

Cranfield University

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An exploration of new methods to assess energy availability in the English oak (*Quercus robur*).

International Ecotechnology Research Centre

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PhD



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Supervisor: Associate Professor S.H.Cousins

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ABSTRACT

Existing methods of measuring the capability of foods to drive ecosystem processes are reviewed and found to be inadequate; these included bomb calorimetry and plant quality assays. This thesis explores two alternative methods, one *in vivo* and one *in vitro*, based on the rate of supply of energy and compares these to plant quality measurements and calorific content. A pilot study was undertaken using cow parsley (*Anthriscus sylvestris*) to determine sampling regime and establish laboratory techniques for fibre content measurement. The energy availability of different parts of the English oak (*Quercus robur*) are compared by measuring their decomposition rates in oak woodland soil. In this form the soil is acting as an *in vivo* microbial digester and the rates at which weight loss occurs in the samples is converted to an energy release rate dimensioned in kW.kg^{-1} . This unit mass power measurement is compared to heat of combustion data derived from bomb calorimetry which is dimensioned in joules.kg^{-1} . Variation in unit mass heat of combustion between plant parts is small at 10% compared to the 400% (at 18 days) and 720% (at 115 days) variation in unit mass power output between plant parts as measured in the tests undertaken. Unit mass power output correlated with conventional plant quality assays of the oak. A third set of experiments has begun the development of the laboratory based measurement of energy availability under standard conditions. Each plant material was macerated in a roller mill for thirty minutes (after hammer milling where required) and subjected to a cellulose digest. A biological oxygen demand measurement was made before and after the cellulose digest. These measurements of metabolisable energy were converted to an energy supply rate by establishing a theoretical gut retention time based on the time a standard amount of cellulose takes to digest the cellulose in a unit mass of sample. The results of the *in vivo* and *in vitro* measurements were qualitatively similar but with the *in vitro* test energy was accessed more quickly and therefore had a higher power output rate. It is suggested that ecosystem or community energetics are dependent on energy availability rather than total energy content and that the importance of this distinction will vary between biomes.

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Glossary

Biomass.	The total dry mass of organic matter of a specified kind e.g. wood biomass.
Biome.	The largest land community usually recognised by the predominant plant community, e.g. tropical forest.
Ecosystem.	A conceptual view of a plant and animal community (of any size with any boundary) and the interaction between living and non-living parts.
Edibility.	How fit a substance is to be eaten, from the substrate perspective.
Food chain.	A number of organisms that form a series through which energy is passed by feeding.
Foodweb.	A description of an ecosystem depicting the flow of energy from organism to organism, as in food chain but represented as an interlinked complex network.
Guild.	A collection or group of organisms from one area made on the basis of similar phenology and morphology.
Decomposition.	The breaking down of substances into smaller molecules or atoms.
Decomposers.	Heterotrophic organisms, mainly bacteria and fungi, which break down organic material in order to acquire energy.
Digestion.	The process by which food material ingested by an organism is rendered soluble and assimilable by the action of enzymes.
Palatability.	Agreeable to taste. How fit a substance is to be eaten from the perspective of the eater (compare with edibility).
Recalcitrant.	The resistance of a substance to decay or degradation.
System.	A group or set of parts that form a complex or connected whole.
Thermodynamics	The mathematical treatment of the relation of heat to mechanical and other forms of energy.
1 st Law	The total energy of a system remains constant although it can be converted to another form, i.e. converted, but not created or destroyed
2 nd Law	No process is possible whose only result is the abstraction of heat from a single heat reservoir for the performance of an equivalent amount of work i.e. the conversion of energy to work can never be 100% efficient
3 rd Law	The entropy of a substance approaches zero as its temperature approaches absolute zero.

Trophic level.

An integer class of organisms within an ecosystem grouped according to their food supply, where the plant is trophic level 1 and the herbivore trophic level 2.

Notation

Notation	In full
N	Nitrogen
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
t	time
K18	Rate constant from t=0 to t=18 days
K115	Rate constant from t=0 to t=115
B1	Rate constant from curve fit
ADL	Acid detergent lignin
ADF	Acid detergent fibre
WSC	Water soluble carbohydrate
VFA	Volatile fatty acid
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
DM	Dry mass
CTAB	Cetyltrimethyl ammonium bromide
TIGER	Terrestrial Initiative in Global Environmental Research
IBP	International Biological Programme
NPP	Net primary productivity
NFP	Net foliar productivity
PQMD	Plant quality minimum dataset

Chapter 1 Ecosystems and trophic chains

This chapter is concerned with the setting of the research. It briefly describes the development of the concepts, identifies the research gap, and illustrates the routes taken to solve these problems.

1.1 Introduction

In 1935 Tansley used the word 'ecosystem' to describe the dependence of organisms on each other and their environment (Hagen 1992). Throughout the history of ecology there has been a need to define and quantify units to allow study of ecosystems and one way of doing this is to use feeding relationships.

Elton (1927 pg56) described the factors limiting the distribution of animal communities. He remarked:

'The primary driving force of all animals is the necessity of finding the right food and enough of it... the whole structure and activities of the community are dependent upon questions of food-supply.'

The recognition of the importance of food on the structure of an ecosystem meant this could be used to explain many of the processes going on within. The size structure of a community is a product of the size relationships of predators and prey organisms (Peters 1983). With basic concepts such as these, ecosystems could be studied to give realistic explanations of their behaviour.

Lindeman (1942) described communities in terms of transfer of energy or 'energy flow' and described the food cycle in terms of efficiency of energy usage at each stage of the trophic chain. Lindeman's trophic transfer model was adopted as a convenient way to study communities and their dynamics. Since then most ecosystems have been defined in these terms with plants described as a homogenous layer of green known as 'producers'. This gave scientists real units to investigate and began the development of ecosystem energetics. To enable science to be of use, future trends must be predictable from a full description of the present. Attempts to discover whether the behaviour of an ecosystem possesses any order began.

This thesis focuses on why the use of energy units as a basis for the study of ecosystem food cycles does not facilitate adequate descriptions of, or understanding of, ecosystems and how improvements to Lindeman's trophic transfer model can be made.

1.1.1 Context

The ecosystem of choice was terrestrial as opposed to aquatic because the plants tend to be more varied in structure and therefore it should be easier to illustrate the difference between these structures. This is a result of the structure of plant material within a fluid medium. The plant requires little support material, and relies on diffusion for surface exchange between tissues and surrounding medium. The compounds produced by terrestrial plants from the energy fixed in photosynthesis are required to support the

leaves for maximum efficiency. This can result in a complex food web making the application of trophic level concepts difficult. It is from the energy fixed in the plant community that all animal communities thrive, and therefore a good understanding of how the energy is transferred is of major importance to ecosystem analysis.

This project is primarily concerned with improvements to the measurement of energy available to herbivores from plant material. In order to describe ecosystems or ecosystem dynamics in terms of energy transfer, a sound basis for measurement is required. It is believed this has been neglected upon adoption of Lindeman's model, as it has been misunderstood by scientists (Cousins 1985). All energy arriving at the earth's surface is derived from the sun and can only be utilised by plants directly. This energy is then used to create various substances through growth or maintenance of the plant and can be made unavailable to the animal community. Because of this plants cannot be viewed as a homogenous layer named 'producers' as used by Lindeman (1942). This project is an attempt to improve this general view of the role of plants in ecosystems. This study was designed to remain on a macro-scale to enable consideration of a full ecosystem and avoid the problems that arise when extrapolating from small-scale studies.

1.2 Effects down the food chain

This study concentrates on the interactions between plants and herbivores. Carnivores are not included. There is a realm of digestive strategies that have evolved to deal with plant tissue, and derive energy from it. This means any measurement of the edibility of plant tissue must be broad based and applicable to most strategies to be of any use. It is argued that all herbivores utilise the services of microorganisms in order to digest plant tissue. From this viewpoint all herbivores are connected, and tests have been developed using the premise that plant materials can be characterised by their ease of digestion by bacteria and other microorganisms. Attempts have been made to keep this study on a macro-scale, avoiding focusing on species-species interactions, although some classification of herbivores and plants is unavoidable and probably useful.

At present herbivores are often classified as to what they eat, for example as specialists or generalists. Plants with a high fibre content are often seen to be nutritionally diluted (Hochuli 1996), because the amount of fibre is very high compared with the amount of nitrogen that is present in the tissue. Within every taxon, there are species whose primary focus is access to cell contents, and others who manage to utilise the fibre component due to their digestive strategies. The cell contents include high levels of nutrients, but the cell walls provide a barrier preventing access. It is the breach of this barrier that is important, whichever food source is required. Cellulose digesters must cleave the chains of glucose monomers joined by β 1-4 links. It has been suggested that herbivores can be classified according to whether they are cell wall or cytoplasm consumers (Abe and Higashi 1991). There is a close relationship between body size and food quality (Demment and Van Soest 1985) and ungulates have been classified according to this (Hofmann 1989). For an animal with a large body size, metabolic demands are high and must be met. Therefore, the food plant must be abundant. An ability to utilise such highly fibrous plants provides an evolutionary advantage. Plant edibility appears to have a far-reaching effect down the food chain. Digestive strategy

can dictate the social structure of ungulates. When body size is small and the high quality parts of the plant are selected, pair bonds are often found socially (Jarman 1974). In the large, free ranging herbivores, herding gives some protection by safety in numbers. Obviously the spatial and temporal availability of the herbivore will then affect the organisms utilising these as prey.

Rate of access to food supply is of primary importance. With a food supply containing readily oxidisable substances, constraints appear to be time spent searching as these foodstuffs are harder to find. Easily digested material as a food source gives rise to a very different way of life. More abundant material has the disadvantage of less easily oxidisable substances in its content and the time required to utilise these substances as food. Between these 2 extremes of body size and food abundance is a sliding scale of relationships where digestibility of food source, rate of access to oxidisable substances, is the basis.

Plants also form the architecture of the ecosystem. For a herbivore living in a forest environment, protection from hunting may be afforded by the furniture of trees. Grasslands provide abundant food, which is necessary to sustain a large body size and population. However visibility of prey to predators is a major disadvantage and provides the main selection pressure on the evolution of a very different predator-prey system: one which relies on the safety provided by the presence and number of herd members.

The key contribution of this thesis is the incorporation of a *rate* to the measurement of the availability of the energy in plant material. When considering feeding relationships, the measurement of the energy supply rate, versus absolute energy content is important. Bomb calorimetry does not include a rate at which the energy is made available and yet this technology is used to apply data for exploration of ecosystem energetics. The absence of a suitable measurement technique for energy availability provides a research gap that this thesis seeks to fill.

1.3 Aim and objectives of the thesis

1.3.1 Aim

The aim of this thesis was to explore alternative methods to measure the energy availability in different plant tissues, to assess the methods already available for doing this, and to devise a new technique with a more biologically representative method. The implications of any new methodology for ecology are considered.

1.3.2 Objectives

- To devise an improved method for measuring the rate at which energy can be accessed from different plant materials.
- To compare existing and new method *in vivo* and *in vitro*
- To develop an approach to standardising the units of measurement for energy availability.

- To explore the potential for the method to provide low cost comparison at the ecosystem level. A method that is inexpensive with small requirement for equipment could be used worldwide making ecosystem measurements comparable even when originating from different biomes.

1.4 Methods development

The methods used to achieve the objectives above were chosen for their suitability for application to a macro-scale study of ecosystem measurement. Support from the literature was sought to emphasise the connection between each and the progression of concepts made clear. Three distinct groups of methods were adopted in the thesis. First, existing plant energy content and quality methods were used to assess the target plant species. Secondly, an *in vivo* method of measuring rate of energy release was used. This was the burial of plant parts in a suitable soil. Thirdly, an *in vitro* study to improve on the repeatability and speed of analysis was developed.

1. A suite of existing tests to characterise biomaterials was selected. Parts of the oak tree (*Quercus robur*) (characteristic of the biome) were subjected to these tests which include:

- Total energy content using a bomb calorimeter
- Analysis of nitrogen, fibre, and water-soluble carbohydrate contents

2. *In vivo* measurement of the availability of energy in the oak tree (*Quercus robur*) parts by the rate of mass loss in soil. This incorporates some of the physical properties of the parts, providing a more holistic approach, and allows a rate dimension to be applied.

3. Development of an *in vitro* method to allow improved measurement of the biologically available energy of oak tree parts. This will be laboratory based to allow standardisation. Attempts will be made to provide a labour saving, low cost method with no need for specialist equipment, which will make it conducive to application around the world.

The measurement of energy availability was made using a power density unit and applied to the data generated in these different ways. This was to enable descriptions of the data generated in comparable terms. This unit was used to illustrate the data in terms of what the materials mean with regard to herbivores and ecosystems.

1.5 Future applications of energy availability measurement

The use of these methods to measure the energy available in plant material, present in an oak woodland ecosystem, demonstrates the utility of the measurement. It enables the:

1. Description of ecosystems and foodweb research enhanced by further division of plants in trophic space.
2. Development of low cost new methods for application to litter quality, ecological research, animal feeds etc.

3. Facilitation of standard methods for analysis of plant materials, allowing comparative ecological studies around the world.

It is thought an improved measure of edibility would facilitate better descriptions of ecosystems and understanding of interactions and of perturbations within the ecosystem. The fact that data on the status of plant material is collected in many different ways is a problem in research. For example, decomposition studies cannot be compared as the data is collected in many different sampling regimes, and the material analysed using different techniques in the laboratory. Comparison of decomposition processes between ecosystems could provide important information.

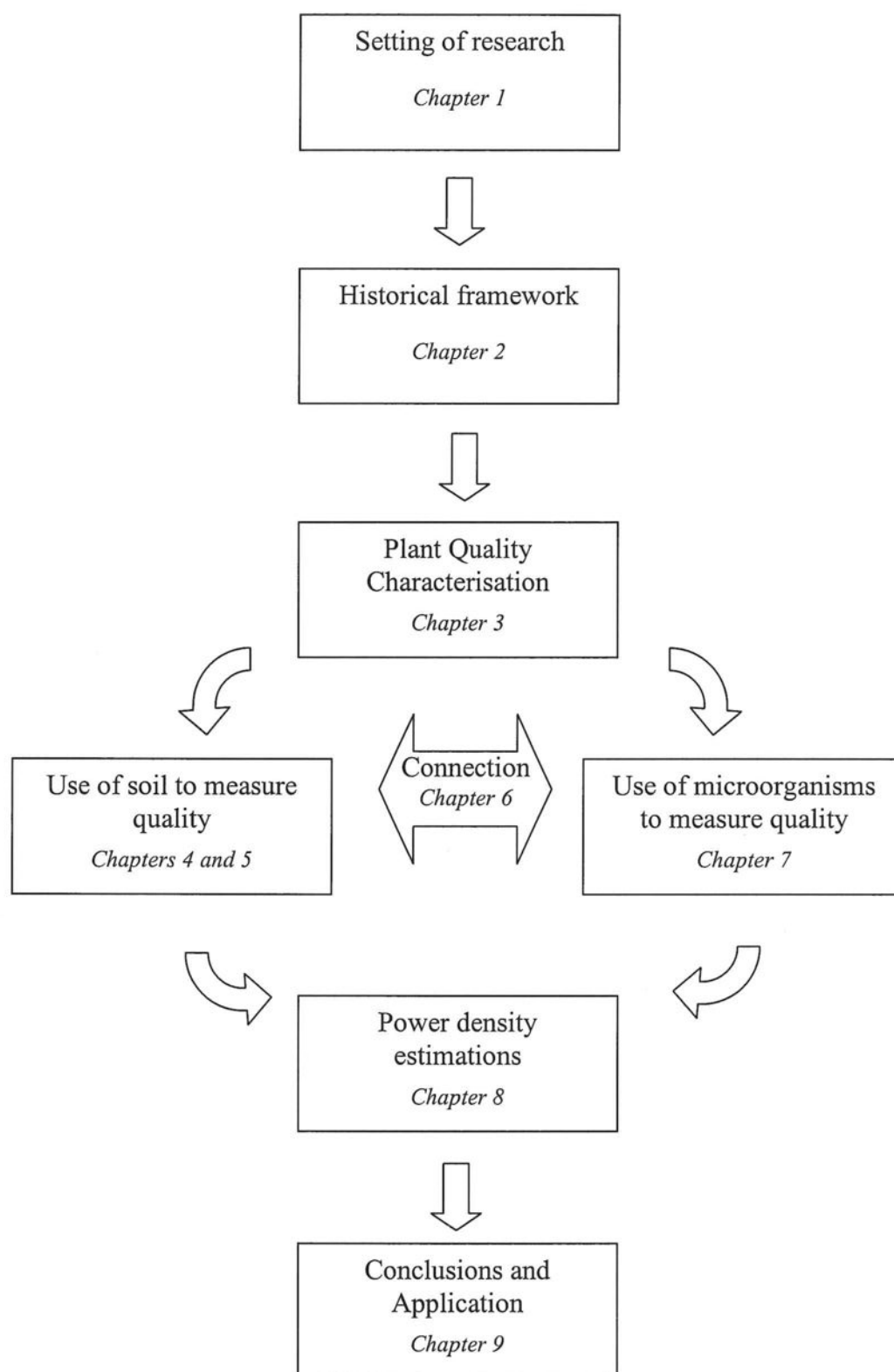
An adequate understanding of plant edibility is important to several disciplines. Agricultural literature is centred on plant quality with respect to the ruminant. There is still much to learn about how rumen microorganisms are affected by variations in quality of foodstuffs and a more holistic approach could be beneficial. Because species specific studies predominate, it is difficult to pinpoint the important parameters that define plant quality in a more general or fundamental way.

Modelling of ecosystem changes has now advanced to incorporate some vegetation parameters, for example, lignin contents of ecosystems. This is because the importance of the type of vegetation cover with regard to hydrological cycles has been recognised (International Council of Scientific Unions 1990). This thesis may provide important information for ecosystem models. An understanding of how plant quality affects the ecosystem is fundamental to predicting change.

1.6 An overview of the thesis

This chapter and the next are concerned with the context of the research. A brief overview and the background of macro-scale ecological studies are given. This includes the development of the science of ecology and the important principles that have been used and developed by researchers. Chapter three is a literature review of the ways in which scientists have viewed plant tissues, and the methods used to analyse plant materials for different purposes. Chapter four contains details of the pilot study with plant tissues from cow parsley (*Anthriscus sylvestris*) used to develop the decomposition technique. The full-scale study developed from the pilot is given in chapter five where the details of the rate of decomposition of 6 different plant tissues derived from the oak tree (*Quercus robur*) provide the *in vivo* measurement of the energy availability of these plant materials. Chapter six links the *in vivo* and *in vitro* measures developed. Chapter seven contains the development of a standard laboratory method to measure energy availability. Chapter eight gives the results of the application of a power density unit applied to the data generated by both the *in vivo* and *in vitro* measurements. Chapter nine is a discussion of the implications this thesis has for various applications. Figure 1.1 gives a pictorial view of the thesis layout.

Figure 1.1. A plan of the format of the thesis



Chapter 2 The development of ecosystem concepts

2.1 Introduction

The ways in which ecosystems are defined and studied are fundamental to the science of ecology. Scientists require patterns in order to predict the behaviour of systems. The nature of random occurrence, means some patterns will be found. It then becomes necessary to discover if these patterns are indicative of future trends.

This study shifts the focus onto the significant or defining features of ecosystems and what causes differences between them. The energy flows through an ecosystem are possibly the most important changes that occur, so the heart of this study is on the changes that occur to the incident energy and the first interaction: the plant herbivore interface.

"...plants alone are able to turn raw sunlight and chemicals into a form edible to animals."

Elton (1927 pg 56)

Plants are the first users of the energy incident on the earth from the sun. They utilise it in the photosynthetic process which produces various organic compounds required for growth. Plant tissue, therefore, is a potential source of energy for heterotrophs. In theory these organic compounds can be oxidised and provide energy for another organism. In reality much of this energy is bound up in complex compounds and is rendered unavailable. Compounds such as lignin are not readily oxidised, and therefore the energy within cannot be utilised in a short space of time.

The enormous variety and distribution of plant and animal communities in the world is so great, there have been many attempts to define and classify communities to find order within the complexity. Biome was a term coined by Frederic Clements in 1916 to describe the combination of plants and animals in a community (Golley 1991). Their plant families or functional types largely separate biomes. By studying the oak, the results have biome significance due to the importance of oak in southern Britain. This study endeavours to provide a new method, which could be used to describe each biome in terms of the plant population. How herbivores access and utilise the energy in plant material is related to the types of plant material present. In 1935 Tansley used the word ecosystem to describe the dependence of organisms on each other and their environment (Hagen 1992). An ecosystem is defined as:

A conceptual view of a plant and animal community, emphasising the interactions between living and non-living parts and the flow of materials and energy between these parts (Wordsworth dictionary 1995).

This study used a temperate ecosystem (natural ancient woodland), and a species that typically characterises this, the oak, *Quercus robur*. Ecosystem processes radiate from the ability of plant material to supply energy to the herbivore population, or looking at it another way, the herbivore population evolved to utilise the energy stored within the plant.

Attempts to simplify the complexity of an ecosystem and to describe patterns were important if ecology was to develop as a science. Ecologists developed ideas in order to explain the associations within a habitat, mainly focusing on feeding relationships. Elton (1927 pg 56) described the factors limiting the distribution of animal communities:

'The primary driving force of all animals is the necessity of finding the right food and enough of it... the whole structure and activities of the community are dependent upon questions of food-supply.'

He also attached some importance to the fact that communities are rarely constant, especially with regard to abundance, and that populations vary cyclically. He described a community as chains of animals linked together by food, all dependent ultimately on plants and refers to all the food-chains in a community as the food-cycle. Elton also wrote of the importance of the size of animals within a community (pg59):

'Size has a remarkably great influence on the organization of animal communities.'

He believed size to be the basis of his 'food cycle' and depicted a cycle for a herring indicating the change in food taken at various ages i.e. size classes. There are lower and upper limits to the size of food an animal can consider.

Lindeman (1942) developed the ecosystem concept by describing communities in terms of transfer of energy or 'energy flow'. He grouped organisms according to their particular sources of energy i.e. plants as producers, herbivores as primary consumers and so on, and described the food cycle in terms of efficiency of energy usage at each stage of the chain. Elton's 'pyramid of numbers' within a community was expressed by Lindeman in terms of biomass, although in fact there is no link between size class and feeding level; all sizes occur at all levels (Cousins 1985). He described succession hypothetically as changes in productivity with time.

In 1953, E.P.Odum applied the laws of thermodynamics to the concept of food chains, pyramids of numbers, biomass, and productivity (Golley 1991) and developed a hypothesis concerning bioenergetics and how it varied with the development of an ecosystem. The concept behind this was to eliminate unnecessary confusion and study ecosystems on a macro scale. Trophic level structure was applied to aid this.

In the 60's, Hairston, Smith, and Slobodkin, usually referred to as HSS, developed a theory to explain how communities were regulated. Predators control the number of herbivores, which in turn control plant communities, both structure and abundance. This approach utilises trophic level structure to explain dynamics and has been applied to theoretical communities by many scientists (Fretwell 1977) but has been challenged by many population ecologists (Oksanen 1991).

Lindeman's theories were also adopted by some scientists, who had become disillusioned by the ways in which some complex ecosystems were tackled. These new concepts allowed scientists to view the system as a whole (Sheail 1987). The International Biological Program (IBP) was developed in 1964 and initially entitled "The Biological Basis of Productivity and Human Welfare". This became an attempt to describe the world's major biomes in terms of trophic levels. 1,800 American scientists, funded by 40-60 million dollars, participated in ecosystem studies (Hagen 1992). However, the IBP failed to meet many of the objectives required of it (Golley 1991),

such as an accurate way of measuring energy flows through ecosystems allowing biome comparisons.

After the end of the IBP in 1974, research continued on the basis of Lindeman's trophic levels, where the application of the theory was possible. This led to 'small-scale' research either in enclosed, fairly static ecosystems or applied to parts of ecosystems. The development of more mechanistic studies introduced modelling attempts, to make the developments applicable to all habitats, or at least more of them. Optimal foraging models appear to be a move towards understanding the role of size in feeding relationships (Cousins 1987).

Criticism of the IBP has been widespread (Hagen 1992; Golley 1991; Cousins 1985; 1987; Lawton 1989). However, the development of such a program has brought much needed funding into the science of ecology and perhaps paved the way for more co-ordinated large scale ecological research such as the Ecosystem Studies Program, largely focusing on systems ecology (Golley 1991).

2.2 The application of trophic levels to systems

Lindeman (1942) structured the ecosystem concept as a system of any size and with any boundary. He discussed the quantitative relationships of the rate of incident solar radiation, the rate of photosynthetic production, rate of primary consumption and so on, with corrections for respiration, predation and decomposition. Rate of change of energy content in a food cycle is:

$$\frac{d\Lambda_n}{dt} = \lambda_n + \lambda'_n, \quad \text{eq1}$$

Where Λ_n = the energy content at level n

λ_n = the rate of contribution of energy from Λ_{n-1} (the previous trophic level)

λ'_n = the rate of energy contribution of Λ_n to Λ_{n+1} i.e. the next level

(Hutchinson in Lindeman 1942).

This theory was applied to illustrate the presumed increased efficiency of energy flux through levels of feeding that occurs throughout succession. He described the changes of energy flow that occur in the various stages of succession. In doing so, Elton's paradigm of body size and abundance relationships was displaced, as the trophic level framework appeared to be a useful way of simplifying complexity. This approach also became the basis of the IBP. Lindeman's paper illustrates the difficulty of simplifying systems without sacrificing essential information (Burns 1989).

2.2.1 The search for order

In systems ecology there has been a search to identify key interactions to predict behaviour of a system. Living systems appear to evolve to complex adaptive systems and an understanding of how this complexity arises is sought (Ruthen 1993). Biologically unrealistic simplifications are common and ignore the problems associated with complexity (Rossiter 1993). Trophic transfer is a prime example. Feeding relationships in an ecosystem are important, and it may be possible to use these to illustrate change over a long period of time.

Patterns have been discovered that show there are similarities between the diverse ecosystems, such as the species relative abundance (SRA) (see May 1985), but the mechanisms behind them remain elusive. Briand's food web catalogue has been used to show the similarities (Briand and Cohen 1987). One similarity is the ratio of numbers of species on each level, which is 0.12: 0.53: 0.29, (primary consumers: intermediate: top predators) (May 1985). It has to be acknowledged that patterns are likely to vary between ecosystems and that extrapolation from one group to another may not be possible.

2.2.2 How productivity is quantified

Plants have obvious primacy in food webs but there is a need to resolve operational definitions when applying food web theories (Power 1992). The productivity of an ecosystem can be measured in terms of the amount of energy from the sun utilised by green plants. Gross primary productivity (GPP) is the rate of photosynthesis of the community and net primary productivity (NPP) involves a correction for the amount of respiration that occurs within the time period. This value can be misleading, as the productivity is not necessarily made available, for example, wood production in forest ecosystems (McNaughton *et al.* 1989).

Empirically, measures used to predict GPP or NPP range from annual precipitation (this is closely linked to production in some systems) in desert ecosystems to amounts of phosphorous in temperate lakes (Power 1992). These give approximate estimations, and form the basis of many studies. As rarely all the plant is eaten, it can be difficult to ascertain how much of the plant is actually assimilated by the herbivore.

The number of levels cannot be predicted by productivity (Hairston and Hairston 1993). However, the herbivore productivity of a system is positively correlated to a revised measure of NPP, which is net productivity to foliage (NFP) (McNaughton *et al.* 1989). This allows for the fact that much of the net productivity of plants is used to produce a poor quality food containing lignin that is largely unavailable to most herbivores (McNaughton *et al.* 1989). In terrestrial systems, only a small proportion of the productivity is consumed by herbivores. Figures range from <10% for some temperate ecosystems up to 60% for African tropical grassland (Sinclair 1975). African grasslands have been shown to support high levels of herbivory (McNaughton *et al.* 1989; Sinclair 1975), which provides evidence to contradict the Hairston, Smith and Slobodkin theory that herbivores are not food limited, especially when it is taken into account that not all productivity can be utilised by the herbivore. In the non-growing season plant tissue can change and become unpalatable. Grasses become lignified, and other leaves chemically

change and have less of a value as food. The apparent abundance of leaves can be misleading (Sinclair 1975). This is probably why energy transfer is sometimes discussed in terms of nutrient transfer (see Oksanen 1991; Leibold 1989).

Recognition of the fact that not all productivity is available to the herbivore is a key step in ensuring measurements of energy transfer are more realistic.

2.2.3 Energy Transfer through systems

2.2.3.1 A limit to number of levels?

There is debate about what limits the number of trophic levels in an ecosystem (Hairston and Hairston 1993; Fretwell 1977). There does not appear to be a correlation between productivity and the number of trophic levels in a system (Leibold 1989; May 1986). High levels of productivity are associated with high levels of folivory and rapid recycling of nutrients through wastes (McNaughton 1989), but not necessarily enhanced transfer of energy to enable creation of further levels. Viewing ecosystems in terms of trophic levels hampers the unravelling of the processes that occur. Increased productivity may affect animals further down the food chain, but the length of chain is probably not a reflection of this (Cousins 1980). It may be that food chains are not limited by energy, except in extreme cases, and this would explain why longer chains do not occur in more productive systems. There may be an advantage in feeding lower in the chain where food is more abundant (Pimm and Lawton 1977). This question only arises because bomb calorimetry is used and as a consequence energy availability dimensioned in kWkg^{-1} is not measured. One of the significant consequences of this thesis is to be able to recast ecology without trophic level concepts

2.2.3.2 Population regulation

To understand what controls a population size it is necessary to understand what constrains an individual and the use of a 'currency' allows this. It is identification of an appropriate currency to use that is important (Hanley 1982). Measures of energy flow are poor indicators of which population interactions are important in regulation of a population (Crawley 1983) possibly because of the difficulty of measuring this in practice. Energy flow is also a poor estimate of feeding impact where there is wastage, where the damage is localised, or where diseases transmitted by herbivores cause damage (Crawley 1983).

The importance of trophic interactions in determining distributions and abundance of organisms is still under debate (Power 1992). Hairston, Smith and Slobodkin (1960) proposed that organisms whose resources are abundant are regulated by predation, and organisms with rare resources are regulated by food. This is supported by McNaughton *et al.* (1989) findings that highly productive ecosystems sustain a larger level of herbivory per unit of primary production than unproductive systems. Abundance, population size, and regulation have been well studied to discover controlling factors (Athey and Connor 1989; Raw 1962; Varley *et al.* 1973; Zimmer and Topp 1997). Whether there is productivity or predation control on herbivore populations is could be

system specific. These are often described as "top-down" or "bottom-up" controls. Aquatic systems have been found to contain herbivorous zooplankton that can have different effects on the responses of the system to nutrients, or predation intensity. Leibold (1989) found nutrients might "cascade up" to the predator level and increase the biomass or abundance of zooplankton. Sinclair (1975) suggests all levels are resource limited. There is considerable controversy surrounding this debate, as predator-prey interactions are widely variable. Models used to describe interactions between predators and prey have undergone development by bear small resemblance to real associations (May 1986). May (1986 pg 1120) explains this with the statement:

"What works for Cyzenis and the winter moth is simply infeasible for lions and zebras".

The development of models represents a scientist's need to understand the regulating factors or processes in order to apply rules.

Parasites and diseases are rarely included in ecosystem process studies unless they impact on man's activities, although it is generally accepted for parasitism to be the norm in nature. These organisms can sometimes have a large influence on the structure of a community. The example of rabies and African wild dogs (*Lycaon pictus*) indicates how important these factors can be when considering population dynamics.

2.2.3.3 *Abundance and body size*

Elton (1927) first recognised that numbers of organisms dwindled as the food cycle advanced. He developed a 'pyramid of numbers' to depict this. This can be applied to populations of organisms involved in a food chain. However, when converted to the number of species in a trophic level, which is often done, the problems of assigning species to a level occurs. This is a misrepresentation of Elton's ideas.

Body size was first recognised as an important factor when describing communities by Elton (1927). Body size and body plan plays a crucial part in the dynamics of ecosystems. Knowledge of a body plan allows prediction of an organism's future actions, for example, if it has wings it can usually fly. Allowing for the inevitable exceptions in nature, such as pack hunters, predators are larger than the species (or plant part) they prey upon. The importance of this notion is now utilised in models such as the cascade model developed by Cohen and Newman (1988). Body size offers a key to understanding structure in food webs (Cousins 1996). Each trophic level contains animals of a wide size range and all trophic levels can be found in one particular size class (Cousins 1985).

2.2.3.4 *Stable isotopes*

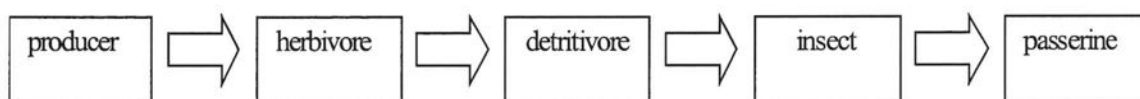
The use of stable isotopes for environmental research has grown since the pioneering work of Urey (1947) who developed the instrumentation for precise isotope abundance measurements (Bowie 1974). Stable isotopes are currently used to study many geochemical processes in the environment and have increased knowledge of the fate of contaminants and pollutants in the biosphere (Gough 1993). More importantly stable isotopes have been used to study aspects of elements and their cycling and have therefore been used to study long term ecosystem processes such as climate change. The

effect of environmental disturbance and fate of contaminants such as nitrogenous pollutants in a forest ecosystem can be evaluated. Linking stable C with stable N isotopic measurements provides a powerful approach towards identifying ecosystem components and biogeochemical processes that are most sensitive to disturbances (Korontzi *et al.* 2000). The use of them in studies of ecosystem processes such as decomposition (Bottner *et al.* 2000) and hydrological pathways (Soulsby *et al.* 2000) is increasing.

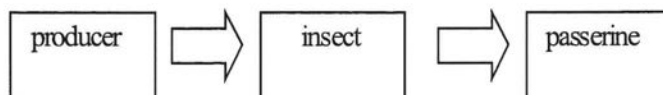
From an ecosystem perspective stable isotopes have a function in food web research as they provide a way in which the path of nutrients and energy flow can be measured. The use of stable isotopes for food web research and ecosystem trophic transfer is increasing. France *et al.* 1998 relate foodweb organism size structure with ratios of isotopes, in order to deal with the non-functionality of trophic levels. Similarly Vander Zanden *et al.* (2000) studied body size and trophic level position using stable isotopes but found no relationship between them. In a review of 102 stable isotope studies, Kelly (2000) found that marine carnivores that ate vertebrates had nitrogen-isotope ratios that were enriched over the ratios of those that ate invertebrates, showing that taxonomic effects may mask any trophic level effects with regard to terrestrial systems. Wood and leaves are both positioned at trophic level 1 and anything feeding on either is positioned at trophic level 2. To revise these categories in a more detailed way using stable isotopes is to ignore the inadequacy of this classification if foodwebs are driven by energy availability.

2.3 The use of food webs to define ecosystems

Figure 2.1. Examples of food chains for a passerine.



4 food chain steps: passerine feeds at 5th trophic level



2 food chain steps: passerine feeds at secondary consumer level

As a central theory in ecology, feeding relationships are often used to define community organisation. It has widespread application but can be refined. Food chain dynamics has usefulness to evolutionary ecology e.g. body size relationships (Fretwell 1987).

Cousins (1985) illustrates how an organism can appear at different levels depending on the history of a particular food item. For example, figure 3.1 shows how this affects the trophic level of a passerine.

There is a problem placing organisms into trophic levels when they feed from different layers (Cousins 1985) and there is evidence to suggest omnivory is common in real systems (Lawton 1989; Power 1992). Specialist feeders can easily be distinguished but generalists or opportunists can possibly cause complications.

Hairston and Hairston (1993) describe the plant layer as producers and argue trophic structure can still be used. However, they mention the different components:

"No seeds are involved in photosynthesis and do not constitute a major energy component of primary production"

And

"Virtually all omnivores in terrestrial communities consume seeds rather than leaves".

Plant material produced from the incident energy is not uniform and can not be assigned to one level of energy quantity or quality. It is well known that population fluctuations of a herbivore can vary due to the abundance of seeds in a certain year. Dead organic matter is treated separately in the paper. The majority of dead matter consists of plant tissue and it is the compartmenting of energy in the producer 'layer' that is important. This dictates the availability of energy in the detritus. The more recalcitrant a tissue, the longer it remains trapped in this pool. Again we encounter the question of rate of supply.

A food web does not recognise changes in state such as growth of animals (Cousins 1985). Elton (1927) depicts the change in the food cycle for a herring as it ages and shows how prey choice changes. A food web includes all species in a given location (Yodzis 1988) and attempts to place every possible feeding combination with no acknowledgement of time. It is difficult to include every stage of a life cycle, especially when some organisms have so many. The food taken will depend on the preferred food of the species, what is available at the time of year of the study, and the abiotic environment, for example, snow cover. Red deer are sometimes forced to take *Molinia* in winter, especially in nutrient poor areas, a plant species normally avoided.

Within any animal population, variation in behaviour, and therefore food choice, will occur. Food webs give no indication of the strength of feeding links. Conversely, individual traits can be important. Regulation of populations can stem from variation within individuals, which can become masked by data processing (Rossiter 1993). Evolutionary or population ecologists who use trophic levels can be said to be studying communities containing an extended guild (Oksanen 1991).

Detritus is a huge source of energy in many ecosystems and cannot easily be assigned to a particular level (Cousins 1987). McNaughton *et al.* (1989) treat detritus as a separate decomposer food web. Hutchinson's original definition of assigning detritus (from Cousins 1987) is to place material according to the number of acts of assimilation it has undergone since being part of a green plant. In the case of herbivore dung, as unassimilated plant material, it remains at the productivity level even though it has been

'processed' and made available to different organisms. Detritivores are also a problem when assigning trophic levels, as many are omnivorous or opportunistic.

Many scientists use a separate decomposer or detritus-based food web. These often lack distinct trophic levels and the fact that detritus based and grazer based systems are often interconnected confuses this issue (Oksanen 1991).

2.3.1 Discussion

There is a growing trend to use trophic level theory as a basis for investigations, but serious difficulty in applying this to the real world (Power 1992; Lawton 1989). It is currently decided arbitrarily which links are too weak or unusual to be included (May 1986). Although this concept facilitates modelling, there are obviously restrictions which should be exercised when applying an "inaccurate tool" to describe feeding and energy relationships.

Several scientists are beginning to doubt the validity of trophic levels (Oksanen 1991; Cousins 1985; Lawton 1989; Golley 1991). Some studies presume that the division of ecosystems into discrete trophic levels will reveal patterns in ecosystem structure or function but ignore the serious operational problems presented by such a division (Peters 1991). Improved measures of energy fixed and transferred through ecosystems are needed for ecology to develop as a science. If measures can be applied widely then global approaches such as the IGBP can provide important information for use in land-use change and climate change modelling. Measures of productivity using methods such as satellite imagery are generally easier to carry out than measures at the consumer level and if they provide information on the resulting food web predictions can be made (McNaughton *et al.* 1989).

Recently there has been a move to understand the implications of Elton's ideas due to the limits imposed by the trophic level concepts. In order for further progress in food web research to be made there is a need for standardisation (Cohen *et al.* 1993) as food web studies contain limited data of highly variable quality (Lawton 1989). Cohen *et al.* (1993) calls for 'more explicitness and more exhaustiveness' in research. Long term studies on selected ecosystems are needed to monitor certain parameters in order to reduce complexity (Rossiter 1993).

Do we need to classify communities? Ecosystem research is a necessary part of present day ecology. Fate of pollutants, bioaccumulation, pest management, and conservation would all benefit from a better understanding of community ecology (Cohen *et al.* 1993). Berryman (1993) argues only one or a few factors determine the dynamics of any particular ecosystem component and that this is all that would be required by any applied ecologist. However, without detailed studies how is a key-factor determined? With short research periods it would be possible to miss interactions with rarer species.

Instead of providing an aid to simplifying dynamics of an ecosystem, trophic levels seem to be an inoperable system. Instead a new approach is proposed, based on current measurement of biological properties, rather than the assignment to unobservable energy categories: trophic levels. This change in outlook is also dependent on change in the technology used to measure energy flow rates as explored further in this thesis.

2.4 Improvements to the trophic level paradigm

Communities can be separated on the basis of what organisms eat. Oksanen (1991) states:

"It takes entirely different kinds of adaptations to photosynthesise, to consume fibre-rich vegetative plant organs and to capture mobile prey"

This conforms to the idea that organisms can be classified on their "body plan" (Cousins 1996). However, the energy captured by the plant can be transformed into many materials, not fibre alone, and it is this idea that encapsulates this thesis. As ecosystems have been described on a plant basis and all energy is fixed there, this is an improvement to the producer concept and how plant communities can be viewed.

It is believed that a better understanding of how energy from the sun can be made available or unavailable to the herbivore via the plant, a true basis for ecosystem description and classification would be formed. A measure of how much energy could be utilised by the herbivore, or functional group of herbivore, would allow prediction of trophic flows or population dynamics.

In order to improve the measurement of energy transfer through the ecosystem, it is necessary to have an understanding of how energy is fixed and how it is transferred by the organisms present in the ecosystem. The next part of this chapter is concerned with this.

2.4.1 Adenosine triphosphate (ATP) as an energy currency

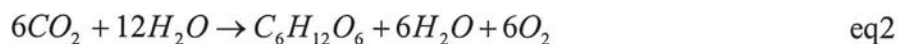
Herbivores must acquire energy from a variety of plant materials for all their energetic requirements. This, therefore, is obtained from the single source of the sun. All animals are heterotrophic. All their energy is derived from autotrophic organisms. Oxidation of carbon compounds yields energy which can then be used to synthesise ATP (energy currency) (Eckert and Randall 1983). Energy is transferred down the food chain in this way. It is commonly written that energy is 'lost' at each step in a food chain as not all can be transferred to the next step (2nd law of thermodynamics).

All biosynthesis requires the use of ATP or other compound containing the reactive phosphate group. The compounds are reactive because of the high-energy linkage between the phosphate groups. ATP can be seen as having the role of trapping a portion of the free energy made available in fuelling reactions, and driving biosynthetic reactions by activating certain intermediates (Stanier *et al.* 1986). In this way all animals are linked. All animals must capture energy released from oxidation of food in high-energy molecules to use in metabolic pathways for maintenance and growth.

2.4.2 Photosynthesis and respiration

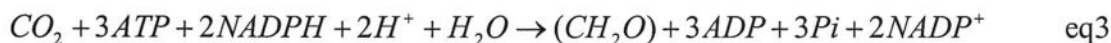
Energy flows through an ecosystem can be viewed in terms of carbon-carbon bonds (energy is passed from one organism to another in organic compounds). Photosynthesis is the process by which energy is fixed in a C-C bond. Respiration is the opposite of photosynthesis and involves the breakdown of C-C bonds, releasing the energy that can then be utilised by the organism. The inorganic nutrients required for this are sourced

from within the system and recycle within it, whereas energy is gained from the sun and later lost from the ecosystem. Every use of chemical energy involves loss of heat. Chemicals cycle within the ecosystem in various forms, whereas energy can be viewed as having a linear flow through the ecosystem.



Equation 2 shows the overall reaction that occurs in photosynthesis. The essence of the reaction is light energy utilised by chlorophyll and used to synthesise ATP and NADPH. The oxygen evolved in photosynthesis comes from the water molecule (and this leaves hydrogen ions and electrons, which are subsequently used in an electron transport system).

Photons are trapped and transferred down the chlorophylls to a reaction centre chlorophyll. This requires the interaction of two processes or photosystems. In photosystem I, when an electron is excited by this energy it is donated to an acceptor and moves through a cyclic pathway which forms ATP. This is cyclic phosphorylation. $NADP^+$ is reduced to NADPH when the electron travels a non-cyclic route. As NADPH is produced from the photosystem I electron, so photosystem II is involved in ultimately donating an electron back to photosystem I. Through this process ATP is generated, and via this route is non-cyclic phosphorylation.

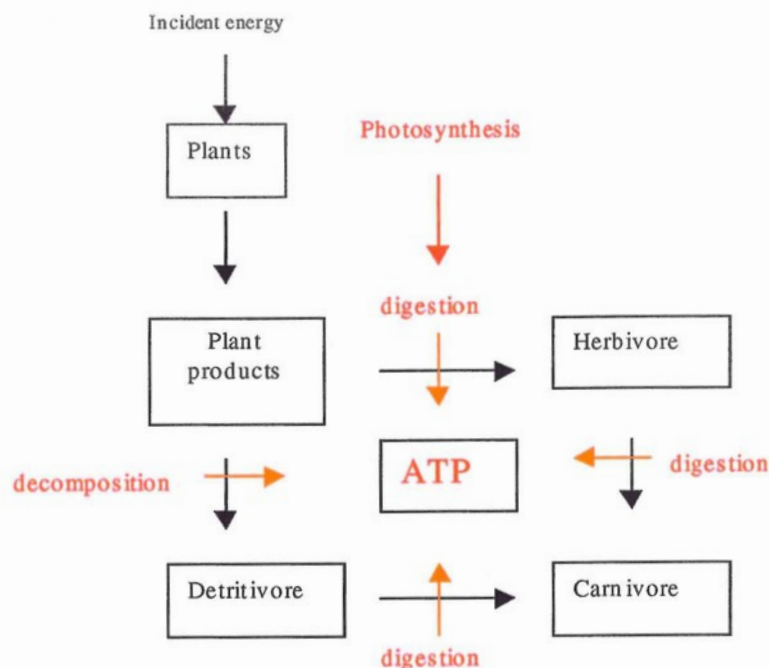


CO_2 and H_2O are fixed

Respiration is the opposite of this and releases these molecules. Respiration is the mechanism which most organisms release energy from molecules for their use. The oxidative systems within this metabolism are very similar in process. All serve to oxidise organic substances, whether aerobically or anaerobically. That is, all respiration processes involve oxidation, and use electron transfer systems within a membrane. This is another reason a biological technology was used as a basis for this thesis. All microbes use electron transfer systems, and all energy derives from the sun (disregarding exceptional circumstances).

