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Development of a sensitive and rapid method for the measurement of total microbial activity using Fluorescein Diacetate (FDA) in a range of soils

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Abstract

Fluorescein diacetate (FDA) hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in a range of environmental samples, including soils. Colourless fluorescein diacetate is hydrolysed by both free and membrane bound enzymes, releasing a coloured end product fluorescein which can be measured by spectrophotometry. The current method for measuring FDA hydrolysis in soils is limited in its application. FDA activity was very low in sandy and clayey soils. The low activity observed for these soil types was made difficult to measure by the original authors' choice of solvent for terminating the hydrolysis reaction. Acetone (50% v/v) was found to be most efficient at stopping the hydrolysis reaction. During this study acetone (50% v/v) was found to cause a decrease of approximately 37% in the absorbance of fluorescein produced by the soil samples measured. Although this colour loss is independent of initial fluorescein concentration, it makes the measurement of FDA hydrolytic activity extremely difficult in soils with low microbial activity i.e. sandy and/or clayey soils. Chloroform/methanol (2:1 v/v) was found to successfully stop the hydrolysis reaction for up to 50 min in a range of soil samples without causing the loss of colour observed with acetone. By changing the solvent used for terminating the hydrolysis reaction, low activity soils could be measured successfully. Other parameters of the hydrolysis reaction were optimised for the measurement of soil samples including effect of pH, optimum temperature of incubation, amount of soil, time of incubation, amount of substrate and preparation of suitable standards. A new, more sensitive method is proposed adapted from the original method, which provides a more accurate determination of FDA hydrolysis in a wide range of soils.

Keywords: Fluorescein diacetate (FDA), enzymic hydrolysis, total microbial activity, soil.

1. Introduction

The use of fluorescein esters as a measure of enzyme activity was first noted by Kramer and Guilbault (1963) where a simple procedure was described for the assay of lipase activity in the presence of other esterases. It was not until 1980 that the use of fluorescein esters as a measure of microbial activity was applied to environmental samples. Swisher and Carroll (1980) demonstrated that the amount of fluorescein produced by the hydrolysis of fluorescein diacetate (FDA) was directly proportional to the microbial population growing on Douglas Fir foliage and a standardised method was developed. This method was later evaluated by Schnürer and Rosswall (1982) who used FDA hydrolysis to determine total microbial activity in soil and straw litter as well as cell density in pure microbial cultures.

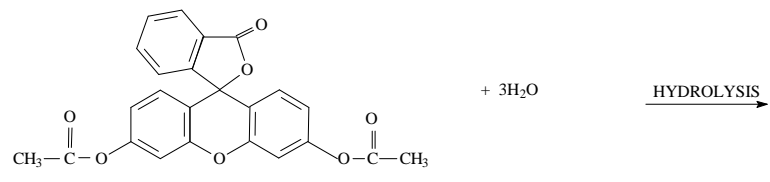
Fluorescein diacetate (3' 6' -diacetyl-fluorescein) is a fluorescein conjugated to two acetate radicals. This colourless compound is hydrolysed by both free (exoenzymes) and membrane bound enzymes (Stubberfield and Shaw, 1990), releasing a coloured end product, fluorescein. Fig. 1 illustrates the enzymic conversion of FDA to fluorescein which appears to be primarily a hydrolysis followed by a dehydration reaction. This end product absorbs strongly in the visible wavelength (490 nm) and can be measured by spectrophotometry. The enzymes responsible for FDA hydrolysis are plentiful in the soil environment. Non-specific esterases, proteases and lipases, which have been shown to hydrolyse FDA, are involved in the decomposition of many types of tissue. The ability to hydrolyse FDA thus seems widespread, especially among the major decomposers, bacteria and fungi (Schnürer and Rosswall, 1982). Generally more than 90% of the energy flow in a soil system passes through microbial decomposers, therefore an assay which measures microbial decomposer activity will provide a good estimate of total microbial activity.

The FDA method was also shown to correlate well with some of the most accurate measures of microbial biomass such as ATP content and cell density studies (Stubberfield and Shaw, 1990) and radio-labelled thymidine incorporation into microbial DNA (Federle *et al.*, 1990). Whereas these methods are time consuming and difficult to perform, enzyme assays are generally rapid and simple.

Since 1982, FDA hydrolysis has been used to measure total microbial activity in a range of samples from mould growth on wood and other building materials (Bjurman, 1993), to plant residues (Zablotowicz *et al.*, 1998), to stream sediment biofilms (Battin,

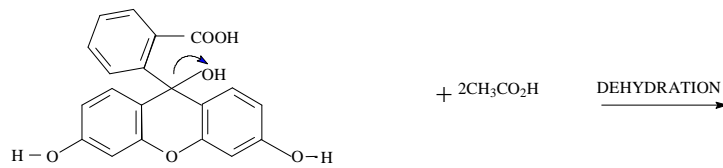
1997), activated sludge (Fontvieille, 1992) and deep sea clay and sand sediment profiles (Gumprecht *et al.*, 1995).

