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DYNAMICS OF FUNGAL GROWTH IN STORED GRAIN

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ABSTRACT

Large and small-scale respirometry apparatus were developed and optimised to measure the respiration of 25 g and 10 kg seed samples stored under a range of environmental conditions, from 0.80 to 0.95 water activity (a_w) and 15 to 35°C in all combinations. Respiration of wheat and barley grains was greater, than that of rapeseed and linseed. Oxygen consumption was linear with time for naturally contaminated wheat grain over the range 0.90-0.95 a_w /15-25°C and 0.80-0.95 a_w /30-35°C, in sterile wheat grain at 0.90 a_w /25°C, and in barley and rapeseed at 0.90 a_w /20°C but was non-linear in wheat grain at 0.80-0.85 a_w /15-25°C, linseed at 0.88 a_w /20°C and in autoclaved, reinoculated wheat grain. Respiration of naturally contaminated wheat grain was determined over the whole range of environmental conditions. Oxygen consumption increased with water activity and temperature. Respiration was comparable whether measured from 25 g or 10 kg samples, allowing the data to be suitable for mathematical modelling. Respiratory quotients (RQ) were generally < 1.0 and closest to 1.0 at 0.95 a_w /20-35°C but at 15°C they exceeded 1.0 and were closest to 1.0 at 0.80 a_w . Respirometry was more sensitive than direct weighing for determining dry matter loss (DML) because fungal biomass was not measured. DML values associated with visible moulding, as calculated by oxygen consumption by wheat and barley, were smaller than those considered acceptable for safe storage. During 7 days storage at 0.85 a_w /25°C and 0.90 a_w /15°C, conditions usually regarded as safe for short term storage, visible moulding and germination loss occurred with, respectively, as little as 0.130% and 0.085% DML. A dose of 10 kGy gamma-radiation destroyed all fungal contamination from wheat grain without affecting percentage germination, although seed vigour and respiration were decreased. Using a new dry spore inoculation method, it was shown that *Eurotium amstelodami* caused more DML in autoclaved than *Penicillium aurantiogriseum* over 28 days at 0.85-0.90 a_w /20°C.

To my family

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NOMENCLATURE

ADFS	-	BBC Master Microcomputer Advanced Disc Filing System
AFB1	-	aflatoxin B ₁
ATP	-	adenosine triphosphate
a _w	-	water activity
BSI	-	British Standards Institute
CFU	-	colony forming units
cv	-	cultivar
db	-	dry basis
DDW	-	distilled deionised water
DFS	-	BBC Master Microcomputer Disk Filing System
DM	-	dry matter
DML	-	dry matter loss
DNA	-	deoxyribonucleic acid
ECU	-	electronic control unit
ELISA	-	enzyme-linked immunosorbent assay
ERH	-	equilibrium relative humidity
Gy	-	Gray, unit of gamma irradiation
GLC	-	gas-liquid chromatography
HPLC	-	high performance liquid chromatography
IAEA-FAO	-	International Atomic Energy Authority-Food and Agriculture Organisation
ISTA	-	International Seed Testing Association
MEA	-	malt extract agar
M90	-	malt extract agar conditioned to 0.90 a _w
McAb	-	monoclonal antibody
NIAB	-	National Institute of Agricultural Botany
nd	-	not determined
STP	-	standard temperature and pressure
PAC	-	phenacridane chloride

ppb	-	parts per billion
rad	-	rad, unit of gamma irradiation
SEM	-	standard error of mean
RQ	-	respiratory quotient
SDDW	-	sterile distilled deionised water
TLC	-	thin layer chromatography
wb	-	wet basis

CHAPTER 1
GENERAL INTRODUCTION

1.1 THE MICROFLORA OF STORED CEREAL GRAIN

Microbial colonisation of grain, predominantly by fungi, starts at anthesis and continues during ripening, harvesting and storage. Fungi colonising grain before harvest have been referred to as 'field' fungi and those growing after harvest as 'storage' fungi (Christensen & Kaufmann, 1969). Representative field fungi include species of *Alternaria*, *Cladosporium* and *Drechslera*, while storage fungi include species of *Aspergillus*, *Eurotium*, *Mucor* and *Penicillium*. Some fungi are considered to be intermediate between the field and storage groups, e.g. *Fusarium* spp. (Pelhate, 1968) which can also be pathogenic to growing cereals in the field. The ecological niches that these fungal taxa occupy are specific to temperate conditions representative of the UK as 'storage' species may be present in the field in tropical crops (Williams & McDonald, 1983). Grain may initially become contaminated with storage fungi before harvest, during harvesting (Flannigan, 1978) or when it is placed in contaminated stores. Their growth is determined by the prevailing environmental conditions, chiefly water availability and temperature, but in stored grain, water is rarely sufficient to support bacterial growth. Water availability in seeds at harvest is determined by the stage of ripening of the crop and on weather conditions, i.e. rain, relative humidity, ambient temperature and wind speed.

Under conducive conditions, fungi may invade grains and metabolise carbohydrates, proteins and lipids. Microbial colonisation may have many harmful consequences including: losses of dry matter, nutritive value, digestibility, seed viability and baking quality; tainting of colour, odour and flavour; heating (occasionally sufficient to ignite the grain); the production of mycotoxins (secondary metabolites which harm livestock and man) by fungi; and the respiratory hazards associated with spore release. In 1983 it was deemed unacceptable in the UK for any feed-stuffs for dairy cows to be contaminated with >10 ppb aflatoxin (Mannon & Johnson, 1985). Stored grain is never completely free of fungi, and both internal and surface fungal growth may occur before it actually becomes visible to the naked eye. However, their growth and deleterious effects can be minimised by manipulating environmental conditions in the

grain store to prevent spore germination, mycelial growth and toxin production.

