Chapter 3 General Materials and Methods

3.1 Introduction

This chapter describes the materials and methods that were repeatedly used in experiments reported in the experimental chapters. Materials and methods that were used less frequently but require a detailed description are also included in this chapter. Specific materials and methods associated with a particular experiment are given in the chapter describing that experiment.

3.2 Experimental Procedures

3.2.1 Preparation of Cobalt-Ethylene Diamine Tetra Acetic Acid Complex

Cobalt as cobalt-ethylene diamine tetra acetic acid (Co-EDTA) complex was used as a marker to ascertain the kinetics of rumen fluid and the outflow of the liquid digesta from the rumen to the lower digestive tract. The Co-EDTA complex was prepared according to the method of Uden *et al.* (1980). Sodium-EDTA (37.2 g), $CoCl_2.6H_2O$ (23.8 g) and NaOH (5.1 g) were dissolved in 200 ml double distilled H_2O with a gentle heating and continuous stirring. After cooling, 20 ml 30% H_2O_2 was added to the solution that was then allowed to stand overnight at room temperature. To this solution, 300 ml 95 % (v/v) ethanol was added and the solution was filtered through Whatman paper No. 42. The residue was washed four times with a total

volume of 500 ml 80 % (v/v) ethanol and then dried at 80°C over night in a forced-air oven. The Co-EDTA.$3H_2O$ obtained was readily dissolved in H_2O and various solutions with appropriate Co concentrations were made for use as a liquid marker.

3.2.2 Measurement of Digesta Flow

The flow of digesta passing the abomasum was estimated using the dual marker technique (Faichney, 1975). The Co-EDTA complex and dietary AIA were used as non-absorbable liquid and particle digesta markers, respectively. A solution containing the Co-EDTA was infused continuously for at least 48 h into the rumen through polyethylene silastic infusion lines with an internal diameter of 1.5 mm. The rate of infusion was regulated by using a peristaltic pump and calibrated pump tubing. The actual rate of infusion was calculated from the rate of change in mass of a reservoir containing the Co-EDTA during the infusion.

Under steady state conditions, the rates of infusion Co and AIA entering the rumen should be equal to that exiting from the rumen and passing with the true digesta through the abomasum (Faichney, 1975): moreover, if the amounts of Co and AIA in true digesta are expressed as a fractions of the daily entry of these markers into the rumen, these fractions should be equal. Inequality indicates that the sample of digesta under investigation is not representative of true digesta. By determining the fractions of the daily entry of both markers in sub-samples of the digesta collected via the abomasal cannula (obtained by separation of the sample into fluid enriched and particle enriched components), true digesta was reconstituted by appropriately re-mixing the two components to contain equal fractions of both markers. An example of this calculation is given in Appendix 5.

3.2.3 Measurement of Rumen Fluid Kinetics

The kinetics of rumen fluid was estimated from the decline over time in the concentration of Co following an injection, or at the end of a continuous infusion of Co into the rumen. The natural log of Co concentration was regressed against time and the coefficient of correlation of the regression line (R^2) was at least 98 %. The

rate of dilution of Co concentration in the rumen fluid followed first order kinetics and was fitted well by the following equation:

$$C_t = C_o \, exp^{-kt}$$

where C_t and C_o are the concentrations of Co at time (t) and zero time, respectively, and k is the rate constant represented by the slope of the regression line.

3.2.4 Sampling of Rumen Fluid

In intact animals, samples of rumen fluid were collected using a stomach tube. In animals equipped with rumen cannulas, each sample was taken via a sampling probe inserted through the cannula bung. The tip of the sampling probe was covered with a nylon material to prevent coarse particles being sampled. To obtain representative samples, about 20 ml of rumen fluid was drawn into a 60 ml syringe, and then flushed back into the rumen 3 – 4 times before a final sample (about 25 ml) was drawn into the syringe and retained. The sample was acidified with 0.25 ml 18 **M** H_2SO_4 and stored at –18 °C until analysis. When the rumen fluid was to be used as a source of bacteria, the rumen fluid was collected into a container placed in ice and transferred to the laboratory for immediate processing to obtain a bacterial isolate (Section 3.2.7)

3.2.5 Sampling of Abomasal Digesta

Prior to collection, any abomasal digesta trapped in the abomasal cannula stem were scraped out and discarded. A John vial was placed underneath the opened cannula and digesta were allowed to flow into it. The collection was at times aided by slowly inserting a smooth plastic tube into the cannula and gently swirling the abomasal digesta. The digesta were stored in the collection container at –18 °C until analysis.

3.2.6 Collection of Rumen Fluid for *In vitro* Experiments

Rumen fluid used as inoculum for *in vitro* experiments was collected from rumen-cannulated steers given a grain-based diet. Details of the diet are given in section 4.2.2.1. The rumen contents were strained through two layers of fly screen and then two layers of muslin cloth. The strained rumen fluid was brought to the laboratory in a 5-litre Schott bottle placed in a thermos container filled with water at about 40°C.

3.2.7 Isolation of Bacteria

The sample containing bacteria (*e.g.* rumen fluid or rumen digesta) was first centrifuged (20,000 x ***g***, 15 min, 4°C; Beckman Model J2-21M) and the supernate was carefully transferred into a wide-neck McCartney bottle, preserved with 18 **M** H_2SO_4 and stored at −18°C until analysed. Bacteria were isolated from the residue by twice resuspending the residue in 20 ml physiological saline (9 g NaCl/l), re-centrifuging and removing the top (bacteria-rich) layers of the residue. The final bacterial isolate was suspended in about 3 ml saline solution and stored at -18°C for analysis of bacterial-N (Section 3.3.4.3) and ^{15}N enrichment (Section 3.3.5). Bacterial isolates were occasionally examined under a light microscope and found to be free of visible feed particles and protozoa.

3.2.8 Measurement of gas production

Gas production was measured in the *in vitro* experiments using a liquid displacement technique (Figure 2). A 1-litre incubation flask held in a water bath, set at 39°C during incubation, was connected by plastic tube to a 500-ml graduated cylinder positioned upside down in a square bucket containing water. The cylinder was completely filled with water at the start of incubation and the volume of gas produced during the incubation was read as the displacement of water in the cylinder. The cylinder was refilled with water when necessary and measurements were continued.

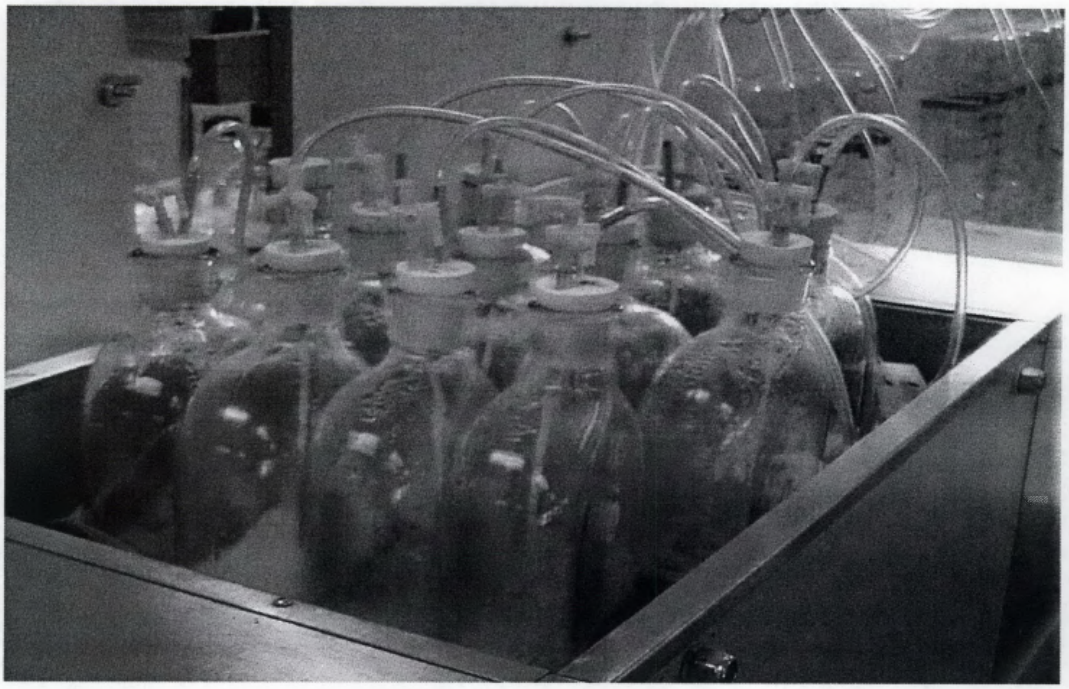

Figure 2 Apparatus used for *in vitro* incubation of duckweed with rumen fluid. Incubation bottles (volume 1 litre) were held in a shaking water bath and tubes passing through the bungs in each bottle were connected to 500 ml measuring cylinders to enable gas production to be determined by a liquid displacement technique.

3.3 Laboratory Analysis

3.3.1 Analysis of Cobalt

Cobalt-EDTA infusion solution and abomasum digesta were subjected to Co analysis following a perchloric acid ($HClO_4$) / hydrogen peroxide (H_2O_2) digestion. The Co concentration was analysed on an Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Varian, Model MPX).

3.3.1.1 Sample preparation and digestion

The abomasum digesta sample was separated into liquid-enriched and particle-enriched fractions by allowing the original digesta (as collected) to stand at room temperature. The more liquid fraction was poured into different container leaving the particle fraction in the original container.

Approximately 0.2 g of sample was weighed into a pre-tared Schott bottle (50 ml) to which 2 ml of freshly prepared 7:3 (v/v) mixture of $HClO_4$ (70 %) and H_2O_2 (30 %) was carefully added in a scrubbed fume cupboard. The bottle cap was put on loosely and the sample was left over night in the fume cupboard. One ml of H_2O_2 was added, the cap was tightened and the bottle was placed in an oven at 80°C for 30 min. The bottle was allowed to cool and a further 1 ml H_2O_2 was added and digested for 1 h at 80°C. The sample was again allowed to cool and diluted with freshly obtained milli-Q water. The dilution was done by adding about 25 g (weighed accurately) of water to the bottle. The solution was mixed thoroughly and allowed to stand at least for 2 h. The solution was filtered through No.1 Whatman paper into a glass vial with a positive snap-on cap and brought to the ICP-OES room 24 h before the analysis. The analysis was done on 3 spectral lines and the best line was selected on the basis of highest recovery of known standards.

To avoid any contamination between batches of samples, the Schott bottles, glass vials and funnels were soaked over night in a bath containing 10 % (v/v) HCl and then next morning rinsed 3 – 4 times with tap water, and finally with milli-Q water.

3.3.1.2 Standards

Stock standard solutions containing 1000 mg of Co in 0.5 mmol nitric acid were purchased. A series of working standards containing different amounts of Co were prepared from the diluted Co stock standards. Because the concentrations of Co in the samples were likely to be low, the stock Co standard was diluted in a 35:65 (v/v) mixture of $HClO_4$ (70 %) and H_2O_2 (30 %). The 35:65 (v/v) mixture of $HClO_4$ and H_2O_2 was chosen because this corresponded with ratio of $HClO_4$ and H_2O_2 remaining at the end of sample digestion.

A known quantity of the diluted stock Co standard solutions was included in the digestion process, with or without digesta sample added, so that quantitative recovery of Co from actual samples could be verified. Representative recovery data are presented in Appendix 4.

3.3.2 Determination of AIA

The AIA concentration in ground (1 mm sieve) feed samples and abomasum digesta was determined according to a method described by Choct and Annison, (1990) and Vogtmann *et al.* (1975). Approximately 2 g of feed or 1 g of digesta was weighed into a pre-tared filtered crucible (porosity 4) and dried in a forced-draught oven at 105°C for at least 8 h. The mass of DM remaining was recorded and the DM was then transferred to an oven and ashed at 480°C for at least 8 h. The sample was allowed to cool and transferred to an evaporating dish. Slowly, 4 **M** HCl was dispensed into the evaporating dish until the sample was wetted from underneath. The crucible was then filled to about 75% of its volume with the acid. The evaporating dish was placed on a hot plate and the acid was allowed to boil for about 15 min. After cooling, the crucible was taken out and the acid was removed by suction, rinsed with the same 4 **M** HCl and then with milli-Q water. The sample was dried in the oven at 105° for 2 h after which the ashing and removal of the acid soluble ash was repeated. The sample was finally dried in an oven at 105°C for 6 h and weighed after cooling in a desiccator.

3.3.3 Analysis of VFAs

Total VFA concentration and the molar proportions of acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acids were determined according to the method of Erwin *et al.* (1961) on samples of the rumen fluid supernatant using a gas liquid chromatograph (Model 427, Packard Instrument Co., USA). Iso-caproic acid was used as an internal standard. The gas chromatograph was connected to a recording data processor (Model 604, Packard Instrument Co., USA). Analysis of N

3.3.3.1 Total N

Total-N content of feed samples was determined on finely ground (1 mm sieve) materials either using an N analyser (Leco FP 2000) or by micro-Kjeldahl digestion followed by steam-distillation and titration of the resulting ammonia.

3.3.3.2 Ammonia N

The concentration of ammonia-N was determined on samples either colorimetrically (Bietz, 1974) on an auto analyser (Technicon Equipment Co., New Jersey, USA) or, as described in Section 3.3.4.1, by steam-distillation of ammonia under mildly alkaline conditions (pH 9) and titration. The latter method was applied in cases where the ^{15}N analysis of ammonia-N was required.