Figure 2.2 shows how energy flows through a system and the processes employed to enable this. Incident energy is used by plants in photosynthesis, and the arrows show the flow of energy. The red arrows depict the processes effecting the energy transfer. The plant material produced is utilised by either the detritivore or the herbivore. In the former, decomposition processes breakdown the plant material to release energy and in the latter, digestion processes break down the plant material. This energy is used to form high-energy molecules such as ATP.

Figure 2.2. The flow of energy through an ecosystem



2.4.3 Power: energy per unit time

In the 19th century, Lotka introduced a fourth law of thermodynamics as the maximum power principle. This is applied to systems and the general idea is that there is a strong selection for systems that can obtain the most energy, and use it efficiently per unit time (Hall and Odum 1995). Odum developed this concept via information theory and coined the use of 'emergy' to mean energy corrected for its quality value but failed to make direct measurements of this. Odum continued to deal with energy flows in a trophic level framework ignoring the fact that trophic levels do not illustrate a system accurately. It is believed a power principle is required, but the fundamental implication of this, the fact that a time or rate *measurement* must be incorporated, has been overlooked.

Energy is measured in joules, courtesy of James Joule. Power units are Joules per second, or energy per unit time. If an organism's rate of access to the energy supply can be measured then, an improvement to the description of energy flows is made.

2.4.4 The effect of edibility on the evolution of digestive strategies in the herbivore

Fibre provides the physical structure or architecture of the community. In a forest biome, although there may be plenty of new green growth in the canopy, it is only available to arboreal species. In a grassland community the new growth is spatially available to grazers. The fibre content (probably the lignin part) of the community is the distinguishing variable. Lignin is a cell wall constituent that is not available to the herbivore community.

Differences in digestion strategies arise from the differences in the constituents of different plant groups. Grasses contain more cell wall than browse species (woody plants) but are less lignified. In different organisms, digestive strategies have evolved to deal with all plant groups.

A herbivore must ingest around 3 times as much dry matter as a carnivore (Karasov 1990), which means more time must be spent by a herbivorous animal in acquiring energy.

2.4.4.1 *Ungulates (hoofed mammals).*

Hofmann (1989) grouped ruminants according to their digestive systems; he studied the physiological differences between species and used three classes:

- i. Concentrate selectors, feeding on high quality parts that are low in fibre,
- ii. Grass and roughage eaters mainly utilising the fibre,
- iii. Intermediate types that can switch between palatable and non-palatable species (adapted to seasonal change in availability).

Hofmann predicted that in order to deal with large amounts of fibre, the grass eaters had to be of large body size (see section 6.5). There are 3 strategies within the fermentation bracket: ruminants as foregut fermenters, colon fermenters such as pigs and equids, and caecal fermenters such as lagomorphs (rabbits and hares). The latter must exercise coprophagy after first passage through digestive tract, as the microbes are located in a region beyond the absorptive area.

As with most herbivores, ruminants are reliant on microbial symbiosis as they are incapable of cellulase enzyme production (Van Soest 1987) and therefore must have large stomachs and a long retention time of food in order to 'ferment' the plant tissue. A long retention time enables the microbial 'culture' to grow and multiply, increasing the amount of cellulase production. Fermentation strategies cannot be utilised by browsing species to a large extent, as the presence of lignin renders much of the cellulose indigestible. Grasses, as they contain large amounts of cellulose compared with other species, are therefore nutritious when viewed from the fermentation perspective.

Herbivores within the browser niche are adapted to the utilisation of higher quality plant parts, which possess less fibre and more cytoplasm. This group tends to have a faster acting digestion strategy. This may be to provide some sort of protection against plant secondary metabolites (Hofmann 1989) or it may be that small retention time is all that is required for a higher quality plant product. Cell contents are highly digestible (about 98%) (Van Soest 1987).

Abe and Higashi (1991) propose that herbivores can be reclassified as to whether they are cell wall or cytoplasm consumers. Cell wall consumers can utilise a high fibre diet that is available in quantity, whereas cytoplasm consumers utilise high quality but low quantity food. Cytoplasm consumers must therefore deal with the secondary metabolites that are present in this compartment. Cell wall consumers must balance the C:N ratio of their food by adding N or eliminating C and microbial symbionts can be used for this. If a combination of these strategies is used then they are termed generalists.

Demment and Van Soest (1985) found herbivores are constrained to a range of foods on a particular 'fiber curve' on which the lower limits are determined by abundance of the food and the upper limits by digestive capacity. An inverse relationship between body size and food quality was found. The efficiency in which large generalist ungulates convert plant protein to animal protein is around 0.4% whereas small mammals and invertebrates are around four or five times more efficient (Sinclair 1975).

Jarman (1974) applied the concept that food items eaten dictate the social organisation of African antelope. He noted selection in feeding was made for both plant species and plant parts and this was largely dependent on body size of the feeder. A large organism requires a large amount of 'energy' to survive and so must select plants or plant parts which are abundant in that ecosystem. A smaller organism can afford to search for higher quality parts such as fruits, which are less abundant but have higher reward. He then grouped the antelope in five classes and noted the complete correspondence between feeding style and social behaviour.

The quality and quantity of food ingested are intimately linked. Low quality food must have a longer retention time in the digestive tract than higher quality, which is limiting to the animal (Iason and Van Wieren 1999). All animals regardless of digestive strategy must be flexible in their foraging, digestive, and metabolic strategies to allow for seasonal fluctuations in ability to digest the food. However, body size is a fixed constraint to retention time and ingestive capacity and probably the most important determinant of the requirements of food. There is an inverse relationship of quality of food eaten with body size (Hofmann 1989; Demment and Van Soest 1985), and the increase in body size is linked with an increase in retention time.

In a fluctuating environment, a herbivore must deal with a period of time where the quality of food decreases. The choices available to the animal are:

- a. Utilise a grass-dominated diet in the wet season and change to a browse diet in the dry season
- b. Utilise grass all year, storing body fat to cope with the decrease in quality in the unfavourable season.

It has been suggested there is strong selection pressure for endurance on a poor diet and therefore for an increased body mass (Gordon and Illius 1996). The animal would therefore have access to abundant food when conditions are good and could store body fat in this period. In forests generalist consumers are scarce but in grasslands they are prevalent (Abe and Higashi 1991).

It should be noted that animal matter has been discovered in small amounts in the digestive tracts of some species considered strict herbivores. This is probably accidental as insects can be ingested with plant material, but some forest duikers have been observed to eat carrion (Tixier and Duncan 1996).

2.4.4.2 Invertebrates

Invertebrates can be split into two groups:

- a. those consuming nutrient rich, low in fibre plant parts
- b. those utilising the cellulose component

The majority of phytophagous insects have been shown to be able to digest cellulose in order to survive on plant material. This is carried out by the use of symbiotic protozoa, bacteria or fungi housed in the hindgut (Martin 1983). Little is known to what extent the fibre component is digestible in insects (Hochuli *et al.* 1993; Slansky 1990; Martin 1991). Insects have evolved to utilise almost any part of the plant, even highly lignified parts such as wood (e.g. the termite *Trinervitermes trinervoides*) and detritus (e.g. the woodlouse *Porcellio scaber*).

Phytophagous insects, which have no association with microorganisms, consume only nutritious parts of the plant such as fruits and young leaves (Abe and Higashi 1991). The leaf chewers, for example, lepidoptera larvae, are probably gaining nutrients from crushing plant cells and liberating the cell contents. This is supported by the fact that secondary metabolites are located in the cytoplasm. It is thought most phytophagous insects do not digest cellulose, although it has been shown in one species that some uncrushed cells can be digested in the tract (Barbehenn 1992). Production of part of the cellulase complex by the dicot feeding silkworm *Philosamia ricini* has been shown (Pant and Ramana 1989). For the majority of species in the foliage feeding Orthoptera, Coleoptera and Lepidoptera, cellulolytic activity again probably occurs mainly courtesy of symbionts. Dicots are generally higher in protein than monocots (Hofmann 1989) and phytophagous insects are not energy limited due to plant proteins (Martin 1991). The dietary factors that limit insect herbivores are nitrogen or water, rather than source of carbon (Slansky and Rodriguez 1987). The carbon compounds are "dumped" in order to concentrate the nitrogen in the organism, which means the throughput of plant material must be high.

Forb foliage is characterised by high water and nitrogen contents (Slansky and Rodriguez 1987). Because of this there is selection pressure on the plant to produce defences, and they are also associated with higher levels of secondary metabolites. This has led to co-evolution of plant and host, and there is a myriad of detoxification enzymes present in the insect community.

The wood feeders have been shown to utilise enzymes to digest cellulose (Kukor *et al.* 1988), although the presence of microbes in the gut does not mean insects are reliant on these for cellulase production. The wood feeders include groups such as the wood boring larvae of cerambycid beetles, termites, and some cockroaches. Cellulase activity has been found in the salivary glands, and more predominantly in the foregut and midgut (Slaytor 1992). Removal of fungi from the gut does not seem to affect the cellulase activity in some organisms (Slaytor 1992) suggesting they are capable of production of some enzyme. The satisfaction of nitrogen requirements in this group is somewhat of a puzzle. Most possess microbial symbionts to access the cellulose as a carbon source but N is thought to be limiting. The hypothesis that atmospheric N can be fixed by some microbial symbionts in termites has shown contradictory findings (Nazarczuk *et al.* 1981; Slansky and Rodriguez 1987).

Gramnivores may also have to cope with low nutrient levels and N is thought to be limiting (Hochuli 1996). Fibre can be viewed as a dilutant. Gramnivorous caterpillars have been shown to digest some fibre, showing access to cell contents is not the only function of leaf chewers, although the mechanical processing probably plays an important role (Hochuli and Roberts 1996). Extensive chewing is probably necessary due to the relatively short retention time in the gut (section 6.4.5).

2.4.4.3 *Birds*

Bird species can be found feeding on many of the tissues produced by the plant. Food enters the crop where it can be stored for some time and in which it is softened by the uptake of water. Most of the retention time for food in birds occurs in the crop (Karasov 1990). Birds have a short retention time compared with ruminants, which possibly restricts the amount of cellulolysis or extraction of nutrients that can occur.

Galliforme birds such as domesticated chickens, grouse and waterfowl possess ceca which contains cellulolytic microbes. Exposure to a high fibre diet causes increase in the size of this organ, as well as the overall size of the gut, and changes in the microbes housed there. These changes result in an alteration of the digestibility of nutrients (Redig 1989). In an extreme season these birds have to meet an increased demand for energy with a possible lowering in quality of the food available. The mechanisms behind this adaptation is not well understood (Redig 1989).

Seed eaters utilise a more complete form of food and may have less of a need to utilise the cellulose component. For frugivores, nectarivores and seed eaters the retention time of food may be the limiting factor on rate of intake (Karasov 1990).

Body size relationships with energy requirements remain true for birds. With small body size, higher metabolic rates per unit body mass means more energy is required for growth and maintenance per unit body mass. However, passerines, as a group, possess higher metabolic rates than expected (Schmidt-Nielsen 1990). With the exception of this group, the energetic requirement of birds remains the same as a mammal with the same body mass.

2.4.5 Discussion

Comparisons across the animal kingdom are difficult when measurements in the literature differ widely. In the case of highly nutritious food such as nectar, it does not seem to matter what the digestion strategy is, because the utilisation efficiency appears to be 100% (Karasov 1990). Throughout the animal kingdom there is a link between the body size and energetic requirements of the herbivore, and the amount of food available in the environment. Digestive strategies have evolved to deal with this.

Without doubt, herbivores can be grouped according to their food choice and/or strategy of overcoming the fibre barrier. In conclusion, food selection has a far-reaching effect down the food chain, and plant availability and quality are fundamental to shaping the ecosystem. Central to this idea is the amount of fibre (cellulose and lignin) present in the plants of the ecosystem. The term primary productivity unifies all plant material, whereas an incorporation of the edibility of this would be a more predictive description. Cellulose is the most abundant organic compound on earth and yet its use in classifying communities has been largely overlooked. If plant tissues could be classified according to their edibility this would facilitate the prediction of trophic relationships.

2.5 Effects of herbivores on ecosystem processes- feedback to the plant community

The size of the home range in mammals is also determined by metabolism. Larger mammals occupy larger home ranges than smaller ones in order to satisfy higher energy requirements (McNab 1963).

Large herbivores appear to have an effect on the vegetation of an ecosystem. This occurs not just by physical means such as bark stripping by deer or tree pushing by elephants, but also by recycling of the productivity via dung and urine. The processes involved in this are poorly understood (McNaughton *et al.* 1988). Urea is hydrolysed to ammonium and after several weeks becomes nitrate and can then be used readily by plants and microbes (Thompson Hobbs *et al.* 1981). Plant responses can be altered by clipping and differences between the organic and inorganic forms of nitrogen in the soil pool (Ruess and McNaughton 1984).

Herbivory has a large effect on plant communities. Grazing of biennials and annuals affects root growth and consequently nutrient absorption and fecundity (Crawley 1983). Clipping grasses produces a lower yield of seeds as the plants respond by producing more leaves (Crawley 1983). Winter moth caterpillars have a reducing effect on the mean summer growth of oak leaves (Varley *et al.* 1973). However, the grazing optimisation hypothesis suggests that plants compensate for effects of defoliation by producing more biomass than ungrazed plants (Thompson Hobbs *et al.* 1981). This may not be true if the plant is genetically not capable of regrowth, or where nutrients or water, for example, limit growth. In some regions, nutrient cycling rates have been found to be depressed by herbivory because the total flow through faecal material is small, and no correlation between dung deposition and net primary production can be found (Pastor and Cohen 1997).

There is contradictory data on the impact of feeding on plants but it is generally agreed that plants have more impact on the dynamics of herbivores than herbivores have on the dynamics of plants, because vertebrate herbivore populations are often limited by the availability of high quality food (Crawley 1983).

2.6 Summary

This chapter describes the various processes involved in energy flow through an ecosystem. The way in which these processes are measured has shaped the development of ecology as a science. The fixing of energy in green plants by photosynthesis and the use of it to produce various plant materials affects the animal community. The energy available in the molecules and the time needed to oxidise them (utilise the molecules as food) defines the energy available to the ecosystem indicating that new measurement techniques are needed. Some groups of organisms and their adaptations to the availability of energy in food are described to give examples.

Chapter 3 Plant edibility

3.1 Introduction

The search for palatable or digestible plant material is of primary importance to herbivores. The linked, cyclic nature of ecology can confuse attempts to define controlling or constraining mechanisms. For example, increased feeding on plant tissue can reduce plant productivity, but reduced plant productivity can affect the population dynamics of herbivore populations (and also affect organisms further down the chain).

On a trophic level basis, herbivores are viewed as feeding on "a pureed cream of vegetable soup" (Pastor and Cohen 1997). In reality the consumer has a choice and should choose the most edible plant or plant part. A consumer that utilises the most available source of these affects nutrient or energy flow within the ecosystem. This heterogeneity can result in opposing conclusions on the factors controlling ecosystem energetics (Pastor and Cohen 1997). A herbivore should choose the most nutritional food available within the parameters dictated by its digestive capacity, i.e. whether the organism requires the cell wall or cell contents. A grazing ruminant would choose young grass over old.

After digestion by a herbivore, the remainder is excreted into the environment. The cell walls have been broken down somewhat and most of the easily digested portion has probably been assimilated by the herbivore. What remains is mostly the less easily digested part. This plant material consists mainly of tougher structural tissue. Through the mastication and digestion processes, this is made more available to the decomposer community. The faeces would contain some of the more easily assimilated nutrients if there has not been time for the animal to process the material adequately. This will happen in the more favourable seasons when plenty of new growth, or other more palatable material, is present in the environment.

3.2 What is edibility?

Plant cell walls are an indigestible barrier that prevents most organisms from utilising plant tissue. Cell walls consist of cellulose, hemicellulose and lignin which are strongly linked chains of carbon (fibre). Cell walls are responsible for the high fixed carbon content of plant tissue (Begon *et al.* 1990). The reason why cellulase enzymes have not evolved in the animal kingdom is still unknown (Martin 1991; Begon *et al.* 1990). If it is advantageous to possess these enzymes, why is it so rare to find a species that can manufacture its own cellulase?

Nitrogen (N) is required by all animals for growth and maintenance, in order to produce and repair proteins. As herbivores are primarily seeking N (Crawley 1983; Coe 1981; Hochuli 1996; Leloup and Marnette 1996) this nutrient is the most likely limiting factor; the highest quality plant parts will be those which contain the most nitrogen and the least fibre (Jarman 1974). In plants carbon is used to form the structure, with supporting structures made up of cellulose and lignin. The C:N ratio in plant material often exceeds 40:1 because of this, whereas in animals, bacteria and fungi it is 8 or 10:1

due to the lack of structural carbohydrate and the replacement of this by chitin or bone as supporting tissue. Cytoplasm (cell contents) is abundant in pollens, seeds, and new leaves, which are rich in proteins, lipids and starches (Abe and Higashi 1991). This supports the hypothesis that some herbivores are seeking the contents of the cell rather than the breakdown products of fibre, which is ultimately glucose. Herbivores select new green growth in preference to old or dry material, which is usually higher in nitrogen, phosphorus, and sugars (Begon *et al.* 1990) and lower in its fibre content. The waste products of herbivores are therefore carbon rich compounds. Excretion of the sugar rich phloem sap (honeydew) in the case of aphids provides evidence that energy is not limiting, and the fast rate of food turnover is probably due to a low concentration of an essential nutrient, probably nitrogen. In order to satisfy requirements for this nutrient, large quantities of other carbon must be ingested and excreted (Hatcher 1994). Organisms that utilise food with low N concentrations must either add N by the use of microbial symbionts, as do termites, or eliminate C by egesting carbon rich compounds allowing the processing of more food (Higashi *et al.* 1992).

Plants are the fixers of energy and the compounds they produce are the foundations for development of the ecosystem. The ability of the subsequent consumers to utilise the potential energy present in these compounds must then be considered. Cellulose is the most abundant organic compound on earth (Abe and Higashi 1991) so it could be argued the majority of energy present in terrestrial ecosystems is fixed within this molecule. A low content of N may be a way in which plants make this material less available to their predators. From a plant perspective the edibility of leaves can be reduced by reallocating the N. N is a mobile nutrient and by placing N in other positions, the value of the tissue can be reduced, by reducing the rate at which energy is biologically available to a herbivore.

3.2.1 Microbial symbionts

Most animals cannot utilise the cellulose compound, as they do not produce a complete set of cellulases. Plant cell walls are an indigestible barrier that prevents most organisms from utilising plant tissue. This section discusses the way in which natural selection has provided herbivores with ways to overcome this obstacle.

Some 78 species of insect do possess at least part of the cellulase enzyme complex (Slaytor 1992; Martin 1991). These are species that occupy a narrow niche mainly feeding on woody plant parts, such as termites and wood lice. All higher herbivores use microbial symbionts to digest cellulose either to utilise the breakdown products, to access the cell contents of the plants, or to feed on the microbes directly. Reasons for the failure of cellulase to evolve in higher organisms may be:

1. The enzymes are costly to produce and yield only small reward, whereas microorganisms are cheap to house and keep,
2. Herbivores are seeking nitrogen primarily and not the breakdown products of cellulose i.e. glucose, or the process of cellulose breakdown is too slow to support organisms requiring a rapid rate of energy production.
3. Microbial proteins provide a substantial part of the diet.

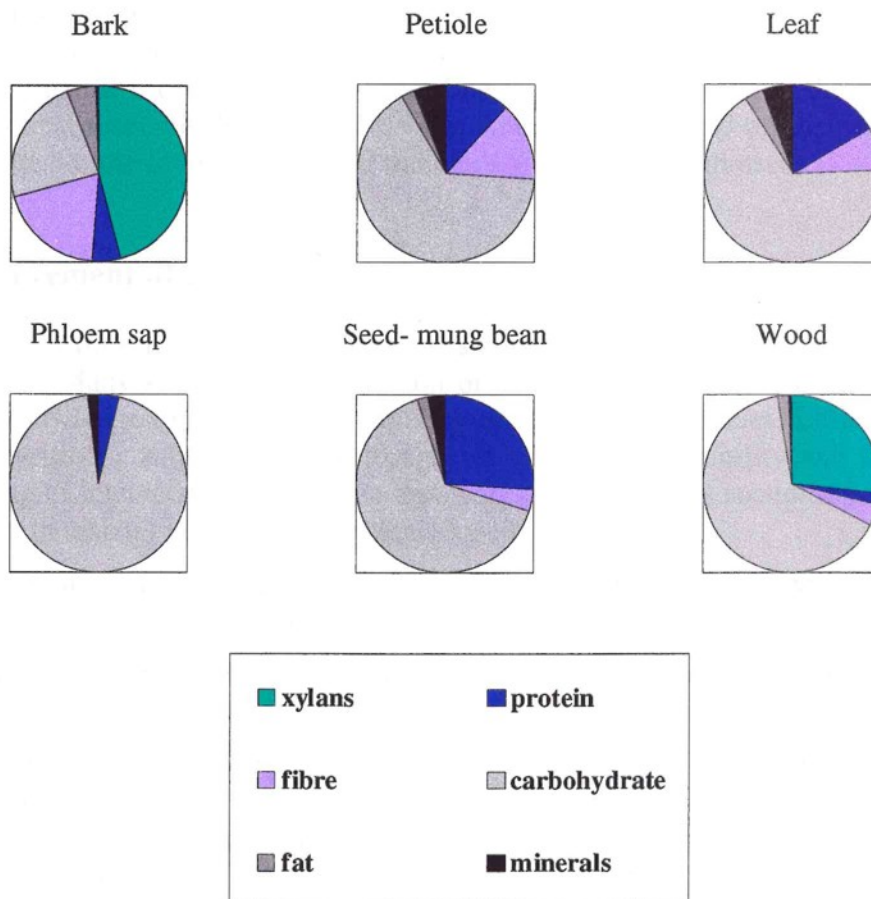
The advantages given to an organism by the presence of microbial symbionts may remove any pressure for an organism to produce it's own enzymes.

The source of the microorganisms seems to vary. In some species there is a stable endogenous micro-flora, while in others it can be associated with diet, not necessarily the presence of cellulose in the gut, as non-herbivores can possess cellulolytic bacteria (Anderson 1991). Cellulose digestion occurs in omnivorous species such as the silverfish, *Ctenolepisma lineata* (Martin 1991). The source of some fungal enzymes isolated is possibly from ingestion with foodstuffs, not from the gut microbes (Kukor *et al.* 1988). This could be true for some detritivores and wood feeders.

3.2.2 Plant components and edibility

Plants are capable of compartmenting energy fixed from the sun into various compounds.

Figure 3.1. Pie charts to show proportions of the various tissue components present in different plant parts.



From Begon *et al.* 1991

Cell wall components are the primary constituent of the plant kingdom. These structures can occur in various combinations and scientists are still discovering new forms of

cellulose. It is an extremely flexible substance, and man has yet to produce something as versatile as this natural compound. Plants with high turnover rates associated with rapid growth and decay possess less secondary cell walls and higher concentrations of N than slower growing plants (Mattson 1980).

Seeds are nutrient rich as they provide all the requirements for the developing seedling. This makes them a desirable food resource. They are protected by secondary metabolites of great diversity. Slansky and Rodriguez (1987) suggest that natural selection would produce this, as any two species with the same defence would present a larger food source to seed predators. The Forb group consists of non-leguminous broadleaf herb plants.

Compared with herbaceous plants the protein and nitrogen contents of grasses tend to be low. This is limiting to the animal, so in order to utilise this family cellulose strategies have developed. The ruminant family demonstrates the utilisation of grasses as a food source. Browse species are defined as woody plants, shrubs, and trees (Van Soest 1987). This group tends to be more lignified, but provides seasonally available high quality new buds and leaves. The N content of nectar is very similar to phloem sap (Mattson 1980) and animals that utilise this part of the plant tend to have fast assimilation rates. No fermentation of the carbon compounds is necessary.

It may be possible to group plants in a more appropriate way to facilitate study of nutritional ecology. One such proposal is the use of the guild concept (de Kroon and Olff 1995). Organisms can be grouped according to their exploitation of the same class of environmental resource (Begon *et al.* 1990). Animals are classed on their body plan, which is related to the food they eat, and therefore, digestive system used.

3.3 Measurement of edibility

At this point in time there appears to be no satisfactory single measure that predicts plant edibility. Many studies into population or community ecology are hampered by the lack of knowledge of the nutritional quality of forage plant species. Little is known about the nutritional value of browse forages in terms of digestibility and this limits understanding of interactions (Guglielmo and Karasov 1995). This section discusses the various ways in which plant quality is characterised.

Two groups of standard measurement were made, and then contrasted with the new *in vivo* and *in vitro* methods proposed. These methods are:

1. the measurement of the energy content of the plant by bomb calorimetry, as shown by separating the plant into different parts, and,
2. the use of a suite of plant quality descriptors to provide a complete, general description of the plant. These are nitrogen by Kjeldahl technique, acid detergent fibre (cellulose and lignin), and soluble sugars. These techniques make up the "plant quality minimum dataset" proposed by Palm and Rowland (1997).

3.3.1 Bomb calorimetry

The bomb calorimeter is an apparatus used to measure the calorific value of a substance. A calorie is the unit of quantity of heat required to raise the temperature of 1g of pure water by 1°C, at 15°C (Wordsworth dictionary 1995). The metric unit is the joule and 1 calorie is equal to 4.1855J. A joule is the unit of work, energy and heat. It is the work done when a force of 1Newton is moved 1m in the direction of the force applied (Wordsworth dictionary 1995).

The energy content of plant tissue is not representative of the energy that is actually available to a herbivore. It measures the energy that is available if a material is burned and completely oxidised to CO₂ and H₂O. In nature this occurs over a very varied time period. In the environment energy is available from a tissue at a characteristic rate and this depends on many factors, such as structure of molecules and presence or absence of nutrients, for example, as well as the 'technology' of the species utilising it. No herbivore can completely oxidise all plant tissue and harness the energy released. An more appropriate measurement would be representative of the way in which the energy in various plant materials is accessed by the herbivore.

The gross energy (GE) content of foods is often used for a basis upon which other energy values are calculated:

$$\text{Digestible energy} = \text{GE} - \text{energy in faeces (FE) or,} \quad \text{eq4}$$

$$\text{Metabolisable energy} = \text{GE} - (\text{FE} - \text{energy in urine}) \quad \text{eq5}$$

From (Louw 1993).

The energy content of herbaceous foliage varies little and is similar to that of woody plant foliage (Slansky and Rodriguez 1987). Variation in energy content does not seem to have much effect on foliage-chewer performance (Slansky and Scriber 1985) and is therefore probably a poor indicator of edibility with regard to invertebrates. Bomb calorimetry of oak tree parts shows a difference of up to 10% only and is in agreement with other findings (see figure 5.2, section 5.3).

The physical structure of a tissue is an important factor when considering edibility. For laboratory analyses, tissue is generally dried and milled. This allows accurate measurement of the content of a substance, as tissues providing a barrier are destroyed. Therefore the physical parts of the plant which have an important effect on whether an animal chooses or disregards the tissue as a foodsource, are lost. This is also true in bomb calorimetry.

The chemical and molecular structure is also largely neglected by bomb calorimetry. The various forms in which cellulose can occur are numerous. This is very much neglected in many quality studies. Long chains of glucose burn as easily as short chains in a high oxygen atmosphere. The terminal subunits are important when considering cellulose, as this is the site of action of part of the cellulase enzyme complex. This biologically important information is not considered in energy content measurements

3.3.2 Proximate analysis of food

A suite of chemical analysis techniques has been developed to characterise plant edibility; not one method adequately supplies information about the value of a foodstuff. This means quite a labour intensive study of an animal feed is required. Palm and Rowland (1997) suggest a particular sequence and method be used for decomposition studies in order to make data comparable. These methods have mainly extended from feed and food quality testing from the agricultural industry and are presented here.

3.3.2.1 N analysis

The Kjeldahl method of analysis is most commonly used. It is recommended by the Association of Analytical Chemists (AOAC 1994) for feed analysis and is often utilised to characterise the quality of plant material in decomposition studies. Some interference from catalysts used for this has been identified (Soon and Kalra 1995). N analysis by this method is expensive, labour intensive and requires specialist equipment. New methods are being developed to address these issues, such as persulphate digestion (Purcell and King 1996).

Total N is a useful measure but does not account for how much and what form the nitrogen is in. The organic N only is measured by this method so nitrate is not included. The total N is therefore only indicative of what is available, and it may not be necessary to perfect a technique that provides only limited information. The total N of the food can be multiplied by 6.25 to give an estimate of the crude protein, because most proteins contain around 16% nitrogen (AOAC 1994; Louw 1993).

3.3.2.2 Fibre analysis

The AOAC (1994) method for analysis of fibre content is similar to the one developed by Van Soest. These are the neutral detergent fibre (NDF) and acid detergent fibre (ADF) and both depend on the capacity of detergents to render proteins soluble by forming complexes so preventing interference with the fibre (cellulose and lignin) (Van Soest and Robertson 1985). This is not a very accurate method and developments have been attempted such as the urea enzymatic dialysis procedure (UED) (Jeraci *et al.* 1988). None have proved labour saving, cheaper or easier to execute. Van Soest *et al.* (1991) describes an updated method of NDF and ADF but states they are not for nutritional use or for the prediction of digestibility. These are established methods that are still widely used, probably as they are suitable for studying substances that contain a wide range of fibre values. When fats are in concentrations above 10% they can interfere with NDF and ADF (Van Soest *et al.* 1991). Measurement of the holo-cellulose and alpha-cellulose is also possible (Rowland and Roberts 1994).

Measurement of the lignin component is possible with separation of the plant cell wall material that is insoluble in 72% w/w sulphuric acid. The residue is termed Klason lignin (Van Soest and Robertson 1985).

Other methods include the use of permanganate as an oxidising agent. This is less common as the endpoint is arbitrary and the lignin value is quoted in terms of how

much permanganate is reduced. The many other methods are not reviewed here, as the problem of measurement of edibility is not solved by the invention of an accurate and precise method to estimate fibre.

3.3.2.3 *Fat content*

This is usually carried out by extraction with ether. This is not included in the plant quality minimum dataset. This is presumably because this constituent of the plant is not considered important in decomposition. The fat contents of plant tissue are low and do not vary widely between plant parts (see figure 3.1).

3.3.2.4 *Water soluble carbohydrates*

These are readily digestible or leached and provide a major energy-yielding component of foodstuffs (Van Soest *et al.* 1991). They are not involved in the cell wall structure and consist of sugars, starches, and other polysaccharides. They can be extracted with cold or hot water or ethanol (Technical association of the pulp and paper industry 1988). The PQMD recommends boiling water and then the filtrate analysed for simple sugars (Palm and Rowland 1997).

The literature reviewed, with the constituents analysed, are contained in a database (Appendix A).

3.3.3 C:N ratios

In animal husbandry, the use of C:N ratios can be traced back to Russell-Hunter in 1970 (Hatcher 1994). He states that the C:N ratio required for animal nutrition is 17:1 because the minimum protein intake for human health is 12% of the calorific value of ingested food and the nutritional requirements of all animals examined so far (up to 1970) excluding ruminants are closely similar to those of man (Hatcher 1994).

Leaf litter decay studies provide us with more ways to measure quality. The use of C:N ratios is widespread and further refined to lignin:N or cellulose:N but these measures have also failed to describe quality in a simple metric (Sinsabaugh and Moorhead 1997). Lignin is considered to be the main constituent determining decay rate (Meentemeyer 1978). Nitrogen content is important, as it can be a limiting factor in the growth of the microorganism population (Sinsabaugh and Moorhead 1997).

C:N ratios are often used to define quality as this incorporates the cell wall content. Nutrient stressed plants have a higher C:N ratio and suffer from lower rates of herbivory, than those fertilised (Feller 1995). It has been suggested that C:Phosphorus (P) or N:P are more informative indices than C:N of nutritional value for some organisms (Hatcher 1994).

3.3.4 In vitro digestibility studies

The metabolism of the carbon substrate and energy production is of use in relation to ruminant feedstuffs, but again is species, or family specific. They also involve isolation

of specific microorganisms from rumen fluid. These studies tend to be carried out in conjunction with nutrient and fibre analyses. Measurements of gas production of feeds with rumen fluid have been shown to produce rapid and reproducible results (Theodorou *et al.* 1995).

In vitro digestion techniques are lengthy, expensive and not very reproducible (Van Soest 1987). They are probably more useful as an indicator of edibility as feed preferences are often positively related to *in vitro* digestibility of the food (Guglielmo and Karasov 1995). Research has focused on the relationship between digestion and chemical composition and a way to predict the edibility from this (AFRC 1993).

3.3.5 Feeding experiments

Measures such as metabolisable energy coefficient (MEC) (Karasov 1990) which involves a relationship between gross energy, intake rate and excretion rate can provide an indication of edibility. Other measures such as dry matter digestibility are formed with feeding trials and chemical determination of the composition of feeds (Osbourne *et al.* 1974).

3.4 Factors affecting the palatability of plant material.

When feeding, herbivores must optimise rate of intake of nutritious food, and therefore must utilise cues in order to do this. Many factors are involved in making up the palatability of a plant tissue. It is probably a combination of several of these that an organism uses for this. Selection in favour of the early detection of food quality would occur and optimal foraging models predict this (Choong *et al.* 1992). By lowering their nutritive quality and digestibility, plants may benefit as animals may behaviourally reduce their fibre intake (Bozinovic *et al.* 1997). The effects of various plant components on palatability are discussed.

3.4.1 Nutrient content

More palatable material can be characterised by high N and P levels (Leloup and Mannetje 1996). Infertile soils limit plant growth and may cause low nutritive quality (Scriber and Slansky 1981). Calcium availability limits the breeding of passerines on poor soils, as they tend to produce defective eggshells (Graveland and Drent 1997). Plants growing on nutrient poor soils may also produce higher amounts of tannins than those on nutrient rich soils (Feller 1995), as they can possibly 'afford' to lose leaves.

Phosphorus fertilisation increased the amount of phenolic compounds present in the plant tissue (Feller 1995). The effects of fertilisation on abundance of invertebrates on a plant can be varied. Nutrient stress can make plants more susceptible to attack by mites, whereas edge chewers were not affected (Orians and Fritz 1996).

Nutrients have been found to be limiting to plant development, although the mechanism of action is not always clear e.g. phosphorus and the growth of dwarf red mangrove trees (Feller 1995). Some organisms may select food based on lipid contents (Jacobsen 1993).

3.4.2 Nitrogen (N)

Nitrogen is discussed separately to other nutrients as it has been identified as an important and limiting nutrient. Spatial and temporal variation in the availability of nitrogen has been implicated as a critical nutritional factor affecting the abundance, food selection and fecundity of herbivores as well as the level of herbivory on the plants (Athey and Connor 1989). N is central to all metabolic processes as well as cellular structure and genetic coding (Mattson 1980). N is necessary to synthesise proteins for growth and maintenance and as all enzymes are proteins N is crucial for metabolism. N is also fundamental to the structure of DNA.

The concentration of nitrogen in a plant tissue, although indicative of quality, does not provide an accurate description. The content of nitrogen can be high in a tissue, but can be present in a non-protein form or protected from digestive enzymes and therefore be of low nutritional value (Claridge and Cork 1994). It occurs in organic (proteins and amino acids) and inorganic forms (nitrates, ammonia and others). Nitrogen availability is closely linked with moisture content of tissues. As growth coincides with increased moisture content and N, this makes it difficult to separate the effects of each of these on herbivory of plant tissues (Mattson 1980).

3.4.3 Tannins and secondary compounds.

High levels of N are also associated with high levels of secondary metabolites. This is because N is incorporated into the structure, but also because N rich plants possibly require extra protection.

In normal feeding generalist herbivores are exposed to a certain amount of plant secondary chemicals. Tannin are high molecular weight polyphenols that have a capacity to bind to proteins (Cooper and Owen-Smith 1985). This prevents utilisation of the proteins present in the digestive tract. Tannins in oak leaves prevent microorganisms housed in the gut from manufacturing cellulase (Moore 1998). There is evidence to suggest generalist herbivores must therefore eat a varied diet in order to prevent the toxins building up to high levels (Moore 1998). This gives the plant an evolutionary advantage, as the damage will be limited unless the predator population is high. Evolution has supplied some herbivores with specialised sets of detoxification pathways; these animals could be called specialists on a range of particular toxins (Freeland and Saladin 1989). Within the insect world there are a large number of specialist feeders. This has sparked a debate on how evolution could have caused this when polyphagy appears to offer more advantages in the survival stakes. It may be that these seemingly specialist feeders are able to adapt to change over a certain number of generations. No one plant food has the optimum level of every nutrient and it is known that caterpillars and grasshoppers can ingest a mixture of plant species to provide themselves with high growth rates (Bernays and Graham 1988).

An increase in tannins decreases the degradation rate of the degradable material (Aharoni *et al.* 1998). It has been suggested that there is a threshold effect. Plant species containing mature leaf concentrations of condensed tannins in excess of 5% are rejected as food (Cooper and Owen-Smith 1985; Gauthier and Hughes 1995). Geese feeding on

willow, cease using it as a food plant once buds have opened and the leaves emerged, which is probably due to the phenol:protein ratio (Gauthier and Hughes 1995).

The effects of tannins on generalist herbivores is now thought to be less influential than previously. It seems there are many ways for herbivores to deal with these compounds, such as enzymes and diet breadth. It is now thought lignin and cellulose components are equally if not more influential (Choong *et al.* 1992; Abe and Higashi 1991).

3.4.4 Silica

There appears to be some debate as to the function of silica incorporated in plant tissue. It has been suggested that silica is a defence compound as amounts increase in clipped plants (McNaughton *et al.* 1985). Certainly, larvae consuming plants high in silica suffer significant mandibular wear (Hochuli 1996) and in concentrations higher than 10% dry weight it causes reduced digestibility in *Spodoptera eridania*, the Southern armyworm (Peterson *et al.* 1988). It has also been suggested that silica deposition is a mechanism by which plants free up carbon as it can be incorporated in secondary thickening and stiffening (McNaughton *et al.* 1985). Whether this occurs in soils where availability of carbon is low has not been tested.

3.4.5 Fibre

Generalist herbivores tend to show a preference for low fibre foods, if available. These are associated with low retention time in passage through the gut and therefore, the animal can generate more energy in a shorter time. Increasing fibre dilutes the concentration of digestible nutrients and energy in a diet (Young Owl and Batzli 1998).

Selection of dicots over monocots occurs in most generalist herbivores due to their higher digestibility. Those that eat mainly fibrous monocots still exhibit preferences for lower fibre varieties or new shoots over old mature stems. Grassland changes in the non-growing season can occur through transport of proteins and carbohydrates to the root leaving a highly lignified and indigestible leaf tissue (Sinclair 1975). Through the growing season, oak leaves lay down tannins so it is only the new growth which can support large invertebrate herbivore populations (Feeny 1970). The leaves in most ecosystems, even though present, and accounted for in measures of productivity, are largely unavailable as food in at least one part of the year. Leloup and Mannetje (1996) found no difference in crude fibre content of browse material in different seasons. However the fibre present could be present in a less easily digestible form in the dry season.

An important constituent of the fibre in plant tissue is the lignin. It has been known since the 1930's that non-lignified feeds are more readily digestible (Keys *et al.* 1970). This is probably because the lignin can prevent access to the cellulose. Even generalist ruminants discriminate among grasses and plant parts on the basis of nitrogen and lignin contents (Jarman and Sinclair 1979). For mammalian herbivores the protein to fibre ratio may determine the food preference (Bozinovic *et al.* 1997).

Leloup and Mannetje (1996) suggest that deciduous species are more palatable than evergreens. They also suggest the evergreenness or deciduousness of the woody plant

community may be a good indicator for the condition of a rangeland. Evergreenness is also an indication that nutrients are limiting in an area.

3.4.6 Stage of growth

When the cells of a plant are rapidly expanding the cell walls are thinner and N concentrations are higher. New growth consists of metabolically active cells with readily degradable primary cell walls. Secondary thickening and lignified tissues are associated with age. N levels drop when growth ceases and continue to drop until senescence. N is a mobile nutrient and translocation from leaves can occur. N demands of the plant and N levels within the plant are highest during growing stages or in growing parts but decline with age as more fibrous tissues are added to the structure (Mattson 1980).

There is evidence that herbivores attack young and vigorous plants more frequently than old and mature plants (Price 1991; Hochuli 1996). Short-lived leaves tend to be more palatable than long-lived ones. This is possibly because it is not worthwhile for the plant to invest in making a leaf tough for a short amount of time (Choong *et al.* 1992).

The availability of plant species varies seasonally and most herbivores have evolved strategies to deal with this, such as diet change or aestivation. Ungulates in the Serengeti show seasonal reliance on more than one species (Jarman and Sinclair 1979).

3.4.7 Physical structure

The architecture of the plant cell wall is extremely complex and has a large impact on the digestibility of the material. Lignin materials provide the structure of the plant. In excess of 15% this can prevent enzyme attack of the cellulose and normally available constituents (Sinsabaugh and Moorhead 1997). Epidermal and vascular structures determine the initial breakdown of organs which is fast in leaves of legumes and high quality temperate grasses but slow in tropical grass leaves (Wilson and Kennedy 1996). All stems require a high chewing effort (Wilson and Kennedy 1996). Tropical grasses are less palatable than temperate grasses due to the larger amount of veins they possess (Wilman *et al.* 1996). A wide angle of attachment of secondary veins to the primary vein in the leaf blade may allow easier breakage of the leaves when eating (Wilman *et al.* 1996). Sheep prefer food with broad leafed species to grasses and temperate grasses to tropical grasses. This may be due to the venal structure (Wilman *et al.* 1996). Tree morphology may have a larger influence on the feeding behaviour of red deer (*Cervus elaphus*) than chemical composition, as more biomass was removed from larger trees (Hartley *et al.* 1997).

Foliage feeders on woody plant species, such as leafcutting ants which are generalist feeders do not seem to be affected by leaf toughness (Howard 1988). However there is some evidence that toughness of leaves is a major determinant of herbivore avoidance of plants (Choong *et al.* 1992). Some studies have attempted to quantify sclerophylly, recognising this as an important factor (Feller 1995—leaf mass/unit area; Feeny 1976—penetrometer). Leaf chewers, where the emphasis of food acquisition is on mechanical processing, are probably highly affected by cell wall structure (Hochuli 1996).

3.4.8 Animal constraints on edibility

Retention time of food within a herbivore's digestive tract is closely linked to the quality of the food within, and has been pinpointed as a major constraint on assimilation of energy (Iason and Van Wieren 1999). If a foodstuff takes a long time to digest, the presence of the food itself prevents the animal from consuming more until the tract is emptied. Therefore, there is a trade-off between quality and quantity of the food ingested. The digestive 'technology' using the food must also be of consideration. See section 6.4 for further explanation.

3.4.9 Discussion

Whether a plant tissue can be regarded as food depends on what energy can be extracted from it and how quickly. This is dependent not only on the chemical constituents of the tissue but also on the digestive strategy acting on the tissue. Biology, or how the body plan affects the digestion process, is an indication of the food that will be chosen. Vertebrate herbivores also seem to select individuals within a plant species, which may reflect differences in the chemical composition or structure at an individual level (Hartley *et al.* 1997). In aquatic systems, measurement of the edibility of phytoplankton is sometimes carried out on a taxonomic basis, using qualitative information (e.g. Leibold 1989). Many methods have been used to assess the quality of plant feed for ruminants including gas production, nylon bag degradation (*in vivo*) (Verbic *et al.* 1995) dry matter degradability (Shem *et al.* 1995), chemical analysis (Kaitho *et al.* 1997).

Edibility is how suitable a substance is to be eaten. This will mean different things to different organisms. Preference for certain groups of plants can be shown by feeding trials. However, data generated by chemical analysis, degradability and microbial gas production fails to separate out the different groups of plants as feeding experiments do (Kaitho *et al.* 1997). This suggests they do not adequately reflect the edibility of plant material. Further research is necessary in order to determine what factors could segregate species into different preference groups (Kaitho *et al.* 1997). Edibility is a complex combination of many factors and the measurement of these is difficult.

