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Nitrogen mineralisation and enzyme activities in Philippine paddy soils

by

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Abstract

Rice soils are known to be of comparatively low fertility, with N supply being the major constraint for rice growth. To examine aspects related to that topic, the International Rice Research Institute (IRRI, Los Baños, Philippines) established a long-term continuous cropping experiment (LTCCE) in 1963, with four different rates of N fertiliser as the mainplots. In order to examine possible differences in processes linked to the nutrient cycling, three methods of examination were applied to the LTCCE soils during this study:

- microplate fluorometric assay to examine the activities of acid phosphatase, *ß*-glucosidase, glucosaminidase, cellobiohydrolase and leucine-peptidase
- ^{15}N isotope dilution technique to estimate gross N mineralisation
- anaerobic incubation for 1 week at 40°C to determine mineralisation under waterlogged conditions

Activities of glucosidase, glucosaminidase and leucine-peptidase were found to increase with increasing rates of fertiliser application, whereas phosphatase and cellobiohydrolase showed no significant differences between the four rates of N amendment. Gross mineralisation rates were around 6 µg N g^{-1} d^{-1} for all samples examined. Negative mineralisation values were obtained from the anaerobic incubation, most probably due to N losses via denitrification and NH_3 volatilisation.

Correlation analyses showed that all enzyme activities were significantly correlated with each other and - except for cellobiohydrolase - with organic matter content estimated by loss on ignition. No correlation between enzyme activities and gross mineralisation rates was found.

It is concluded that the fluorometric enzyme assay has the potential of a fast and useful predictive tool for nutrient cycling in paddy soils, especially if future studies should reveal correlations between enzyme activities and the results obtained by applying the version of anaerobic mineralisation which has been modified by IRRI for waterlogged soils.

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1 Introduction

1.1 Paddy fields and soil fertility

Approximately 75% of the world's rice is produced in puddled fields where irrigation is guaranteed for at least one crop per year. Of this, 92% is produced in Asia, primarily for domestic consumption. In 1990, the average rice yield on 74 million ha harvested in Asia was 4.9 t ha^{-1} (IRRI, 1991). Continuous annual double- or triple-crop systems are established on more than 12 million ha and account for about 25% of global rice production (CASSMAN & PINGALI, 1995).

It is a remarkable feature of paddy soils that they have a basic inherent fertility due to biological nitrogen fixation, which allows grain yields of about 3 - 5 t ha^{-1} without any addition of mineral fertiliser. Achieving yields above that figure, however, has often proved to be difficult, and DE DATTA & CRASWELL (1982) point out that "the low fertility of rice soils and a limited supply of inorganic fertilizers are serious constraints to increased rice yields in South and Southeast Asia."

Rapid population growth leads to the demand for increased production. It has been estimated that an increase of almost 70% over the next 35 years has to be accomplished if the supply of rice is to match the demand. Taking into account the relatively low yields (around 2 t ha^{-1}) and the fragility of rain-fed and upland rice systems, the present lack of investment in irrigation. and the pressures caused by urbanisation, most of this extra production will have to come from increased productivity in the irrigated systems of today.

In order to increase yields, about 7 Mt of elemental N was applied as fertiliser to irrigated rice in Asia in 1990. Yields, however, did not always respond to fertiliser

application rates. In the long-term continuous cropping experiment (LTCCE) of the International Rice Research Insitute (IRRI, Los Baños, Philippines) for example, grain yields of triple-cropped rice declined from 1968 to 1991 at an annual average rate of 1.4 to 2.0% (DIZON, 1995). This occurred in spite of regular fertilizer applications and the goals of maximum annual grain production and nitrogen use efficiency. At the same site, yields started to increase after significant changes in crop management (DOBERMANN *et al.*, 2000).

At the basis of the discussion about sustainable rice production is the search for reliable indicators to show the efficiency of nutrient cycling and thus availability of nutrients to the crop. Numerous authors have suggested various soil parameters, most of them linked to the nitrogen dynamics prevailing in a paddy field.

1.2 Nitrogen dynamics in a paddy field

1.2.1 A complex system

CASSMAN *et al.* (1998) described the nitrogen dynamics in the soil-floodwater-rice system as "perhaps one of the most complex biophysical systems in agriculture". The main reason for this lies in the distribution of O_2 in a flooded paddy field: the top few millimetres (1-20 mm) and regions in close proximity to roots are aerobic, whereas reductive sites exist immediately under (and in) the aerobic layer. Accordingly, several metabolic processes, i.e. mineralisation, assimilation of NH_4^+ and NO_3^-, nitrification, NO_3^- reduction and N fixation occur simultaneously in the surface layers of a paddy field. All together, these processes form an interrelated complex, and finally affect N absorption by rice plants.

1.2.2 N inputs

^{15}N experiments have shown that rice obtains half to two-third of its N requirement from the soil mineralisable nitrogen pool. Even in fertilised paddies the mineralisable N plays a dominant role in the nitrogen nutrition of rice (SAHRAWAT, 1983). The LTCCE soil at the IRRI Los Baños site contains approx. 0.2% total N (= approx. 2.5 t N ha^{-1}).

Mineralisation of organic N in paddy fields is affected by several environmental factors, most importantly by temperature and moisture; BROADBENT (1979) also mentions organic amendments, nature and amount of organic matter and clay content.

The influence of temperature was examined by IRRI. A study showed that mineralisation of organic nitrogen in four soils under anaerobic incubation increased with the increase in temperature from 15°C to 45°C (IRRI, 1974).

The influence of the moisture regime will be viewed in more detail. It is critical for mineralisation of soil N because the pattern of mineral N release is affected by soil drying. In order to simulate field conditions, mineralisation has mostly been determined anaerobically. ANDO *et. al.* (1992) incubated Maahas clay samples from IRRI lowland paddy fields at 20, 25 and 30°C. During the first week they obtained mineralisation rates of 0.006, 0.009 and 0.019 µg NH_4-N g^{-1} soil d^{-1}. These rates remained fairly constant over the whole 13-week period of anaerobic incubation. SAHRAWAT (1980*b*) added 100 µg g^{-1} NH_4-N to Maahas clay and incubated anaerobically (200% water content) at 30°C for several weeks. As it can be seen in Table 1-1, the NH_4^+ concentrations remained constant over time. Ammonification was obviously impeded by waterlogging, whereas decreasing NO_3^- values indicated denitrification losses. N serve did not have a significant influence on anaerobic mineralisation.

N-serve [ppm]	weeks incubated									
	0		1		3		5		7	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-
0	82	12	79	1	84	1	79	1	81	0
2	82	12	89	1	85	0	87	0	83	0

Table 1-1: concentration of NH_4-N and NO_3-N (µg g^{-1} soil) at different times of extraction

The same author also examined the effect of air-drying prior to anaerobic mineralisation in four Histosols from Laguna, Philippines (SAHRAWAT, 1981). Figure 1-1 shows that ammonification has taken place during the air-drying process, which also continues for approximately four weeks after flooding. The NH_4-N however stayed constant over the whole 8-week period and even decreased after 6 weeks of submergence.

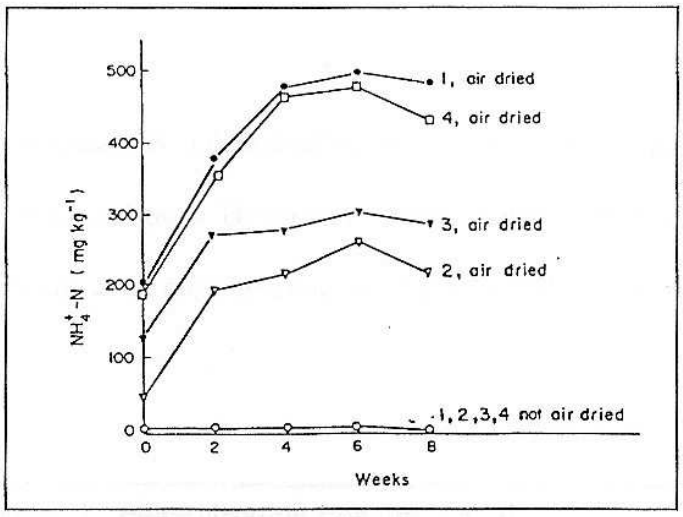

Figure 1-1: effect of air-drying on ammonification in four water-logged Histosols (SAHRAWAT, 1981)

When the author incubated air-dried Philippine paddy soils (SAHRAWAT, 1983), values varied between 17-428 µg g^{-1} (at 30°C for 2 weeks) and 13-522 µg g^{-1} (at 40°C for 6 days) mineralisable NH_4^+. The fraction of mineralisable NH_4^+ was found to be 1.4 to 11.8% of the total N in the soils examined.

NISHIO *et al.* (1993) examined heavy clay soils from Japanese paddy soils using $(^{15}NH_4)_2SO_4$ as a tracer at two different concentrations (0.25 mM, 0.50 mM). The samples were incubated anaerobically at 25°C without previous air-drying. During the initial 8 days, amounts of NH_4^+ increased very slowly - from 12 to 14 µg N g^{-1} at 0.25 mM and from 18 to 19 µg N g^{-1} at 0.50 mM. With time, production of NH_4^+ in the submerged soils then accelerated and reached a maximum after 40-60 days after which they declined again. As likely reasons for this observation the authors suggested that a) soil microorganisms grew in the stable conditions during submergence, and/or b) there was a time lag before NH_4^+ was produced under anaerobic conditions because of the slowness and incompleteness of the anaerobic decomposition of the native organic matter.

NISHIO (1994) compared N mineralisation rates for Japanese paddy soils under anaerobic and aerobic conditions. He set up a simple nitrogen transformation model and calculated the mineralisation rates by using the derived mathematical equations and ^{15}N tracers.

treatment	**mineralisation rate [µg N g^{-1} d^{-1}]**
aerobic [a]	*at 25°C*: 12 (0-20 h), 12 (20-44 h), 10 (44-69 h)
aerobic [b]	*at 25°C*: 9 (0-7 h), 10 (7-12 h), 9.5 (12-24 h) *at 30°C*: 12 (0-7 h), 13 (7-12 h), 12 (12-24h)
anaerobic	*0-1 cm*: 11.3, *1-10* cm: 2.3, *10-20* cm: 1.9

[a] 3 g of soil incubated; 5 mg N l^{-1} of $(^{15}NH_4)_2SO_4$ and $K^{15}NO_3$ at 20.1 at.-% added
[b] 20 g of soil incubated; 10 mg N l^{-1} of $(^{15}NH_4)_2SO_4$ and $K^{15}NO_3$ at 20.1 at.-% added

Table 1-2: rates of mineralisation determined by NISHIO (1994)

Under aerobic conditions, mineralisation rates were found to be constant within one treatment and highest for the incubation at 30°C. The rates of N transformations under the anaerobic condition were dependent on the sampling depth, with the values for the top layer being significantly higher than for the completely anaerobic subsurface layers. This indicates that N mineralisation in the surface layer of paddy soils is quantitatively of great importance even though the process only happens in a small fraction of the overall soil mass.

All authors working with labelled substances used emission spectrometry in order to determine ^{15}N concentrations of ammonium and nitrate. No literature could be found on past determinations of gross mineralisation rates in paddy soils by using the ^{15}N isotope dilution technique.

Other significant sources of N supply in paddy fields besides the mineralisation of SOM are:

- **fertiliser application**: prilled urea and ammonium sulfate are the least expensive and most common fertiliser sources used on rice; Appendix 1 gives an overview over the amounts of N fertiliser added at the LTCCE site since 1968
- **atmospheric deposition:** previous measurements indicate very small amounts at the IRRI LTCCE site; APP ETAL. (1984) found total N amounts of 2.45 kg ha^{-1} a^{-1} in 1978 but values below 1 for the three following years
- **irrigation water:** estimated to be less than 3 kg ha^{-1}, because N in well water is very low (CASSMAN *et al.*, 1996)

- **incorporated plant residues:** all straw and stubble residues were always removed at the LTCCE site; root biomass and stem bases, however, still account for 0.6 Mt of N annually on 74 Mha of harvested irrigated rice in Asia (= 8.1 kg N ha^{-1}), assuming root dry matter and stem bases represent 10% of the above-ground biomass at maturity and have an N concentration of 0.8% (CASSMAN *et al.*, 1998)
- **biologically fixed N:** estimated at 15-70 kg N ha^{-1} per rice crop cycle (ESKEW *et al.*, 1981); without N application, grain yields of about 3 - 5 t ha^{-1} can be supported; major source of N in unfertilised wetland rice
- **subsoil N:** this fraction is difficult to measure, because it would involve soil disturbance by placing a root barrier; microplot studies using this method in flooded rice soils gave estimates of 5-30% of total plant N derived from subsoil
- **release of fixed NH_4-N** from micaceous minerals: release and uptake of fixed NH_4-N was found to occur in lowland rice soils with NH_4-fixing minerals (KEERTHISINGHE *et al.*, 1984); ammonium fixation in the LTCCE however is supposed to be negligible

Finally it should be noted that organic sources of N in soils differ from fertilizer sources in that the mineralisation process plays a more important role in controlling initial N availability from the added nitrogen source. This is of special significance in flooded soils where the relatively low N requirement and low synthetic efficiency of anaerobic microorganisms usually lead to a more extensive mineralisation and lower critical limit for N in organic substrates. Measurements of available N in flooded soils, amended with organic and fertilizer N, have shown a gradual increase over time for the former and decreases for the latter (SAHA *et al.*, 1982).

1.2.3 N outputs (losses)

The main reason for low nitrogen efficiency in paddy fields is the rapid immobilisation of N fertilizer occurring immediately after flooding. It has been estimated that 10 to 66% of the added N source is immobilised within 20 d after flooding. KAI & WADA (1979) reported that a third of the initially immobilized N is remineralised and actually available for plant use. The rates of immobilisation depend on factors such as method of application, soil type, presence of organic substrate, and previous moisture status of the soil (REDDY & PATRICK, 1980).

Because flooded soils tend to stabilise at pH values just above 6.5 or higher, added NH_4^+ might be lost locally by NH_3 volatilisation. BURESH & DE DATTA (1990) reported that most gaseous losses in puddled Philippine rice soils resulted from NH_3 volatilisation rather than from losses due to denitrification.