The advantage of this method being simple, rapid and sensitive, coupled with the widespread acceptance of FDA hydrolysis as a measure of total microbial activity, suggests this would be a good method to optimise to include a wide range of soils. The original Schnürer and Rosswall (1982) method, which most authors use today, is limited in its application. Schnürer and Rosswall found that FDA activity was very low in sand and clay samples. The low activity observed for these soil types was made more difficult to measure by the authors' choice of solvent for terminating the hydrolysis reaction. Because of the rapidity of FDA hydrolysis, it is necessary when working with many samples to find a way of terminating hydrolysis at a specific time. Schnürer and Rosswall found acetone (50% v/v) to be most efficient, totally stopping hydrolysis in a soil sample for 2 h. However, a substantial decrease in the absorbance of fluorescein produced by the soil samples was observed when acetone was added. This dramatic colour loss is independent of initial fluorescein concentration but makes the measurement of FDA hydrolytic activity very difficult in soils with low microbial activity i.e. sandy and/or clayey soils. Therefore, a new, more sensitive method is proposed adapted from the original Schnürer and Rosswall method which will provide a more accurate determination of FDA hydrolysis in a wide range of soils.

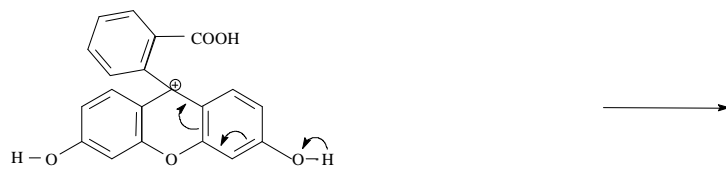


step 1

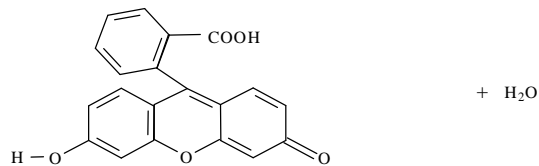
fluorescein diacetate – colourless



step 2



step 3



fluorescein – coloured acid yellow

visible 490 nm

Fig. 1 Enzymic conversion of fluorescein diacetate (FDA) to fluorescein.

The enzymic conversion of fluorescein diacetate (FDA) to fluorescein appears to be primarily a hydrolysis reaction followed by a dehydration reaction. The two acetate groups are hydrolysed at their ester linkage and the lactone part of the structure is cleaved at its internal ester link (step 1). The resultant OH group leaves, creating a positively charged bond (step 2). This charge must be satisfied so the above intermediary step occurs starting from a loss of H at the terminal position (step 3). This results in an overall loss of water.

2. Material and Methods

2.1 Soils

A total of five surface soils and one manufactured soil were selected to obtain a range of textural properties within the sandy and /or clayey textural class and cover a range of enzymic activities. Particle size analysis for the determination of textural class was carried out as described by the modified method of Khan (PhD thesis, University of Glasgow 1987) from the ADAS method 57 (1981b). Dehydrogenase activity was assessed by the method of Casida *et al* (1964). Total nitrogen content of the soils was determined by the digestion method of Bremner and Mulvaney (1982) coupled to an automated determination of nitrogen in the digests. Soil pH was determined according to ADAS method 32 (1981a). Organic matter content was assessed by loss on ignition (LOI). 5 g soil was weighed, in triplicate, into silica basins and dried overnight at 105°C. The soils were reweighed to obtain the oven dry soil weight. The soils were then placed in a muffle furnace and ignited at 500°C for 6 h. The samples were then reweighed and the weight of ignited soils calculated. The % organic matter by loss on ignition was calculated by subtracting the weight of ignited soil from the weight of oven dry soil. This value was then divided by the weight of oven dry soil and the resulting value multiplied by 100 to obtain % LOI. Table 1 and 2 show the textural, chemical and biological properties of the six soils chosen.

Table 1 Textural properties of the soils used in the study. (Coarse sand > 0.18 mm, fine sand 0.18 – 0.05 mm, silt 0.05 – 0.002, clay < 0.002 mm).

Soils	% coarse sand	% fine sand	% silt	% clay	Textural class
Barassie†	77.7	14.2	4.1	4.4	Sand
Bargour‡	39.4	27.5	11.3	21.8	Sandy loam
Caprington‡	29.2	22.0	25.7	23.1	Sandy clay loam
Dreghorn†	32.7	35.3	16.3	15.7	Sandy loam
Garscube	51.7	20.8	12.8	15.2	Loamy sand
John Innes compost	72.2	17.6	4.9	8.4	Sand

† Textural properties taken from Metwaly (PhD thesis, University of Glasgow, 1999).

‡ Textural properties taken from Khan (PhD thesis, University of Glasgow, 1987).

Table 2 Chemical and biological properties of the soils used in this study

Soils	pH (water)	LOI (%)	Total N (%)	Dehydrogenase activity ($\mu\text{g TPF g}^{-1}$ oven dry soil 24 h^{-1})
Barassie†	7.20	5.7	0.20	173.59
Bargour‡	5.46	6.9	0.18	145.79
Caprington‡	6.46	10.5	0.28	151.16
Dreghorn†	6.86	6.5	0.17	67.80
Garscube	7.23	9.6	0.35	224.70
John Innes compost	7.06	10.2	0.19	76.88

† Total N (%) taken from Metwaly (PhD thesis, University of Glasgow, 1999).