Foraging strategy has a major effect on the flow of nutrients or energy to consumers and therefore what enters the detritus pool. This means viewing plants as a homogenous layer cannot facilitate understanding of ecosystems. In terms of ecosystem energetics measurement of total energy content of plant tissue does not give an accurate picture of food content or value. Chemical analysis techniques are quick and relatively cheap, but mainly due to the complexity of the cell wall fraction, do not provide comprehensive descriptions of quality.

It is clear from this that the processes controlling and constraining animal feeding are complex and interdependent. The relationship between plant structure and food choice can not be explained by a few easily measured parameters.

3.5 Towards a test for edibility

Is it possible to develop a test that can incorporate all the palatability factors and which could also account for the multitude of feeding and digestive strategies that allow the animal kingdom to be categorised? Can such a test be both integrated and simple?

The many studies on the quality of plant material as food for herbivores show there is no single one-parameter test that can be used to measure edibility. Many studies present contradictory results (Feller 1995) and a complex 'general' description of plant quality has recently been proposed (Palm and Rowland 1997). A different approach is to utilise a biological technology, which can take into account all the different digestion strategies in the animal kingdom and chemical composition of the plant kingdom. Microorganisms provide a potential technology to do this. They allow the utilisation of the plant kingdom as food for herbivores, particularly the compound cellulose. Microorganisms provide the metabolism for decomposition also and it is suggested that measurement of a rate of energy usage can be carried out using degradation of plant materials in soil. In theory, all parts of the plant contain energy which is accessible only if enough time passes to access it. Animals have to meet daily metabolic demands; there is a deadline that has to be met. An improved basis for studying plant/herbivore interactions is suggested by measuring the *availability* of plant energy to a herbivore and the next chapter describes a pilot method for doing this with the emphasis on the time in which this occurs.

Chapter 4 A Pilot Study

4.1 Introduction

It is rapidly being recognised that litter decomposition rate in soils is dependent on the quality of that litter. The more edible the plant species the faster the rate of decay. Decay rates are also further complicated by the fact that all are site specific and regulated by edaphic and climatic variables (Sinsabaugh and Moorhead 1997). In October 1996, the hypothesis that decomposition would be a synoptic measure of plant quality was devised. Chesson (1997) writes on the similarities of degradation of litter in soils to that of degradation in ruminants.

Ingested food is attacked by rumen microorganisms in fermentation processes. These processes yield more microbes and volatile fatty acids, which can then be utilised as food for the herbivore. In soils, the attack of plant materials occurs in an aerobic environment which yields different substances (volatile fatty acids) and results in a different amount of energy extracted. The animal can then oxidise the volatile fatty acids to carbon dioxide and water aerobically. The rumen is an evolved ecosystem which has resulted in the prevalence of optimum conditions for degradation of a foodsource as rapidly as possible. Decomposition in soils is also an evolved system, which results in degradation over a longer period of time, due to a succession of microorganism interactions. Both systems revolve around microorganism processes, and qualities of the plant affect both similarly.

Plant material is disrupted by mastication. This results in destruction of the integrity of the cuticle and some cell walls. Once in the digestive tract, the high population sizes of microbes and the conditions mean energy is rapidly extracted from the constituents. Litter on the forest floor is more intact and colonisation is slower to occur. The physical aspect of plant material affects both processes. Tougher structures are harder to chew, more intact on reaching the rumen or stomach, and will present less free ends for enzyme attachment. In the litter layer, enzyme attachment will also be inhibited, and the number of species that can immediately colonise the material will be less, where more specialised metabolism is required. The structural tissues of the plant contain more lignified material, which is combined with cellulose and prevents access to it (section 3.4.7). The heterogeneous nature of plant material makes measurement of these factors difficult. This needs to be considered for a new plant quality measure.

Similarities in decomposition and digestion processes are:

- Both are microbially mediated
- The factors affecting the processes occurring are the same
- Both require a cellulase enzyme complex

Differences between the two processes are:

- The time taken to access energy within the plant material
- The amount of energy extracted (aerobic versus anaerobic) by the microbial population

See chapter 6 for more detail on the factors affecting digestion and the processes involved. The hypothesis is that the soil microorganism community can be viewed as a single compartment for this purpose, and microorganism metabolism can be used to measure the energy availability in a plant part.

There is a recommendation for a plant quality minimum dataset (PQMD) when considering rate of decomposition of litter (Palm and Rowland 1997). This would then facilitate comparisons of rates between ecosystems. The PQMD requires the measurement of lignin, soluble carbon total carbon, soluble phenolics (if nitrogen contents are in excess of 1.8%), nitrogen, phosphorus, and ash free dry weight. The lack of a physical quality measurement is acknowledged. The methods used to analyse the constituents must be the same to enable comparisons, and the recommended ones were used in this study.

An alternative experimental method was considered using broad bean plants, *Vicia faba*. Plants were grown in a controlled environment in order to prevent herbivory affecting the quality of foliage due to induced chemical defences. One seed was established in each pot with a specified amount of John Innis compost no.3. These were grown at 20°C, with the natural photoperiod of that time of year (March to April 1997). Sufficient material for a decomposition study could not be produced, so these plants were separated into leaves, roots and stem parts and used to establish the method for fibre analysis (section 4.2.2.1). Results are given in Appendix B.

A pilot study was designed to incorporate:

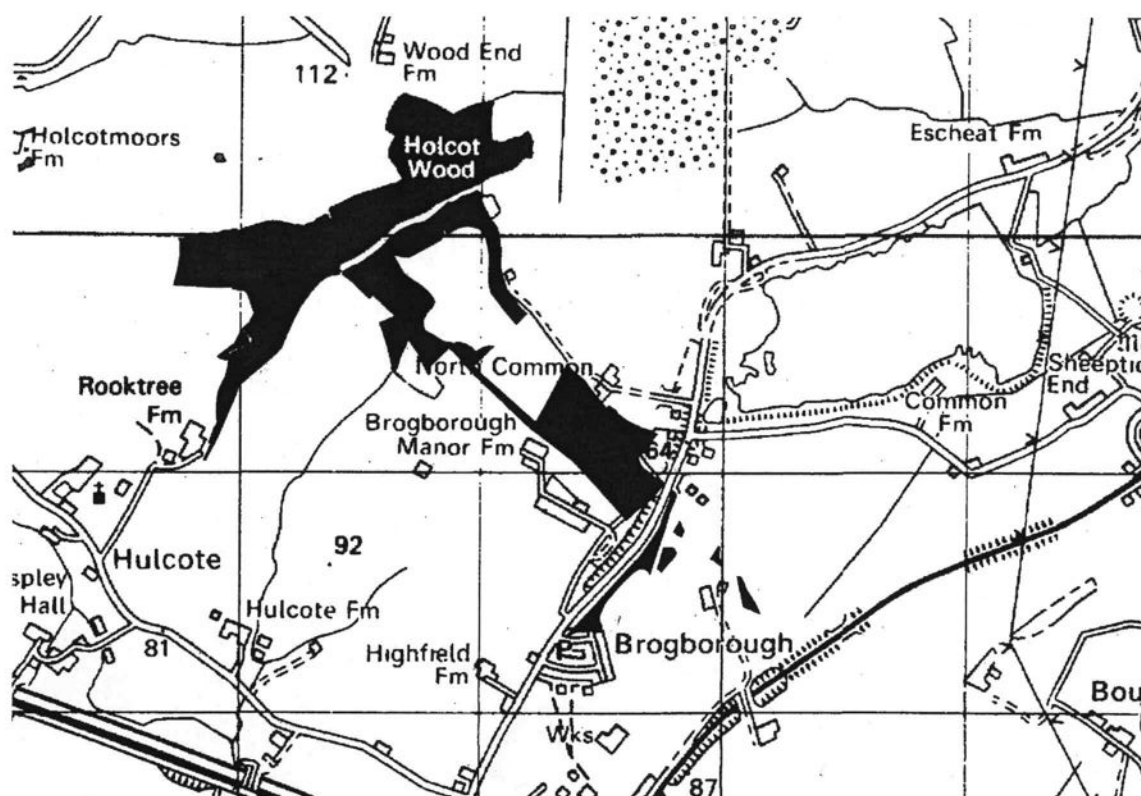
1. a decomposition study in order to incorporate a rate of energy supply parameter. H_0 =Loss of mass of a plant part will vary according to its edibility and,
2. analysis of a food plant according to the plant minimum dataset (Palm and Rowland 1997).

A site was chosen in Holcot Wood close to Cranfield University. Holcot Wood is semi-natural ancient woodland over 400 years old and dominated by ash and field maple. It is owned and managed by The Woodland Trust. The areas owned by the Woodland trust are shaded in fig.4.1. The site was located under the '1' in Holcot in figure 4.1.

The woodland edge perennial plant cow parsley, *Anthriscus sylvestris*, was chosen as the subject for a decomposition study as it is fast growing and theoretically should have a low content of protective compounds (Cornelissen and Thompson 1997) and the rapid rate of decomposition required for a one-season study. Because this plant is an annual, it has the added advantage of all plant parts being present at once.

The plants were collected from Holcot Wood, Cranfield, Bedfordshire and the surrounding hedgerows. It is important the plants are collected from the proposed site of the decomposition experiment, because of the connection between plant growth, plant quality and microorganism community in the soil. Because organic matter decomposition and, therefore nutrient mineralisation, is mediated by the microorganism population, the availability of nutrients to the plant relies on this. Nutrient availability affects the growth of the plant in terms of number, size of leaves, and fecundity. This affects the amount and quality of leaf litter produced that returns to the soil and is available again to the microorganism community. Soil properties can change appreciably over long periods of time and associations are likely to occur between certain plant and microbe groups.

Figure 4.1. Map of the area including the field site chosen. Reproduced courtesy of The Woodland Trust



4.2 Method

4.2.1 Decomposition

The method chosen was the commonly used litter bag technique due to its ease of use, the fact it is an established method and because it requires little in the way of technical equipment. Various methods were employed in order to establish the sample rate, optimum litter bag size and mass of sample. The plant parts were distinguished as being structurally or functionally distinct and separated on the basis of this as described in table 4.1.

Two types of mesh were used: 1.5mm and 0.3mm mesh manufactured by 'House and Company'. Bags were made up using polyester thread and measured 70x70mm. They were individually weighed and tagged with laminated cards for identification. This was to prevent any need to open the bags and remove the contents. They were filled with a specified amount of the fresh plant part (table 4.1). Empty bags were also used as a control to correct for soil entering or adhering to the bag. This also accounted for any loss of weight of bag through decomposition or drying.

Table 4.1. The preparation of each of the fresh plant parts for burial in the decomposition experiment.

Plant part	Preparation	Mass per bag (g) $\pm$ 0.01
Root	tap root only, cut in cross section into 5-10 mm lengths	3
Leaves	petiole removed so only leaf blade remained	1
Stem	sample taken 10cm above ground level and cut into lengths ranging from 5-10mm	2
Flowers	removed from the final stalk of the umbel	1
seeds	separated from flowers and the whole pod used	0.5

The bags were then buried in Holcot Wood in grids (1 for each plant part) which were marked out by nylon string. The site chosen was away from paths to avoid human disturbance and edge effects.

Figure 4.2. Photograph of one of the decomposition grids in Holcot wood.



The bags were assigned random positions by the use of co-ordinates and buried at 5cm depth in the topsoil. This was to ensure aerobic decomposition ensued. The bags were buried with the tag above ground, as can be seen in fig.3.2, so co-ordinates could be picked at random for retrieval. Five bags of each type, repeated for each plant part, were chosen at random using co-ordinates and retrieved every 20 days. These were dried in

an oven at 80°C until a constant weight of bag and contents was reached on a top pan balance.

4.2.2 Laboratory analyses

A subsample of each plant part was taken and dried in an oven at 80°C for 2 to 3 hours, until a constant mass was reached. These samples were used for analysis of fibre and nitrogen levels.

4.2.2.1 Fibre

Several methods are available for analysis of fibre content, mainly developed for analysis of dietary feeds for the agricultural industry. These include the urea enzymatic dialysis procedure (UED) (Jeraci *et al.* 1988), neutral detergent fibre (NDF) (Van Soest *et al.* 1991), and acid detergent fibre (ADF) (Association of Analytical Chemists (AOAC) 1994; Rowland and Roberts 1994). It is suitable for low and high levels of fibre and it was estimated there would probably be a wide range of levels in the samples collected. The ADF method was chosen, as this appeared most suited to the range of samples to be analysed.

0.5-1.0g of dry material (**w1**) was placed in a 250ml conical flask. 100ml of cetyltrimethyl ammonium bromide (CTAB) solution (50g of CTAB in 5.0 litres of 0.5M sulphuric acid) and a few drops of antifoaming agent were added. The CTAB was general purpose reagent obtained from Merck Ltd., Lutterworth, Leics., UK. This was heated under reflux for an hour and filtered hot through porosity P100 filter (old no.2 sinter filter) under gentle suction. The filter was pre-weighed and gives **w2**. The residue was washed three times with 50ml aliquots of boiling deionised water and then with 50ml acetone until no more colour was removed. The resulting fibre was sucked dry and then placed in an oven for 2 hours at 105°C, then cooled in a desiccator and weighed (**w3**). The percentage acid detergent fibre was then calculated using equation 6.

$$\%ADF = (w3 - w2) * 100 / w1 \quad \text{eq6}$$

In order to estimate the percentage of lignin and cellulose the sample can be further analysed. 72% w/v sulphuric acid at a temperature of 15°C was added to the sinter filter and the sample mixed to a paste. Sulphuric acid was continually added as it drained away so that acid was in contact with the sample for 3 hours. The residue was then washed with hot water and acetone, dried, and weighed (**w4**) as before. The ash content was determined by igniting the sinter at 550°C in a furnace for 2 hours. This was then cooled in a desiccator and weighed (**w5**).

$$\% \text{ lignin} = (w4 - w5) * 100 / w1 \quad \text{eq7}$$

$$\% \text{ cellulose} = (w3 - w4) * 100 / w1 \quad \text{eq8}$$

The whole procedure was repeated in triplicate for each plant part except for seed and flowers. This procedure could only be carried out once for these due to shortage of sample.

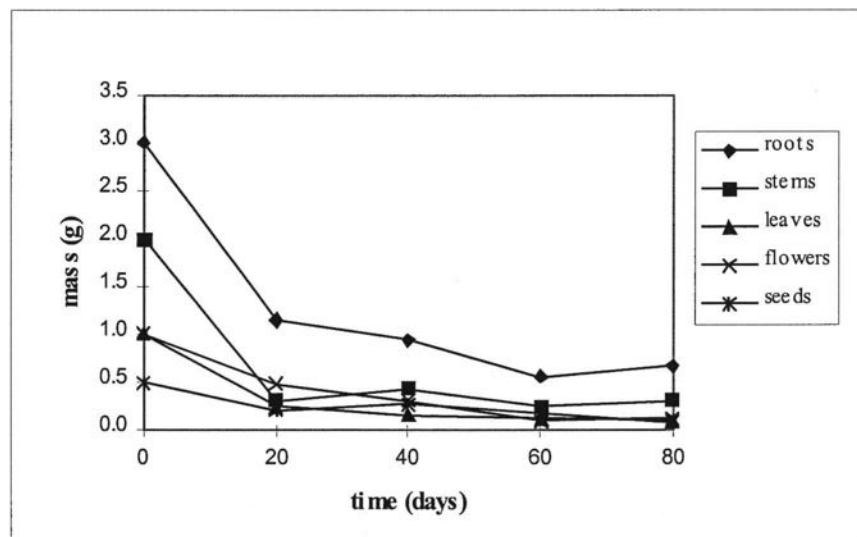
4.2.2.2 Nitrogen

Herbivores can possibly utilise nitrogen in several forms from the plant. Therefore, the decision to use a method to estimate total nitrogen was made. The commonly used Kjeldahl method determines only organic nitrogen without nitrate (Matejovic 1995) and is a laborious and time-consuming procedure. Recent developments include the Dumas method which does account for nitrate and is also accepted by the AOAC in analysing feeds (AOAC 1994) and, as they can be automated or semi-automated, are preferred. Low cost alternatives include persulphate digestion in which organic nitrogen can be estimated colorimetrically (Purcell and King 1996) but nitrate nitrogen must be estimated separately (usually using an autoanalyser). It was decided the Kjeldahl method was most appropriate as this is the most commonly used in decomposition studies and samples were sent to Natural Resource Management Ltd. (NRM), Berks., UK. for contract analysis. This is also the method which is recommended in the PQMD (Palm and Rowland 1997).

4.3 Results

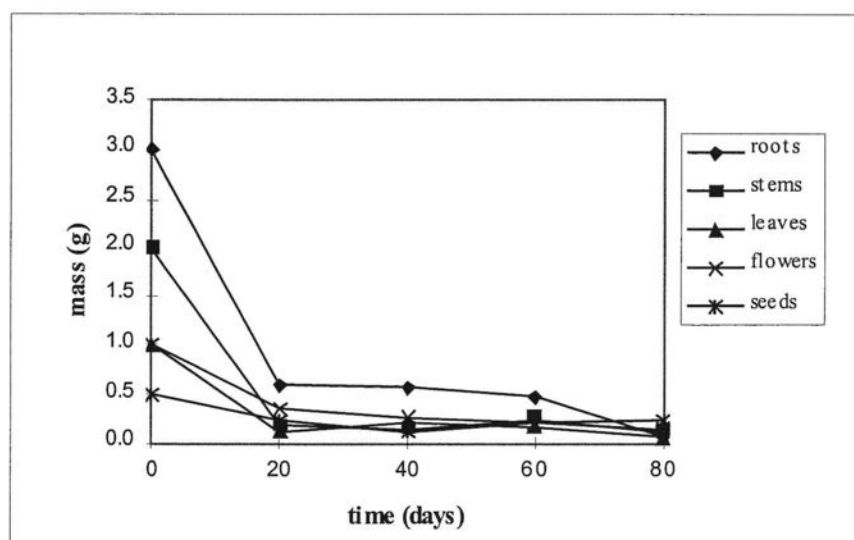
4.3.1 Decomposition

Figure 4.3. The decline in weight of cow parsley, *Anthriscus sylvestris*, plant parts in 0.5mm mesh litter bags



The sample sizes used were five bags for each plant part at each date of retrieval. Figures 4.3 and 4.4 show the decay of most of the mass of material occurs within the first 20 days of the study. This occurs in both size mesh bags although this may happen faster for roots in the 1.5mm mesh (fig.4.4). There is little difference between the materials as the masses subsequently remain largely unchanged. The raw data is given in Appendix B.

Figure 4.4. The decline in weight of cow parsley, *Anthriscus sylvestris*, plant parts in 1.5mm size mesh litter bags



4.3.2 Laboratory analyses

Figure 4.5. The mean percentage of Kjeldahl nitrogen in the plant parts of cow parsley *Anthriscus sylvestris*

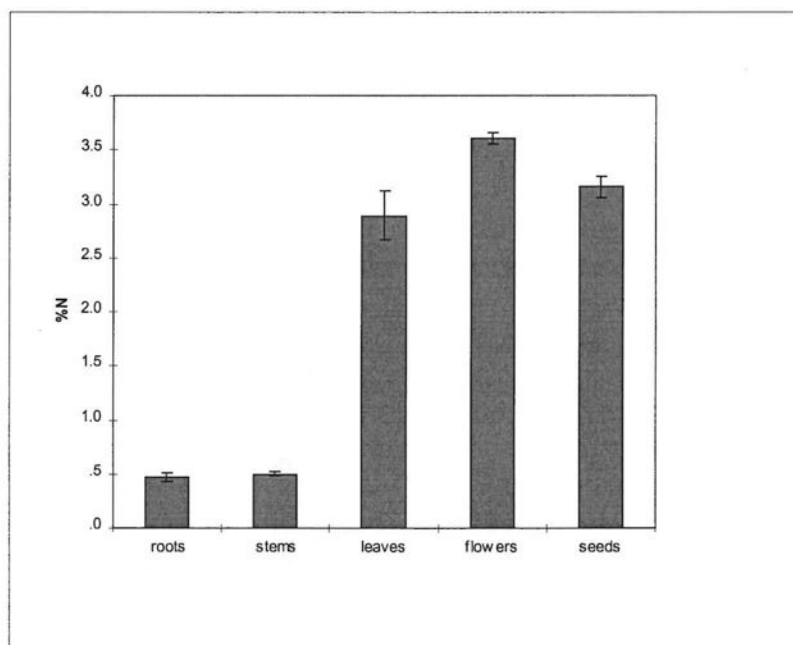


Figure 4.5 shows cow parsley roots and stems possess only a small amount of nitrogen when compared with the other parts. Leaves, flowers and seeds have more than five times this amount. Confidence limits in the leaves are larger than for other samples. In

figure 4.6, it can be seen that stems have a large amount of fibre as expected for a structural tissue. The other plant parts do not vary by more than 5%. The amount of lignin included in the %ADF value was negligible for each plant part, except stems where it consisted of 26%. Figure 4.7 is a representation of a C:N ratio for each part where a value for fibre is substituted for total carbon. The ratio is very high for stems, roots being only half this value and flowers, leaves and seeds have very low values assigned to them.

Figure 4.6. The mean percentage of fibre determined using the acid detergent method in cow parsley *Anthriscus sylvestris*

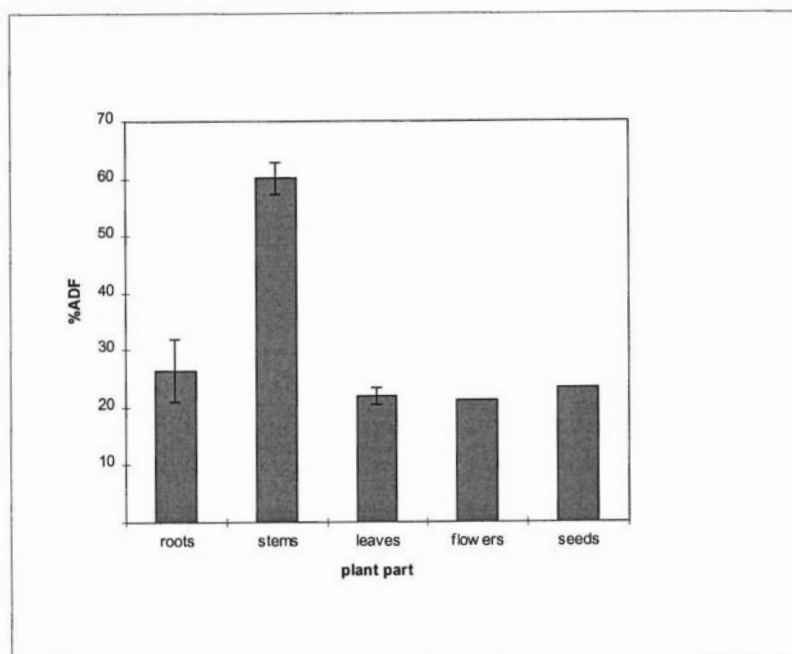
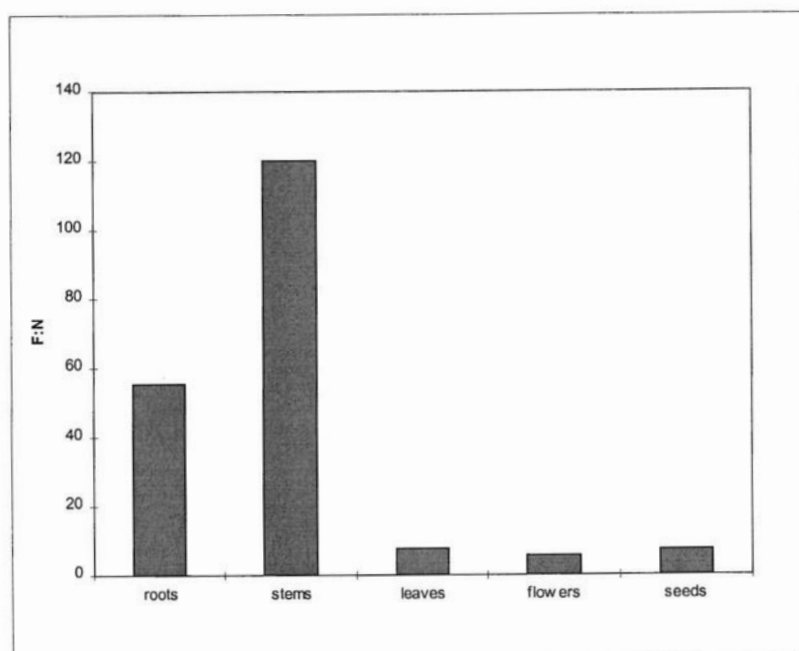


Figure 4.7 The ADF:N ratio for the plant parts of *Anthriscus sylvestris*



4.4 Discussion

4.4.1 Rate of mass loss

The size of the mesh bags (within the range used in this study) appeared to make only a small difference to the rate of mass loss. It is clear from the results that decay of the most available parts of the plant occurred in the first 20 days. The initial rapid leaf weight loss is probably due to leaching of soluble materials (Elliott *et al.* 1993). Any differences between the plant parts are not apparent from the graphs due to the time-scale of sampling. The disappearance of large amounts of plant material was probably due to excessive rainfall and high temperatures which occurred shortly after burial of the samples. This would cause rapid loss of mass due to leaching of the soluble compounds and would also provide conditions that favour the growth of microorganisms (Wild 1988).

The cow parsley plant was chosen for its rapid rate of decomposition, being an annual forb. As this is central to the design of a study, the sampling dates were set according to this but were inappropriate due to exceptional environmental conditions. This also could have been an inappropriate plant for such a study as it may lack the full range of cell types that characterise plant material. Cow parsley is an annual plant so it may possess less secondary cell walls and lignified material. It does not possess the full spectrum of features as it is lacking in materials such as bark and wood.

The mass of sample was fairly small and varied between the plant parts so the results were reported as the percentage of mass lost (figs. 4.3 and 4.4). Fresh material was used to avoid loss of soluble sugars and phenols, which are important when considering food value or energy flow (Taylor and Barlocher 1996). The seeds used in this experiment were buried in their protective sheath on account of their size. Due to shortage of this sample, only three replicates per sampling date were drawn.

This study provided important information towards the design of a more appropriate study and therefore served its purpose as a pilot. Whether rates of mass loss can be used to indicate the edibility of a plant part remains inconclusive. Three attributes of plant material are likely to affect the palatability: physical toughness, nutritional quality, and chemical defence and these are also likely to affect the decomposition of litter (Cornelissen and Thompson 1996; Grime *et al.* 1996). Lignin, tannin and carbohydrates all have significant correlations with decomposition rates (Meentemeyer 1978). Litter decay is also correlated with nitrogen contents (Hobbie 1992). Herbaceous dicots decompose much faster than graminoid monocots and deciduous species have faster rates than evergreens in woody plants (Cornelissen 1996). This would be expected as deciduous species have greater input into the detritus pool. Herbaceous dicots are associated with higher amounts of fibre and N and this is probably why certain nutrients are traditionally measured, but do not encompass edibility.

4.4.2 Fibre and nitrogen

Rowland and Roberts (1994) found methods based on the acid detergent fibre extraction fulfilled the criteria of a simple method that is easy to implement which produces

precise results and is applicable to a range of study materials (such as faeces, wood, and bark with ADF levels exceeding 35%). The CTAB solution hydrolyses hemicellulose and dissolves protein leaving a residue containing cellulose, lignin, and insoluble ash. The methods were extended to include separation of the cellulose and lignin fractions according to Rowland and Roberts (1994).

Leaves are more likely to vary in quality than a structural tissue. This may account for the larger confidence limits associated with this material (fig.4.5). The high amounts of nitrogen associated with flowers are likely to be a result of the presence of pollen and nectar.

Fibre content and nitrogen values provide valuable information on the edibility of plant parts (figs.4.5 and 4.6). Stems are lacking in nutritional value since this material contains large amounts of fibre and small amounts of nitrogen. Roots contain less fibre than expected of a root material (see results for beans appendix B) and the same amount of nitrogen as stems. The other parts possess the same amount of fibre as roots, but more than six times the amount of nitrogen. Leaves, flowers and seeds appear to be more edible than stems and roots.

The plant parts can be positioned or ranked according to their edibility using the fibre:N ratio (fig.4.7). However, this is obviously not of a high enough resolution to distinguish between the three more edible parts. Seeds are known to be the most nutritious food available to herbivores (Begon *et al.* 1990) and this is not reflected in this measurement. Other facets of the plant, rather than nutrient content alone must be represented in a measurement of quality.

4.4.3 Improvements to be made

This study was used to aid the development of a full-scale study of mass loss of plant tissue in a temperate climax community. It is believed that the rate of decomposition in soils can supply important information about the quality of the plant parts as food.

The small initial masses of plant material were not suitable for such a study, especially as corrections were included for the mass of the bag. The initial mass was fresh weight and subsequent masses are of dried material. Even though fresh to dry weight ratios were established, it cannot be assumed these remained constant throughout the study.

A larger amount of material in the bags initially would minimise errors that occur during weighing. Some error occurred in the larger mesh sized bags as soil particles entered the bags or small particles of plant material escaped affecting the masses recorded. In the full-scale study a small mesh was used to minimise this.

The depth the bags were buried in the soil was not sufficient as surfacing of some bags occurred through the study. It is advisable to bury bags deeper than 5cm. Empty bags, used to correct for soil adhesion or particles accumulating, caused too many errors so these were not used. Instead the contents should be inspected carefully for contamination and weighed. This was advised by The Institute of Terrestrial Ecology, Merlewood Research Station, Cumbria, UK (personal communication).

Measuring the fresh to dry weight ratio, allowing burial of the fresh sample is advantageous in that the tissue is not altered in any way by the method of drying. However it is impractical for a larger study due to the amount of time it takes to weigh

out and fill the bags. Decomposition would occur in the laboratory and it is important that the bags be buried quickly.

The plant chosen for the next part of the study should be one which degrades less rapidly. The design of the sampling dates should allow for a high initial loss of mass and subsequently little change. Five bags should be collected at each site visit to allow for variance in mass loss between samples.

4.5 Summary

This chapter describes a pilot study using the annual plant cow parsley (*Anthriscus sylvestris*) and its rate of decomposition in soil for various fresh tissues: stems, roots, leaves, seeds, and flowers. It has been established in the literature there is a link between the rate of digestion in ruminants and the rate of mass loss of plant tissue in soils (Chesson 1997). This study begins the assessment of energy availability in plant material. It reports a pilot study used mainly to develop laboratory analyses and field methodology. It provided the means to test the method chosen, and provided valuable information for the design of a large study with an appropriate sampling regime. Among the factors modified were the depth of the topsoil used, the mesh size, initial preparation of the plant material, and initial masses used. An improved decomposition experiment was designed using a biome characteristic plant, the English oak (*Quercus robur*) and this is described in the next chapter. The study is designed to provide valuable data on the availability of energy from different oak tree parts to an oak woodland ecosystem (Wytham Wood, Oxford, UK.).

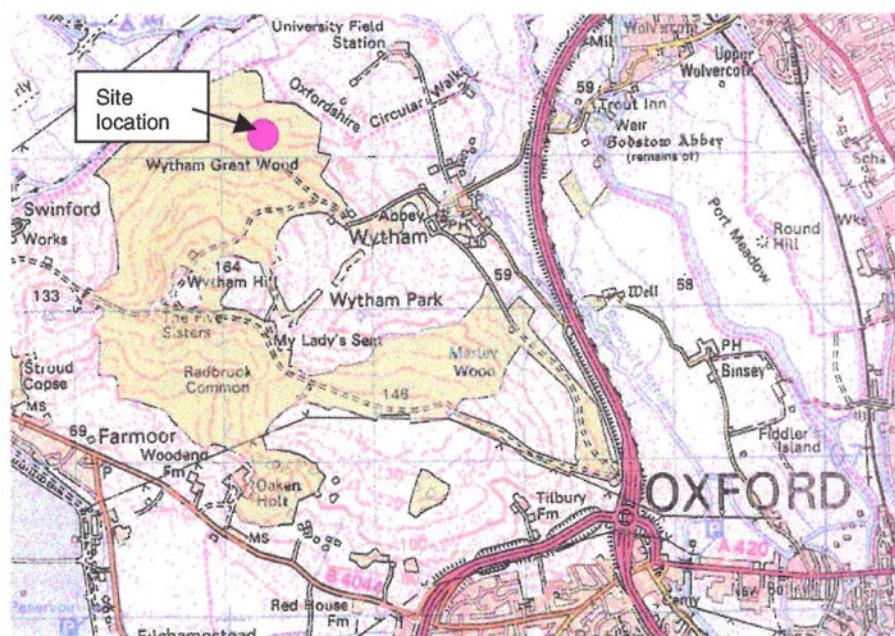
Chapter 5 Wytham Wood study

It is rapidly being recognised that litter decomposition rate in soils is dependent on the quality of that litter. The more edible the plant species the faster the rate of decay. In chapter 4 a pilot study using cow parsley *Anthriscus sylvestris* was described. The rapid rate of decomposition of cow parsley made it an unsuitable subject for such a study, but allowed the litter bag technique to be evaluated and subsequently modified. A full-scale study is warranted, as the data generated can be used for the development of edibility units with the incorporation of a time dimension. This chapter describes a rate of decay study using a tree characteristic of a temperate biome in southern England, the English oak (*Quercus robur*).

5.1 Introduction

The rate of decomposition of oak tree (*Quercus robur*) parts in an ancient woodland climax community was established. Wytham Wood was chosen as it is one of the most studied sites in the world and because of its status as an Environmental Change Network (ECN) site. The ECN monitor a broad range of environmental variables in order to detect climate change and provided useful data for this study. The site consists of a

Figure 5.1. Map to show position of field site and Wytham Wood, from Ordnance Survey Landranger 164.



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wooded hill rising from 60m to 165m. The soil type varies from alluvium overlaying Oxford Clay by the Thames, to exposed clay and deep heavy soils and sandstone at higher elevations. The wood itself consists of ancient, secondary and plantation areas. An area was chosen within the plantation (at least 200 years old), sufficiently far enough up the hill to prevent waterlogging in winter, where beech and oak were widespread. The co-ordinates on landranger map 164 are 4609.

5.1.1 Decomposition processes in soils

Decomposition is the breaking down of a substance into simpler molecules or atoms. In soils this occurs with the help of enzymes produced by microorganisms. Decomposition is also defined as the gradual disintegration of dead organic matter and the conversion of elements from organic to inorganic form.

Degradation of plant material in soil occurs due to the varied flora of bacteria and fungi, as they rarely act alone and the process would be much slower if this occurred (Begon *et al.* 1990). Soils contain varying proportions of bacteria, Actinomycetes, fungi, algae, and protozoa. Total characterisation of the microorganism population of a soil is not possible as yet (Wild 1988), although identification methods are constantly improving. Studies show that most soils contain all the main groups of microorganisms (Trehen *et al.* 1993).

Bacteria and fungal spores are omnipresent (Begon *et al.* 1990). Many taxa are widespread in soils but environmental conditions determine which species predominate (Giller and Day 1985). Microorganisms contribute to the mineralisation, of almost all organic substances and 90% of carbon dioxide released by biological processes into the atmosphere is the result of their metabolic activity (Sorlini 1991).

Initially, when senescent plant tissues reach the soil, there is leaching of the soluble compounds into the surrounding soil. These can be utilised by the microorganism populations that colonise the plant material. As the structure of the decomposing material changes the colonising or dominant microorganisms change. In general, the succession begins with simple sugar fungi, such as Phycomycetes Fungi Imperfecti followed by septate fungi (Ascomycetes, Basidiomycetes and Actinomycetes) with more specialised metabolism. The recalcitrant gradient shown by plant tissues is as follows:

sugars<starch<hemicelluloses<pectins and proteins<cellulose<lignin<suberins<cutins
(from Begon *et al.* 1990).

The process of decomposition is extremely complicated and often site specific in organisation and is therefore difficult to measure. It involves communities of microorganisms metabolising the products of enzymatic reactions, whilst constantly experiencing population changes through mechanisms such as competition and predation.

5.1.1.1 Adapted soil microorganism community

For the purposes of this study, an holistic view of the microorganism community must be taken. The soil is viewed here as an '*in vivo* digester' and the microorganisms within it as a tool with which to compare the plant parts i.e. using the soil as a biological technology. Any assay developed to predict or measure edibility should be centred on microbial activity. This is certainly the mediator for decomposition and nutrient cycling (Sinsabaugh 1993). Sinsabaugh (1993) also hypothesises that microbial communities behave as an 'economic' unit, maximising production by optimising resource allocation between macronutrient acquisition activities. This is possible, as natural selection would drive populations toward this. It is thought that by using ancient woodland the

microorganism community has been exposed to oak defence compounds. Evolution of microorganism metabolism which can utilise the nutrients in the leaves despite the presence of such compounds has had time to occur. This then provides the 'best technology' for degradation of such plant materials. It is suggested that the varied populations contained in soil are best suited to degradation of the plant material that grows there and the time taken for this to happen gives an indication of that plant's edibility. This provides a 'biological technology' more suited to ecosystem measurements.

5.1.2 The Action of cellulase and ligninase

Enzymatic disruption is an aerobic process (Wild 1988). The aerobic fungal species *Trichoderma reesei* and *Penicillium pinophilum* have been implicated as important in decomposition (Wood 1985) although the process is complex as many enzymes act synergistically. Studies of stream ecosystems are prevalent due to rapid rate of decomposition and therefore period of study. In streams, fungi are the dominant and highly cellulolytic initial decomposers of leaf litter (Suberkropp and Klug 1976).

Cellulose has been implicated as one of the important compounds defining rate of decomposition of tissues in soils. The largest fraction of plant litter is the structural compounds that are resistant to degradation (Sinsabaugh *et al.* 1994). These include cellulose and lignin. Cellulase produced by microorganisms is an important aspect of organic matter decomposition (Walters and Smock 1991) as many species of fungi can utilise cellulose as their main carbon source (Wild 1988). For these reasons it was decided that it would be advantageous to include a cellulose standard. Raw cotton, which is usually over 85% cellulose was included and treated as the other plant parts for this purpose.

The relative importance of bacteria and fungi to decomposition differs among forest types although the more recalcitrant the litter, the greater the proportion metabolised by fungi (Elliot *et al.* 1993). Soils under coniferous canopies tend to have a higher ratio of fungal to bacterial production than deciduous soils (Sinsabaugh *et al.* 1992). This is probably because of the greater amount of lignin in recalcitrant litter. Fungi also show a higher metabolic rate than bacteria indicating that they are of greater importance to forest floor decomposition (Elliott *et al.* 1993). Cellulose in plant tissue is not always accessible due to lignin between and within plant cell walls (Kirk 1988), therefore bacteria would not necessarily find this an available source of carbon. Bacteria are unable to degrade lignin and it is degraded in the environment by fungi, but at a very slow rate (Sorlini 1991). The lignin fungi decompose their substrate more easily if there is a high cellulose content still associated with it (Wild 1988). Soils of Wytham Wood are known to contain viable populations of both bacteria and fungi (see MSc thesis Atree 1998).

Most of the cellulolytic fungi belong to the Ascomycetes, Deuteromycetes or Basidiomycetes and the lignin fungi are generally species of the higher Basidiomycetes (Wild 1988). Basidiomycetes have been isolated as important decomposers of lignin in straw in arable soils, although generally the lignin degraders are not well characterised (Harper and Bowen 1988). Ascomycetes and Basidiomycetes are able to mineralise all the structural components of wood. The presence of lignin would prevent access to the

cellulose for the cellulolytic fungi, and the presence of these lignin degraders within a community would render more available over time.

Multi-enzyme complexes exist in the cellulase systems elaborated by aerobic fungi (Wood *et al.* 1988). The enzyme system of *Penicillium pinophilum* consists of 2 major cellobiohydrolases, a glucohydrolase, 5 major endoglucanases, and 2 beta-glucohydrolases (Wood *et al.* 1988). Most cellulases are surface enzymes requiring physical contact between the decomposer and the resource. *Trichoderma reesi* possesses a binding exocellobiohydrolase and 2 endonucleases (Schulein 1988).

5.1.3 Litter quality and nutrient cycling

The annual litter fall in deciduous woodland provides the major source of energy and nutrients for the soil and litter organisms (Lousier and Parkinson 1976). The concentration of nutrients returned in plant material to the soil is important because of its influence on both the rate of litter decomposition and the amounts of nutrients liberated during decomposition (Jenzen 1974). Nutrient turnover is fairly complex, and as the rate at which this occurs affects the availability to growing plants, studies of nutrient cycling are common. For example, carbon turnover in soil depends on many aspects of the soil organisms that facilitate this. The dynamics depend on feeding habits, growth and metabolic rates, annual and seasonal abundance, generation time, competition and predation (Lousier and Parkinson 1975). Resource quality affects the rate of growth of microflora, and hence has effects down the detritus food chain (Heal and Dighton 1985). The rate of release of organically bound nutrients into inorganic forms is of particular importance when considering growth of plants that utilise these, and therefore ecosystem dynamics. Immobilisation occurs when elements are incorporated into an organic form and they are rendered unavailable to plants. There is also a growing body of evidence to suggest the decomposer organisms closely affect the characteristics of the plants grown in that environment (Grime *et al.* 1996). Herbivory and damage to leaves change the quality of the living plant tissue. In some species stimulation to growth occurs. Conversely, reduction in growth can cause fibre content to increase or nutrient levels to be reduced. Damage to leaves causes changes in the content of secondary chemicals, phenolics, tannins and nitrogen. This would then change the quality of the leaf litter produced (Findlay *et al.* 1996). Samples were therefore collected from the area, or as close to as possible, proposed for the study.

Nutrient recycling occurs in 3 phases: *i.* nutrient release through leaching, *ii.* immobilisation by decomposer organisms, *iii.* release (Prescott *et al.* 1993). The period of time a nutrient may spend in each phase is variable with site and substrate. Attempts have been made to model nutrient release, but patterns can be very variable and not simply related to the quality of the litter.

Nitrogen (N) release from plant material is considered important as this is a major nutrient required for plant and decomposer growth. N is important for herbivores and their symbiotic microbes. N may limit decomposer activity in early stages (Swift *et al.* 1979). Nitrogen content of leaf litter is thought to have a large effect on rates of decomposition although sometimes contradictory. Mellilo *et al.* (1982) found a positive effect between N content and rates of decomposition whereas Findlay *et al.* (1996) found no significant difference. Kemp *et al.* (1994) found N fertilisation had only a

small effect on the rate of decay of leaf litter. The role of N in decomposition is complex and cannot be generalised by measures of total N (Sinsabaugh *et al.* 1993). Factors contributing to high quality are high concentrations of N and low concentrations of lignin and polyphenols. Nutrient release is related to nitrogen content of litter and also probably nitrogen availability on forest floor (Prescott *et al.* 1993). Urea excreted by animals provides a substrate that is readily available to microbes and consequently can enhance conditions for decomposition of organic matter by providing N (Ruess and McNaughton 1984). The use of total N incorporated into a measure of quality, such as C:N, does not provide an adequate index of quality.

5.1.4 Role of energy in driving ecosystems

The bulk of organic matter in temperate forests is dead, and the majority is plant material (Hairston and Hairston 1993). Dead plant material (herbivore faeces is included in this term as it can be described as processed dead plant material) is often separated out from the food web and termed a decomposer food web. Flows of energy to this web are often large (see section 9.2 fig.9.1). Predictions are that as annual production increases, flow to decomposers decreases (McNaughton *et al.* 1989). This is presumably because these systems support a larger population of herbivores. Litter accumulation in the absence of large mammals affects decomposition processes by affecting the soil temperature, water, and nitrogen content (McNaughton *et al.* 1988). Wytham Wood has a considerable litter layer which probably helps to buffer temperature and water loss.

There is also a recommendation for a plant quality minimum dataset (PQMD) when considering plants rate of decomposition (Palm and Rowland 1997). This would then facilitate comparisons of rates between ecosystems. The PQMD would require the measurement of lignin, soluble carbon total carbon, soluble phenolics (if nitrogen contents are in excess of 1.8%), nitrogen, phosphorus, ash free dry weight. These methods are also used to characterise the quality of a plant material as food. These methods are therefore used in this study and provide information to support the rate of decay data. Thus, this study also incorporates the impact of the physical structure of plant parts on the rate of decomposition, as efforts have been made to keep this as intact as possible.

5.2 Method

The plant parts collected were green leaves, brown leaves, twigs, bark, roots, and acorns. These were collected on several dates throughout the summer and autumn of 1997. These were collected from a population of trees situated on line transects from an entrance on the edge of the wood. A small amount from each tree only was collected in order to prevent damage and to provide a varied genetic background. Bark was collected from both trunk and large lower branches. Most samples came from the lower branches as there was a limit on the height reached. Roots were dug within a radius of 2m of the trunk, and there was a limit to the size due to the tools available. They were narrow enough to be cut with a spade. Brown leaves were collected from the ground after senescence, but were undamaged and only collected from the top of the litter layer, and appeared to be freshly fallen. They were judged by eye to be over 90% brown. Green

leaves were assessed to be 100% green and undamaged and were collected from the lower branches. Some were collected from the edge of the wood, to avoid collecting purely shade leaf.

The samples were stored in airtight containers during transport to the laboratory. Raw cotton was included as a cellulose standard obtained from Ralli Brothers and Co., Liverpool. Before drying the plant parts were picked clean of insects, parasites and extraneous material. The leaves were separated from their petioles. Any damaged or diseased leaves were discarded. Twigs, roots and bark were cut into appropriate sized pieces (see table 5.1). In order to subject the parts of the plant to the same climatic variation, burial at the same time of year was necessary. As collection of the various parts was possible only when available, this necessitated drying of samples. This was carried out at a temperature of 80°C overnight and then cooled in a desiccator.