To assess the potential for fungal growth in a grain bulk it is necessary to accurately monitor environmental conditions. The simplest measure of water in grain is water content (Christensen & Kaufmann, 1969), expressed as a percentage of the wet or dry weight of the grain. Although water content describes the amount of water in a material, it does not indicate its availability to microorganisms nor the potential for biodeterioration. Equilibrium relative humidity (ERH), water activity (a_w) and water potential (Ψ , in units of MPa) provide better measures of the availability of water (Scott, 1957; Griffin, 1981; Table 1.1), but are less easy to determine than water content. ERH is the relative humidity of the intergranular air in equilibrium with water in the grain substrate and Ψ is the sum of osmotic, matric and turgor potentials.

Conventionally, water availability in stored grains is expressed as a_w , on a scale of 0 - 1.0 (where 1.0 = pure water). The relationship between a_w and Ψ is expressed in the equation:

$$\psi = \frac{RT}{V} \ln a_w + p$$

where; R = ideal gas constant, T = temperature (K), V = volume of 1 mole of water, p = atmospheric pressure.

The relationship between water content and a_w can be expressed by a moisture sorption isotherm, which takes the form of a sigmoid curve. Different materials give dissimilar isotherms, for example, oilseeds generally equilibrate to a particular a_w at a lower water content than starchy seeds (Hunt & Pixton, 1974). Varieties of the same crop can also differ in their water sorption characteristics (Henderson, 1977; Pixton & Warburton, 1977). It is thus essential to determine this relationship for each new substrate studied. A hysteresis effect, which can be quite large in some products, is evident, depending

Table 1.1 Relationships between water activity, equilibrium relative humidity, water content and water potential for wheat grain at 25°C

Water Activity	0.60	0.70	0.80	0.90
ERH (%)	60	70	80	90
Water Content (% wet basis) (Pixton, 1982)	13.5	14.5	16.5	20.5
Water Potential (MPa) (Papendick & Mulla, 1986)	70.3	40.1	30.7	14.5

on whether the water content has been reached by adsorption or desorption (Ayerst, 1986). The relationship between a_w and water content also changes with temperature. At constant water content in the range 10-20%, a 10°C temperature rise increases a_w by approximately 0.03, or ERH by 3% (Pixton, 1982).

The temperature of stored grain is determined both by ambient temperatures and also by the amount of respiratory heat produced by the grain and its microflora (Lacey, 1980), which is again determined by water availability. Temperature and a_w together determine which species of fungi can grow and their activity, including the ability to produce mycotoxins. Bacteria require $>0.90 a_w$ for growth while some fungi can grow with as little as $0.65 a_w$ (Griffin, 1981). The majority of fungi colonising grain are either mesophilic, thermotolerant or thermophilic (Magan & Lacey, 1988). Some fungi and actinomycetes require high temperatures e.g. *Aspergillus fumigatus* (12-55°C) and *Thermoactinomyces* spp. (35-65°C), and may be present in grain. These include species that can cause respiratory allergy in humans and livestock. This subject has been reviewed extensively (for example, in Lacey *et al.*, 1991).

1.2 GRAIN DRYING METHODS

Producers and dealers need to store high quality grain in large quantities, especially as a result of recent increases in cereal production (Wilkin & Rowlands, 1988). Water content is a major factor limiting their ability to achieve and maintain high quality standards. Increased mechanisation of harvesting has allowed crops to be harvested at higher water contents than was previously possible, which are too high to allow safe storage of grain without quality loss and the development of storage moulds and insects. A number of recently developed systems have become available allowing grain storage at high water contents (Abel, 1992). These employ the principle of sealing the silo or bin and introducing preservative gases. However, these are not always economically possible because of their high cost and effective drying regimes remain critical to prevent spoilage.

In the UK, wheat and barley grain are usually dried to 14-15% (w.b.) water content before storage (Anon., 1982a) although, to prevent all mould development, barley should be dried to below 13.5% water content (Hill & Lacey, 1983). The EC requires 15% water content or less for grain to be placed in intervention stores and wetter grain is rejected (Smith, 1984a). In Canada, maize is dried to 15-16% (Bereza *et al.*, 1981). Kreyger (1972) has suggested that grain could be stored with 1 to 1.5% higher water than in bulk stores if it were stored in sacks. However, such water contents would still be sufficient to allow slow moulding.

The three main categories of grain driers mainly used in the UK have been classified into the following groups (Nash, 1978):

1.2.1 Low temperature and ambient air in-store drying

Ambient air drying can be the least expensive drying system under optimal operating conditions, and hence it has become the most popular type in the UK (MAFF, 1984). The stored crop is ventilated with air at ambient or near-ambient temperatures to dissipate respiratory heat and water before grain deterioration occurs. Air is usually directed through ducts in the silo or barn floor and then passes upwards through the grain bed. Heat from gas or electrical fan heaters adjacent to the air inlets may be used to dry the incoming air. The amount of heating required is determined by the temperature and relative humidity of the ambient air. Grain dries from the bottom of the bulk upwards while the relative humidity of the moving air equilibrates with the water content of the grain as it moves from drier to wetter areas until it becomes saturated. Consequently grain at the surface can remain damp for a long period after the store is filled during which time it is highly susceptible to fungal spoilage. To optimise drying, air must be retained within the grain bed for as long as possible to allow it to pick up the maximum amount of water while ensuring that the drying front reaches the surface before spoilage occurs (Nellist, 1988).

Computer models have been developed to predict safe drying periods in near-ambient dried grain in the UK (Sharp, 1983; Bowden *et al.*, 1983, Smith & Bailey, 1983 and

Brook, 1987). However, their predictions of spoilage have often been inaccurate (Sanderson *et al.*, 1989), and the models need to be developed further by compiling more information on mould development and quality loss during the drying process. Smith (1984b) used a previously determined formula (Bowden *et al.*, 1983) to relate the appearance of mould to grain temperature and water content culminating in a computer model that successfully calculated the minimum energy required to dry grain but little account was taken of biological processes in the grain bulk.