‡ Total N (%) taken from Khan (PhD thesis, University of Glasgow, 1987).

2.2 Reagents

2.2.1. 60 mM potassium phosphate buffer pH 7.6

8.7 g K_2HPO_4 (Riedel-de Haën, Sigma-Aldrich Co. Ltd., Analar) and 1.3 g KH_2PO_4 (Merck, BDH Analar) were dissolved in approximately 800 ml deionised water. The contents were made up to 1 l with deionised water. The buffer was stored in the fridge (4°C) and pH checked on day of use.

2.2.2. 2:1 chloroform/methanol

666 ml chloroform (Fisher Scientific UK Limited, analytical grade) was added to a 1 l volumetric flask. The flask was made up to 1 l with methanol (Fisher Scientific UK Limited, analytical grade) and the contents mixed thoroughly.

2.2.3. 1000 $\mu\text{g FDA ml}^{-1}$ stock solution

0.1 g fluorescein diacetate (3' 6'-diacetyl-fluorescein., Sigma-Aldrich Co. Ltd.) was dissolved in approximately 80 ml of acetone (Fisher Scientific UK Limited, analytical grade) and the contents of the flask made up to 100 ml with acetone. The solution was stored at -20°C.

2.2.4. 2000 $\mu\text{g fluorescein ml}^{-1}$ stock solution

0.2265 g fluorescein sodium salt (Merck, BDH Analar) was dissolved in approximately 80 ml of 60 mM potassium phosphate buffer pH 7.6 and the contents made up to 100 ml with buffer.

2.2.5. 20 $\mu\text{g fluorescein ml}^{-1}$ standard solution

1 ml of 2000 $\mu\text{g fluorescein ml}^{-1}$ stock solution was added to a 100 ml volumetric flask and the contents made up to the mark with 60 mM potassium phosphate buffer pH 7.6. 1-5 $\mu\text{g ml}^{-1}$ standards were prepared from this standard solution by appropriate dilution in 60 mM potassium phosphate buffer pH 7.6.

2.3 Methods

The individual parameters of the fluorescein diacetate hydrolysis reaction were studied to optimize the assay for the measurement of soil samples. These factors included effect of pH, amount of soil, amount of substrate, time of incubation, optimum temperature of incubation, choice of solvent for terminating the hydrolysis reaction and

preparation of suitable standards. The results from each parameter studied were culminated to produce the final assay procedure. To determine the effect of each parameter on the FDA hydrolysis reaction, changes were made to the final procedure but these changes will be explained where appropriate in the results and discussion section.

2.4 Final Procedure

2 g soil (fresh weight, sieved < 2 mm) was placed in a 50 ml conical flask and 15 ml of 60 mM potassium phosphate buffer pH 7.6 added. 0.2 ml 1000 $\mu\text{g FDA ml}^{-1}$ stock solution was added to start the reaction. Blanks were prepared without the addition of the FDA substrate along with a suitable number of sample replicates. The flasks were stoppered and the contents shaken by hand. The flasks were then placed in an orbital incubator (Gallenkamp Orbital Incubator, 100 rev min^{-1}) at 30°C for 20 min.

The following steps involving chloroform/methanol were carried out in a fume cupboard. Once removed from the incubator, 15 ml of chloroform/methanol (2:1 v/v) was added immediately to terminate the reaction. Stoppers were replaced on the flasks and the contents shaken thoroughly by hand. The contents of the conical flasks were then transferred to 50 ml centrifuge tubes and centrifuged at 2000 rev min^{-1} for approximately 3 min (MSE Scientific Instruments, Coolspin 2 centrifuge). The supernatant from each sample was then filtered (Whatman, No 2) into 50 ml conical flasks and the filtrates measured at 490 nm on a spectrophotometer (Hitachi U – 1100 spectrophotometer).

The concentration of fluorescein released during the assay was calculated using the calibration graph produced from 0 – 5 $\mu\text{g fluorescein ml}^{-1}$ standards which were prepared from a 20 $\mu\text{g fluorescein ml}^{-1}$ standard solution. The 0 $\mu\text{g ml}^{-1}$ fluorescein standard was used to zero the spectrophotometer before each set of blanks and samples were read.

2.5 Statistical analysis

Sample standard deviations were used to assess standard error and replicate variability was measured by the coefficient of variation (CV) using an EXCEL statistical package (Microsoft). One-way analysis of variance (ANOVA) was prepared using MINITAB (for WINDOWS 10.1). Probability values were set at 0.05 level for all statistical

measures. FDA hydrolysis values were expressed as μg fluorescein released g^{-1} oven dry (105°C) soil unless stated otherwise.