The litter bags were made of net curtain material of mesh size 0.05mm. These were stitched with polyester thread. The bags were then filled with sample as described in table 5.1.

Table 5.1. Table to show how each plant part was prepared and the content of each litter bag.

Plant part	Preparation	Mass per bag (g $\pm$ 0.01)
Cotton	Raw cotton from Sudan, California, Pakistan and Senegal	2
Acorn	Milled with hammer mill and 6mm seive, then small particles removed with 425 μ m seive	2
Root	All between 5mm and 20mm diameter and lengths of 20-40mm	5
Bark	Large pieces broken to under 3cm in all dimensions and sieved with 425 μ m	8
Twig	Between 5 and 20mm in diameter and lengths of 20-40 mm	4
Green leaves	Crushed lightly so no leaves intact and smaller particles removed with 425 μ m seive	4
Brown leaves	As green leaves	4

Seventy bags were filled for each plant part making a total of 490 bags and each was buried in a separate grid at 10cm depth in the topsoil. Grids measure 145 cm by 160 cm and the bags were separated by 5cm on all sides. The grids were dug within a 10m radius of an oak tree. The soil was well aerated, crumbly in texture and any clay patches were avoided to ensure aerobic decomposition ensued. The grids were placed on flat land to avoid being destroyed by flooding. The top soil and litter layer was replaced on each grid. 5 bags of each plant part were collected on each site visit.

Figure 5.2. Photograph of decomposition grid in Wytham Wood

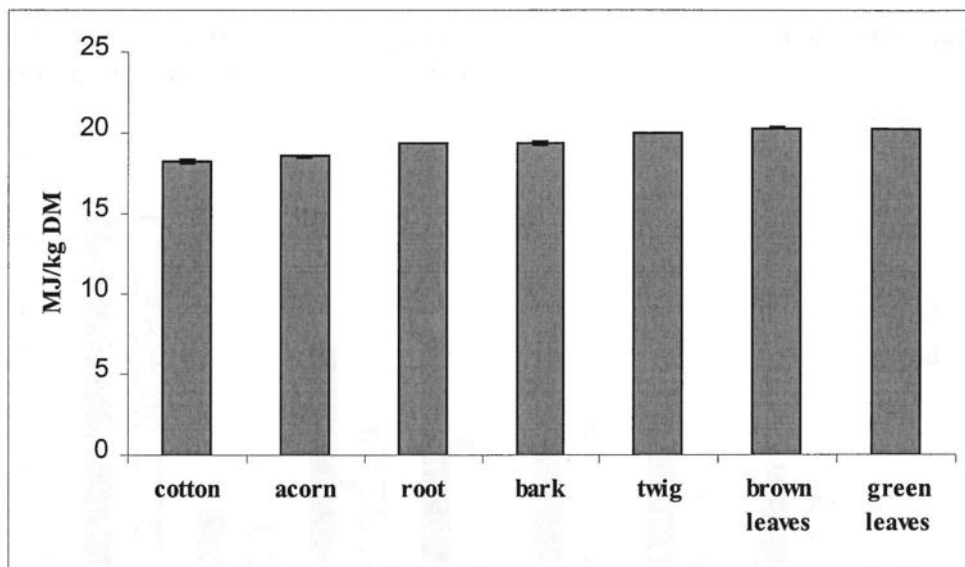


Subsamples were sent off for contract analysis to Natural Resource Management Ltd., Bracknell, for the following constituents. Nitrogen was determined using the Kjeldahl technique and water soluble carbohydrate by boiling with sulphuric acid and anthrone mixture. Sucrose and the water soluble fructans were hydrolysed by the acid to glucose and fructose, which react with anthrone at 100°C to form a coloured complex which absorbs at 620nm. Acid Detergent Fibre (ADF) was determined by the method described in chapter 4 (section 4.2.2.1). To determine lignin content, the sample was oxidised with potassium permanganate buffered with acetic acid, containing trivalent iron and silver as catalysts. Deposited manganese and iron oxides were dissolved with a solution of oxalic and hydrochloric acids. On ashing, the weight loss and permanganate lignin gives the cellulose content.

Samples were also sent to UK Agricultural Development and Advisory Service (ADAS), Stratford-upon-Avon, UK, for measurement of total energy content. This was carried out using a Parr 1261 Isoperibol bomb calorimeter.

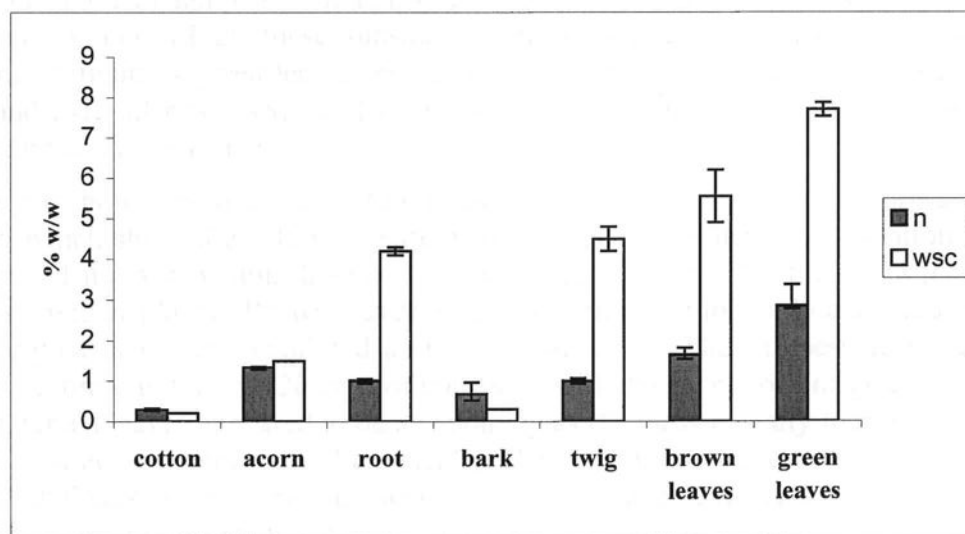
5.3 Results

Figure 5.3 Mean gross energy content (MJ kg^{-1} dry mass) of the oak tree parts



There appeared to be very little variation in the energy content of oak tree parts (fig. 5.3) when measured by bomb calorimetry. The number of samples is 2 and the bars show these 2 points. All plant parts varied between 17 and 21 MJ per kg of material. Cotton and acorn showed the lowest values within this range and brown and green leaves appeared to contain the most energy of all the plant parts.

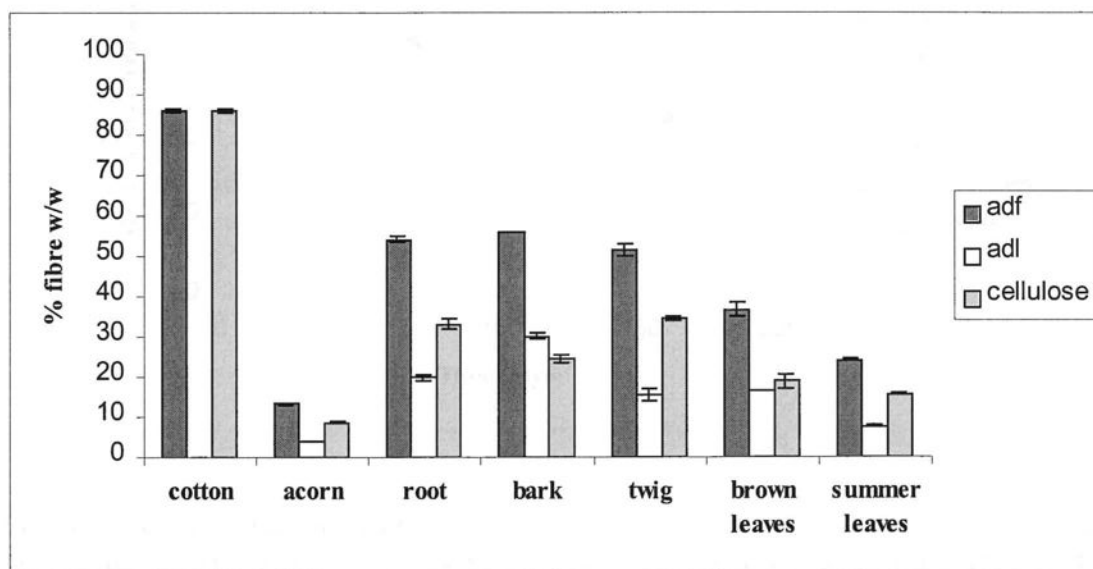
Figure 5.4 Nutrient status of oak tree parts as distinguished by water soluble carbohydrate (wsc) and nitrogen (n) contents (% of dry matter)



The highest values found for both nitrogen and water soluble carbohydrates were in the content of leaves (fig.5.4). $n=3$ for nitrogen and $n=2$ for water soluble carbohydrates. The bars depict the maximum and minimum data values (i.e. contain the range of the data). As would be expected for a mobile nutrient, nitrogen values were lower in brown

leaves than green. Lowest values were found in cotton and bark. Cotton contained very small amounts of these substances. Roots and twigs were very similar in their contents of both nitrogen and water soluble carbohydrates. The mean nitrogen and the mean water soluble compound contents were significantly correlated to one another ($r = 0.821$, $p < 0.05$).

Figure 5.5 Fibre status of oak tree parts measured by acid detergent fibre (adf), acid detergent lignin (adl), and cellulose content



The bars show the range of the data in figure 5.5, and $n=2$ for all plant parts. Figure 5.5 shows cotton contained a large amount of fibre, but none of this was present as lignin. Cotton contained more than 85% cellulose. Lowest overall fibre values were found in and acorn the leaf samples, although slightly more in brown leaves. Acorn showed the smallest amounts of all these substances. Brown leaves contained about twice the amount of lignin as green leaves, but only a small increase in cellulose content. Root, bark and twig all possessed similar amounts of fibre, but the proportion consisting of lignin and cellulose varied.

Figure 5.6 shows that the rate of decomposition was fastest for cotton, acorn and green leaves, which showed a different pattern to the rest of the plant parts. Cotton lost the majority of its mass within this time period. A smooth curve for this plant part, along with acorn is displayed. Brown leaves, bark, root, and twig lost only a small amount of their original mass, and exhibited a slow rate of decay. The sharpest decrease in the curves occurred in the first 20 days of the study, with the exceptions of green leaves and cotton. Green leaves appeared to be an anomaly as the mass actually increased at 2 data points, and at one point quite dramatically. Bars are omitted due to complexity in the graph, but this is given in the statistical analysis (section 5.4, figure 5.8). The actual data collected is given in Appendix C.

Figure 5.6 Graph to illustrate the mean decline in mass with time for each oak tree part, where $n=5$ at each data point

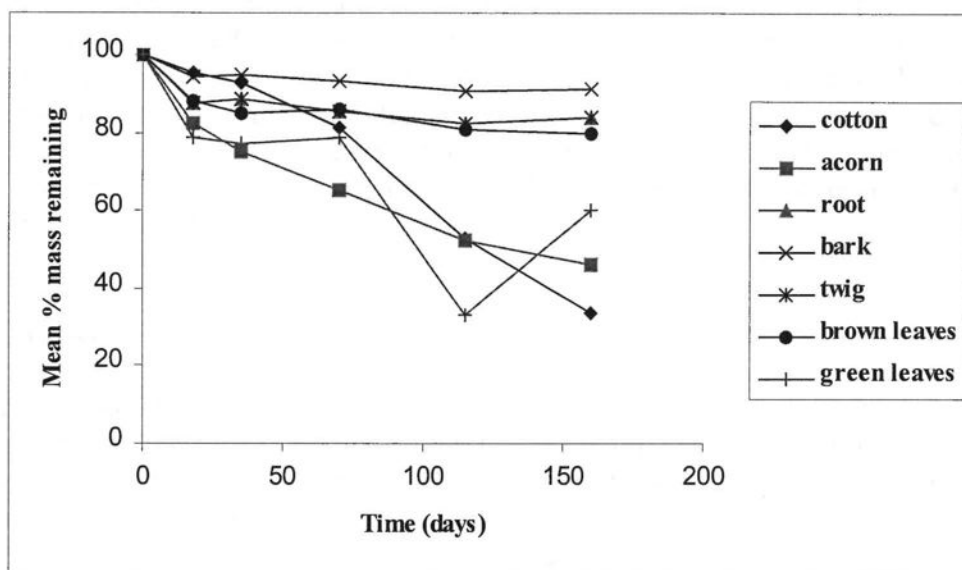
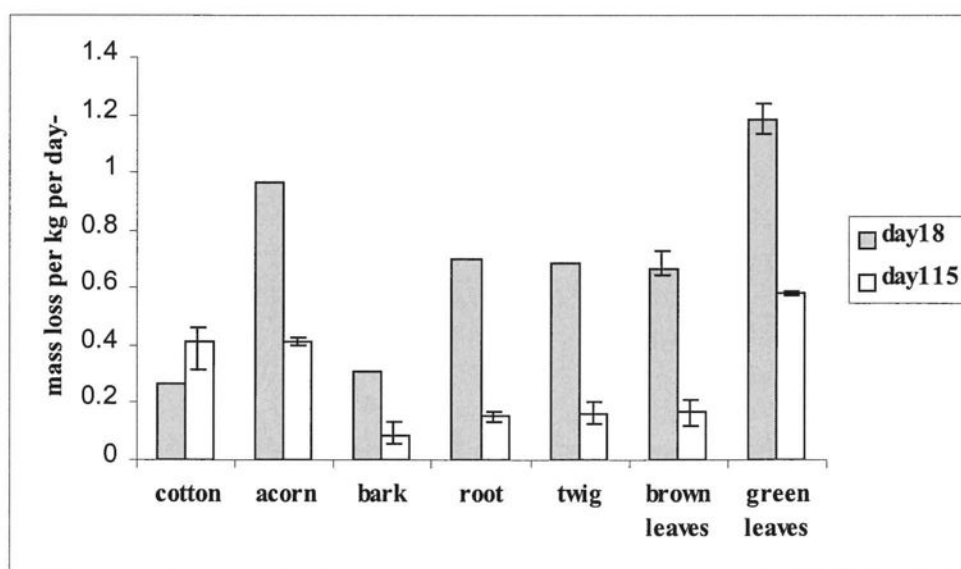


Figure 5.7 depicts the daily rate of mass loss of each plant part. The calculation of daily rate, by mean percentage mass loss over number of days, was carried out with 2 sections of the data, one using data from t_0 to t_{18} , and one using t_0 to t_{115} . The percentage of mass remaining was converted to a mass by treating the initial 100% as 1kg. Each percentage was then converted to represent the proportion of this 1kg:

$$[(\text{initial mass} - \text{mean mass remaining (kg)}) / \text{initial mass}] / \text{time (days)} = K_t (\text{kg} \cdot \text{kg}^{-1} \text{day}^{-1})$$

eq9

Figure 5.7 Graph to show the change in the mass lost per day for each plant part using 2 site visits, day 18, and day 115, $n=5$ (K_t in $\text{kg} \cdot \text{kg}^{-1} \text{day}^{-1}$)



The bars in fig. 5.7 show the spread of values produced from the 5 samples for each plant part at each date. 100% of the data is contained within these bars. For both dates chosen, the ranking of plant parts according to the rate of mass loss per day was the same. Green leaves and acorn lose most mass, root, twig and brown leaves are almost the same, and bark loses the least mass per day of all the plant parts at both stages. The data was used subsequently for calculation of a power density (Chapter 8) and also as rate constants K_{18} and K_{115} for correlations in section 5.4. K_{115} provides an estimation of a rate of decay following a linear pattern and K_{18} is a rate constant over the first 18 days between 2 data points.

5.4 Statistical tests

The use of non-parametric tests was necessary due to the distribution of the sample population. The increase in heteroscedascity throughout the study can be seen in figure 5.8 (page 57) and shows that the use of parametric tests in this case would be invalid. This is discussed fully in section 5.6.

The decision to use the data from site visit 0-5 was made because of the subsequent scatter of data points. The boxplots (fig.5.8) show that throughout the data collection period, spread from central tendency increases. The wide bars show the largest values observed that are not classed as outliers. The boxes themselves show the median and 50% of the values. If the line is not central in the box then the observed values are skewed. A 'o' indicates an outlier value and * and extreme value. There are several outliers and extremes for most of the plant parts except cotton. The data is skewed in many of the collection dates. The Levene test for homogeneity of variance (table 5.2) shows that for most plant parts (only twig excepted) the null hypothesis, that all the group variances are equal, is rejected at $<0.05\%$ significance.

Figure 5.8 Boxplots of the decomposition data for all seven plant materials to show increased heteroscedascity in sample masses from site visit 1 to 6

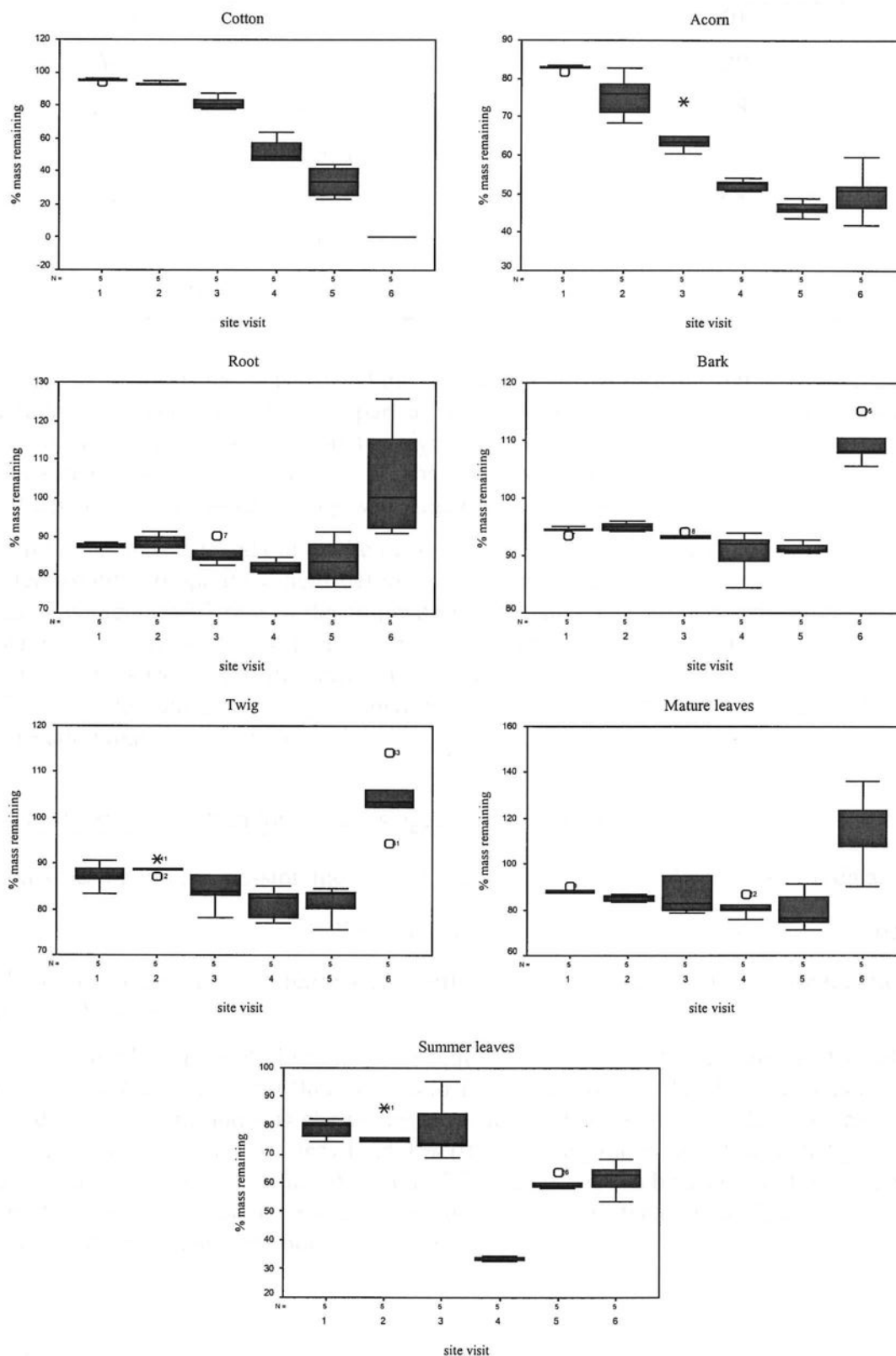


Table 5.2 The Levene statistics for variation between samples on each site visit for each plant part

Plant part	Levene statistic (based on mean)	Significance
Cotton	7.989	0.001
Acorn	3.380	0.029
Root	4.655	0.008
Bark	6.999	0.001
Twig	1.405	0.268
Brown leaves	10.116	0.000
Green leaves	6.917	0.001

The Kruskal-Wallis test was carried out on the percentage of mass remaining, up to and including site visit 5, with plant part as the grouping variable. The mean ranks were generated for each plant part and a significant difference between these was shown (Chi-square = 48.7, $P < 0.001$). The H_0 that there is no difference between the plant parts in the percentage mass remaining with time (up to day 160) was rejected.

Correlations were carried out on the means of all quality parameters. These are nitrogen, water soluble compounds, acid detergent fibre, acid detergent lignin, cellulose, gross energy content and 3 rate of decomposition constants. The percentage mass remaining data was converted into 2 values for each plant part: the mass lost per day using the data up to 18 days (K_{18}), and the mass lost per day from $T=0$ to $T=115$ (K_{115}) (see figure 5.7). The rate constants (b_1) generated by the curve fit model were also used. For the correlation matrix see appendix C.

5.4.1 Curve estimation for using a single exponential model

A curve estimation regression model was applied to the rate of decomposition data.

$$Y = b_0(e^{b_1 t}) \quad \text{eq10}$$

Where: b_0 = intercept, b_1 = regression coefficient, or decay constant, e = the natural log base, and t = time

This is a single exponential model where the R^2 value shows the goodness of fit of the curve generated. The nearer this value is to 1 the better the fit. The b_1 value can be used as a decay constant and this shows the percentage of mass loss per day. For all seven plant parts these values are less than 1% (0.01). The graphs are shown in figure 5.9, page 60 and the figures displayed in table 5.3, page 61. It can be seen that for most plant parts the exponential model explains the observed rate of decay well. The rate of decay of root and green leaves do not seem to be explained by this.

Figure 5.9. Curve fit estimations using a single exponential model for the rate of decomposition of each of the seven plant materials.

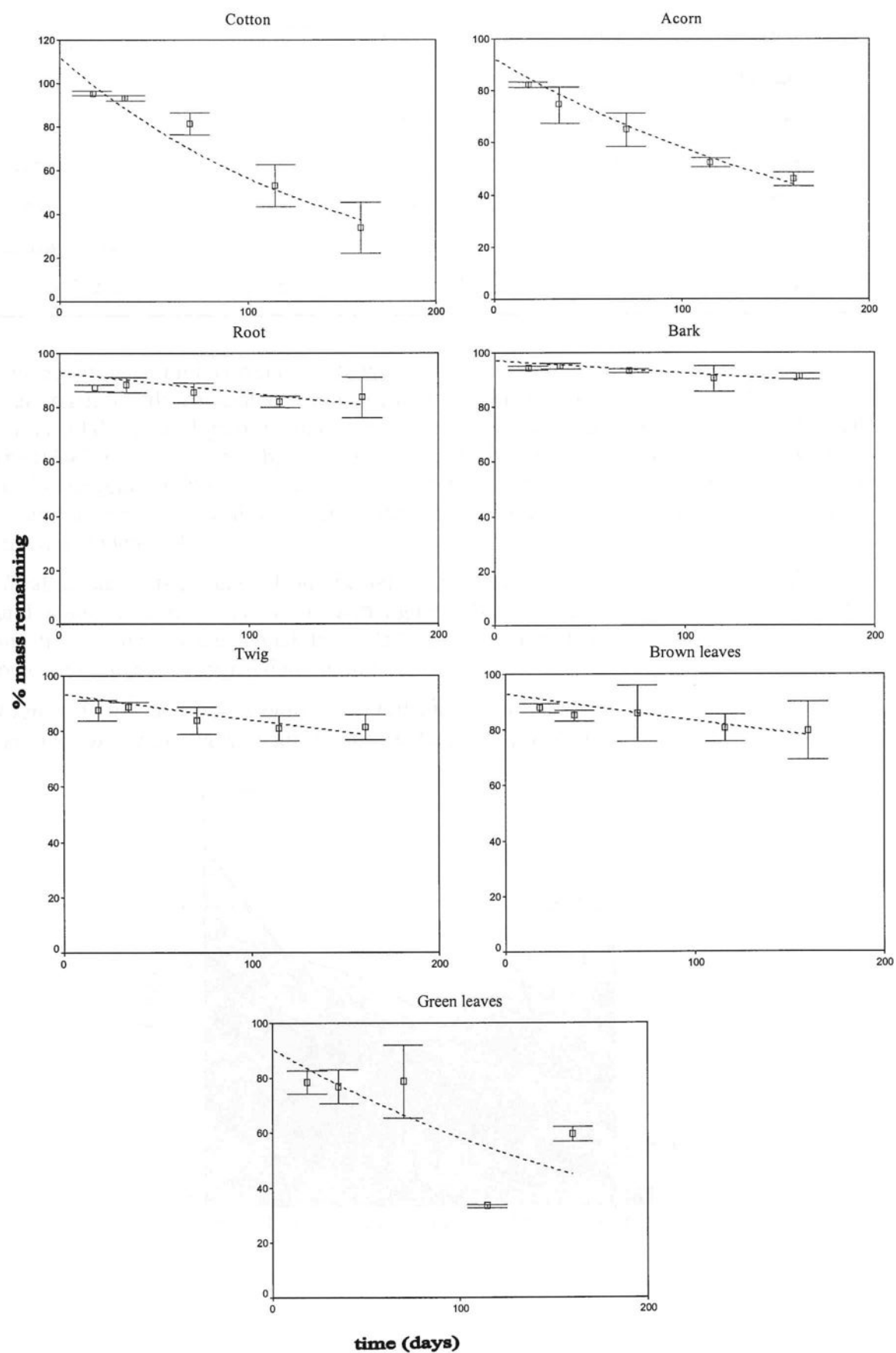


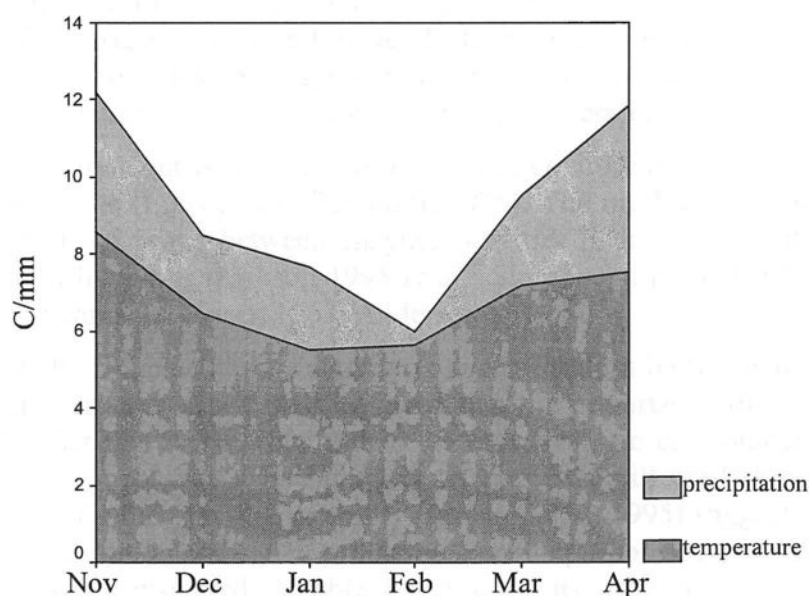
Table 5.3 Table to show the R^2 value and rate constant calculated using the curve fit model

Plant part	F value	Sig f	df	R^2	Rate constant (b1)
Cotton	65.6	0.001	4	0.943	-0.0069
Acorn	112	0.000	4	0.966	-0.0046
Root	5.48	0.079	4	0.578	-0.0009
Bark	10.2	0.033	4	0.717	-0.0005
Twig	8.83	0.041	4	0.688	-0.0011
Brown leaves	9.30	0.038	4	0.699	-0.0011
Green leaves	3.88	0.120	4	0.493	-0.0044

The Spearman's rank correlation shows that the rate of mass loss per day measurements were significantly correlated with some quality variables. K_{18} was negatively correlated with acid detergent lignin ($r=-0.886$, $p < 0.05$). K_{115} was negatively correlated with ADF ($r=-0.943$, $p<0.01$) as was b_1 ($r=-0.986$, $p<0.01$). b_1 was also negatively correlated with acid detergent lignin ($r=-0.812$, $p<0.05$) and positively correlated with K_{115} . K_{115} is also positively correlated with nitrogen content ($r=0.886$, $p<0.05$). The correlation matrix is given in appendix C.

Weather data was collected from the nearby Environmental Change Network (ECN) site and is shown as monthly averages in figure 5.10. Monthly average temperature under the tree canopy is calculated from daily minimum and maximum temperatures and precipitation was recorded daily in mm.

Figure 5.10 The mean monthly rainfall under the canopy (mm) and mean monthly temperature taken 10cm under the soil surface ($^{\circ}\text{C}$) for the study period.



5.5 Discussion

5.5.1 Methods used

The various methods which are employed to dry plant material are thought to have different effects. Air drying is probably the most common as this is thought to have the least effect on the constituents. Effects of air drying on leaching losses from leaves in decomposition studies varies from species to species. In some it can increase leaching losses whilst in others it can decrease (Taylor and Barlocher 1996). If old leaves are used for the study then leaching losses probably are not that different as to what would be experienced naturally as leaching of nutrients can occur while the leaves are still on the trees, where they are washed down to the litter layer. The method of drying should be standardised for decomposition studies to enable comparisons. Cornelissen (1996) found most leaves stored in open paper bags in a lab at 20°C reached their equilibrium moisture content (7-8%) within four days, whereas Palm and Rowland (1997) recommend 40°C to preserve phenolics and for the PQMD. The procedure used was to dry the samples in an oven at 80°C for 2 to 3 hours (until 2 constant weights are achieved) and then cool in a desiccator. This may have affected the secondary metabolites in the samples.

All plant parts had to be prepared differently due to the nature of the material. Milling was not possible for some parts such as bark due to the brittle nature and non-uniform structure. Incorporation of the physical nature of each material was considered important so efforts were made to prevent change of this within reason. Acorns were milled in order to prevent pilfering of the decomposition bed.

The litter bag technique used in this study has some inherent problems. The bags allow fine particles to wash out and allowed some invertebrate access (Boulton and Boon 1991). Reduction in the mesh size to the point where microinvertebrate access is prevented results in problems relating to water flow through the bag (Cornelissen, personal communication). Other problems include penetration of the bags by roots and colonisation of the bag by fungi and algae (Jackson *et al.* 1995). The main problems found in this study were badger digging of the beds, and surfacing of the litter bags. Enough bags were buried to allow for the occurrence of these problems.

The litter bag environment is different to the natural environment because of the more homogenous substrate (Lousier and Parkinson 1975). The mesh can act as a filter and differences have been found between enzyme activities in free particulate matter and that confined to a litter bag (Jackson 1995 *et al.*; Sinsabaugh *et al.* 1992; Bocock and Gilbert 1957; Witcamp and Olsen 1963; Anderson 1973).

A laboratory study of lignocellulase activity in decomposition beds was impossible due to lack of equipment and space. It is believed that the importance of a field study is reflected in the fact that it is carried out in the most realistic environment, as near to natural conditions as possible. The litter bag technique is still used to calibrate some enzymatic models for their predictive ability (Jackson *et al.* 1995) suggesting it is still a valid technique despite its associated problems. For the purpose of this study, the litter bag technique was considered suitable because of its ease of use and it allows comparisons between materials. Plant species richness also affects microbial biomass

present (Wardle and Nicholson 1996) providing another reason for use of decomposition study in the field rather than laboratory. The number of bags used was 490, and the small sample sizes had an effect on the statistics used (section 5.5.8).

5.5.2 Energy and nutrient contents

The total amount energy contained in the parts is no indication of their edibility (Fig.5.3). A high carbon value assigned to a plant part (i.e. a good carbon source) is no reflection of its potential to supply energy. The rate at which the nutrients can be accessed is of great importance. Nitrogen is probably limiting to growth and colonisation by microorganisms, closely linking carbon and nitrogen in importance. N levels are seldom less than 1% in plant tissues and plant litter (Mattson 1980). In litter, levels of N decrease very rapidly with colonisation of microorganisms (Kemp *et al.* 1994) suggesting the nitrogen content may be associated with the initial rapid decay rate. N and carbon levels are only indicative of quality, as the structure and availability of each nutrient, and the changes of this in time are not accounted for in many decomposition studies.

Nitrogen levels are a reflection of the amount of phenols present in a plant material (Palm and Rowland 1997). Some resources of nitrogen are given over to secondary metabolite production in the plant when supplies are plentiful. The amount of N in the green leaf sample indicates the presence of these substances is likely (fig.5.4). The rate of decay of the green leaf sample is very variable (figure 5.6) and this may have been a contributory factor.

Acorn shows a surprisingly low level of both water soluble carbohydrates and nitrogen. For the most nutritionally complete plant part, the levels of water soluble carbohydrate do not compare to those of root, twig, or leaves. N levels are lower in acorn than both brown and green leaves. However, if the fibre values are compared (figure 5.5) then it can be seen that acorn possesses less overall fibre, so it may be that more of this N is available.

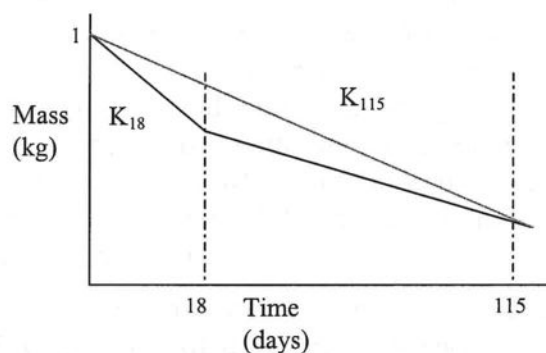
Fibre is present in plant material in many different forms. The methods used to analyse the fibre content of the materials in this study give a sketchy picture of the various structures present (figure 5.5). The amount of fibre present as lignin and cellulose can give an indication of the availability of this as a food source. Lignin contents are highest in the most recalcitrant plant parts, which are also the least palatable.

Cotton was included as a reference material for observation of the decay of the cellulose component. Cotton strips assays were used as an index of decomposition in soils by the Natural Environment Research Council (Harrison *et al.* 1988). It can be seen that cotton consists mainly of cellulose, more than 85% (figure 5.5) and very small amounts of N and water soluble carbohydrates (figure 5.4). The decay of cellulose occurs steadily over the time period without a high initial rate.

5.5.3 Rate of Decay

Figure 5.11 shows that a sampling date in each section can be chosen to establish a gradient. K_{18} gives an estimation of the readily available energy in plant materials. K_{115} treats the entire decomposition project as if it were linear.

Figure 5.11. Graph to show how the decomposition data was treated to obtain 2 decay constants



The rate of mass loss of the plant parts is studied using the mean daily percentage of original mass lost at 18 days after burial and 115 days (site visit 4) (figure 5.7). By using these dates, it is hoped both the rapidly leached substances and readily available sugars are represented as well as the more slowly available cellulose, and structural components. It is suggested that a rate of decay curve for a substance is a 2 stage curve, where the initial mass loss consists of easily accessed energy and some leaching of nutrients. Two stage decomposition curves were produced for most samples but a simple approach can be adopted, where the gradient of each slope gives the mean decomposition rate. An improvement on the model used in this thesis would be if the second decay constant was calculated using the time period 18 days to 115 days. K_2 would then give an approximation of the energy that is not immediately available. K_{115} gives the decay constant for a linear decay pattern. Few datapoints mean the relationship between the variables in each section of the graph (whether linear or other) cannot be established and therefore the simplest approximation is used.

The rate of mass loss was fastest for cotton, acorn, and green leaves (figures 5.6 and 5.7) which as expected for materials theoretically containing more available energy. Green leaves would be predicted to have a faster rate of decay than brown leaves as the latter probably contain more tannins and are broken down more slowly. (Cornelissen 1996). The more recalcitrant tissues may not follow the path of exponential decay (see fig. 5.9). Sinsabaugh *et al.* (1992) found mass loss of wood did not follow this, as after an initial period of several months, the pattern formed was linear. If this is the case, the rate of mass loss may not be suitable for the comparison of such heterogeneous materials, although in the initial phase a difference between all the plant parts has been demonstrated. The steeper curve associated with leaves, acorns and cotton is probably also connected with the physical structure of these parts, making the nutrients more available than the more structural components such as twigs and roots. The difference found between the plant parts also relates to the structural components and the action of enzymes on them (Sinsabaugh *et al.* 1992).

Figure 5.9 and table 5.3 clearly show the variation in rates of decay in each plant part. The R^2 value shows that the decay of cotton and acorn closely follow the exponential pattern. The data for green leaves is variable and in one site visit actually increases, suggesting there has been a large source of error in the later stages of decomposition. This may be due to incorrectly assessing soil and leaf particles prior to weighing.

5.5.4 Statistical treatment

The small sample sizes meant little attention must be paid to results that are not highly significant, as the chances of a type 1 error are large. A type 1 error is to reject H_0 when in fact it is true, and α gives us the probability of doing this (Siegel 1956). The exploratory data analysis (fig.5.8 and table 5.2) suggest this dataset is typical of decomposition studies. Scatter increases with time making the application of parametric tests impossible. T-tests are often used where small samples are concerned (Taylor and Barlocher 1996). Multiple T-tests are invalid as there is a high chance of committing a type 1 error (Boulton and Boon 1991).

Anova or Ancova tests are commonly used to compare means and explain differences between the rate of decomposition of different plant species (see Wise and Schaefer 1994; Gessner 1991; Franck *et al.* 1997). The power of this statistical test is dependent on how well the data meets the assumptions. For parametric tests such as these the data is assumed to have been drawn from a population which has the same variance as the data. The residuals must also be examined to check they are normally distributed. If error varies widely between the groups the power of a parametric statistical test is reduced (Wieder and Lang 1982). Anova can be a useful test when comparing plant species decay rates, but can only be used if all dates are considered (Weider and Lang 1982). The nature of decomposition data is not very well suited to a test such as this as heteroscedascity (scatter) tends to increase in later samples. Anova also requires an equal number of replicates and often researchers increase the number of samples with time to compensate for scatter. The data must be normally distributed and the data collected for the purposes of this study is not (figure 5.8).

It was decided that for the purposes of this study, non-parametric statistics should be used as the methods are more appropriate and are no less powerful than parametric (Boulton and Boon 1991). They are often called distribution-free tests as there is no need to satisfy population distribution assumptions, and are also suitable for application to data containing small sample sizes.

The Kruskal-Wallis one-way analysis of variance by ranks is a non-parametric alternative to the Anova test. This test helps to decide whether K independent samples are from different populations or whether they represent chance variations in random samples (Siegel 1956). It is the most efficient of the non-parametric tests for k independent samples (apart from F test) and it preserves the magnitude of the scores more fully than most other tests (Siegel 1956). It was used here is because of its ability to test the significance of difference between more than 2 groups.

For the purposes of this thesis, the data is studied to incorporate a time dimensions into energy acquisition. The rate of decay literature provides detailed exploration of models in order to do this.

A single exponential decay function which characterises the loss of mass was the first step and daily decay rates are used by Olson (1963).

$$dx / dt = I - kX \quad \text{eq11}$$

where I is organic matter input, X is organic matter accumulated, t is time, and k is the decomposition constant (Olson 1963).

Decay constants are a useful tool and often applied to enable comparisons of datasets. Development of this idea has led to double exponential decay model because of the two step nature of decomposition (Wieder and Lang 1982). However these do not account for cellulose being decomposed to a more available material (Carpenter 1981). A good compromise, a single exponential model that tends to a positive constant rather than to 0, is an asymptotic model. The application of this to decomposition data is questioned by Wieder and Lang (1982) as the recalcitrant tissue is not completely resistant to the action of micro-organisms.

An exponential decay curve allows comparisons between the plant parts as the duration of the study does not allow mass to equal 0. The R^2 value shows the goodness of fit and the rate constant shows the percentage of mass loss per day. For an accurate model of decomposition as 2 stage curve is necessary incorporating 2 rate constants (e.g. k_{1d} and k_{2d}) (Powlson *et al.* 1996). The model used here is not an exact fit (see y axis intercept <100, fig.5.9) but follows closely the observed rate of decay in the majority of samples. The rapid initial rate of decay may be responsible for the axis intercept.

Correlations suggest rate of decay may be largely dependent on the fibre content of a material. b_1 was correlated with ADL and ADF suggesting the curve fit model was predictive of the long term pattern of decay observed. K_{18} is significantly correlated with ADL, and K_{115} was significantly correlated with fibre and nitrogen content. This is consistent with other findings (Wise and Schaefer 1994; Tripathi and Singh 1992; Mafongoya *et al.* 1998). See appendix C for correlation matrix.

5.5.5 Climatic effects

The decay rate depends entirely on the nature of the original material if environment is not considered. In this case all plant parts were subjected to the same environmental fluctuations and can therefore be compared to each other.

Degree days have been suggested for use to factor out temperature and compare rates of decay in various biomes (Irons *et al.* 1994). This has mainly been used for aquatic systems where water temperature has been implicated as an important variable. Irons *et al.* (1994) found latitudinal gradients in rate of breakdown are attributable to the proportion of microbial decomposition relative to macroinvertebrate feeding. It seems litter can still easily be broken down at temperatures near freezing point. Degree days have also been incorporated into models of decomposition (Boulton and Boon 1991). As all plant parts in this study are subjected to the same variables, corrections are not necessary.

Dry periods may also affect decomposition therefore degree days above a minimum soil moisture deficit would be useful. More importantly this shows that standardisation under laboratory conditions is required in order to represent plant quality measurement.

5.5.6 Soil process modelling

There have been several attempts at modelling decomposition processes in order to establish a way of predicting the rate of decay in ecosystems. Respiration measurements are useful but limited in their usefulness as only a portion of the organisms present are involved in the degradation (Sinsabaugh *et al.* 1992).

Lately, there has been a focus on the enzymatic level i.e. mass loss with enzyme levels. Lignocellulases have been implicated as one of the most important factors to incorporate into soil decomposition or ecosystem processes models. When compared to the litter bag technique used in this study, enzyme studies have certain advantages, mainly that there is no error from loss of fine particles (Jackson *et al.* 1995). Enzymatic decomposition models retain predictive value even when viewed from the ecosystem perspective (Sinsabaugh *et al.* 1994). If extracellular enzyme activity is controlled by site and substrate characteristics then the relationship between mass loss and lignocellulase activity should transcend site differences (Sinsabaugh *et al.* 1992). Models such as these should be centred on microbial activity (Sinsabaugh *et al.* 1993). There is now a body of research which goes some way to bridging the gap between the biochemistry of fibre dynamics, and large scale ecosystem nutrient flow studies.

5.6 Conclusions

Decomposition processes are fundamental to ecosystem composition. Many processes of ecosystem functioning converge at the microbial community. The importance of microorganisms in ecology, especially for prediction of processes such as climate change, cannot be ignored. The decomposition of litter contributes to the formation of soils and determines community structure. Relationships between resource quality and decomposition are well known (Begon *et al.* 1990; Chesson 1997; Neely *et al.* 1991). This chapter has shown that litter bags can be used to measure energy release rate as a significant correlation was found between the different rate of energy release coefficients: K_{115} and b_1 .

Decomposition of plant material depends greatly on its physico-chemical properties (Cornelissen 1996) as does digestion. Features of plant tissue which are negatively associated with decomposition rate, also affect digestion e.g. lignin content, tannin content, nitrogen content, and physical structure (Cornelissen and Thompson 1997). This is probably because both processes rely on the actions of microbes, both fungal and bacterial. It is the diversity of microbial species that allows the decomposition of plant material, as many can cope with only a limited number of substrates (Begon *et al.* 1990). Grime *et al.* (1996) found decomposition rates of leaf litter corresponded with the palatability of the leaves to generalist herbivores. Begon *et al.* (1990) use the term 'external rumen' to explain the action of cellulases of soil microorganisms acting on plant detritus before ingestion of detritivores.

It is a fundamental concept of this thesis that general thermodynamic principles underlie the properties of plant materials as an energy source and the processing of these energy sources to release energy by heterotrophs. From the autotroph perspective, energy captured via the photosynthetic pathways is irreversibly changed from sugars to long chain molecules such as cellulose and lignin. Thus, this is a change in the 2nd law of

thermodynamics description (ability to do work in a system) which is important to the organism which attempts to release that energy. The bomb calorimeter gives a 1st law description (number of Joules) of the plant material. For there to be a general description of the ability of the plant materials to do work the system in which the work is applied also needs defining. The decomposition rate of different plant parts by microorganisms is a general or universal description of energy availability. The method of doing this constitutes a technology: an equivalent of the bomb calorimeter. At another level the microbes can be considered enzyme factories and enzyme technology represents another approach.