1.2.2 Batch drying

Discrete batches of grain are ventilated with slightly heated air before placing in store. Radially ventilated bins are perhaps the most popular type of batch drier, and are usually operated at 6-12°C above ambient temperatures. Output rates have been slow until the recent development of fully automated systems which can rotate and thoroughly mix the grain.

1.2.3 Continuous flow drying

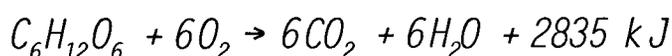
In this process, thin layers of grain are exposed to high temperatures for relatively short periods of time. Grain still warm after continuous flow drying must be allowed to cool, usually aided by intermittent aeration, to prevent moisture migration during subsequent storage. The method can be applied to most crops of virtually any water content, but is expensive in its energy requirement unless high temperatures are used and the crop is dried by only 4-5 %. However, if the grain is heated too much, seed viability and protein quality can be severely affected rendering it unsuitable for both seed and baking (Lupano & Añón, 1987). Its hygroscopic properties may also be affected, increasing its susceptibility to fungal invasion compared to grain dried in the field (Tuite & Foster, 1963). Maximum drier air temperatures have been defined for different crops and uses (Anon., 1982a). To ensure viability is not affected, the upper limit for seed barley dried from 20% to 15% water is 75°C. However, milling wheat can be dried at up to 90°C before bread making qualities are affected (Anon., 1982b) and wheat, barley and

oats for feed can be dried at up to 104°C.

1.3 RESPIRATION AND DRY MATTER LOSS

1.3.1 Patterns of grain respiration

Respiration has been used to measure the deterioration of stored produce for many years (Bailey & Gurjar, 1918). During aerobic respiration of grain, carbohydrates are oxidised to carbon dioxide and water according to the equation:



The intensity of this process is largely governed by water availability and temperature and, to a lesser extent, by oxygen concentration, microbial contamination (Bailey, 1940), mechanical damage (Steele *et al.*, 1969), the conditions and length of previous storage (Milner & Geddes, 1945b) and the degree of mite and insect infestation. However, insect infestation is generally of minor importance in temperate climates (Nash, 1978).

It is well known that microorganisms, especially fungi, play a major role in the deterioration of stored grain (Norman *et al.*, 1941; Christensen, 1955). However, the grain itself is also living and respire in a similar way to the microorganisms it supports (Hummel *et al.*, 1954). The relative contributions of these two components to total respiration remains controversial (Pomeranz, 1974). In some studies with maize at high water contents (22-27%), respiration by the grain itself was considerably greater than that of its surface mycoflora (Seitz *et al.*, 1982b). Conversely, other workers recorded relatively low and constant levels of respiration from mould-free wheat grain at 12-35% water content (Larmour *et al.*, 1935; Hummel *et al.*, 1954) although the sterilants used (sodium hypochlorite, carbon tetrachloride) could have affected physiological processes in the seed. Sterilising maize grain with combinations of sodium hypochlorite, phenacridane chloride (PAC), and gamma-irradiation (Woodstock & Combs, 1965), eliminated fungi from up to 80% of seeds. Respiration was decreased by up to 10%

and, although some viable fungi remained after these treatments, it was assumed that the seed-borne fungi probably contributed about 10% of the total respiration observed.

Production of CO₂ or consumption of O₂ by stored cereal grains has often been recorded (Milner *et al.*, 1947a, b; Scholz, 1962 and White *et al.*, 1982a, b) and some of the published data on respiration of naturally-contaminated (i.e. untreated) wheat grain at a range of a_w and temperatures are presented in Figure 1.1. The data therein is derived from a wide range of wheat grain varieties, apparatus and sampling intervals. An example of published data on wheat grain respiration *outside* the range of Figure 1.1 is that from Kittock & Law (1968) who recorded 75.35 l O₂/kg dry grain at 0.95 a_w and 20 - 25°C over 7 days. Girish (1970) also reviews some of the published data in this area. Attempts have also been made to fit mathematical formulae to grain respiration:

a)

$$y = ae^{bW} + c$$

where: y = CO₂ respired (mg CO₂ 24 h⁻¹ 100 g dry grain⁻¹); a, b = constants; c = constant (0.4926); W = water content (%) (Bailey, 1940).

b)

$$\alpha = \frac{Ke^{aT}}{0.107}$$

where: α = CO₂ respired (mg kg dry grain⁻¹ 24 h⁻¹); K = constant determined by water content; a = constant (0.1385 for cereals); T = temperature (°C) (Srour, 1988).

c)

$$\text{Log}_{10}\alpha = -4.054 + 0.0406T - 0.0165\theta + 0.0001(\theta^2) + 0.2389W$$

where: α = CO₂ respired (mg kg dry grain⁻¹ 24 h⁻¹); T = temperature (°C); θ = time in storage (d); W = water content (%) (White *et al.*, 1982a).