SAHRAWAT (1980*b*) however notes that an appreciable part of both native and applied N in flooded rice soils is lost by nitrification-denitrification processes. In the aerobic surface layer of the paddy field, NH_4^+ released from SOM or added as fertilisers is converted to NO_3^-. After entering the anaerobic zone by mass flow or diffusion, it is rapidly lost to dissimilatory denitrification, serving as an electron acceptor for microbial anaerobic respiration; the resulting products, N_2 and N_2O, both of which are gases, are then lost from the system. Recent research from ZHANG *et al.* (1999) has shown that increasing concentrations of non-exchangeable NH_4^+ after flooding were closely correlated with declining redox potentials This would mean that Eh may indeed have an important impact on the fate of NH_4^+ in paddy soils. As mentioned before, the profile of a paddy soil can be divided into an aerobic and an anaerobic layer, which leads to an Eh

gradient from the top to the bottom of the plough layer. This gradient can cause differences in NH_4^+ fixation between oxic and anoxic zones of the flooded soil.

Only NH_4^+ which is fixed by soil minerals or transformed to resistant organic matter will not be immobilised rapidly. The percentage of NH_4^+ thought to be fixed on micaceous minerals is mostly negligible. Furthermore, MENGEL & SCHERER (1981) have shown that the so-called "fixed NH_4^+" of clay minerals can be released or exchanged in considerable quantities and thus enter into the general soil N cycle of a paddy field. Additionally, fixation of ammonium could be inhibited by SOM, because organic molecules of low molecular weight may hinder the diffusion of NH_4^+ ions into the interlayers of clay minerals or the contraction of the interlayers.

1.2.4 Practical consequences

NISHIO *et al.*, (1993) nicely summarise the above by stating that the continued recycling of N between the organic and inorganic pools of a paddy soil is a distinct property of an anaerobic soil, compared to an aerobic soil, where NO_3^- produced by nitrification is most often not readily immobilised by soil microorganisms.

As a matter of fact, data from long-term experiments at several locations in the Philippines (DE DATTA *et al.*, 1988) and India (NAMBIAR & GOSH, 1984) prove that N is the most limiting nutrient in continuous annual double- or triple-crop systems. A reliable indicator for N availability must be identified to enable fertiliser recommendations to be made effectively.

According to several workers the incubation methods, though time consuming, provide a good index of soil nitrogen availability (e.g. KOYMA 1971). Ammonia released during incubation at 30°C for 2 weeks or at 40°C for 6 days has been repeatedly suggested as

an index of nitrogen availability to rice (e.g. SIMS *et al.*, 1967). CHANG (1978) reported that the initial NH_4-N plus the ammonia released after waterlogged incubation for 1 week gave the best assessment of the soil nitrogen availability to rice.

Chemical indeces proposed include measurement of organic carbon and total N contents or of mineral nitrogen released by extraction or digestion with neutral, acid or alkaline reagents. The ammonium released from SOM by boiling with alkaline permanganate was proposed by TRUOG (1954).

CASSMAN *et al.* (1996) evaluated the relationship between the indigenous N supply (N_i) and total N (N_t) in surface soil of long-term fertility experiments (LTFE) at IRRI, Philippines. They obtained a tight linear correlation between yield and N uptake in N_0 (no added fertiliser) plots, but immense variation in all other plots. Most probably this was due to N inputs from other sources than N mineralisation of SOM (see Section 1.2.2). Differences in SOM quality related to intensive cropping in submerged soil might have also been of influence. They conclude that soil organic carbon or total nitrogen are not sensitive indices of N_i in tropical lowland rice systems. Actual N uptake from the soil-floodwater system was selected as the most useful index of indigenous nutrient supply because a) its measurements is relatively easy and b) it is tightly correlated with grain yields.

The ability to predict the indigenous nitrogen supply of paddy fields would help farmers to adjust their N rates accordingly to increase N fertiliser efficiency, and would help to develop practices to maintain or increase this indigenous N supply.

At present, recommendations for irrigated rice transplanted in the Philippines call for 50 to 67% of total fertiliser-N inputs to be broadcast and incorporated before transplanting and the remainder topdressed at 5 to 7 days before panicle initiation.

1.3 Enzyme activities

Perpetuation of life on earth is conditioned by the mineralizing action of soil and water microorganisms on the plant and animal residues. This mineralizing action is inseparably related to the activity of enzymes. These two statements are easily formulated, but in a more detailed discussion many open questions remain: do the enzymes accumulated in soil precisely reflect the decomposition and mineralization processes of the proliferating microorganisms? Do they therefore have a real significance in the biological cycle of elements? Do these enzymes contribute to soil fertility and to the creation of conditions favourable for the nutrition of higher plants?

Because of their important role in nutrient cycling, hydrolytic enzymes are indeed most adequate when we look for possible indicators of soil fertility. Almost thirty years ago, KHAZIEV (1972) stated that P-hydrolysing enzymes play an important role in soil fertility. He had found a positive correlation between phosphatase and nuclease activities and soil mobile P content as well as P uptake from the soil by wheat, barley and millet. He finally suggested the measurement of four activities, including phosphatase, invertase, catalase and urease.

Table 1-3 shows a short characterisation of the five soil enzymes examined during my study.

Enzyme	involved in	function
phosphatase	P cycle	catalyses the hydrolysis of organic phosphate esters and anhy-drides to ortho-phosphate; thus constitutes an important link between biologically unavailable and mineral P pools in soil
ß-glucosidase	C cycle	catalyses the hydrolysis of glucosides; cellodextrins and cellobiose are attacked and transformed to glucose
glucosaminidase	C cycle	catalyses the reaction of glucosaminic acid to 2-keto-3-deoxygluconate + NH_3
cellobiohydrolase	C cycle	attacks the ends of cellodextrin chains and leaves cellobiose as final product; this is further degraded by *ß*-Glucosidases
leucine-peptidase	N cycle	catalyses the rapid hydrolysis of L-leucylglycine and L-leucin-amide and other peptides with NH_2-terminal leucine; releases free amino acids from the N terminus of the peptide substrate

Table 1-3: enzymes examined in this study and their characteristics

Enzyme activities are influenced by many factors, with waterlogging probably being the most important one in paddy fields. However, little information is available on the effect of waterlogging on enzyme activities. In spite of considerable interest in the chemistry of reduced soils, especially in redox-reactions, studies have not been extended to include enzyme-catalysed reactions in soils.

From a theoretical point of view, changes in redox potential taking place after flooding should have a considerable effect on enzyme activities in soils because a) changes in microbial populations take place in adaption to a reduced environment, resulting also in the synthesis of additional enzymes, and b) the reduced metal ions produced could serve as inhibitors or activators of enzymes.

PULFORD & TABATABAI (1988) measured the activities of eight enzymes involved in C, N, P, and S cycling in soils before and after water-logging for times ranging from 0 to 10 days at room temperature (22°C). Surface samples (0-15 cm) from common soil series in Iowa were used, which displayed clay contents between 14-29%. In their experiment, redox potential (Eh) decreased after flooding, which had a striking effect on the reaction rates of the examined enzymes. Changes in acid phosphatase activity in the 10 soils, for example, ranged from +2 to - 59% (average -29%). Changes in *ß*-glucosidase activity were partly positive (6 soils, +4 to +43%), partly negative (4 soils, -11 to -43%); the soils which originally contained relatively low *ß*-glucosidase activity, showed an increase of the activity of this enzyme; no significant correlation with Eh_7 could be detected.

Decreases in enzyme activities were suggested to occur due to inhibition by the reduced metal ions produced upon waterlogging of soils. These ions have greater solubilities in water than their oxidised counterparts. Fe^{2+}, Mn^{2+} and other metal ions are already known to be strong inhibitors of phosphatases in soils (STOTT *et al.*, 1985).

Most authors have determined soil enzyme activities by using the methods described in TABATABAI (1982). The application of fluorometric assays on enzyme activities in flooded soils has been extremely rare. FREEMAN *et al.* (1995) examined Welsh peat soils for several enzymes, using the MUB-method (see Section 2.4). Table 1-4 shows the results of their measurements.

depth (cm)	MUB release (µmoles min^{-1} g^{-1})			
	Cerrig yr Wyn peat		Ogwen valley peat	
	phosphatase	glucosidase	phosphatase	glucosidase
0	30	7	1.9	0.5
10	4	2	2.2	0.6
20	6	3	1.1	0.4

Table 1-4: enzyme activities measured by FREEMAN *et al.* (1995)

A distinct feature of paddy soils is their high clay content (61% at the LTCCE site). Although many hydrolases are intracellular, they are also found associated with clay, cell debris and organic colloids. Mechanisms by which enzymes can be immobilised on clay minerals include cation exchange, physical adsorption and ionic binding. It is commonly anticipated that enzymes bound to clay and humic colloids - the so-called immobilised enzyme fraction - have a residual activity not found in enzymes free in the soil aqueous phase. SARKAR *et al.* (1989) immobilised various enzymes (among them acid phosphatase and ß-D-glucosidase) on clays minerals. After immobilisation the enzymes retained a large amount of their original activities. They also found that activities of phosphatase and glucosidase decreased with increasing clay content.

In paddy fields as well as in all other agricultural systems, sustainable management is highly important to preserve “soil quality“. It is therefore important to determine the effect of different organic and inorganic inputs on SOM. Changes in the quality and quantity of soil organic matter are known to occur slowly and to be difficult to quantify in short-term studies, especially because they are small in relation to the large background of organic matter and the natural soil variability. Due to their dynamic nature, soil microbial biomass and soil enzymes respond quickly to changes in organic matter and fertiliser input.

DEBOSZ *et al.* (1999) examined glucosidase and cellobiohydrolase for low and high-OM treatments. The high-OM treatment showed consistently higher cellobiohydrolase activity (24-92%) than the low-OM treatment. *ß*-glucosidase followed a similar pattern and was approx. 40% higher in the high-OM treatment than in the low-OM treatment on all sampling dates.

BOLTON *et al.* (1985) measured biomass and selected enzyme activities to see the effect of fertilisation and cropping practices. All three enzyme activities measured were significantly higher in the soil from management system 1 (only N input since 1909 from leguminous green manure crops) than in the one from management system 2 (regular applications of anhydrous ammonia, P and S at recommended rates for the last 20 years).

The contrasting behaviour of enzyme activities to an oganic N source (vetch) compared to $(NH_4)_2SO_4$ as an inorganic N source was examined by WESTCOTT & MIKKELSEN (1985). When they regressed available N against cumulative CO_2 production across all vetch treatments and controls in flooded soil, they got highly significant correlations (r^2 = 0.79 - 0.91, depending on incubation time). However, regression of apparent soil N mineralisation against cumulative CO_2 production did not show any significant relationship (r^2 = 0.00 - 0.28). The authors therefore concluded that the priming effect of $(NH_4)_2SO_4$ on soil-N mineralization was not due to a stimulation of microbial activity as defined by CO_2 production.

LADD (1978) proved that in case of maize, phosphatase activities of field soils showed little or no variation with season, but are influenced by the cropping system or the nature of the plant cover. Activities generally increased after the addition of inorganic and organic fertilizers to the system.

1.4 Objectives of my study

The literature review has shown that understanding soil fertility in paddy fields is based on a complex mixture of biological, physical and chemical properties. In order to assess the fertility status of a flooded soil and to be able to give fertiliser recommendations, therefore no single soil property alone can be used as reliable indicator.

During the present study attention was focused on three measurements, which all could be possible "mosaic pieces" to provide insight into the fertility of the examined soils:

- ^{15}N isotope dilution technique to estimate gross mineralisation; this method has been characterised as the most powerful method to analyse the N cycle in soil, particularly when several processes are responsible for temporal changes in the pool size of each N compartment
- anaerobic mineralisation at 40° for 1 week
- determination of enzyme activities involved in the soil C, N and P cycle, using a recently developed fluorimetric microplate enzyme assay

As hydrolytic enzymes are directly responsible for nutrient availability, correlation studies will be done between the outcomes of the above experiments and also involving yield data and fertiliser application rates.

The outline of the present study is in line with ZAMAN *et al.* (1999) who noted that "understanding the regulatory mechanisms of extracellular enzymes and microbial activity in relation to N mineralization in soil may provide insights into N mineralization dynamics and help the efficient utilization of organic wastes with minimized environmental impacts."

2 Materials and Methods

2.1 Origin and nature of the soil samples

The samples were sent from IRRI (International Rice Research Institute), Laguna, Philippines. At their Los Baños site (14°11' N, 121°15' E, 21 m asl), a long-term continuous cropping experiment (LTCCE) has been established in 1963, which now is the longest running experiment of triple-cropped rice (*Oryza sativa* L.). The dry season (DS) crop is transplanted in January and harvested in April, the early wet season crop (EWS) from May to August and the late wet season crop (LWS) from September to December. Mean annual rainfall is 2030 mm with pan evaporation exceeding rainfall in 5 months from January through the first week of June. Mean air temperatures are 26°C (DS), 28°C (EWS) and 27°C (LWS).

At present, the LTCCE experimental set-up involves a 2-factorial split-plot with N fertiliser rates as mainplots and rice varieties as subplots with 4 replications. During the examination no regard was given to the subplots, as IRRI was only interested in differences between the four N rate treatments. The most recent N fertiliser rates were F1 = 0 kg ha^{-1}, F2 = 45 kg ha^{-1}, F3 = 90 kg ha^{-1} and F4 = 135 kg ha^{-1}. All crop residues were removed. Appendix 1 shows the detailed N management of the LTCCE and the changes made over time since 1968. All other nutrients are applied uniformly to all plots to avoid deficiencies.

Following the USDA classification (Soil Survey Staff, 1994), the soil has been classified as Aquandic Epiquoll containing amorphous materials and smectites as dominant clay minerals. According to CASSMAN *et al.* (1996), the clay content is 61% and the CEC is 37 $cmol_c$ kg^{-1}.

The soil provided by IRRI was sampled on 10 May 2000, two days after planting the EWS crop. For harvest the soil had been drained 2 weeks before and flooded again shortly after sampling. The sampling depth was 0-15 cm with a given bulk density in that layer of 0.63 g cm^{-3} (CASSMAN *et al.*, 1998). Nine soil samples of at least 2 kg were sampled in each of the 16 plots, mixed and a subsample of 1 kg for each plot put into a plastic bag. During the transport, leakage did not occur and the samples arrived in Reading in a moist state.

All work related to this study was performed between May and July 2000.

2.2 Soil processing

Approximately 500 g of each sample have been dried and ground.

Photo 2-1: soil sample during the drying process

For drying, the soil was placed onto acid-washed sand. A nylon mesh (2µm) was put in between to avoid contamination of the soil. All samples were oven-dried at 35°C until they reached a water content which was supposed not to endanger microbial activity on the one hand, but on the other hand allow grinding.

Pestle and mortar were used to grind the samples. Subsequent sieving proved impossible, as due to the clayey structure of the soil, aggregates of approximately 1 cm in diameter were still present which could not be crushed any further.

All samples were stored in the cold room (2°C) before and after processing. In the following, I will either refer to "fresh" (= unprocessed) or "processed" (= dried and ground) soil.

