Chapter 7 General Discussion

7.1 Analysis of ^{15}N

Quantification of ^{15}N abundance on an isotope-ratio mass spectrometer was a critical laboratory analysis performed in this study. The ^{15}N abundance on various N samples from the rumen and the abomasum, after or during the provision of an exogenous ^{15}N source, was determined in order to understand the kinetics of N among those samples. It was therefore important that the procedures for preparing the samples for analysis and for the ^{15}N analysis on the purified samples were verified at an early stage of the study and then re-inspected throughout the study. The procedures used for isolating N for ^{15}N analysis must provide a pure sample of the N in the original sample (*e.g.* rumen bacteria) and the N must be delivered to the mass spectrometer without contamination with extraneous N in the preparatory steps. It is also important that between-sample ^{15}N contamination in the laboratory is prevented, *e.g.* by adsorption of ^{15}N on glassware or distillation apparatus. The procedures used were those described by Nolan (1971) where details of the necessary precautions that were followed in the work described in this thesis are outlined and discussed in detail.

Systematic checking was performed on the accuracy of the analysis against a commercial standard of known ^{15}N abundance that was calibrated and supplied by the International Atomic Energy Agency. The repeatability of the analysis over time and the linearity of the analysis for samples of increasing abundance were also examined.

Results of the analyses, which are given in Appendix 3, indicate that the analysis was accurate, repeatable and linear in the range of abundance used in this study. Figure 38 which shows the similarity of the estimates of enrichment in ammonia derived from fluid-rich and particle-rich components separated from single digesta samples, supports this conclusion.

7.2 Rumen Degradability of Duckweed

Duckweed can contain protein as high as 45 % DM, (Leng *et al.*, 1995), and it is potentially a valuable source of amino acids for domesticated animals. Studies to confirm the nutritional value of duckweed have so far been done mainly with simple-stomach animals (see Chapter 2) but there was little information available when my candidacy commenced on whether duckweed could also provide protein for ruminants.

The overall objective of the work recorded in this thesis was to answer the question of whether duckweed could also be used as an amino acid source for ruminants. Two roles played by amino acids needed to be considered simultaneously for this purpose: the provision of rumen degradable N for the synthesis of microbial protein in the rumen (the main amino acid source in ruminants) and the provision of additional amino acids to the small intestine for absorption and utilisation by the host animal ('escape' or 'bypass' protein; AFRC, 1992). Therefore, a series of interrelated *in vitro* and *in vivo* experiments was carried out encompassing these two aspects.

Results of experiments in which the rumen degradability of duckweed was estimated using the *in sacco* technique showed that duckweed, whether fresh or dried, was quite resistant to rumen degradation. Experiment 1.2 (Chapter 4) showed that the degradability of duckweed N was reduced by drying it: the N fraction that was insoluble in water but fermentable in the rumen was about 30 % lower in dried than in fresh duckweed (47 versus 67 %). This may have been due to the general effect of drying in reducing the degradability of feeds in the rumen (McDonald, 1982) or to the hydrolytic effects of active plant proteolytic enzymes (Zhu *et al.*, 1999) in the fresh duckweed.

Even though the *in sacco* technique has probably been the most widely used technique for predicting feed degradability in the rumen (see Broderick and Cochran, 1999), results obtained with this technique have to be interpreted with care. This technique is not suitable for feeds with fine particles, but on the other hand, the particle size of feed samples put into the bags for incubation in the rumen should be large enough to be retained in the bag and sufficiently uniform to avoid the effects of particle size differences. In reality, these criteria are quite difficult to fulfil, and even if they are achieved, they may not mimic particles in the rumen from feeds that have been subject to mastication by the animal. In the studies reported in Chapter 4, the duckweed and cottonseed meal test samples were not milled, so the existing proportions of fine and coarse particles may have affected the estimates of degradability. The degradability obtained for cottonseed meal, which was chosen as a 'standard' representing 'escape' protein sources thought to have a degradability of about 60 %, might have been over-estimated due to the large proportion of fine particles in this feed.

In vitro techniques that predict rumen degradability of feed protein based on ammonia production are relatively simple and readily performed, but may under-estimate N degradability because microorganisms use some of the ammonia that is produced for cell growth. The introduction of ^{15}N-labelled ammonia into the *in vitro* incubation system, not only enables the microbial assimilation of ammonia to be quantified but also gives an indication as to whether or not the incubation system is capable of supporting efficient microbial growth. Results of Experiments 3.2 and 3.3 (Chapter 4) showed that it was important to ensure a sufficiency of energy substrates for microbial growth: more bacterial-N was synthesised when energy was added to an energy-deficient *in vitro* system. The estimate of the degradability of feed N was also increased as a result of energy addition, suggesting that additional energy enhanced proteolytic activity, probably due to increased numbers of microbes. The degradability of protein at 6 h in the presence of additional energy, however, was very high (>85 % for both cottonseed meal and dried duckweed), probably because there was no capability to allow for the effect of rumen outflow that occurs *in vivo*.

7.3 Rumen Utilisation and Abomasum Flow of Duckweed N

It is noteworthy to point out that the utilisation of N for bacterial synthesis observed in Experiment 3.3 (Chapter 4) appeared to differ between cottonseed meal and duckweed. Whereas the amount of bacterial-N synthesised was similar between the two feeds, the proportion of the bacterial-N synthesised from ammonia-N was lower for duckweed than for cottonseed meal, or conversely, more bacterial-N was synthesised from NAN (*i.e.* peptides and amino acids) in the case of duckweed than for cottonseed meal. It is difficult to know whether this was due to different properties of the NAN in cottonseed meal and duckweed that affected their utilisation by bacteria or to a different availability of the NAN (due to different rates of release) between the two feeds. Any direct bacterial incorporation of feed NAN (in contrast to assimilation of ammonia) will reduce the inefficiency of dietary protein fermentation in the rumen because the bacterial-N formed will eventually be available to the host (whereas some ammonia is absorbed across the rumen wall). Moreover, the efficiency of microbial protein synthesis itself often appears to be higher when amino acids rather than ammonia are utilised for microbial protein synthesis (Maeng *et al.*, 1976).

