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Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates

Qualité du sol — Mesure en microplaques de l'activité enzymatique dans des échantillons de sol en utilisant des substrats fluorogènes

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*.

This second edition cancels and replaces the first edition (ISO/TS 22939:2010), which has been technically revised. The main changes compared to the previous edition are as follows:

— Clause 3 “Terms and definitions” added;

— 6.2.4: unit corrected in (40 ml to 40 µl);

— 6.2.6, Table 1 (Chitinase change E.C. 3.2.1.30 to E.C.3.2.1.52 and Alanin-aminopeptidase E.C. 3.4.11.12 to E.C. 3.4.11.2).

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Micro-organisms are responsible for many key processes in the cycle of elements. Enzymes play key roles in the degradation and mineralization of organic macromolecules. The main postulate is the microbial origin of soil enzymes, even if plant root exudates include enzymes. The simultaneous monitoring of several enzyme activities important in the biodegradation of organic compounds and mineralization of C, N, P and S in soil may reveal harmful effects caused by chemicals and other anthropogenic impacts (e.g. acidification, compaction). However, the measurements carried out under selected laboratory conditions using artificial substrates cannot be a substitute for the actual rate of enzymatic processes in soil in situ.

Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates

1 Scope

This document specifies a method for the measurement of several enzyme activities (arylsulfatase, α –glucosidase, β -glucosidase, Cellubisidase, β -Xylosidase, phosphodiesterase (PDE), chitinase, phosphomonoesterase (PME), leucine-aminopeptidase, Alanine-aminopeptidase) simultaneously (or not) using fluorigenic substrates in soil samples. Enzyme activities of soil vary seasonally and depend on the chemical, physical and biological characteristics of soil. Its application for the detection of harmful effects of toxic chemicals or other anthropogenic impacts depends on the simultaneous comparison of enzyme activities in a control soil similar to the test soil, or on exposure tests with chemicals or treatments.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

4 Abbreviated terms

E.C. Enzyme code number defined by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)

SOM Soil organic matter content

MUB Modified universal buffer

5 Principle

This document describes a method for the simultaneous measurements of several enzymes in soil samples. It is based on the use of soil samples diluted in buffer containing fluorogenic substrates, which

are incubated for 3 h at (30 ± 2) °C in multi-well plates. After the incubation the enzyme activities are measured as fluorescence with a plate-reading fluorometer [1][2]. The method described is based on dried standard and substrate plates enabling storage and limiting bias due to differences between reagent batches, and also enabling comparison between reagent batches. Annex A describes a method utilizing freshly prepared reagents, which has a clearly defined and exact incubation period. The advantage of the use of freshly prepared substrates is that an instrument for lyophilization is not required.

6 Reagents

6.1 Buffers

6.1.1 General

The selection of the buffer depends on the soil sample because the pH strongly affects enzyme activities. Sodium acetate buffer, 0,5 mol/l, at pH 5,5 has been used for acid soils with a high organic matter content. The use of the modified universal buffer (MUB) at the pH of the soil sample gives the flexibility necessary for coverage of a broad spectrum of different soils. Adequate stability of substrates at different buffers needs to be ensured. Good stability has been observed in 0,5 mol/l sodium acetate buffer at pH 5,5[3].

6.1.2 **Sodium acetate buffer**, 0,5 mol/l, pH 5,5.

— sodium acetate trihydrate (CAS N°: 6131-90-4 – 136,08 g/mol): 68,04 g;

— deionized water 1 000 ml;

— acetic acid (CAS N°: 64-19-7 – 60,05 g/mol): >99,8 %.

Dissolve sodium acetate trihydrate in water (e.g. 800 ml) and adjust the pH to 5,5 with concentrated acetic acid (>99,8 %; pro-analysis). Fill up to 1 000 ml. Sterilize in an autoclave at (121 ± 3) °C for 20 min. Store in a refrigerator for a maximum of two weeks.

6.1.3 **Modified universal buffer (MUB)**[4].

