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Use of isotope and radiation methods in soil and water management and crop nutrition

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FOREWORD

This publication is a replacement for the IAEA Training Course Series No. 2 "*Use of Nuclear Techniques in Studies of Soil-Plant Relationships*" published in 1990. This edition, prepared by staff of the Soil Science Unit, Seibersdorf, and the Soil and Water Management & Crop Nutrition Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, differs in many respects from its predecessor both in terms of content and objectives. The earlier publication provided basic information for use in interregional training courses held at regular intervals at the Seibersdorf Laboratories. Since the discontinuation of these training courses in 1996, the need for dissemination of up to date information to Member States has become more acute, particularly in view of the evolution of new methodologies during the past decade and new applications of existing methodologies to monitor the dynamics of soil, water and nutrients in cropping systems, and to pilot test interventions to conserve the natural resource base and optimize the availability of water and nutrients to crops. The present publication attempts to fulfill a part of this need.

The manual provides an overview of the use of nuclear techniques in soil science and plant nutrition, balancing the need for a comprehensive coverage of a multitude of techniques involving isotopic tracers and sealed or unsealed sources, while giving sufficient depth to be of practical value to the end-users — students, technicians, scientists in national agricultural research systems and fellowship trainees. In this respect it is important to emphasize that nuclear techniques do not in themselves provide solutions to real world problems — they provide tools which when used in conjunction with other techniques, provide precise and specific information necessary to understand system dynamics and hence the value of alternative management practices to improve system productivity and resource conservation. This publication covers selected aspects of the use of nuclear techniques in studies of soil-plant relationships. However, the need for a series of detailed guidelines on the theory and practical applications of nuclear techniques in soil and water management and crop nutrition, including methodologies, case studies, comparative advantages with non-nuclear techniques and comprehensive bibliographies, is recognized.

Rebecca Hood, Soil Science Unit and Graeme Blair, visiting scientist at the Unit, compiled the manual. Several staff of the Soil and Water Management & Crop Nutrition Section, and the Soil Science Unit, reviewed the contributions and edited the publication. The list of contributors is provided on page 247 of the manual.

EDITORIAL NOTE

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Table of Contents

CHAPTER 1

OVERVIEW OF THE THEORY AND USE OF NUCLEAR TECHNIQUES

1.1 INTRODUCTION

1.1.1 Stable and radioactive isotopes

A Introduction

The nucleus of an atom contains two sub-atomic particles, namely protons (p) and neutrons (n). The atom of a given element has a set number of protons and this is termed the atomic number. The number of protons+neutrons is referred to as the mass number. A particular element can have differing numbers of neutrons and therefore have a different mass number.

Examples of isotopes with the same atomic number (subscript) but with different mass number (superscript) are ${}_{15}{}^{31}P$, ${}_{15}{}^{32}P$, ${}_{15}{}^{33}P$.

A nucleus contains protons, which are positively charged so they should repel. The presence of neutrons, however, keeps the protons together and so stabilises the nucleus. Stability depends upon the neutrons:protons (n:p) ratio.

For light elements, the number of neutrons greatly exceeds the number of protons for stability. For heavier elements more neutrons than protons are necessary for stability.

When the ratio of neutrons to protons is outside a particular number, which varies with each atom, the nucleus becomes unstable and spontaneously emits particles and/or electromagnetic radiation and such a substance is called radioactive. If the ratio of n:p is not outside the "belt of stability" then the isotope does not spontaneously emit particles and is said to be stable eg. ${}^{15}N$, ${}^{34}S$, ${}^{13}C$.

Three types of particles can be emitted from an unstable nucleus

Alpha emission (α)

This is a cluster of 2 neutrons and 2 protons, ${}^{4}He^{2+}$, a helium nucleus. They are heavy, slow-moving, have low energy and are easily stopped by a sheet of paper or a few cm of air. They are highly charged and so are very harmful.

Beta emission (β)

These are fast moving, high-energy electrons, resulting from a neutron decaying into a proton and an electron. They can travel further than an alpha particle and, a few meters of air or a sheet of aluminium is needed to stop them.

Gamma emission (γ)

This is not a particle, but a burst of very high-energy electromagnetic radiation of a very high frequency. γ rays are very dangerous, and require large amounts of lead or concrete to absorb them.

The exact type of emission from a given isotope is a fixed property of that isotope — there will be a fixed pattern of decay until a stable product is reached.

B Half-life

The amount of radioactivity from a radioisotope is measured as a rate i.e. number of disintegrations per unit time. This rate declines with time and the rate of decline is a characteristic of the isotope, e.g. for ${}^{32}P$, a β emitter, the rate drops by 50% every 14.7 days so this is the half-life (see Table 1.1)

The decay curve for any α or β emission is exponential. The rate of decline of the curve is characterised by its half-life. Half-life is independent of mass and, furthermore, is independent of all prevailing conditions (e.g. temperature, pressure, concentration) and of whether it is an atom, part of a molecule, in a solution etc.

The mathematical equation for decay is exponential in nature. The half-life of a substance can be calculated as follows:

$$T = \frac{\log_e 2}{\lambda}$$ where T = half-life

λ = decay constant

$\log_e 2$ = 0.6931

This equation can be used to determine the activity of a particular nuclide at a particular time.

That is:

Decay factor (DF) = $2^{-T/T1/2}$

where T = days since T_0 (i.e.T_0 = activity date)

$T_{1/2}$ = half-life in days

Hence, to determine the activity of a particular substance:

Counts at time *x* = counts at T_0 x D.F.

e.g. Determine the activity of ^{35}S 20 days after its activity date, when the activity was 2200 Bq.

so, T = 20 days

$T_{1/2}$ = 87.2 days

$\therefore$ Decay factor (DF) = $2^{-T/T1/2}$

$= 2^{-20/87.2}$

= 0.8529

Therefore, the ^{35}S has an activity after 20 days of 2200_{Bq} x 0.8533 = 1877.3 Bq

A useful rule of thumb is that 1% of radioactivity remains after 7 half-lives and 0.1% after 10 half-lives

Table 1.1. Half-life calculations for ^{32}P:

Number of half-lives	0	1	2	3	4	5
Mass of ^{32}P (g)	10	5	2.5	1.25	0.625	0.3125
Mass of emissions (g)	0	5	7.5	8.25	9.375	9.6875
Fraction of original mass	1/1	1/2	1/4	1/8	1/16	1/32
Number of ^{32}P atoms	$1.88x10^{23}$	$9.40x10^{22}$	$4.70x10^{22}$	$2.35x10^{22}$	$1.175x10^{22}$	$5.875x10^{21}$
Cumulative number of β particles emitted	0	$9.40x10^{22}$	$1.41x10^{23}$	$1.645x10^{23}$	$1.7625x10^{23}$	$1.82125x10^{23}$

C Units of Radioactivity

The SI unit of activity is the *becquerel* (Bq) which is defined as 1 nuclear disintegration per second. Before the introduction of SI units the most commonly used unit of radioactivity was the *curie* (Ci) defined as 3.7×10^{10} nuclear disintegrations per second. The commonly used units used to express the activity are as shown in Table 1.2.

Table 1.2. Units of radioactive decay

Unit	Fraction of unit	Disintegrations per second (DPS)	Disintegrations per minute (DPM)
Becquerel (Bq)	10^0	1	60
Kilobecquerel (KBq)	10^3	$1x10^3$	$60x10^3$
Megabecquerel (MBq)	10^6	$1x10^6$	$60x10^6$

Some useful conversion factors are:

1 becquerel (Bq) = 60 disintegrations per minute (dpm)

1 curie (Ci) = 3.7×10^{10} becquerels (Bq)

= 37 gigabecquerels (GBq)

1 millicurie (mCi) = 3.7×10^7 becquerels

= 37 megabecquerels (MBq)

1 microcurie (μCi) = 3.7×10^4 becquerels

= 37 kilobecquerels (kBq)

1.1.2 Radioactive isotopes as tracers

Radioactive isotopes can be used to follow a particular element through various pathways and quantitative measurements may be made. They have the advantage of behaving in the same way that their stable counterparts do, but they can be readily traced. Radioactive isotopes can be likened to a coloured dye. They have a wide range of uses and are particularly valuable in plant nutrition research.

The physical properties of a radioactive nuclide determine its usefulness as a tracer. The three most important are half-life, mode of decay and decay energy. If the half-life of a nuclide is very short, any compound labelled with it will be difficult to prepare, use and measure within the time of decay. The mode and energy of decay determine how the nuclide will be measured.

1.1.3 Stable isotopes

Stable isotopes are used in the same way as radioactive isotopes in soil/plant studies. Whereas radioactive isotopes emit particles which are captured in photomultiplier tubes and counted stable isotopes are separated from each other by passing a gas containing them through a strong magnetic field, which deflects them differentially according to their mass. The most common stable isotope used is ^{15}N but a large number of other stable isotopes are produced which are increasingly being used in agricultural studies (Table 1.3).

Table 1.3. Useful stable isotopes in commercial production

Atomic Number	Isotope	Natural abundance %	Possible enrichment %	Other isotopes possibly present
6	^{13}C	1.07	>95	
7	^{15}N	0.37	>99	
8	^{18}O	0.21	>96	
12	^{25}Mg	0.13		
14	^{28}Si	92.21	>99	^{30}Si
	^{29}Si	4.70	>99	^{30}Si
	^{30}Si	3.09	>99	^{28}Si
16	^{33}S	0.76	>99	^{32}S
	^{34}S	4.29	>99	
	^{36}S	0.02	>90	
17	^{35}Cl	75.78	>99	^{37}Cl
	^{37}Cl	24.22	>99	^{35}Cl
26	^{54}Fe	5.85	>99	
	^{57}Fe	91.75	>95	
	^{58}Fe	0.28	>95	
30	^{68}Zn	18.75	>90	
34	^{74}Se	0.89	>95	^{76}Se
	^{76}Se	9.37	>95	^{74}Se
35	^{79}Br	50.69	>99	^{81}Br
	^{81}Br	49.31	>99	^{79}Br
80	^{196}Hg	0.15	>90	
	^{198}Hg	9.97	>99	
	^{199}Hg	16.87	>90	^{202}Hg
	^{200}Hg	23.10	>95	
	^{201}Hg	13.18	>90	^{202}Hg
	^{202}Hg	29.86	>99	
	^{204}Hg	6.87	>95	
82	^{204}Pb	1.40	>85	
	^{206}Pb	24.10	>95	
	^{207}Pb	22.10	>95	
	^{208}Pb	52.40	>99	

1.2 ISOTOPE TERMINOLOGY

Absorbed dose

The energy absorbed by a patient from the decay of a radionuclide given for diagnostic or therapeutic purposes. Unit is the gray (Gy).

Absorber

Any material that stops ionizing radiation. Some examples are lead, concrete, and steel attenuate gamma rays. A thin sheet of paper or metal will stop or absorb alpha particles and most beta particles.

Absorption

The process by which the number of particles or photons entering a body of matter is reduced by interaction with matter. Also the process in which energy is absorbed from the particles or photons even if the number is not reduced.

Activation product

A radioactive material produced by bombardment with neutrons, protons, or other nuclear particles.

Alpha decay

Radioactive decay in which an alpha particle (He4 nucleus) is emitted.

Alpha particle (alpha ray)

A positively charged particle (a Helium4 nucleus) made up of two neutrons and two protons. It is the least penetrating of the three common forms of radiation, being stopped by a sheet of paper. It

is not dangerous to living things unless the alpha emitting substance is inhaled or ingested or comes into contact with the lens of the eye.

Alpha radiation

Emission of an alpha particle from a radionuclide. More damaging than the same dose of beta or gamma radiation but can be stopped by a sheet of paper. It has a positive charge.

Atom

A particle of matter indivisible by chemical means. It is the fundamental building block of elements. An atom has a relatively heavy nucleus made up of positively charged protons and neutral neutrons surrounded by orbiting electrons with a negative charge balancing that of the protons in the nucleus. The number of electrons (from I to 92) determines the chemical characteristics of the atom. The number of neutrons and protons (from I to 238) determines the weight and isotope of the atom.

Atomic mass

Originally defined as the number of protons and neutrons in the nucleus of an atom. Now measured in atomic mass units which are exactly one twelfth the mass of a neutral atom of carbon 12 (u7l.660 x 1027 kg.)

Atomic number

The number assigned to each element on the basis of the number of protons found in the element's nucleus.

Background radiation

Radiation from cosmic sources, naturally occurring radioactive materials and global fallout as it exists in the environment from testing of nuclear explosive devices. It does not include radiation from source, byproduct, or special nuclear materials regulated by the NRC. The typically quoted average individual exposure from background radiation is 360 millirems per year.

Becquerel (Bq)

The unit of radioactive decay equal to I disintegration per second. 3.7 X 1010 Bq =1 Curie.

Beta particle

A charged particle emitted from a nucleus during radioactive decay, with a mass equal to 1/1837 that of a proton. A negatively charged beta particle is identical to an electron. A positively charged beta particle is called a positron. Large amounts of beta radiation may cause skin burns, and beta emitters are harmful if they enter the body. Beta particles may be stopped by thin sheets of metal or plastic.

Beta decay

Radioactive decay in which a beta particle is emitted.

Charged particle

Parts of an atom possessing a small charge of static electricity. A proton has a single unit of positive charge. An electron has a single unit of negative charge. A very large number of electrons passing through a conductor is called an electrical current.

Contamination

The deposition of unwanted radioactive material on the surfaces of structures, areas, objects, or personnel. It may also be airborne or internal (inside components or personnel).

Cosmic radiation

Penetrating ionizing radiation, both particulate and electromagnetic, originating in outer space. Secondary cosmic rays, formed by interactions in the earth's atmosphere, account for about 450 to 500 millisievert of the 3600 millisievert (see Sievert) background radiation that an average individual receives in a year.

Counter

A general designation applied to radiation detection instruments or survey meters that detect and measure radiation. The signal that announces an ionization event is called a count.

Daughter product(s)

The nuclide(s) formed by the radioactive decay of a different parent nuclide. In the case of radium 226, for example, there are 10 successive daughter products, ending in the stable isotope lead 206.

Decay (radioactive)

The change of one radioactive nuclide into a different nuclide by the spontaneous emission of alpha, beta, or gamma rays, or by electron capture. The end product is a less energetic, more stable nucleus. Each decay process has a definite half life.

Decay chain

A series of nuclides in which each member decays to the next member of the chain through radioactive decay until a stable nuclide has been formed.

Decay product

Nuclide or radionuclide produced by decay.

Decontamination

Activities taken to remove unwanted (typically radioactive) material from facilities, soils, or equipment by washing, chemical action, mechanical cleaning, or other (treatment) techniques.

Deuterium (D)

A stable naturally occurring hydrogen isotope. Its natural abundance is about one part in 7000 of hydrogen. Used in the form of deuterium oxide as a moderator.

Disintegration energy (Q-value)

The amount of energy released in a particular nuclear disintegration. This is usually expressed in MeV/disintegration.

Disintegration

Used to describe any process in which the arrangement of particles in the nucleus of an atom is disrupted, producing a change in the nature of the atom. It may happen spontaneously, in the case of a radioactive atom, or as a result of bombardment by other particles.

Dose

A general term denoting the quantity of radiation or energy absorbed in a specific mass. The energy of radiation can damage human living tissue by causing chemical changes in cells which can have biological effects. The amount of damage depends on the energy of the radiation, how much of it is absorbed, which parts of the body absorb it and the timing and duration of the absorption over lifetimes and generations. It is expressed numerically in millisievert or grays.

Dosimeter

A portable instrument for measuring and registering the total accumulated dose to ionizing radiation.

Effective half life

The time required for the amount of a radioactive element deposited in a living organism to be diminished by 50% as a result of the combined action of radioactive decay and biological elimination.

Electromagnetic radiation

A travelling wave motion resulting from changing electric or magnetic fields. Familiar electromagnetic radiation range from X rays (and gamma rays) of short wavelength, through the ultraviolet, visible, and infrared regions, to radar and radio waves of relatively long wavelength. All electromagnetic radiations travel in a vacuum with the velocity of light.

Electron

An elementary particle with a negative charge and a mass 1/183.7 that of the proton. Electrons surround the positively charged nucleus and determine the chemical properties of the atom.

Electron capture

A radioactive decay process in which an orbital electron is captured by and merges with the nucleus. The mass number is unchanged, but the atomic number is decreased by one.

Exposure

Being exposed to ionizing radiation or to radioactive material.

Film badge

A pack of photographic film used for measurement of radiation exposure for personnel monitoring purposes. The badge may contain two or three films of differing sensitivities, and it may contain a filter that shields part of the film from certain types of radiation.

Gamma radiation

High energy, short wavelength radiation emitted from the nuclei of atoms. Less damaging than the same dose of alpha radiation, but much more penetrating. Can be stopped by thick slabs of lead or concrete.

Gamma ray

A highly penetrating type of nuclear radiation, similar to X rays, except that it comes from within the nucleus of an atom, and, in general, has a shorter wavelength.

Geiger Mueller counter

A radiation detection and measuring instrument. It consists of a gas filled tube containing electrodes, between which there is an electrical voltage, but no current flowing. When ionizing radiation passes through the tube, a short, intense pulse of current passes from the negative electrode to the positive electrode and is measured or counted. The number of pulses per second measures the intensity of the radiation field. It was named for Hans Geiger and W. Mueller, who invented it in the 1920's. It is sometimes called simply a Geiger counter or a G/M counter.

Glove box

A closed glass, plastic, or metal chamber, with gloves attached to the chamber wall, used for handling hazardous or weakly radioactive materials. Highly radioactive materials require robotic arms and hot cells.

Gray

SI unit of absorbed dose. Named after L. H. Gray, the British radiation biologist, and equal to the absorption of one joule of energy in one kilogram of tissue. The older unit rad is one hundred times smaller than the gray.

Half life

The time in which one half of the atoms of a particular radioactive substance disintegrates into another nuclear form. Each radioactive isotope has a characteristic half life, e.g. U235 = 0.7038 x 109 years; U238 = 4.468 x 109 years.

Half value layer

The thickness of any given absorber that will reduce the intensity of a beam of radiation to one half of its initial value.

Hot spot

The region in a radiation/contamination area in which the level of radiation/contamination is noticeably greater than in neighboring regions in the area.

Ion

An atom that has too many or too few electrons, causing it to have an electrical charge, and therefore, be chemically active.

Ionization

The process of adding one or more electrons to, or removing one or more electrons from, atoms or molecules, thereby creating ions. High temperatures, electrical discharges, or nuclear radiations can cause ionization.

Ionizing radiation

Any radiation capable of displacing electrons from atoms or molecules, thereby producing ions. Some examples are alpha, beta, gamma, X rays, neutrons. High doses of ionizing radiation may produce severe skin or tissue damage.

Irradiate

To expose to some form of radiation.

Isomer

One of several nuclides with the same number of neutrons and protons capable of existing for a measurable time in different nuclear energy states.

Isotope

Isotopes of a given element have the same atomic number (same number of protons in their nuclei) but different atomic weights (different number of neutrons in their nuclei). Uranium 238 and uranium 235 are isotopes of uranium.

keV

One thousand electron volts

Mass number

The total number of protons and neutrons in the nucleus of an atom.

Megacurie

One million curies

MeV

One million electron volts.

Microcurie curie

One millionth of a curie

Molecule

A group of atoms held together by chemical forces. A molecule is the smallest unit of a compound that can exist by itself and retain all of its chemical properties.

Monitoring

Periodic or continuous determination of the amount of ionizing radiation or radioactive contamination present in an occupied region, as a safety measure, for the purpose of health protection.

Nanocurie

One billionth of a curie.

Neutron activation

The process of irradiating a material with neutrons so that the material itself is transformed into a radioactive nuclide.

Neutron

One of the particles found in the nucleus of an atom, so called because of its neutral electric charge. Free neutrons, released by fissioning or radioactive disintegration of atoms, are very penetrating. When they do collide with the nuclei of other atoms they are likely to cause a wide variety of changes, or transmutations, in the physical characteristics of the atoms they strike.

Nuclide

Any species of atom that exists for a measurable length of time. A nuclide can be distinguished by its atomic weight, atomic number, and energy state.

Picocurie

One trillionth of a curie

Pig

A container (usually lead) used to ship or store radioactive materials. The thick walls protect the person handling the container from radiation.

Pocket dosimeter

A small ionization detection instrument that indicates radiation exposure directly. An auxiliary charging device is usually necessary.

Proton

Elementary particle with positive electrical charge which forms part of the nucleus of every atom. The number of protons determines what element the atom is.

Rad

Acronym for radiation absorbed dose, the basic unit of absorbed dose of radiation. A dose of one rad means the absorption of 100 ergs (a small but measurable amount of energy per grain of absorbing tissue).

Radiation absorbed dose

The basic unit of an absorbed dose of ionizing radiation. One rad is equal to the absorption of 100 ergs of radiation energy per gram of matter.

Radiation area

Any area with radiation levels greater than 50 millisievert.

Radiation sickness syndrome

The complex of symptoms characterizing the disease known as radiation injury, resulting from excessive exposure of the whole body (or large part) to ionizing radiation. The earliest of these symptoms are nausea, fatigue, vomiting, and diarrhea, which may be followed by loss of hair (epilation), hemorrhage, inflammation of the mouth and throat, and general loss of energy. In severe cases, where the radiation exposure has been relatively large, death may occur within two to four weeks. Those who survive six weeks after the receipt of a single large dose of radiation may generally be expected to recover.

Radiation

Energy given off by atoms when they are moving or changing state. Can take the form of electromagnetic waves, such as heat, light, X rays, or gamma rays, or streams of particles such as alpha particles, beta particles, neutrons or protons.

Radiation shielding

Reduction of radiation by interposing a shield of absorbing material between any radioactive source and a person, work area, or radiation sensitive device.

Radioactive contamination

Deposition of radioactive material in any place where it may harm persons or equipment.

Radioactive dating

A technique for estimating the age of an object by measuring the amounts of various radioisotopes in it.

Radioactive waste

Materials which are radioactive and for which there is no further use.

Radioactive

Exhibiting radioactivity or pertaining to radioactivity.

Radioactivity

The spontaneous emission of radiation, generally alpha or beta particles, often accompanied by gamma rays, from the nucleus of an unstable isotope.

Radioisotope

An unstable isotope of an element that decays or disintegrates spontaneously, emitting radiation. Approximately 5,000 natural and artificial radioisotopes have been identified.

Radionuclide

A radioactive nuclide. An unstable isotope of an element that decays or disintegrates spontaneously, emitting radiation.

Rate meter

An electronic instrument that indicates, on a meter, the number of radiation induced pulses per minute from radiation detectors such as a Geiger-Mueller tube.

Roentgen (R)

A unit of exposure to ionizing radiation. It is the amount of gamma or X rays required to produce ions resulting in a charge of 0.000258 coulombs/kilogram of air under standard conditions. Named after Wilhelm Roentgen, German scientist who discovered X rays in 1895.

Scintillation counter

An instrument that detects and measures gamma radiation by counting the light flashes (scintillations) induced by the radiation.

Scintillation Detector

The combination of phosphor, photo multiplier tube and associated electronic circuits used to count light emissions produced in the phosphor by ionizing radiation.

Sievert

Effective dose in humans of 1 joule/kg of gamma radiation. Millisievert (mSv) = 10^{-3}Sv

Shielding

A protective barrier, usually a dense material, which reduces the passage of radiation from radioactive materials to the surroundings.

Tracer

A small amount of radioactive isotope introduced into a system in order to follow the behavior of some component of that system.

X ray

Penetrating electromagnetic radiation (photon) having a wavelength that is much shorter than that of visible light. These rays are usually produced by excitation of the electron field around certain nuclei. In nuclear reactions, it is customary to refer to photons originating in the nucleus as gamma rays, and to those originating in the electron field of the atom as X rays. These rays are sometimes called roentgen rays after their discoverer, W.K. Roentgen.

1.3 MEASUREMENT OF STABLE ISOTOPES

Isotopes have identical chemical properties but some slightly different physical properties. Detection methods use one of these properties such as mass, emission spectrum, IR absorption. The most common and most precise method to measure stable isotopes is mass spectrometry. For the determination of ^{15}N emission spectrometry can also be used, but with much less precision.

1.3.1 Mass spectrometer

Mass spectrometry (MS) is an analytical technique in which atoms or molecules from a sample are ionized, separated according to their mass-to-charge ratio (m/z), and then recorded. There is a wide range of mass spectrometers for different type of samples with different ionization and separation methods. This chapter focuses on instruments capable of determining the isotope ratios of light element stable isotopes (H, C, N, O and S). Instruments of this type are often called Isotope Ratio Mass Spectrometers (IRMS) (*Figure 1.1*). The sample has to be converted to a gas (N_2, CO_2, H_2, SO_2) by means of a suitable preparation system. This gas is fed into the mass spectrometer where the ratios of the isotopes of interest are determined.

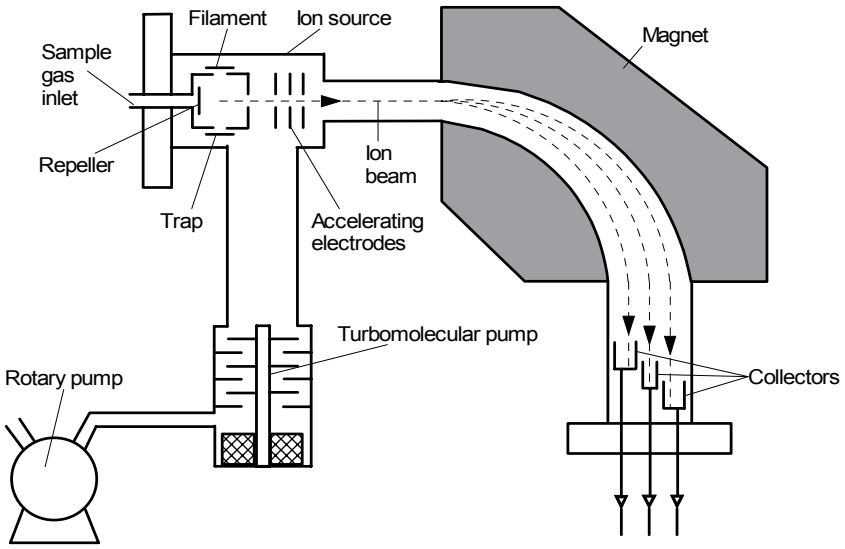

Figure 1.1. Schematic diagram of an isotope ratio mass spectrometer.

Companies which currently market IRMS include:

- Finnigan MAT, Bremen, Germany
- Micromass, UK
- PDZ Europa, UK

The main parts of an IRMS are:

A Ion source

All modern IRMS use an electron impact source based on a design proposed by Nier (1947). Electrons from a hot wire filament (W or ThO_2-coated Ir) interact with the gas molecules producing positively charged ions of the sample. These ions are extracted from the ion source and focused to an ion beam.

B Mass analyzer

The mass analyzer or ion optic is responsible for dispersing the ion beams to different paths according to there mass-to-charge-ratio (m/z). Only magnetic sector analyzers can produce the flat-toped peaks necessary for high precision isotope ratio measurements. The magnet can be either a permanent or electro-magnet.

C Ion collector

The ions separated by the mass analyzer fall on a collector where the ions are discharged and a small current ($<10^{-8}$A) proportional to the amount of ions is produced. IRMS instruments usually have 3 collectors. A setup with two wide collectors and one small collector in the middle can be used to tune the instrument to N, C or S (Figure 1.2).

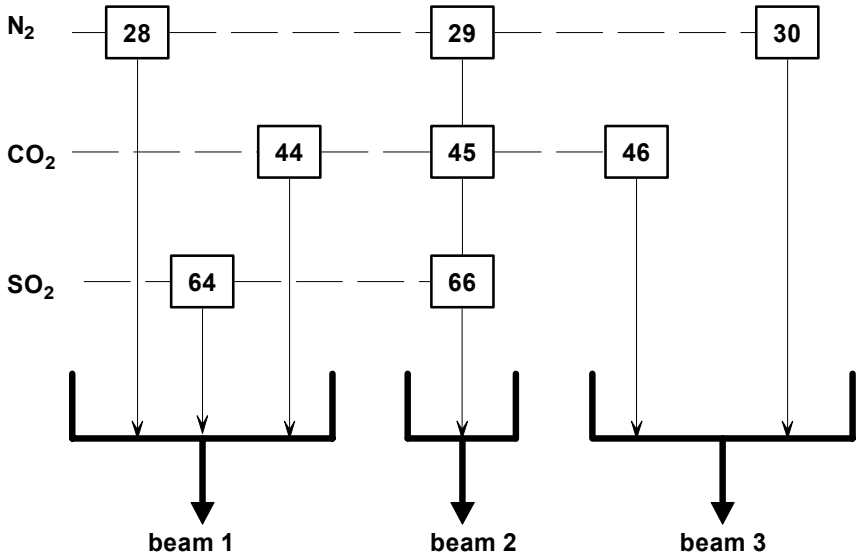

Figure 1.2. Universal triple collector scheme.

For deuterium measurements an additional collector is necessary because of the large spatial difference between the m/z 2 and m/z 3.

D Vacuum system

The ion source, flight tube and the collectors of the mass analyzer have to be under high vacuum (~10^{-8} mbar). Higher pressure broadens the ion beams because of collisions of the ions with gas molecules. This high vacuum is achieved in two stages:

1. A rotary pump evacuates from atmospheric pressure down to 10^{-2} mbar.

2. A turbomolecular pump is used to pump down from 10^{-2} mbar to 10^{-8} mbar. In older mass spectrometers this pump was often and oil diffusion pump.

Because it takes at least a day to establish the high vacuum, the pumping system is left running all the time and is never switched off under normal circumstances. A reliable power line or an uninterruptable power supply (UPS) unit is essential to run an IRMS.

E Sample inlet system

Two systems are available:

i *The dual inlet system (DI)*

Sample gas and a reference gas are stored in two separate variable volume containers. From each of the variable volumes a steel capillary runs to a changeover valve which switches at regular time intervals to either the sample or the reference gas at the inlet of the IRMS. A measurement consists of a set of sample-reference determinations (usually about 8). This method has the highest precision but the measuring time is long (20 min per sample) and a rather large sample is needed (1mg of N or C).

ii *The continuous flow interface (CF)*

A small capillary samples a small portion (~0.5%) of gas out of the He carrier/sample gas stream coming from the preparation system. The very popular connection of an elemental analyzer to an IRMS uses this interface. Analysis time is much faster (4min for N, 7min for C) but precision is not as good as with DI.

F Sample preparation systems

Depending on the isotope of interest and the type of sample different preparation systems and methods have to be used to produce the sample gas. All of the following preparation systems can be connected online (CF or DI) to the IRMS for automatic sample measurement.

i *Elemental analyzer*

Can be used to measure N, C and S in a wide range of sample types (plant material, soil, filter disks, almost all organic materials and some inorganic substances like $SrCO_3$ or NH_4^+ salts). It not only gives isotope ratio values but also total element values.

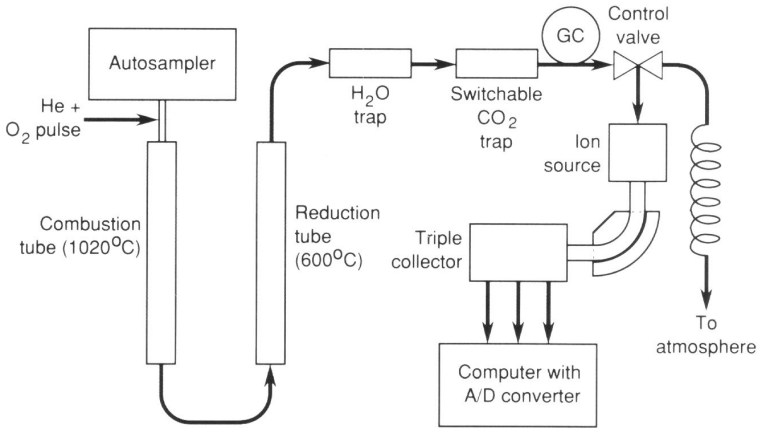

Figure 1.3. Elemental analyzer with CF interface online.

The sample is weight into small Sn containers and put into an autosampler wheel. After purging the sample with He the sample drops into the hot combustion tube (1000°C) partly filled with Cr_2O_3 as an oxidation catalyst. Prior to the sample drop the tube was filled with O_2 gas. The sample and the Sn undergo a flash combustion which oxidizes the sample material. The combustion gases (CO_2, H_2O, N_2, SO_2 and N-oxides) are moved with a He carrier gas to the reduction tube (600°C) which is filled with metallic Cu wires. The N-oxides are reduced to N_2 and the access of O_2 reacts with the hot Cu forming CuO. A trap filled with Mg-perchlorate removes the water out of the gas stream. CO_2 is absorbed by a chemical trap (Carbosorb). If C isotopes want to be measured this trap can be bypassed and the CO_2 and N_2 can be separated by a chromatographic column. The sample gases are introduced into the mass spectrometer by a continuous flow (CF) interface (*Figure 1.3*).

ii *Pyrolysis (high temperature conversion in the absence of oxygen)*

Is used to determine ^{18}O in organic compounds, water and selected inorganic materials like CO_3^{2-}, NO_3^- or SO_4^{2-} and hydrogen isotope ratios from organic compounds and water. The instrument is similar to an elemental analyzer with some major differences. The reaction tube is made of glassy carbon and filled with graphite. The conversion temperature has to be higher (1300°C) than in an elemental analyzer. The sample is converted into CO. This reaction gas is introduced into the mass spectrometer with a CF interface where ^{18}O is measured.

iii *Gas equilibration of water samples*

The equilibration technique can be used to determine D or ^{18}O in water. To determine ^{18}O small amounts of water (~1ml) are equilibrated in closed containers with CO_2 gas at room temperature. The oxygen from water equilibrates with the oxygen from CO_2. After the equilibration period the CO_2 is sampled and introduced to the IRMS by either DI or CF. The same principle can also be used to measure D in water if H_2 is used as the equilibration gas. In this case a Pt catalyst has to be used and because of the strong temperature dependency of the equilibration the reaction has to be carried out in a temperature controlled water bath.

iv *Reduction of water to H_2*

Small water samples (10µl) are brought in contact with hot metals (Zn, U, Mn, Cr) where the water is reduced to H_2 gas. This can be done either offline in sealed tubes or online in a reaction column.

v *Carbonates device*

Can be used to determine C and O ratios in carbonates. H_3PO_4 is added to the sample and the produced CO_2 is passed to the IRMS with a DI or CF interface. To measure ^{18}O, water-free phosphoric acid has to be used to prevent an oxygen isotope exchange between the sample CO_2 and the water.

1.3.2 Emission spectrometer

Emission spectrometers are much simpler than mass spectrometers. They can be maintained much easier but they can be used only to determine $^{15}N/^{14}N$ ratios.

Nitrogen gas at low pressure (10 torr) is excited to higher electronic levels by absorbing energy from an external radio-frequency (RF) source (electrodeless discharge) and emits radiation in the VIS-UV region when the electrons fall back to ground state. A monochromator resolves the spectrum. The variation of light intensity with wavelength at the exit slit is detected by a photomultiplier tube (PM) or a photo diode array (PDA) (*Figure 1.4*).

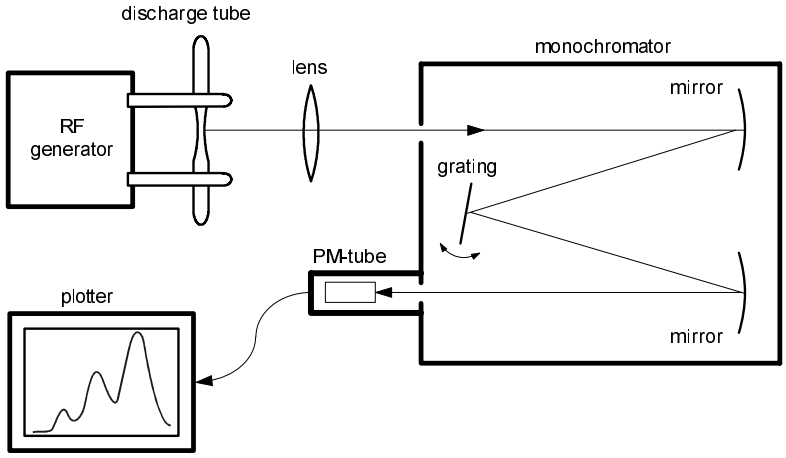

Figure 1.4. Simple emission spectrometer for sealed discharge tubes.

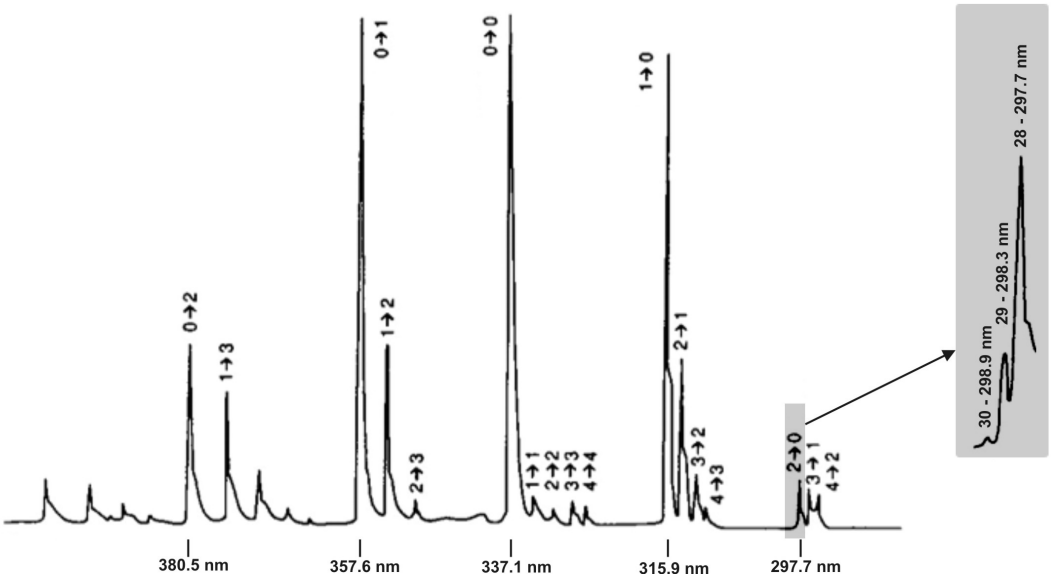

Figure 1.5. Emission spectrum of molecular nitrogen.

The emission bands (*Figure 1.5*) of molecular nitrogen which are used for measurement are:

$^{14}N\ ^{14}N$	297,7 nm (2977Å)
$^{14}N\ ^{15}N$	298.3 nm (2983Å)
$^{15}N\ ^{15}N$	298.9 nm (2989Å)

The monochromator must be able to scan over this wavelength region. In older types of emission spectrometers (NOI-5, Jasco, Sopra) this is carried out by moving the prism or grating in the monochromator slowly with an electro motor. A photomultiplier (PM) tube is used to record the different light intensities over time. One scan of a spectrum takes about a minute. The more advanced NOI-6 emission spectrometer uses a vibrating slit in front of the PM-tube which scans

the region of interest about 50 times per second which results in an life display of the spectrum on the computer. The NOI-7 machine is current state of the art as this detects the spectrum simultaneously with a photo array diode detector (PDA). Each cell of the PDA represents a small part of the spectrum and can be read out by the computer. An advantage of this design is the absence of moving parts, which improves the robustness of the instrument.

The measured values have to be converted to the true atom% values by means of a calibration curve. This curve has to be prepared beforehand with a set of known standards. The NOI-6 and NOI-7 are equipped with a data acquisition unit (PC) which stores the calibration data and automatically converts the measured to the true value.

A Off-line sample preparation

The older type of emission spectrometer (NOI-5, Jasco, Sopra) need sealed discharge tubes to be prepared off-line on a separate vacuum line. A small amount of NH_4^+ sample representing about 10μg N is inserted together with CuO and CaO into a glass tube. The tube is evacuated, sealed and put in a muffle furnace at 600°C to convert NH_4^+ to N_2 gas. Measuring time (including the preparation of the tubes) is more than 20 minutes per sample. The standard deviation is more than 3%.

B On-line sample preparation

The NOI-6 and NOI-7 emission spectrometers have a built-in sample preparation system which converts NH_4^+ nitrogen to N_2 gas on-line by the Rittenberg conversion. The N_2 sample and He carrier gas passes through a drying tube and a discharge tube where the pressure is adjusted to about 10 torr by means of a vacuum pump and two flow restrictors. Measurement time is about 1 minute per sample with a standard deviation of about 1 %.

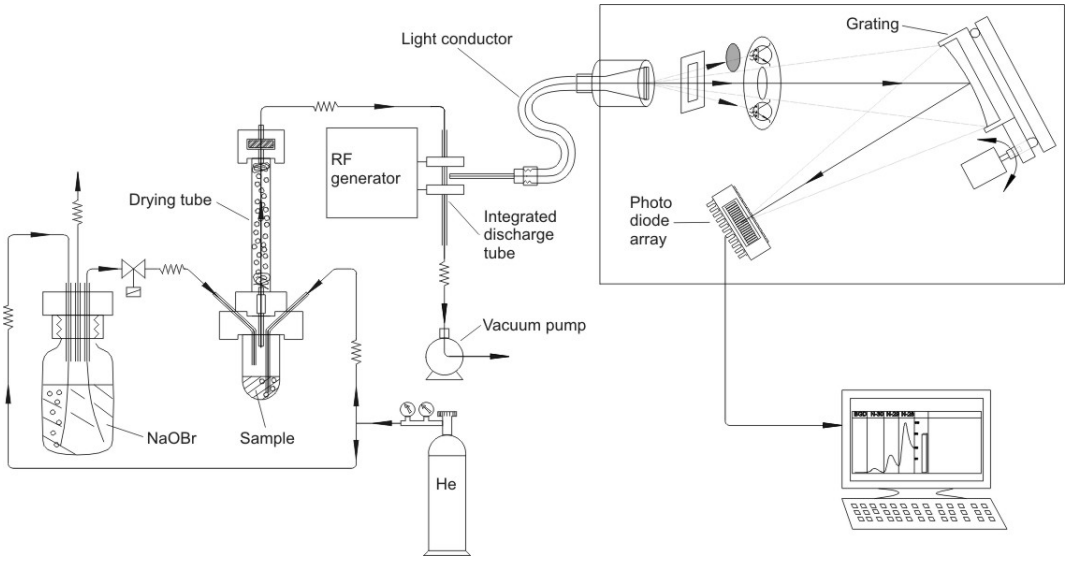

Figure 1.6. Modern emission spectrometer with integrated sample preparation system (NOI-7).

1.3.3 Summary of features of commercially available emission spectrometers

Emission spectrometer	Sealed tubes	Integrated preparation system	Wavelength scanning	Detector	Output device
NOI-5	yes	no	motor	PM-tube	plotter
JASCO					
SOPRA					
NOI-6	yes	yes	vibrating slit	PM-tube	computer
NOI-7	yes	yes	n.a.	Photo diode array	computer

1.3.4 Comparison of IRMS versus optical emission spectrometer (OES)

IRMS	OES
Can measure isotope ratios of H, C, N, O and S	For N only
Can be used to determine total element values (depending on preparation system)	No total N values
High precision (SD < 0.1 δ per mill); suitable for natural abundance work	Low precision (RSD 1%); not suitable for natural abundance work
Expensive (> US$ 100.000,-)	Less expensive (US$ 60.000,-)
One point calibration (with one known standard)	Calibration curve necessary (with a set of known standards)
Instrument is running all the time; reliable power line or UPS necessary	Instrument can be switched off if it is not needed; eventually a constant voltage transformer is necessary
For ^{15}N analyses no Kjeldahl digestion is necessary (elemental analyzer)	Most samples have to be converted to NH_4^+ by Kjeldahl digestion
High operator skills required	Medium operator required

1.4 MEASUREMENT OF RADIOISOTOPES

A detailed treatment of this can be found in L'Annunziata (1998)

1.4.1 Scintillation Counting

A Principles

Beta decay occurs when an unstable nucleus returns to stability through the conversion of a neutron to a proton with the emission of an electron and an antineutrino as follows

$$\text{Neutron} \longrightarrow \text{Proton} + \text{Electron} + \text{Antineutrino}$$

This process occurs within the nucleus of a compound like carbon-14, which has 6 protons and 8 neutrons but as a result of beta decay, transforms into stable nitrogen with a nucleus of 7 protons and 7 neutrons.

The total energy of beta decay, Emax, consists of the combined energy of the emitted the beta and antineutrino particles. Very few of the emitted beta particles have maximum energy, as energy is shared between the beta particle and the antineutrino. Most of the emitted beta particles have an energy of approximately one third of Emax (Figure 1.1)

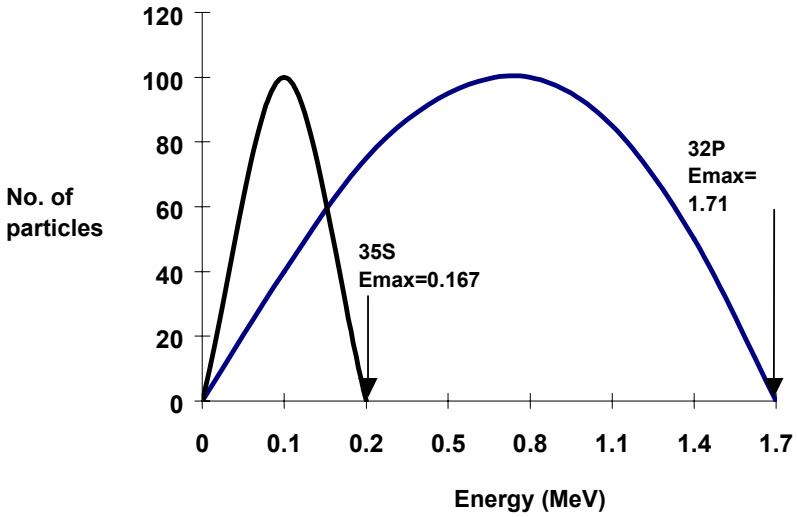

Figure 1.7. Energy spectra for ^{32}P and ^{35}S.

The windows can be set on the liquid scintillation counter LSC to capture β^+ particles of particular energies. These window settings can also be used to simultaneously count two or more isotopes that have different energy spectra such as the ^{32}P and ^{35}S isotopes in *Figure 1.7*. When two isotopes with almost identical spectra eg. ^{35}S, E_{max}= 0.167 and ^{14}C, E_{max}= 0.156 need to be counted together the differing half lives (^{35}S = 87.4 days, ^{14}C = 5730 years) are used to distinguish the isotopes. To achieve this the same samples are counted at different times and the decrease in total radioactivity can be attributed to the decay of ^{35}S. The following equations are used to calculate for radioactivity of ^{14}C and ^{35}S using the counts obtained from two separate dates:

At T1: Total Bq = Bq ^{14}C + Bq ^{35}S (1)

At T2: Total Bq = Bq ^{14}C + λ Bq ^{35}S (2)

Where λ the decay factor is equal to:

$$\lambda = e^{-0.693(t/t_{1/2})}$$

In liquid scintillation counting a solvent is used to ensure that the liquid to be counted is in solution. An emulsifier or detergent is added to enhance the mixing of organic and inorganic solutions and a compound which fluoresces when struck with an electron (fluor) is added. In some instances the fluorescence that occurs cannot be detected by a photomultiplier tube, so a secondary wave shifter is added.

The solvent is the first compound in the scintillation cocktail to capture the energy of the beta particle and the solvent molecule achieves an excited state. The excess energy is transferred from solvent molecule to solvent molecule. The solvent remains in the excited state for an extended period of time, decaying into the ground state without the emission of light. The fluor then absorbs the excitation energy of the solvent, and quickly returns to the ground state by emitting light. If a wave shifting fluor is used this absorbs the signal of the first solute and emits a second burst of light at a longer wavelength and this is detected by a photomultiplier in the counter.

B Preparation of Scintillation Mixture for LSC

There are many "cocktails" used for the preparation of radioactive samples. Following, are the details for the preparation of a cocktail, which can be successfully used with most samples (Till et al., 1984).

i *Reagents*

Toluene — scintillation grade
p-terphenyl
POPOP
teric

ii *Method*

1. Heat 1L of toluene in a beaker on a heater/stirrer.
2. Add 16.92 g of p-terphenyl and 0.73 g of POPOP (1,4-bis-2-(5-phenyloxazolyl)benzene or phenyl-oxazolyl-phenyl-oxazolyl-phenyl) to the toluene.
3. Heat and stir until dissolved.
4. The toluene/p-terphenyl/POPOP solution is then added to 2080 mL Teric (A detergent manufactured by Shell) in a 5 L volumetric flask.
5. Make the solution to volume with toluene once the solution is cool.

iii *Sample/scintillant ratio*

This mixture can be used at a ratio of 3mL sample:17mL of scintillant, to give a final volume of 20mL. However, if the mixture of the sample and scintillant prove immiscible, the ratio of scintillant to sample can be varied (e.g. 18–19mL scintillant to 2-1mL sample) or the mixture acidified using a concentrated mineral acid (e.g. 2mL sample, 1mL acid and 17mL of scintillant). These ratios may be altered until a clear emulsion results.

C The Phenomenon of Quenching

Chemical constituents and colour in the sample can result in a reduction in the number of β^+ particles leaving the sample. This is termed quench. This can be accounted for by adding known quantities of the isotope to the matrix and using this count data to correct the counts measured in the samples. This is termed quench correction.

To compensate for quenching losses, a calibration curve must be prepared against which the unknown samples can be compared. The quench curve can be established by counting a series of samples containing a known constant amount of radioactivity, but varying concentrations of a quencher.

In theory, the construction of one calibration curve should suffice for all mechanisms quenching the same isotope, provided that the calibration is for the same scintillator and constant settings. However, most users calibrate for each particular sample preparation, unless using a sophisticated machine which can compensate for colour and photon quenching.

A prepared quench curve should cover the range in which the unknown samples are to be measured. The standards should be prepared in duplicate and there should be 5–10 calibration points. The activity of the radioactive isotope used should lie between 60 and 600 mBq/vial.

Two substances that can be used as quenching agents are chloroform and acid. Chloroform is a very effective quencher, but, due to its volatility, it can be difficult to measure accurate volumes. When using chloroform, use in a cool, draught-free area, to minimise errors.

Table 1.4 contains a suggested method of preparing a quench curve. Chloroform is used, but the user can modify this to suit the particular sample matrix required. The isotope activity and volume of quench can also be varied.

Table 1.4. Volumes used in preparing quench standards

Identifier	Volume of quench (eg cloroform) (mL)	Volume of water (mL)	Volume of isotope solution (eg.1500mBq/mL) (mL)	Volume of scintillant (mL)
Background 1	0	3.00	0	17.00
Background 1	0	3.00	0	17.00
Quench 1a	0	2.00	1.00	17.00
Quench 1b	0	2.00	1.00	17.00
Quench 2a	0.05	1.95	1.00	17.00
Quench 2b	0.05	1.95	1.00	17.00
Quench 3a	0.10	1.90	1.00	17.00
Quench 3b	0.10	1.90	1.00	17.00
Quench 4a	1.15	1.85	1.00	17.00
Quench 4b	0.15	1.85	1.00	17.00
Quench 5a	0.20	1.80	1.00	17.00
Quench 5b	0.20	1.80	1.00	17.00
Quench 6a	0.25	1.75	1.00	17.00
Quench 6b	0.25	1.75	1.00	17.00
Quench 7a	0.30	1.70	1.00	17.00
Quench 7b	0.30	1.70	1.00	17.00
Quench 8a	0.35	1.65	1.00	17.00
Quench 8b	0.35	1.65	1.00	17.00

When running the quench curve on the LSC, set-up the measurement conditions and load the curve so that the background samples are read first, followed by the unquenched standards, then the quenched standards in increasing volumes of quench.

To measure dual labelled samples (e.g. both ^{32}P and ^{35}S), two quench curves must be prepared for each isotope, i.e. the two isotopes are not mixed together in the same standard. This is because the LSC cannot differentiate between the two different radioisotopes; it simply combines the energies. (Many modern LSCs use sophisticated software to then separate the counts for the different nuclides.) To run a quench curve for dual labelling, load the lowest energy nuclide first (e.g. ^{35}S), followed by the highest energy nuclide (e.g. ^{32}P).

1.4.2 Geiger-Müller counting

In contrast to the scintillation counter, the Geiger-Müller counter is a device for measuring ionization. Its efficiency is usually low and its use has declined markedly since the introduction of sophisticated scintillation counters. However, sample preparation is very simple; no solvent is used and the radiation from the sample interacts directly with the ionization chamber. As the source material does not interfere with the operation of the GM tube, this technique can be readily used on soil providing the samples are uniformly treated.

1.4.3 Cerenkov counting

When β particles are emitted they leave the nucleus at speeds approaching that of light in a vacuum. However, in the surrounding medium the speed of light is lower and consequently the passage of particles through the medium causes shock waves from which light photons are emitted. This light can be counted to give a measure of the radioactivity present. The minimum energy required to produce light in an aqueous solution is 0.263 MeV. This means that only those radionuclides with high-energy emissions, such as ^{32}P, may be counted by this method.

1.4.4 Autoradiography

Autoradiography may be used to give a qualitative estimate of the amount of activity present. The degree of darkening of the developed emulsion is related to the activity present.
It can be used to show events ranging from individual tracks due to disintegration of cellular materials through root growth, to the movement of phosphorus from fertiliser granules into the soil.

1.4.5 Gamma counting

Gamma rays are electromagnetic radiation similar to light but with much shorter wavelengths. Both have well defined energies. Gamma emissions are mono-energetic so they are not like beta emission where there is a broad spread of energies. Most gamma emitters produce gamma particles with a single energy but some like ^{137}Cs, emit two or even more gamma rays of different energies, which arise from different decay processes. The energy of the gamma photon is three to six orders of magnitude higher than that of the light photon. The energies measured in a gamma counter are in the range 20,000 eV to 2,000,000 eV (20 keV to 2 MeV).

The gamma counter has two detector elements, a sodium iodide crystal and the photomultiplier. The detector type commonly used is a single crystal of thallium-activated sodium iodide, NaI (Tl). In the energies below 2 MeV interaction of gamma rays with the crystal may take place by two principal mechanisms. In the photoelectric effect a gamma photon disappears and a (photo) electron is ejected from one of the atomic electron shells with a kinetic energy, which is the difference between the gamma photon and the orbital electron binding energy. The photoelectric absorption is used in the measurement of the energy of a gamma photon.

Soil and liquid samples can be counted. The sample is introduced into the counter and is brought alongside the sodium iodide crystal and the photomultiplier and the gamma rays emitted from the sample counted.

1.5 REFERENCES AND FURTHER READING

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CHAPTER 2

APPLICATIONS OF NUCLEAR TECHNIQUES IN SOIL FERTILITY AND PLANT NUTRITION STUDIES

2.1 PRINCIPLES AND APPLICATIONS OF ISOTOPES IN FERTILISER EXPERIMENTS

2.1.1 INTRODUCTION

Fertilisers are one of the essential inputs for maintaining or increasing the soil fertility level in intensive agricultural systems. The purpose of applying fertilisers is primarily to supply the crop with essential plant nutrients to ensure normal plant growth. The major plant nutrients (N, P and K) have to be applied regularly to compensate for the amounts exported from the soil during harvest. Other plant nutrients such as Ca, Mg, S and the micronutrients eg. Zn, Mo, B may also need to be added to maintain adequate levels of these nutrients, or to correct deficiencies (FAO, 1983 b, 1984).

Fertilisers are applied to facilitate plant uptake of a particular nutrient. Increased uptake can lead to a yield response if the particular nutrient is a limiting factor. It is important though to note that the fertiliser is not applied to obtain a yield response but to feed the plant. The yield response is a consequence of the additional uptake of the nutrient when other production factors are adequate.

A combination of all the production factors and conditions in an agricultural system results in a given yield and only if all factors are optimized (fertiliser, soil, plant, water, pest control, etc.) will yield be maximized. In fact the contribution of fertiliser to increased yield is perhaps of the greatest importance among the purchased inputs. Fertiliser, when used in combination with the other adequate inputs such as high-yielding varieties and irrigation water, can result in a positive interaction thereby further increasing its contribution to increased yield (Fried, 1978).

In the decade of 1980 due to the substantial increases in the cost of the fertilisers and their limited supplies to resource-poor farmers, enhanced nutrient management was pursued through maximising the efficiency of nutrient uptake from various inorganic and organic sources utilising two complementary approaches: i) identification, and /or selection of plant genotypes efficient at low levels of soil available nutrients and tolerant to predominant stress conditions, and ii) development of integrated plant nutrition systems to maximise yield responses and to reduce environmental contamination and degradation of natural resources (Zapata and Hera, 1995).

Since 1996 the strategic objective of the subprogramme Soil and Water Management & Crop Nutrition of the Joint FAO/IAEA Programme is to develop and promote the adoption of nuclear-based technologies for optimising soil, water and nutrient management in well-defined cropping systems and agro-ecological zones, which support intensification of crop production and preservation of the natural resource base. An integrated approach to soil, water and nutrient management has been adopted and implemented in the Co-ordinated research Projects and whenever possible in the Technical Co-operation Projects.

2.1.2 FERTILISER USE EFFICIENCY

Fertiliser use efficiency is a quantitative measure of the actual uptake of fertiliser nutrient by the plant in relation to the amount of nutrient added to the soil as fertiliser. A common form of expression of fertiliser use efficiency is plant recovery or “coefficient of utilization” of the added fertiliser. This is shown in equation (1):

$$\%\ \text{utilization of added fertiliser} = \frac{\text{Amount of nutrient in the plant derived from the fertiliser}}{\text{Amount of nutrient applied as fertiliser}} \times 100 \quad \text{(Equation 1)}$$

The concept of fertiliser use efficiency, however, is much broader. It implies not only the maximum uptake of the applied nutrient by the crop but also the availability of the applied nutrient under variable climatic and edaphic conditions. Environmental issues, such as pollution resulting from the fertiliser application, should also be considered. It is important to study the efficient use of fertilisers because we are interested to obtain the highest possible yield with a minimum fertiliser application.

The crop responds to the application of nutrients such as nitrogen and phosphorus when the soil is deficient in such nutrients. The objective should be to apply the fertiliser to the crop, not to the soil and to avoid the fertilisers becoming unavailable to the crop, i.e. sorbed in the case of phosphorus

or lost as nitrate by leaching or as gaseous losses due to denitrification and/or volatilization in the case of nitrogen.

It is, therefore, essential to ensure that the applied fertiliser is taken up by the crop to the highest possible extent. This is done in field trials by assessing the best fertiliser practices such as sources, timing, placement and their interactions in different farming systems (FAO, 1980; 1983a; 1985).

2.1.3 MEASUREMENT OF FERTILISER USE EFFICIENCY

The best combination of fertiliser practices can be established for each crop by carrying out field experiments under different environmental conditions. When these experiments have been conducted, with identical design and layout, it is possible to determine what generalization can be made with respect to placement, timing and source of fertiliser that result in the most efficient fertiliser uptake by the crop.

Therefore, in practice, a series of carefully designed field experiments, in several representative locations are carried out over a period of time for estimating the effect of placement, timing and source on fertiliser nutrient uptake. Yield, particularly economic yield, is generally the most important criteria for the farmer but it is equally important that this yield is obtained with a minimum of fertiliser investment (minimum cost).

The following methods can be utilised to assess the efficiency of fertiliser practices:

A The classical or conventional method based on yield

This measures the biological response or the effect of increasing fertiliser rates on crop yield. Yield is however dependent on a series of factors: some controllable, others not controllable. They all will influence, to a variable extent, the yield and quality of the product.

B Methods based on nutrient uptake

i *Difference method*

In this indirect method the nutrient uptake by the crop in a control plot (without fertiliser application) is subtracted from that of the fertilized treatments. It is assumed that the nutrient uptake of the control plot measures the amount of nutrient available from the soil, whereas that of the fertilized treatments, the amount of nutrients available from soil and fertiliser. This method, furthermore, assumes that all nutrient transformations i.e. mineralisation, immobilization and other processes in the case of nitrogen, are the same for both fertilized and unfertilized soils. Obviously, this is an erroneous assumption, and can account for gross differences between recoveries calculated by non-isotope and isotopic methods (Broeshart, 1974; Westerman and Kurtz, 1974; Harmsen and Moraghan, 1988). Recovery data estimated using this method are best referred to as “apparent utilisation”

ii *Isotopic method*

The only direct means of measuring nutrient uptake from the applied fertiliser is through the use of isotopes. Extensive work has been conducted using N-fertilisers labelled with the stable isotope ^{15}N and P-fertilisers labelled with the radioactive isotopes ^{32}P or ^{33}P. This does not mean that K and the other plant nutrients are not important. Initial work has been done with N and P utilising isotopic methods, and many studies have also been conducted with the others, as researchers in developing countries became experienced and confident with the methodology (Broeshart, 1974; Fried, 1978; IAEA, 1970a, 1970b, 1971, 1974, 1975, 1978a, 1980, 1983a; FAO, 1980; Zapata and Hera, 1995).

The principal tracer isotopes used in soil-plant relationships studies are shown in Table 1. The chemical elements have been grouped into 3 categories. The first two groups refer to the essential plant nutrients i.e. macro and micronutrients, respectively, while the third one consists of a miscellaneous group of trace elements and others used in soil-plant relationships and related studies. It is often argued that the labelled fertilisers loose their identity in the soil since they became incorporated into the organic matter, soil solution, ion exchange processes, etc. resulting in just one pool of nutrients. The only basic assumption made when utilising isotopically labelled fertiliser is that the behavior of the isotope and the carrier is identical in the soil-plant system. In other words there should not be any isotope effect.

The isotopic labelling of the fertiliser is best done during the manufacturing process by specialized firms.

Table 2.1. Isotopes useful in soil/plant studies

Element	Most abundant isotope	Tracer isotope	Characteristics	Typical Applications
I - MACRONUTRIENTS				
Nitrogen	^{14}N	^{13}N	R, T ½ = 10 min β emitter (1.2 MeV) and γ (0.511 MeV)	Limited because of short half-life. Very short term studies on N_2 fixation, denitrification
		^{14}N	S, natural abundance = 99.634 % $^{15}N/^{14}N$ ratio by MS	N-14 enriched (N-15 depleted) materials for single season fertiliser use efficiency studies
		^{15}N	S, natural abundance = 0.366 % $^{15}N/^{14}N$ ratio either by MS or ES	Fertiliser N use efficiency, biological nitrogen fixation, N balance, N transformation in soils, N availability from organic-materials, animal nutrition studies
Phosphorus	^{31}P	^{32}P	R, T ½ = 14.3 d β emitter (1.71 MeV) GM, Cerenkov or LSC	Fertiliser P use efficiency, residual P fertiliser studies, exchangeable P in soils, root activity patterns of crops, root distribution in soils, agronomic evaluation of rock phosphates, residual P fertiliser availability.
		^{33}P	R, T ½ = 25 d β emitter (0.248 MeV) LSC	Root autoradiography, diffusion in soil, double labelling for root activity patterns, fertilisers P use efficiency
Potassium	^{39}K	^{40}K	R, T ½ = 1.3 x 10^9 y β (1.3 MeV) LSC, Cerenkov γ (1.46 MeV) emitter NaI (T1) Natural Radioisotope, Natural abundance - 0.0118 %	Exchangable K in soils
		^{41}K	S, natural abundance = 6.77 %	Potentially useful
		^{42}K	R, T ½ = 12.4 h β (3.5 and 2.0 MeV) LSC, Cerenkov γ (1.52 MeV) emitter NaI (T1) 2”/3”	Ion uptake mechanisms. Limited because of short half-life
Rubidium		^{86}Rb	R, T ½ = 18.7 d β (1.8 and 0.7 MeV) Cerenkov, LSC and γ (1.08 MeV) Counting by GM or LSC NaI (T1) 2”/3”	Substitute tracer for K. Only qualitative studies like placement
Calcium	^{40}Ca	^{45}Ca	R, T ½ = 165 d β emitter (0.252 MeV) LSC	Soil Ca (ion uptake, exchangeable Ca) and plant Ca movement (root autoradiography)
Strontium		^{85}Sr	R, T ½ = 64 d γ emitter (0.514 MeV) LSC, Cerenkov	Cation exchange capacity of soil, ion uptake mechanisms
		^{89}Sr	R, T ½ = 52.7 d β emitter (1.463 MeV) Counting by LSC, Cerenkov	Substitute tracer for Ca

Magnesium	^{24}Mg	^{26}Mg	S, natural abundance = 11.29 %	Potentially useful. Environmental pollution. Ecological and medical research
		^{28}Mg	R, T ½ = 21.3 h β emitter (0.5 MeV) and also γ emitter (0.03; 0.4; 0.95; 1.35 MeV)	Movement in plants
Sulfur	^{32}S	^{34}S	S, natural abundance = 4.25 % $^{34}S/^{32}S$ ratio by MS	Potentially useful, environmental pollution, ecological and medical research
		^{35}S	R, T ½ = 87 d β emitter (0.165 MeV) LSC	Uptake from atmosphere (SO_2), S cycling studies, availability from soil
II - MICRONUTRIENTS				
Iron	^{56}Fe	^{55}Fe	R, T ½ = 2.6 y Electron capture (EC), LSC	
		^{59}Fe	R, T ½ = 45.6 d β emitter (0.475; 0.273 MeV) LSC γ emitter (1.1; 1.29 MeV) NaI (T1) 2"/3"	Erosion studies, soil and plant movement, availability from soil
Copper	^{63}Cu	^{64}Cu	R, T ½ = 12.8 h β emitter (0.6; 0.7 MeV) and γ emitter (1.34 MeV) EC	Complexing in soil solution, soil and plant movement
		^{65}Cu	S, natural abundance = 30.9 %	Animal nutrition studies. Potentially useful
		^{67}Cu	R, T ½ = 58.5 h β emitter (0.58; 0.48; 0.40, 0.091 MeV) and γ emitter (0.092; 0.184 MeV)	
Manganese	^{55}Mn	^{52}Mn	R, T ½ = 5.7 d β emitter (0.6 MeV) and γ emitter (1.43; 0.94; 0.74; 0.84 MeV)	
		^{54}Mn	R, T ½ = 314 d γ emitter (0.835 MeV) EC	Complexing in soil solution, availability from soil, soil and plant movement
Zinc	^{64}Zn	^{65}Zn	R, T ½ = 245 d β emitter (0.327 MeV) and γ emitter (1.115 MeV) from EC	Complexing in soil solution. Availability from soil and fertiliser. Soil and plant movement.
Boron	^{11}B	^{10}B	S, natural abundance = 19.7 %	Foliar absorption, neutron activation, neutron moderation (soil moisture studies), soil chemistry
Molybdenum	^{96}Mo	^{99}Mo	R, T ½ = 66.7h β emitter (1.2; 0.45 MeV) and γ emitter (0.74; 0.18; 0.78; 0.37 and 0.041 MeV)	Plant nutrition
III - OTHER ELEMENTS				
Hydrogen	^{1}H	^{2}H	S, natural abundance = 0.01492 % $^{2}H/^{1}H$ ratio by MS	Water movement, biochemical studies, water cycling
		^{3}H	R, T ½ = 12.3 y Very weak β emitter (0.0181 MeV) LSC	Water movement, metabolism, leaching

Carbon	^{12}C	^{11}C	R, T ½ = 20.5 min β emitter (1 MeV)	Limited because of short half-life
		^{12}C	S, natural abundance = 98.892 %, $^{13}C/^{12}C$ ratio by MS	C-12 enriched (C-13 depleted) Organic matter reaction mechanisms work
		^{13}C	S, natural abundance = 1.108 % $^{13}C/^{12}C$ ratio by MS	Soil organic matter studies in ecosystems, photosynthesis, C translocation, C cycling
		^{14}C	R, T ½ = 5720 y β emitter (0.156 MeV) Counting by LSC	Photosynthesis and C translocation ,Soil organic matter studies, C balance studies
Oxygen	^{16}O	^{16}O	S, natural abundance = 99.759 %	
		^{17}O	S, natural abundance = 0.037 %	
		^{18}O	S, natural abundance = 0.204 % $^{18}O/^{16}O$ ratio by MS	Photosynthesis, respiration, soil organic matter studies, ecological studies, hydrology
Chlorine	^{35}Cl	^{35}Cl	S, natural abundance = 75.53 %	
		^{36}Cl	R, T ½ = 0.38 x 10^5y β emitter (0.714; 0.115 MeV) LSC	Solute movement in soils.
		^{37}Cl	S, natural abundance = 24.47 % ^{37}Cl /^{35}Cl ratio by MS	Herbicidal and insecticidal effects on life forms, water, air and soil
Iodine	^{127}I	^{125}I	R, T ½ = 60 d EC conv. el., LSC γ (0.035 MeV), NaI (T1) 2" X ray (0.027 MeV), MLSC	
		^{131}I	R, T ½ = 8.05 d β emitter (0.61; 0.25; 0.81 MeV) and γ emitter (0.36; 0.08; 0.72 MeV)	
Sodium	^{23}Na	^{22}Na	R, T ½ = 2.6 y β emitter (0.5 MeV) LSC and γ emitter (1.27 MeV) NaI (T1) 2"/3"	
		^{24}Na	R, T ½ = 15 h β emitter (1.4 MeV) LSC, Cerenkov γ emitter (2.75; 1.35 MeV) NaI (T1) 2"/3"	
Cobalt	^{59}Co	^{57}Co	R, T ½ = 270 d EC conversion el. LSC γ emitter (0.122; 0.014 MeV) NaI (T1) 2"/3"	
		^{58}Co	R, T ½ = 71 d β emitter (0.48 MeV) LSC γ emitter (0.81; 1.64 MeV) NaI (T1) 2"/3"	
		^{60}Co	R, T ½ = 5.3 y β emitter (0.31 MeV) and γ emitter (1.33; 1.17 MeV)	

Cesium	^{133}Cs	^{134}Cs	R, T ½ = 2.046 y β emitter (0.662; 0.089 MeV) and γ emitter (0.57; 0.605; 0.796 MeV)	Soil erosion. (Spike for sediment movement and deposition)
		^{137}Cs	R, T ½ = 30 y β emitter (1.176; 0.514 MeV) and γ emitter (0.662 MeV)	Soil erosion and sedimentation (Fallout radionucleiotide)
Barium	^{138}Ba	^{131}Ba	R, T ½ = 11.6 d EC and γ emitter (0.5; 0.122; 0.216 MeV)	
		^{133}Ba	R, T ½ = 7.5 y EC and γ emitter (0.082; 0.36; 0.30; 0.80 MeV)	
		^{140}Ba	R, T ½ = 12.8 d β emitter (1.02; 0.48 MeV) and γ emitter (0.54; 0.16 MeV)	
Arsenic	^{75}As	^{73}As	R, T ½ = 76 d γ emitter (0.054; 0.14 MeV)	
		^{74}As	R, T ½ = 17.5 d β emitter (0.9; 1.36 MeV) and γ emitter (0.06; 0.64; 2.53 MeV)	
		^{76}As	R, T ½ = 26.8 h β emitter (2.97; 2.41 MeV) and γ emitter 0.56; 1.21; 0.66 MeV)	
Selenium	^{80}Se	^{75}Se	R, T ½ = 120d EC and γ emitter (0.265; 0.136; 0.280; 0.240; 0.58 MeV) LSC, NaI (T1) 2”	
Aluminium	^{27}Al	^{26}Al	R, T ½ = 7.4 x 10^5 y β emitter (3.21; 1.16 MeV) and γ emitter (1.83; 1.12 MeV)	
Chromium	^{52}Cr	^{51}Cr	R, T ½ = 27.8 d EC and γ emitter (0.32 MeV) LSC, NaI (T1) 2”/3”	
Bromine	^{79}Br	^{79}Br	S, natural abundance = 50.54 %	
		^{81}Br	S, natural abundance = 49.46 %	
		^{82}Br	R, T ½ = 35.7 h β emitter (0.44 MeV) LSC γ emitter (0.55; 1.47 MeV), NaI (T1) 2”/3”	
Mercury	^{200}Hg	^{203}Hg	R, T ½ = 47 d γ emitter (0.3 MeV)	
Nickel	^{58}Ni	^{63}Ni	R, T ½ = 92 y γ emitter (0.067 MeV)	

Description		
R	=	Radioactive isotope
S	=	Stable isotope
T ½	=	Half-life expressed in time units, e.g. minutes (min), hour (hr), day (d) and year (y)
β	=	Beta radiation
γ	=	Gamma radiation
EC	=	Electron capture
LSC	=	Liquid scintillation counting
MLSC	=	Metal-loaded liquid scintillation counting
GM	=	Geiger Müller
MS	=	Mass spectrometry
ES	=	Emission spectrometry
MeV	=	Energy intensity in Mega-electron volts

2.2 ISOTOPIC TECHNIQUES IN N FERTILISER USE EFFICIENCY STUDIES

In isotopic-aided fertiliser experiments, a labelled fertiliser is added to the soil and the amount of fertiliser nutrient that a plant has taken up is determined. In this way different fertiliser practices (placement, timing, sources, etc.) can be studied.

The first parameter to be determined when studying the fertiliser uptake by a crop by means of the isotope techniques is the fraction of the nutrient in the plant derived from the (labelled) fertiliser, i.e.: fdff.

Often this fraction is expressed as a percentage, i.e.:

% dff = fdff x 100 (Equation 2)

The procedure followed in the calculation of this fraction and other derived parameters for nitrogen using ^{15}N labelled materials is given below:

2.2.1 MEASUREMENTS NEEDED FOR EXPERIMENTS WITH ^{15}N

In summary, for all field and greenhouse experiments with ^{15}N (or any other stable isotope) labelled materials, the following basic primary data need to be recorded for each plot:

1. Dry matter (D.M.) yield for the whole plant or sub-divided into plant parts.
2. Total N concentration (% N in dry matter) of the whole plant or plant parts as in point 1.This is done by chemical methods, e.g. Kjeldahl or by combustion (Dumas).
3. Plant % ^{15}N abundance, which is analysed by emission or mass spectrometry.
4. Fertiliser % ^{15}N abundance.
5. ^{15}N labelled fertiliser(s) used and N rate(s) of application.

2.2.2 CALCULATIONS FOR EXPERIMENTS WITH ^{15}N

% ^{15}N abundance is transformed into atom % ^{15}N excess by subtracting the natural abundance (0.3663 atom %N) from the % N abundance of the sample. Afterwards the following calculations can be made:

$$\%Ndff = \frac{atom\,\%^{15}N \;\; excess_{plant}}{atom\,\%^{15}N \;\; excess_{fertilizer}} \times 100 \quad \text{(Equation 3)}$$

Dry matter yield per unit area:

$$DM \;\; yield\,(kg/ha) = FW(kg) \times \frac{10000\,(m^2/ha)}{area\,harvested\,(m^2)} \times \frac{SDW\,(kg)}{SFW\,(kg)} \quad \text{(Equation 4)}$$

Where FW is fresh weight per area harvested and SDW and SFW are subsample dry and fresh weight, respectively.

$$N\ yield\,(kg/ha) = DM\ yield\,(kg/ha) \times \frac{\%N}{100} \qquad \text{(Equation 5)}$$

$$Fertilizer\ N\ yield\,(kg/ha) = N\ yield\,(kg/ha) \times \frac{\%Ndff}{100} \qquad \text{(Equation 6)}$$

$$\%\ fertilizer\ N\ utilization = \frac{Fertilizer\ N\ yield}{Rate\ of\ N\ application} \times 100 \qquad \text{(Equation 7)}$$

2.2.3 QUANTIFICATION OF FERTILISER N UPTAKE

The nitrogen isotope composition, i.e. the ^{15}N/total N ratio, of any material can be expressed as atom % ^{15}N (a) or simply % ^{15}N abundance. This isotopic ratio of a sample is measured directly in a single determination by optical emission or mass spectrometry. Since the % ^{15}N natural abundance (a_0) is 0.336 atom % ^{15}N this has to be subtracted from the % ^{15}N abundance (a) of any enriched material to obtain the atom % ^{15}N excess (% ^{15}N a.e. = a') or ^{15}N enrichment.

What then is Ndff? It is the fraction of N in the plant derived from the ^{15}N labelled fertiliser. From simple isotope dilution principles, the following relationship may be written:

$$Ndff = \frac{atom\ \%^{15}N\ excess_{\ plant\ sample}}{atom\ \%^{15}N\ excess_{\ labelled\ fertilizer}} = \frac{a'_{\ plant\ sample}}{a'_{\ labelled\ fertilizer}} \qquad \text{(Equation 8)}$$

or as percentage

$$\%Ndff = \frac{atom\ \%^{15}N\ \ excess_{\ plant\ sample}}{atom\ \%^{15}N\ \ excess_{\ fertilizer}} \times 100 \qquad \text{(Equation 9)}$$

Therefore, for the calculation of % Ndff it is necessary to determine the atom % ^{15}N excess of the plant samples and of the fertiliser(s) used in the experiment.

For instance if Ndff = 0.25 this means that 1/4 of the nitrogen in the plant came from the fertiliser. If soil and fertiliser were the only sources of N available to the plant, then the remaining 3/4 of the nitrogen in the plant came from the soil. If these fractions are expressed in percentages then %Ndff = 25% and %Ndfs = 75 %, where %Ndfs is % N derived from soil.