The significant findings of this study arose from experiments reported in Chapter 5 in which rumen N kinetics were studied *in vivo* by supplying ^{15}N-ammonia or ^{15}N-labelled duckweed into the rumen. When the same quantity of ^{15}N in these forms was administered into the rumen, the resulting rumen ammonia-N enrichment was always lower when ^{15}N-duckweed was given rather than ^{15}N-ammonia. This was clear evidence that ^{15}N in ingested duckweed was not completely degraded to ammonia in the rumen: it must therefore have escaped undegraded from the rumen or have been utilised in NAN forms for the synthesis of microbial protein. The proportion of bacterial-N synthesised from ammonia-N was 51 – 70 % when ^{15}N-ammonia was administered into the rumen, suggesting that 30 - 41 % of the bacterial-N was synthesised from NAN (*i.e.* peptides and amino acids). These values are, generally, in agreement with those reported previously (Nolan *et al.*, 1976; Nolan and Stachiw, 1979) and suggest, by inference, that when peptides and amino acids are

available in relatively high concentrations in rumen fluid they may be extensively used by microorganisms for microbial protein synthesis.

Direct evidence for the microbial utilisation of plant N in NAN forms was obtained from the studies in which ^{15}N-labelled duckweed was administered into the rumen. The enrichment ratio of bacterial-N to that of ammonia-N was always higher when ^{15}N-duckweed rather than ^{15}N-ammonia was introduced into the rumen: it follows that there must have been a specific uptake of ^{15}N in forms other than ammonia. This was clear and direct evidence that N sources more complex than ammonia were used for bacterial protein synthesis in the rumen. Cottle (1980) came to a similar conclusion after infusing sheep intra-ruminally with ^{15}N-labelled amino acids derived from labelled algal protein. The relatively low concentrations of intermediary products of dietary protein degradation that are often found in the rumen has been attributed to their rapid deamination to ammonia rather than to their microbial uptake (Lewis, 1955; Warner, 1956) and, in such cases, the microbial requirements for peptides and amino acids in animals given diets containing mainly NPN sources are thought to be satisfied by 'cross-feeding' (release of microbial-NAN into the rumen fluid). The higher enrichment ratio (bacterial-N to rumen ammonia-N) when ^{15}N-duckweed rather ^{15}N-ammonia was supplied to the rumen in the present study was not, however, only due to 'cross-feeding' because this would have given similar results for both forms of tracer.

The percentage of the ^{15}N in duckweed escaping the rumen fermentation and passing to the lower gut in Experiment 4.3 was calculated to be about 30 % and most (75 %) of the ^{15}N from the component that was digested in the rumen was converted to bacterial-N. The remainder was probably mainly absorbed as ammonia. Some amino acids may also be absorbed (Cook *et al.*, 1965), but this may be quantitatively insignificant because of the low rumen concentration of amino acids. Because the percentage of bacterial-N being synthesised from ammonia-N was about 50 %, it follows that about 50 % of the ^{15}N found in bacteria must have been incorporated as NAN. The direct incorporation of dietary peptides and amino acids during bacterial protein synthesis in the rumen is a desirable process because this would reduce the

potential loss of the ingested protein by its further degradation to ammonia that is readily absorbed through the rumen wall. Less bacterial-N was derived from NAN (about 22 %) in the experiment in which the ^{15}N label was provided as a single dose. In addition, to reasons for why ammonia-N or NAN is utilised for bacterial protein synthesis that have already been discussed in Chapter 5, it is possible that the frequency of feeding may affect the relative amounts of ammonia or NAN used for bacterial synthesis. More frequent feeding, in which there is constant release of peptides and amino acids through the degradation of dietary protein, may ensure a more continuous availability of these substances for microbial incorporation. Thus, the efficiency of dietary protein conversion into products in ruminants may be improved by increasing the frequency of feeding. On the other hand, it is possible that ammonia may be a more important source of bacterial-N in ruminants that are given feed once each day.

7.4 Duckweed and Ruminant Production

Beneficial effects of feeding duckweed on the production of ruminants were shown in experiments presented in Chapter 6 in which wool growth and wool fibre characteristics were determined in response to the inclusion of duckweed in a hay diet. Wool growth in sheep is highly responsive to the amounts of total amino acids absorbed from the small intestine (Ferguson, 1975). Wool growth can therefore be used to provide an indication of the availability of total amino acids of microbial or dietary origin in the small intestine of normally fed animals.

In Experiment 5.1 sun-dried or fresh duckweed was included to form an iso-energetic maintenance diet for Merino sheep. Wool growth and wool fibre characteristics did not to differ as a result of duckweed supplementation. It was calculated in this experiment that duckweed supplementation only slightly increased, the total digestible protein leaving the stomach (sum of microbial protein synthesised in the rumen and the rumen undegraded or partially degraded duckweed protein relative to non-supplemented sheep) and this may have not been readily detectable in wool growth. The extra amino acids derived from duckweed protein may have been metabolised to maintain live weight of the animals rather than being used for wool

