper.

Interpretation of soil enzyme activity

Enzyme activity vs. microbial activity In the Discussion sections of papers that have used enzyme assays, it is very common for the authors to refer to enzyme activities as an indicator of microbial activity or other soil biological properties, which results in contradictory interpretation and confusing discussions of enzyme activity results (Skujiņš 1967). Dehydrogenase activity is considered an indicator of microbial oxidative activity because it only occurs in viable cells, is rapidly inactivated following cell death, and often correlates with respiratory activity in soil. However, Skujiņš (1978) long ago reported that dehydrogenase activity does not always correlate with microbial activity, as shown by Howard (1972) where oxygen consumption was not closely related to dehydrogenase activity.

Many papers using a variety of enzyme assays, other than dehydrogenase activity, are routinely interpreted to be equivalent to microbial activity, but this is not necessarily the case (Skujiņš 1978). The discrepancy between microbial parameters and enzyme activity can be due to (1) inadequate methods that do not accurately reflect microbial activity, community composition, or biomass; (2) specificity of many enzyme assays (i.e., only a small fraction of the population may possess and express the measured enzyme at any given time); and (3) activity of many enzymes in soil is a composite of activities from many sources and locations (external cell membranes, soil solution, microbial debris, or stabilized in the soil matrix—as discussed below) (Skujiņš 1967; Nannipieri et al. 2012).

Abiontic enzyme activity Understanding the potential for extracellular catalysis by enzymes in soil no longer under the regulation or associated with viable cells is poorly understood or not discussed in many papers reporting soil enzyme activity data. Here in brief, we present a central and well-established concept for many enzymes found in soil that has a long research history, which John Skujiņš defined as abiontic enzymes (Skujiņš 1978). Full discussions of this

Table 1 Milestones in the history of soil enzymology research

Year	Milestone	Seminal citation
1899	First report of enzyme activity in soil (peroxidase activity).	Woods (1899)
1957	Conclusive evidence that enzymes are stabilized in the soil matrix and remain catalytic.	McLaren et al. (1957)
1969	Beginning of rigorous vetting of soil enzyme assays and use of *p*-nitrophenyl-based substrates.	Tabatabai and Bremner (1969)
1972	Use of fluourogenic substrate to determine enzyme activity in soil, later allowing for the development of multi-enzymatic microplate protocols.	Pancholy and Lynd (1972)
1975	Most abiontic enzymes are stabilized as humus-protein complexes by ionic, hydrogen, or covalent binding.	Ladd and Butler (1975)
1978	Debunking of enzyme activities as soil fertility tests and that the activity of many enzymes does not consistently correlate with microbial activity.	Skujiņš (1978)
1982	Elucidation of the role of abiontic enzymes in soil microbial ecology.	Burns (1982)
1990	Enzymes artificially stabilized on organo-mineral supports could be put in soil and remain catalytic.	Boyd and Mortland (1990)
2002	Detection of gene encoding enzymes and first attempts to relate them with the activity of the corresponding in soil.	Metcalfe et al. (2002)

Many other soil enzymologists (including E. Hofmann and G. Hoffmann in Germany, S. Kiss in Rumania, J. Mayaudon in Belgium, D.J. Ross and T. Speir in New Zealand, Hayano in Japan) have greatly contributed to the advancement of soil enzymology

concept, the potential role in soil ecology, and the historical research on this topic can be found in the following reviews: Burns (1978b, 1982), Dick and Burns (2011), and Skujiņš (1978). As discussed by Skujiņš (1978), the term abiontic was chosen because it conveys the idea of catalytic enzymes that are of biological origin but no longer controlled or associated with viable cells, and derived from the Greek a-, the alpha privative meaning "removal or absence of a quality," and the Greek suffix -biontic, meaning "not having a form of life."

Abiontic enzymes can have various locations in soils that include soil solution after excretion during cell growth and division, or release from extant cells or lysed cells, and cell debris and dead cells (Burns 1982). Additionally and importantly, abiontic enzymes can exist in stabilized forms primarily in two locations: adsorbed to inorganic surfaces (mainly clay and iron oxides and hydroxides) and complexed with organic colloids through adsorption, entrapment, or co-polymerization during soil organic matter genesis (Boyd and Mortland 1990; Nannipieri et al. 1996).