Exercise 1

In a field experiment 80 kg N/ha in the form of ^{15}N labelled urea was applied to a maize crop. The maize was harvested at tasseling time with a dry matter yield of 4 tons/ha and a plant sample had 0.67 % ^{15}N abundance and 3 % total N. The applied fertiliser had 1.37 % ^{15}N abundance.

Questions:
What was the fraction of N in the plant, which was derived from the fertiliser or % Ndff?
What was the fraction of N in the plant, which was derived from the the soil?
What was the total N yield of the crop?
What was the fertiliser N yield of the crop?
What was the fertiliser N utilization or recovery by the crop?
Calculations:
% N derived from the fertiliser:
% atom ^{15}N excess plant = 0.67 - 0.37 = 0.30
% atom ^{15}N excess fertiliser = 1.37- 0.37 = 1.00
The fraction of fertiliser N in the plant is calculated using Equation 12:

$$\%Ndff = \frac{0.30}{1.00} \times 100$$

%Ndff = 30%

% N derived from the soil: Since the crop had only two sources of nutrients the % N derived from the soil is obtained by difference is as follows:

% Ndfs = 100 - % Ndff
100 - 30 = 70 %

The nitrogen yield of the crop:

The total amount of N contained in the crop during the experimental period is obtained by recording the dry matter yield and multiplying it by the % total N in the crop as follows:

$$N\ yield = 4000 \times \frac{3}{100} = 120\ kgN/ha$$

The fertiliser N yield of the crop:

The amount of fertiliser N taken up by the crop is calculated by multiplying the total N yield by the fraction of Ndff:

$$Fertilizer\ N\ yield = \frac{30}{100} \times 120 = 36\ kgN/ha$$

Fertiliser N utilization or recovery by the crop

The fraction of the fertiliser nutrient taken up by the plant in relation to the rate of fertiliser nutrient applied is commonly expressed as percentage:

$$\%\ Fertilizer\ N\ utilization = \frac{36}{80} \times 100 = 45\%$$

Exercise 2

In a field experiment, 60 kg N/ha as ^{15}N labelled ammonium sulfate was applied to hybrid sorghum. The ^{15}N treated plots were harvested at the grain milky stage of development. The harvest consisted of gathering all above ground plant material in the harvesting area of the isotope plots and separating them into shoots and panicles. The fresh weight of each component was recorded. Adequate sub-samples were taken and chemical and isotopic analyses were performed on each sub-sample separately.

Question? What is the fertiliser N utilization of sorghum in this experiment?

Calculations:

The basic primary data are listed in the following Table 2.2:

Table 2.2. Experimental data

Plant part	DM yield tons/ha	Total N %	N yield kg/ha	Ndff %	Fertiliser N yield kg/ha
Shoots	5.0	1.2	60	27.4	16.4
Panicles	2.2	2.1	46	19.6	9.1
TOTAL			106		25.5

As shown in the Table 2.2 N yield and fertiliser N yield of each plant part has to be calculated. Add up these data to obtain total N yield and total fertiliser N yield for the entire crop.

The next step is to calculate the weighted average % Ndff for the entire crop.

$$\%Ndff\ (weighted\ average) = \frac{25.5}{106} \times 100 = 24$$

Finally the % fertiliser N utilization is calculated using the total fertiliser N uptake as follows:

$$\%\ Fertilizer\ N\ utilization = \frac{25.5}{60} \times 100 = 42.5$$

2.2.4 QUANTIFICATION OF FERTILISER N UPTAKE WITHOUT PLANT-FERTILISER INTERACTION

Fertiliser N management practices such as sources, timing, placement, etc. may be studied in the presence and absence of the effects of the fertiliser treatments on plant development, root distribution and crop yield. The use of a special design with isotope techniques allows the study of the fertiliser uptake without plant-fertiliser interaction These are called “Single-treatment” fertility experiments (Broeshart, 1974; Fried et al., 1975).

Example 1: N timing

Timing (T) of fertiliser N application (90 kg N/ha applied to maize at 3 different times T_1, T_2 and T_3). N rates marked with an asterisk * denote ^{15}N labelled fertiliser.

Design 1: Fertiliser treatments with interaction

Treatments	T_1	T_2	T_3
1	90*	-	-
2	-	90*	-
3	-	-	90*

In Design 1, treatments 1, 2, and 3 each receive 90 kgN/ha at T_1, T_2 and T_3, respectively. In this case the effect is measured with plant-fertiliser interaction.

Design 2: Fertiliser treatments without interaction

Treatments	T_1	T_2	T_3
A	30*	30	30
B	30	30*	30
C	30	30	30*

In Design 2, all fertiliser treatments A, B and C are identical with regard to the total N rate (90 kg N/ha) and three-split of 30 kg N/ha at all timing treatments. Only the position of the labelled fertiliser changes, thus the effect of timing would be measured in the absence of any plant-fertiliser interaction effect. By utilising design 2, the partial fertiliser use efficiency of the 30 kg N/ha applied at each timing, and the total fertiliser use efficiency of a three-split application of 90 kg N/ha can be measured without any interaction effect.

Example 2: N placement

In this experiment 80 kg N/ha was applied to sorghum in 2 different placements.

Design 1: Fertiliser treatments with interaction

Treatments	Surface broadcast over plot area	Band at 5 cm depth in a furrow
1	80*	-
2	-	80*

Design 2: Fertiliser treatments without interaction

Treatments	Surface broadcast over plot area	Band at 5 cm depth in a furrow
A	40*	40
B	40	40*

Example 3: N sources

In this experiment the efficiency of NO_3^- and NH_4^+ sources of N were compared in rice.

Design 1.: Fertiliser treatments with interaction

Treatments	NO_3^-	NH_4^+
A	$NaNO_3^-$	-
B	-	$(NH_4^+)_2SO_4$

Design 2: Fertiliser treatments without interaction

Treatments	NO_3^-	NH_4^+
A	$NH_4{}^*NO_3$	
B		${}^*NH_4NO_3$

In this design one single N source (NH_4NO_3) is used but in treatment A ${}^*NO_3$ is labelled whereas in treatment B, ${}^*NH_4$ is the labelled ion.

Exercise

Data from an experiment where $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ was applied to wheat either in the autumn or spring are presented in Table 2.3. When the labelled fertiliser was applied in the autumn unlabelled fertiliser was applied in the spring and *vice versa*. This allowed the calculation of %Ndff, N uptake from fertiliser and the fertiliser N utilization coefficient (%) to be calculated.

Table 2.3. Measured parameters from an experiment where nitrogen uptake from NO_3^- and NH_4^+ supplied as labelled ammonium nitrate by wheat

Time and rate of N application (kg/ha)		%Ndff			N uptake from fertiliser (kg/ha)			Fertiliser N utilization coefficient (%)		
Autumn	***Spring***	Grain	Straw	Total	Grain	Straw	Total	Grain	Straw	Total
$^{15}NH_4NO_3$										
60*	60	8.0	7.9	**15.9**	7.6	6.1	**13.7**	25.3	20.3	**45.7**
60	60*	10.4	9.0	**19.4**	9.8	7.0	**16.8**	32.7	23.3	**56.0**
	Total	**18.4**	**16.9**	**35.3**	**17.4**	**13.1**	**30.5**	**29.0**	**21.8**	**50.8**
$NH_4{}^{15}NO_3$										
60*	60	10.2	10.6	**20.8**	9.6	8.3	**17.9**	32.0	27.7	**59.7**
60	60*	12.2	13.0	**25.0**	11.3	10.2	**21.5**	37.7	34.0	**71.7**
	Total	**22.2**	**23.6**	**45.8**	**20.9**	**18.5**	**39.4**	**34.8**	**30.8**	**65.7**

These data shows that the application of either nitrate or ammonium in spring resulted in greater N fertiliser use efficiency.

2.2.5 THE CONCEPT OF AVAILABLE AMOUNTS OF A NUTRIENT

When comparing different nutrient sources, the term "available amount of a nutrient" has first to be defined. Only the plant can judge what is available since no chemical extraction can determine what is available to a plant. However, if the plant is used to measure which source of nitrogen or phosphorus is available and to which extent, one has to be able to discriminate between the sources, which is conveniently done by labelling one of the sources with an appropriate isotope.

Also, when comparing nutrient sources one wants to know how much available nitrogen or phosphorus in a given source the plant sees in comparison with well-known fertilisers. Thus, if a rock phosphate is applied we want to know what is the available amount of phosphorus in the rock phosphate in terms of equivalent units of superphosphate or in other words how many kg of rock phosphate supplies the same amount of phosphorus to a crop as one kg of superphosphate. However, since the soil is a source of nutrients, the first question which may be asked is: What is the available amount of phosphorus in the soil in terms of equivalent units of superphosphate or what is the available amount of nitrogen in the soil in terms of equivalents units of ammonium sulfate? Thus by utilising a labelled fertiliser source it is possible to determine the plant available amount of nutrient in the soil and this amount is expressed relative to the amount available in the fertiliser source.

For example, if 100 kg N/ha as ^{15}N labelled ammonium sulfate is added to the soil and plant analysis gave %Ndff = 50% then %Ndfs = 50%. It could then be said that the soil had the same amount of available nitrogen as the 100 kg N/ha added as fertiliser or in other words the soil had an available amount of 100 kg N/ha in terms of ammonium sulfate equivalent units.

What will be the consequence when 200 kg N/ha are added to the same soil? Since the soil has an amount of available nitrogen equivalent to 100 kg N/ha and the fertiliser 200 kg N/ha, the proportion taken up by the plant from the soil will be one third and two thirds from the fertiliser.

What will be the consequence when 300 kg N/ha are added? The proportion taken up from the soil will be one fourth and three fourths from the fertiliser.

Thus the following assumption may be formulated: "When a plant is confronted with two or more sources of a nutrient element, the nutrient uptake from each of these sources is proportional to the amounts available in each source" (Fried and Dean, 1952; Fried, 1964; Broeshart, 1974; Fried, 1978b and Vose, 1980). Implicit in this assumption is that there is complete mixing of the isotope or isotopic equilibrium.

The above relationships, which are also called fractional utilization ratios, can be expressed in the form of an equation. In a situation where soil and fertiliser are the only sources of nutrient available to a plant, the equation is as follows:

$$\frac{Total\ nutrient_{plant}}{Total\ nutrient_{supply}} = \frac{Fertilizer\ nutrient_{plant}}{Fertilizer\ nutrient_{supply}} = \frac{Soil\ nutrient_{plant}}{Soil\ nutrient_{supply}} \qquad \text{(Equation 10)}$$

or

$$\frac{Total\ nutrient_{plant}}{Total\ nutrient_{supply}} = \frac{Fertilizer\ nutrient_{plant} + Soil\ nutrient_{plant}}{Fertilizer\ nutrient_{supply} + Soil\ nutrient_{supply}} \qquad \text{(Equation 11)}$$

The fertiliser N supply is equivalent to the rate of N applied as fertiliser. The soil N supply corresponds to the amount of soil N in terms of fertiliser units which is available to the plant during the growth period. Both soil and fertiliser N, as well as total N supply, are expressed in the same way, i.e. as equivalent units of applied fertiliser, for example, in kg N/ha of equivalent units of ammonium sulfate, if this was the fertiliser used.

The following exercises are given to illustrate the determination of the available amount of a nutrient in the soil.

Exercise 1 (Greenhouse experiment)

To determine the available amount of N in a soil, ^{15}N labelled urea (1% atom^{15}N excess.) was applied at a rate of 80 mg N/pot, each containing 1 kg air-dried soil. Barley was used as the test crop but oats, ryegrass or any other fast-growing plant could have been used.

Plant sample taken at harvest had 0.25% atom^{15}N excess.

$$\%Ndff = \frac{0.25}{1.00} \times 100 = 25\%$$

Therefore Ndfs = 100 - 25 = 75%.

Thus the fractional utilization ratio is as follows:

$$\frac{25\%}{80\,mg\ N\ as\ urea} = \frac{75\%}{x}$$

$$x = \frac{75\% \times 80\,mg\ N}{25\%}$$

x = 240 mg N as urea

Therefore, this soil has an available amount of 240 mg N/kg soil as urea equivalent units.

Exercise 2 (Field experiment)

100 kg N/ha was applied as ^{15}N labelled ammonium sulfate to a sorghum crop. After harvest, the plant and fertiliser samples were analysed for total N and for nitrogen isotopic ratio. It was found that the sorghum crop had 120 kg N/ha total N yield and 40 kg N/ha fertiliser N yield.

Questions:

1. What was the available amount of N in the soil as ammonium sulfate equivalent units?
2. What was the Ndff in the plant sample?

Calculations:

120 kg N/ha total	(total N yield)
40 kg N/ha from fertiliser	(fertiliser N yield)
80 kg N/ha from soil	(soil N yield), by difference

Then the following relationship may be written:

$$\frac{40\,kg\,N/ha_{fertilizer}}{100\,kg\,N/ha\,as\,ammonium\,sulphate} = \frac{80\,kg\,N/ha_{soil}}{x}$$

Therefore the available amount of N in the soil is 200 kg N/ha as ammonium sulfate equivalent units.

The Ndff in the plant sample would be

$$Ndff = \frac{40}{120} = \frac{1}{3}$$

The available amount of nutrient in soil or the soil nutrient supply, measured in equivalent units of the fertiliser standard has been referred to as the "A-value" (Fried and Dean, 1954).

In a simplest case, when a plant is confronted with only two nutrient sources, i.e. the native soil nutrient pool (ndfs) and the labeled fertiliser nutrient (ndff) supplied as a given rate.

Then

%ndfa + %ndff = 100

and

%ndfs = 100 - %ndff (Equation 12)

As mentioned before the calculation is based on the fractional relationship as shown in Equation 10 where fertiliser and soil nutrient in the plant are the respective proportions of nutrient taken up from each source. The percentage nutrient in the plant derived from the fertiliser is experimentally determined using isotopically labelled fertiliser.

The available amount of fertiliser nutrient is the rate of nutrient application as fertiliser standard. It should be noted that as far the plant is concerned, one fertiliser unit is the same as any other fertiliser unit. Thus, for instance, two fertiliser units contain twice as much available nutrient as one fertiliser unit.

The available amount of soil nutrient, or the A value of the soil for the particular nutrient under study, is expressed in equivalent units of the applied fertiliser.

With reference to Equation 8 the above Equation 10 may be written as follows:

$$\frac{\%ndff}{Available\,amount\ of\ fertilizer\,nutrient} = \frac{100 - \%ndff}{Available\,amount\,of\ soil\,nutrient\ (A)}$$

and solving for A

$$A = \frac{100 - \%ndff}{\%ndff} \times Available\,amount\,of\ fertilizer\,nutrient$$

where the available amount of fertiliser nutrient is the rate of fertiliser nutrient applied.

In determining A values, it is important to note the following:

- Since the available amount of nutrient in the soil is an inherent property of the soil, it will be constant for any set of experimental conditions.
- The “A value” is a yield-independent parameter. It is only necessary to determine the respective proportions absorbed from each source, so as to determine the A value of the soil. No yield data need to be recorded. The absolute amounts of nutrient taken up from either source do not appear in the equation.
- The A value for a particular soil remains constant even at different rates of application of the same fertiliser standard. In other words, the available amount of nutrient in the soil is independent of the rate of fertiliser applied. Thus, in soil fertility studies, it is sufficient to use only one rate of application to assess the nutrient supply of a soil and make relative comparisons of fertiliser treatments (Aleksic et al., 1968; Broeshart, 1974).
- Any change in the set of experimental conditions (nature, source, placement, timing, etc) will affect the magnitude of the A value of the soil. Also changes in harvesting times are important, since the plant samples reflect the nutrient isotopic composition of the soil from the seeding time until harvesting time. These changes of the A value of a soil with time can be easily observed in a time course study of nutrient uptake using labelled fertilisers (Rennie, 1969; Smith and Legg, 1971; Broeshart, 1974; Zapata et al., 1987).
- The determination of the A value of the soil has a number of practical applications, such as the quantitative evaluation of fertiliser practices, in particular fertiliser sources, and the design of further isotope-aided experiments (Rennie, 1969; Broeshart, 1974; Fried, 1978b; IAEA, 1983).
- Extensive research work using A values has been done for most plant nutrients, both macro-and micronutrients (Fried, 1954; IAEA, 1976; IAEA, 1980; Vose, 1980; IAEA, 1981; Wagner and Zapata, 1982).

2.2.6 QUANTITATIVE EVALUATION OF N FERTILISER PRACTICES

The nutrient supply from several fertiliser management practices can be quantitatively evaluated using isotope techniques:

1. Method of placement
2. Timing of application
3. Chemical and physical nature of sources, including symbiotic nitrogen fixation
4. Interaction among topics I, 2 and 3, and of these with cultural practices (irrigation, mulching, tillage, etc.)

For instance one may ask how placement method A compares with method B. Also when comparing different fertiliser sources, one wants to know how much available phosphorus or nitrogen the plant sees from a given nutrient source in comparison with a manufactured fertilisers.

The following series of examples illustrate the quantitative evaluation of fertiliser practices with annual crops. It essentially consists of equating the two fertilisers with each other and is based on the principle that the available amount of nutrient in the soil, which is identical for all treatments, is expressed in units of each fertiliser treatment. This enables a direct comparison of fertiliser equivalent units among different treatments.

A Method of N fertiliser placement

A maize field experiment was carried out to compare a band placement to a surface-broadcast application of 120kg N/ha as ^{15}N-labelled ammonium sulfate.

Results:

	Banding	Surface broadcast
% Ndff	65	46
% fertiliser N utilization	78	48

The farmer used to apply 120 kg N on the surface. If in the future he decides to apply the fertiliser in a band, what should be the rate of N application in order that the maize crop takes up the same amount of N from banding as previously from surface application?

i *Principle*

The available amount of soil N seen by the maize crop is the same for banding and surface treatments. Therefore we estimate the available amount of soil N in equivalent banding and surface units, which will enable us to calculate how many kg N given in a band supply the same amount of N to the maize as 120 kg N on the surface.

ii *Banding treatment*

%Ndff = 65% and %Ndfs = 35%.

From the fractional utilization relationship:

$$\frac{65}{120} = \frac{35}{x}$$

Thus x or the available amount of soil N is 65 kg N/ha in banding equivalent units.

iii *Surface treatment*

%Ndff = 46% and %Ndfs = 54%

From the fractional utilization relationship:

$$\frac{46}{120} = \frac{54}{x}$$

Thus x, or the available amount of soil N, is 141 kg N/ha in surface equivalent units.

The experimental soil has an available amount of N equivalent to 65 kg N/ha as banding units or 141 kg N/ha as surface units. In other words, 65 kg N/ha in a band supplies the same amount of N to the maize crop as 141 kg N/ha on the surface.

65 kg N in a band = 141 kg N on the surface

X = 1 kg N on the surface

$$x = \frac{65}{141} = 0.46\, kg\ N\ in\ a\ band$$

Instead of the application of 120 kg N/ha on the surface, the farmer should apply 0.46 x 120 = 55 kg N/ha in a band to get the same amount of fertiliser taken up by the maize crop.

B Timing of fertiliser N application

A field experiment was carried out to compare the available amounts of N in various ^{15}N-labelled urea treatments on winter wheat. One single application of 100 kg N/ha urea in the autumn was compared with a two split applications of 50 kg N/ha at the tillering stage + 50 kg N/ha at the heading stage.

Results:

	Single application	Two-split application
% Ndff	37	44
A values	170	127

The two-split application was found to be superior to the single application. By how much is the two-split application better than the single application?

The soil has an available amount of N equivalent 170 kg N/ha as single application units or 127 kg N/ha as two-split application units.

Therefore

170 kg N in a single application = 127 kg N in a two-split application.

1 kg N in a single application = X

1 kg N in a single application is equivalent to $x = \frac{127}{170} = 0.75\,kg\,N$ in a two-split application. Therefore, the two-split application is 33% better than the single application.

C Comparison of fertiliser N sources

Comparison of N sources, i.e. two N fertilisers such as urea and ammonium sulfate.

Treatments:

^{15}N- labeled urea, and ^{15}N labeled ammonium sulfate, both applied at the same rate of 100 kgN/ha.

Results:

Urea treatment:

N yield = 120 kg N/ha

Plant sample = 1.2 atom % ^{15}N excess

Fertiliser sample = 2.0 atom % ^{15}N excess

$$\%Ndff = \frac{1.2}{2.0} \times 100 = 60\%$$

%Ndfs = 40%

From the fractional utilization relationship

$$\frac{60}{100} = \frac{40}{x}$$

x = available amount of soil N or 66.7 kg N/ha in urea equivalent units.

Ammonium sulfate treatment:

N yield = 105 kg N/ha
Plant sample = 0.6 atom % ^{15}N excess
Fertiliser sample =1.2 atom % ^{15}N excess

$$\%Ndff = \frac{0.6}{1.2} \times 100 = 50\%$$

%Ndfs = 50%

From the fractional utilization relationship

$$\frac{50}{100} = \frac{50}{x}$$

x = available amount of soil N or 100 kg N/ha in ammonium sulfate equivalent units.

Quantitative comparison of urea and ammonium sulfate.

66.7 kg N as urea = 100 kg N as ammonium sulfate

x = 1 kg N as ammonium sulfate

1 kg N as ammonium sulfate is equivalent to $\frac{66.7}{100} = 0.67\ kg\ N$ as urea

2.3 ISOTOPIC TECHNIQUES IN P FERTILISER USE EFFICIENCY STUDIES

2.3.1 INTRODUCTION

Phosphorus has one stable isotope (^{31}P) and several radioisotopes (from ^{26}P to ^{30}P and from ^{32}P to ^{38}P), but only two of them (^{32}P and ^{33}P) are suitable for agronomic studies. The main characteristics of these radioactive P isotopes are shown in Table 2.4.

Detection efficiencies for both radioisotopes are high using modern liquid scintillation counters/analysers. The high beta energy of the photons emitted by ^{32}P allows the use of water as the counting medium using the Cerenkov process, which makes the technique cheaper. Conversely, the low beta energy of the photons emitted by ^{33}P requires the use of scintillation fluors or cocktails, which are commercially available and more expensive. The lower energy emitted by ^{33}P presents less radiation hazard, and its longer half-life (24.4 days) allows studies to be conducted for relatively longer time periods. The ^{32}P can be easily monitored because of its high beta energy and its use is limited to P uptake studies with duration of 60 up to 90 days due to its short half-life (14.3 days).

The availability of these two isotopes also makes it possible to use double labeling techniques in root activity studies and P placement experiments (Broeshart and Netsinghe, 1972; IAEA, 1975). An additional factor to be considered is the cost of the isotopes, the ^{32}P being far cheaper than ^{33}P and also easier and faster to obtain.

It should be noted that the handling and use of radioisotopes require compliance with regulations concerning radiation protection and safety set by international standards (FAO et al., 1996 a, b; IAEA and ILO, 1999) and the National Radiation Protection Authorities. This involves, among others, special laboratory facilities and trained personnel to perform all the activities.

Table 2.4. Summary of main characteristics of P isotopes used in plant nutrition studies

Isotopes	Half-life	Radiation characteristics		Typical applications
		Type	Energy	
^{32}P	14.3 days	β^-	1.71 MeV(Emax)	Exchangeable P in soils P availability from P fertilisers Plant root distribution / activity Residual P fertiliser availability Auto-radiography Diffusion in soils Double labeling with ^{32}P
^{33}P	24.4 days	β^-	0.248 MeV(Emax)	

The first extensive use of isotopes as tracers in plant nutrition was made in the 1940's. The radioactive isotope of P (^{32}P) was used to study the utilization of P fertilizers by various crops in a series of greenhouse experiments and field trials. Essentially, a tracer was incorporated into a fertiliser material or the nutrient source of interest, and the amount of tracer taken up by the plant was measured directly (Fried and Dean, 1952; Larsen, 1954; Russsell et al., 1954).

An ideal tracer should have exactly the same behavior as the material being traced, and should have no effect on the components of that material in the system under study. Since tracer detection is very sensitive, the amount of radioactivity added to the system in a tracer experiment is usually very small compared with the amounts already present in, or added to the system. Thus, very little disturbance of the system is made.

In phosphate studies, ^{32}P carrier-free materials, i.e. contain only ^{32}P atoms, are normally utilized as tracers. They can also be used for labeling commercial P fertilisers such as superphosphates during manufacturing or for preparing ^{32}P labeled solutions of known concentration for use in greenhouse and field experiments. High specific activity ^{32}P labeled orthophosphate solutions are also commercially available, upon request.

In case of natural fertiliser sources, like phosphate rocks (PR), it is not possible to directly label these materials with P isotopes, because of the changes induced in their physical and chemical characteristics during labeling.

Therefore, techniques based on reverse isotopic dilution have been widely used to investigate P availability from PR sources to plants (Fried, 1954; Kucey and Bole, 1984; Zapata et al., 1986; Zapata and Axmann, 1995; Kato et al., 1995; Fardeau et al., 1995).

2.3.2 MEASUREMENTS NEEDED FOR EXPERIMENTS WITH ^{32}P AND/OR ^{33}P

The following basic primary data need to be recorded for field and greenhouse experiments with labelled P fertilisers:

1. Dry matter (D.M.) yield
2. Total nutrient concentration (% P in dry matter) -Analysed by a conventional chemical method
3. Plant specific activity (S.A. plant)
4. Fertiliser specific activity (S.A. fertiliser)

For the points 3 and 4, two independent measurements are required in each aliquot, i.e. the determination of the ^{32}P activity by Cerenkov counting and the content of the element i.e. amount of P.

5. Radioisotopically-labelled fertiliser(s) used and the rate(s) of application

Calculations for experiments with ^{32}P and/or ^{33}P

The following calculations need to be made:

1. The S.A. of plant and fertiliser
2. %Pdff = (S.A. plant / S.A. fertiliser) x 100
3. Dry matter yield per unit area:
   DM yield (kg/ha) = FW (kg) x (10 000 m^2 / area harvested m^2) x (Sample DW kg/Sample FW kg)
4. P yield (kg/ha) = DM yield (kg/ha) x (%P / 100)
5. Fertiliser P yield (kg/ha) = P yield (kg/ha) x (%pdff / 100)
6. % fertiliser P utilisation = (Fert P yield / Rate of P application) x 100

2.3.3 QUANTIFICATION OF FERTILISER P UPTAKE

The phosphorus isotopic composition, i.e. the ^{32}P/total P ratio, of any material is called specific activity (S.A.). The determination of the specific activity (S.A.) of a sample requires two independent measurements:

1) Determination of the activity (Bq) of the radioisotope by radio-assay techniques using appropriate detectors, i.e. proportional detector, Geiger Muller (GM) detector, liquid scintillation counting, Cerenkov counting (for high-energy β emitters) or sodium-iodide scintillation detectors.

2) Determination of the total nutrient content by any conventional chemical method, i.e. total P by spectrophotometric (methavanadate yellow) method. It is customary to express the specific activities of plant samples and fertiliser in Bq ^{32}P/g P, at the time the samples were counted. It is important to note that the concept of specific activity (ratio ^{32}P/total P) for radioisotopes is identical to that of' ^{15}N atom excess (ratio ^{15}N/total N) for stable isotopes.

The fraction of P in the plant derived from the ^{32}P or ^{33}P labelled fertiliser material is termed Pdff. It also follows from the isotope dilution principle that:

Pdff = (S.A. plant sample / S.A. labelled fertiliser)

or as a percentage:

%Pdff = (S.A. plant sample / S.A. labelled fertiliser) x l00

How is Pdff measured?

Both the activity and total P content in the plant and fertiliser samples must be determined to measure Pdff.

Exercise 1

An aliquot of a digested plant sample containing 8 mg P gives an activity of 800 Bq. If an aliquot of the dissolved ^{32}P labelled fertiliser containing 10 mg P gives an activity of 4000 Bq then the following relationship can be written:

4000Bq = 10 mg P in the fertiliser

800 Bq = X mg P in the plant, and

X = (800 x 10) /4000 = 2 mg P in the plant came from the fertiliser

The next step is to calculate the Pdff by relating the amount of P in the plant which came from the fertiliser (2 mg P) to the total amount of P in the plant sample (8 mg P). Thus 2 mg out of 8 mg was derived from the fertiliser and:

Pdff = 2/8 = 0.25

or % Pdff = 25%

Exercise 2

In a greenhouse experiment, 20 mg/kg P as ^{32}P labelled single superphosphate was applied to pots containing 2 kg soil with barley as the test crop. After 2 months, the plant material was harvested and analysed for ^{32}P activity and total P concentration. The ^{32}P labelled single superphosphate (standard) used in this experiment was analysed in the same way.

Results:

Plant sample: 5 g dry matter yield

0.2 % total P

An aliquot containing 2 mg P gave 250 Bq ^{32}P by Cerenkov counting. Since the counting efficiency was 50% the activity of the plant sample was 500 Bq ^{32}P.

Thus the S.A. plant = 500 Bq ^{32}P / 2 mg P = 250 Bq ^{32}P/mg P.

Fertiliser: An aliquot containing 10 mg P counted by Cerenkov gave 6250 Bq ^{32}P. Considering also 50% counting efficiency the activity of the fertiliser sample was 12500 Bq ^{32}P.

Thus the S.A. fertiliser = 12500 Bq ^{32}P / 10 mg P = 1250 Bq P/mg P

Questions:

1. What was the fraction of P in the plant which was derived from the fertiliser or % Pdff?
2. What was the fraction of P in the plant which was derived from the soil?
3. What was the total P content of the crop?
4. What was the fertiliser P content of the crop?
5. What was the fertiliser P utilization or recovery by the crop?

Calculations:

1. % P derived from the fertiliser:

   %Pdff = (S.A. plant / S.A. fertiliser) x l00

   =(250/1250) x 100 = 20%

2. % P derived from the soil

   %Pdfs = 100 - %Pdff

   = 100-20 = 80%

3. P content of the crop = Yield (g DM) x (%/100)

   = 5 g x (0.2/100) = 0.01g P/pot

   = 5000 mg x (0.2/100) = 10 mg P/pot

4. Fertiliser P uptake by the crop = P content of the crop x (%Pdff/100)

   = 10 x (20/100) = 2 mg P/pot

5. Fertiliser P utilization or recovery by the crop: Since 20 mg P/kg soil was applied to each pot containing 2 kg soil, thus the P rate was 40 mg P/pot and

   % Fertiliser P utilization = (Fert. P content of the crop / Fert. P applied) x 100

   = (2/40) x 100 = 5%

Exercise 3

In a rice field, 20 kg P/ha as ^{32}P-labelled single superphosphate was applied at transplanting time. After about six weeks, plant samples were harvested from these treated plots. The dry matter yield was 2500 kg/ha with a total P concentration of 0.30 %.

A representative plant dry matter sample was ashed and extracted with a known amount of 2N HCl. An aliquot of this extract containing 0.2mg P was counted by Cerenkov and was found to give a count rate of 320 Bq. At the same time an aliquot of an acid extract of the ^{32}P labelled superphosphate containing 0.3 mg P was counted and was found to have a count rate of 3450 Bq.

The counting efficiency was found to be 40 %.

Question: What was the fertiliser P utilisation?

Calculations:

1. % Pdff is calculated as follows:

   Plant sample activity = 320 / 0.4 = 800 Bq
   S.A. plant sample = 800 / 0.2 = 4000 Bq/mg P
   Fertiliser activity = 3450 / 0.4 = 8625 Bq
   S.A. fertiliser = 8625/ 0.3 = 28750 Bq/mg P
   % Pdff = 4000 / 28750 x l00 = 13.9%

2. The total P yield or uptake

   P yield = 2500 x (0.3 /100) = 7.5 kg P/ha

3. The fertiliser P yield:

   Fertiliser P yield = 7.5 x (13.9 / 100) = 1.04 kg P/ha

4. The fertiliser P utilisation:

   % Fertiliser P utlIlsation = (1.04/20) x 100 = 5.2%

2.3.4 QUANTIFICATION OF FERTILISER P UPTAKE FROM FERTILISERS THAT CAN BE LABELLED

A Introduction

Fertiliser management practices such as source, timing, placement, etc. may be studied in the presence and absence of the effects of the fertiliser treatments on plant development, root distribution and crop yield. The use of special designs using isotopes allows the study of fertiliser uptake without a plant-fertiliser interaction (Single treatment fertility experiments, Broeshart,1974; Fried *et al*. 1975). Refer to section 2.2.3.

B Comparison of two ^{32}P labelled fertilisers such as superphosphate and nitro-phosphate (Greenhouse experiment)

Treatments

^{32}P labelled superphosphate applied at a rate of 50 mg P/kg soil

^{32}P labelled nitro phosphate applied at a rate of 50 mg P/kg soil

Results

Superphosphate treatment:

% Pdff = 20% and % Pdfs = 80%

From the fractional utilization relationship 20/50 = 80/x

Where X = available amount of soil P or 200 mg P in superphosphate equivalent units.

Nitro-phosphate treatment:

% Pdff = 10% and % Pdfs = 90%

From the fractional utilization relationship 10/50 = 90/x

Where X = available amount of soil P or 450 mg P in nitro-phosphate units

Quantitative comparison of superphosphate and nitro-phosphate.

200 mg P as superphosphate = 450 mg P as nitro-phosphate
1 kg P as superphosphate = X
1 kg P as superphosphate is equivalent to 450/200 = 2.25 kg P as nitro-phosphate.

C Comparison of the efficiency of powdered and granulated triple superphosphate

i *Treatments*

Powdered ^{32}P-labelled triple superphosphate and granulated ^{32}P-labelled triple superphosphate applied at a rate of 40 kg P/ha.

ii *Results*

Powdered ^{32}P labelled triple super (TSP)

% Pdff = 15% and % Pdfs = 85%
From the fractional utilization relationship 15/40 = 85/x
Where x = available amount of soil P or 227 kg P as powdered TSP units

Granulated ^{32}P labelled TSP

% Pdff = 20% and % PdfS = 80%
From the fractional utilization relationship 20/40 = 80/x
Where x = available amount of soil P or 160 kg P as granulated TSP units

Quantitative comparison of powdered and granulated TSP

227 kg P as powdered TSP = 160 kg P as granulated TSP
1 kg P as powdered TSP = X
1 kg P as powdered TSP is equivalent to 160/227 = 0.70 kg as granulated TSP.

If the application of 40 kg P/ha as powdered TSP was the farmer's practice and he decides to change the formulation, the farmer should now apply 28 kg P/ha as granulated TSP to get the same amount of fertiliser taken up.

2.3.5 QUANTIFICATION OF FERTILISER P UPTAKE FROM SOURCES WHICH CANNOT BE LABELLED (INDIRECT OR REVERSE DILUTION METHOD)

A Introduction

This methodology can be used in the following cases:

a) When it is impossible to label fertiliser sources such as natural products (rock phosphates) and organic materials, i.e: guano, green and animal manures, compost, agricultural residues, etc.

b) When it is impractical to label nutrient sources such as atmospheric N_2, which can be biologically fixed by field-grown legume crops (refer to chapter on this topic).

c) When the cost of the required labelled fertilisers for the experiment; e.g. study of residual effect, is too high or simply, the required labelled fertilisers for a particular study are not available.

The reverse dilution technique is used in this case. It essentially consists of labelling the soil with an isotopically labelled solution and utilise the plant to measure the isotopic ratio (atom%^{15}N excess for nitrogen or specific activity for phosphorus) of the N or P supplied by the labelled soil. This method is usually referred to in the literature as "the isotope dilution technique", although this is not a true isotope dilution as defined in isotope tracer chemistry. The experiments can be conducted in the greenhouse and field conditions and several crops have been used as test plants (Zapata et al., 1994; Zapata and Axmann, 1995; Fardeau et al., 1996).

The isotopic ratio in the fertilised treatment will decrease as a result of nutrient uptake from the added unlabelled fertiliser source. A standard treatment (without application of the unlabelled source) is also required as reference.

B Isotope solutions

In case of P, a solution of KH_2PO_4 or NaH_2PO_4 (at low P concentration: 10-50 mg P/L) labelled with ^{32}P carrier free or a high specific activity orthophosphate solution (directly available from commercial firms) can be used. The rate of isotope application (irrespective of the P rate of application) should be about 7-18 x $l0^6$ Bq ^{32}P /m^2 to field plots and 4-7 x $l0^6$ Bq ^{32}P /kg soil in greenhouse experiments.

C Treatments

I: Soil + ^{32}P labelled solution
II: Soil + superphosphate (60 kg P/ha) + ^{32}P labelled solution
III: Soil + rock phosphate (200 kg P/ha) + ^{32}P labelled solution

D Results

Specific activities (S.A.) of the harvested plant material per treatment.

I: S.A. plant = 2000 Bq/mg P
II: S.A. plant = 1200 Bq/mg P
III: S.A. plant = 1400 Bq/mg P

E Calculations

% Pdff = (S.A. plant sample / S.A. labelled fertiliser) x l00
% Pdfl = P derived from labelled source = (S.A. plant sample/ S.A. labelled source) x l00
Since S.A. labelled source = S.A. labelled soil
% Pdfl = (S.A. plant sample / S.A. labelled soil) x l00
and S.A. labelled soil = S.A. of the plant in treatment 1.

Treatment II

% Pdfl = (1200/2000) x l00

= 60%

% Pdf. unlabelled fertiliser (super) = 100 - 60

= 40%

From the fractional utilization relationship 40/60 = 60/x

Where x = available amount of soil P or 90 kg P/ha as superphosphate equivalent units.

Treatment III

% Pdfl= (1400/2000) x 100
= 70%
% Pdf unlabelled fertiliser (rock phosphate) = 100 - 70
= 30%
From the fractional utilization relationship 30/200 = 70/x

Where x = available amount of soil P or 467 kg P/ha as rock phosphate equivalent units.

Quantitative comparison of rock phosphate and superphosphate

90 kg P as superphosphate = 467 kg P as rock phosphate
1 kg P as superphosphate = x
1 kg P as superphosphate is equivalent to 467/90 = 5.2 kg P as rock phosphate

2.4 THE USE OF ISOTOPES OF S IN SOIL/PLANT STUDIES

2.4.1 INTRODUCTION

Two isotopes of S are useful in soil/plant/animal studies. Radioactive ^{35}S has proven to be an extremely useful tool in such studies because it has a convenient half-life (87.2 days) and is a relatively soft β emitter (E_{max} 0.167 MeV). The stable isotope of S, ^{34}S, occurs naturally (4.29%) and is becoming increasingly available, although still extremely expensive. The stable form has the

advantage that it remains indefinitely in the system, except when lost by leaching, volatilization and product removal. The disadvantage is that an isotope ratio mass spectrometer has to be used to detect it and this is more expensive, and not as sensitive, as detecting ^{35}S by liquid scintillation counting.

2.4.2 APPLICATIONS USING RADIOACTIVE ^{35}S

A Direct labelling

Numerous studies have used ^{35}S labelled elemental S or sulfate sources to follow the fate of S supplied in fertilisers. Samosir et al., (1993) used ^{35}S labelled fertilisers to investigate the best form to apply (elemental or sulfate) and the best placement (surface or deep) for flooded rice.

A pot experiment was conducted with a factorial design to include the complete combination of the following factors:

Fertiliser sources (^{35}S labelled): a) K_2SO_4 in solution and

b) elemental S (size 425 to 142 μm).

Fertiliser placement: a) broadcast onto the soil surface in the presence of 0.5 cm of surface water soon after transplanting and

b) mixed with the bottom layer soil (depth 7-21 cm) 30 days before transplanting (total soil depth was 21 cm).

In the 0-42 days after transplanting (dat) period both total and fertiliser S uptake was highest in the Surface Sulfate treatment and lowest in the Deep Elemental treatment (Table 2.5). In the 42 dat-maturity period total and fertiliser S uptake was highest in the Surface Elemental treatment and again lowest in the Deep Elemental treatment. At 42 dat 82.1% of the S in the plant was from the added sulfate in the Surface Sulfate treatment and this was higher than in the other treatments. By maturity, this had declined to 66.8%, which was not different than that in the Surface Elemental treatment. At both sampling times the % of S in the plant derived from the fertiliser was higher with surface than with deep placement for each source.

Table 2.5. Plant sulfur uptake (mg/ pot).

Fertiliser treatment	Total S uptake		Fertiliser S uptake		% S in plant derived from fertiliser	
	0-42 days	42d - maturity	0-42 days	42d - maturity	At 42 dat	At maturity
Surface Elemental	13.4	81.9	7.5	57.3	56.0	68.0
Surface Sulfate	24.6	63.9	20.2	38.9	82.1	66.8
Deep Elemental	5.5	27.2	0.4	5.5	7.3	18.0
Deep Sulfate	11.3	65.1	3.9	33.4	34.5	48.8

B Reverse dilution

In situations where it is not possible to apply a label to the fertilisers as with natural deposits of elemental S or with commercial fertilisers a reverse dilution procedure must be used.

In the technique presented, ^{35}S is used to label the soil available sulfur pool which is subsequently diluted with unlabelled S released from fertiliser. Dilution of the S in the soil by sulfur released from the fertilisers is monitored by measuring the changes in specific radioactivity of sulfur in plants growing in the soil.

The general applicability of the method depends on the fulfillment of two requirements.

The first requirement is that the radiotracer becomes distributed in the same pool from which the plant obtains its supply of sulfur. In this experiment, the phosphate added to the radioactive sulfur solution reduced the likelihood of the $^{35}SO_4$ being adsorbed on surface layers of organic matter and soil before it could mix in the available pool.

The second requirement is that the rate of movement of the tracer from the plant available pool into other soil pools is not greatly affected by the treatments (i.e. the rate of sulfate release from the fertiliser does not affect the rate of movement between soil sulfur fractions).

When both requirements are met it is possible to calculate release rates from the fertilisers. If both requirements are not met it is possible to determine relative release rates from different fertilisers provided that adequate information is available to enable suitable precautions to be taken, e.g. the adjustment of application rates in this experiment.

In the procedure used by Shedley et al., (1979) carrier-free $Na_2\ ^{35}SO_4$ was diluted with 0.1 M KH_2PO_4, to give a solution containing 122 kBq/mL and 3.1 mg P/mL. The phosphate was added to ensure that the carrier-free sulfate was not left adsorbed onto the glass container used for dilution, to aid penetration by reducing adsorption of the sulfate ion onto soil components, and to provide adequate phosphate for plant growth. A syringe was then used to apply 20 mL of the radioactive solution evenly to the surface of each pot.

After fourteen days, the plant tops were clipped to 1 cm above the soil surface to remove any foliage that had been contaminated during the application of radioactivity. Fertiliser treatments shown in Table 2.6 were applied to the pots.

Plant tops were harvested every 14 days by clipping 1 cm above the soil surface. Ten harvests were taken, and the specific radioactivity (SR) of the S in each plant sample was measured. This SR measurement was used to determine the amount of dilution of the S in the plant available sulfur pool. At any particular time, the lower the plant SR the more S that was in the plant derived from the unlabelled fertiliser.

However, because SR. was decreasing with time in all treatments, the ratio of treatment S.R. to control SR (SRR) was the parameter used to compare sulfate release from fertilisers in various treatments over time. Thus the SRR and the rate of release of sulfate are inversely related to each other. This can be a difficult concept to grasp so Dana et al., (1994) used 1-SRR to directly estimate the contribution of fertiliser S to total plant S.

Table 2.6. Sulfur application rates and mean particle diameter of Mexican dark elemental S used in the experiment

Fertiliser	Particle diameter	Application Rate (kg/ha)		
	(mm)	Level 1	Level 2	Level 3
Elemental sulfur	0.05	2	4	16
Elemental sulfur	0.1	4	8	32
Elemental sulfur	0.2	16	32	128
Elemental sulfur	0.4	30	60	240
Elemental sulfur	1.0	200	400	1600
Na_2SO_4 in solution		2	4	16
Control		0	0	0

Some results are presented here to demonstrate the effectiveness of the technique for comparing the rates of nutrient release from different fertilisers and the subsequent uptake of these nutrients by plants.

By the time of the first harvest the sodium sulfate fertiliser had caused a large depression in SRR (*Figure 2.1*) indicating that the fertiliser was supplying a large proportion of the plant sulfur. By comparison, the two elemental sulfur treatments did not release appreciable amounts of sulfate until 42 days after the start of the experiment. The similar shapes of the SRR curves for the two elemental sulfur treatments during the first 70 days indicates that sulfate release from both particle sizes was similar over this period, even though they were applied at different application rates (16 kg/ha and 32 kg/ha).

After decreasing for 70 days the SRR from the 0.05 mm sulfur treatment began to increase in a manner similar to the sulfate treatment, indicating that the 0.05 mm sulfur had finished oxidising after 70 days.

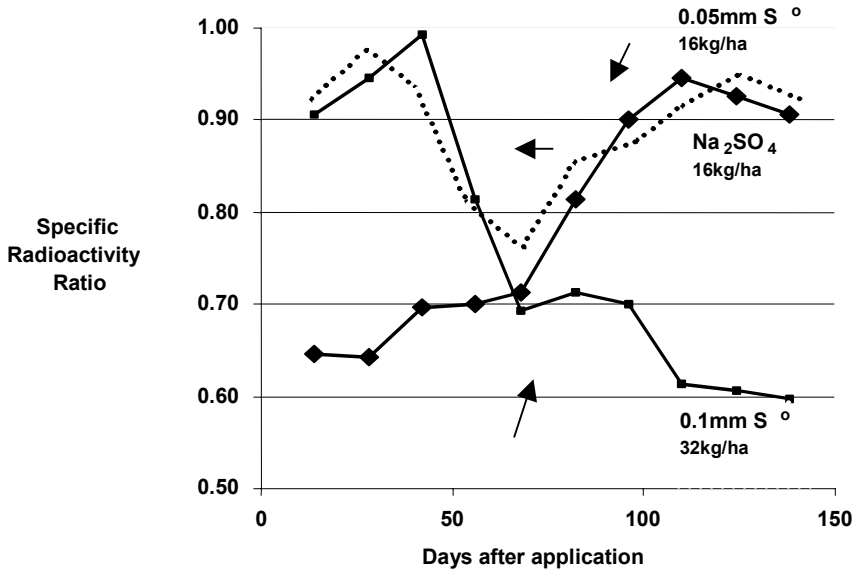


Figure 2.1. Effect of form and particle size of sulfur on its availability to plants.

*SRR is the ratio of the S.R. of sulfur in plants from fertiliser treatments to the S.R. of sulfur in plants from the controls.

At the end of the experiment neither treatment was making a significant contribution to the sulfur available to the plants. Conversely, the continuing decline in the SRR curve for the 0.1 mm treatment indicated that sulfur was being released and taken up by the plants throughout the experiment.

Data from the experiment has been used to demonstrate the relationships between application rate, particle size and sulfate release from fertilisers. Table 2.7 contains mean plant SRR's and 1-SRR's for some selected treatments.

Table 2.7. The effect of particle size and application rate on the supply of S to plants

Particle Size	Application Rate	Mean SRR x 100		Mean (1-SRR) x 100	
(mm)	(kg/ha)	0-140 day	126-140 day	0-140 day	126-140 day
0.1	32	75	57	25	43
0.2	32	87	66	13	34
0.4	30	92	85	8	15
0.4	60	82	68	18	32
0.4	240	74	58	26	42

For treatments with similar application rates (0.1 mm, 0.2 mm and 0.4 mm at about 30 kg/ha), the plant SRR increased as particle size increased. This in accordance with the inverse relationship between particle size and oxidation rates of sulfur reported in the literature.

The three 0.4 mm treatments show the relationship between application rate and sulfate release for one particle size.

It is interesting to note that 240 kg/ha of 0.4 mm particles supplied the same amount of plant S as 32 kg/ha of 0.1 mm particles, indicating the agronomic importance of particle size distribution in elemental sulfur fertilisers.

2.4.3 APPLICATIONS USING STABLE ^{34}S

Either the radioactive ^{35}S or the stable isotope ^{34}S can be used to monitor the movement of S in the soil-plant system. This study outlines the ^{34}S technique used by Wen (1999) and the results obtained.

A Measurement of S leaching

i *The experiment*

The experimental area was located 20 km south of Armidale, New South Wales, Australia. There were three pasture treatments (degraded, phalaris dominant and phalaris/white clover) each with two replicates. Each of the 6 experimental plots was divided into three strata, namely the top, the middle and the lower slope areas. Two 1 m by 0.5 m areas were selected in each stratum, by visual assessment of pasture biomass and species to be representative areas within each stratum. Elemental S, 90% ^{34}S enriched, was ground to pass a 250 μm sieve, and 50 mg of the sieved sulfur was mixed with 30 g of similarly sieved sand. This mixture was evenly applied by hand on each labelled area to give a rate equivalent to 1 kg S/ha and thus would have had only a negligible effect on S dynamics, but provided a pulse of ^{34}S which could be measured as it moved through the soil-plant system under grazing

Soil cores were taken from each labelled area with a 3 cm diameter hand auger and the holes were re-packed with the soils collected from the same site. Whole soil cores were carefully laid on a PVC tube cut longitudinally in half to avoid contamination between depths and then sectioned into 0-5, 5-10 and 10-20 cm soil layers. After significant summer rainfall events the sampling depth was extended to 60 cm in February.

Pasture tops were harvested from two sampling locations within the labelled area at the same time as the soils were sampled by cutting the plants with clippers. Samples were not sorted into component species. Pasture roots were separated from the soil cores and washed.

ii *Calculation of $\delta^{34}S$ and ^{34}S recovery in soil and plant*

From the ratios (R) of mass 50 and mass 48 ion beams measured in an Automated Nitrogen and Carbon Analyser connected to a Mass Spectrometer (ANCA-MS).

$\delta(50)$ was calculated as follows: $\delta(50) = (R_{sample}/R_{standard} - 1) \times 1000$

The laboratory standard used was methionine. The $\delta^{34}S$ value in the sample was then calculated based on the method of Eriksen (1996):

$$\delta^{34}S = \delta(50) \times C + \delta^{34}S \text{ (lab. standard)} + 1/1000 \times C \times \delta^{34}S \text{ (lab. standard)}$$

where C is the correction factor for oxygen normally considered as 1.046 (Eriksen 1997 pers. comm.). The atom % ^{34}S was then calculated using the $\delta^{34}S$ value of all soil and plant samples based on the method of Peoples *et al*. (1989). Using the calculated atom % ^{34}S of all samples, the ^{34}S recovery in soil and plant samples is calculated in the same way as is done for ^{15}N.

iii *Results*

^{34}S was recovered from the top 0-5cm soil layer at all samplings but only in the 5-10cm layer in August 1995 and February 1996. The result from further soil analysis for the samples down to 10-20 cm indicated no further movement of ^{34}S down the soil profile as $\delta\ ^{34}S$ in December 1995 and February 1996 samplings at 10-20 cm soil layer were similar to the background value (*Figure 2.2*).

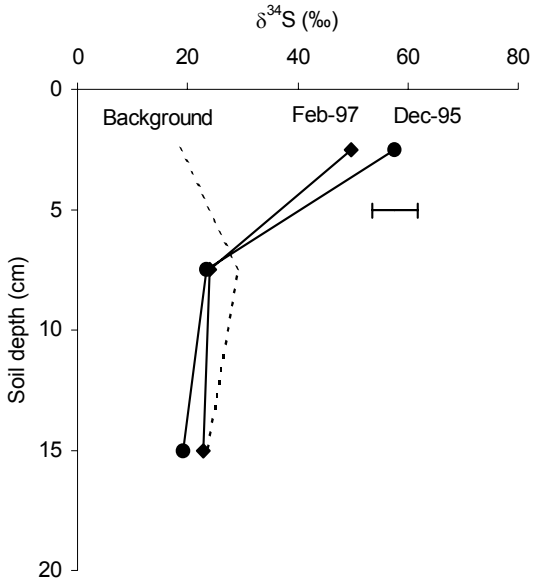


Figure 2.2 $\delta^{34}S$ over three samplings at three depths averaged over pasture types. Horizontal bar is lsd (P = 0.05).

^{34}S recovery in pasture tops varied from 0.5 to 1.6% of that applied throughout the experiment without any difference between the three pastures. There was an effect of time with ^{34}S recovery being higher in the year of application than in the second year and generally higher in summer than winter.

Three months after ^{34}S application (August 1995), 53% of the fertiliser ^{34}S was recovered in soil and plant and 47% was unaccounted for. By the end of this experiment, only 23% was recovered whilst 77% was unaccounted for.

The majority of the applied ^{34}S could not be accounted for in the soil and pasture. The mean proportion of ^{34}S unaccounted for was 58 and 73% for 1995 and 1996 respectively. These values are comparable to the estimates of pasture S utilisation by grazing sheep. In the early experiment using ^{35}S, Till and May (1971) suggested that as much as 80% of the dose applied could be removed by grazing sheep from the "hot spots" to the remainder of the pasture.

B Rainfall S contributions to soil and herbage

Zhao et al., (1998) used the $^{34}\delta$ S of pasture samples from the Rothamstead Park Grass Experiment to investigate the contribution of atmospheric SO_2 to plant S uptake. They found a significant negative correlation between herbage $^{34}\delta$ S and SO_2 emissions over a 135 year period. They also found that topsoil total and extractable S were less sensitive to the change in pollutant S inputs. They estimated that anthroprogenic S contributed up to 50% of herbage S content at the peak of SO_2 emissions and in 1998 accounted for about 30% of S stored in the topsoil.

2.5 ^{13}C AND ^{14}C ISOTOPE STUDIES

2.5.1 INTRODUCTION

The study of soil organic matter (SOM) is becoming increasingly important as world agriculture attempts to increase sustainability of soils while at the same time increase production to feed an ever-increasing population. The use of green manures, the return of residues to the soil, the use of pasture leys and additions of organic amendments are often used in attempts to increase SOM which can have large benefits on both the chemical and physical fertility of the soil. Carbon is the energy source, which drives many of the nutrient cycles that occur in the soil. A ready supply of accessible carbon is necessary for a continuous supply of soil nutrients and to maintain soil structure.

It can often be difficult to study the effect of these procedures on the SOM pools and soil chemical and physical fertility because of the large amount of background carbon present in the soils. The

use of carbon isotopes can provide an easy method of tracing the additions of different plant materials to soil carbon fractions and its influence on soil properties such as soil nutrients and soil structure.

2.5.2 NATURAL ABUNDANCE OF C ISOTOPES

The ratio of ^{13}C to ^{12}C in the atmosphere can vary with different physiographic parameters such as altitude, latitude and temperature as well as by some biological processes (Lefroy et al., 1995). When plants fix carbon during photosynthesis there is a degree of discrimination between the amount of ^{13}C and ^{12}C. Discrimination occurs during the carboxylation step in photosynthesis, with greater discrimination against ^{13}C in C3 (Calvin cycle) plants than in C4 (Hatch-Slack cycle) plants, due to the greater discrimination in the primary carboxylation step of C3 plants. This primary carboxylation step is catalysed by the enzyme ribulase biphosphate carboxylase (RuBP) resulting in a lower ^{13}C:^{12}C ratio in C3 plants than in C4. CAM plants (crassulacian acid metabolism) plants show variable discrimination, but it is more often similar to C4 plants.

The ^{13}C:^{12}C ratio is generally measured as $\delta^{13}C$. A C4 species such as maize will have a $\delta^{13}C$ value of approximately $-12^{o}/_{oo}$ whereas in a C3 species such as wheat or rice it will be approximately –26‰. The $\delta^{13}C$ of SOM is comparable to that of the source plant material (Schwartz et al., 1986) and thus every change in vegetation between C3 and C4 plants results in a corresponding change in the $\delta^{13}C$ value of the SOM (Lefroy et al*.,* 1995). This means that when C3 plants are grown in soils, which had previously been under C4 vegetation (or *vice versa*) there is virtually an *in situ* labeling of the organic matter incorporated into the soil. Cerrie et al. (1985) first used this method in order to measure the turnover rate of organic matter in a 50-year-old sugarcane field, after forest clearing. Schwartz et al. (1986) used this principle to investigate changes in vegetation in the Congo while Skjemstad et al. (1990) studied the turnover of organic matter in pastures using this method. This principle has also been used by Balesdent et al. (1987) and Lefroy et al. (1993) to investigate changes in SOM as a result of cropping, while Bonde et al. (1992) used it to quantify maize root derived soil C.

2.5.3 MEASUREMENT OF ^{13}C

^{13}C is most often determined in CO_2 produced from a solid sample combusted in a stream of oxygen. The two pieces of equipment most commonly used are the Leco and Carlo-Erba furnaces linked to a mass spectrometer set to measure the mass 45/44 ratio. The results are expressed as $\delta^{13}C$ (‰), which is not the absolute isotope ratio but that relative to a standard. The original standard used was a limestone fossil of *Belemnitella americana* (PDB) from the Cretanaceous Pee Dee formation in South Carolina, USA. Since this material is no longer available other standards which have been cross calibrated are used.

2.5.4 CALCULATION OF PROPORTION OF ADDED RESIDUES REMAINING IN THE SOIL

The proportion of soil C derived from the C3 (or C4) plant can be calculated from (Equation 13

$$\chi = \frac{(\delta f - \delta s)}{(\delta r - \delta s)} \qquad \text{(Equation 13)}$$

Where δf is the $\delta^{13}C$ value of the soil at time t after the addition of the residues, δs is the $\delta^{13}C$ of the original soil or soil of the control treatment and, δr is the $\delta^{13}C$ of the C3 (or C4) plant residue added to the soil.

If the total C content C of the soil is known then the absolute quantity X of carbon from the C3 (or C4) plants can be determined from (Equation 14.

$$X = \chi \times C \qquad \text{(Equation 14)}$$

The absolute quantity Y of residual carbon from the initial soil can be determined from (Equation 15.

$$Y = C(1 - \chi) \qquad \text{(Equation 15)}$$

Example calculation

In an incubation experiment 7.50 g of maize leaf with –11.5 ‰ δ^{13}C was mixed into 1000g of soil, which had a total C concentration of 10.00 mg/g and –23.5‰ δ^{13}C and incubated for 60 days at 75% of field capacity. At the end of the experiment the total C concentration was 13.80 mg/g and the δ^{13}C value was –20.26 ‰ δ^{13}C. What proportion of soil C was made up of the added maize leaf?

$$\chi = \frac{-20.26-(-23.5)}{-11.5-(-23.5)}$$

$$= \frac{-20.26+23.5}{-11.5+23.5}$$

$$= \frac{+3.24}{+12.00}$$

$$= 0.27$$

Therefore the added maize residue contributed 27% of the soil C after 60 days.

The total content of residue C remaining is $X = \chi \times C$

= 0.27 x 13830 (i.e. 13.83 mg/g x 1000g)

= 3734 mg

The total quantity of native soil C remaining is $Y = C(1-\chi)$

= 13830 (1- 0.27)

= 10096 mg

or

The total concentration of residue C in the soil is $X = \chi \times C$

= 0.27 $\times$ 13.83 mg/g

= 3.73 mg/g

The total concentration of native soil C remaining is $Y = C(1-\chi)$

= 13.83 (1-0.27) mg/g

= 10.10 mg/g

Exercise

Lefroy et al. (1993) measured changes in soil organic C and the δ^{13}C signature of surface soil under different vegetation on a Vertisol at Warra in SE Queensland. The site was originally under brigalow scrub (C3 photosynthetic pathway) with an organic C content of 30.1 g kg^{-1} and δ^{13}C of -24.6 ‰. The site was clear felled in the early 1900s and soon became heavily infested with prickly pear (*Opuntia inermis*) which rendered it useless for any agricultural enterprise. The prickly pear is a CAM species with a δ^{13}C signature of - 11.4 ‰. It was destroyed by the larvae of an introduced moth, *Cactoblastis cactorum*, in the 1930s. Between then and 1992 the site produced about 40 annual crops of winter cereals (C3 pathway). The organic C content in 1992 was 7.8 g kg^{-1} with a δ^{13}C of - 22.0 ‰.

Using the information in this section answer the following questions.

Q. 1. What percentage of the soil organic C in 1992 was prickly pear C ?

Q. 2. What percentage of the original brigalow C had been lost in 1992 ?

Answers.

Q. 1. 20 %

Q. 2. 79 %

2.5.5 THE "BOMB" ^{14}C TECHNIQUE

During the 1950s and 1960s ^{14}C and ^{137}Cs were deposited over the entire surface of the earth from atmospheric testing of nuclear devices. The ^{137}Cs technique has been widely used to assess the amount of soil deposition that has occurred by measuring how deep the ^{137}Cs layer is beneath the current soil surface.

Rafter and Stout (1970) and Stout and O'Brien (1973) pioneered the "bomb" ^{14}C technique. O'Brien (1984) used this method to investigate the effects of pasture improvement and earthworms on carbon input rate and decomposition time and diffusivity down the soil profile. This technique is potentially very valuable to monitor the stability of an agricultural system and hence its sustainability. The main problem in using the method in soil studies is the very low amount of ^{14}C present in the soil and the consequent difficulty and expense of analysis (Lefroy et al., 1995).

O'Brien (1984) provides the methodology for the "bomb" ^{14}C calculations and has used the technique to study the impacts of earthworms on organic C dynamics in a New Zealand soil. The calculations used in this procedure are also presented in Goh (1991)

The results obtained in the study of O'Brien (1984) are presented in Table 2.8 to illustrate the utility of the technique.

Table 2.8 Organic C dynamics of a New Zealand soil with and without introduced earthworms (O'Brien, 1984).

	Without introduced earthworms	With introduced earthworms
Amount of "bomb" ^{14}C in the profile (Tk) $gcm^{-2} \times 10^6$	36.4	120.9
New C added from herbage (Fo) gcm^{-2}/yr^{-}	0.0048	0.0174
Total amount of C/unit area of soil (W) gcm^{-2}	0.071	0.91
Soil C turnover time (T) yr	149	52
Soil C diffusivity (K) cm^{-2}/yr	3.9	9.0

It is notable that the carbon input rate Fo is larger by a factor of more than 3 on the site with worms. The profile without earthworms had a surface mat of plant litter, the carbon of which was not included in the 0-10 cm layer. Thus Fo only includes carbon entering the soil profile and does not include carbon that respires in the litter mat. Where earthworms were present they obviously fed on this litter and thereby transported organic carbon into and down the soil profile, resulting in a much larger value of the input rate Fo and a greater amount of carbon (W) in the profile. The profile with introduced worms exhibited a much shorter decomposition time (T) of 52 years as against 148 years for the wormless pasture, indicating the presence of more metabolically active organic carbon and more bacterial activity in the worm infested soil. The diffusivity parameter (K) is increased by more than a factor of 2 in the worm infested soil, showing the part that earthworms play in transforming organic carbon down the soil profile.

Data on the change in ^{14}C down the profile ($\delta^{14}C$) were also obtained in this study (Table 2.9).

Table 2.9 Changes in $\delta^{14}C$ values in the soil profile of a New Zealand soil with and without introduced earthworms (O'Brien, 1984)

Soil Depth (cm)	Without Introduced Earthworms	With Introduced Earthworms
	$\delta^{14}C$ (‰)	
0-10	+89	+194
10-17	-11	+102
17-24	-62	-22
24-40	-121	-127
40-50	-250	-224

These data show a greater amount of "bomb" ^{14}C in the profile and deeper mixing of the bomb ^{14}C organic C in the 0-17 cm horizons of the soil with introduced earthworms.

O'Brien (1984) states "The analysis carried out in this study, indicates that it is possible to calculate organic carbon input rate, decomposition time and carbon diffusivity down a soil profile from data

on the ^{14}C, total carbon and soil density. This should enable this method to be more widely used in studies on the transport and turnover of organic carbon in soils. Further work is needed on the validity of the method in different soils and in particular to examine how widespread is the uniform distribution of "old" carbon in soil profiles."

2.5.6 THE USE OF ^{14}C AND /OR ^{13}C IN SOM STUDIES

Bingeman et al. (1953) and Hallam and Batholomew (1953) were some of the first researchers to use ^{14}C in soil chemistry studies of SOM. Since that time ^{14}C has been used in numerous aspects of SOM research. By using substrates that were labelled with ^{14}C and/or ^{13}C it became possible to trace the decomposition of added plant residues with considerable accuracy, even when there were relatively large amounts of native organic matter present. Stevenson (1986) showed that by using ^{14}C labelled materials it was possible to identify plant C as it became incorporated into different fractions of the soil humus. Fu et al. (2000) used ^{14}C labeled corn residues to study the carbon dynamics and respiration of soil organisms in soils under different tillage regimes and were able to estimate the carbon budget and carbon partitioning during a short period after residue application.

Plant roots grown in a ^{13}C-depleted CO_2 atmosphere were used by Van Vuuren et al. (2000) to study the amount of C mineralized from decomposing wheat (*Triticum aestivum* L. cv. Tonic) roots grown under ambient and elevated CO_2 concentrations. The plant roots had $\delta^{13}C$ values of $-38.3‰$ and $-41.1‰$ (Van Vuuren et al., 2000). By using these ^{13}C-depleted roots they were able to distinguish between the root-derived C and native soil C that was mineralized. A study by Angers et al (1997) showed that the use of ^{13}C labelled wheat (*Triticum aestivum* L.) straw made it possible to determine the fate of organic matter in water stable soil aggregates of differing sizes during the decomposition of newly added crop residues. Wheat plants were grown in a growth chamber containing 2% ^{13}C-CO_2 to produce a uniformly labelled straw with an enrichment of 9.060 atom% ^{13}C excess (Angers et al., 1997). Trinsoutrot et al., 2000 used ^{13}C labelled oilseed rape (*Brassica napus* L., cv. Star) to investigate the decomposition of the residues added to soil and to determine how the breakdown of the plant materials related to N content, soluble C compounds, and the cellulose and lignin content of the plant material. The labelled plant material was produced by transferring 2-4 leaf stage spring oilseed rape plants to an airtight growth chamber. The plants were then grown with continual labelling of $^{13}CO_2$ with an isotopic excess of 3.13%, throughout the whole growth cycle (Trinsoutrot et al., 2000).

2.5.7 LABELLING PLANTS WITH ^{14}C AND/OR ^{13}C

Many techniques have been developed to label plants with ^{14}C and/or ^{13}C. It is important to define the purpose of the labelling as this will determine if the plant material needs to be uniformly labelled or not. If the study is to trace the fate of C added in the residues then it is essential that the material added be uniformly labelled. An example from the paper of Sanchez et al. (2000) of a simple labelling chamber and the additions of ^{14}C and ^{13}C necessary to prepare labelled plant residues that can be successfully traced when added to soil is presented in Chapter 4.

2.5.8 CARBON ISOTOPE FRACTIONATION AS A TOOL FOR SCREENING FOR WATER USE EFFICIENCY

Biological and physical process "prefer" lighter isotopes, or discriminate against heavier ones. $^{12}CO_2$ diffuses more rapidly than $^{13}CO_2$ and enzymes react more readily with $^{12}CO_2$. In C3 plants, rubisco discriminates against $^{13}CO_2$ thus, C3 plants generally have a $\delta^{13}C$ of -22 to -29‰ less than the atmosphere (-6 to -8‰).

The discrimination, or fractionation, of plant material in water use efficiency or plant breeding studies is usually described by $\Delta^{13}C$ (Farquhar and Richards, 1984). This means that values are reported as positive, the greater the $\Delta^{13}C$ value the greater the extent of discrimination, lower $\Delta^{13}C$ values indicate less discrimination against ^{13}C. To convert from $\delta^{13}C$ to $\Delta^{13}C$ the following equation is used.

$$\Delta = \frac{R_s}{R_p} - 1 = \frac{\delta_s - \delta_p}{1 + \delta_p}$$

R is the ratio of the source and product usually reported with reference to PDB and where δ_s and δ_p are the source (air –6.5 to 8.0 ‰ dependent on season and location) and product δ ^{13}C of plant material, also reported with reference to PDB.

Carbon dioxide enters the leaves through stomata, however stomatal opening also allows for water loss through transpiration. The rate of diffusion of $^{13}CO_2$ across the stomatal pore is less than that of $^{12}CO_2$ causing a discrimination of about 4.4 $\times 10^{-3}$. In C3 plants lower $\Delta^{13}Cs$ are known to be related to the decrease in the ratio of the leaf intercellular space CO_2 concentration over the ambient concentration (i.e. an increase in the CO_2 diffusion gradient into the leaf) and therefore increased water use efficiency (WUE). For C3 plants discrimination Δ can be partitioned into the fractionation due to carboxylation (b) ca. 27×10^{-3} and the fractionation due to diffusion (a) 4.4×10^{-3} and the difference in internal CO_2 partial pressure p_i and atmospheric CO_2 partial pressure p_a.

$$\Delta = a + (b + a)\frac{p_i}{p_a}$$

Plants with high water use efficiency have mechanisms that reduce transpiration by temporal stomatal closure and improved CO_2 scavenging. Both these processes will lead to less discrimination against the heavier $^{13}CO_2$. Thus under water limiting conditions there is an inverse relationship between $\Delta^{13}C$ and WUE. Farquhar and Richards 1984 showed that in wheat an increase in $\Delta^{13}C$ of 1 $\times 10^{-3}$ corresponded to a decrease in water use efficiency of 19%. Hubrick et al. (1986) showed a close negative relationship between WUE and Δ in diverse peanut (*Arachis*) germplasm. There is significant variation in water use efficiency within species due to a number of morpho-physiological traits related to drought tolerance, including rate of gas exchange, osmotic adjustment, and stomatal density. By using the $\Delta^{13}C$ value it is possible to get an integrated indicator of water use efficiency under water limiting conditions.

These relationships do not necessarily hold true in C4 plants as shown in experiments with sorghum (Hammer et al., 1997).

Measurement of discrimination by plants requires measurement of the carbon abundance ratio in the dry matter and the atmosphere. Carbon dioxide in air can be collected by slowly passing air through an ethanol/dry ice trap then through liquid nitrogen traps, then bleeding it into the mass spectrometer. The ^{13}C abundance in the plant material is analysed using a total combustion unit linked to the mass spectrometer. Collection of plant samples for analysis can be done by taking the whole plant and grinding it, or in some cases where this is not possible taking samples of comparable tissues (i.e. tissues with same age). Experiments conducted at the Soil Science Unit, Seibersdorf, have shown that good results can be obtained taking samples using an office hole punch from comparable leaves. The advantage of this method is that it is non destructive.

2.6 ROOT ACTIVITY STUDIES USING ISOTOPE TECHNIQUES

2.6.1 INTRODUCTION

Root studies are becoming increasingly important component of crop improvement and selection programs. The roots are responsible for plant water and nutrient uptake. Plant and microbes can also form associations at the root level, which may significantly affect crop productivity in different ways. There is a continuous development of new methods for studying root systems. The suitability of one or another method depends basically on the objective(s) of the study and the available resources. Most classical methods (visual observations and/or physical separation of roots) are aiming at determining rooting pattern of crops but do not provide information on root activity, growth and physiological responses to environmental factors. Isotope techniques are unique tools to provide information to many of these. For example they offer a quick and reliable means for determining the distribution pattern of active roots.

2.6.2 ISOTOPE TRACER TECHNIQUES FOR ROOT ACTIVITY STUDIES

A Introduction

The tracer methodology consists basically of injecting a suitable isotope either to the soil or to the plant. Two approaches have been adopted in the development of these isotope tracer techniques, as follows:

a) An isotope such as ^{32}P or ^{86}Rb is injected into the plant stem and the *pattern of root distribution* is determined by taking soil-root cores. and measuring the radioactivity in them.

b) A ^{32}P -labelled phosphate solution is injected into the soil at the various positions (distances and depths) and by measuring the radioactivity in the plant samples the *root activity* (*patterns of root activity*) at the various positions is evaluated against a standard (reference) position.

Both isotope tracer techniques have been applied extensively to field crops. Part of this work has been done by the FAO/IAEA program. A soil injection technique for determining the root activity distribution of various tree crops of economic importance to developing countries was developed within the framework of a Co-ordinated Research Program of the Joint FAO/IAEA Division. The FAO/IAEA Laboratory at Seibersdorf was instrumental in the improvement of the technique by working out the injection and sampling aspects (IAEA, 1975).

B Definitions

Root activity: Relative term used to compare various parts of a root system. It is expressed as a function of the amount of nutrient taken up from a common source of nutrient supply.

Root activity ratio: The relationship of the amount of nutrient taken up by two different parts of a root system. The activity ratio may change with time and space.

Root activity distribution pattern: It is the root activity ratio of any part of the root system related to that of an arbitrary standard location.

C Applications

The injection technique for root activity studies may be used for the following applications:

To study the effect of land preparation methods on root activity (tillage/plowing techniques for seedbed preparation and erosion control).

To study the effect of cultivation and other methods for weed control.

Evaluating methods of fertiliser placement/timing for orchard and plantation trees as well as wide-row planted annual crops.

Genotypic differences in response to environmental stresses, i.e. crop cultivars response to moisture stress.

Definition of crop rotation/ association systems, i.e. combination of crops with different root depth or trees/ shrubs with annual crops in agroforestry systems.

i *Root activity patterns of tree crops*

Quantitative measurement of fertiliser practices using isotope techniques cannot be readily applied to tree crops. The quantities of labelled fertiliser required are much larger than those for annual crops and therefore, high costs are involved in field experimentation of tree crop plantations.

Moreover, it is not practical to harvest and sample whole trees. The approach adopted has been to reduce costs through a careful selection of a small number of treatments and replications. More recently, further improvement has also been achieved by reducing the amount of labelled fertiliser required to label a tree through the use of advanced analytical equipment for isotopic analysis. This has, furthermore, been possible by developing and applying a soil injection technique for determining the root activity distribution of various tree crops of economic importance to developing countries (IAEA, 1975).

Rationale

Application of fertiliser in close proximity to the zone of highest root activity and at a time when the roots are most active can be expected to result in a higher fertiliser nutrient uptake by the crop. Information on the distribution pattern of root activity are, therefore, an essential prerequisite for the formulation of sound fertiliser practices for tree crop plantations.

Injection procedure

Single trees are used as experimental units. Particular care must be taken to ensure the selection of trees with uniform vegetative characteristics (girth, height, foliage, fruiting capacity).

Each tree is treated with a given total ^{32}P activity distributed in equal aliquots of approximately 5ml containing 1000 mg P L^{-1} solution. These are inserted into 15-20 holes in a ring around the tree at a specific depth and distance from the tree to be tested.

A wide variety of equipment and devices can be used for preparing the holes and injecting the solution in the holes (injection points).

Plant sampling and analysis

- Sampling is the main difference between annual (whole plants) and perennial (leaves) crops. In the case of tree crops, two types of leaf sampling may be considered:

  Bulk sample: Representative sample obtained by systematic sampling of leaves from the entire tree, i.e.: counting the leaves and taking every fourth or tenth leaf (depending on the type and size of tree) from every twig and branch.

  Standard sample: Representative sample of leaves from a well-defined morphological position. The sample recommended for foliar diagnosis is also used for this purpose.

- Sampling time interval: Sufficient time (a few weeks) after the injection should elapse so that the nutrient can be translocated and redistributed uniformly throughout the entire canopy. This is a condition for the method to be valid. When unknown, 2 or 3 samplings are made at two weekly intervals.

- Sampling size: It is a function of the activity of the sample. Since the counting rates are low, usually 5-10 g of oven-dry leaf material is used for analysis.

- Analysis: The isotope ($^{32}P/^{33}P$) activity of leaf samples digested in HCl is determined by counting using a liquid scintillation counter.

- Interpretation: For each experiment, the count rates are corrected for decay to a pre-set time and the results are expressed in Bq ^{32}P /g dry matter.

  The activity of the sample is a measure of the root activity. A relative comparison of the root activity at different locations (injection treatments) can be made.

Sources of error

- Soil (spatial) variability across the plantation.
- Plant variability (genetic origin).
- Sampling factors (type, size, time, etc.). They are the main source of experimental error. It can be reduced to some extent by increasing the number of sub-samples, particularly in the case of "standard" sampling. In practice, bulk sampling results in a significant reduction of the sampling variation but it is more laborious.
- Injection factors. Unequal probability of contact between roots and the applied isotope. This may occur if the numbers of injection points are too small.
- Eccentricity factor. Unequal probability of contact between roots and the applied isotope at different distances tested. This is the case when the number of injection points per unit length of circumference for distances close to the trunk is greater than that for circumferences further out from the trunk.

ii *Single labelling technique*

This exercise is to illustrate the single labelling technique. A ^{32}P labelled phosphate solution was injected in regular patterns into the soil. The ^{32}P activity in the leaves of the tree is a measure of the corresponding root activity of the location where the ^{32}P was injected into the soil.

Objective

To determine the area of highest root activity in a 3-year old oil palm plantation.

Application treatments

- Inside a clean weeded ring
- At the crown's diameter
- Outside the crown's diameter

1 palm = 1 experimental unit, 3 treatments x 6 replications = 18 palms

Experimental procedure

Injection: About 11 840 KBq ^{32}P/palm as 16 injections of 5 ml 1000 mg P L^{-1} solution containing 740 KBq ^{32}P each were injected in rings around the base of the palm into the top 5 cm of the soil.

A bulk sample of leaves was taken by systematic sampling of the entire crown. Each branch was sampled systematically by taking every tenth leaf. Midribs were removed and only centre 10 cm of each leaflet taken as subsample. After quartering, the final subsample was weighed, oven-dried at 70°C, weighed again for dry matter determination, ashed and dissolved in 50 ml 2M HCl. The activity was determined by Cerenkov counting in a liquid scintillation analyser.

Results

The final results, expressed in Bq ^{32}P /g dry matter, are shown in Table 2.10.