In the steam-distillation and titration method, a sub-sample of well-mixed N-containing materials, such as rumen fluid supernatant (3 ml) or rumen digesta (2 ml), was transferred to the base of a micro-Kjeldahl digestion flask that was designed to be attached (by a ground glass joint) to a distillation apparatus. To this was added 2 drops of silicone anti-foaming solution and 4 drops of universal indicator to provide a visual indication of the pH. Any solution in the stem of the flask wall was washed down with a minimum volume of H_2O. The sample was made to pH 9 with 4 ml of saturated Na-tetraborate and then immediately steam-distilled for 3.5 to 4 min: the distillate (containing the liberated ammonia-N) was collected into a beaker containing 3 ml 0.025 **M** (standardised) H_2SO_4. The distillate was continuously stirred and titrated to pH 5.0 with standardised 0.025 **M** NaOH (Autoburett ABU80, Radiometer, Copenhagen). The titration value for a relevant blank sample (H_2O) was always subtracted from that of the sample. Recovery of ammonia N was checked by distilling a standard $(NH_4)_2SO_4$ solution. The distillation apparatus was cleaned between successive distillations by distilling ethanol (15-20 ml) for 5 min. This was done to displace any ammonia adsorbed onto glass surfaces in the glass distillation apparatus thereby minimising ^{15}N cross-contamination between samples.

3.3.3.3 Bacterial-N

The micro-Kjeldahl technique was employed to digest bacterial samples. A representative sample (0.5 ml) of bacterial isolate (Section 3.2.1) was transferred to a digestion flask. A tablet of low selenium catalyst was added followed by 3.5 ml of 18 **M** H_2SO_4. The contents were mixed and digested at 450°C for approximately 3 h, or until the solution was clear. After cooling, approximately 10 ml of H_2O was carefully

added to increase the volume and the sample was sealed, left overnight and ammonia was distilled from the contents the following day.

Before the contents were distilled, anti-foaming solution and universal indicator were added. The contents were then made alkaline by slowly adding approximately 10 ml of 40 % (w/v) NaOH and steam-distilled as for ammonia-N. The bacterial-N, now converted to $(NH_4)_2SO_4$ was processed and retained for ^{15}N analysis as described in Section 3.3.5.

3.3.3.4 Non-ammonia N

The NAN was determined in samples from which ammonia-N had first been removed by steam distillation. The digestion, distillation and titration processes for NAN, and its subsequent analysis for ^{15}N, were the same as for bacterial-N.

3.3.4 Analysis of ^{15}N

3.3.4.1 Preparation of N samples

Following the steam-distillation and titration procedures, the N-containing samples were retained and re-acidified with 1 ml of 0.025 **M** H_2SO_4 and dried in an oven at 95°C to a minimum volume. The content of the distillation beaker was transferred to a 5 ml plastic vial and dried again at the same temperature. The dried $(NH_4)_2SO_4$ was re-dissolved in 0.1 ml of H_2O and an appropriate volume of the resulting solution, calculated to contain 80-100 µg N, was carefully transferred into a 8 x 5 mm tin capsule (Elemental Microanalysis Ltd., Cat. No. D1008). The common volume of solution transferred to this tin size was less 30 μl. For volumes larger than this, it was found to be more convenient to use a larger tin capsule (8 mm x 12 mm).

The open capsule was placed in a plastic or aluminium block with a well that was designed to hold the capsule in an upright position while solution was dispensed into it. After samples were added to a number of capsules, the contents of the capsules were re-dried overnight by placing them in a rack in a vacuum desiccator. Care was taken in the initial stages of evacuation of the desiccator to lower the pressure slowly

thereby minimising the rate of out-gassing, bubbling and potential splattering of the sample solutions in the capsules. When the contents were completely dry, each tin capsule was closed using tweezers and then folded and squashed to make it small enough for loading into a sample carousel on an automatic N/carbon/sulphur analyser (Carlo Erber Instruments; Model NA1500).

3.3.4.2 Oxidation of samples and estimation of ^{15}N abundance

The samples contained in the squashed tin capsules were dropped automatically, by the N analyser, into a furnace filled with pure O_2 at 1030°C where the ammonium salt was oxidised by flash combustion to N_2 gas. The resulting N_2 gas was then dried and passed into the analyser tube of an isotope-ratio mass spectrometer (Tracermass; Europa Scientific) and its ^{15}N abundance was determined.

The samples were loaded in a sequence that commenced with 3 empty (blank) capsules, followed by 2 'conditioning' samples (approximately 500 μg $(NH_4)_2SO_4$ laboratory grade) followed by the first reference standard (natural abundance). The same reference standard was placed after the last sample of each analytical run. Any 'drift' in the estimates of sample abundance resulting from changes in the analytical conditions within the mass spectrometer during that analytical run could then be detected, and allowed for, by assuming the drift was linear over time.

3.3.4.3 Calculation of ^{15}N abundance and enrichment

Abundance of samples was calculated from the ion currents in the mass spectrometer derived from the ion collectors for mass/charge (m/e) 28 and 29. These currents reflected the relative proportions of the singly-charged molecular species of N_2 gas, *i.e.* $^{14}N^{14}N$ and $^{14}N^{15}N$, respectively. The proportion of the molecular species $^{15}N^{15}N$, which was not determined in this isotope-ratio instrument, was determined by assuming the proportions of the three possible types of diatomic molecules were binomially distributed (Nolan, 1971).

Abundance (A, %) was calculated as:

$$A = \frac{Number\ of\ atoms\ of\ ^{15}N}{Number\ of\ atoms\ of\ (^{15}N + ^{14}N)}\ x\ 100$$

The enrichment (E, %) of any sample was calculated from the determined A of that sample minus the A for a corresponding sample collected before ^{15}N-labelled material was made available to the system.

3.3.4.4 Checks of the precision and accuracy of the ^{15}N analysis methods

A series of standards with a range of known ^{15}N abundances was initially analysed to check the linearity of the ^{15}N analysis. The precision and accuracy of the ^{15}N analysis were determined by including a ^{15}N standard obtained from the International Atomic Energy Agency (IAEA), Vienna, in each run. Also, in each run, two standards with different ^{15}N abundance were included to enable the repeatability of the analysis to be determined. Details and results of these quality assurance procedures are presented in Appendix 3.

3.3.5 Analysis of Wool

3.3.5.1 Rate of fibre elongation

A dye-banding technique (Chapman and Wheeler, 1963) was used to determine the growth of wool staple during a particular period of time. The distance on the staple from the base of the first dye-band (inserted at the start of a period) to the base of the second band (inserted at the end of that period), divided by the number of days, gave the daily rate of fibre elongation. For example, when the first and the second dye band were inserted at the start and the end of a pre-experimental period, the distance from the base of the first dye-band to the base of the second band indicated the length of the wool staple grown during that pre-experimental period. Measurements of the mean inter-band distance were made using 5 randomly chosen wool staples from each sheep.

3.3.5.2 Diameter of wool fibre

For measurement of fibre diameter of the wool grown during a particular period of time, the staples were guillotined at the base of the last inserted dye-band for that period to give a 2 mm sample immediately above the cut. These samples were washed twice with *n*-hexane, after which the hexane was removed by double washing with hot water (60°C). The sample was dried in a forced-draught oven at 75°C for 1.5 h, and left overnight in the wool laboratory. The sample was then mini-cored to provide 2,000 snippets of fibre, which were subjected to optical fibre diameter analysis (IWTO, 1995).

3.3.5.3 Wool volume

Wool volume was calculated from the data of rates of wool fibre elongation and fibre diameter as:

$$\text{Fibre volume} = \pi r^2 \times E$$

where r is the radius of the wool fibre (half of the wool fibre diameter) and E is the rate of fibre elongation.

Chapter 4 Degradation and Fermentation of Duckweed in the Rumen

4.1 General

Feeds ingested by ruminants undergo extensive microbial degradation and fermentation in the rumen. Carbohydrates and proteins are broken down to simpler monomers which are then either used by the microbes as carbon and N sources for cell synthesis or fermented to obtain the energy required for cell integrity and synthesis. Fermentation results in the formation of VFA (mainly acetate, propionate and butyrate) and gas (mainly carbon dioxide and methane). The VFA are absorbed from the rumen and become the major energy source for tissue metabolism while the gases are mainly released through eructation. Ammonia is also released when protein is fermented and it disappears from the rumen either through microbial assimilation for the synthesis of cell protein, absorption across the rumen wall or passage to the lower digestive tract.

The nutritional value of a protein supplement for ruminant animals is to a large extent determined by its degradability in the rumen. Supplements containing proteins that are rapidly and highly degradable in the rumen will provide nitrogenous substances that are potentially used and converted into microbial protein which is the major source of amino acids for the host under most feeding systems (see Dewhurst *et al.* 2000). For such supplements, there is a need to optimise microbial growth and utilisation of the products resulting from degradation of dietary protein, thereby preventing an excessive loss and inefficient use of dietary proteins.

Knowledge on the rumen degradability of a protein dictates whether or it is necessary to provide additional N from other sources for both the rumen microbes and the host. For a supplement with high rumen degradability, an additional amount of 'escape' protein may be required to complement the flow of microbial protein that can be insufficient for high-producing animals (Orskov, 1970). On the other hand, a protein supplement that is too resistant to rumen degradation may limit the availability of degradable N required for an optimum synthesis of microbial protein in the rumen.

Even though duckweed contain a relatively high concentration of N and is therefore a potential source of dietary protein for ruminants, as discussed in Section 2.3.4, little is known about the degradability of its protein in the rumen and the subsequent microbial utilisation of its degradation products or about the extent to which its protein may escape fermentation in the rumen. The experiments currently reported were undertaken to investigate the rumen degradability of duckweed using both *in sacco* and *in vitro* techniques.

4.2 Experiment 1: Degradability of Duckweed in the Rumen Determined with the *In Sacco* Technique

4.2.1 Introduction

A study to estimate the rumen degradability of duckweed was undertaken by Huque *et al.* (1996) using the *in sacco* technique. Sun-dried duckweed of three different genera (*Spirodella*, *Lemna* and *Wolffia*) was incubated in the rumen of bulls fed a diet of straw and green grasses supplemented with a concentrate mix. These workers found that the rates of the DM degradation (%/h) for the three duckweed genera were 2.2, 3.6 and 5.7, respectively, while the corresponding rates for CP degradation were 5.1, 4.2, and 6.0. The effective degradabilities for DM (%) were 85.3, 72.3 and for CP (% DM) were 80, 87 and 94, respectively. In similar study (Khan *et al.*, 2002), it was found that the protein of dried *Lemna* species (*L. trisulaca and L. perpusila*) was approximately 65 % degraded after incubation in the rumen for 48 h.

Apart from these two studies, there are no other published investigations of the rumen degradability of duckweed. The approach taken in the study now reported was

to expand the studies of Huque *et al.* (1996) and Khan *et al.* (2002). The degradability of fresh as well as dried duckweed was investigated using the *in sacco* technique. Cottonseed meal, which is considered to be an effective source of 'escape' protein (Leng *et al.*, 1983) was included in the current experiments as a reference feed.

4.2.2 Materials and methods

4.2.2.1 Animals and their diets

Two Hereford steers, each equipped with a rumen cannula, were used in these experiments. The animals were kept in individual cages indoors at the University of New England. The steers had a continuous access to drinking water and received a basal diet of oaten chaff (2 kg) and a grain-based supplement (3 kg) that was given once a day at about 10.00 h. The supplement consisted of (g/kg) 174 each of cracked barley, wheat, sorghum, oat, and maize, 87 oaten chaff, 17 molasses, 14 slaked lime, 8.0 urea and 1.0 mineral mix.

4.2.2.2 Bags and procedure of incubation

Bags used were made of a monofilament polyester material with a size of 7 m x 4 cm and a pore size of 44 µm x 44 µm (Swiss Screen, Seven Hills, NSW 2147). The tested feeds were first weighed into a tared John's vial, poured into pre-weighed bags that were then reweighed and tied up with a monofilament fishing line (20 lb / 9 kg). Bags with the same period of incubation were enclosed in a mesh bag, which was then tied up. A metal chain was attached to each of the mesh bags to ensure that they remained in the liquid phase of the rumen contents during incubation and to facilitate their removal from the rumen when the desired incubation periods had been reached.

Preceding the incubation, the mesh bags containing the polyester bags (and the tested feed) were first hydrated by soaking them in warm water (39°C) for 15 min. Each set of mesh bags was then introduced into the rumen of each animal in reverse order, *i.e.* the bags collected last were introduced first, and all sets were collected from the rumen at the same time. At least 6 incubation periods were obtained within 36 h.

Immediately after withdrawing the mesh bags from the rumen, they were transferred into a bucket containing cold water. The metal chain was detached, and the polyester bags containing feed residues were removed from the mesh bag, cleaned of coarse feed particles and transferred to another bucket containing clean cold water. A cleaning under running tap water was done to ensure a complete removal of the coarse particles trapped around the mouth of the bag. The bags were gently squeezed and washed with cold water using an automatic washing machine with a washing time of about 40 min. The washed bags were gently squeezed, placed in an aluminium tray and dried in a forced-draught oven at 80°C for 48 h during which time each bag was opened. The dried bags were cooled in a desiccator and weighed (for DM calculation) and the residues were retained for N analysis.

Bags used for zero time loss were not incubated in the rumen but otherwise were treated exactly the same as the bags placed in the rumen.

4.2.2.3 Laboratory analysis

DM content of feeds and their residues in the bags after rumen incubation was determined by drying them in a forced-draught oven at 80°C for 24 h. Their total-N content was determined by steam distillation of ammonia after a micro-Kjeldahl digestion.

4.2.2.4 Calculation and statistical analysis

Rumen degradability of feed DM or N was expressed as the fraction (%) of each that disappeared from the bag during incubation. Data describing DM loss were fitted using the 'Neway' Program, version 5 for MicroSoft Excel (Chen, 1997) using

the equation of Orskov and McDonald (1979), *i.e.*

$$P_t = a + b(1 - \exp^{-ct})$$

where P_t is the degradation at time t, a is the zero time intercept, b is the asymptote of the curve and c is the degradation rate constant. The water insoluble percentage was calculated as the sum of a and b less the percentage that disappeared during washing.