The concept of abiontic enzyme activity was extensively discussed in various review chapters and papers (Ladd and Butler 1975; Nannipieri et al. 1996; Dick and Burns 2011). The evidence for this is that (i) various sterilization techniques (such as radiation or use of antiseptics and antibiotics) that negatively affect microbial cells either had no or only a partial negative effect on enzyme activity and (ii) extractable levels of certain enzymes were very low compared to the measured activity. A major pioneer that conclusively established the occurrence of the abiontic enzyme fraction was Douglas McLaren, at University of California at Berkley. His lab showed that irradiation of soil at the right intensity resulted in soils that could not culture microorganisms but yet had highly measurable urease and phosphatase activities (McLaren et al. 1957, 1962; Skujiņš 1978; Dick and Burns 2011). Another approach supporting the concept of abiontic enzymes comes from research where enzymes are artificially immobilized on clay minerals, humic substances, or organo-mineral complexes but remain catalytic (Ladd and Butler 1975; Nannipieri et al. 1996). Besides providing evidence for abiontic enzymes from a different perspective, these model systems developed mechanisms for enzyme stabilization on inorganic or organic surfaces and entrapment into organo-mineral complexes. For a more detailed discussion on these mechanisms, see Ladd and Butler (1975), Stotzky (1986), Boyd and Mortland (1990), Nannipieri et al. (1996), and Gianfreda and Rao (2011). Although all of these lines of evidence conclusively establish the existence of abiontic enzymes, it is still not possible to routinely and conclusively separate abiontic enzyme activities from those regulated by viable and active microbial cells (Nannipieri et al. 2012).

Unfortunately, in spite of the long history and ongoing debate on this issue, as shown by many chapters dealing with the topic (e.g., Gianfreda and Ruggero 2006; Dick and Burns 2011; Nannipieri et al. 2012), much of the published research using soil enzyme activity methods do not acknowledge this concept and often misinterpret such data. For example, McDaniel et al. (2013) determined hydrolase activities in post-harvest forest soils but generically referred to this as extracellular activity as did previous papers such as Waldrop et al. (2003), Allison and Vitousek (2005), Hassett and Zak (2005), and Henry (2013). Too often, when the term "extracellular enzyme activity" is used, the discussion and interpretation assumes that this is equivalent to microbial activity. This has two issues that are often not recognized: (1) activity may not be extracellular but could be intracellular or at the cell surface and/or (2) the presence of enzyme stabilized in the soil matrix that are not associated with viable and active cells.

In recent years, one line of rationale for equating enzyme activity with microbial activity is that the typical short incubation (hours) is not long enough to produce a product from intracellular enzyme activity, and therefore, all the activity must be extracellular. However, this has never been proven and does not account for externally attached enzymes on cell surfaces or mucigels. High molecular weight substrates (for example, casein and derivatives of 4-methylumbelliferone (MUF)) justified to show true extracellular enzyme activities of soil by the argument that these substrates are too large to be taken up by microbial cells. However, this needs to be experimentally confirmed, especially considering that microbial cells can take up compounds such as amino acids or oligopeptides, of comparable size to MUF derivatives (Nannipieri and Paul 2009). Also, these high molecular weight substrates could be hydrolyzed by enzymes attached in the outer surface of viable cells with the active sites extending into the surrounding soil solution.

In any case, even if it was true that the short-term enzyme assay reflects extracellular enzyme activity, this ignores simultaneous activity that could be coming from enzymes stabilized in the soil matrix. Several papers use the term extracellular meaning that this equals microbial community activity at the time of sampling (Allison and Vitousek 2005; Henry 2013), when in fact a large amount of the activity could be due to enzymes in the soil matrix that likely have been there for many years. For example, Knight and Dick (2004) using microwave irradiation to sterilize soil showed that 34 to 75% of β-glucosidase activity, across three soil types, came from the fraction stabilized in the soil matrix. Thus, for the majority of papers in recent years that have enzyme activity data, the interpretation in the Discussion section does not acknowledge that a significant amount of activity and treatment effects on activity could be due to the stabilized fraction which has accumulated over the long term. Unfortunately, it is not possible to know in an activity assay how much of the activity is coming from enzyme produced by viable microbial cells or the stabilized fraction when the soils were sampled. Thus, when

interpreting activities of enzymes, both of these sources of activity should be considered in an attempt to explain treatment effects, which all too often is not the case in papers using enzyme activity data. It is very plausible that one might have a very different explanation for treatment effects on enzyme activity if it is thought to be only due to microbial activity compared to the situation that a significant contribution of the determined enzyme activity is due to the activity of the stabilized fraction. The activity coming from the stabilized fraction builds up slowly over the years and therefore reflects long-term management effects.

Enzyme activity vs. biogeochemical functions A fundamental principle that is being ignored in several soil enzyme publications as pointed out long ago by Burns (1978b) is that enzyme assays give potential rather than actual in situ enzyme activity rates (see next section for discussion on flaws to use enzyme assays to mimic in situ rates).