2.3 General soil parameters

2.3.1 pH

pH has been measured in deionised water and 0.01 M $CaCl_2$. A soil suspension was made up in deionised water using a 1:2.5 soil:solution ratio (5g processed soil, 12.5 ml solution). The soil suspensions were shaken for 15 minutes on a rotational shaker and the pH subsequently measured using a glass electrode. The electrode was calibrated with pH 4 and pH 7 buffer solutions. 125 µl of 1 M $CaCl_2$ were then added to each sample and after shaking for 30 min the pH values were determined again.

2.3.2 Water content

10.00 g of fresh soil were transferred into a weighed porcelain crucible and placed in an oven at 105°C overnight. After cooling in a desiccator, the crucibles were re-weighed and the water content calculated as follows:

$$water\ content[g\ H_2O\ g^{-1} oven-dry\ soil] = \frac{mass\ of\ water\ lost[g]}{mass\ of\ oven-dry\ soil[g]} \qquad \textbf{Equation 2-1}$$

2.3.3 Loss on ignition

To get a rough measure of the present organic matter content, loss on ignition has been determined for all samples. The samples used for determination of the water content were placed in a furnace at 500°C overnight. After cooling in a desiccator, the crucibles were re-weighed and the loss on ignition calculated as follows:

$$loss\ on\ ignition\ [g\ 100\,g^{-1}\ oven-dry\ soil] = \frac{100\cdot(mass\ od\ \ soil\ -\ mass\ ignited\ \ soil)}{mass\ od\ \ soil}$$

Equation 2-2

2.4 Measurement of enzyme activities

A recently developed fluorimetric microplate enzyme assay (MARX *et al.*, 2000) has been applied: conjugates of the highly fluorescent 4-methylumbelliferone (MUB) and 7-amino-4-methyl coumarin (AMC) are chemically attached to the enzyme substrates causing the loss of the fluorescent property; when the bond between MUB and the attached component is hydrolysed, the MUB is liberated and its fluorescence can be measured and subsequently related to the activity of the hydrolytic enzymes.

All samples have been examined for phosphatase, glucosidase, glucosaminidase, cellobiohydrolase and leucine-peptidase (for short description of the enzymes, see Section 1.3). No kinetic studies have been made.

2.4.1 Preparation of samples

2 g of fresh soil were transferred into an autoclaved honey-jar and a soil suspension made by adding 98 ml of sterilised water. For dispersion, the suspension was vigorously shaken by hand and afterwards sonicated at 50 J s^{-1} for 120 seconds (ultrasonic disintegrator MSE Soniprep 150).

Three suspensions (= "true" replicates) have been prepared for each sample.

2.4.2 Preparation of reagents

The substrates used were 4-MUB-phosphate, 4-MUB-β-D-glucoside, 4-MUB-N-acetyl-β-glucosaminide, 4-MUB-β-D-cellobioside and L-leucine-7-AMC (obtained from Sigma-Aldrich Co. Ltd.). Under sterile conditions, 10 mM stock solutions were prepared in deionised water and 1mM working solutions in a buffer.

1µM MUB and AMC standard working solutions were prepared using existing 10 mM stock solutions.

The following buffers have been used to standardise the method: 0.1 M MES (2-[N-morpholino]ethanesulfonic acid; pH 6.1) for glycosidases and phosphatase; 0.05 M Trizma buffer (pH 7.8) for leucine-peptidase.

2.4.3 Microplate set-up

Due to two different substrates being used, two microplate set-ups had to be established. In the case of the MUB substrate, a set-up has been chosen which allows the examination of 2 soils per microplate. As Figure 2-1 shows, each enzyme was tested on 3 samples of 3 suspensions, which gave a total number of 9 replicates for each sample.

Figure 2-1: microplate set-up for MUB substrates

The amount of soil suspension filled into each well was 20 µl which equals 400 µg soil. In the case of all substrates, 60 µl of the working solution were used in each well, equalling 60 nmoles and a concentration of 300 µM. The standard ranged from 0 µl (blank) up to 140 µl (= 140 pmol MUB). All wells were filled up to 200 µl using MES buffer. For all pipetting processes automatic pipettes have been used (FinnPipette; Jencons Sealpette 1200). Pipette tips were sterilised prior to usage.

In the case of the AMC standard, where only leucine-peptidase was examined, six samples were fitted on one microplate (Figure 2-2). Three soil suspensions with 2 replicates each provided a total of 6 replicates for each soil sample. The amounts of soil suspension, substrate, standard and buffer were the same as for the MUB microplate set-up.

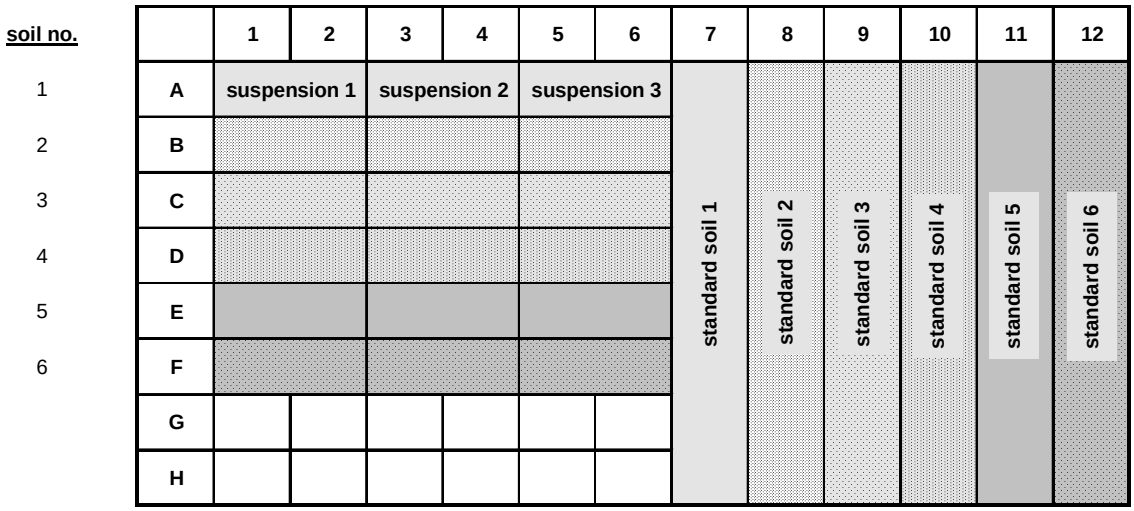

Figure 2-2: microplate set-up for AMC substrate

2.4.4 Measurement of fluorescence and calculation of results

A computerised microplate reader (Biolumin™ 960, Molecular Dynamics Inc.) was used to determine the developing fluorescence. MUB fluoresces at 450 nm after excitation at 330 nm. The temperature was adjusted to 30°C. The machine reads the rate of fluorescence increase in each of the 96 micro-wells for 35 minutes and afterwards automatically calculates a regression line of the collected data.

By using the standard readings, a calibration curve was established for each soil sample. After correcting for background fluorescence the equation of the calibration curve was used to transfer the readings for the rate of fluorescence increase in each well (fluorescence min^{-1}) into units of pmoles MUB min^{-1}. Plotted against the soil used, the results were displayed in units of nmoles MUB min^{-1} g^{-1} oven-dry soil.

2.5 Determination of mineralisation rates

2.5.1 ^{15}N isotope dilution technique

The rate of gross mineralisation under aerobic conditions has been studied by using the stable nitrogen isotope ^{15}N as a tracer (BARRACLOUGH *et al.*, 1985). A small quantity of ^{15}N is added to the soil. Given that it mixes with the indigenous soil NH_4^+, the decline in the ^{15}N abundance in the NH_4^+ pool over time will be a direct function of the rate at which mineralisation introduces unlabelled NH_4^+ into the pool.

Processed soil was used for this experiment.

2.5.1.1 Sample preparations

Incubations were conducted using the equivalent of 30 g oven-dry soil. The soil was weighed and packed into 2 cm sections of 6.5 cm diameter plastic cylinder, which were covered at the base with 280 µm nylon mesh (Stanier and Co., Manchester). Following NISHIO (1994) the water content was adjusted to 60% (0.6 g water g^{-1} oven-dry soil) by adding the necessary amount of deionised water. To allow free gas exchange the samples were kept uncovered and therefore had to be watered each day to maintain the optimum water content. The incubation temperature was 26°C; the samples were kept in the dark to avoid uptake of nitrate by the photosynthetic microorganisms.

When rice seedlings unexpectedly started growing after approximately seven days, all of them were removed as soon as they were discovered. However, in some cases fine root material had to remain within the cylinder, as it was interwoven with the nylon mesh at the bottom.

Photo 2-2: ^{15}N samples during incubation

Six replicates (three for each of the two extractions) were made for most of the samples. However in some cases due to lack of soil only five replicates could be employed.

After 16 days of incubation, 10 µg N g^{-1} oven-dry soil as $(^{15}NH_4)_2SO_4$ at 5.1 at.% were added to each cylinder. To achieve uniform distribution, the prepared liquid was spread in a grid pattern onto the sample by slowly releasing it as small drops from a 1 ml syringe. The solution was readily absorbed by the bigger aggregates of soil, making an injection of the liquid into the soil unnecessary.

Three cores from each sample were sampled after 1 day, the other three after 4 days. All of the soil from each core was shaken with 200 ml 1M KCl for 1 hour at 200 rotations min^{-1}. Vigorous shaking was chosen to disrupt the soil clumps present and thus guarantee optimum extraction conditions. The extracts were filtered through Whatman GF/A glass microfibre filter paper and stored in the cold room (2°C) until analysis

2.5.1.2 Analyses

All extracts were examined colorimetrically for NH_4^+ and NO_3^- using a Tecator FIAstar 5010 flow injection auto-analyser.

To measure the ^{15}N enrichments, the extracts were prepared following BROOKES ETAL (1989): 50 ml of each extract were transferred into Erlenmeyer flasks. A GF/D glassfibre disk ("acid trap") acidified with 10 µl 2.5 M $KHSO_4$ was placed on a hook at the bottom of the flask's bung. The diffusion process was started by adding 0.4 g MgO and the flasks left at room temperature for 7 days. Three blanks were prepared, containing 50 ml 1 M KCl and 0.4 mg MgO. Each day, all flasks were swirled shortly in order to optimise the diffusion and remove all the NH_4^+ present from solution. After diffusion the disks were transferred into the wells of a microplate and put into a desiccator to dry for several days.

Subsequently, each disk was put into a 8 x 5 mm tin capsule and shaped into a small package. ^{15}N standards were prepared using atropine (^{15}N enrichment at 2.36 at.%) and acetanilide (2.27 at.%). All samples were analysed for ^{15}N:^{14}N isotope ratios using a VG 622 mass spectrometer coupled to a Europa Scientific Roboprep combustion analyser.

In addition, ^{15}N enrichments in the NO_3^- pool were determined after the first diffusion process by adding 0.4 g Devarda's alloy to the extracts and thus reducing all the NO_3^- present to NH_4^+, triggering a second diffusion onto a new set of acidified disks.

2.5.1.3 Determination of gross mineralisation rates

The mass spectrometer results were corrected for the KCl blank and internal machine drift. The following equations of KIRKHAM & BARTHOLOMEW (1954) were used:

- gross mineralisation rate

$$m = \frac{M_0 - M_1}{t} \frac{\log\left(\frac{H_0 M_1}{H_1 M_0}\right)}{\log\frac{M_0}{M_1}} \qquad \textbf{Equation 2-3}$$

- consumption rate

$$c = \frac{M_0 - M_1}{t} \frac{\log\left(\frac{H_0}{H_1}\right)}{\log\frac{M_0}{M_1}} \qquad \textbf{Equation 2-4}$$

m = rate of gross mineralisation (unit: µg N g^{-1} oven-dry soil d^{-1})

c = rate of gross consumption = sum of immobilisation, autotrophic nitrification, volatilisation, and other possible fates (unit: µg N g^{-1} oven-dry soil d^{-1})

M_0 = $^{14+15}NH_4$-N pool at day 1 (unit: µg N g^{-1} oven-dry soil d^{-1})

M_1 = $^{14+15}NH_4$-N pool at day 4 (unit: µg N g^{-1} oven-dry soil d^{-1})

H_0 = ^{15}N pool at day 1 (unit: µg N g^{-1} oven-dry soil d^{-1})

H_1 = ^{15}N pool at day 4 (unit: µg N g^{-1} oven-dry soil d^{-1})

t = time between the two extractions = 3 (unit: d)

For reasons of comparison, an improved equation (ZAMAN *et al.*, 1999) has also been applied:

$$m = \theta \frac{\log\left(\frac{A_0^*}{A_t^*}\right)}{\log\left(1+\frac{\theta t}{A_0}\right)} \qquad \textbf{Equation 2-5}$$

m = rate of gross mineralisation (unit: µg N g^{-1} oven-dry soil d^{-1})

θ = rate at which the NH_4-N pool size changes = $[(A_t\text{-}A_0)/t]$ (unit: µg NH_4-N g^{-1} d^{-1})

A_0^* and A_t^* = ^{15}N excess abundance in the NH_4-pool at day 1 and day 4 (unit: at.%)

t = time between the two extractions = 3 (unit: d)

2.5.2 Anaerobic mineralisation

Mineralisation under waterlogged conditions has been examined following the method described by SSSA (1994), which is based on WARING & BREMNER (1964).

The equivalent of 5 g oven-dry soil and 12.5 ml deionised water were transferred into a 50 ml universal plastic tube. To guarantee complete covering of the "sticky" soil by water, all samples had to be shaken on a rotational shaker for 90 minutes. Six replicates were made, three for each of the two extractions. For the first (= time 0) extraction, 12.5 ml of 2 M KCl was added to half of the replicates. The samples were shaken on a rotational shaker and filtered through Whatman GF/A glass microfibre filter paper. The KCl extracts were then stored in the cold room (2°C).

The second half of the samples was incubated at 40°C for 1 week and extracted the same way. All samples were examined for NH_4^+ using a Tecator FIAstar 5010 flow injection auto analyser.

The rate of anaerobic mineralisation was calculated by

$$m = \frac{A_t - A_0}{t} \qquad \textbf{Equation 2-6}$$

m = rate of anaerobic mineralisation (unit: µg NH_4-N g^{-1} oven-dry soil d^{-1})

A_0 and A_t = amount of NH_4-N at time 0 and 1 week (unit: µg NH_4-N g^{-1} od. soil)

t = duration of incubation at 40°C = 7 days (unit: d)

2.6 Statistical analyses

GENSTAT® 5 for Windows Release 4.1 (fourth edition, 1998), was used to analyse the resulting data. To analyse differences between the four main plots (fertiliser treatments), one-way analyses of variance (ANOVA) were applied to water content, loss on ignition, pH, enzyme activities and gross mineralisation results. A difference was considered significant when $P < 0.05$.