3. Results and Discussion

3.1 Effect of pH

The rate of hydrolysis of fluorescein compounds reaches a maximum between pH 7.0 and 8.0 (Guilbault and Kramer, 1964). Fluorescein diacetate was found to exhibit a maximum rate of hydrolysis at pH 7.6 (Swisher and Carroll, 1980). Carrying out the enzymic reaction at this pH was advantageous for many reasons. At high and low pHs, solubilisation of organic matter in the soil samples caused interference problems with the measurement of fluorescein released, by creating blanks with very high background absorbances. Carrying out the reaction at pH 7.6 removed this interference problem. Spontaneous hydrolysis of fluorescein esters is known to occur at high pHs (Guilbault and Kramer, 1964). At pH 7.6 no spontaneous hydrolysis of fluorescein diacetate was observed. Finally, the product of FDA hydrolysis, fluorescein, exhibits a maximum fluorescence at about pH 8.0 (Guilbault and Kramer, 1964). This was verified when standards were prepared in buffers at different pHs. The absorbance values measured for the same concentration of fluorescein standards were more than double in the pH 7.6 potassium phosphate buffer compared with the pH 5.6 buffer. This illustrates that fluorescein is near its maximum absorbance at pH 7.6.

3.2 Effect of temperature

The rate of hydrolysis of a substrate by an enzyme depends on the temperature of incubation. A study of FDA activity in soil as a function of temperature showed maximum activity occurred at 30°C . This is in agreement with findings by Breeuwer *et al* (1995) who observed maximum FDA activity by yeast esterases at this temperature. The activity rapidly decreased just above 30°C suggesting inactivation of the enzymes involved at this elevated temperature (Fig 2). At high temperatures considerable spontaneous hydrolysis of fluorescein esters can occur (Guilbault and Kramer, 1964), adversely affecting the accuracy and reproducibility of the method. No spontaneous hydrolysis of FDA occurred between $20\text{-}40^\circ\text{C}$ which covers the range around the temperature chosen for this assay.

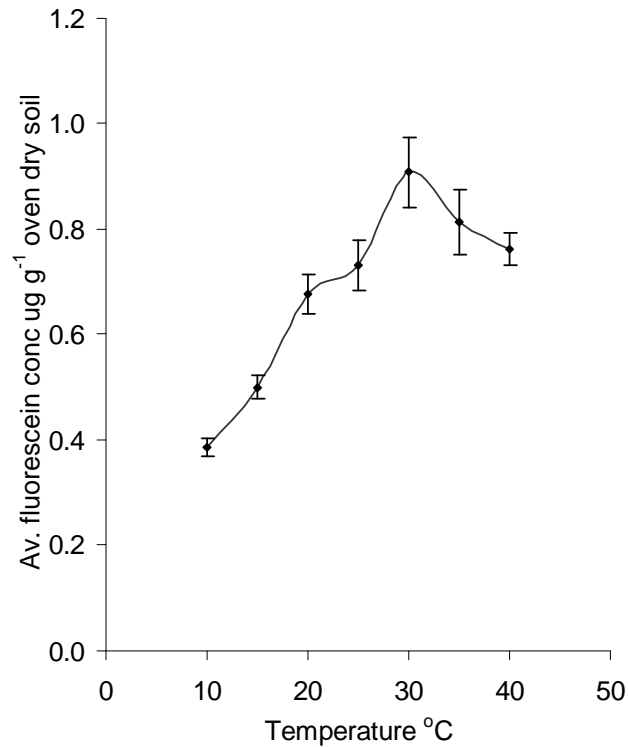


Fig. 2 Effect of temperature on FDA hydrolysis by enzymes present in a sandy soil (Barassie). Mean values with standard error bars, n = 4.

3.3 Amount of soil

The initial reaction rates of soil enzymes are usually proportional to the amounts of soil added to the assay (Frankenberger and Johanson, 1983). Soil weights (fresh weight, sieved < 2 mm) ranging from 0.5-5 g were incubated to discover the optimal amount of soil required for the reaction to proceed at a steady rate without substrate becoming limiting. A linear relationship was observed between soil weight and fluorescein released up to 2.5 g (Fig. 3). The deviation from linearity when soil weights greater than 2.5 g were used indicates substrate concentration was probably becoming a limiting factor.

A soil weight of 2 g was used for the final assay. This allowed the reaction to proceed at a steady rate, for all the soils tested, without substrate becoming limiting. It also ensured that the amount of fluorescein hydrolysed during the assay fell within the sensitivity range of the spectrophotometer (0.1-1.0). The three soils chosen to illustrate this in Fig. 3 were: (a) a manufactured compost (John Innes Compost No 2) which represents a sandy soil with low microbial activity; (b) Dreghorn which represents a

sandy loam with an intermediate microbial activity and (c) Garscube soil which is a loamy sand with high microbial activity. The low activity soil released enough fluorescein during the assay to be measured accurately by spectrophotometry (absorbance values greater than 0.1) and the high activity soil released enough fluorescein to lie within the range of the spectrophotometer without dilution (absorbance values below 1.0). These three soil types hopefully represent the range of microbial activities encountered in most soils.