One facet of this was the hope that enzyme activities could be used to assess soil fertility as a test to aid fertilizer recommendations in combination with the extractable soil nutrient test which was pursued as early as the 1940s. The goal was to determine nutrient mineralization rates (particularly N, S, and P which have large organic pools in soil) in an attempt to predict the availability of nutrients that would become available to crops and improve the ability to determine crop nutrient requirements prior to planting. However, by 1978, John Skujiņš wrote "In the 1970s, obtaining a fertility index by the use of abiontic enzyme activity values seems unlikely. It is evident that enzymes are substrate specific and individual measurements cannot reflect the total nutrient status of the soil. Individual soil enzyme measurements, however, might answer questions regarding specific decomposition processes in the soil or questions about specific nutrient cycles" (Skujiņš 1978).

Recently, this same concept has been used by measuring the rate of hydrolysis of a single peptide substrate such as glycine or alanine, in order to represent the rate of organic N mineralization in total (as just a few of the examples Dong et al. 2007; Grandy et al. 2007). To make such a conclusion assumes that the release of NH_4^+-N from an organic N source, such as an amino acid, is a single enzyme reaction when in fact there are several enzymes involved. Take protein N as just one example of the complex organic N pool: there is a sequential hydrolysis of endopeptidases and exopeptidases to oligopeptides and amino acids, which are then converted to NH_4^+-N by amino acid oxidases and amino acid dehydrogenases (Nannipieri et al. 2012). Urease activity is also often used in this way to somehow represent organic N mineralization in general when in fact urea is just a very small part of soil organic N.

Despite the extensive early research, that was later pointed out by Gil-Sotres et al. (2005) and Nannipieri et al. (2012), among others, single enzyme activities are still interpreted as an index of the rates of nutrient mineralization which in fact is controlled by many enzymes (Dong et al. 2007). A more appropriate approach would be to conduct multi-enzyme assays representing key reactions in the stepwise hydrolysis of a given nutrient during the mineralization process (Sinsabaugh et al. 1992; Kiss et al. 1998; Trasar-Cepeda et al. 1998).

In summary, the flawed interpretation in much of the soil enzymology literature reflects the complexity of biogeochemical processes and ignores the well-established research of the past and fundamental biochemistry.

Mimicking in situ reaction rates Another problem related to proper use of soil enzymology is the attempt to mimic in situ conditions, particularly in terms of in situ pH or temperatures (as just a few of the examples Freeman et al. 2004; Drouillon and Merckx 2005; German et al. 2011; Peacock et al. 2015), by using incubation solutions that are unbuffered or adjusted to pH of the assayed soil and/or run assays at field soil temperatures (German et al. 2011).

The first problem is whether one can even mimic in situ pH because (i) pH values have spatial distribution at micro scales as H^+ or OH^- ions swarm at mineral surfaces causing a pH gradient across the water films of minerals and (ii) the target in situ pH is not the actual pH because pH is typically measured in a soil suspension (e.g., 1:2.5 soil/water suspension). Burns (1978b) pointed out the flaw of using just water instead of a buffer as follows: (a) soil pH can change during the year due to factors such as plant growth and agricultural practices, (b) pH of distilled water can range from 6 to 8 depending on water source, and (c) the use of buffer is needed not only to obtain the optimal pH value but also to maintain a constant pH during the assay—all of which would give variable results. This is in general, but there are some exceptions like for determining the impact of heavy metals on enzyme activities, for which it has been suggested the use of water instead of buffers because the presence of both H^+ or OH^- as well as the presence of high affinity organic ligands in the buffer can alter the metal concentration and activity in the soil solution (Lessard et al. 2013). Our recommendation is to do both water and optimal pH buffer-based assays to enable better chance to draw firm conclusions, as was done by Lessard et al. (2013). Another factor of not buffering pH at the optimal level for a given enzyme is that it may change the ranking of treatments or mask treatment effects of enzyme activities compared to if the assay had been run at an optimal pH. In this case, the farther away the incubation solution pH is from the optimal pH, the greater the effect on the treatment outcomes because enzyme activity will go down accordingly. Therefore, it is possible that treatment effects easily detected at the optimal pH are no longer detected, or treatment differences are smaller when the unbuffered in situ pH is different than the optimal pH. This latter issue for mimicking soil pH is also a problem for using incubation temperatures that are similar to field

conditions. Here again, the problem could be that if temperatures are too low, this becomes a rate-limiting factor and obscures any treatment effects being investigated.

Finally, no matter how one attempts to mimic in situ pH or temperatures, the actual results cannot reflect in situ rates of reactions because so many other facets of the lab assay are different than in situ conditions. This includes saturating the soil sample with substrate, adding co-factors, and adding the substrate in a slurry solution, greatly diluting—all of which greatly changes the chemical makeup and conditions of the assay compared to field conditions. Therefore, the only way for mimicking the in situ conditions is to repeat the same assay under optimized condition.