The effective degradability at different rumen outflow rates (k, %/h) was calculated as follows (Orskov and M^{c}Donald, 1979):

$$a+b[c/(c+k)]$$

4.2.3 Experimental procedures

4.2.3.1 Experiment 1.1: The effects of DM quantity of fresh duckweed on its degradability in the rumen

The objective of this experiment was to determine the optimum quantity of fresh duckweed that should be placed in a polyester bag for the determination of its rumen degradability. With the *in sacco* method, the amounts of DM of dried feeds used are usually 3–5 g per bag which allows the feed can move freely during the incubation and the rumen microbes can enter the bag without restriction to invade and degrade the feeds and exit the bag through its pores. These quantities, however, may not be applicable for high-moisture feeds such as fresh duckweed that has a typical DM content of less than 10 %. For such feeds, lower quantities of DM per bag may be required.

Fresh duckweed (*Spirodella punctata*) was originally obtained from Scone, NSW and was maintained at the University of New England in a galvanized-iron tank (diameter 175 cm) containing approximately 800 l of nutrient solution. The duckweed required for incubation was harvested with a strainer, wrapped with a nylon cloth and gently hand-squeezed to remove most of its water content. Different quantities of duckweed, fresh and not milled, were enclosed in each bag for incubation in the rumen for 3, 6, 9, 12, 24 and 36 h. The amount of DM enclosed in each of duplicated bags was 1.0, 1.7 or 3.3 g. Cottonseed meal, also not milled, was included (4.8 g DM per bag) as the reference feed.

4.2.3.2 Experiment 1.2: The effects of drying on the degradability of duckweed DM and N in the rumen

Once the optimum quantity of fresh duckweed to be enclosed in a bag had been determined, this experiment was carried out to compare the rumen degradability of

fresh and dried duckweed. Fresh duckweed (*Spirodella punctata*), obtained from the BioTech Waste Management, Armidale, was harvested and squeezed with a nylon cloth to increase its DM content to approximately 12 %. The N content was 2.43 g N/100 g DM. Some of the fresh duckweed was oven dried at 80°C for 24 h, and some was kept fresh in a cool room (-4°C). A quantity of dried and fresh duckweed (not milled) corresponding to approximately 2 g DM for dried duckweed and 1 g for fresh duckweed was enclosed in each of duplicated bags and incubated in the rumen. Cottonseed meal (2 g DM per bag) was also included as the reference feed. The incubation was conducted for periods of 3, 6, 10, 16, 24 and 36 h.

4.2.4 Results

4.2.4.1 Experiment 1.1: The effects of quantity of fresh duckweed on its degradability in the rumen

Degradability estimates were not repeatable when the DM quantity of fresh duckweed placed in individual bag was 3.3 g. These data, therefore, were omitted and not included in the data handling and analysis.

The DM degradability of fresh duckweed for the two smaller amounts of DM placement (1.0 and 1.7 g per bag), as well as that of cottonseed meal (4.8 g DM per bag), was consistent with the length of incubation (Figure 3). The data were fitted to the exponential equation of Orskov and McDonald (1979) and the fitted degradation parameters obtained are given in Table 3.

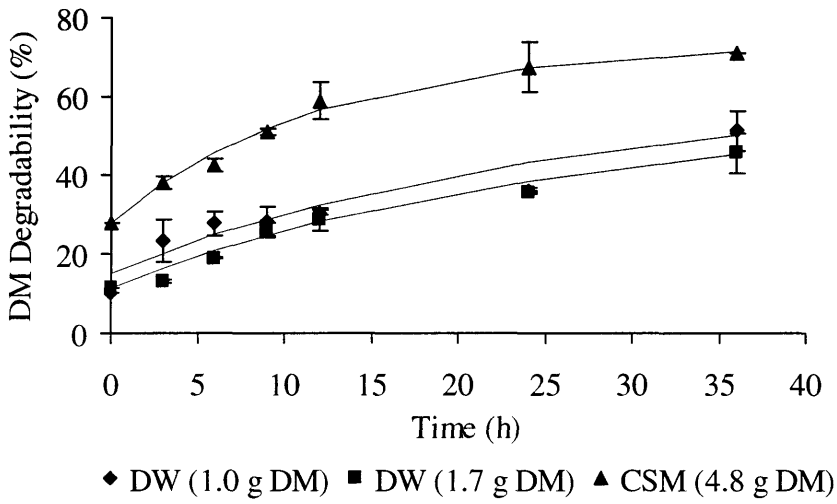

Figure 3. *In sacco* DM degradability (%) of fresh duckweed (determined with quantities of 1.0 and 1.7 g per bag) and cottonseed meal (4.8 g DM per bag) were incubated for different periods in the rumen (mean ± SD, n = 2). The lines represent the fitted curves (Orskov and McDonald, 1979).

Table 3. *In sacco* degradation parameters for DM of fresh duckweed and cottonseed meal and their effective degradabilities at assumed rumen outflow rates of 2, 5 and 8 %/h.

Items	Duckweed (1.0 g DM)	Duckweed (1.7 g DM)	Cottonseed meal (4.8 g DM)
Degradation parameters			
Washing loss (%)	10.4	11.5	27.7
Water insoluble but fermentable fraction (%)	52.3	45.1	46.2
Rate constant (fraction/h)	0.033	0.038	0.082
Effective degradability (%) at outflow rate (%/h) of			
2	46.1	41.0	64.8
5	35.5	31.0	56.3
8	20.3	26.0	50.9

The degradability for fresh duckweed was lower than for cottonseed meal during the incubation. The amounts of DM enclosed in the bag (1.0 or 1.7 g) appeared to have little effect on the observed degradability of fresh duckweed. The fraction of

DM that disappeared during washing (the water soluble fraction) for the fresh duckweed was similar for both levels ($P>0.05$; 10.4 and 11.5 % for the 1.0 and 1.7 g DM, respectively) and these percentages were lower ($P<0.01$) than those for cottonseed meal. The intercept for the curve of best fit for bags with 1.0 g DM duckweed increased to about 15.1 % due to variation in the observed data points.

4.2.4.2 Experiment 1.2: Rumen DM and N degradability of dried and fresh duckweed

The respective DM and N degradability of dried and fresh duckweed and cottonseed meal are presented in Figures 4 and 5, and the degradation parameters obtained after fitting the data into the equation of Orskov and McDonald (1979) are presented in Table 4.

The DM and N fractions removed by washing were lower ($P<0.01$) for fresh duckweed, than for dried duckweed or cottonseed meal for which the disappearance due to washing did not differ. The degradability during incubation in the rumen was higher ($P<0.01$) for cottonseed meal than for dried or fresh duckweed. At 24 h of incubation, the observed DM degradability (means ± SD, %) were 31.7 ± 0.68 for dried duckweed, 30.4 ± 2.15 for fresh duckweed and 66.0 ± 1.51 for cottonseed meal, while the corresponding values for N were 44.8 ± 0.88, 44.3 ± 1.58 and 74.7 ± 1.03.

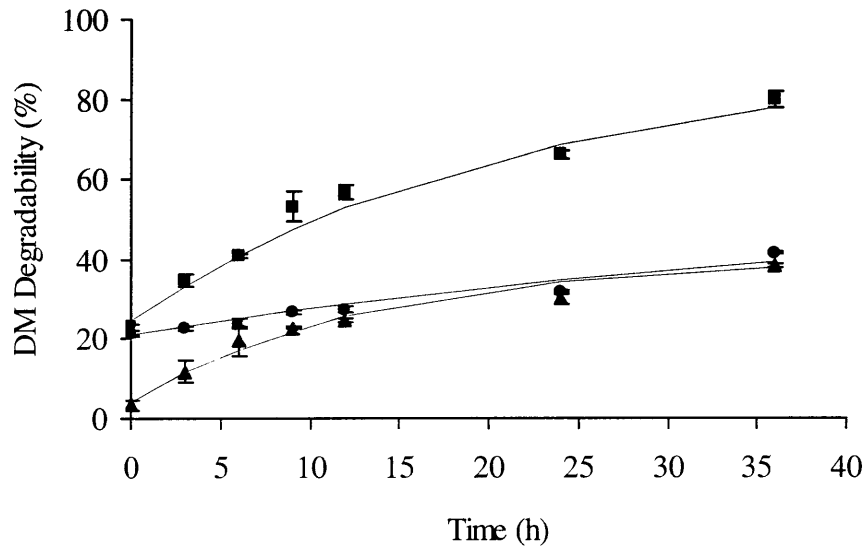

Figure 4. *In sacco* DM degradability (%) for cottonseed meal (■), dried (●) and fresh (▲) duckweed when incubated for different periods in the rumen (mean ± SD, n = 2). The lines represent the fitted curves (Orskov and McDonald, 1979).

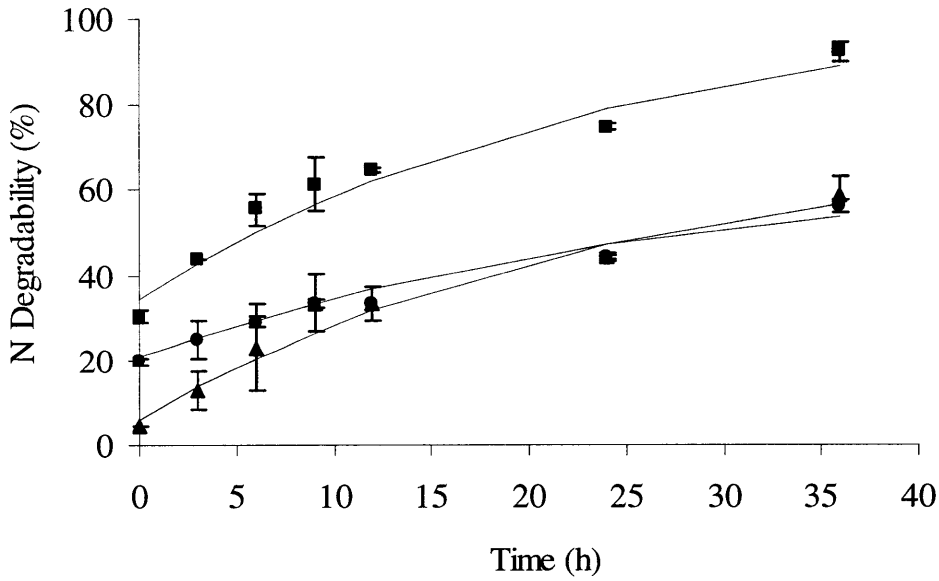

Figure 5. *In sacco* N degradability (%) for cottonseed meal (■), dried (●) and fresh (▲) duckweed when incubated for different periods in the rumen (mean ± SD, n = 2). The lines represent the fitted curves (Orskov and McDonald, 1979).

Table 4. Degradation parameters of DM and N for dried and fresh duckweed and cottonseed meal and their effective degradabilities at assumed rumen outflow rates of 2, 5 and 8 %/h.

Items	Fresh Duckweed		Dried Duckweed		Cottonseed Meal	
	DM	N	DM	N	DM	N
Degradation parameters						
Washing loss (%)	3.30	4.40	21.0	19.7	23.0	30.3
Water insoluble but fermentable fraction (%)	37.0	66.9	32.7	47.3	66.5	74.2
Rate constant (fraction/h)	0.075	0.041	0.023	0.035	0.048	0.042
Effective degradability (%) at outflow rate (%/h) of						
2	32.7	50.1	38.5	50.2	70.4	82.0
5	25.8	35.7	31.4	39.8	56.4	66.5
8	21.6	28.4	28.4	34.8	49.0	58.6

4.3 Experiment 2: Determination of N Solubility

Soluble proteins tend to be rapidly or completely degraded in the rumen (Annison, 1956; Henderickx and Martin, 1963) although there are exceptions to this. The proportion of soluble protein in a feed may therefore be used to obtain preliminary information about the degradability of different feeds in the rumen. Determination of protein solubility is simple and readily performed and it is therefore a fast and convenient means for comparing the potential rumen degradability of feeds.

4.3.1 Experimental procedure

The solubility of N in McDougall's buffer solution (Table 5) was determined for of three dried duckweed samples (*Spirodella punctata*; 80°C, 24 h) as well as fresh duckweed and cottonseed meal. The dried duckweeds (Duckweeds 1, 2 and 3) were obtained from different sources with different growth conditions and they had different concentrations of N.

The dried duckweeds and the reference feed (cottonseed meal) were finely ground to pass a 1 mm sieve and an appropriate quantity of each was weighed into duplicate 250 ml conical flasks. The amount of N from the dried feeds added into each flask was between 9.3 and 12.4 mg for duckweed and cottonseed meal, respectively. Fifty ml of the buffer solution was then added to each flask. For the fresh duckweed (DM = 11.7 %), about 5.5 g was first macerated for 10 min with 100 ml of the buffer solution and the resulting fresh duckweed–buffer mixture was equally divided into 2 portions; each portion was then weighed into one of two (duplicate) pre-tared flasks. The flasks containing feed samples were transferred into a temperature-controlled water bath (39°C) and incubated for 1 h during which the flasks were continuously shaken. At the end of incubation, the filtrate containing the soluble N was obtained by filtering the flask contents through Whatman filter paper No. 42 and stored at –18°C to await total-N analysis (Section 3.3.2).

Table 5. Chemical composition (g/l) of McDougall's buffer solution

Chemical	Concentration (g/l)
$NaHCO_3$	9.76
Na_2HPO_4	2.8
NaCl	0.47
KCl	0.57
$CaCl_2$ anhydrous	0.04
$MgCl_2.6H_2O$	0.13

4.3.2 Calculation and statistical analysis

The total amounts of N recovered in the buffer solution divided by the amounts of feed N added indicated the percentage of total N that was soluble. An analysis of variance was applied to test between-feed differences in N solubility.