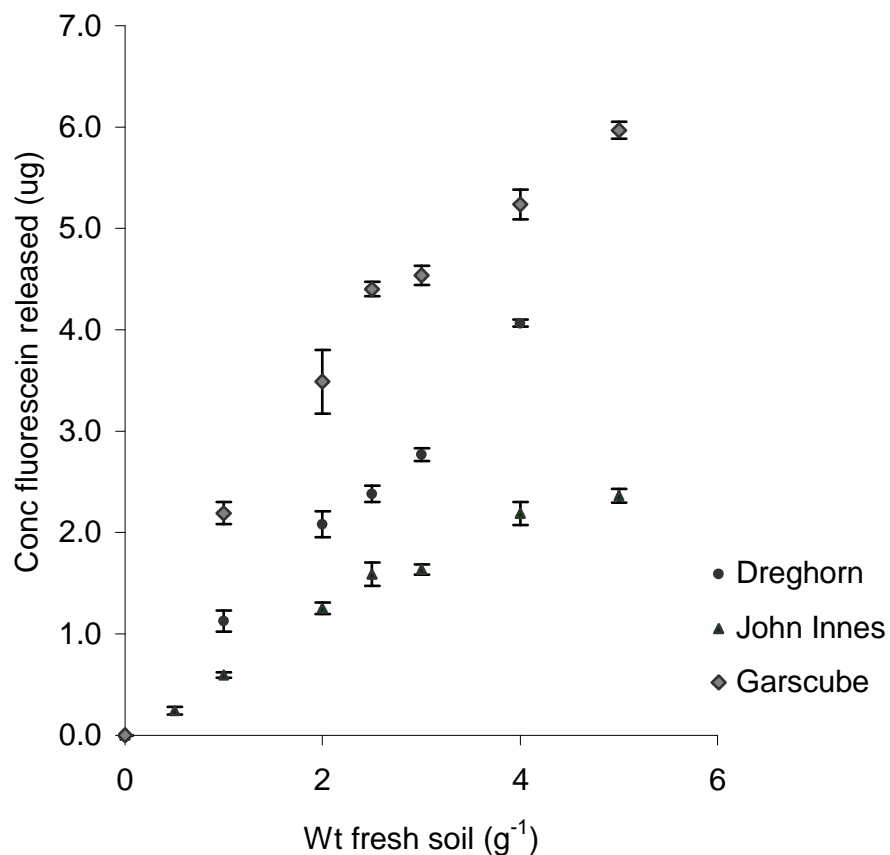


Fig. 3 Relationship between soil weight and FDA hydrolysis. Mean values with standard error bars, n = 3.

3.4 Adsorption of Fluorescein onto soil

The amount of fluorescein adsorbed onto soil was considered before carrying out the enzyme assay. When a new soil is investigated, the amount adsorbed onto soil should be calculated so the values obtained for the assay can be corrected for the loss. Soil samples were incubated using the conditions described for the final assay procedure in 15 ml fluorescein standard at each concentration (0-5 µg ml⁻¹) (Table 3). Blanks were

prepared without the addition of soil. Samples and blanks were centrifuged and filtered as described in the final assay procedure. The amount of fluorescein adsorbed at each concentration is shown as % fluorescein adsorbed and an average total value is given for each textural class. Generally the amount of fluorescein adsorbed is less than 5%. This observation was noted by the original authors who found the adsorption of fluorescein to soil did not exceed 7% and was mostly lower than 5% (Schnürer and Rosswall, 1982). A soil with a high silt–clay ratio and high organic matter content, such as Caprington can however adsorb up to 13.7% fluorescein, a large proportion of the total released.

Table 3 Adsorption of fluorescein onto soils with differing textural properties.

Percentage fluorescein adsorbed by soil (mean \pm S.E., n = 3)			
Fluorescein conc ($\mu\text{g ml}^{-1}$) in blank	Barassie sand	Dreghorn sandy loam	Caprington sandy clay loam
0	0	0	0
1	5.7 \pm 0.83	3.3 \pm 0.69	20.2 \pm 0.47
2	4.3 \pm 0.60	7.1 \pm 2.57	14.6 \pm 0.39
3	3.2 \pm 0.15	5.8 \pm 0.27	11.1 \pm 0.93
4	4.1 \pm 0.29	3.3 \pm 0.74	10.5 \pm 0.26
5	2.7 \pm 0.24	2.4 \pm 1.07	11.7 \pm 1.53
Average total adsorbed %	4.0	3.6	13.7

3.5 Time of incubation

It has been suggested that an assay for soil enzymes should not require a long incubation time because the risk of error through microbial proliferation increases with increasing incubation time. Part of this error can be minimised by the addition of toluene as a bacteriostat to enzyme assays although many authors do not favour the use of toluene for this purpose. Toluene has been shown to inhibit some enzymes as well as having an activating effect on others due to increased permeability of the cell membrane in the presence of toluene, allowing entry of the substrate (Skujins, 1967). In this study the use of toluene as a bacteriostat in the assay of FDA hydrolysing enzymes was dismissed as it was found to inhibit FDA hydrolysis in the soil samples investigated by

approximately 35%. It was therefore decided to keep the incubation time as short as possible.

The hydrolysis reaction was linear with time up to 40 min for the soils investigated using the conditions described for the final assay procedure (Fig. 4). The assay was not limited by substrate concentration over this time period.