The rate of decay was chosen as an holistic measurement to incorporate a rate value from a microorganism viewpoint. The data generated from this are used further in chapter 8. Decomposition is a complementary process to digestion and an adequate understanding of the mechanisms involved is central to ecosystem functioning but beyond the realm of this thesis. A more standardised procedure that can be carried out quickly and used in the most basic of laboratories would be preferable for the purpose of energy availability assessment. The next portion of the thesis concentrates on a way to assess edibility *in vitro* as opposed to *in vivo*.

Chapter 6 Digestion and rate of energy supply

This chapter deals with the relationship between energy contained in plant material and how access to this is achieved by herbivores. It contains explanations of the biochemical processes involved in digestion and absorption and demonstrates how similar the processes used by all organisms are. This forms the basis of the new tests, as microorganisms are used to demonstrate differing rates of access to different quality foodstuffs. Food is a question of oxidisable products and how quickly these can be assimilated.

Cellulose is the most abundant carbohydrate in the world (Abe and Higashi 1991; Van Soest 1987) and therefore potentially provides an extremely important foodsource for herbivores. The ability of ruminants to utilise cellulose has facilitated their evolution and possible co-evolution with their foodsource. Because ruminants have been domesticated and become an important food source for man, their metabolic requirements and processes are well studied. The literature available provides relevant information on the quality of the plant as food for ruminants.

The amount of cellulose in a tissue is a key factor in determining edibility as it is an important substrate for microorganisms. Herbivores are known to require N in large amounts (Van Soest 1987), as do microbes, which are known to make up a large amount of the supply of energy to ruminants. Most herbivores utilise microorganisms for digestion.

6.1 Digestion.

Digestion is a process by which molecules in the gut are hydrolysed until they are small enough to cross cell membranes (Eckert and Randall 1983). These molecules are then assimilated by the process of absorption.

Some components of foods are insoluble in water, such as fats, cellulose and starch. In order to be absorbed they must be made into small molecules and hydrolysed by enzymes to render them soluble (Schmidt-Nielsen 1990). The main products of digestion are amino acids, from proteins, sugars from carbohydrates, and fatty acids from fat and cellulose. Most animals utilise these products, in varying quantities

Ruminant digestion occurs in an anaerobic environment (Orpin 1988). Ruminants (as with all higher animals) digest plant cellulose with the use of symbiotic microorganisms (Hungate 1966). The animal's rumen has often been described as a large fermentation vat, where the microorganisms are kept in controlled conditions with a constant supply of fibrous substrate (Van Soest 1987). The evolution of ruminants allowed them to eat large amounts of food and then chew at a more convenient location or time.

Rate of digestion varies with quality and type of diet, microbial population present and their pop sizes. The efficiency of microorganisms in digesting cellulose appears to be variable although fungi are often implicated for their importance in cellulolysis. Only 25% of the cellulose and hemicelluloses in straw are available to microorganism cellulases in the soil, as the rest is inaccessible due to the presence of lignin (Harper and Bowen 1988).

The presence of a certain microbial species in the rumen of an animal has been shown to be partially dependent on the presence of that organism in the environment or other members of the herd. The rumen contains a complete ecosystem that is presently partially characterised. The upper portion of the stomach expands to form a rumen which contains cellulolytic anaerobic bacteria, fungi, and protozoa. All species indigenous to the rumen are anaerobic. The eubacteria (prokaryotes) ferment carbohydrates to fatty acids, carbon dioxide and hydrogen. Archaeobacteria (methanogens) produce methane from carbon dioxide and hydrogen. Anaerobic fermentation can result in methane and carbon dioxide production as the sole endproducts with the action of methanogenic bacteria (Van Soest 1987).

Anaerobic fungi implicated in cellulolysis in ruminants are *Neocallimastix frontalis*, *Piromonas communis*, *Sphaeromonas communis*, *Trichoderma viride*, *T. koningii*, *Fusarium solani*, *Penicillium funiculosum*, *Sporotrichum pulverulentum*, *Talaromyces emersonii* (from Wood 1985; Wood *et al.* 1988). Many of these organisms were previously thought to be protozoan flagellates, but are now known to be zoospores of anaerobic phycomycetous fungi. These fungi remain classified as protozoa (Wood *et al.* 1988). Plant material in the rumen is quickly colonised by zoospores which attach and produce hyphae. The lignin fungi decompose their substrate more easily if there is a high cellulose content still associated with it (Wild 1988). Species frequently present include *Bacteroides succinogenes*, *Bacteroides amylophilus*, *Clostridium thermocellum*, *Clostridium locheadii*, *Ruminococcus albus*, *R. flavefaciens*, *Methanobacterium ruminantium* (from Begon *et al.* 1990; Wood 1985). Some bacteria are facultative anaerobes which utilise oxygen that has diffused through the substrate and hence protect the obligate anaerobes from exposure to free oxygen (Annison and Bryden 1998).

Microorganisms in the rumen produce cellulases which hydrolyze the β (1-4) linkages between successive D-glucose residues of cellulose and release glucose. This is then fermented to organic acids (acetate, butyrate, propionate) which are the true energy source for the ruminant. As discussed with reference to decomposition, the cellulase complex consists of three classes of hydrolytic enzymes: endoglucanases (or C_x -cellulases), cellobiohydrolases (or C_1 -cellulases) and B-glucosidases (cellobiases). It is believed the endoglucanases attack the amorphous regions of crystalline cellulose and in doing so create more sites for enzyme attack within the chains. These are attacked by cellobiohydrolases which destroy the structure in the cellulose and so the cycle continues, as more sites for action of endoglucanases appear. Cellobiose is liberated along with linear oligosaccharides and these are both ultimately degraded to glucose by cellobiases. The action of the cellulase complex is reported to be very similar throughout the literature (see Schulein 1988; Slaytor 1992; Wood 1989).

Fermentation and degradation products from soluble sugars, starch, pectin, hemicellulose and cellulose are mainly volatile fatty acids (VFA's), such as acetic, propionic, butyric, formic, and valeric, which provide the main food source for the animal. Complex lipids are hydrolysed by rumen bacteria and liberate long chain fatty acids. These are readily absorbed and oxidised or converted to other readily oxidised products and carbon dioxide, methane and microbes (Iason and Van Wieren 1999). The gases produced are lost by eructation. In the other fermenter groups, the VFA's provide less than 40% of their energy requirements but are the main source of energy for the ruminants (Iason and Van Wieren 1999). In comparison with ruminants, some insects

show evidence of producing VFA's from glucose derived from cellulose fermentation (Martin 1991).

Energy trapped as ATP during fermentation is used to maintain the microbes and these microbes themselves are an important source of amino acids in ruminants. The microbes produce most of the vitamins required by the animal and the microbes are digested by enzymes produced by the ruminant to amino acids and sugars and provide additional nutrition. The amount of energy provided for microbial cell growth is difficult to measure and provides another complication to studies of the fate of plant substances.

6.2 Absorption

Microorganisms and organisms with an alimentary canal must absorb food or/and nutrients through their body surface or surface membrane. Cell membrane structure is mainly lipid and proteins, although amounts vary. The phospholipid molecules which make up the membrane have polar heads which are hydrophilic and non-polar tails (hydrophobic). This forms the basis of an interface between an non-aqueous lipid environment (across the membrane) and the aqueous intra-cellular and extra-cellular phases, by orientation of the molecules. In 1972 a fluid mosaic model was proposed by Singer and Nicolson to explain the organisation of molecules within a membrane (Stryer 1995). The essence of this is described by Stryer (1995 p278) as:

"membranes are two-dimensional solutions of oriented lipids and globular proteins".

This is the phospholipid bilayer. The fluid nature of membranes allows globular proteins to move laterally within it. Integral proteins are thought to be amphipathic (ie polar and non-polar sections) as well as the phospholipids. This fluid mosaic model appears to offer the most acceptable structural picture of membranes and is supported by freeze-fracture studies. These are common to all heterotrophs.

Absorption of molecules can occur by various mechanisms. Substances that are in an aqueous phase can diffuse passively, or actively with sodium pumps, through channels in the membrane. Molecules can also be incorporated into the lipid layer, diffuse across this and re-enter the aqueous phase. Facilitated diffusion occurs with combination of the molecule with an ionophore. When there is a large concentration gradient, energy must be utilised for active transport. Carrier proteins bind to the molecule which then interact with membrane transport proteins to move the solute molecule inside the cell. In most microorganisms and the animal kingdom the energy for this is derived from ATP.

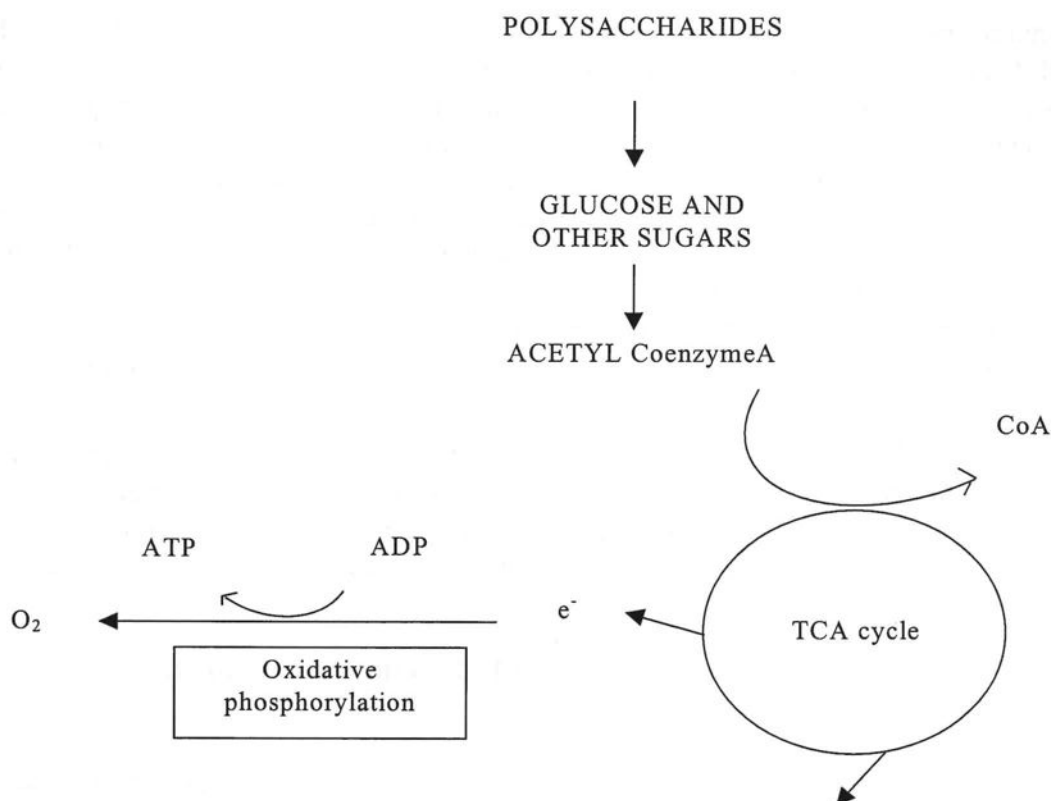
In the mammalian intestine, glucose is carried across the membrane by cotransport mechanism (Eckert and Randall 1983). This is driven by the electrochemical gradient of the sodium ion (from lumen into absorptive cell). Short chain fatty acids are probably primarily absorbed passively in the rumen and hindgut as there is evidence to suggest there is movement from a high to low concentration (Annison and Bryden 1998). Lipid absorption in the small intestine appears to be similar in ruminants and non-ruminants.

Micro-organisms can utilise almost any organic carbon molecule as a source for carbon, although the nutritional requirements of microorganisms vary enormously between species.

6.3 ATP as an energy currency.

Figure 6.1 shows the pathways involved in accessing the energy present in food. Polysaccharides, such as cellulose, are broken down by enzymes into smaller chains or monomers. These are then converted from these simpler units into the acetyl part of acetyl CoA. The final stage is the citric acid cycle and oxidative phosphorylation which completely oxidise the acetyl unit of acetyl CoA to generate ATP. The TCA cycle releases 4 electrons for each acetyl group. These reduce electron carriers (such as FAD and NAD^+) and as they flow down the chain to reduce oxygen, ATP is generated.

Figure 6.1. The metabolism involved in the extraction of energy from plant materials



Energy is the capacity to do work or cause particular changes. The ultimate source of all energy in food chains is trapped by photosynthesis and derived from the sun. ATP is a high-energy molecule and is used to generate energy in higher organisms. It breaks down completely to the products ADP and P_i with a standard free energy change of 7.3Kcal/mole (Prescott *et al.* 1996). It can then be broken down further to AMP and P_i . ATP has a high energy potential phosphate group and readily transfers its phosphate to water. ATP is formed in energy-trapping and energy-generating processes such as photosynthesis, fermentation and aerobic respiration. ATP is still generated in anaerobic respiration but by a less efficient method as a different electron acceptor molecule must be utilised. Therefore the basis of energy transfer is the use of ATP in all organisms. ATP is not the only compound used for this, but all possess the phosphate group with high energy bond (see section 2.4.1).

Symbiotic microorganisms benefit from the reduction of a carbohydrate substance to volatile fatty acids (VFA's) with generation of a little ATP. The host organism however can make use of these VFA's by aerobically oxidising them to carbon dioxide and water. One mole of hexose generates 5 moles of ATP anaerobically, whereas 38 moles ATP are generated aerobically (Chesson 1997).

6.3.1 Factors affecting digestibility

6.3.1.1 Chemical unpalatability factors

Factors that determine nutritional value of food are both qualitative and quantitative (Dzowela *et al.* 1995) and are often inextricably combined making assessment difficult and help to explain the many conflicting reports produced. Many studies have used the chemical composition of feedstuffs as a measure or predictor of nutritional value. Analysis of crude protein and fibre give an indication of feed value especially in herbaceous and browse legume species where secondary metabolites can restrict digestibility (Dzowela *et al.* 1995). Secondary plant compounds such as phenolics and tannins constrain utilisation of the food either by binding the bacterial enzymes or forming complexes with cell wall carbohydrates (Dzowela *et al.* 1995). There is considerable variation between individuals of the same species and this may be related to the tannin contents of their diets (Kaitho *et al.* 1997).

Nitrogen is derived from the plant material and incorporated into the microbial cells after conversion to ammonia (Dzowela *et al.* 1995). If nitrogen is suboptimal (<50 mg/l) for microbial requirements, the rate of digestion of cell wall by the microbes will be depressed (Wilson and Kennedy 1996)

6.4 Animal constraints on edibility

6.4.1 Retention time

Retention time of the food has been isolated as the main constraint on an animal feeding. Many physical characteristics affect this, such as shape and buoyancy of particles. Air can be trapped within and between plant tissues and this can be inhibitory to strictly anaerobic microorganisms, restricting the amount of microbes colonising the material. Large particles of plant tissue have a smaller surface area to volume ratio reducing the area that is available for microbial attachment and therefore a longer time is taken to digest large particles. Ten to twelve hours a day can be spent ruminating food.

The rate of digestion of plant cell wall by the rumen microbes is depressed by the supply of nutrients, especially N, if this becomes limiting (Wilson and Kennedy 1996). Particle passage from the reticulo-rumen is an inverse function of particle size (Wilson and Kennedy 1996). The more lignified a tissue the more this will have an effect. The amount of slowly digested fibrous particulate matter in the rumen has been pinpointed as one of the main constraints on a herbivore's intake rate of food. (Wilson and

Kennedy 1996) The lignin content of food is probably the most limiting factor in its digestibility (Orpin 1988). A large amount of lignin and xylans inhibits cellulolysis.

As the cell wall fraction of food is the most significant contributor to rate of digestion it has been the subject of most studies looking at plant constraints on rate. Much of this material contains undigested components that survive to the faeces (Van Soest 1987). The rate of disruption may be affected by the density and geometry of the vascular network. Sheep show a higher intake rate of leaves with a small number of, and small size of, vascular bundles in the stems and leaves (Wilman *et al.* 1996). Epidermal and vascular structures partly determine the initial breakdown of plant material.

All organic substances can become oxidisable eventually but the constraint to the animal is due to its energy requirements. There is a lower limit to the supply of energy an animal receives from its food. It is the rate of access to this which is the main constraint.

6.4.2 Particle size

Particle size is not often measured as a forage characteristic due to the difficulty of doing this (Van Soest 1987). The particle size produced from plant material will vary depending on the proportions of lignin and cellulose. Plants with a higher lignin content, due to its rigidity providing properties, would require more chewing and the particles generated would be less fibrous and larger. Older plants that have more thickened and lignified secondary layers will also give larger particles. This will subsequently affect rate of digestion due to the decreased surface area available to the membrane bound cellulases, as well as access to cellulose itself. The morphological characteristics of the particles determine the surface area and the interstitial spaces contained within (Van Soest 1987).

6.4.3 Intake rate

Intake rate is often associated with palatability. Lower intake rates are associated with higher proportion of fibre and lower digestibility and make the leaves more difficult to eat quickly (Wilman *et al.* 1996). The main constraint to intake rate is the high volume of slowly digested fibrous particulate matter and the slow passage of this from the rumen (Wilson and Kennedy 1996).

6.5 Scaling

As body mass increases, metabolic demand increases, but this is not a linear relationship. The metabolic requirements of a small animal are higher than a larger animal per unit mass. Indications are that resting metabolic rates increase approximately with the 0.75 power of body mass. There is evidence to suggest there is enhancement of glucose absorptive mechanisms in larger animals (humans compared to rats) (Pappenheimer 1998). Retention time of food particles also appears to be related to body weight (Demment and Van Soest 1985).

6.6 Discussion

Herbivores must acquire energy from a variety of plant material for all their energetic requirements. This therefore is obtained from the single source of the sun. All animals are heterotrophic. All their energy is derived from autotrophic organisms. Oxidation of carbon compounds yields energy that can then be used to synthesise ATP. Energy is transferred down the food chain in this way. Energy is given off as heat at each step in a food chain. The costs of biological maintenance and metabolism mean that not all energy can be transferred to the next step in the chain.

All aerobic organisms, whether large herbivores or microorganisms use the same biochemical pathways to oxidise foodstuffs. Foodstuffs viewed in this way consist of oxidisable compounds. The ways in which they are rendered oxidisable differ sometimes between organisms. A test which can measure the amount of oxidisable substances in a substrate will provide some information on how valuable this substance is as a food source. The total amount of oxidisable substances is measured by bomb calorimetry. It is the rate of access to oxidisable substances that is important from the point of view of a consumer. A 2nd law description of a substance provides a measure of the quality of the energy available, whether it can be accessed quickly. In higher organisms there are two steps to oxidising substances. Microbial substances are oxidised by microorganisms anaerobically and the products then oxidised aerobically by the animal. Measurement of which substances in a food source can be oxidised by these paths provides the basis of a new test.

A measure of edibility cannot be focused purely on the constituents of a plant tissue. The quality of a plant as food depends also on the digestive 'technology' utilising it. A measure of edibility should take this into account. Digestive technology seems to be dependent on the suite of microorganisms housed in the tract and whereabouts they are housed: whether before or after absorptive surfaces. Identification of key substances required by organisms is possible from the literature and this is what scientists have focused upon. The analysis of animal feed is usually carried out by standardised methods that have been developed over many years (see Association of Analytical Chemists 1994). However, the various methods used in laboratories to analyse a plant material do not reflect the nature of the material. The presence of a nutrient extracted in the laboratory does not make the nutrient available to a herbivore. The nutrient may be present but may be in a form such as present in a complex with lignin. Again, the methods in general use can be regarded as akin to measurement of instantaneous energy content. Descriptions of a plant material in this manner are often not relevant to ecosystem energetics when considering the animal 'technology' that would normally utilise the material.

The development of a more appropriate technique for measuring energy assimilation possibilities has implications to the field of agriculture and improvement of yield from ruminants. The conversion of plant material to animal products has been the subject of much research, and with an improved basis of measuring an animal's digestibility, could be built upon.

"...plant material should be characterized by the component fractions that show uniform reaction to digestive enzymes"

(Demment and Van Soest 1985).

The overwhelming aspect of digestion, absorption, body size relationships and energy acquisition is the fact that rate is important. In the past, scientists have focused on content or status of energy at one particular moment in time. This is where the problem of measurement arises. The rate of rendering available, and assimilating, nutrients is dependent on all the factors discussed in this chapter.

Herbivores can adjust to change in the nutritional status of their food to a certain extent by diet change and seasonal movement. Many studies have been undertaken to allow predictions from anatomy and body size to diet choice. In practise this has proved difficult and scientists do not wholly agree on how to classify digestive strategies; it seems digestive technologies can come into play in emergencies ie extreme but rare situations, and that animals prove themselves to be very flexible in difficult circumstances. In general, the relationship of body size and diet choice is an increase in the amount of cell wall in the diet with the larger body size. This material is abundantly available and therefore can satisfy the greater metabolic requirements of the larger herbivore with the employment of microorganisms.

The information in this chapter was used to design an appropriate laboratory study to measure the energy available to heterotrophs from plant tissues. The method produced was an holistic measurement of this with intended application to ecosystems research. Most herbivores use microbial symbionts and most utilise the cell wall for maintenance of the population. Most aerobic microorganisms absorb similar compounds and use ATP as an energy currency, so the test is based on the use of aerobic microorganisms for this. All organisms are limited by time. They must assimilate nutrients at a certain rate in order to survive. This concept forms the basis of the laboratory test.

Chapter 7 The development of a laboratory method to measure energy availability

In chapter 5 it was established that the rate at which microorganisms digest plant materials is an appropriate calculation of energy availability. The problem that remains is how to standardise such a measure so that plants in ecosystems can be compared. This was the apparent strength of bomb calorimetry and why it formed the basis of the International Biological Programme. One solution may be to adapt a standardised technique developed for a seemingly very different purpose in the water industry. The test concerned, the measurement of the Biochemical Oxygen Demand of water, provides a good measure of the digestion of plant materials by microbes.

This chapter discusses the way in which water quality tests have been adapted to serve the purpose of establishing plant quality. The utilisation of the substrates by microorganisms, and the possible problems that may arise in the BOD test, are described. The process of method development, and preliminary results from this, are given. The discussion includes improvements that could be made to this method to enable it to become a standard technique in general use.

7.1 Biochemical Oxygen Demand (BOD)

Biochemical oxygen demand (BOD) is defined as the amount of oxygen utilised by microorganisms while metabolising decomposable organic matter aerobically. It originates from The Royal Commission on Sewage Disposal, where it was used to estimate effects on streams from discharged effluent. *Decomposable* can be taken to mean the organic matter that is available to microbes, and can therefore derive energy from its oxidation (Sawyer *et al.* 1994). The application of the BOD test is mainly in measuring waste loadings of effluents from treatment plants. It estimates the oxidisable fraction in wastewater (Gray 1989).

The test is carried out in conditions as close to nature as possible. 20°C is set as the incubation temperature. The microbes or 'seed' used are often native to the soil (Sawyer *et al.* 1994). They usually consist of large numbers of heterotrophic bacteria and also nitrifying bacteria, which could deplete dissolved oxygen levels. It has been found that a large proportion (70-80%) of the total BOD occurs in five days so the test has developed to be the BOD₅ test. This period of time is also selected in order to minimise interference from the oxidation of ammonia. The BOD test thus measures the amount of carbonaceous compounds that can be oxidised (when the nitrogenous oxidation is suppressed by an inhibitory chemical, in this case allylthiourea). It shows which compounds are biologically available to microorganisms present in the solution. The microbes in the solution are utilising the compounds present as a feed stock, and the amount of dissolved oxygen used in the process gives an indication as to the amount of 'food' present.

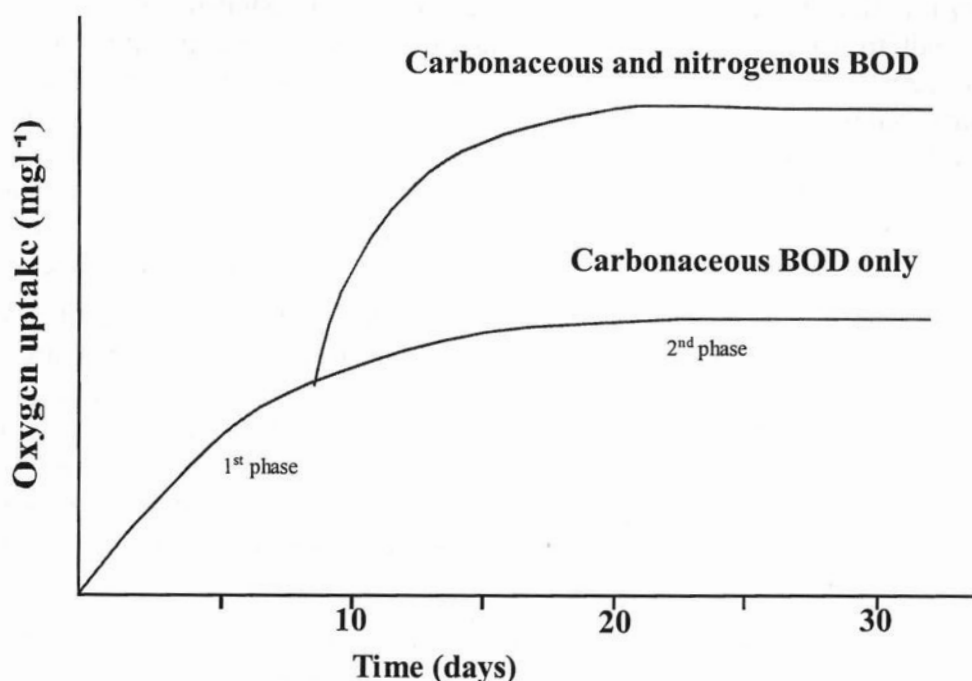
Animals also obtain energy through the oxidation of foodstuffs. The rate of oxygen consumption is often used synonymously with the rate of energy metabolism (Schmidt-Nielsen 1990). If the rate of oxygen consumption by microorganisms can be seen to be

similar to higher animals, then the BOD test may provide us with an appropriate technology to measure edibility.

7.1.1 Action of microorganisms.

The BOD bottle contains a microbial ecosystem with the environmental conditions stabilised. It is generally believed microbial growth rate is a first order reaction, although this has been disputed (Stones 1982).

Figure 7.1 Graph depicting the progress of oxygen uptake in a BOD bottle over time by microorganisms.



Heterotrophic bacteria utilise the organic matter present for maintenance and growth. The first phase results in an increase in the microbial population and depletion of the available organic matter and oxygen. This can be summarised by:



This phase generally lasts between 24 and 36 hours, and in this time period approximately 50% of the organic matter is oxidised (Gray 1989). When the substrate becomes limiting there is a period of slower oxygen uptake usually referred to as endogenous respiration (Her Majesty's Stationery Office 1988) where the cells produce energy by self-oxidation. This is also referred to as the stationary growth phase and is shown on fig.7.1 by the plateau.



The above is carried out by endogenous respiration.

The time taken for this to occur varies, dependent on the type and nature of the organic material (Her Majesty's Stationery Office 1988). The rate constant for a first order reaction such as this is usually between 0.05 and 0.3 per day (Phelps 1909). The presence of this plateau (fig 7.1) is also linked to predation upon the microbial biomass by protozoans (Bhatla and Gaudy 1965). For this study, the amount of oxygen taken up by the organism's metabolism is represented by the 1st phase of the graph, when a linear relationship between the oxygen uptake and time was expected.

The nitrogenous BOD, when not suppressed, doubles the amount of oxygen used in the bottle. Figure 7.1 shows that this does not usually occur before day 5, although a chemical inhibitor is normally added to prevent this happening (see section 7.1.2).

The nature of sewage is similar to plant tissue from the point of view of the oxidisable compounds in the substrate. Organic matter in sewage comprises of carbon, hydrogen and oxygen with nitrogen frequently present. In settled sewage 50% of the organic carbon and 35-50% of the organic nitrogen is in solution (Painter 1983). Three quarters of the organic carbon is made up of carbohydrates, fats, proteins, amino acids and volatile acids (Gray 1989). Fats are not water soluble but are important as they may contribute up to 50% of the total carbon present (Gray 1989). All these substances are present in plant tissue making application of the BOD test valid. Many constituents of sewage are decomposed biologically at slow rates, which is also true of plant tissue.

A process, to enable use of the BOD test, was designed and this is depicted in Fig.7.2. Several attempts were made to solubilise the tissue adequately to simulate processes present in nature. This was necessary in order to provide a standardised digestion procedure. The material must be in solution in order for an organism to absorb the nutrients, whether within a digestive tract, or from an external source as with microorganisms. A dilution factor was then established to bring the concentration of the solution within a range measurable by the BOD test.

Figure 7.2. Diagram to show development of the procedure for preparation of plant part for application of the BOD test



7.1.2 Interferences

Nitrifying organisms were inhibited by the addition of allylthiourea (ATU) to the dilution media. This was to prevent use of the dissolved oxygen by these organisms, which would provide high results. ATU prevents the onset of nitrification for approximately 9 days with no effect on carbonaceous oxidation (Gray 1989). Without ATU the oxidation of nitrogenous matter would occur (see below) exerting an extra oxygen demand, although this may not occur to any detectable extent in the 5 day BOD determination (Department of the Environment 1972).



In order to guard against varying pH, phosphate buffer is added to the dilution media. As the pH of the plant part solution used is 5 (because the enzyme used for the digest procedure requires this), the pH of the BOD solutions was measured to check that the addition of this did not cause too much variation from the recommended 7. The pH of the solution for a BOD test should ideally be between 6.5 and 8.5 (Her Majesty's Stationery Office 1988).

As any substance toxic to microorganisms will affect the BOD result, it is probable that plant phenols and other secondary metabolites have affected the results reported. This is not seen as error as these compounds have a detrimental affect on microorganisms involved in digestion. These substances are incorporated correctly into this measurement of plant edibility.

7.1.3 Modifications

The BOD test was adapted to the BOD₃ test for this study as there is no requirement to make the data comparable to water quality tests. This also allowed an increased experimental throughput. The dissolved oxygen of a known amount of fluid is measured before and after 3 days.

7.1.4 Cellulose digest

A standard digest procedure was carried out on all plant parts. This was thought necessary as, in order to perform BOD and COD test, particulate matter must be filtered out. This means that an important constituent is not present for the tests. Cellulose is not soluble in water but a digest would show how much of the cellulose could be made available to an organism, or suite of microorganisms. Cellulose is a key substance when considering digestion in many organisms.

A measure of the amount of cellulose that can be solubilised is afforded with the use of a cellulose digestion procedure. The BOD test is carried out before and after this digest and provides a crude measure of this. Cellulose is insoluble so the BOD test carried out prior to the digest (preBOD results) gives the oxygen demand of the non-cellulose component. The digest renders the cellulose soluble so the BOD test carried out after the digest (postBOD) gives a measurement of the oxygen demand from both the cellulose which is available to an enzyme in that plant part, and the non-cellulose component.

7.2 Chemical Oxygen Demand (COD)

This test is widely used as a measure of the organic strength of industrial and domestic wastes. It provides a measure of the amount of oxygen required in oxidising the compounds to carbon dioxide and water. Most organic compounds are oxidised by

agents under acid conditions. The compounds that are not oxidised include aromatic hydrocarbons and pyridine (Her Majesty's Stationery Office 1986).

Glucose and lignin are converted to carbon dioxide and water. These very important and different (in terms of their relative energy availability) compounds are not distinguished by the test. It measures that which is oxidisable in a very short period of time, as does the bomb calorimeter, but the results are given in terms of mg l^{-1} of oxidisable compounds instead of energy units. COD tests are, therefore, always of a higher value than BOD tests and if a large amount of biologically resistant matter is present (for example, in wood pulping wastewater) the COD may be much higher than the BOD (Sawyer *et al.* 1994). The limitation of the COD test is the fact that there is no differentiation between biologically oxidisable compounds and biologically inert organic compounds. In this case, the recalcitrant molecules that have not been cleaved or ground finely enough to fit through the syringe filters are not present. This means the samples mostly consist of soluble sugars. The lignin and unavailable cellulose would be removed in both the pre and post-digest products. The test kits consist of potassium dichromate as an oxidising agent in a strongly acidic environment. The vials are incubated at 148°C in order for oxidation to take place. The advantage of this test over the BOD is the rapidity of the test kit (i.e. 2 hours as oppose to 5 days). If a relationship between the two tests can be established, the more laborious BOD test can be subsequently disregarded.

The COD test will always give a higher reading than the BOD, as it provides a measure of the non-biodegradable compounds as well as the biodegradable ones. The ratio of COD to BOD is often used as a guide to the proportion of biodegradable material in wastewaters. It is often assumed to fall around 2:1 (Ormesher personal comm.) but as this has never been carried out on plant material this cannot be assumed to be true. For substances over 100mg/l the correlation is usually expressed as:

$$\text{COD} = a\text{BOD}_5 + b \quad \text{eq15}$$

where a and b are constants (Gray 1989).

The COD test was carried out on the plant parts along with the BOD test. The plant part solutions were prepared as for the BOD test, and the appropriate dilution found for the range $0\text{--}1500\text{mg l}^{-1}$ COD test kit. This was found by trial and error. The plant parts were prepared as shown in fig.7.2.

7.3 Method

Subsamples of the plant samples used for the decomposition experiment were taken for these tests.

7.3.1 Materials and equipment used

Equipment:

Pestle and mortar
Waring blender
Magnetic stirrer

Astell Hearson autoclave AAJ040
Nalgene syringe filters obtained from Merck Ltd., Lutterworth, Leics. 0.45µm catalogue number 402/0800/12. 0.2µm catalogue number 402/0800/11.
Camspec M350 Double Beam UV-Visible Spectrophotometer
Kartell polystyrene standard cuvette from Merck Ltd. Catalogue number 307/3802/02
Soniprep 150 (MSE) sonicator
Roller mill, Pascall engineering Co. Ltd., machine number 21533
Hach COD test kit vials high range 0-1500mg l⁻¹ obtained from Camlab Ltd., Cambridge. Catalogue number HH/21259-51
Camlab portable datalogging spectrophotometer Hach DR/2010
Orion pH meter model 410A
Jenway portable DO2 meter 9071
LMS cooled incubator
Merck Thermoreactor TR200
Corning 252 colorimeter
Shaking machine- LH Engineering Co. Ltd., Bucks, UK
Stomacher- Lab-Blender 400

Chemicals:

Protein assay (Bradford method) Bioquant testing kit obtained from Merck Ltd., Lutterworth, Leics. Catalogue number 43681 2W
Cellulase enzyme derived from *Trichoderma reesi* obtained from Sigma chemicals, Poole, Dorset (cat. no. C8546).
All other chemicals used were AnalaR reagents obtained from Merck Ltd. unless stated otherwise.

7.3.2 Maceration of the plant parts

Large herbivores must masticate their food for an extended period of time in order to increase the surface area available for microbial action. Smaller herbivores, such as forb chewing insects, must still rupture the cell wall to access the cell contents. In order to perform these tests, a reproducible method of attaining similar particle sizes was necessary. Ideally all cell walls would be broken to expose the cell contents, but practically this is difficult to achieve. Several methods of maceration were attempted.

7.3.2.1 Pestle and mortar.

1g of green leaves with 40ml of water were ground in a pestle and mortar with 0.5g of glass balls to increase abrasive action for 30 minutes. In order to establish the optimum time for cell breakage, a sample of 2ml was taken every 5 minutes. This was pushed through a syringe filter of 0.45 microns and the absorbance read at 450nm (the main peak of absorbance of chlorophyll). It was assumed that when no more cell walls were intact then the absorbance reading would remain constant. This could only be carried out for green leaves due to sufficient amounts of chlorophyll being present.

Table 7.1 The absorbance of the plant sample over time at 450 nm

Time (mins)	Mean absorbance 450nm
5	0.137 ± 0.030
10	0.168 ± 0.076
15	0.103 ± 0.088
20	0.150 ± 0.110
25	0.150 ± 0.119

Table 7.1 shows the amount of chlorophyll in the sample is variable with time. It is thought the variance in the reading was caused by the heterogeneity of sample and the difficulty this caused in pipetting a representative sample. The sample was prepared by centrifuging and this gave source for further error. This method was later modified with the use of a blender for 10 minutes prior to use of pestle and mortar. A sample of this was placed under a microscope for inspection and to study the state of cell breakage. The sample appeared to be heterogeneous as can be seen in figs 7.3 and 7.4.

Figure 7.3. Green leaves at x200 magnification after grinding in a pestle and mortar for 15 minutes

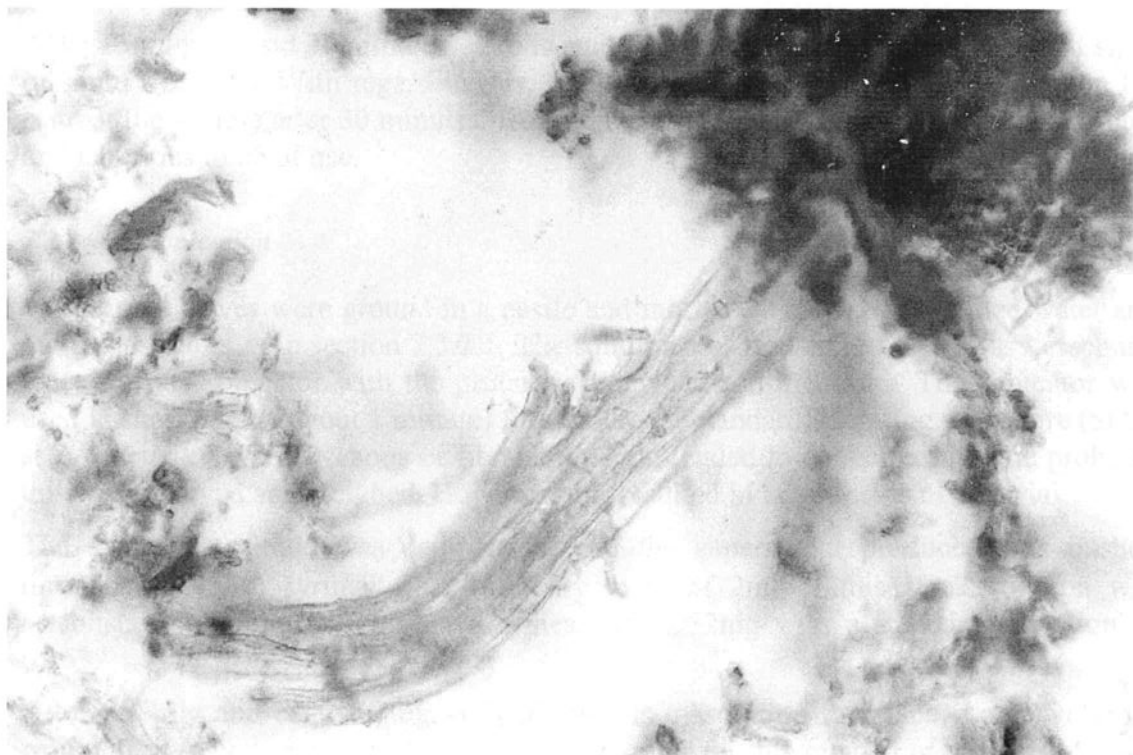
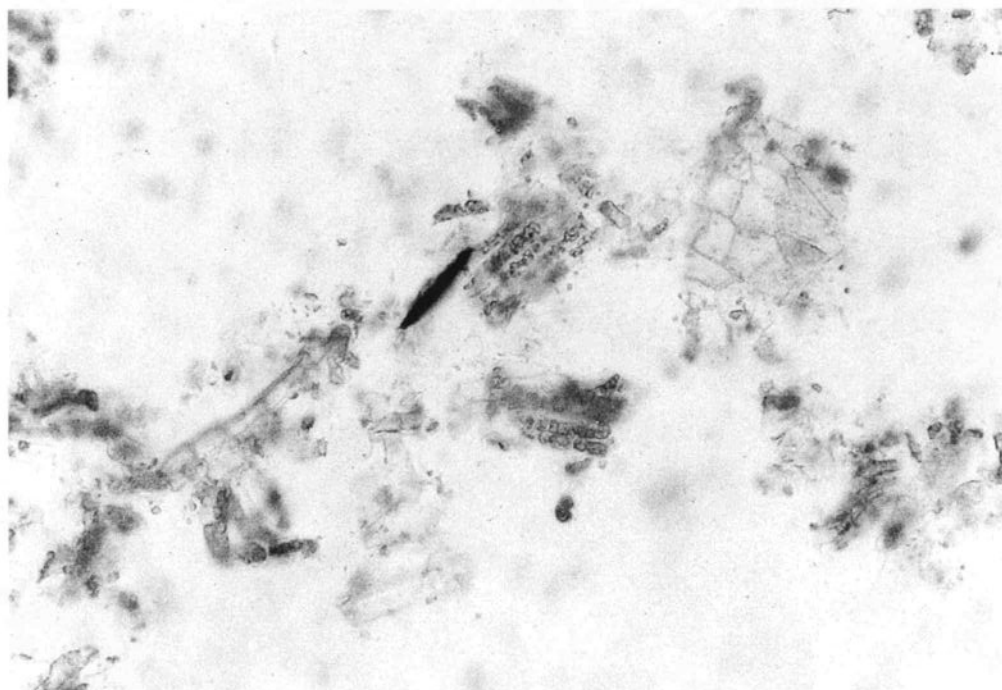


Figure 7.4. Green leaves (x200 magnification) after grinding in a pestle and mortar for 30 minutes



After 15 minutes and 30 minutes there is evidence of much recalcitrant tissue and signs of some free cells. With regard to this, Fig7.14 did show a slight improvement on the state of the sample after 30 minutes. However, this method was found to be too variable and laborious to be of use.

7.3.2.2 Sonication

2g of green leaves were ground in a pestle and mortar with 20ml of distilled water and 1g of glass balls as in section 7.3.2.1. The solution was transferred to a glass vessel and placed in the sonicator with the probe just touching the meniscus. The sonicator was used in short bursts (about 1 minute) for 1 hour (for standard operating procedure (SOP) see Appendix D). The venous or fibrous material tended to collect around the probe so this was removed and weighed. However, this resulted in large losses of material.

The sonicated sample was centrifuged, and the supernatant produced was pushed through a 0.45 μ m syringe filter. The dry mass of 2ml of this stock solution was established in triplicate. This gave a mean of 0.952mg with a standard deviation of 0.163.

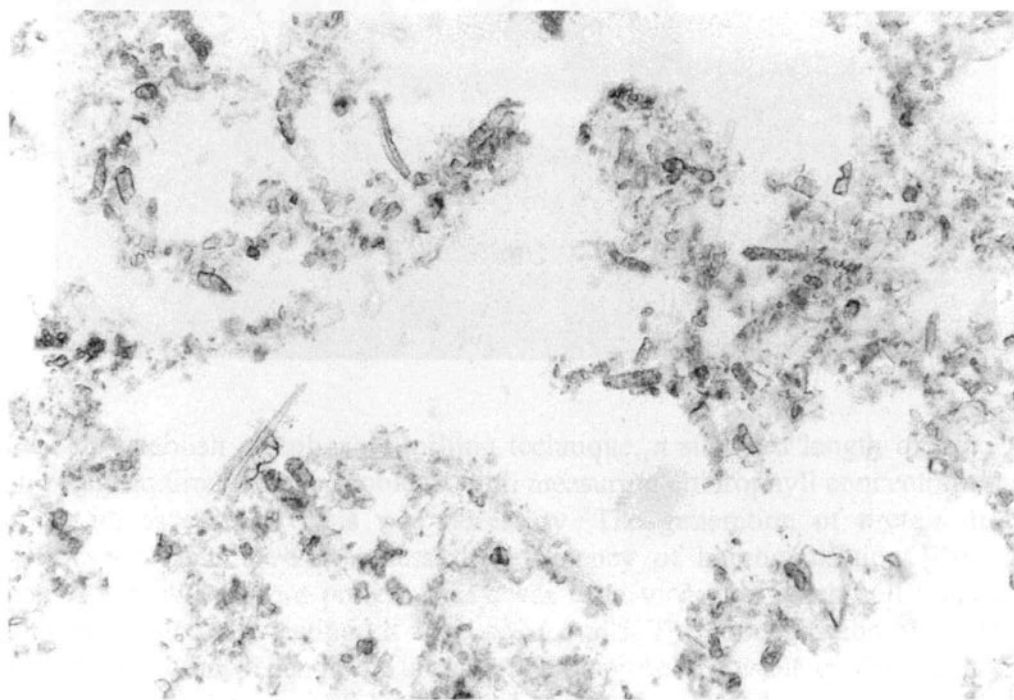
After filtering and centrifuging, only a few mls of solution remained. Large volumes would need to be processed in this manner, in order for this method to be useful for the purposes of assessing plant quality. It was thought that the solution produced would be far too variable to be of use. Samples were taken regularly and inspected for cell breakage under a microscope.

Figure 7.5. Green leaves after sonicating for 15 minutes (x200)



In figure 7.5, there is much evidence of venous material and large clumps of cells. To the naked eye, the material appeared to be heterogeneous.