6.1.3.1 Stock solution

— tris(hydroxymethyl)aminomethane (CAS N°: 77-86-01 – 121,14 g/mol): 12,1 g;

— maleic acid (CAS N°: 110-16-7 – 116,07 g/mol): 11,6 g;

— citric acid (CAS N°: 77-92-9 – 192,12 g/mol): 14,0 g;

— boric acid (CAS N°: 10043-35-3 -61,83 mol/l): 6,3 g;

— sodium hydroxide (CAS N°: 1310-73-2 – 40,00 g/mol): (1 mol/l) 488 ml;

— deionized water 1 000 ml.

Dissolve the ingredients and store the solution in a refrigerator.

6.1.3.2 Final buffer

— hydrochloric acid (CAS N°: 7647-01-0 -36,46 g/mol): (0,1 mol/l);

— sodium hydroxide (CAS N°: 1310-73-2 – 40,00 g/mol): (0,1 mol/l).

Place 200 ml of the stock solution (6.1.3.1) in a 500 ml beaker containing a magnetic stirring bar and place the beaker on a magnetic stirrer. Set the required pH with hydrochloric acid or with sodium

hydroxide. Adjust the volume to 1 000 ml with deionized water. Sterilize in an autoclave at (121 ± 3) °C for 20 min.

6.2 Substrates and standards

6.2.1 Preparation of standard solutions

6.2.1.1 4-Methylumbelliferone (MUF) solution

— 4-methylumbelliferone (MUF) (CAS N°: 90-33-5 - 176,17 g/mol): 0,022 g;

— dimethylsulfoxide (DMSO) (CAS N°: 67-68-5 – 78,13 g/mol): add 25 ml.

MUF in powder form can be stored at room temperature but protected from light. Weigh MUF carefully and dissolve it in DMSO in a brown volumetric flask, avoiding exposure to daylight. The solution cannot be stored.

6.2.1.2 7-Amino-4-methylcoumarin (AMC) solution

— 7-amino-4-methylcoumarin (AMC) (CAS N°: 26093-31-2 – 175,18 g/mol): 0,021 9 g;

— dimethylsulfoxide (DMSO) (CAS N°: 67-68-5 – 78,13 g/mol): add 25 ml.

AMC as powder can be stored in the refrigerator. Weigh AMC carefully and dissolve it in DMSO in a brown volumetric flask, avoiding exposure to daylight. The solution cannot be stored.

6.2.2 Preparation of substrate solutions

Commercially available fluorogenic substrates are delivered as powders that can be stored deep-frozen at (–20 ± 2) °C. On the day of use, weigh the amount required for a 1 000 µmol/l, 2 500 µmol/l or 2 750 µmol/l concentration in a volume of, for example, 50 ml, avoiding exposure to light. Weigh the powder into a brown volumetric flask and fill to the required volume with DMSO.

The volume should be big enough for reliable weighing and measurement of volumes. It also depends on the number of plates needed.

The commonly used dispensers are able to distribute simultaneously just one volume (e.g. 40 µl) to eight rows. To facilitate the use of these instruments enabling good volumetric precision, 2 500 µmol/l solutions of the substrates should be prepared. However, for 4-MUF-β-D-glucopyranoside and for 4-MUF-phosphate substrates, a solution with the centration of 2 750 µmol/l is needed in order to produce the same final concentration of 500 µmol/l. These two solutions are further diluted simultaneously with the addition of the sample; 20 µl dimethylsulfoxide is added to the wells of these two substrates to facilitate dissolution. For chitinase activity measurement, a lower concentration is needed in order to avoid substrate inhibition, and the preparation of a solution with a concentration of 1 000 µmol/l 4-MUF-*N*-acetyl-β-D-glucosaminide can be used to produce the final concentration of 200 µmol/l.

6.2.3 Preparation of multi-well plates

The substrate and standard solutions are added to multi-well plates as solutions and dried (e.g. freeze-dried) on the multi-well plates directly after dispensing. Dry plates can be stored at (–20 ± 2) °C for a year. Exposure to light shall be avoided during handling and storage of substrates, standards and multiwell plates. A separate multi-well plate for substrates and standards has proved to be convenient.

6.2.4 Preparation of standard plates

Adequate replicate measurements, e.g. three to four replicates, are necessary due to the small sample volume. Standardization requires several concentrations of MUF or AMC, in replicate. Exposure to light shall be avoided during the dilution of standards. Calculate the required volume that depends on the number of samples and multi-well plates prepared. One example for the preparation of standards

covering a wide range of enzyme activities is given below, but modifications can be made depending on the range of enzyme activities in the samples studied.