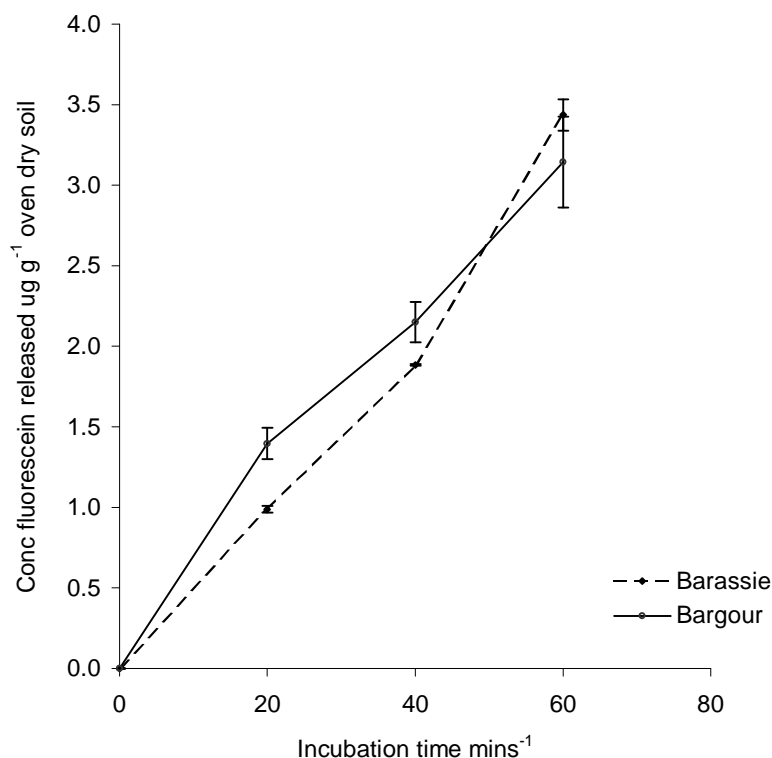


Fig. 4 Determination of optimum incubation time. Means and standard error bars, n = 3.

An incubation time of 20 min was chosen for the final assay procedure to allow the concentration of substrate hydrolysed to lie within the range of the spectrophotometer for all the soil types investigated without the need for dilution.

3.6 Choice of solvent for terminating hydrolysis

Schnürer and Rosswall found acetone (50% v/v) to be most efficient, totally stopping hydrolysis in a soil sample for 2 h. The addition of acetone to the soil samples did terminate hydrolysis but it also caused a decrease in the amount of fluorescein measurable by spectrophotometry. Although the drop in colour was by the same ratio each time (ranged from 39.8-41.0% in 1-5 μ g ml⁻¹ fluorescein standards where acetone

was added), samples with low microbial activity which only release a small amount of fluorescein were made increasingly more difficult to measure. This decrease in colour, which was more than a dilution effect, was, on average, 37% of the colour developed in a sandy soil. Table 3 illustrates the decrease in fluorescein concentration of samples where acetone has been used to stop the hydrolysis reaction compared to samples where chloroform/methanol (2:1 v/v) has been used. The samples sometimes dropped below the range of the spectrophotometer when acetone was added. The absorbance values for the samples where chloroform/methanol (2:1 v/v) has been used to terminate hydrolysis lie just above 0.1, the minimum absorbance that can be measured accurately by spectrophotometry. The sample values where acetone has been used to stop the reaction have fallen below the range that can be measured accurately. The relative precision of the method, defined by the coefficient of variation of replicate measurements, is also increased when chloroform/methanol (2:1 v/v) is used instead of acetone. Therefore chloroform/methanol (2:1 v/v) was proposed as the new solvent for terminating FDA hydrolysis in soil samples.

3.7 Change in hydrolysis over time

Changing from acetone to another means of terminating the hydrolysis reaction involved finding a substitute that would stop hydrolysis successfully without causing the same loss of colour observed with acetone. A 2:1 ratio of chloroform/methanol (v/v) was most efficient, stopping hydrolysis from continuing for up to 50 min after its addition (Fig. 5). The length of time the reaction was terminated for was sufficient to allow the measurement of a large number of samples without changes occurring in the samples. In addition, chloroform will help solubilise cell membranes, as acetone did, facilitating the extraction of fluorescein. FDA, being non-polar, readily penetrates into the cell and is hydrolysed to fluorescein. The polarity of fluorescein impedes its transport back through the cell membrane causing intracellular accumulation. Fluorescein is liberated into the environment only after the storage capacity of the cell has been exceeded and the excess is excreted (Rotman and Papermaster, 1966). Chloroform will help solubilise cell membranes aiding the extraction of fluorescein. The presence of methanol will help the chloroform interact with the moist soil hence increasing its ability to terminate the reaction effectively. Fluorescein released during the incubation also moves preferentially into the more polar potassium phosphate buffer/methanol phase, which increases the efficiency of the extraction procedure. Acetone also removed a lot of dissolved organic matter from the samples producing blanks with very high background absorbances whereas the chloroform/methanol (2:1

v/v) does not. All these advantages make chloroform/methanol a more beneficial solvent to use in terminating the hydrolysis reaction.