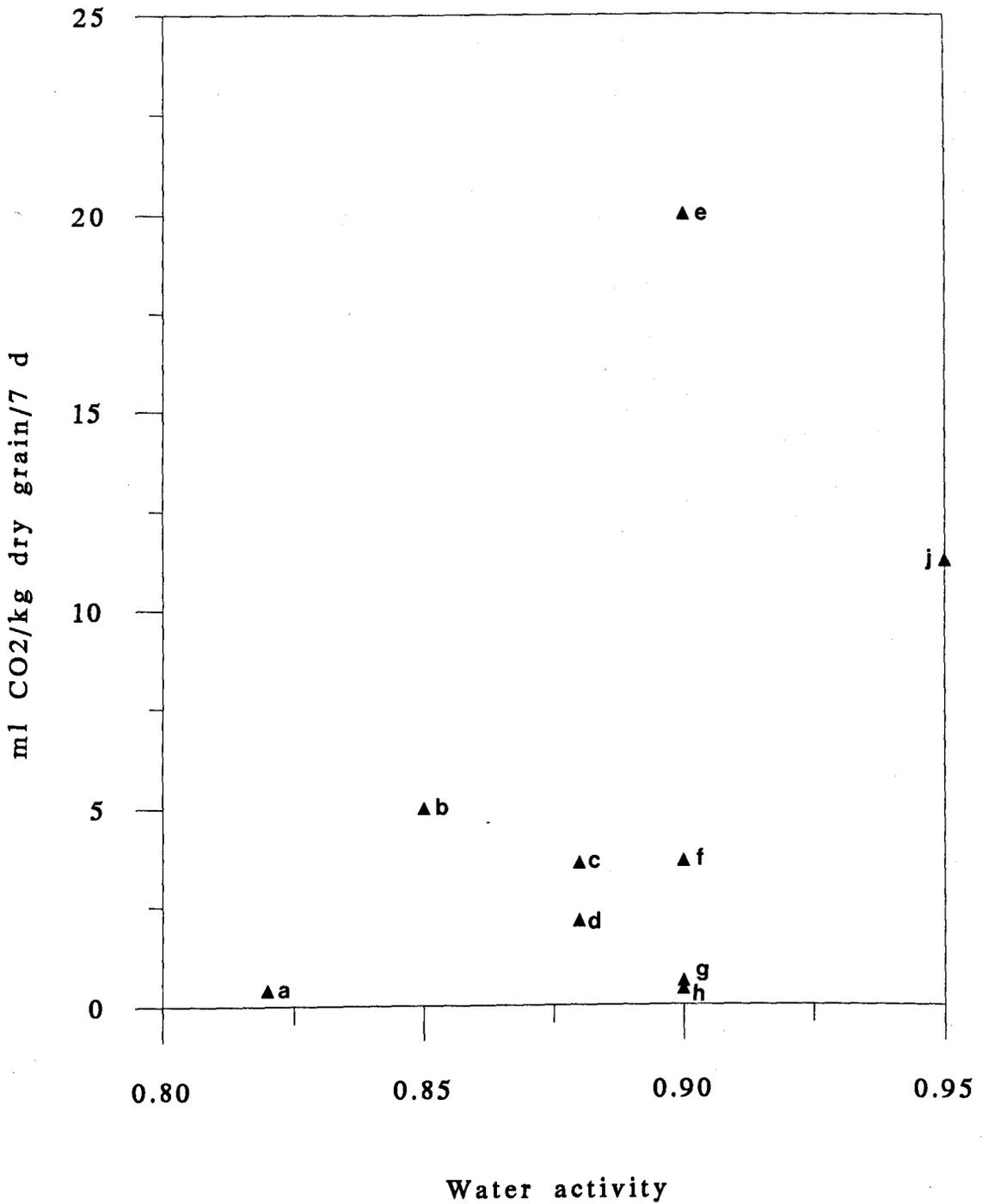


Figure 1.1 Published data on the respiration of wheat grain. Data points represent the following published findings: a) Bailey (1940); b), e) Scholz (1962); c), f) Milner *et al.* (1947a); d) Larmour *et al.* (1935); g), h) White *et al.* (1982a); j) Woodstock & Justice (1967).

The large differences between results of different respiration studies (Figure 1.1) may result, in part, from the use of grain of different varieties (Cantone *et al.*, 1983), ages (Kittock & Law, 1967), sizes and qualities or from the use of diverse methods to determine respiration. Also, during even the shortest periods between experiments the microflora may change making interpretation of the results of different experiments and methods difficult. The numbers and types of fungi may differ with origin of the grain, with more fungal taxa isolated from grain grown and stored in warmer climates than from that in temperate regions. It has been shown that different fungi contribute in different ways to the colonisation, nutrient utilisation and respiration of grain (Kaspersson, 1986) and other substrates like silage (Muck *et al.*, 1991) so that the history of the grain used must always be considered before comparing experiments. Grain for experiments should be as fresh, dry and clean as possible.

1.3.2 Methods used to measure respiration

Respired CO₂ can be absorbed in pre-weighed ascarite (Steele *et al.*, 1969) or alkaline materials, e.g. soda lime (Kaspersson, 1986) or hydroxides of barium (Larmour *et al.*, 1935), sodium (Tribe & Maynard, 1989) or potassium (Fernandez *et al.*, 1985). CO₂ is then quantified by titration of the alkaline material to neutrality with acids. Alternatively, gas chromatography (White *et al.*, 1982a) or infrared gas analyzers (Kaspersson, 1986) can be used. Methods of CO₂ and O₂ detection have been devised which exploited changes in the conductivity of heated wires or in alkaline solutions on exposure to different gas concentrations (Leach, 1932).

When sampling of respiratory gases is discontinuous, it is important that the sampling interval is short enough to prevent gases accumulating sufficiently to inhibit respiration. Larmour *et al.* (1935) showed that more CO₂ was produced in grain aerated continuously than in that aerated intermittently. Similarly, decreasing the sampling frequency of intergranular gases in sealed experiments from daily to every four days decreased the volume of CO₂ produced (Bailey, 1940).

O₂ uptake can be measured using Warburg-type respirometer systems, gas

chromatography (Kaspersson, 1986; White *et al.*, 1982a) or paramagnetic detection apparatus (Paster *et al.*, 1990). Sometimes sealed respirometer units have been used in which the O₂ used has not been replaced and CO₂ can accumulate to inhibitory levels (Pomeranz, 1974) while other methods have been destructive of the whole sample, prohibiting continuous monitoring. A respirometer designed by Milner and Geddes (1945a) overcame these problems by the controlled aeration of grain.