As mentioned above, enzyme activity rates measured in the laboratory are not the same nor even close to in situ rates, and thus, caution is required to use them for modeling biogeochemical processes, such as simulating nutrient mineralization rates or modeling C cycling relative to C sequestration in soils. To use enzyme activities for improving model algorithms is fundamental, but enzyme activities should be measured under the field conditions, for example, using in situ substrate concentration.

In conclusion, with an enzyme assay, we get a snapshot of the *potential* of the soil to perform a specific reaction by catalytic isoenzymes, that could have been produced in the past by metabolically active microorganisms and stabilized in the soil matrix, and/or those directly associated with viable cells at the time of sampling. Essentially, the attempt to use enzyme assays to mimic in situ rates of reaction is fundamentally flawed biochemistry. The only way to sound approach is under optimized conditions, which does not reflect in situ rates. Moreover, the other major reason for following optimized assays is that it allows for comparisons with other studies where the same optimized method was used, and therefore, meta-analysis of data is possible.

Standardization and accuracy of assays

Biochemistry and bench-scale enzyme protocols Standardization and accuracy of methods in soil enzymology are still problems despite having been discussed and debated since the 1960s. In 1978, Burns wrote "Most of the primary obstacles impeding advances in soil enzymology are associated with methodology because, as any evolving scientific discipline, those methods have yet to stabilize and throw up a cogent series of experimental techniques." Unfortunately, despite this long history, the importance of protocol standardization today is too often overlooked, as shown in the review of Burns et al. (2013).

Currently, there are less than 50 internationally recognized methods available for determining enzyme activities of soil. The most comprehensive enzyme methodology book was edited by Dick (2011a), which has about 35 bench-scale assays, all of which have been optimized. However, for some important enzyme activities, such as nuclease activity, no accurate protocols are available. As an aside, development of such an assay could be useful as corroborating data for determining the fate of nucleic acids in soil and the efficiency and yield of extracted nucleic acids which is important for downstream microbial diversity or gene expression analyses (Bakken and Frostegård 2006).

To establish an optimized enzyme assay that is reproducible by any lab operator, a vetting process should follow the classical protocol of Tabatabai and co-workers, who developed many of the available soil enzyme assays (Dick 2011b). A detailed description of this enzyme method vetting process is provided by Dick (2011b). In brief, to develop a fully vetted enzyme assay, the following should be done on adequate number (usually five to six) of soils that have contrasting chemical and physical properties to confirm: (1) high extraction efficiency of the product or substrate, (2) accurate measurement of the product formation or substrate disappearance (generally the sensitivity of the assay is greater by measuring the product), (3) the optimal pH value, (4) the appropriate buffer that maintains pH throughout the incubation and is not inhibitory, (5) the minimum substrate concentration to enable zero-order kinetics, (6) the appropriate amount of soil to detect measurable enzyme activity, (7) the minimum incubation time to minimize microbial growth and production of enzymes during the assay, (8) optimal temperature (usually it ranges between 25 and 37 °C), and (9) the proper control(s) (accounting for end products derived from reagent contaminants or soil solutions not catalyzed by the enzyme of interest).

Other factors that need to be tested are shaking soil-substrate reaction mixtures during the incubation, pre-assay soil handling and storage, preparation of standards with soil (to account for the adsorption of the compound being measured occurring in some soil types), and need for cofactors—all extensively discussed by Trasar-Cepeda et al. (2003) and Dick (2011b).

However, there are many enzyme methods used that have not been optimized for diverse soil types. Not surprisingly therefore, researchers have developed methods that ignore the principles presented above or modified methods (change of the buffer, for example) without vetting these methods for optimization and often do not report these modification or the details of a new method in published papers.

Fluorogenic microplate method Pancholy and Lynd (1972) first used a fluorogenic substrate, an ester of MUF, to determine lipase activity of soil which is sensitive to low activity levels. Since then, substrates releasing MUF or 7-amino-4-methyl coumarin (7-AMC) have been widely used to measure the activity of many enzymes, including simultaneous activity

determination of multiple soil enzymes on microplates, mainly developed by Freeman et al. (1995).

The microplate method is promoted as a high-throughput method that can measure activity of several enzymes on a single microplate from the same soil suspension. Aside from misuse or ignoring rigorous assay conditions (as discussed below), the micro-scale method has great potential for operator error because of (1) the preparation of the soil suspension, (2) the very small amounts of soil placed in each microplate well, (3) pipetting error because microliter volumes are required, and (4) the need for standard curve calibration with every sample to account for fluorescence quenching.