protein synthesis. In Experiment 5.2, wool growth and wool fibre characteristics were again measured in response to the provision of an iso-nitrogenous diet of oaten chaff supplemented with either urea, sun-dried duckweed or cottonseed meal. The total digestible protein leaving the stomach and the resulting wool growth were assumed not to be affected by sources other than the supplemental protein escaping the rumen fermentation. The rumen ammonia-N concentrations were above the concentration required for an optimum microbial growth in the rumen (Satter and Slyter, 1974) or for optimum feed digestion (Leng *et al.*, 1993). Therefore, the microbial protein contribution to the total protein available in the small intestine or feed digestion in the rumen may have been optimum and similar across the supplements. The similar wool growth observed for duckweed and cottonseed meal, which was higher than that for urea, was considered to be due to the escape of undegraded protein from the duckweed and cottonseed meal. It appears from the results of Experiment 5.2 that the assumed rumen degradability (80 %) for duckweed used in the GrazFeed calculation may have been higher than the true value. It is likely that the true rumen degradability of duckweed protein may not exceed 70 % as obtained in Experiment 4.3 using ^{15}N-labelled duckweed. Using a value for rumen degradability of duckweed of 70%, and 60 % for cottonseed meal, both supplements used in Experiment 5.2 would have provided a similar amount of escape protein (about 11 g/d.

The duckweed used in Experiment 5.1 and 5.2 was obtained from a sewage treatment works in which the duckweed was principally used as a water purifier rather than produced as an animal feed. The duckweed could therefore have contained substances that are toxic to animals or humans. However, during the conducts of these experiments, the animals readily ingested the duckweed in either dried or fresh forms and no clinical ill-effect was detectable. It was concluded that the nutritional benefits of duckweed out-weighed any toxic or other detrimental effects that may have been present. A similar conclusion was drawn by Skillicorn (2002), *i.e.* that duckweed (*Lemna* spp.) grown on sewage effluent is chemically safe for use as an animal feed. The author found some pathogenic bacteria in recently harvested (fresh) duckweed, but not in dried duckweed. While pathogen-containing sewage-grown fresh duckweed may represent some risk for monogastric animals, it may be less problematic for

ruminants as pathogenic bacteria would probably not survive in the rumen. Research is required to confirm this hypothesis.

It is clear, that duckweed can benefit ruminants by supplying amino acids available for absorption in the small intestine that augment those contributed by the microbial protein synthesised in the rumen. This conclusion is valid mostly for dried duckweed. The degradability of fresh duckweed was not determined in these studies, but the possibility of feeding fresh duckweed exists as the study in Chapter 6 showed that sheep that were familiar with duckweed avidly ingested up to 1 kg/d of fresh duckweed. Unless this duckweed was ruptured by mastication, it would be likely to float in the rumen and perhaps persist alive for some time in the rumen. Its subsequent fate is completely unknown, but it is possible that the percentage of escape protein from fresh duckweed could be even higher than for dried material and this could be especially advantageous for highly producing ruminants.

In field situations, the choice of whether to use dried or fresh duckweed may depend on a variety of considerations. Fresh duckweed could be offered to animals thereby reducing the cost associated with drying. Fresh duckweed may also be more beneficial for straw-based diets where high water content could help to moisten and soften the straw before and during presentation to the animals. For duckweed to be used in the fresh form, a duckweed 'plantation' has to be conveniently close to the animal house because transportation would be less practical for the fresh duckweed. Drying duckweed is not likely to be a major problem is tropical areas, but a drying area would be required that is free from wind disturbance. Dried duckweed could be stored for future use by the grower or sold and transported to where it is required.

7.5 Further Studies

These studies showed that it is easy to label duckweed with ^{15}N by growing it on a synthetic medium containing ^{15}N-ammonia. In the present study, the resulting ^{15}N-labelled duckweed was used for studying the degradation of dried plant-N in the rumen and its subsequent utilisation by the microbes. It would be possible to extend the results reported in this thesis by conducting a study similar to that in Chapter 5

using ^{15}N-labelled fresh duckweed. The outcomes might be different to those reported in Chapter 5 due to the different physical properties of the fresh compared with the dried material. For this to be done, a continuously growing ^{15}N-labelled duckweed culture with sufficient harvestable material to supplement the experimental animals on a continuous basis would be required.

It was believed in that part of the rumen ammonia-N enrichment when ^{15}N-labelled duckweed was used in experiments reported in Chapter 5 was due to the presence of ^{15}N-ammonia as an integral component of the washed ^{15}N-labelled duckweed. It is possible to fractionate the ^{15}N label in duckweed into ^{15}N-labelled ammonia and ^{15}N-labelled NAN. Introducing only the latter into the rumen and analysing ^{15}N-ammonia should provide an indication of the rate of NAN degradation to ammonia. The ^{15}N-labelled NAN from duckweed may also be fractionated further into ^{15}N-labelled peptides and amino acids and these could be used as substrates in N metabolism studies.

Field experiments in which dried or fresh duckweed is fed to ruminants are to be encouraged. Such experiments may be directed to building an integrated duckweed-ruminant system in which the complementary roles of plant and animal could be exploited. The duckweed is an input to the animals while the animals' excreta may be used as a fertilizer to support the growth of the duckweed. Such systems require relatively few external inputs and are therefore suitable for farmers with less capital in developing countries. Such systems would ensure the maximal use of each of the natural resources and, at the same time, such integrated systems also benefit the environment.

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Appendices

Appendix 1 Publications incorporating work presented in this thesis

Damry, J.V. Nolan, R.E. Bell and E.S. Thomson, 2001. Duckweed as a protein source for fine-wool Merino sheep: its edibility and effects on wool yield and characteristics. *Asian Australasian Journal of Animal Sciences* **14**: 507-514.

Damry and J.V. Nolan, 2001. *In sacco* determination of rumen degradability of fresh and dried duckweed. In *Recent Advances in Animal Nutrition in Australia*, Vol. 13 (Ed, J. L. Corbett). Animal Science, University of New England, Armidale, NSW 2351, Australia, pp. 15A.