The stock solution of MUF with a concentration of 5 mmol/l is used to produce the dilutions containing 1 000 µmol/l, 500 µmol/l, 250 µmol/l, 125 µmol/l, 50 µmol/l, 25 µmol/l and 5 µmol/l MUF. Distribute the volumes needed (e.g. 40 µl) into a multi-well plate for concentrations of 0 nmol/well, 0,2 nmol/well, 1,0 nmol/well, 2,0 nmol/well, 5,0 nmol/well, 10 nmol/well, 20 nmol/well and 40 nmol/well, in replicate. This step is critical for the measurement uncertainty.

NOTE 1 This set of stock solutions enables the use of automatic dispensers, which yield a significantly better precision than manual pipetting.

The stock solution of AMC with a concentration of 5 mmol/l is used to produce the dilutions containing 250 µmol/l, 125 µmol/l, 50 µmol/l, 25 µmol/l, 5 µmol/l, 2,5 µmol/l and 0,5 µmol/l AMC. Distribute the volumes needed (e.g. 40 µl) into a multi-well plate for concentrations of 0 nmol/well, 0,2 nmol/well, 1,0 nmol/well, 2,0 nmol/well, 5,0 nmol/well, 10 nmol/well, 20 nmol/well and 40 nmol/well, in replicate. This step is critical for the measurement uncertainty.

NOTE 2 This set of stock solutions enables the use of automatic dispensers, which yield a significantly better precision than manual pipetting.

6.2.5 Preparation of substrate plates

Exposure to light shall be avoided during dilution of substrates. When using multi-well plates with dry substrates with the final substrate concentration of 500 µmol/l and the sample volume of 200 µl, a volume of 40 µl of the 2 500 µmol/l solution for the substrates is added, in replicate, to the wells. For 4-MUF-β-D-glucopyranoside and for 4-MUF-phosphate, a 2 750 µmol/l substrate solution is added, in replicate, to the wells. For chitinase activity measurement, 40 µl of the substrate 4-MUF-*N*-acetyl-β-D-glucosaminide is added as a 1 000 µmol/l solution to reach the final concentration of 200 µmol/l.

This is the concentration that has been used for several different soils with the assumption of an approximate saturation level. In validation tests for a broad spectrum of soils, appropriate substrate concentrations should be checked and/or an enzyme kinetic approach considered.

If a plate with 96 wells and an automatic dispenser are used for eight substrates, 12 replicates are conveniently available. When using four replicates, it is possible to analyse three different samples or dilution levels on one plate.

6.2.6 Fluorogenic substrates

Table 1 gives a list of fluorogenic substrates and standards that are available commercially[1)].

Table 1 — Fluorogenic artificial substrates available commercially for the enzyme activity measurements

Enzyme	NC-IUBMB (see Clause 4)	Substrate	Element	Macromolecule degraded
Arylsulfatase	E.C. 3.1.6.1	4-MUF-sulfate	Sulfur	Mineralization of organic sulfur
α-Glucosidase	E.C. 3.2.1.20	4-MUF-α-D-glucopyranoside	Carbon	Starch and glycogen
MUF = 4-methylumbelliferone AMC = 7-amino-4-methylcoumarin				

1) Glycosynth and Sigma are examples of producers of fluorogenic molecules. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the producer named. Equivalent products may be used if they can be shown to lead to the same results.