Figure 7.6. Green leaves after sonication for 40 minutes (x100)



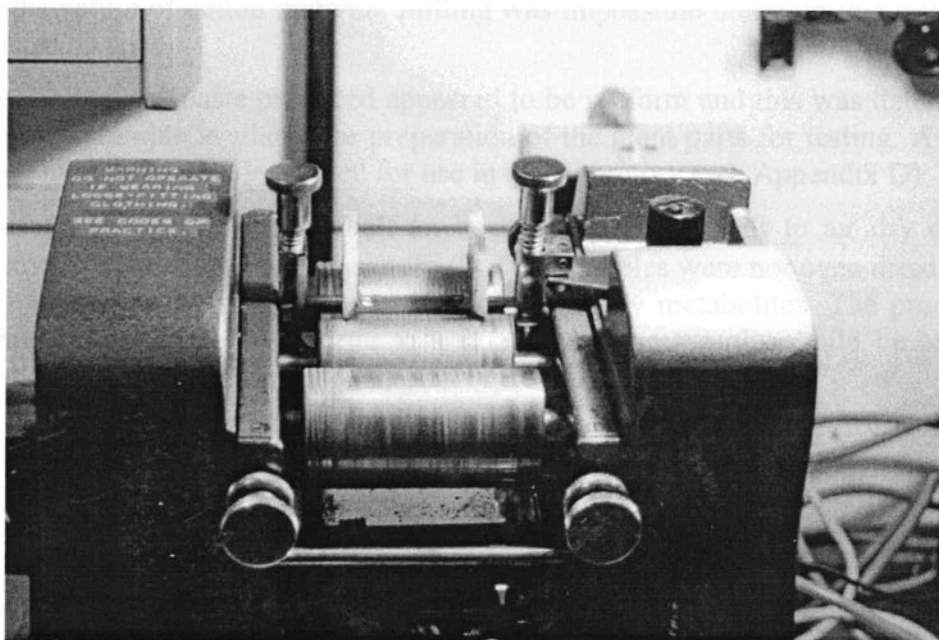
As fig.7.6 shows, there is an improvement in the amount of cell breakage with the length of time of sonication. Recalcitrant tissue is visible in both, but after 30 minutes the particles of tissue appear smaller in size. However much of the recalcitrant material has been removed due to processing problems, so this would be expected. This was not a suitable method of preparing the plant parts as the venous material had a tendency to collect around the probe. This was removed and weighed but constituted a large part of the mass and would have severely affected a measurement of edibility of the more recalcitrant plant parts.

This method also proved time consuming as the sonicator could only be used for 15 minutes at one time with 30 minutes cooling period between each use. This meant a one hour sonication period actually took 2 and a half hours to execute, and the volumes generated were small.

7.3.2.3 Milling

The use of a mill fulfilled the requirements of low cost, and efficient production of a suitable amount of substrate.

Figure 7.7. The roller mill used to macerate the plant tissues



In order to establish a replicable milling technique, a standard length of time for the milling was required. Due to problems with measuring chlorophyll concentration, a new technique to assess cell lysis was necessary. The generation of protein from cell disruption has been used to assess the efficiency of homogenisation (Pandolf and Kinney 1998). A Bradford protein assay was used to decide when cell breakage was optimal and a Bioquant testing kit was used for this. The concentration of the Bradford protein test solution depends on the amount of protein present in the solution to be tested. For concentrations of 0.1 to 1.4 mg of protein per ml, the test solution is used undiluted. It was assumed the plant parts would contain very small amounts of protein judged on their total nitrogen contents. Therefore, the Bradford protein test solution was

diluted to 1 in 10 concentration as instructed. A small sample was taken from the mill and pushed through a 0.45 μ m syringe filter. 0.05ml of the filtrate was placed in a 4.5ml capacity plastic cuvette with 2.5ml of Bioquant reagent solution. This was left for 2 minutes and then the absorbance read at 595nm. This was carried out every 10 minutes until the generation of protein remained constant. 0.05ml of distilled water was used for a blank.

It was found the machine had to be covered to prevent evaporation and drying out of the sample. The fact that distilled water had to be added to produce a paste, probably affected the concentration of protein in the sample. Therefore the milling procedure was carried out twice on green leaves and bark to establish length of time for milling. See Appendix D for results.

The time required for adequate crushing of green leaves was established as 30 minutes, as after this time the increase in protein is negligible and the shortest time should be used to prevent overheating of the sample. Due to the heat generated by the milling technique, it was decided to treat all plant parts the same and mill for 30 minutes and no longer. This was also to ensure that some of the structural tissue of the more recalcitrant plant parts remained intact and would be incorporated into the measure of energy availability. Organisms are also constrained in the length of time they can masticate. Due to the nature of cotton material, milling was impossible therefore this was omitted from the BOD tests.

When collected, the paste produced appeared to be uniform and this was determined to be the best technique available for preparation of the plant parts for testing. A standard operating procedure was developed for use in the laboratory (see Appendix D)

All plant parts were milled for half an hour, collected and left to air dry on watch glasses for 36 hours at room temperature (20 °C). Samples were not oven dried to avoid any further denaturing of proteins or loss of secondary metabolites. The product was then ground in a pestle and mortar to a fine powder so samples could be accurately weighed out and resuspended in citric acid buffer, pH5.

Figure 7.8. Close up of the plant paste (from leaves) produced in the roller mill

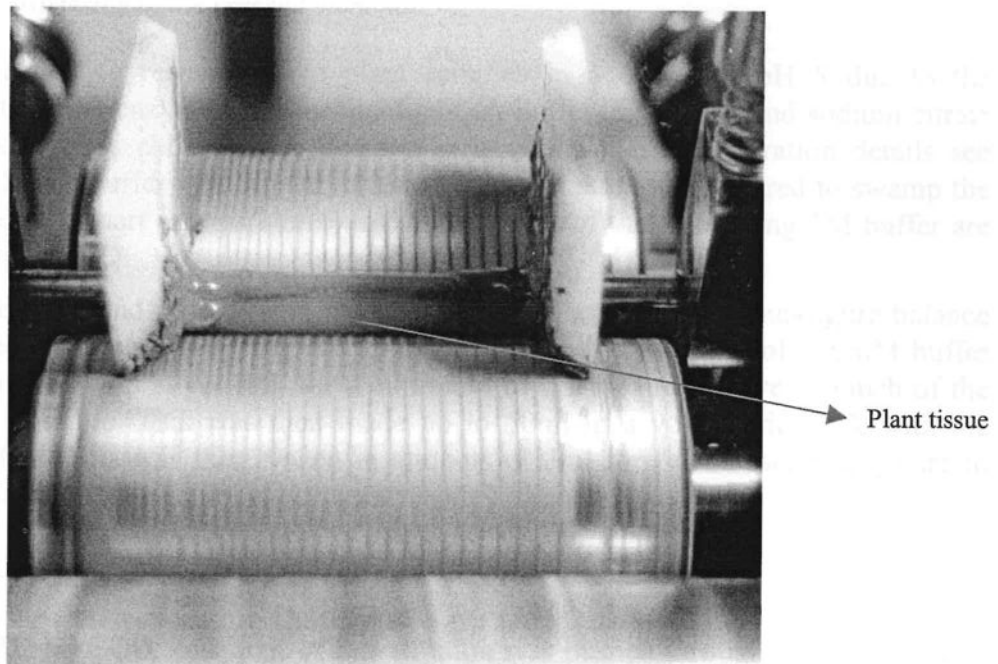
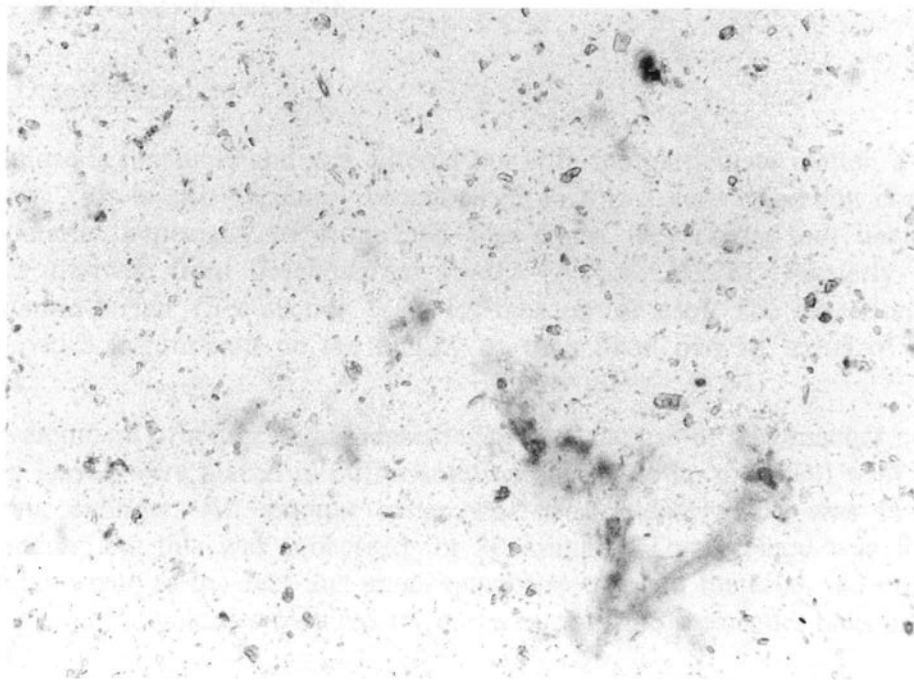


Figure 7.9. Green leaves (x10) after milling for 30 minutes in a roller mill



Figures 7.8 and 7.9 show that the paste produced appears to be more suitable for testing than with the other methods of maceration. To the naked eye it appears more homogenous as a smooth paste, and under a microscope there are signs of lots of free cells, fewer clumps of cells and smaller particles of venous material.

7.3.3 BOD test method

7.3.3.1 Resuspension of the crushed plant part

It was necessary to resuspend the plant samples in a buffer at pH 5 due to the requirements of the enzyme used for the digest. A buffer, citric acid and sodium citrate buffer solution, was chosen from Dawson *et al.* (1987). For preparation details see Appendix D. The buffer was diluted to 100mM, as full strength appeared to swamp the effect of the plant part as a carbon source. The results of the tests using 1M buffer are given in Appendix D.

0.25g of the dried and milled plant material was weighed out on a four-figure balance and recorded. This was then placed on a magnetic stirrer with 20ml of 100mM buffer solution, covered with silver foil, for a minimum of 2 hrs to redissolve as much of the material as possible. This was then made up to 50ml in a volumetric flask with the 100mM buffer. This was then frozen in aliquots of 2ml for future use, taking care to ensure the solution was homogenous before taking aliquots.

7.3.3.2 Syringe filtering

For the BOD and COD tests, part of this solution was pushed through a 0.2 μ m syringe filter to remove microorganisms and particulate matter, which may interfere with the tests. Suspended solids can affect dilution, and also may absorb some microorganisms, leading to stratification of dissolved oxygen concentration in the BOD bottle (Her Majesty's Stationery Office 1988).

7.3.4 Digest procedure

As cellulose is insoluble and was filtered out with the particulate matter, a digest was necessary. This is also necessary because cellulose is a very important constituent of plant material, especially to ruminants. The digest was carried out using cellulase enzymes derived from *Trichoderma reesei* (ATCC 26921), formerly known as *Trichoderma viride*. (See section 5.1.6 for reasons for use). The digestion procedure also provides information on the quality of each plant part in terms of a cellulose provider.

The development of a digest procedure began with the use of a stomacher machine. 5g of green leaves were placed in buffer solution and made up to 100ml with buffer in a measuring cylinder. An amount of enzyme was added which was in excess of requirements and this was processed for 30 minutes. This method was found to be unacceptable due to the fact that small quantities of plant material and enzyme were available, and the volumes required for correct use of the stomacher bags are in excess of 100ml.

A test run with the use of conical flasks on a shaking machine was attempted. The amount of enzyme required for a complete digest of the cellulose in 20 hours was calculated. This appeared to be more appropriate. Accurate calculation of the amount of enzyme needed was then required:

The rate of reaction given with the enzyme purchased was 1 unit of enzyme liberates 1 μ mole of glucose in 1 hour at a temperature of 37°C at a pH of 5. The reaction must not be enzyme limited, so the highest value for cellulose content found in that plant part was used in the calculation. The mass of sample in 1litre divided by 100 and multiplied by the percentage of cellulose in that plant part gives the amount of cellulose in 1litre of solution.

Assuming this cellulose can all be converted into glucose by the enzyme and allowing for the loss of 1 molecule of water per glucose unit in the reaction, the molecular weight used is adjusted:

$$180 \text{ (molecular weight of glucose)} - 18 \text{ (molecular weight of H}_2\text{O)} = 162$$

Using this the mass can be converted to mole equivalents in solution

$$\text{Cellulose content (g)/162} = \text{number of mole equivalents in solution}$$

This was converted to μ moles and this is equivalent to the number of units required to convert all the cellulose in 1litre of sample per hour. This can then be adjusted for a longer time period of incubation and smaller amount of sample if necessary.

For example, for green leaves in a concentration of 5g l⁻¹:

With cellulose content not more than 16%,

$$(5\text{g}/100) * 16 = 0.8\text{g}$$

$$0.8/162 = 0.00494 \text{ mole equivalents of glucose}$$

This is equivalent to 4940 μ moles of glucose, which, according to the rate of reaction (1 μ mole of glucose generated per unit of enzyme), would be generated with the use of 4940 units of enzyme.

Therefore, for a 10ml sample size, 4940 divided by 100 = 49.4 units,

and for a 10 hour incubation period, 49.4 units divided by 10 = 4.94 units

For green leaves, 4.94 units would be required on a 5g l⁻¹ stock solution for a 10 hour incubation period at 36°C, and a pH of 5. A spreadsheet in excel was written for calculation of the number of units of enzyme required for each plant part with the concentration of 250mg per 50ml of buffer. A copy of this can be seen in Appendix D.

There are 2500 units in 0.4g of cellulase enzyme. 19.7 mg of enzyme were weighed out and made up to 25ml in a volumetric flask with distilled water. Aliquots of 1ml containing 4.9644 units were frozen for convenience.

The controlled temperature room was slightly below optimum temperature at 30°C so the incubation time was increased to compensate for this. The number of enzyme units and incubation time was standardised for each plant part, regardless of the differing concentrations of cellulose present. Therefore, only one control was necessary. It was ensured the enzyme was present in excess quantities except for root and twig, where the amount of cellulase required is only slightly more than what was used. The incubation time was therefore in excess of what was required.

10ml of the stock solutions of plant parts were incubated with 1ml of enzyme solution for 48 hours at 30°C. 10ml of buffer and 1ml of enzyme was used as a control. The Standard Operating Procedures for this are given in Appendix D. The digested solution

was then syringe filtered and diluted accordingly for the post-digest BOD and COD tests (preBOD and postBOD).

7.3.5 Establishment of dilution factor and amount of seed for the BOD test

The BOD test was carried out on various dilutions of the plant part stock solutions and with various amounts of seed to achieve a BOD with a minimum depletion of dissolved oxygen of 2.0 mg l^{-1} . This is recommended as the limit of detection in the government guidelines (Her Majesty's Stationery Office 1988). For diluted samples the depletion of oxygen should lie between 30 and 70% of the initial oxygen concentration for accuracy.

The seed used was humus effluent from Cranfield University Sewage Works. A volume of 200-300ml was allowed to settle for 30 minutes before use and the sample taken from the centre of the volume. Green leaves were used to establish a technique, as this sample was not in short supply. The samples were resuspended in buffer medium as described in section 7.3.3.1. The tests were conducted on a range of stock solutions from 200 mg l^{-1} to 5 g l^{-1} . Small volumes were prepared as the sample was limited. Series dilutions were prepared with distilled water for the BOD test. Various amounts of seed were used. Various quantities of these dilutions were used with the dilution media (prepared as described in section 7.3.5.1). Results within the range required were eventually obtained for green leaves at a concentration of 250mg per 50ml of buffer diluted to 1 in 10 and with 5ml of seed.

Table 7.2. The $\text{BOD}_3(\text{ATU})$ results for green leaves at a concentration of 250mg per 50ml of 100mM buffer.

Sample (1in10 dilution)	Volume (ml per litre)	Seed (ml)	Day 0 reading (mg l^{-1})	Day 3 reading (mg l^{-1})	BOD (mg l^{-1})
Blank	0	0	9.0	8.0	-
Green leaves	15	5	9.0	6.9	80.9
Green leaves	25	5	9.1	5.8	96.9
100mM buffer	15	5	9.1	7.6	40.9
100mM buffer	25	5	9.1	7.4	32.9

Table 7.2 shows that a BOD reading within the range required could be obtained for the plant part green leaves. This sample shows more dissolved oxygen is used by microbes over 3 days than with the buffer solution alone. The BODs for the buffer are around half that of the sample and this is statistically significant ($F=33.8$, $P<0.05$).

7.3.5.1 BOD Procedure.

Table 7.2 was used as a basis for design of a standard method to apply to all the plant parts before and after a standard digestion procedure. This is described in this section.

Preparation of dilution media required five litres of distilled water to allow the measurement of 4 samples and the blank. This was incubated for 24hrs at 20°C . The

incubator used was an air incubator with fan assisted air circulation. The water was then aspirated for $1\text{ hr} \pm 10\text{ mins}$ to saturate with oxygen, keeping the temperature as close to 20°C as possible. 5 mls each of ferric chloride, calcium chloride, magnesium sulphate, phosphate buffer, and allylthiourea was added to the aspirated distilled water. The standard operating procedure for this is given in Appendix D. These reagents provide osmotic balance, buffer the pH and provide essential nutrients (other than carbon) and trace metals for microbial growth (Her Majesty's Stationery Office 1988). 5ml of humus effluent to act as seed, and the appropriate quantity and dilution of plant part solution, was made up to 1litre with the dilution media. For all plant parts this was 30ml of 1 in 10 dilution from the stock solutions. For the post-digested plant material, which also contained some enzyme, the amount was adjusted to a volume of 20ml of 1 in 10 dilution. For the blank determination the dilution media alone is used.

Two 250ml glass stoppered BOD bottles were filled with the solution. The dissolved oxygen of one was measured with a dissolved oxygen (DO_2) meter to give the day 0 value. The vessel was placed on a magnetic stirrer and the probe inserted to just above. This was left for a few minutes for the display to stabilise and the reading taken. The meter was calibrated before every use. The other bottle was incubated at 20°C for 3 days and then measured as above. This gives the day 3 value. The difference between the day 0 and day 3 measurements (minus a correction given by the blank) gives the BOD_3 value. A spreadsheet in excel was designed for this (Appendix D).

BOD equation for diluted samples:

$$\text{BOD}_n = f[(D_0 - D_n) - \frac{(f-1)}{f}(B_0 - B_n)] \text{mgO}_2 \text{ l}^{-1} \quad \text{eq16}$$

where f = dilution factor, given by v_1 divided by v_2 , where v_1 is the sample aliquot in ml, and v_2 is the volume in ml of the diluted sample.

Where, D_0 = dissolved oxygen at day 0, D_n = dissolved oxygen at day n (in this case 3), B = blank

Allylthiourea (ATU) was used for suppression of nitrogenation and the results are reported as BOD_3 (ATU). This was carried out on the plant solutions before and after the digest procedure.

7.3.6 COD testing

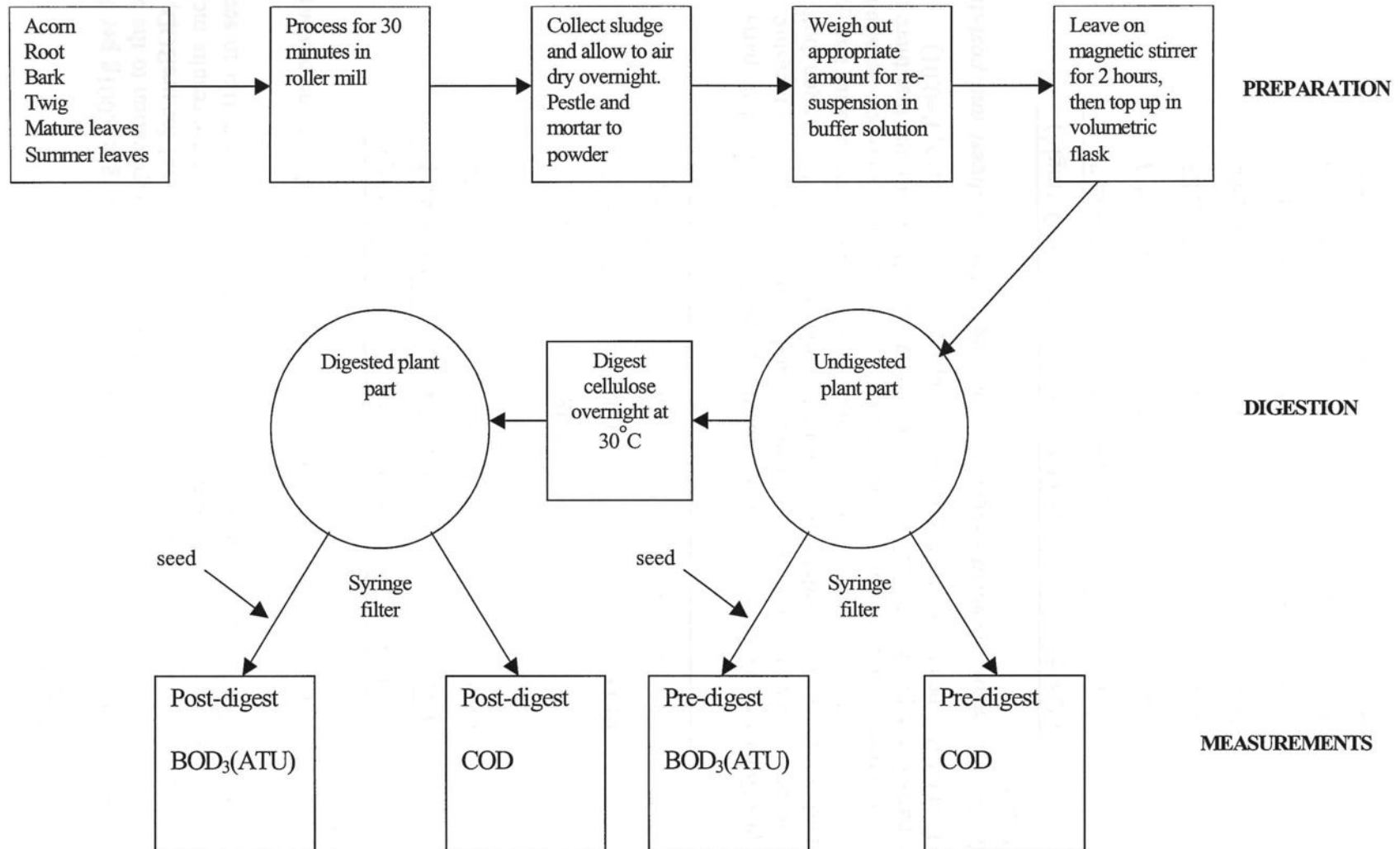
It was assumed the COD reading from the plant solution would be at least twice as much as the BOD and that establishing the COD would indicate the dilution required for the BOD. However, it became apparent that the dilution would need to be established for each test separately. Hach test kits with the range of $0\text{--}1500 \text{ mg l}^{-1}$ were used. Green leaves were used to establish the correct dilution and procedure. The standard operating procedure is given in Appendix D. Results within range were obtained from a stock solution of 0.5 g l^{-1} for both green and brown leaves

Chemical oxygen demands were then established for all the plant parts from the filtered plant part stock solutions at a concentration of 5 g l^{-1} of buffer. The dilution used for this was 1 in 10, for both the pre- and post-digest plant material. This was replicated. The

buffer alone was used as a control for the preCOD and the buffer with enzyme for the postCOD. This was subtracted to give the COD value for the amount of plant material alone.

Fig 7.10 depicts the whole laboratory process. Preparation of the plant material consisted of maceration and re-suspension in citric acid buffer, pH5, to create a stock solution. Part of this stock solution is then subjected to a digest with cellulase enzyme. These 2 solutions were then prepared by filtering through a syringe filter and diluting to the appropriate concentration required. The BOD and COD tests were then performed on both solutions.

Figure 7.10. The whole procedure to assess the energy available in plant materials



7.4 Results

7.4.1 The BOD and COD results

The stock solutions of the plant parts were of a concentration $0.25\text{g} \pm 0.001\text{g}$ per 50ml of buffer. These were diluted to 1 in 10 with distilled water for application to the tests. The biological oxygen demand was calculated for a volume of 30ml for preBOD, and 20ml for postBOD. A copy of the spreadsheet is in Appendix D. These results include the BOD of the buffer. The figures are adjusted for the effect of the buffer in section 7.4.2.

Table 7.3. The mean biochemical oxygen demand (mg l^{-1}) for pre-digest and post-digest plant parts

Plant part	PreBOD (mg l^{-1})	PostBOD (mg l^{-1})
Acorn	118 ± 4.74	157 ± 41.2
Root	74 ± 9.40	152 ± 4.74
Bark	51 ± 14.1	132 ± 2.33
Twig	75 ± 0.78	146 ± 13.6
Brown leaves	91 ± 10.1	150 ± 5.87
Green leaves	132 ± 2.33	167 ± 1.20

The postBOD results show an increase on the preBOD for all the plant parts. The enzyme present exerts some oxygen demand and is included in this measure. The standard deviation varied widely. Bark exerts the smallest demand for oxygen, both pre- and post-digest, whereas the highest demands can be found with acorns and green leaves. A one-way analysis of variance showed no significant difference between the plant parts in the postBOD results; for the preBOD the null hypothesis, that there is no significant difference between the groups, was rejected ($F=0.797$, $df=5$, $P=0.01$).

Table 7.4. The mean chemical oxygen demand (mg l^{-1}) for pre-digest and post-digest plant parts

Plant part	PreCOD (mg l^{-1})	PostCOD (mg l^{-1})
Acorn	298 ± 4.95	447 ± 34.7
Root	288 ± 95.5	382 ± 11.3
Bark	197 ± 7.78	387 ± 2.83
Twig	220 ± 5.66	362 ± 25.4
Brown leaves	265 ± 32.5	382 ± 12.0
Green leaves	284 ± 3.53	415 ± 38.9

As with the BOD test, the postCOD shows an increase on the preCOD for all plant parts. Again, the standard deviation varies quite widely. The smallest demand for oxygen is found with bark material pre-digest, but with twig post-digest. Acorn and green leaves are associated with the highest oxygen demands in both the pre and postCOD. A one-way analysis of variance showed no significant difference between the means for neither the preCOD nor the postCOD.

7.4.2 Calculation of biological oxygen demand for a gram of plant material with corrections for presence of enzyme

The mean BOD of the buffer and seed, established in triplicate, was 51.5 mg l^{-1} with a standard deviation of 6.39. This was used as a correction to establish the biological oxygen demand of a gram of plant material alone. For the postBODs, a buffer with enzyme control was used. This gave a mean BOD of 99.9 mg l^{-1} , with a standard deviation of 22.7.

The mean COD of the buffer alone was 161 mg l^{-1} (SD=12.2), and the mean COD of the buffer and enzyme was 308 mg l^{-1} (SD=24.9).

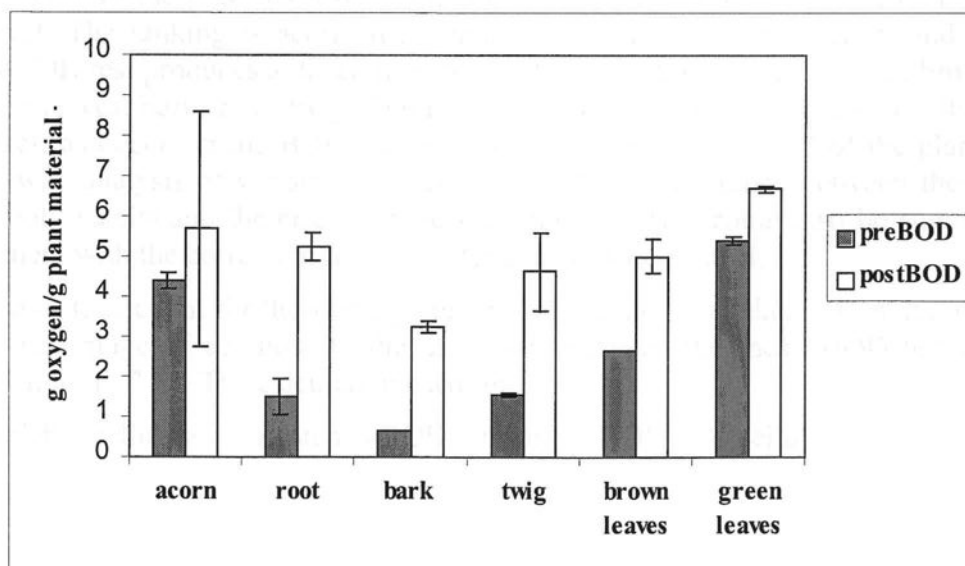
These mean values were subtracted from their corresponding plant part BOD results to give the biological oxygen demand of the plant part alone. 30ml of 1 in 10 stock solution was added for the pre-digest BODs and 20ml of the post-digested stock solution and this enables calculation of the mass that produces each oxygen demand. The spreadsheet is in Appendix D and an example of this follows:

Mass in 1ml of stock solution = 0.25 divided by 50 = 0.005 g

Mass in 10ml of 1 in 10 solution = 0.005 g therefore, mass in 30 ml = 0.015 g l^{-1}

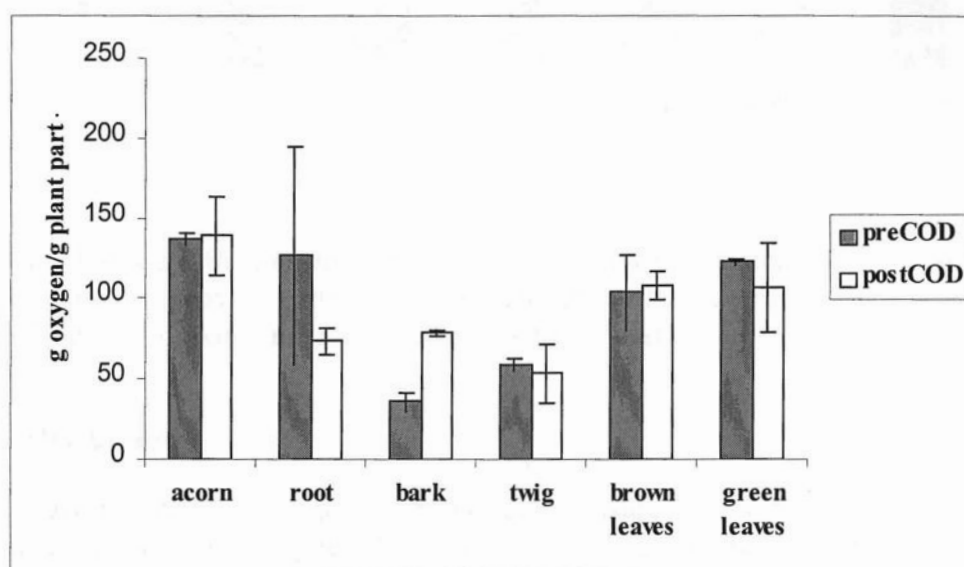
There is a mass of 15mg of green leaves in 1litre of dilution media (as 30ml was used for the pre-digest BOD). The BOD of the green leaves solution is 46.6 mg l^{-1} , therefore 15mg of green leaves uses 46.6 mg of oxygen.

Figure 7.11. The mean biological oxygen demand (g O_2) given per gram of material over 3 days before (preBOD) and after (postBOD) a cellulase digest



The error bars show the spread of results and illustrate that the error involved in BOD tests can vary widely. The postBOD results give an estimate of the energy availability of the soluble components after a cellulose digest for each plant part. As fig.7.11 shows, the highest BODs are given for green leaves and acorn. The lowest BOD is given by bark. The ordering of plant parts for the postBOD test is green leaves, acorn, root, brown leaves, twig, and bark. This is slightly different for the preBOD test in that root and twig are much the same and come after brown leaves. The postBOD results show an increase on the preBOD. This would be expected as these results incorporate an oxygen demand from the cellulose that has been rendered soluble in the digest. When corrected for the buffer content the means of the preBOD show a significant difference between the plant parts ($F=26.3$, $P=0.001$). The postBOD results are not significantly different between the plant parts, even when corrected for buffer and enzyme content.

Figure 7.12. Chemical oxygen demand (g O_2) for 1 gram of each plant part



Again the error involved, for some of the plant parts such as root preCOD, is quite large. The amount of oxygen used in the COD test is 10 fold that of the amount used in the BOD. In the preCOD test, acorn exhibits the highest demand and bark is still the lowest. The ranking is acorn, root, green leaves, brown leaves, twig, and bark. The postCOD test produces a different ranking of the plant parts again: acorn, brown leaves, green leaves, bark, root, twig. The postCOD should show an increase on the preCOD values, as occurs in the BOD test, but this can be seen in only 3 of the plant parts. A one-way analysis of variance showed no significant difference between the plant part for both results and the error may account for this. This could also be suggestive of a problem with the correction for the buffer and enzyme control.

In order to account for the varying contents of cellulose in relation to mass, the ratio of the percentage of cellulose to the difference between the mean BOD test results are shown in fig.7.13. The calculation used for this is:

$$\text{Available cellulose} = [\text{mean postBOD} - \text{mean preBOD}] / \% \text{ cellulose}$$

Figure 7.13. Histogram to show the ability of each plant part to provide cellulose, using the mean difference between the preBOD and postBOD divided by the mean initial percentage of cellulose

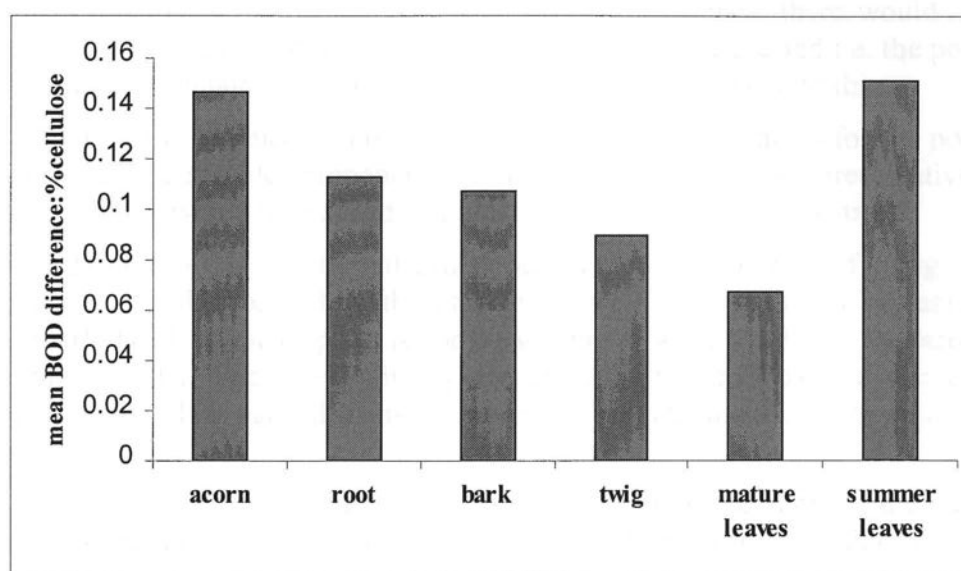


Fig.7.13 shows a different profile to fig.7.11 and gives an indication of the potential of the plant part to provide cellulose. Acorn and green leaves are the highest but bark is now of a higher ranking and mature leaves is the lowest.

7.5 Discussion

The COD results do not give a biological measure of oxidisable substrate, but a chemical measure. The oxygen demands are measured by the oxidation of the soluble constituents by phosphoric acid in a period of two hours. The BOD test measures the oxidation of the soluble component over a period of three days by microorganisms. This can be seen as a more biologically sympathetic measure. It provides results that are actually measured from the utilisation of the substrate by microbes.

The COD test was originally designed to produce a rapid method for prediction of the BOD, as it can for wastewater, but so far a relationship has not been established. The postCOD results do not demonstrate the increase on the preCOD that would be expected after a cellulase digest. This is possibly because the COD of the enzyme and buffer solution is high, so the correction is too large. There is only 1 replicate for each plant part and this makes it difficult to apply any statistical test. The postBOD and postCOD do not show a significant correlation (Spearman's Rank Correlation Coefficient 0.322, $P > 0.05$).

The pre-digest BOD values (preBOD) are representative of the soluble components of the plant parts and therefore it would be predicted that differences in edibility would be apparent. The filtered plant part solution probably contained mostly readily available soluble carbohydrates. The post-digest BOD values (postBOD) should contain additional components that were rendered soluble or available from the cellulase digest. This would consist of glucose generated from the cell walls, and other compounds from

further cell lysis. The difference between the two tests gives a crude measure of the amount of cellulose that is available (i.e. not bound up with lignin) in that plant part.

The results show the ratio of grams of oxygen used per gram of plant material for each of the plant parts (figs.7.11 and 7.12). After a cellulose digest, there would be more glucose present in solution so a higher BOD result would be expected i.e. the postBOD. All the plant parts contain some cellulose and the increase is related to this.

The preBOD results produce a ranking of the plant parts the same as for the postBOD, but is related to the soluble components of the plant parts. This is representative of the components which would be leached from leaf litter early in decomposition.

The postBOD results (fig.7.11) are the most pertinent to the question of energy supply. The highest postBODs exerted by the plant parts are for green leaves and acorn. The lowest postBOD of the plant parts is for bark. This is what would be expected when considering edibility. The digest slightly decreases the range of oxygen demands that occur. For all the plant parts there is an increase in biochemical oxygen demand after the digest.

Fig.7.13 is a ratio of this difference in BODs and the cellulose content of the plant part. Although the BOD difference is not only due to the glucose produced from the cellulose, but also increased amounts of cell contents from cell lysis, this still provides a crude measure. The postBOD results still stand, as this would occur with fermentation, or cellulolysis in the digestive tract. Fig.7.13 shows a very different profile to that of the BODs. As cellulose is a very significant dietary component for many herbivores, such as ruminants, this diagram may show a more realistic picture of energy availability. The fact that the energy availability is depressed in mature leaves may be due to the release of tannins into solution, and therefore restricting microbial metabolism. Conversely, it may be that the increased amount of lignin present in this tissue restricts access to the cellulose. Acorns and green leaves are still shown to be of superior food value compared to the other plant parts in this diagram.

All heterotrophs must utilise substrates that contain macromolecules. These cannot pass through a cell membrane and therefore can not be absorbed in the digestive tract. This means they must be hydrolysed to smaller molecules. The processes involved in extracellular digestion by microorganisms are similar to those in which complex foods are broken down in the digestion tract (Stanier *et al.* 1986). Therefore the BOD measure of energy availability may be appropriate to all forms of digestion and represents an improvement to the use of bomb calorimeters.

In order to test the edibility of a substance, the incorporation of the physical structure of a plant tissue is important. The more recalcitrant plant tissues would have retained more of their structure than the more malleable plant tissues. Fibres with low lignin to cellulose ratios tend to bend rather than break, to give long fibrous chains as opposed to smaller particles. Cellulose provides tissues with tensile strength and lignin provides rigidity to the structure (Van Soest 1987) which may explain this. The only variation in the preparation is the fact that the tougher structures were hammer milled prior to roller milling in order to fit them into this mill. However, hammer milling would probably have shortened the chains of lignin and cellulose, but not changed the complex or crystal structure of these (Van Soest 1987). It is important to have a 'simulated chew' as the importance of chewing and its subsequent effect on the plant tissue has been recognised. There is evidence to suggest smaller herbivores can compensate for a lower

digestive efficiency associated with body size by chewing for longer (Iason and Van Wieren 1999).

The effects of body size and scaling can produce a different view of plant material. Some insects may only need to rupture the cell wall to access the nutritious contents. If the cell contents are sought by the herbivore e.g. an insect foliage chewer, then it may be possible to use the preBOD results alone, saving time and costs. If the cellulose part is also utilised by the herbivore and its symbionts, the postBOD results are probably more representative. In designing this test, the application of energy availability measurements to a whole ecosystem was in mind. However, the test may become useful for application to a single species interaction, such as a crop pest.

Filtering will possibly have removed some of the important edibility characteristics of the plant tissues such as cellulose, lignin and large particles. These were accounted for by the digest procedure. The cellulose, which is not available due to binding with lignin, would not contribute to the food value of a plant part. Therefore, the fact that these tissues are lost is of no importance. The digestion of the cellulose that is available implies the postBOD results show how much of this is important when considering edibility. Some valuable nutrients may also have been filtered out, such as nitrogen incorporated into the cell wall. Some of this may have been released in the digest procedure but some may have been lost. Some large particles may have become smaller and in doing so released more cell contents.

In settled sewage 50% of the organic carbon and 35-50% of the organic nitrogen is in solution (Painter 1983). This means that at least 50% of the nitrogen and 50% of carbon is not accounted for in the BOD test. When considering wastewater, which the BOD test has been developed in order to do, it is not regarded as important that the polluting effects of these solids cannot be accounted for probably because large particles will settle out. Unfortunately there was not time to measure how much of each of the principal constituents were in solution and how much was filtered out with the suspended solids.

It is also hypothesised that the digestion procedure accounts for any physical defences present in the plant tissue such as waxes and resins, as well as physical structure. The presence of these compounds would prevent access to the substrate by cellulase. The resulting post-BOD would be suppressed as less cellulose is converted to glucose. The physical structure of a plant tissue is normally not considered in tests of food quality, as laboratory tests tend to begin with destruction of the structure to access all nutrients present (AOAC 1994). It is thought that milling for a standard time of 30 minutes may not alter the physical structure of the more recalcitrant plant parts as much as the less structural materials. This is of great importance when considering rate of access to energy or nutrients. For example, components such as silica, which are known to restrict access of microorganisms in the rumen to plant tissues (Vicari and Bazely 1993), can be measured. An improvement to this would be made if the restriction imposed by the component is measured, rather than reporting the amount of silica as a percentage of dry mass.

The enzyme complex consists of a synergy between exocellobiohydrolase (CBH1) and endoglucanase. The CBH1 plays the major role in binding to insoluble cellulose, acting as a specific β 1,4-glucanase. It is thought this action allows water to enter the crystal cellulose structure and the two endoglucanases can then attack the polymer and render it

soluble as cellobiose and glucose (Schulein 1988). As the structure of the cellulose present (and lignin preventing access) is a major part of the digestibility of plant tissue, this digest should allow differences between the parts to show.

The BOD test should also have taken into account the amount of tannins, phenols and other components that are known to suppress digestibility of plant tissues. Any complex formations by tannins and proteins should affect the availability of these to microorganisms in the BOD bottle, as would occur in the gut affecting availability to the herbivore. This is one possibility why the rank of brown leaves changes between the 2 tests, as there would be no suppressing effect on the oxygen demand of the chemicals in the COD test. The wide variety of secondary compounds produced by plants have variable effects on different herbivores, probably as they evolve or co-evolve in response to specific feeders. The effects of each one on various digestive strategies cannot be measured but the effects of these on a microorganism ecosystem can be, and is, in the BOD test.

Acorns can be shown to be of superior food value, compared to the other plant parts. This can be supported by the evidence of concentrate selectors, such as roe deer (*Capreolus capreolus*), that eat acorns in preference to fresh foliage of palatable plants. They are known to select for the most digestible plant part (Tixier and Duncan 1996). Acorns appear to contain the most energy as measured by the COD test, but are a close second to green leaves in the BOD. Mature leaves contain the highest concentration of phenolic compounds, such as tannins, and lignin (Cornelissen 1996) and it would be expected that the oxygen demand of microbes would be smaller than for green leaves. Acorn and green leaves also appear to possess the most available cellulose of the plant parts. This may be related to their low levels of lignin. Brown leaves appear to possess the lowest amounts of cellulose but this is probably unavailable due to the presence of lignin. The BOD test (especially the post dig) seems to give the results that would be expected from an energy availability test.

These results show that it may be feasible to apply BOD and COD tests to plant samples as the results reveal differences between the plant parts. However, repeatability has not been tested. As a feasibility study, a difference between the BODs has been demonstrated that seems to be consistent with the rate of decay data. This possibility of utilising a modified BOD test to measure energy availability in tissues appears to have potential.

7.6 Future refinements

Maceration of the tissues is standardised to allow for differences in the structure to appear. However, it may be possible to measure this in some way. For example, in syringe filtering, the dry mass extracted could be weighed and cellulose and lignin content estimated.

Increased masses should be used for stock solutions to obtain a more precise dilution. In weighing out small masses of plant material, a source of error is created. In order to avoid this, a large amount should be weighed out and resuspended. The volume can then be adjusted to provide the desired concentration. This would also increase the chances of having a representative sample with which to apply the BOD test.

As consistency and repeatability with the BOD test is notoriously difficult to achieve (Ormesher, personal communication), it may be better to carry out one set of BOD tests from the same stock of dilution media. This means the correct amount of distilled water must be incubated and aspirated in the same vessel.

Further work on this subject is required to establish repeatability of all stages. This method should be repeated from the preparation of stock solution point, through the digest procedure to the BOD and COD tests. In this case the same stock solutions were used throughout the study to examine feasibility.