Correlation was tested with the same statistical package. Pearson product moment correlation tables (one-sided test) were used to assess the significance of the findings. For the parameters yield and fertiliser application respectively, only four data were available. Although not stated each time in detail, correlations results obtained at $n = 4$ (d.f. = 2) have been interpreted with care because of their restricted meaningfulness.

3 Results

3.1 General soil parameters

3.1.1 pH

Table 3-1 shows the results for all pH measurements. Each figure represents the mean of the four subplots, which have all been sampled separately (e.g. “F1“ is the mean of F1-1, F1-2, F1-3 and F1-4).

soil sample (N treatment)	pH measured in deionised water	pH measured in 0.01 M $CaCl_2$
F1	6.91	6.53
F2	6.76	6.37
F3	6.57	6.17
F4	6.63	6.22

Table 3-1: Results of pH measurements

The results of an analysis of variance are displayed in Table 3-2. For deionised water as well as for $CaCl_2$, no significant differences were found between the four different main treatments (N fertiliser rates).

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatment	3	0.282	0.094	2.22	**0.139**
residual	12	0.508	0.042		
total	15	0.790			

Table 3-2: analysis of variance (ANOVA) results for pH in deionised water

The measurements in deionised water prove the fact that pH values tend to stabilise at about 6.5 - 7.0 in soils after flooding (ROWELL, 1988).

Values in 0.01 M $CaCl_2$ are approximately 0.4 pH units lower than in deionised water which suggests that the examined soil is acidic and has a net negative charge.

3.1.2 Water content

The mean values were F1: 1.09, F2: 1.16, F3: 1.17, F4: 1.20 g H_2O g^{-1} oven-dry soil.

An analysis of variance (Table 3-3) showed no significant differences between the main treatments. The amount of mineral fertiliser added therefore seems to have no significant effect on water content.

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatments	3	0.030	0.010	0.48	**0.703**
residual	12	0.249	0.021		
total	15	0.279			

Table 3-3: analysis of variance (ANOVA) results for water content

3.1.3 Loss on ignition

The following values were determined (g 100 g^{-1} oven-dry soil):

sample	subplot 1	subplot 2	subplot 3	subplot 4	mean
F1	7.28	7.59	8.37	7.86	7.78
F2	9.74	9.08	9.52	9.90	9.56
F3	9.75	9.59	10.73	10.48	10.14
F4	10.59	10.62	10.92	10.38	10.63

Table 3-4: Loss on ignition results

It can be observed that the loss on ignition - and therefore the organic matter content - is positively correlated with the amount of N fertiliser application. The significance and meaning of this correlation will be discussed in Section 4.3.

An analysis of variance proved highly significant differences between the four different N treatments:

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatments	3	18.618	6.206	35.68	**<0.001**
residual	12	2.087	0.174		
total	15	20.705			

Table 3-5: analysis of variance (ANOVA) results for loss on ignition

3.2 Measurement of enzyme activities

3.2.1 Calibration curves

For all soil samples examined with MUB substrates, high correlation coefficients have been found. The lowest correlation was found for F1-3 (r^2 = 0.9534), the best one with the coefficient of determination r^2 = 0.9980 for F2-1, the average was r^2 = 0.9843.

In case of AMC substrate, r^2 ranged from 0.8965 to 0.9988 with a mean of 0.9869.

Figure 3-1 and Figure 3-2 show typical examples of calibration curves obtained from analysing the fluorescence data:

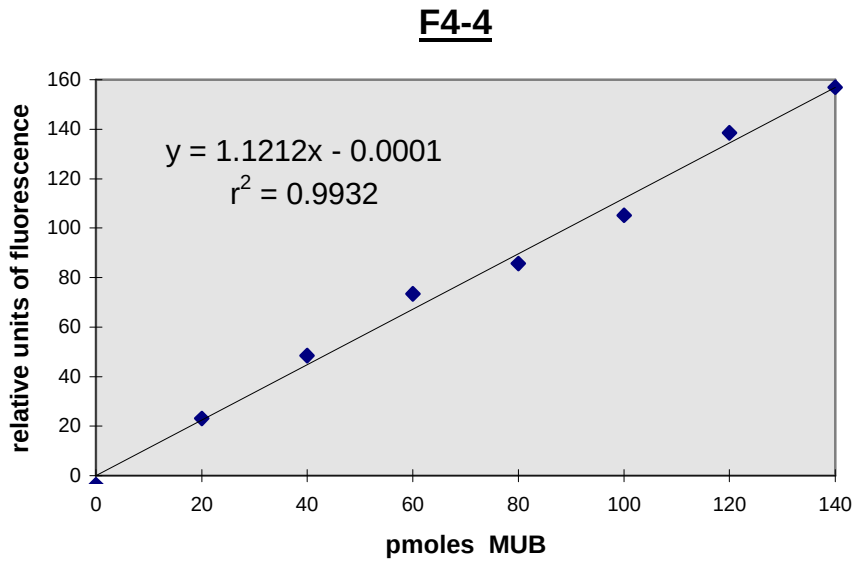

Figure 3-1: example of a MUB calibration curve (sample F4-4)

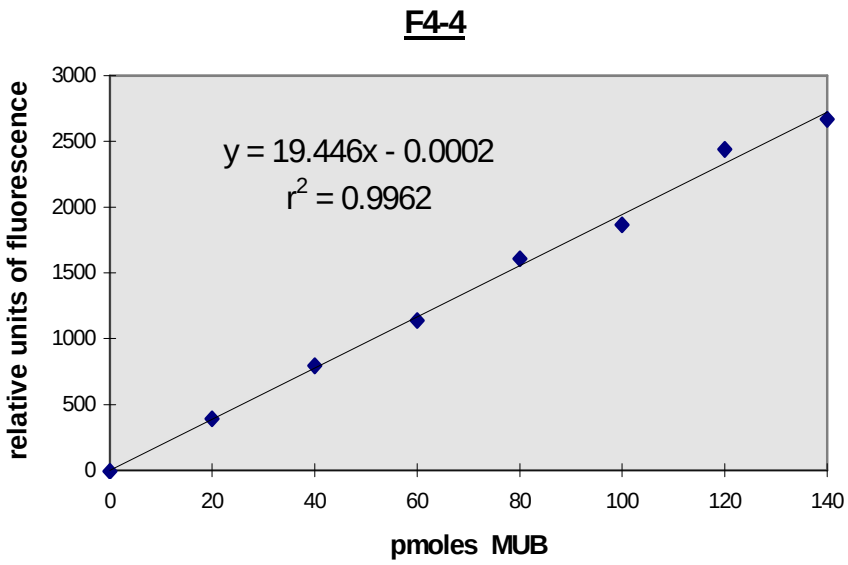

Figure 3-2: example of a AMC calibration curve (sample F4-4)

3.2.2 Enzyme activities

3.2.2.1 Phosphatase

Phosphatase showed the highest activities of all 5 enzymes tested. Mean rates of fluorescence increase ranged from 20.34 nmoles MUB min^{-1} g^{-1} oven-dry soil to 28.64 nmoles MUB min^{-1} g^{-1} oven-dry soil.

sample	result
F1-1	20.33
F1-2	21.14
F1-3	23.22
F1-4	25.52
F2-1	26.52
F2-2	22.92
F2-3	22.75
F2-4	25.35
F3-1	25.40
F3-2	21.18
F3-3	27.76
F3-4	22.34
F4-1	26.15
F4-2	26.68
F4-3	28.66
F4-4	24.57

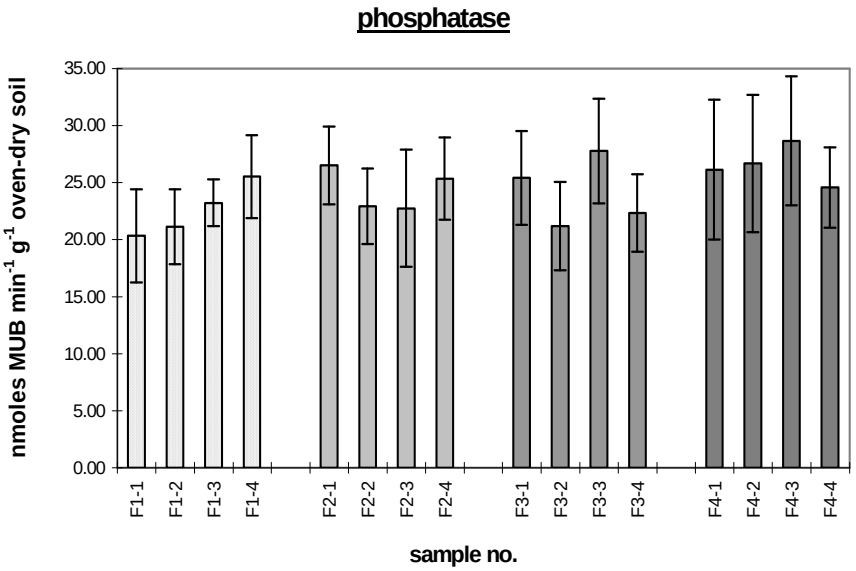

Figure 3-3: numerical and graphical phosphatase results

The graphical results indicate no significant differences between the four fertiliser treatments and the analysis of variance confirms it:

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatments	3	31.758	10.586	2.06	**0.159**
residual	12	61.730	5.144		
total	15	93.488			

Table 3-6: analysis of variance (ANOVA) results for phosphatase

3.2.2.2 Glucosidase

Results for glucosidase proved to be lower than for phosphatase. In contrast to phosphatase, clear variation between the four N treatments and also within each of the mainplots was found. The highest value was nearly three times the lowest one.

sample	result
F1-1	7.36
F1-2	7.93
F1-3	10.12
F1-4	9.99
F2-1	9.21
F2-2	8.06
F2-3	10.57
F2-4	11.52
F3-1	9.52
F3-2	8.79
F3-3	16.66
F3-4	11.23
F4-1	12.43
F4-2	21.02
F4-3	22.23
F4-4	19.25

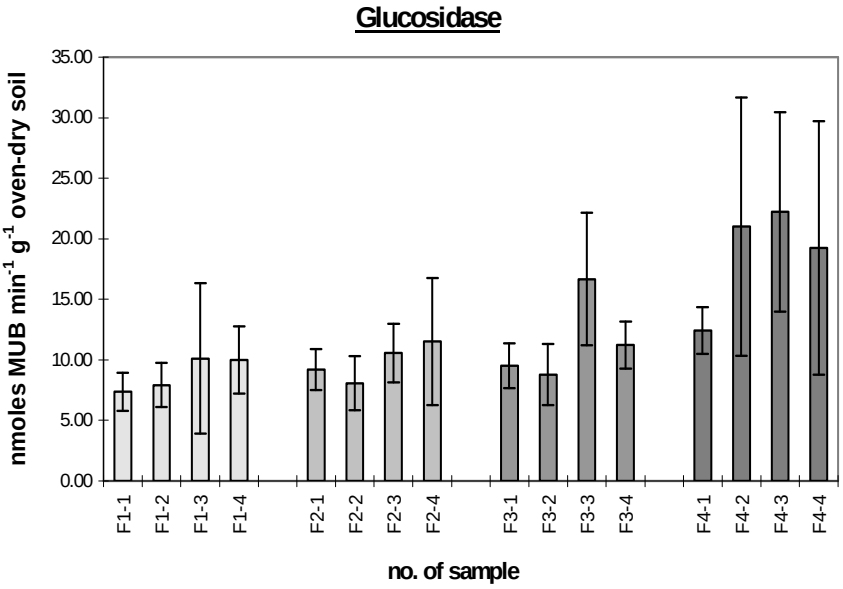

Figure 3-4: numerical and graphical glucosidase results

Unlike for phosphatase, the glucosidase results showed significant differences for the four N treatments (Table 3-7).

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatments	3	239.523	79.841	8.85	**0.002**
residual	12	108.307	9.026		
total	15	347.830			

Table 3-7: analysis of variance (ANOVA) results for glucosidase

3.2.2.3 Glucosaminidase

Although the values for glucosaminidase were only half the ones for glucosidase, they showed a similar pattern.

sample	result
F1-1	4.59
F1-2	4.55
F1-3	4.65
F1-4	6.69
F2-1	5.14
F2-2	5.13
F2-3	7.53
F2-4	6.42
F3-1	6.40
F3-2	4.77
F3-3	8.52
F3-4	6.22
F4-1	11.23
F4-2	11.02
F4-3	12.91
F4-4	11.03

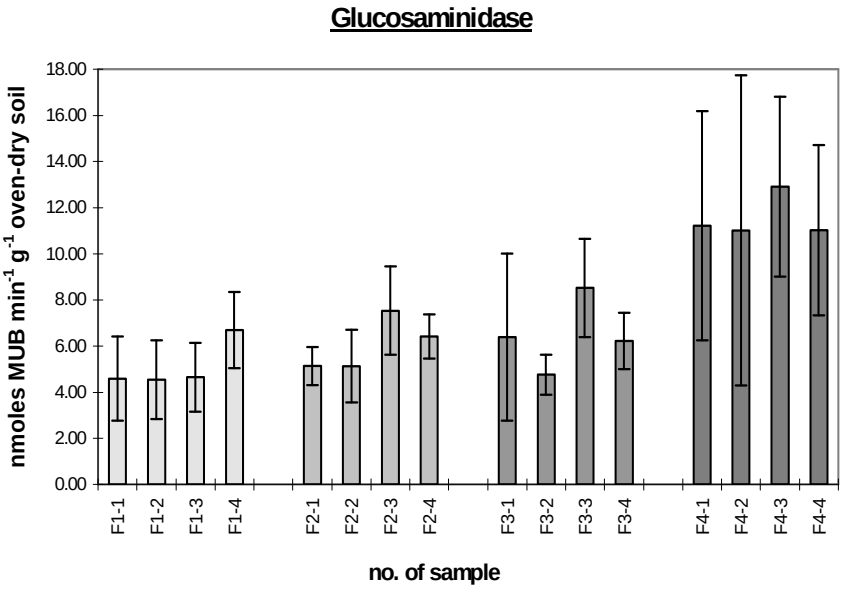

Figure 3-5: numerical and graphical glucosaminidase results

An analysis of variance showed highly significant differences between the four N treatments:

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatments	3	100.081	33.360	23.61	**<0.001**
residual	12	16.956	1.413		
total	15	117.037			