4.3.3 Results

About half of the N in cottonseed meal was soluble in buffer (Table 6). For dried duckweeds, the N solubility was variable but was not related to the N content of

the duckweed. The N solubility for dried duckweed was found to be either similar (Duckweed 2), higher (Duckweed 1) or lower (Duckweed 3) than that for cottonseed meal. Oven drying at 80°C for 24 h reduced ($P<0.01$) the solubility of duckweed N (*i.e.* Duckweed 3 versus fresh duckweed).

Table 6 N contents (% air-dry) and solubility (%, mean ± SD) in McDougal's buffer solution of dried duckweed, fresh duckweed and cottonseed meal

Feed	N content (% air-dry)	N solubility (% ± SEM)**
Duckweed 1	3.61	60.3 ± 1.81^{a}
Duckweed 2	4.80	51.2 ± 0.88^{b}
Duckweed 3	2.97	23.4 ± 2.26^{c}
Fresh duckweed	2.97*	31.7 ± 1.89^{d}
Cottonseed meal	6.34	50.7 ± 0.90^{b}

*% DM
**Different subscripts denote significant difference ($P<0.05$)

4.4 Experiment 3: *In Vitro* Degradation and Fermentation of Duckweed in the Rumen Fluid

4.4.1 Experiment 3.1: Ammonia and Gas Production from Duckweed and Cottonseed Meal when Incubated with Rumen Fluid

4.4.1.1 Introduction

Ammonia is an end-product of protein degradation in the rumen. Determination of ammonia production when feeds are incubated with rumen fluid is therefore also a common approach that has been used to predict the rumen degradability of protein and the potential loss of the protein as ammonia from the rumen. Gases, methane and CO_2, are produced in the rumen as a result of microbial fermentation of dietary OM. The quantity of gas produced when feeds are incubated with rumen fluid (Menke and Steingass, 1988) has also been used as an alternative method for estimating the degradability of feed in the rumen.

4.4.1.2 Experimental procedures

This experiment was carried out in triplicate using thin-shaped incubation bottles with a nominal volume of 1 litre. The rubber stopper of each bottle was equipped with a bunsen valve which was connected through a silastic tube to an apparatus for reading gas production during incubation (Section 3.2.8).

Approximately 5 g each of finely ground (1 mm sieve) dried (80°C, 24 h) duckweed (*Spirodella punctata*; 3.83 % N air-dry) and cottonseed meal (6.46 % N air-dry) were transferred into one of the triplicate incubation bottles. For fresh duckweed (*Spirodella punctata*; 9.88 g N/100g DM, N = 5.26 g N/100g DM), about 215 g fresh material were weighed into a 500 ml beaker and macerated for 30 min with 400 g milli-Q water using a blender set at low speed. The resulting fresh duckweed–water mix was evenly divided into three portions: each portion was then weighed into one of the triplicate incubation bottles. The original proportion of duckweed and water before maceration was used to calculate the amount of duckweed and water in each bottle.

Strained rumen fluid (500 ml) was transferred into each of the incubation bottles containing the feed samples as well as the 'blank' flasks (containing rumen fluid only). The rumen fluid was obtained from two steers, about 1 h after they were offered their diet, by a procedure described in Section 3.2.6.

The incubation was run for about 3 h during which gas production was continuously monitored and 5 samples of contents were taken from the incubation flasks. When sampling, 15 ml of sample was taken (after, for 4 – 5 times, sucking the bottle contents into the sampling syringe and flushing them back into the bottle for the purpose of mixing). The sample was immediately centrifuged (20,000 x ***g***, 15 min, 4°C) to obtain the supernatant fraction that was acidified with 0.25 ml 18 **M** H_2SO_4 and stored at –18°C until analysed for ammonia-N (section 3.3.2.2).

4.4.1.3 Calculation and statistical analysis

Ammonia production (mg ammonia-N/g feed N) was calculated as the difference in the ammonia concentration for flasks containing the test feed and that for the blank. Ammonia production data were analysed by analysis of variance.

4.4.1.4 Results

Production of ammonia (mg N/g feed N) during the incubation is presented in Figure 6. Recovery of N was relatively low from all three materials but less ammonia-N was produced from cottonseed meal than from dried or fresh duckweed. Only about 4.84 % of the cottonseed N was recovered as ammonia-N at the end of incubation. The corresponding values for dried and fresh duckweed N were 11.9 and 7.57 %, respectively.

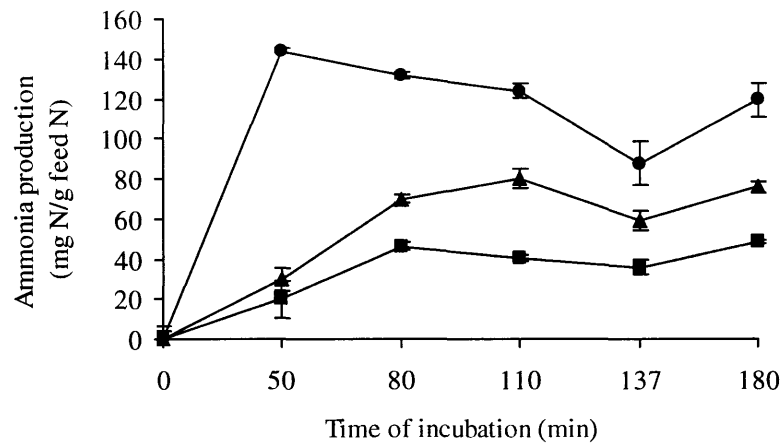

Figure 6. Net ammonia production (mg N/g feed N) for cottonseed meal (■), dried (●) and fresh (▲) duckweed at different times (min) during incubation with strained rumen fluid.

Cumulative gas production (ml/g feed DM) is presented in Figure 7. The gas production for cottonseed meal at the end of incubation was about 21.0 ml/g DM which was higher ($P<0.01$) than for dried or fresh duckweed.

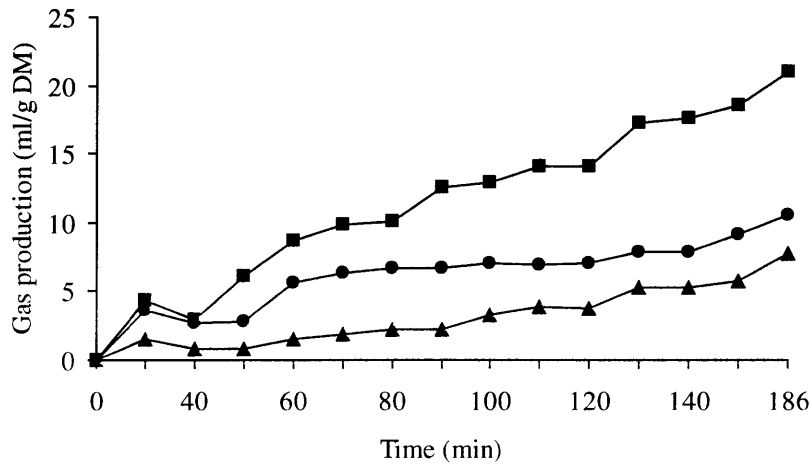

Figure .7 Cumulative gas production (ml/g feed DM) for cottonseed meal (■), dried (●) and fresh (▲) duckweed at different times (min) during incubation with strained rumen fluid.

4.4.2 Experiment 3.2: N Degradation from Duckweed and Its Utilisation for Microbial Protein Synthesis

4.4.2.1 Introduction

Results of Experiment 2.2 showed that duckweed, whether dried or fresh, produced less gas than cottonseed meal during the 3-hour incubation. On the other hand, the apparent ammonia production was higher for dried or fresh duckweed than for cottonseed meal, suggesting that the degradation of N in duckweed and its conversion to ammonia was higher for duckweed than for cottonseed meal. It is known, however, that degradation of feed-N and utilisation of the degradation products for microbial-N synthesis occur simultaneously (Broderick, 1978). Therefore, prediction of feed-N degradability based on ammonia production alone will not necessarily provide an unambiguous indication of feed protein degradability.

Several techniques have been developed to address the problem posed by microbial utilisation of the released feed-N when determining the degradability of feed N *in vitro*. Broderick (1987) added hydrazine sulphate and chloramphenicol into the incubation medium to inhibit microbial growth and therefore microbial uptake of the released N. Raab *et al.* (1983) developed a technique based on the relationship between

fermentation of carbohydrates and microbial-N synthesis that enabled the degradation of feed-N to be predicted on the basis of production of gas and ammonia. An energy source was added in graded amounts and it was suggested that back-extrapolation to zero added energy gave an estimate of the net production of ammonia-N.

A more direct *in vitro* technique (*e.g.* Hristov and Broderick, 1994) is the addition of ^{15}N-ammonia into the incubation medium to trace the ammonia-N utilisation by microbes. During the incubation, microbes will assimilate the ^{15}N label and therefore become labelled with ^{15}N. The ^{15}N enrichment ratio between microbes and ammonia indicates the proportion of microbial-N synthesised from ammonia-N.

To extend the results of Experiment 2.2, the next experiment was carried out to study the degradability of duckweed protein and the utilisation of the degraded nitrogenous materials for bacterial-N synthesis, using a ^{15}N dilution technique. Based on the results of Experiment 3.1, it was hypothesised that microbial-N synthesis would be lower with duckweed than with cottonseed meal, because gas production was lower (i.e. less ATP) and net ammonia production was higher for duckweed than for cottonseed meal.

4.4.2.2 Experimental procedures

Approximately 1.5 g each of finely ground (1 mm sieve) dried (80°C, 24 h) duckweed (*Spirodella punctata*, 3.62 g N/100 g DM) or cottonseed meal (7.20 g N/100 g DM) was weighed into duplicate conical flasks (250 ml) that were used as incubation vessels. For the fresh duckweed, about 20 g fresh material (DM = 9.12 g N/100 g DM, 4.73 g N/100 g DM) was transferred into a 200 ml beaker containing 100 g double distilled H_2O and macerated for 30 min. The resulting fresh duckweed–water mix was then evenly divided into 2 portions and each portion was transferred into one of the duplicate flasks. The flasks were then placed in a water bath at 39°C.

Strained rumen fluid (150 ml) was obtained from a cannulated steer 1 h before feeding (Section 3.2.6) and added to each flask. One ml ^{15}N solution, made by dissolving 0.182 g $(^{15}NH_4)_2SO_4$ (5.39 atom % ^{15}N abundance, Amersham International, U.K) in 9.010 ml of deionised H_2O, was also added. The ^{15}N solution contained 13.3

μmol of ^{15}N. Two flasks containing no added feed were used as blanks, but only one of them accompanied the feed containing flasks during the incubation. The other blank was stopped at 0 h and mixed and sampled immediately after the ^{15}N solution was added.

Incubation was allowed to proceed for 3 h during which time the flasks were automatically shaken. A manual swirling was applied to the flasks every 20 min for the first 1 h and then every 30 min thereafter. At the end, the incubation was stopped and samples for analysis of ammonia, NAN and bacterial-N in the flask contents were immediately taken and processed. To mix the flask contents well before sampling, the contents were sucked into the sampling syringe and flushed back into the flask four times before a final sample was taken. The sample was centrifuged (20,000 x ***g***, 15 min, 4°C) and the supernatant fraction was acidified and stored to await analysis for VFA (Section 3.3.3). A bacterial isolate was obtained from the residues (Section 3.2.7) and stored frozen at -18°C until analysed for N content (Section 3.3.4.3). A sub-sample of flask contents (16 ml) was also obtained and acidified with 0.3 ml of 18 **M** H_2SO_4 and stored frozen until analysed for ammonia-N (Section 3.3.4.2) and NAN (Section 3.3.4.4). Each N containing sample was analysed for ^{15}N enrichment (Section 3.3.5).

The transactions of N during incubation are illustrated in Figure 8.

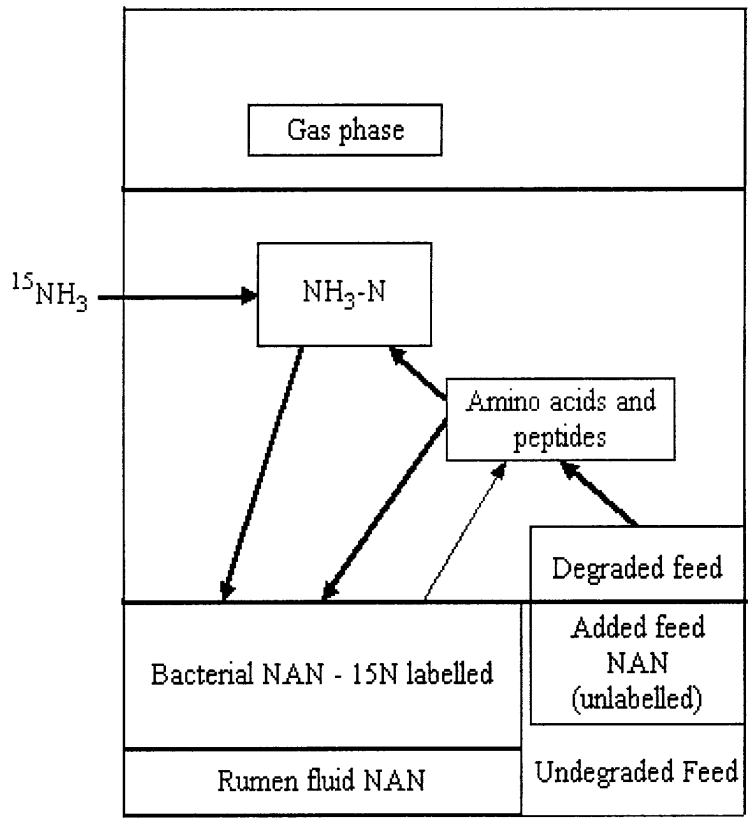

Figure 8. A diagram depicting the transactions of N during *in vitro* incubation of feeds (duckweed and cottonseed meal) with rumen fluid (Hristov and Broderick, 1994). The ammonia-N pool was initially labelled with ^{15}N-ammonia and the enrichment decreased over time as the labelled ammonia-N was diluted by incoming unlabelled ammonia-N released from feed N (and possibly also from degradation of microbial compounds). Bacterial-N became labelled with ^{15}N as the cells grew and assimilated the ^{15}N-ammonia. The NAN pool consisted of undegraded feed N, the NAN that originated from rumen fluid and bacterial-NAN; and the ^{15}N found in this pool was therefore due to the enrichment of bacterial-N.