It is questionable whether in fact this is actually a "high-throughput" method because of the need to create soil suspensions (labor intensive and not needed in conventional bench assays), for high analytical replication, for more controls, and to have a standard curve for every soil sample. Nonetheless, for valid data comparison and interpretation, there is clearly a need to have a strict and agreed-upon enzyme assay protocol to standardize the microplate-based method that currently is not the case.

First of all, there is no standardized method that has been fully vetted by the "Tabatabai protocol" using MUF-based substrate. Preliminary validation involves establishing performance characteristics such as specificity, sensitivity, reproducibility, and accuracy based on comparative testing with a reference method as discussed above. Evaluative studies of various fluorimetric microplate assays using MUF-based substrates have been done in several laboratories (e.g., Deng et al. 2013; Marx et al. 2001; Trap et al. 2012), and most were done in the same laboratories that developed the methods. However, the resulting data have not consistently had the same outcomes when the fluorescent microplate and the colorimetric *p*-nitrophenol (*p*NP) bench-scale methods were directly compared on the same soil samples (e.g., Deng et al. 2013). Deng et al. (2013) investigated the colometric (PNP) microplate method and concluded that the presence of the soil suspension in the wells interfered with absorbance and resulted in increased standard errors to levels that were unacceptable. Other problems in the use of MUF-conjugated substrates are that researchers often do not follow the protocols that have been established by (1) not running a standard curve on every sample to account for quenching of fluorescence by soil particles (necessary because quenching varies from one sample to the next and varies temporally and spatially; Freeman et al. 1995); (2) use of a single point standard curve instead of multiple range standard curve (German et al. 2011); (3) not ensuring a pH of 10 or greater after the incubation or use of NaOH instead of THAM buffer (pH 10–12) that affects the optimal fluorescence intensity for detecting and maintaining the signal (Deng et al. 2013); (4) not reporting the exact protocol for creating the soil suspension (variations between labs in preparing soil suspensions with the type of homogenizer, size of glassware and stir bars, and differences in mixing speed and the time the sample is in suspension all affect the outcome; Dick et al. 2013); (5) not controlling incubation temperature which is important since MUF florescence is very sensitive to temperature (Guilbault 1990) (it is important to have all reagents at the incubation temperature prior to starting the substrate-soil incubation because it could take nearly 30 min of the incubation time to allow reaction mixtures to reach the protocol temperature of 37 °C); and (6) using the same buffer pH across all microplate wells that have several enzyme assays which have different optimal pH levels, which is likely due to convenience of using the same pH across all wells.

Therefore, it is our recommendation to develop a strict and agreed-upon enzyme assay protocol using the MUF-conjugated substrates to standardize the microplate-based method for valid data comparison and interpretation. This would mean doing this for every enzyme of interest by following all the vetting required in the Tabatabai protocol.

In the last years, some enzyme methods have been proposed to measure soil quality ISO/TS 22939:2010. Clearly, also for the development of ISO methods, our recommendation is that only enzyme assays vetted and standardized by the Tabatabai's protocol should be utilized.

Functional gene analysis: a future for soil enzymology

The advent of nucleic acid analyses offers a new avenue for studying enzymes in soils, potentially overcoming the limitations of classical enzymatic methods. The detection of microbial enzyme-encoding genes combined with relative enzyme activity can give insights into the origin of the specific enzyme in soil (Nannipieri et al. 2012). Metcalfe et al. (2002) observed that the increase in chitinase activity by sludge addition to a brown forest soil was associated with the prevalence of actinobacterium-like chitinase sequences. Other studies have related β-glucosidase (Cañizares et al. 2012; Pathan et al. 2015) and protease (Fuka et al. 2008b; Baraniya et al. 2015) activities to their respective enzyme-encoding genes in soil. This provides a means to identify the microbial origin of an active enzyme relative to environmental or agricultural treatment effects.

However, here again, improper use of these methods or interpretations can have negative impacts on the veracity of soil enzyme research. For example, the approach to run both enzyme activity and analysis for the abundance of enzyme-encoding genes, it is important to recognize that detection of gene does not mean that it is expressed; both transcription (synthesis of mRNA) and translation (synthesis of proteins) should be monitored. In particular, the protein enzyme should be detected as well as microbial species synthesizing it. Soil proteomics offers great potential to overcome these

shortcomings, but this technique is in its infancy due to a series of unresolved methodological problems related to extraction from soil, purification, separation, and determination of proteins (Renella et al. 2014). Furthermore and again, there is the issue of interpretation where comparative results from these two approaches have to take into account all the potential locations of where a catalytic enzyme can exist in soil. The detection of microbial sources of stabilized extracellular enzymes may give insights on past microbial events occurring in the monitored soil layer (Nannipieri 2006).