Table 3-8: analysis of variance (ANOVA) results for glucosaminidase

3.2.2.4 Cellobiohydrolase

This enzyme showed the lowest activities. Values oscillated around 3 nmoles MUB min^{-1} g^{-1} oven-dry soil. No significant variation could be observed regarding the different N

treatments. Sample F3-4 however, the highest-ranking sample in all of the measured enzymes, was a surprise with a value approximately three times as high as the average.

sample	results
F1-1	3.10
F1-2	3.65
F1-3	2.99
F1-4	3.93
F2-1	3.46
F2-2	3.35
F2-3	4.06
F2-4	4.18
F3-1	2.75
F3-2	2.53
F3-3	3.62
F3-4	4.05
F4-1	4.09
F4-2	3.75
F4-3	9.20
F4-4	4.31

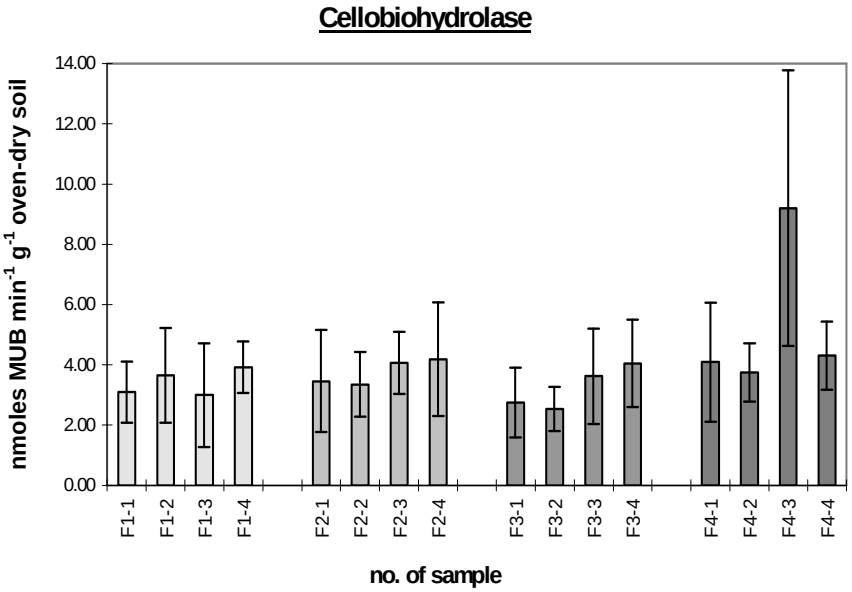

Figure 3-6: numerical and graphical cellobiohydrolase results

The value obtained for F3-4 is more than double the next highest value. Most probably, this is due to a sampling mistake (e.g. wrong depth, wrong location), as the sample bag did not contain any unusual contents and the value has been generated from 3 solutions of three replicates each.

No statistically significant differences between the four N treatments could be found:

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatments	3	11.005	3.668	1.94	**0.177**
residual	12	22.720	1.893		
total	15	33.725			

Table 3-9: analysis of variance (ANOVA) results for cellobiohydrolase

In another ANOVA analysis, the value of F3-4 was substituted by the mean of the other three values in the F4 treatment. An F value of 0.138 was obtained.

3.2.2.5 Leucine-peptidase

Compared to the other enzymes, leucine-peptidase is on a medium rank and shows a slight increase for increasing N application. Especially within the F4 treatment comparatively large variations were obtained:

sample	results
F1-1	6.87
F1-2	6.80
F1-3	5.84
F1-4	7.57
F2-1	7.96
F2-2	10.76
F2-3	8.42
F2-4	9.62
F3-1	9.55
F3-2	10.06
F3-3	10.81
F3-4	11.24
F4-1	9.00
F4-2	10.52
F4-3	15.63
F4-4	11.80

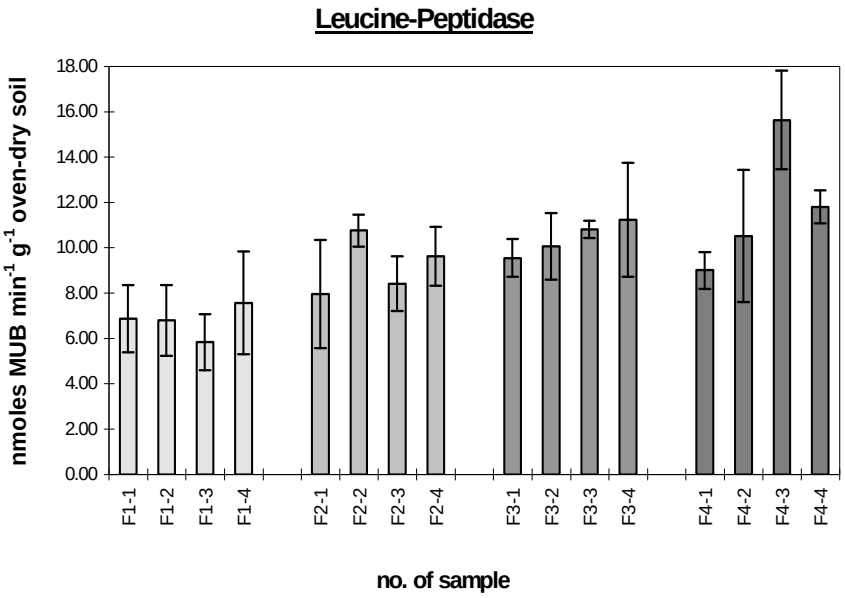

Figure 3-7: numerical and graphical leucine-peptidase results

An analysis of variance proved a significant difference for the four N treatments but evaluated the variation between the replicates as not significant.

source of variation	d.f.	s.s	m.s.	v.r.	F pr.
treatments	3	53.558	17.853	6.67	**0.007**
residual	12	32.114	2.676		
total	15	85.672			

Table 3-10: analysis of variance (ANOVA) results for leucine-peptidase

3.3 Determination of mineralisation rates

3.3.1 ^{15}N isotope dilution technique

3.3.1.1 Colorimetric determination of NH_4^+ and NO_3^-

At day 1 (1st extraction) and day 4 (2nd extraction) after adding the ^{15}N component, the size of the NH_4^+ and NO_3^- pools were determined. Figure 3-8 shows the changes which occurred within these three days.

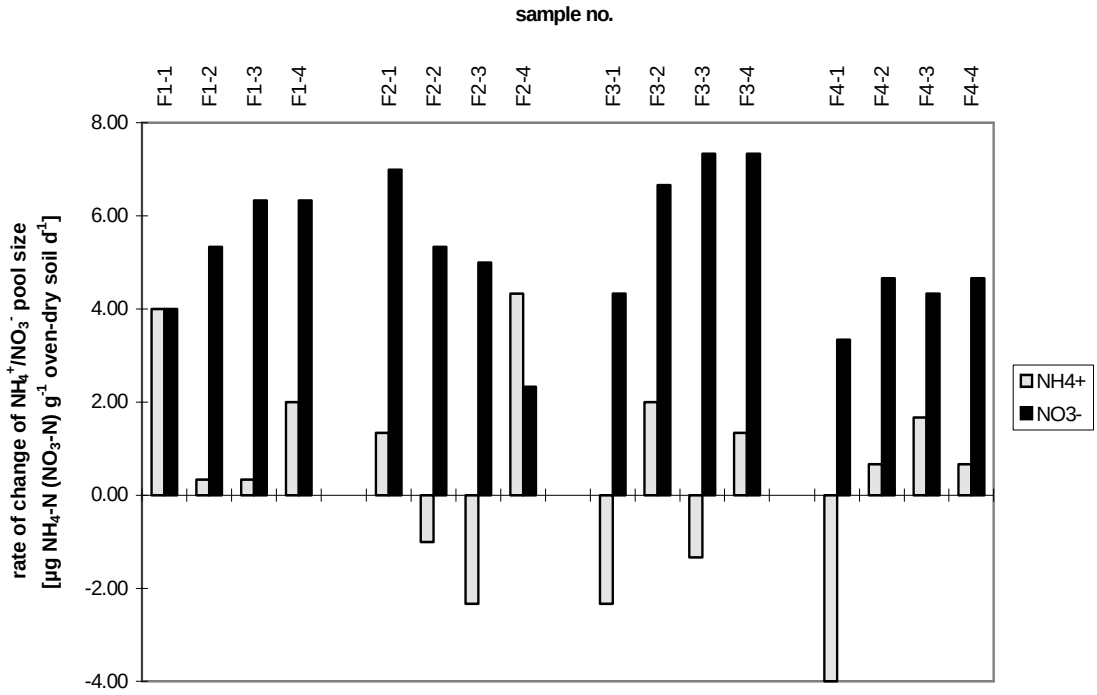

Figure 3-8: change of NH_4-N and NO_3-N pool sizes between the two extractions

The ammonium pool slightly increased in most cases but decreased for 5 of the 16 samples. Maximum increase was 4 µg NH_4-N g^{-1} oven-dry soil d^{-1} and the maximum decrease 4 µg NH_4-N g^{-1} oven-dry soil d^{-1}. No general pattern could be found, as parallel decreases and increases of the NH_4^+ pool were even found for replicates within the same main N treatment.

For all samples, the nitrate pool was higher at the time of the second extraction compared to the first one. Increases ranged from 4 to 32 µg NO_3-N g^{-1} oven-dry soil d^{-1}. Compared to ammonium, all rates of change were significantly higher.

3.3.1.2 ^{15}N enrichments; gross mineralisation

Figure 3-9 shows the ^{15}N enrichments for each of the tested samples. A significant decrease in enrichment took place over the 3 days, reducing the percentage of the tracer for approximately one third in all four N treatments.

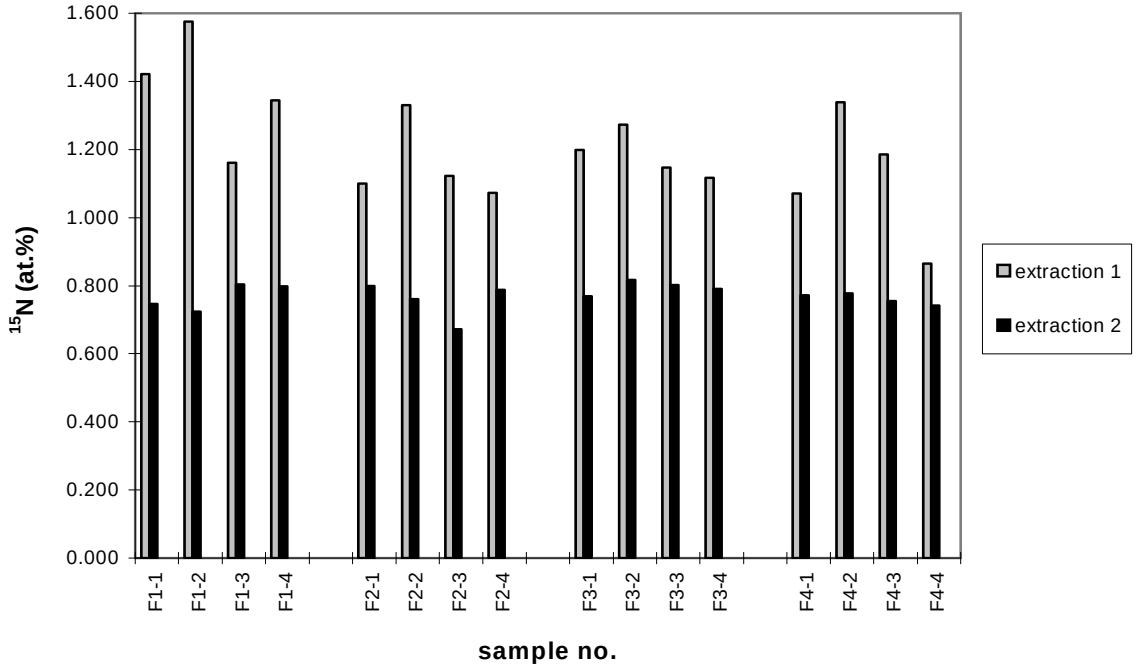

Figure 3-9: enrichments of ^{15}N at the time of extractions 1 and 2

This is clear evidence that the labelled NH_4^+ has been diluted with ammonium produced during the mineralisation process. Taking into account these changes and in addition the size of the unlabelled NH_4^+ pool, gross mineralisation rates were calculated using Equations 2-3, and 2-5 (Table 3-11).

sample	gross mineralisation [µg N g^{-1} oven-dry soil d^{-1}] KIRKHAM & BARTHOLOMEW (1954)				gross mineralisation [µg N g^{-1} oven-dry soil d^{-1}] ZAMAN *et al.* (1999)			
F1-1	6.13				6.18			
F1-2	4.86				4.82			
F1-3	5.39				5.36			
F1-4	5.37				5.43			
F2-1	5.58				5.58			
F2-2	7.59				7.58			
F2-3	7.78				7.77			
F2-4	4.86				4.94			
F3-1	6.33				6.53			
F3-2	5.31				6.44			
F3-3	5.61				5.22			
F3-4	5.22				5.56			
F4-1	5.22				5.18			
F4-2	4.98				5.00			
F4-3	5.48				5.51			
F4-4	4.41				4.20			
	F1	**F2**	**F3**	**F4**	**F1**	**F2**	**F3**	**F4**
mean	5.44	6.45	5.62	5.02	5.44	6.47	5.94	4.97
std	0.52	1.45	0.51	0.46	0.56	1.42	0.65	0.56
cv	9.57	22.50	9.00	9.08	10.28	21.93	10.94	11.18

Table 3-11: gross mineralisation rates

The two equations gave very similar values.

Figure 3-10 represents a graphical display of the data calculated by KIRKHAM & BARTHOLOMEW (1954). The same data were used to calculate mean gross mineralisation rates for the four N treatments and their internal error caused by the four replicates (Figure 3-11).