Table 2.10. Results of Cherenkov counting of leaves from palm trees after injection of ^{32}P in different locations

Treatment	Replicate						
	1	2	3	4	5	6	Mean
	Activity Bq ^{32}P/g DM						
Inside a clean weeded ring	312	110	570	480	410	220	350
At the crown's diameter	730	942	751	1160	1012	852	908
Outside the crown's diameter	120	90	260	20	213	160	144

Conclusions

- The highest activity was found to be at the crown diameter.
- Despite the high standard deviation, the differences between treatments were highly significant.

iii *Double labelling technique*

In this example the double labelling technique with ^{32}P and ^{33}P will be illustrated.

Trees are individuals and despite the fact that all the trees in a plantation block are of the same genetic origin, age, etc, the variability in nutrient uptake among individual trees may be large. Improving sampling methods cannot solve this. It could be improved by substantially by increasing the number of replications for each treatment, but this is usually too costly. Therefore, benefitting of the availability of two radioisotopes for phosphorus, a double labelling technique was developed.

This technique consists of injecting solutions labelled with ^{32}P in the locations to be studied and ^{33}P in a given location around the same tree. The difference between areas of root activity is measured in terms of the ratio of uptake from ^{32}P and ^{33}P.

Objective

To determine the root activity of oil-palm trees in six different locations (combinations of distance and depth).

Experimental procedure

Injection: 16 injections of 5 ml 1000 mg P L^{-1} ^{32}P labelled solution were given in rings according to the experimental treatments listed in Table 4. Similarly 16 injections of 5 ml 1000 mg P L^{-1} ^{33}P labelled solution were applied in the same "standard" location as shown in Table 2.11.

Table 2.11. Injection points for ^{32}P and ^{33}P

Treatment	^{32}P injections		^{33}P injections	
	Distance (m)	Depth (cm)	Distance (m)	Depth (cm)
1	1	10	1	10
2	2	10	1	10
3	3	10	1	10
4	1	20	1	10
5	2	20	1	10
6	3	20	1	10

For this experiment 6 treatments x 4 replications = 24 palms were used

Results

The activity of ^{32}P and ^{33}P was determined in a liquid scintillation counter and the data are presented in Table 2.12.

Table 2.12. Activities of ^{32}P and ^{33}P in palm leaves.

Treatment	Isotope	Replicate			
		1	2	3	4
		Activity (Bq/g DM)			
1	^{32}P	1050	830	417	610
	^{33}P	352	279	140	205
2	^{32}P	3517	3210	1509	2012
	^{33}P	360	330	140	195
3	^{32}P	512	780	309	121
	^{33}P	334	520	190	100
4	^{32}P	412	815	1025	517
	^{33}P	343	660	702	412
5	^{32}P	1103	938	1269	713
	^{33}P	370	302	470	260
6	^{32}P	0	110	50	29
	^{33}P	358	390	260	345

Calculations

In order to compare the root activity in the 6 locations, it is necessary to determine the ratio corresponding to the actual amounts of phosphorus taken up. For this purpose, the ^{33}P counts had to be transformed into equivalent ^{32}P counts. The following calculations were made:

The conversion factor to transform ^{33}P counts into equivalent ^{32}P counts was determined. Using the data of treatment 1 (standard) where the ^{32}P and ^{33}P injections were made in the same location. The activity of ^{32}P represents the same amount of phosphorus (carrier P) taken up as the activity of ^{33}P. Thus in Treatment 1, replication 1, 1050 Bq ^{32}P represents the same uptake of carrier P as 352 Bq ^{33}P and the corresponding ratio of $^{32}P/^{33}P$ is 1050/352 = 3. A similar calculation was performed for the other replicates and this resulted in a mean weighting factor of 2.98.

The transformation of ^{33}P counts into equivalent ^{32}P counts and the calculation of activity ratios for each treatment are shown in Table 2.13.

Table 2.13. Transformation of ^{33}P counts into equivalent ^{32}P counts by multiplying the ^{33}P counts by 2.98 and calculation of the ratio for each treatment.

Treatment		Replicate			
		1	2	3	4
			Activity (Bq/g DM)		
1	^{32}P	1050	830	417	610
	^{33}P to ^{32}P	1050	830	417	610
	Ratio	1.00	1.00	1.00	1.00
2	^{32}P	3517	3210	1509	2012
	^{33}P to ^{32}P	1080	990	417	581
	Ratio	3.30	3.30	3.60	3.50
3	^{32}P	512	780	309	121
	^{33}P to ^{32}P	995	1550	566	298
	Ratio	0.51	0.50	0.55	0.41
4	^{32}P	412	815	1025	517
	^{33}P to ^{32}P	1022	1967	2092	1228
	Ratio	0.40	0.41	0.49	0.42
5	^{32}P	1103	938	1269	713
	^{33}P to ^{32}P	1103	900	1401	775
	Ratio	1.00	1.04	0.90	0.90
6	^{32}P	0	110	50	29
	^{33}P to ^{32}P	1067	1162	2247	1028
	Ratio	0	0.09	0.02	0.03

Table 2.14. Summary of results (Ratio $^{32}P/^{33}P$)

Depth (cm)	Distance (m)				
	1.0		2.0		3.0
	Ratio	Significance	Ratio	Significance	Ratio
10	1.00	*	3.40	**	0.50
Significance	*		*		**
20	0.40	*	1.00	**	0.05

* significant at p = 0.05, ** significant at p = 0.01

Conclusions

- The zone of highest root activity is located 2.0 m from the base of the tree and at 10 cm depth.
- Root activity at 10 cm depth was always higher than at 20 cm depth.
- The variability of ratios is less than that of activities.

2.7 USE OF ^{15}N TO QUANTIFY BIOLOGICAL NITROGEN FIXATION IN LEGUMES

2.7.1 INTRODUCTION

Legumes play an important part in the diets of most of the people of the world, and they are second only to cereals as a source of human and animal food. Grain legumes are a particularly important source of protein. Although in terms of dry matter production, legumes (food legumes and leguminous oil seeds) account for only 10% of the combined world yield of cereals and legumes, they constitute as much as 24% of the total protein yield of these crops because of the latter's high protein content.

One of the most important characteristics of legumes are their ability, in symbiosis with *Rhizobium* bacteria, to form nodules on the root system (*Figure 2.3*) and to fix atmospheric nitrogen for growth. Legumes having effective biological nitrogen fixation (BNF) can therefore be grown without nitrogen fertiliser application. Having these characteristics, they are particularly important in developing countries due to the often high cost and/or restricted availability of nitrogen fertiliser.

Figure 2.3. Common bean (Phaseolus vulgaris L.) nodulated by rhizobial bacteria.

For proper management and a full realization of the benefits of this plant-microbial association, it is necessary to estimate how much nitrogen is fixed under different field conditions. It is only after this is known that various factors can be manipulated so as to increase the amount of N a crop derives from the atmosphere through biological nitrogen fixation. A suitable method for accurately measuring the amount of N crops derive from the atmosphere is therefore an important requirement in any program aimed at maximizing biological nitrogen fixation. The objective of this chapter is to illustrate the use of ^{15}N methodologies to quantify above and below ground N derived from the atmosphere through biological nitrogen fixation.

2.7.2 METHODOLOGIES

There are several methods available to measure N_2 fixation (Bergersen, 1980) based on

- increment in N yield and plant growth,
- nitrogen balance,
- acetylene reduction and
- the use of isotopes of N.

The selection of methodology will depend on the objective of the work. The various methods are compared in Table 2.15. The dry matter yield, total N, ARA and xyleme-solute methods are simple, rapid and relatively cheap (Hardarson and Danso, 1993). These methods may be used in breeding programs where many analyses have to be performed on plant populations and precision is of secondary importance. When selecting plants for N_2 fixation related traits, it may be important to evaluate plants non-destructively for later production of seed from the same plants. For those plants that can be transplanted, ARA, xylem-solute and fresh instead of dry matter yield methods may be used. Time integrated measurement of N_2 fixation and quantification of %Ndfa (percentage **N** **d**erived **f**rom the **a**tmosphere) and total Ndfa will be particular important for field measurements of various agronomic treatments and of breeding lines after the selection process. Total N, ^{15}N isotope dilution, A-value and ^{15}N natural abundance methods will be the most suitable for such a task, when only few and relatively accurate measurements have to be made.

Table 2.15. Comparison of methods to quantify symbiotic nitrogen fixation (Hardarson and Danso, 1993)

	Dry Matter yield	Total N difference	Nodule observations	Acetylene Reduction	Xylem-solute	$^{15}N_2$	Isotope Dilution	A-value	Natural Abundance
Direct	-	-	-	-	-	+	-	-	-
No reference plant needed	-	-	+	+	+	+	-	-	-
Simple, rapid and cheap	++	+	+	++	+	-	-	-	-
Non-destructive	-(+)	-	+	+	(+)	-	-	-	-
Time integrated measure	+	+	-	-	-	-(+)	+	+	+
% Ndfa measured	-	+	-	-	-	-(+)	++	++	+
Measure of kg N/ha fixed	-	+	-	-	-	-	++	++	+
Small field variability	+	+	-	-	?	-	+	+	?

In this chapter only some of the isotopic methods, i.e. the isotope dilution and A-value methods are illustrated in detail and reference is made to publications describing the other methods.

2.7.3 USE OF ^{15}N TO ESTIMATE BIOLOGICAL NITROGEN FIXATION

In N isotopic tracer studies, the system under investigation is supplied with materials containing $^{15}N/^{14}N$ ratios measurably different from the ^{15}N natural abundance. It is also essential that the nitrogen isotope ratio should again be measurably different from ^{15}N natural abundance at the time the system under investigation is sampled.

A Use of $^{15}N_2$

The earliest application of $^{15}N_2$ in N_2 fixation studies was by Burris and Miller (1941). This method has been used to provide direct evidence for N_2 fixation since the ^{15}N concentration in plants exposed to $^{15}N_2$ is greater than the 0.3663% natural abundance if fixation occur. The extent to which ^{15}N is detected in the plant provides an estimate of the proportion of the plant's N that was derived from fixation, and is thus a direct method for quantifying N_2 fixed. The use of $^{15}N_2$ involves the enclosure of plants in chambers filled with the enriched gas (Witty and Day, 1978). The environment within the chamber is, however, likely to be different from that in a field situation. Also, it is difficult to confine plants in these chambers for long periods without affecting the growth conditions as compared to the field environment. The isotope composition of the chamber can also change with time due to leakage of air. Results obtained from such studies therefore tend to be short term (hours to days) and subject to errors associated with extrapolating data from short term studies to a growing season which involves diurnal, daily and seasonal variations (Knowles, 1980).

B Use of enriched fertiliser or substrates

i *Introduction*

The so called ^{15}N isotope dilution method and other methods based on the same principle involves the growth of N_2 fixing (F) and non-fixing reference (NF) plants in soil fertilized with ^{15}N enriched inorganic or organic fertilisers. It relies on differential dilution in the plant of ^{15}N-labelled fertiliser by soil and fixed nitrogen (McAuliffe et al., 1958; Fried and Broeshart, 1975; Fried and Middelboe, 1977). It provides an integrated measurement of amount of fixed N accumulated by a crop over the growing season. The uptake of ^{15}N enriched fertiliser added to soil will result in a $^{15}N/^{14}N$ ratio greater than 0.3663% within the plant, the extent of which is a reflection of uptake of the labelled ^{15}N fertiliser. A decrease in the atom $\%^{15}N$ excess of the fertiliser nitrogen within the plant is an indication of the extent to which the plant took up N from other unlabelled N sources, e.g. air.

The measurement of atom $\%^{15}N$ excess or % N derived from the fertiliser (%Ndff) is necessary before BNF can be calculated. The next examples illustrate the calculation of %Ndff for non-fixing and fixing crops:

Example 1

In a field experiment 50 kg N/ha of 2.501 atom $\%^{15}N$ excess ammonium sulfate was applied to a cereal crop. At the end of the growing season plant sample from the harvested material had 0.534 atom $\%^{15}N$ excess. What was the % N derived from fertiliser (% Ndff)?

Calculation:

$$\%Ndff = \frac{atom\ \%^{15}N\ \ excess\ (plant)}{atom\ \%^{15}N\ \ excess\ (fertilizer)} \times 100 \qquad \text{(Equation 16)}$$

$$\%Ndff = \frac{0.534}{2.501} \times 100 = 21\%$$

21% of the N in the plant was derived from labelled fertiliser and the remaining 79% was derived from unlabelled soil N.

The same type of calculation as in Example 1 can be made for legume crops as shown in Example 2.

Example 2

20 kg N/ha of 5.231 % ^{15}N atom excess was applied to fixing (F) and a non-fixing (NF) crops in a field experiment. Plant samples from the harvested materials yielded 0.702 and 1.251 $\%^{15}N$ atom excess for F and NF crops, respectively. What was the % Ndff for the two crops?

Calculation:

$$\%Ndff = \frac{atom\ \%^{15}N\ \ excess\ (plant)}{atom\ \%^{15}N\ excess\ (fertilizer)} \times 100$$

$$\%Ndff_F = \frac{0.702}{5.231} \times 100 = 13.4\%$$

$$\%Ndff_{NF} = \frac{1.251}{5.231} \times 100 = 23.9\%$$

In the NF crop the remaining 76.1% was derived from unlabelled soil N. However in the F crop the remaining 86.6% was derived from the atmosphere (%Ndfa) through biological nitrogen fixation and from soil (%Ndfs) as

% Ndff $_F$ + % Ndfs $_F$ + % Ndfa = 100

The question therefore remains what were the proportions derived from air and soil in the F crop. To be able to calculate the relative proportion derived from these two sources the following assumption has to be introduced:

Assumption: *It is assumed that both non-fixing and fixing crops take up N from soil and fertiliser in the same ratio,* i.e.

$$\frac{\%Ndff_{NF}}{\%Ndfs_{NF}} = \frac{\%Ndff_F}{\%Ndfs_F} \qquad \text{(Equation 17)}$$

Using equation 17 the calculation of example 2 can be continued as illustrated in the following table:

	Atom %^{15}N excess	%Ndff	%Ndfs	%Ndff/%Ndfs	%Ndfa
NF	1.251	23.9	76.1	0.314[a]	0
F	0.702	13.4	42.7[b]	0.314[a]	43.9[c]
Fertiliser	5.231				

According to the above assumption:

a $$\frac{\%Ndff_{NF}}{\%Ndfs_{NF}} = \frac{23.9}{76.1} = 0.314 = \frac{\%Ndff_F}{\%Ndfs_F}$$

Thus

b $$\%Ndfs_F = \frac{\%Ndff_F}{0.314} = \frac{13.4}{0.314} = 42.7$$

and

c %Ndfa = 100 - %Ndff$_F$ - %Ndfs$_F$

%Ndfa = 100 - 13.4 - 42.7 = 43.9

In this way the proportions of N from all available sources have been quantified, i.e. for the non-fixing crop (NF)

%Ndff = 23.9

%Ndfs = 76.1

and for the fixing crop (F)

%Ndff = 13.4

%Ndfs = 42.7

%Ndfa = 43.9

The methodology is certainly not accurate enough to measure decimal points so one would report the %Ndfa = 44%. But what is the accuracy of the ^{15}N isotope dilution method? That question will be covered in section D below.

There are six main variations in the use of ^{15}N labelled substrates:

- the ^{15}N isotope dilution method (ID) which was partly illustrated above (McAuliffe et al., 1958; Fried and Middelboe, 1977),
- the A-value method (AV) (Fried and Broeshart, 1975),
- the single treatment method (ST) (Fried and Broeshart, 1981),
- yield dependent model (Smith et al, 1992),
- yield independent method (Chalk and Ladha, 1999) and
- the natural abundance method (Amarger et al., 1979; Kohl et al., 1980).

C The ^{15}N isotope dilution method (ID)

This is the case when both fixing and reference plants are grown in soil to which the same amount of fertiliser having the same ^{15}N enrichment, has been applied, as illustrated in example 2. Thus, in the absence of any supply of N other than soil and ^{15}N labelled fertiliser, a fixing plant and a non-fixing reference plant will contain the same ratio of $^{15}N/^{14}N$, since they are taking up N of the same $^{15}N/^{14}N$ composition, but not necessarily the same total quantity of N. In both plants, the $^{15}N/^{14}N$ ratio within the plant is lowered by the N absorbed from the unlabelled soil. However, in the presence of N_2, and because of atmospheric N_2 fixation by the fixing plant the $^{15}N/^{14}N$ ratio is lowered further. The extent to which the $^{15}N/^{14}N$ ratio in the fixing crop is decreased, relative to the non-fixing plant can be used to estimate N_2 fixed in the field.

The determination of N_2 fixation using this approach is depicted in *Figure 2.4*. By using ^{15}N labelled fertiliser, 50% of the N in the NF reference crop was derived from the applied fertiliser. Since there are only two sources of N available to this crop,

$$\%Ndff_{NF} + \%Ndfs_{NF} = 100 \qquad \text{(Equation 18)}$$

or

$$a + b = 100$$

It follows from Equation 18 that the other half or 50% of the N in the plant came from the soil. This then establishes that the ratio of soil to fertiliser N available to the non-fixing plant in this example was 1:1.

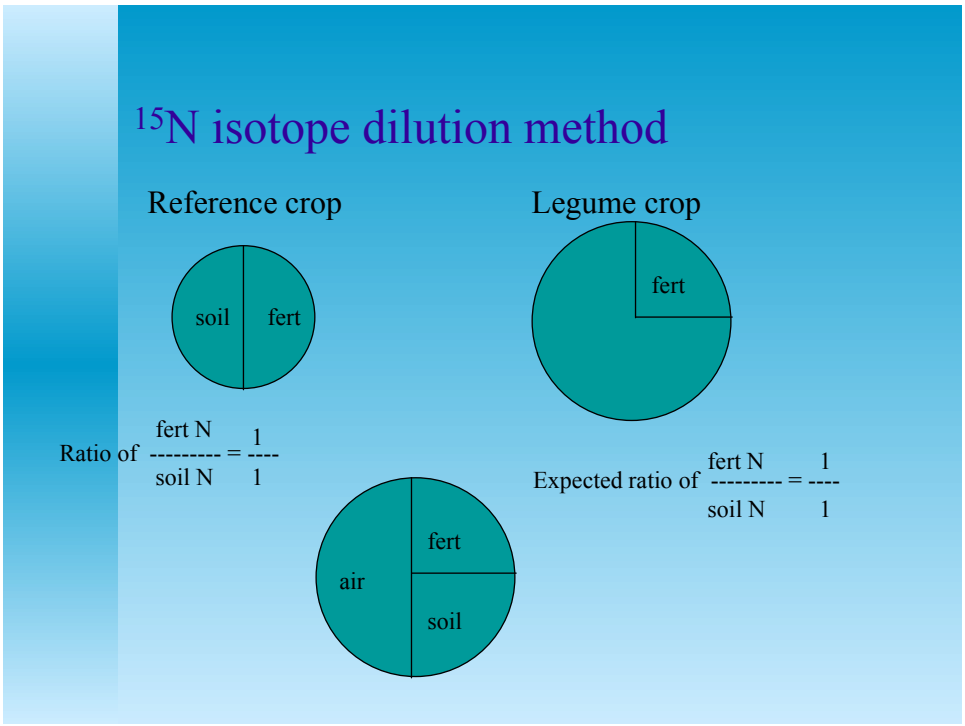


Figure 2.4. A simplified example of how the ^{15}N isotope dilution technique is used to measure nitrogen fixation by grain legumes.

For the N_2 fixing crop in *Figure 2.4*, there is a third source of N available to the plant, i.e. N_2 from the atmosphere. The total N in the plant can therefore be represented by the following equation:

$$\%Ndff_F + \%Ndfs_F + \%Ndfa = 100 \qquad \text{(Equation 19)}$$

or

$$c + d + e = 100$$

The non-fixing reference crop took up N from soil and fertiliser in the ratio 1:1, and it is assumed, as shown in Equation 17, that the same occurs in the fixing crop, i.e.

$$\frac{a}{b} = \frac{c}{d}$$

In the example %Ndff in the fixing crop was 25%. Therefore, according to Equation 17 the %Ndfs in the fixing crop is also 25%. The remainder of the N taken up (50%) was derived from atmosphere, since according to Equation 19: % Ndfa = 100 - ($\%Ndff_F$ + $\%Ndfs_F$)

%Ndfa, as quantified by the ^{15}N isotope dilution method, is usually calculated by the following equation:

$$\%Ndfa = (1 - \frac{\%Ndff_F}{\%Ndff_{NF}}) \times 100 \qquad \text{(Equation 20)}$$

Derivation of Equation 20.

Equation 20 is derived from Equation 17, Equation 18 and Equation 19 as follows:

From Equation19

$$e = 100 - c - d \qquad \text{(Equation 21)}$$

and from Equation 17

$$d = \frac{c \times b}{a} \qquad \text{(Equation 22)}$$

where according to Equation 18

$$b = 100 - a$$

From Equation 18 and Equation 22

$$d = \frac{c}{a} \times (100 - a)$$

or

$$d = \frac{100 \times c}{a} - c \qquad \text{(Equation 23)}$$

From Equation 19 and Equation 23

$$e = 100 - c - (\frac{100 \times c}{a} - c)$$

or

$$e = (1 - \frac{c}{a}) \times 100 \qquad \text{(Equation 24)}$$

or

$$\%Ndfa = (1 - \frac{\%Ndff_F}{\%Ndff_{NF}}) \times 100$$

Equation 24 can also be written as:

$$\%Ndfa = (1 - \frac{atom\ \%^{15}N\ excess_F}{atom\ \%^{15}N\ excess_{NF}}) \times 100 \qquad \text{(Equation 25)}$$

since

$$\%Ndff = \frac{atom\ \%^{15}N\ excess_{(sample)}}{atom\ \%^{15}N\ excess_{(fertilizer)}} \times 100 \qquad \text{(Equation 26)}$$

The amount of N_2 fixed can be calculated according to:

$$Amount\ of\ N_2\ fixed = \frac{\%Ndfa \times total\ N\ in\ fixing\ crop}{100} \qquad \text{(Equation 27)}$$

The use of Equation 24, Equation 25 and Equation 27 is illustrated in Table 2.16

Table 2.16. The following data were recorded for nodulating (F) and non-nodulating (NF) soybean under field condition at the Seibersdorf Laboratory. 20 kg N/ha of ^{15}N labelled fertiliser was applied to the fixing and non-fixing crops. Only one replicate from five is shown in this example.

Fixing crop	Dry matter yield[a] (kg/ha)	N[b] (%)	N yield[d] (kg/ha)	atom ^{15}N excess[c] (%)	Ndff[e] (%)	N fert. yield[f] (kg/ha)	Ndfa[h] (%)	Fixed N[i] (kg/ha)
Stems	4478	0.63	28.2	0.152	3.16	0.89		
Leaves	2743	1.90	52.1	0.158	3.28	1.71		
Pods	1867	2.58	48.2	0.132	2.74	1.32		
Total	9088		128.5		3.05[g]	3.92	26	33

The values needed for the calculation are:

- Atom $\%^{15}N$ excess of fertiliser: 4.18
- $\%Ndff_{NF}$: 4.14 (calculated by the same method as $\%Ndff_F$)

Measured values in Table 2.16

[a] Dry matter yield of plant parts (kg/ha)
[b] %N of each plant part in [a]
[c] atom % ^{15}N excess of each plant part in [a] and of fertiliser applied

Calculated values in Table 2.16

[d] $$N\ yield\ (kg/ha)\ of\ each\ plant\ part = \frac{D.M.of\ each\ plant\ part \times \%N}{100}$$

[e] $$\%Ndff = \frac{atom\ \%^{15}N\ \ excess\ of\ sample}{atom\ \%^{15}N\ \ excess\ of\ \ fertilizer} \times 100$$

[f] $$N\ \ fertilizer yield (kg/ha) = \frac{N yield (kg/ha) \times \%Ndff}{100}$$

[g] $$\%Ndff\ (weighted\ average) = \frac{Total N\ fertilizer\ yield}{Total\ N yield} \times 100$$

[h] $$\%Ndfa = (1 - \frac{\%Ndff_F}{\%Ndff_{NF}}) \times 100$$

or

$$\%Ndfa = (1 - \frac{atom\ \%^{15}N\ excess_F}{atom\ \%^{15}N\ excess_{NF}}) \times 100$$

[i] $$N_2\ fixed\ (kg/ha) = \frac{\%Ndfa \times total\ N\ in\ fixing\ crop}{100}$$

Example 2 continued

It is possible to use Equation 24 or Equation 25 to calculate %Ndfa for the above example

$$\%Ndfa = (1 - \frac{\%Ndff_F}{\%Ndff_{NF}}) \times 100$$

$$\%Ndfa = (1 - \frac{13.4}{23.9}) \times 100 = 43.9\%$$

or

$$\%Ndfa = (1 - \frac{atom\ \%^{15}N\ excess_F}{atom\ \%^{15}N\ excess_{NF}}) \times 100$$

$$\%Ndfa = (1 - \frac{0.702}{0.251}) \times 100 = 43.9\%$$

i *Assumption made by the ^{15}N isotope dilution methodology*

The assumption made in Equation 17 is the only assumption made in the ^{15}N isotope dilution methodology. It is assumed that both fixing and non-fixing plants take up nitrogen from soil and fertiliser in the same ratio. For this to be true the fixing and the non-fixing crops have to meet the following conditions (Witty, 1984):

- Either fertiliser distribution is even with depth or that the legume and reference crops have spatially similar nutrient uptake profiles, i.e. the root systems should be similar.
- The contribution of seed N should be negligible, which is not always true especially if the plants are harvested early in the growing season.
- It is implicit in the calculation that the enrichment of plant available soil N remains constant with time or that the legume and control have similar N uptake patterns. In practice when fertiliser N is added as a single application the enrichment of plant available soil N declines with time; and this decline can vary between the legume and the control plant. Depending on whether the control takes up soil nitrogen faster or slower than the legume, the calculated nitrogen fixation rate will be greater or less than the true value (Witty, 1983). Errors due to making this assumption may be reduced by the use of slow-release N fertiliser and by choice of a control plant which closely parallels the legume in its nitrogen uptake. (Witty, 1984).

D Accuracy of measurements of N_2 fixation

The accuracy and precision of the isotope dilution method depends to a great extent on selecting a suitable NF reference crop. The selection of the appropriate reference plant is crucial, and it is essential to observe the following:

- That the reference crop does not itself fix nitrogen. This can, if necessary, be checked very quickly, using the acetylene reduction assay.
- The rooting depths of both reference and fixing crops should be similar, or both crops should derive all of their N from the same zone.

- Both N_2 fixing and reference crops should go through similar growth or physiological stages, and mature at about the same time.
- Both N_2 fixing and standard crops should be planted and harvested at the same time.
- Both crops should be affected in similar fashion by changes in environmental conditions, such as temperature and water, during growth period.

For estimating N_2 fixed in grain legumes, the following NF reference crops have been used:

- A non-legume, non-fixing plant.
- A non-nodulating legume plant.
- An uninoculated legume plant in soils devoid of the appropriate strains of *Rhizobium.*

Although one does take care of the above conditions there is no guarantee that the measurement of BNF is correct. Only through several experiments one can gain confidence in the system one is working with and a feeling for the accuracy of measurements.

Methods based on the dilution in the plant of ^{15}N labelled fertiliser by N derived from the atmosphere and soil potentially offer accurate methods to quantify symbiotic nitrogen fixation. Variations are, however, often found depending on the non-fixing standard crop (Wagner and Zapata, 1982). This has been found to be mainly due to differences in N uptake patterns of the legume and reference plants, together with a decrease in the $^{15}N/^{14}N$ ratio of the substrate with time (Witty, 1983).

It has been observed at the Seibersdorf Laboratory that the N methodology is particularly accurate when large proportions of the N in the fixing plant is derived from the atmosphere (Reichardt et al., 1987, Hardarson et al., 1988). This prompted us to model the percentage of N derived from the atmosphere in relation to ^{15}N enrichment in the fixing and non-fixing standard crops and to investigate where major errors in estimates of N_2 fixation can be expected.

As shown previously, the percentage N derived from atmosphere (e) is calculated using the ID method using Equation 24, i.e.

$$e = (1 - \frac{c}{a}) \times 100 \qquad \text{or} \quad \%Ndfa = (1 - \frac{\%Ndff_F}{\%Ndff_{NF}}) \times 100$$

where *c* and *a* are % Ndff of the fixing and non-fixing crops, respectively.

Using this equation the modelled curves for various *c* and *a* for both fixing and non-fixing crops are shown in *Figure 2.5*. When c is 1% e increases very rapidly up to 80% with increased value of *a*. At higher *c* values differences in *a* will not affect *e* to the same extent. A 10% coefficient of variation of *a* (% Ndff of reference crop) produces much larger variation in *e* (% Ndfa) at low fixation values (*Figure 2.5* and Table 2.17).

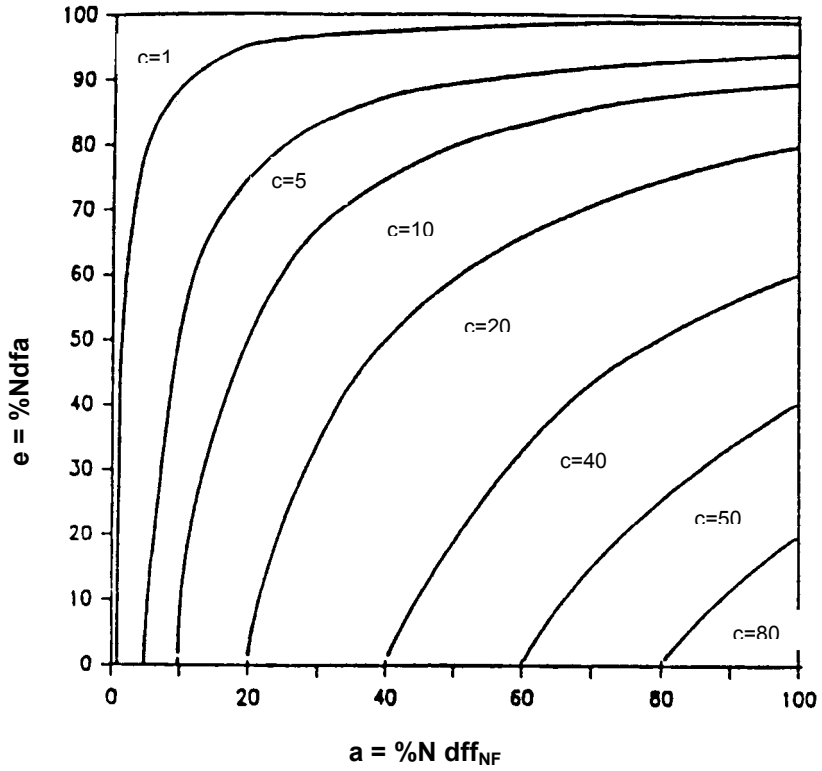


Figure 2.5. Modelled curves for e (% Ndfa) at various a (%Ndff$_{NF}$) and c (% Ndff$_F$) values.

Table 2.17. Calculated values for *e* (%Ndfa) from various values of *c* (%Ndff of fixing crop) when *a* (%Ndff of reference crop) has 10% coefficient of variation.

Example	a	c	e	Range (%)
1	20 ± 2	18	0 - 18	18
2	20 ± 2	12	33 - 45	12
3	20 ± 2	8	55 - 64	9
4	20 ± 2	4	78 - 82	4
5	20 ± 2	2	89 - 91	2

It is clear from the above modelling that methods based on the dilution in the plant of ^{15}N labelled fertiliser by N derived from atmosphere are potentially accurate methods to quantify N_2 fixation when a large proportion (> 70%) of the N in the fixing crop is derived from the atmosphere. However, at lower N_2 fixation levels (< 30%) the methodology is much less accurate and, under these conditions the selection of a reference crop, and the stability of the $^{15}N/^{14}N$ ratio of the substrate, is particularly important.

E The A-value method (AV)

Often, it is necessary to apply different doses of N to fixing and non-fixing plants. As high levels of inorganic N can depress N_2 fixation it is necessary to apply low amounts of labelled N fertiliser to the fixing crop in order to estimate N_2 fixed. However, such amounts may be too low to support the proper growth of the reference plants, especially in soils of low fertility. For these reasons it is practical to give a reasonable dose of ^{15}N labelled fertiliser (40-80 kg N/ha) to the reference crop, while the fixing crop receives a low quantity (5-20 kg N/ha) (Fried and Broeshart, 1975). It is recommended to use the ID method whenever it is possible and the A-value method when the reference crops are not able to grow due to lack of N in the soil.

When different fertiliser rates are applied to the F and NF crops *n* is the relative amount of fertiliser applied, i.e. *n* = amount of fertiliser applied to the F crop divided by the amount of fertiliser applied to the NF crop.

The assumption (Equation 17) which was previously presented for the ^{15}N isotope dilution method is also used for the A-value methodology but *n* $\%Ndff_{NF,}$ which is the estimated $\%Ndff_{NF}$ at the rate of N applied to the F crop has to be calculated in the following equation:

$$\frac{n \times \%Ndff_{NF}}{\%Ndfs_{NF}} = \frac{\%Ndff_{F}}{\%Ndfs_{F}} \qquad \text{(Equation 28)}$$

Example 3

In a field experiment two different rates of ^{15}N labelled ammonium sulphate were applied to F and NF crops, i.e. 20 kg/ha of 5.6 atom $\%^{15}N$ excess to the F crop and 60 kg N/ha of 2.5 % atom $\%^{15}N$ excess to the NF crop. What was the %Ndfa for the F crop?

Calculation:

	Fert. rate Kg N/ha	$\%^{15}N$ a.e. (fert.)	$\%^{15}N$ a.e. (plant)	%Ndff	%Ndfs	%Ndff/ %Ndfs	%Ndfa
NF	60	2.50	0.40	16	84	0.063[a]	
F	20	5.60	0.08	1.4	22.1[b]	0.063[a]	76.5[c]

a $$\frac{n \times \%Ndff_{NF}}{\%Ndfs_{NF}} = \frac{0.33 \times 16}{84} = 0.063 = \frac{\%Ndff_{F}}{\%Ndfs_{F}}$$

b $$\%Ndfs_{F} = \frac{1.4}{0.063} = 22.1\ \%$$

c $\%Ndfa = 100 - \%Ndff_{F} - \%Ndfs_{F}$

$\%Ndfa = 100 - 1.4 - 22.1 = 76.5\ \%$

Example 3 can also be calculated using the following equation:

$$\%Ndfa = 100\left(1 - \frac{\%Ndff_{F}}{n \times \%Ndff_{NF}}\right) + \%Ndff_{F}\left(\frac{1}{n} - 1\right) \qquad \text{(Equation 29)}$$

$$\%Ndfa = 100\left(1 - \frac{1.4}{0.33 \times 16}\right) + 1.4\left(\frac{1}{0.33} - 1\right) = 76.5\%$$

Equation 20 of the ^{15}N isotope dilution method is a particular case of Equation 29 when *n* is equal to 1.

Derivation of Equation 29 is shown in Hardarson et al. (1991).

Table 2.18. Data were recorded for fababean (F) and barley (NF) at the Seibersdorf laboratory. 20 kg N/ha of ^{15}N labelled fertiliser was applied to the faba bean and 100 kg N/ha to the barley. Only one replicate from five is shown here as an example.

	N fert. rate kg N/ha	Total N yield[a] (kg/ha)	%Ndff[b]	%Ndfa[c]	Fixed N[d] kg N/ha
F	20	151.7	0.877	79	120
NF	100		18.17		

Measured values (not shown)

Dry matter yield of plant parts (kg/ha)

%N of each plant part

atom $\%^{15}N$ excess of each plant part and of the fertiliser applied

The values needed for the calculation:

- Atom %^{15}N excess of fertiliser: F: 5.64
  NF: 1.00

Calculated values in Table 2.18

[a] $$N\ yield\ (kg/ha)\ of\ each\ plant\ part = \frac{D.M.of\ each\ plant\ part \times \%N}{100}$$

[b] $$\%Ndff = \frac{atom\ \%^{15}N\ excess\ of\ sample}{atom\ \%^{15}N\ excess\ of\ fertilizer} \times 100$$

[c] $$\%Ndfa = 100\left(1 - \frac{\%Ndff_F}{n \times \%Ndff_{NF}}\right) + \%Ndff_F\left(\frac{1}{n} - 1\right)$$

[d] $$N_2\ fixed(kg/ha) = \frac{\%Ndfa \times total\ N\ in\ fixing\ crop}{100}$$

This method was originally presented using the A-value concept of Fried and Broeshart (1975) with a slightly different way of calculating BNF.

F Single treatment method

The third variation of the isotope dilution method was introduced by Fried and Broeshart (1981). As this method is not commonly used it will not be explained further here.

G Yield dependent and independent models

These approaches are based on measurement of the temporal change in the isotopic composition of the pool of available soil N which is being exploited by the legume roots. The integrated pool enrichment is used as the reference criterion instead of the N taken up by a non-fixing plant as is done in the isotope dilution method. The models and the equations used are explained in detail by Smith et al (1992) and Chalk and Ladha (1999).

H Natural abundance method

As a result of isotope discrimination effects occurring during soil formation, most soils have a slightly higher ^{15}N abundance than the atmosphere. As an outcome of this difference in ^{15}N abundance between soil and atmospheric N_2, nitrogen fixing plants have been found to have a lower ^{15}N enrichment than non-fixing plants, and this has been used to measure BNF (Amarger et al., 1979; Kohl et al., 1980).

In expressing the level of natural ^{15}N abundance the more sensitive expression of $\delta\ ^{15}$N % is often used (Vose et al., 1982)

$$\delta\ ^{15}N‰ = \left(\frac{^{15}N/^{14}N_{\ sample}}{^{15}N/^{14}N_{\ standard}} - 1\right) \times 1000 \qquad \text{(Equation 30)}$$

If N_2 fixing plants are grown in soil which has a higher $\delta\ ^{15}$N value than the atmosphere then % Ndfa can be calculated according to the following equation:

$$\%Ndfa = \left(1 - \frac{\delta\ ^{15}N‰_{fixingcrop} - \delta\ ^{15}N‰_{air}}{\delta\ ^{15}N‰_{\ non-fixing\ crop} - \delta\ ^{15}N‰_{air}}\right) \times 100 \qquad \text{(Equation 31)}$$

The main advantage of this method is that no tracer has to be applied. The method is therefore particularly useful for natural ecosystems, e.g. trees, for which it is very difficult to label the substrate. However, the main limitations are the rather small differences in ^{15}N abundance being traced requiring a highly precise isotope ratio mass spectrometer and the often small difference between ^{15}N abundance in soils and air.

2.7.4 BELOW GROUND NITROGEN

Most studies on BNF have concentrated on the above ground plant parts and they have largely ignored the below ground N. One might consider this a major limitation to the previous BNF work. However, it all depends what are the objectives. It is certainly not necessary to measure below ground N if one is screening for BNF potential in various crops or when the effect of various agronomic practices are being investigated. If one is only interested in the measurement of %Ndfa then sampling of below ground N is of no importance. The reason is that the atom % ^{15}N excess in the above and below ground plant parts are usually similar and the %Ndfa will be similar whether above ground material or both above and below ground material was used. For enhancing BNF in various grain legumes it is not necessary to include measurements of below ground N. However, if the objective is to measure total amount of N fixed or N balance then it is necessary to measure below ground N, both in the roots and in the substrate, if there is rhizodeposition occurring.

The work on below ground N has concentrated on N transfer from legumes to non-legumes. Several isotope techniques, have been used in these studies including the ^{15}N isotope dilution, $^{15}N_2$ labelling, split root ^{15}N labelling and leaf or stem ^{15}N feeding. Most of these methods have shown very little or no direct transfer of N from legumes to non-legumes when grown in mixed cropping systems. However, some studies have been able to quantify N rhizodeposition by legumes and the N transfer when the root system of a leguminous plant is decomposing, e.g. during or after cutting or stress.

The stem ^{15}N labelling technique of McNeill (1999) has been successfully used to label the root systems of leguminous plants (*Figure 2.7*) using two ml of 0.075M urea (~20 % ^{15}N atom excess) solution.

Figure 2.6. Experimental set-up for stem ^{15}N labelling.

Figure 2.7. ^{15}N stem labelling.

Few studies have concentrated on the below ground N but it is clear that it has to receive much more attention if N dynamic in cropping systems are to be fully understood. ^{15}N methodologies may become very useful in these studies.

2.8 MEASUREMENT OF SOIL N MINERALISATION USING ^{15}N TECHNIQUES

2.8.1 NET MINERALISATION

Mineralisation is the breakdown of soil organic matter into inorganic plant available forms (*Figure 2.8*) . During nitrogen mineralisation organic matter is broken down to ammonium by the soil macro and micro-organisms in a process termed ammonification. Ammonium can be further oxidised to nitrate (nitrification) by nitrifying bacteria. Both ammonium and nitrate can be immobilised by the microbial biomass and re-mineralized to ammonium.

2.8.2 MEASUREMENT OF NET MINERALISATION USING NON-ISOTOPIC METHODS

Using non-isotopic methods it is only possible to measure net mineralisation by mass balance as shown in Equation 32.

$$\text{Net mineralisation} = (\Delta A + \Delta N) + \Delta\text{plant} + \text{loss} \qquad \text{(Equation 32)}$$

However, if plant uptake and losses are zero then net mineralisation rates can be calculated using (Equation 33).

$$\text{Net mineralisation rate} = \frac{(A_t + N_t) - (A_0 + N_0)}{t} \qquad \text{(Equation 33)}$$

Where A is the ammonium N pool size and N is the size of the nitrate N pool, t=time after initial sampling which is denoted by 0.

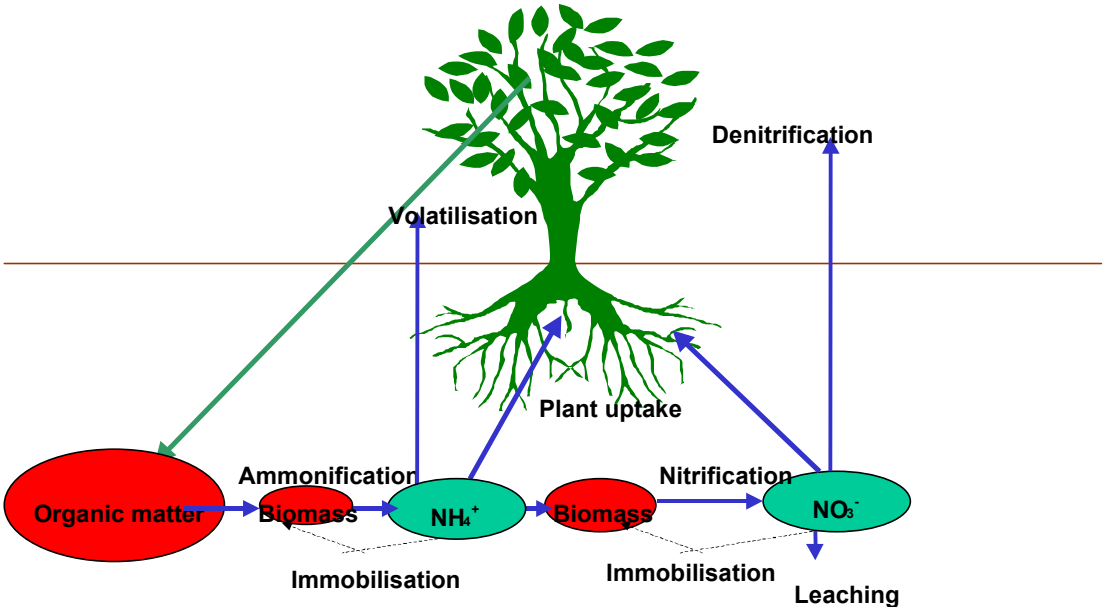


Figure 2.8. A schematic of the nitrogen cycle.

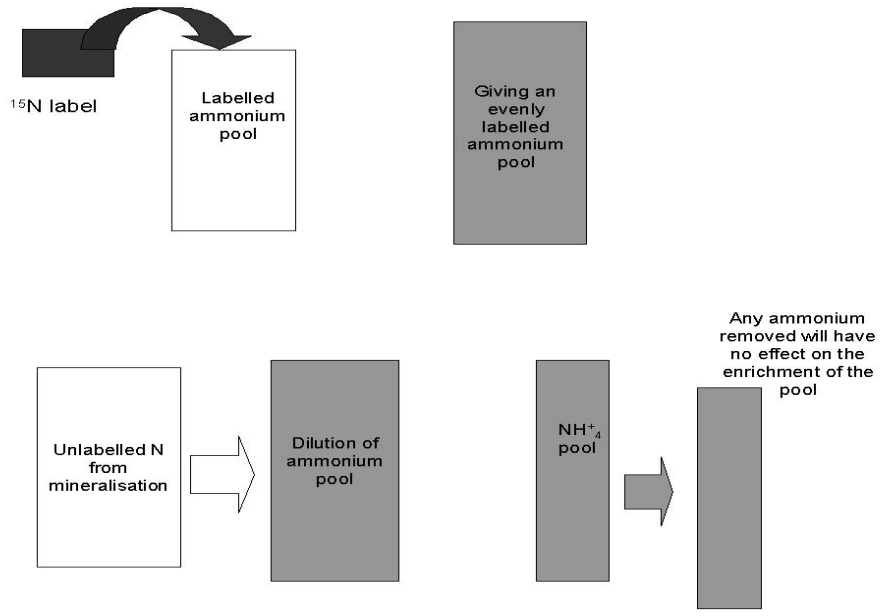
Various methods have been used to measure net mineralisation in soil. The simplest is to take soil samples over a set period and measure the change in ammonium and nitrate concentrations. This is adequate if you can account for the N taken up by the plants and N losses which is not necessarily easy to do. Therefore another approach is to eliminate the complication caused by plants and N losses, in laboratory incubations or field incubations. In field incubations soil cores are taken from the field, sub-sampled for analysis and then the remaining material is returned to the soil in a plastic bag at the same depth. These are re-sampled are measured after several days and net mineralisation rate calculated using Equation 33 (Rees et al., 1994.).

Other methods of mineralisation measurement include preparing soil cores using either plastic tubing or side perforated inverted aluminium soft drinks cans buried into the soil. These can be sealed at the top to preventsoil leaching losses. Cores are sampled sequentially throughout the growing season and inorganic N measured. In all methods, adequate replication is required to take account of spatial variability and it is recommended that measurements of net mineralisation are made over several days or weeks rather than hours. Another variation of this method is the use of anion and cation exchange resin bags at the bottom of the core to determine the leaching loss Hübner (1991).

2.8.3 GROSS MINERALISATION

Net mineralisation measurements are limited and yield little insight into the processes controlling N turnover in soils and ignore the interaction of plant roots in N turnover. Gross mineralisation measurements on the other hand allow us to determine the controlling parameters of organic matter breakdown irrespective of N uptake by plants, immobilisation or leaching. Gross mineralisation is the conversion of organic matter to ammonium and is determined using an isotope dilution technique. The main assumption is that when organic matter is broken down it is primarily converted to ammonium prior to nitrate (*Figure 2.8*) (Barraclough 1991; 1996, Kirkham and Bartholomew 1954).

The principle of the gross mineralisation measurement is that the ammonium pool is labelled with ^{15}N and the decline in the ^{15}N enrichment and change in the pool size are monitored. It is assumed that the ammonium pool is uniformly labelled and that any losses from the ammonium pool have the same ^{15}N abundance as the whole pool. That is, those processes removing ammonium from the ammonium pool will remove ^{15}N and ^{14}N in proportion to their presence in the pool and they will not in themselves alter the abundance of the pool.



Therefore, the incoming gross amount of N that is mineralized can be simply determined using isotope dilution and zero order kinetics (i.e. not dependant on the initial concentration). The rate of mineralisation can be calculated from Equation 34 assuming that the size of the ammonium pool is not constant. Conceptually it is simple to think of the ammonium pool as a glass containing water to which some dye is added (^{15}N label), the processes of mineralisation adds more unlabelled water (ammonium at natural abundance) causing a dilution effect.

$$m = \theta \frac{\mathrm{Log}(A_0^* / A_t^*)}{\mathrm{Log}(1 + \theta\, t/A_0)} \qquad \text{(Equation 34)}$$

Where is the size of the ammonium pool, usually expressed in mg N kg soil^{-1}, A* is the atom % ^{15}N excess in the ammonium pool, (t) time in days after (0) initial sampling, θ is the observed rate at which pool size changes, i.e. $(A_t - A_0)/t$, and m is the mineralisation rate.

2.8.4 MEASUREMENT OF GROSS NITRIFICATION

The process of nitrification is usually not assumed to be limiting. Indeed Jarvis et al., (1996) concluded that in temperate soils, with high pH it is unusual to find accumulations of ammonium, as the nitrification rate exceeded the mineralisation rate. In some agricultural soils the nitrification rate is so rapid it is often difficult to estimate gross mineralisation due to the rapid transition of ammonium to nitrate, with not enough N in the ammonium pool to make an analysis.

The pool dilution technique can also be used to determine gross nitrification. However in this case only the nitrate pool is labelled and the decline in the ^{15}N enrichment of the nitrate pool and in the pool size is monitored. The nitrate values are substituted for the ammonium values and thus Equation 34 becomes Equation 35 (Barraclough 1991; Hart et al., 1994; Stark and Hart 1997). Using this technique Stark and Hart (1997) showed that net nitrification rates significantly underestimated the turnover rates of forest soils.

$$\text{Nitrification rate} = \frac{\text{Log}(N_0^*/N_t^*)}{\text{Log}(1+\ t/N_0)} \qquad \text{(Equation 35)}$$

Where N is the size of the nitrate pool, N* is the atom % ^{15}N excess of the nitrate pool and θ is the observed rate at which pool size changes.

2.8.5 MIRROR IMAGE OR CROSS LABELLING METHODS

The principle of the mirror image or cross labelling method is that experiments are identical in all but the ^{15}N label. For example soils are labelled with ammonium nitrate at 5 atom % ^{15}N excess in the ammonium moiety, the mirror image being soil labelled with ammonium nitrate at 5 atom % ^{15}N excess in the nitrate moiety. This would allow simultaneous determination of mineralisation, nitrification and an assessment of re-mineralisation. These types of experiments can be very useful in determining the complexities of nitrogen cycling (Barraclough 1997). One of the problems with this method is that it can lead to an overestimate of the basal nitrification rate, as the ammonium pool is made artificially larger by the addition of label which drives the process of nitrification.

2.9 THE USE OF ISOTOPES IN ORGANIC RESIDUE STUDIES

2.9.1 ORGANIC RESIDUES, GREEN MANURES AND FERTILISERS

The use of organic residues as a source of nutrients is increasingly important in many parts of the humid tropics where inorganic N fertilisers have not proved to be economically viable. At a time when there is increasing concern about the decline in organic matter content in tropical soils, the use of organic residues may play a dual role as fertiliser and as a source of nutrients and organic matter (Figure 2.9). There are three soil quality-enhancing effects of the addition of residues recognised: i) direct nutrient supply; ii) the indirect effects of residues on the soil microclimate and iii) the effect of residues on soil structure.

It has been shown that crop N recovery from organic inputs such as plant residues or manures is often less than 20% (Haggar et al., 1993; Vanlauwe et al., 1996). However, it has been widely accepted that organic inputs play a significant role in the long-term build-up of soil organic matter and associated soil stabilisation. To maximise the potential N benefit of organic inputs it is necessary to be able to predict the amount of N supplied to the crop from the organic inputs. This demands an understanding of the factors controlling N supply and plant N uptake.

Figure 2.9. Organic residues being grown in the field.

The amount of N supplied to the crop from an organic input is dependent on the mineralisation of plant unavailable organic forms, to plant available inorganic forms of N, ammonium and nitrate. Mineralisation is a complex process dependent on many environmental factors (Azam et al., 1993; Muller, 1988) in addition to quality parameters of organic inputs such as N concentration, C:N ratio, lignin and polyphenol concentrations (Seligman et al.,1986; Thomas and Asakawa, 1993; Tian et al., 1992; Weeraratna, 1979).

The use of ^{15}N in organic fertiliser studies has significantly advanced our understanding of N release from organic materials. There are two main approaches to the use of ^{15}N in organic residue studies, the use of direct techniques, whereby plant residues or organic materials are labelled directly and the fate of the ^{15}N is traced, and indirect techniques in which the soil is labelled, and the dilution of ^{15}N in the crop receiving the residue is measured.

2.9.2 ^{15}N DIRECT LABELLING TECHNIQUES

A Crop residues or green manures

Crop residue or green manure studies using the direct method are relatively simple. Green manures can be easily obtained by growing crops fertilised with ^{15}N tracer, the above-ground or below ground material is then harvested and added as residue to unlabelled soil where the next crop is grown. This crop is then harvested and the percentage nitrogen in the crop derived from added residue calculated using Equation 36 (Hauck and Bremner 1976).

$$\%\text{Ndfr} = \frac{\text{atom }\%^{15}\text{N excess in the crop}}{\text{atom }\%^{15}\text{N excess in the residue added}} \times 100 \qquad \text{(Equation 36)}$$

The quantity of N derived from the residue can be calculated as follows

$$\text{Ndfr kg} = \frac{\%\text{Ndfr}}{100} \times \text{N in the crop} \qquad \text{(Equation 37)}$$

Percentage nitrogen derived from the applied residue can be calculated using Equation 37.

$$\frac{\text{Ndfr (kg)}}{\text{amount of N added as residue}} \times 100 = \%\,\text{N recovery} \qquad \text{(Equation 38)}$$

B Production of labelled material

Production of labelled plant material by application of ^{15}N fertiliser to soil or sand is the simplest way of labelling plant material, however some precautions should be taken. The plant material should be evenly labelled and the ^{15}N tracer should be applied throughout the growing period. Thus split or multiple applications of ^{15}N fertiliser to the soil are recommended. The target enrichment of the residue should be considered when designing such production systems. For most studies an ^{15}N enrichment of approximately 1 atom % ^{15}N excess is adequate. However, higher enrichments may be required if emission spectrometry is used to determine the isotope ratios of the crop, or if the material will be subsequently used as animal feed in manure experiments. In addition, a higher initial enrichment of the plant material is required when studying the release of residue N over a number of cropping seasons. When labelling nitrogen fixing crops the percentage nitrogen fixed should be accounted for to obtain the final target enrichment of the residue.

The production of labelled residues in the field is ideal, however it can prove to be expensive as much of the fertiliser will be immobilised by the soil microbial biomass or lost by leaching, etc. Labelled materials can also be produced by growing plants in sand, or other inert media, supplemented with low-N-^{15}N labelled nutrient solution, either in the greenhouse or in pots outside. The only problem with this approach is that it is often difficult to obtain material representative of field conditions. Efforts should be made to obtain material, which has similar characteristics to field residues.

The use of ^{15}N labelled residues in agronomic studies has enhanced our understanding of the N turnover,(Table 2.19). In addition to these studies labelled materials have been used to determine the parameters which control N release from mulches or green manures and this has led to a clearer understanding of residue characteristics which determine N release (Cadisch et al., 1998). In tropical tree species polyphenols have been identified as one of the main parameters controlling N release.

Table 2.19. Studies in which the ^{15}N direct technique has been used to measure Ndfr.

Following crop	Residue added	N added as residue	Ndfr %	Ndfr (amount)	Reference
Sorghum bicolor	*Acacia saligna* (Labill.)	94 kg/ ha	28	6±2 kg/ ha	Lehmann et al., (1999)
Wetland rice *Oryza sativa*	*Sesbania rostrata*	19.55 mg/pot	20.9	2.6 mg/ pot	Rahman and Parsons (1999)
Maize *Zea mays*	*Casuarina equisetifolia*	500 mg/pot	20.9	100 mg/ pot	N'Goran et al., (1998)
Maize *Zea mays*	Goundnut *Archis hypogaea* L Tainan 9	100 kg/ ha	8.3	9.6 kg/ ha	Mc Donagh et al., (1993)
Maize *Zea mays*	Goundnut *Archis hypogaea* LKK60-1	110 kg/ ha	9.3	8.7 kg/ ha	Mc Donagh et al., (1993)
Winter barley (first season)	Pea *Pisum sativum*	82.8 kg/ ha	15	10.9 kg/ ha	Jensen (1994)
Oil seed rape *Brassica napus oleifera*	Pea *Pisum sativum*	82.8 kg/ ha	13	11.4 kg/ ha	Jensen (1994)
Rhodes grass *Chloris gayana*	Siratro	391.5 mg/ pot	50.5	46.1 mg/ pot	Yaccob and Blair (1980)
Rhodes grass *Chloris gayana*	Soybean *Glycine max*	222 mg/ pot	19.8%	11.7 mg/ pot	Yacob and Blair (1980)

C Dual labelling of residues

Dual or multiple labelling of residues allows the study of mineralisation of different elements simultaneously. ^{13}C and ^{15}N dual labelling enables a more detailed understanding of soil mineralisation processes; there are some interesting studies using this technique (Broadbent and Nakashima 1974; Anger et al., 1997; Aita et al., 1997). Dual labelling can be achieved by either growing plants in ^{15}N labelled medium in an enclosed chamber continually supplied with $^{13}CO_2$, or pulse labelled with $^{13}CO_2$ Figure 2.10 (Aita et al., 1997). Care must be taken to ensure that the material is uniformly labelled. Although there has been some dual labelling with ^{14}C (Amato and Ladd 1980; Jawson 1989; Broadbent and Nakahima 1974), there appears to be few publications using other types of multiple labelling procedures for residue turnover studies.

Figure 2.10. Tent used for $^{13}CO_2$ labelling.

D Labelling of residues using tree injection

The tree injection technique is a relatively recent advance in ^{15}N methodology and has evolved due to the problems of labelling trees using the soil fertilisation approach. Tree injection is a relatively easy method to study N transformations and cycling in undisturbed tree–soil systems *Figure 2.11* and Figure 2.12 (Horwath et al., 1992). Labelled nitrogen is injected into the active xylem stream of a growing tree, followed by a period of equilibration of the label, after which the labelled leaf biomass is collected. This method allows large quantities of plant material to be labelled relatively inexpensively; it also allows studies of below-ground N input to be undertaken. Seiter and Horwath (1999) showed during the first season after harvesting of alder (*Alnus sinuta*) that 18% of the ^{15}N injected into the alder was taken up by the maize companion crop, 12% coming from the above ground fraction of the alder. They found that by the end of two growing seasons that 80% of the injected ^{15}N was in the soil fraction. However, both above-and below-ground components only supplied 3% of the N recovered in the maize crop.

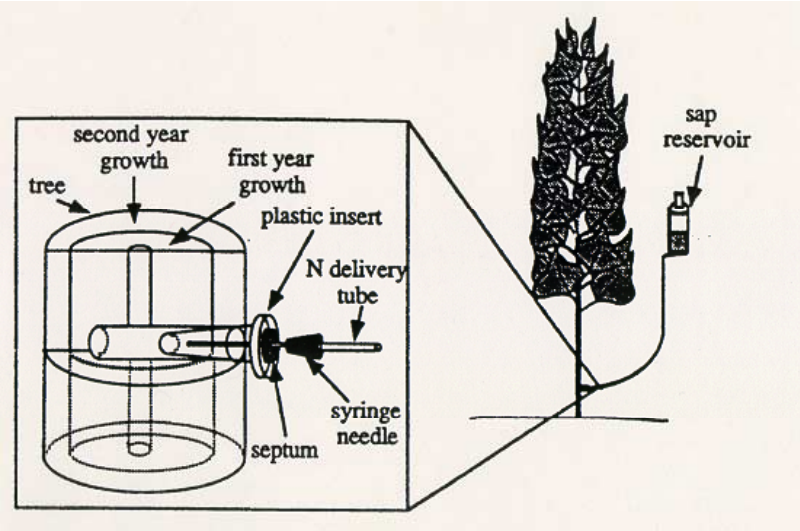


Figure 2.11. Tree injection principle.

Figure 2.12. Tree injection technique showing needle inserted into the stem.

E Leaf labelling or stem injection techniques for annual crops

Although there is substantial evidence of increased soil N availability following grain or pasture legumes quantifying the N benefit remains difficult. Non-isotopic methods based on N fertiliser equivalents do not take into consideration other benefits that legumes may confer within rotations (Chalk 1998). McNeill et al., (1998) and Russel et al., (1996) developed isotopic methods which allow direct *in situ* estimations of N benefit to the following crop to be measured using a leaf labelling or stem feeding techniques.

McNeill et al., (1998) showed that wheat following subterranean clover recovered 27 % of the labelled below-ground legume biomass N present at sowing, whereas wheat following serradella only recovered 13% of the labelled legume biomass present at sowing. On a per hectare basis the below ground N benefit to the wheat was equivalent to 31 kg N ha^{-1} or 80% of the grain N yield following the clover. Following seradella the N benefit to the wheat was 16 kg N ha^{-1} or 56 % of the N grain yield.

The potential of using these techniques to measure below ground N benefits has yet to be realised and it is envisaged that over the next few years this will become a major area of research.

F Labelling of animal manures

^{15}N techniques have been used to determine N release from organic residues such as animal manures. The principle is that plant material is labelled and this is fed to the animal and the manure collected. The manure is then applied to the crop and the nitrogen derived from the manure determined using Equation 39. The production of labelled manure is a complex and expensive operation. The manure must be evenly labelled both temporally and chemically. It is also recommended that urine and faeces be collected separately. This technique has been used to study plant N uptake from pig, goat, poultry and sheep manures (He et al., 1994; Kirchmann 1991; and Sørensen *et al.,* 1994 Sørensen and Jensen 1998). The advantage of this approach is that it allows direct measurements of plant N uptake and N loss by mass balance and allows management strategies to be investigated.

% Nitrogen in the crop derived from the manure = Ndfm

$$\%\text{Ndfm} = \frac{\text{atom }\%^{15}\text{N excess in the crop}}{\text{atom }\%^{15}\text{N excess in the manure added}} \times 100 \qquad \text{(Equation 39)}$$

Another approach is to label manure or urine by spiking with ^{15}N. The manure or urine is spiked with ^{15}N fertiliser following collection, urine is normally spiked with urea. Stockdale and Rees (1995) attempted this approach by labelling a variety of manures followed by anaerobic incubation, however they concluded that there was uneven distribution of the label in the manure, which led to difficulty in interpreting the results. Labelling of urine appears to be more successful, Bronson et al., (1999) labelled sheep manure and showed that one third of the urine N was lost as ammonia gas on application to a sandy soil in an Australian summer.

2.9.3 INDIRECT TECHNIQUES

Indirect techniques have been used to study plant N uptake from organic residues. ^{15}N tracer is added to the soil and treatments with and without residues (no-residue controls) are set up. The no residue controls will have an ^{15}N enrichment that reflects the soil ^{15}N pool and the residue treatments should have a lower ^{15}N enrichment due to the input of the unlabelled N coming from the decomposingresidue. This is the same principle as the ^{15}N dilution method for estimating biological nitrogen fixation. Nitrogen derived from residue (Ndfr) is calculated using Equation 40. In practice it has been shown that if ^{15}N label and residues are applied at the same time this causes errors associated with pool substitution (Hood *et al.,* 1999). Thus it is recommended that the soil be pre-labelled with ^{15}N and left to equilibrate for up to 6 months prior to the application of residues.

$$\%\text{Ndfr} = \left(1 - \frac{\text{atom }\%\ ^{15}\text{N excess}_{\text{residue treatment}}}{\text{atom }\%\ ^{15}\text{N excess}_{\text{no residue control}}}\right) \times 100 \qquad \text{(Equation 40)}$$

Soil pre-labelling can be achieved by adding a carbon source and ^{15}N fertiliser simultaneously to soil. This technique has been tested and yielded good results in the greenhouse and compares well with the direct technique (Hood et al., 2000). However care must be taken to ensure the correct C:N ratio of material is added as the pre-label and that the pre-labelling takes account of the inorganic N initially present in the soil. The ideal scenario is that all the inorganic N is immobilized and that it is fully incorporated into the soil microbial biomass. Only when the soil has returned to the initial levels of inorganic N concentration is the second phase of the experiment initiated where residues or manures are added. Experiments carried out suggested that a C:N ratio of 1:24 is approximately the correct ratio for short term experiments (Hood et al., 2000). Field studies showed that due to problems of uniformly labelling the soil profile this method of pre-labelling was not ideal. An alternative pre-labelling method was tested in which the ^{15}N fertiliser was applied to the crop proceeding the one that received the residues. This allowed the fertiliser to be taken up by the initial crop and the ^{15}N to be distributed throughout the rooting zone. The above ground component was then removed and then left to equilibrate over winter. After this period the second phase of the experiment was initiated in which the residues were added. This method was tested against the direct method in the field and yielded promising results.

2.10 ^{15}N AS A TOOL IN N LOSS STUDIES

2.10.1 INTRODUCTION

Efficient use of nitrogen by crops requires an understanding of the loss pathways and crop demands for N throughout the growing season. One of the areas in which stable isotope techniques have been extremely useful, is in the assessment of N losses from agricultural systems. There are three major pathways of N losses: nitrate leaching, denitrification and ammonia volatilisation (*Figure 2.13*).

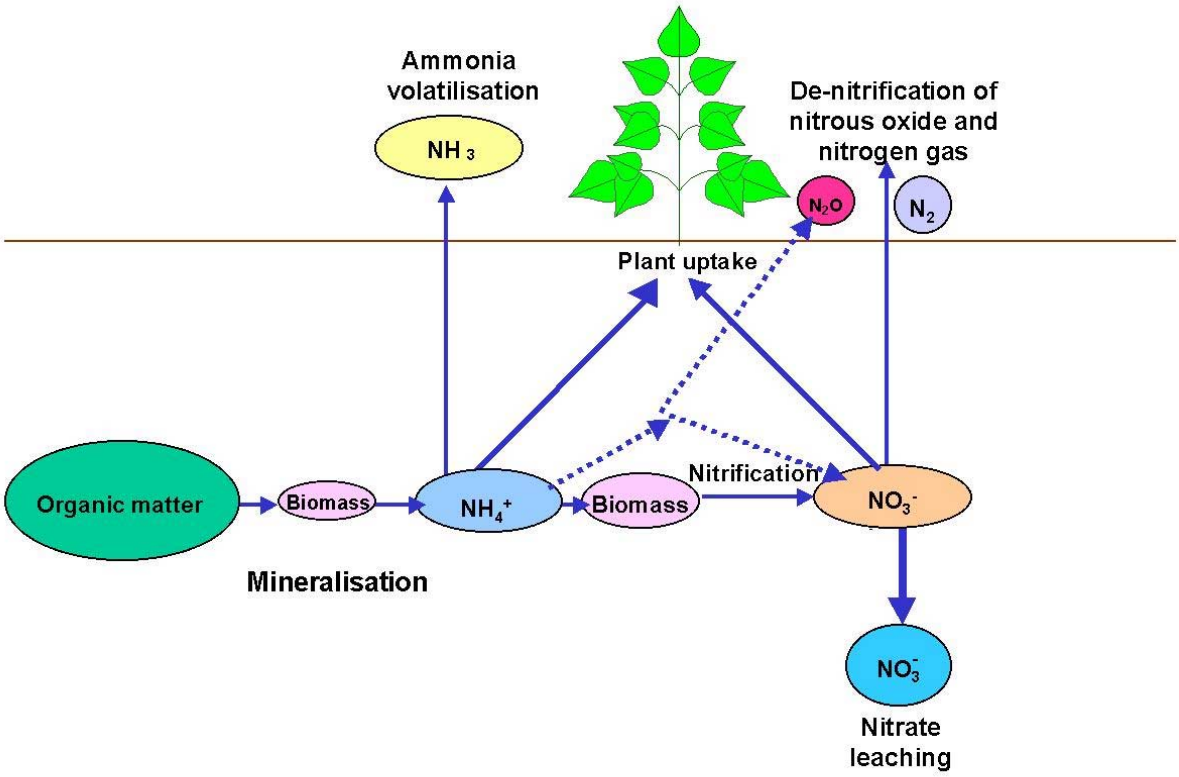


Figure 2.13. Pathways of N loss.

One of the simplest ways to determine loss of labelled N from a system is to carry out a mass balance of the ^{15}N fertiliser applied. However, care must be taken to sample every available N pool and minimise N losses during the sample preparation procedures, so that all possible N can be accounted for.

2.10.2 LEACHING

Efficient use of soil N requires minimal losses by leaching. Not only is nitrate leaching a loss of a valuable resource it is also environmentally detrimental. Nitrate can contaminate ground water and causes accelerated soil acidification. The WHO safety limit for nitrate in drinking water is 11.3 mg NO_3 –N/L.

Nitrogen is usually leached in the form of nitrate, as ammonium is more strongly bound to the cation exchange sites. In soils with net negative charge nitrate moves freely with the water. Two factors determine the rate of leaching: 1. The quantity of water passing across the surface of interest, 2. The concentration of nitrate in the water. Rainfall intensity and surface soil conditions determine the rate at which the water enters the soil. The soil structure and size and shape of the soil pores determine the rate and distance that the water moves through the soil and thus the rate of nitrate leaching. In heavier textured soils water moves through cracks and macropores, and the rate of movement through the soil aggregates may be very different than through the pores.

Understanding these complex processes has been aided by computer models as discussed in Chapter 6. However models require validation and isotopic techniques have been extremely useful in this regard.

By labelling the soil with ^{15}N-labelled nitrate or urea it is possible to trace the fate of fertiliser-derived nitrate down the soil profile. This can be achieved by taking sequential soil cores or by using suction cups, tensionic samplers, mini lysimeters and other techniques to sample the nitrate in the soil water (Figure 2.14). Often the nitrogen determined in soil sampling is expressed as g/g soil while nitrate in the soil solution is g/mL. It is therefore important that the correct unit is used when calculating nitrate leaching.

Nitrate in the soil water can be determined by a variety of analytical techniques described later in the manual. The ^{15}N abundance of the nitrate samples can be determined by mass spectrometry or emission spectrometry following sample preparation using diffusion or distillation techniques (Moutonnet and Fardeau 1997). This is a relatively simple procedure and the percentage of fertiliser is leached over the growing season can be determined.

One of the main advantages of the ^{15}N technique over the traditional chloride or bromide non-reactive tracer techniques is that it accounts for nitrogen transformations during the growing season whether they are through plant uptake, immobilisation, volatilisation losses etc. Another advantage is that it allows the impact of fertiliser as distinct from indigenous soil N to be determined allowing better nutrient management strategies to be developed.

There have been numerous studies using these techniques showing that % loss of fertiliser N applied is dependent on a variety of edaphic and environmental factors. In the study of Barraclough et al., (1984) it was shown that fertiliser N leached ranged from 0.2 -18 % of applied N, increasing with rate of fertiliser applied.

Figure 2.14. Set-up used for suction cups.

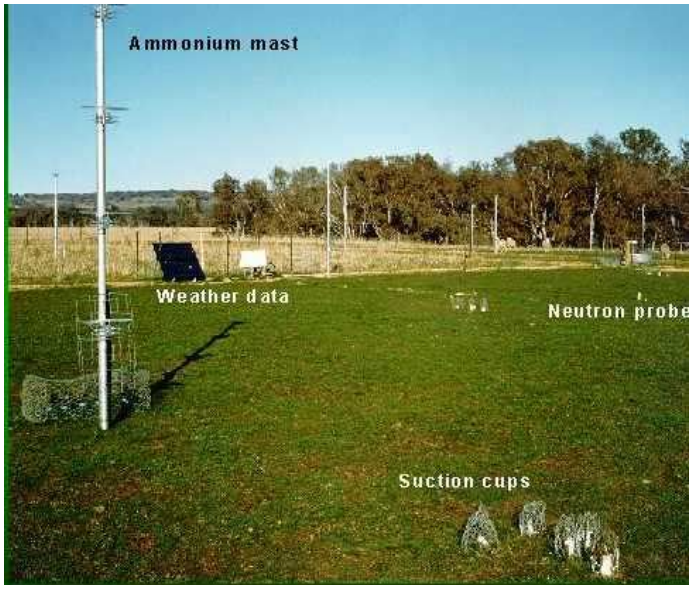
2.10.3 AMMONIA VOLATILISATION

Volatilisation is the loss of ammonia from soil to the air, usually under alkaline conditions. The factors which control ammonia volatilisation include, fertiliser form, method of application, soil pH, soil water content, soil cation exchange capacity, wind speed, air temperature, crop and stage of crop growth (Freney et al., 1983). Isotopic techniques have been used to determine the percentage of ammonia volatilised that is derived from the labelled fertiliser or urine applied therefore allowing the primary source of the ammonium to be identified. Micro meteorological techniques are probably the best way to determine the amount of ammonia volatilisation (Figure 2.15). Using the two techniques in parallel has yielded a better overall understanding of ammonia losses from agricultural systems.

The two main agricultural systems in which volatilisation losses are a problem are wetland rice and fertilised grazing systems. By labelling urine with ^{15}N labelled urea, or labelling the fertiliser applied, it is possible to trace the fate of urine N in addition to fertiliser N in grazing systems. Losses of nitrogen from urine affected and unaffected soils were found by Black et al., (1984) to be 27 and 7% of the fertiliser N applied, respectively. Using the ^{15}N balance method in a sheep grazing system Bronson et al. (1999) showed that one third of urine applied N can be lost as ammonia during the first three days. Monaghan and Barraclough (1993) concluded from ^{15}N studies that these large losses in urine N were probably due to increased soil water-soluble carbon, resulting from urine addition.

In irrigated wetland rice, ammonia volatilisation is a major N loss pathway from applied fertiliser. Work undertaken by Fillery and de Datta (1986) showed that an indirect estimate of ammonia losses based on the rate of ^{15}N loss from nitrapyrin and urea treatments (33% of applied N) was similar to total N loss computed with micro-meteorological techniques (36% of applied N). Again one of the main advantages of the ^{15}N technique is that it allows assessment of the impact of fertiliser N, as distinct from native ammonia losses.

Sampling head space gas from a chamber for denitrification experiments



Micrometerological tower used for ammonia volatilisation experiments

Figure 2.15 Equipment used for measuring gaseous N losses.

2.10.4 DENITRIFICATION

Denitrification is the process by which soil nitrogen is reduced to N_2 gas or the gaseous oxides of nitrogen. Estimates of N losses by denitrification vary from 3-62 % of applied N in arable soils and loss is controlled by three primary factors oxygen, nitrate and carbon. Denitrification is an anaerobic process, however there is evidence to suggest that there are sufficient anaerobic micro-sites in soils for denitrifcation to occur even in dryer soils (Smith and Arah 1992). The losses of N_2 by denitrification represent a loss of fertiliser and losses of N_2O are an environmental problem. Denitrification losses of N_2 and N_2O have been determined using isotopic techniques and are reviewed by Smith (1987). The advantage of these techniques is that they are direct and do not require the use of inhibitors that may interfere with other soil processes.

A Measuring losses of N_2

One of the major problems with measuring N_2 losses is the extremely high background of N_2 in the atmosphere. However techniques have been developed using the relative abundance of the ^{14}N and ^{15}N in the N_2. N_2 losses can be determined by labelling the soil with highly enriched ^{15}N nitrate and then determining the abundance of the N_2 over time in the head space of a closed chamber

above the labelled plot (Hauck and Bouldin 1961; Mulvaney and Boast, 1986; Arah 1992). If it is assumed that the initial N_2 is in equilibrium and that the source of the N_2 is from a single pool, the loss of N_2 can be calculated; however a triple collector mass spectrometer is required to determine the molecular fraction of the $^{30}N_2$. Modifications of the equations for a dual collector mass spectrometer are detailed by Arah (1992) and Mulvaney and Boast (1986) and are beyond the scope of this Chapter.

The proportion of N_2 derived from denitrification (d) is given by equation 41 where the a_a equals the atom fraction of the initial gas (i.e. atmosphere), a_m is the atom fraction of the mixture (i.e. gas collected in the chamber), and X_m is the molecular fraction of the $^{30}N_2$. Atom fraction is the atom % ^{15}N excess divided by 100.

$$d = \frac{(a_m - a_a)^2}{(X_m - a_a^2 - 2a_a a_m)} \qquad \text{(Equation 41)}$$

This technique offers the potential to study N_2 losses directly, however it may have been under utilised due to the complex mathematics presented in the literature. There may also be additional problems with gas entrapment which can affect estimates of denitrification when studying flooded systems (Chen et al., 1998)

B Nitrous oxide emissions

Nitrous oxide (N_2O) is a greenhouse gas that also contributes to stratospheric ozone depletion (Granli and Bøckman, 1994). Its increasing concentration (0.25% yr^{-1}) in the atmosphere has caused serious environmental concern. Nitrous oxide is present in relatively low concentrations about 300 ppb (v/v) and is 310 times more radiatively active than CO_2 and has a life time of 150 years. Soils are thought to be responsible for approximately half the annual N_2O load on the atmosphere (Bouwman, 1990). The methods for measuring nitrous oxide emissions from soils are discussed by Smith and Arah (1992) and IAEA (1992). The principle sources of N_2O in the soil are the microbial processes, nitrification and denitrification. Until the 1970's denitrification was thought to be the only important source of N_2O; however, it was demonstrated that nitrification could also be a significant source (Bremner and Blackmer 1978; 1980; 1981).