Recently an innovative system was designed for monitoring respiration of soil, consisting of an electrolysis cell containing CuSO₄ (Tribe & Maynard, 1989). O₂ uptake by the sample together with absorption of respired CO₂ in the NaOH solution, caused pressures to drop and the electrolyte level to rise to make contact with the electrodes. The current flowing generated oxygen at the anode, until pressure was equalised, contact at the anode was broken and current ceased to flow. The cycle repeated as long as respiration continued. The periods of operation of the cell were recorded electronically and converted into volumes of O₂ produced.

Insulated Dewar flasks of up to 4.5 l capacity have been widely used to measure respiratory heating of grain, using thermometers, thermistors or thermocouples to measure temperature change (Festenstein *et al.*, 1965; Lacey, 1980; Hill & Lacey, 1983). Hill and Lacey (1983) studied the spontaneous heating and changes in fungal colonisation of barley grain stored in polyethylene bags, Dewar flasks, fibre drums and plastic bins. As Dewar flasks may differ in their insulating properties (Carter & Young, 1950), adiabatic incubation has been used to minimise heat loss to the atmosphere (Norman *et al.*, 1941; Currie & Festenstein, 1971). Some researchers have used grass or silage as substrates for the study of microbial thermogenesis. Such substrates have a high surface to mass ratio, and decompose at a faster rate than grain. However, grain has a larger nutrient reserve, allowing a longer period for microbial colonisation and decomposition (Lacey, 1980). Microcalorimetry has been used to measure small changes in heat output of the soil biomass (Sparling, 1981) and could also have possible applications in studies of grain fungi.

1.3.3 Respiratory quotients and dry matter loss

Respiration converts carbohydrate to CO₂, water and energy resulting in dry matter loss (DML). DML can be measured as decreases in the dry weight of a standard mass, volume or number of grains (Reed, 1987) or by accurate measurements of O₂ uptake, CO₂ evolution or energy release. The complete aerobic oxidation of carbohydrates, as described in the respiration equation, gives a respiratory quotient (RQ; CO₂ evolved: O₂ absorbed) of 1.0. The stoichiometry of the respiration equation allows the mass ratio between glucose utilised and CO₂ evolved to be calculated:

$$\frac{\text{molecular weight } C_6H_{12}O_6}{\text{molecular weight } 6CO_2} = \frac{180}{264} = 0.682$$

From this equation, the weight of dry matter (DM) utilised can be estimated by multiplying the weight of CO₂ are produced by 0.682 (Rees, 1982). Similarly, when equivalent masses of O₂ are utilised and CO₂ produced in cereal grains, 14.7 g CO₂ is evolved per kg of substrate for every 1% DML (Steele *et al.*, 1969). Assuming a linear relationship between respiration and time, predictions can thus be made of the time taken for grain to attain a given loss in DM under known conditions.

RQs can also show whether anaerobic activity or lipid or protein metabolism contribute to respiration. During anaerobic fermentation only 0.493 g CO₂ is evolved for every 1% DML, resulting in a RQ >1.0 (White *et al.*, 1982a), while lipid and protein metabolism by some grain fungi results in a RQ <1.0. Tripalmitin metabolism, for example, gives an RQ of 0.7 (Pomeranz, 1974). However, Schmidt & Jacobsen (1982) monitored O₂ and CO₂ in 56 experiments with stored rapeseed, and they recorded a maximum RQ of 7.4 and a minimum of 0.30.

Dry wheat grain is composed of approximately 80% carbohydrate, 16% protein and 4% oil while dry rapeseed contains approximately 34% carbohydrate, 23% protein and 43% oil (White *et al.*, 1982b). However, in experiments with cereal grains, metabolism of

lipids and oils has so far been neglected (Saul & Lind, 1958; Steele *et al.*, 1969; White *et al.*, 1982a) since carbohydrate is the predominant constituent. With rapeseed the large lipid content gives an RQ < 1.0 and makes CO₂ measurement impracticable for assessing DML unless a conversion factor can be used (White *et al.*, 1982b).

DML in stored grain comes from two sources; grain metabolism and microbial activity. Acceptable levels of DML during storage may depend on which route is responsible. For example, slow loss through low respiratory activity by grain tissue over long periods of storage may affect seed quality less than rapid loss caused by sporulating, metabolite-producing fungi even though the DML is equal.

1.3.4 Acceptable levels of dry matter loss during storage

a) **Maize:** In maize grain, CO₂ evolution was closely correlated with the level of fungal contamination and only 0.5% DML was sufficient to render the grain unfit for use (Saul & Lind, 1958; Saul & Steele, 1966). This was later confirmed by Seitz *et al.* (1982a) who showed that such a loss could occur before moulding was visible. According to Steele *et al.* (1969), DML and quality in aerated shelled maize are functions of kernel damage, temperature and water content which can be expressed in the simple formula:

$$\theta = \theta_R \times M_T \times M_W \times M_D$$

where: θ = estimated allowable storage time before 0.5% DML; θ_R = time taken to 0.5% DML at 25% moisture content, 15.5°C and 30% mechanical damage; M_T , M_W , M_D = constants relating to temperature, water content and mechanical damage, obtained from graphs.

Respiration losses predicted using the formula of Steele *et al.* (1969), based on the earlier work of Saul & Steele (1966) were compared with experimental data (Fernandez *et al.*, 1985). Depending on water content, differences of 2-30% between predicted CO₂ production and experimental data were found. Respiration in grain stored at a

range of temperatures before measurement differed from that of unstored grain because different fungi developed. To eliminate these differences, freezing at -10°C was recommended to maintain microbial populations closest to that of freshly harvested grain, and prevent fungal growth prior to experiments.

b) *Wheat and Barley:* Acceptable DMLs depend on the proposed use of the seed, with stricter standards applied to grain for seed than to that for baking. Current predictions of safe storage times for cereals are based almost entirely on the work of Kreyger (1972). Kreyger's predictions are still used in many publications to determine safe storage periods for stored cereals (e.g. Anon, 1982a; Anon, 1987) although other studies have suggested that these storage periods are excessive and a more precise definition is still required.