Table 4 Decrease in fluorescein concentration ($\mu\text{g ml}^{-1}$) of Barassie soil in acetone terminated samples compared with chloroform/methanol (2:1 v/v) terminated samples.

	chloroform/methanol (2:1 v/v)		Acetone (50% v/v)		
replicate ^a	abs 490 nm	conc ($\mu\text{g g}^{-1}$) soil	abs 490 nm	conc ($\mu\text{g g}^{-1}$) soil	Corrected conc ($\mu\text{g g}^{-1}$) soil
1	0.129	0.543	0.060	0.249	0.332
2	0.125	0.533	0.063	0.268	0.356
3	0.109	0.467	0.061	0.255	0.340
4	0.109	0.455	0.043	0.180	0.239
5	0.133	0.568	0.068	0.288	0.383
6	0.124	0.518	0.052	0.221	0.294
Final vol. of filtrate (ml) ^b		20		30	20
av. conc ($\mu\text{g g}^{-1}$) soil		0.514		0.244	0.324
CV ^c		8.61			15.74

^a All replicate absorbance values and concentration values in $\mu\text{g fluorescein g}^{-1}$ oven dry soil are given.

^b The final volumes of filtrate collected were 20 ml in the chloroform/methanol (2:1 v/v) samples and 30 ml in the acetone samples. Due to the differences in the final volumes a conversion factor of 0.33 was used on the acetone values to counteract this dilution effect. The corrected concentration values for the acetone samples give fluorescein concentrations ($\mu\text{g g}^{-1}$ oven dry soil) in a 20 ml final volume. These values can be compared directly to the values obtained for the chloroform/methanol (2:1 v/v) samples.

^c CV = coefficient of variation.

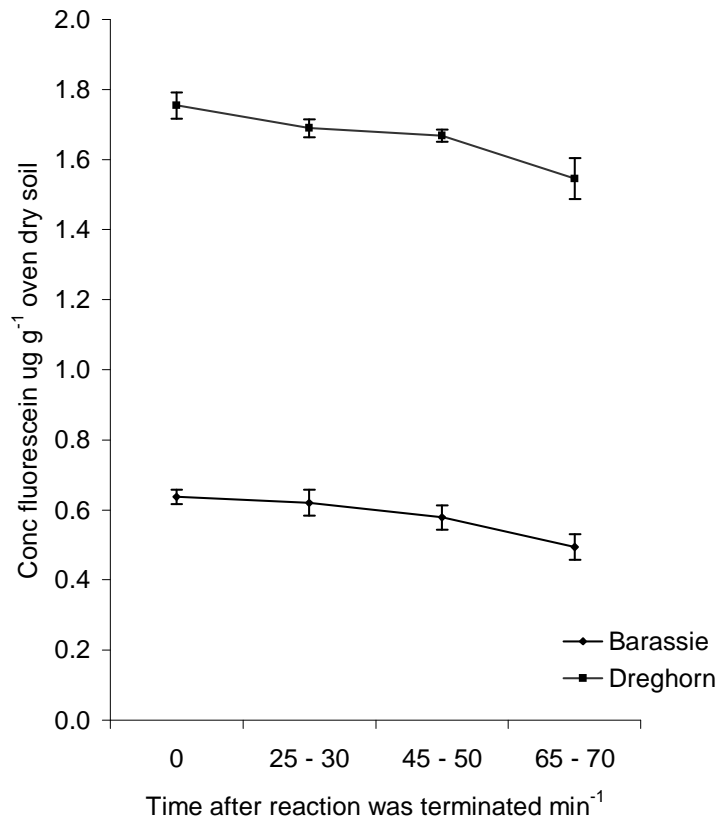


Fig. 5 Change in fluorescein concentration over time after termination of the hydrolysis reaction. Mean values and standard error bars, $n = 5$. Means followed by the same letter are not significantly different at $P < 0.05$.

3.8 Substrate concentration

2000 $\mu\text{g ml}^{-1}$ fluorescein diacetate (FDA) solution was used by most authors as the substrate for the reaction. By adding 0.2 ml of 2000 $\mu\text{g ml}^{-1}$ FDA, 400 μg FDA was achieved in each replicate. This amount was unnecessarily high for the conditions chosen for the final procedure. High concentrations of FDA should be avoided as FDA is poorly soluble in water and other polar solutions (Breeuwer *et al.*, 1995). Even in acetone, high concentrations of FDA produce slightly cloudy solutions suggesting not all the FDA added is in solution, hence available to the microorganisms. Instead a 1000 $\mu\text{g ml}^{-1}$ FDA solution was chosen to start the reaction. This supplied 200 μg FDA to each replicate which can, in turn, release a maximum of 160 μg of fluorescein. This 160 μg of fluorescein is diluted in 15 ml 60 mM potassium phosphate buffer pH 7.6 then a further 5 ml of methanol (from 15 ml chloroform/methanol (2:1 v/v) only the methanol is added to the filtrate). This gives a final possible fluorescein concentration

of $8 \mu\text{g ml}^{-1}$. The maximum concentration of fluorescein is never released by the conditions described for the final procedure therefore standards are prepared covering a range of $0\text{-}5 \mu\text{g fluorescein ml}^{-1}$. All the soils investigated, using the conditions set for the assay, were within the range described by the standards.