Using ^{15}N tracers it is possible to apportion N_2O fluxes between nitrification and denitrification using a greenhouse gas preparation system linked to the mass spectrometer. This is a relatively simple procedure using a two source mixing model and is described in more detail by Stevens et al.(1997) and Arah (1997). ^{15}N nitrate fertiliser is applied to the soil and left for 24 h to equilibrate. The ^{15}N abundance of the N_2O (a_m) of the head-space of a closed chamber (closed for approximately one hour) above the labelled soil is determined. Simultaneously the ^{15}N abundance of the inorganic nitrate and ammonium pool is sampled. It is assumed that N_2O, which is derived from the ammonium pool of ^{15}N atom fraction (a_n), is the product of nitrification and that the N_2O which is derived from the nitrate pool of ^{15}N atom fraction (a_d), comes from denitrification. This may not be strictly true, but the intermediate pools will have a similar ^{15}N abundance to the main pools, and therefor the hypothesis should hold true. Thus the proportion of N_2O derived from denitrification (d) can be calculated using Equation 22:

$$d = \frac{(a_m - a_n)}{(a_d - a_n)} \qquad \text{(Equation 42)}$$

The model assumes that the ^{15}N from the ammonium and nitrate pools are typical of the nitrous oxide producing pools. In a field situation care must be taken to distribute the label evenly down the soil profile ensuring that the N_2O emissions from the unlabelled sections of the soil do not dilute the N_2O pool and thus invalidate the equations. Fortunately it is also recommended that the label be applied in the form of nitrate which makes uniform labelling easier.

This technique was used to demonstrate that N_2O emissions from nitrification were significantly correlated with nitrification rate and that N_2O from denitrification increased with increasing soil moisture content (Stevens et al., 1997).

2.11 THE NEUTRON PROBE FOR WATER MEASUREMENTS

2.11.1 INTRODUCTION

Soil water is critical for crop production and yields are often more closely related to soil water than to any other soil or meteorological variables. Various methods of measuring soil water are available, including time-domain reflectometry (TDR) and frequency-domain reflectometry (or capacitance sensors) as well as the neutron scattering/moderation methods. Soil water measurement based on neutron scattering has been a valuable tool for the past 50 years because it possesses many qualities such as simplicity, reliability, repeatability, cost-effectiveness, and the method is non-destructive.

Research on soil water and irrigation often depends on the determination of actual soil moisture at many different experimental sites, at different depths in the soil, and with different irrigation and other treatments. Before the development of the soil moisture neutron probe (SMNP) such measurements were laborious and time consuming. The development of SMNP has enhanced our ability to do research in this area and made it much easier to measure soil water in the field.

2.11.2 PRINCIPLES OF OPERATION

The neutron method of measuring soil water content uses the principle of neutron thermalization. Hydrogen nuclei have a marked propensity for scattering and slowing neutrons. During operation, high-energy neutrons emitted from a radioactive substance, such as radium-beryllium or americium-beryllium, slow down by elastic collisions with atomic nuclei (thermalization). The energy of the neutrons is reduced to about the thermal energy of atoms in a substance at room temperature. Hydrogen, which has a nucleus of about the same size and mass as the neutron, has a much greater thermalizing effect on fast neutrons than any other element. Subsequently, water has a marked effect on slowing or thermalizing neutrons. Thermal neutron density is easily measured with a detector, and may be calibrated against water concentration on a volume basis.

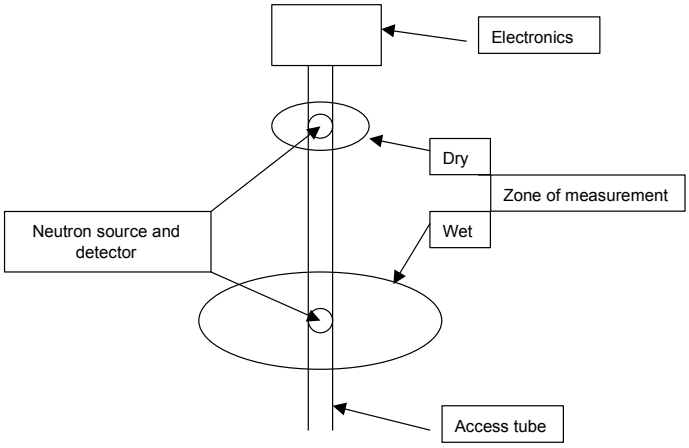


Figure 2.16. Diagrammatic of a neutron probe in an access tube showing the effect of soil moisture on the zone of measurement.

The neutron probe (*Figure 2.16*) consists of a source of fast neutrons centered on a tube-counter of slow neutrons. The sealed source contains a mixture of Americium 241, emitting alpha particle, and Beryllium acting as a target. The slow neutron detector is usually filled with boron trifluoride (BF_3 enriched in ^{10}B) or helium-3. The fast neutron source and the slow neutron detector are both contained in the probe carrier.

When alpha particles bombard beryllium nuclei, the following reaction takes place:

$$ {}^{4}_{2}\alpha + {}^{9}_{4}Be \rightarrow {}^{1}_{0}n + {}^{12}_{6}C \qquad \text{(Equation 43)} $$

The neutrons, which are products of the above reaction, have an average value of about 4.5 MeV. Because of its high-energy, the source is protected in a shield. The strength of the sources is generally expressed by the activity of the alpha emitter, in milli Curie (mCi) or in Becquerel (Bq). Typical sources have activities in the range 370 to 1,850 MBq (i.e.10 to 50 mCi).

During measurement the source is lowered into the soil inside an access tube. Collisions of fast neutrons produce slow neutrons; this process occurs most rapidly when neutrons collide with hydrogen nuclei because their masses are almost equal. The number of thermal neutrons registered by the detector per unit time (the count rate) is a measure of the volumetric concentration of hydrogen nuclei (and therefore the water content) in the soil around the probe.

The shape of the soil measured by the neutron probe is approximately a sphere. The radius of measurement is a function of moisture content, decreasing with increasing moisture. 95% of the neutrons counted by the detector vary from 0.1 m to 0.2 m in wet soil to 0.8 m or more in dry (sandy) soil.

2.11.3 INSTALLATION OF ACCESS TUBES

A wide range of materials, including aluminium, steel and PVC can be used for neutron probes access tubes. However, thin wall aluminium is recommended because it is most permeable to neutrons and does not generally corrode in soils. Steel tubing and PVC contain iron, chlorine and hydrogen and these elements affect the sensitivity due to absorption of neutrons. Once a particular tubing material is chosen, calibration and all experimental work must be done with the same material.

The inside diameter of access tubes should be chosen according to the outside diameter of the neutron probe. The outside diameter of access tubes determines the size of the soil auger used for their installation. Tube length depends on the depth to which measurements are to be made and should be at least 10 to 20 cm longer than the maximum measurement depth to allow the “active center” of the probe to be placed at the desired depth.

Access tubes should also extend 20 to 40 cm above soil surface to allow the positioning of the shield case on top of the tube. The top of the tube should be covered with a rubber stopper or inverted aluminium can to avoid entry of water or debris. The bottom end should also be sealed to exclude water entry. The tubes should be checked to make sure that they are free of water before measurements are made.

The installation of the access tube requires making a hole to the desired depth and of sufficient diameter to closely accommodate the access tube. A hand operated soil auger should be used, as motor powered mechanical augers may cause drastic disturbance of the soil structure causing soil compaction, churning with caught-up stones, and over-sizing. After making the hole, the access tube should be slowly driven in. Care must be taken to ensure close contact between soil and tube as erroneous results can occur due to air gaps, which can also allow water run down the side of the tube.

The access tube should be installed at locations representative of the immediate surroundings and care should be taken to avoid surface water from concentrating near the tube. A platform should be used to prevent damage to vegetation and soil compaction (radial and below) when installing a tube.

The following equipment is needed for a good installation of access tubes:

- Aluminium plate
- Guiding –tube made of steel with a cutting edge
- Hand auger
- Tube extractor
- Access tubes.

Access tubes and their installation are discussed in detail by Eeles (1969) and Greacen (1981).

2.11.4 CALIBRATION

The neutron probe only measures slow neutrons. As it is difficult to describe the interactions between slow neutrons and soils, a calibration curve is needed to convert the neutron counts to volumetric soil water content. The relationship between counts per minute/second and the percent moisture by volume is used. For a linear equation calibration:

$$\theta = a + b(\frac{R}{R_s}) \qquad \text{(Equation 44)}$$

where θ is the volumetric water content (cm^3/cm^3), *a* is the intercept, *b* is the slope, *R* is the actual count rate and R_s is the standard count rate, measured in the shield. R/R_s is the count ratio.

The volumetric soil water content θ is normally evaluated indirectly as the product of soil water content, on a weight basis, and the dry soil bulk density.

STEPS NECESSARY TO PRODUCE A NEUTRON PROBE CALIBRATION (EVETT, 2000)

- Make sure there is a wide range in the water content data by having both dry and wet sites. Wet sites can be created by flooding. As soon as the profile is wetted to the desired depth allow it to drain to 'field capacity'.
- Ensure adequate numbers of samples by installing at least 3 access tubes in both the wet and dry sites, and by taking 4 samples around each tube at each depth measured with the neutron probe. This should give enough samples so that calibration equations can be broken into soil layers or horizons and the slopes of the calibration lines can be reliably compared between horizons. The top 10 cm depth always requires a separate calibration equation due to loss of neutrons to the atmosphere.
- Ensure that samples are in as good a condition as possible. Check for compression by observing the surface and height of soil in the cores. Likewise, see if the sample is shattered, which would result in bulk density being too low for that sample. Samples not fulfilling these criteria should be discarded on the spot and replaced by new ones. With 4 samples per depth per tube, outliers can be discarded later, and there will still be enough samples to give good average water content at each depth.
- Ensure that the probe is at the correct depth for each reading. Stands can be built that slide over the access tubes and keep the gauges a constant height above the soil surface (*Figure 2.17*). Cable stops can be set to give the desired depths of measurement.
- Ensure that standard counts in the shield are not influenced by soil water content. Standard counts can vary depending on whether the soil was wet from heavy rain or dry. Standard counts have also been shown to vary with temperature. Lower standard counts are normally observed with higher temperatures. A change of 30^oC has been shown to give a change in measured water content of 0.006 m^3/ m^3.

2.11.5 FACTORS AFFECTING NEUTRON PROBE MEASUREMENTS

- Soil water content measured by direct soil sampling does not necessarily represent the soil water content within the sphere of influence of a neutron probe.
- The field calibration relationships are influenced by soil horizons, which differ in chemical composition and soil bulk density.
- Bound water in clay minerals and soil organic matter.
- Soil compaction.
- Calibration in gravelly soil.
- Soil cracking.

Instructions for the operation of SMNP and field calibration methods are described in several publications including Gardner (1986), Greacen (1981) and Bacchi et al. (2001).

Figure 2.17. A CPN model 503DR neutron probe mounted on a stand, which has been placed over an access tube. The feet of the stand are designed to fit between plants in a row, yet provide enough surface area to not sink into the soil. The protrusion of the access tube above the soil surface prevents the stand from falling over (Evett, 2000).

2.11.6 NUMBER OF MEASURING SITES

The number of sites needed to achieve a given accuracy can be calculated, if the standard deviation of estimated water storage is known:

$$N = t^2 s^2 / D^2 \qquad \text{(Equation 45)}$$

where *N* is the number of measuring sites, *t* is the tabulated value of *t* for the probability level *p*, *s* is the variance of the measurements and *D* is the specified deviation from the true mean which is to be estimated.

For 95% probability, using an approximate *t* value of 2.0, for *s* = 10 mm and *D* = +5 mm

N = 4 x 100/25 = 16 sites.

In studies that compare different treatments, the number of measuring sites needed in each treatment, to achieve a specified least significance difference (*LSD*) between treatments, is given by:

$$N = 2t^2 s^2 / (LSD)^2 \qquad \text{(Equation 46)}$$

where *LSD* is the 5% least significant difference.

For example, if *s* = 10 mm, to achieve a 5% LSD of 10 mm, one would need

N = 2 x 4 x 100/100 = 8 measuring sites

2.11.7 ADVANTAGES

- Simplicity, repeatability, dependability and reliability.
- Equipment is relatively portable.
- Fast, economical, non-destructive way of monitoring soil water.
- Spatial (depth) and temporal soil moisture changes can be monitored.
- Relatively easy to install.
- A large volume of soil is measured, providing more accurate measurements of soil moisture than most other methods in large-scale studies.

2.11.8 DISADVANTAGES

- Escape of neutrons to the atmosphere at shallow depths (<15 cm) can result in underestimated soil moisture unless a special calibration curve is established for these depths.

- The large sphere of influence can render small-scale moisture measurements inaccurate, e.g. in the presence of abrupt moisture changes or in layered soil.
- Health risks, since it contains a radioactive source.
- Licensing, training and safety regulations pertaining to radioactive sources make their use expensive and do not allow operations such as unattended monitoring. Only trained personnel are allowed to operate the equipment.
- Transport, storage and disposal of gauges are also becoming expensive.

2.11.9 APPLICATIONS

The amount of soil water stored between two depths L_1 and L_2 at any time t is given as:

$$S_{L_2-L_1}(t) = \int_{L_1}^{L_2} \theta(z,t)dz \qquad \text{(Equation 47)}$$

where *S* is the equivalent depth of soil water stored measured in cm, θ is volumetric water content ($cm^3\ cm^{-3}$) and *z* is vertical position coordinate (cm), positive downwards from the soil surface.

When L_1 = 0, i.e. from the soil surface, the integration is then made over the entire soil profile to depth L_2.

Equation (47) can be written as:

$$S_{L_2-L_1}(t) = \overline{\theta}(L_2 - L_1) \qquad \text{(Equation 48)}$$

where $\overline{\theta}$ is the average soil water content in the interval L_1-L_2.

Example 1

Given the count ratio and soil water contents in Table 2.20, calculate the soil water storage between depths.

Table 2.20. Count ratios and soil water contents as a function of soil depth for a cereal crop.

Depth (cm)	Count Ratio (CR)	Soil water content ($cm^3\ cm^{-3}$)
25	0.494	0.420
50	0.485	0.410
75	0.503	0.429
100	0.473	0.398
125	0.465	0.389
150	0.471	0.396

Using equations (5) and (6), the soil water storage between various depth intervals can be calculated:

$$S_{0-150} = 0.407(150-0) = 61.1\,cm$$

$$S_{50-100} = 0.412(100-50) = 20.6\,cm$$

where 0.407 and 0.412 are the average moisture contents between intervals 0-150 cm and 50-100 cm, respectively.

Example 2

The soil water balance is a computation of water gains and losses of a given agro-ecosystem during a specified time interval (Δt) for a thickness *L* of soil. The soil water balance can be written as:

$$P + I - E - R - D = \Delta S \qquad \text{(Equation 49)}$$

where *P* is precipitation, *I* is irrigation water, *E* is evapotranspiration, *R* is runoff and *D* is deep drainage. ΔS is the change in the amount of water stored in the soil profile during interval Δt. All terms are expressed in mm of water.

The rainfall over a 10-day period was 15 mm, a farmer irrigated his crop with 10 mm of water. Assuming deep drainage was 2 mm and the water in the soil profile decreased 5 mm, what was the average daily evapotranspiration rate of the crop?

Using equation (49)

$$P + I - E - R - D = \Delta S_L = 15 + 20 - E - 0 - 2 = -5$$

Answer: With E = 38 mm during the 10-day period, the E rate was 3.8 mm/ day

Example 3

The initial soil water content θ_i under a crop with roots distributed within the profile to a depth of 80 cm is given in Table 2.21, calculate the net irrigation depth if it is to irrigate to field capacity θ_{FC}.

Table 2.21. Initial and field capacity water contents of the soil

Soil Layer	Depth (cm)	θ_{FC}	θ_i
1	0-20	0.30	0.18
2	20-50	0.28	0.19
3	50-100	0.27	0.19

θ_{FC} volumetric soil water content at field capacity

θ_i initial volumetric water content of the soil

Because the soil is stratified, the net irrigation depth is calculated separately for each layer according to equation (49):

For layer 1, I_1 = (0.30 - 0.18) x 20 = 2.4 cm

For layer 2, I_2 = (0.28 – 0.19) x 30 = 2.7 cm

For layer 3, I_3 = (0.27 – 0.19) x 30 = 2.4 cm

Therefore the total net irrigation depth for the rooting depth of 80 cm is 2.4 + 2.7 + 2.4 = 7.5 cm.

2.11.10 SAFETY

Exposure to radiation, including neutrons, especially at high doses, can cause detrimental health effects. In order to achieve operational radiation safety protection and safety standards have to be followed. The design and manufacture of commercially available neutron moisture gauges are such that risks to the health of the user have been greatly reduced. The major concern is radiation escape from the soil during measurement, especially in dry conditions and when the radius of influence is large. With appropriate work practices, recorded occupational doses have been well below recommended annual limits.

Rules for utilisation of radioactive equipment vary with each country but the general instructions are as follows:

- The operator of the neutron probe should wear a radiation detection badge that is a dose measurement device, every time the neutron probe is used or moved from one location to another. The badge can be obtained from your national Atomic Energy Commission or Authority through the designated nuclear licensing and regulatory section and must be developed every month.
- If the operator has any doubt about use of the neutron probe, call the Safety Office or the Person in Charge of Security in the Institute.
- The probe should have a wipe test every year to check for leakage.

- Transport requires securing the neutron probe in the trunk or boot of a car or in the back of a pickup or utility using a tie strap. A transport certification must be obtained. A wipe test must also be conducted.
- When used properly neutron gauges pose minimal or negligible health and safety risks (Oresegun, 2000).

A detailed discussion on the various aspects of the soil moisture neutron probe (SMNP) can be found in (IAEA, 2000)

2.12 EXPERIMENTAL DESIGN, ANALYSIS OF VARIANCE (ANOVA), LINEAR CORRELATION AND REGRESSION

2.12.1 INTRODUCTION

The purpose of this is section to demonstrate the need to ask the right questions, to adopt the appropriate design and to interpret statistical analyses correctly. Data should be carefully checked before undertaking statistical analysis.

2.12.2 EXPERIMENTAL DESIGN

A design consists of two basic structures: the treatment structure (which is defined by the factors and their level combinations, called treatments) and the design structure (which is specified by the random assignment of treatments from the treatment structure of the experimental units). Constructing an experimental design means designating the number of experimental unit and the order of applying treatments to experimental units.

It is critical that the appropriate experimental design is used. Prior knowledge about the source of variation in the glasshouse or in the field site is of considerable advantage. If there is a gradient of light and/or temperature in one direction across a glasshouse, or a fertility gradient down the slope in a field site, then the treatments must be blocked across that gradient so that the differences between blocks is accounted for in the block effect in the ANOVA and not confounded with the treatment effect. If a gradient exists in two directions at the experimental site then this can be accounted for by using a latin square design.

The number of factors to be investigated in an experiment will determine if a one way or two way ANOVA is used.

In some situations it may be preferable to consider many levels of application of one or more factors rather than a few levels replicated several times. In this instance regression analysis would be appropriate.

2.12.3 ANOVA

When you wish to compare more than two treatments or sample means, a null hypothesis is a useful technique to make decisions about hypotheses.

Null hypothesis H_0: A=B
Alternative hypothesis H_1: A≠B

ANOVA - statistical technique for analyzing observations depends on the simultaneous operation of a number of effects. The total variance, expressed as the sum of squares of deviation from the grand mean, is partitioned into components corresponding to the various sources of variation in the data. The goal is to estimate the magnitude of the effects and their interactions, and to decide which ones are significant. In most experimental situations where isotopes are used a 5% level of probability is used.

A summary ANOVA table is presented in Table 2.22.

Table 2.22. Summary table of analysis of variance

SOURCE OF VARIATION	Term	Number of degrees of freedom	Sum of squares	Mean square	Observed F	Required F for significance	
		Df	**SS**	**MS**	**F Exp**	**P=0.05**	**P=0.01**
Replicates	R	r-1					
Factor A	A	i-1					
Factor B	B	j-1					
Interaction	AB	(i-1)(j-1)					
Error		Total SS – (SS of all other terms above)		**VARIANCE**			
TOTAL		n-1					

Because of the prominence of analyses in Excel outputs from Excel for various experimental designs are presented below.

A The completly randomized design Single factor ANOVA

This is a design in which the runs are assigned randomly to treatments. The runs are viewed as a random sample from a normal distribution.

The one-way analysis of variances contains only one effect A , each level having K observations:

Example 1.:

The Complete Randomized Design

		T1	T2	T3	T4	
	R1	80	90	80	90	
	R2	70	80	70	100	
	R3	60	85	75	110	
	R4	50	100	65	120	
	R5	60	95	85	100	
	Σ	320	450	375	520	1665
mean	mean	64	90	75	104	

Anova: Single Factor from Excel

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
T1	5	320	64	130
T2	5	450	90	62.5
T3	5	375	75	62.5
T4	5	520	104	130

ANOVA				F exp.		0.05	0.01
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	*F crit*
Between Groups	4573.8	3	1524.58	**15.84	0.00005	3.24	5.29
Within Groups	1540	16	96.25				
Total	6113.8	19					

VARIANCE $\mathbf{s}^2$

From the F-distribution table

** denotes significant at 1% level of probability

B The randomized complete block design (RCBD)

Anova: Two-Factor Without Replication IN EXCEL

This is a design in which the runs are grouped into blocks such that the runs are assumed to be homogenous with blocks. Runs are assigned randomly to treatments within blocks. The effect of blocking is assumed to be orthogonal to the effect of factors. It is important to form the blocks on the basis of a variable which is logically related to the outcome of the experiment because the purpose of blocking is to control the variation of this variable. Effectively blocking reduces the residual error, i.e. the denominator of the F test. This designed is preferred over completely randomized design if the within-block variability is smaller than the between-block variability.

Example 2.:

The randomized complete block design

	T1	T2	T3	T4		
R1	90	100	100	85	375	
R2	70	85	90	55	300	
R3	75	80	90	50	295	
R4	90	100	100	75	365	
	325	365	380	265		1335
mean	81.25	91.25	95	66.25		

Anova: Two-Factor Without Replication from Excel

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
R1	4	375	93.75	56.25
R2	4	300	75	250
R3	4	295	73.75	289.5833
R4	4	365	91.25	139.5833
T1	4	325	81.25	106.25
T2	4	365	91.25	106.25
T3	4	380	95	33.33333
T4	4	265	66.25	272.9167

ANOVA				F exp.		0.05	0.01
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	*F crit*
Rows	1329.69	3.00	443.23	**17.61	0.0004	3.86	6.99
Columns	1979.69	3.00	659.90	**26.21	0.0001	3.86	6.99
Error	226.56	9.00	25.17				
Total	3535.94	15					

VARIANCE s^2

From F-distribution table

F value for both Rows and Columns exceeds
F crit at p=0.01 so effects are significant at p=0.01

C The Latin square design

Design for studying the effect of one factor and two blocking variables. The blocking variables and factor must have equal number of levels. These are normally in the range of 4x4 to 12x12. The assumption is that the two blocking variables and factor are additive, i.e. there is not interaction among them. The levels of the factor are assigned randomly to the combinations of blocking variable levels such that each level of a blocking n variable receives a different level of the factors. This design eliminates two extraneous sources of variation. Examples of the layout of treatments in rows and columns is shown below:

4X4 5X5, 6X6, 7X7, 8x8

ABCD ABCDE ABCDEF ABCDEFG ABCDEFGH
BCDA BCDEA BCDEFA BCDEFGA BCDEFGHA
CDAB CDEAB CDEFAB CDEFGAB CDEFGHAB
DABC DEABC DEFABC DEFGABC DEFGHABC
EABCD EFABCD EFGABCD EFGHABCD
FABCDE FGABCDE FGHABCDE
GABCDEF GHABCDEF
HABCDEFG

Example 3.:

The Latin Square Design

		column				
	row	T1	T2	T3	T4	
	R1	B 41	A 32	D 43	C 37	153
	R2	C 40	B 40	A 36	D 40	156
	R3	D 45	C 36	B 39	A 33	153
	R4	A 35	D 40	C 27	B 40	142
		161	148	145	150	
mean		40.25	37	36.25	37.5	

ANOVA				F exp.	0.05	0.01
Source of Variation	SS	df	MS	F	F crit	F crit
Rows	28.5	3	9.50	0.969	4.76	9.78
Columns	36.5	3	12.16	1.240	4.76	9.78
Treatments	179	3	59.60	***6.080**	4.76	9.78
Error	59	6	9.80			
Total	303	15				

VARIANCE s^2

Fexp for Rows and Columns is less than Fcrit at p=0.05 so effects are non-significant

D 2 factor ANOVA

Anova: Two-Factor With Replication

Two-level factorial design in which each factor has only two levels. This design is of special importance because with relatively few runs it can indicate major trends and can be a building block in developing more complex designs (MANOVA).

A two -level complete factorial design is denoted 2x.

Example 4.:

variable or column

		A1		A2		A3		A4		
		B1	B2	B1	B2	B1	B2	B1	B2	
case or row	R1	8	8	12	10	12	11	14	17	
	R2	10	9	13	12	10	9	17	19	
	R3	11	8	11	10	13	11	13	17	
	R4	10	10	14	11	12	11	14	16	
	R5	7	9	10	7	14	12	17	21	
	Σ	46	44	60	50	61	54	75	90	480
		90		110		115		165		
	mean	9.2	8.8	12	10	12.2	10.8	15	18	
	B1	46		60		61		75		
	B2		44		50		54		90	

		ANOVA from Excel				F exp.		0.05	0.01
		Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	*F crit*
df=(B-1)	B	Sample	0.4	1	0.4	0.16	1	4.15	7.50
df=(A-1)	A	Columns	305	3	101.67	40.07	** 0	2.90	4.46
df=(B-1)*(A-1)	AB	Interaction	37.4	3	12.47	4.91	** 0	2.90	4.46
df=B*A*(R-1)	ERROR	Within	81.2	**32**	2.54				
df=variable-1		Total	424	39					

VARIANCE s^2

From F-distribution table

Fexp for Columns and Interaction >Fcrit at p=0.01

so these effects significant

Conclusions:

- The effect of samples averaged over columns is non-significant
- The effect of columns, averaged over samples, is significant at p=0.01
- The interaction between samples and columns is significant at p=0.01. This means that the sample effect is different in the different columns. When the interaction effect is significant any main effect of any of the interacting terms must be ignored as the magnitude of the main effect differs at the different levels of the other factor.

Factor (treatment) A is significantly larger than treatment B at the 5% significance level

	A1	A2	A3	A4	mean A
B1	9.2	12	12.2	15	12.10
B2	8.8	10	10.8	18	11.90
mean	9	11	11.5	16.5	12.00

LSD test for factor A

sd-A	0.71

t-table

2.04

2.75

LSD test for interaction AB

sd-AB	1.01

LSD(0.05)*sd-A	1.45
LSD(0.01)*sd-A	1.96

LSD(0.05)*sd-AB	2.06
LSD(0.01)*sd-AB	2.77

		A1	A2	A3	A4
		9	11	11.5	16.5
A4	17	**7.5	**5.5	**5.0	
A3	12	**2.5	0.5		
A2	11	**2.0			
A1	9				

interaction on the B1 level

		A1	A2	A3	A4
		9.2	12	12.2	15
A4	15	**5.8	**3.0	**2.8	
A3	12	**3.0	0.2		
A2	12	**2.8			
A1	9.2				

interaction on the B2 level

		A1	A2	A3	A4
		8.8	10	10.8	18
A4	18	**9.2	**8.0	**7.2	
A3	11	2.0	0.8		
A2	10	1.2			
A1	8.8				

CONCLUSION after LSD-test

- On the basis of the examined factors, and their interaction, we see in the case of B1 in all combinations except A2 and A3 the differences are very significant (**).
- In the case of B2 differences are high significant(**) only between A4 in relation to all other levels; and the B2 difference is NOT significant in relation to A1, A2 and A3

2.12.4 LINEAR CORRELATION AND REGRESSION

CORRELATION is interdependence between two variables, x and y. The correlation coefficient rxy is a measure of this interdependence and it ranges between -1 and +1. A zero value indicates absence of correlation.

REGRESSION is a collection of statistical methods using mathematical equations to model the relationship among measured or observed quantities. The goal of this analysis is modelling and predicting. The relationship is described in algebratic form as y=f(x)+e

Example 5.:

	y yield	x fertilizer	xy	x2	y2	pred.y	residuals yi-pr.y	(yi-pr.y)2
1	26	5	130	25	676	26	0	0
2	32	7	224	49	1024	31	1	1
3	35	9	315	81	1225	36	-1	1
4	38	10	380	100	1444	38.5	-0.5	0.25
5	40	11	440	121	1600	41	-1	1
6	45	12	540	144	2025	43.5	1.5	2.25
Σ	216	54	2029	520	7994	216	0	5.5
mean	36	9						

from Excel

Regression Statistics	
Multiple R	0.987
R Square	0.975
Adjusted R Square	0.968
Standard Error	1.173
Observations	6

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	212.5	212.5	154.55	0.0002
Residual	4	5.5	1.375		
Total	5	218			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept=a	13.5	1.872	7.211	0.002	8.302	18.698	8.302	18.698
fertiliser=b	2.5	0.201	12.432	0.000	1.942	3.058	1.942	3.058

2.12.5 COMPUTER SOFTWARE

There are many commercial packages available which can calculate the statistics required for analysis of experiments. However, few are directed specifically towards experimental design. Most tend to concentrate on producing statistics, which are only really relevant to passively accumulated data.

ChemStat, SigmaStat 2, MiniTab 12, SigmaPlot 4, StatGraphics plus 4, Statistica, SysStat 9,

TableCurve 2D 4, S-plus, SAS, SPSS

2.13 REFERENCES AND FURTHER READING

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CHAPTER 3

A PRACTICAL GUIDE TO USING NUCLEAR TECHNIQUES IN THE LABORATORY, GLASSHOUSE AND FIELD

3.1 GENERAL LABORATORY PRACTICE

3.1.1 CLEANING LABORATORY GLASSWARE

Good laboratory practice demands clean glassware. Glassware is the basic tool in laboratory analysis and if dirty can lead to inaccurate results and wasted time. The following washing procedures are sufficient for glassware to be used in most macro- and micro-nutrient analyses.

A. General glassware

i Initial rinsing

Immediately after use, rinse glassware in tap water to remove contaminants. For more stubborn stains, soil residues, etc., rinsing may not be adequate and thorough scrubbing will be needed. It is good practice to have a complete set of brushes on hand to fit large and small test tubes, funnels and various flasks and bottles for this purpose.

ii Soaking

Completely immerse rinsed glassware in a 2% solution of phosphorus-free detergent and tap water. Detergent concentration may be increased depending upon contamination.

Normal contamination - 2%
Heavy contamination - 5%
Persistent contamination up to 20%

Soaking should be for 2–24 hours; any longer than this and the glassware becomes slimy and the soaking water smelly. Soaking can be conveniently carried out in plastic tubs which are easily moved about and emptied every 2 days. Before the tubs are refilled they should be scrubbed to remove the sediment and slime which quickly builds up during use. Ideally, water used for soaking should be warm, say 50^{O}C, though obviously this will cool after a few hours.

iii Final rinsing

After soaking, the glassware must be thoroughly rinsed to remove all traces of detergent and contaminants. This is done by rinsing three times in warm tap water, followed by three rinses in distilled water.

iv Drying

Non-volumetric glassware (e.g. beakers, plasticware, bottles, etc.) is dried by placing in a clean oven at temperatures of up to 140^{O}C or by air drying. The oven shelves should be contamination-free (e.g. not rusty) and, ideally, stainless steel. The glassware can also be placed in stainless steel baskets for oven or air drying.

Volumetric glassware, e.g. volumetric flasks, cannot be dried at temperatures above 80^{O}C. The constant expansion and contraction experienced at temperatures above this renders accurately calibrated glassware useless.

Hanging glassware on pegs to air dry should be avoided as they are a source of contamination. An alternative is to hang the glassware through holes which are large enough to fit most of the glassware through but small enough to hold the base, e.g. volumetric flasks and measuring cylinders. Glassware that is unsuitable for this method of drying, e.g. beakers, can be left in a clean, dust free area to air-dry.

v Storage

Glassware should not be put into storage until completely dry otherwise fungal growth can occur. As a further precaution against contamination, glassware can be covered with plastic film or lids may be fitted.

B. Pipettes

Due to their narrow bore, pipettes require special treatment when considering cleaning methods. Cleaning can be facilitated by the use of a pipette soaker and washer which allows for the pipettes to fill and empty several times until clean.

The following procedure is used for pipette cleaning:

i Initial rinsing

Rinse the pipettes in tap water immediately after use.

ii Soaking

Completely immerse pipettes in a 2% solution of detergent and tap water and leave to soak for 2–24 hours. Ideally, the pipettes should be soaked vertically, with the tip up, in a pipette soaker.

iii Final rinsing

The pipettes must be rinsed several times with tap water, inside and out, until all detergent is removed. Following this, rinse 3 times in distilled water. This can easily be done in a pipette washer. Alternately, connect the pipette to a water or mechanical vacuum pump, using rubber tubing of suitable bore, and suck the water through the pipette.

iv Drying

Dry the pipettes with 2 or 3 quick rinses of acetone. This should be done soon after the distilled rinse but before the pipettes have air-dried and formed water marks inside the tube. This is best done by sucking through small volumes of acetone and air successively. Allow all acetone to evaporate; drain by leaving pipettes upright before storing.

C. Acid soaking

Glassware, including pipettes, which is to be used for micro-nutrient analysis should be acid-washed to minimise contamination. It is important to note that acid should not be allowed to come into contact with a piece of glassware before the detergent is thoroughly removed. If this happens, a film of grease may be formed and, in the case of pipettes, this causes inaccurate delivery.

When working with acid observe the usual safety procedures by wearing protective glasses and gloves. It is important to note that coloured rubber gloves sometimes contain trace elements which may contaminate the glassware when handled. An alternative is to use disposable plastic gloves.

To acid-soak glassware, follow the washing procedures described, including *Rinsing* step above. Then:

i Acid soak

Completely immerse the glassware in 10% nitric acid or 10% hydrochloric acid made up in distilled water. The glassware is left to soak overnight.

ii Rinsing

After soaking rinse 3 times in ultrapure (i.e. distilled-deionized water).

iii Drying

Dry glassware in a clean, stainless steel shelved oven or air-dry in a warm dust-free environment. Pipettes should be air-dried. Do not use acetone as it may leave trace contamination.

3.1.2 EQUIPMENT MAINTENANCE

NOTE: It is essential that the Instruction Manuals supplied with laboratory equipment be read, as they generally give a simple and concise guide to maintenance, use and problem-solving.

A. Care of balances

Never move the balance when the power is on or if the balance has not been correctly locked.

Check that the balance is level; if not, operate the adjustable feet until the spirit level bubble is centrally located in the viewer. Adjustments to the level should only be done when the balance is turned off.

Ensure balance is clean and tared prior to use.

Ensure all doors on the weighing chamber of an analytical balance are fully closed and the reading is stable prior to recording the weight.

Never allow any liquid or solid chemical to come into contact with the pan as corrosion and incorrect weighing will result.

Ensure balance has been recently calibrated prior to use and that both accuracy and precision are good.

Cover the balance, if possible, when not in use.

Ideally, the balance should be left connected to the power supply and switched on so that thermal equilibrium is maintained. If balances must be switched off, allow for a warming-up period to achieve thermal equilibrium.

Locate all balances on a solid base in an area away from direct sunlight, air currents (e.g. fans, doors, open windows) and vibrations.

B. Care of pH meters

Locate the meter in an area of the bench which is free from other instruments to minimise electrical interference.

If possible, store the meter in a moisture-free environment. (This is important for all electrical equipment.)

Standardise the pH meter daily before carrying out pH measurements.

Always calibrate with a buffer which is as close as possible to the unknown pH range.

i Maintenance of pH electrodes

After measurement, remove electrode(s) from sample, wash and blot dry.

When an electrode is used intermittently, disconnect and leave in an upright position immersed in pH buffer 4. The salt bridge should not be allowed to dry out.

Keep reference chamber topped up with electrolyte when not in use for short periods of time.

For **long term storage**, drain electrolyte and wash reference chamber several times with distilled water. Stand electrode(s) in distilled water for 12 hours to remove potassium chloride from the salt bridge. Dry and store in a box. Before use, rinse and fill with electrolyte solution, then, soak in pH buffer 4.

ii Cleaning of pH electrodes

Both the membrane and salt bridge must be clean at all times. Do not allow fats, oil, proteins, soil, etc. to dry on the membrane or salt bridge.

The membrane may be cleaned with solvents, detergents or acid. Place a small amount of acetone, ethanol, etc. on cotton wool and gently wipe the membrane. **Abrasive materials must not be used.** For acid cleaning, use 20% sulfuric acid initially, followed by chromic acid if this is unsuccessful. After cleaning, wash well with tap water and soak before use in pH 4 buffer.

To clean the salt bridge, apply a vacuum or immerse the salt bridge in boiling water for 5–20 seconds.

iii Rejuvenation

Some improvement in the response of the aged electrodes may be obtained by the following treatment:

Alternate the electrode between 0.1M hydrochloric acid and 0.1M ammonia for 5 min. intervals. Rinse with water.

Recalibrate using appropriate buffers.

iv Precautions with glass electrodes

The sensitivity of the glass electrode will be affected by:

Continuous use; after which the electrode may need regenerating;

Protein buildup on the membrane, which must be removed;

Dehydrating agents (e.g. ethanol) or concentrated acids, which dehydrate the membranes. The presence of water in the glass is essential; pH function is impaired when the glass is dehydrated, but can be restored by subsequent immersion in distilled water for several hours or overnight.

Temperature increase will cause a fall in pH and this must be accommodated for by either (a) a temperature probe in the sample for automatic adjustment, or (b) knowing the temperature of the sample when the pH is measured, then manually correcting.

Scratching or fracturing of the glass membrane. Under these circumstances, a new glass electrode will be required. New electrodes will need conditioning by placing them in 0.lM HCl overnight.

v Sources of error

The following are likely sources of error in pH measurement.

Solutions should be stirred when taking readings. This minimises the effect of gradients and reduces the response time.

For best results the standard buffers and sample should be at the same temperature. If the temperature differential is too great it may take up to 30 min for the system to regain stability.

Solutions with low conductivity, e.g. distilled H_2O or non-buffered solutions, take a considerable time to equilibrate and the pH readings tend to drift.

The salt bridge (porous plug) must be completely immersed in the solution being measured.

If the porous plug clogs up, the electrolyte will not flow freely into the sample resulting in erratic readings.

The reference filling solution should occupy at least 2/3 of the chamber.

The electrode should be stored wet when not in use (except for long term storage, see previous notes).

Sensitivity to $[H^+]$ is reduced if the pH sensitive glass membrane is dirty.

3.1.3 LABORATORY SAFETY

No laboratory guide can be complete without the inclusion of a segment on laboratory safety. The following notes outline general safety instructions and chemical hazards encountered in an analytical laboratory.

A. General safety instructions

i Dress and protective clothing

Laboratory coats and closed shoes should be worn at all times.

Protective equipment must be worn when handling chemicals and dangerous equipment (e.g. faceshields, safety glasses, gloves, film badges, etc.)

ii Conduct in the laboratory

Note location of safety showers, fire blankets, first aid boxes, fire extinguishers and fire exits at places of work.

Be aware of hazards related to working conditions.

Smoking, eating and drinking should not be permitted in laboratories due to the presence of flammable substances and the risk of ingesting poisonous substances.

Hands must be washed after handling any chemicals.

Persons engaged in any process requiring careful attention should not be distracted.

B. Housekeeping

Good housekeeping is essential. General cleanliness reduces exposure to hazards and promotes safety, efficiency and accuracy.

Fire doors must be kept closed at all times and not obstructed.

All containers must be clearly and appropriately labelled.

Chemicals to be discarded must be disposed of immediately

(i) Inorganic — down the sink with plenty of water
(ii) Organic — in residue container

C. Laboratory procedures

Glassware should be used with care. Clean up all breakages.

Never pipette hazardous chemicals by mouth Use a pipette filler or an automatic dispenser.

Corrosive liquids, e.g. acids, alkalis, must be handled with care.

Hazardous or toxic chemicals, particularly those which give off strong, irritating or poisonous vapours, should be handled in the fume cupboard.

Make up solutions by gradually adding solid to solvent. Always add acid to water when diluting.

Solvents, e.g. acetone, chloroform, are often highly flammable and sometimes poisonous. Avoid inhaling vapour, use in small amounts and keep away from sources of ignition.

Bunsen burners must be operated on the open bench. Do not leave unattended. Flammable chemicals must not be left near a lighted burner or hotplate. Try to eliminate "hot spots" by heating slowly and continuously stirring. Care must be taken when heating test tubes containing solution; point away from persons nearby.

Any electrical apparatus must be kept dry.

Winchester bottles and other large glass vessels must not be carried by the neck. One hand should be placed below the vessel and the other around the neck.

Ventilate the work area.

D. Safety and first aid

All chemicals should be considered as potentially harmful substances. When considering the use of any chemical for an experiment, thought should be given to any possible hazard associated with its use. Where a dangerous substance is to be used, alternative safer chemicals should be contemplated.

Information regarding the hazardous nature and safe use of chemicals not mentioned in these notes may be found in one of the references given at the end of the notes. If no information is available in the reference books, then the manufacturer should be contacted. In general, the following rules for handling chemicals will minimize any hazard. Do not substitute these rules for obtaining definite information. Some chemicals are more dangerous and may require special precautions and first aid treatment.

Avoid inhaling vapour or dust.

Avoid contact of the chemical with the eyes or skin. The use of safety glasses and gloves will facilitate this.

Do not allow mouth contact with the chemical.

The following general advice regarding first aid in the event of an accident with a chemical will be found useful.

Vapour or dust inhaled: remove patient from exposure, rest and keep warm. If necessary obtain medical attention.

Affected eyes: irrigated thoroughly with a copious supply of running water. In severe cases or where splashing has occurred, obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. It may be necessary to obtain medical attention.

If swallowed: wash mouth out thoroughly with water. Obtain medical attention.

ACETONE

Identification: Colourless liquid with characteristic odour. Miscible in water.

Health hazard: Inhalation of the vapour may cause dizziness, narcosis and coma. The liquid irritates the eyes and may cause severe damage. If swallowed, may cause gastric irritation, narcosis and coma.

Fire hazard: Flammable. Flash point -18^{o}C. Extinguish with water spray, dry powder, carbon dioxide or vaporising liquids.

Precaution: Avoid inhaling vapour and avoid contact with eyes. Wear safety glasses. Use in small quantities and keep well away from sources of ignition.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm.

Affected eyes: irrigate thoroughly with water; in severe cases or where splashing has occurred, obtain medical attention.

If swallowed: give plenty of water to drink. If a large amount has been swallowed, obtain medical attention.

Spillage disposal: Shut off possible sources of ignition. Wear face shield and gloves. Mop up with plenty of water and run to waste, diluting greatly with running water. Ventilate area well to evaporate remaining liquid and dispel vapour.

AMMONIUM HYDROXIDE (NH_4OH)

Identification: Colourless liquid with a pungent small.

Health hazard: Corrosive to all tissues if inhaled, ingested or spilt on eyes or skin.

Fire hazard: None.

Precaution: Avoid breathing vapour and prevent contact with eyes and skin. Wear a face shield and gloves and use a fumehood when handling the concentrated reagent. Safety glasses may be worn instead of the face shield when handling dilute solutions.

Release the cap of bottles of concentrated reagent with care because of pressure buildup in bottle.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention.

Affected eyes: irrigate thoroughly with a copious supply of water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive immediate medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink followed by vinegar or 1% acetic acid. Obtain medical attention.

Spillage disposal: Wear breathing apparatus and gloves. Mop up with plenty of water. Run this to waste and dilute greatly with running water.

AMMONIUM META VANADATE (NH_4VO_3)

Identification: Colourless crystalline powder, soluble in water.

Health hazard: Harmful dust which may cause irritation of the respiratory system, chest constriction and coughing. Dust irritates eyes and may cause conjunctivitis. If ingested, causes vomiting, excessive salivation and diarrhoea. Large doses may damage the nervous system. The dust may cause skin irritation.

Fire hazard: None.

Precaution: Avoid inhaling dust. Wear safety glasses.

First aid:

If inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention.

Affected eyes: irrigate thoroughly with water. Obtain medical attention.

Skin contact: wash off with a copious supply of water.

If swallowed: wash out mouth thoroughly with water. Obtain medical attention.

Spillage disposal: Dissolve in water and mop up and run to waste diluting greatly with running water.

ANTIMONY POTASSIUM TARTRATE ($KSbO.C_4H_4O_6$)

Identification: White crystal, soluble in water.

Health hazard: Poisonous. Some antimony compounds cause skin irritation and dermatitis. If taken internally, antimony compounds may cause burning of the mouth and throat, choking, nausea and vomiting.

Fire hazard: None.

Precaution: Avoid eye and skin contact. Wear safety glasses.

First aid:

Affected eyes: irrigate with a copious supply of water.

Skin contact: wash thoroughly with soap and water.

If swallowed: wash out mouth thoroughly with water. Obtain medical attention.

Spillage disposal: The disposal of antimony compounds in any quantity must be considered in the light of local conditions and regulations.

Burial in an isolated area can be considered, as can gradual disposal with very high dilution, into a sewage system.

BROMINE (Br)

Identification: Rhombic crystals or dark red liquid with characteristic odour and toxic fumes. Slightly soluble in water.

Health hazard: Highly toxic — even the fumes cause serious burns and blisters to the skin. Fumes cause serious irritations to the eyes of mucous membranes and to the respiratory system. Severe exposure may cause pulmonary oedema or death. Bromine substances can produce depression, emaciation and, in severe cases, psychoses and mental deterioration.

Fire hazard: Moderate in the form of liquid or vapour by spontaneous chemical reaction with reducing agents. May react violently with a number of substances, some of the more common being acetone, ammonia, copper, ethanol, phosphorus, hydrogen, potassium, sodium, lithium and mercury.

Precaution: Highly dangerous — requires special handling. When heated, emits highly toxic fumes; will react with water or steam to produce toxic and corrosive fumes; can react vigorously with reducing agents.

Avoid breathing vapour and prevent contact with eyes and skin. Keep ammonia water on hand to neutralize any drops that contact the skin. Wear gloves and safety glasses. Work in fume hood.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention. Apply artificial respiration if breathing has stopped.

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water and then give plenty of water to drink. Obtain medical attention.

Spillage disposal: Cover with a saturated solution of sodium bisulfite and 3M sulfuric acid. Mix. Spray with H_2O and scoop into a large container of H_2O, neutralize with sodium carbonate and flush to sewer with a large excess of H_2O. Wash site with a solution of sodium bisulfite followed by soap and H_2O.

CHLOROFORM (trichloromethane)

Identification: Colourless volatile liquid with characteristic colour. Immiscible with water.

Health hazard: The vapour has anaesthetic properties, causing drowsiness, giddiness, headache, nausea, vomiting and unconsciousness. The vapour and liquid irritate the eyes causing conjunctivitis. The liquid is poisonous if taken by mouth.

Fire hazard: Slight when exposed to high heat, otherwise practically none.

Hazardous reactions: Vigorous reaction with acetone in the presence of KOH or $Ca(OH)_2$; may react explosively with fluorine, N_2O_4, Al, Li, Na, Na/methanol, NaOH/methanol.

Precaution: Avoid breathing vapour. Avoid contact with eyes and skin. Wear safety glasses and use only in small quantities.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention. Apply artificial respiration if breathing stopped.

Affected eyes: irrigate thoroughly with water. In severe cases or where splashing has occurred, obtain medical attention.

Skin contact: drench with water and wash thoroughly with soap and water. Remove and air thoroughly any contaminated clothing.

If swallowed: wash out mouth thoroughly with water. Obtain medical attention.

Spillage disposal: Instruct others to keep at safe distance. Wear breathing apparatus and gloves. Apply dispersing agent, if available, and work to an emulsion with brush and water. Run this to waste, diluting greatly with water. If dispersant is not available, absorb onto sand, shovel into bucket(s) and transport to safe open area for atmospheric evaporation. Site of spillage should be washed thoroughly with water and detergent. Ventilate area of spillage thoroughly to dispel vapour.

HYDROGEN PEROXIDE (H_2O_2)

Identification: Colourless liquid, miscible with water.

Health hazard: Corrosive to all tissues. If swallowed, sudden evolution of oxygen may cause injury by acute distension of stomach. May cause nausea, vomiting and internal bleeding.

Fire hazard: Strong oxidizing agent. Promotes fire. Avoid mixing with organic substances.

Precaution: Prevent contact with eyes and skin. Wear face shield and gloves when handling the concentrated reagent. Safety glasses may be worn instead of a face shield when handling dilute solutions.

Release the cap of bottles of concentrated reagent with care because of pressure buildup in bottle.

First aid:

Affected eyes: irrigate thoroughly with copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water and then give plenty of water to drink. Obtain medical attention.

Spillage disposal: Wear face shield, gloves. Mop up with plenty of water. Run to waste, diluting greatly with running water.

HYDROCHLORIC ACID (HCl)

Identification: Colourless, fuming liquid with pungent small; miscible with water.

Health hazard: Corrosive to all tissues if inhaled or ingested or spilt on the skin.

Fire hazard: None.

Precaution: Avoid breathing vapour and prevent contact with eyes and skin. Wear face shield and gloves when handling the concentrated reagent. Safety glasses may be worn instead of a face shield when handling dilute solutions.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention.

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink, followed by milk of magnesia. Obtain medical attention.

Spillage disposal: Instruct others to keep at a safe distance. Wear breathing apparatus and gloves. Spread soda ash liberally over spillage and mop up cautiously with of water. Run this to waste and dilute greatly with running water.

MERCURIC THIOCYANATE ($Hg(SCN)_2$)

Health hazard: Highly toxic. Mercury is a general protoplasmic poison; it circulates in the blood and is stored in the liver, kidneys, spleen and bone. In high doses it affects the central nervous system.

Fire hazard: None.

Precaution: Dangerous when heated to decomposition or on contact with acid or acid fumes. Emits toxic fumes.

Avoid breathing vapour when heated and avoid breathing dust. Prevent contact with eyes and skin. Wear safety glasses and gloves when handling.

First aid:

Dust inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention.

Affected eyes: irrigate thoroughly and continuously with water until medical attention has been obtained.

Skin contact: remove affected clothing and thoroughly wash affected part of body with a copious supply of running water.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink. Obtain medical attention.

Spillage disposal: Small quantities of the compound can be swept up, dissolved in water or acid and run to waste at very high dilution. If burial is carried out in an isolated area, the solid compound should first be diluted 10–20 times by weight with sand.

NITRIC ACID (HNO_3)

Identification: Colourless or pale yellow fuming liquid, miscible with water.

Health hazard: Corrosive to all tissues if inhaled or ingested or spilt on the skin.

Fire hazard: Strong oxidizing agent. Promotes fire. Avoid mixing with organic substances.

Precaution: Avoid breathing vapour and prevent contact with eyes and skin. Wear a face shield and gloves when handling the concentrated reagent. Safety glasses may be worn instead of a face shield when handling dilute solutions.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention.

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink, followed by milk of magnesia. Obtain medical attention.

Spillage disposal: Instruct others to keep at a safe distance. Wear breathing apparatus and gloves. Spread soda ash liberally over spillage and mop up cautiously with of water. Run this to waste and dilute greatly with running water.

PERCHLORIC ACID ($HClO_4$)

Identification: Colourless liquid, miscible with water.

Health hazard: Corrosive to all tissues if inhaled or ingested or spilt on the skin or eyes.

Fire hazard: Strong oxidizing agent. Promotes fire. Avoid mixing with organic substances.

Precaution: Prevent contact with eyes and skin. Wear a face shield and gloves when handling the concentrated reagent. Safety glasses may be worn instead of a face shield when handling dilute solutions.

First aid:

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink, followed by milk of magnesia. Obtain medical attention.

Spillage disposal: Instruct others to keep at a safe distance. Wear breathing apparatus and gloves. Spread soda ash liberally over spillage and mop up cautiously with of water. Run this to waste and dilute greatly with running water.

PHENOL (carbolic acid)

Identification: Colourless to pink crystals, having a distinctive odour and being somewhat soluble in water.

Health hazard: Vapour irritates the respiratory system and eyes. Skin contact causes softening and whitening followed by the development of painful burns. Its rapid absorption through the skin may cause dizziness, rapid and difficult breathing, weakness and collapse. If swallowed, it causes severe abdominal pain, nausea, vomiting and internal damage.

Fire hazard: Flammable at high temperature. Flashpoint 85^{O}C.

Precaution: Avoid inhaling vapour. Avoid contact with eyes and skin. Wear face shield.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention.

Affected eyes: irrigate thoroughly and continuously with water until medical attention has been obtained.

Skin contact: remove affected clothing, drench with water and swab affected skin with glycerol for at least l0 minutes. If contamination is other than slight, obtain medical attention.

If swallowed: induce vomiting by placing finger far back in throat, summon medical attention immediately. After vomiting has ceased, mouth should be thoroughly washed out with water.

Spillage disposal: Wear face shield or goggles, and gloves. Mix with sand and transport to safe open area for burial. Site of spillage should be washed thoroughly with water and soap or detergent.

POTASSIUM HYDROXIDE (KOH)

Identification: White, deliquescent pieces, lumps or sticks having crystalline fracture. Soluble in water.

Health hazard: Highly toxic. Highly irritant to skin, eyes and mucous membranes. A very powerful caustic. If taken by mouth results in severe internal irritation and damage.

Fire hazard: Moderate.

Precaution: Will react with water or steam to produce caustic solution and heat.

Prevent contact with eyes and skin. Wear a face shield and gloves when handling the concentrated reagent. Safety glasses may be worn instead of a face shield when handling dilute solutions.

First aid:

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink. Obtain medical attention.

Spillage disposal: Wear face shield or goggles and gloves. Shovel into a large volume of water in an enamel or polythene vessel and stir to dissolve; run the solution to waste diluting greatly with running water.

SODIUM HYDROXIDE (NaOH)

Identification: White to colourless pellets soluble in water.

Health hazard: The solid and strong solutions of the compound are extremely corrosive to all tissues.

Fire hazard: None.

Precaution: Prevent contact with eyes and skin. Wear face shield and gloves when handling the concentrated reagent. Safety glasses may be worn instead of a face shield when handling dilute solutions.

First aid:

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink, followed by vinegar or 1% acetic acid. Obtain medical attention.

Spillage disposal: Wear face shield and gloves. Shovel solid into a polythene bucket and add a little at a time to a large volume of water. Run this to waste, diluting greatly with running water. Strong solution: mop up with water. Run this to waste, diluting greatly with running water.

SODIUM HYPOCHLORITE (bleach, NaClO)

Identification: Colourless solution with strong chlorine smell.

Health hazard: Corrosive and irritant via ingestion and inhalation. Bleaches and may burn the skin.

Fire hazard: None, but anhydrous salt is highly explosive.

Precaution: Violent reaction with amines, ammonium acetate $(NH_4)_2CO_3$, NH_4NO_3, ammonium oxalate $(NH_4)_3PO_4$, cellulose.

Prevent contact with eyes and skin. Wear safety glasses and gloves when handling.

First aid:

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink. Obtain medical attention.

Spillage disposal: Wear face shield, goggles and gloves. Mop up with plenty of water and run to waste, diluting greatly with running water.

SULFURIC ACID (H_2SO_4)

Identification: Colourless, viscous liquid which reacts vigorously with water.

Health hazard: Corrosive to all tissues if inhaled or ingested or spilt on the skin.

Fire hazard: None.

Precaution: Prevent contact with eyes and skin. Wear face shield and gloves when handling the concentrated reagent. Safety glasses may be worn instead of a face shield when handling dilute solutions.

Great care should be taken when diluting the acid with water. Small amounts of acid should be added cautiously with stirring to water.

First aid:

Affected eyes: irrigate thoroughly with a copious supply of running water. Obtain medical attention.

Skin contact: remove affected clothing and thoroughly wash affected part of the body with a copious supply of running water. Burns must receive medical attention.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink, followed by milk of magnesia. Obtain medical attention.

Spillage disposal: Instruct others to keep at a safe distance. Wear breathing apparatus and gloves. Spread soda ash liberally over spillage and mop up cautiously with plenty of water — remember vigorous reaction with water. Run this to waste and dilute greatly with running water.

TOLUENE (methylbenzene, phenylmethane) $C_6H_5CH_3$

Identification: Colourless liquid, benzol-like odour; immiscible with water.

Health hazard: Toluene is derived from coal tar and commercial grades usually contain small amounts of benzene as an impurity (N.B. benzene is highly toxic whether inhaled as a vapour or absorbed via the skin; it is a carcinogen). Acute poisoning resulting from exposures to high concentration of vapours are rare with toluene. Depending on exposure concentration, symptoms observed can be headache, nausea, loss of appetite, a bad taste, lassitude, impairment of co-ordination and reaction time.

Fire hazard: Slight when exposed to heat, flame or oxidizers. To fight fire, use foam, CO_2 or dry chemical. Flash point 4.4°C

Precaution: When heated emits toxic fumes which can react vigorously with oxidizing materials. Avoid breathing vapour and avoid contact with eyes and skin. Wear safety glasses and gloves, Work in a fume hood.

First aid:

Vapour inhaled: remove from exposure, rest and keep warm. In severe cases, obtain medical attention.

Affected eyes*:* irrigate thoroughly and continuously with water until medical attention has been obtained.

Skin contact*:* remove affected clothing. Thoroughly wash affected part of body with a copious supply of running water.

If swallowed: wash out mouth thoroughly with water. Give plenty of water to drink. Obtain medical attention.

3.2 BASIC DESIGN FEATURES OF RADIATION INSTALLATIONS

The following has largely been extracted from IAEA Radiation Protection Procedures (IAEA 1973) and Austrian Radiation Protection Regulations (Moser 1978)

3.2.1 INTRODUCTION.

The application of radioisotopes as tracers in agricultural research requires suitable facilities to handle these nuclides safely, ensuring that radiation workers are not exposed to undue external or internal radiation hazards.

The following suggestions for the proper design of a radioisotope lab focuses on the most common radiotracer nuclides (β^--emitters) used in agricultural research, i.e. ^{32}P (β^-, 1.7 MeV, t/2=14.3d), ^{33}P (β^-, 0.25 MeV, t/2=25.3d), ^{35}S (β^-, 0.17 MeV, t/2=87d) and ^{14}C (β^-, 0.15 MeV, t/2=5730a), being handled as so called “open sources”, i.e. not in closed containments.

Note: that the following features are partly based on international (FAO/IAEA) and partly on national (Austrian) safety regulations. The latter should therefore not be taken as mandatory, but as

additional suggestions and evidence for designing a laboratory for handling radionuclides. National safety regulations have to be observed.

3.2.2 CLASSIFICATION OF LABORATORY TYPES

The large range and characteristics of radionuclides makes it impossible, to give a single general safety rule for handling open sources. The basis for the classification of laboratory types is the grouping of radionuclides according to their relative radiotoxicities per unit activity as well as the intended chemical operations and treatment of the radionuclides.

The classification of isotopes according to their relative radiotoxicity results in four groups. Group No. 1 showing radio nuclides of highest radiotoxicity, group No. 4 containing the least hazardous nuclides. The quantity of radionuclede that can be handled in each laboratory type is shown in Table 3.1.

All radiotracer nuclides mentioned above, i.e. ^{32}P , ^{33}P, ^{35}S and ^{14}C, belong to radiotoxicity group No.3.

Table 3.1. Classification of laboratories for handling radionuclides.

Group of radionuclide (radiotoxicity group)	Type of laboratory required for levels of activity specified below [1 Ci = 3.7×10^{10} Bq]		
	Type 1	Type 2	Type 3
1	< 10 μCi [< $3.7 \cdot 10^5$ Bq]	10 μCi to 1 mCi [$3.7 \cdot 10^5$ - $3.7 \cdot 10^7$ Bq]	> 10 mCi [> $3.7 \cdot 10^8$ Bq]
2	< 100 μCi [< 3.7·MBq]	100 μCi to 100 mCi [3.7 - $3.7 \cdot 10^2$ MBq]	> 100 mCi [> $3.7 \cdot 10^9$ Bq]
3	< 1 mCi [< $3.7 \cdot 10^7$ Bq]	1 mCi to 1 Ci [$3.7 \cdot 10^7$ –$3.7 \cdot 10^{10}$ Bq]	> 1Ci [> $3.7 \cdot 10^{10}$ Bq]
4	<10 mCi [< $3.7 \cdot 10^8$ Bq]	10 mCi to 10 Ci [$3.7 \cdot 10^8$ – $3.7 \cdot 10^{11}$ Bq]	> 10 Ci [> $3.7 \cdot 10^{11}$ Bq]

Modifying conditions	Multiplication factors for activity levels
Simple storage	x 100[a]
Very simple wet operations (e.g. preparation of aliquots of stock solutions)	x 10
Normal chemical operations (e.g. analysis, simple chemical preparations)	x 1
Complex wet operations (e.g. manipulation of powders) and work with volatile Radioactive compounds	x 0.1[a]
Dry and dusty operations (e.g. grinding)	x 0.1[a]

[a] These figures could be increased by one or more orders of magnitude if operations are carried out in closed boxes

Example.

As specified in Table 3.1, very simple wet operations with radionuclides of radiotoxicity group No.3 (e.g. ^{32}P, ^{33}P, ^{14}C and ^{35}S) can be performed in a type 1-laboratory up to an activity level of 10 mCi (or 3.7×10^8 Bq).

3.2.3 SAFETY REQUIREMENTS FOR RADIATION INSTALLATIONS

i General considerations.

When choosing open radioactive substances for specific applications, care should be taken

- To minimise levels of radioactivity and radiotoxicity.
- To minimise external exposure to radiation and the risks of radiation workers incorporating radioactive substances into their bodies.
- To avoid uncontrolled spreading of these substances by scattering, spillage or formation of gases, vapours, aerosols or dust; (e.g. by training in the handling with inactive substances, blind tests).
- Work with the risk of air contamination has to be performed in a fume hood.
- Air sucked from fume hoods, digesters, closed working chambers etc. may only be released following the national safety regulations.

- Radioactive substances should only be present in the working area in such quantities necessary for the intended chemical operation.
- Radioactive substances not needed for the chemical operation have to be stored in a fume hood or in a separate storage room following the relevant national safety regulations.
- Equipment and used materials may only be removed from the radiation area after removal of any unacceptable level of contamination (see Table 3.2).
- Radioactive waste has to be collected in separate containers for liquid and solid burnable, and non-burnable waste.
- Radioactive waste may only be disposed of following the relevant national safety regulations. A(as rule of thumb ^{32}P, ^{33}P and ^{35}S can be stored in appropriately labelled containers in appropriate storage facilities for a minimum shelf time of ten half-lifes and then disposed of in the normal, uncontaminated waste after the remaining activity has been checked to be negligible. **Note:** National safety regulations for radioactive waste disposal must be observed.

ii Responsibilities of an overall radiation safety organisation

The organisation in charge of an overall radiation safety program should be responsible for

- The formulation and implementation of appropriate protection regulations.
- The siting, location and design of radiation installations with particular reference to (i) the types of radiation sources to be used, (ii) environmental factors related with the disposal of radioactive material and to its dispersal both under normal and emergency conditions and (iii) the presence of occupied areas in the vicinity of the installation.
- Structural design features which would have a bearing on (i) the possible spread of contamination throughout the area and (ii) ease of decontamination.
- The setting up of house rules and well-defined operational procedures.
- The proper instruction of personnel in these rules and procedures.
- The provision of all necessary facilities for (I) personal monitoring, (ii) area monitoring, (iii) medical supervision and (iv) the maintenance of all relevant records.
- The drawing up of procedures for meeting emergencies and the provision of all the facilities necessary for carrying out these procedures.
- The maintenance of proper liaison with external agencies, such as the fires, police, transport and public health authorities.
- The maintenance of cumulative whole-body radiation exposure records covering both internal and external exposures.
- The initiation of appropriate action in cases of excessive exposures or radiation emergencies.

iii Location of a radiation installation in a building

When a radiation installation is part of a large building, the following points should be borne in mind when deciding on the location of such an installation.

- The installation should be located in a relatively unfrequented part of the building so that access to the area can be easily controlled.
- Fire hazard potential should be minimal in the area chosen.
- The location of the installation and the ventilation facilities provided should be such, that possibilities for the spread of both surface and airborne contamination are minimal.
- The location should be judiciously chosen so that, with minimum expenditure on shielding, radiation levels can be effectively maintained within permissible limits in the immediate vicinity.
- Separate ventilated radioactive waste storage facilities have to be designed depending on the expected levels of radiation[1]. Where only small quantities of radioactive materials are handled, they can be conveniently stored inside a fume hood, provided they are adequately shielded.

iv General safety rules for working with radionuclides

- A health physicist familiar with emergency procedures must be on the site during working hours.
- All operational personnel involved in radiation work should be trained by participation in appropriate radiation safety training programs in applying principle radiation protection procedures and the use of protective devices.
- Personnel should be furnished with written copies *of* radiation protection procedures.
- Personnel should be provided with all instruments and equipment (here in particular pocket dosimeters, film badges and β-contamination monitors, e.g. GM-counters) necessary to implement these procedures.

- Open radioactive substances never should be touched with bare hands, i.e. sufficient amounts of disposable rubber gloves should be available.
- Radioactive solutions never should be pipetted with the mouth, i.e. hand — or mechanical pipetting and dispensing devices should be available.
- Objects not necessary for the intended operations should be removed from the laboratory, especially food, tobacco products, medicines, and cosmetics.
- Foot operated radioactive waste bins (for burnable, non-burnable and liquid waste) should be provided.
- Paper towels and handkerchiefs should be available and used appropriately.
- Used paper towels etc. have to be disposed of as radioactive waste (solid, burnable).
- Regular checks for contamination of all workbenches has to be performed.
- Radiation workers have to check for contamination of hands, clothes, shoes etc. before leaving the radiation area.
- Relevant actions for decontamination have to be taken in when the permissible level of contamination is exceeded (seeTable 3.2).

v Special requirements for type 1-laboratories

- Warning signs with the label “RADIOACTIVE” placed at the entrance.
- Access only for authorised persons.
- Walls, floor and lab furniture should have smooth surfaces, easy to clean and non liquid absorbent (e.g. workbenches can be covered with disposable PVC foil, tightly fixed with tape).
- Only absolutely necessary furniture may be present.
- Portable radiation monitor (e.g. β-monitors i.e. GM-counters for ^{32}P,^{33}P and ^{35}S) available.
- Laboratory well ventilated and illuminated.
- Suitable washing facilities and possibly showers available.
- Relevant shielding against radiation (e.g plexiglass or perspex shielding with 0.6 cm (0.25 inch) wall thickness for shielding of β^--radiation up to 1 MeV, 2.5 cm (1 inch) for shielding of β^--radiation up to 4 MeV).
- Special washbasin for decontamination of used equipment should be available (generally minimal amount of washing water should be used).
- Depending on the nuclide and activity levels separate effluent lines leading to specially built storage/delay tanks of adequate capacity should be provided following the relevant national safety regulations.

vi Special requirements for type 2-laboratories

- All rules applying for type 1-laboratories.

Additionally:

- Workbenches made of fire inhibiting materials.
- Surface of floors may not absorb liquids and must be resistant to acids, organic solvents etc.
- Walls have to be painted with washable paint up to 3 m height; if the height of the room is less the ceiling has to be painted accordingly.
- Work where a risk of air contamination is possible have to be performed in closed decompression chambers including continuous control measurements of the surrounding air.

vii Special requirements for type 3-laboratories

- All rules applying for type 1 and type 2 laboratories.

Additionally:

- Type 3-laboratories are required to be located in special, separate buildings with continuous surveillance monitoring.
- Low pressure conditions, air filters, air continuously monitored for contamination.
- Building constructed of fire inhibiting and walls, floors etc.
- Access restricted to authorised, specially skilled personnel.
- Special protective clothes and shoes obligatory.
- Change rooms.
- Hand- and foot monitors at the entrance to the working area.
- Specially trained radiation protection personnel and health physicists present during operation and standby outside working hours.

Table 3.2. Maximum permissible surface contamination levels.

(a) Maximum gamma dose rate of contaminated surfaces: 0.5mR/h in 5 cm distance from surface

(b) Maximum activity levels of surfaces or objects which are contaminated with α- or β–radiation:

Surfaces or objects	Maximum activity levels per unit of surface (μCi/cm^2, [Bq/cm^2]) contaminated by:		
	Toxicity class 1		Toxicity class 2-4
	α-radiation	β-radiation	α-and β-radiation
Surfaces < 100 cm^2 ,	10^{-4} [3.7]	10^{-3} [37]	10^{-3} [37]
Lab equipment, glassware, tools surfaces $\geq$ 100 cm^2	10^{-5} [0.37]	10^{-4} [37]	10^{-4} [3.7]

(c) Maximum activity levels of clothes or shoes contaminated with α- or β–radiation:

Clothes	Maximum activity levels per unit of surface (average value over 150 cm^2 in μCi/cm^2 , [Bq/cm^2]) contaminated by:		
	Toxicity class 1		Toxicity class 2-4
	α-radiation	β-radiation	α-and β-radiation
Underwear, gloves	10^{-5}[0.37]	10^{-4}	10^{-4} [3.7]
Clothes, protective clothes, shoes	10^{-4}[3.7]	5 x 10^{-4} [18.5]	5 x 10^{-4}[18.5]

(d) Maximum activity levels of skin contaminated with α- or β–radiation:

skin area	Maximum activity levels per unit of surface contaminated by:		
	Toxicity class 1		Toxicity class 2-4
	α-radiation	β-radiation	α-and β-radiation
Hands	3 x 10^{-3} μCi [111 Bq] per hand	3 x 10^{-2} μCi [1.11·10^2 Bq] per hand	3 x 10^{-2} μCi per hand [1.11·10^2 Bq]
Skin on other parts of body (average over 30 cm^2)	10^{-5} μCi /cm^2 [0.37 Bq/cm^2]	10^{-4} μCi /cm^2 [3.7 Bq/cm^2]	10^{-4} μCi /cm^2 [3.7 Bq/cm^2]

[1] *Radiation Protection Procedures*, IAEA, 1973, Safety Series No.38, table 12.2.

3.3 PREPARATION OF RADIOACTIVELY-LABELLED FERTILISERS

3.3.1 PREPARATION OF RADIOACTIVELY-LABELLED MONO-CALCIUM PHOSPHATE

Mono-calcium-phosphate is prepared by dissolving the 'cold' phosphate in 5N HCl, adding the ^{32}P, then precipitating the MCP out of solution by increasing the pH with 10N NaOH. Great care must be taken to keep the MCP precipitate below 30^{O}C. This can be achieved by working with the solutions in ice and by adding the 10N NaOH very slowly. Once formed, the precipitate is rinsed through a Buchner funnel with a vacuum pump attached, and left to dry.

The recovery of MCP is low. Only about 50–60% can be achieved so this must be accounted for when calculating the amount required.

^{32}P is a dangerous isotope. Care must be taken at all times when handling.

Before attempting to make the labelled MCP, it is best to have a trial run with cold chemicals. This allows you to become familiar with the method and recovery.

To ensure safety when working with the isotope, you should wear full safety gear (gloves, mask, laboratory coat, plastic apron, film badge, etc.), work behind a perspex shield and work with everything in plastic, paper lined trays.

A. Equipment/materials

- Calcium tetrahydrogen d-orthophosphate [$CaH_4(PO_4)_2.H_2O$] (BDH G.P.R. chemical was found to give best results)
- Sodium hydroxide.
- Hydrochloric acid 32%.
- Potassium dihydrogen phosphate.
- Phosphoric acid.
- Beakers.
- Stirring rods.
- Trays.
- Tub.
- Ice.
- Plastic bags.
- Plastic sheeting.
- Perspex shielding.
- Elastic bands.
- Radiation stickers.
- Geiger Muller counter.
- Buchner funnel.
- Filter paper.
- Buchner flasks.
- Vacuum pump.
- ^{32}P, carrier free.

B. Method

- Determine amount of MCP to be prepared. Add 40–50% to allow for sample loss during preparation.
- Weigh MCP into a beaker.
- 5N HCl and 10N NaOH are required for the preparation of MCP. The volumes needed can be determined from the following ratios:
- for 1g MCP - 2.5mL 5N HCl and
  - 2.0mL 10N NaOH
- Add the 5N HCl to MCP, stirring continually to dissolve. This reaction is endothermic so doesn't need to be mixed on ice.
- Prepare approximately 50mL of 5% H_3PO_4. This is used for rinsing the ^{32}P into the MCP/HCl solution. It also aids in desorbing the ^{32}P from the glass.
- Carefully transfer the ^{32}P into the MCP/HCl solution using a pasteur pipette. Rinse the pipette and glass ^{32}P stock bottle copiously with 5% H_3PO_4 to remove all ^{32}P.
- Alternately, having transferred the majority of ^{32}P to the MCP/HCl solution and rinsing the vial several times with 50% H_3PO_4, the 'hot' vial can be immersed in the MCP/HCl solution. The vial can be left for approximately 30 minutes, by which time the ^{32}P should be evenly spread through the solution. Carefully remove the ^{32}P vial from solution and discard.
- Place the now labelled MCP/HCl solution into a tub of ice. Add the 10N NaOH. This must be added very slowly to ensure that the MCP does not get too hot. Stir continually.
- The pH of the solution should be around 7. Check with pH paper. As the NaOH is added, the MCP should precipitate out of solution and form a thick slurry.
- Filter the precipitate through a buchner funnel with a vacuum pump attached. The best set-up for this procedure is as follows:
- place a large filter paper (No. 42 Whatman) in a Büchner funnel so that the base and sides of funnel are covered by the paper. The paper should form a basin inside the funnel so that the MCP can be poured into the paper and not leak over the sides of the paper.

- place the funnel in a Büchner flask. The Büchner flask should be attached to another Büchner flask. This second flask should contain a saturated solution of CaOH and cottonwool to act as a ^{32}P trap should any isotope be sucked through.
- the vacuum pump is attached to the second flask. When the pump is operating, air should be pulled through the filter paper, through the ^{32}P trap and then to the pump.
- Rinse the slurry with distilled/deionised H_2O as it is being drained on the filter paper. Water equivalent to about half the volume of the MCP slurry should be sufficient for rinsing.
- Leave the precipitate to air dry. This may take several days. When dry, transfer to a labelled container stating:
  (i) contents
  (ii) user
  (iii) activity
  (iv) date
- Due to the higher energy of the ^{32}P the MCP should be stored in a glass dessicator to contain the beta emission.

3.3.2 PREPARATION OF RADIOACTIVELY-LABELLED GYPSUM

Gypsum ($CaSO_4.2H_2O$) is prepared by mixing calcium chloride and sulfuric acid. A thick white precipitate is formed which is dried and ground to a fine white powder.

If care is taken with precipitate transferral and rinsing, a recovery of >95% can be achieved.

Before attempting to make the labelled gypsum it is best to have a trial run with 'cold' chemicals. This allows you to become familiar with the method and recovery.

Safe working practices must be adhered to at all times.

A. Equipment/materials

- $CaCl_2.2H_2O$.
- H_2SO_4 (98%).
- Beakers.
- Stirring rods.
- Evaporating dish.
- Wash bottle.
- ^{35}S - as sulfate in aqueous solution.
- Trays.
- Geiger Muller counter.
- Pasteur pipettes and bulbs.
- Plastic bags.
- Plastic sheeting.
- Radiation stickers.
- Elastic bands.

B. Method

- Quantities of calcium chloride and sulfuric acid required must be determined. This can be calculated from the following equation:
- $CaCl_2.2H_2O + H_2SO_4 \rightarrow CaSO_4.2H_2O + 2HCl$
- Molecular weights 147.02 g + 100.08 g → 172.17g
- If 6.885 g $CaSO_4.2H_2O$ is to be prepared, then
- Amount of $CaCl_2.2H_2O$ required $= \frac{147.02}{172.17} \times 6.885\text{g} = 5.88\text{ g } CaCl_2.2H_2O$
- Amount of H_2SO_4 required $= \frac{100.08}{172.17} \times 6.885\text{ g} = 4.00\text{ g } H_2SO_4$
- Weigh $CaCl_2.2H_2O$ into a beaker and dissolve in a minimum amount of distilled/deionised H_2O.

- Weigh H_2SO_4 into a beaker. H_2SO_4 needs to be diluted approximately 1:6 with H_2O otherwise the precipitate forms too quickly when mixed with $CaCl_2.2H_2O$.
- Divide the H_2SO_4 into two containers, one containing the majority. Mix the ^{35}S with the H_2SO_4. This can be done by adding the ^{35}S into the larger of the two amounts of H_2SO_4 and then rinsing the hot vial several times with the other H_2SO_4 into the now labelled H_2SO_4.
- Slowly add the $CaCl_2.2H_2O$ to the labelled H_2SO_4 with gentle stirring. The precipitate should develop gradually. Rinse the $CaCl_2.2H_2O$ into the H_2SO_4 with H_2O.
- Mix well to get a smooth, sloppy paste.
- Carefully transfer the precipitate to a preweighed evaporating dish. Precipitate can be dried at room temperature, or can be placed in a fan-forced oven at 25–30^{o}C to speed up drying.
- While gypsum is drying, stir regularly to prevent precipitate drying into a hard lump. As it dries, crush up finely.
- When the gypsum is completely dry, weigh dish and contents to determine % recovery.
- Store gypsum in a clearly labelled vial stating:
  (i) contents
  (ii) user
  (iii) activity
  (iv) date
  The labelling is important. It should alert other persons to the content's radioactive danger.