Kreyger's (1972) predictions relied on the appearance of visible moulding, but this can be unreliable as an indicator of unacceptable spoilage (Seitz *et al.* 1982a; Armitage & George, 1986). Data from several sources, including those of Scholz (1962), were extrapolated to predict safe storage times for different seeds, including cereals. Up to 2% DML and decreased germinability were accepted as it was assumed that the grain would be used as animal feed. Subsequent research has suggested that acceptable storage losses may be much smaller than those verified by Kreyger (1972).

Hall & Dean (1978) used data published by Steele *et al.* (1969) and appropriate water content/ERH/temperature relationships (Morey *et al.*, 1981) to predict safe storage periods for cereals and to produce a model for safe storage of wheat. They assumed that up to 1% DML over 12 months was acceptable (Hall & Dean, 1978) and that DML in wheat and maize at the same a_w and temperatures would be similar (Morey *et al.*, 1981). However, Kreyger (1972) stated that wheat could be stored without loss of germination more easily than maize and 0.5% (half of 1.0%) thus formed an acceptable, if not conservative DML. Additionally, Morey *et al.* (1981) had concluded that aerated wheat grain could be safely stored for twice as long as Kreyger (1972) had predicted for sealed storage.

The intergranular gas composition of wheat grain stored under a range of environmental conditions in experimental silos was monitored (White *et al.*, 1982a) and the data were fitted to equations to predict storage times allowing 0.1% DML. However, although it was predicted that wheat at 18.4% water content could be stored safely for 55 days, visible moulding appeared after 23 days, suggesting that only 0.04% DML was the limit for acceptability. Unless the methods used were inaccurate or RQs differed significantly from 1.0 (making the assumption of its equalling 1.0 inaccurate), it appears that only very small DMLs can be accepted in wheat grain. Error could have been introduced into these predictions by the non-uniform method in which CO₂ was sampled on only three days a week at 10°C and 20°C and daily at 30°C and 40°C (White *et al.*, 1982a). Brook (1987) considered that the equation used by Morey *et al.* (1981) was a better method of prediction than that used by White *et al.* (1982a).

A model of fungal growth on barley, under constant storage conditions, was described by Bowden *et al.* (1983) which related the period of time for visible mould to appear, temperature (5-25°C) and water content (16-26%). Visible moulding was successfully predicted but calculations of germination damage were unsatisfactory. Further studies are needed on the effects of the early stages of moulding on seed germination.

Most studies have neglected the effects of differing kernel size between different cereals. This affects not only the surface area available for gas exchange and fungal colonisation but also the ratio of pericarp to endosperm (i.e. the available substrate per unit weight). Bailey (1940) suggested that respiration rate may be proportional to kernel size. Brook (1987) then adapted Kreyger's (1972) predictions for wheat in near-ambient grain driers to compensate for this, and calculated that 0.5% DML in maize was equivalent to 0.085% DML in wheat. Unfortunately, measurement of such small DML directly by weighing is prone to errors because the weight of fungal mycelium on the grain should be subtracted from the total DM.

c) **Oilseeds:** The respiration of stored rapeseed was related to a_w (Schmidt & Jacobsen, 1982) and a linear relationship was found between the log of respiration rate

divided by temperature against water activity. At 20°C and 0.90 a_w , 4.12 g O₂ were consumed kg dry grain⁻¹ 7 d⁻¹. Magan (1991) showed that at 25°C and 0.95 a_w that 70.58 ml O₂ kg grain⁻¹ were consumed over 7 d which related to a dry matter loss, separately determined, of 0.55%. Nellist (1993) showed that when modelled similarly, the data of Magan (1991) and Schmidt & Jacobsen (1982) were in close agreement and the times for safe storage with only 0.1% DML were less than predicted by Kreyger (1972).

1.3.5 Inhibition of respiration by carbon dioxide accumulation

If gaseous diffusion within the grain bulk is restricted, respired CO₂ may accumulate sufficiently to inhibit first insect activity and then aerobic respiration of microorganisms, although still permitting anaerobic fermentation. Where water contents are high, anaerobic bacteria may grow (Shejbal & Boislambert, 1988) outcompeting the few anaerobic or micro-aerophilic filamentous fungi and yeasts present (Christensen & Kaufmann, 1974). The absence of O₂ in the intergranular atmosphere affects the type of respiration more than increases in CO₂ concentration (Nash, 1978; Shejbal & Boislambert, 1988). Usually CO₂ concentrations seem to stabilise at between 15 and 20% in sealed silos, although the initial concentration may be greater (Hyde, 1974). Srour (1988) considered 10% CO₂ sufficient to inhibit respiration during airtight storage of grain.

Increased CO₂ and decreased O₂ concentrations generally slow the rate of fungal spore germination but some fungi may grow faster at concentrations of up to 5-10% CO₂ (Magan & Lacey, 1984b). Although the effects of CO₂ concentration have usually been tested in the presence of 20% O₂, and of O₂ concentrations in the near absence of CO₂; in practice O₂ concentrations decrease as CO₂ concentrations increase and act synergistically on fungal growth. In addition, gas composition may act synergistically with a_w and temperature in their effects on fungal growth.

1.3.6 Moisture migration and hot spots

Moisture migration can be induced by hot spots or by seasonal or diurnal fluctuations

in ambient temperature which cause water to be moved by convection or down a temperature gradient to cooler areas where intergranular humidity is increased and eventually water condenses making grain able to support fungal growth (Burrell, 1974). Grain is a poor conductor of heat (Sinha, 1973; Pomeranz, 1974) and changes in ambient temperature chiefly affect grains adjacent to silo walls, causing the movement of water from the sun-warmed side to the opposite side. Similar effects occur during the shipping of grain across different climatic zones. Cargoes "sweat" as ships enter cooler waters, intergranular air is cooled below dew point and water condenses on the vessel walls and peripheral grain (Milton & Pawsey, 1988). Moving from cool to warm climates, the peripheral grain warms and dries so water then condenses on the cooler grain within.