Conclusions

This position paper raises the issue of non-standardized methods and improper interpretation of soil enzymology research which is very common in the literature, and in turn is affecting the reliability of research produced by these methods. In brief, we report on the long history of enzymology research that is often ignored when enzyme methods are used in recent research papers.

A fundamental flaw for too many papers is the use of enzyme assays that have not been optimized (particularly the fluorogenic microplate method) to ensure reliable and reproducible results which includes the goal of mimicking in situ conditions that results in suboptimal assay conditions. Data from such studies is not accurate from a rate perspective and might even result in different treatment effects than optimized assays. Furthermore, this negates meta-analysis and comparisons of papers studying the same enzyme.

Another major problem is not recognizing the various locations for a specific enzyme, which includes intracellular, cell surface, soil solution, or stabilized in the soil matrix (abiontic). For example, a common misperception is that enzyme activity equals microbial activity, which may or may not be the case, depending on the particular enzyme. In particular, this is a problem for many enzymes that can exist and accumulate as protected enzymes in the soil matrix that remain catalytic. Thus, measured activity can come from both viable cells and stabilized enzymes. A further problem for interpreting enzyme activity is to imply that a single enzyme assay can represent a complex biogeochemical cycle such as nutrient mineralization that in fact is a multi-enzyme stepwise process.

The advent of nucleic acid-based analytical methods such as gene expression offers a new research tool for developing a mechanistic understanding on the functioning and ecology of soil enzymes. However, here again, improper use of these methods or interpretations can have negative impacts on the veracity of soil enzymic research—in particular interpreting such data without a recognition of all the potential locations of where a catalytic enzyme can exist could lead to inappropriate interpretations or conclusions.

In summary, we are urging that scientists first consider the long history of soil enzymology knowledge base before embarking on soil enzymology research and use this as basis for interpreting enzyme data which also means understanding the limitations of the data generated by the methods available today and, secondly, that they select methods that have been fully optimized and stick to these exact protocols. And if there is no such procedure, then that they take the time to develop an optimized method as laid out by Dick (2011b).

References

Allison SD, Vitousek PM (2005) Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biol Biochem 37:937–944

Bakken LR, Frostegård A (2006) Nucleic acid extraction from soil. In: Nannipieri P, Smalla K (eds) Nucleic Acids and Proteins in Soil. Springer, Berlin, pp 49–73

Baraniya D, Puglisi E, Ceccherini MT, Pietramellara G, Giagnoni L, Arenella M, Nannipieri P, Renella G (2015) Protease encoding microbial communities and protease activity of the rhizosphere and bulk soils of two maize lines with different N uptake efficiency. Soil Biol Biochem 96:176–179

Boyd SA, Mortland MM (1990) Enzyme interactions with clays and clay-organic matter complexes. In: Bollag J-M, Stotzky G (eds) Soil Biochemistry, vol 6. Marcel Dekker, New York, pp 1–28

Burns RG (1978a) Soil enzymes. Academic Press, New York

Burns RG (1978b) Enzyme activity in soil: some theoretical and practical considerations. In: Burns RG (ed) Soil Enzymes. Academic Press, London, pp 295–340

Burns RG (1982) Enzyme activity in soil: location and a possible role in microbial ecology. Soil Biol Biochem 14:423–427

Burns RG, Dick RP (2002) Enzymes in the environment. Activity, Ecology and Applications. Marcel Dekker, New York

Burns RG, DeForest J, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, Weintraub MN, Zoppini A (2013) Soil enzymes in a changing environment: current knowledge and future directions. Soil Biol Biochem 58:216-234

Cañizares R, Moren B, Benítez E (2012) Biochemical characteristics with detection and expression of bacterial β-glucosidase encoding genes of a Mediterranean soil under different long-term management practices. Biol Fertil Soils 48:651–663

Deng S, Popova I, Dick L, Dick RP (2013) Bench scale and microplate format assay of soil enzyme activities using spectroscopic and fluorometric approaches. Appl Soil Ecol 64:84–90

Dick RP (2011a) Methods of soil enzymology. Soil Science Society of America. Madison, WI

Dick WA (2011b) Development of a soil enzyme reaction assay. In: Dick RP (ed) Methods of Soil Enzymology. Soil Science Society of America, Madison, WI, pp 71–84

Dick RP, Burns RG (2011) A brief history of soil enzymology research. In: Dick RP (ed) Methods of Soil Enzymology. Soil Science Society of America, Madison, WI, pp 1–34

Dick LK, Jia G, Deng S, Dick RP (2013) Evaluation of microplate and bench-scale β-glucosidase assays for reproducibility, comparability, kinetics, and homogenization methods in two soils. Biol Fertil Soils 49:1227–1236