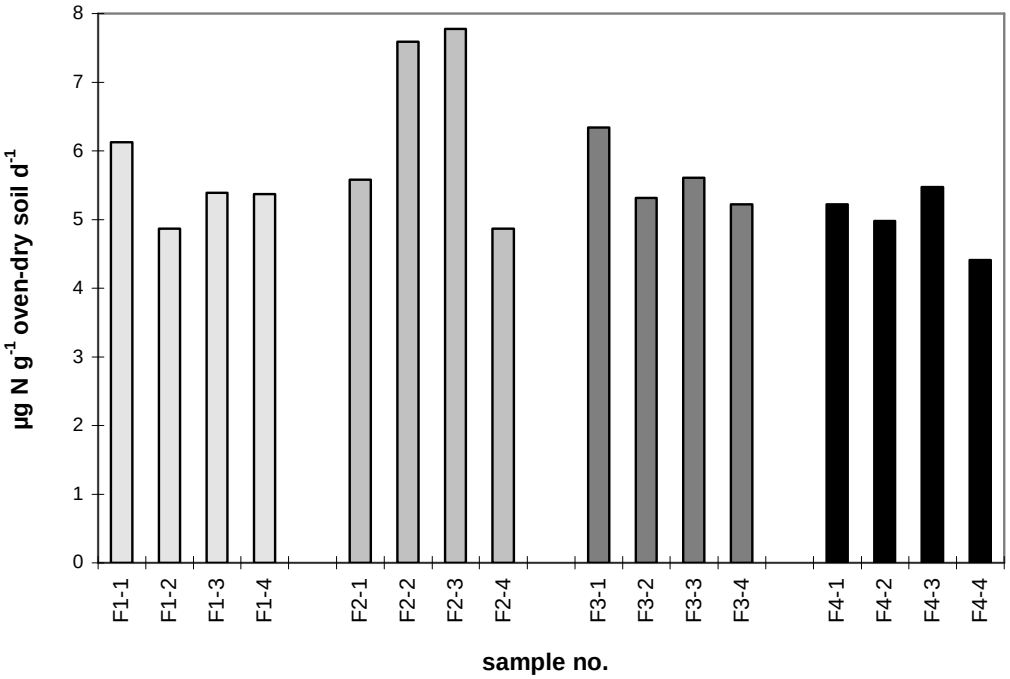

Figure 3-10: graphical display of gross mineralisation rates

From an one-way analysis of variance (ANOVA), the F pr. value of 0.144 was obtained. This means that there are no significant differences in gross mineralisation rates between the four N treatments.

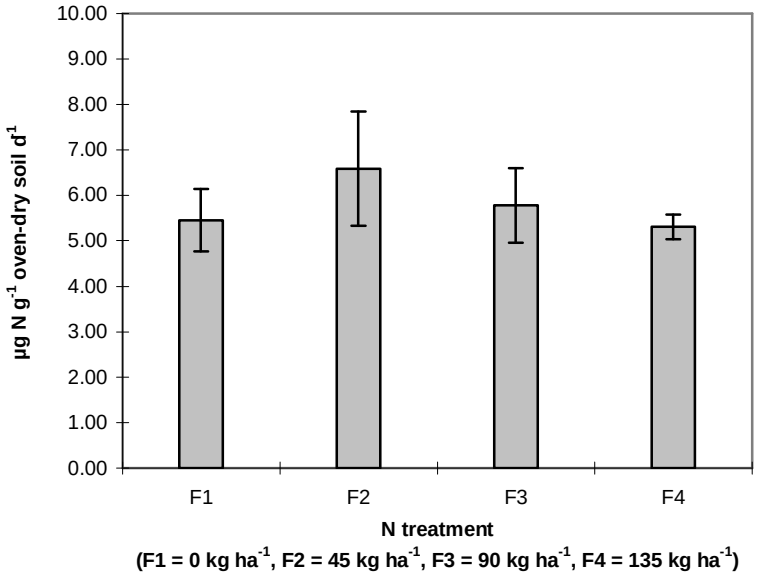

Figure 3-11: mean gross mineralisation rates and their errors

3.3.1.3 Consumption rates

Consumption rates for NH_4^+ were calculated using Equation 2-4. Net mineralisation was calculated as the difference between gross mineralisation and gross consumption.

sample	gross consumption [µg N g^{-1} oven-dry soil d^{-1}] KIRKHAM & BARTHOLOMEW (1954)	net mineralisation [µg N g^{-1} oven-dry soil d^{-1}]
F1-1	3.02	3.11
F1-2	4.80	0.07
F1-3	5.26	0.13
F1-4	3.92	1.45
F2-1	4.47	1.11
F2-2	8.55	-0.96
F2-3	9.73	-1.95
F2-4	1.53	3.34
F3-1	8.54	-2.21
F3-2	4.89	0.42
F3-3	6.55	-0.94
F3-4	4.68	0.54
F4-1	8.24	-3.02
F4-2	4.58	0.40
F4-3	4.15	1.32
F4-4	3.95	0.45

	F1	**F2**	**F3**	**F4**	**F1**	**F2**	**F3**	**F4**
mean	4.25	6.07	6.17	5.23	1.19	0.38	-0.55	-0.21
std	0.99	3.77	1.79	2.02	1.43	2.35	1.29	1.92
cv	23.36	62.18	29.04	38.67	120.44	612.52	235.76	907.45

Table 3-12: gross consumption rates and net mineralisation rates

Gross consumption rates ranged from 1.5 to 9.7 µg N g^{-1} oven-dry soil d^{-1} and net mineralisation rates from -3 to +3 µg N g^{-1} oven-dry soil d^{-1}. There was no recognisable pattern regarding the four N treatments.

3.3.2 Anaerobic mineralisation

The results of mineralisation under waterlogged conditions are displayed in Table 3-13.

sample no.	**anaerobic mineralisation** ($\mu g\ NH_4\ g^{-1}$ oven-dry soil d^{-1})	**sample no.**	**anaerobic mineralisation** ($\mu g\ NH_4\ g^{-1}$ oven-dry soil d^{-1})
F1-1	-1.19	F3-1	-1.19
F1-2	-1.20	F3-2	-1.26
F1-3	-0.88	F3-3	-0.50
F1-4	-1.40	F3-4	-0.69
F2-1	-1.06	F4-1	-0.99
F2-2	-0.16	F4-2	-1.01
F2-3	-0.77	F4-3	-0.84
F2-4	-0.81	F4-4	0.18

means	**F1**: -1.17	**F2**: -0.70	**F3**: -0.91	**F4**: -0.76
st.d.	0.21	0.38	0.37	0.39
c.v.	18.39	54.60	40.99	51.76

Table 3-13: rates of anaerobic mineralisation

Except for one of the 16 samples, all rates were negative. As we would expect positive values only, a detailed discussion of these results can be found in Section 4.2.3.2. No pattern can be seen in response to the different N treatments.

4 Discussion

4.1 Discussion of the methods

4.1.1 Fluorometric enzyme assay

The newly developed microplate assay has several advantages when compared to previous methods. Most importantly, formation of the products can be measured directly in the microplate without the need for prior extraction and purification of the product. The assay also allows the analysis of a large number of soil samples and / or enzymes in a comparatively short time. Data-processing is PC-supported and the total data output is much higher than actually needed.

Another main advantage is directly linked to the measurement of enzyme activities of flooded paddy soils: the highly fluorescent substrates allow the detection of even very low activities, whereas conventional methods for studying hydrolytic soil enzymes tend to be of limited value due to their dependence on relatively insensitive colorimetric detection.

However, there are some restrictions. The actual activity observed is dependent upon the degree of competition between the MUB substrates and any naturally occurring substrates. It is therefore recommended that operators should work at the maximum possible substrate saturation. Due to lack of time, the effects of increasing substrate concentrations could not be examined. The optimum values for acid phosphatase and *ß*-glucosidase found by FREEMAN *et al.* (1995) for Welsh peat soils, however, was in the proximity of the 300 µM MUB substrate concentrations established in my micro-wells. No substrate inhibition studies for the AMC substrate were found.

Furthermore, MUB substrates are dimeric rather than polymeric, and so the extent to which their enzymatic hydrolysis is representative of that of natural materials is yet uncertain.

The applied fluorometric assay is cost-intensive, as it involves a computer-linked fluorescence reader, expensive substrates and multi-channel pipettes.

4.1.2 ^{15}N istotope dilution technique

This method to estimate gross mineralisation can only be applied, if the following assumptions can be fulfilled:

- microorganisms do not discriminate between ^{14}N and ^{15}N; this statement is in fact not true, but DAVIDSON *et al.* (1991) noted that it is probably valid in the case of transformations of enriched samples for incubations of only a few days
- rates of processes measured remain constant over the incubation period; BJARNASON (1988) found that this assumption caused minor errors that were probably insignificant relative to experimental error
- ^{15}N assimilated during the incubation period is not remineralised; in my study, a 3-day incubation period was assumed to be a safe time during which the immobilised ^{15}N would not be remineralised

The high clay content of the soil caused the material to be extremely sticky and difficult to work with. It is for that reason that the soils could not be sieved through a 2 mm sieve and aggregates of approx. 1 cm diameter had to be included in the calibration of the samples. This might have caused partially anaerobic zones and thus have influenced the measurement.

Mineralisation results have been calculated by using two different equations. The results have been very similar, but it is still questionable if these equations may be applied uniformly to aerobic and anaerobic soils. TORIYAMA & MIYAMORI (1989) modified the original equation for paddy soil conditions, but their publication unfortunately is written in Japanese.

Further experimental error may have been caused by

- sampling; I relied on correct sampling and labelling of the soils sent from IRRI, Philippines
- microbiological contamination of the samples; although ethanol was always used when several samples were handled after one another, transfer of microorganisms through appliances used for drying, grinding or transferring, or through the air during incubation time can never be totally excluded
- growth of rice plants; in some cases, small amounts of organic matter were added where grown roots could not be completely removed; furthermore, each removal of a seedling was necessarily combined with the loss of a very small amount of soil

4.1.3 Anaerobic mineralisation

A simple method was applied to a complex system. The results produced were not satisfactory. Obviously, the procedure was apt for aerobic conditions but not suitable in case of flooded, anaerobic soils. According to DOBERMANN (personal communication), results like this show the weakness of standard mineralisation methods when applied to paddy soils. If the mineralised N is not constantly removed, it is prone to loss mechanisms and the accumulation of mineral N will additionally slow down the true potential mineralisation rates.

DOBERMANN also mentioned that a PhD student at IRRI has enhanced the method by adding cation exchange resins to the tubes to absorb all N generated during the mineralisation proces. The mineralisation rates he got were significantly higher compared to the standard method.

Experimental error may have been caused by shaking all samples on a rotational shaker for 90 minutes prior to incubation. This had to be done to guarantee that all the soil is actually covered by water and does not stick to the walls of the tubes. However, this might have thoroughly mixed O_2 (volume of approx. 30 ml in each tube) into the soil suspension and delayed the rapid establishment of anaerobic conditions. As a consequence, some of the NH_4^+ present might have been nitrified. This could not be verified, however, as NO_3^- concentrations were not determined after the incubation period.

To avoid the possible influence of residual O_2, NISHIO *et al.* (1993) stripped dissolved O_2 by bubbling the samples with helium gas.

^{15}N labelling studies as applied in some of the work mentioned in the literature review - though more involved and time-consuming - would have been more suitable as a means of examining the mineralisation process under anaerobic conditions.

4.2 Discussion of the results

4.2.1 Loss on ignition

Table 4-1 shows soil organic carbon and total N measurement results from the LTCCE site (CASSMAN *et al.*, 1998). Samples were taken from the surface (0-20 cm) of treatment plots without applied N (N_0) or with 150/90/90 kg N ha^{-1} (N_{330}) applied to the dry season, early and late wet season rice crops.

Year	**organic carbon (g kg^{-1})**		**total N (g kg^{-1})**		**C:N ratio**	
	N_0	N_{330}	N_0	N_{330}	N_0	N_{330}
1963	18.3	18.3	1.94	1.94	9.5	9.5
1978	18.8	21.4	1.97	2.22	9.6	9.7
1983	18.7	21.4	1.95	2.14	9.6	10.0
1985	20.4	23.9	2.07	2.38	9.9	10.0
1991	20.4	23.5	1.97	2.27	10.3	10.4
1992	20.7	23.0	2.09	2.30	9.9	10.0

Table 4-1: soil organic carbon and total N (see above)

My SOM estimates by loss on ignition range from 72.8 to 109.2 g kg^{-1}. Assuming that C accounts for 58% of the organic matter present (which might not be true for paddy soils), my values are significantly higher than CASSMAN's.

The main reason of this discrepancy might be seen in the nature of the loss on ignition measurement. It does only give a rough estimate for SOM content and is less accurate when applied to clayey soils as they can lose considerable amounts of structural water during the measurement and thus reduce the meaningfulness as a direct measure for SOM. The fact that organic matter content is rising with increasing amounts of fertiliser, will be discussed in Section 4.3.3.

4.2.2 Enzyme activities

The observed activities do not necessarily have to reflect the actual values under field conditions. More correctly, they represent optimum values measured under optimum conditions (optimum pH, constant temperature, excess substrate availability). What has been measured therefore reflects maximum potential rather than actual activity.

On the other hand, the soils have not been dried or processed in any way before the examination and thus are as close to natural conditions as possible *in vitro*.

The range of values generated is similar to values obtained from aerobic soils from the Reading area (results not included in this study), but especially phosphatase "activity" was significantly lower than values found by MARX (personal communication) for aerobic silty clay loam soils from Devon.

Low phosphatase results, however, would have been expected. The enzyme phosphatase is produced mainly by plants and microorganisms in response to low P availability in soil, and its production and activity is halted when P is abundant. CASSMAN *et al.* (1995) mention that nutrients other than N did not limit yields in the LTCCE experiment at IRRI; the present P concentrations should have therefore been high enough to make high levels of phosphatase production unnecessary. In addition, earthworms which could release acid phosphatases are not present in flooded paddy soils.

Thus, it is not surprising that my values are extremely low compared to the results obtained by FREEMAN *et al.* (1995) for Welsh peat soils, where P most probably was highly deficient. Whereas these authors found phosphatase activities ranging from 30,000 to 1,000 nmoles of MUB release min^{-1} g^{-1}, the highest value for the Philippine paddy soils was 29 nmoles of MUB release min^{-1} g^{-1}.

OLK *et al.* (1996) provide another explanation why microbial activities and therefore also enzyme production might be restricted under flooded conditions. They point out that cations such as Fe^{2+} with high charge density form stable complexes with humic substances and subsequently protect them from degradation. As a consequence, lignin and the phenolic subunits of lignin are highly resistant to degradation without the presence of O_2. And even "young" humus develops greater phenolic character and makes it more recalcitrant to microbial degradation, thus increasing the potential for abiotic immobilization of available N.

Correlations of the observed enzyme activities to other parameters will be given in Section 4.3. To draw conclusions concerning the relationship of soil enzymes to essential agricultural and environmental aspects like soil productivity or degrading potential etc. is dangerous, unless it is known which of the many components of the total activity of an enzyme are being measured during the MUB assay and what contribution those components actually make to the overall breakdown of the substrates. BURNS (1981) is even more pessimistic when he states that "an ill-defined property as the fertility of a soil is almost certainly related to a composite of biological and non-biological events rather than to an individual enzyme or even to a number of enzymes". NANNIPIERI & LANDI (2000) however point out that enzyme measurements answer qualitative questions related to specific metabolic processes. Therefore, even if enzyme measurements represent potential rather than actual activities, they are of great value in screening procedures for determining the influence of e.g. agrochemical amendments, cultivation practices, and environmental and climatic factors on soil processes.