3.3.3 PREPARATION OF RADIOACTIVELY-LABELLED ELEMENTAL SULFUR

Elemental sulfur is prepared by dissolving cold crystalline elemental sulfur in toluene, adding ^{35}S, then recrystallising the sulfur. Once dry, the sulfur is sieved to size (150 □m-250 □m).
If care is taken with sulfur preparation and sieving, a recovery of >90% can be achieved.
Before preparing the labelled elemental S, a trial run should be carried out.

A. Equipment/materials

- S-free toluene
- ^{35}S - as elemental sulfur.
- Beakers.
- Hotplate.
- Foil.
- Pasteur pipettes and bulbs.
- Trays.
- Plastic bags.
- Plastic sheeting.
- Radiation stickers.
- Elastic bands.
- Geiger-Müller counter.

B. Method

- Determine amount of elemental S fertiliser required. Add l0–20% to allow for sample loss during preparation.
- Weigh cold, crystalline elemental S into a small beaker.
- Take a small portion of this elemental S and transfer into another beaker. This portion will be used for rinsing the ^{35}S into the larger portion.
- To both beakers of elemental S add just enough toluene to dissolve crystals. Generally, 1mL toluene/150 mg elemental S. A third container of approximately 5–10mL toluene is also required.
- On a foil-covered hotplate, gently heat the larger elemental S portion to dissolve. Agitate gently and avoid splashing crystals up the sides of beaker.
- When the larger portion is almost dissolved, start heating the smaller portion. This should dissolve very quickly. The extra toluene can also be heated at this stage.
- Keep both portions warm but do not boil. Do not allow to cool, otherwise the sulfur will recrystallise.

- Using a pasteur pipette, transfer one addition of the warmed smaller portion to the ^{35}S crystals. The ^{35}S vial may be placed upon the hotplate to aid dissolution if the warmed addition is not effective. Once dissolved, transfer the ^{35}S to the larger portion. Continue rinsing the ^{35}S vial by adding the warmed smaller portion and transferring to the larger portion. Rinse the ^{35}S vial several times with the third container of warmed toluene to ensure maximum transferral.
- Mix the final solution well. Remove from heat. Crystals should start developing quickly but it may take several days for the excess toluene to evaporate. To speed up evaporation, leave sample uncovered in fume hood with fan operating.
- When crystals are completely dry sieve to size. Any crystals which fall outside the required dimensions may be redissolved and sieved again to boost recovery.
- Keep a check of the recovery by noting (i) final weight of elemental S liberated, and (ii) activity of the elemental S. This can be done by taking a known weight, dissolving in scintillation fluid, heating if necessary, and counting by liquid scintillation.
- Store crystals in a clearly labelled vial stating:
  - (i) contents
  - (ii) user
  - (iii) activity
  - (iv) date

3.4 PREPARATION OF PLANT SAMPLES FOR ANALYSIS

3.4.1 INTRODUCTION

Plant analysis is carried out for two basic reasons: for monitoring the nutrient status of plants or for diagnosis of a problem. It is important that the reasons for taking particular plant samples for analysis are appreciated, as they affect the way the samples are taken. However, in many respects the principles behind the collecting, handling and analysing of samples are the same irrespective of the aims of the sampling. Unless due care is taken with all these steps, the results will be unreliable and may result in misleading interpretations.

The following are broad guidelines for sampling and preparing plants for chemical analysis.

A more detailed discussion, from which much of this section is taken, is provided by Reuter et al., (1986).

3.4.2 SAMPLING

A. Collecting representative samples

The principal objectives in any sampling programme are to collect samples, which are representative of the situation being examined and which contain sufficient material for a laboratory to analyse. For a full range of tests many testing laboratories suggest they require around l00g of fresh material. With careful sampling and sub-sampling or when only a small number of analyses are required, much smaller quantities can be used.

When samples are being taken for monitoring purposes (that is, to assess management practices, such as fertiliser applications and irrigation, for comparison with previous crops), the samples must be collected to represent the average for the particular crop or pasture. When samples are being taken for diagnostic or troubleshooting purposes (that is, a problem is perceived which may be due to a nutritional imbalance), the samples should be taken from plants with the perceived problem and, where possible, nearby plants without the problem. In this "point" (for diagnostic) as opposed to "average" (for monitoring), sampling the collected samples are assumed to be representative of the healthy and the less healthy populations.

In either case, samples should be collected from plants distributed evenly throughout the particular area or areas of interest in the crop, pasture, etc. The selection strategy adopted should be systematic, repeatable and convenient.

There are various techniques for sampling large areas of crop or pasture. (These are most important when sampling for monitoring purposes but may be used for diagnostic sampling where there are large areas of affected plants.) In the most complex techniques, the area to be sampled is divided into a number of blocks (say 4), then each block is sub-sampled, either systematically throughout or from a small uniform sampling area within each block. These samples are then bulked and, if necessary, mixed and sub-sampled, before analysis. Less complex techniques

involve collecting samples from a single strip or small area within the crop or pasture and assuming this to be representative of the whole area. Where a single sampling area is chosen as representative of a large area, this monitoring plot can be established as a permanent monitoring plot.

If the crop or pasture to be monitored is not very uniform, then the area should be divided up into sections that are more uniform, perhaps on the basis of soil type or aspect, and each area sampled and analysed separately.

Plants that are obviously poor in health because of disease, insect damage or some other non-nutritional problem, should not be sampled unless they are of particular interest. These factors influence tissue composition and so may mislead a diagnosis based on the tissue analysis.

Unless specifically required, only one species of plant should be sampled to find the nutrient status of a pasture. Pasture species can differ markedly in their nutrient composition, even when plants are growing side-by-side. Therefore, in mixed swards or crops, either one species should be sampled and analysed, or different species should be sampled and analysed separately.

B. Stage of maturity and tissue type

All plants selected for sampling should be at a similar stage of maturity. Nutrient composition of plant tissues change with maturity; for example, concentrations of nitrogen, potassium and many other nutrients are highest in very young plants. These nutrients become diluted by subsequent growth and so are lower in mature plants. Differences in the stage of maturity can, as a result, complicate the interpretation of tissue analyses. The effect is minimised by restricting sampling to a specific developmental stage, generally when tissue composition is most stable. For most crops this is usually around early flowering, although if the results of sampling are to be used for corrective fertilizing, this may be too late. For pastures, a particular stage of maturity is generally translated to a particular season. Since these results are more commonly for assessing next year's fertiliser program, samples are usually taken late in the season when the plants are still growing rapidly, but after the initial very rapid growth in early spring.

The nutrient status of whole plants changes with maturity and so does the nutrient status of the constituent parts. For most crops tissue analysis is conducted on a sample of leaves or petioles. The nutrient composition of leaves changes with physiological age, position on the plant (i.e. height, aspect) and with development of fruit or grain. For this reason most published information on plant nutrient status is only for a particular stage of maturity but, for particular tissue types, the most commonly sampled tissue is the most recently matured leaves. The nutrient composition of young leaves is usually changing very rapidly and it is therefore difficult to assess adequate, deficient or toxic levels. The nutrient levels of old leaves is largely a product of nutrient and carbohydrate re-translocation. However, very young or old tissue is sometimes sampled for particular nutrients, depending on their mobility around the plant. If the appropriate sample tissue is not known, it is customary to sample only recently mature leaves, avoiding leaves which are very young or very old.

C. Other considerations when sampling

Avoid sampling soiled, diseased and insect or mechanically-damaged plants, and exclude senescing and dead tissue from the gathered material.

Avoid sampling plants growing within areas which have unusual features (e.g. rocky areas, animal resting areas, headlands, firebreaks, etc.).

Do not sample when plants are under water or temperature stress; also avoid sampling waterlogged plants. Sampling should normally be undertaken when plants are growing vigorously.

Avoid sampling vegetative organs after flowering in non-woody determinate species since many nutrients are redistributed to developing fruits and grain.

Minimise sample contamination. Take particular care when sampling for trace element determinations. Use clean plastic gloves and stainless steel cutting implements when sampling and avoid contact with soil, copper and galvanised materials. If samples have to be washed deionised water must be used. Such things as foliar sprays, fungicides, dust, etc., which may contain trace elements, can be reduced by rinsing, but this must be done when the sample is still fresh. More refined decontamination procedures may be carried out in the laboratory, if required.

If subsamples taken for dry weight measurement and nutrient analysis are taken to be representative of larger samples weighed in the field when fresh, then the fresh weight of the subsample must be measured at the same time, with the same balance and under the same conditions as the main sample.

Standardise sample collection during certain periods of the day for elements for which the concentration is known to vary diurnally (Reuter and Robinson, 1986).

i Sample transport

Delays and adverse conditions during transport of fresh samples can cause substantial respiratory losses in weight or enhanced enzymatic activity, both of which produce corresponding errors in nutrient determinations (e.g. Bradfield and Bould 1963; Mayland, 1968; Leece 1972).

The following guidelines are aimed at reducing transport errors:

sampler should wash his/her hands before collecting samples, or use disposable gloves;

place material in labelled open paper bags and place immediately in cooled containers or car refrigerators (at 5^{o}C) for transport from the field;

soil adhering to the plants should be removed by washing basal shoots briefly in deionised water or by brushing with tissue paper;

on return from the field, samples may be separated into appropriate parts for analysis. Roots should be washed thoroughly in several rinses of running water and placed in labelled paper bags;

if appropriate, a fresh weight measurement of shoots and roots may be done at this time, prior to drying (see "Other Considerations when Sampling").

ii Sub-sampling

Sub-sample preparation

Samples harvested from field plots are generally large so as to overcome site variation and more accurately measure yield, etc. Consequently, there is usually a need to take representative sub-samples. Prior to sub-sampling and drying the fresh weight of the total sample must be recorded so that all analyses can be converted back to the original field samples.

Procedure

Spread sample out on a large clear plastic sheet and mix well

Halve the sample and then quarter it

Discard 3 of the 4 quarters

Mix remaining quarter, then half and quarter it as before

Discard 3 of the 4 quarters. This "halving and quartering" procedure is continued until the desired sub-sample size is obtained (usually 20–300g)

Record the fresh weight of the final sub-sample so field fresh weights can be converted back to oven-dry (or constant moisture %) values.

NOTE: As the ratio of sub-sample weight to total sample weight decreases, greater care in sample mixing is required.

A data sheet for plant sampling and analysis procedures encourages clearly labelled and well organised data which increases efficiency.

Botanical separation

Where a sub-sample contains different species (or different plant tissues), a further separation stage is often required. Examples of two methods of sample separation are given below.

METHOD I - Spread the sub-sample on a table, mix and divide in half. Place one half in a labelled bag marked "complete sample" and dry. Separate the other half on the basis of botanical species (or tissue types) into different bags for each species and dry.

METHOD 2- Spread the sub-sample on a table and separate out desired species (or tissue types). Place in labelled bags and dry.

Care should always be taken to make sure that labels are both complete and correct.

3.4.3 SAMPLE DRYING

Normally two drying cycles are necessary before plants can be analysed chemically.

A. Initial drying cycle

This rapidly de-activates all plant enzymes and removes all water from plant tissue; the sample reaches an "oven-dry" state, in which its weight remains unchanged with renewed drying.

Generally samples are dried at 65–70^{O}C in a forced draught, stainless steel-lined oven, which allows adequate circulation of air between samples, for a minimum of 24 hours, depending on sample size. Prolonged drying or drying at temperatures in excess of 80^{O}C can promote thermal decomposition and appreciable loss of volatile constituents.

B. Final drying cycle

Following initial drying, samples may be weighed and then ground and stored for a period prior to analysis. The finely ground material may absorb moisture that will necessitate a second drying cycle (e.g. 70^{O}C for 12 hours) immediately before sub-samples are weighed for chemical analysis. Plant concentrations can then be expressed on an oven-dry basis.

3.4.4 GRINDING AND STORAGE

Dried samples are customarily ground to reduce field samples to manageable sizes for storage and to facilitate the preparation of homogeneous sub-samples for chemical analysis. However, grinding is not essential. Small samples need not be ground, instead the whole sample can be cut into small pieces or simply crushed by hand and then weighed for analysis.

Mechanical grinding can be carried out in a variety of mills. These should have stainless steel grinding surfaces to minimise contamination. Where iron is to be determined, an agate mortar and pestle or a tungsten carbide mill with teflon-coated blades should be used.

During grinding, particular care must be taken to ensure that the sample is not contaminated and that it does not separate into coarse and fine particles.

Separation on the basis of particle size can be a significant source of error, particularly with samples containing fibrous stems and succulent leaves which pulverise at different rates and contain markedly different nutrient concentrations.

It is recommended that samples be ground to a particle size of less than I mm and mixed thoroughly before sampling for analysis.

Samples should be stored in clearly labelled, airtight containers. Glass or polycarbonate containers are convenient as they can withstand the heating involved in a second drying of the sample, if required.

For convenience, the container should also be of sufficient size to enable thorough mixing of the sample prior to weighing (i.e. container should be approximately half full). Mixing is necessary since settling occurs on storage, resulting in unrepresentative samples being taken for analysis.

The samples should ideally be stored in a dark, cool and dry environment.

When weighing a sub-sample for analysis, care should be taken to ensure that the ground material is representatively sampled by taking small spatula quantities from throughout the container, with thorough mixing between sub-sampling.

A typical sample weight sheet is as follows:

DATE:				NUTRIENT		DIGEST		
Sample code	Sample weight	Tube #	Vial #	Reading	Conc.	Reading	Conc.	Comments

3.5 PREPARATION OF SOIL SAMPLES FOR ANALYSIS

A. Sampling

Many of the principles that apply to sampling of plants (Section 3.4) also apply to the sampling of soils.

Soil sampling can be done for diagnostic or troubleshooting reasons, where poor crop or pasture growth is noted in one area, but more commonly, samples are collected to monitor current fertiliser and other management practices and to plan for future fertiliser applications.

The main aim in sampling is that the sample be truly representative of the soil in the area being sampled. Soil properties often vary markedly over short distances and with depth, and different properties vary to different extents. Sampling techniques must be used which minimise these variations.

B. Where to sample

The area to be sampled should be as homogeneous as possible. Different areas should be separated on the basis of soil type, topography, crop or pasture growth and management history. As with plants, there are various techniques for sampling large areas of soil. One approach is to divide each homogeneous area into a number of blocks, then sample these using a systematic grid or zigzag sampling system, and bulk these. Another approach is to set up a monitoring site, or several sites, within each homogeneous sample area. A number of samples are taken from these monitoring sites and bulked for analysis. Where sampling is going to continue over time, it has been argued that these monitoring plots should be set up and the same sites used over time (Friesen and Blair, 1984). This should allow more reasonable estimates to be made of soil test values with time.

When sampling, atypical sites should be avoided such as dung or urine patches, paths, waterlogged areas (unless these are the main interest), minor water courses or irrigation channels, tree stumps or their ash residue after burning, fertiliser dumps, and even fertiliser granules where recently applied.

The depth of sampling depends largely on the agricultural system. For pastures, samples are usually taken to 7.5 or 10cm, for crops to 10 or 15cm, and for deeper-rooted tree crops, samples to 1m may be required. Particularly for deeper samples, or for specific soil characteristics such as salinity or certain physical characteristics, separate samples should be taken from different depths. This can be done on the basis of fixed intervals (often smaller intervals are sampled near the surface) or, if possible, on the basis of soil horizons.

C. When to sample

Temperature, moisture, crop growth and crop residue levels will all affect soil characteristics, although different characteristics will be affected to different degrees. The result is that soil test levels will vary over time.

When samples are taken depends on the characteristics being measured and the reason for the sampling. For most annual crops, where fertiliser and other management strategies are being planned, samples are normally taken before land preparation. For pastures, an appropriate growth phase/soil organic matter turnover phase, such as the rapid growth and organic matter turnover

period in spring, is usually selected. In either case, if soils are being monitored repeatedly, it is important that the samples are taken at the same time each year.

D. Number of samples to take

As the variability of soil properties differ, so do the number of samples required to adequately characterise an area. Some studies have shown that several hundred samples may be required to adequately characterise some soil properties. This is generally impractical with around 20 samples for a 20ha area being more normal. With this sort of variability, the choice of a systematic and representative sampling technique is vital and makes the monitoring site technique attractive, where appropriate.

E. How to sample

Samples are usually collected using corers (tube), augers or spades/trowels. To avoid contamination, the sampling equipment must be clean, the samples should be placed in clean containers which can be sealed as soon as possible, and handling of the samples should be avoided or kept to a minimum, especially when exchangeable sodium and potassium are being measured since perspiration can affect these.

It is also important to remember to clearly label the samples with a permanent marker and to record as much information as possible about the sample site. This information should include a detailed description of the location (perhaps including a grid reference), the date, the depth sampled, the current and past cropping programmes, including fertilisers and other soil amendments, the reasons for the analyses and which characteristics are to be measured.

3.5.1 DRYING

Once collected, soil samples must be treated with care. If contained in plastic bags, samples must be kept cool to prevent sweating, and hence chemical change, before drying and analysis. Soil samples need to be dried as quickly as possible to minimise biological, chemical and physical changes that can occur when stored moist. The amount of drying required ultimately depends on what analyses are to be determined.

If drying is carried out carefully, biological and chemical changes can be limited so that the soil characteristics remain similar to those in the field. Particular problems are experienced when working with flooded soils as obviously any degree of drying will significantly affect the soil conditions and therefore chemical and physical characteristics. Special consideration must be given to such soils.

Soil samples are usually partially air-dried (until not sticky) at a temperature of 25–35^{O}C and a relative humidity of 20–60%. Alternatively, soils can be dried in a fan-forced oven at 40^{O}C for at least 24 hours. The soil should be in open plastic bags, or similar containers, to minimise contamination. Once dry, soil can be stored in clearly labelled, capped vials, ideally in a cool room at <5^{O}C.

Many determinations are not significantly affected by complete air drying for storage purposes however, there are exceptions. Some analyses must be carried out on moist samples immediately after collection. Examples are: determinations of exchangeable ferrous iron and manganese; exchangeable potassium, acid extractable phosphorus and nitrate nitrogen. Storage of soils to be analysed for these determinations is best carried out by freezing or freeze drying.

Soil samples can be dried in an oven at a higher temperature, say 105^{O}C, for 24 hours. However, this is only recommended for limited analyses such as total element determinations. Air drying is by far the best for the majority of analyses.

3.5.2 GRINDING

Once dry, soil samples need to be ground to break up aggregates and form a homogeneous sample for many analyses. Clay soils are best crushed to pass the sieve before they reach complete air dryness, otherwise the crushing process is difficult. Crushing the primary sand and gravel particles is generally avoided. For most of the analyses outlined in this Handbook the soil samples need only be ground to pass a 2mm sieve, however, other determinations may require much finer grinding.

3.5.3 SIEVING

Some determinations call for soil fractionation, however for the analyses outlined in this Handbook, this is not necessary.

If samples are to be ground and sieved, they should be ground before sieving otherwise bias may arise from fractionating the soil portions. In most cases the whole soil sample should be passed through the sieve. The bias that arises from only sieving a portion of the soil sample usually results in increased concentrations of most elements measured to assess soil fertility. After sieving, the remaining coarse material should be re-ground and re-sieved. Continue the screening and crushing until all aggregate particles are fine enough to pass a 2mm sieve. Discard the stones and organic residues that remain.

For trace element analyses, such as copper, zinc and other heavy metals, copper and brass utensils must be avoided. Stainless steel sieves are an excellent alternative. Aluminium and iron can be used, but these are not as robust as stainless steel and may cause contamination if aluminium and iron are being studied.

3.6 CONDUCTING A FIELD EXPERIMENT USING STABLE ISOTOPES

3.6.1 INTRODUCTION

Some stable isotopes can be used under field condition. ^{15}N is the most used isotope but other, e.g. ^{13}C and more recently ^{2}H, ^{18}O and ^{34}S are gaining importance. The main limitation to the use of isotopes is the cost, which tends to limit the size of the experiments. Therefore it may not be possible to use the same sized plots as in conventional field experiments. However, isotope measurements tend to have lower coefficient of variation as compared to yield measurements.

3.6.2 PLANNING OF THE EXPERIMENT

First step before setting up an isotope field experiment is to carefully formulate the objective. It is important that the aim is clear and that only one or two questions are to be answered. An experiment with ten objectives will probably not give any answer.

The second step will be to design the treatments that are likely to give solutions to the problem being investigated. Do not include unnecessary treatments as they will only make the experiment larger. Do not forget to include control treatments.

At this stage the experimental design will have to be selected (Little and Hills, 1975) and the number of replicates determined. Will you use a completely randomised design, randomised complete block design, latin square design, split-plot design, split-split-plot design or another design. It is important to select the design based on the statistical analyses to be done. Bear in mind that you need to receive an answer to your questions, which is supported by statistics.

3.6.3 SOIL PREPARATION

The land selected for the experiment should be as homogenous as possible and representative for the area or conditions to be studied. It is important to consider the history of the experimental area. What was the previous crop? Is it likely that previous crops or treatments will affect the experiment? Was an isotope experiment previously conducted in the field? Was there a crop grown on the experimental area to remove any residues of the tracer? All these questions are important when selecting an area for a field experiment. After selecting the area the land can be prepared. This may include conventional tillage or no-tillage.

3.6.4 PLOT SIZE

It is difficult to give a general rule on the size of the isotope plots. Increasing the size of the plots is likely to reduce experimental error but at the same time increase the cost. In general it is sufficient to harvest about 30 plants/plot. This will be easy if the crops being grown are wheat, barley, alfalfa or any type of grass or pasture. For larger plants such as maize or any tree crops the number of harvested plants will have to be reduced which will probably increase experimental error. *Figure 3.1* shows a schematic layout of an isotope plot. The ^{15}N should be applied to the whole plot (a x b) and only the inner area (c x d) should be harvested leaving the borders. The yield should be calculated based on the harvested area.

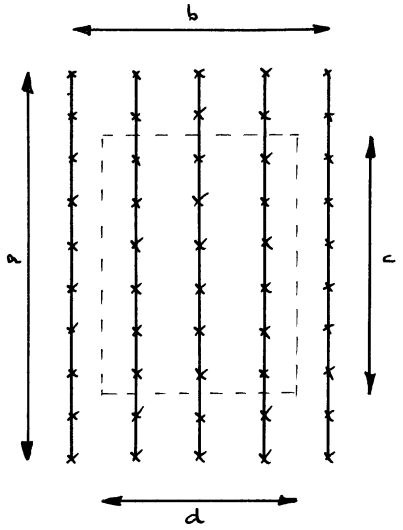

Figure 3.1. A schematic layout of an isotope plot.

It is important to note that the plants being cultivated should not be able to take up any N outside the labelled area hence the rooting patterns are important. In some cases it has been necessary to dig drenches and mount barriers (plastic or metal) between the plots to limit root growth. For most crops this is not needed.

Most isotope plots will be 1–3 m^2. These isotope plots will not be large enough to give a reliable measure of yield. For that purpose 10–12 m^2 will be needed. The isotope plots may be located within the yield plots as shown in *Figure 3.2*.

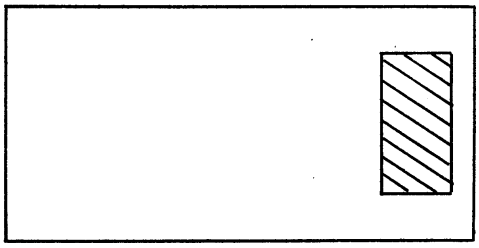

Figure 3.2 Isotope plot placed within an yield plot.

3.6.5 LAYING OUT OF PLOTS

After the experiment has been planned draw a schematic plan of the experiment including all the treatments, size of plots, inter-row spacing, size of alleys between plots etc. Depending on experimental design the treatments within each block should be randomised at this stage using a table of random numbers.

3.6.6 LABELLING OF POTS AND PLOTS IN GREENHOUSE AND FIELD EXPERIMENTS

The labelling of the pots/plots in any agricultural experiment is crucial for the identification of treatments and the samples collected. It is not possible to give comprehensive guidelines on this subject here but only few suggestions. In general the labelling has to be very simple to avoid any mixing of the samples.

Table 3.3 gives an example how pots/plots could be labelled. In this experiment five treatments are to be tested with four replicates. Individual pots/plots will have two number, the first indicating the treatment and the second the replicate. This type of table can be extended as needed.

Table 3.3. Labelling of pots/plots in greenhouse or field experiments. Example 1.

	a) TREATMENTS				
Replicates	1	2	3	4	5
1	1-1	2-1	3-1	4-1	5-1
2	1-2	2-2	3-2	4-2	5-2
3	1-3	2-3	3-3	4-3	5-3
4	1-4	2-4	3-4	4-4	5-4

It may be useful to skip some of the numbers if there are related treatments. For example, as shown in Table 3.4 treatments 1, 2 and 3 are cultivars without N and 11, 12 and 13 are the same cultivars with N and all treatments are replicated four times.

Table 3.4. Labelling of pots/plots in greenhouse or field experiments. Example 2

			TREATMENT			
Replicate	1	2	3	11	12	13
1	1-1	2-1	3-1	11-1	12-1	13-1
2	1-2	2-2	3-2	11-2	12-2	13-2
3	1-3	2-3	3-3	11-3	12-3	13-3
4	1-4	2-4	3-4	11-4	12-4	13-4

In our experience it is best to use pencil to label the individual pots in greenhouse experiments as writing by some markers tends to disappear with time.

It will be necessary to randomise the treatments within each replicate before conducting the experiments. Use tables of random numbers.

3.6.7 SOWING

Most isotope experiments are sown by hand as it is difficult to find suitable sowing machines for such small experiments. Try in this case to limit the error by having one person or a group of people doing each replicate.

If the soil is dry water after sowing to facilitate germination.

3.6.8 ISOTOPE APPLICATION

There are various methods of isotope application varying timing, placement and incorporation. In general it is sufficient, if a mass spectrometer is used for the analyses, to apply 0.1 g $^{15}N/m^2$. Table 3.5 shows the rates and suggested enrichments. If an older type emission spectrometer is being used for the analyses the enrichments may have to be doubled.

Table 3.5. Rates and suggested enrichments for a field experiment

Rate of application (kg N/ha)	Enrichments (% ^{15}N atom excess)
200	0.5
100	1
20	5
10	2.5

In most fertiliser recovery experiments the tracers have to be applied according to the fertiliser treatments, taking care of placement, split application or fertiliser form. This may be performed after the sowing, or during the growing period.

In most isotope dilution experiments the isotopes are applied as tracers and not as fertiliser. The objective is in this case to label the substrate so that nutrients from other sources, e.g. atmosphere through biological nitrogen fixation, crop residues or sewage sludge, can be quantified. The best method for labelling will be to apply the tracer well in advance of the experiment, e.g. incorporate the tracer into a preceding crop, which is ploughed under. This may, in fact, be necessary when organic residues are being incorporated into the soil (Hood et al., 1999).

It is very important that the tracer is applied uniformly to the plots. Dissolving the fertiliser in water and using aliquots for each plot can facilitate this. These aliquots may be diluted further into 3–5 litres of water that is applied uniformly to the plots. Precaution should be taken not to apply the fertiliser when the soil temperature is very high due to direct ammonia volatilization.

The experiment should be well maintained with the objective to be able to measure the effect of treatments used in the experiment. All unwanted external factors, which are not part of the treatments, should be limited as far as possible, e.g. water has to be applied if there is not enough rain; spraying has to be done if the experiment is affected by any type of infection; a fence has to be used if animals are grazing in the neighborhood etc.

3.6.9 HARVESTING

Harvesting can be done sequentially, during physiological maturity or during full maturity. The time of harvest will depend on the objectives of each experiment. Sequential harvesting may be needed, if one likes to understand processes during crop growth. Most experiments measuring biological nitrogen fixation or fertiliser recovery will be harvested at physiological maturity, i.e. before senescence and loss of leaves and experiments having the objectives to measure yields will be harvested at full maturity.

Harvesting is usually done by cutting the inner area of the experimental plots leaving boarders all around. At the time of harvest, the plots of reference crop should always be harvested at the same time as the plots with N fixing crops. The pods and straw usually have very different %N and $\%^{15}N$ a.e. Therefore it is not possible to take directly representative sub-sample from those plant parts when mixed. The plants should therefore be separated into pods and straw. These parts are then weighed and sub-sampled after chopping into small fragments. Materials with lower $\%^{15}N$ a.e. should be chopped first to minimize contamination from one sample to another. In any case, the forage chopper, if used, has to be cleaned thoroughly between treatments. Sub-samples should be ground after drying at 70^0C. These samples can then be analysed for total N by the Kjeldahl procedure. The $^{15}N/^{14}N$ ratio can be analysed by either emission or mass spectrometer as described by (this volume).

3.6.10 CALCULATION

After the analyses of % ^{15}N abundance in the plant and fertiliser samples, $\%^{15}N$ atom excess has to be calculated by subtracting the $\%^{15}N$ natural abundance (0.3663%) from the $\%^{15}N$ abundance in the sample. The $\%^{15}N$ atom excess or derived values are used for all the following calculations, e.g. recovery from various sources.

3.7 CONDUCTING A POT EXPERIMENT USING STABLE ISOTOPES

3.7.1 INTRODUCTION

Only some brief guidelines on how to conduct pot experiment using stable isotopes will be mentioned in this chapter and reference made to further reading. The same guidelines will apply to any pot experiments whether they are conducted in a greenhouse or outdoors.

It is important to know that pot experiments have certain limitations and they should only be used to test hypothesis, methods or material. It is very unlikely that the results from a pot experiment can be interpolated directly to field conditions. However, it may be useful to test various isotopic methods in pots before a large scale field experiment is conducted. Also, due to environmental considerations, it may only be possible to conduct pot experiments with certain isotopes.

3.7.2 POTS

There are many different possibilities in terms of material (clay or plastic) or sizes (0.5 kg to 10 or 20 kg or larger pots). It is usually better to use as large an amount of soil as possible but the space in the greenhouse is usually the limiting factor. When selecting the pots it is important to decide whether there should be drainage or not. Holes in the pots will allow drainage but it might be necessary to place saucers under each pot.

3.7.3 SUBSTRATE

Various substrates can be used in pot experiments and this will depend on the objective of the study. Inert material (vermiculite or washed sand) may be used in nutrient studies and the nutrients for plant growth supplied by solutions (See section 3.2.5).

Any type of soil, depending on the objective of the experiment, may be used for a greenhouse experiment. The soil should be sieved (2 cm mesh) to remove the largest stones or gravel. It may be useful to mix the soil with sand (50:50) if roots are to be excavated from the substrate during harvest.

The amount of substrate in each pot will have to be weighed.

3.7.4 AMENDMENTS

These can be anything between a complete nutrient solutions (See nutrient solutions) to amendments of missing elements (N, P, K or micro-nutrients) or amendments to correct or modify physical or chemical characteristics ($CaCO_3$). Amendments should always be reported as mg or g / kg soil. Most scientists are used to expressing fertiliser rates in terms of kg/ha. These have to be converted to mg/kg soil in a greenhouse experiment. This is usually done by assuming that one ha has 2 million kg of soil (Table 3.6).

Table 3.6. Relationship between rates of amendments in field and pot experiments assuming that 1 ha has 2 million kg of soil.

Field experiment (kg/ha)	Pot experiment (mg/kg soil)
200	100
100	50
50	25
20	10

Do not use kg/ha when reporting data from pot experiments.

3.7.5 NUTRIENT SOLUTIONS

It is important that, as far as possible, the concentration of ions in the solution does not vary between treatments, except for the concentration of the treatment ion. To this end, a series of 21 stock solutions (Table 3.7)) have been developed by Lisle et al., (2000) that allows the removal of a particular macro- or micro-nutrient with no or little affect on the concentration of other nutrients in the solution. In this way, any affects can be directly attributed to the omission of the particular ion and the relevant fertility status of the soil.

Table 3.7. Concentration of the stock solutions and the dilutions made for the nutrient solutions used in the study

Compound	Amount added	Conc.	Complete	-N	-P	-K	-S	-Ca	-Mg	-Zn
	(g/L)	(M)	----------mL/L ----------							
Macronutrients										
$Ca(NO_3)_2.4H_2O$	236.10	1.00	3.0	-	3.0	3.0	3.0	-	3.0	3.0
KNO_3	101.10	1.00	2.0	-	2.0	-	-	4.0	0.5	2.0
$NH_4H_2PO_4$	115.00	1.00	2.0	-	-	2.0	-	2.0	2.0	2.0
$MgSO_4.7H_2O$	184.80	0.75	1.0	1.0	1.0	1.0	-	1.0	-	1.0
KCl	149.00	2.00	1.0	1.0	1.0	-	1.0	-	1.0	1.0
KH_2PO_4	136.00	1.00	-	2.0	-	-	2.0	-	-	-
K_2SO_4	87.10	0.50	-	-	-	-	-	-	1.5	-
$CaCl_2.6H_2O$	109.50	0.50	-	-	-	-	-	-	-	-
NH_4NO_3	40.00	0.50	-	-	2.0	-	2.5	2.0	1.5	-
$Mg(NO_3)_2.6H_2O$	128.20	0.50	-	-	-	-	1.5	-	-	-
$MgCl_2.6H_2O$	101.65	0.50	-	-	-	-	-	-	-	-
NH_4Cl	106.80	2.00	-	-	-	1.0	-	1.0	-	-
$Ca(CH_3COO)_2$	158.20	1.00	-	3.0	-	-	-	-	-	-
$K(CH_3COO)$	98.10	1.00	-	-	-	-	-	-	-	-
$(NH_4)_2SO_4$	132.10	1.00	-	-	-	-	-	-	-	-
Micronutrients		(mM)								
Fe sequestrene	64.360	150.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$MnCl_2.4H_2O$	2.969	15.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$ZnCl_2$	0.204	1.50	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-
$CuCl_2$	0.134	1.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
H_3BO_3	0.031	0.50	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.012	0.01	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Note: The Fe, Mn, Zn, Cu, and B and Mo can be combined into one stock solution if a minus trace elements treatment is to be used.

The final concentrations of the nutrients in the solutions is presented in Table 3.8.

Table 3.8. Nutrient Concentrations in the solutions added to the pot.

Nutrient	Complete	-N	-P	-S	-K	-Ca	-Mg	-Zn
	----------	----------	----------	-------mM	----------	----------	----------	----------
N	10.00	0.00	10.00	10.00	10.00	10.00	10.00	10.00
P	2.00	2.00	0.00	2.00	2.00	2.00	2.00	2.00
K	4.00	4.00	4.00	4.00	0.00	4.00	4.00	4.00
S	0.75	0.75	0.75	0.00	0.75	0.75	0.75	0.75
Ca	3.00	3.00	3.00	3.00	3.00	0.00	3.00	3.00
Mg	0.75	0.75	0.75	0.75	0.75	0.75	0.00	0.75
Cl	2.04	2.04	2.04	2.04	2.04	2.04	2.04	2.04
Acetate	0.00	6.00	0.00	0.00	0.00	0.00	0.00	0.00
	----------	----------	----------	----------	----------	----------	----------	----------μM----------
Fe	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00
Mn	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Zn	1.50	1.50	1.50	1.50	1.50	1.50	1.50	0.00
Cu	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mo	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07

3.7.6 ISOTOPE APPLICATION

The isotopic tracers have to be, in most cases, uniformly applied and mixed with the whole substrate. As an example ^{15}N labelled ammonium sulfate is weighed for the whole experiment and dissolved in water allowing aliquots to be mixed with the soil in each pot. In most cases it will be sufficient to apply 0.5 mg ^{15}N/kg soil. Table 3.9 gives some guidelines as to the amounts and enrichments for a pot experiment. It should be possible to detect the ^{15}N in the crop if these rates and enrichments are used and the analyses are performed by mass spectrometry. The enrichments may have to be doubled if an older type emission spectrometer is used for the analyses.

Table 3.9. Sufficient % ^{15}N atom excess enrichments of various N rates when applied to a pot experiment.

N rates to pot experiments (mg/kg soil)	Enrichments (% ^{15}N atom excess)
100	0.5
50	1.0
25	2.5
10	5.0

3.7.7 TREATMENT APPLICATION

If the treatments are soil amendments then these have to be mixed with the soil before the soil is put into the pots.

3.7.8 SOWING

All the pots should be sown on the same day, i.e. all cultivars and controls and the pots watered to facilitate germination. If it is not possible to complete the sowing on the same day then complete

the sowing of replicates and leave the remaining replicates to be sown later. In this way any environmental effects will be analysed as a replicate effect in statistical analyses.

The viability of the seed should be checked before sowing and more seed than is required should be sown and the seedlings thinned to the desired number after emergence. Leave the removed seedlings on the soil as they may contain some ^{15}N.

3.7.9 WATERING

Depending on the experiment the pots will have to be watered, e.g. to a certain weight, by capillary rise by placing water in the saucers or by watering from the top and allowing drainage into the saucers. In all cases water should not be allowed to drain out of the pots or the saucers as this may mean loss of the tracer.

3.7.10 GREENHOUSE CONDITIONS

Light and temperature will have to be maintained to facilitate plant growth. Depending on the location additional lighting, heating or cooling might be required. The conditions should be registered and reported.

3.7.11 MAINTENANCE

The experiment should be looked after carefully and any unwanted external effects reduced to the minimum. If the plants become infested by insects try to spray them with the appropriate chemical. Remember that the various treatments should limit the growth and the development of the plants and not any unwanted external effects.

3.7.12 HARVESTING

After conducting a greenhouse or pot experiment with isotopes samples have to be taken for analyses. The experiment may include sequential harvesting or all the plant may be harvested at the completion of the experimental period. In most pot experiments the whole material is harvested and placed in appropriate (labelled) paper bags. If the roots are removed these will have to be washed carefully before they are processed. All samples should be chopped or cut into 1–2 cm pieces. If the fresh material is more than 200 g then sub-samples (see below) will have to be taken otherwise the whole material may be used as a sample. The next step will be to dry the material in the open paper bags at 70^{o}C. Do not use higher temperature, as some N may be lost.

The following measurements will have to be taken:

Total fresh weight/pot
Sub-sample fresh weight
Sub-sample dry weight*

Note: Sub-sample fresh or dry weights are only needed if the total fresh weight is more than approximately 200 g and it will be inconvenient to dry such a large amount of material. The sample will now be ready for milling and isotope analyses

3.7.13 USING THE ^{15}N ISOTOPE DILUTION METHOD IN GREENHOUSE EXPERIMENTS

The ^{15}N isotope dilution method can be used to quantify biological nitrogen fixation in pot experiments under greenhouse conditions. In this case one has to uniformly label the soil or substrate with the ^{15}N tracer.

i Pots/soil

To measure nitrogen fixation in, for example, in soybean inoculated with 10 different strains of *Bradyrhizobium japonicum* under greenhouse condition one would need at least 48 pots, i.e. 4 replicates of each inoculation treatment, 4 for the uninoculated control and 4 for the non-fixing reference plant. You might select pots that will hold 10 kg of soil, i.e. you will need 48 pots x 10kg soil = 480 kg of soil. If the pots have drainage holes use saucer to limit ^{15}N losses.

ii ^{15}N tracer

Equivalent of 10 kg N/ha of 10% ^{15}N atom excess as ammonium sulfate or urea will be sufficient to evaluate nitrogen fixation in such an experiment. The calculation of how much N is needed for such a pot experiment is as follows:

1 ha x 15 cm of soil weighs 2 x 10^6 kg.

i.e. 10 kg N/ha is 10 x 10^3 gN/2 x 10^6 kg soil

for 480 kg of soil $\frac{1\times10^4}{2\times10^6}\times 480\,g\,N = 2.4\,g\,N$

If you use ammonium sulfate, which is approximately 21 % N, you will need 11.43 g of A.S./480 kg soil. Dissolve the 11.43 g of A.S. in water to make 2400 ml and add 50 ml aliquots to the soil for each pot and mix thoroughly.

iii Treatments/sowing

The soybean seed will have to be inoculated with the various strains to be tested and the pots have to be sown. One should take care not cross contaminate the strains and not to contaminate the uninoculated treatment. The non-nodulating soybean (reference crop) should not be inoculated. You may sow four seed/pot and later thin to two plants/pot. The pots should be randomised and watered to below field capacity to avoid drainage. Water should be applied carefully to avoid cross contamination by soil splashing.

iv Harvest/analyses

After the growing period the plants should be harvested and analysed as outlined in Section 3.7.12

3.7.14 USING THE ^{15}N ISOTOPE DILUTION METHOD IN FIELD EXPERIMENTS

A similar type of experiment could be done under field conditions. Usually the number of treatments to be tested would be lower than in the greenhouse experiment, e.g. select the five best bradyrhizobial strains previously tested under greenhouse conditions.

i Plots

After preparation of the experimental area the plots have to be measured and laid out. It is best to mark the border of plots with string. To be able to measure N_2 fixation of a legume crop, a plot of legume and another with a non-fixing reference crop are required. One reference plot in each replication is sufficient if strains of *Rhizobium* are being tested. However, if P application or time of harvesting are being studied, the reference crop has to be treated the same way as the fixing crop. In this case, one plot of reference crop is needed for each plot of fixing crop. 1–3 m^2 isotope plots with at least 20–30 plants to be harvested per plot, are usually sufficient.

ii ^{15}N application

Approximately 0.1 g N/m^2 , i.e. 10 kg N/ha of 10 % ^{15}N a.e.; 20 kg N/ha of 5% ^{15}N a.e. or 100 kg N/ha of 1% ^{15}N a.e. is usually enough for detection. Urea, ammonium nitrate or ammonium sulfate fertiliser can be applied in a solid or liquid form (fertiliser dissolved in at least 500 ml water/m^2). Precaution should be taken not to apply the fertiliser when the soil temperature is high to avoid direct ammonia volatilization.

iii Harvest/analyses

At the time of harvest, the plots of the reference crop should always be harvested at the same time as the plots with fixing crops. The pods and straw usually have very different %N and %^{15}N a.e. Therefore it is not possible to directly take representative subsample from those plant parts when mixed. The plants should therefore be separated into pods and straw. These parts are then weighed and sub-sampled after chopping into small fragments. Material with lower %^{15}N a.e. should be chopped first to minimize contamination from one sample to another. In any case, the forage chopper, if used, has to be cleaned thoroughly between treatments. Sub-samples should be ground after drying at 70^{0}C. These samples can then be analysed for total N by the Kjeldahl procedure. The $^{15}N/^{14}N$ ratio can be analysed by either emission or mass spectrometer as described in Section 1.3.

iv Calculation

After the measurement of % ^{15}N abundance in the plant and fertiliser samples, %^{15}N atom excess has to be calculated by subtracting the %^{15}N natural abundance (0.3663%) from the %^{15}N abundance in the sample. The %^{15}N atom excess values are used for all the following calculations. % N derived from fertiliser (%Ndff) is the first derived value.

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CHAPTER 4

LABORATORY METHODS

4.1 NITROGEN

4.1.1 TOTAL N DETERMINATION – SEMI-MICRO - KJELDAHL - ANALYSIS

A Scope and field of application

To determine the total amount of nitrogen in a plant sample.

B Principle

The acid digestion procedure breaks down all the organic nitrogen in the sample to ammonium. The digested sample is distilled after addition of a strong alkali, liberating ammonia which is collected in a known amount of acid. A back titration is then performed to determine the amount of acid consumed by the ammonia and to calculate the amount of N in the sample.

C Typical sample

- Ground plant sample, particle size < 1 mm, dried at 70 ° C, min. 48 hours.
- Material stored in paper bags (=>air dry) and the bags covered with plastic

D Apparatus

- Standard laboratory equipment.
- Standard laboratory glassware.
- Aluminum digestion block (e.g. from Tecator, Büchi) or similar.
- Automatic distillation unit (e.g. from Tecator or Büchi) or a standard laboratory distillation unit.
- Digestion tubes (volume 100 mL).
- Balance, readablility +/- 0.1 mg.
- Set of reference weights (e.g.:100g, 5 g, 2 g, 1 g, 500 mg, 200 mg, 100 mg, 50 mg) to check the balance calibration.
- Drying oven at 70°C +/- 2°C.
- 10 - 20 mL dispenser mounted on a 1-2 liter -glass bottle.
- Vortex shaker.
- Burette, preferably digital, volume 25 mL.
- 10 mL volumetric pipette, class A (calibrated)
- 100 mL digestion tubes, e.g. Tecator tubes or 100 mL traditional Kjeldahl digestion flasks.
- 200 mL titration flasks.

E Reagents

- Sulfuric acid, conc. (w = 95-97%), p.a. , Merck Cat. No. 731.
- Distilled or de-ionised water.
- Kjeldahl catalyst tablets (“Kjeltabs”, 95.5% K_2SO_4 1.5, $CuSO_4$, 2% Se), Merck Cat. No. 10958 or Selenium Reaction Mixture, Merck Cat. No.8030.
- Perhydrol^R (H_2O_2 , 30 %), Merck Cat. No.7210
- NaOH, solid, p.a., Merck Cat. No. 6482.
- Tashiro indicator (0.25g of methylene blue, Merck Cat. No. 15 943, and 0.5g of methyl red, Merck Cat. No. 6076, in 100 mL of 70% ethanol)
- Titrisol^R standard solution of hydrochloric acid, HCl c= 1.0 mol/L , Merck Cat. No. 1.09970.
- Titrisol^R standard solution of sodium hydroxide, NaOH c= 0.1mol/L, Merck Cat. No. 1.09961.

F Procedure

i *Kjeldahl - Digestion*

- Check balance calibration with a set of reference weights and re-calibrate the balance, if out of range.

- Weigh an amount of air dry plant material corresponding to about 7–10 mg N into a 100 mL digestion tube (approximately 300–1000 mg plant material); the estimated range of N-content of a specific plant material is based on the analyst's experience.
- Note the sample air dry weight (to +/- 0.1 mg) in the datasheet.

NOTE: a **correction for the moisture** content of the air dry sample has to be performed on a separate sample to correct the final %N-result, which has to be based on **oven dry material** (see 4.1.1(f) ii)).

- Rinse tube walls with 1–2 mL of distilled water.
- Dispense approximately 8 mL of concentrated H_2SO_4 using a dispenser.
- Shake to get all plant material into contact with the acid.
- Add 1 Se- catalyst -tablet or about 800 mg of Selenium Reaction Mixture.
- The digestion equipment should be set up in a fume cupboard and the fan switched on
- In addition a water jet pump should be connected to the digestion system to suck the developing acid vapours.
- The tube is put into a cold digestion block.
- The temperature is slowly increased to approximately 200°C.
- At this first step watch the reaction continuously to avoid boiling over , shake from time to time to destroy developed foams.
- When foaming ceases, increase temperature to 360–380° C.
- The sample is maintained at this temperature until a colourless solution is obtained (typically 2–3 hours).
- In case the sample does not turn colourless, cool down the digest and carefully add few drops of H_2O_2, 30 % (Perhydrol^R) to the **hand warm digest** to oxidize remaining organic compounds
- Place the tubes back in the digestion block and continue digestion until colourless and approximately 30 minutes more.
- Cool samples to room temperature.
- Add approximately 40 mL of distilled-distilled water to the sample and mix (e.g. with Vortex shaker).
- Cool samples to room temperature.
- Add 2–3 drops of Tashiro indicator (reason: see 9 below).

NOTE: Generally 2 blank samples per day, which receive all treatments and reagents, except plant material, should be run with each batch of samples.

If the blank value exceeds 0.2 mg N (i.e. 2% of a 10 mg N sample), the distilled water and the reagents should be checked for N-contamination. (The problem is that the blank N from the reagents is normally natural abundance N (i.e.99.634% ^{14}N) and this dilutes the ^{15}N enrichment of the enriched plant sample.)

ii ***Correction for moisture in air dry plant sample***

- Weigh a small dry glass dish and note the tare of the glass dish.
- Zero the balance.
- Add about 3 g of air dry plant material and note the precise net weight of the air dry sample.
- Dry in drying cabinet (preferably without ventilation to avoid loss of powder) for 18 hours (overnight) at 7075°C.
- Cool to room temperature in a dessicator with silica gel.
- Weigh precisely the dish with the oven dry sample and note the gross weight.
- Calculate % dry matter and correct the weight of the air dry plant material.

Calculation example:

1) tare of dish……..55.1234 g
2) net of air dry plant material……..3. 2876 g
3) gross after drying…….58.2352 g
=> net oven dry plant material = 58.2352 g - 55.1234 g = 3.1118 g
=> % dry matter = 3.1118 x 100 / 3.2876 = 94.653 %
=> moisture correction factor: 94.653 / 100 = 0.94653

4) Weight air dry plant sample for Kjeldahl analysis:…… ……512.6 mg
=> [mg] oven dry plant sample = 512.6 mg x 0.94653 = 485.2 mg

- note this oven dry weight in the datasheet under "**W**" (see Table 4.1)

iii ***Preparatory work for distillation and titration :***

NOTE: it is advisable to prepare the NaOH — titrant solution from Titrisol^R (see iv below), but if this is not available, the titrant can also be prepared from solid NaOH and a titer determination has to be performed using oxalic acid, see H below.)

iv ***Preparation of NaOH titrant solution, c=0.1 mol/L, t (titer) = 1.000 from Titrisol^R***

- Prepare a clean 1-liter-volumetric flask.
- Place the Titrisol^R ampoule in the neck of the volumetric flask.
- Follow the instructions on the package.
- After careful rinsing of the empty ampoule fill the volumetric flask up to the mark with distilled water.
- Close the flask and mix the solution thoroughly.
- Transfer the solution to a clean, ***dry*** glass bottle and connect the burette.
- Rinse the burette carefully with the NaOH-solution to eliminate air bubbles.

v ***Preparation of 10 liters of HCl- solution, c=0.1 mol/L, from Titrisol^R, c= 1 mol/L***

- Prepare a clean 10 liter glass-bottle and mark at 10 liter volume.
- Place the Titrisol^R ampoule in the neck of the volumetric flask.
- Follow the instructions of the package.
- Rinse ampoule thoroughly with distilled water.
- Fill up to the 10 L - mark with distilled water.
- Close bottle and mix.
- Make a titer determination of the HCl solution with a "nominal" concentration of $c_{nominal}$ = 0.1 mol/L
- Transfer 1–2 liters of the solution to a clean, dry glass bottle and connect a 10 mL dispenser.
- Rinse dispenser carefully with HCl to remove air bubbles.

vi ***Determination of the titer "t_{HCl}" of the HCl- solution, $c_{nominal}$ =0.1 mol/L***

- Prepare a calibrated volumetric pipette and 3 200-mL-titration-flasks.
- Pipette 10.0 mL of HCl, $c_{nominal}$= 0.1 mol/L into each titration flask.
- Add 2–3 drops of Tashiro indicator.
- Titrate the solutions and calculate the mean consumption of NaOH-solution, c= 0.1 mol/L.
- The endpoint is the color change from violet to green.

Calculation of t_{HCl}
mean consumption of NaOH for 10.0 mL HCl: 10.09 mL

- => if 0.1 mol NaOH …… 1000 mL
- => Y mol NaOH ……10.09 mL
- => Y = 0.001009 mol OH^- which is equal to 0.001009 mol H^+
- => c_{actual} = 0.001009 mol H^+ in 10.0 mL or:
  c_{actual} = 0.1009 mol / l

  $c_{nominal}$ = 0.1 mol / l

  $c_{actual} = c_{nominal} \times t_{HCl}$

  $t_{HCl} = c_{actual} / c_{nominal}$

  => t_{HCl} = 0.1009 / 0.1 = 1.009

- note this titer t_{HCl} in the data sheet

vii ***Preparation of NaOH, w=40% (e.g. for Tecator system)***

[w=40 % means 40 g NaOH per 100 g solution = 40 g NaOH + 60 g H_2O]

Attention: protect your eyes for this operation

- Weigh 600 g of solid NaOH into a glass beaker.
- Measure a volume of 2400 mL of distilled water.
- Prepare a 3-Liter-Erlenmeyer flask and add about 800 g of the pre-weighed solid NaOH.
- Add about 1000 mL of the measured volume of distilled water and mix thoroughly , cool the solution slightly in a water bath
- After dissolution of the first amount of NaOH add more NaOH and more water.
- Continue until all 1600 g of NaOH are mixed with all 2400 mL of distilled water.
- Cool solution to room temperature.
- Transfer the alkali-solution to a plastic (e.g.polyethylene) storage bottle, not to glass bottles.

viii ***Determination of the dispensed volume of HCl***

- Prepare 3 titration flasks, e.g. 200 mL Erlenmeyer flasks.
- Dispense 10 mL of HCl into each titration flask.
- Add 2–3 drops of Tashiro indicator.
- Titrate with NaOH, c = 0.1 N (e.g. Titrisol ^{R}, t_{NaOH} = 1.000).
- The endpoint is the point of color change from violet to green.
- Calculate the mean consumption of NaOH.
- The mean volume of NaOH consumed for neutralisation of the HCl is equal to the mean dispensed volume of HCl.
- Note this volume in the datasheet for all samples under "**A**" in Table 4.1.

ix ***Distillation of digest using the Tecator Distillation system***

- Switch on the distillation unit and the cooling water(flow rate 1.5 l / min) and generate steam.
- Distill about 50 mL of distilled water or ethanol, p.a. to clean the system.
- Place a titration flask with known volume (10 mL) of HCl, $c_{nominal}$ = 0.1N underneath the outlet tip of the distillation stand and lift to immerse the tip.
- Connect the sample tube to the distillation apparatus and let the steam bubble through the digest.
- Add an excess (about 35 mL) of NaOH-solution, w= 40% :

NOTE: If the added indicator does NOT turn green (before the indicator molecules are destroyed by the strong alkali), the amount of NaOH has to be increased to really add NaOH in excess.

- The sample is distilled for minimum 4 minutes plus 1 minute with lowered titration flask to rinse the glass tip inside (about 70 mL of distillate should be collected).
- Rinse the tip and remove the flask from the distillation stand.

x ***Titration***

- Homogenize the titrant solution: NaOH, c = 0.1 N (e.g. Titrisol ^{R}, t_{NaOH} = 1.000).
- Fill the burette with titrant solution, remove airbubbles from the tubes.
- Set the burette display to zero.
- Back titrate the H^+ remaining after reaction with NH_3 ($NH_3 + H^+ \Rightarrow NH_4^+$) to the end point manually or by automatic titration: the endpoint is the point of color change from violet to green.
- Note the consumption of [mL] NaOH in the datasheet under "B" in Table 4.1.

If the sample is to be analysed by emission spectrometry , the following procedures are carried out:

- The sample is re-acidified with a few drops of 0.1N HCl
- The sample is evaporated to the desired concentration of 1 mg N / mL on a hot plate at ~200°C. (calculation, see G and H below)

NOTE: Care should be taken to avoid the drying out of samples as this will result in loss of the ammonia.
It is advisable to transfer the solutions to clean glass vials of about 15 mL content after they have been evaporated to this volume, and continue the evaporation to the desired volume in a drying cabinet at a maximum temperature of + 100 ° C.

G Calculation of % N in plant material

[mg] N in plant sample:

$$[\text{mg}]\ N_{\text{plant sample}} = [([\text{ml}]\ HCl_{\text{sample}} \cdot c_{HCl} \cdot t_{HCl}) - ([\text{ml}]\ NaOH_{\text{sample}} \cdot c_{NaOH} \cdot t_{NaOH})] \cdot M(N)$$

[mg] N in blank:

$$([\text{mg}]\ N_{\text{blank}} \cdot c_{HCl} \cdot t_{HCl} - ([\text{ml}]\ NaOH_{\text{blank}} \cdot c_{NaOH} \cdot t_{NaOH})] \cdot M(N)$$

% N in plant material:

$$\%N = 100 \cdot \frac{([\text{mg}]\ N_{\text{plant sample}} - [\text{mg}]\ N_{\text{blank}})}{[\text{mg}]\ \text{ovendry plant sample}}$$

NOTE: The calculation is based on **oven dry** plant material, and **NOT on air dry** plant material ! Therefore a correction for the moisture content of the air dry material has to be done.

- Example for [mg] N and % N calculation (assuming, that t_{NaOH} = 1.000):

1) [mg] ovendry plant material……..485.2
2) mean [mL] HCl dispensed in titration flasks (samples and blank):……..10.35
3) concentration of HCl, $c_{nominal}$ [mol/L]……..0.1
4) concentration of NaOH, $c_{nominal}$ [mol/L]]……0.1
5) titer t_{HCl}……..1.009
6) titer t_{NaOH}……..1.000
7) [mL] NaOH consumed for sample titration ……5.12
8) [mL] NaOH consumed for blank titration ……10.27
9) molar mass of nitrogen , M(N) [g/mol]…… ……14

$$[\text{mg}]\ N_{\text{plant sample}} = [(10.35 \cdot 0.1 \cdot 1.009) - (5.12 \cdot 0.1 \cdot 1.000)] \cdot 14 = \underline{7.45\ \text{mg N}}$$

$$[\text{mg}]\ N_{\text{blank}} = [(10.35 \cdot 0.1 \cdot 1.009) - (10.32 \cdot 0.1 \cdot 1.000)] \cdot 14 = \underline{0.17\text{mg N}}$$

NOTE: If the blank value is higher than 0.2 mg, try to reduce the blank by preparing new reagents.

$$\%\ N = 100 \cdot \frac{(7.45 - 0.17)}{485.2} = \underline{1.50\ \%\ N}$$

Calculation of volume needed for ^{15}N determination by OES

The required N-concentration for OES (No 16) is approximately 1 mg N / mL:

=> For about 7mg N found in the plant sample the volume has to be reduced to approximately **7** mL (for preparation see below).

H Preparation of NaOH titrant from solid NaOH and related calculations

NOTE: This procedure is only necessary, if no Titrisol^R is available for the NaOH titrant solution.

i *Preparation of NaOH titrant solution, $c_{nominal}$=0.1 mol/L, from solid NaOH*

- Prepare a clean 1-liter-volumetric flask.
- Weigh in 4.0 g of solid NaOH.
- Add about 600 mL of distilled water, mix and dissolve completely.
- Make up to mark and mix thoroughly.
- Perform a titer determination (see II below.).

ii *Determination of the titer "t_{NaOHl}" of the NaOH titrant- solution, $c_{nominal}$ =0.1 mol/L*

- Place about 0.5 g of oxalic acid ($C_2H_2O_4$* 2 H_2O, Merck Cat. No. 495) in a clean glass dish.

- Dry in drying cabinet at 105°C for min. 3 hours.

NOTE: No transparent crystals should remain after drying — all oxalic acid should be completely white, i.e. all water of crystalisation removed.

- Cool the oxalic acid to room temperature in a dessicator with silica gel.
- Weigh 3 times exactly 45.0 mg (this amount corresponds to 1mmol of H^+) of solid, dry oxalic acid into 3 200-mL-titration flasks.
- Add about 10 mL of distilled water to dissolve.
- Add 2 drops of phenolphthalein indicator (Merck Cat.No. 7233, 0.1 g in 100 mL Ethanol,96%).
- Place the titration flasks on a hotplate at about 100 °C for 5 minutes to warm the solution to about 50 °C; avoid boiling.
- Titrate with the titrant NaOH solution, $c_{nominal}$ = 0.1mol/L until the colourless solution turns to the first stable pink color.
- Repeat with 2 more replicates and note consumptions in the data sheet.
- Calculate the mean consumption of NaOH, $c_{nominal}$ = 0.1mol/L.

NOTE: If the titer t_{NaOH} would be 1.000, exactly 10.0 mL of NaOH would be needed to neutralize 1 mmol of H^+ from the oxalic acid.

Calculation of t_{NaOH} , example:

example: mean consumption of NaOH for 45.0 mg of oxalic acid: 9.87 mL

=> since 9.87 mL NaOH- neutralize 1 mmol of H^+

- = 1 mmol OH^- ……..9.87 mL
- > x mmol OH^- …… . 1000 mL
- => x = 101.3 mmol OH^- which is equal to 0.1013 mol OH^-/ 1000 mL
- **=>** c_{actual} = 0.1013 mol /L

  $c_{nominal}$ = 0.1 mol / l

  $c_{actual} = c_{nominal} \times t_{NaOH}$

  $t_{NaOH} = c_{actual} / c_{nominal}$

- => t_{NaOHl} = 0.1013 / 0.1 = 1.013

iii ***Calculation of %N***

% N in plant material, if no Titrisol® was used, i.e. if the titer of NaOH is not 1.000:

$$\%N = 100 \cdot \frac{([ml]\,HCl_{sample} \cdot c_{HCl} \cdot t_{HCl}) - ([ml]\,NaOH_{sample} \cdot c_{NaOH} \cdot t_{NaOH}) - [mg]\,N_{blank}] \cdot M(N)}{[mg]\ ovendry\ plant\ sample}$$

$$[mg]\,N_{blank} = [([ml]\,HCl_{blank} \cdot c_{HCl} \cdot t_{HCl}) - ([ml]\,NaOH_{blank} \cdot c_{NaOH} \cdot t_{NaOH})] \cdot M(N)$$

Table 4.1. Example of data sheet for total N determination by Kjeldahl

Sample			Moisture correction factor	W	A	t_{HCl}	B	t_{NaOH}	N_{sample}	N_{blank}	% N
Code	Repl.	Weight airdry [mg]		weight, oven dry [mg]	[mL] 0.1N HCl	Titer of HCl	[mL] 0.1N NaOH	Titer of NaOH	[mg] N in sample	[mg] N in blank	in oven dry sample

4.1.2 KCL EXTRACTION FOR NITRATE AND AMMONIUM DETERMINATION

A Scope and field of application

To extract the nitrate and ammonium from the soil by KCl extraction so that analysis of NH_4^+ and NO^-_3 can be made.

B Principle

An extraction of the soil is made with KCl solution, the K^+ ions substitute on the soil cation exchange sites and thus NH_4^+ is released into solution and can be measured. The nitrate is extracted at the same time.

C Typical sample

Fresh soil sample.

D Apparatus

500 mL polythene bottles, 300 mL polythene bottles for collection of samples, polythene funnels, fibre glass filter papers (or acid washed cellulose papers), mechanical shaker, or large volume capacity centrifuge.

E Reagents

1 M Potassium chloride (KCl) high grade, lower grades may be contaminated with N (74.5 g in 1L).

F Procedure

- Take a 30 g sub-sample of soil sample to determine the moisture content at 105°C till constant weight.
- Weigh 40 g aliquots of fresh soil into a 500 mL plastic containers.
- Add 200 mL of 1M KCl.
- Shake for 1 hour on a mechanical shaker.
- Using Whatman GF/A glass microfibre filters paper or acid washed cellulose papers
- Filter gravimetrically.
- Retain the filtrate solution for inorganic analysis.
- Retain an adequate amount of the extractant KCl for blank N determination.

Notes: Samples can also be centrifuged rather than filtered at 3000 rpm for 15 minutes, however if using a flow injection apparatus this procedure is not recommended as the soil particles may block the tubes.

KCl extracts can be stored in the refrigerator for up to a week or in at –18°C indefinitely.

4.1.3 SIMPLE AMMONIUM CONCENTRATION DETERMINATION

A Scope and field of application

This is a simple laboratory method developed to determine the amount of ammonium in KCl soil extracts with a spectrophotometer.

B Principle

Ammonium is extracted with KCl and determined by a colourimetric reaction. The intensity of the colour is measured at 655nm with a spectrophotometer.

C Typical sample

1M KCl soil extract.

D Apparatus needed

- Volumetric flasks (100mL, 250mL, 1000mL, 2000mL).
- Pipettes.
- Balance ± 0.1mg readability.
- Spectrophotometer.
- Vortex mixer.
- Test tubes.

E Chemicals needed

- Sodium citrate (citric acid sodium salt dihydrate).
- Sodium hydroxide (NaOH).
- Sodium hypochlorite (NaOCl) solution, 5% available Cl.
- Sodium nitroprusside (sodium nitroferricyanide dihydrate) **Caution: Poison.**
- Sodium salicylate (salicylic acid sodium salt dihydrate).
- Sodium tartrate (tartaric acid sodium salt dihydrate).
- Potassium chloride (KCl).
- Ammonium sulfate ($(NH_4)_2SO_4$, 99,9%).

Reagents to prepare

- Reagent N1: Dissolve 68g sodium salicylate, 50g sodium citrate, and 50g sodium tartrate together in about 1500mL deionized water. Add 0.24 g sodium nitroprusside, make up to 2 liters with deionized water.
- Reagent N2: Dissolve 60g sodium hydroxide in about 1500mL deionized water. Allow to cool, add 20mL sodium hypochlorite solution and make up to 2 liters with deionized water.

NOTE: Reagent N1 should be made at least 24 hours before use and stored in an amber bottle. Reagent N2 should be made immediately before use. It can be stored in an amber bottle for a few days, but its effectiveness diminishes rapidly due to decomposition of the sodium hypochlorite.

- Stock standard ammonium-N solution (1000 mg N/L): dry about 7 g ammonium sulfate at 105 °C for 2 hours, cool in the desiccator and dissolve 4.714 g in 1 litre distilled water. Store in a refrigerator.
- Ammonium working standards in 1M KCl (0, 0.25, 0.50, 1.00, 2.00 mg N/L): prepare an intermediate stock standard ammonium-N solution (50 mg N/L, pipette 25 mL of the stock standard into a 500 mL volumetric flask, dilute to the volume with distilled water, store in a refrigerator.)
- Pipette 0, 0.5, 1, 2, and 4 mL of the intermediate stock standard into 100 mL volumetric flasks, dilute to volume with 1M KCl, store in a refrigerator.

NOTE: Prepare new ammonium working standards every 7 days. Solution to be pipetted must be at room temperature.

Measurement procedure:

This ammonium method is very sensitive therefore all glassware has to be very clean (rinsed with 5% (v/v) HCl) and distilled water has to be used

- Pipette 2 mL of the KCl extract (or 2 mL of the standards) into a test tube.
- Add 5 mL of reagent N1 with a dispenser or pipette, mix well using a vortex mixer, leave for 15 minutes.
- Add 5 mL of reagent N2, mix well and leave for 1 hour for full colour development (the colour is stable for one day).
- Before reading the absorbance at 655 nm mix the samples/standards with the vortex mixer.
- Use a blank to set the absorbance to zero.

Calculation:

Calculate the linear regression of the standards, use the equation to calculate the ppm ammonium-N of your samples. To convert the ppm to µg N g dry soil^{-1} the following equations must be used:

$$\%\text{MC of fresh soil} = \frac{(\text{FW - DW})}{\text{FW}} \text{X } 100$$

$$\text{Amount of N (µg) x g of dry soil}^{-1} = \text{ppm X } \frac{\text{VE} + (\text{AS(MC/1000)})}{\text{AS - (AS(MC/100))}}$$

Weight of dry soil sample (dried at 105°C until constant weight) = DW
Weight of fresh soil sample = FW
Amount of fresh soil used in extraction = AS
% Moisture content of soil = MC
Amount of extractant used = VE
ppm of extractant given from the above equation

4.1.4 SIMPLE NITRATE CONCENTRATION DETERMINATION

A Scope and field of application

Determination of the amount of nitrate in KCl soil extracts.

B Principle

The UV absorbance of soil nitrate, which has been extracted with 1M KCl is measured at 210 nm. Two measurements are made: one prior to the reduction of nitrate by copperised zinc and one after. The concentration of nitrate is determined by difference.

C Typical sample

1M KCl soil extract (as described in 4.1.2))

D Apparatus needed

- Volumetric flasks.
- Pipettes.

- Balance ± 0.1mg readability.
- Spectrophotometer.
- Quartz cuvettes.

E Reagents needed

- Diluted sulfuric acid: dilute concentrated H_2SO_4 (96–98%) 1:10 with distilled water.
- Copper sulfate solution, 2.5% (w/v): dissolve 3.9g of $CuSO_4$ $5H_20$ in 100mL distilled water.
- Granulated zinc (approximately 2–5 mm granule size).
- Nitrate standard stock solution, 100 μg NO_3^- - N/mL: dissolve 722 mg of dry (oven dried at 70 °C), analytical grade KNO_3, make up accurately to 1 L with distilled water in a volumetric flask.
- Prepare nitrate calibration standards 0; 0.5; 1; 1.5 μg NO_3^- - N/mL from the stock solution in distilled water.

F Procedure

- Production of copper coated zinc: wash 250 g of zinc granules with 10%(v/v) H_2SO_4, until the surface is polished and wash 4 times with distilled water; add 150mL distilled water and 25 mL of copper sulfate solution drop by drop, continue stirring. The zinc must be well coated with the black copper. Remove granules from the solution, and wash again with distilled water, dry the granules at room temperature, store them in a closed glass vessel.

i *Measurement procedure*

- Pipette 5 – 25 mL of the soil extract into a series of 50 mL volumetric flasks.
- Add 2 mL of diluted sulfuric acid.
- Make up to 50 mL with distilled water.
- Read the absorbance at 210nm (use quartz cuvettes!), set zero with reagent blank (2mL of diluted sulfuric acid made up to 50 mL with distilled water) =reading 1.
- To obtain the standard curve, read the nitrate standards (0; 0.5; 1; 1.5 μg NO_3^- - N/mL) at 210 nm and note the values.
- Add 3–4 zinc granules to the samples and allow to react for 24 hours.
- Read the absorbance at 210 nm a second time and note the values =reading 2.
- Determine the moisture content by drying about 30 g of fresh soil at 105^oC for 24 hr

ii *Calculation*

Calculate the ppm nitrate-N value with the linear regression of the standard curve

Concentration of nitrate-N= (ppm reading 1 – ppm reading 2)

Correct with the dry weight factor:

Moisture content MC (%) = (fresh weight — dry weight / fresh weight) x 100

Dry matter correction: Amount of N (μg) x g of dry soil^{-1} = ppm $X \frac{VE + (AS(MC/1000)}{AS - (AS(MC/100))}$

4.1.5 MICRODIFFUSION FOR PREPARATION OF KCL SAMPLES FOR ^{15}N ANALYSIS

A Principle

The KCl extract is measured into a gas tight jar, magnesium oxide is added and the jar sealed. The pH of the solution is increased and this slowly liberates ammonium from the solution in a gaseous ammonia form, which is collected on an acidified glass fibre disc, which is sealed in teflon (polytetrafluoroethylene) tape. The discs are later dried and analysed using mass spectrometry. The jars are then opened and kept for one day in a dark room to liberate any trace of ammonia. Another acidified disc is then added to the solution and Devarda's alloy added and the jar once again sealed. The Devarda's alloy reduces the nitrate to ammonium, which in turn is liberated from the solution as ammonia and is collected on the acidified disc. This too is then dried and analysed for ^{15}N using mass spectrometry.

This method should give a 100 % recovery of N, however, it is always good to check your N recovery with a labelled nitrogen standard.

B Apparatus

- 100mL plastic containers (disposable urine collection containers with screw tops are ideal, or glass pickling jars (if these are to be used they should be acid washed with 10% (v/v) HCl to remove any trace Devarda's alloy).
- Teflon tape (available in most plumbing shops).
- Quartz filter paper cut into discs using a standard office paper punch.
- Desiccator with silica gel and containing a small open bottle of conc. H_2SO_4.
- 10µl micro pipette.
- Tin cups.
- A constant temperature room (or a place for storage with minimal temperature fluctuation).
- Horizontal shaker, temperate at 25°C (if shaking option is used).
- Analytical balance, readability ± 0.1 mg.

C Reagents

- 2.5 M $KHSO_4$
- If available: NO_3^- and NH_4^+ standards (e.g. $^{14}NH_4\ ^{15}NO_3$ 5 atom%, 100µg of NO_3^-N in 1mL distilled water = 0,571g $^{14}NH_4\ ^{15}NO_3$ in 1000mL and $^{15}NH_4\ ^{14}NO_3$ 5 atom%, 100µg of NH_4^+-N in 1mL distilled water = 0,571g $^{15}NH_4\ ^{14}NO_3$ in 1000mL) to assess the N recovery and contamination.
- Devarda's alloy (ball milled to pass through a 300 mesh screen).
- Magnesium oxide.
- Ethanol.

D Procedure

- Add 50g of 1M KCl soil extract into a 100mL plastic jar.
- Flatten and cut teflon tape into strips place a quartz filter disc on to the strip (*Figure 4.1*).
- Add of 10µl $KHSO_4$ (*Figure 4.1*) and place a second tape on the top of the first tape to cover the filter papers (*Figure 4.2*).
- With a pipette tip with a diameter bigger than the filter papers seal the two teflon tapes rocking in a circular motion to create a perfect seal (Figure 4.2).
- Add trap to the KCl sample and 0.2g MgO, immediately close the bottle tightly.
- Shake the jars 72 h at 25°C (or room temperature) on a horizontal shaker at 100 rpm (or leave standing for 5 days).
- Remove the teflon-traps with forceps, open the trap and place in a multi-well plastic tray and put in the desiccator to dry (*Figure 4.3*).
- If the filter papers are to be analysed using mass spectrometry, when dry put them into tin cups (Figure 4.3) ensuring that you clean the forceps with ethanol after each sample.
- Close the tin cups and form into a round shape, do not touch the samples with your fingers (Figure 4.4) then analyse with total N analyser linked to a mass spectrometer.
- Leave the jars open but covered in a dark room over night.
- To diffuse the nitrate add a fresh teflon-acid-trap and 0.2g of Devarda's alloy and continue the procedure in the same way as for the diffusion of the ammonium.
- To prove the recovery you use 1mL of the each standard (described above) in 49mL 1M KCl and follow the same procedure as for the samples.

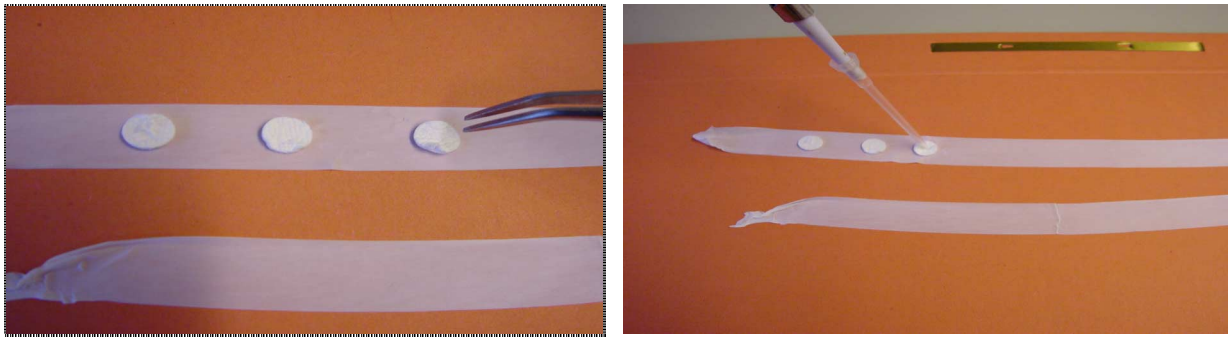

Figure 4.1. Filter papers placed on the teflon strips (left) and $KHSO_4$ pipetted onto the filter papers (right).

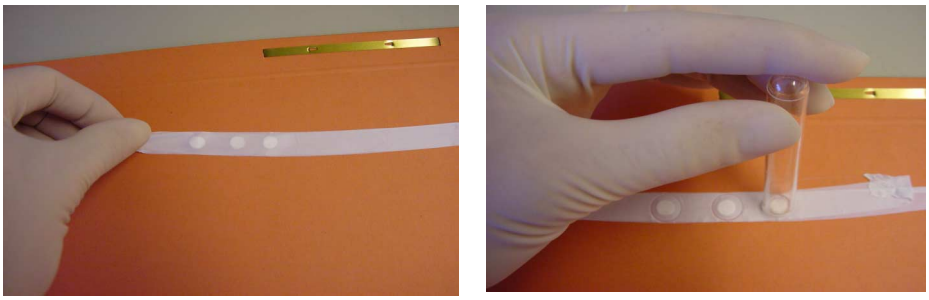

Figure 4.2. Filter papers being covered (left) and sealed (right).

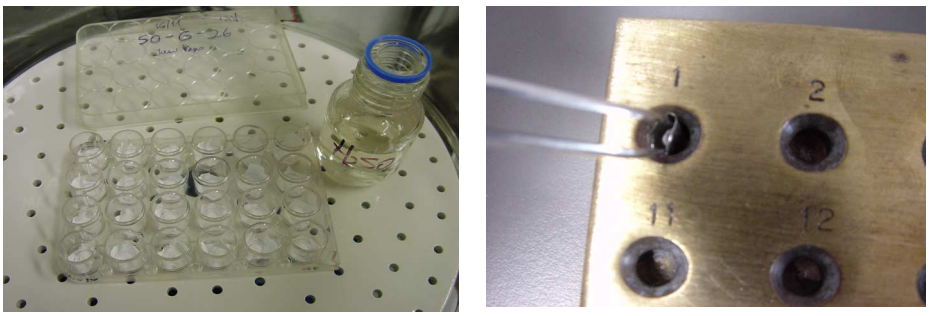

Figure 4.3. Filter papers being dried in the desiccator (left) and being placed in tin cups (right).

Figure 4.4. Tin cups being formed into a round shape.

E Calculation of N concentration from diffusion

It is possible to get a value of the amount of N on the disc from the total N analyser/mass spectrometer if it is correctly calibrated and recovery standards are included in the experimental setup. The maximum amount of N which the disc can absorb is 350µg N.