4.4.2.3 Calculation and statistical analysis

The proportion of NAN that was of bacterial origin was calculated as the ratio of NAN enrichment and bacterial-N enrichment. Multiplying that ratio by the NAN gain (*i.e.* the NAN concentration for the 'feed' flask subtracted for that of the blank) gave the amount of bacterial-N that was newly synthesised from feed N. Similarly, the enrichment ratio, rumen fluid ammonia-N: bacterial-N, indicated the proportion of bacterial-N that was synthesised from ammonia-N.

Ammonia production was calculated as the difference in ammonia-N concentration between the 'feed' flask and that of the blank. The total feed N released was calculated as the sum of the released ammonia-N and the bacterial-N synthesised from feed N. The rate of protein degradation was calculated according to Raab *et al.* (1983).

Production of total VFA from feeds (mmol/g DM) was calculated as the difference in the concentration of total VFA between feed flask and blank. Production of ATP was then calculated assuming that 2, 3 and 2 mol ATP would be generated in the production of 1 mol acetate, propionate and butyrate, respectively (Czerkawski, 1986).

All data were analysed by analysis of variance.

4.4.2.4 Results

a Distribution of ^{15}N label

The total amount of ^{15}N added at the start of incubation, calculated from ^{15}N enrichments (mol ^{15}N/100 mol total N) and concentrations of ammonia-N and NAN on samples taken from the blank flask (containing rumen fluid only) at 0 h, was 13.33 µmol. At the end of incubation, most of the ^{15}N remained in the ammonia-N pool, and only about 11 % of the ^{15}N was recovered in the NAN fraction. The enrichment of ammonia-N decreased from 1.267 at the start of incubation to, at the end, 0.6698 for the

blank (RF) flask and to a mean ± SEM of 0.3774 ± 0.0096 for the 'feed' flasks. The enrichment of ammonia-N at the end of incubation for the 'feed' flasks was lower ($P<0.01$) than for RF flasks due to the dilution of the ^{15}N-ammonia with the ^{14}N-ammonia released from the feeds.

b Concentration of N

The concentration of ammonia-N (mM) increased from 6.9 at the start of incubation to 11.7 at the end of the 3-h incubation for the blank flask, and to 19.5 ± 0.49 (mean ± SEM) for flasks with feeds added. The concentration of NAN (mM) increased from 77.4 to 77.8 for the blank flask, and to 101.6 ± 3.77 for the flasks to which feeds had been added. The concentrations of ammonia-N and NAN at the end of incubation for flasks containing feeds were higher ($P<0.05$) than for the flasks containing RF only. There was difference in the ammonia-N concentration in the feed-containing flasks. More than 80 % of the total-N in the flasks was found in the NAN pool at the end of the incubation (Figure 9).

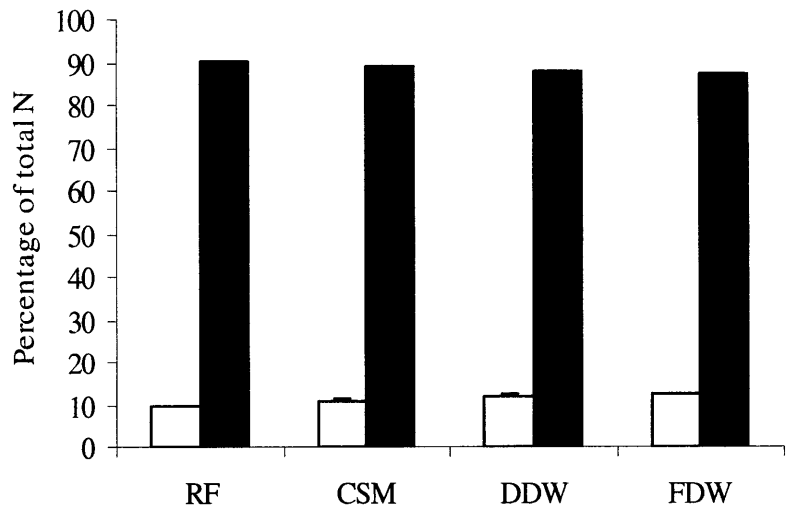

Figure 9. Proportion (% total-N) of ammonia-N (□) and NAN (■) at the end of a 3-h incubation of rumen fluid (RF) and RF to which had been added cottonseed meal (CSM), dried duckweed (DDW) or fresh duckweed (FDW).

c Fermentation

VFA concentrations (mmol/flask), production (mmol/g feed DM), molar proportion (%) of individual acid and calculated ATP production (mmol/g DM) at the end of the 3-hour incubation are presented in Table 7. The production of total VFA and the calculated ATP did not differ (P>0.05) among feeds. Dried duckweed generated a lower (P<0.05) molar proportion of propionate and a lower acetate : propionate ratio than fresh duckweed or cottonseed meal for which these values did not differ. The proportion of isobutyrate was also lower (P<0.05) for dried duckweed than for cottonseed meal or fresh duckweed.

Table 7. Means (± SEM) of total VFA concentration (mmol/flask), production (mmol/g feed DM), molar proportion of individual VFA (%) and calculated ATP production (mmol/g DM) at the end of a 3-h incubation of rumen fluid (RF) and RF to which either cottonseed meal (CSM), dried (DDW) or fresh duckweed (FDW) had been added.

VFA	Feeds			
	RF	CSM	DDW	FDW
VFA Concentration (mmol/flask)	8.44[a]	9.61 ± 0.72[a]	11.7 ± 2.89[a]	9.73 ± 0.91[a]
Molar proportion (%)				
Acetate	74.2[a]	72.1 ± 0.70[a]	74.3 ± 0.32[a]	71.3 ± 1.13[a]
Propionate	13.1[a]	14.1 ± 0.16[b]	13.1 ± 0.04[a]	13.9 ± 0.13[b]
Isobutyrate	1.33[a]	1.30 ± 0.02[a]	1.11 ± 0.05[b]	1.44 ± 0.02[a]
Butyrate	8.75[a]	9.52 ± 0.31[a]	9.23 ± 0.15[a]	10.2 ± 0.64[a]
Valerate	0.77[a]	1.12 ± 0.09[a]	0.79 ± 0.03[a]	1.04 ± 0.12[a]
Isovalerate	1.86[a]	1.85 ± 0.11[a]	1.55 ± 0.05[a]	2.08 ± 0.22[a]
C2 : C3 ratio		5.13 ± 0.11[a]	5.69 ± 0.04[b]	5.13 ± 0.13[a]
VFA production (mmol/g DM)	-	0.70 ± 0.51[a]	2.09 ± 0.20[a]	1.21 ± 0.95[a]
Calculated ATP production (mmol/g DM)	-	1.74 ± 1.08[a]	4.66 ± 0.43[a]	2.86 ± 2.00[a]

Means within a row without a common superscript differ (P<0.05)

d Bacterial-N synthesis

The enrichment (mol ^{15}N/100 mol total-N) of bacterial-N and NAN are given in Figure 10. The ratio, NAN enrichment : bacterial-N enrichment, which indicated the fraction of NAN that was bacterial-N, did not differ (P>0.05) among feeds.

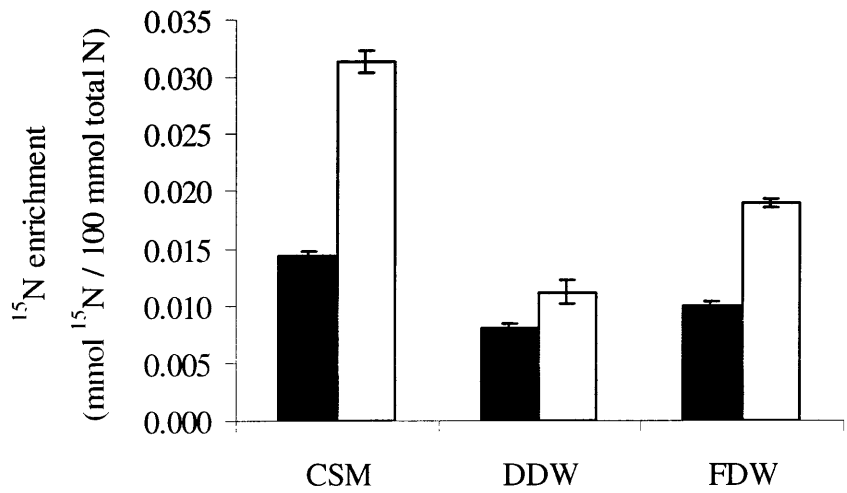

Figure 10. Means (± SEM) of enrichment (mol ^{15}N/100 mol total-N) of NAN (■) and bacterial-N (□) for cottonseed meal (CSM), dried duckweed (DDW) and fresh duckweed (FDW) at the end of a 3-hour incubation with strained rumen fluid.

Bacterial-N newly synthesised from feed-N (mol N/100 mol feed N; Figure 11) differed between test feeds ($P<0.01$), being highest for the dried duckweed followed by the fresh duckweed and then the cottonseed meal. The percentage of the newly synthesised bacterial-N derived from ammonia-N also differed significantly ($P<0.01$), being highest for cottonseed meal (9.0 %), followed by the fresh duckweed (4.8 %) and the dried duckweed (2.9 %).

e Degradation of feed N

Microbial degradation of feed protein results in the release of peptides, amino acids and then ammonia-N. These products of degradation either remain in the rumen fluid or are utilised by microbes during microbial protein synthesis. The total feed N released, therefore, can be calculated as the sum of the net gain in ammonia-N and the feed N newly synthesised into bacterial materials.

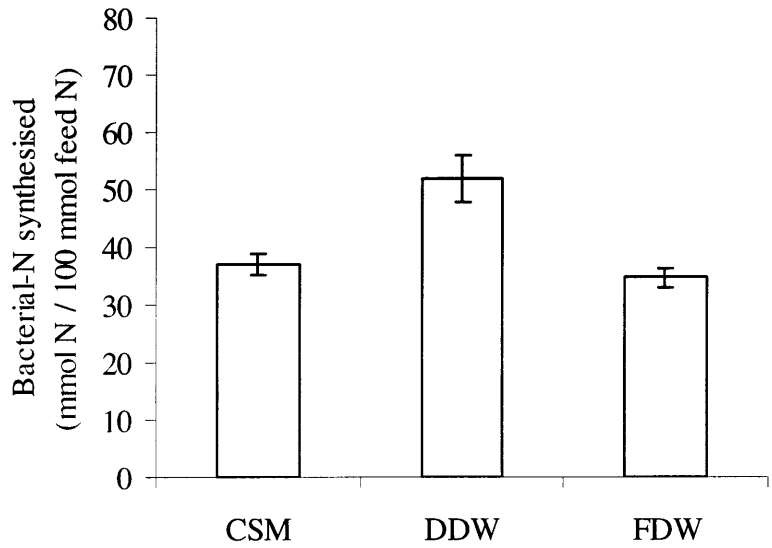

Figure 11. Means (± SEM) of amounts of bacterial-N synthesised from feed N (mol N/100 mol feed N) at the end of a 3-hour incubation of cottonseed meal (CSM), dried duckweed (DDW) and fresh duckweed (FDW) with strained rumen fluid.

The amount of ammonia-N released from feed N was higher ($P<0.001$) for dried or fresh duckweed than for cottonseed meal. The total feed N released (mol N/100 mol feed N) was highest for dried duckweed followed by fresh duckweed and cottonseed meal (Figure 12).

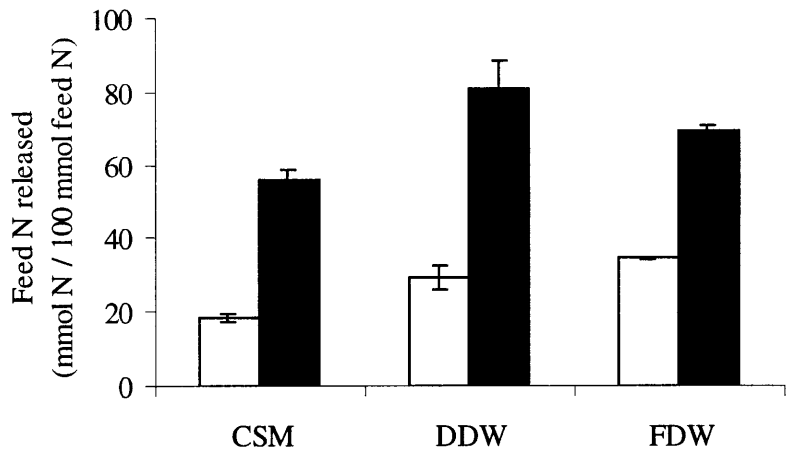

Figure 12 Amounts (mol N/100 mol feed N; means ± SEM) of ammonia-N (□) and total-N (■) released from cottonseed meal (CSM), dried duckweed (DDW) and fresh duckweed (FDW) after incubation for 3 hour with rumen fluid.

The extent of feed N degradation (%), degradation rate (%/h) and calculated escape (%), assuming a rumen outflow rate of 5 %/h, are presented in Table 8. Dried duckweed tended (P=0.08) to have higher N degradation than cottonseed meal. At an assumed a DM outflow rate of 5%/h, duckweed N had less (P<0.01) estimated rumen N 'escape' than cottonseed meal, *i.e.* the duckweed protein was more ruminally degradable.

Table 8. Means (± SEM) of N degradation parameters for cottonseed meal (CSM), dried duckweed (DDW) and fresh duckweed (FDW) after incubation for 3 h with strained rumen fluid.