Table 1 *(continued)*

Enzyme	NC-IUBMB (see Clause 4)	Substrate	Element	Macromolecule degraded
Cellobiosidase	E.C. 3.2.1.91	4-MUF-β-cellobi-opyranoside	Carbon	Cellulose
β-Xylosidase	E.C. 3.2.1.37	4-MUF-β-D-xy-lopyranoside	Carbon	Xylane, xylobiose
β-Glucosidase	E.C. 3.2.1.21	4-MUF-β-D-glu-copyranoside	Carbon	Cellulose
Phosphodiesterase (PDE)	E.C. 3.1.4.1	bis-(4-MUF)-phos-phate	Phosphorus	Hydrolysis of phosphate diesters
Chitinase	E.C. 3.2.1.52	4-MUF-*N*-acetyl-β-D-glucosaminide	Carbon	Breaking β-1-4-glycosid-ic bonds in *N*-acetyl-glu-cosaminide (chitin) and chitobiose
Phosphomonoesterase (PME)	E.C. 3.1.3.2	4-MUF-phosphate	Phosphorus	Hydrolysis of phosphate monoesters
Leucine-aminopeptidase	E.C. 3.4.11.1	L-leucine-AMC	Nitrogen	Hydrolysis of oligopeptides → aminoacids
Alanine-aminopeptidase	E.C. 3.4.11.2	L-alanine-AMC	Nitrogen	Hydrolysis of oligopeptides → aminoacids
MUF = 4-methylumbelliferone AMC = 7-amino-4-methylcoumarin				

7 Apparatus and materials

7.1 Equipment for the homogenization of the soil samples.

7.1.1 Sieves, with grid size, e.g. 4 mm.

NOTE Other grid sizes can be used depending on the soil texture.

7.1.2 Mechanical homogenizer[2].

For some soil types, an ultrasonic disaggregator has been used.

7.2 Usual laboratory glassware.

7.3 Multi-well plates, with covers.

There are differences in background fluorescence between brands and MUF and AMC analyses may require different brands.

7.4 Automatic dispenser for reagents (optional)[3].

NOTE Compared with manual pipetting, an automatic dispenser decreases significantly the uncertainty of volumes dispensed.

2) Bamix rod homogenizer and OmniMixer are examples of suitable homogenizers. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

3) Wallac 1298-003 Delfia is an example of a suitable instrument. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this instrument. Equivalent instruments may be used if they can be shown to lead to the same results.

7.5 Instrument for drying of the multi-well plates (e.g. by lyophilization).

7.6 Incubators, set at (30 ± 2) °C and allowing the use of a plate shaker at 450 min^{-1} to 700 min^{-1}.

7.7 Orbital plate shaker.

7.8 Plate-reading fluorometer, with the excitation wavelength set at 355 nm and the emission wavelength set at 460 nm, with the excitation lamp energy set appropriately[4)].

8 Procedure

8.1 Sampling

Take and handle soil samples as specified in ISO 18400-206. A composite sample consisting of 20 sub-samples taken in the field and homogenized by sieving through the selected sieves has been observed to yield reasonably low uncertainty of measurement for soil samples.

NOTE This method has been applied to boreal forest soil samples with a high soil organic matter (SOM) content and to agricultural soils with a high clay or silt content. For coarse samples with a relatively big particle size, a different sample handling could be necessary.

Soil pH is an important characteristic affecting enzyme activities and shall be measured in accordance with ISO 10390. This information can be used in the evaluation of enzyme activity results, even if the measurements are not carried out at in situ pH. The soil organic-matter content correlates strongly with soil enzyme activities. It shall be measured in accordance with ISO 10694.

Storage in a refrigerator after sieving is not suitable but samples can be stored deep-frozen at (–20 ± 2) °C for at least four months.

8.2 Sample preparation

8.2.1 Homogenization

The sieved soil is homogenized, and a test sample of 4 g is added to 120 ml of the selected buffer at (22 ± 2) °C. Treatment with the homogenizer (7.1.2) for 3 min in an ice bath and treatment with the homogenizer using 9 600 min^{-1} for 3 min in an ice bath have been used. The homogenized sample is adjusted into a final volume of 200 ml (dilution 1:50).

For some soil types the use of an ultrasonic disaggregator is more appropriate: disperse the soil sample in the buffer (dilution dependent on the activity of the soil) and homogenize the soil suspension using an ultrasonic disaggregator for 120 s at an output energy of 50 $J \cdot s^{-1}$[5].

8.2.2 Preparation of dilutions

The optimal dilution level depends on the soil sample and enzyme. Dilutions 1:100 and 1:1 000 are usually adequate. It is advisable to use the same dilution level for each enzyme because different dilutions do not give exactly comparable results. This may be due to the differences in exposure of enzymes to substrates in microsites.