The ppm concentration of the solution will be calculated by the following equation:

$$\text{The ppm concentration of the solution} = \frac{\text{Value given from the total N analyser in µg}}{\text{Volume of extractant diffused}}$$

To get the ppm value in to a µg N g dry soil^{-1} the following equations must be used:

$$\%\text{MC of fresh soil} = \frac{(\text{FW - DW})}{\text{FW}} \text{X } 100$$

$$\text{Amount of N (µg) x g of dry soil}^{-1} = \text{ppm X} \frac{\text{VE} + (\text{AS(MC/1000)})}{\text{AS - (AS(MC/100))}}$$

Weight of dry soil sample (dried at 105°C until constant weight) = DW
Weight of fresh soil sample = FW
Amount of fresh soil used in extraction = AS
% Moisture content of soil = MC
Amount of extractant used = VE
ppm of extractant given from the above equation

4.1.6 MEASUREMENT OF UREA IN KCl EXTRACTS

A Scope and field of application

Determination of the amount of urea N in KCl soil extracts

B Principle

$H_2N\text{-}CO\text{-}NH_2 + 2\ H_2O \xrightarrow{urease} 2\ NH_4^+ + CO_3^{2-}$

The soluble N-compounds of the soil are extracted with a 1M KCl solution. Urea is broken down to ammonium by the enzyme (urease) and the concentration is measured. Initial ammonium concentrations are determined and the urea –N concentration is calculated by difference.

C Typical sample

1M KCl soil extract

D Apparatus needed

- Volumetric flasks.
- Pipettes.
- Balance $\pm$ 0.1mg readability.
- Incubator, 30^{o}C / water bath.
- Parafilm.
- Flow injection analyzer.

E Reagents

- Potassium chloride solution (KCl), 1M.
- Potassium phosphate buffer, pH about 8.0: dissolve 165.5g of K_2HPO_4 and 6.8g of KH_2PO_4 in 800mL of distilled water and dilute the solution to 1L.
- Urease solution, 1% (w/v): dissolve 0.5g of urease (3500 – 4500 U/g, ICN Biomedicals Inc.) in 50mL of distilled water. Prepare daily as needed.
- Standard urea solution, 25μg of urea-N/mL: dissolve 0.1072g of pure, dry urea in 1M KCl, dilute the solution with 1M KCl to 2000 mL in a volumetric flask, and mix the solution thoroughly. Store in a refrigerator.

F Procedure

i *Urea standards*

- Pipette 7.5; 6.5; 5.5; 4.5; and 2.5mL of the potassium phosphate buffer into test tubes.
- Add 0; 1; 2; 3; and 5mL of the urea standard (the volume rises up to 7.5mL in all test tubes).
- Add 0.25mL urease solution.
- Close the test tubes with parafilm and mix the solution.
- Place in the incubator or waterbath for 2hr at 30^{o}C and measure the ammonium concentration with the flow injection analyzer.

ii *Soil samples*

- Pipette 2.5mL of the potassium phosphate buffer into test tubes.
- Add 5mL of the soil extract.
- Add 0.25mL urease solution.
- Close the test tubes with parafilm and mix the solution.
- Incubate for 2hr at 30^{o}C and measure the ammonium concentration (A).
- Measure the ammonium concentration (B) of 5mL soil extract + 2.75mL potassium buffer.

G Calculation

ppm urea-N = [ppm NH_4 – N (A) minus ppm NH_4 – N (B)]

To get the value in to a µg N/ g dry soil the following equations must be used:

$$\%MC \text{ of fresh soil} = \frac{(FW - DW)}{FW} X\, 100$$

$$\text{Amount of N}\,(\mu g)\ x\ \text{g of dry soil}^{-1} = \text{ppm } X\, \frac{VE + (AS(MC/1000)}{AS - (AS(MC/100))}$$

Weight of dry soil sample (dried at 105°C until constant weight) = DW
Weight of fresh soil sample = FW
Amount of fresh soil used in extraction = AS
% Moisture content of soil = MC
Amount of extractant used = VE
ppm of extractant given from the above equation

4.1.7 SOIL MICROBIAL BIOMASS N DETERMINATION

A Introduction

The soil microbial biomass is the process engine of the soil, mediating the processes of mineralisation nitrification and organic matter turnover. The microbial biomass acts as a source and sink of nitrogen in the soil and therefor in turnover studies it is often useful to determine the size and enrichment of this pool.

B Soil microbial biomass determinations

There are many methods that can be used to measure microbial biomass in soils, soil respiration Anderson (1982), ninhydrin reactive nitrogen determinations Amato and Ladd (1988) and chloroform fumigation Jenkinson and Powlson (1976). A method that is most useful when working with ^{15}N labelled samples (Widmer et al., 1989) is presented here. The method includes several steps, which are described in detail below. First the soil is pre-extraction with KCl to remove the inorganic N fraction and then the sample is fumigated with chloroform which kills off and lyses the microbial cells in the soil. The microbial N is then extracted by a second potassium sulfate extraction and the organic nitrogen extracted is converted to nitrate by potassium persulfate digestion. The nitrogen concentration and ^{15}N enrichment of the solution are determined using the diffusion method. There are many steps in this method that can be adapted to the available equipment and to various levels of expertise. For example nitrate in the sample can be measured using distillation and the ^{15}N of the distillate can be measured using emission spectrometry.

i *Step 1. KCl soil extraction with a buchner funnel*

Introduction

In ^{15}N studies the ^{15}N enrichment of the inorganic pool is often higher than that of the microbial pool so it is useful to remove the interference from the inorganic N pool prior to determination of the enrichment of the microbial N. In this procedure, inorganic N is removed from the soil so it is possible to fumigate and extract only the microbial N from the soil sample.

Scope and field of application

The nitrate, ammonium and soluble organic N can be removed by KCl extraction, the extractant is retained for the analysis of NH_4^+ and NO^-_3.

Principle

An extraction of the soil is made with KCl solution, the K^+ ions substitute on the soil cation exchange sites and thus NH_4^+ is released into solution and can be measured. The nitrate is extracted at the same time. The soil is retained for fumigation with chloroform.

Typical sample

Fresh soil sample.

Apparatus

500 mL polythene bottles, polythene bottles for collection of samples (300 mL), fibre glass filter papers, orbital shaker, Buchner funnels, Vacuum pump, Vacuum conical flasks (500 mL).

Reagents

1 M Potassium chloride (KCl).

Procedure

- Take a 30 g sub-sample to determine the soil moisture content (dry at 105 °C until constant weight).
- Weigh 40 g aliquots of fresh soil into a 500 mL plastic containers.
- Add 200 mL of 1M KCl to the soil in the plastic container.
- Shake vigorously for 1 hour on a rotary shaker.
- Retain 500 mL of KCl for blank N determination.
- Using Whatman GF/A glass microfibre filters paper or acid washed cellulose papers filter under vacuum using Buchner funnels until the soil is dry. Note: if the soil adheres to the side of the bottle swill it out with a small amount of the collected filtered extract and filter it. Do not use water.
- Remove collection vessel and retain the filtrate solution in labelled plastic bottles for inorganic N analysis.
- Replace the empty collection vessel and pour an additional 100 mL of clean KCl solution over the sample and vacuum filter until the soil is relatively dry, discard the additional KCl collected at this point (it is only to ensure that all the inorganic N is removed from the soil).
- Retain the soil for fumigation.

Figure 4.5. Equipment used for soil extraction.

ii ***Step 2. Chloroform fumigation***

Warning: This is a fairly dangerous procedure and should not be performed alone. On no account should one sniff the chloroform. All efforts to expel chloroform to the outside should be made. This procedure should be carried out in a fume hood.

Introduction

The collected soil is now free of inorganic N, when the soil is fumigated with chloroform it kills and splits open the cells so the cell contents are extractable.

Scope and field of application

The chloroform fumigation kills the microbes in the soil and this allows the microbial N to be subsequently extracted and measured.

Principle

The inorganic N fraction is extracted from the soil by KCl/vacuum extraction. The soil is then placed in a desiccator or vacuum oven and fumigated with chloroform for 24 hours.

Typical sample

A soil in a Buchner funnel, which has been KCl/vacuum extracted. It should be as dry as possible.

Apparatus

A glass ground seal vacuum desiccator fitted with a vacuum pump with an outlet to the outside, or a vacuum water pump, or a vacuum oven fitted with a vacuum pump with an outlet to the outside.

Glass or ceramic wide mouthed beakers (numbered with either a diamond marker or ceramic paint) or aluminium cup cake cases which can be marked by indentation with a pencil. Do not use indelible markers for marking or rubber seal desiccators as the chloroform destroys them.

Reagents

- Chloroform, $CHCl_3$ (if this procedure is used for biomass C analysis as well it is necessary to use alcohol free chloroform).
- Anti bumping granules.

Procedure

- Remove the soil and the filter paper from the Buchner funnel and place into the fumigation beaker or aluminium cup.
- Note the sample number and the identification number of the fumigation beaker in a logbook or on a data sheet.
- Put the fumigation beakers, containing the soil samples, into the vacuum desiccator.
- Put 10–20 anti bumping granules in a small Pyrex beaker and add about 30 mL of $CHCl_3$.
- Put this in the desiccator.
- Put the lid on the desiccator and evacuate until all the chloroform boils off (taking care not to allow the escape of any chloroform to the room where you are working).
- Close the valve of the desiccator so that the vacuum is maintained.
- Leave for 24 hours, preferably in the dark, as light destroys the chloroform.
- Open the desiccator, remove the samples and extract using the K_2SO_4 extraction procedure.

Notes

- Note this is the procedure for nitrogen biomass determination only, the procedure for carbon biomass determination is different and the appropriate methodology must be used.
- The chloroform damages the integrity of rubber and may damage the seal on a vacuum oven.
- It is preferable to use a water pump to a normal vacuum pump as the chloroform tends to damage the mechanics of the pump.

iii *Step 3. K_2SO_4 soil extraction*

Introduction

Having killed and lysed the microbial cells it is now necessary to extract the newly soluble organic N from the soil sample using potassium sulfate.

Scope and field of application

To extract the organic nitrogen from the fumigated soil.

Principle

An extraction of the soil is made with K_2SO_4 solution, the K^+ ions substitute on the soil cation exchange sites and thus NH_4^+ is released into solution In addition, organic nitrogen is extracted. The extract is subsequently digested and analysed for N determination.

Typical sample

Chloroform fumigated soil sample.

Apparatus

500 mL polythene bottles, polythene bottles for collection of samples (300 mL), polythene funnels, glass fibre filter papers, orbital shaker.

Reagents

0.5 M potassium sulfate (K_2SO_4)

Procedure

- Put the fumigated soil into the polythene bottle
- Add 200 mL of 0.5 M K_2SO_4
- Shake for 1 hour on a rotary shaker
- Filter gravimetrically using Whatman 42 filter papers
- Retain the solution for digestion
- Retain an adequate amount of the extractant K_2SO_4 for blank N determination

iv *Step 4. Persulfate digestion of K_2SO_4 extracts for microbial N and ^{15}N determinations*

Introduction

The organic N in the extract is digested and oxidised to nitrate. The amount of nitrate and the enrichment of the nitrate determined by mass spectrometer and prepared using a diffusion procedure. It is also possible to determine the concentration of nitrate in the sample using conventional procedures and the ^{15}N of the sample using emission spectrometry.

Scope and field of application

To breakdown the organic N into an inorganic form, which is then subsequently diffused onto a quartz or fibre glass filter disc for analysis.

Principle

The persulfate digestion procedure breaks down all the nitrogen in the sample to nitrate. The digested sample is diffused with magnesium oxide and Devarda's alloy which liberates the nitrate as ammonia and this is collected on an acid disc trap.

Typical sample

A K_2SO_4 extract.

Apparatus

- Standard laboratory equipment,
- Plastic conical centrifuge tubes 50 mL,
- PTFE tape, paint brush, scissors,
- Diffusion jars which could be, 0.5 l Kilner jars (glass food storage jars, approximately120mL) or small plastic pots,
- Glass fibre or quartz filter disks cut into circles using a office paper punch,
- A desiccator containing silica gel and a small open bottle of concentrated H_2SO_4 to absorb any atmospheric ammonium,
- Micro pipette 10µl,
- Tin cups,
- A constant temperature room (or a place for storage with minimal temperature fluctuation).

Reagents

Potassium persulfate ($K_2S_2O_8$), 0.5 M K_2SO_4, 2.5 M $KHSO_4$ (dissolve 68.1g of dry $KHSO_4$ in 180 mL of de-ionised distilled water and make up to 200 mL in a volumetric flask, some re-crystalisation may occur), NO_3^- and NH_4^+ standards, Devarda's alloy (milled to pass through a 300 mesh screen), magnesium oxide.

Procedure

- Pipette or weigh 25 mL of extract into the centrifuge tube.
- Label the tube with a strong marker or pencil, which will not come off in the autoclave.
- Add 0.84g of potassium persulfate.
- Put caps loosely on tubes.
- Autoclave at 121 °C for 40 minutes.
- Remove from autoclave and allow to cool.
- Make up to 50mL with 0.5 M K_2SO_4.
- Tighten cap and shake by hand or using a mechanical shaker allow to cool.
- Transfer all the solution into the diffusion jar or use the centrifuge tube as the diffusion vessel.
- Flatten a strip of teflon tape with a paint brush onto a pile of 3 or 4 sheets of clean paper.
- Place punched filter papers on the tape.

- Add 10µl $KHSO_4$ to the filter paper.
- Lay a second tape over the first tape to cover the filters.
- Seal the tape with a pipette tip which has a bigger diameter than the filter.
- Sealing the tape requires that the pipette tip is rocked smoothly from front to side to back to front. This requires some practice to perfect.
- Cut from strip to give one filter per envelope.
- Add one filter envelope per jar.
- Add 1g of magnesium oxide and 0.5g of Devarda's alloy.
- Shake the jars for 72 h at 25^{o}C on a horizontal shaker at 100 rpm, or leave static for 7 days.
- Remove the teflon-traps with a pair of forceps, labelled appropriately, open and put in the desiccator to be dried.
- Once dried they can be loaded up into a plastic micro well tray and shipped for analysis.
- Seal the micro well tray, and place in a plastic bag containing silica gel, enclose full sample description.
- Or if you have mass spectrometer facilities on site, put the dried filter discs into tin cups and measured with the mass spectrometer within 24 hours as the acid of the trap degrades the tin of the cup.

Alternatively the concentration of N in the digested sample can be determined using the procedure described for nitrate determination or steam distillation. It is assumed that all ammonium is converted to nitrate during the autoclave persulfate digestion procedure. The ^{15}N of the distillate can be determined using emission spectrometry. It is possible to re-dissolve the N on the diffusion disc for emission spectrometry analysis, however N concentration of the solution must be determined separately.

v ***Step 5. Calculation of biomass N***

It is assumed that all the N in the extract is from the biomass as the soil has been pre-extracted with KCL. It is also assumed that all organic N in the extract is digested to either ammonium or nitrate and this is all collected on the disc.

The amount of N on the disc is the N from 25 mL of K_2SO_4 extract. This will be the µg value given by the total N analyser connected to the mass spectrometer (DN) digested nitrogen. Thus to get a value per mL i.e. ppm value we divide this by the amount of extract digested in the above example 25 which example.

$$\text{ppm N} = \frac{\text{DN}\,(\mu\text{g})}{\text{amount of extract diffused (mL)}}$$

To get the value in to a µg N g dry soil^{-1} the following equations must be used
Weight of dry soil sample (dried at 105°C for until constant weight) = DW
Weight of fresh soil sample = FW
Amount of fresh soil used in extraction = AS
% Moisture content of soil = MC
Amount of extractant used = VE
ppm of extractant given from the above equation

$$\%\text{MC of fresh soil} = \frac{(\text{FW - DW})}{\text{FW}}\,\text{X}\,100$$

$$\text{Amount of N (µg) g of dry soil}^{-1} = \text{ppm X}\,\frac{\text{VE} + (\text{AS}(\text{MC}/100))}{\text{AS - (AS}(\text{MC}/100))}$$

C Calculation of the microbial biomass N in the soil

Using the above procedure it is possible to calculate the amount of N in the biomass extracted. The extraction procedure is not 100 % efficient (that is, not all the soil biomass N is extracted) thus an efficiency factor must be used. This is known as the K_N factor, Brookes (1985) suggested that an extraction efficiency of 0.68 (K_N = 1/0.68) across a range of several fumigated soils. This is a generally accepted value, and is acceptable for use for inter-comparative studies using the same soil. However more detailed measurements to determine the K_N should be made if one is making comparisons between soils (Horwath and Paul, 1994).

N extracted X K_N = Total soil biomass N

N extracted X 1.4705 = Total soil biomass N

D Alternative methods for measuring the size of the microbial N pool

An alternative method to measure N immobilisation in soils, which have received ^{15}N labelled inorganic fertiliser is described in detail by Recous et al (1988) and Rice and Smith (1983). The theory being that in non-ammonium-fixing soils, labelled inorganic N is immobilised is in the microbial biomass N fraction. Basically 10g of fresh soil is mixed with 100 mL of 2M KCl solution in a centrifuge tube and shaken for 1 hr, centrifuged at 3000 rpm for 15 minutes and the supernatant discarded. This washing procedure is repeated twice and the residual KCl washed out using the same procedure with 100 mL of distilled water. The washed soil is then dried at 70°C and ground for total N - mass spectrometer analysis.

4.1.8 LEAF OR PETIOLE LABELLING USING ^{15}N

A Scope and field of application

Labelling with ^{15}N via leaf or petiole feeding is a technique that has been used for a number of years in plant physiology experiments (Oghoghorie and Pate 1972, Pate 1973 and Palta et al., 1996, Mc Neill et al 1997,1998). This method achieves isotope labelling of shoots and roots *in situ* however the amount of ^{15}N solution taken up by the plants can be 1.5–2.5 times lower than with the stem feeding technique. The technique allows determination of below ground N (Rochester et al., 1998), and it is simple and easy to use in the field where a large number of plants need to be labelled. Leaf-labelling has also been used to label roots for experiments showing the effect of earthworms on root N turnover (Schmidt and Curry, 1999).

B Principle

The principle is that a portion of the leaf or petiole is submerged in a solution containing ^{15}N (e.g as urea), the solution is taken into the transpiration stream and the ^{15}N is assimilated by passive and active N uptake processes.

C Apparatus

- Putty, Plasticene (e.g.Terostat or Blutak).
- 0.5 or 1 mL Micro pipette.
- Sticky tape.
- Scissors.
- Aluminium foil and paper towels.

i *Petiole feeding*

- Eppendorf tubes.

ii *Leaf feeding*

- Tissues or paper towels.
- Corner cut from plastic bags or leaf size plastic bags made using heat sealer.
- Short sticks.

D Reagents

- Normal unlabelled urea to assess plant N tolerance.
- Usually per plant 1–2 mL of highly enriched (95–98% atom % ^{15}N excess) urea solution at concentrations of 0.5% (w/w) is sufficient. A single leaf feed (2 mL) in pasture plants resulted in clean root enrichment of 0.7858 atom % ^{15}N excess.
- **Note:** It is best to assess the tolerance of the plants to be labelled and test the method using unlabelled urea solutions, as high N concentrations can cause necrosis and death of the plants. This is done using the same procedures for ^{15}N labelling. It is always best to add no more than about 5% of the total plant N as urea. If there are no spare plants available to test for tolerance then 1 mL of 0.5% (w/w) solution should not cause any problems in most plant

species that are older than a few weeks. The practical constraints and aim of the experiment will determine the time and frequency of feeding, generally young plants take up solution more rapidly and apportion more to the root systems than older plants.

E Procedures

- Make up required amount of ^{15}N solution in a volumetric flask with distilled water.
- Ensure that the plant growing conditions are good and that transpiration is maximum i.e. make sure the plants are well watered and feeding is carried out at the beginning of a photoperiod.
- Cover the soil with a layer of aluminium foil then paper towel (this will absorb any spilt ^{15}N).
- Have solutions, pipettes eppendorfs and putty material ready.

i *Leaf / Petiole feeding*

- Fill the plastic eppendorf with required amount of ^{15}N solution (usually 1–1.5 mL) have it at the ready.
- Find the youngest newly fully expanded leaf.
- Cut the leaf tip off under water (top 1–2mm in wheat, or tips of all the leaflets in a compound leaf such as clover) (if you are quick enough it is not necessary to cut the tip under water especially if you have many samples to do).
- Insert the cut tip(s) into the eppendorf and seal the petiole to the edge of the eppendorf with the putty, then securely place the eppendorf in the soil or on to a supporting stick (with sticky tape).
- Wash your hands and then remove the tin foil and tissue avoiding contact with the plant or soil.
- Leave the plants to take up solution (this can be 24hours under high temperature and light intensity for 3–4 days it is ideal that all the solution added is taken up from each vial.
- When you remove the feed it is advisable to remove the leaf also as it may be highly labelled due to contact with the solution.

ii *Leaf feeding*

- Find the youngest newly fully expanded leaf.
- Wrap the leaf in a small amount of paper towel or tissue.
- Put the wrapped leaf still attached to the plant in the plastic bag.
- Using the pipette add the required amount of ^{15}N solution usually about 1mL.
- Close the bag securely with sticky tape and support it with a stick so that no ^{15}N solution can escape.
- Wash your hands and then remove the tin foil and tissue avoiding contact with the plant or soil
- Leave labelling set up on the plant for one week.
- Remove leaf and labelling device.

Note: although this method is quick and maybe more suitable for the field, it is not as quantitative as the other method, since it is relatively difficult to assess how much of the ^{15}N solution is left in the bag and on the leaf. The entire leaf set up can be shaken in 50 mL of water or 1M KCl to measure the urea and ammonium concentrations but this takes time.

4.1.9 STEM FEEDING USING ^{15}N

A Scope and field of application

Stem feeding with ^{15}N urea solution achieves isotope labelling of shoots and roots *in situ* (Russell and Fillery 1996a). This allows the contribution of root N to the N cycle to be assessed. The technique has proved to be a particularly powerful tool in determining residual root N benefit to following crops (Russel and Fillery 1996b) and allows N transfer studies to be undertaken. The technique is particularly useful in greenhouse experiments however it is not so easy to do in the field. In field situations leaf labelling or petiole feeding may be more appropriate.

B Principle

The principle is that a wick passed through the stem of the plant is then immersed in a solution labelled with ^{15}N. The ^{15}N solution is drawn up with the transpiration stream and it is assumed that the urea is assimilated normally in the plant. Urea is generally used as it is a non-polar un-dissociated molecule with a low salt index and a high concentration of N that is readily metabolised by plants that have the urease enzyme.

C Apparatus

- Sewing needle.
- Micro drill – dentist or hobby drill with drill bits of 0.25–0.5 mm diameter (for plants which the needle does not pass through the stem easily alone).
- Thick cotton thread (embroidery thread which is 100% cotton is ideal).
- Flexible plastic tubing (thin PVC pipes from continuous flow apparatus are ideal) cut into about 2–3 cm pieces.
- Plasticene or putty (Terosat, Blutack).
- Small plastic vials with push-on or screw tops (drilled with 3 small holes of 2–3 mm diameter).
- 0.5 or 1 mL Micro pipette.
- Aluminium foil and paper towels.
- Scissors.

D Reagents

- Normal unlabelled urea to assess plant N tolerance.
- Usually per plant 1–4 mL of highly enriched (95–98% atom % ^{15}N excess) urea solution at concentrations of 0.5% (w/w) is used, however solutions of up to 4% have been used for lupins.
- **Note:** It is best to assess the tolerance of the plants to be labelled and test the method using unlabelled urea solutions, as high N concentrations can cause necrosis and death of the plants. This is done using the same procedures for ^{15}N labelling. It is always best to add no more than about 5% of the total plant N as urea. If there are no spare plants available to test for tolerance then 1 mL of 0.5% (w/w) solution should not cause any problems in most plant species that are older than a few weeks. The practical constraints and aim of the experiment will determine the time and frequency of feeding, generally young plants take up solution more rapidly and apportion more to the root systems than older plants. A single stem feed (1mL) of young plants in the greenhouse enriched the clean root systems of lupin, faba bean and field pea within a range of 0.2294 –1.3613 atom % ^{15}N excess.

E Procedure

- Make up the stem feeding vials, pass two pieces of plastic tubing through the two adjacent holes in the top of the vial.
- Make up required amount of ^{15}N solution in a volumetric flask with distilled water.
- Ensure that the plant growing conditions are good and that transpiration is maximum i.e. make sure the plants are well watered and that injection is carried out prior to a photo-period or early in the morning.
- Cover the soil with a layer of aluminium foil then paper towel (this will absorb any spilt ^{15}N).
- Have solutions, pipettes and putty material ready as this next stage should be carried out as quickly as possible to prevent sealing of the xylem vessels.
- Pass the needle threaded with about 20 cm of with cotton through one of the pieces of plastic tubing.
- For thick stems, drill the stem with a dentist drill 1–3 cm from the soil surface.
- Pass the needle threaded with cotton through the plant stem then through the second piece of plastic tubing.
- Tie a knot at the bottom of the two tubes so that the tubing fits tightly against the stem and cut the cotton one or two cm after the knot so that the cotton reaches the bottom of the vial.
- Screw or push the vial on to the top, (NB if screwing the vial on keep the top stationary).
- Add the ^{15}N solution to the vial through the 3rd hole using the pipette (note the volume of ^{15}N solution added).
- Seal the hole with the putty.
- Seal the joints between the plant and the plastic tubing with the putty.
- Cover the vial with aluminium foil to avoid condensation.
- Wash your hands and then remove the tin foil and tissue avoiding contact with the plant or soil.
- Leave the plants to take up solution for 3–4 days it is ideal that all the solution added is taken up from each vial.

4.1.10 NOTES AND CALCULATIONS ON LEAF, STEM OR PETIOLE ^{15}N - FEEDING

If total ^{15}N recovery is to be determined then confined systems such as PVC cylinders or pots need to be used which have sealed bases to prevent leaching of mobile nitrate ions. In field studies anion exchange resin bags at the base of any cylinder will trap any ^{15}N leached.

It is important for assessment of below-ground N that contamination from falling leaves is prevented this can be done using leaf traps or making the assessments prior to any senescence event.

A Soil core fractions

Bulk soil (BS) material which passes through the mesh of the sieve when the core is broken apart (the mesh should be fine enough to facilitate good root recovery. All material on the sieve should be dried using a freeze drier (samples can be frozen prior to drying) or in the oven at 70 °C.

Rhizosphere soil (RH) is soil easily separated from the roots (sometimes accumulates at the bottom of the bag during drying).

Clean root (CRT) root material, which is thoroughly cleansed of soil using a dry brush or cleaning in a small amount of water then re-drying the plant sample at 70 °C and then drying down the water.

Residual fraction (RF) very fine root fragments and fine soil remaining after root cleaning.

To reduce number of samples separating samples into bulk soil, clean root and residue fraction is also possible (bulking rhizosphere soil and residual fraction together, however this should not be used to estimate rhizodeposition as it contains a high content of fine root fraction).

All fractions should be finely ground and analysed for ^{15}N enrichment using either continuous flow GC/MS or digestion emission spectrometry procedures.

Calculation of total below-ground N.

Assuming that the ^{15}N is uniformly distributed throughout the root system then the source ^{15}N should be the enrichment value of the clean root fraction, then root-derived N (RdfN) can be calculated for each fraction from the measured ^{15}N enrichment of each fraction.

$$\%\text{RdfN} = \frac{\text{atom \% } ^{15}\text{N excess of BS}}{\text{atom \% } ^{15}\text{N excess of CRF}} \times 100$$

$$\text{RdfN (BS)} = \frac{\text{atom \% } ^{15}\text{N excess of BS}}{\text{atom \% } ^{15}\text{N excess of CRF}} \times \text{total of N in BS}$$

$$\text{RdfN (RH)} = \frac{\text{atom \% } ^{15}\text{N excess of RH}}{\text{atom \% } ^{15}\text{N excess of CRF}} \times \text{total of N in RH}$$

$$\text{RdfN (RF)} = \frac{\text{atom \% } ^{15}\text{N excess of RF}}{\text{atom \% } ^{15}\text{N excess of CRF}} \times \text{total of N in RF}$$

Then total below-ground N can be calculated from the following equation

Where Ncrt = total N of clean root fraction

Total below ground N = Ncrt + RdfN(BS) + RdfN(RH) + RdfN(RF)

Quantifying decomposition of N in terms of N benefit to the subsequent crop.

Pots or cylinders in which the root fraction has been labelled, and from which the labelled shoot has been removed, can be used to determine the N benefit of the roots to the subsequent crop.

There are two calculation approaches that have been used to determine N benefit and these essentially result in the same outcome:

1. Russell and Fillery (1996b) used the average ^{15}N enrichment of the total belowground N.

$$\text{N derived from previous crop} = \frac{\text{atom \% } ^{15}\text{N excess crop grown}}{\text{average atom \% } ^{15}\text{N excess}} \times \text{total of N o}$$
$$\text{of below - ground N of previous crop}$$

2. McNeill et al., (1998) calculated the benefit using the actual amounts of excess ^{15}N in the total BG N of the previous crop and that recovered by the subsequent crop.

$$\text{N derived from previous crop} = \frac{\text{Amount of excess } ^{15}\text{N in subsequent crop}}{\text{Amount of excess } ^{15}\text{N in total below ground N of previous crop}} \text{ xTotal of N in subsequent crop}$$

4.1.11 TREE INJECTION USING ^{15}N

A Scope and field of application

In recent years the role of below ground N in agroforesty systems has raised many questions. One way of studying the below ground N cycling is to use an in situ ^{15}N tree injection technique. This technique was pioneered by Horwarth et al (1992) and allows the contribution of root material to N flux and organic matter to be assessed.

B Principle

A hole is drilled in the tree and ^{15}N solution is injected into the active transpiration stream. The distribution of ^{15}N in the plant is determined by sampling through out the canopy. This technique can be used to study above and below ground N cycling.

C Typical sample

Trees with ring porous xylem structure are the only trees suitable for this procedure. That is, trees with a xylem that is evenly distributed throughout the stem and not only on the surface. The location of the xylem can be determined easily. Cut an active fresh stem and place it in a shallow solution of Fuchsia basic for several hours. After some time has elapsed, cut the stem and examine the cross section, from this it is easy to determine where the water moves in the stem from the stain of the dye. If the xylem is only on the surface of the tree this technique will not work so well as if the xylem is evenly spaced through out the stem.

D Apparatus

- Electric drill,
- Drill bit 4mm (type used for wood drilling)
- 2 syringe needles
- 2mL and 50mL syringes
- Tube connectors
- Subaseal, size 17
- Plastic pipe, 30cm long, fitting onto the tube connector
- Paper punch
- pH meter, or strips
- Autoclave or pressure cooker for heat sterilization
- 1000 mL and 500mL volumetric flasks
- Parafilm
- Fuchsia basic indicator

E Reagents

- ^{15}N solution, autoclaved at 120 ^{o}C for 15min, (This is a 70 mM solution of $(NH_4)_2SO_4$:dissolve 9.353g of $(NH_4)_2SO_4$ in 1 litre of distilled water). This gives a total of 0.204 g N per 100mL of solution. Use between 10–100 mL of ^{15}N solution per tree depending on the initial tree size.

Ensure that you do not add more than 5% of the total tree N content, more N will lead to burning of the leaf material.

- Artificial sap solution (5.0 mM KCl and 0.4 mM malic acid adjusted to pH 5.4 with dilute potassium hydroxide (KOH) or sodium hydroxide (NaOH) solution, autoclaved at 120 ^{o}C for 20min)
- To prepare a stock sap solution: Disolve 3.75g KCl and 0.54g malic acid in 1 litre of distilled water. To obtain the correct sap solution dilute the stock solution 1:10 with distilled water and adjust to pH 5.4 with a dilute alkali solution (0.01M).

F Procedure:

Cover the soil with aluminum foil and paper to avoid liquid dropping onto the soil. Note it is best to carry out the tree injection early in the morning when there is maximum transpiration. It may be worth watering the trees before hand to really pump up transpiration. The technique relies on having an active transpiration stream.

Prepare the pipe system: Cut the body of a 2 mL syringe at approximate 2 cm and connect with a Subaseal. Then connect about 20 cm of piping to the body of the 50 mL syringe, on the other end attach a tube connector and a syringe needle. Attach the 50 mL injection system to the tree with tape and flush through with sap solution (note this is not labelled with ^{15}N). Fill another syringe with unlabelled sap solution.

- Measure the width of the stem of the tree, label wood drill at 2/3 of the diameter of the tree with a small piece of tape. Drill a hole (2/3 of tree diameter). Immediately after the hole is drilled connect the syringe body/subaseal connector. Put a second needle in the subaseal, flush the system using the syringe filled with sap solution to expel all the air. This should lead to the solution coming out of the second needle, then remove both needles.
- Connect the needle with the 50 mL syringe system, mark the level of the sap solution to determine the uptake of solution cover the open 50 mL syringe with parafilm.
- Once you have determined that there is uptake of the sap solution add the labelled ^{15}N solution to the syringe, and again keep topping up with the ^{15}N solution until you have injected the desired amount (10–100ml). Again mark 50ml syringe to monitor uptake.
- Keep topping up the syringe with artificial sap solution to ensure the ^{15}N gets into the tree and keep doing this until uptake stops. If there is substantial uptake it may be best to take a plastic bottle and insert the end of the tube into this. This uptake of sap solution can be as much as 2 litres.

4.1.12 EXPERIMENTAL PROTOCOLS FOR GROSS MINERALISATION AND GROSS NITRIFICATION EXPERIMENTS

A Measuring gross mineralisation.

i *Scope and field of application*

The simultaneous processes of mineralisation immobilisation turnover make it extremely difficult to determine the fate or turnover rates of nitrogen in the soil. In some soils the net gains of nitrogen are so low that the soils appear to be dormant, however when the gross processes are studied the turnover rates are high and rapid. Thus it is only with the use of isotopes that the gross processes can be studied, which in turn will allow us to identify the parameters governing N release from organic matter.

ii *Principle*

The principle of the isotope dilution technique is that the ammonium pool is ^{15}N labelled and is assumed to give a homogeneously labelled ammonium pool. The ammonium pool is subsequently monitored for size and enrichment. Any incoming N will cause a dilution of the pool any removal processes will cause the size of the pool to change but not affect the enrichment of the pool. From the enrichment and concentration data it is then possible to calculate the rate of unlabelled N coming into the ammonium pool or the mineralisation rate as shown in equation below. Labelling is achieved by adding a ^{15}N label to a soil core as a solution or gas so that uniform labelling is achieved (Figure 4.6). The concentration of ammonium label added should not be greater than the

original ammonium pool (thus usually about 2–10 µg N/g soil is added of about 2–10 atom % ^{15}N excess).

$$m = \theta \frac{\text{Log } A_0^* / A_t^*}{\text{Log}(1 + \theta\, t/A_0)}$$

Where is the size of the ammonium pool, usually expressed in mg N kg soil^{-1}, A* is the atom % excess in the ammonium pool, (t) time in days after (0) initial sampling, θ is the observed rate at which pool size changes, i.e. $(A_t - A_0)/t$, and m is the mineralisation rate.

There are three assumptions that should be noted when using the isotope dilution approach to measure gross mineralisation or nitrification.

- That the ^{15}N and ^{14}N exhibit the same behaviour in soil
- There is adequate mixing of the N added as label and the native soil pool
- There is no remineralisation of previously immobilised nitrate N into the ammonium pool

Pots used for incubation

Ammonium gas injector

Simple soil solution injector

Figure 4.6. Equipment used for mineralisation experiments.

In dry soils gaseous application of ammonia is recommended (see gas injector in Figure 4.6) and in moist soil minimal quantities of solution should be added. The added label must mix uniformLy with the indigenous soil N, this is an underlying assumption in the method. It is best to apply the label and take the first samples 24 hours after application to avoid problems associated with pool substitution and to carry out the second sampling no longer than 5 days after application to avoid remineralization. This also allows all processes to be described by zero order kinetics. Paired cores for initial and final sampling are prepared and at final sampling the entire core is mixed and sub-sampled for analysis. The exchangeable nitrate and ammonium are extracted and measured in 1 or 2 M KCl and sufficient extract is sub-sampled to ensure adequate nitrogen for mass spectrometer or emission spectrometer analysis. These and further details are discussed by Murphy et al.,(1997); Monaghan (1995) and Murphy et al., (1999).

iii *Apparatus*

- A labelling device, liquid injection device or gas injection device see Figure 4.6.
- For field work, soil corer, sticks with flags on to identify the centre of the injected area
- For laboratory work incubation pots, cut PVC pipe (7–8cm diameter) sealed with gauze make good incubation pots (Figure 4.6).
- Equipment and resources to do inorganic N analysis and ^{15}N determinations.

iv *Reagents*

- ^{15}N labelled ammonia solution (ammonium sulfate is usually used) the enrichment and concentration of this solution will be determined by ^{15}N available and the moisture content of the soil, it is best not to change the size of the ammonium pool too drastically. Additions of 10 ppm N g soil^{-1} at 5 % ^{15}N atom % excess should give enough N of high enough enrichment for analysis and to determine the decline in enrichment in the pool. It is always best to label with the least amount of solution possible. In arid soils it is probably best to use a gas injection device.

- For nitrifcation measurement ^{15}N labelled nitrate solution is required.

v *Procedures*

- Prepare eight cores for each measurement.
- Label all eight cores with ^{15}N, label and note position of core.
- Incubate for 24 hours (this allows some time for equilibration of the label added)
- After 24 hours sample each core separately mixing the soil and subsampling each core separately.
- Extract the four soils individually with 1M KCl and determine ammonium and nitrate concentrations and enrichments. See sections 4.1.2, 4.1.3, 4.1.4.
- After 72–120 hours extract the second set of cores with 1M KCl and determine ammonium and nitrate concentrations and enrichments.
- Calculate mineralisation rates or nitrification rates using equation above.

4.2 PHOSPHORUS

4.2.1 BRAY 1 EXTRACTABLE PHOSPHORUS (BRAY AND KURTZ 1945)

A Reagents

Ammonium fluoride: (NH_4F) 1N: dissolve 37g of NH_4F in distilled water and dilute to 1 litre. Store this solution in a polythene bottle. Solution 1.

Hydrochloric acid: (HCl) 0.5N: dilute 20.2mL of concentrated (32%) HCl to 500mL with distilled/deionised water. Solution 2.

Extracting solution: Add 15mL of NH_4F (Solution 1) and 25mL of 0.5N HCl (Solution 2) to 460mL of distilled/deionised water. This gives a solution of 0.03N NH_4F and 0.025N HCl. Store in glass for up to a year.

B Method

- Weigh out 2g of 2mm sieved, oven-dried soil into a 30mL capacity vial.
- Add 14mL of extracting solution and shake continuously for 5 minutes.
- Filter through a Whatman No. 42 paper and collect filtrate.

C Analysis

See Section 4.2.6

D Discussion

The Mo blue methods are the most sensitive and, as a result, are widely used for soil extracts containing small amounts of P as well as for total P in soils. These methods are based on the principle that in an acid molybdate solution containing orthophosphate ions, a phosphomolybdate complex forms that can be reduced by ascorbic acid, $SnCl_2$ or other reducing agents to a Mo blue colour. The intensity of the blue colour varies with the P concentration, but is affected also by other factors such as acidity, arsenates, silicates and substances that influence the oxidation-reduction conditions of the system.

4.2.2 OLSEN P DETERMINATION USING 0.5M $NaHCO_3$ EXTRACTION (OLSEN ET AL., 1954)

A Reagents

0.5M sodium bicarbonate solution ($NaHCO_3$): weigh out 42g of $NaHCO_3$ and make to 1 litre with distilled/deionised water. Prepare when required, do not store for long periods. Immediately before use, bring solution to pH 8.5 ($\pm$0.05) with concentrated NaOH solution.

Concentrated sodium hydroxide: prepare approximately 10–20mL of concentrated NaOH by dissolving NaOH in distilled/deionised water until saturated.

B Method

- Weigh 5g air-dried and 2mm sieved soil into a 125mL screw-top polythene bottle. Record weight to 2 decimal places.
- Add 100mL of 0.5M $NaHCO_3$ at pH 8.5.
- Screw lids on tightly and tumble for 30 minutes at 25^{o}C on an end-over-end tumbler.
- After tumbling, filter solutions through Gelman glass fibre filters (a leur lock filter and syringe apparatus is a convenient method of doing this).
- The filtered extract should be analysed immediately. If this is not possible, the extracts can be frozen for up to 7 days, or stored at 4^{o}C for 2 days, however, this is not recommended.

C Comments

Reproducibility to within $\pm$ 1ppm can be achieved by the following:

- Shaking temperature 25^{o}C $\pm$ 0.5^{o}C.
- pH 8.5 $\pm$ 0.5 - the solutions increase in pH with time, thus if a large bulk of extractant is made up, only the amount required each day should be brought to the correct pH with NaOH.
- Once extracted, the solutions should be analysed that day.

D Analysis

See Section 4.2.6.

4.2.3 COLWELL P DETERMINATION USING 0.5M $NaHCO_3$ EXTRACTION (COLWELL, 1965)

A Reagents

0.5M sodium bicarbonate solution ($NaHCO_3$): weigh out 42g of $NaHCO_3$ and make to 1 litre with distilled/deionised water. Prepare when required, do not store for long periods. Immediately before use, bring solution to pH 8.5 ($\pm$0.05) with concentrated NaOH solution.

Concentrated sodium hydroxide: prepare approximately 10–20mL of concentrated NaOH by dissolving NaOH in distilled/deionised water until saturated.

B Method

- Weigh 1g air-dried and 2mm sieved soil into a 125mL screw-top polythene bottle. Record weight to 2 decimal places.
- Add 100mL of 0.5M $NaHCO_3$ at pH 8.5.
- Screw lids on tightly and tumble for 16 hours at 25^{o}C on an end-over-end tumbler. Tumbling overnight (5 p.m.to 9 a.m.) fits conveniently into the laboratory routine.
- After tumbling, filter solutions through Gelman glass fibre filters (a leur lock filter and syringe apparatus is a convenient method of doing this).
- The filtered extract should be analysed immediately. If this is not possible, the extracts can be frozen for up to 7 days, or stored at 4^{o}C for 2 days.

C Comments

Reproducibility to within ±1ppm can be achieved by the following:

- Shaking temperature 25^{o}C $\pm$ 0.5^{o}C.
- pH 8.5 $\pm$ 0.5 - the solutions increase in pH with time, thus if a large bulk of extractant is made up, only the amount required each day should be brought to the correct pH with NaOH.
- Once extracted, the solutions should be analysed that day. The solutions can be stored as previously mentioned, but it is not recommended.

- Note that the Colwell soil:solution ratio is 1:100 and the Olsen soil:solution ratio is 1:20.

D Analysis

See Section 4.2.6.

4.2.4 TOTAL, TOTAL INORGANIC AND TOTAL ORGANIC PHOSPHORUS IN SOILS

A Introduction

Phosphorus occurs in soils in organic and inorganic forms, both of which are important to plants as sources of this nutrient. Between 25 and 80% of the total phosphorus content is present in the organic form. The proportion is highest in the topsoil where the organic matter level is also the highest. The phosphorus content of a soil is also dependant on parent material. Inorganic phosphorus exists as:

- Strongly adsorbed species on the surface of clay minerals and sesquioxides, and
- Insoluble compounds of aluminium, iron and calcium.

The iron and aluminium compounds occur in acid soils. Above pH 6.5 phosphates form insoluble salts with calcium such as hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$.

An estimate of inorganic phosphorus can be obtained by extracting a soil sample with 0.5M sulfuric acid. If a second sample of the soil is ignited to convert the organic phosphorus to inorganic phosphorus, extraction with 0.5M sulfuric acid will remove both forms as inorganic phosphorus. The difference between the second and the first extraction represents organic phosphorus.

B Reagents

0.5M H_2SO_4: To approximately 900mL of distilled/deionised water in a 1 litre beaker add 49mL of sulfuric acid (98%) slowly and with much stirring. Allow to cool and make to 1 L.
2.35M sulfuric acid: Add 225mL conc. H_2SO_4 (98%) to 1475mL distilled/deionised water in a 2 L beaker. Do this slowly whilst stirring continuously.

Colour Developing Reagents: (**POISONOUS**):

- Dissolve 9.6g ammonium molybdate (MW = 1235.86) in 200mL distilled/deionised water.
- Dissolve 0.22g antimony potassium tartrate (M.W. = 333.903) in 100mL distilled/deionised water.

Add these reagents to 1700mL 2.35M sulfuric acid. Mix thoroughly. Dissolve 8.45g ascorbic acid (M.W. = 176.13) in this mixed solution. This reagent must be freshly prepared.

Working phosphate standard solution, 2.5μg P/ mL in distilled water.

mL of 2.5 μg P/ mL	Total Volume	μg P/ mL
0	50	0
4	50	0.2
8	50	0.4
12	50	0.6
16	50	0.8
20	50	1.0

C Method

i Total Phosphorus

- Accurately weigh 2g soil into a crucible and label accordingly.
- Place crucibles into a muffle furnace and ignite at 450^{o}C for 16 hours.
- Allow to cool and transfer all of the sample to a 100mL polythene screw-top bottle.
- Add 100mL of 0.5M H_2SO_4 and place on a tumbler at 12rpm for 16 hours at 25^{o}C.

- Filter through double Whatman No. 41 filter paper. If filtrate is still not clear, refilter using No. 42 paper.
- Analyse extracts as per manual method following.

ii Inorganic Phosphorus

- Accurately weigh 2g soil into a 100mL polythene screw-top bottle. Label accordingly.
- Place 100mL 0.5M H_2SO_4 to each bottle.
- Place on tumbler at 12rpm for 16 hours at 25^{o}C.
- Filter through double Whatman No. 41 filter paper.
- Analyse extracts as per manual method following.

D Measurement of phosphorus

The method of analysis for phosphorus requires that the various forms of phosphorus in the soil be extracted and converted, where necessary, to the inorganic form, as the orthophosphate series of species PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^{-}$. Addition of the Colour Developing Reagent to the extract allows combination of the molybdate ions in that reagent and orthophosphate ions in the extract to form a colourless phosphomolybdate complex; the ascorbic acid in the reagent reduces the colourless complex to a "molybdenum blue" complex.

Phosphate concentration is determined colorimetrically by reading the absorbance of the fully developed molybdenum blue complex with a spectrophotometer.

E Procedure

- Pipette 5–15mL of each extract and standard into 50mL volumetric flasks.
- Add 10mL Colour Developing Reagent and mix well.
- Make to volume with distilled/deionised water.
- Stand flasks at room temperature for 20 minutes.
- Read off the absorbance value at 882mm of all standards and solutions against a reagent blank on a spectrophotometer.
- Plot absorbance vs. concentration for the standards and read off the P concentration of the unknown solutions in μg P/ mL.

Phosphate may also be determined by: ICP or autoanalyser method (Section 4.2.6)

4.2.5 COMBINED PHOSPHORUS AND SULFUR DIGEST METHOD FOR SOILS AND FERTILISERS (TILL ET AL., 1984)

This method utilises an already established analytical technique for phosphorus and combines it with sulfur, thus eliminating many of the sampling problems and equipment.

A Reagents

Unless specified otherwise all chemicals are of analytical reagent grade.

Digestion Mixture: Dissolve 15g potassium dichromate in 730mL of deionised water and add to a mixture of 2170mL of perchloric acid (70%) and 2100mL concentrated nitric acid (70%) to make 5 litres. 50mL bromine is finally added to the 5 litres and mixed thoroughly.

B Sample Preparation

- Weigh a 50mL Erlenmyer flask with 3–5 anti-bumping granules; record this weight. Into this flask weigh 1.5–3g dry soil (or 0.1–0.5g fertiliser); record the sample weight accurately.
- Add 10mL of the digestion mixture using a plastic syringe. Ensure this is done in a fume hood and all safety equipment, i.e. gloves, glasses, lab coat, etc. must be worn.
- Place a small funnel in the neck of the flask to allow refluxing of the mixture.
- After allowing the initial oxidation by nitric acid (brown fumes) to subside, the flasks are heated on a hotplate at a low temperature.
- The temperature is gradually raised until fumes of nitrogen dioxide (white fumes) cease to be evolved and the perchloric acid begins to reflux.

The dichromate in the mixture turns green (reduced to Cr^{3+} during the early stages and then is reconverted to orange-red as oxidation nears completion. This change is a very convenient indicator and after the change to red, the heating should be continued for about 1 hour at approximately 190^{O}C.

If heating is discontinued as soon as the colour change occurs, resistant materials such as methionine will not be completely oxidized. At the completion of oxidation, there should be approximately 1 mL of perchloric acid remaining.

- Allow flasks to cool.
- Wash glass funnels and inside of flask with distilled water using a squeeze bottle, and make volume up to 50g by weight.
- Mix thoroughly. Allow to stand overnight and then transfer solution (minus particulate matter) to a vial ready for analysis by ICP or autoanalyser.

C Washup

Rinse flask in running tap water and leave to soak overnight in a 2% Decon solution. Rinse three times in tap water, then rinse in distilled/deionised H_2O three times, invert and dry.

D Safety

Bromine is corrosive or vesicant to skin and other tissues. Its vapours should not be inhaled. Instruct others to keep at a safe distance. Use fume hood or wear breathing apparatus and gloves.

Spread soda ash or sodium bicarbonate liberally if a spillage occurs, and mop up cautiously with plenty of water; run this to waste diluting greatly with running water.

4.2.6 DETERMINATION OF P IN SOLUTION

The following methods of determining phosphate are based on either the colorimetric analysis of the blue phospho-molybdate complex or the yellow phosphovanado-molybdate complex. These methods can be used for a range of solutions containing phosphate, including total P in plant material after digestion with nitric/perchloric acid or sulfuric acid/hydrogen peroxide , total P in soil after digestion with potassium dichromate/nitric/perchloric acid or phosphate in solution from soil extracts (e.g. sorption.

The method chosen for analysis depends on:

- The extract (background matrix)
- Sensitivity required
- Whether manual or automated analysis is desired

A Manual Murphy and Riley (1962) method for P determinations

i *Introduction*

The soil be extracted and converted, where necessary, to the inorganic form, as the orthophosphate series of species PO_4^{-3}, HPO_4^{-2}, $H_2PO_4^{-}$. Addition of the colour developing reagent to the extract allows combination of the molybdate ions in that reagent and orthophosphate ions in the extract to form a colourless phosphomolybdate complex; the ascorbic acid in the reagent reduces the colourless complex to a "molybdenum blue" complex.

Phosphate content is determined colorimetrically by reading the absorbance of the fully developed molybdenum blue complex with a spectrophotometer at 882 nm.

ii *Reagents*

Sulfuric acid (5N): Dilute 70mL of concentrated sulfuric acid (98%) to 500mL.

Ammonium molybdate: Dissolve 20g of A.R. ammonium molybdate in water and dilute to 500mL. Store the solution in a pyrex glass bottle.

Ascorbic acid (0.1M): Dissolve 1.32g of ascorbic acid in 75mL of water. This solution should be prepared on the day it is required as the ascorbic acid easily becomes oxidized.

Potassium antimonyl tartrate (1mg Sb/mL): Dissolve 0.2734g of potassium antimonyl tartrate in distilled water and dilute to 100mL.

Mixed reagent: Mix thoroughly 125mL of 5N sulfuric acid and 37.5mL of ammonium molybdate. Add 75mL of ascorbic acid solution and 12.5mL of potassium antimonyl tartrate solution. This solution should be prepared as required as it does not keep for more than 24 hours.

iii *Standard Preparation*

Using 1000 ppm stock phosphate solution (1000 μg/mL), dilute 2mL to 1 litre. This solution now contains 2ppm (2μg/mL). To prepare solutions for a standard curve dilute as shown in Table 4.2.

Table 4.2. Solutions for a standard curve

mL 2ppm solution	ML distilled water	Final concentration (ppm)
10	0	2.0
8	2	1.6
6	4	1.2
4	6	0.8
2	8	0.4
0	10	0.0

iv *Procedure*

- Pipette 1mL aliquots of the samples into individual test tubes and add 9mL of distilled/deionised water. Mix well.
- Add 2mL of mixed reagent to the standards and samples and mix well.
- After not less than 10 minutes measure the optical density of the solution at 882nm using 10mm cells. Determine the reagent blank in the same manner using distilled/deionised water.
- Calibrate the method using the standards as listed above. The calibration curve only needs occasional checking as it remains constant and appears to be independent of changes in the batches of reagents.
- Once all results have been recorded and checked, rinse all glassware in tap water and leave to soak overnight in a 2% detergent solution.

B Manual Method for Phosphorus Determination in 0.5N $NaHCO_3$ Extracts

i *Reagents*

Phenolphthalein: Dissolve 0.05g of phenolphthalein in 50mL ethanol and make up to 100mL with distilled water. Keep in a sealed container when not in use.

Ammonium molybdate: Dissolve 20g ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) in approximately 400mL distilled/deionised H_2O. In a separate beaker carefully add 100mL concentrated H_2SO_4 to 400mL distilled/deionised H_2O. Cool both solutions and add the molybdate to the acid mixture in a 1 L volumetric flask. Make up to 1 L with distilled/deionised H_2O and store in an amber bottle.

Ascorbic acid: Dissolve 2.5g of ascorbic acid in distilled H_2O in a 25mL volumetric flask. Needs to be freshly made each day.

Ammonium hydroxide: Dissolve 100mL of NH_4OH in 100mL of distilled water (1:1).

ii *Method*

Add, in the following order, to a 50mL volumetric flask:

- 5mL of either standard or sample.
- Dilute to approximately 25mL with distilled H_2O.
- Add 2 drops of phenolphthalein and neutralise (until just pink) with ammonium hydroxide.

- Make up to approximately 30mL with distilled H_2O.
- Add (pipette) 4mL ammonium molybdate and stir.
- Immediately add 1mL ascorbic acid.
- Make to final volume of 50mL with distilled water.
- For a reagent blank the above procedure should be carried out using reagents only (i.e. no sample) plus 2mL of distilled H_2O.
- Read the absorbance at 882 nm.
- A standard curve should be prepared ranging from 0–1 μg/mL P, and hence sample values determined. (Remember to subtract the reagent (blank) value from the sample value.)

NOTE: It is obviously important that all glassware be free of phosphorus contamination and hence must be washed in a phosphorus-free detergent and rinsed in double distilled water before drying.

C Automated Total Phosphorus Determination (0–20 μg/mL) (Thomas et al., 1967)

NOTE: Total phosphorus determination is only possible when the sample is treated so that all the phosphorus exists in the inorganic form, e.g. plant material which has undergone complete acid digestion.

i *Reagents*

Ammonium Molybdate: Place approximately 600mL of distilled/deionised water in a clean beaker and, with the aid of a wash bottle, wash in 20g of ammonium molybdate powder

$[(NH_4)_6\ Mo_7O_{24}.4H_2O]$. Add slowly and carefully 225.0mL of concentrated H_2SO_4 with much stirring.

NOTE: Keep beaker half submerged in cold water during preparation and wear protective clothing, gloves and glasses at all times.

Make up to 1 litre when cool. Store for up to 1 month in an amber bottle in the refrigerator to prevent reagent breakdown due to UV light.

Ascorbic Acid: Add 1.76g of ascorbic acid and dilute to 200mL with distilled water. A fresh solution needs to be prepared each day.

NOTE: The bottle of ascorbic acid crystals should be stored in a refrigerator to prevent oxidation and consequent deterioration.

P Diluent: Add 5mL Wetting Agent A to 2.5 litres of distilled water. This solution should be prepared fresh each day.

Sample Wash Solution: Either 6.6% H_2SO_4 or 6.6% $HClO_4$, depending upon digestion technique used. Store in refrigerator when not in use.

ii *Manifold Design*

The manifold design is given in *Figure 4.7*

Phosphorus analyses are based on ammonium molybdate reduced with ascorbic acid as adapted for the auto-analyser by Colwell(1965).

Ensure the ascorbic acid reagent is added before the molybdate to ensure baseline stability.

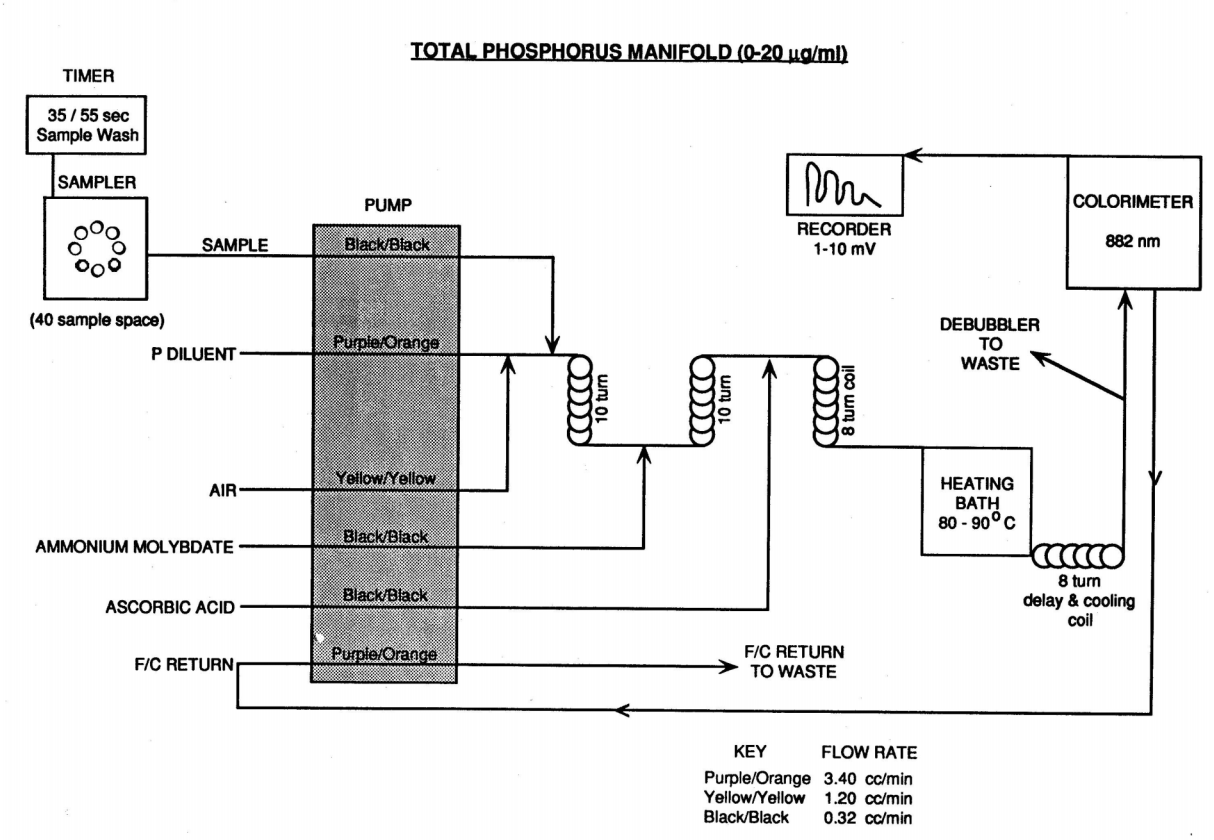

Figure 4.7. Autoanalyser manifold to determine 0–20ppm total P in an acid digest.

D Automated Determination of Phosphate in Extracts of Soil made with 0.5M $NaHCO_3$ and 0.01M (Salt, 1968; Fogg and Wilkinson, 1958)

i *Sample Preparation*

0.5M $NaHCO_3$ extracts: 5 g air-dried soil that had passed a 2mm sieve was shaken for 30 min. with 10mL 0.5M $NaHCO_3$ solution, filtered and phosphorus determined in the filtrate (Olsen *et al.*, 1954).

OR

1g air-dried soil that had passed a 2mm sieve was shaken for 16 hrs. with 100mL 0.5M $NaHCO_3$ solution, filtered and phosphorus determined in the filtrate (Colwell, 1965).

P in equilibrium with 0.01M $CaCl_2$: 20g air-dried soil passing a 2mm sieve was shaken at 5 minute intervals for 1 minute over 15 min. with 100mL 0.01M $CaCl_2$ solution — filtered and phosphorus determined in the filtrate (Schofield, 1955).

ii *Chemical Solutions*

Stock standard phosphorus solutions: Dissolve 1.098g oven-dried KH_2PO_4 in 250mL distilled water. This solution contains 1000 ppm P. Dilute with the appropriate extracting solution to 0–0.1ppm for 0.01M $CaCl_2$ extracts and 0–5 ppm P for 0.5M $NaHCO_3$ extracts.

0.5M $NaHCO_3$ buffered to pH 8.5 with sodium hydroxide solution.

0.01M $CaCl_2$ solution.

1M sulfuric acid OR 2N HCl (if sulfur is to be determined in the extract as well).

iii *Chemicals for Murphy & Riley Method*

Molybdate Reagent: Mix 50mL 5N H_2SO_4, 15mL 4% w/v ammonium molybdate, 5mL 0.273% w/v potassium antimonyl tartrate, and dilute to 100mL with distilled water. Prepare the tartrate and reagent fresh each day.

Ascorbic Acid:

(a) For 0.01M $CaCl_2$ extracts

Dissolve 2g ascorbic acid in approximately 50mL distilled water, add 5.4mL concentrated HCl, 25mL ethyl alcohol and dilute to 100mL with distilled water.

(b) For 0.05M $NaHCO_3$ extracts

Dissolve 2g ascorbic acid in approximately 50mL distilled water, add 25mL alcohol and dilute to 100mL with distilled water.

Prepare (a) or (b) fresh each day and add a few drops of Levor IV wetting agent.

iv *Manifold Design*

The manifold design for 0.5M $NaHCO_3$ phosphorus is given in Figure 4.8 and for 0.01M $CaCl_2$ in Figure 4.9.

1. The Murphy & Riley method is approximately one and a half times more sensitive than the Fogg and Wilkinson method.

2. The Murphy & Riley method was adapted to the auto analyser by dividing the single reagent into two parts:

(a) Sulfuric acid, ammonium molybdate and potassium antimonyl tartrate, and

(b) Ascorbic acid.

The concentration of ascorbic acid was increased and ethyl alcohol and hydrochloric acid were added to it to prevent the blue complex precipitating within the mixing coils. The baseline was then stabilized even when phosphorus ranged ten times in the 0.01M $CaCl_2$ extracts, providing the ascorbic acid reagent was added before the molybdate. Hydrochloric acid was omitted from the ascorbic acid reagent used for analysing 0.5M $NaHCO_3$ extracts, because these were neutralised with excess acid.

3. 1M sulfuric acid replaces the 2N hydrochloric acid as it has a more stable acidity plateau and greatly reduces the precipitate buildup within the manifold.

NOTE: The extra water-line is required on the 0.01M calcium chloride manifold to dilute the standard range and samples, otherwise the colour developed would swamp the manifold.

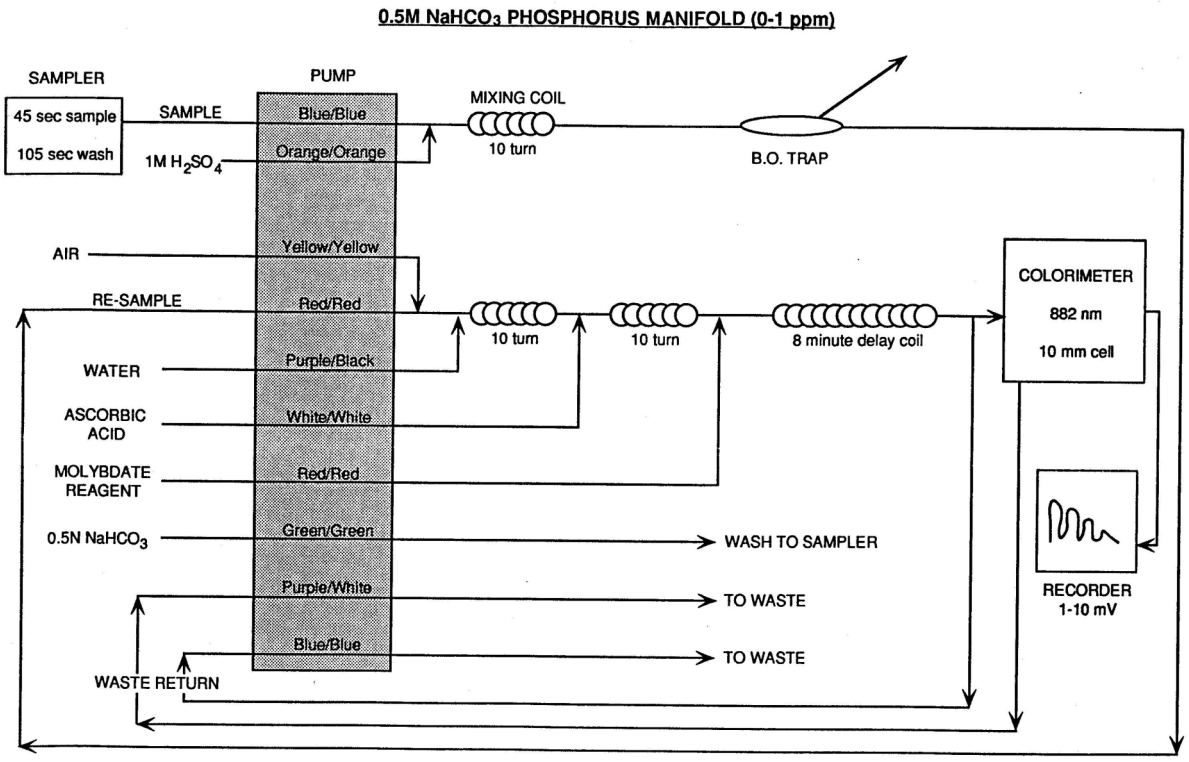

Figure 4.8. Autoanalyser manifold to determine 0–1 ppm P in $NaHCO_3$.

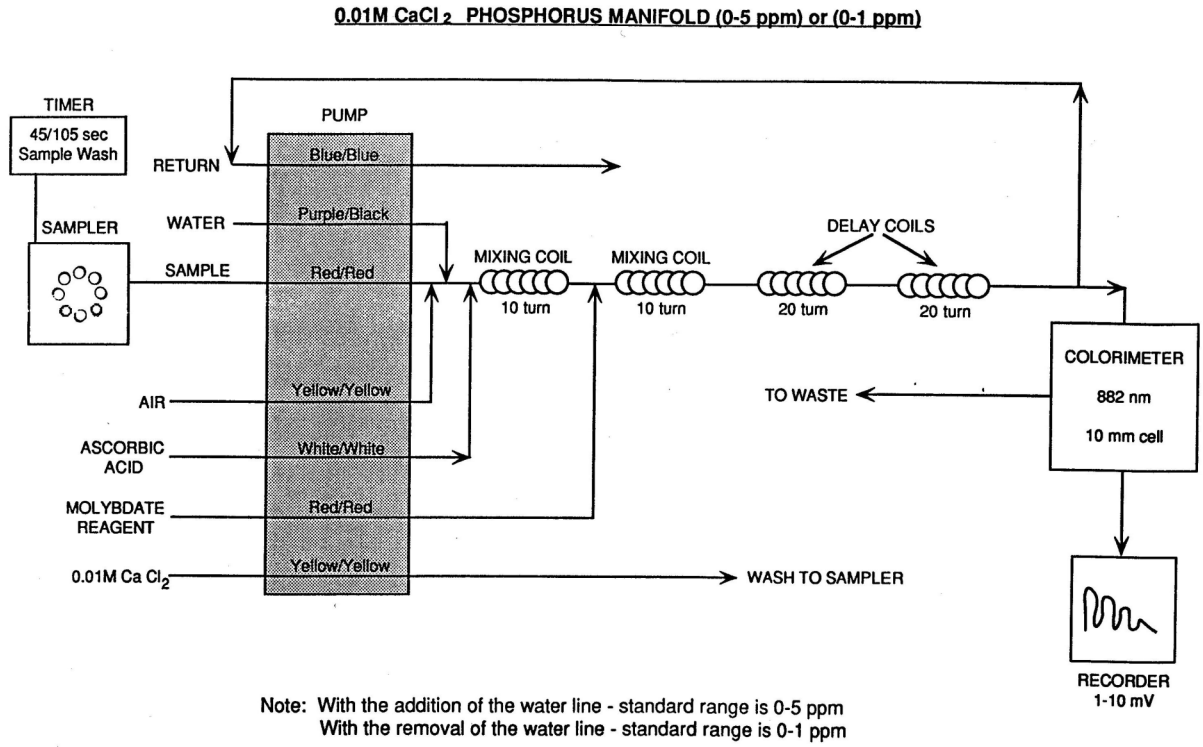

Figure 4.9. Autoanalyser manifold to determine 0–5 or 0–1 ppm P in 0.01M $CaCl_2$.

4.2.7 AUTOMATED DETERMINATION OF MICRO AMOUNTS OF PHOSPHATE IN DILUTE CALCIUM CHLORIDE EXTRACTS OF SOILS (0–1 μG/ML) (WARRELL AND MOODY, 1984)

A Sample Preparation

For soil only: 20g of air-dried soil (<2mm) is shaken (end-over-end) with 100mL of 0.01M $CaCl_2$ solution for 18 hrs. at 25^{o}C $\pm$ 2^{o}C. Following filtering through acid washed Whatman No. 42 paper, aliquots are taken for phosphate determination.

B Chemical Solutions

Ammonium Molybdate: Dissolve 20g ammonium molybdate [$(NH_4)_6$ $Mo_7O_{24}.4H_2O$] in 400mL distilled H_2O. Warm if necessary, and cool. Add 125mL concentrated H_2SO_4 to 400mL distilled water, and cool. Combine ammonium molybdate and acid solutions and make up to 1 litre.

Ascorbic Acid: Dissolve 1g L- ascorbic acid ($C_6H_9O_6$; LR grade) in distilled water and make up to 50mL. Prepare fresh each day.

Antimony Potassium Tartrate: Dissolve 0.23g antimony potassium tartrate ($KSbOC_4H_4O_6$) in distilled water and make up to 100 mL.

Mixed Reagent: Add 50mL ascorbic acid solution (2) to 70mL ammonium molybdate solution (1). Mix and add 10mL antimony potassium tartrate solution (3). Mix 25mL of 2% Levor IV solution with 12.5mL ethanol and add to mixed solution. Make mixed solution up to 250mL with distilled water. Prepare fresh each day.

Acid Diluent: 3mL of concentrated H_2SO_4 in 200mL distilled water.

0.01M $CaCl_2$: Dissolve 14.7g of calcium chloride in 10 litres of distilled water. Make up as required.

C Manifold Design

The manifold design is given in Figure 4.10.

- This manifold is used for phosphorus sorption curves or dilute salt extracts of soils.
- The phosphorus concentration in $CaCl_2$ extracts is generally less than 100 μg P/L and, with colorimetric methods, this necessitates the use of cells of long light paths or concentration of the colour complex into an alcoholic phase.
- The heating of reagents to speed colour development must be avoided, as Salt has shown that hydrolysis or organic phosphate occurs under heated conditions.
- Solutions were read at 882 nm using a 50mm flow-through cell.
- Time delay in the mixing soils to allow colour development was approximately six minutes.
- The acid diluent was necessary to adjust the final acidity for colour development to approximately 0.3N, which is the suitable plateau when transmittance of a phosphorus solution was plotted against acidity.
- A sample = wash time of 45–105 sec, respectively, is used.

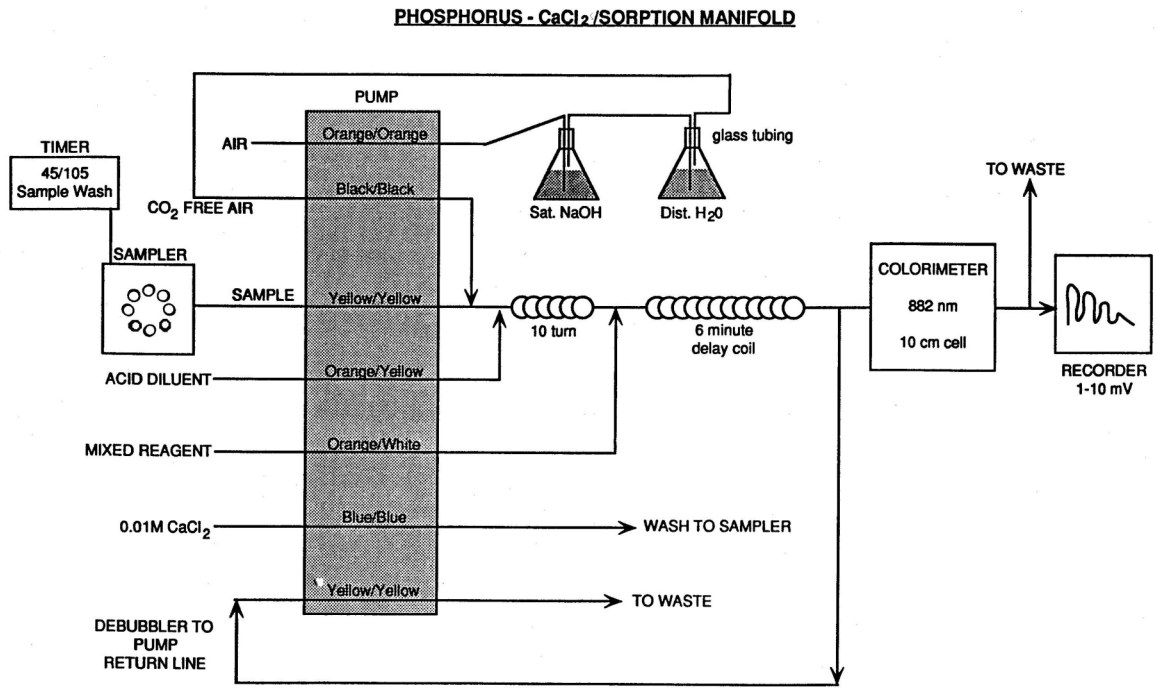

Figure 4.10. Autoanalyser manifold to determine P in dilute salt extracts of soil.

4.2.8 AUTOMATED DETERMINATION OF PHOSPHATE IN FERTILISERS USING THE PHOSPHOVANADO-MOLYBDATE COMPLEX (HANSON, 1950)

A Introduction

This rapid method makes use of the stable yellow colour complex developed when an excess of molybdate solution is added to an acidified solution of a vanadate and an orthophosphate; all three reagents required can be used in a composite solution. Details for the determination of water soluble and total phosphate are given below, along with adaptations for use on a Technicon autoanalyser.

B Reagents

i *Composite reagent*

For each litre there are required 140mL of concentrated HNO_3, 1.0g ammonium vanadate, (NH_4VO_3) and 20g ammonium molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$.

- The ammonium molybdate is dissolved in about 400mL distilled/deionised water at about 50^{O}C, and then the solution is cooled.
- The ammonium vanadate is dissolved in about 300mL boiling distilled/deionised water, cooled, and then the nitric acid added gradually with stirring.
- The ammonium molybdate solution is then added gradually to the other solution (b) with stirring. The mixed solution is finally diluted to 1 litre with distilled/deionised water.

ii *Mixed Reagent*

Dilute the composite reagent **1:4** for autoanalyser use.

Standard Range

0–500 μg/mL of phosphate as KH_2PO_4 made in the appropriate background solution.

Method

- Prepare samples as per required, i.e. water extraction or acid digest.
- Filter samples to remove particulate matter.
- Set up autoanalyser as given in Figure 4.11, using standards for calibration.
- Run samples.

NOTE: The composite reagent is stable for long periods of time and the optimum acidity is 0.5N. There is little interference from other ions, compared with the molybdenum blue method, especially as regards ferric ion and silicate.