4.2.3 Determination of mineralisation rates

4.2.3.1 Under aerobic conditions

Why do the colorimetric measurements of NH_4^+ / NO_3^- suggest significant differences in mineralisation, whereas ^{15}N reductions are similar among all samples?

The colorimetric measurements of ammonium and nitrate at day 1 and 4 after adding the ^{15}N isotope (Fig. 3-8) have shown highly different production rates of both N components between and also within the four different N treatments. Whereas some NH_4^+ production rates were negative, others within the same main treatment were positive. All NO_3^- production rates however were clearly positive.

Most of this irregularity may be explained by the variation between the three-fold replicates used for each sample. Especially the samples where negative NH_4^+-rates were calculated showed huge variation. In fact, for each sample, positive values could have been calculated if some of the replicates had been neglected. Therefore it is assumed that positive rates of NH_4-N pool change occurred in each sample, and that most of the produced NH_4^+ was then quickly nitrified.

Figure 4-1 shows the size of the NH_4^+-pool over the whole period of equilibration including the 4-day-incubation. The zero-treatment (F1) shows significantly lower values for KCl-extractable NH_4^+. However, rates of ^{15}N reduction over the 4-day-incubation period were comparable to those from the other N treatments. This suggests that the amounts of NH_4^+ produced to dilute the added $^{15}NH_4^+$ were also comparable between all four treatments. If losses via NH_3 volatilisation had occurred, $^{14}NH_4$ as well as $^{15}NH_4$ would have been lost and therefore the $^{15}N/^{14}N$ ratio remained unchanged.

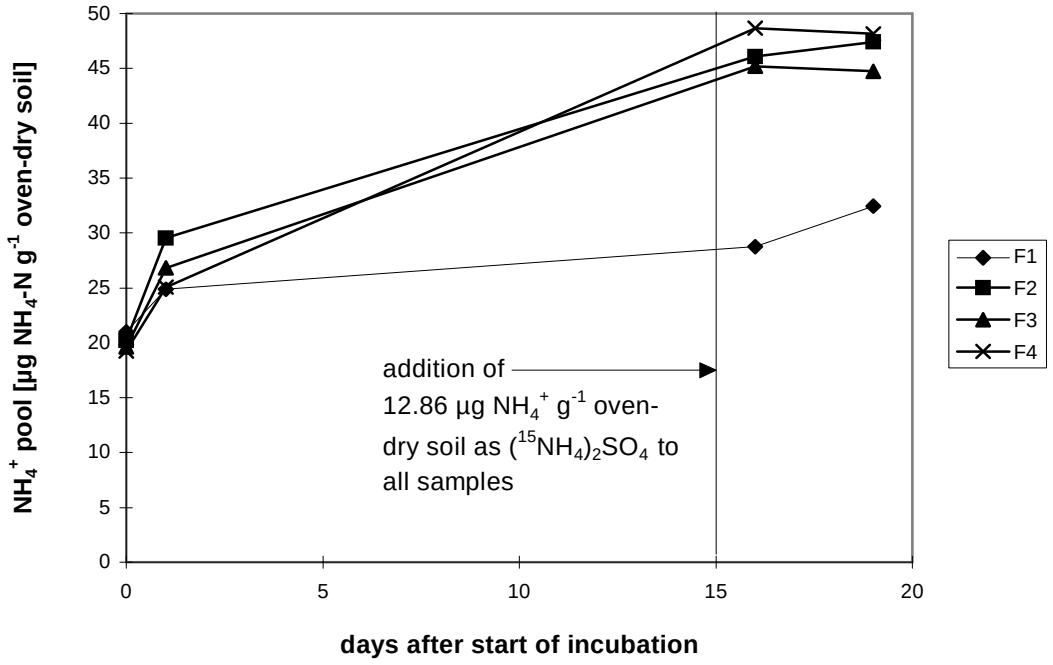

Figure 4-1: change of ammonium pool size over time during equilibration at 26°C

Compared to the amounts of $^{14}NH_4^+$ present, the $(^{15}NH_4)_2SO_4$ added may seem to be a considerable amount. However, it has to be emphasised that estimation of gross mineralisation is unaffected by the problem of substrate stimulation because the pools increased by ^{15}N injection are the products of the process not the substrate.

Why are the rates of gross mineralisation similar in spite of very different rates of fertiliser application?

No correlation has been found between rates of applied fertiliser and gross mineralisation rates ($r = -0.388$ at $n = 4$ and d.f. = 2).

The last rice harvest before sampling the soils was on 24 April, puddling of the soil was performed approximately on 02 May, and the transplanting of the new rice plants was done on 08 May. Sampling took place on 10 May which is 2 days after transplanting (DAT) and 8 days after puddling.

The first fertiliser application of 20/40/60 kg ha^{-1} (F2/F3/F4) was made on 15 DAT, two weeks after sampling and therefore could not influence the measurements.

But what about the fertiliser applied regularly since 1963? As a matter of fact, due to the processes described in Section 1.2., not much N is left after the crop season, independent from the fertiliser application rate. WITT *et al.* (1998) noted, that regardless of the N rate applied in the dry season (DS), only a small amount of NH_4-N was found after harvest of the DS rice and maize crops. As a consequence, the amounts of total mineral nitrogen the rice plants start with, are roughly the same each year.

It can be observed that differences between the replicates *within* a N treatment sometimes are higher than the variations *between* the main treatments. They might be explained by quantitative and qualitative differences in the organic material present. and/or differences in amount and types of microbial communities. The latter might have been caused by the effects already mentioned of the germination of rice seedlings or contamination from outside the samples.

Why are the rates of gross mineralisation (Table 3-11) still considerably high?

The highest levels of mineralisation in paddy fields usually occur after puddling and then decrease rapidly as the labile N pool is depleted and soil becomes reduced. This rapid phase lasts approximately 12-17 days; at the same time, crop N demand is negligible until 15-20 days after transplanting (CASSMAN *et al.*, 1994); the soils used in this study were sampled 8 days after puddling and therefore in the period of highest mineralisation.

Mineralisation therefore must have constantly taken place in the samples since sampling, initially under partly anaerobic conditions until the start of the equilibration

(37 days) and then enhanced under aerobic conditions during equilibration and incubation (19 days). The fact that mineralisation rates are still equally high after such a long period indicates that enough readily available SOM must have been present to support mineralisation in the case of all four N fertiliser treatments.

Estimated organic matter content has been shown to increase with fertiliser application rates (also see Section 4.3.3). Assuming that the amounts of readily available SOM also increase with N application rates, you would expect the mineralisation rate of the zero N treatment (F1) to decrease first. It is not known, how long the equilibration should have been continued to see these possible differences between the four N treatments.

When compared to the values NISHIO (1994) obtained from aerobic incubation of Japanese paddy soils (approx. 10 µg N g^{-1} d^{-1}), my rates appear comparatively low (approx. 6 µg N g^{-1} d^{-1}). Temperatures during equilibration and SOC / total soil nitrogen were comparable. However, NISHIO used equations based on his model to calculate gross mineralisation whereas I applied the equations established by KIRKHAM & BARTHOLOMEW (1954).

How can the gross consumption and net mineralisation rates (Table 3-12) be interpreted)?

According to DAVIDSON *et al.* (1991), gross consumption includes the sum of immobilisation (i.e., microbial assimilation), autotrophic nitrification, volatilisation and other possible fates.

The same authors warn that gross consumption values should be viewed with caution because substrate has been added to the soil. If as in my case the total amount of

inorganic-N substrate is increased significantly, consumption rates could be overestimated. NH_4^+ consumption rates would be suspect, because measured consumption rates would nearly always exceed calculated mineralisation rates which obviously could not be sustained. Unfortunately all methods involving addition of inorganic ^{15}N have the limitation that microbial assimilation could be stimulated by increasing the substrate pool.

More precise immobilisation rates could have theoretically been determined as the difference between ^{15}N not recovered in the soil or plant at maturity and NH_3 losses determined by micro-meteorological techniques.

When consumption rates are overestimated, net mineralisation will automatically be underestimated. The negative values obtained for some of the samples are the unavoidable outcome. These figures are of course unrealistic, especially because high net mineralisation rates would be anticipated in an anaerobic system. In contrast to aerobic soil, where microbial biomass is C-limited and net mineralisation reflects the nature of the organic substrates being decomposed, the N requirement of anaerobic metabolism is lower and net N mineralisation rates therefore expected to be higher.

Has the application of this measurement to flooded soil been sensible?

Yes and no.

Yes in so far as it could be shown that rates of mineralisation in paddy fields are significantly higher under aerobic compared to anaerobic conditions. This indicates that N mineralisation and NH_4^+ assimilation in the surface (0-20 mm) layer are quantitatively of great importance even though this only represents a small portion of the whole soil mass in a paddy field.

No in so far as fully aerobic conditions as created in vitro will never occur under field conditions. Even when soil dries between two crop cycles, deep soil cracking only dries the sides and edges of the dense peds that are formed. Mineralisation occurring under anaerobic condition is therefore of no use in the search for reliable indicators of N availability to the rice crop.

4.2.3.2 Under anaerobic conditions

Except for one sample, all obtained mineralisation rates shown in Table 3-13 were negative. This indicates that KCl-extractable NH_4-N present at time 0 has been lost from the system.

Clay fixation, NH_3 volatilisation or denitrification may theoretically be responsible for the observed phenomenon.

As mentioned before, NH_4^+ fixation only plays a minor role in the LTCCE soil. That ammonium was lost via denitrification is more likely, as the potential for denitrification is high: waterlogged conditions, easily available C compounds, high incubation temperature and pH at the necessary optimum (6-8) would all favour this process. Furthermore, it has to be considered that mineralisation was already going on at time zero, and therefore nitrification could have generated NO_2^- and/or NO_3^- which was then prone to losses when anaerobic conditions were established. The oxygen mixed into the suspension by vigorous shaking prior incubation might have supported this process. However, as the soil in the bags was supposed to be mostly anaerobic and no further processing took place, nitrification rates are supposed to be small.

NH_3 volatilisation can be seen as the most likely reason accounting for the observed decrease in NH_4^+ concentrations. Ammonification might have been taken place to a certain degree, but the produced ammonium was lost as NH_3. The pH measurements in deionised water showed that values are between 6.5-7.0, and ammonia production usually requires pH values above 7. MIKKELSEN *et al.* (1978) mention that algae play an important role in determining the pH of the floodwater. They report that growing algae can cause pH to rise to levels around 10. Again, the incubation temperature of 40°C would have stimulated the growth of algae.

The influence of temperature was also reported by WESTCOTT & MIKKELSEN (1985), who added labelled $(^{15}NH_4)_2SO_4$ to flooded soils in a laboratory experiment. They observed that fertiliser was initially lost or immobilised; they also found that increasing the temperature stimulated the disappearance of $(^{15}NH_4)_2SO_4$.

4.3 Inter-relationships

4.3.1 Inter-relationships between enzyme activities and mineralisation

It has been one of the aims of this study to find possible correlations between the measured parameters. A correlation between enzyme activities and gross mineralisation would be of particular interest, as the enzyme assay provides a rapid measurement whereas the isotope dilution technique to estimate gross mineralisation takes several weeks.

Positive correlations between mineralisation and enzyme activities under aerobic conditions were found by several authors. For example, ZAMAN *et al.* (1999) examined rates of gross mineralisation and their relationship to enzyme activities and soil microbial biomass in sandy loams treated with dairy shed effluent (DSE) and ammonium fertilizer (NH_4Cl). In the case of DSE addition, significantly higher rates for gross mineralisation were obtained, whereas in case of NH_4Cl results were lower or similar compared to the control. Protease, urease and deaminase activities were significantly higher for the DSE-treatment compared to the "inorganic" NH_4Cl-treatment or control. Water potential had high impact on enzyme activities, with the lowest activity observed at 0 kPa due to low O_2 availability. In the DSE-treatment gross N mineralisation rates were positively correlated with the activities of protease and urease (r = 0.86 and 0.61) and the amount of microbial biomass C and N, reflecting the fact that N mineralisation involves a sequence of microbial and enzymatic activities. No such correlation was found for the NH_4Cl-treatment.

Positive correlation between the N mineralisation rate and protease activity have also been reported by ALEF *et al.* (1988).

Figure 4-2 and Figure 4-3 show the correlation between my gross mineralisation rates (Table 3-11) and the “activities“ of glucosidase (see Figure 3-4) and leucine-peptidase (see Figure 3-7) respectively.