Dong S, Brooks D, Jones MD, Grayston SJ (2007) A method for linking in situ activities of hydrolytic enzymes to associated organisms in forest soils. Soil Biol Biochem 39:2414–2419

Drouillon M, Merckx M (2005) Performance of *para*-nitrophenyl phosphate and 4-methylumbelliferyl phosphate as substrate analogues for phosphomonoesterase in soils with different organic matter content. Soil Biol Biochem 37:1527–1534

Fenner N, Freeman C, Lock MA, Harmens H, Reynolds B, Sparks T (2007) Interactions between elevated CO_2 and warming could amplify DOC exports from peatland catchments. Environ Sci Technol 41:3146–3152

Freeman C, Liska G, Ostle NJ, Jones SE, Lock MA (1995) The use of fluorogenic substrates for measuring enzyme activity in peatlands. Plant Soil 175:147–152

Freeman C, Ostle NJ, Fenner N, Kang H (2004) A regulatory role for phenol oxidase during decomposition in peatlands. Soil Biol Biochem 36:1663–1667

Fuka MM, Engel M, Gattinger A, Bausenwein U, Sommer M, Munch JC, Schloter M (2008a) Factors influencing variability of proteolytic genes and activities in arable soils. Soil Biol Biochem 40:1646–1653

Fuka MM, Engel M, Haesler F, Welzl G, Munch JC, Schloter M (2008b) Diversity of proteolytic community encoding for subtilisin in an arable field: spatial and temporal variability. Biol Fertil Soils 45: 185–191

García C, Gil F, Hernández T, Trasar C (2003) Técnicas de Análisis de Propiedades Bioquímicas en Suelos: Medida de Actividades Enzimáticas y Biomasa Microbiana. MundiPrensa Editorial, Madrid

German DP, Weintraub MN, Grandy AS, Lauber CL, Rinkes ZL, Allison SD (2011) Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol Biochem 43:1387–1397

Gianfreda L, Rao M (2011) Stabilizing Enzymes as Synthetic Complexes. In: Dick RP (ed) Methods of Soil Enzymology. Soil Science Society of America, Madison, pp 319–369

Gianfreda L, Ruggero P (2006) Enzyme activities in soil. In: Nannipieri P, Smalla K (eds) Nucleic Acids and Proteins in Soil. Springer, Berlin, pp 257–311

Gil-Sotres F, Trasar-Cepeda C, Leirós MC, Seoane S (2005) Different approaches to evaluating soil quality using biochemical properties. Soil Biol Biochem 37:877–887

Grandy AS, Neff JC, Weintraub MN (2007) Carbon structure and enzyme activities in alpine and forest ecosystems. Soil Biol Biochem 39: 2701–2711

Guilbault (1990) Practical fluorescence, 2nd edn. Marcel-Dekker, New York

Hassett JE, Zak DR (2005) Aspen harvest intensity decreases microbial biomass extracellular enzyme activity and soil nitrogen cycling. Soil Sci Soc Am J 69:227–235

Henry H (2013) Reprint of "Soil extracellular enzyme dynamics in a changing climate". Soil Biol Biochem 56:53–59

Howard PJA (1972) Problems in the estimation of biological activity in soil. Oikos 23:235–240

ISO/TS22939, (2010) Soil quality: Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates. ISO Central Secretariat, Geneva, Switzerland.

Kiss S, Pasca D, Drăgan-Bularda M (1998) Enzymology of Disturbed Soils. Elsevier Science, Amsterdam

Knight TR; Dick RP (2004) Differentiating microbial and stabilized β-glucosidase activity relative to soil quality. Soil Biol Biochem 36: 2089-2096

Ladd JN, Butler JHA (1975) Humus-enzyme systems and synthetic organic polymer-enzyme analogs. In: McLaren AD, Paul EA (eds) Soil Biochemistry, vol 5. Marcel Dekker, New York, pp 143–194

Lessard I, Renella G, Sauvé S, Deschênes L (2013) Metal toxicity assessment in soils using enzymatic activity: can water be used as a surrogate buffer? Soil Biol Biochem 57:256–263

Marx MC, Wood M, Jarvis SC (2001) A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biol Biochem 33:1633-1640.