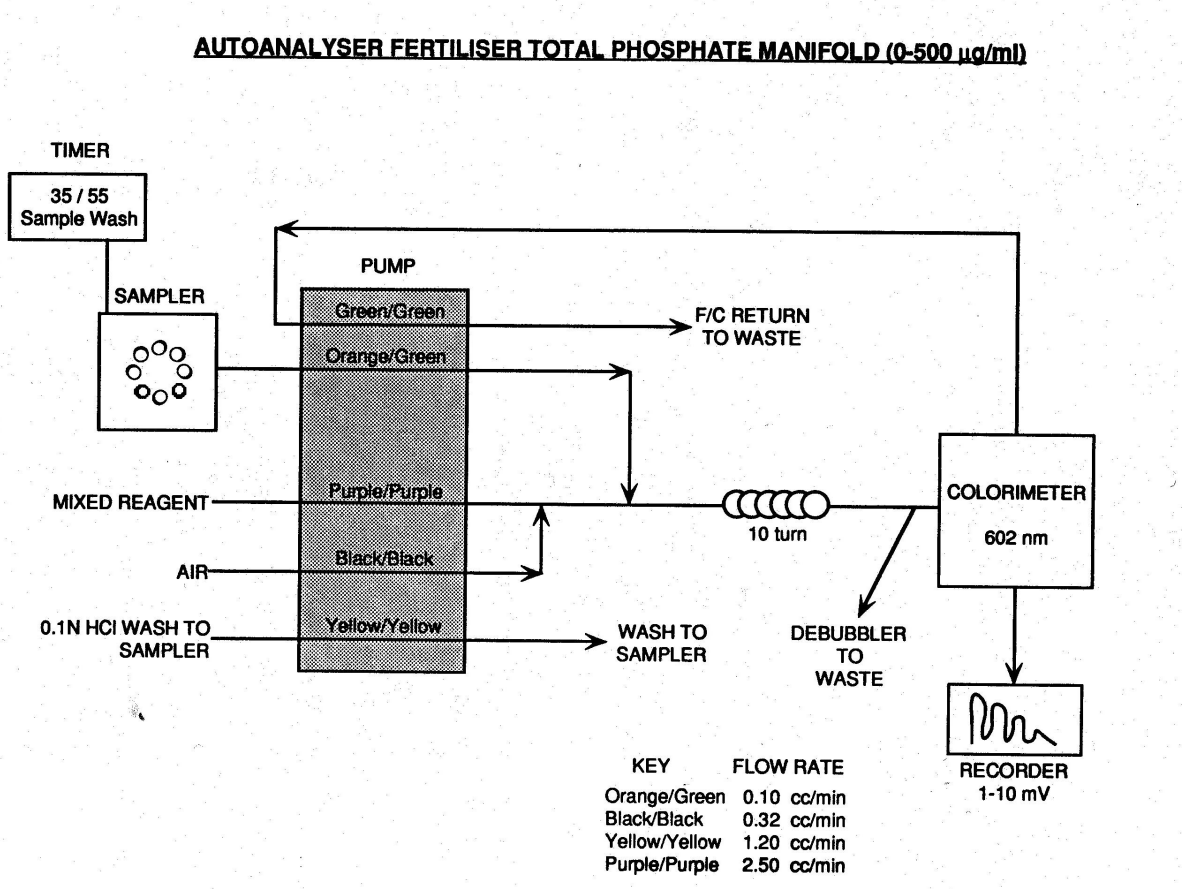

Figure 4.11. Autoanalyser manifold to determine 0–500 ppm P in fertilisers.

4.2.9 BIOAVAILABLE PHOSPHORUS IN SOIL – INTRODUCTION TO THE ISOTOPIC EXCHANGE KINETICS (IEK) METHOD

A Introduction

Plants take up phosphorus in the form of phosphate ions and P uptake is a time dependent process. The quantity of phosphate ions present in the soil solution at a given time represents only a small proportion of the total P taken up by the plants during this time. This fact indicates, that most of the phosphorus available to the crop is derived from the solid phase of the soil.

It has been shown that bioavailable soil P can be determined by defining three soil status parameters. The first parameter, the intensity factor, represents the concentration of phosphate ions in the soil solution (c_p).

The second parameter, the so called quantity factor, reflects the proportion of the total soil P that can possibly enter the soil solution and therefore is potentially available for plant uptake e.g. phosphate ions in the soil-water suspension. The P isotopically exchangeable P (E_1) within one minute is a good measure for the quantity factor.

The ratio of the quantity factor to the intensity factor, i.e. the quotient of P potentially in solution, divided by the P actually in solution [E_1 / c_p] called the capacity factor, is representing the P-buffering capacity of the soil, and describes the ability of a soil to maintain the intensity factor constant when P is added or removed from the soil by P-application or P uptake by plant roots respectively.

Available soil P fertility must be interpreted taking into account the three factors: the intensity factor c_P, i.e. the concentration of P in the soil solution present as phosphate ions, the quantity factor estimated by the E_1 - value and the capacity factor E_1/c_P. Each of them can be limiting for crop yield, even, if the two other two factors are not limiting.

B Intensity factor

As previously explained this factor has been identified to be the concentration of phosphate ions in the soil solution. This concentration can vary from about 0.002 mg P l^{-1} to 4 mg P l^{-1}. The intensity factor is the most sensitive parameter of the soil P fertility. When the concentration C_P of phosphate ions is lower than 0.02 mg P l^{-1}, P will be limiting, and can actually be considered as one of the major limiting factors of crop growth. There are two main reasons accounting for too low P-concentrations in soil solutions:

- A continuous soil mining, as observed in many developing countries;
- A high fixing capacity of the soil, i.e. the ability of certain soil components to instantaneously transform soluble P into fixed, insoluble forms of P. The available soil P will remain a limiting factor in soils with a P-concentration in soil solution lower than 0.02 mg l^{-1} irrespective of the quantity factor. Such high to very high fixing capacities are frequently observed in Andosols.

The P concentration in the soil solution can only be increased by P applications exceeding the quantities of P taken by crops (i.e. by a net excess). However it is very difficult to increase the P concentration in soil solutions of soils with low to very low P-status. In such cases the P-application should rather be done in bands than broadcasted or incorporated to increase the P-concentration near the root zone..

C Quantity factor

It was shown that the true quantity factor represents a pool of phosphate ions, which are loosely attached to soil particles and can instantaneously exchange with phosphate ions in the soil solution. This pool can only be determined by double isotopic dilution method. This method is time- and material intensive. In fact it was shown that this pool is of the same order of magnitude as the E_1 pool. The fraction of soil P that can instantaneously be isotopically diluted, i.e. exchanged, has been found to be slightly lower than the E_1 value. Therefore the E_1 pool, i. e. the quantity of P isotopically exchangeable during the first minute of isotopic exchange, is a good measure of the quantity factor.

The quantity factor can vary from about 0.1 mg P kg^{-1} to 50 mg P kg^{-1}. E_1 values lower than 3–4 mg P kg^{-1} indicate available soil P to be a limiting factor for crop production. E_1 values higher than 10 mg P kg^{-1} indicate soil P fertility to be sufficient, provided that the intensity factor exceeds 0.02 mg P l^{-1}.

D Capacity factor or buffering capacity

This factor varies from 10 L $(kg\ soil)^{-1}$ to 2000 L $(kg\ soil)^{-1}$ in different soil types. No theoretical value can be given. The capacity factor represents the volume of soil solution, which can be obtained from a soil showing a constant P concentration

A low value is obtained in soils with a low P buffering capacity. In such soil types the concentration of P in the soil solution can easily be altered by P application and by P uptake mainly from plants. A low buffering capacity corresponds to a low fixing capacity, i.e. a low ability of the soil particles to fix phosphate ions and convert them to non-mobile forms.

A capacity factor of 50 L $kg^{-1}$and higher will not allow easy respectively inexpensive enhancement of soil P fertility

E Time factor

The effect of time can only be studied applying the isotopic exchange method by determination of the various isotopically exchangeable pool sizes of phosphate ions. No other experimental

procedure can estimate the quantity of P that can leave the soil particles and enter into the soil solution with time. The advantage of the isotopic exchange method is therefore the possibility to study the ability of a soil to deliver P to the crop by releasing P from the solid phase into the soil solution compared to the changing crop demand for P during the growth period.

The most important pool for P uptake of annual crops is the pool [1 minute – 1 day]. A pool size lower than 10 mg P $(kg\ soil)^{-1}$ indicates P to be a limiting factor for crop yield.

F Kinetic factors

Of highest interest to agronomists is the mean P-flux F_m, i.e. the rate of P-transfer from soil particles to the soil solution. When this flux is lower than 20–30 mg P $(kg\ soil)^{-1}$ $(min)^{-1}$, the ability of this soil to release phosphate ions to the soil solution can be considered as a limiting factor for crop production.

4.2.10 ISOTOPIC EXCHANGE KINETICS (IEK) – REFERENCE METHOD FOR THE ASSESSMENT OF BIOAVAILBALE SOIL P

A Introduction

The isotopically exchangeable phosphate ions of a soil-solution system have been shown to be equivalent to the available soil P.

Therefore, to characterize the available P of a soil, it is necessary to determine the physico-chemical characteristics of the isotopically exchangeable phosphate ions of a soil solution system.

In this connection some recommendations concerning the use of isotopes to study complex systems as well as some theoretical constraints should be considered:

1. The isotopic tracer must be applied to the system at a precise time in a unique chemical and physical form and in a known quantity. This means that the isotopic tracer applied to the soil solution system for determining the isotopically exchangeable phosphate ions has to be applied as a known volume of a solution exclusively containing orthophosphate ions in a known quantity at a defined time .

2. The system has to be maintained in steady state during the experiment, otherwise unreliable data will be obtained and the results will be difficult to interpret. The introduction of the isotopic tracer must neither modify the equilibrium status, nor modify the system itself, e.g. change the total P quantity in the system. Consequently, despite the complications it is recommended to apply the isotope without carrier.

3. The subsampling for determination of the radioactivity has to be precisely timed, out of a precisely defined pool in a precise chemical form. As the radioactivity is applied to the soil solution as phosphate ions, also the subsampling of has to be done only of the phosphate ions and only from the soil solution. This particular point is of a great importance to obtain reliable results and will have consequences on the choice of the experimental procedure.

B Calculation of the quantity of isotopically exchanged P and of isotopically exchangeable P at time T

If measurements of isotopically exchangeable phosphate ions in soil solution systems have been carried out in steady state, which is a basic requirement for interpretable experimental results and:

- R is the quantity of radioactivity applied to the soil solution system as phosphate ions,

- $r_{(t)}$ is the quantity of radioactivity after a time t of isotopic exchange,

- $E_{(t)}$ is the quantity of isotopically exchanged P after a time t of isotopic exchange, and the quantity $E_{(t)}$ is calculated based on the assumption, that the isotopic composition, the specific activity, i.e. the ratio between the quantity of the radioactivity and the quantity of P as phosphate ions (both of these synonymous expressions can be utilized), is the same for phosphate ions of the soil solution and all isotopically exchanged phosphate ions in the system. Thus,

$$R / E_{(t)} = r_{(t)} / q_s \qquad \text{(Equation 1)}$$

and therefore:

$$E_{(t)} = q_s\, R / r_{(t)} \qquad \text{(Equation 2)}$$

To obtain good results R , $r_{(t)}$ and q_s have to be determined accurately. R and $r_{(t)}$ are determined by counting the radioactivity and q_s is determined by measuring the concentration of P as phosphate ions in the soil solution. It is of utmost importance that only phosphate ions are present in the filtered soil solution, i.e. it must be absolutely free of soil particles!

The quantity of isotopically exchanged P in solution, $E_{(t),}$ increases when time t increases, since the radioactivity $r_{(t)}$ in the solution decreases with increasing time of exchange. It was shown that the relation between $r_{(t)}$ and t , expressed in minutes, can be described by the following formula:

$$r_{(t)}/R = r_1/R \, [\, t + (r_1/R)^{1/n} \,]^{-n} + r_{eq}/R \qquad \text{(Equation 3)}$$

In the above equation r_{eq} is the radioactivity of the soil solution being in equilibrium

It has been shown that:

$$r_{eq}/R = q_s/P_T \qquad \text{(Equation 4)}$$

where P_T is the total P of the soil.

Therefore substituting r_{eq}/R we can write:

$$r_{(t)}/R = r_1/R \, [\, t + (r_1/R)^{1/n} \,]^{-n} + q_s/P_T \qquad \text{(Equation 5)}$$

Refering to equation [2] equation [4] shows that, for calculation of the isotopically exchangeable P, $E_{(t)}$ at any time t of isotopic exchange the following four parameters have to be determined:

- q_s , the P quantity, present as phosphate ions in soil solution;
- P_T , the total P in the soil;
- r_1/R, i.e. the ratio of the radioactivity found in the soil solution after 1 minute of isotopic exchange to the total radioactivity applied;
- n, i.e. the exponent of the power function respectively the slope of the logarithmic function, describing the rate of exchange of the radioactivity in solution with time t.

C Determination of isotopically exchangeable phosphate ions in soil – Laboratory studies

The items listed below consist of a set of materials and the equipment needed for the measurement of the specific activity of one soil solution sample after isotopic exchange at four different sampling times.

i *Preparation of soil suspension:*

Material / equipment	Procedure	Remarks
• min. 10 g soil, sieved (2mm), dried at room temperature (RT) or a quantity of soil equal to 10 g of airdry soil	• weigh 10.0 g of soil into a plastic bottle	• this soil has to be representative for the soil to be studied, i.e. the soil sampling should follow an (officially accepted) standard procedure • the storage time before the IEK-experiment should be kept short and the soil should be stored in a cool, dry and dark place • **important**: if the soil has not been dried for special reasons (mainly, because the drying could modify the P status of the sample), the [g] water have to be determined on another sub-sample of this soil and the amount of soil corresponding to 10 g dry soil have to be weighed in and less water has to be added accordingly!!
• Plastic bottle with screw cap, capacity ~250 mL, liquid tight, preferably with rubber fitting		• generally: all plastic- and glassware should be free of P-contamination; it is recommended to treat P-contaminated vessels with dilute acid (e.g. ~ 2M HCl) and then rinse it thoroughly with distilled water
• distilled water	• add 99.0 mL of distilled water and close the bottle tightly	• the water should be checked for being free of any P-contamination, since we are working at the ppb-level [= μg/L]
• shaker for the 250 mL plastic bottles (possibly 6–12 positions)	• fix the bottle in the shaker and shake 8–12 hours (e.g. overnight)	• the aim of the shaking is to obtain an equilibrium state for all ions including the phosphate ions released into the soil solution

ii ***Preparation of " ^{32}P mother solution":***

Material / equipment	Procedure	Remarks
• β - monitor, gloves, labcoat, overshoes , wastebin for radioactive waste, labels to mark radioactive items etc.		• see safety precautions according to the authority's regulations for working with radioisotopes
• two 20-mL plastic vials • small lead container for vial	• place one 20-mL vial in the lead container add about 15 mL of distilled water to the vial	• lead container used as shielding against radiation from the ^{32}P stock solution, which should be placed near the soil suspension, and to avoid spillage
• variable automatic pipette, 0.2-1.0 mL plus suitable disposable tips		• pipettes and tips e.g. from Eppendorff, Gilson or Socorex
	! use gloves for the following operations!	
• carrier-free ^{32}P solution, min. 20 μCi or 750 kBq • 50 mL volumetric flask	• add a calculated volume of "solution **A"** containing about 20μCi or 750 kBq ^{32}P stock-solution into the 15 mL of distilled water => " solution **B**"	• carrierfree ^{32}P as orthophosphate in dilute HCl, e.g. from Amersham Buchler GmbH, D-38110 Braunschweig example: if 1 mCi has been ordered, dilute with 1mL H_2O => "solution **A**" piupette 20 μl of "solution **A**" to 15 mL of distilled water=>soln. "**B**"
• 20-mL plastic syringe • disposable filter to be fixed to the syringe, pore size 0.2μm	• filter " solution **B**" through the 0.2 μm filter into the second clean plastic vial => "solution **C**" = "P-32 mother solution" • replace the empty first vial in the lead container with the new vial containing "solution **C**" discard the contaminated filter, tip, vial and syringe	• disposable filter units e.g. from Fa. Millipore S.A.,67 Molsheim, France, Millex-GS, 0.22 μm • filtration is made to eliminate labeled particles other than ^{32}P labeled phosphate ions , which are sometimes present in the ^{32}P containers supplied by the producer

iii *Counting of radioactivity of the"^{32}P mother-solution":*

Material / equipment	Procedure	Remarks
• 100 mL volumetric flask • min. 25 mL of KH_2PO_4-solution, 1 mg P l^{-1}	• pipette about 25 mL of the KH_2PO_4-solution into the volumetric flask • pipette 1 mL of "solution **C**" into the volumetric flask • make up to the mark with distilled water and shake => "solution **D**"	• the KH_2PO_4-solution is added to avoid fixation of the radioactive phosphate ions to the glass wall of the volumetric flask • solution **D** is a 1:100 dilution of the " ^{32}P mother solution **C**" and is used to count accurately the radioactivity applied to the soil solution
• min. 4 counting vials for LSC- or Cerenkov counting • distilled water or LSC cocktail (see remarks)	• take 4 replicate subsamples of exactly 1.0 mL of " solution **D**" • add 9.0 mL of distilled water or LSC cocktail respectively • count and calculate the mean of the 4 replicates to obtain the actual activity, which will be applied to the soil samples: the activity should range between 40000 and 10 000 cpm	• the advantage of ^{32}P is the possibility of Cerenkov counting in aqueous solution, which is much cheaper than Liquid Scintillation counting (LSC), but a quench correction curve for color quench correction has to be prepared before counting the solutions • the lower counting efficiency of about 50 % in Cerenkov counting has to be taken into account • in case of LSC a suitable LSC-cocktail has to be used • in case of too high count rates the mother solution can be diluted accordingly if the counting rate is too low a new mother solution has to be prepared • R is 100 times the radioactivity of "solution **D**"

iv *Isotopic Exchange Kinetics experiment*

Material / equipment	Procedure	Remarks
• nitrate cellulose membrane filters, 0.2 and 0.025 μm calibrated pore size; fitting into the filter holders (see remarks below)		• filter membranes, 47 mm diameter e.g. from Schleicher&Schüll D-37582 Dassel Germany, Ref. No. for 0.2 μm: 406 970, for 0.025 μm: 402 212 pore size depending on the soil type; the filtrate should be absolutely clear and possibly colorless
• 4 filter holders (for 4 different sampling times), 50 mm diameter	• place a nitrate cellulose membrane filter in the filter holder • close and screw tightly	• filter holders e.g. from 1)Millipore, model Swinnex 47, i.e. 47 mm in diameter; 2) Micro Filtration System (MFS) model propylene in line, 47 mm in diameter, Ref. Cat.No. 501200 • it is recommended to use the MFS-filter holders for two reasons: 1) membrane position clearly defined => no destroying of filters during closing 2) less expensive a shop made tool to close the MFS filter holders is of advantage
• 4 clean 15–20 mL plastic vials to collect the filtrates • 4 Liquid Scintillation vials	• label the vials	• usually the filtration is done by pushing down the piston of the syringe with the hand; however this may be difficult, when using the 0.025 μm-filters with tropical soils and for 15–17 mL of the t_4-subsample of I.E., which will be used for P-determination (see below) => in this case a specially designed (shopmade) “pressing device” can be utilized for pushing down the piston of the syringe containing the sampled soil solution, which is connected to the filter holder after sampling (see below) instead of the pressing device for MFS-system a vacuum system with different filter holders can be used to filter the soil solution sample
• magnetic stirrer • teflon coated magnetic bar, ~1-2 cm length	• remove the soil suspension from the shaker • open and add a magnetic bar without splashing place the solution on the magnetic stirrer and stir with ~200 rpm	

Material / equipment	Procedure	Remarks
• 3 plastic disposable 10mL- syringes (for t_1 - t_3)		• syringes e.g. Fa Terumo Europe N.V., 3030 Leuven, Belgium it is recommended to use 1 new syringe for each sampling time to avoid troubles with cross-contamination and the time limitation to clean the syringe.
	• 52 to 53 seconds after addition of the P-32 solution switch off the magnetic stirrer and subsample about 7 to 8 mL of soil suspension using a 10 mL syringe	**! important !** : • the aim is quick filtration to obtain the quantity "r" of radioactivity after exactly 1 min of isotopic exchange => "r_1"
	immediately switch on the stirrer again and quickly connect the filled syringe to the filterholder, place the outlet in the opening of the vial and filter a little more than 1 mL of the suspension by manually pushing down the piston for about 10 to 15 seconds depending on the number of sub sampling times repeat the above steps using new vials, syringes and filter holders (sub-sample always 7 to 8 seconds before the desired exchange time is over and try to filter within 10 seconds maximum) *see Figure 4.12, Figure 4.13*	• the filtrate must be absolutely clear (!), if not, take a membrane filter with smaller pore size (i.e. 0.025 μm instead of 0.2 μm) ; there might be a slight yellowish color, if the soil has a high clay or organic matter content (for instance in tropical soils) • a minimum of 2 sub-sampling times is required for an isotopic exchange kinetic study: 1 and 10 min); the number of sub-sampling times depends on the skill and practice of the analyst • the following time grid is suggested: t_1=1 min, t_2=3 min, t_3=7 min and t_4=10 min
• plastic disposable 20 mL- syringe (for t_4)	• for the t_4-value use a 20 mL syringe and sub-sample about 15 to 17 mL of the soil suspension (use **a 0.025 μm membrane filter**)	• the t_4-subsample is also used for colorimetric determination of the total P in the soil solution present in the form of phosphate ions (see below) K^+, Ca^{++}, Mg^{++} and Na^+ can be determined in the solution using atomic absorption spectroscopy (AAS), to obtain some additional information on nutrients in the soil(see below)
• Liquid scintillation cocktail or distilled water • 1-mL automatic pipette plus disposable tips	• pipette 1.0 mL of the filtrate into a LSC vial onto an aluminum plate and evaporate slowly for GM counting) add about 9 mL of Liquid Scintillation cocktail for LSC or 9 mL of distilled water for Cerenkov-counting to the vial,close and shake to mix	• if the volume of filtrate is lower than 1 mL , measure the volume of solution using a variable micropipette and note the volume used for counting! • complete with distilled water to 1 mL for GM-counting and LSC or to 10 mL for Cerenkov counting to achieve the same counting conditions in all samples
GM-counter or: LSC	count the radioactivity r_1, (r_3, r_7,) and r_{10} of the filtrates • use the "t_4- filtrate" for total P determination *see Figure 4.14*	• Liquid Scintillation Counter, ***NOTE:*** **store the soil- filtrates at least for 10 halflifes (i.e. about 5 months) before disposal!**

Figure 4.12. Setup of the IEK-assay.

Figure 4.13. Filtration of labelled soil suspension for Cerenkov counting or LSC.

Figure 4.14. Filtration of soil suspension for determination of intensity factor, c_p.

v *Determination of the P-concentration in soil solution using the Malachite Green Method (Ohno and Zibilske, 1991; Novozamsky et al.,1993; Van Veldhoven et al.,1983)*

The proposed malachite green method allows for the determination of P in small volumes (2 mL) and in concentrations (ppb level) too low for the standard Murphy and Riley method and generally all “blue methods” based on the reduction of molybdate.

Two reactants are to be prepared: (1) containing the molybdate in sulfuric acid solution (2) containing the malachite green.

The colorimetric reaction is taking place at nearly neutral pH, which is the case in soil solutions.

Preparation of reactants:

Material / equipment	**Procedure**	**Remarks**
	reactant 1:	
• H_2SO_4 conc.,p.a. • ammonium heptamolybdate tetrahydrate , p.a. $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ • distilled (or deionized) water • lab glassware • deep freezer • refrigerator • magnetic stirrer , teflonized magnetic bar	• dissolve 308 g (or 168 mL) of concentrated sulfuric acid in about 500 mL of distilled water • place solution in the deepfreezer (-18° C) for 2 to 3 hours to achieve a temperature of about +12° C • dissolve 17.55g of ammonium heptamolybdate in about 250 mL of distilled water • place solution in the deep freezer (-18° C) for 2 hours to achieve a temperature of about +12° C when both solution are at the required temperature, transfer the ammonium heptamolybdate solution into the cold sulfuric acid solution and stir to mix completely, make up to 1 L and transfer to a dark bottle	• H_2SO_4 conc : e.g. Merck, Cat.No. 731 • ammonium heptamolybdate tetrahydrate, p.a.: Merck, Cat.No. 1182 • reactant 1 must be stored in a colored bottle in the refrigerator at about + 4 °C to avoid molybdate reduction and is stable for long time (> 12 months)

Colorimetric P-determination

Preparation of a standard curve

Material / equipment	Procedure	Remarks
• disposable plastic cuvettes with caps, d= 10 mm (or [quarz-] glass-flow through-cuvette, d=10mm) • KH_2PO_4, p.a. • lab glassware • dessicator with silica gel	• dry 1–2 g of KH_2PO_4 at 105°C for 2 hours • cool to RT in a dessicator with silica gel • prepare a 100 ppm (= 100mg l^{-1}) P-stock solution: weigh 439 mg dried KH_2PO_4 into a 1000 mL volumetric flask, dissolve in distilled water and make up to mark prepare a 1 ppm (=1 mg/mL) P-standard solution: pipette precisely (!) 10.0 mL of the 100 ppm P stock solution into a 1000 mL volumetric flask add distilled water, swirl and make up to mark with distilled water into each cuvette pipette the reactants as follows	• plastic cuvettes e.g. Fa. Elkey Ultra-Vis,d=10mm, Elkey-Products Inc. MA01545, Boston USA ***NOTE:*** if the reading of color density is done in **glass cuvettes**, the cuvettes have to be cleaned with ethanol between each measurement to remove the color attached to the walls; strictly avoid using water for cleaning !! • KH_2PO_4, p.a.: e.g. Merck Cat.No. 4873 • store both solutions in the refrigerator at +4°C, but they have to be at room temperature before pipetting precise volumes !!
• disposable cuvettes with caps • automatic pipettes, preferably variable: 0.2–1.0 mL and 0.05–0.5 mL plus disposable tips • UV/VIS-spectrophotometer, wavelength 610 nm	• 2.0 mL of dilute standard solution as indicated in table IV.4.2.2. • 0.4 mL of reactant (1) containing the ammonium molybdate in sulfuric acid • wait about 10 min • 0.4 mL of reactant (2) containing the malachit green dye • close cuvettes and shake once by turning over the cuvette to mix • wait for minimum 1 hour and read the optical density at 610 nm • prepare a standard curve by linear regression and plot it in a graph	• plastic cuvettes e.g. Fa. Elkey Ultra-Vis,d=10mm, Elkey-Products Inc. MA01545, Boston USA, • Spectrophotometer, e.g. Specord UV/VIS - S10, Zeiss, Germany • if using glass cuvettes, prepare the sample solutions in polyethylene vials with caps , mix in those vials and pipette the colored solution into cuvettes afterwards • the color of the sample solution is stable for about one week at room temperature ***NOTE:*** the optical density of cuvette 2 containing 0.05 ppm P, i.e. 0.1 mg P in 2 mL, should read about 0.125 absorbance • with this very sensitive method it is possible to measure concentrations down to 0.002 mg l^{-1} (ppm) or 2 μg/L (ppb)

Pipetting scheme for Standard Curve

Cuvette number	1	2	3	4	5	6
P-concentration [mg P/L]	0	0.05	0.1	0.15	0.20	0.25
[mL] distilled water	2.0	1.9	1.8	1.7	1.6	1.5
[mL] of 1 mg/L- P-standard solution	0	0.1	0.2	0.3	0.4	0.5

Measurement of the phosphate ion concentration c_p in the soil solution

Material/ equipment	**Procedure**	**Remarks**
• disposable cuvettes with caps • automatic pipettes, preferably variable: 0.2–1.0 mL and 0.05–0.5 mL plus disposable tips • UV/VIS-spectrophotometer, wavelength 610 nm	• pipette 2.0 mL of the "t_4-filtrate" of each soil sample in a cuvette • add 0.4 mL of reactant (1) • wait about 10 min • add 0.4 mL of reactant (2) • close cuvette, mix and wait for minimum 1 hour before reading the optical density at 610 nm • plot the absorbance reading of the soil solution on the standard curve and read out the P-concentration in mg P/L (=ppm)	• ***NOTE:*** if the absorbance value is too high (> 1.5), less filtrate has to be pipetted and the total volume in the cuvette has to be adjusted with distilled water example: 1.5 mL soil filtrate + 0.5 mL H_2O + reactants or: 0.5 mL of soil filtrate + 1.5 mL H_2O+reactants etc. ***NOTE:*** the reading of the standard curve and the soil-solutions should be done after the same time of color development and in the same session!!

vi *Determination of total phosphorus content P_t of the soil*

Material/ equipment	Procedure	Remarks
• fume cupboard • balance, readability +- 0.1g • lab glassware • 100 mL Erlenmeyer • $HClO_4$ conc. • hotplate • boiling granules • blue band filter paper • funnel • 100 mL volumetric flask	• add exactly 2.0 g of dry soil to 10 mL of $HClO_4$ • swirl to mix and add some boiling granules to avoid splashing of hot acid • gently heat until the acid starts to evaporate white fumes • when the soil turns to white or yellow color (after evaporation of min. 4 mL of acid) stop heating • cool the mixture slightly (not too much) • slowly (!!) add about 25–30 mL of distilled water and mix • filter the solution after cooling to RT • transfer the solution quantitatively to a 100 mL volumetric flask and make up to mark • determine the P-concentration of the solution by using a colorimetric method	• preferably work in a fume cupboard • $HClO_4$ conc., e.g. Merck No. 518 • filter: blue band • the time needed for evolution of white fumes and the developed color of the residue is depending on the soil type and composition • the total P-concentration P_t of the mineralized soil sample is generally determined using a yellow vanado-molybdate method (e.g. Murphy Riley) • calculation of P_t : measured P-concentration [mg l^{-1}] *50 = P_t [mg P kg^{-1} soil], if a soil to solution ratio of 1: 10, e.g. 10 g soil : 100 mL solution was used

D DETERMINATION OF THE MOST PROBABLE VALUES FOR r_1/r AND n

The general equation [5] describes the decrease of radioactivity $r_{(t)}$ in the soil solution with time t.

This formula can be simplified for any time of isotopic exchange not higher than 10 minutes for all soil types. In some soils time to reach equilibrium can be more than 1000 minutes, but to avoid complication of the procedure it is recommended to use only the data recorded during the first 10 minutes of isotopic exchange.

- The values of n range from 0.05 to 0.5.
- The values of r_1/R range from 0.01 to 0.8.

For a time $\leq$10 minutes, the value of $(r_1/R)^{1/n}$ is generally lower than 0.02 for any n and r_1/R values and can therefore be neglected.

Thus the equation [4] can be simplified as:

$$r_{(t)}/R = r_1/R\ [\ t\ ]^{-n} + q_s/P_T \qquad \text{(Equation 6)}$$

Generally for any time t $\leq$ 10 minutes, the ratio q_s/P_T, which reflects the equilibrium value for the radioactivity remaining in the solution for an infinite time is negligible compared to the $r_1/R\ [\ t\ ]^{-n}$ value.

Therefore the general equation [5] can be simplified further:

$$r_{(t)}/R = r_1/R\ [\ t\ ]^{-n} \qquad \text{(Equation 7)}$$

Equation [7] can also be written as:

$$\log [r_{(t)}/R\ ] = \log [r_1/R] - n \log t \qquad \text{(Equation 8)}$$

n and $\log [r_1/R]$ and can be considered as the "most probable values" of slope and intercept respectively of the linear regression line between $\log [r_{(t)}/R\ ]$ and log t for the values of $r_{(t)}/R$ obtained for each sampling at the time t, for any time t between 1 minute and 10 minutes.

Therefore a minimum of two samplings of the labeled mixture have to be made to determine *n* and *log r_1/R*. In fact very good results can be obtained with four samplings performed at 1, 3, 7 and 10 minutes. For many soils other time intervals can be utilized; for example 1, 4, 10 (and 20) minutes. The choice of the applied time intervals depends on the practice and experience of the analyst.

E DETERMINATION OF Q_s and P_T

i *Phosphorus in the soil solution*

The quantity q_s is the quantity of P in soil solution, present as phosphate ions. This quantity is the product of the P concentration times the volume of soil solution**.** The concentration of phosphate ions in the soil solution is determined by a colorimetric method. Many colorimetric methods can be applied; but presently the most sensitive, accurate and reproducible method known is using malachite green as a dye. This method is described in section 4.2.10, C, 5.

In the standard procedure, using 10 g of soil suspended in 100 mL of soil solution the quantity q_s , expressed in mg P/kgsoil, will be:

$$q_s = 10\ c_P \qquad \text{(Equation 9)}$$

where c_p is the concentration of P in the soil solution expressed in mg/L [ppm].

Example:

- ⇨ c_p = 0.13 mg P $l^{-1}$of soil solution
- ⇨ 0.013 mg P in 100 mL of soil solution
- ⇨ 0.013 mg P in 10 g soil
- ⇨ 1.3 mg P kg^{-1} soil

ii *Total P in the soil sample*

The quantity P_T , expressed in mg P kg-1 soil is the quantity of total P of the soil sample. Various methods for total P- determination in soil are described in literature. A quick and easy method,

using $HClO_4$, is described in section 4.2.10, C, 7.2, although with this method the total P can be slightly underestimated.

Calculation of the pool sizes

After having determined the values of r_1/R, n, q_s and P_T, the $E_{(t)}$ values can be calculated using the two following equations [2] and [5].

First the $r_{(t)}/R$ value is calculated using equation [5] for the chosen time t and secondly the $E_{(t)}$ value is calculated, using equation [2].

For all the following calculations t is expressed in minutes.

Generally, the sizes of the following 5 pools are to be determined:

Pool E_1

This pool is isotopically exchangeable during the first minute of exchange and reflects the instantaneously isotopically exchangeable P. It represents the quantity of P, as phosphate ions, that can move from the soil to the solution almost instantaneously and can be considered as the pool of phosphate ions directly available to the plants without chemical transformation. Its size can be calculated as follows:

$$E_1 = 10\ C_P\ [R/r_1] \qquad \text{(Equation 10)}$$

Pool [1 day] - Pool E_1

This pool corresponds to the quantity of phosphate which can be taken up by a given root zone, which can actively take up P within a time, limited between 1 and 2 days. 1 day was chosen for the calculations The size of this pool is:

$$\text{Pool [1 day] - Pool } E_1 = 10\ C_P\ [\ R/r_{1440} - R/r_1\ ] \qquad \text{(Equation 11)}$$

where r_{1440} (= r_{1day})is calculated using formula [5]

Pool [1 day - 3 months]

This pool is explored during the time of root functioning of a crop. Its size is estimated the following:

$$\text{Pool [3 months] - Pool [1 day]} = 10\ C_P\ [R/r_{129600} - R/r_{1440}\ ] \qquad \text{(Equation 12)}$$

Pool [3 months - 1 year]

This pool corresponds to the quantity of phosphate ions that can be released from soil into the soil solution in less than 1 year.

$$\text{Pool [1 year] - Pool [3 months]} = 100\ C_P\ [R/r_{518400} - R/r_{129600}] \qquad \text{(Equation 13)}$$

Pool exchangeable in more than 1 year

This pool corresponds to very slowly exchangeable P which can be released into the soil solution over the years under the condition of zero P fertilization It reflects the ability of soil to release phosphate ions to plants in many developing countries, where no P is added for crop production.

Its size can be estimated by the below equation:

$$\text{Pool} > 1\ \text{year} = P_T - 10\ C_P[R/r_{518400}] \qquad \text{(Equation 14)}$$

Calculation of the mean rate of exchange, the mean sojourn time and the flux of exchange

Three kinetic parameters can be determined using the various parameters previously obtained.

F Calculation of the mean rate of exchange, the mean sojourn time and the flux exchange

i *Mean rate of exchange between soil and solution*

The mean rate of exchange k_m of phosphate ions between the liquid phase and the solid phase follows a fundamental kinetic law. It value is expressed in t^{-1} i.e. per unit of time, here being 1 minute. Its numerical value gives the number, of entries of a quantity of phosphate ions into the soil solution and the number of exits out of the soil solution per minute. Its values is calculated using the following formula:

$$K_m = n\ /\ [r_1/R]^{1/n} \qquad \text{(Equation 15)}$$

ii *Mean sojourn time of phosphate ions in the soil solution*

The mean sojourn time T_m is the time required for a quantity of phosphate ions in the soil solution to enter or leave the soil solution ("mean residence time") This time is calculated the following:

$$T_m = 1/K_m = [r_1/R]^{1/n} / n \qquad \text{(Equation 16)}$$

T_m is generally expressed in minutes.

iii *Mean flux of exchange between the solid phase and the liquid phase of the soil solution system*

This mean flux represents the amount of P, which enters, and leaves the soil solution. Its value is calculated as follows:

$$F_m = 10\ c_p\ k_m \qquad \text{(Equation 17)}$$

F_m is generally expressed in mg P min^{-1}. The factor 10 is only valid for a soil: solution ratio of 1: 10, e.g. 10 g soil in 100 mL solution.

4.2.11 PREDICTION OF THE EFFICIENCY OF A P FERTILISER USING A PREDICTED %PDFF OBTAINED BY IEK EXPERIMENTS – POOL SIZE

A Definition of P fertiliser efficiency

No unique definition of the efficiency or effectiveness of any kind of P fertiliser can be given.

Two parameters indicating the benefit of a fertiliser to a crop can be derived by either applying the direct or the indirect method, i.e. either labeling the fertiliser or the available soil P:

1. The percentage of P utilization of an applied fertiliser, %PU, i.e. the ratio of P in the crop derived from the fertiliser over the amount of P fertiliser.
2. The percentage of P derived from a P fertiliser in the crop, % Pdff, i.e. the ratio of the same P-quantity as above, but over the total P quantity in the crop derived from both P sources, the fertiliser and the available soil P.

The first parameter is highly depending on the root exploration of the soil and on the climatic conditions, whereas the second parameter appears to be almost independent from the total plant P uptake. Therefore the %Pdff provides a good estimate of the effectiveness of a P-fertiliser.

B Prediction of the efficiency of a P fertiliser using a predicted %pdff

Although it is a useful information to evaluate P-efficiency of a fertiliser by determining the %Pdff after crop growth, it is of greater interest to agronomists to predict the efficiency of a P-fertiliser prior to application in the field:

The following prerequisites are necessary to be able to predict P-fertiliser efficiency:

- A P-fertiliser can only be effective if available soil P is so low, that it is the major limiting factor to crop growth.
- Provided P is the main limiting factor, only if %Pdff exceeds 30%, the P- fertiliser can be called "efficient".
- Plants, that have been grown on a P-fertilized soil and whose available P- fraction has been labeled by isotopic exchange, must have the same isotopic composition of P as the P in the soil solution.

To find out, if — under field conditions — P is the main limiting factor in the studied soil, is very difficult. Under standard conditions, excluding special constraints like water- or nitrogen stress, aluminum toxicity, presence of pollutants etc., the 3 factors intensity, quantity and capacity which characterize the availability of soil P and therefore indicating if P is limiting, can easily be determined. Under field conditions one or the other abovementioned constraints can change the results significantly, as well as for example essential cations being below the critical concentration.

Therefore the analysis of available soil P must be complemented by other general data on soil- and crop conditions, i.e. data on cation concentrations in soil.

Secondly it needs to be determined, whether the applied P-fertiliser yields a %Pdff higher than 30%, indicating that the fertiliser was efficient in enhancing P nutrition in the crop.

There are two ways to determine the %Pdff. One way is to conduct a greenhouse experiment, comparing the isotopic composition (or specific activity S.A.) of P in plants grown on soil having a labelled available soil P pool (indirect approach) either with or without P fertiliser application:

$$\%Pdff = 100\ [1-(S.A._{\text{plants in soil with fertiliser}})(S.A._{\text{plants in soil without fertiliser}})^{-1}] \quad \text{(Equation 18)}$$

As it was shown that the isotopic composition of P in plants is equal to the isotopic composition of P in soil solution, a laboratory method to predict %Pdff , can be used instead of time consuming and labour intensive plant studies applying the following equation:

$$Pdff\ \% = 100\ [E_{1F} - E_{1C}] / E_{1F} \quad \text{(Equation 19)}$$

where

- E_{1F} is the E_1 value, after a time of contact between the soil and fertiliser of the same order of magnitude as the time of crop growth and
- E_{1C} value is the E_1 value of the soil after the same time, which did not receive P (control).

The E_{1F} value decreases with time. This decrease can be described by two kinds of mathematical functions, an exponential and a power function.

The decline can be predicted measuring the values of E_{1F} at two different incubation times, the soil being kept moist for this incubation period in order to simulate the effect of contact time between the soil and fertiliser. Suggested incubation times are 1 day and 1 month. The decrease of E_{1F} with increasing time of contact can be described mathematically as follows:

$$E_{1Ft} = E_{1F1}\ t^{-m} + \beta \quad \text{(Equation 20)}$$

where:

- E_{1Ft} is the E_1 value for a P fertilized soil after time t expressed in days of incubation
- E_{1F1} is the E_1 value for the soil which received the P fertiliser after 1 day of incubation
- β is the equilibrium value for E_1 of a fertilized soil after an infinite time of contact between the soil end the fertiliser.

It has been shown that under agricultural conditions in which the P applications are limited by the funds available to the farmers, i.e. under minimum fertilization conditions, the β value is similar to the E_{1C} value.

Thus equation 20 becomes:

$$E_{1Ft} = E_{1F1}\ t^{-m} + E_{1C} \quad \text{(Equation 21)}$$

m, the slope of the logarithmic relation, is obtained by conducting two times (e.g. t_1= 1day and t_2=1 month) the experimental determination of E_{1f}.

Then equation [19] can be modified to:

$$Pdff\ \% = 100\ [E_{1F1}\ t^{-m}\ ] / E_{1F1}\ t^{-m} + E_{1C} \quad \text{(Equation 22)}$$

This equation can be used to predict % Pdff after time t of crop growth, respectively contact time between soil and fertiliser.

It can be concluded, that:

1. The % Pdff decreases, when the native soil P fertility increases
2. The % Pdff decreases when the time t increases
3. The % Pdff is depending on the fixing capacity of the soil since E_{1F1} depends on the fixing capacity
4. The % Pdff is depending on the quantity of P applied since E_{1F1} depends on the P application rate and the applied chemical form.

C Example of results obtained using isotopic exchange determinations

The following experiment was carried out on a soil sampled in Ghana. The aim of the experiment was to analyse the effect of a heavy P fertilization applied as phosphate rock (PR) of Tunisia and to evaluate the « recapitalisation » effect of such heavy application on a fresh P application of Triple Super Phosphate (TSP)

i *Experimental procedure*

An experiment with two fertiliser treatments was carried out as described in the standard procedure.

- *Treatment K and L* : 10 g of soil , no fertiliser added
- *Treatment M and N*: 10 g soil and 1000 mg P $(kg\ soil)^{-1}$ as PR
- These four pots were incubated in moist conditions (3 mL of deionized water added to each soil sample) during 1.5 months.
- After 1.5 month of incubation, 50 mg P $(kg\ soil)^{-1}$ were applied to treatment L, which did not receive P fertiliser before and treatment N, which previously received PR. The TSP was applied as 5 mL of monocalcium phosphate solution.

96 mL of water were added to treatments K and M. 91 mL of water were added to treatments L and N. All pots were shaken overnight and an isotopic exchange kinetics experiment was performed on the next morning. The following experimental results were obtained.

ii *Experimental results*

For the determination of the applied radioactivty R , 4 replicate vials containig 1 mL of the ^{32}P "solution D", which is a 1:100 dilution of the " ^{32}P mother solution C" were counted and the following count rates were recorded: 22193, 22099, 22055 and 22449 cpm. The mean value is 22199 cpm per mL. Therefore the total radioactivity applied to each soil solution is 22199 10^2 cpm. As this amount of radioactivity was applied to 99 mL + 1 mL = 100 mL of soil solution, which means that each mL of soil solution at the beginning received: 22199 10^2 / 10^2 = 22199 cpm per mL. This is the R value.

The following count rates (Table 4.3) were obtained for each sampling time for each soil sample:

Table 4.3. Example of results of counting on the four treated soil samples, three of which (L, M, N) received P fertilisers.

Treatments	K	L	M	N
Time (minutes)	Control	TSP 50	PR 1000	PR 1000 + TSP 50
1	9873	14522	14748	16038
3	6360	11750	12001	14070
7	4590	10020	10360	12700
10	4066	9429	9656	12178
C_P (mg P/ L)	0.03	0.91	1.5	3.3

The concentrations of P present as phosphate ions, were determined, using the malachite green method (see 4.2.12,C,7)

iii *Calculations*

Most probable r_1/R and n values. Determination of correlation coefficient r_2

To determine the most probable value for r_1/R and n, the linear regression between log $r_{(t)}$ and t has to be determined. As an example this evaluation is performed step by step with the K treatment. Data are recorded in Table 4.4.

Table 4.4. Data to determine r^2 and the most probable r_1/R and n.

$r_{(t)}$	log $r_{(t)}$	t	log t
9873	3.99	1	0
6360	3.80	3	0.477
4590	3.66	7	0.845
4066	3.61	10	1

Calculating the linear regression between log $r_{(t)}$ and log t the most probable slope, i.e. n, is 0.38 and the Y intercept 3.987. This means that Log r_1 =3.987 i.e. r_1/R = 9705 cpm. This result allows to calculate r_1/R = 9705 / 22199 = 0.44

Table 4.5. Most probable values for r_1, r_1/R and n.

Treatment Parameters	K	L	M	N
r_1 (cpm/mL)	9705	14493	14726	16040
r_1/R	0.44	0.65	0.66	0.72
n	0.38	0.188	0.183	0.12

Calculations of the pool sizes

These results allow to calculate the size of the various pools using formula [2] and formula [5]. Examples are explained for the K treatment.

In this experimental procedure the quantity q_s of P as phosphate ions present in the soil solution, is calculated as $q_s = 10\ C_P$, since 10 g soil are mixed with 100 mL of water. When the soil to solution ratio is modified for any reason, this factor 10 must be modified accordingly.

⇒ ***Pool E_1***, i.e. P isotopically exchangeable during the first minute

Applied to the K treatment using equation [2], the value for E_1 is:

$$\text{pool } E_1 = 10 \times 0.03 \times [1/0.44] = \underline{0.7\text{mgP/kg soil}}$$

⇒ ***Pool 1 minute - 1 day***

Application of the formula [5] for 1 day i.e. 1440 minutes:

As t is big compared to $(r1/R)^{1/n}$, the formula is reduced to:

$$r_{(t)}/R = r_1/R\ [\ t\ ]^{-n} + q_s/P_T \qquad \text{(Equation 23)}$$

For 1 day r_1/R = 0.44; n = 0.38; q_s = 0.3 mg P/kg soil and P_T = 130 mg P/kg soil.

Thus:

$$r_{1440}/R = 0.44\ [1440]^{-0.38} + 0.3/130 = 0.0301$$

and as $E_{1day} = q_s\ R/r_{1440}$,

$$E_{1day} = 0.3\ [1/0.0301] = \underline{10\text{ mg P/ kg}}$$

Applying equation [11] pool 1 minute - 1 day = 10 - 0.7 = <u>9.3 mg P/kg soil</u>.

⇒ ***Pool 1 day - 3 months***

Application of the formula [5] for 3 months i.e. 129600 minutes.

$$r_{129600}/R = 0.44\ [129600]^{-0.38} + 0.3/130 = 0.0073$$

and as $E_{3\ months} = q_s\ R/r_{129600}$,

$$E_{3\ months} = 0.3\ [1/0.0073] = \underline{41\text{ mg P/kg}}.$$

Applying equation [12] pool 1 day - 3 months = 41 - 10 = <u>31 mg P/kg soil</u>.

⇒ ***Pool 3 months - 1 year***

Application of the formula [5] for 1 year i.e. 518400 minutes

$$r_{518400}/R = 0.44\ [518400]^{-0.38} + 0.3/130 = 0.0053$$

and as $E_{1year} = q_s\ R/\mathbf{r}_{518400,}$

$$E_{1year} = 0.3\ [1/0.0053] = \underline{57\ mg\ P/kg\ soil}.$$

Applying equation [13] pool 3 months - 1 year = 57 - 41 = <u>16 mg P/kg soil</u>.

⇒ ***Pool > 1 year***

The size of this pool is calculated as follows:

pool > 1 year = Total P - pool 1 year (Equation 24)

Thus

pool > 1 year = 130 - 57 = <u>73 mg P/kg soil</u>.

The same calculations were made for the other treatments and the results are given in Table 4.6.

Table 4.6. Size of the various pools of decreasing mobility with time.

Treatments	K	L	M	N
total P (mgP/ kg)	130	180	1130	1180
q_s=10 C_P (mgP/ kg)	0.3	9.5	15	33
P pools [mg P/(kg soil]				
E_1	0.7	14.6	22.6	45.8
1 mn - 1 day	9.3	28.5	57	55
1 day - 3 months	31	38	86	62
3 months - 1 year	16	12	40	35
> 1 year	73	92	923	992

D Agronomic comments

The treatment K, i.e. the native “control soil”, can be considered as a soil of low P status, that means, it is too low in available soil P. Two complementary reasons can be identified:

1. the P concentration in the soil solution is near the critical value of 0.02 mg P/L.
2. the low E_1 value of this soil, i.e. the quantity of P directly available to crops can be considered as a limiting factor, since it is below the critical value of 3–4 mg P/kg.

It can be noted that this soil has a rather low fixing capacity for phosphate ions and therefore the available P can easily be modified by P fertiliser application.

The capacity factor of this soil, i.e. the volume of soil solution, that can be obtained without change in P concentration, can be calculated as follows:

C = Quantity factor (E_1)/ Intensity factor(c_p)[kg/ L] (Equation 25)

For treatment K follows:

$$0.7\ mg\ P\ kg^{-1} / 0.03\ mg\ P/\ L = \underline{23.3\ L/kg\ soil}$$

provided there are no other main limiting factors.

In our experiment two modes of fertilization were studied: a heavy fertilization with phosphate rocks to « recapitalize » the soil, as recommended by some researchers and a lower fertilization rate using Triple Super Phosphate.

The results shown in Table 4.6 indicate, that applications of TSP and PR significantly increased the soil P fertility since, irrespective of the P fertiliser applied, the fixing capacity decreased, whereas the E_1 value (quantity factor) and C_P value (intensity factor) increased. After fertiliser application in the two ways mentioned above, phosphorus does not remain a limiting factor for crop production.

Two further results can be calculated:

1. The expected %Pdff with the three fertiliser application modes
2. The P-fertiliser efficiency, which can be calculated as the ratio between the increase of the E_1 value in each treatment and the quantity of P applied.

The expected %Pdff was calculated according to equation18

$$\% \text{ Efficiency} = 100\,[E_{1F}-E_{1C}]/\text{mg P (kg soil)}^{-1} \qquad \text{(Equation 26)}$$

Table 4.7. Expected %Pdff and P fertiliser efficiency of the various P fertilisers applied.

Treatments	Expected Pdff %	Efficiency %
L(50 TSP)	95	27.8
M (1000 PR)	97	2.2
N (1000 PR + 50 TSP)	98	4.3

Conclusions

As the %Pdff is higher than 30 % in all fertiliser treatments, P fertilization will be efficient in terms of crop yield. The PR is efficient in this soil, because the pH is near 5.1.

Concerning the recapitalization effect, the results show, that a fraction of the P applied as TSP, even after a high application rate, is always transformed into a less mobile form than the applied form, which is soluble. In this example the increase of E_1-value after P application as TSP on soil which previously received PR is 45.8-22.6 = 23.2 mg P/kg and was 14.6 - 0.7 =13.9 mg P/kg without previous PR application. The results of the pool size variations show that the applied P was transformed partly into very slowly available forms.

4.3 LABELLING PLANTS WITH ^{13}C AND/OR ^{14}C AND ANALYSIS OF C IN SOIL AND PLANT SAMPLES

A Preparation of labelled plant material

- A simple method for uniform labeling of plant material using a pulse labeling technique is shown.
- If continuous labeling is required a suitable air-tight growth chamber and bottled ^{13}C enriched or depleted CO_2 will be required.

B Labelling chamber

- A chamber such as is shown in Figure 4.15 will be necessary.
- This chamber measures 2.5 m long, 1.3 m wide and 1m high, and is large enough to produce large quantities of plant material needed in incubation studies.
- The frame is made from aluminum and PVC pipes and the sides from clear, gas proof ethyl-vinyl alcohol film.
- The three sides of the chamber are permanently taped to the floor and sandbags are used to produce a permanent and gas tight seal. The front is left unsealed to allow watering of the plants.
- A commercial air-conditioning unit is attached on one side to circulate the air inside the chamber and at the same time regulate the temperature to about 25 °C

- If additional lighting is required such as when cloudy or in short daylight hours, lights such as mercury vapour lamps should be used above the chamber.
- The CO_2 concentration inside the chamber should be monitored by an infra red gas analyser (ADC Type 225 Mk3 CO_2 analyser or similar).

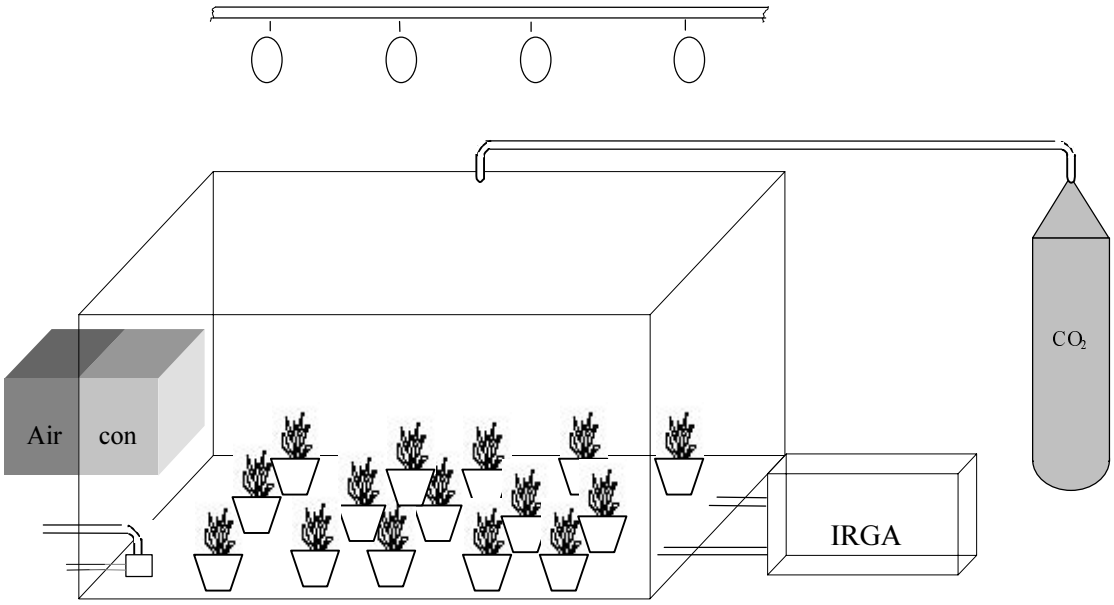

Figure 4.15. Set-up of the labelling chamber (Sanchez et al, 2000).

C Growth and Maintenance of Plants

- The required plants should be grown in a 1:1 vermiculite: sand mixture or similar, in plastic lined pots (to prevent isotopic contamination of the surroundings when using ^{14}C).
- The pots should be watered to field capacity regularly by removing them from the chamber.
- A nutrient solution such as Aquasol supplemented with $MgSO_4$ should be applied, once every fortnight then increased to weekly as plant growth increases.
- Pesticides should be applied if and when necessary.
- Unless labelling commences when the plants are small, such as at the 2-4 leaf stage, it may be necessary to heavily defoliate or severely prune them prior to labelling to ensure that the newly formed biomass will have a high enrichment.

D ^{13}C and ^{14}C labelling

- The $^{13}CO_2$ and $^{14}CO_2$ can be generated from the reaction of $Na_2{}^{13}CO_3$ and $Na_2{}^{14}CO_3$ with lactic acid injected through a thin plastic tube that runs through the side of the labelling chamber.
- The pulses of labelled CO_2 should be administered at such times as when photosynthetic activity is expected to be high such as between 9.00 and 10.00 am.
- During cloudy days or when day length is short, the lights above the chamber should be turned on to maintain a day length of 12 hours.
- The CO_2 concentration inside the chamber is allowed to drop from 350 ppm to 300 ppm before the labelled CO_2 pulse is introduced.
- When the CO_2 concentration has declined to approximately 180 ppm, following the introduction of the labelled CO_2, and became steady, $^{12}CO_2$ is introduced into the chamber from a gas cylinder, to return the concentration to 350 ppm.
- Sequential $^{12}CO_2$ pulsing should be continued for the rest of the day, or until 6.00 pm.
- Artificial lighting should be used, to maximize the uptake of the labelled CO_2 remaining in the chamber.
- The chamber should be closed overnight to contain any labelled CO_2 released during respiration and prevent any labeled CO_2 leaking into the atmosphere.
- Additional pulses of $^{12}CO_2$ should be administered the next day before opening the chamber to expose the plants to natural conditions.

- In the experiment of Sanchez et al (2000) 2.5MBq of ^{14}C was administered at each labelling time and the frequency of labelling increased from once a week, to four times a week as the plant biomass increased.
- The plants were pulse-labelled with $^{14}CO_2$ fifteen times during the growing season.
- The pots should be rotated prior to each labelling to ensure uniformity of labelling among pots.
- The amount of $Na_2{}^{13}CO_3$ (99%) used by Sanchez et al (2000) for each pulse was increased from 0.5g in the first week to 1.0 g in the second and third weeks then to 1.9 g for the succeeding weeks. It was then reduced to 1.0 g when only the rice plants were left to mature.
- The plant materials produced by Sanchez et al (2000) had the enrichments shown in Table 4.8. Rice straw contained the lowest ^{13}C and ^{14}C enrichment due to translocation of label to the grains as below ground translocation of assimilated carbon was reduced (less than 5%) during grain filling.

Table 4.8. Carbon and nitrogen concentration and isotopic enrichment of flemingia, medic and rice straw after labelling with $^{13}CO_2$, $^{14}CO_2$ and $^{15}NH_4Cl$.

Plant Material	C (%)	$\delta^{13}C$ (‰)	^{13}C (atom %)	Sp. Act ($kBq^{14}C/g$ C)
Flemingia	44.14	107.38	1.2291	13.09
Medic	43.65	117.01	1.2396	8.73
Rice	40.79	86.44	1.2061	7.33

E Preparation of soil samples for total C, ^{13}C and ^{14}C analysis

- The soil samples should firstly be prepared as in Section 3.7.
- If only a portion of the sample is to be used for C analysis or if the soil sample is large a sub-sample should be taken using a sample splitter similar to that shown in Figure 4.16. A sample splitter is necessary to ensure you obtain a representative sub-sample with the same particle size distribution as the original sample.

Figure 4.16 A type of sample splitter.

- The larger the sample used the more representative it will be but usually the sample to be ground need not be any larger than 100 g. In many cases it will be necessary to use smaller sample sizes, but try to avoid sample sizes of less than 10 g.
- Oven dry the soil sample at 70^{o}C for 24 hours prior to grinding.

- Soil samples should be ground to less than 500 μm (or less than 150 μm for determination of ^{14}C as shown in Section 3.5. If the sample is large a micro hammer-cutter mill with a maximum screen size of 500 (or 150) μm may be used. It is essential that the soil is ground for sufficient length of time so that all of the sample passes through the screen. There should be no sand grains remaining on the screen at the completion of grinding. The only soil remaining in the grinder should be that distributed around the edges. This will ensure that a representative sample is obtained. Smaller samples should be ground to less than 500 (or 150) μm using a ball mill. After grinding, the sample should be passed through a 500 (or 150) μm sieve to check that it has been ground sufficiently. If the entire sample does not pass through the sieve it should be returned to the ball mill and ground until such time as the whole sample passes through the sieve. Soils with high clay content may form lumps during the grinding process. These are easily broken to pass through the sieve by using the back of a spatula or similar.
- Both types of grinders should be thoroughly cleaned after each sample to avoid cross contamination. A brush and compressed air will be necessary to clean the micro hammer-cutter mill. Ball mills should be cleaned by washing with water, followed by rinsing with distilled water to remove contaminants in the tap water. They can then be dried with paper towel followed by a final drying with compressed air.
- The sample is now ready to send to the Seibesdorf Laboratory or for your own analysis. If sending to Seibesdorf, larger samples should be sub-sampled to approximately 10 g using the sample splitter as above. The sample size sent should be not less than 1 g to ensure a representative sample.

F Pretreatment of carbonate soils prior to combustion or analysis for total C.

i *Introduction*

The measurement of carbon by high frequency induction furnace techniques or by complete digestion results in the complete conversion of carbonates into carbon dioxide. As a result it is necessary to remove these in advance by acidification of the soil sample, prior to introduction to the furnace or the addition of the digestion mixture.

ii *Reagents*

- Orthophosphoric acid (H_3PO_4) 85%: Make to approximately 2% w/v by taking 2mL of concentrated orthophosphoric acid and make to 100mL total volume with distilled/deionised water.

iii *Method*

- Prepare the soil samples as per Section 3.5
- Weigh the amount of soil required into the tin capsules used for the combustion or the digestion tubes to be used for total C determination but do not crimp the tin cups closed. Place them into the tin capsule holder.
- Using a fine tipped pasteur pipette add 2 drops of 2% orthophosphoric acid to the tin cups containing the soil. Watch for any visible signs of effervescence. For the digestion tubes add the acid drop wise until the soil is completely wet by the acid.
- Place the holder containing the tin cups or digestion tubes and soil into a 70^{o}C oven for 1-2 hours. This will assist the reaction and drive off the remaining liquid.
- Remove the samples from the oven and add the 2% orthophosphoric once again, look for effervescence. If none occurs dry as above and then crimp the tin cup ready for analysis on the mass spectrometer or continue with the digestion. If effervescence does occur, repeat the acid additions until effervescence cannot be detected.

iv *Comments*

- Orthophosphoric acid (2%) was chosen as it did not cause significant corrosion of the tin capsules but at the same time allowed sufficient reaction of the soil to occur.

- When crimping the tin capsules after acid treatment care should be taken not to pierce or break them. If this does occur it may be necessary to place them inside another tin capsule and then crimp it. If this is required, standards and blanks must be treated in the same way to ensure continuity.

G Measurement of ^{14}C in soil and plant samples

- Because open digestion procedures lose C from plant and soil samples closed systems or solubilisation procedures have been developed to measure ^{14}C in soil and plant samples
- The general procedure recommended for soil samples is essentially that of Amato (1983) in which digestion takes place in a sealed vial, which contains a CO_2 trapping disc or solution.
- Once digestion is complete the tubes are left overnight and the absorbing solution or disc placed in a scintillant and counted in a liquid scintillation counter.
- Plant samples can be solubilised in proprietary solutions and counted in a liquid scintillation counter after addition of a scintillant.
- An alternative procedure is to combust the sample in a furnace as described for ^{13}C measurement and the evolved CO_2 trapped in NaOH and this counted by liquid

i *Wet digestion*

- The wet digestion and counting procedure below is taken from Voroney et al (1991)
- As in all analytical determinations each batch of samples analysed should contain a ^{14}C standard to determine the efficiency of the digestion mixture.
- As well a blank samples should also be included in each batch to measure background levels.
- When determining total C in calcareous soils all carbonates should be removed by acid treating as per Section 4.3 D prior to measurement.

Digestion Apparatus

- A block digester.
- Digestion tubes (250 X 25 mm), with thick walls (1.5 mm) to withstand pressure (Figure 4.17).
- A subaseal (size 49) to stopper the digestion tube (Figure 4.17).
- A 2-mL glass vial, or a 10-mL graduated tube (if total C is desired) (Figure 4.17).
- A glass support rod (5-mm diameter X 170-mm length) bent at one end to support a glass vial, or a shorter rod (95 mm) if graduated tubes are used (Figure 4.17).

Reagents

- 1 mL ethanolamine, or 5 mL 2M NaOH to trap CO_2.
- Digestion mixture:
- 189.4 g CrO_3
- 250 mL 14.7M H_3PO_4
- 500 mL I8M H_2SO_4

Scintillation Cocktail for Counting Ethanolamine (1L)

- 700 ml toluene
- 400 mL methoxy ethanol
- 4 g 2,5-diphenyloxazole (PPO)
- 100 mg 1,4-bis(5-phenyloxazole-2-yl) benzene (POPOP)

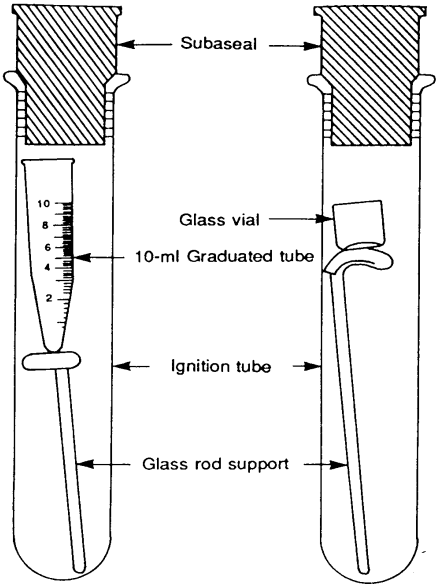

Figure 4.17. Digestion tube set-up for total and labeled C determination. (Voroney et al, 1991).

ii *Procedure*

Preparation of the Digestion Mixture

- Place the H_3PO_4 and H_2SO_4 into a 500-mL Pyrex Erlenmyer flask and heat to 145–150°C, using a hot plate and a magnetic stirrer to facilitate dissolution of the CrO_3.
- When 150°C has been reached, turn off the heat.
- As the temperature starts to fall from 150°C, add CrO_3 and continue to stir during cooling. Keep covered with an inverted beaker to prevent water vapor from entering the flask since water diminishes the oxidizing efficiency of the mixture.
- The digestion mixture should be brown colored; prolonged heating produces a green solution of oxidized chromium.

Digestion of Plant and Soil for Total ^{14}C

- Weigh into the digestion tube 0.3–1.0 g of soil (oven dried at 60°C and ground to finer than 0.15 mm), or 0.04 g of plant material (oven dried at 60°C and ground to finer than 0.25 mm). Samples should contain less than 20 mg C. If determining total C soil samples containing carbonates should then be pretreated with acid.
- Prepare the CO_2 trap by adding 1 mL of ethanolamine to the 2 mL vial.
- Add 6 mL of cool digestion mixture to each tube, quickly add the glass support rod and CO_2 trap, and stopper the tube with a subaseal
- Place the tube in a heating block for 1 hr at 130°C (but no higher), then cool and leave overnight to allow complete CO_2 absorption.

CAUTION: The contents of the tube are under pressure

- In a fumehood and wearing protective eyeglasses, remove the CO_2 trapping vial from the digestion tube and place the contents into a glass scintillation vial.
- Add 11 mL of scintillation cocktail, cap tightly with a foil-backed plastic cap and swirl gently until the contents are thoroughly mixed; the cocktail should appear clear.
- Storage of the samples for 24 hr prior to counting slightly increases the counting efficiency.

Digestion of Plant and Soil for Total ^{14}C and Total C

- Weigh plant or soil and add to a digestion tube as in b above.
- Prepare the CO_2 trap by adding 5 mL of 2M NaOH to a 10-mL graduated tube.
- Add the digestion mixture, CO_2 trap, stopper and digest, cool, and store as in b above.
- Remove the CO_2 trap from the digestion tube and add an aliquot (e.g., 0.1 mL) of the NaOH solution to a scintillation cocktail.

Measurement of Total C

- After removing the aliquot for scintillation counting in c. above, dilute the remaining NaOH solution in the graduated tube to 10 mL with CO_2-free distilled water and seal the tube to prevent further exposure to CO_2.
- Total C can be determined by titration of the NaOH solution using the method described by Anderson (1982).

Solubilisation of plant material

- ^{14}C in plant samples can be determined by solubilising the plant material in 3 mL of soluene-350 (Packard) and counted in liquid scintillation counter after addition of 17 ml of liquid scintillation consisting of p-terphenyl, POPOP, teric and toluene.

- A quench curve, consisting of a series of spiked plant materials with varying amounts (5–25 mg) solubilized with 2 mL soluene-350, needs to be prepared to correct for counting interferences due to Soluene-350 and to the color resulting from the solubilisation.

4.4 SIMPLE METHODS FOR ORGANIC RESIDUE QUALITY ASSESSMENT

4.4.1 MEASUREMENT OF TOTAL PHENOLICS

A Scope and field of application

Polyphenols are one parameter to quantify plants N release characteristics. They are heterogeneous group of compounds that have a hydroxyl group bonded to an aromatic ring. All tannins and flavonoids are examples of this group.

The following method is adapted from King and Health (1967) and Allen *et al*. (1974) and allows the determination of the total amount of soluble phenolics.

Principle:

Total soluble polyphenolics are analysed by the Folin-Ciocalteus method:

Hydrolysable tannins, condensed tannins and non-tannin polyphenols build with the reagents a blue colour complex in alkaline media, the absorbance of this colour can be measured with the spectrophotometer at 760nm.

Typical sample:

Fresh or dried material, dried at room temperature, not higher than 55^{o}C, ideally 50–52^{o}C with a forced air oven) and ground to 0.3 mm or to pass through a 60 mesh.

Fresh material should be kept on ice, not exposed to light because the enzymes may react with polyphenols.

Dried material should not be exposed to light and be kept dry (ideally in a desiccator)

B Apparatus

- Spectrophotometer.
- Balance $\pm$ 0.001g.
- Volumetric flasks 50mL, 500mL.
- Beakers 50mL.
- Funnels.
- Filter paper (Whatman nr.1).
- Water bath 77–80 ^{o}C.
- Parafilm or watch glasses.

C Reagents

- Tannic acid-standard solution, 0.1mg/mL: dissolve 0.050g tannic acid (obtained from Merck company) in 500mL of demineralized water (use a volumetric flask); the solution has to be prepared freshly.
- Folin-Ciocalteus Reagent, store at 4^{0}C.

- Sodium carbonate, 17% (dissolve 17g sodium carbonate in demineralized water and make up to 100mL with demineralized water).
- Aqueous methanol 50% (v/v).

D Procedure

- Weigh about 0.75 ± 0.001g of material in a beaker.
- Add 20mL aqueous methanol, cover with parafilm or a watch glass and place in a water bath at 77–80 °C for 1 hour.
- Filter through the Whatman filter.
- Rinse the beaker a few times with a small amounts of 50% methanol to transfer the sample quantitatively on the filter paper (so that there is no sample left in the beaker), leave a margin of about 0.5 cm to wash the filter paper.
- Wash the filter paper three times with 50% methanol (use a wash bottle to rinse at the margin and make sure that all liquid went through the filter paper to collect all the filtrate).
- Remove first the filter paper then the funnel.
- Make up to the mark of the volumetric flask with water and mix well.
- *Standard curve:* pipette 0, 1mL, 2mL, 3mL, 4mL of the standard into a 50mL volumetric flask, add about 20mL demineralized water, 2.5mL Folin-Ciocalteus reagent and 10mL sodium carbonate solution; make up to the mark with water, mix well and read the absorbance at 760nm after 30 minutes
- Subtract the blank value from the other values or set the blank to zero (depending on your spectrophotometer).

Samples:

- Pipette 1mL of the filtrate into a 50mL volumetric flask and continue as the standard.

Note: if the absorbance is higher than 1 use a smaller aliquot.

Calculation:

- Calculate the linear regression of the standards, read mg / mL of the samples.

Total extractable polyphenolics (%) = (mg / mL x 5) / sample weight

If fresh material is used make a dry weight correction.

4.4.2 ADF (ACID DETERGENT FIBRE) DETERMINATION

A Scope and field of application

ADF is one of a variety of plant characteristics, which have been linked to organic matter breakdown and N release. The procedures described have been tested against the ANKOM techniques, which in turn have been tested against the classical methods (Van Soest and Wine 1968). The described methods save considerable time and are probably less open to operator error than the classical ones. The ANKOM bags are about 30 US cents each. Another advantage of these methods is that they also allow determination of ADF, lignin and cellulose from complex residues such as manures.

B Principle

Plant material is digested in a solution of boiling sulfuric acid and cetyltrimethyl ammonium bromide (CTAB). The CTAB dissolves nearly all the nitrogenous constituents, it hydrolyses hemicellulose and dissolves protein. The acid hydrolysis the starch and leaves lignin, cellulose and ash as a residue. The content of ADF is measured gravimetrically.

C Typical sample

Any plant material, sample must be dried to constant weight and ground to pass through a 1mm screen. If you want to determine polyphenols of the same material do not dry the material higher than 55°C.

D Apparatus

- 250mL round bottom flasks with.
- Fitting rubber stoppers with two holes for two glass tubes.
- Tubes for condenser.
- 600mL beakers without spout.
- Hot plates.
- Anti-bumping granules.
- ANKOM filter bags.
- Balance, ±0.0001g.
- Desiccator.
- Oven (set at 105 ^{o}C).
- Heat sealer.
- Sieve or tea strainer.
- Tweezers

Figure 4.18. Apparatus for ADF determination.

E Reagents

- Acetone.
- Octan-2-ol (as an antifoaming reagent).
- Sulfuric acid/CTAB solution (dissolve 100g cetyltrimethyl ammonium bromide in 5 litres 0.5M sulfuric acid, filter if cloudy – to make 0.5M sulfuric acid: add 26.6mL of conc. H_2SO_4 to 973.4mL of deionised water).

Caution: CTAB will irritate the mucous membranes. A dust mask and gloves should be worn when handling this chemical. The waste has to be collected and treated as toxic waste.

F Procedure

- Pre-weigh the dried filter bag which has been stored in the desiccator (W2)
- Weigh about 0.5 - 1g ± 0.0001g of dry plant material into a filter bag and note the weight (W1).
- Heat-seal the bag, label it with a pencil.
- Put 2–4 filter bags into a 600mL beaker.
- Add a few anti-bumping granules.
- Add 100mL of CTAB/sulfuric acid solution per bag.
- Add a few drops of antifoaming reagent (e.g. octan-2-ol)
- Connect the round bottom flasks as a condenser (fix the rubber stopper and the water pipes)
- Once boiling, reflux for 1.5 hr.
- Let the system cool down.

- Remove filter bags with tweezers (**collect reagent and treat as toxic waste**).
- Put under running hot tap to wash off most of the CTAB (use the sieve).
- Repeatedly wash the filter bags with boiling demineralized water until the pH is neutral (this is about 5 washes).
- Wash the bags with acetone until no more colour is removed (about 3 times): place bags in a beaker, add acetone to cover the bags, use a smaller beaker to agitate the bags by pushing and lifting the smaller beaker carefully.
- After the acetone is evaporated, dry the filter bags in the oven at 105 ^{o}C for 2 hr.
- Remove the bags from oven and immediately transfer to a desiccator and leave to cool.
- Re-weigh the filter bags at room temperature and note the weight (W3).

Calculation:

Ash containing ADF (%) = {(W3 – W2 x factor) / W1} x 100

Factor = W3-blank / W2-blank

This classical method is described in the following references: Tropical Soil Biology and Fertility (1993); Rowland and Roberts,(1994); Van Soest and Wine (1968).

4.4.3 CELLULOSE DETERMINATION

A Scope and field of application

The measurement of cellulose in plant material is of particular importance as it is an indicator of the degradability of plant material.

B Principle

After an acid-detergent fibre pre-extraction cellulose is removed with 72% sulfuric acid. This is a gravimetric method and the percentage of lignin is calculated by weight difference.

C Typical sample

Continued from ADF determination.

D Apparatus

- Balance, ±0.0001g.
- Desiccator.
- Hot plate,
- Oven, 105 ^{o}C.
- Beakers, 2 L and 3 L (or aliquot smaller ones if you have less samples).
- pH paper.

E Reagents

Acetone, sulfuric acid, 72% (w/w add 1000mL conc. H_2SO_4 = 1840g to 663 g demineralized water).

F Procedure

- Continue the procedure following on from the ADF determination:
- Put the filter bags into a 3 L beaker.
- Cover the bags with cold sulfuric acid (approximately 250 mL per 25 samples)
- Use a 2L beaker to agitate the bags by pushing and lifting it up and down, repeat the agitation in 30min intervals
- After 3hr take the bags out, put them under a running hot tap, then wash the bags with boiling demineralized water until the water is neutral (check with pH-paper)
- Wash with acetone until no more colour is removed
- After the acetone is evaporated, dry the bags at 105 ^{o}C for 2 hr
- Cool in a desiccator
- Re-weigh the bags at room temperature (W4)

G Calculation

Cellulose (%) = {(W3 – W4) / W1} x 100

4.4.4 ACID DETERGENT LIGNIN DETERMINATION

A Scope and field of application

Lignin is an important plant characteristics and plays a major role in the decomposability and digestibility of plant material.

B Principle

Subsequent to an acid-detergent fibre pre-extraction, and lignin removal-determination by hydrolysis with 72% sulfuric acid, cellulose is determined by weight loss upon ashing.

Typical sample:

Continued from cellulose determination.

C Apparatus

- Balance, ±0.0001g.
- Desiccator.
- Oven, 525 ^{o}C.
- Labelled (NOT WITH A WATERSTABLE PEN BUT HEATSTABLE OR SCRATCH WITH A GLASS CUTTER) beakers or porcelain bowls

D Reagents

None

E Procedure

- Continue the procedure following on from the cellulose determination (section 4.4.3).
- Lable the beakers or porcelain bowls: put them in the oven at 525 ^{o}C for 1hr, let them cool to room temperature in the desiccator, take the weight of the empty bowls (W5).
- Put the filter bags into the porcelain bowls or beakers and place them in the oven at 525^{o}C for 3 hr (consider the time the oven takes to reach 525 ^{o}C and let it cool down to about 150^{o}C).
- Leave the bowls/beakers to cool in a desiccator.
- Re-weigh the bowls at room temperature (W6).

F Calculation

Acid detergent Lignin (%) = {[(W4 – W2)-(W6-W5)] / W1} x 100

Figure 4.19. Bag before ashing.

4.4.5 PROTEIN BINDING CAPACITY OF TANNINS BY FILTER PAPER ASSAY

A Scope and field of application

This method allows distinguishing the tannins as protein binding and non-protein binding tannins

B Principle

The tannins of the plant extracts are bound with the protein BSA (Bovine serum albumin), the excess of the BSA is washed off and the protein-tannin complex is stained with Ponceau S. The absorbance of the colour is read at 525nm.

C Typical sample

Fresh or dried (at room temperature not higher than 55^{o}C, ideally 50–52^{o}C with a forced air oven) and ground material (very fine to pass through a 60 mesh).

Fresh material should be kept on ice not exposed to light because the enzymes may react with polyphenols.