Damry and J.V. Nolan, 2002. Degradation of duckweed protein in the rumen of sheep. *Animal Production in Australia* **24**: 45-48

Appendix 2 Growth of duckweed and production of ^{15}N-labelled duckweed

a. Introduction

As duckweed is a potential protein supplement for farm animals, it is important to determine the optimum conditions required for the plant to grow efficiently. As discussed in Chapter 2, many factors affect duckweed growth and therefore its production of protein. One of those factors is the availability of assimilable nutrients in the growth medium at an optimum concentration. In regard to the production of protein, the N concentration in the water *per se* is also an important factor: there is a close relationship between the concentration of N in the water and that of CP in duckweed produced (Leng *et al.*, 1995; Rodriguez and Preston, 1996).

Another potential application of duckweed is the possibility of labelling it with ^{15}N and using it as a model plant material in tracer studies aimed at elucidating the metabolism of plant protein by animals. In ruminants, for example, tracer studies (*e.g.* (Nolan, 1971) have mostly used simpler ^{15}N-labelled compounds such as urea or $(NH_4)_2SO_4$. Higher plants labelled with ^{14}C or ^{15}N have also been used (Chapman and Norton, 1984; Nugent and Mangan, 1978), but labelled plant experiments have been limited by potential health hazards (for ^{14}C) and the time and expense required to produce appreciable quantities of labelled plant material.

The main objective of this laboratory scale experiment was to determine the optimum concentrations of macro- and micro-nutrients in a growth medium for the growth of duckweed. The experiment was also designed to investigate whether the growth rate of duckweed would respond differently to different N sources at the same N concentration. Results of this experiment were used as a basis for selecting a medium for producing ^{15}N-labelled duckweed for the experiments described in Chapter 5. The ^{15}N-labelled duckweed was used as the substrate in other experiments for studying the rumen metabolism of duckweed protein, in particular, and of plant protein, in general.

b. Materials and Methods

Stock growth medium

Four individual stock solutions (A, B, C and D) containing different individual macronutrient were prepared as shown in Table 19. A stock micronutrient solution containing different concentration of elements (solution E) and a Fe-EDTA stock solution (solution G) were also prepared (Table 20).

Table 21 Stock solutions of individual macronutrients and their concentrations

Solution	Ingredients	Concentration (g/100 ml)
A	$MgSO_4.7H_2O$	24.6
B	$Ca(NO_3)_2.4H_2O$	23.6
C	KH_2PO_4	13.6
D	KNO_3	10.1

Table 22 Stock solutions of micronutrients (g/l) and Fe-EDTA (g/250ml)

Solution	Ingredients	Concentration
E	Micronutrients	(g/l)
	H_3BO_3	2.86
	$MnO_4.H_2O$	1.82
	$ZnSO_4.7H_2O$	0.22
	$Na_2MoO_4.2H_2O$	0.09
	$CuSO_4.5H_2O$	0.09
F	Fe-EDTA	(mg / 250 ml)
	$FeCl_3.6H2O$	121.0
	EDTA	375.0

Duckweed

Two strains of fresh duckweed were obtained locally from Bio-Tech Waste Management, Pty Ltd. Armidale. The duckweeds were identified to have more than 1 but less than 7 roots, and therefore they were categorised as *Spirodela punctata*

species (Les and Crawford, 1999). The two duckweed strains were mainly different in the size of their leaves (fronds), and they were marked as 'big leaves' and 'small leaves'.

Growth mediums

The individual stock nutrient solutions in Table 20 and 21 were mixed at appropriate volumes and used as the nutrient source for growing duckweed. The composition of the individual stock nutrient solutions is presented in Table 22.

Table 23 Composition of individual nutrient stock solution (ml/l) in the original medium used to grow duckweed

Stock solution	Nutrients	Composition (ml / l)
A	$MgSO_4.7H_2O$	1.0
B	$Ca(NO_3)_2.4H_2O$	2.3
C	KH_2PO_4	0.5
D	KNO_3	2.5
E	Micronutrients	0.5
F	Fe-EDTA	20.0

In this experiment, the original growth medium with composition shown in Table 22 was used as the control, and it was identified as treatment '1.0 strength'. The concentrations (mg/l) of individual macro-elements in this solution were as follows: 24.6 magnesium, 32.1 sulphur, 92.2 calcium, 100.2 N, 16.1 phosphorous and 118.8 potassium. The solution also contained a mixture of micro-elements and Fe-EDTA. Two other growth mediums were made by diluting the '1.0 strength' solution so that the concentrations of nutrients were reduced to one-half and one-quarter of their original strength. These growth mediums were termed as '0.5 strength' and '0.25 strength', respectively.

As indicated above, KNO_3 was used as the source of N in the original growth medium. To investigate the effects of different N sources on the growth rate of duckweed, the $NO_3^=$ in the '0.25 strength' solution was replaced with either urea or ammonium chloride.

The pH of all growth mediums was adjusted to 7.0.

Experimental procedures

Two consecutive experiments were conducted indoors in a temperature-controlled room (25 ± 2.8 °C). The growth rate of the 'big leaves' duckweed was observed in the first experiment, and that of the 'small leaves' was studied in the second experiment.

Three round plastic food containers were used for each growth medium. Each container was filled with approximately 200 ml of one of growth medium. On the first day, five individual duckweed leaves were placed into each container and the container was placed in a random place on a table. The room was continuously lit, and additional light sources were provided and placed at about 100 mm above the containers to supply approximately 100 lux of light intensity.