3.9 Preparation of standards

The original method stated that standards should be prepared using hydrolysed fluorescein diacetate (FDA). This was achieved by boiling FDA solutions of known concentrations in a water bath for 30 min (Schnürer and Rosswall, 1982). Other authors increased the boiling time to 60 min (Chen *et al.*, 1988). This method for obtaining reproducible standards proved too variable. $0\text{-}200 \mu\text{g}$ concentrations of FDA were added to 5 ml of 60 mM potassium phosphate buffer pH 7.6 in screw top vials (Fig 6A). The lids were replaced and the standards placed in a boiling water bath for a set time (30 or 60 min). Once cool a further 10 ml of 60 mM potassium phosphate buffer pH 7.6 was added to keep all volumes the same as the final procedure. 15 ml of chloroform/methanol (2:1 v/v) was added and the standards were centrifuged and filtered as described by the final procedure. The standards were measured at 490 nm and the results plotted to produce a standard calibration graph. The results differed for both hydrolysis times. Fluorescein diacetate can in fact be hydrolysed for up to 6 h in a water bath, although a slight plateau is reached after 4 h. Fig 6B illustrates the continued hydrolysis of a $100 \mu\text{g}$ FDA standard. Replicate $100 \mu\text{g}$ FDA standards in 5 ml 60 mM potassium phosphate buffer pH 7.6 were placed in a boiling water bath. A duplicate set of replicates were removed every hour and the fluorescein released measured as described above. Whether FDA is continuing to be hydrolysed or whether it is being degraded in the prolonged heating is unclear but the method for preparing standards is clearly unacceptable.

Sodium fluorescein salt was chosen instead to prepare the standards. Sodium fluorescein salt released the same acid yellow coloured fluorescein as FDA released allowing direct measurement of fluorescein released from FDA in soil by a standard calibration graph prepared from sodium fluorescein salt. Certain pure fluorescein preparations should be avoided as this compound is generally solvent yellow coloured and adds an error to the absorbance measurements. The fluorescein salt can be weighed accurately and known concentrations of fluorescein obtained so standard results rarely differ. Standards prepared by this method did not alter significantly over three months.

By using the boiling water bath method for preparing standards the amount of fluorescein hydrolysed by the samples and the amount of FDA hydrolysed during standard preparation was continually underestimated and was not always consistent. Preparing standards from sodium fluorescein salt is a much more accurate method.

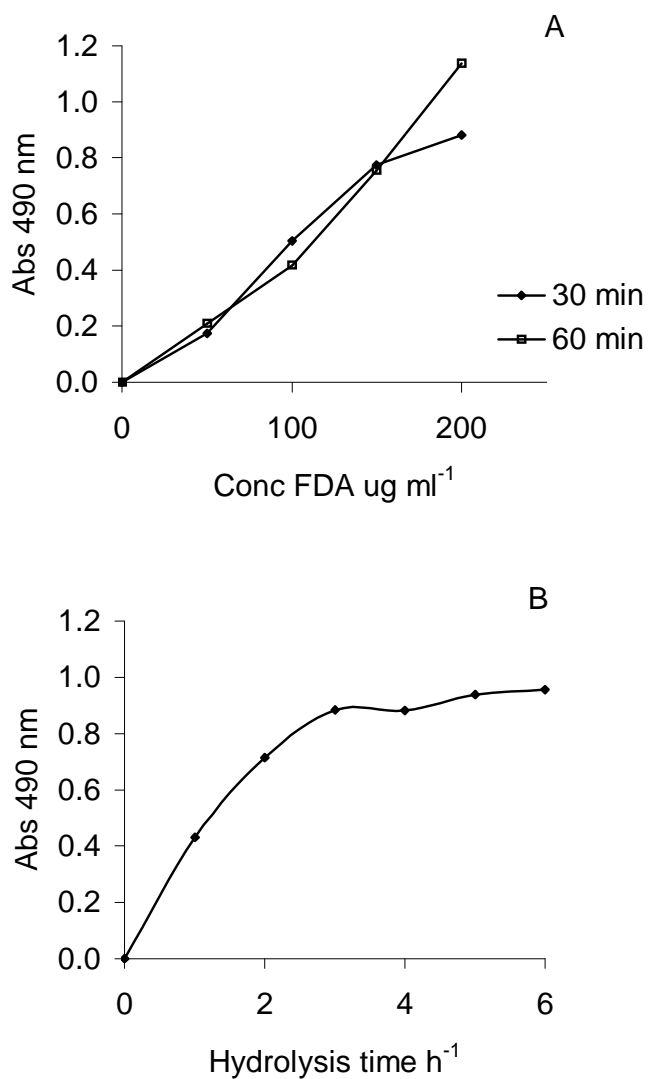


Fig. 6 (A) Fluorescein diacetate (FDA) hydrolysed in boiling water bath (diluted 1 : 1 in 60 mM potassium phosphate buffer pH 7.6) and (B) continued hydrolysis of 100 µg FDA standard in boiling water bath.

4. Conclusions

The potential of fluorescein diacetate (FDA) hydrolysis as a measure of total microbial activity has been recognised by many authors and used on a wide range of samples. The most frequently used method for measuring FDA activity in soil was found to be limited in the range of soil types it could measure successfully. The method described in this study critically assessed each individual parameter of the FDA hydrolysis assay and optimised each one for the measurement of a wide range of soils.

The most important parameter assessed during this study was the choice of solvent for terminating the reaction. By changing the solvent from acetone (50% v/v) to chloroform/methanol (2:1 v/v) low activity soils, such as sandy and clayey soils, could be measured successfully. This increased sensitivity was achieved as no loss of colour was observed when chloroform/methanol (2:1 v/v) was used.

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