Dried material shouldn't be exposed to light and be kept dry (ideally in a desiccator)

D Apparatus

- 1MM Whatman paper or Whatman filter paper no.1.
- Volumetric flasks.
- Pipettes.
- Balance ± 0.0001g.
- Centrifuge, 2500g.
- Centrifuge vials.
- Vortex mixer.
- Petridish or other suitable pot to dye the strips.
- Tweezers, pencil, scissors.

E Reagents

- Tannic acid solution: dissolve 100 mg tannic acid (obtained from Merck) in 100 mL of 50% aqueous methanol. 100 mg ascorbic acid should be added to minimise the oxidation of the tannic acid.
- Acetate buffer (pH 5; 0.05M): pipette 2.85 mL glacial acetic acid to about 800 mL demineralized water, adjust pH of this solution to 5.0 with sodium hydroxide and bring to final volume of 1 L.

- Bovine serum albumin (BSA) solution: dissolve 200 mg of bovine serum albumin (fraction V, obtained from Sigma) in 100 mL of 0.05 M acetate buffer.
- Dye solution: dissolve 0.2 g Ponceau S dye in 100 mL of a 3% (w/v) trichloroacetic acid.
- Acetic acid solution (0,2%,v/v): add 2 mL of galacial acetic acid to 998 mL demineralized water.
- Sodium hydroxide solution (0.1 N): dissolve 4 g sodium hydroxide in about 500 mL demineralized water, make up the volume to 1 L.
- Acetic acid solution (10%, v/v): add 10 mL of galacial acetic acid to 990 mL of demineralized water.

F Procedure

- Prepare a methanol plant extract as described in 4.3.
- Cut the chromatography sheet or filter paper into appropriate size and draw squares of approximately 2 cm using a light pencil.
- Apply different aliquots (5 to 25 μl) of tannic acid solution on the sheet, apply only small amounts at one time to avoid that the liquid is spreading over the borders you made with the pencil. It is useful to apply only about 10 μl at one time and wait till the solution is dry.
- Each aliquot has to be applied at least in triplicates.
- Similar apply appropriate aliquots of the plant extracts (make replicates, useful volume is 10 to 50 μL)
- Leave two squares empty and use them as blanks.
- Allow the spots to dry, and spray immediately with BSA solution until the paper is wet.
- After 30 min wash the paper with acetate buffer (pH 5.0 , 0.05M): shake slightly from time to time for 10min, repeat the washing 2 times.
- Dip the paper for 10 min in the stain solution (Ponceau S) (the staining solution should not be used in successive experiments).
- Wash the stained strips in 0.2% acetic acid solution until no more colour is eluted from the strip. This requires about three washings to make the background clear.
- Air dry the strips and cut the stained areas
- Transfer the strips one by one into centrifuge vials.
- Elute the colour by adding 3 mL of 0.1N NaOH solution and vortexing.
- Add 0.3 mL of 10% acetic acid and centrifuge at approximately 2500 g.
- Read the absorbance of the colour at 525 nm against the corresponding blank (take the mean of the blanks).
- Convert this absorbance to protein content by using a standard curve.

Standard curve:

- Apply 5 to 50μl of BSA solution (1mg/mL BSA solution in acetate buffer) as separate spots (at least in triplicate for each concentration) and proceed as above described.

Note: The tannic acid is used to test the method but not needed for the calculation

Calculation:

- Subtract the mean blank of the sample- and the standard values.
- Calculate the linear regression of the BSA absorbance values and use the equation to calculate the concentration of the tested samples.
- Convert the results into ppm (using the applied volume).
- Convert to % tannins with protein binding capacity by using the initial weight of the plant extract.

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CHAPTER 5

QUALITY ASSURANCE

5.1 QUALITY CONTROL

5.1.1 INTRODUCTION

The analytical techniques used by each laboratory must be proven to be precise and reproducible, both within and between laboratories. Satisfactory quality control measures must be implemented, maintained and monitored constantly to ensure that all reported analytical results are reliable and do not alter with time, instrument or operator. Moreover, laboratory staff must be well trained in their specific tasks and re-trained when new procedures or instruments are introduced.

Success in achieving high standards of analytical accuracy and precision involves the appropriate selection and testing of laboratory methods, as well as the introduction of quality control in all phases of the routine testing service.

Such quality control procedures include:

(a) Preparation of blank solutions;
(b) Duplication of unknown samples;
(c) Inclusion of internal and external standard samples, both soil and plant material;
(d) Use of recovery procedures for method testing.

A Blank solutions

These are prepared by performing the complete analysis but with the soil/plant sample omitted. All chemical tests except electrical conductivity, pH and lime requirements require the use of blanks.

The main purpose of using blanks is to account for contamination by reagents. For this reason, it is important to prepare a fresh blank solution with each batch of samples and run this blank with each batch of samples. The sample value in each batch should be corrected for the blank value.

B Sample duplicates

Samples may be in duplicate but this reduces output. Alternatively, if the recoveries are good and the analyses accurate and repeatable, single sample analysis may be carried out and any unusual results repeated. Another alternative is to duplicate some unknowns, for example, two out of every 40 samples. The means of the duplicate values can then be calculated and the values regarded as satisfactory if they lie within $\pm$3–5% of the mean.

C Standard plant/soil material

This may be prepared by laboratory staff and be used as an internal reference. Rigorous testing of the material must be done prior to its use in the laboratory so as to obtain accurate and repeatable element concentrations. Once mean values for all elements have been obtained, the internal standard should be included in all runs of each technique.

The standard plant/soil material must undergo exactly the same treatment as the unknown material being analysed. By including duplicates of these in each set of samples, checks between sets of samples can be performed. The number of sets of duplicate standards should be increased as the batch size increases. The value of the standard should not vary from the mean value by more than 3–5%; if it does, extensive investigation to determine likely causes should be undertaken. If no cause can be found, the batch should be repeated.

It is important to always check blanks and standard plant/soil results prior to checking unknowns as these are the main indication of any potential problems.

D External standards

External standard plant and soil materials that are of known and usually published values can also be used as a method-to-method and lab-to-lab comparison. These are generally commercially

prepared and can be purchased, along with the analytical details and results, from national quality control laboratories.

Internal and external standards should be digested/extracted and analysed at regular intervals to provide checks on techniques being used. A detailed record of the results should be kept to indicate any variations with time which will then require correction and investigation.

The International Plant and Soil Exchange systems (IPE and ISE, respectively), run from Wageningen, are examples of widely used quality control systems which allow internal and external comparisons of analytical quality, across a large number of laboratories, 4 times per year on a range of materials.

E Recovery procedures

These are one of the most important laboratory techniques, particularly during method development or the adoption of a new method. This involves the use of "spikes" of known nutrient content which are added to blanks and plant/soil samples to indicate if a problem exists with the particular method.

Generally, approximately half of the nutrient that is already present in the plant/soil sample is added. For example, the digest of a subsample of 0.2g of plant material containing 0.2% P would contain 200 x 0.002 or 0.4mg P. The added spiking solution would therefore contain 0.2mg P. 100% recovery is the aim of this procedure $\pm$3–5%.

In order to track down where a problem arises, a sequential recovery procedure can be implemented, i.e. spikes of known nutrient content are added to the plant or soil sample at different stages of the procedure to see where the problem develops or disappears.

In plant and soil analyses there are a number of steps where analytical problems can develop and all should be investigated. Inaccuracies could result from:

- Dirty glassware.
- Contaminated plant or soil samples.
- Contaminated digestion or extraction mixtures.
- Incomplete digestion (e.g. sulphur).
- Contaminated water or reagents used in analysis.
- Malfunction of equipment, i.e. autoanalyser, ICP, etc.
- Chemical interferences.
- Inaccurate standard solution preparation.
- Elemental interference in solution matrix.
- Inaccurate or inappropriate fitting of calibration curve (sometimes resulting from inappropriate use of computer software).

Reliable plant and soil analysis requires that the best possible techniques are used in each stage from collection to analysis, and that appropriate quality control measures are rigorously adhered to.

AN EXAMPLE OF A RECOVERY RUN FOR A PLANT DIGESTION AND ANALYSIS

Sample	Replicate	Sample Weight (gm)	Quantity of spike added (mL)	Result
(a) Blank	1	-	-	Should equal zero. A positive
	2	-	-	reading indicates contamination
	3	-	-	in reagents, water, vials, etc.
(b) Internal Standard	1	0.2054	-	Allows for comparison with lab
	2	0.2010	-	mean and calculation of a mean
	3	0.2009	-	for this run.
(c) Half weight of the Internal Standard	1	0.1008	-	Enables mean to be calculated for
	2	0.1003	-	half the quantity of the internal
	3	0.1010	-	standard. This should give the same concentration and half the amount found in (b) otherwise digestion procedure is being influenced by the sample weight or the calibration of other standard curve does not hold for different mean ranges.
(d) Spike Solution	1	-	1.003	Allows mean to be calculated for
	2	-	1.008	all the elements used and %
	3	-	1.012	recovery of amount added.
(e) Half volume of spike solution	1	-	0.511	Should give the same concentraion
	2	-	0.502	and half the amount as in (d).
	3	-	0.509	
(f) Half weight of internal standard + half volume of spike solution	1	0.1010	0.503	Should equal (c) + (e).
	2	0.1002	0.514	
	3	0.1005	0.511	

Note: In (a) to (f) the samples are taken through the whole digestion/analysis procedure; (g) to (i) include some additions at different stages in the digestion/analysis process.

(g) Blank + Spike solution added after digestion	1	-	1.011	Should give same result as (d).
	2	-	1.013	This comparison tests if digestion
	3	-	1.005	or analysis is the problem.
(h) Blank + half volume of spike solution added after digestion	1	-	0.510	Should give same concen-
	2	-	0.508	tion and half the amount of (g).
	3	-	0.514	Compares with the (d) to (e) comparison.
(i) Half the weight of internal standard + half the volume of spike added after digestion	1	0.1001	0.500	Should equal (f) which should
	2	0.1021	0.503	equal (c) and (e). Indicates if
	3	0.1013	0.498	there is a matrix interference.

5.2 INTRODUCTION TO QUALITY ASSURANCE (QA). BASIC REQUIREMENTS FOR ANALYTICAL LABORATORIES WITH EMPHASIS ON TOTAL N AND ^{15}N ANALYSES OF PLANT MATERIALS

5.2.1 ENVIRONMENT

A A proper working area

Absolutely necessary:

- Strictly separate fertiliser preparation area and analytical area
- Clean (uncontaminated) work benches
- Distilled water for reagent preparation and analysis

Advisable:

- Separate rooms for (a) wet chemistry
  (b) balance - and instrument room
- Air conditioned instrument room
- Fume hood for digestion unit

Of advantage:

- Dish washer with demineralized water for glassware
- Fridge for storage of reagents
- Drying cabinet (for moisture determinations and evaporation of Kjeldahl-distillate; temperature up to 120° C)

B Suitable storage facilities for plant materials

- Dry, well ventilated, cool room
- No direct sunlight
- Shelves
- Plant samples in properly labelled paper bags (short and clear coding, best with non erasable marker)
- Archived samples: covered with plastic sheets or in labelled plastic buckets which are “mouse- and insect proof” for long term storage of minimum 1 year!
- **Important:** no fertiliser storage in the same room

Figure 5.1. Samples suitably packaged for storage.

5.2.2 MATERIALS AND SUPPLIES

A Well prepared, representative plant samples

Figure 5.2. Finely ground, homogeneous plant sample suitable for analysis.

Homogeneity is a prerequisite for a representative analysis therefore plant samples should be prepared in the following way:

- Particle size: ≤ 0.5 mm.
- Powder dry.
- Visibly homogeneous (colour).
- Not infested (fungi, cops, insects etc.).
- About 5–10 mg N (i.e. 300–1000 mg plant powder, depending on expected N-content) for one Kjeldahl analysis.
- Plant parts with different N and ^{15}N – content (e.g. grain and straw, pods and leaves) should be separated before drying and analyzed separately => homogeneous ^{15}N-enrichment.

To minimize the risk of cross contamination at grinding:

- Estimate the approximate^{15}N content of the plant-material and sort samples in ascending or descending order of ^{15}N-content before grinding.
- Clean the mill properly between each sample (with brush or compressed air).

B Certified reference materials for traceability

Figure 5.3. Certified reference materials for total N analysis.

Analytical measurements should be referred to internationally or nationally recognized standards (primary standards). If your internal working standard (IRM) is characterized by the IAEA Soil Science Unit in Seibersdorf, traceability to a CRM can be provided.

A Certified Reference Material (CRM) is a primary standard and is certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation issued by the certifying body (ISO Guide 30, 1981).

A Working Standard or Internal Reference Material (IRM) is calibrated against a CRM and then used to calibrate measurements of actual samples.

Examples for certified reference materials in total-N- and ^{15}N- analysis:

1. Plant - CRMs for total N (by Kjeldahl):
- CRM129 (Commission of the European Communities, BCR, Belgium): Hay powder
- GBW 07603 (The People's Republic of China): Bush leaves
- NIST 8436 (National Institute of Standards & Technology, Canada): Durum wheat

2. Organic N- compound CRMs (used e.g. in dry combustion analysers):
- Organic Analytical Standards (OAS), Microanalysis, UK: Cat.No. B2000, Acetanilide
- Organic Analytical Standards (OAS), Microanalysis, UK: Cat.No. B2002, Atropine

3. Inorganic ^{15}N-compound CRMs:
- IAEA-305A: ^{15}N- labelled Ammonia Sulfate (0.0145 atom % ^{15}N vs. air)
- IAEA-305B: ^{15}N-labelled Ammonia Sulfate (0.1368 atom % ^{15}N vs. air)
- IAEA-310A: ^{15}N-labelled Urea (0.0172 atom % ^{15}N vs. air)
- IAEA-310B: ^{15}N-labelled Urea (0.0892 atom % ^{15}N vs. air)
- IAEA-311: ^{15}N-labelled Ammonia Sulfate (2.05 atom % ^{15}N vs. air)

4. ^{15}N-labelled plant - CRM : Not yet commercially available.

- ^{15}N labelled ryegrass leaves (1.2 atom %^{15}N vs. air) are in preparation (IAEA, Soil Science Unit, Seibersdorf):

C Correctly calibrated instruments and functioning lab equipment

General Rule: All instruments and equipment, which provide numbers used in the calculation of the analytical result need to be calibrated correctly (e.g. balances, burettes, Emission Spectrometers, Mass Spectrometers etc.).

One needs to distinguish between (1) *calibrating* and (2) *checking the calibration*.

i Calibration

- Process of referring an instrument response (measured signal) to an analytical result, usually by means of a calibration curve; modern, microprocessor controlled instruments calculate and apply the calibration curve automatically.

Figure 5.4. Certified reference weights (left) are used to calibrate the balance after careful cleaning (right).

Examples for instruments that can be calibrated

- Analytical balance (min. required readability +/- 0.0001g): switched to "CAL-mode" the balance is calibrated by internal (electronic) weights or by external certified reference weights (usually one or two).
- Optical Emission Spectrometer (OES): a calibration curve is prepared with a set of ^{15}N- labelled Ammonia Sulfate standard solutions prepared from solid, ^{15}N enriched A.S.- Reference Materials (e.g. from FAN GmbH, Germany or IAEA) with different enrichments (usually 7–12 Stds.)

ii Checking the calibration and procedure control

- A working standard (IRM, traceable to a CRM) is treated and analyzed like a routine sample ("Control sample") and the displayed result is compared with the theoretical result — if the actual and the theoretical result are equal, the instrument is calibrated correctly
- A set of certified reference weights is weighed and the displayed value is compared with the value on the certificate.

NOTE: If the result of the calibration check is unsatisfactory, re-calibration is needed, eventually after maintenance of the instrument / equipment!

Examples for calibration check of instruments

- *Analytical balance:* weigh a set of certified reference weights (e.g. 5 mg, 10 mg, 50 mg, 100 mg, 500 mg, 1 g, 50 g, 100g)
- *OES:* measure several ammonia sulfate standard solutions with different enrichments, 3 digits required (e.g. natural abundance=0.366, 0.4.., 0.5.., 0.6.., 0.7.., 0.8.., 1.0.., 1.5.., 2.0.., 4.0.., 6.0.. atom % ^{15}N abundance)
- *Volumetric pipettes:* weigh a pipetted volume of distilled water on a calibrated analytical balance
- *Digital or glass burettes:* weigh a dispensed volume of distilled water on a calibrated analytical balance
- *Volumetric flasks:* weigh the volume of distilled water filled up to the mark on a calibrated top load balance with a readability of +/- 0.1g.

Purified chemicals and water

The quality of reagents and distilled water is of great importance for the quality of the analysis.

If natural abundance N is present in the reagents, it lowers the ^{15}N-result of the plant material! Therefore the reagents and the water, which is used for rinsing of glass ware should be checked to be free of Nitrogen.

This can be done by (1) direct blank determinations or (2) isotope dilution:

(1) Direct blank- determination

All ingredients except plant material are added (all reagents, same amount of water, boiling stones etc.), and is treated and analyzed like a routine sample; if the blank value does not exceed 2% of total N in the sample (e.g. 0.2 mg N per 10 mg N) the result can be corrected for the blank

(2) Isotope dilution

A known amount of ^{15}N-enriched AS-solution is pipetted into a digestion vessel (e.g. 10.0 mg N as AS-solution, 2.00 % ^{15}N atom excess) and then treated like a routine sample: (=>the recovery should not exceed 10.0 mg N and should not be lower or higher than 2.00 % ^{15}N a.e. otherwise the reagents, the glass ware or the water are possibly contaminated with natural abundance N (99.634%^{14}N) or ^{15}N-enriched N).

5.2.3 QUALITY SYSTEM

A Standard operating procedures (SOP) of validated methods

In a modern, “quality controlled” laboratory, it is obligatory to document all steps to be taken in the analysis in Standard Operating Procedures and follow these procedures strictly.

There are many different formats how these SOP’s should look and how detailed they should be. As a help: “An SOP should be understood by analysts, who are not necessarily involved in this type of analysis.”

A suggested format for an SOP is the following (similar to the ISO standard format):

- Scope and field of application of the described analytical method.
- Principle of the method.
- Typical sample.
- Apparatus.
- Reagents.
- Procedure (description of all steps taken).
- Calculation of result.
- Delicate points and precautions.
- References.

The methods described in the SOPs should be locally validated, i.e. checked to give correct results.

B Documentation: Up to date laboratory log books. Quality control charts

Laboratory log books and QC-charts should be used daily while analysis is performed. In routine analysis it can help identify problems with the analytical results even months or years after the analysis has been performed. The logbook should be used to track samples from receipt to disposal.

i The laboratory log book should contain

- All relevant data for the calculation of the analysis result.
- All background information about the nature and origin of the samples.
- All unusual observations or conditions, that could affect the quality of the results (e.g. high room temperature in the instrument room, because air condition did not function on that morning, etc.).
- The date and the name of the analyst.

ii A special instrument log book, for each instrument separately, should contain

- All changes of instrument settings.
- All maintenance activities.
- All calibration data (QC-charts, see below).
- The date and the name of the analyst.

iii Quality control (QC-) charts

These should be used to monitor the instrument performance and give evidence for the whole analysis to be under control. Their use is illustrated in Figures 5.5–5.8.

- Plot the % deviation (∆%) from reference value of an IRM-sample ("Control sample") into the chart daily (Table 5.1).

Table 5.1. Example for QC-chart, list of data for method control

Date of analysis	Reference value (IRM) [%N]	N found [%N]	∆% 100* (N found -ref.val) / ref.val.
3/10/98	2.75	2.73	- 0.7
3/11/98	- "-	2.79	+ 1.5
3/12/98	- "-	2.67	- 2.9
3/13/98	- "-	2.76	0.1
3/14/98	- "-	2.81	2.3

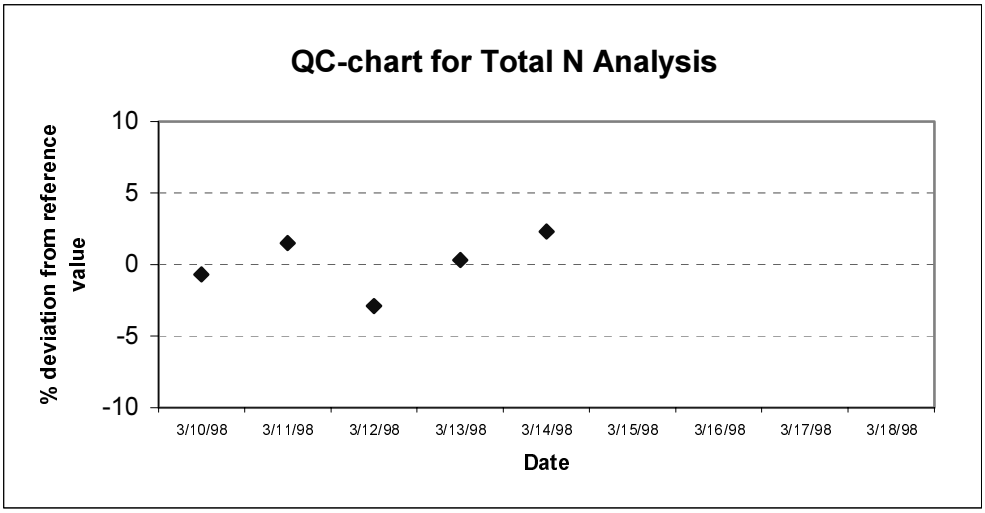


Figure 5.5. QC chart for method control. The values in a QC-chart for method control should not exceed +/- 5% deviation.

iv Examples of the benefit of QC-charts:

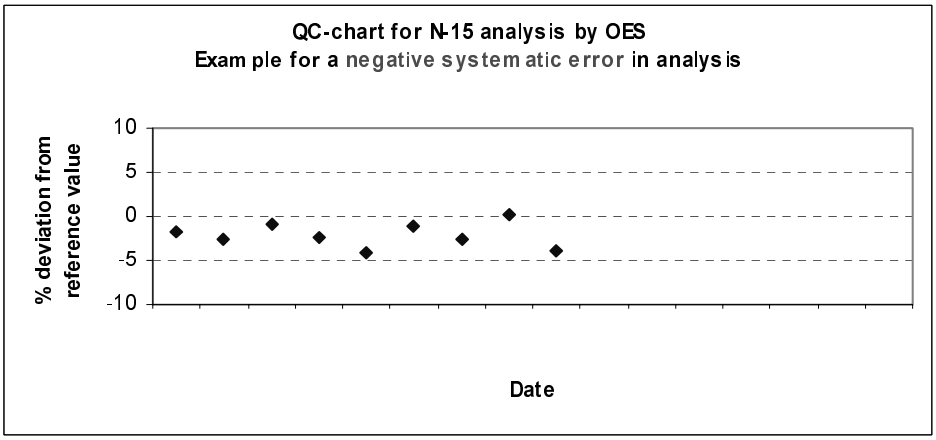


Figure 5.6. Example for negative systematic error.

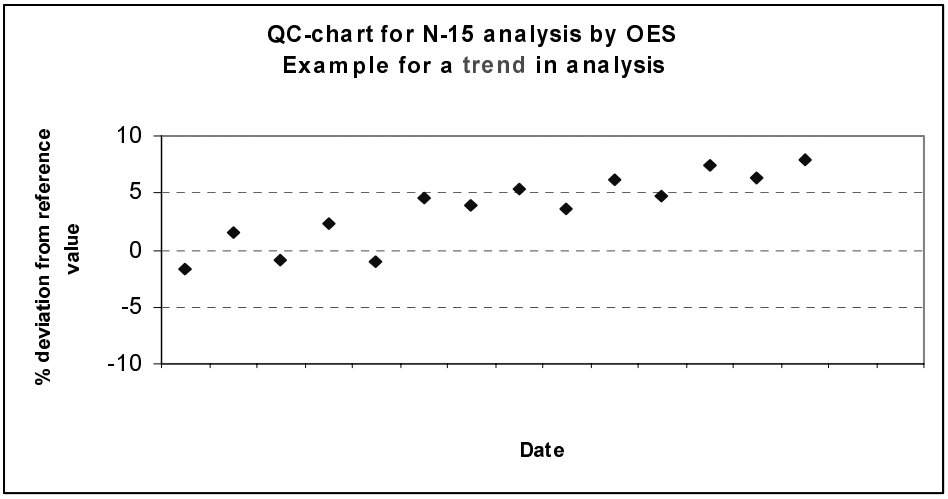


Figure 5.7. Example for positive trend.

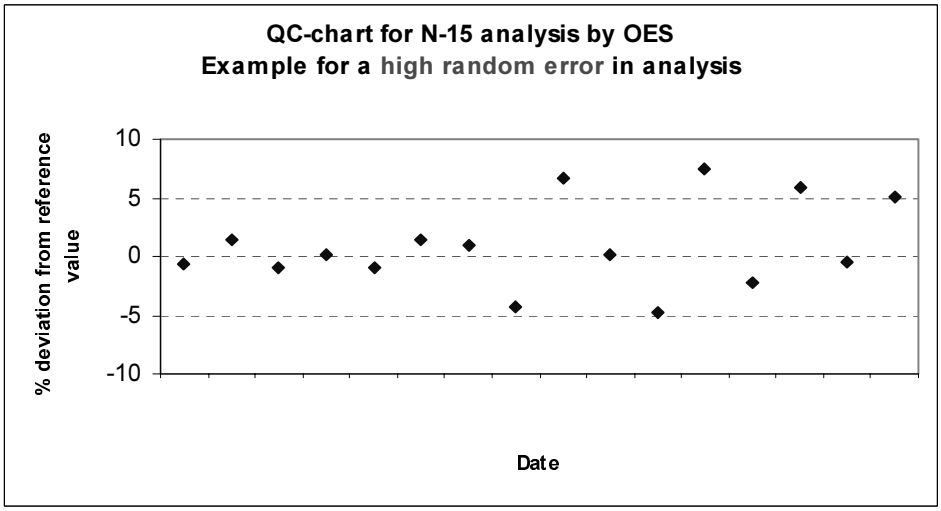


Figure 5.8. Example for increasing random error. QC-charts for instrument-control.

- Calculate the calibration curve of the emission spectrometer by linear regression analysis => y = ax + b, a= slope, b= intercept, r= coefficient of correlation
- Daily plot the slope and the intercept of the calibration curve into a QC-chart.
- Take the slope and intercept of a "good" calibration curve (after maintenance of the instrument) as a "reference" and monitor the variation around these values to identify changes in the instrument performance (Table 5.2).

Table 5.2. Example for a QC-chart, list of data for instrument control.

Date	Slope (a)	Intercept (b)	Correlation coefficient (r)
3/10/98	1.104	- 0.145	0.99991
(after maintenance)			
3/11/98	1.099	- 0.142	0.99974
3/12/98	1.107	- 0.139	0.99986
3/13/98	1.098	-0.150	0.99899
3/14/98	1.135	-0.149	0.99829
3/15/98	1.132	-0.154	0.99769
3/16/98	1.130	-0.159	0.99739

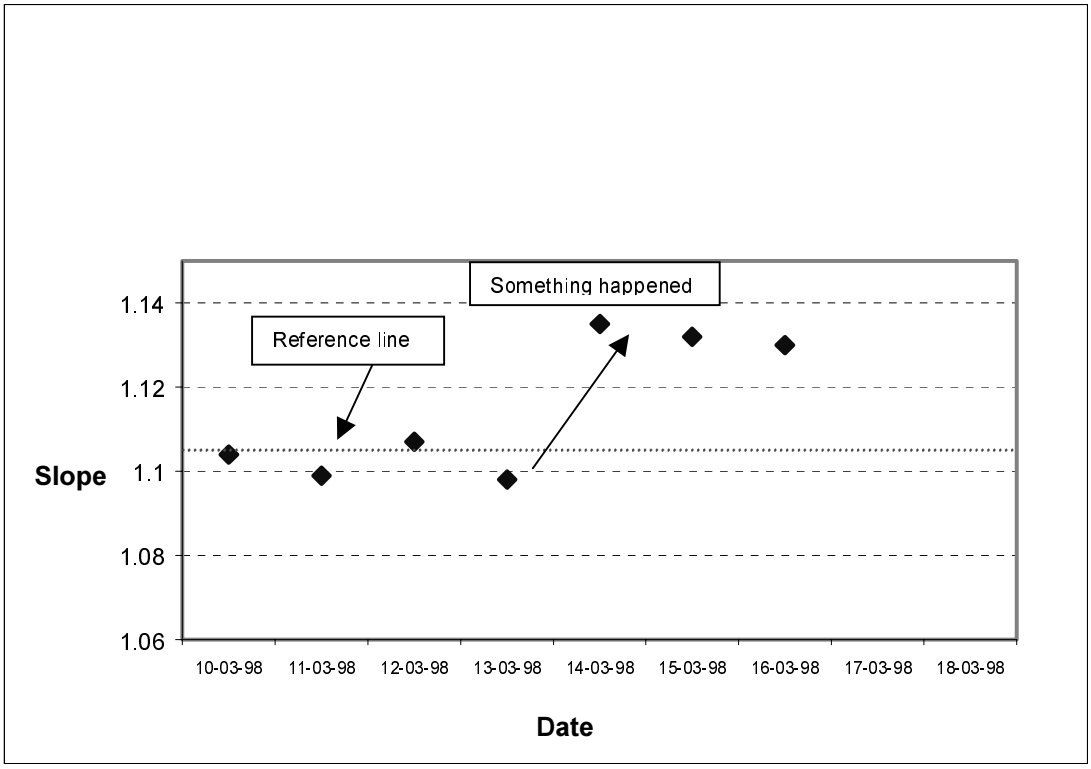


Figure 5.9. QC chart for instrument control.

C Staff: Well trained and motivated

- It is of utmost importance that the analyst and all technicians involved in the analysis feel responsible for their contribution to the final result of the analysis.
- The analyst should keep track of the sample from the receipt in the laboratory to the reporting of the result => "trackability".

- Be aware, that even if all Quality Assurance measures have been implemented and followed perfectly and the typist clerk mixes up the coding on the result reporting form all effort was in vain.
- This feeling of responsibility cannot be imposed – but it can be reached best by a good working atmosphere and a positive recognition of the contribution of each staff member involved in the process of analysis.

  Always remember: (1) "A chain is only as strong as its weakest link."
  (2) "Too many cooks spoil the soup."

D Regular participation in proficiency testing exercises or intercomparisons.

- For gaining self confidence in the analytical capability and to demonstrate proficiency in the field of N- and ^{15}N analysis it is essential to participate *regularly* in External Quality Assurance (EQA) programmes

NOTE: The Soil Science Unit, FAO/IAEA Biotechnology and Agriculture Laboratories, Seibersdorf, Austria, organises annual interregional proficiency testing exercises for total N- and ^{15}N analysis of plant materials.

Figure 5.10. Test panel as dispatched from IAEA with plant samples of unknown N- and ^{15}N-concentration.

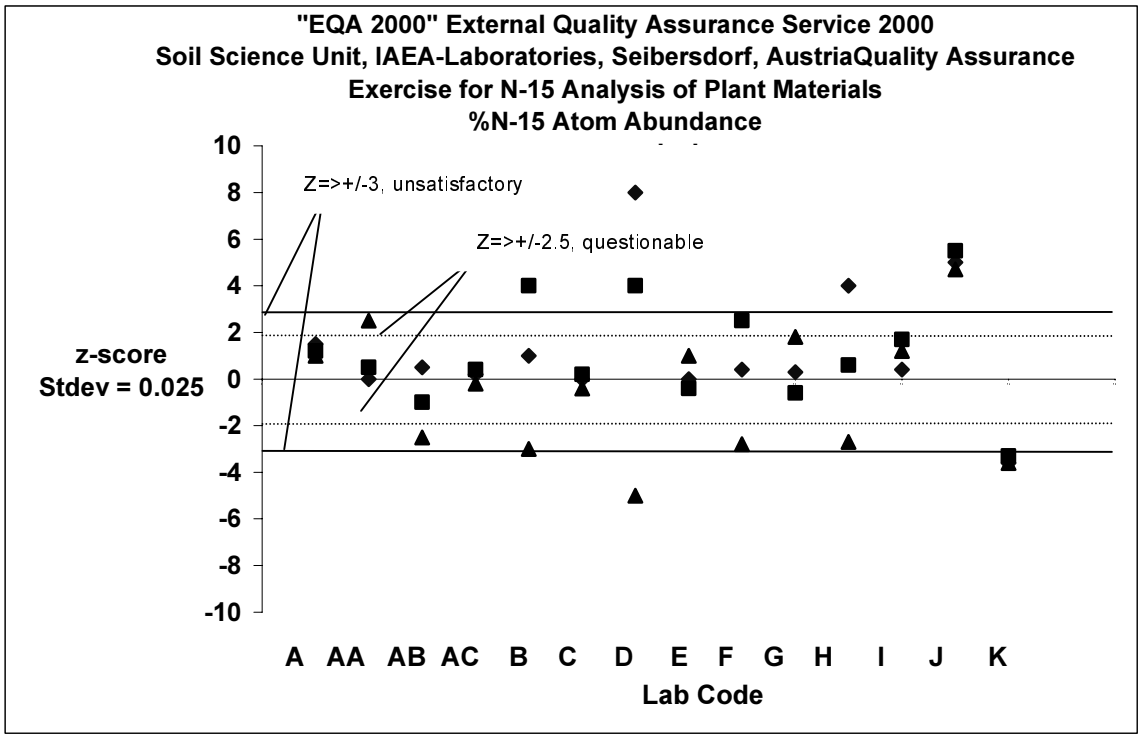


Figure 5.11. Representation of IAEA inter-laboratory QA report form.

5.3 QUALITY CONTROL (QC). MEASURES APPLIED IN TOTAL N AND ^{15}N PLANT ANALYSIS. ASSESSMENT OF THE ANALYTICAL PERFORMANCE

5.3.1 SUMMARY ("QA-CHECKLIST")

A Check the suitability of the environment

- ✓ Check the facilities for storage of the plant material
- ✓ Check the laboratories and instrument rooms
- ✓ Check the quality of the plant material

B Check the calibration of instruments and equipment

- ✓ Check the calibration of the burette
- ✓ Check the calibration of the volumetric pipette

C Check your analytical method – Part of "method-validation"

- ✓ Check the N-recovery of your Kjeldahl method
- ✓ Check the ^{15}N-recovery of your analytical method (i.e. sample preparation by Kjeldahl + OES)

D Determine the performance of your analytical method

- ✓ Determine the accuracy of your Kjeldahl method
- ✓ Determine the precision, i.e. repeatability and reproducibility, of your Kjeldahl method
- ✓ Determine the accuracy of your ^{15}N –Analysis method (OES alone)
- ✓ Determine the accuracy of your ^{15}N –Analysis method (plant sample preparation + OES)
- ✓ Determine the precision, i.e. repeatability and reproducibility, of your ^{15}N –Analysis method (OES alone)
- ✓ Determine the precision, i.e. repeatability and reproducibility, of your ^{15}N –Analysis method (plant sample preparation + OES)

5.3.2 "QA-CHECKLIST" IN DETAIL

A Check the suitability of the environment

- ✓ *Check the facilities for storage of the plant materials:*
  Storage of plant material should be:
- In a dry, well ventilated and cool room.
- Samples possibly in labeled paper bags.
- Batches of samples kept in large plastic buckets or covered with plastic sheets to prevent from moisture uptake.

- ✓ *Check the laboratories and instrument rooms:*

- Work benches, glass ware, automatic burettes, pipettes, balances, spatulas, digestion- and distilling units, hotplates etc. should be clean and free of contamination with N-compounds (both: ordinary N- and ^{15}N- compounds).
- Outlet tips of the distilling unit and burette for HCl-titrant solution have to be very clean and free of traces of (highly concentrated) NaOH.
- Chemicals for reagent- and standard preparation should be closed and touched only with clean spatulas.
- Standards and reference materials should be kept in a separate place.
- Aqueous N- and ^{15}N standard solutions have to be stored in the fridge.
- Reagents should never be exposed to direct sunlight, best kept in a fridge or in a cool and dark place (< 20°C).
- All standards and reagents should be labelled with:

- Date of preparation
- Name of compound
- Concentration of analyte
- Name of responsible person.

- Electronic instruments and measuring equipment with metal parts (e.g. balances, digital burettes, automatic pipettes etc.) should not be placed in corrosive atmosphere (e.g. near the digestion unit).
- The digestion unit should be placed in a fume hood or at least connected to a water jet pump.
- The emission spectrometer and possibly the analytical balance should be placed in a separate, air conditioned room.

B Check the quality of the plant material

- Plant powder shall be visibly homogeneous: particle size of ground material should not exceed 1 mm for semimicro Kjeldahl procedure (300-1000 mg material for 1 analysis depending on expected N-content)
- If the plant powder is infested with fungi, insects, cobwebs etc., the material cannot be used for analysis

C Check The Calibration of Instruments and Equipment

✓ *Check the calibration of the analytical balance:*

- Regular calibration check (suggestion: weekly) by weighing a set of Certified Reference Weights in the range of the routine sample weights (e.g. 5 mg, 10 mg, 50 mg, 100 mg, 500 mg, 1 g, 50 g and 100 g).
  : never touch reference weights with the fingers – use cotton gloves or plastic tweezers.
- Proper documentation of weighing data in a computer file or logbook.
- Regular control (suggestion: weekly) of weighing data by the responsible person.
- Re-calibration of balance, if out of range (acceptable deviation from reference weight has to be agreed upon, suggestion: +/- 0.1 % for weights from 5 g - 500 mg and +/- 0.5% for weights < 500 mg).
- Documentation of weighing-data of reference -weights after re-calibration.
- Regular renewal of certificate of certified reference weights by the relevant authorities (Government Office of Weights and Measures).
- Yearly service of balance and filing of service-data sheets.

✓ *Check the calibration of the burette:*

- Fill a clean burette with distilled water (remove air bubbles).
- Prepare 8 clean and dry vials.
- Note tare of vials to +/- 1 mg.
- Dispense 5.00 mL of distilled water from the burette into each glass vial.
- Note gross weights of vials plus water to +/- 1mg.
- Note the room temperature.
- Calculate the net weights, mean and standard deviation of the dispensed water.
- Note all data in the log book.

The density of (air free) water is :0.99823 g/cm^3 at 20°C and 0.99707 g/cm^3 at 25°C
Therefore 5.00 mL dispensed water should weigh theoretically:

- 5.00 cm^3 x 0.99823 g/cm^3 = 4.99_1 g at 20° C
- 5.00 cm^3 x 0.99707 g/cm^3 = 4.98_5 g at 25°C

BUT: The weight displayed on the balance has to be corrected for bouancy:

$(1-\rho_{air}/\rho_{bal})/(1-\rho_{air}/\rho_{H20\ 20°C})$ (Equation1)

where ρ_{air} = density of air= 0.0012 g/cm^3, ρ_{bal} = density of balance weights= 8.0 g/cm^3, $\rho_{H20\ 20°C}$ = density of water at 20 °C = 0.99823 g/cm^3

⇨bouancy correction factor:

=> (1 - 0.0012 / 8.0) / (1- 0.0012 / 0.99823) = <u>1.00105</u>

⇨"apparent density" of H_2O at 20°C: 0.99823 / 1.00105 = <u>0.99715</u>

⇨"apparent density" of H_2O at 25°C: 0.99707 / 1.00105 = 0.99602

Taking into consideration the variation of burette dispensing and the uncertainty of weighing, 5.00 mL of dispensed distilled water should weigh:

- 5.000 g * 0.99715 = 4.98_6 g +/- 0.005g at 20° C
- 5.000 g * 0.99602 = 4.98_0 g +/- 0.005 g at 25°C

If the deviation of the mean net weight from the calculated weight is higher than +/- 0.1% (+/- 0.005 g for 5 mL dispensed volume), the burette has to be re-calibrated.

If the standard deviation of the net weights of the 8 replicates is higher than +/- 0.5% (s=+/- 0.025 g for 5 mL dispensed volume), the burette should be serviced or renewed!

✓ *Check the calibration of the volumetric pipette:*

- Prepare a clean 10 mL volumetric pipette (calibrated , class A, ex 15s).
- Prepare 8 clean and dry glass vials.
- Note tare of glass vials to +/- 1 mg.
- Pipette 10.0 mL of distilled water into each vial.
- Note gross weights of vials plus water to +/- 1mg.
- Note the room temperature.
- Calculate the net weights, mean and standard deviation of the pipetted water.
- Note all data in the log book.

Taking into consideration the variation of pipetting and the uncertainty of weighing, 10.00 mL of pipetted distilled water should weigh:

- 10.000 g * 0.99715 = 9.97_2 g +/- 0.01g at 20° C
- 10.000 g * 0.99602 = 9.96_0 g +/- 0.01 g at 25°C

If the *deviation of the mean net weight* from the calculated weight is higher than +/- 0.1% (+/- 0.01 g for 10 mL pipetted volume), the pipette has to be de-greased or exchanged.

If the *standard deviation* of the net weights of the 8 replicates is higher than +/- 0.1% (s=+/- 0.01 g for 10 mL pipetted volume), pipetting should be trained better, or the pipette should be de-greased or renewed!

D Check your analytical method - (Part of "method-validation")

Keywords: recovery, accuracy, precision, repeatability, reproducibility

✓ *Check the N-recovery of your Kjeldahl method:*

By using an ammonia sulfate (AS) standard solution with known amount of N prepared from commercially available solid ammonia sulfate p.a., e.g. Merck Cat.No.1217.

- Pipette a precise volume of the standard solution into a digestion vessel and treat it like a routine plant sample.

Example:

- Dissolve 4.715 g ammonia sulfate p.a. (dried at 105°C, 2 hours) in a 1Liter- volumetric flask with about 700 mL distilled water, homogenize well and make up to 1000 mL.
- Using a *calibrated* volumetric pipette transfer exactly 10.0 mL of the standard solution containing 10.0 mg N (N_{taken}) into a Kjeldahl digestion vessel and analyze like a routine plant sample (N_{found}).

calculation of % recovery:

[mg] N_{taken} …… 100 %

[mg] N_{found} …… …… Y %

$$Y\ (\%\ \text{recovery}) = 100 \times [\text{mg}]\ N_{found} / [\text{mg}]\ N_{taken} \qquad \text{(Equation 2)}$$

If the recovery is lower than 100 %, check for:

- Leaks in the distillation unit.
- Bad calibration of balance, burette and/or pipette.
- Insufficient amount of NaOH to make the digest alkaline before distillation.
- Wrong titer of HCl , c = 0.1N.
- Wrong titer of the NaOH, c= 0.1N.
- Old Tashiro indicator (sluggish endpoint).

If the recovery is higher than 100 % (=> N- blank), check for:

- N-contamination of distilled water, reagents, glassware etc.
- Bad calibration of balance, burette and/or pipette.
- Wrong titer of HCl , c = 0.1N.
- Wrong titer of the NaOH, c= 0.1N.
- NaOH-contamination of outlet tip of distilling unit (*explanation:* if NaOH is neutralizing part of the HCl prior to titration, the NaOH consumption of the titrant is too low and the N-amount of the sample is overestimated).

: only, if N-recovery with ammonium sulfate-solution is satisfactory (between 98 and 102%), continue with plant material (IRM).

By analyzing a minimum of 6 replicate samples of an IRM (traceable to a CRM) plant material treating it like a routine sample.

If the recovery is lower than 100%, check for:

- Bad calibration of balance, burette and/or pipette.
- Insufficient moisture correction of IRM sample.
- Quality of IRM sample (=>infested, inhomogeneous etc.).
- Loss of material (=> during transfer of powder to digestion vessel or due to splashing or boiling over during digestion).
- Insufficient digestion time (=>until *colorless* digest is obtained).
- Insufficient amount of H_2SO_4 applied.
- Too low temperature during digestion (check temperature of the digestion block with a mercury thermometer => should be 360 to 380°C after foaming ceased).

If the recovery is higher than 100 %, check for:

- Bad calibration of balance, burette and/or pipette.
- N- contamination of used reagents or distilled water.
- N-contamination of spatula or glassware.

: N-blank determinations have to be done on each analysis day.

: “Isotope Dilution” can be used for more precise blank N- determinations (see below)

✓ *Check the ^{15}N-recovery of your analytical method* (i.e. sample preparation and OES):

By checking the N- and ^{15}N-recovery analyzing a ^{15}N labelled ammonia sulfate standard solution (e.g. solid compound from IAEA, FAN or JASCO) with known amount of N and known isotopic composition.

: The ^{15}N-solution should undergo the complete Kjeldahl procedure, not only the isotope determination part by OES.

Example:

- Dissolve 0.472 g purified ^{15}N labelled ammonia sulfate with known ^{15}N atom abundance in a 100 mL-volumetric flask with about 70 mL distilled water.
- Homogenize well and make up to 100 mL.
- Using a calibrated volumetric pipette transfer exactly 10.0 mL of the standard solution containing 10.0 mg N into a Kjeldahl digestion vessel and analyse like a routine plant sample, measure ^{15}N atom abundance by OES

  % recovery = 100 * [%]^{15}N ab. $_{found}$ / [%]^{15}N ab. $_{taken}$ (Equation 3)

- *If the ^{15}N atom abundance is higher than the reference value*:*

- ^{15}N-contamination due to messy, contaminated working area e.g. spatula, glassware and/or reagents contaminated with traces of enriched fertilisers or enriched plant materials
- *If the ^{15}N atom abundance is lower than the reference value:*
- ^{14}N-contamination due to messy, contaminated working are e.g. spatula, glassware and/or reagents contaminated with traces of ordinary fertilisers or unlabelled plant materials

: Only, if the ^{15}N-recovery with Ammonia Sulfate-solution is satisfactory (between 98 and 102%), continue with point 2.

By checking the ^{15}N-recovery analysing a labelled IRM plant material with known amount of N and known isotopic composition treating it like a routine plant sample

Reason for high or low recovery: see above.

: the reason for high or low^{15}N-results could of course also be a wrong calibration of the OES.

Method of "Isotope Dilution" for blank determination:

- To determine the [mg] natural abundance N (blank N) of reagents, glass ware, used water etc.:

Example: (*NOTE:* calculation is done with atom excess values, *not* with atom abundance values)

^{15}N labelled AS- solution taken: 10.0 mg N, 2.73 % ^{15}N a.e.

^{15}N atom excess found by OES: 2.68 % ^{15}N a.e.

Calculation of blank N (natural abundance, i.e. 0 % 15N a.e.):

2.73 % ^{15}N a.e. x f = 2.68 % ^{15}N a.e. => f = $\frac{2.68}{2.73}$ = 0.9817

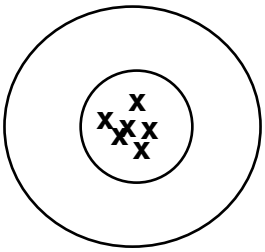
f = $\frac{10mgN}{(10+x)mgN}$ = 0.9817

(10 +x) = $\frac{10mgN}{0.9817}$ = 10.19 mg N

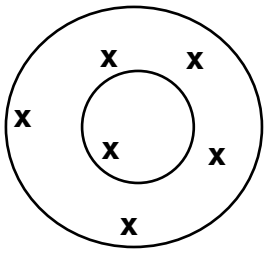
x = (10.19 – 10) = <u>0.19 mg N</u> ("blank N")

E Determine the performance, i.e. accuracy and precision of your analytical method

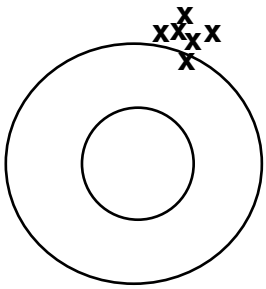
Case 1. Accurate + precise => no “systematic error”, “low random error”:



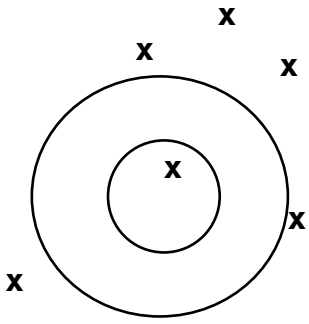
Case 2. Accurate , but not precise => high “random error”:



Case 3. Not accurate , but precise => “systematic error”:



Case 4. Not accurate , not precise => “high random error”:



✓ *Determine the accuracy of your Kjeldahl-method*

- "Accurate" results are "correct" results, i.e. they do not differ significantly from the "true" Nitrogen assay in the sample, the difference is within pre-defined, accepted limits.
- Usually, the "true" value is unknown. For a CRM, the certified reference value is assumed to be the true value.
- Presently, the Soil Science Unit of the FAO/IAEA – Laboratories is regarding a difference of = +/- 5 % as acceptable accuracy limits for Kjeldahl-analysis. (Difference between the mean result and the reference value).

% deviation (%) n:

$$\% = [(\text{mean analysis result} - \text{reference-value}_{CRM})/ \text{reference-value}_{CRM}] \times 100 \qquad \text{(Equation 4)}$$

The upper and lower bounds of the confidence interval have to lie within these accepted limits to be sure (with 95% probability) that the accuracy of the method is ±5% (relative error) or better

Example:

- 6 replicates of a CRM with a reference value of 2.00 % N were analyzed.

a) Calculate the mean N-content and the standard deviation "s":

1. result 2.04 % N
2. result 1.99 % N
3. result 2.05 % N
4. result 2.07 % N
5. result 1.97 % N
6. result 2.05 % N

mean result: $\bar{x}$ = 2.03 % N
stdev: s = 0.04

b) Calculate the confidence interval:

$$\bar{x} \pm t \frac{s}{\sqrt{n}} \qquad \text{(Equation 5)}$$

- $\bar{x}$ = mean analysis result
- t= one-sided (!) t-value for (n-1) degrees of freedom and 95% probability
- s = standard deviation of analysis results
- n= number of analysis result

In the example:

- $\bar{x}$ = 2.03
- t_{table} = 2.02
- s = 0.04
- n=6

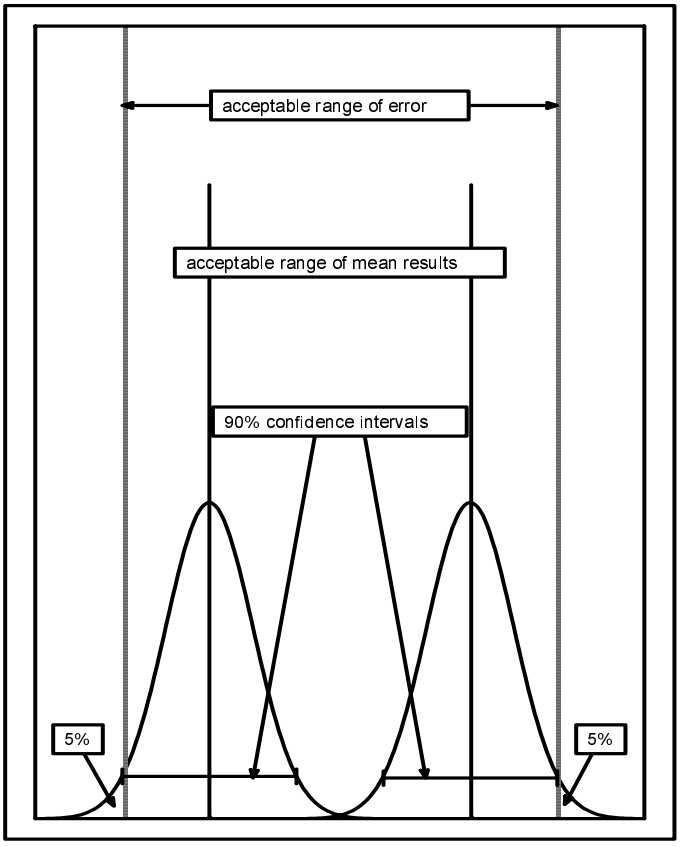
=> *confidence interval:* $2.03 \pm 2.02 \frac{0.04}{\sqrt{6}} = 2.03 \pm 0.03$ %N (Equation 6)

- The reference value of the CRM is 2.00 % N.
- The accepted limits are +/- 5% deviation, that means, results between 1.99 and 2.10 %N are deemed as acceptably accurate.
- The actual mean analysis result lies within 2.00 % N and 2.06 % N (with 90% probability), i.e. there is a 5% chance that the result is higher than 2.06 % N or lower than 2.00 % N (note: the result cannot be too high and too low at the same time).
- There is less than 5% chance that the result is higher than 2.10 % N or lower than 1.99 % N (actually much less).

c) Check if the interval is smaller than the accepted limits:

- With a probability of more than 95%, the applied Kjeldahl method is accurate to ±5% (relative error).

Graph explaining the acceptable limits and the confidence interval:



✓ *Determine the precision (i.e. repeatability and reproducibility) of your Kjeldahl-method:*

The precision of a method can be expressed by the standard deviation (s) or the relative standard deviation (c.v.%).

Example for reporting the precision of the analysis:

(1) "mean result +/- stdev":

example: 2.03 +/- 0.04 (1s)

(2) *"mean result +/- %c.v.":* ("coefficient of variation" or "relative standard deviation"):

$$\% \text{ c.v.} = s \times 100 / \bar{x} \Rightarrow 0.04 \times 100 / 2.03 = \underline{1.97} \%$$

example: 2.03 +/- 1.97 (%c.v.)

An estimate of the repeatability of your method can be obtained by analyzing a minimum of 6 replicates of one plant material with known N-content (Internal Reference Material, IRM) in one particular analysis session and calculate the standard deviation or the c.v.% (i.e. the "precision" within one particular analysis session)

The reproducibility of your method can be estimated by analyzing different batches of a plant material with known N-content (Internal Reference Material, IRM) at different times, with different reagent batches, analyzed by different technicians. Calculate the mean and standard deviation or the c.v.% of all results of one IRM collected from the different analysis sessions (i.e. the "precision" over a long period and under different conditions)

✓ *Check the Accuracy of your ^{15}N-Method by OES:*

- Prepare an ^{15}N-labelled Ammonium Sulfate Standard solution (e.g. prepared from solid compound from IAEA, FAN or JASCO) like for the ^{15}N –recovery determination (see above).
- Analyse the ^{15}N atom abundance of 6 replicates of the A.S.-Standard solution and 6 replicates of an IRM plant sample (presently no ^{15}N labelled plant CRM available) for % 15N atom abundance.

- Calculate the mean results, standard deviations and confidence intervals (calculations, see "Determination of Accuracy of the Kjeldahl Method").
- Check, if your result confidence intervals lie within the acceptable limits of the IAEA Laboratories, i.e. a deviation of Δ = +/- 5% from the reference value.

: different accuracy results will be found for ^{15}N determination of AS-Standard solution and plant IRM (these are two different methods).

✓ *Check the precision, repeatability and reproducibility of your ^{15}N-analysis by OES:*

- Calculate the standard deviations and relative standard deviations of analysis results of one IRM (i.e the precision) in one session (i.e. the repeatability) and over a long period of analyses (i.e. the reproducibility); explanations see above

: The performance of the Kjeldahl-method and the ^{15}N analysis by OES can be evaluated in one time using one ^{15}N labelled IRM.

5.4 PRODUCTION OF PLANT REFERENCE MATERIAL

5.4.1 LABORATORY PROCEDURE: PRODUCTION OF AN ^{15}N LABELLED PLANT INTERNAL REFERENCE MATERIAL (IRM)

A Scope and field of application

To produce a homogenous working reference material of a known reliable and constant N and ^{15}N content.

B Principle

A large quantity of homogeneous plant material is prepared so that it may act as the long term laboratory standard. A suitable crop with an appropriate expected N content is selected and grown in large field plots. The crop is homogeneously labelled with ^{15}N enriched fertiliser to achieve a desired ^{15}N abundance. The crop is then harvested and separated into the constituent fractions (i.e. leaves, grain, shoots). The desired fraction of the material (e.g. the leaves) is then weighed and dried in an oven at 70°C. The fraction required is finely ground into a powder (<0.5mm). This may be done in batches. All the batches are combined and sample is then mixed for two days on a custom built mixer and transferred into a polythene container.

i Basic requirements

Plant material for standards is usually selected so that it fulfils the following criteria:

- There is sufficient plant material available to act as a long term standard (3- 4 kg dried material).
- The plant material selected has an appropriate N content and ^{15}N atom excess (i.e. similar to the samples to be analysed in routine).
- The material is homogenous (it is usual that a particular plant constituent is selected e.g. grain standard or straw standard); a homogeneity test should be applied (see below).
- The material can be finely ground (e.g. no oil-containing seeds).
- The material will not degrade with time (e.g. low content of fat, which can be oxidized).

ii Typical Sample

- Large quantity of homogenous plant material of even ^{15}N abundance.

iii Apparatus

- Drying oven, large enough to dry ~25–30 kg of fresh plant material at 70 °C.
- Harvest chopper for cutting fresh plant material to ~1–2 cm.
- Cutting mill for cutting dry plant material to a particle size of ~2 mm.

- Rotary grinder or ultracentrifugal mill (e.g. ZM-1000, Fa. Retsch, Germany), for grinding ground plant material (2 mm pieces) to a particle size of <0. 5 mm.
- Top load balance, readability (+/- 1g), analytical balance, readability (+/- 0.1mg).
- 3–4 large metal trays (e.g. aluminium or stainless steel) to hold about 5–10 kg of fresh plant material, fitting into the drying oven.
- Rotating mixer or a mixing device, shop made.
- Polythene jars with screw caps.
- Polythene balls, 2–3 cm diameter.

iv Reagents: not applicable

v Procedure

Field propagation

- Select a crop suitable for the intended purpose and calculate the seeding density and field plot size in order to achieve a sufficiently high yield; a 50 % loss of material during harvest, separation, drying and grinding procedure can be expected.
- Apply the appropriate rate of ^{15}N-labelled fertiliser in 3 splits
  - After germination
  - During vegetative growth
  - Early reproductive stage.
- It is advisable to apply the fertiliser in the form of a solution and to divide this solution into equal portions for uniform distribution on ~ 5 m^2 subplots (in case of beans or maize being the selected crop the second and third split application should preferably be applied in bands near the plants and not onto the leaves)

Harvest

- Harvest carefully without contaminating the plant material with ^{15}N- labelled soil.

***NOTE*:** In producing ^{15}N-labelled leaves the harvest should take place before translocation of the N to the seeds!

NOTE: The border rows should not be harvested to avoid non-homogeneous ^{15}N – concentrations.

- Pods, grain, cops etc. are separated from leaves and treated separately.
- The constituent plant part is weighed at harvest, giving the fresh weight.
- Rough chopping to 1–2 cm pieces may occur at this point.

Production of reference material

- The plant material is transferred into large open metal trays.
- The samples are dried in a drying oven set at 70 °C +/- 2 °C, until constant weight (normally 48 hours).
- Trays are removed from the oven.

NOTE: The plant material is weighed and ground immediately after drying

- The dry weight is noted and % dry matter calculated.
- The material can then be cut to a particle size of about 2 mm (if necessary) using, the cutting mill.

NOTE: Make sure, that the chopping and grinding devices are cleaned thoroughly between materials with different N- and ^{15}N contents, e.g. pods and straw.

- The samples are then finely ground in batches using the rotary grinder to a fine flour consistency to <0.5 mm particle size.
- The entire material is put into a large polythene jar, which can be closed airtight to avoid moisture uptake during mixing and having at least three times the volume of the plant material, which shall be mixed.
- Metal or teflon balls (about 2–3 cm in diameter) can be added to the jar to aid mixing.
- This is mixed for 48 hours using a custom built machine which constantly rotates the jar or an alternative mixing system.

- The homogeneity of the plant material is determined by taking a minimum of five 5g-subsamples from different places / depths in the jar and analysing five times 1 g of the subsamples the ^{15}N-atom abundance (= 25analyses).
- If the variance between the five 5-g-subsamples is equal to the variance within each 5-g-subsample (according to one way ANOVA, F-test) the material is sufficiently homogeneous for the intended purpose.
- Store the plant material in several airtight polythene 2-liter-containers and re-homogenize every 12 months.
- Check regularly for infestation or moisture uptake.

***NOTE**:* Never use contaminated spatulas for weighing out subsamples from the plant reference material

Homogeneity Test

Formula for a one-way ANOVA:

1. Within sample estimate of variance: . $s_0^2 = \sum_i \sum_j (x_{ij} - \bar{x}_i)^2 / h(n-1)$

2. Between sample estimate of variance: $s_0^2 = n\sum_i (\bar{x}_i - \bar{x})^2 / (h-1)$

Where
I = index of 5-g-subsample
j = index of replicate measurement of one 5-g-subsample
h = number of 5-g-subsamples taken from different places in the drum(here:h=5), and
n = number of replicate measurements on one 5-g-subsample (here: n=5)

Generalization Table:

Subsample number taken from 5 different places in the jar:	5 replicate measurements* [% ^{15}N atom abundance]	Mean
subsample 1	x11…..x12……..x1j……x1n	$\bar{x}_1$
subsample 2	x21…..x22……..x2j……x2n	$\bar{x}_2$
subsample i:	xi1…..x22……..xij…… ……xin	$\bar{x}_i$
subsample h	xh1…..xh2……..xhj……xhn	$\bar{x}_h$
	overall mean	$\bar{x}$

Example for testing the homogeneity of a ^{15}N labelled plant material:

Subsample number taken from 5 different places in the jar:	5 replicate measurements* [% ^{15}N atom abundance]	mean
5-g-subsample 1	0.885, 0.888, 0.879, 0.874, 0.883	0.882
5-g-subsample 2	0.880, 0.884, 0.881, 0.895, 0.892	0.886
5-g-subsample 3	0.875, 0.880, 0.888, 0.878, 0.889	0.882
5-g-subsample 4	0.895, 0.880, 0.892, 0.887, 0.878	0.886
5-g-subsample 5	0.887, 0.880, 0.876, 0.892, 0.881	0.883
	overall mean $\bar{x}$	0.884

$$\text{Variance 1}=\frac{(0.885-0.884)^2+(0.888-0.884)^2+(0.879-0.884)^2+(0.874-0.884)^2+(0.883-0.884)^2}{5-1}=0.000036$$

$$\text{Variance 2}=\frac{(0.880-0.884)^2+(0.884-0.884)^2+(0.881-0.884)^2+(0.895-0.884)^2+(0.892-0.884)^2}{5-1}=0.000053$$

$$\text{Var. 3}=\frac{(0.875-0.884)^2+(0.880-0.884)^2+(0.888-0.884)^2+(0.8978-0.884)^2+(0.889-0.884)^2}{5-1}=0.000044$$

$$\text{Variance 4}=\frac{(0.895-0.884)^2+(0.880-0.884)^2+(0.892-0.884)^2+(0.887-0.884)^2+(0.878-0.884)^2}{5-1}=0.000061$$

$$\text{Variance 5}=\frac{(0.887-0.884)^2+(0.880-0.884)^2+(0.876-0.884)^2+(0.892-0.884)^2+(0.881-0.884)^2}{5-1}=0.000041$$

1. Within-sample estimate of

$s_0^2 = (0.000036 + 0.000053 + 0.000044 + 0.000061 + 0.000041) / 5 = 0.000047$

This estimate has 20 degrees of freedom: each subsample has (5 - 1) =4 and there are 5 subsamples => 4 x 5 = 20

2. Between-sample variation:

$$\text{Sample Mean Variance}=\frac{(0.882-0.884)^2+(0.886-0.884)^2+(0.882-0.884)^2+(0.886-0.884)^2+(0.883-0.884)^2}{5-1}=\frac{0.000021}{4}$$

Between-sample estimate of $s_0^2 = \frac{0.000021}{4} * 4 = 0.000021$

This estimate has (5-1)= 4 degrees of freedom, since it is calculated from 5 sample means.

Summary:

- Within-sample estimate of variance: 0.000047 with 20 degrees of freedom
- Between-sample estimate of variance: 0.000021 with 4 degrees of freedom

F-test:

If the plant material is homogeneous, the two estimates of s_0^2 should not differ significantly ➔ the null hypothesis is correct ! If it is incorrect, the between-sample estimate of s_0^2 will be greater than the within-sample estimate because of between-sample variation*. To test whether it is significantly greater, a one-tailed F-test is used:

$F_{4,20}$ = 0.000047 / 0.000021 = 2.202

*NOTE: the higher value has to be the within-sample estimate and has to be the numerator of the F-value $F_{4,20\ table}$ (P=0.05) = 2.866

Result:

$F_{4,20} < F_{4,20\ table}$ for P=0.05 ➔ no significant difference ➔ the material is homogeneous.

5.5 REFERENCES AND FURTHER READING

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CHAPTER 6

MODELLING

6.1 What is a model?

A model is defined as a mathematical representation of a system. Modelling is the process of developing that representation.

6.2 Fundamental goals of model

There are basically two fundamental goals for crop and agricultural system models:

- To better understand the cause-effect relationships in a system and to provide improved qualitative and quantitative interpretations of the behaviour of that system. The result of this type of effort is an increase in knowledge so this is a research-oriented goal. An example of such a model is MACRO (Jarvis et al., 1991). MACRO is a one-dimensional, finite difference numerical model for non-steady state water flow and solute transport in macro porous field soils.
- To better predict system behaviour for immediate use in improving control or management of the system. The end result could be a tool/system (software/hardware product) designed for a specific application. One example is NLEAP (Shaffer *et al*., 1991), which is a nitrate leaching and economic analysis model, developed to provide a rapid and efficient method of determining potential nitrate leaching associated with agricultural practices.

6.3 Strengths and weaknesses of models

6.3.1 STRENGTHS

- Models may identify knowledge gaps, gain insights into situations where experimental results are lacking or are incomplete.
- Can be used to generate and test hypotheses, an aid in the design of experiments.
- They may determine the most influential parameters of a system and provide a framework for understanding a system and for investigating how manipulating it affects its various components (sensitivity analysis).
- Evaluate long term impact of particular interventions.
- Provide quicker and cheaper answers than is possible with traditional experimentation by its ability to interpolate and extrapolate a limited set of data so that repetitive, laborious and time-consuming experimentation can be reduced.
- Provide a medium for better communication between researchers, experimenters and producers in different disciplines to solve common problems.

6.3.2 WEAKNESSES

- While a model makes a situation easier to define and manage by considering only the most important aspects and ignoring all the less important details, it is also its weakness in that all the less important contributing factors are ignored.
- Some models are so parameter-intensive that it is impossible to obtain all the required information.

Although models are useful, they are considered complimentary to experimentation, and are not meant to take its place. A model cannot be developed or verified without a good data set. Often much effort is spent in developing a model, while less effort is put into conducting appropriate experiments to parameterise and test the model.

6.4 Classification of models

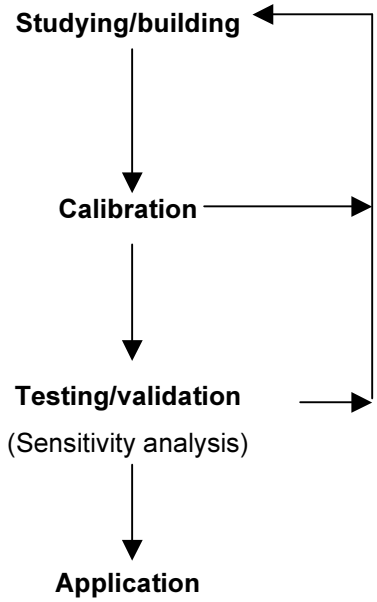
There are many different classes of models. One classification is based on whether they are developed primarily for research purposes or as guides to agricultural management. The first type of model tends to be more mechanistic and is mainly for helping to understand how the processes underlying the system interact. The second type of model is more "functional" or empirical in nature, in that the treatment of soil processes is simplified, with little account is taken of the mechanisms of the system. Models of these types are simplified enough for them to be used for practical management decision. The input information and computer expertise requirement for their use is also less demanding.

The second classification is based on the outcome it predicts. In this classification, models are either deterministic or stochastic in nature. Deterministic models ignore random variation, and so predict the same outcome for a given set of events. On the other hand, stochastic or probabilistic models are more statistical in nature and may predict a distribution of possible outcomes.

Passioura (1996) classified models into scientific models and engineering models. The first is to help with understanding; the second is for applying science to solve a problem. Matthews *et al*. (2000) extended the classification and divided model applications into (1) those used as tools by researchers, (2) those used as tools by decision-makers, and (3) those used as tools by those involved in education, training, and technology transfer. No classification is perfect, there is bound to be overlap between groups.

6.5 Processes of modelling

The process of modelling can be divided into four broad stages: studying/building, calibration, testing and application in this order. Often problems encountered at the calibration and testing stages are returned back to the building stage to be corrected, and the whole process needs to be repeated.



6.5.1 MODEL STUDYING/BUILDING

- Problem analysis - Recognising problem and defining objectives.
- Recognising client/server
- Cost benefit analysis
- Process analysis – data flow diagrams/charts, decision tables
- Requirements (prototype, pseudocode, system specifications)

6.5.2 MODEL CALIBRATION

- Choosing appropriate tools/mathematical equations
- Calibrating the system and evaluating the performance. This is necessary to minimize parameter errors and to determine the extent of conceptual model error. Calibration requires input parameters to run the model, together with data on the performance or field conditions.

6.5.3 MODEL TESTING/VALIDATION AND SENSITIVITY ANALYSIS

This involves comparing outputs of a fully calibrated model to real data and determining the suitability of the model for its intended purpose, resulting in the approval (or disapproval) for a particular use, or as a documentation of the precision and accuracy of the model for specified environments.

Data required for validation include a complete data set required to run the model. For crop simulation models, these are the initial conditions and the field information on the aspects that model is desired to predict, and for which the model is being validated. The dataset should not have been used previously for calibration and should represent the complete array of environments in which the model will be applied.

Sensitivity analysis is the study of the behaviour of the model, where the parameters or inputs of the model are evaluated with regards to their importance, relative to the simulated results. The advantage in this exercise is the ability to simulate a complete growing season(s) in a matter of seconds, allowing both “what if” questions to be answered and a great range of treatments to be examined, rather discovering it by trial and error.

6.6 Model application

Model application can only be carried out after it has successfully gone through the above stages.

6.7 Data requirements

The application of simulation models to problems in the real world depends not only on the availability of models and application software, but also on the availability and quality of information that would make it possible to run the models. It is therefore important that the appropriate data requirements, data collection and experimental procedures are specified so that data handling structures and analytical approaches can be defined and developed.

6.7.1 MINIMUM DATA SETS (MDS)

It is the minimum data set that is required to run a model and validate the outputs. For functional or management type of model, very few input parameters are needed, often only basic soil, crop and weather information is required. However, for many crop models, validation requires weather data for the duration of growing season, crop and soil data and management and experimental data.

A typical minimum weather data set includes: latitude and longitude of the weather station, incoming solar radiation, maximum and minimum air temperature, and rainfall, usually collected on a daily basis in order to calculate evapotranspiration and computing water balance.

Soil data includes soil classification, particle size distribution (% sand, silt, and clay), bulk density, organic carbon, pH, aluminium saturation, and root density.

For more complicated models such as APSIM and CERES-Wheat (see later), slope, infiltration and drainage information, and soil profile depth data by horizons are also needed. In addition, management data includes planting date, dates of soil samplings, planting density, row spacing, planting depth, crop variety, irrigation, and fertiliser practices are also required for both model validation and strategy evaluation. Experimental data such as crop growth, soil water and fertility measurements are often needed for model validation.

6.7.2 DATABASES

A database is an integrated collection of data, or a set of relations which describe the interrelated sets of persons, places, things and/or events (entities). Databases may also be called files. Databases generally comprise records as individual items of information. These can vary in size

and complexity. There are several different types of databases and they are classified by the type of information they contain, how they are structured, or a combination of the two.

The four databases categories are: textural, numeric, bibliography and directory databases.

- Textural databases: contain records/references of the objects.
- Numeric database contains very little textual information, but have various fields of numeric data. What separates numeric database from those containing mostly words is the need to perform complex calculations on the data in a field, rather than merely retrieving a piece of information contained in the text.
- Bibliographic databases contain bibliographic citations.
- Directory databases present factual information on organisations, companies, products etc. the files are used to create lists or to find a profile of a known entity.

Databases are also described by the way the information is structured. The two common structures are flat file and relational databases. In flat files, the individual records are designed to hold and organise all the information you have about the topic. These records are all contained in one database. In a relational database, multiple records in different files may be linked together – related – to organise the information.

Advantages of database processing are that duplication of data can be reduced and consistency of data can be improved. In addition, broader data sharing, and improved productivity in application development and maintenance can be achieved.

Some of the commercially available databases are: ACCESS, ORACLE, DB2.

Below is a list of URL/websites for soil-related databases:

- The database of the USDA-NRCS Soil Survey Laboratory (SSL), National Soil Survey Center, contains analytical data for more than 20,000 pedons of U.S. soils and about 1,100 pedons from other countries. Data consists of soils, soil survey investigations, soil characterisation data, soil laboratory data, soil research data, physical soil data, chemical soil data, mineralogical soil data, and pedon descriptions.

  The data are available on CD-ROM disk and on-line at

  http://www.statlab.iastate.edu/soils/ssl/natch_data.html.

- Food and Agriculture Organization (FAO)

  Various databases are available, however some of them are not freely available.

  1. Digital Soil Map of the World (SMW) Global Soil Profile Databases

  Contain map sheets of Africa, North America, Central America, Europe, Central and Northeast Asia, Far East, Southeast Asia, and Oceania and derived soil properties files.

  http://www.fao.org/landandwater/agll/dsmw.htm

2. "FAOSTAT".

   FAO's on-line, multi-lingual database containing more than 1 million time-series records covering international statistics in areas such as agricultural production and trade, producer prices, land use, irrigation, agricultural machinery, fertilisers and pesticides.

http://www.fao.org/WAICENT/FAOINFO/AGRICULT/guides/resource/data.htm

6.8 Models as tools in decision-making (Decision support systems (DSS))

Many problems faced in agriculture are multi-disciplinary in nature, this means it is necessary to consider, besides the soil-plant atmosphere continuum, also socio-economic and biophysical aspects. A system approach is therefore necessary as perturbation in one part often affects all other parts of the system.

Decision support systems are interactive computer-based systems that utilize data and models to solve multi disciplinary problems. The main aim is to improve performance of decision-makers while reducing the time and human resources required for analysing complex decisions. DSS should support all phases of a decision making process, characterised by: i) identifying possible

problems or opportunities, ii) creating and analysing possible courses of action, and iii) suggesting a course(s) of action from those analysed.

Various DSS have been developed, but two comprehensive DSS for soil/crop modelling are DSSAT version 3 (Decision Support Systems for Agro-technology Transfer, Tsuji *et al*., 1994; Jones *et al*., 1998) and APSIM (Agricultural Production Systems Simulator, McCown *et al*., 1996). These DSS allow 'experiments' to be created on computers, and outcomes of the complex interactions between various agricultural practices, soil, and weather conditions simulated, as well as appropriate solutions to site-specific problems. One good tip for a successful DSS (Matthews *et al*., 2000): Address real problems (often complex) not readily solved by rule-of-thumb (e.g. pest management and irrigation scheduling require decision making on issues that vary from one season to the next). The cost of making a mistake is high and therefore use of DSS maybe worthwhile.

Expert systems (ES) are another computer tool for decision-making, however, it uses qualitative rather than quantitative reasoning. The rules of thumb on which they are based are often formulated with the use of crop simulation models.

6.9 Good modelling practice

It is important that Good Modelling Practice (GMP) is exercised. GMP is defined as the development, maintenance, distribution and use of computer simulation models whereby the integrity of the model, its various improvements and utilization is assured (Estes & Coody, 1993). It is also important that enough information is documented so that it is possible for an independent user to reproduce the model results. A good model must possess the following characteristics:

- Must be easy to use and output oriented
- Must be targeted at the client.
- Must not require an experienced computer programmer to operate, or must be part of a system where the operator works as a consultant passing on the relevant outputs in a useable manner.
- Must be introduced to the client with a thorough training package and continued support.
- Need to be maintained and updated with changing technology and in response to user demand.

6.10 Internet discussion groups involving in modelling agricultural systems

- AGMODELS listserver (AGMODELS-L@UNI.EDU)
- DSSAT listserver (DSSAT@LISTSERV.UGA.EDU)
- ESA-AGMODELS listserver (ESA-AGMODELS@ESA.UDL.ES) and
- FAO-AGROMET listserver (AGROMET-L@MAILSERV.FAO.ORG).

6.11 References and further reading

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1.3	LM	4.1.6	MH, RH
1.4	GB	4.1.7	RH, MH
2.1	FZ	4.1.8	RH, AM, MH
2.2	FZ	4.1.9	RH, AM, MH
2.3	FZ	4.1.10	RH, AM, MH
2.4	GB	4.1.11	MH, RH
2.5	NB, RH	4.1.12	RH, MH
2.6	FZ	4.2.1	LL
2.7	GH	4.2.2	LL
2.8	RH	4.2.3	LL
2.9	RH	4.2.4	LL
2.10	RH	4.2.5	LL
2.11	LH	4.2.6	LL
2.12	MM, GB	4.2.7	LL
3.1	LL	4.2.8	LL
3.2	MA	4.2.9	MA, JF
3.3	LL	4.2.10	MA, JF
3.3	LL	4.2.11	MA, JF
3.4	LL	4.3	NB, RH
3.5	LL	4.4	MH,RH
3.6	GH	5.1	LL
3.7	GH	5.2	MA
4.1.1	MA, RH	5.3	MA
4.1.2	MH, RH	5.4	MA
4.1.3	MH, RH, RB	6	LH

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