If the test is to be developed to be applied to dicots rather than monocots, it is suggested further study of the fate of tannins in the gut and BOD bottle would be advantageous, due to the larger concentrations of these in dicots. Tannin concentrations in grasses are generally low in comparison (Vicari and Bazely 1993). This can be carried out by the use of polyethylene glycol (PEG) which bind to tannins preferentially to protein. The PEG-binding capacity of plant samples is not affected by pre-drying at 90°C (Silanikove *et al.* 1996). It may be the case that these substances have very little effect on energy availability. Findings show that tannins may not reduce digestibility of plant tissues because the pH within a digestive tract prohibits the formation of the complexes found in the laboratory (Martin *et al.* 1985).

In order to develop these tests for general use, attention must be paid to some aspects of this which, due to the constraints on an interdisciplinary study, have been neglected here, such as the effect of allylthiourea in the BOD.

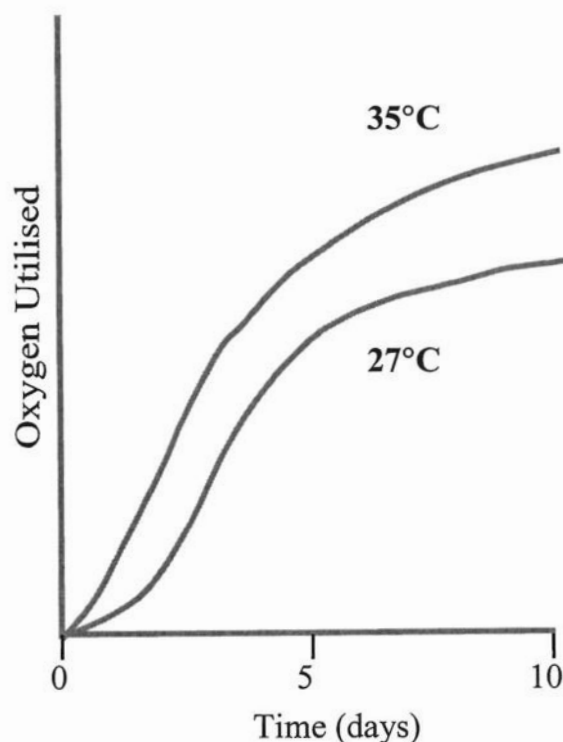
7.6.1 Development of the BOD test for this purpose

As this test has not yet been widely used for the purposes of establishing plant quality, it seems sensible to modify the test to maximum efficiency before this happens. It would be of use to know the content of nitrogen that was filtered out before carrying out the BOD and COD tests. Presumably more nitrogen would be made available to the filtrate after the digestion procedure, from the lysing of cell wall tissue liberating cell contents and the proteins bound up in the cell wall.

Temperature is one way in which the time taken for the BOD test can be minimised. The incubation temperature can be raised to as high as 37°C. There is close agreement between the 5 day BOD test at 20°C, and a 2.5 day test at 35°C (Gray 1989) see figure 7.14). Further work is necessary to establish the effect of elevated temperature on the microorganisms themselves, and not just reaction rate.

For some wastewaters where the effluents are toxic or inhibit microbial reactions, it is necessary to use acclimatised seed. It is possible that improved inocula could be discovered for these purposes. It is possible to isolate microorganisms from soils, or ruminants, that are more suited to degradation of plant products. If the test was to be performed at a more elevated temperature, there is a possibility that microbial symbionts can be used. If cellulolytic bacteria can be incorporated in standard inocula for use in a modified BOD test then this would negate the need for a cellulase digest. The use of a digest was necessary as sewage organisms acted as seed in this case. Sewage organism had advantages in that samples were easy to collect, and there was prior knowledge of their action. As it was not known if a BOD test could be used for this purpose, the development of suitable inocula was disregarded.

Figure 7.14. The pattern of oxygen uptake in the BOD bottle at 2 different temperatures



Adapted from Gray (1989).

7.6.2 Development of COD test

One of the problems found with the COD test is that nitrite NO_2^- exerts a COD of 1.1 mg O_2 per $\text{NO}_2^- \text{N}$ (Greenberg *et al.* 1992). In wastewaters, concentrations rarely exceed 1 or 2 mg l^{-1} but corrections for plant material may need to be used. This may have been a reason close agreement between the BOD and COD could not be reached. This requires further investigation.

Some other inorganic compounds, including sulphide and manganese, are also oxidized in this test and corrections for this were not developed in this thesis.

7.7 Conclusions

This study shows that it may be possible to utilise a modified BOD test to assess the energy availability of different plant tissues. A standardised method that can be used in any laboratory could be developed. Once this has been carried out, use of this test for application to ecosystems research may be possible.

As a feasibility study, the results are conclusive; a difference between the oxygen demand for each plant part has been demonstrated. Criticism of this measurement can

be made on a few points, for example differences in the absorption process between microbes and higher organisms, but it is argued that this more biological approach goes some way towards the development of more appropriate technology. The chemical constituents in a foodstuff are simply viewed as present in a certain quantity, but the truth is quantities tell us little. Van Soest stated:

"Fibre must be regarded as a biological unit and not as a chemical entity"
(VanSoest 1987 pg32)

and this project attempts to treat fibre as such.

Chapter 8 The power density calculations of the plant parts and applications

The calculation of a power density index for the plant parts is a convenient way to view the energy availability in a material and incorporates a time dimension. Many ecosystem studies require units in order to explain the dynamic processes at work. The unit joule was initially used to facilitate these studies. Energy flux within a system could then be measured and the factors affecting these processes identified more easily. Subsequently, study has been directed to finding a limiting nutrient or factor which could help explain the processes within a food chain or cycle. This is known as Leibig's law of the minimum.

The power density unit allows the study of the energy that can be used to do work in an ecosystem. It provides a measure of the energy available to a biological organism. The technology originally used to measure energy contents allows standardisation of methods to do this, but does not reflect the 'technology' that utilises these materials in reality. When measuring energy stored in materials, the purpose to which this is applied should be remembered. For application to a biological system, an improved technique for edibility measurement is suggested with the use of a modified BOD test.

Figure 8.1. The amount of work produced from energy depends on the efficiency of the machine utilising it

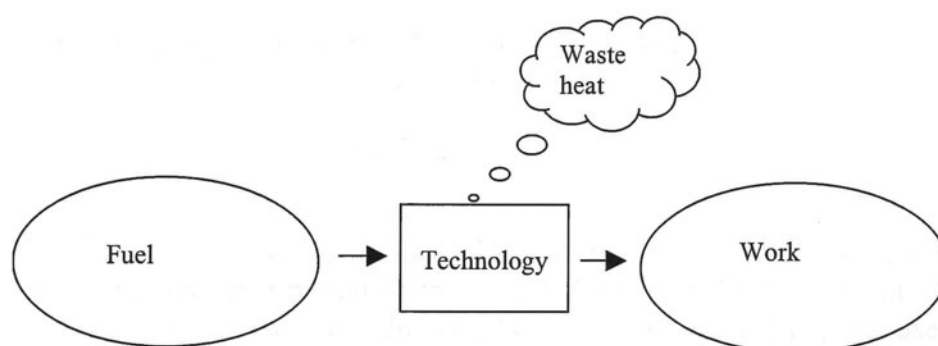


Figure 8.1 shows work produced via a machine from an energy source. The machine can be a bomb calorimeter or a vertebrate herbivore but in each case the work produced is not the same. Standardisation of the technology used to measure energy and work produced allows comparisons between energy sources.

The rate of decay data was an initial step used to generate energy availability data incorporating time. This is supported by the fact that decomposition processes are well correlated in the literature with ruminant digestion (Chesson 1997). It appears the same plant quality variables inhibit or promote both processes equally. Because of this the rate of decay data can be used for the same purposes as rate of digestion data and it has the added advantage of being easier to collect in terms of expense and labour. The *in vivo* decay processes are slow and this method emphasises or exposes the time dimension effects of plant quality.

The modified BOD test sought to improve upon the rate of decay technique. It involved the use of microorganisms and their rate of energy assimilation to assess the quality of

foodstuffs. This was carried out in controlled conditions, and is therefore a standard method which can be carried out quickly (in comparison) and requires no specialised equipment. This means it is an inexpensive technique which could be carried out in any laboratory.

8.1 Power from the decay rate data

Due to the double exponential decay curve normally exhibited by decomposition data, it was decided to use the data from 2 site visits as a basis for the calculation; the first was taken from the rapid initial decay (18 days, site visit 1) and the second from further into the experiment (day 115, site visit 4). The decay data is treated as a linear process for both of these decay constants (see section 5.1.8). The mean percentage mass lost per day for both dates ($n=5$ for each plant part) was standardised to an initial amount of 1kg, to give an amount lost per day per kg (see section 5.3 page 56 for full explanation). The power density is calculated using the decay constants K_{18} and K_{115} and the gross energy contents.

The calculation is based on $1W=1Jsec^{-1}$

For the rate of mass loss data:

Power density =

$$\frac{[\text{mean mass loss per day (kg)} * \text{calorific value (Jkg}^{-1})] * 10^3}{\text{time (60*60*24(secs))}}$$

$$= \text{kWkg}^{-1} \quad \text{eq 17}$$

The power density was based on an initial mass of the plant part of 1 kg and the mass lost in 1 day over the time period of the first 115 days of the experiment. An Excel spreadsheet was used to calculate this coefficient for all the plant parts used and is shown in table 8.1.

Table 8.1. Table showing calculation of the power density coefficient produced from the rate of mass loss data from day 0 to day 18(K_{18})

Plant part	Gross Energy (MJkg ⁻¹)	Mass loss per day up to 18days (g)	Energy loss per day ⁻¹ up to day 18 (MJ)	Power density for data up to day 18 (Wkg ⁻¹)
Cotton	18.2	0.26	0.0473	0.547
Acorn	18.6	0.97	0.1804	0.209
Root	19.4	0.70	0.1358	1.572
Bark	19.4	0.31	0.0601	0.696
Twig	20.0	0.69	0.1380	1.597
Brown leaves	20.3	0.66	0.1339	1.551
Green leaves	20.2	1.18	0.2383	2.759

Table 8.2 Table showing calculation of the power density coefficient produced from the rate of mass loss data from day 0 to day 115 (K_{115})

Plant part	Gross Energy (MJkg^{-1})	Mass loss per day up to 115 days (g)	Energy loss per day ⁻¹ up to day 115 (MJ)	Power density for data up to day 115 (Wkg^{-1})
Cotton	18.2	0.41	0.0746	0.864
Acorn	18.6	0.42	0.0781	0.904
Root	19.4	0.15	0.0291	0.337
Bark	19.4	0.08	0.0155	0.179
Twig	20.0	0.16	0.0320	0.370
Brown leaves	20.3	0.17	0.0345	0.399
Green leaves	20.2	0.58	0.1172	1.356

Tables 8.1 and 8.2 show how the power density was calculated. Figure 8.1 shows the order of the plant parts in terms of the power supplied to the ecosystem remains the same as the rate of mass loss per day. The units used are Watts per kg of plant material. The energy is released from the materials as the constituents are oxidised and, as this occurs over an extended period of time, Watts are more appropriate units.

Figure 8.2. The power density coefficient (Wkg^{-1}) calculated from 2 dates in the decomposition experiment for each plant part

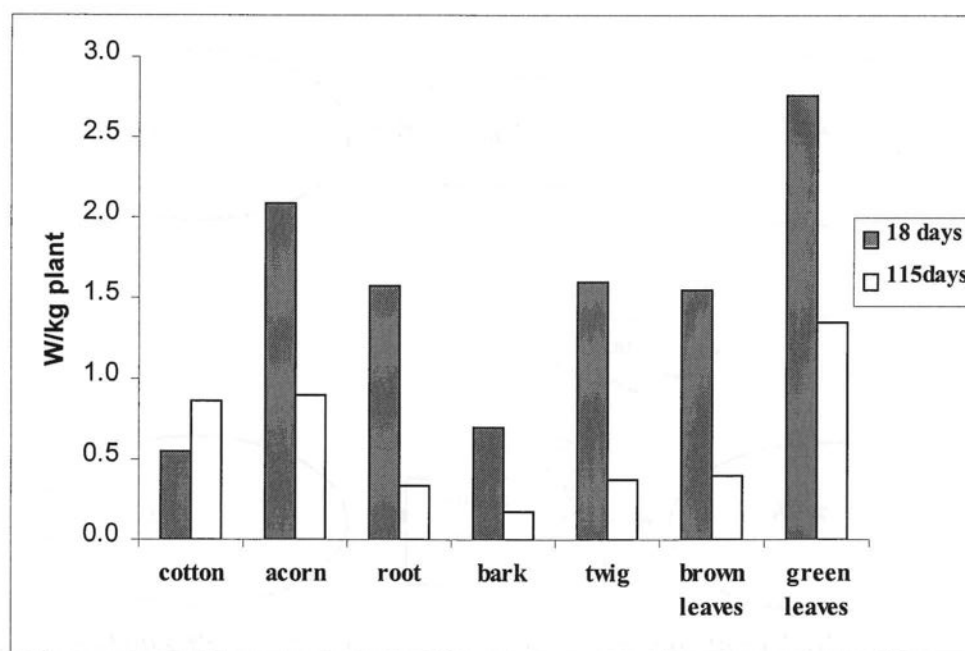


Figure 8.2 also shows that the power density from K_{18} is more than that of K_{115} . This is as expected for a longer time period. The energy extracted per second becomes less.

8.2 From the field to the laboratory

The decomposition experiments established a way of measuring the power density of plant materials using the soil and its microbes as a black box 'technology'. Heat is given off by microbial respiration and chemical work performed as a result of the energy extracted. However, altering the technology used to perform work will change the energy production rate and therefore provide a different measure of the power density of the plant material. Therefore, a standardised method is required where the efficiency of the technology is held constant and the output is a result of the fuel used i.e. plant material.

Figure 8.3. A representation of different power outputs as a result of different technologies for extracting energy biomaterials

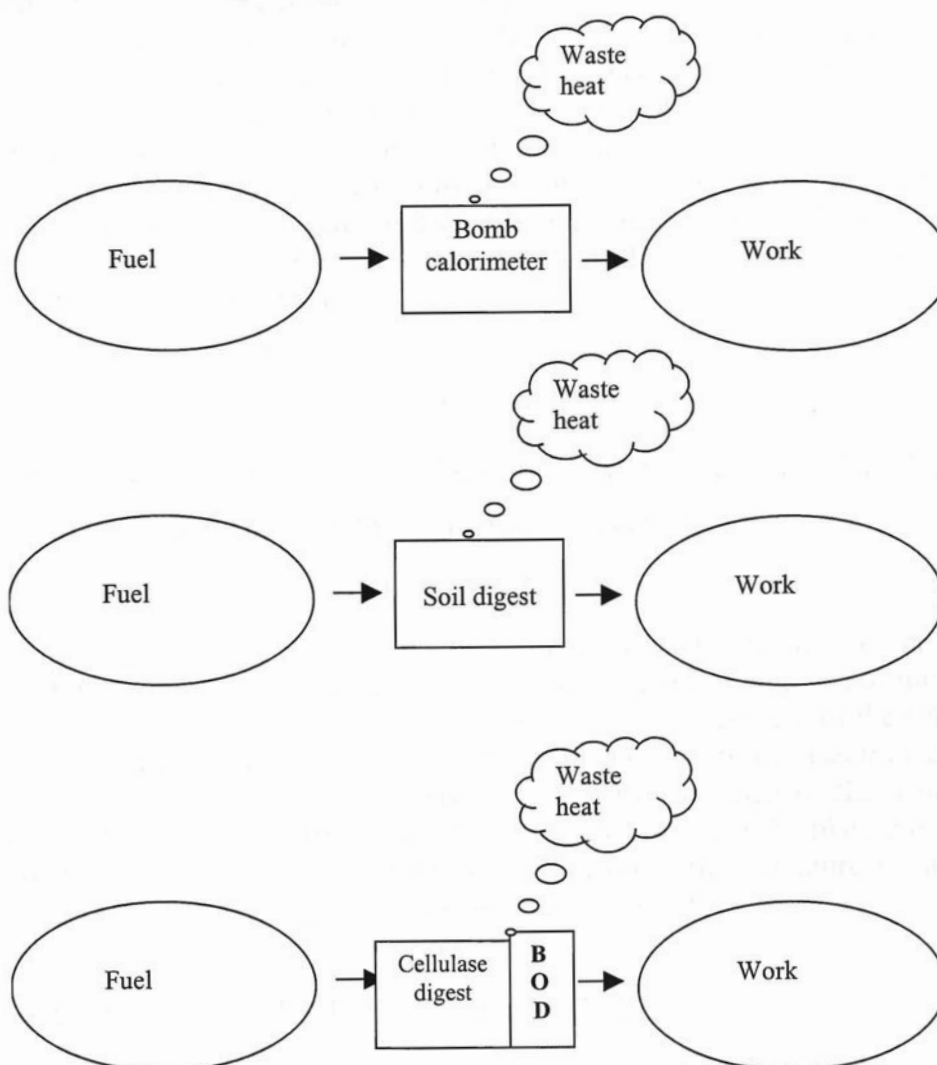


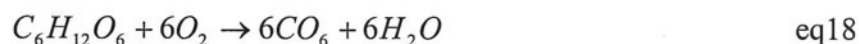
Figure 8.3 shows the technology used to extract energy from biological materials as fuels controls the rate at which energy is extracted and therefore the measurement of the

power density of the fuel. In the case of the bomb calorimeter the energy production is assumed to be instantaneous. For the soil digest, which is acting as a model for herbivory, the time dimension is considerably exaggerated taking over 100 days rather than a period of hours in the herbivore digestive tract. In taking the experimental procedure from the field to the laboratory, a cellulase was used as the digestive process that models herbivore digestion and retention time. The time base for the power density calculation was established by estimating the relative reaction times for the different plant parts dependent on their cellulose.

8.3 Power from the BOD data

The calculation of the power density coefficient was based on the amount of energy produced by the microorganisms per mole of oxygen used in their respiration. This depends on the metabolism pathways used by the microbes in the BOD bottle. The pathways used by microbes include aerobic respiration, fermentation, anaerobic respiration. It is the aerobic respiration pathway which is of interest, as this is probably responsible for the use of the majority of the dissolved oxygen. All respiration pathways involve the synthesis of molecules containing high energy bonds such as ATP, which can then be utilised as and when required. ATP can be described as energy currency. The commonly used example of respiration in textbooks is the generation of ATP by an electron transport chain from glucose through the TCA cycle. In the respiration chain oxygen atoms are reduced to water molecules (equation 18) which releases energy then utilised for an electron transport chain. About 40% of the energy in a glucose molecule can be trapped in ATP in this manner.

The equation for respiration is:



with a typical energy yield of 36 ATP. The synthesis of ATP requires 7.3 kcal/mole.

In the electron transport chain the following reaction is created.



This applies only to aerobic respiration which utilises NADH to reduce oxygen to water. For the purposes of this study, it is assumed only aerobic respiration has occurred in the BOD bottle over the 3 day period. In truth, there may have been use of the substrate to fuel fermentation pathways, or other pathways utilising different electron acceptors, resulting in a different net energy release. It is not the accuracy of the actual figure which is of interest, but the difference which is shown between the plant parts and the impact of their differing fuelling abilities. For a hypothetical creature using aerobic respiration to acquire its energy from glucose, these results hold.

8.3.1 The calculation of amount of energy generated

For 1 mole of glucose, or 1 pass of equation 18, yields 36 ATP through glycolysis using NADH oxygen linked oxidation in the electron transport chain.

1 mole of ATP requires 30.56364 kJ for its production (Stanier *et al.* 1986).

As 1 mole of glucose produces 36 ATP, $36 * 30.56364 =$ the amount of energy produced from 1 mole of glucose = 1100.29 kJ.

This requires 6 moles of oxygen. 1 mole weighs 16g, therefore 96g of oxygen is required to produce 36 moles of ATP, so 183.382 kJ are produced per mole of oxygen in this manner. The BOD results are given in grams of O_2 , per g of plant material (figure 7.11) and these figures were converted to mole equivalents (table 8.2):

Grams of oxygen used divided by the molecular weight of oxygen = mole equivalents

N.B. the results for the BOD need to be multiplied by 1000 convert from g to kg.

Mole equivalents * 183.382 kJ = number of kJ generated

Table 8.3. The calculation of the amount of energy generated in the BOD test for 1kg of material

Plant part	Mean postBOD (kg O_2 per kg material)	Mean mole equivalent	Mean number of kJ generated
Acorn	5.69	316	57988
Root	5.26	293	53639
Bark	3.26	181	33250
Twig	4.64	258	47259
Brown leaves	5.01	273	51051
Green leaves	6.69	372	68133

8.3.2 Incorporation of a time dimension

In order to account for the effect of retention time and cellulose fermentation, a theoretical 'retention time' is used for the incorporation of a time dimension in the equation. This enables expression of a power density measurement in $kWkg^{-1}$. This is based on the time it would take for all the cellulose to be converted to glucose, given a standard number of enzyme units, and knowledge of the rate of reaction of the enzyme used in the laboratory digest. It assumes that all the cellulose is available, which may not be the case in reality. This is impossible to test accurately in the laboratory as the methods for analysing cellulose and lignin contents are crude (Van Soest 1987).

1 unit of the enzyme used in this experiment produces one micromole of glucose in 1 hour at 36°C. Assuming all cellulose can be converted to glucose, the molecular weight of glucose can be used to give mole equivalents. The cellulose chain consists of glucose monomers joined by $\beta 1,4$ linkages therefore the actual molecular weight used for the calculation is 162 (180 – the molecular weight of 1 water molecule).

16,200,000 micromoles = 1 mole of glucose from cellulose

1 micromole weighs 0.00018g,

The average amount of cellulose between plant parts is 31.6%, which is 316g in a kilogram so the average number of micromoles of glucose is $316 / 0.00018 = 1755555.5$

1 μ mole of glucose is liberated in 1 hour per unit of enzyme in optimum conditions, so this figure is equivalent to the number of units required for the digestion of the average amount of cellulose in the plant parts, For the purposes of convenience, it is assumed that the mean 'retention time' for these materials is 12 hours, therefore:

$1755555.5 / 12 = 146,296$ units required for a twelve hour incubation time.

This figure is required only to calculate the number of units that would theoretically be added to the solutions. It is not used in the power density calculation

The amount digested per hour by this number of units is = 316 (average amount) / $12 = 26.3$ g per hour

Therefore:

mass of cellulose (g) in a kg / 26.3 = number of hours required for all the cellulose to be digested in that plant part.

For green leaves $156 / 26.3 = 5.93$ hours = 21327 seconds

The calculation of this for the plant parts is shown in table 8.3

Table 8.4. Table showing calculation of theoretical retention time for each plant part.

Plant part	% cellulose	Mass of cellulose in 1kg (g)	Incubation time (hours)	Incubation time (secs)
Acorn	8.75	87.5	3.3	11962
Root	33.2	332	12.6	45456
Bark	24.7	247	9.4	33767
Twig	34.3	343	13	46960
Brown leaves	18.9	189	7.2	25906
Green leaves	15.6	156	5.9	21327

8.3.3 The power density calculation

The time dimension calculated can then be inserted into the power density equation:

Power density = number of kJ generated over 3 days from the plant part rendered soluble (table 8.3) from 1kg / incubation time theoretically required to convert all cellulose to glucose (table 8.4)

i.e. power density = energy extracted from 1kg divided by time dimension

$$\text{Power density} = \text{kJ} / \text{kg} / \text{sec} = \text{kWkg}^{-1} \quad \text{eq20}$$

Figure 8.3 summarises the progression of the calculation of a power density from the laboratory data and figure 8.4 shows the result of this for each plant part. Cotton, used

as a reference material, is not present in the data collected in the laboratory. This is because of problems in macerating and mixing a solution from such a fibrous material.

It can be seen that the graphs produced (figures 8.2 and 8.4) do have similar profiles. Green leaves and acorns appear to contain the most available energy of the plant parts when measured in this way. Bark seems to contain the least, although in the lab measurements, there is little difference between bark, roots and twigs. The power density measurement using the rate of decay data at day 115 also shows little difference between these.

Figure 8.4. Steps involved in calculating the power density coefficient from the BOD test data

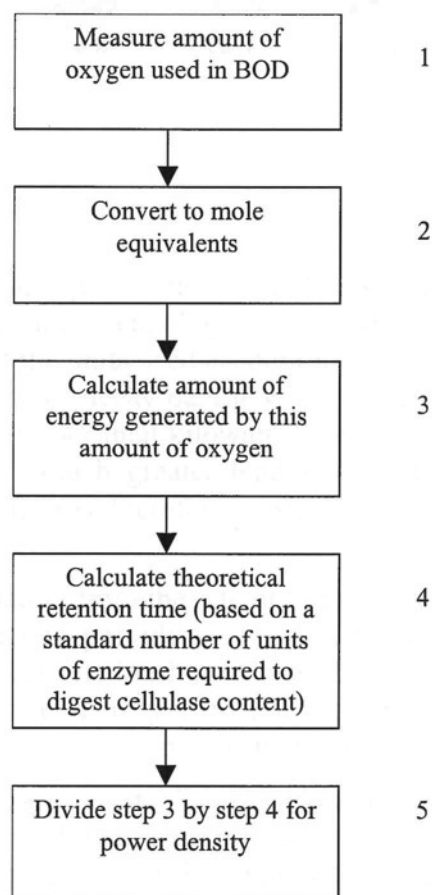
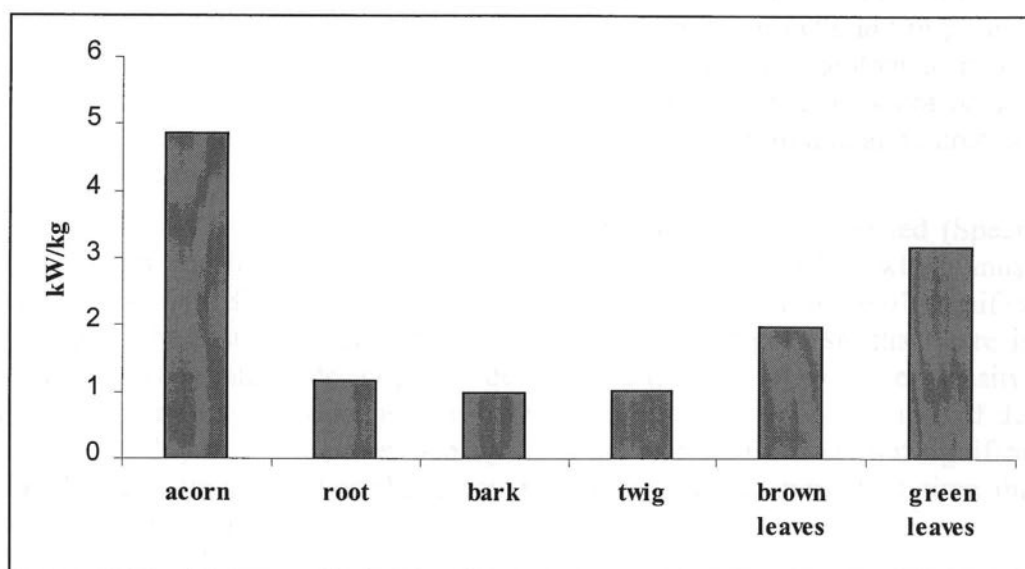


Figure 8.4 The power density of each plant part using the laboratory data



8.4 Discussion

When the data is viewed as a power density it allows comparison between the methods used to collect the data, as the 'technology' used determines the work produced (figure 8.1). It must be noted that the units used account for a difference between the 2 methods of data collection. The rate of decay data gave a power density measurement in Watts per kilogram of material, rather than kilowatts. This is because the energy in the plant parts was released over a much greater length of time, so the amount released per second was much less. It was therefore more appropriate to use watts rather than kilowatts.

The power density calculation from the rate of decay data could be improved by the use of a more accurate model to provide an improved decay constant. The data clearly cannot be treated as a linear relationship of mass loss over time, as carried out for the estimation of K_{115} . The power density using K_{18} is probably more useful as a measure of work gained from the plant materials by this method.

The calculation that was used to establish the amount of energy involved in respiration in the BOD bottle assumed that the only substrate available was glucose. Other substrates were probably present, and the amount of energy involved in the metabolism of these was not accounted for. The figures used were derived from the BOD of the soluble products after milling, and contained the glucose produced from a cellulose digest. This meant, the energy supplied by the materials was measured in a way that assessed its availability to a system which utilised the cellulose component as well as the soluble carbohydrates present. This may mean the measurement is more appropriately applied to digestibility of material by ruminants, which utilise this portion.

A connection between ruminant digestion and decomposition in an ecosystem has been made in the literature (Begon *et al.* 1991; Chesson 1997) therefore, the rate of decay data can be used as a standard. The modified BOD test can be seen as more appropriate as it allows study of the energy available in a more convenient timescale under standard

conditions. This is suggested as an improved method, simplified and integrated, compared to what is currently available for ecosystem research, for example, the PQMD (Palm and Rowland 1997). This is because it is based on the growth and respiration of microorganisms. Hence the test uses a technology incorporating biological pathways involving respiration and oxidation, rather than a technology which is not present in nature to measure contents, such as bomb calorimetry or by combining an indirect series of plant quality measurements.

Two power density measurements (K_{115} and BOD) are closely correlated (Spearman rank correlation coefficient 0.943, $P < 0.01$). The critical value of r_s which must be reached for $N=6$ is 0.829 at 0.05 significance level, and 0.943 at the 0.01 significance level meaning the result is just in this bracket. The null hypothesis that there is no association between rate of decay power density and the post BOD power density can be rejected. There may be a connection between ruminant digestibility, rate of decay, and the new quality test. The power density from K_{18} and the BOD are not significantly correlated, suggesting that it is the decay rate over a longer period of time that is comparable to digestibility.

The incorporation of time into the calculation is straightforward for the rate of decay data. The time dimension was incorporated with knowledge of the mean percentage cellulose in each plant part. The theoretical retention time required for each of the plant parts was calculated using this, and knowledge of the rate of action of the enzyme from *Trichoderma reesi*. This was then used in the power density equation as the rate determinant. Although rate of decay and ruminant digestion are similar processes, the main difference between them is the rate of reaction. This led to a difference in the units used. The amount of energy released or used up in one day in the rate of decay experiment was very small compared to the rate of energy release from the materials in the laboratory in one day. In the laboratory, conditions were standardised for the microbes, and the plant material was prepared as a substrate. The laboratory test rates are closer to the rate of digestion for a herbivore, than the decomposition rates.

The use of this test is seen as an improvement on the use of Joules to explain energy flux in a food chain. It also represents an improvement on the use of nutrient cycles, for the purpose of ecosystem energetics, where measurement of amounts of N or other nutrients are used to explain enhanced or suppressed productivity down a food chain. The need for these nutrients is not ignored as microbial requirements are the driving force of the test and nutrient shortage slow rates of oxygen consumption and be reflected as a reduced power density value.

8.5 Summary

The power supply from plant materials was measured in 2 different ways: one from field decomposition and one from sewage microorganism growth and respiration. Both use oxidation of food as the basis of the measure, although the rate of reaction is very different. Power density coefficients were calculated the data generated. The calculations are described step by step and the results compared. The power supplied by these materials is different depending on the method of extraction. It is thought that the power density description is an improvement on tests such as gross energy content or analysis of chemical constituents.

Chapter 9 Conclusions and Applications

This thesis has begun the development of new, more appropriate methods to the calorimeter for measurement of edibility. It offers the prospect of more correctly describing energy inputs to ecosystems and, therefore, has the potential to form the basis for an effective International Biological Programme 2 (IBP2). The first IBP aim's to do this had limited success as a result of inappropriate use of the unit joule. The development of more appropriate units to standardise measurements made in real systems is the first step towards this reappraisal of methods.

The conversion of glucose to lignin is a universal entropic change that affects all species via the 2nd law of thermodynamics. According to this, energy conversion of thermal energy into work cannot be 100% efficient (Hudson and Nelson 1990). Therefore heat energy is lost when plants utilise the energy incident on the earth to manufacture tissues. At each subsequent step in the food chain, thermal energy is lost. Approximately 2% of the sun's incident energy is utilised by plants (Colinvaux 1973) and only part of this is available to the herbivore. The plant requires energy for maintenance and reproduction so only part of the energy fixed is available to the herbivore.

Lignin is a compound that is remarkably resistant to degradation. This means that when lignin is produced using the energy fixed in photosynthesis, the energy is essentially trapped in the molecule. The energy can only be released slowly, or a greater effort is required in order to release it. This provides an example of the way the 2nd law of thermodynamics affects ecosystems.

Energy can be sequestered in materials that are subsequently largely unavailable, or only slowly available, to the ecosystem. The fate of this plant material is either to enter the decomposer chain, where the energy is released over a period of time, or to enter a herbivore. In the latter case, the readily available energy is used by organisms in a relatively short period of time, and the rest excreted, so joining the decomposer community. In the detritus pool, a succession of microorganisms and detritivores have evolved to utilise the various compounds present as food. Rates of decomposition and colonisation of detritus indicate that the readily available energy is utilised preferentially as shown in this thesis.

9.1 The utility of a new measurement

There is a problem when measuring ecosystems on a large scale as their size can prevent manipulation. It is therefore expected that research be carried out on ecosystems that can be managed in a controlled environment. Problems then arise when behaviour does not conform to expectations when scaling up. If measurements can be made and applied on a large scale this can be overcome.

A microbial measurement based on the action of microbes was designed. The principle of microbial infallibility is that microbes exist which, in the right conditions, can oxidise any substance that can theoretically be oxidised (Stanier *et al.* 1986). However, individual species tend to be restricted to a narrow niche. They can use only a few carbon sources for substrate. Their versatility lies in the way that the substrate is

changed for the succession of microbial populations that subsequently are able to utilise it. Mineralisation of cellulose is carried out by specialised microorganisms, such as the *cytophaga* group. They can utilise only cellulose as a carbon source. After the action of this group, the substrate can be colonised by the next in succession. It is the interaction of functional groups which allows the full mineralisation of organic complexes. In the digestive tract of animals, a suite of microorganisms exists allowing digestion to occur.

9.2 Implications for ecology

It is of interest to know how to estimate how much energy is contained in this plant material, and how much passes on to the animal community. The difficulty in doing this has been compounded with the use of advanced technology. In using instruments such as the bomb calorimeter, the amount of energy in a plant material could be established. However, in application of this technique, only a small amount of information was gained. The amount of energy that can be assimilated by an organism in a finite period of time, and not pure content, is a principle detail. The energy in an ecosystem can be viewed in terms of the 2nd law of thermodynamics. It is the quality of the energy that is important and this determines how much work can be done.

The function of trophic levels was to simplify descriptions of ecosystems, which would then lead to an improved understanding of an organism's role within the ecosystem. Due to the difficulties of assigning species to one particular trophic level, this has extended to the idea of guilds of organisms with a specific role. Guilds can be used to simplify system complexity and provide a way of studying utility of organisms. Ecological engineering relies on functional classification (Browne 2000) which can only be done if a common basis for simplification can be used.

For ecosystem research, a way of measuring energy availability to a high resolution could be useful tool. If amounts of energy entering the food web or decomposer web can be measured, then processes and disturbances can be better studied. The energy that drives a dynamic system such as this is derived from solar radiation. The importance of detritus in driving ecosystems is becoming apparent. Odum (1953) demonstrated that energy cycling could be paralleled with a nutrient cycle. He developed Lindeman's trophic flow concept and stated:

"Cycling of nutrients is vital to sustain energy flow"

Plant material makes up 90% of the detritus pool. Some of this will, however, have been processed and rendered more available due to increased surface areas, as herbivore dung is also classed as detritus. Few herbivores can extract more than 50% of the available energy in food which they consume so this part of the detritus is probably fairly energy rich. This thesis also has implications for soil carbon sinks and cycles. The amount of carbon in soil is a product of the vegetation present and its productivity. The loss of this carbon is attributable primarily to heterotrophic respiration (Post *et al.* 1982).

This study measures the power that each compartment of plant can provide the ecosystem with. This will obviously depend on the plant tissue and therefore power density must be given relative to this. The power density measurement could prove to be a better way of studying energy flux relationships of an ecosystem. Using the power

density measurement it may be possible to show the fate of energy fixed in a temperate biome forest, oak woodland.

Figure 9.1. Routes of energy through an ecosystem

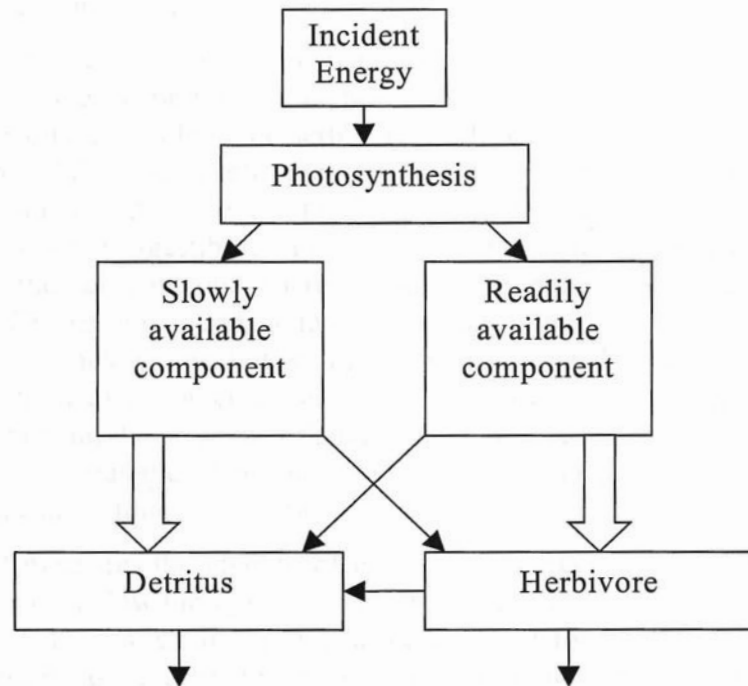


Fig 9.1 shows the various routes that energy can flow through an ecosystem from plants. The amounts that flow through particular channels vary between ecosystems. Some compounds are resistant to degradation or oxidation by microorganisms. The lignin component of detritus can accumulate in the detritus pool. Humus from some soils has been shown to be thousands of years old by radiocarbon dating (Stanier *et al.* 1986). The amounts of detritus accumulating as soil and the amounts of energy flowing through a herbivore channel will depend on the plant community.

A herbivore is limited by time. In its search for food, a problem that faces every organism is, where can the most food be found in the shortest length of time. There are several strategies that have evolved to cope with this predicament. A herbivore of a large body size must access abundant food in order to avoid being limited by energy assimilation. This means there is no time to seek out the higher quality parts of the plant, so energy is assimilated from fermenting the cellulose. In utilising the respiratory and fermentation pathways of microorganisms the energy within the material is accessed. A herbivore of small body size and energetic requirement has time to seek out the more edible parts of the plant, and need retain these in the digestive tract for less time to access the energy. This means the amounts of easily available energy and largely unavailable energy (or unavailable within a certain time limit) in an ecosystem is an important factor or constraint.

The composition of diet in herbivores is one way in which size and digestive strategy have been studied. This affords a clear distinction between grazers and browsers. If the term browse is widened to encompass herbaceous as well as woody browse this would

aid the distinction even more (Owen-Smith 1997). Among mixed feeders, the plentiful season diet choice seems to be concentrated towards a grazing or browsing extreme (Owen-Smith 1997). Time is the crucial factor in a herbivore's energy budget. The amount of time it takes to access the energy in a plant material determines whether the material can be considered food. This means in any estimation of food quality a time dimension must be incorporated.

Many of the compounds produced by plants are completely digestible. Most of the cell contents such as sugars, protein, other forms of nitrogen, lipid, organic acids, soluble minerals are readily available to the herbivore. In the cell wall component all unlignified cellulose, hemicellulose and pectin are available. Lignin, highly lignified cellulose and hemicellulose, silica and cuticle are totally indigestible (Iason and Van Wieren 1999). The factors that affect digestibility are, therefore, lignin in the cell wall and secondary metabolites in the cell contents. There are several evolutionary strategies available to the herbivore. Grasses are abundant and contain less lignin than browse and little in the way of secondary metabolites, but are of low nutritional value (Iason and Van Wieren 1999). Eating food of a higher quality, such as browse, the constraints of increased amounts of lignin and the presence of tannins have influence. Some herbivores opt for a mixture of the two strategies depending on the food availability with season. The latter requires the digestive strategy to be flexible.

A method that measures the amount of energy in each component could improve the way in which energy flow through ecosystems is perceived. The presence of plants in an ecosystem has led scientists in the past to believe that food is not limiting. However, food web research has developed to include studies on the limiting factors on herbivore abundance. Herbivores play a central role in most ecological communities (Krebs *et al.* 1999). They have direct effects on the vegetation, but also cause indirect changes through nutrient displacement and alter forces on vegetation structure such as competition. Most ecosystems contain a variety of herbivores both invertebrate and vertebrate, so a holistic view of their effects is advantageous. Many studies are conducted on a species-species basis, but on transferring the concepts developed to a real situation information can be lost. Multiple species can have compensatory or additive effects (Ritchie and Olff 1999). The availability of energy in the tissues is possibly one of the most important factors on the presence or absence of herbivore species and the gradient between. This is probably not an isolated factor and may be related to the spatial availability of nutrients or energy also.

9.3 Future Development of the test

The buffer used was not suitable for this study as it supplied an extra carbon source for the microorganisms. The use of a digest meant a buffer was necessary, but this method should be developed with the use of a carbon-free buffer. The extra carbon source given meant a large correction factor was needed, and this is a large source of error in the calculations.

Tannin contents are an important consideration of the quality of dicotyledons. It was not seen as necessary to assess levels in the oak samples as the measurement of quality relies upon the action of microorganisms. Suppression of the activity of microorganisms

by tannins may have occurred in the test. It may be necessary to have a greater understanding of the way in which tannins affect the outcome of the tests. There are several methods for assessment of tannin concentration, most of which involve heating with butanol-HCl (95:5 v/v) reagent (Makkar and Singh 1995). The use of polyethylene glycol (PEG) could be used to assess the value of tannins when considering edibility.

Rates of decomposition of oak litter have been found to vary depending on the species of fungus colonising the litter (Hering 1967). A standard inoculum for the BOD test would prevent any variation in rate of degradation within a BOD bottle. Standard inocula are currently available from companies such as Merck for water quality testing and it more appropriate microbes could be developed for this purpose.

In this thesis, the biological technology chosen was a modified biochemical oxygen demand test. There is a possibility that other tests may prove more useful for this application. The term biodegradability is usually taken to mean biodegradability under aerobic conditions (Eden *et al.* 1968). As the amount of energy acquired from anaerobic pathways is different to aerobic it may be of interest to measure anaerobic biodegradability. The main product of respiration is carbon dioxide, and this is used as a test for ready biodegradability in the Sturm test. The modification of this test to a plant quality perspective may be possible and this test has already been used to explore the energy status in a simple food chain (Atree 1998). There is evidence to suggest that the CO₂ produced by the microorganisms in the test may not reflect accurately the amounts of dissolved organic carbon removed (Weytens *et al.* 1994). Although remedies for this can be made, the problems of entrapment mean measured rates of CO₂ production are always lower than real ones (Weytens *et al.* 1994). Insufficient knowledge of the fate of CO₂ means oxygen usage rather than CO₂ evolution is probably a better way to measure the rate of metabolism in a solution. It is suggested that a modified BOD is the best way to proceed.

For optimum cell breakage various methods are used. Information can be gained from the use of homogenisers and mills for improved degradation of sewage and sludge. Although the effects of these methods are generally measured only on bacteria and yeast, the results have implication for the break up of plant cells. Measurement of the disruption of cells can be estimated using particle size distribution. Rupture of microorganism cell walls was found to occur with the use of an ultrasonic homogeniser at a high-energy input (Kopp *et al.* 1997). This may have an advantage over the mechanical mills currently used in that heat generated in the processing may change constituents. Various methods for cell disruption need to be investigated. Incidentally, Kopp *et al.* (1997) found the best degree of cell disruption by mechanical means, with a stirred ball mill.

The intrinsic value of the BOD test is the fact that it is a low cost, holistic method. Because of this measurement of the fate of constituents was not carried out. It may be that a better understanding of the fate of carbohydrates, lipids and proteins is necessary, especially if use of the test was extended to the agricultural feed industry.

9.4 Applications

Within the area of ecosystem management and conservation, the trophic aspects of relationship, cause and effect, is of importance. The fragility of ecosystems can only be judged with adequate understanding of the responses of a system to extinction, invasions and other disturbances (Nilsson and Grelsson 1995).

Macroecology is developing as a valid attempt to produce statistical order from ecosystems whether on a local or global scale (Lawton 1999). The IBP had limited success in its attempts to do this with a systems approach, largely because the trophic level concept is difficult to apply in reality. With new concepts in the trophic routes through systems, and the development of methodology to measure this, success may be within reach. ECOPATH (Christensen and Pauly 1993) was designed to describe the structure of ecosystems and incorporates a routine that aggregates the entire system into discrete trophic levels. However, ECOPATH does not include a time dimension and is not dynamic. Trophic level structures are still used for ecosystem models. Modellers have noted the importance of trophic position on ecosystem stability and species extinction (Nilsson and Grelsson 1995).