McDaniel MD, Kaye JP, Kaye MW (2013) Increased temperature and precipitation had limited effects on soil extracellular enzyme activities in a post-harvest forest. Soil Biol Biochem 56:90–98

McLaren AD, Reshetko L, Huber W (1957) Sterilization of soil by irradiation with an electron beam, and some observations on soil enzyme activity. Soil Sci 83:497–502

McLaren AD, R. A. Luse RA, Skujins J (1962) Sterilization of soil by irradiation and some further observations on soil enzyme activity. Soil Sci Soc Am Proc 26:371-377

Metcalfe AC, Krsek M, Goodday GW, Prosser JI, Wellington EM (2002) Molecular analysis of a bacterial chitinolytic community in an upland pasture. Appl Environ Microbiol 68:5042–5050

Nannipieri P (2006) Role of stabilized enzymes in microbial ecology and enzymes extraction from soil with potential applications in soil proteomics. In: Nannipieri P, Smalla K (eds) Nucleic Acids and Proteins in soil. Springer, Berlin, pp 75–94

Nannipieri P, Paul EA (2009) The chemical and functional characterization of soil N and its biotic components. Soil Biol Biochem 41: 2357–2369

Nannipieri P, Sequi P, Fusi P (1996) Humus and enzyme activity. In: Piccolo A (ed) Humic substances in terrestrial ecosystems. Elsevier, Amsterdam, pp 293–328

Nannipieri P, Giagnoni L, Renella G, Puglisi E, Ceccanti B, Masciandaro G, Fornasier F, Moscatelli MC, Marinari S (2012) Soil enzymology: classical and molecular approaches. Biol Fertil Soils 48:743–762

Pancholy SK, Lynd JQ (1972) Quantitative fluorescence analysis of soil lipase activity. Soil Biol Biochem 4:257–259

Pathan SI, Ceccherini MT, Hansen MA, Giagnoni L, Ascher J, Aremella M, Sørensen SJ, Pietramellara G, Nannipieri P, Renella G (2015) Maize lines with differing nitrogen use efficiency select bacterial communities with different β-glucosidase encoding genes and glucosidase activity in the rhizosphere. Biol Fertil Soils 51:995–1004

Peacock M, Jones TG, Airey B, Johncock A, Evans CD, Lebron I, Fenner N, Freeman C (2015) The effect of peatland drainage and rewetting (ditch blocking) on extracellular enzyme activities and water chemistry. Soil Use Manag 31:67–76

Rao MA, Scelza R, Gianfreda L (2014) Soil enzymes. In: Gianfreda L, Rao MA (eds) Enzymes in Agricultural Sciences. OMICS Group eBooks, pp 10-43

Renella G, Landi L, Ascher J, Ceccherini MT, Pietramellara G, Nannipieri P (2006) Phosphomonoesterase production of persistence and composition of bacterial communities during plant material decomposition in soils with different pH values. Soil Biol Biochem 38:795–802

Sinsabaugh RL, Antibus RK, Linkins AE, McClaugherty CA, Rayburn L, Repert D, Weilnad T (1992) Wood decomposition over a first-order watershed: mass losses a function of lignocellulose activity. Soil Biol Biochem 24:743–749

Skujiņš JJ (1967) Enzymes in soil. In: McLaren AD, Peterson GH (eds) Soil Biochemistry, vol 1. Marcel Dekker, New York, pp 371-414

Stotzky G (1986) Influence of soil mineral colloids and metabolic processes, growth adhesion, and ecology of microbes and viruses. In: Huang M, Schnitzer M (eds) Interactions of Soil Minerals with Natural Organics and Microbes. Special Publication 17, Soil Science Society of America, Madison, WI, pp 305-428

Tabatabai MA (1994) Soil Enzymes. In: Weaver RW, Angle S, Bottomley P, Bezdicek D, Smith S, Tabatabai A, Wollum E (eds) Methods of soil analysis, Part, vol 2. Microbiological and Biochemical Properties. Soil Science Society of America, Madison, pp 775–833

Tabatabai MA, Bremner JM (1969) Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Trap J, Riah W, Akpa-Vinceslas M, Bailleul C, Laval K, Trinsoutrot-Gattin I (2012) Improved effectiveness and efficiency in measuring

soil enzymes as universal soil quality indicators using microplate fluorimetry. Soil Biol Biochem 45:98–101

Trasar-Cepeda C, Leirós C, Gil-Sotres F, Seoane S (1998) Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties. Biol Fertil Soils 26:100–106

Trasar-Cepeda C, Leirós de la Peña MC, Gil-Sotres F (2003) Consideraciones Generales sobre la Determinación de las Actividades Enzimáticas en Suelos. In: García C, Gil F, Hernández T, Trasar C (eds) Técnicas de Análisis de Propiedades Bioquímicas en Suelos: Medida de Actividades Enzimáticas y Biomasa Microbiana. MundiPrensa Editorial, Madrid, pp 2–26

Waldrop MP, McColl JG, Powers RF (2003) Effects of forest postharvest management practices on enzyme activities in decomposing litter. Soil Sci Soc Am J 67:1250–1256

Woods AF (1899) The destruction of chlorophyll by oxidizing enzymes. Zentralbl Bakteriol Parasitenkd Abt 2(5):745–754