Degradation parameter	CSM	DDW	FDW	P value
Degradation (%)	55.6 ± 2.88^{a}	80.7 ± 7.53^{a}	68.9 ± 1.71^{a}	= 0.08
Degradation rate (%/h)	0.23 ± 0.01^{a}	0.82 ± 0.10^{b}	0.55 ± 0.00^{a}	< 0.05
Estimated escape (%)*	17.9 ± 0.35^{a}	5.82 ± 0.67^{b}	8.37 ± 0.01^{b}	< 0.01

*Calculated at an assumed DM outflow rate of 5 %/h
Means within a row without a common superscript differ (P<0.05)

4.4.3 Experiment 3.3: Metabolism of Duckweed N in the Rumen Fluid with or without Additional Energy Source

4.4.3.1 Introduction

Experiment 3.2 showed that the enrichments of microbial-N and NAN were low in relation to the enrichment of ammonia-N (*i.e.* the enrichment ratios were less than 0.1). This indicated that microbial assimilation of the ammonia and its utilisation for microbial-N synthesis was low in this experiment. Microbial growth might have been limited by factors other than ammonia because the ammonia-N concentration was above the concentration that is thought to be optimum for microbial growth (Satter and Slyter, 1974). The proteolytic activities of the microbes and thus the degradation of feed N might have also been different if they had achieved optimum growth. Nevertheless, the N degradation for the reference feed (cottonseed meal) obtained in this experiment (56 %) was in agreement with that reported in the literature (Goetsch and Owens, 1985).

The low microbial growth observed in Experiment 3.2 was most likely to be due to the limited availability of fermentable substrate in the rumen fluid. The rumen fluid was collected from an animal that had been fasting for 24 h at which time the fermentation of the last meal might have almost been completed. No additional energy source was provided to the incubation system. The current experiment was carried out to address whether degradability was affected by substrate availability and, if degradability was under-estimated during periods of energy restriction, to obtain a more reliable estimate of the degradability and microbial utilisation of N in duckweed. An additional energy source was therefore provided to the incubation system to ensure an optimum microbial growth and proteolytic activity.

4.4.3.2 Experimental procedures

Incubation was carried out for dried duckweed and cottonseed meal in the presence or absence of added energy, using thin-shaped, one-litre incubation vessels (Figure2).

An amount of finely ground (1 mm sieve) oven-dried (60°C, 24 h) duckweed (*Spirodella punctata* 4.73 g N/100 g air-dry) and cottonseed meal (N = 6.13 g N/100 g air-dry) providing approximately 35 mg N in each duplicate flask (30 mg N/100 ml rumen fluid) was initially weighed into the flask. The flask was then transferred into a water bath that was set at 39°C. Into each flask, 375 ml buffer solution (pH = 7) containing $NaHCO_3$ (0.98 g/l) and $Na_2H_2PO_4$ (3.74 g/l), with or without dissolved energy, was added. The energy source was a mixture of glucose and maltose (3.07 g/l buffer solution). The contents of the flasks were well mixed with a spatula, flushed with CO_2, and the flasks were allowed to stand for approximately 1 h before the rumen fluid was added.

One ml $(^{15}NH_4)_2SO_4$ solution (^{15}N abundance 5.39 atoms %; Amersham International, U.K), providing an additional 7.03 mg N/flask, was dosed into each of the flasks. Strained rumen fluid (115 ml/flask), obtained from one steer 24 h after the last meal of a diet as described in Section 3.2.7, was added. The incubation was allowed to proceed for 6 h at a temperature of 39°C during which gas production was monitored and the flasks were continuously shaken.

Sampling of the flask contents was done for the flasks containing rumen fluid immediately after the addition of $(^{15}NH_4)_2SO_4$ solution (0 h) and for those containing feed supplements at 3 h and at 6 h, using the sampling procedures described previously (see Experiment 3.2). A 20 ml sample was taken from each flask, which was then divided into 2 portions: 15 ml was centrifuged (20,000 x ***g***, 15 min, -4 °C) to obtain the supernatant fraction while the remaining 5 ml was preserved with 0.1 ml of 18 **M** H_2SO_4 and stored frozen for analysis of NAN (Section 3.3.4.4). The supernatant was retained, preserved with 0.3 ml of 18 **M** H_2SO_4 and stored frozen for analysis of ammonia-N (Section 3.3.4.2) and VFA (Section 3.3.3), while the residue was used to obtain a pure sample of bacteria (Section 3.2.7) that was frozen and later analysed for N (Section 3.3.4.3). Enrichment of was determined (Section 3.3.5) in all nitrogenous samples.

4.4.3.3 Calculation and statistical analyses

Calculations were generally similar to those described for Experiment 3.2. Data were first analysed with an analysis of variance as a 3 x 2 factorial design with 3 diets and 2 energy levels.

4.4.3.4 Results

a Fermentation

The original cumulative gas production results are presented in Figure 13, while VFA concentration, VFA proportions, and VFA production are presented in Table 9.

Gas production increased markedly in response to added energy in the incubation system. At the end of incubation there was 84 ± 6.9 ml/g DM gas produced for cottonseed meal and 42 ± 10.5 ml/g DM for duckweed. Production of VFA (mmol/g DM) did not differ ($P>0.05$) between cottonseed meal samples incubated with or without additional energy.

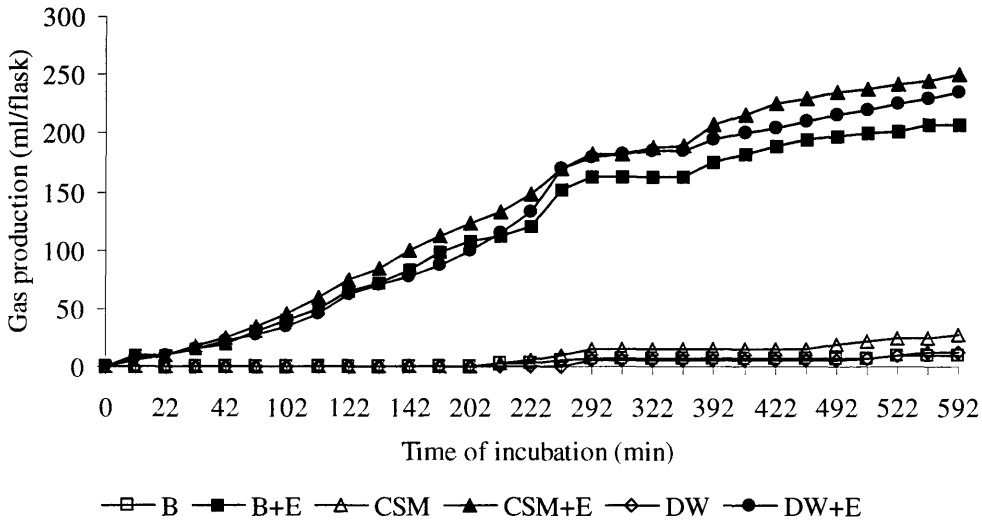

Figure 13. Gas production (ml/flask) for rumen fluid (RF), RF added with cottonseed meal (RF+CSM) or duckweed (RF+DDW) during the incubation in the presence (filled symbols) or absence (unfilled symbols) of additional energy substrate. (B represents flask + rumen fluid, but no added substrate; B+E represents B+added energy substrate; CSM = cottonseed meal; DW = dried duckweed.)

Table 9. Total VFA concentration (mmol/flask) and individual VFA percentages (%) at 3 and 6 h of incubation of rumen fluid (RF), RF added with cottonseed meal (RF+CSM) or dried duckweed (RF+DDW) in the presence (+E) or absence (–E) of additional energy sources.

Time and VFA	+E			–E			Effect of Feed			Effect of Energy	
	RF	RF+CSM	RF+DW	RF	RF+CSM	RF+DW	RF	RF+CSM	RF+DW	+E	–E
3 h											
VFA concentration (mmol/l)	47.7	47.5	49.6	33.1	34.3	35.0	40.4^{a}	40.9^{a}	42.3^{b}	48.3^{a}	34.2^{b}
Proportion (%)											
Acetate	59.7	59.7	59.5	65.3	65.3	64.8	62.5^{a}	62.5^{a}	62.1^{a}	59.6^{a}	65.1^{b}
Propionate	25.0	24.7	25.2	17.2	17.3	17.4	21.1^{a}	21.0^{a}	21.3^{a}	25.0^{a}	17.3^{b}
Isobutyrate	0.34	0.38	0.33	1.32	1.28	1.26	0.83^{a}	0.83^{a}	0.80^{a}	0.35^{a}	1.29^{b}
Butyrate	12.8	13.0	12.8	13.4	13.4	13.6	13.1^{a}	13.2^{a}	13.2^{a}	12.9^{a}	13.5^{b}
Valerate	1.16	1.20	1.27	1.27	1.09	1.27	1.22^{a}	1.14^{a}	1.27^{a}	1.21^{a}	1.21^{a}
Isovalerate	1.01	1.03	0.97	1.56	1.64	1.66	1.29^{a}	1.34^{a}	1.31^{a}	1.01^{a}	1.62^{b}
VFA production (mmol/g DM)		0.39	1.79		1.13	1.40		0.76^{a}	1.60^{a}	1.09^{a}	1.26^{a}
6 h											
VFA concentration (mmol/l)	54.2	55.0	53.0	35.0	36.9	37.4	44.6^{a}	45.9^{a}	45.2^{a}	54.1^{a}	36.4^{b}
Proportion (%)											
Acetate	57.0	56.9	56.0	64.8	65.5	65.0	60.9^{a}	61.2^{a}	60.5^{a}	56.7^{a}	65.1^{b}
Propionate	26.2	26.0	26.0	16.3	16.4	16.6	21.2^{a}	21.2^{a}	21.3^{a}	26.0^{a}	16.4^{b}
Isobutyrate	0.32	0.32	0.33	1.00	0.91	0.99	0.66^{a}	0.62^{a}	0.66^{a}	0.32^{a}	0.97^{b}
Butyrate	14.3	14.4	15.2	14.7	14.3	14.4	14.5^{ab}	14.4^{a}	14.8^{b}	14.6^{a}	14.5^{a}
Valerate	1.29	1.37	1.43	1.35	1.40	1.32	1.32^{a}	1.38^{a}	1.38^{a}	1.36^{a}	1.36^{a}
Isovalerate	0.99	0.98	1.08	1.80	1.57	1.76	1.39^{a}	1.28^{a}	1.42^{a}	1.02^{a}	1.71^{b}
VFA production (mmol/g DM)		0.72	1.34		1.88	1.80		1.30^{a}	1.57^{a}	1.03^{a}	1.84^{a}

Means within a row without a common superscript differ ($P<0.05$)

b Ammonia concentration

The proportions of ammonia-N (% of total-N) are given in Figure 14. Provision of an additional energy source to the incubation system markedly reduced the net ammonia-N release from feed N. The net ammonia-N fraction was generally higher ($P<0.05$) for duckweed than for cottonseed meal, except at 6 h in the flasks with additional energy in which case the fraction of feed N appearing in the net ammonia-N production did not differ ($P>0.05$) between duckweed and cottonseed meal.

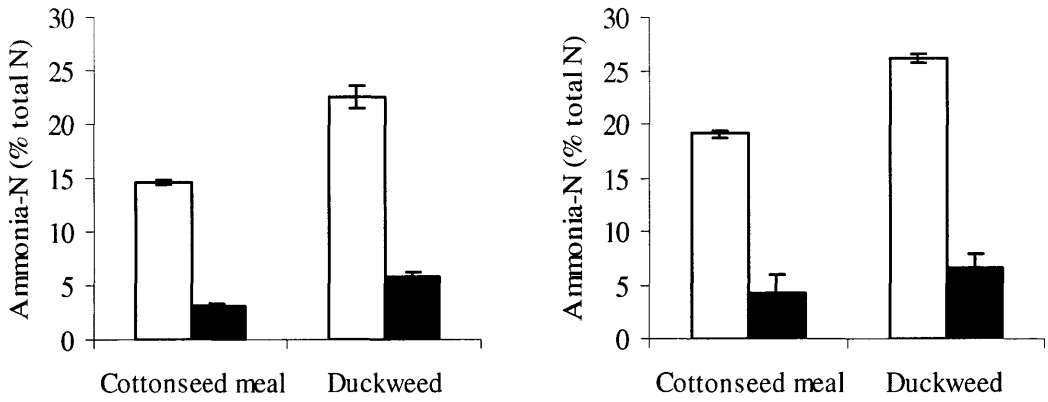

Figure 14. Net ammonia-N release (means ± SEM; % total-N) for cottonseed meal and duckweed at 3 h (left) and 6 h (right) of incubation with strained rumen fluid with (■) or without (□) additional energy.

c Enrichment

The enrichment of ammonia-N, NAN and bacterial-N are given in Figures 15, 16 and 17, respectively. The provision of an additional energy source to the incubation system reduced the enrichment of ammonia-N and increased that of NAN and bacterial-N. The reduction in ammonia-N enrichment in response to energy addition was more evident for cottonseed meal than for duckweed. Cottonseed meal had a lower ($P<0.05$) ammonia-N enrichment than duckweed at 6 h in the presence of an additional energy source. NAN enrichment or bacterial-N enrichment at 6 h for the duckweed treatment did not differ ($P>0.05$) from that for the cottonseed meal treatment when the incubation was supplied with an additional energy source.