The growth of the duckweed, which was indicated by the appearance of new leaves, was recorded for about two weeks. Any new daughter leaves emerging from their mother leaves were counted as independent leaves. The number of leaves counted every day was plotted over time and the data were fitted with an exponential equation. Doubling time, that is the time (d) required for the number of leaves to double, was calculated as:

$$Doubling\ time(\ d\ ) = 0.693 / exponential\ power$$

c. Results

The first daughter leaves emerged from the original mother leaves on the second or third day of each experiment. Subsequently, grand-daughter leaves were then produced from the daughter leaves. The newly produced leaves were green in colour, but a lighter green than that of the mother leaves. The new leaves usually did not separate from the mother leaves, and there were up to 10 leaves observed together in one lobe.

Changes in the number of duckweed leaves for the 'big leaves' variant and 'small leaves' variant grown in a medium with a similar nutrient content but different nutrient concentrations are presented in Figure 33 and 34, respectively. At the end of experiment, the concentration of nutrients did not affect the number of leaves for the 'big leaves' (grand mean ± SEM of 56 ± 3.4 leaves). For the 'small leaves' variant, however, the '0.25 strength' medium produced more leaves ($P<0.05$) than the '1.0 strength' solution. When growth of the two variants was examined on the same nutrient solution, the 'big leaves' variant produced more leaves ($P<0.05$) than the 'small leaves' variant by the end of the observation period.

The effects of source of N (KNO_3, urea or and NH_4Cl at approximately 25 mg N/l), on the number of leaves for the two duckweeds are shown in Figure 35 ('big leaves') and Figure 36 ('small leaves'). The N source did not affect the number of leaves produced by either duckweed variant. The fitted exponential equation for the growth of these duckweeds on different nutrient concentrations and their doubling times (d) are indicated in Table 23. The effects of N sources are presented in Table 24.

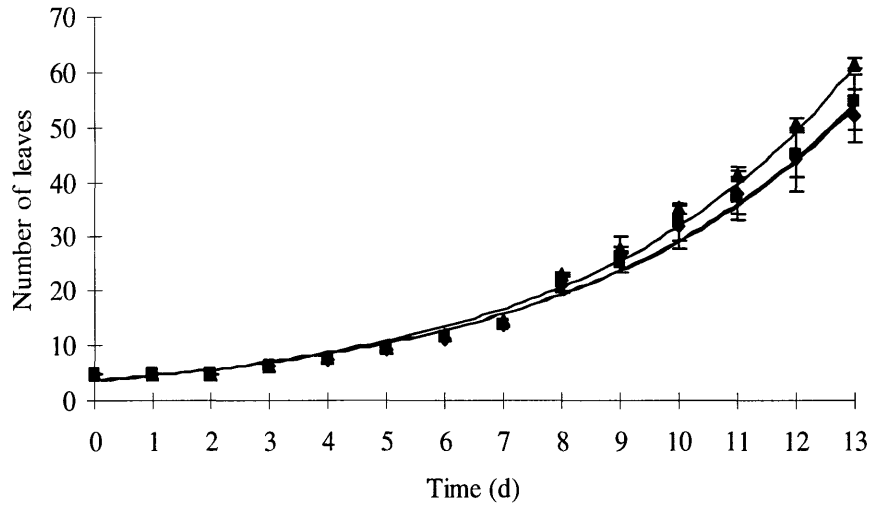

Figure 33 Changes in the number of duckweed fronds for the 'big leaves' variant when grown on a medium at nutrient concentration of either '1.0 strength' (◆), '0.5 strength' (■) or '0.25 strength' (▲)

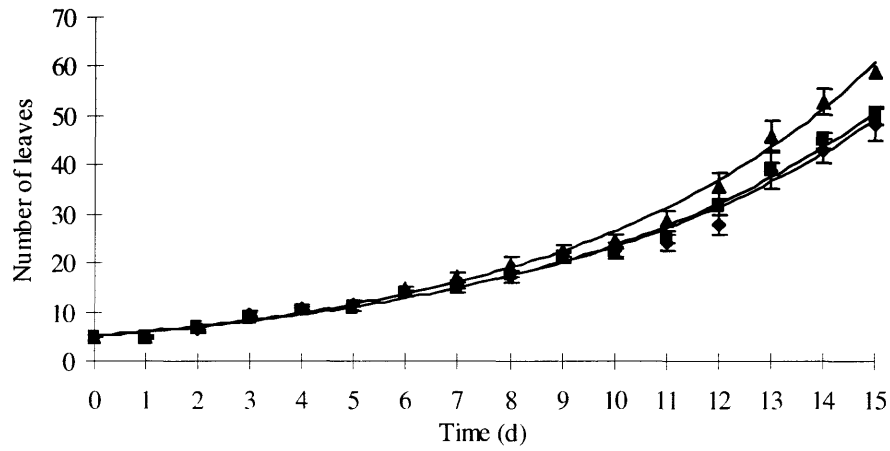

Figure 34 Changes in the number of duckweed fronds for the 'small leaves' variant when grown on a medium at nutrient concentration of either '1.0 strength' (◆), '0.5 strength' (■) or '0.25 strength' (▲).

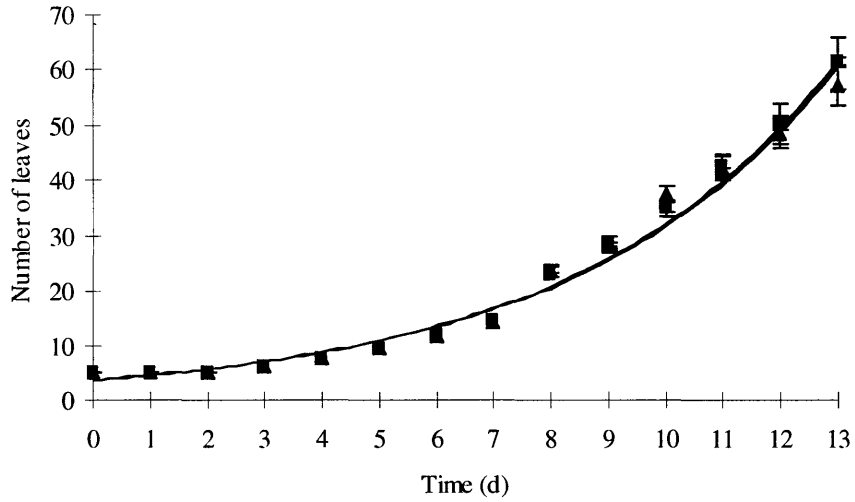

Figure 35 Changes in the number of duckweed leaves for the 'big leaves' when grown on the '0.25 strength' medium having a N concentration of about 25 mg/l provided as KNO_3 (◆), $CO(NH_2)_2$ (■) or NH_4Cl (▲)