When using plates with dried substrates and standards.

a) Prepare the dilution of 1:100 by adding 20 ml of tempered buffer (6.1.2 or 6.1.3) at (22 ± 2) °C to 20 ml of homogenized sample (8.2.1).

4) Wallac Victor is an example of a suitable instrument allowing excitation lamp energy to be set optimally. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this instrument. Equivalent instruments may be used if they can be shown to lead to the same results.

b) Prepare the dilution of 1:1 000 by adding 36 ml of tempered buffer (6.1.2 or 6.1.3) at (22 ± 2) °C to 4 ml of the dilution 1:100 prepared above.

8.2.3 Sample distribution

In four replicates, add 200 µl of the diluted soil sample to each well of the multi-well plate containing freeze-dried substrates, to yield substrate concentrations of 500 µmol/l. Add 20 µl of DMSO to the wells containing substrates for ß-glucosidase and phosphomonoesterase. Place the cover on each plate.

Add 200 µl in three or, preferably, four replicates of the homogenized, diluted sample to the wells with each standard. Place the cover on each plate.

A separate standard curve is necessary for each soil sample and dilution for MUF and AMC separately.

The blank values are obtained by measuring the substrate plates immediately after adding the sample.

NOTE This kind of blank does not reveal chemical instability of fluorogenic compounds. This is the reason why the stability of the compounds in sterile buffer needs to be tested separately (see [3]).

8.3 Incubation

Incubate the multi-well plates for 3 h at (30 ± 2) °C while shaking continuously, e.g. at about 700 min^{-1}.

NOTE The incubation temperature affects reaction rates and the optima depends on the enzymes. Depending on the study, a different temperature, as described in this document, can be used, e.g. the in situ temperature.

8.4 Fluorescence measurements

Measure the fluorescence directly after adding the sample to the multi-wells and after a 3 h incubation, using excitation at 355 nm and emission at 460 nm with adjusted excitation lamp energy.

NOTE Fluorogenic compounds dried in the multi-wells can yield elevated fluorescence directly after addition of the sample to the micro-wells because of undissolved compounds on the well surfaces. In soil samples, this is usually not a significant source of measurement uncertainty. However, it is also possible to measure fluorescence repeatedly to yield information on the dissolution, reaction rates and kinetic parameters[6].

The stability of the substrates shall be tested for each substrate batch (powder from the manufacturer) by measuring the change in fluorescence in the sterile buffer during incubation[1].

9 Calculation of results

The standard curve is plotted for MUF or AMC molar concentration (µmol/l) versus fluorescence. The MUF or AMC concentration of blank (*cb*) and sample (*cs*) are read from tempered the standard curve.

An example of a graph is given in Annex B.

The result is calculated by subtracting the average of four measurement replicates of blanks from the sample and multiplying the difference by the dilution factor, using either soil volume, soil fresh mass, dry mass or soil organic matter (SOM).

10 Expression of results

The results are expressed as micromoles per litre (µmol/l) of MUF or AMC released per soil volume or per gram (g) of soil fresh mass and/or dry mass and/or soil organic matter during 3 h, depending on the study. The measurements necessary for each expression, volumetric or gravimetric measurements or the determination of loss on ignition for soil organic matter shall be carried out.

Soil characteristics vary widely due to geography, climate and land use. The interpretation of results cannot, currently, be based on set limit values for each enzyme activity. The experimental design shall facilitate comparisons with a control soil or between samples from relevant sites.

11 Test report

The test report should include the following information:

a) a reference to this document, i.e. ISO/TS 22939;

b) adequate identification of the sample;

c) details of storage temperature and duration;

d) type of soil and soil physical and chemical characterization;

e) pH value of the soil sample;

f) buffer and incubation conditions applied;

g) test results;

h) any details not specified in this document or which are optional, as well as any incident which may have influenced the results.

Annex A
(informative)

Guidance on the use of freshly prepared substrates

A.1 Introduction

The fluorogenic substrates can also be applied as freshly prepared substrates according to [7]. It is preferable to analyse fresh soil samples directly after sampling and homogenization. However, because the reagents are not stable in solution and variation between batches may be significant, it is sometimes advisable to store all the soil samples to be compared as weighted deep-frozen aliquots, and to carry out the measurements of enzyme activities on all the soil samples to be compared simultaneously, rather than to carry out repeated measurements using separate batches of reagents. The weighing of substrate and standard compounds is a potential source of measurement uncertainty and an increase in the mass to be weighed decreases the measurement uncertainty.