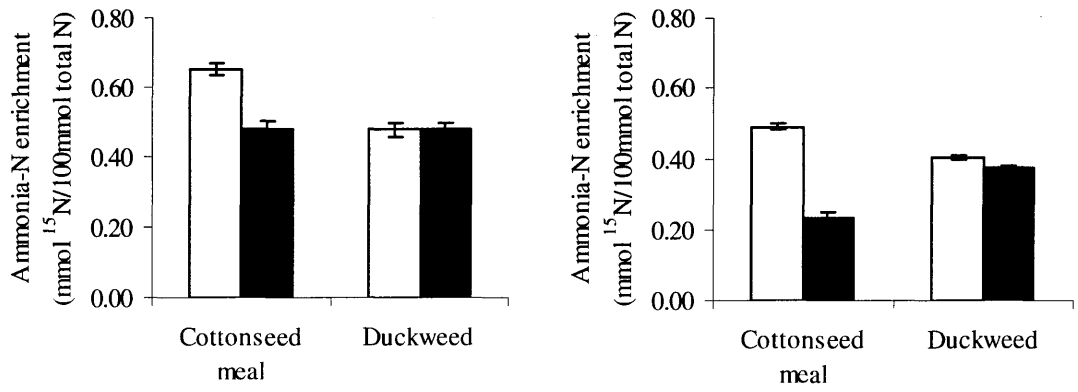

Figure 15. Enrichment of ammonia-N (means ± SEM; mmol ^{15}N/100 mmol total-N) for cottonseed meal and duckweed at 3 h (left) and at 6 h (right) of incubation with strained rumen fluid with (■) or without (□) additional energy source.

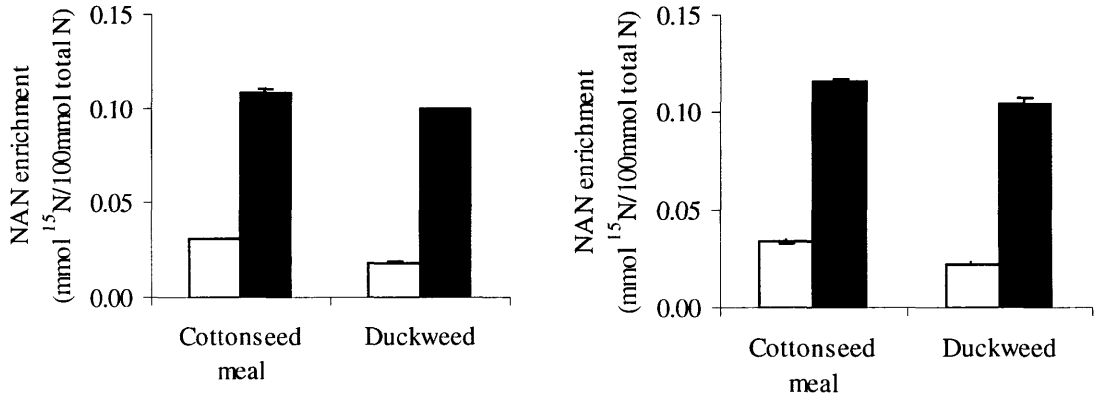

Figure 16. Enrichment of NAN (means ± SEM; mmol ^{15}N/100 mmol total-N) for cottonseed meal and duckweed at 3 h (left) and at 6 h (right) of incubation with strained rumen fluid with (■) or without (□) additional energy source.

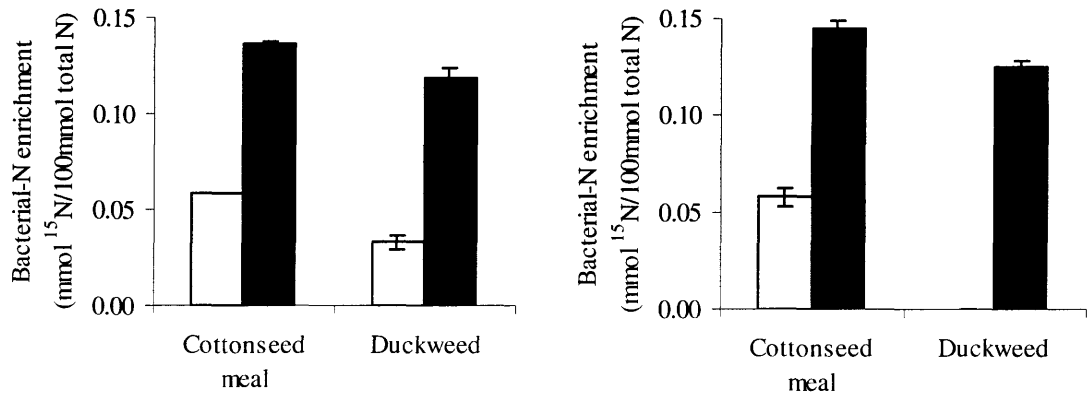

Figure 17. Enrichment of bacterial-N (means ± SEM; mmol ^{15}N/100 mmol total-N) for cottonseed meal and duckweed at 3 h (left) and at 6 h (right) of incubation with strained rumen fluid with (■) or without (□) an additional energy source.

d Bacterial-N synthesis

The percentage of the NAN that was bacterial-N, the amounts of bacterial-N synthesised from feed N (mg N/100 mg feed N) and the percentage of bacterial-N synthesised from ammonia-N are given in Figures 18, 19 and 20, respectively. In the absence of added energy, it was not possible to isolate bacterial samples for duckweed at 6 h and so no data associated with these samples were available. The percentage of NAN that was bacterial-N and the amounts of bacterial-N synthesised did not differ ($P>0.05$) between flasks containing cottonseed meal and duckweed at 6 h when an energy source was added. However, the percentage of bacterial-N synthesised from ammonia-N at 6 h was lower ($P<0.05$) for duckweed than for cottonseed meal.

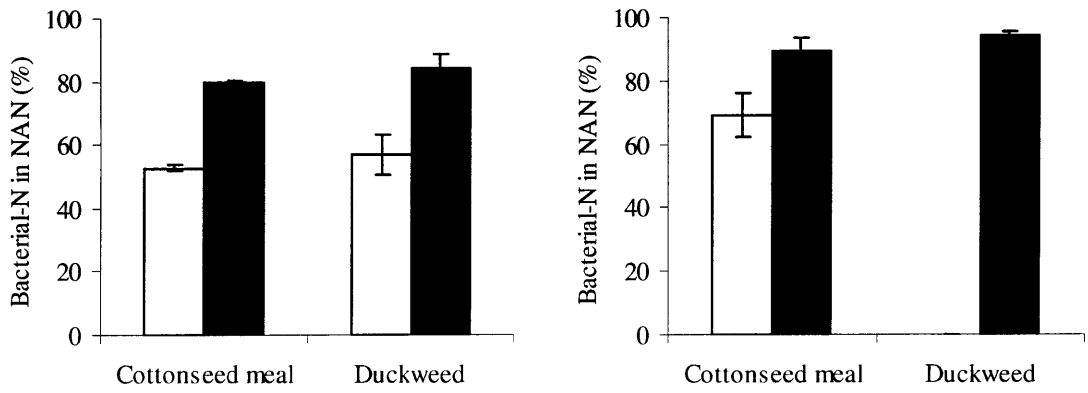

Figure 18. Percentage of NAN that was bacterial-N for cottonseed meal and duckweed after 3 h (left) and after 6 h (right) of incubation with strained rumen fluid, with (■) or without (□) an additional energy source.

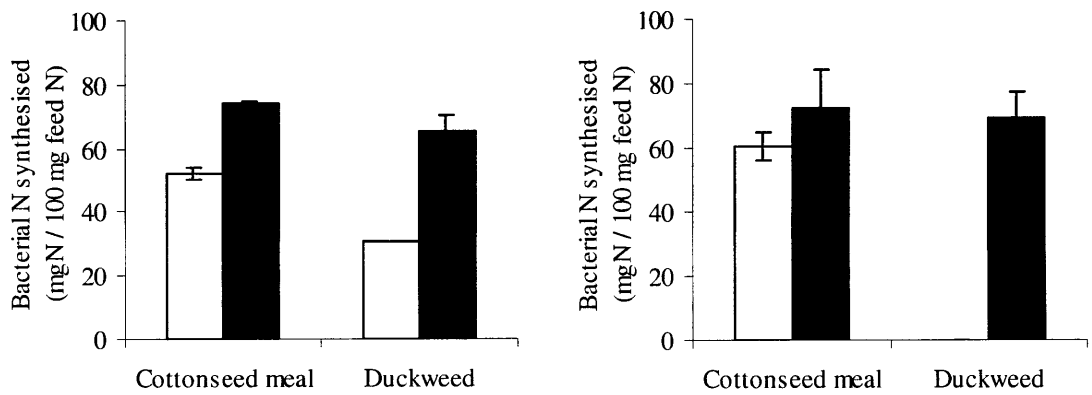

Figure 19. Bacterial-N synthesis (mg N/100 mg feed N) for cottonseed meal and duckweed at 3 h (left) and at 6 h (right) of incubation with strained rumen fluid, with (■) or without (□) an additional energy source.

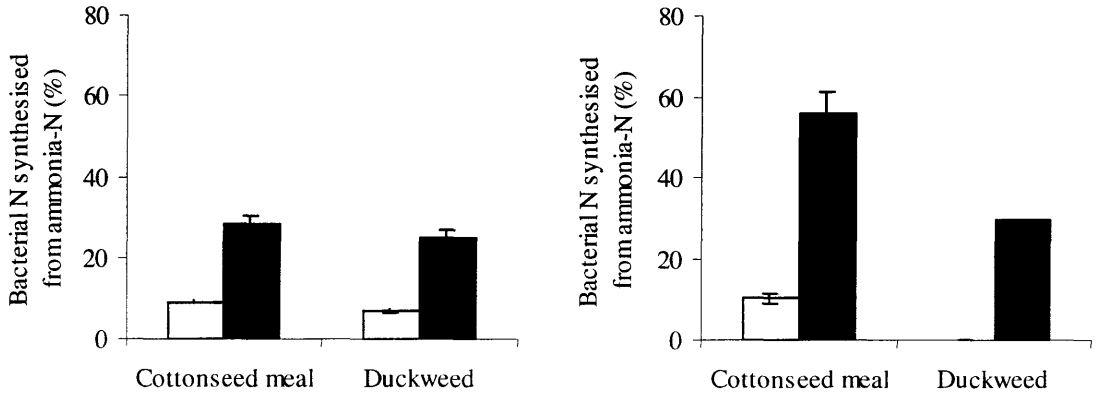

Figure 20. Percentage of bacterial-N synthesised from ammonia-N for cottonseed meal and duckweed at 3 h (left) and at 6 h (right) of incubation with strained rumen fluid with (■) or without (□) an additional energy source.

e Degradation of feed N

The addition of an energy source to the incubation system appeared to affect the production of ammonia for the cottonseed meal and duckweed treatments in a different way. The effect of an interaction between feed and energy on ammonia production, however, was only significant at 3 h of incubation. Production of ammonia at 6 h in the presence of additional energy did not differ between the two feeds.

Degradation of feed N (%) is shown in Figure 22. The degradation of N tended ($P=0.08$) to be affected by the interaction between feed and energy. At 3 h, duckweed N was more completely degraded ($P<0.05$) than cottonseed meal whether or not the incubation was supplied with an additional energy source. The presence of additional energy at 6 h further increased the degradation of feed N, and this was higher for duckweed than for cottonseed meal ($P = 0.05$).

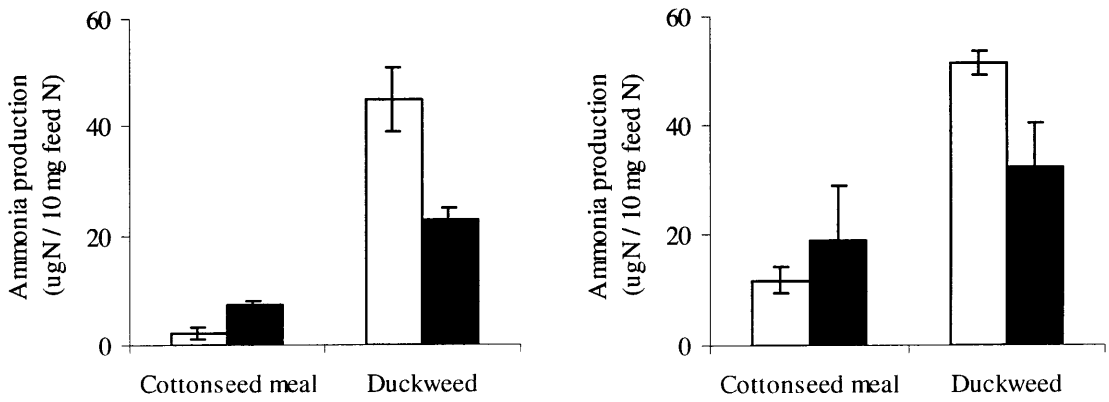

Figure 21. Production of ammonia (mg N/100 mg feed N, mean ± SEM) for cottonseed meal and duckweed after 3 h (left) and after 6 h (right) of incubation with strained rumen fluid, with (■) or without (□) an additional energy source.

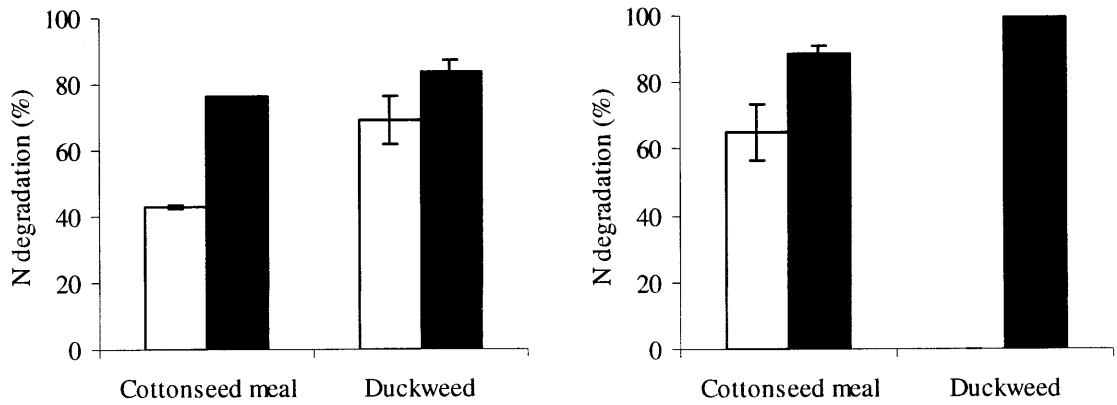

Figure 22. Degradation of N (%, mean ± SEM) for cottonseed meal and duckweed after 3 h (left) and after 6 h (right) of incubation with strained rumen fluid, with (■) or without (□) an additional energy source.