Convection currents are formed in grain silos in response to temperature differences between the inner and outermost grain (Muir, 1973). Currents rise at the sides of the silo when these are warmer, perhaps resulting in condensation at the base. Alternatively, currents may rise in the centre when the sides are cooler, condensing at the top, which may be marked by sprouted grains. Hot spots also cause convection carrying respiratory heat and water upwards through intergranular air (Sinha, 1973).

Hot spots are localised areas of high temperature (Bailey & Gurjar, 1918) resulting from the release of heat energy from the respiration of fungi, insects and mites. Such activity increases with water content, stimulating respiration and producing more water, which may be moved to other parts of the bulk by convection and diffusion, thus initiating a cycle of further microbial growth, respiration and heating. Temperatures up to 70°C may result and eventually spoil the entire bulk. Hot spots are initiated within grain bulks when localised zones of high a_w or poor quality material provide foci for spontaneous heating. Grain handling practices, for example loading by auger, can often cause dust and broken kernels to accumulate in discrete zones in the grain bulk, encouraging the development of hot spots (Milton & Pawsey, 1988).

Hot spots can be detected by regular monitoring of relative humidity, airflow, and

especially temperature at many points within the grain bulk during storage (Bereza *et al.*, 1981). Detection of an area of high temperature or humidity then requires rapid action to prevent further damage. Ventilation with cool air removes heat, water and CO₂ and buffers temperature changes thus increasing the period over which grain may be safely stored (Smith & Bailey, 1983). However, without care, aeration can dissipate water only as far as the cooler surface layers of the grain bulk where resulting condensation may lead to sprouting and clumping due to mould growth which inhibits air movement and exacerbates the risk of further heating.

Superficial fungal material may be removed by redrying mouldy grain in a batch or continuous flow drier. This improves grain cosmetically, but any mycelium or mycotoxins within the grains may remain undetected (Seitz *et al.*, 1982a) and the airborne spores released may present health hazards to farm workers and livestock (Lacey, 1975). These problems are almost inevitable while grain is traded on a wet weight basis. This encourages traders to sell grain with as large a water content as possible, whereas dealing with materials on a dry matter basis could prevent spoilage problems.

1.4 QUANTIFICATION OF FUNGAL BIOMASS IN STORED GRAIN

1.4.1 Traditional methods

Microorganisms in grain have traditionally been quantified by direct plating of whole grains or by plating diluted washings or suspensions of comminuted grain onto agar media which can then be incubated under a range of environmental conditions. Such plating methods favour the isolation of heavily sporulating and fast growing fungi and provide, at best, semi-quantitative information on the species contaminating the grain (Jarvis *et al.*, 1983) and comparison of different samples (Lacey *et al.*, 1980) since propagule numbers, not biomass, are being measured. Comparison of a range of culture methods to examine the microflora of high-moisture barley from partially sealed silos showed that dilution plating was unsuitable for the detection of slimy and sticky spored fungi since the spores remained attached to the grain surface, but was

satisfactory for dry spores and for grains with a polished, smooth pericarp, such as maize (Mulinge & Chesters, 1970).

Alternative methods for quantifying fungal biomass have been described, based on measurement of respiration (Section 1.3), analysis of biochemical markers or immunoassay. Biochemical assays to identify fungal materials in grain have been developed over the last 15 years but, like respiration do not allow the contributions of different species to be distinguished. Immunoassays are more specific and may allow distinction to genus or species level, depending on the specificity of the antibody. Future biochemical and immunological research should disclose more specific compounds, markers (Clarke *et al.*, 1986) or metabolic products (Magan & Lacey, 1989; Jain *et al.*, 1991; Magan *et al.*, 1993), that could enable rapid quantification of total fungi or particular taxa. Techniques for determining the species composition of the grain microflora are generally more time-consuming than rapid methods that do not allow species identification. The use of complementary methods, dictated by the objective of the study, are often useful (Cahagnier & Poisson, 1988).

1.4.2 Biochemical assays

Biochemical assays that have been utilised to study moulding of grain include chitin and ergosterol analysis and assay of adenosine triphosphate (ATP) and extracellular enzymes. Whipps *et al.* (1982) has described the range of compounds that have been assayed as markers of fungal growth in plant tissues and concluded that chitin has the widest application.

a) Chitin: Chitin is a polymer of β -1,4 N acetyl glucosamine and is a constituent of the cell walls of many fungi. It has been used to assess invasion of corn and soybean seeds by moulds (Donald & Mirocha, 1977). During analysis, chitin is hydrolysed to glucosamine, then deaminated to an aldehyde which is measured colorimetrically (Ride & Drysdale, 1972). However, quantification of fungal growth in diseased plant tissues by chromatography of the glucosamine is limited by the low chitin contents of some pathogenic fungi (Stahmann *et al.*, 1975). The chief disadvantages of the chitin assay

are firstly, that chitin is a major cuticular component of grain storage insects, secondly, glucosamine comprises a large part of the bacterial cell wall and thirdly, the glucosamine content of fungi, e.g. *Aspergillus oryzae*, can change with colony age (Sakurai *et al.*, 1977). Thus, the relationship between chitin content and fungal biomass in grain may be difficult to determine. Jarvis *et al.* (1983) commented that "where non-homogeneity of contamination occurs the variance of the results is such as to render the method (i.e. chitin assay) of little value".