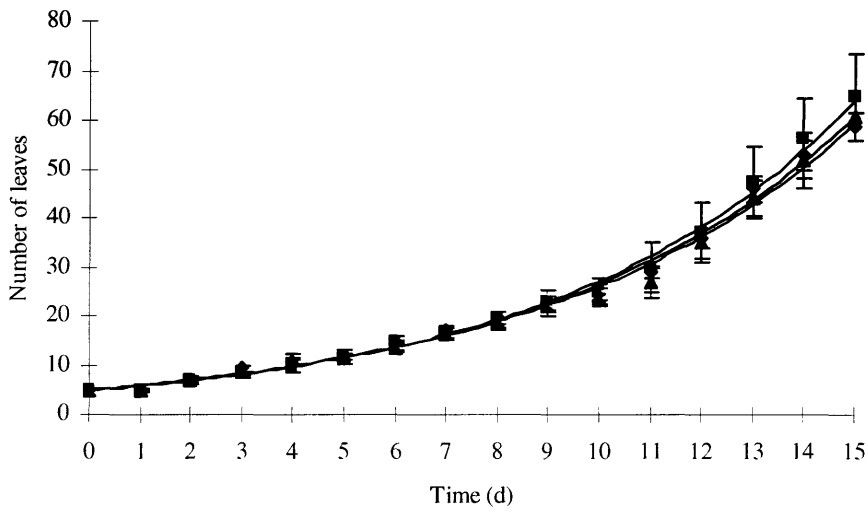

Figure 36 Changes in the number of duckweed leaves for the 'small leaves' when grown on the '0.25 strength' medium having a N concentration of about 25 mg/l provided as KNO_3 (◆), urea (■) or NH_4Cl (▲)

Table 24 Exponential equation for the growth of duckweed on different nutrient concentration and time (d) required for the frond to double in number

Nutrient concentration	Exponential equation	Doubling time (d)
'Big leaves'		
1.0 strength	$y = 3.4572\ e^{0.198x}$; R^2=0.99	3.5
0.5 strength	$y = 3.4314\ e^{0.1995x}$; R^2=0.99	3.5
0.25 strength	$y = 3.365\ e^{0.2093x}$; R^2=0.99	3.3
'Small leaves'		
1.0 strength	$y = 4.4773\ e^{0.1501x}$; R^2=0.97	4.6
0.5 strength	$y = 4.3669\ e^{0.1535x}$; R^2=0.99	4.5
0.25 strength	$y = 4.2422\ e^{0.1668x}$; R^2=0.99	4.2

Table 25 Exponential equations for the growth of duckweed on a nutrient solution of '0.25 strength' (25 ml N/ l) and the time (d) required for the fronds to double in number

Source of N	Exponential equation	Doubling time (d)
Bigger leaves		
KNO_3	$y = 3.365\ e^{0.2093x}$; R^2=0.99	3.3
$CO(NH_2)_2$	$y = 3.3746\ e^{0.2097x}$; R^2=0.99	3.3
NH_4Cl	$y = 3.4131\ e^{0.2077x}$; R^2=0.99	3.3
Smaller leaves		
KNO_3	$y = 4.2533\ e^{0.1664x}$; R^2=0.99	4.2
$CO(NH_2)_2$	$y = 4.0793\ e^{0.1722x}$; R^2=0.99	4.0
NH_4Cl	$y = 4.2569\ e^{0.1648x}$; R^2=0.99	4.2

d. Discussion

The growth of duckweed observed in these experiments supports the view that, in optimum growth conditions, the plant will exhibit an exponential growth. According to (Landolt and Kandeler, 1987), the growth of duckweed occurs in several phases, namely the lag phase, the exponential growth phase, the transitional phase and the equilibrium phase. The lag phase occurs when fresh duckweed is transferred to a new growth medium and prepares itself to grow in response to the nutrients available in the medium. The exponential growth phase, which follows the lag phase, is a period where the plant is growing rapidly as indicated by the generation of new fronds.

(Cheng *et al.*, 2002) found there was a lag phase of approximately 4 days for *Spirodela punctata* when it was grown on water obtained from a swine lagoon with an ammonia concentration of 240 mg N/l. According to (Landolt and Kandeler, 1987), one factor that affects the duration of each phase is the origin of the duckweed. Duckweed that is in the equilibrium phase when it is transferred to a rich-nutrient medium will have a longer lag period than duckweed transferred when in its transitional phase. On the other hand, the lag phase may be very short or skipped if the original duckweed is obtained from a culture in its exponential growth phase.

In this experiment, the lag phase was not apparent, suggesting that the duckweed culture might have been in the rapid growth phase. It was noted that the duckweed did not have any roots when first obtained and the absence of roots in a duckweed colony is also an indication of an abundant availability of nutrients (Landolt and Kandeler, 1987).