4.5 Discussion

4.5.1 Concentration and production of ammonia-N

It has frequently been proposed that the concentration of ammonia-N in the rumen can be used as an indicator of whether the N available from the diet is adequate for microbial protein synthesis. For example, it has been suggested that the minimum ammonia-N concentration for optimal microbial protein synthesis *in vitro* is about 50 mg N/l (Satter and Slyter, 1974). The concentration of ammonia-N at any time during

the incubation, however, is a balance between its rates of production and removal from the medium. In an *in vitro* system, deamination of intermediary products of proteolysis (amino acids) and probably secretion of ammonia by protozoa are processes that produce ammonia, while the uptake of ammonia by bacteria is the only route of ammonia-N removal from the medium. It is possible therefore that, when the incubation system allows an extensive microbial growth, the concentration of ammonia-N in the medium may be lower than the concentration suggested as being limiting for microbial growth. It was shown in Experiment 3.3 that the concentration of ammonia-N was relatively low, *i.e.* only between 2.53 and 30.4 mg N/l, when energy sources were added to the incubation system. This was unlikely to mean that the availability of ammonia-N for microbial protein synthesis was inadequate, but rather to indicate there was an extensive microbial growth and uptake of ammonia-N for cell N synthesis at a rate that was faster than the rate of its release from feed N.

Because the ammonia-N concentration at the end of incubation could sometimes be lower than the concentration at the start, as observed in Experiment 3.3 when energy was added, it was not appropriate to present the ammonia-N production over time. The concentrations of ammonia-N at the end of incubation were, however, always higher for flasks containing feed samples than for the flasks containing rumen fluid only (blanks), indicating there was net ammonia-N production from feed N. Net production of ammonia (total ammonia-N produced not accounting for ammonia-N that had been incorporated by bacteria) was calculated, as this is an indicator of the portion of feed N that has the potential to 'escape' from the rumen. The net production of ammonia-N was generally higher for duckweed than for cottonseed meal, and more ammonia-N could, therefore, be lost from the rumen for duckweed than for cottonseed meal. The implication from these results is that cottonseed meal may be a better 'escape' protein supplement than duckweed.

4.5.2 Fermentation

Microbial fermentation of OM in the rumen results in the formation of VFA and gases. Blummel *et al.* (1997) found there was a linear relationship between *in vitro* gas production and feed degradability. Gas production in the experiments reported in this Chapter was lower for duckweed (fresh or dried) than for cottonseed

meal. In Experiment 3.1, for example, the gas production (ml/g DM) for cottonseed meal was about 21 ml/g DM, whereas that for dried and fresh duckweed was about 9 and 6 ml/g DM, respectively. This was supported by data obtained from Experiment 3.3 which showed that the fermentation rate for dried duckweed was about half that of cottonseed meal. Taken together, these results indicated that duckweed may be less fermentable in the rumen than cottonseed meal. Another interpretation, however, is that more substrate was used for microbial biomass synthesis from duckweed than from cottonseed meal because microbial biomass yield and gas production were inversely related (Blummel *et al.*, 1997). The gas production, however, was inconsistent with the data for VFA production for which cottonseed and duckweed did not differ.

Experiment 3.3 indicated that the addition of readily fermentable substrates (glucose and maltose) to the incubation vessels increased gas production markedly. When these substrates were not added, negligible amounts of gas were produced. It appeared that the microbial populations required the readily fermentable substrate to meet their immediate requirements so that they could more quickly begin to ferment the feeds being tested.

4.5.3 Bacterial-N synthesis

During the incubation, unfermented feed N and microbial N would have been the N fractions comprising the NAN fraction. Any ^{15}N label in the NAN fraction, however, must have signified uptake of the ^{15}N-ammonia, probably mainly by the bacteria, during the synthesis of their cell proteins. Protozoa might also have become labelled due to the engulfment of labelled bacteria (Coleman and Sandford, 1979). (Ammonia-N is the not the main source of N for rumen protozoa but is an end-product of their intermediary metabolism (Eadie *et al.*, 1970)). Only bacterial cells were isolated in these experiments and this was performed with care, as any unlabelled non-bacterial-NAN in the isolate would have reduced the enrichment of the bacterial-N and affected the validity of conclusions being drawn. The bacterial isolate was occasionally inspected under a light microscope and was always found to be visually free of feed particles or protozoa.

As bacteria grew, ^{15}N-ammonia (as well as unlabelled ammonia) would have been incorporated into bacterial cells. Only the growing or newly synthesised bacterial cells would, therefore, become enriched. It was impossible, however, to isolate only this fraction of newly formed bacterial cells and the bacterial isolate would inevitably contain an unknown amount of 'old' bacterial cells originating from the rumen fluid present at the start of the incubation period. The ^{15}N label in the bacterial isolate would therefore be diluted not only by the unlabelled N in the newly synthesised bacterial cells, but also by the unlabelled bacterial-N present at the start of the incubation. The actual enrichment of the newly produced bacterial-N would, therefore, have been higher than the observed value for 'new' plus 'old' bacteria. This under-estimation of bacterial-N enrichment would result in an over-estimation of bacterial-N synthesis and of the contribution of NAN in the newly synthesised bacterial-N. Given that a consistent procedure for isolation of bacterial cells was applied in these experiments, differences between actual and observed enrichments in bacterial isolates would likely to be higher when microbial growth was less optimum than when it was unrestricted, *i.e.* without energy addition, or with extra energy, respectively.

Bacterial growth is dependent on the availability of energy and N precursors. ATP is required to transport the N precursors into cells, to maintain cell integrity and to synthesise new cellular proteins and other materials from the transported N precursors. In Experiment 3.2, the incubation system was likely to be deficient in energy rather than N (or other essential nutrients). The strained rumen fluid used was obtained from animals that had been fasting for 24 h where probably limited amounts of fermentable materials were still available to be used during the incubation. During the incubation, production of VFA and calculated ATP were negative for the flask containing rumen fluid only, and although production rates increased when feeds were added, the increases were statistically non-significant. Production of ammonia-N, on the other hand, was about 50 % for rumen fluid and it was higher for rumen fluid added with feeds.

Bacteria did not extensively incorporate the ammonia released from feed N in Experiment 3.2. Most of the released ammonia remained in the rumen fluid and bacteria incorporated only about 12 % of the available ammonia-N. The ratio,

bacterial-N : ammonia-N, was only about 5.6 %. This was far below the reported values obtained *in vivo* (Nolan and Leng, 1972). The low proportion of bacterial-N arising from ammonia-N in Experiment 3.2 could not simply be accepted to mean that most of the bacterial-N was synthesised from NAN, and that NAN was preferentially utilised for bacterial-N synthesis. This was more likely to be due to the slow growth of bacteria and the diluting effect of ^{14}N that was already present in rumen fluid. The dilution of ^{15}N label in the bacterial isolate by the 'old' bacterial-N would have reduced the observed ^{15}N enrichment of the bacterial isolate and over-estimated bacterial growth.

The suggestion that bacterial growth in Experiment 3.2 was likely to be restricted by the limited availability of energy was confirmed by results obtained in Experiment 3.3. Incorporation of ammonia-N into bacterial-N generally remained low when no additional energy source was provided whereas provision of additional energy sources markedly increased bacterial-N synthesis and the proportion of bacterial N synthesised from ammonia. At 3 h, for example, only 8 % of the bacterial-N was derived from ammonia-N when there was no energy added, in contrast to 30 % when energy was added.

Experiment 3.3 showed that bacterial-N synthesised from feed N via ammonia-N was always higher for cottonseed meal than for duckweed, irrespective of whether or not additional energy was provided. This was consistent with the results of Experiment 3.2. The higher proportion of bacterial-N synthesised from ammonia for cottonseed meal could not have been related to the availability of energy, as the calculated ATP formation was similar for the two feeds. Neither could it be quantitatively attributed to the availability of ammonia-N, as the ratio of ammonia-N to total-N released from feed N was either similar for the two feeds, or higher or lower for duckweed than for cottonseed meal.

It appeared that, in these experiments, NAN compounds (probably present mostly as peptides and/or amino acids) were preferentially used for microbial growth on duckweed whereas ammonia-N (resulting from degradation of peptides and amino acids) was preferentially utilised for bacterial-N synthesis when cottonseed meal was tested.

4.5.4 Degradation of feed protein

The basic principle of the *in sacco* technique in predicting the rumen degradability of feeds is that the rumen microbes are allowed to freely enter and pass out of the bag during the incubation, giving them the opportunity to invade and degrade the enclosed feed under test. The microbes and the feed under test must itself also be allowed to move freely inside the bag. The amount of feed enclosed in a bag is likely to be one major factor that affects the free movement of microbes and the incubated feed. Excessive amounts of feed enclosed in a bag could restrict the accessibility of the microbes to the feed, and this could invalidate the results of degradability obtained. This was likely to be the case in Experiment 1.1 when the size of fresh duckweed sample used was 3.3 g DM per bag and inconsistent degradability estimates were obtained. Results suggest that for less dense feeds such as fresh duckweed with a DM content of about 10 %, it is more convenient to use about 1–2 g DM per bag. This corresponds to a ratio of 10-20 mg DM per cm^2 bag surface.

The *in sacco* technique is able to predict and fractionate the feed under test into water soluble, and insoluble but potentially ruminally degradable fractions, as well as the rate at which the latter is degraded. The water-soluble fraction is usually obtained from the fraction that disappears during the washing of samples obtained before incubation. Fine feed particles, *i.e.* those smaller than the pore size of the bag, would also be extensively removed during washing and contribute to an over-estimation of the soluble fraction. On the other hand, the estimate of solubility may be low if large particles in the sample are dominant. Variations in the proportions of fine particles to larger size particles in the samples enclosed in the bag may contribute to variations in the estimate of the soluble fraction with the *in sacco* technique. The experimental results showed that the percentage of water-soluble fraction obtained for cottonseed meal in Experiment 1.1.was higher than in Experiment 1.2 (27.7 *v.* 23.0 %) and this led to the prediction of a lower estimate of the fraction that is water insoluble but potentially fermentable in the rumen for cottonseed meal in Experiment 1.1 than in Experiment 1.2. This might be a result of higher proportions of fine particles in the larger sized samples used in Experiment 1.1 (4.8 g DM per bag) than in Experiment 1.2 (2.0 g DM per bag). Nevertheless, the predicted effective degradability of DM at

each of the assumed rumen outflow rates between the two experiments was very similar.

Errors in the *in sacco* prediction of the size of the soluble fraction due to variations in the size of feed particles and the physical disappearance of fine feed particles from the bag were eliminated in the Experiment 2 when ground or macerated feed samples were used. The soluble N fraction determined in this way was higher than the fraction that disappeared during washing in the *in sacco* experiments. For dried duckweed, there were significant variations in the N solubility of samples that had been dried under similar conditions (80°C for 24). Samples of dried duckweed used in this experiment were obtained from different duckweed colonies at different stages of growth and grown in different conditions. The differences were reflected in their different N concentrations. The experiment was partly intended to indicate whether the N concentration of duckweed could be used as an initial indicator of its solubility and degradability in the rumen. However, data indicated that factors other than the N concentration, possibly those associated with the growth conditions of the duckweed, affected duckweed N solubility and possibly its degradability in the rumen.

The *in sacco* experiments showed that duckweed, either fresh or dried, was less soluble and was degraded at slower rates and to lesser extents in the rumen than cottonseed meal. These experiments suggest that the quantity of protein from duckweed that escapes rumen fermentation may be higher than in cottonseed meal. Oven-drying duckweed reduced the insoluble but degradable N fraction and the rate at which this fraction was degraded in the rumen, but it increased the effective N degradability (Experiment 1.2). For dried duckweed, the crude protein degradation rate was slower and the effective degradability lower than determined by Huque *et al.* (1996) with a similar technique. These authors reported a protein degradation rate of 5.14 %/h for dried *Spirodela spp.* with an effective degradability of 52.4 % at rumen outflow rate of 5%/h. These differences may have been due to various factors such as different growth conditions of duckweed and different ruminal environments in which samples were incubated. Results of the current experiments suggest that about 50 % of the duckweed protein may leave the rumen unfermented and become available for digestion and absorption in the small intestine.

In the *in vitro* experiments, the total feed N released was calculated as the sum of ammonia-N produced and N utilised for microbial protein synthesis. The total feed N released from dried duckweed as compared to cottonseed was not consistent. When the availability of energy was limited, the total-N released from dried duckweed tended to be higher (Experiment 3.2) or lower (Experiment 3.3) than that from cottonseed meal. This was probably due to differences between batches of dried duckweed used in both experiments. In Experiment 3.3, in the presence of added energy, the amount of total feed N released was similar at 3 and 6 h for dried duckweed and cottonseed meal. Bacterial-N synthesis, on the other hand, tended to be higher for cottonseed meal than for dried duckweed in the presence of added energy at 3 h, but similar at 6 h. It appeared that bacterial growth and thus the ability of bacteria to degrade feed N were less for dried duckweed than for cottonseed meal at the earlier times of incubation, but they became more similar as the incubation proceeded.

The degradation of feed N in my *in vitro* experiments suggested a need to optimise the incubation conditions when using the method in predicting the degradability of feed N. Less optimum conditions of incubation, for example when the availability of energy was limited, would lead to underestimation of microbial growth and feed N degradability. It was shown in Experiment 3.3 that the addition of energy to the incubation system markedly increased bacterial-N synthesis and degradation of feed N. However, the degradation of feed N estimated with the *in vitro* technique (Experiment 3.3) appeared to be very high for both cottonseed meal and duckweed (>85 % degradable). Given that *in vivo* experiments have usually given lower values, questions arise as to the precision of the *in vitro* technique in predicting rumen degradability of feeds.