Size relationships have also been recognised as important in ecosystem modelling. Species loss has an effect on ecosystem structure, but there is debate on the effect of trophic position. Species loss has an effect on ecosystem structure but there is debate on the effect of trophic position. Large organisms that are low in population size, such as top predators, can be as important in shaping ecosystems as small but abundant organisms, such as aphids.

Detecting climate change is currently of great importance. The debate on whether climate change is occurring, or going to occur, continues but is beyond the scope of this thesis. Vegetation cover is of significance within this field, and also measurement of carbon sinks and sources, so ways in which to measure the fate of substances could have implications. Soil carbon is strongly correlated with climatic variables (Post *et al.* 1982) and world climate models usually result in maps of potential natural vegetation in a given environment (Prentice *et al.* 1992) which suggests these are closely linked. Land use changes are coupled to the climate system through patterns of transpiration and runoff and the processes governing the role of vegetation at continental scales are being investigated (International Council of Scientific Unions 1990). Underpinning all models is the concept of the plant functional type (cross ref) (Prentice *et al.* 1996). The International Geosphere-Biosphere Programme (IGBP) suggests that this concept is developed and this thesis may have implication for this. There is no standard unit to measure processes and changes within ecosystems. This project will ultimately address the need for standardisation in ecosystem measurements (Cohen 1993) and re-position the 'producers' in terms of a re-dimensioned trophic space.

With the development of the science of ecology has come an increase in the number of ways to describe ecosystems. They are regarded in terms of fragility, stability, diversity, persistence, resilience and resistance to name a few. An interesting aspect of this is how value is placed on an ecosystem. These words relate to conservation of certain ecosystems, and recently there have been attempts to place economic value on this

(Schoon 1997). There are difficulties in calculating the value of an ecosystem's service to mankind, but minimum estimates equal or exceed global gross national product (Pimm 1997).

The agricultural feed industry has incorporated a time dimension with its use of metabolisable energy (ME) with the SI units MJ per day, or MJ per kg dry matter (DM). The importance of time has been indicated, with the use of retention times, and retention of net energy into the calculations of feeding levels (AFRC 1993). This test could provide an improvement on the huge collection of laboratory measurements and calculations that are currently used within this industry. The test could provide information on the energy available in different foodstuffs with one method reducing the need for labour and equipment. The amounts of nutrients in plant material can provide information about whether the plant material can be regarded as food. In the agricultural feed industry, the amounts of nitrogen and fibre are considered crucial factors (AOAC 1994). These tests are laborious and often inaccurate and do not allow adequate comparisons due to their resolution. The measurements using the new methods have shown differences in energy supply from oak parts to detritus pools and herbivores. With these methods, the power rating of the materials when utilised by these two processes have been shown to be up to 7 times different (green leaves compared with bark).

9.5 To Conclude

This project has explored various methods to improve upon the measurement of energy inputs to the ecosystem. It has been shown that conventional bomb calorimetry does not distinguish between the energy availability of very different plant components such as bark and seed. The total energy content for a unit mass varies little between plant tissues. The rate of decay experiment successfully demonstrated different energy availability between plant components when respired by microorganisms, whereas calorific differences between the most different plant parts were of the order of 10%, energy availability, as expressed in the decomposition experiment were up to 700% different. This was due to the incorporation of a measure of the rate at which energy is available in the different plant tissues. This shows the potential of developing standardised methods in order to quantify energy inputs to ecosystems.

A further aspect of the decomposition experiment was to examine the relationship between new orthodoxy of plant quality measurement (Palm and Rowland 1997) where a given set of tests provides a general measure of plant quality for detritivores. This decomposition experiment showed that there were correlations, both positive and negative, between components of the PQMD method and the measurement of energy availability developed in this thesis. In addition, the two power density measurements calculated in chapter 8 from both methods chosen are closely correlated. This establishes a relationship between plant quality and ecosystem energetics for the first time.

The attempt to standardise measurements for application to macroecology led to the development of a cellulose digest and BOD test. The development of the power density coefficient from this was necessary to facilitate the study of energy fluxes in food webs and ecosystems. These measurements, applied to a plant which is characteristic of a

temperate biome, successfully separated the plant parts in terms of energy availability. The power density calculated from these results appears to be comparative to the power density calculated from the rate of decay data.

9.6 The wider view

The progression of research and ideas in ecology has led to simplification of actual ecosystem complexity. However the basic principles had to be identified in order to teach ecology. Lindeman (1942) pinpointed the importance of dynamic processes of succession and defined trophic levels in order to generate data to measure energy flows he thought drove succession. But for his untimely death his ideas may have progressed and become more developed concepts (Willis 1997). Since Elton (1927) trophic structure became a way in which the ecosystem concept could be understood theoretically and teaching of this continues at present.

Odum (1964) is also a commonly used teaching-text and is relevant as it retains a holistic viewpoint where energetics is targeted as the important process for measurement. Energy incident on the earth is fixed by photosynthesis and thus made potentially available to the animal kingdom. Plant biomass is therefore identified as a fundamental part of an ecosystem. Ecologists often calculate this in order to measure and predict energy flows within ecosystems. The development of the net primary productivity (NPP) model was a way in which different biomes could be compared though mapping the productivity of different regions (see Lieth 1975).

However measures such as NPP are limiting in that there is little or no recognition of plant energy availability. Nevertheless, these measures meant ecology could be studied at a macro-scale and attempts to predict and construct models could be made. More recently the NPP measure has been recognised as only a partial descriptor and subsequently modified. The net foliar productivity (NFP) measure proposed by McNaughton *et al.* (1989) recognises the difference between wood and leaf. This is one step towards acknowledging the importance of different energy availabilities of plant materials at an ecosystem level.

The laws of thermodynamics with regard to ecosystems are of fundamental importance and have been recognised as such from Lindeman (1942), via Odum (1964), and currently (Jørgensen 1992; Hall and Odum 1995); the 'quality' of energy flows is reported to have an effect on the thermodynamic equilibrium of an ecosystem. The recognition of the 'quality' of energy flows is in accordance with this thesis when high quality energy is viewed as easily assimilated.

It is proposed that measurements of energy availability should be put into practise and this thesis provides one way in which this can be achieved. The application of the BOD method to assess the energy availability within an ecosystem could provide valuable data from which global or biome processes can be researched. Programmes of research into large-scale ecology appear to be waning. One contribution of this thesis is the recognition of the need for wider collaboration between research groups. The TIGER programme (Terrestrial Initiative in Global Environmental Research) was designed to do this for researchers with varying aims (Cummins and Watkinson 1999). However this has resulted in many small-scale studies and the effects of climate change upon

these. Few food chain steps and no webs were studied. The incorporation of a measure which could accurately assess the rate at which energy is available to an ecosystem would contribute greatly to programmes such as TIGER, or a new International Biological Programme, IBP2.

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Appendix A

Database illustrating how quality is characterised in various studies.

Nitrogen	Fibre (any)	Lignin	Carbon	Secondary metabolites	Other nutrients	Physical characteristic	Digestibility /palatability	Silica	Other	Reference
1	1	0	0	0	1	0	0	0	1	Leloup and Mannetje 1996
1	1	0	1	1	1	1	0	0	1	Feller 1995
0	1	0	0	0	0	0	1	0	0	Young Owl and Batzli 1998
1	1	0	0	0	0	0	0	0	0	Claridge and Cork 1994
1	1	1	0	1	0	0	0	0	0	Gauthier and Hughes 1995
1	1	0	0	1	0	0	1	0	0	Keys <i>et al.</i> 19
1	0	0	0	0	0	0	0	0	0	Jacobsen 1993
1	1	0	0	1	0	0	0	0	0	Bozinovic <i>et al.</i> 1997
0	0	0	0	0	0	1	0	0	0	Choong <i>et al.</i> 1992
1	0	0	1	0	0	0	0	0	0	Higashi <i>et al.</i> 1992
0	0	0	0	0	0	0	0	1	0	McNaughton <i>et al.</i> 1985
0	0	0	0	1	0	0	0	0	0	Janzen 1974
0	1	0	0	0	0	0	0	1	0	Peterson <i>et al.</i> 1988
1	0	0	0	0	0	0	0	0	0	Mattson 1980
0	0	0	0	0	0	1	1	0	0	Wilman <i>et al.</i> 1996
1	0	0	0	0	0	0	1	0	0	Athey and Connor 1989
0	1	0	0	0	0	0	0	0	0	Hatcher 1994
0	0	0	0	0	0	0	1	0	0	Hochuli 1996
0	0	0	0	0	0	0	1	0	0	Barbehenn 1992
0	1	0	0	0	0	0	1	0	0	Hochuli and Roberts 1996
0	0	0	0	0	0	0	1	0	0	Wilson and Kennedy 1996
0	1	1	0	0	0	0	1	0	0	Osbourne <i>et al.</i> 1974

Appendix B

Mass loss of cow parsley *Anthriscus sylvestris* plant parts with time.

Root

name	NET1.0	NET1.20	NET1.40	NET1.60	NET1.80	NY1.0	NY1.20	NY1.40	NY1.60	NY1.80
time	0	20	40	60	80	0	20	40	60	80
	3.00	.84	.76	.52	.84	3.00	.69	.52	.36	-.08
	3.00	1.04	.81	.47	.82	3.00	.73	.72	.44	-.02
	3.00	1.20	.76	.69	.62	3.00	.27	.69	.47	.35
	3.00	1.38	1.01	.60	.55	3.00	.66	.34	.40	.15
	3.00	1.32	1.39	.57	.48	3.00	.67	.58	.68	-.12
mean	3.00	1.16	.95	.57	.66	3.00	.60	.57	.47	.06

Leaf

name	NET2.0					NY2.0	NY2.20	NY2.40	NY2.60	NY2.80
time	0	20	40	60	80	0	20	40	60	80
	2.00	.30	.33	.24	.35	2.00	.09	.13	.42	.16
	2.00	.33	.51	.18	.19	2.00	.22	.13	.31	.07
	2.00	.38	.60	.24	.40	2.00	.22	.18	.14	.12
	2.00	.32	.33	.49	.46	2.00	.25	.19	.18	.11
	2.00	.29	.38	.11	.18	2.00	.15	.10	.15	.16
mean	2.00	.32	.43	.25	.32	2.00	.19	.15	.24	.12

stem

name	NET3.0	NET3.20	NET3.40	NET3.60	NET3.80	NY3.0	NY3.20	NY3.40	NY3.60	NY3.80
time	0	20	40	60	80	0	20	40	60	80
	1.00	.18	.32	.26		1.00	.20	.22	.04	.02
	1.00	.25		.04	.08	1.00	.09	.25	.26	.01
	1.00	.27	.00	.26	.11	1.00	.10	.02	.06	-.01
	1.00	.29	.14	.05	.01	1.00	.16		.38	.12
	1.00	.24	.22	.05	.25	1.00	.09	.31	.11	.13
mean	1.00	.25	.17	.13	.11	1.00	.13	.20	.17	.05

flowers

name	NET4.0	NET4.20	NET4.40	NET4.60	NET4.80	NY4.0	NY4.20	NY4.40	NY4.60	NY4.80
time	0	20	40	60	80	0	20	40	60	80
	1.00	.43	.25	.04	.05	1.00	.29	.42	.18	.30
	1.00	.41	.63	-.03	.23	1.00	.38	.12	.09	.12
	1.00	.37	.25	.13	.05	1.00	.35	.29	.43	.10
	1.00	.70	.32	.27	.21	1.00	.34	.15	.17	.13
	1.00	.51	.08	.13		1.00		.40	.18	.12
mean	1.00	.48	.31	.11	.14	1.00	.34	.28	.21	.15

seeds

name	NET5.0	NET5.20	NET5.40	NET5.60	NET5.80	NY5.0	NY5.20	NY5.40	NY5.60	NY5.80
time	0	20	40	60	80	0	20	40	60	80
	.50	.37	.17	.12	.14	.50	.16	.05	.20	.34
	.50	.22	.45	.15	.04	.50	.22	.12	.33	.20
	.50	.05	.20	.29	.05	.50	.27	.22	.07	.17
mean	.50	.21	.27	.19	.08	.50	.22	.13	.20	.24

Mean mass loss over time of cow parsley *Anthriscus sylvestris* plant parts in 1.5mm size mesh litter bags.

time	0	20	40	60	80
roots	3.00	1.16	0.95	0.57	0.66
stems	2.00	0.32	0.43	0.25	0.32
leaves	1.00	0.25	0.17	0.13	0.11
flowers	1.00	0.48	0.31	0.11	0.14
seeds	0.50	0.21	0.27	0.19	0.08

Mean mass loss over time of cow parsley *Anthriscus sylvestris* plant parts in 0.5 mm size mesh litter bags

time	0	20	40	60	80
roots	3.00	.60	.57	.47	.06
stems	2.00	.19	.15	.24	.12
leaves	1.00	.13	.20	.17	.05
flowers	1.00	.34	.28	.21	.15
seeds	.50	.22	.13	.20	.24

Results of fibre analysis of bean plant *Vicia faba* grown in controlled conditions

plant part	W1	W2	W3	W4	W5	%ADF	%lignin	%cellulose
stems	0.7911	23.9332	24.2116	23.9785	23.8961	35.19	10.42	29.46
stems	0.8261	23.3191	23.6465	23.3164	23.2629	39.63	6.48	39.96
roots	0.9274	21.9339	22.606	22.2925	21.9244	72.47	39.69	32.8
roots	0.965	148.9934	149.7318	149.4389	149.432	76.52	0.715	30.35
leaves	0.8975	23.8902	24.0486	24.6755	23.889	17.65	87.63	-69.8
leaves	0.9209	23.2315	23.2802	23.8898	23.2314	16.15	71.49	-55.4

Results of laboratory analysis of cow parsley, *Anthriscus sylvestris*, plant parts

component	N1	N2	N3	N4	N5	ADF1	ADF2	ADF3	ADF4	ADF5
part	roots	stems	leaves	flowers	seeds	roots	stems	leaves	flowers	seeds
	.44	.48	2.87	3.56	3.21	25.50	60.70	21.60	21.10	23.30
	.50	.51	3.23	3.62	3.11	31.40	62.20	23.40		
	.49	.51	2.70	3.64		22.00	57.50	20.90		
			2.77							
mean	.48	.50	2.89	3.61	3.16	26.30	60.13	21.97	21.10	23.30

Fresh to dry weigh mass for each plant part

part	fresh weight	dry weight
roots	6	3.61
roots	6.3	3.76
leaves	2.31	1.34
leaves	4.32	2.12
stems	8.88	2.27
stems	17.37	4.37
flowers	12.66	8.01
flowers	11.86	7.91
seeds	3.95	2.66
seeds	2.09	1.42

Appendix C

Mass loss of oak parts and sampling dates

date	no. of days	cotton	acorns	root	bark	twigs	brown leaves	green leaves
15/11/97	0	2	2	5	8	4	4	4
15/11/97	0	2	2	5	8	4	4	4
15/11/97	0	2	2	5	8	4	4	4
15/11/97	0	2	2	5	8	4	4	4
15/11/97	0	2	2	5	8	4	4	4
03/12/97	18	1.91	1.65	4.35	7.55	3.56	3.49	3.3
03/12/97	18	1.88	1.63	4.4	7.48	3.62	3.49	3.23
03/12/97	18	1.91	1.67	4.42	7.58	3.33	3.53	3.2
03/12/97	18	1.92	1.66	4.3	7.57	3.46	3.61	3.05
03/12/97	18	1.9	1.65	4.37	7.6	3.56	3.49	2.97
20/12/97	35	1.87	1.37	4.44	7.64	3.64	3.34	3.44
20/12/97	35	1.85	1.42	4.56	7.56	3.49	3.47	2.97
20/12/97	35	1.84	1.57	4.5	7.69	3.54	3.45	3.03
20/12/97	35	1.89	1.65	4.36	7.53	3.56	3.35	3
20/12/97	35	1.84	1.52	4.28	7.6	3.54	3.43	2.98
24/01/98	70	1.55	1.48	4.23	7.44	3.5	3.18	3.37
24/01/98	70	1.62	1.27	4.52	7.48	3.36	3.15	2.93
24/01/98	70	1.57	1.25	4.2	7.54	3.12	3.3	3.81
24/01/98	70	1.67	1.21	4.13	7.44	3.32	3.79	2.75
24/01/98	70	1.75	1.3	4.32	7.46	3.5	3.8	2.94
10/03/98	115	1.28	1.02	4.16	6.76	3.08	3.02	1.31
10/03/98	115	0.93	1.06	4.23	7.42	3.41	3.46	1.37
10/03/98	115	0.98	1.05	4.04	7.51	3.34	3.22	1.35
10/03/98	115	1.15	1.08	4.03	7.36	3.12	3.18	1.32
10/03/98	115	0.93	1.01	4.13	7.13	3.3	3.29	1.33
24/04/98	160	0.68	0.87	3.95	7.42	3.02	2.85	2.55
24/04/98	160	0.89	0.91	3.85	7.26	3.35	3.06	2.34
24/04/98	160	0.47	0.98	4.18	7.24	3.21	3.66	2.4
24/04/98	160	0.51	0.95	4.56	7.27	3.38	2.98	2.37
24/04/98	160	0.83	0.92	4.41	7.34	3.34	3.42	2.33
09/10/98	292	0	0.93	5.77	8.63	3.78	4.81	2.14
09/10/98	292	0	0.84	4.54	8.45	4.24	3.6	2.74
09/10/98	292	0	1.04	6.29	8.66	4.57	4.93	2.51
09/10/98	292	0	1.19	4.62	8.84	4.14	4.3	2.59
09/10/98	292	0	1.02	5.02	9.2	4.09	5.45	2.35

Percentage of mass loss with sampling dates for oak parts

number	date	no. of days	cotton		acorns	% origm	root	%	bark	%	twigs	%	brown leaves	%	green leaves	%
0	15/11/97	0	2	100	2	100	5	100	8	100	4	100	4	100	4	100
0	15/11/97	0	2	100	2	100	5	100	8	100	4	100	4	100	4	100
0	15/11/97	0	2	100	2	100	5	100	8	100	4	100	4	100	4	100
0	15/11/97	0	2	100	2	100	5	100	8	100	4	100	4	100	4	100
0	15/11/97	0	2	100	2	100	5	100	8	100	4	100	4	100	4	100
1	03/12/97	18	1.91	95.5	1.65	82.5	4.35	87	7.55	94.375	3.56	89	3.49	87.25	3.3	82.5
1	03/12/97	18	1.88	94	1.63	81.5	4.4	88	7.48	93.5	3.62	90.5	3.49	87.25	3.23	80.75
1	03/12/97	18	1.91	95.5	1.67	83.5	4.42	88.4	7.58	94.75	3.33	83.25	3.53	88.25	3.2	80
1	03/12/97	18	1.92	96	1.66	83	4.3	86	7.57	94.625	3.46	86.5	3.61	90.25	3.05	76.25
1	03/12/97	18	1.9	95	1.65	82.5	4.37	87.4	7.6	95	3.56	89	3.49	87.25	2.97	74.25
2	20/12/97	35	1.87	93.5	1.37	68.5	4.44	88.8	7.64	95.5	3.64	91	3.34	83.5	3.44	86
2	20/12/97	35	1.85	92.5	1.42	71	4.56	91.2	7.56	94.5	3.49	87.25	3.47	86.75	2.97	74.25
2	20/12/97	35	1.84	92	1.57	78.5	4.5	90	7.69	96.125	3.54	88.5	3.45	86.25	3.03	75.75
2	20/12/97	35	1.89	94.5	1.65	82.5	4.36	87.2	7.53	94.125	3.56	89	3.35	83.75	3	75
2	20/12/97	35	1.84	92	1.52	76	4.28	85.6	7.6	95	3.54	88.5	3.43	85.75	2.98	74.5
3	24/01/98	70	1.55	77.5	1.48	74	4.23	84.6	7.44	93	3.5	87.5	3.18	79.5	3.37	84.25
3	24/01/98	70	1.62	81	1.27	63.5	4.52	90.4	7.48	93.5	3.36	84	3.15	78.75	2.93	73.25
3	24/01/98	70	1.57	78.5	1.25	62.5	4.2	84	7.54	94.25	3.12	78	3.3	82.5	3.81	95.25
3	24/01/98	70	1.67	83.5	1.21	60.5	4.13	82.6	7.44	93	3.32	83	3.79	94.75	2.75	68.75
3	24/01/98	70	1.75	87.5	1.3	65	4.32	86.4	7.46	93.25	3.5	87.5	3.8	95	2.94	73.5
4	10/03/98	115	1.28	64	1.02	51	4.16	83.2	6.76	84.5	3.08	77	3.02	75.5	1.31	32.75
4	10/03/98	115	0.93	46.5	1.06	53	4.23	84.6	7.42	92.75	3.41	85.25	3.46	86.5	1.37	34.25
4	10/03/98	115	0.98	49	1.05	52.5	4.04	80.8	7.51	93.875	3.34	83.5	3.22	80.5	1.35	33.75
4	10/03/98	115	1.15	57.5	1.08	54	4.03	80.6	7.36	92	3.12	78	3.18	79.5	1.32	33
4	10/03/98	115	0.93	46.5	1.01	50.5	4.13	82.6	7.13	89.125	3.3	82.5	3.29	82.25	1.33	33.25
5	24/04/98	160	0.68	34	0.87	43.5	3.95	79	7.42	92.75	3.02	75.5	2.85	71.25	2.55	63.75
5	24/04/98	160	0.89	44.5	0.91	45.5	3.85	77	7.26	90.75	3.35	83.75	3.06	76.5	2.34	58.5
5	24/04/98	160	0.47	23.5	0.98	49	4.18	83.6	7.24	90.5	3.21	80.25	3.66	91.5	2.4	60
5	24/04/98	160	0.51	25.5	0.95	47.5	4.56	91.2	7.27	90.875	3.38	84.5	2.98	74.5	2.37	59.25
5	24/04/98	160	0.83	41.5	0.92	46	4.41	88.2	7.34	91.75	3.34	83.5	3.42	85.5	2.33	58.25
6	09/10/98	292	0	0	0.93	46.5	5.77	115.4	8.63	107.875	3.78	94.5	4.81	120.25	2.14	53.5
6	09/10/98	292	0	0	0.84	42	4.54	90.8	8.45	105.625	4.24	106	3.6	90	2.74	68.5
6	09/10/98	292	0	0	1.04	52	6.29	125.8	8.66	108.25	4.57	114.25	4.93	123.25	2.51	62.75
6	09/10/98	292	0	0	1.19	59.5	4.62	92.4	8.84	110.5	4.14	103.5	4.3	107.5	2.59	64.75
6	09/10/98	292	0	0	1.02	51	5.02	100.4	9.2	115	4.09	102.25	5.45	136.25	2.35	58.75

Spearman's rank correlation for plant quality parameters and rate of decay constants

Correlations		ADF	ADLCELLULO	S	N	WSC	ENERGY	DAY18	DAY115	B1
Spearman's rho	ADF Correlation Coefficient	1.000	.829	.371	-.771	-.257	-.058	-.714	-.943	-.986
	Sig. (2-tailed)	.	.042	.468	.072	.623	.913	.111	.005	.000
	N	6	6	6	6	6	6	6	6	6
	ADL Correlation Coefficient	.829	1.000	.200	-.714	-.429	.232	-.886	-.771	-.812
	Sig. (2-tailed)	.042	.	.704	.111	.397	.658	.019	.072	.050
	N	6	6	6	6	6	6	6	6	6
	CELLULO S Correlation Coefficient	.371	.200	1.000	.086	.600	.493	-.143	-.257	-.406
	Sig. (2-tailed)	.468	.704	.	.872	.208	.321	.787	.623	.425
	N	6	6	6	6	6	6	6	6	6
	N Correlation Coefficient	-.771	-.714	.086	1.000	.714	.493	.657	.886	.696
	Sig. (2-tailed)	.072	.111	.872	.	.111	.321	.156	.019	.125
	N	6	6	6	6	6	6	6	6	6
	WSC Correlation Coefficient	-.257	-.429	.600	.714	1.000	.551	.600	.486	.232
	Sig. (2-tailed)	.623	.397	.208	.111	.	.257	.208	.329	.658
	N	6	6	6	6	6	6	6	6	6
	ENERGY Correlation Coefficient	-.058	.232	.493	.493	.551	1.000	-.116	.290	.000
	Sig. (2-tailed)	.913	.658	.321	.321	.257	.	.827	.577	1.000
	N	6	6	6	6	6	6	6	6	6
	DAY18 Correlation Coefficient	-.714	-.886	-.143	.657	.600	-.116	1.000	.771	.754
	Sig. (2-tailed)	.111	.019	.787	.156	.208	.827	.	.072	.084
	N	6	6	6	6	6	6	6	6	6
	DAY115 Correlation Coefficient	-.943	-.771	-.257	.886	.486	.290	.771	1.000	.928
	Sig. (2-tailed)	.005	.072	.623	.019	.329	.577	.072	.	.008
	N	6	6	6	6	6	6	6	6	6
	B1 Correlation Coefficient	-.986	-.812	-.406	.696	.232	.000	.754	.928	1.000
	Sig. (2-tailed)	.000	.050	.425	.125	.658	1.000	.084	.008	.
	N	6	6	6	6	6	6	6	6	6
* Correlation is significant at the .05 level (2-tailed).										
** Correlation is significant at the .01 level (2-tailed).										

* Correlation is significant at the .05 level (2-tailed).

** Correlation is significant at the .01 level (2-tailed).

Appendix D

SOP 1

The preparation of sonicated sample 16/11/98

1. Weigh out 2g of sample
2. Pestle and mortar with approximately 20ml of distilled water and 0.5g of 0.25mm diameter glass balls until a paste (is not smooth due to venous material)
3. Rinse out with distilled water to retrieve sample
4. Place in a glass vessel
5. Clamp in the sonicator so the probe is just beneath the surface of the solution
6. Sonicate sample for 15 minutes in short bursts of no longer than one minute
7. Allow sonicator to cool for 30 minutes
8. Check regularly for cell breakage under microscope and, if necessary, continue sonicating at 15 min intervals
9. Freeze sample at -5°C in 2ml aliquots for future use

SOP 2

The Bradford protein test 9/2/99

1. Place plant material in centre of rollers
2. Add distilled water and switch on mill
3. When sample is churning, begin timing.
4. Pipette 0.25ml for T=0 sample
5. Continue to sample at 10 minute intervals until T=40
6. Centrifuge sample for 5 minutes
7. Pipette 0.05ml of the supernatant into a cuvette
8. Add 2.5ml of the Bradford protein stock solution (solution 1) to the cuvette
9. Use 0.05ml of distilled water and 2.5ml of the stock solution for the blank and measure the absorbance at 595nm

SOP 3

The preparation of milled sample 12/12/98

1. Tighten rollers to almost maximum
2. Switch on and add some broken, dried material
3. Loosen and tighten rollers as appropriate
4. When crushed add citrate buffer (pH 5 due to requirements of cellulase used) in drops until a paste is formed
5. Begin timing of milling
6. Collect sample at appropriate intervals for Bradford protein test
7. When protein content is constant, collect paste
8. Place 1ml on pre-weighed filter paper for estimation of dry matter content

SOP 4

Milling plant parts 23rd Jan 1999

1. Add sample (previously hammer milled to pass through a 4mm sieve) and distilled water to rollers
2. Loosen and tighten rollers until a paste is formed, making sure all sample is collected in the centre and therefore being crushed
3. Begin timing
4. Add distilled water as needed to prevent the sample drying out
5. After 30 mins collect sample on a watch glass
6. Allow to air dry
7. Collect sample and crush in pestle and mortar to a fine powder, in case any separation of particle sizes has occurred
8. Store in air-tight container until needed
9. Clean rollers with 60% methanol ready for next use
10. Collect the milled sample at the appropriate time i.e. when the concentration of protein remains constant

SOP 5

Preparation of buffer solution (from Dawson et al 1987)

1. Weigh out 21.01g (± 0.001) of citric acid monohydrate
2. Dissolve in distilled water and make up to 1 litre in a volumetric flask
3. Weigh out 29.41g (± 0.001) of trisodium citrate dihydrate
4. Make up to 1 litre in a volumetric flask
5. Calibrate pH meter
6. Place 650ml of the trisodium citrate soln in a 1l beaker
7. Measure out 350ml of the citric acid soln
8. Add this gradually to the beaker until the pH meter is stable at 5
9. Autoclave buffer in a Dumas bottle and store in fridge
10. The buffer can then be diluted to a 1 in 10 concentration from this for

SOP 6

SOP for simulated digestion procedure 2/2/99

1. Using milled sample, place 200mg in conical flask
2. Add 100ml citrate buffer pH5
3. Add a magnetic flea, cover flask neck with aluminium foil to prevent evaporation and leave stirring overnight
4. With buffer, make up the solution to 1litre in a volumetric flask
5. Determine BOD:COD for this solution as per SOP's and also for buffer solution.
6. Depending on amount of cellulose in sample add enough units of cellulase so reaction is not limited (1 μ mole of glucose is liberated by 1 unit of enzyme in 1 hr at 37 °C). The reaction must not be enzyme limited, so use highest value for cellulose content in the calculation

Mass of sample in 1l/100*% of cellulose in that plant part
=amt of cellulose in 1l

Assuming this cellulose can all be converted into glucose by the enzyme and allowing for the loss of 1 molecule of water per glucose unit, the molecular wt used is adjusted.

180 (molecular weight of glucose)-18 (mwt H₂O)=

potentially:

cellulose content(g)/162 =no. of moles in soln
convert to μ moles and this is equivalent to the no of units required to convert all the cellulose in 1litre of sample per hour
This amt can then be adjusted for a longer time period of incubation and smaller amount of sample if necessary

e g for summer leaves

Cellulose content not more than 16% Therefore:

$(0.2\text{g}/100)*16=0.032\text{g}$

$0.032/162=0.0002$ mole equivalents of glucose

=200 μ g equivalent to 200 units.

Therefore for 2 hour incubation period and for 100ml sample size, require $200/2/10=10$ units (to 3 s.f.)

For my enzyme solution 1ml contains 2.55 units so therefore require 8ml in each conical flask

7. Take out 0.075ml for initial Bradford protein assay
8. Set up control as above using 100ml buffer instead of plant part solution
9. Place conical flasks on shaker in 30°C room
10. At half hour intervals, take out 0.075ml, centrifuge and carry out Bradford Protein assay (SOP no) in order to assess stage of reaction
End point reached when protein conc is same for 2 consecutive samples
At this point assume all cell walls lysed
- 5 Centrifuge sample for 5 mins and store supernatant for further tests
11. Carry out BOD and COD tests as per SOP's

SOP 7

SOP for digestion procedure 2/3/99

1. Using milled sample, place 200mg in conical flask
2. Add 100ml citrate buffer pH5
3. Add a magnetic flea, cover flask neck with aluminium foil to prevent evaporation and leave stirring overnight
4. With buffer, make up the solution to 1litre in a volumetric flask
5. Make up control solution of buffer and enzyme at correct concentration without plant part.
6. Push through 0.45µm syringe filter to clean
12. Determine BOD:COD for this solution as per SOP's and also for buffer solution.
13. Depending on amount of cellulose in sample add enough units of cellulase so reaction is not limited (1µmole of glucose is liberated by 1 unit of enzyme in 1 hr at 37 °C). The reaction must not be enzyme limited, so use highest value for cellulose content in the calculation

Mass of sample in 1l/100*% of cellulose in that plant part
=amt of cellulose in 1l

Assuming this cellulose can all be converted into glucose by the enzyme and allowing for the loss of 1 molecule of water per glucose unit, the molecular wt used is adjusted.

180 (molecular weight of glucose)-18 (mwt H₂O)=

potentially:

cellulose content(g)/162 =no. of moles in soln
convert to µmoles and this is equivalent to the no of units required to convert all the cellulose in 1litre of sample per hour
This amt can then be adjusted for a longer time period of incubation and smaller amount of sample if necessary

e g for summer leaves

Cellulose content not more than 16% Therefore:

$$(0.2\text{g}/100)*16=0.032\text{g}$$

$$0.032/162=0.0002 \text{ mole equivalents of glucose}$$

$$=200\mu\text{g equivalent to 200 units.}$$

Therefore for 2 hour incubation period and for 100ml sample size,
require $200/2/10=10$ units (to 3 s.f.)

For my enzyme solution 1ml contains 2.55 units so therefore require
8ml in each conical flask

14. Place conical flasks on shaker in 30°C room

15. Assay for glucose generation at half hour intervals

15. Carry out BOD and COD tests as per SOP's on digestion products

SOP 8

SOP for digest procedure 23/7/99

1. Place 10ml of plant part solution used for the preBOD test in a universal bottle
2. Add aliquot of previously made up enzyme solution
3. Repeat for each plant part and add a control of 11mls of buffer alone, and a control of 10ml of buffer and aliquot of enzyme.
4. Check pH is around 5
5. Leave all universals on shaking machine for 48 hours
6. Check pH has not strayed too far from 5
7. Freeze for future use

SOP 9

SOP of stock reagent solutions for BOD dilution media.

1. Ferric chloride solution 0.0125%.

Dissolve 0.125 ± 0.005 g of ferric chloride hexhydrate in distilled water and make up to 1l in a measuring cylinder.

2. Calcium chloride solution 2.75%

Dissolve 27.5 ± 0.5 g calcium chloride in distilled water and make up to 1l in a measuring cylinder

3. Magnesium sulphate solution 2.5%

Dissolve 25.0 ± 1.0 g of magnesium sulphate heptahydrate in water and dilute with water to 1l

4. Phosphate buffer solution pH 7.2

Dissolve 52.5 ± 0.5 g potassium dihydrogen phosphate in 700 ± 10 ml water in a beaker and add 8.8 ± 0.1 sodium hydroxide. Add 2.0 ± 0.1 g ammonium sulphate and when dissolved make up to 1l. The pH of this should be checked

5. Allyl thiourea solution 0.1%

Dissolve 1.00 ± 0.02 g allyl thiourea in water and make up to 1l

SOP 10

USE OF COD TEST KIT AND HACH READER

1. Turn on COD reactor and preheat to 150°C
2. Using 1 in 10 dilution of filtered plant part stock solutions, slowly pipette 2ml into a COD reagent vial as this gets hot
3. Hold by the cap and shake gently to mix contents
4. Heat in reactor for 2 hours
5. Allow to cool to room temperature
6. Using Hach spectrophotometer, dial in program number for high range test kit (0-1500 ppm) and set to wavelength 620nm
7. Wipe the outside of the vial to remove fingerprints and place in reader with Hach logo facing the front. Zero with blank
8. Readings are in mg/l
9. Save used vials for correct disposal

SOP 11

BOD test on pre or post-digest material

1. Aspirate 5 litres of distilled water for 1 hour
2. Prepare plant part solutions by filtering with 0.2 micron syringe filter.
3. Dilute by one part plant to 9 parts distilled water- minimum 40ml volume so enough for BOD and COD tests
4. Aspirate the distilled water, previously incubated for 24hrs at 20 °C for 1 hr $\pm$ 10mins
5. Prepare dilution media using the aspirated distilled water by adding 5ml of each of the stock reagents
6. Fill 2x250ml BOD bottles with pure dilution media for the blank.
7. Assign 2 bottles for each sample
8. Place 30ml of the pre-digested, filtered and diluted (1in10) plant part solution to a jug, or 20ml of post-digested, filtered, 1in10 solution
9. Add 5ml of seed and make up to 1 litre with dilution media
10. Fill the 2 bottles to the brim from the jug
11. When all are prepared, incubate one set, and measure the dissolved oxygen of the other set with a calibrated meter
12. After 3 days, measure the DO₂ of the other set

Bradford protein assay

The plant parts were crushed in a roller mill for a period of 40 minutes. Samples were taken every 10 minutes and subjected to a Bradford protein test. This was used to assess how effective the milling technique was in crushing cells and releasing the contents.

Table to show the generation of protein from some of the plant parts with length of time in the mill

Plant part	Absorbance at 595nm				
Time milled	0	10	20	30	40
Green leaves	0.377	0.409	0.453	0.458	0.460
Green leaves	0.458	0.491	0.453	0.377	0.409
Bark	0.112	0.118	0.150	0.173	0.180
Bark	0.129	0.173	0.135	0.115	0.118
Twig	0.267	0.267	0.270	0.267	0.221

Some of the variability in the absorbance readings can be explained by the fact than distilled water needed to be added to prevent drying out of sample and mixing may have taken a while to occur. This resulted in a paste which was not homogenous when samples were taken.

This did not appear to be a very accurate test, but this is probably due to the fact that plant material is made up of a very diverse population of cell types.

Results of initial tests leading to decision to dilute the concentration of the buffer used.

Green leaves were used for these tests as this was the most plentiful sample.

BODs and CODs post-digestion

CODs of the universal products were way over range.

BODs were carried out on the products of digest- 27ml in 1l dilution media with 10ml humus effluent as seed.

	Day 0	Day 5
A	7.8	0
B	7.9	0
C	7.9	0
D	7.9	0

CODs were then used to establish dilution needed. Digests were repeated as follows using 1.4996g/l green leaves solution:

A	B	C	D	E
21ml buffer	20ml buffer+ 1ml enz	20mlpp stock + 1ml enz	20 ml of pp stock +1ml enz	20ml of ppstock +1 ml enz

C,D, and E, are replicates

After digesting for 24hrs the products were pushed through a 0.45 μ m syringe filter and dilutions made for COD tests.

	A	B	C	D	E
1in10	1596	1577	1584	1573	1591
1in50	328	308	290	304	307
1in100	176	181	185	176	182

1 in 10 dilutions are all over range

1 in 50 gives best values but evidently the green leaves sample shows no change when compared with the buffer.

Dry Mass of pre and post-digested green leaves solution

Mass of vessel	7.1820	8.1575	7.3820
Vessel+buffer10ml	6.9106	7.8870	7.1122
Mass of buffer10ml	0.2714	0.2705	0.2698

Mass in 10 ml of green leaves pre digest, filtered, initial concentration of 1.4996mg/l

Mass of vessel	8.7624	8.7568	8.7054
Vessel +10sl	8.5189	8.5297	8.4527
10ml of SL	0.2435	0.2271	0.2527

Therefore there is very little difference in the mass of the plant part solution and the buffer alone.

Postdigest dry weights.-1.4966 g/l stock solution of green leaves. 10ml volume dried

A	B	C	D	E
21ml buffer	20ml buffer + 1ml enz	20mlpp stock + 1ml enz	20 ml of pp stock +1ml enz	20ml of ppstock +1 ml enz
0.2697	0.2660	0.2583	0.2659	0.2614

No difference in dry mass between control (pure buffer) and plant part in buffer.

Possible solution- plant part is swamped by the buffer so difference in the CODs and dry weights is negligible. Change one thing at once and start by reducing concentration of buffer by a factor of 10.

100mM down to 10mM

Fig. Excel spreadsheet to demonstrate the amount of enzyme required for each plant part for digestion of available cellulose completely and their associated incubation times.

	mass(g) /50ml vol	% cellulose	mass cellulose (g)	micromol equivalent	incubation time	for sample size 10ml
green leaves	0.2499	16	0.04	246.81	12.34	2.47
brown leaves	0.2502	21	0.05	324.33	16.22	3.24
root	0.25	35	0.09	540.12	27.01	5.40
bark	0.2501	26	0.07	401.40	20.07	4.01
twig	0.2498	34	0.08	524.27	26.21	5.24
cotton	0.2492	87	0.22	1338.30	66.91	13.38
acorn	0.2497	9	0.02	138.72	6.94	1.39

Cotton has a very high cellulose content so requires the most enzyme. However, this material was not included in the BOD tests or digest procedure.

The percentage cellulose is obtained from the laboratory analysis (see chapter 5).

Excel spreadsheet to show calculation of the BOD result

Blank Diff.	Vol. of Sample	Initial Dissolved oxygen	Final dissolved oxygen	B.O.D.
(ml)	per litre (ml)	(mg/l)	(mg/l)	(mg/l)
0.9	30	8.6	3.7	163.3
0.8	25	8.2	2.3	236.0

Excel spreadsheet containing the calculation of the oxygen demands for each plant part for a gram of material from the pre-digest BOD results.

	mass/50ml (mg)	actual Bod	correction for buffer	corrected BOD	mass in 10ml (mg)	mass in 30ml (mg)	BOD(mg/l) per g	mean BOD
twig	249.8	74.2	51.5	22.7	4.996	14.99	1.515	1.551
twig	249.8	75.3	51.5	23.8	4.996	14.99	1.588	
summer leaves	249.9	130.9	51.5	79.4	4.998	14.99	5.295	5.405
summer leaves	249.9	134.2	51.5	82.7	4.998	14.99	5.516	
mature leaves	250.2	98.5	51.5	47.0	5.004	15.01	3.131	2.655
mature leaves	250.2	84.2	51.5	32.7	5.004	15.01	2.178	
root	250.0	67.6	51.5	16.1	5.000	15.00	1.073	1.517
root	250.0	80.9	51.5	29.4	5.000	15.00	1.960	
bark	250.1	40.9	51.5	-10.6	5.002	15.01	-0.706	
bark	250.1	60.9	51.5	9.4	5.002	15.01	0.626	0.626
acom	249.7	114.2	51.5	62.7	4.994	14.98	4.185	4.409
acom	249.7	120.9	51.5	69.4	4.994	14.98	4.632	

		actual	buffer	corrected	mass (mg)	
summer leaves	15ml/l	80.9	40.9	40	7.497	7497
	25ml/l	96.9	32.9	64	12.495	12495
	30ml/l	134.2	54.2	80	14.994	14994
buffer	30ml/l	44.2				
		54.2 mean		51.5		
		56.1				

The buffer was in 100mM concentration

Excel spreadsheet showing calculation of biological oxygen demand per gram of plant material with a correction for the enzyme and buffer solution after a cellulase digest

	mass/50ml (mg)	actual BOD	buffer + enz	corrected BOD	mass in 10ml (mg)	mass in 20ml(mg)	BOD(mg/l per g	average
twig	249.8	136.6	99.9	36.7	4.996	9.992	3.673	4.639
twig	249.8	155.9	99.9	56.0	4.996	9.992	5.604	
summer leaves	249.9	167.6	99.9	67.7	4.998	9.996	6.773	6.688
summer leaves	249.9	165.9	99.9	66.0	4.998	9.996	6.603	
mature leaves	250.2	154.2	99.9	54.3	5.004	10.008	5.426	5.011
mature leaves	250.2	145.9	99.9	46.0	5.004	10.008	4.596	
root	250.0	149.2	99.9	49.3	5.000	10.000	4.930	5.265
root	250.0	155.9	99.9	56.0	5.000	10.000	5.600	
bark	250.1	134.2	99.9	34.3	5.002	10.004	3.429	3.264
bark	250.1	130.9	99.9	31.0	5.002	10.004	3.099	
acom	249.7	127.6	99.9	27.7	4.994	9.988	2.773	5.692
acom	249.7	185.9	99.9	86.0	4.994	9.988	8.610	
	84.2							
buffer+enzyme	125.9 mean		99.9					
	89.6							

The buffer was in 100mM concentration

Excel spreadsheet for the calculation of chemical oxygen demands from both COD results, pre-and post-digest, with the appropriate correction.

PRE	mass/50ml (mg)	actual COD	correction for buffer	actual COD	mass in 10ml(mg)	mass used(mg)	COD per gram	mean
twig	249.8	216	161	55	4.996	0.999	54.74	58.75
twig	249.8	224	161	63	4.996	0.999	62.75	
summer leaves	249.9	287	161	126	4.998	1.000	125.75	123.25
summer leaves	249.9	282	161	121	4.998	1.000	120.75	
mature leaves	250.2	242	161	81	5.004	1.001	80.64	103.62
mature leaves	250.2	288	161	127	5.004	1.001	126.60	
root	250.0	356	161	195	5.000	1.000	194.70	127.20
root	250.0	221	161	60	5.000	1.000	59.70	
bark	250.1	192	161	31	5.002	1.000	30.69	36.19
bark	250.1	203	161	42	5.002	1.000	41.68	
acom	249.7	295	161	134	4.994	0.999	133.86	137.36
acom	249.7	302	161	141	4.994	0.999	140.87	

cod		
buffer	172	mean
buffer	148	161.33
buffer	164	

POST	mass/50ml (mg)	actual COD	correction buff+enz	actual COD	mass in 10ml	mass used	COD per gram	mean
twig	249.8	344	308	36	4.996	0.999	35.70	53.71
twig	249.8	380	308	72	4.996	0.999	71.73	
summer leaves	249.9	388	308	80	4.998	1.000	79.70	107.21
summer leaves	249.9	443	308	135	4.998	1.000	134.72	
mature leaves	250.2	425	308	117	5.004	1.001	116.58	108.08
mature leaves	250.2	408	308	100	5.004	1.001	99.59	
root	250.0	374	308	66	5.000	1.000	65.67	73.67
root	250.0	390	308	82	5.000	1.000	81.67	
bark	250.1	385	308	77	5.002	1.000	76.64	78.64
bark	250.1	389	308	81	5.002	1.000	80.64	
acom	249.7	423	308	115	4.994	0.999	114.81	139.34
acom	249.7	472	308	164	4.994	0.999	163.87	
buff+enz	280							
buff+enz	327	mean						
buff+enz	318	308.33						