The growth rates of the 'big leaves' duckweed were higher than the 'small leaves' variant as indicated by, on the same nutrient solution, the higher number of leaves produced and shorter doubling time. This indicated there was a more efficient uptake of nutrients and conversion into biomass in the 'big leaves' than in the 'small leaves' duckweed. In duckweed, nutrients are absorbed through the underside surface of the leaves rather than through the roots. The higher efficiency may be related to a larger surface area of leaves for nutrient absorption in the 'big leaves' than in the 'small leaves' variant.

Under the conditions of these experiments, there was no growth rate advantage obtained by increasing the concentration of nutrients providing N (as $NO_3^=$) and phosphorous (as $PO_4^=$) at concentrations above 25 and 4 mg/l, respectively. It appeared that the '0.25 strength' solution provided the optimum nutrient concentration and composition for the growth of duckweed in these experiments. The concentrations of N and phosphorous in this medium were within the concentrations found by (Leng *et al.*, 1995) to support a growth of duckweed with a maximum CP content of about 40 %. The '1.00 strength' solution inhibited the growth rate of the 'small leaves' variant suggesting that, at this concentration, the nutrients might have been toxic to the growth of this duckweed.

It was shown in these experiments that changing the source of N, at a concentration of 25 mg N/l, from nitrate to ammonia, did not affect the growth of duckweed. *Spirodela punctata* is therefore able to utilise either nitrate or ammonia equally well in the medium.

Outcome

The '0.25 strength' solution was finally adopted as the optimum medium for the growth of duckweed that was used to produce the ^{15}N-labelled duckweed used in the experiments described in Chapter 5. The N source in the original solution was replaced with $(^{15}NH_4)_2SO_4$ with an ^{15}N abundance of 5.92 % (Amersham International, U.K). The ^{15}N-labelled duckweed was grown in a square container (0.90 x 0.90 x 0.25 m) filled with approximately 20 l of growth medium. The container was kept indoors in a temperature-controlled room with conditions similar to those applied during the experiment, or outdoors during the summer season when sunlight and temperatures were appropriate to support the growth of duckweed. Restocking of the nutrient solution and harvesting the ^{15}N-labelled duckweed were carried out regularly. The harvested ^{15}N-labelled duckweed was washed about 5 times with water before it was dried in a forced- draught oven at 60°C for 24 h. The typical ^{15}N abundance of the ^{15}N-labelled duckweed produced was between 2 and 3 %. This material has also successfully been used as substrate to study the N partitioning in poultry (Li, 2002; Widodo, 2001).

Appendix 3 Checks of the precision and accuracy of the ^{15}N analysis methods

a. Precision of ^{15}N analysis

The precision and accuracy of the mass spectrometer (Tracermass; Europe Scientific) as a means of measuring ^{15}N abundance was evaluated by measuring the ^{15}N abundance of an $(^{15}NH_4)_2SO_4$ standard issued by the International Atomic Energy Agency (IAEA), Vienna. The standard (standard IAEA No. 311A) had ^{15}N abundance of 2.0500 % and it was included randomly in the analyses. Results obtained on different occasions are shown below:

Table 26 Results of ^{15}N abundance analyses performed on various occasions of an IAEA ^{15}N standard (standard No. 311A; theoretical ^{15}N abundance 2.0500 %)

Dates of analysis	Measured ^{15}N abundance (%)
4 September 2001	2.0435
	2.0437
	2.0430
25 October 2001	2.0490
	2.0470
	2.0498
	2.0506
30 October 2001	2.0508
	2.0518
7 November 2001	2.0462
	2.0478

The lowest individual ^{15}N abundance (%) was 2.0430 and the highest was 2.0518, with an overall mean ± stdev of 2.0476 ± 0.0032. These results were within the abundance range of 2.03 to 2.07 % that the IAEA regards as acceptable for this standard.

b. Repeatability of ^{15}N abundance measurement

Repeatability of ^{15}N analysis was inspected by including two standards with different ^{15}N abundances in every run of ^{15}N analysis (usually in 50-100 analyses done on one day). ^{15}N abundance analyses for the standards obtained on various occasions are presented in Table 27.

Table 27 Representative results of ^{15}N abundance analyses for standards

Standard 1		Standard 2	
Date	^{15}N Abundance (%)	Date	^{15}N Abundance (%)
17 April 2000	0.3662	17 April 2000	0.5376
17 April 2000	0.3650	28 July 2000	0.5424
17 April 2000	0.3656	2 August 2000	0.5443
28 July 2000	0.3646	2 August 2000	0.5437
28 July 2000	0.3650		
28 July 2000	0.3652		
2 August 2000	0.3653		
2 August 2000	0.3650		

c. Linearity of ^{15}N analysis

The linearity of the mass spectrometer in the ^{15}N analyses was inspected by determining the ^{15}N abundance on a series of 8 standards having different calculated ^{15}N abundance of between 0.3692 and 1.4738 %. The standards were made by mixing solutions of laboratory grade $(NH_4)_2SO_4$ and $(^{15}NH_4)_2SO_4$ (^{15}N abundance = 5.39 %, Amersham International, U.K). Relationship between calculated and measured ^{15}N abundance of those standards is given in Figure 37.

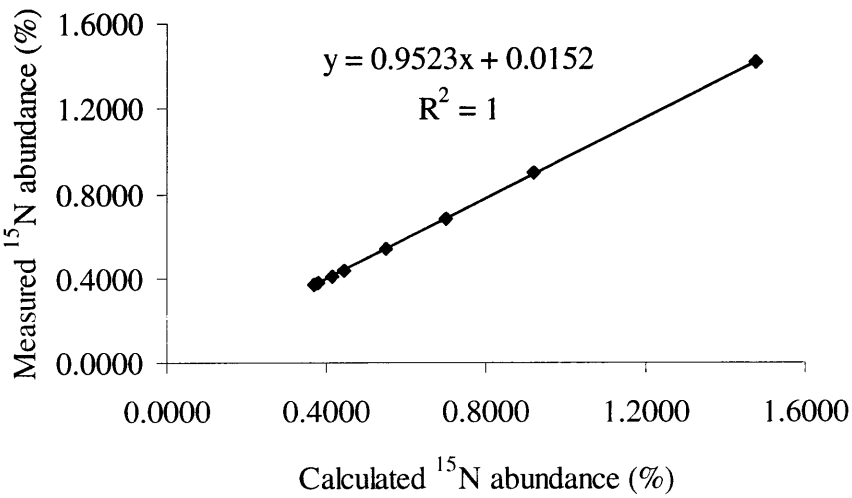

Figure 37 Relationship between calculated and measured ^{15}N abundance of a series of standard differing in ^{15}N abundance.