A.2 Reagents

A.2.1 Buffer

The following buffers have been applied for the measurement of enzyme activities using freshly prepared substrates.

The selection of the buffer is critical. The buffering capacity shall be adequate to enable the set pH to be constant throughout the measurement. Either a constant pH or a pH optimized for the soil in question, or optimized separately for each enzyme in question, can be selected. However, the feasibility of enzyme activity pattern analysis supports the use of the same buffer for all the enzyme activities measured simultaneously. The same buffers as those described in 6.1.2. and 6.1.3 can also be used for freshly prepared substrates and the soil sample can be diluted directly in the buffer as in 8.2.1. MES (2-[*N*-morpholino]ethanesulfonic acid) buffer at pH 6,1 for the majority and Trizma buffer[5)] at pH 7,8 for the aminopeptidase activities are commonly used whith freshly prepared substrates.

Use MES buffer for phosphatase and enzymes involved in C cycling (substrates containing MUF as the fluorescent compound, see Table 1). To obtain 0,1 mol/l MES buffer (pH 6,1), dissolve 22,1 g in 1 l of water.

Use Trizma buffer for peptidases (substrates containing AMC as the fluorescent compound, see Table 1). To obtain 0,05 mol/l Trizma buffer (pH 7,8), dissolve 0,985 g of Trizma base and 2,66 g of Trizma HCl in 0,5 l of water.

Sterilize in an autoclave at (121 ± 3) °C for 20 min.

A.2.2 Substrates

Stock solution (10 mmol/l): dissolve substrates in 300 µl of dimethylsulfoxide (DMSO) and adjust the volume to 10 ml with autoclaved water.

5) Trizma buffer is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Working solutions (1 mmol/l): dilute stock solution with autoclaved MES buffer for MUF substrates and autoclaved Trizma buffer for AMC substrates. The total volume depends on the number of samples. For one sample series, all the substrate solutions should be produced at once.

NOTE When the sample volume is equal to the substrate volume in each well, the final substrate concentration is 500 µmol/l.

A.2.3 Standards

Dissolve standards in dimethylsulfoxide to concentrations of 5 mmol/l; subsequently dilute with MES buffer (for substrates containing MUF as the fluorescent compound) or Trizma buffer (for substrates containing AMC as the fluorescent compound) to a final concentration of 10 µmol/l. The total volume depends on the number of samples. For one sample series, all the standard solutions should be produced at once.

A.3 Procedure

A.3.1 Substrate plates

The substrate solutions can be added to multi-well plates as solutions, following the addition of the sample dilution to the wells.

Disperse 1 g of soil with 100 ml of sterile deionized water (for optimal dilution, see 8.2.2) using an ultrasonic disaggregator (50 $J \cdot s^{-1}$ for 120 s). Mix 50 µl of the soil suspension with 50 µl of the appropriate autoclaved buffer (MES buffer for MUF substrates, Trizma for AMC substrates) and 100 µl of substrate solution in microplates. Three or four replicates of each sample is preferable.

NOTE Manual pipetting of 50 µl volumes of substrates, and especially pipetting of soil samples in suspension in 50 µl aliquots, causes very high measurement uncertainty.

A.3.2 Standard plates

The standard solutions can be added to multi-well plates as solutions following the addition of the sample dilution to the wells.

Mix standards with 50 µl of soil suspension (separate standard curve for each sample, see 8.2.3) and the appropriate amount of buffer to obtain final concentrations of 0 µmol/l, 0,5 µmol/l, 1 µmol/l, 2,5 µmol/l, 4 µmol/l and 6 µmol/l in a final volume of 200 µl. To account for quenching, mix 100 µl of buffer with 100 µl of substrate for each substrate.

A.3.3 Incubation

For incubation and fluorescence measurements, see 8.3 and 8.4.

When freshly prepared plates are used with the fluorogenic compound already in solution, even a very short incubation period can yield measurable enzyme activities and the change in fluorescence shall be measured in precise constant time intervals.