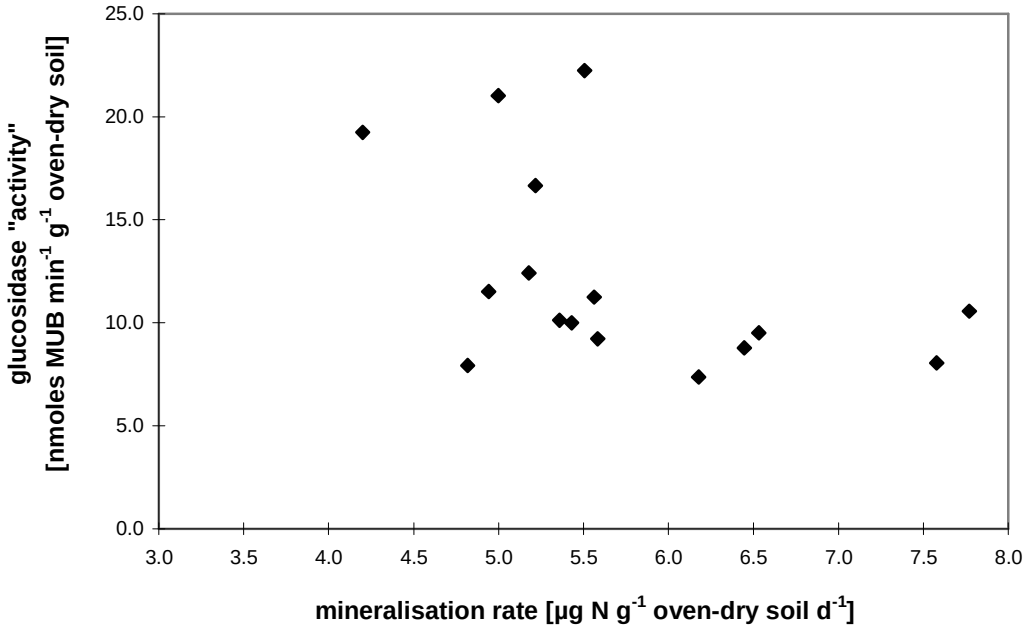

Figure 4-2: glucosidase results plotted against mineralisation results

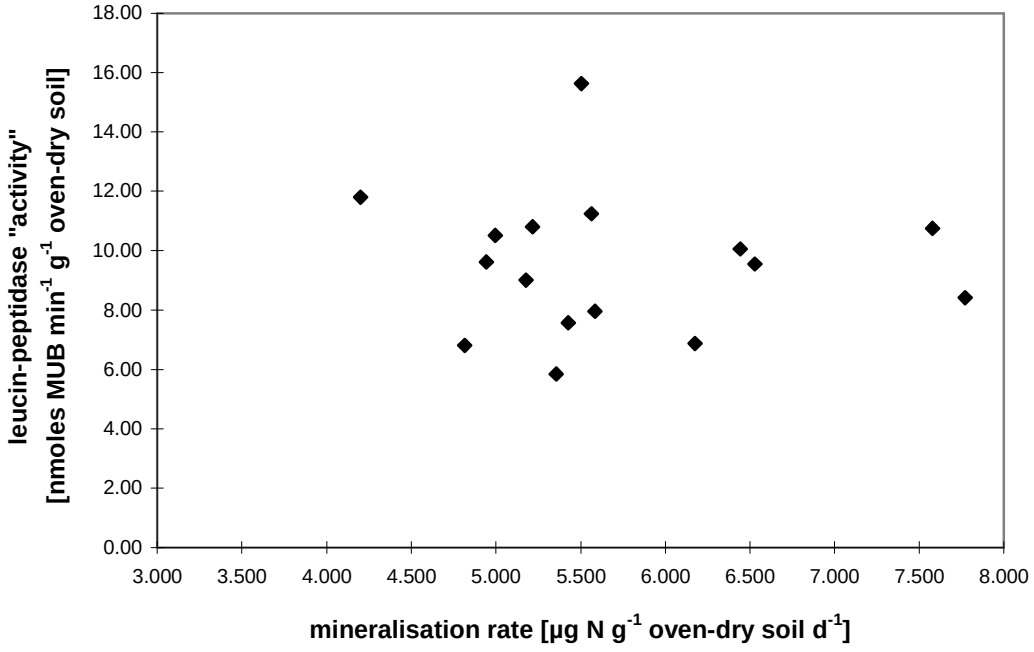

Figure 4-3: leucine-peptidase results plotted against mineralisation

A correlation analysis in GENSTAT proved that no statistic relationship exists between gross mineralisation rates and measured enzyme "activities":

	mineral.	**leucin-pep**	**cellobio**	**glucosamin**	**glucoside**	**phosphat**
phosphatase	-0.347	0.508[a]	0.529[a]	0.701[b]	0.694[b]	-
glucosidase	-0.472	0.729[b]	0.656[b]	0.898[b]	-	
glucosidase	-0.366	0.686[b]	0.679[b]	-		
cellobiohydrolase	-0.193	0.709[b]	-			
leucin-peptidase	-0.068	-				
gross mineralisation	-					

Table 4-2: *r* values of correlations between enzyme activities and gross mineralisation, and between the enzymes tested (n=16); [a] $p \leq 0.05$, [b] $p \leq 0.01$

The main reason for non-correlating results is most probably that mineralisation has been determined with processed soil whereas the samples used for enzymatic measurements were fresh (unprocessed). Due to negative results for anaerobic mineralisation, a correlation between these results and enzyme activities was not sensible.

As it can be seen from Table 4-2, correlation among the enzymes themselves was mostly highly significant. The raw data (see Section 3.2.2) show that the average activity of each enzyme is higher in the F4 treatment and lowest in the F1 (zero) treatment. Therefore the following correlation was examined:

4.3.2 Inter-relationship between enzyme activities and fertiliser addition

I have mentioned above that at the end of a cropping season virtually no fertiliser N is left over at the Philippine LTCCE site. As my samples were taken before the addition of the first fertiliser addition of the new season, no direct correlation would be expected .

A correlation analysis produced the following results:

	phosphat.	glucosidase	glucosamin.	cellobioh.	leucin-pept.
fertil. appl. rate	0.926[a]	0.906[a]	0.881	0.502	0.986[b]

Table 4-3: *r* values of correlations between fertiliser application rates and enzyme activities (n=4); [a] $p \leq 0.05$ [b] $p \leq 0.01$

Phosphatase, glucosidase and leucin-peptidase show significant correlation and glucosaminidase also has a high *r* value. It has to be taken into account however, that only four fertiliser application rates could be tested against 16 possible values for each enzyme. This reduces the meaningfulness of the above correlations.

An important question has to be asked at this stage: if enzyme activities are significantly related to fertiliser application rates, but the total amounts of N available are assumed not to be too different in all four treatments, what causes the observed correlation?
I propose that the different rates of fertiliser at the LTCCE site applied since 1963 have caused differences in the quantity and quality of the organic matter present. Further correlation analyses were made to examine this hypothesis.

4.3.3 Inter-relationships between fertiliser addition and SOM content

Loss on ignition results indeed seem to increase with increasing rates of fertiliser application. A correlation *r* value of 0.947 was found which is significant at a p level of 0.05. As only four data could be analysed (n = 4), the figure as such is questionable. Nevertheless, a strong correlation can be imagined for several reasons: Firstly, fertiliser addition triggers algal growth. One of the main reasons why fertiliser additions to paddy fields are split into three partial applications is that the immediate growth of algae would otherwise smother the young rice plants during the seedling stage. Over long time

periods, the amounts of algal biomass added to the soil organic matter pool cause this significant variation in SOM content which can be observed today.

Secondly, ROWELL (1988) suggested that in soils of possibly low biological activity a proportion of the added nitrate persists for several weeks and becomes incorporated in the SOM.

4.3.4 Inter-relationships between organic matter and enzyme activities

It is generally recognised that the major portion of readily decomposable organic matter originates from microbial biomass, as for example, the mentioned algae. Newly immobilised N is mineralised faster than native organic nitrogen, which is decomposed within thousands of years only. GAUNT *et al.* (1995) accordingly divided humic compounds into

a) humic acid fractions that are more accessible to microorganisms and more active in nutrient cycling = mobile humic acids (MHA), and
b) the more recalcitrant calcium-bound humic acids (Ca-HA).

They quantitatively determined these fractions in the LTCCE soil used for this study:

carbon (g kg^{-1} soil)			**nitrogen (g kg^{-1} soil)**		
total	MHA	Ca-HA	total	MHA	Ca-HA
28.8	3.74 (13%)	2.82 (10%)	2.44	0.30 (12%)	0.19 (8%)

Table 4-4: humic acid fractions at the LTCCE site, GAUNT *et al.* (1995)

Compared to an aerobic soil, where MHA only accounted for approx. 3% of total SOC, the authors concluded that younger humic acids may play a more important role in flooded soils. This again would speak for the hypothesis that algal growth and its

reaction to different rates of N application had an important influence on the formation of SOM.

Because all crop residues are constantly removed at the LTCCE site, algal biomass might indeed be mainly responsible for additions to organic matter. Especially the readily decomposable SOM fraction should therefore have a strong correlation to the measured enzyme activities. As Table 4-5 shows, even the total organic matter content - measured by loss on ignition - was highly correlated with enzymatic activity:

	phosphat.	**glucosidase**	**glucosamin.**	**cellobioh.**	**leucin-pept.**
loss on ignition	0.658[a]	0.704[a]	0.711[a]	0.408	0.763[a]

Table 4-5: *r* values of correlations between enzyme activities and loss on ignition (n=16); [a] $p \leq 0.01$

My findings are supported by other authors: MARTENS *et al.* (1992) found that soil enzyme activity increased with increasing SOM content. Activities of acid phosphatase, *ß*-glucosidase, N-acetyl-*ß*-glucosaminidase increased by an average of 2- to 4-fold by incorporation of 4 organic amendments when compared with the unamended soil (tillage alone).

Only recently, CHAKRABARTI *et al.* (2000) found that the acid phosphatase activity in soils was significantly higher for a manure compared to a no input treatment. The chemical fertiliser treatment, however, was found to be statistically similar to the no input treatment. This would emphasise the importance of organic inputs into the paddy soil system like those obtained from algal growth.

CASSMAN *et al.* (1998) mention that there is general agreement in the literature that the N supplying capacity of lowland rice soils is positively correlated with SOM content. It has to be taken into account, that most of these studies were laboratory- or greenhouse

based. From the measured enzymes in this study, only leucin-peptidase is involved in the N cycle. It indeed shows the highest correlation to the organic matter estimates compared to the other enzymes.

There is no doubt that soil microbial biomass and enzyme activities are closely related (ZAMAN *et al.*, 1999). Extracellular enzyme activities are therefore sometimes used as indices of microbial growth and activity in soil. However it is less well-known, which of the extra-cellular soil enzymes most accurately reflect N mineralization or microbial growth and activity on a quantitative basis.

5 Conclusions

The applied microplate enzyme assay worked well for the measurement of the extremely low activities found in the examined paddy soils.

For glucosidase, glucosaminidase and leucine-peptidase, significant differences were found between the four fertiliser N treatments, showing increasing activities with increasing rates of fertiliser application. The different N rates applied since 1963 have caused significant changes in quantity and maybe quality of the SOM, for example via allowing for different amounts of algal growth. The observed differences in SOM are suggested to be the main reason for the correlation with the measured hydrolytic enzyme activities.

Thus, the enzyme activities did not only reflect short-term nutrient availability in the examined paddy soils, but also gave information about long-term changes due to management practices.

This is the more remarkable as the isotope dilution technique did not suggest any significant differences regarding the gross mineralisation rates between the four N treatments. Only a three-day incubation with ^{15}N was monitored which might have been outside of the time period where expected differences between the N treatments would have occurred due to quality and quantity of the organic matter present. However, it is generally questionable in how far an aerobic incubation can give clues about the actual nitrogen availability under field conditions. The anaerobic mineralisation is widely regarded as a suitable means in that case, but produced negative and therefore useless results in the context of this study.

For future research it is recommended to perform comparative measurements of the recently modified anaerobic mineralisation assay (IRRI, not yet published), and enzyme activities as performed in this study. The focus should be on enzymes involved in the soil C and N cycle. As inorganic P is applied at the LTCCE site in sufficient amounts, the phosphatase activity is not expected to give high variations and therefore no useful indication.

ZAMAN *et al.* (1999) mention that the relationship between N mineralisation, extracellular soil enzymes and microbial biomass is not yet fully understood, and that an immediate relationship must not necessarily be anticipated.

This study has shown that activities of hydrolytic enzymes, with the exception of cellobiohydrolase, were significantly correlated with loss on ignition as a rough measure for organic matter content. The obtained positive correlation with fertiliser application rates has to be interpreted carefully, as no detailed data on N application rates were available.

As fertiliser application rates at the LTCCE site are positively correlated with rice yields, the enzyme microplate assay may evolve as a useful predictive tool in the near future. Compared to mineralisation assays and their time-consuming incubation procedure, the MUB assay would also have a great time-saving potential.

6 References

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7 Appendices

Appendix 1: Changes in rice crop management in the long-term continuous cropping experiment at Los Baños, Philippines; from: DOBERMANN *et al.* (2000)

Year	N rate (kg ha^{-1}) †	N management ‡	Other measures ¶
1968 §	DS: 120, 60, 40, 0	120	Water buffalo (carabao) used for plowing and harrowing.
	WS: 80, 60, 40, 0	80	
1969-75	DS: 150, 100, 50, 0	125+25	Highest N rate in 1969 DS was 140 kg N ha^{-1}
	WS: 100, 75, 50, 0	80+20	
1976-79	DS: 150, 100, 50, 0	125+25	
	WS: 90, 60, 30, 0	60+30	
1980-90	DS: 150, 100, 50, 0	100 + 50	Mechanized soil tillage since 1986 (landmaster and hydrotiller).
	WS: 90, 60, 30, 0	60+30	
1991	DS: 150, 100, 50, 0	100+50	Removal of IR8. Only two crops grown (DS, Feb.-May and EWS, July-Oct.). After harvest of the DS crop the field dried partially and remained fallow from May to July 1991. Field was fallow-flooded (Oct.-Dec.) until the 1992 DS crop.
	WS: 90, 60, 30, 0	60+30	
1992	DS: 189, 126, 63, 0	54+36+60+39	Prophylactic fungicide application (2 sprays) to prevent sheath blight (*Rhizoctonia solani*).
	WS: 120, 80, 40, 0	45+45+30	
1993	DS: 216, 144, 72, 0	60+60+60+36	Only two crops grown (DS, Jan.-Apr. and EWS, July-Oct.). After harvest of the DS crop the field dried and remained fallow from May to July 1993. Field was fallow-flooded (Oct.- Dec.) until planting of the 1994 DS crop. DS: N applied at flowering when SPAD was below 35. Furadan (1 x) and fungicide applied (2 x).
	WS: 108, 72, 36, 0	54+54	
1994	DS: 216, 144, 72, 0	60+60+60+36	Only two crops grown (DS, Jan.-Apr. and EWS, July-Oct.). After harvest of the DS crop the field dried partially and remained fallow from May to July 1994. Field was fallow-flooded (Oct.-Dec.) until planting of the 1995 DS crop. DS: N applied at flowering when SPAD-was below 36.5. EWS: N applied after 20 DAT when SPAD reading was below 36. Furadan (1 x) and fungicide applied (2 x).
	WS: 117, 78, 39, 0	36+36+45	
1995	DS: 195, 130, 65, 0	60+60+45+30	Standard N split applications at critical growth stages, not SPAD-based. Furadan (1 x) and fungicide applied (2 x).
	WS: 110, 73, 36, 0	40+40+30	

† N rates for all four N levels are shown. DS, dry season; WS, wet season.

‡ N splitting of the highest N treatment in the DS and WS is shown. From 1969 to 1991, N was applied in two splits: basal and 5-7 days before panicle initiation (PI). From 1992 to 1994, N was applied in two to four splits at basal, midtillering, PI, and flowering. In 1995 N was applied in four splits at midtillering, late tillering, PI, and flowering.

§ Start of the experiment with its present basic design (rice-rice-rice, N rates x varieties).

¶ DAT – days after transplanting. EWS – early wet season. SPAD: chlorophyll meter reading (dimensionless, SPAD 502 chlorophyll meter, Minolta).

Appendix 2: Abbreviations used in the text:

AMC	7-amino-4-methyl-coumarin
approx.	approximately
CEC	cation exchange capacity
c.v.	coefficient of variation
DAT	days after transplanting
IRRI	International Rice Research Institute
LTCCE	Long-term continuous cropping experiment
MUB	4-methylumbelliferone
nmoles	10^{-9} moles
od	oven-dry
pmoles	10^{-12} moles
SOM	soil organic matter
st.d.	standard deviation