A representative sample of the results of ^{15}N enrichment analysis performed on ammonia-N in components of abomasal digesta taken from Sheep C in Period 2 of Experiment 5.3 and taken through the various steps used to prepare a purified sample of ammonia-N in the laboratory, is shown in Figure 38. The samples of ammonia from the fluid phase and particle-rich fractions of abomasum digesta were shown to have very similar enrichments when analysed in different analytical runs.

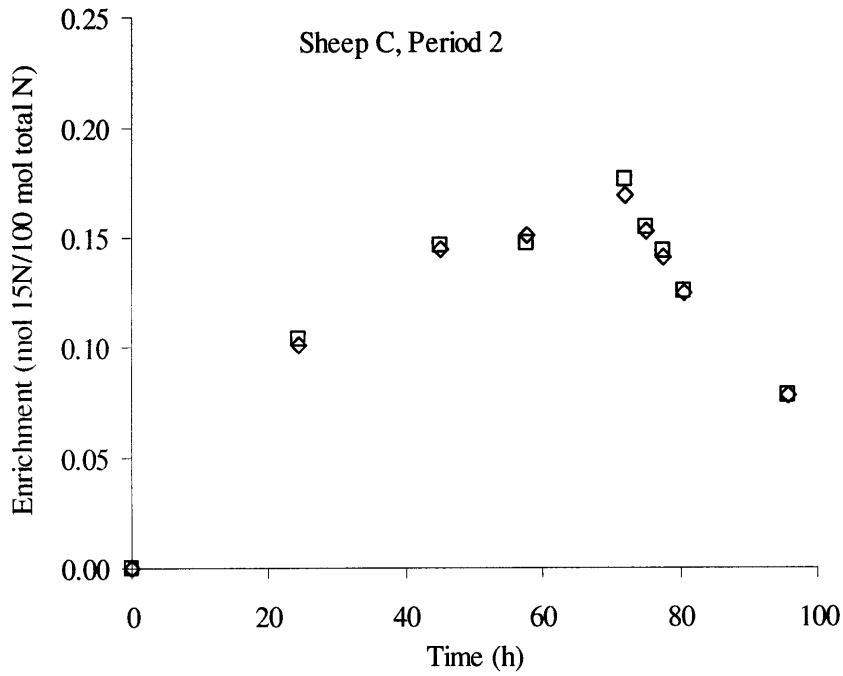

Figure 38 The enrichment of various N-containing materials from the fluid (□) and particle (◇) fractions of the abomasum digesta.

Appendix 4 Recovery of Co standards in Co analysis

In the earlier stages of Co analysis on the ICP-OES, recovery of standards containing a known amount of Co was inspected. Those standards were digested on their own or spiked into samples of fluid or particle-rich abomasum digesta containing no extraneous Co. Details of digestion process are given in section 3.3.1.1. Representative recovery data are presented in Table 27. It was concluded that the Co analyses was accurate with a mean percentage recovery 100.6 and a coefficient of variation of less than 1.5 %.

Table 28 Recovery of Co standards analysed on an ICP-OES

Co concentration (mg/kg)		
Calculated	Measured	Recovery (%)
0.454	0.463	101.9
0.392	0.391	99.8
0.792	0.796	100.6
0.772	0.770	99.7
0.798	0.822	103.0
0.794	0.782	98.6
0.797	0.800	100.4
0.787	0.791	100.6
	Mean	100.6
	CV	1.36

Appendix 5 The flow of abomasum digesta and its constituent: a representative calculation

The flow of digesta and its constituents through the abomasum was estimated using a dual marker technique (Faichney, 1975) with Co and AIA as the fluid and particle digesta markers, respectively. An example of calculation is given in Table 27. The digesta is separated into particle-rich and fluid-rich fractions and the amount of marker in each phase is expressed as a fraction of the daily amount administered. A reconstitution factor (R) is then determined which is the fraction of the fluid phase that has to be added to, or removed from, the particle phase to obtain true digesta, *i.e.* digesta that contains the same fraction of both markers. If digesta constituents such as DM, NAN or ^{15}N concentrations are analysed on both phases of the digesta, their concentration in the true digesta can be calculated mathematically using the same reconstitution factor.

Table 29 A representative calculation of the amount and abomasum flow of true digesta (fresh and DM) in sheep given a mixed diet of (g/d) 400 oaten chaff and 300 lucerne chaff

	Concentration¶		True Digesta	Abomasum Flow (kg/d)
	Particle fraction	Fluid fraction		
Co	0.1234	0.1347	$\frac{(0.1234+3.572\,x\,0.1347)}{(1+3.572)}=0.132$	7.56
AIA	0.6044	0.00005	$\frac{(0.6044+3.572\,x\,0.00005)}{(1+3.572)}=0.132$	
	$R=\frac{0.6044-0.1234}{0.1347-0.00005}=3.572$			
DM	193.3	16.4	$\frac{(193.3+3.572\,x\,16.4)}{(1+3.572)}=55.2$	0.416

¶ Fraction of the daily dose per kg digesta for markers, g/kg for DM