b) Ergosterol: Ergosterol is the major sterol in all fungal membranes (Deacon, 1984) but is absent from most higher plants (Seitz *et al.*, 1979). It may be analysed by HPLC (Seitz *et al.*, 1977) or TLC (Naewbanij *et al.*, 1984) but the apparatus required is more expensive and complex than that for assaying chitin. However, it takes less time than the chitin assay and detection of non-fungal material is unlikely. Both viable and non-viable mycelia are detected enabling the total biomass to be quantified. 1 g dry wheat grain has been found to contain between 2 - 5 μg ergosterol (Cahagnier *et al.*, 1991; Tothill *et al.*, 1992). Ergosterol assay has been preferred to the chitin assay in wheat (Kaspersson, 1986), barley (Seitz *et al.*, 1977), rice (Cahagnier *et al.*, 1983) and maize grains (Seitz *et al.*, 1979). However, ergosterol content, like chitin content changes during growth and with fungal species (Marfleet *et al.*, 1991). Ergosterol may be a more sensitive and accurate measure of the *initiation* of mycelial growth on substrates like grain than traditional dilution plating techniques (Tothill *et al.*, 1992).

c) Adenosine triphosphate (ATP): ATP is present in all living cells but its activity can be affected by environmental conditions, e.g. low a_w . ATP is assayed by measuring the bioluminescence produced during the oxidation of luciferin in the presence of luciferase and ATP. Kaspersson (1986) used the ATP assay to measure biomass during colony growth in artificial media and in grain. ATP activity increased during exponential growth but declined to zero once growth ceased because of its presence only in metabolically active cells (Kaspersson, 1986). Living grain is also metabolically active, although generally quiescent during storage, with low ATP content. If the assay is to detect only fungal biomass, ATP in grain tissues must not

be extracted, thus preventing the quantification of biomass from mycelia within the grain. Efficient extraction must also release ATP quantitatively from fungi and all enzyme activity in the extract must be inactivated. Kaspersson also found that the ATP content of grain correlated with dilution plate counts of microorganisms, although the correlation with colony forming units (CFU) may not be ideal because it favours heavily sporulating fungi and failure to assay for internal mycelium.

d) Extracellular Enzymes Fungi produce extracellular enzymes to solubilise nutrients and the identification and quantification of those specific for fungal colonisation could be useful for detection and assay. Of a range of enzymes assayed, N-acetyl- β -D-glucosaminidase and α -D-galactosidase were most indicative of fungal colonisation of grain under some a_w conditions when compared to control unmoulded grain and could form the basis of a rapid test (Jain *et al.*, 1991).

e) Fungal Volatiles A number of studies have chemically analysed and quantified the dominant fungal volatiles in moulding grain, characterised by off-odours. 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone have been found to be the volatiles most commonly produced by post-harvest spoilage fungi e.g. *Aspergillus* and *Penicillium* spp. in *in vitro* studies and in damp stored grain (Abramson *et al.*, 1980). Others are characteristic of mite infestation (Tuma *et al.*, 1990; Curtis *et al.*, 1981). Studies have indicated that monitoring of such volatiles may detect and quantify the initiation of fungal activity in stored grain (Sinha *et al.*, 1988; Tuma *et al.*, 1990). Recently, Borjesson *et al.* (1990) compared the production of fungal volatiles by *P. aurantiogriseum* with ergosterol production and total CFU on oat grain. Volatiles were first produced within 2 d of the start of the experiment and were highly correlated with CO₂ production and moderately correlated with ergosterol contents. This and subsequent studies (Borjesson *et al.*, 1992) showed that there was little, if no, correlation with total numbers of CFU and that differences in the production of volatile metabolites depended more on fungal species rather than on grain type. Tuma *et al.* (1990) suggested that the detection of odour volatiles may serve as a useful bioindicator for the prevention of hot spots in stored grain.

1.4.3 Immunoassay

Warnock (1971, 1973) emphasised the shortcomings of culture methods by comparing them with fluorescent antibody techniques that specifically detected mycelia of *Alternaria*, *Aspergillus* or *Penicillium* species in barley grains. Culture methods often underestimated *Alternaria* species and suggested that *Aspergillus* and *Penicillium* predominated. Immunofluorescence assay showed all three genera to be present in all samples, with mycelium of *Aspergillus* and *Penicillium* species comprising a smaller proportion of the total fungal biomass than predicted by isolation in culture.

Monoclonal antibodies have been raised to *Penicillium islandicum* and three immunoassays using monoclonal antibodies have been used to detect and quantify *Humicola lanuginosa* in rice (Dewey *et al.*, 1989). The monoclonal antibodies were highly specific and allowed potentially indefinite production of identical preparations. This contrasts with polyclonal antibodies which may cross-react with other fungi and host plant tissues. Immunological detection of fungi in plant tissues was reviewed by Clarke *et al.* (1986), who described the development of polyclonal and monoclonal antibodies to *Penicillium viridicatum*, a mycotoxin-producing species common in stored grain.

1.5 GRAIN STERILISATION

Sterile grain inoculated with defined populations of fungi and bacteria can be used to study specific interactions, the effects of different storage conditions and effects on seed quality more clearly than the study of mixed populations on natural grain. Surface sterilisation of maize, wheat and barley seeds have been compared by Cuero *et al.* (1986), Niles (1978), Ramakrishna (1990) and Ramakrishna *et al.* (1991). The methods used included heat, chemicals and gamma-irradiation, which are discussed below. Interactions of fungi with bacteria, yeasts and filamentous fungi have been studied on irradiation-sterilised maize (Cuero *et al.*, 1987b; Ramakrishna, 1990 and Ramakrishna *et al.*, 1991) and autoclaved wheat grains (Magan & Lacey, 1985).

1.5.1 Heat

Autoclaving with high pressure steam at 121°C for 25-30 min has often been used to sterilise seeds (Magan *et al.*, 1984; Magan & Lacey, 1985; Smith *et al.*, 1987; Dewey *et al.*, 1989). However, autoclaving not only kills all seed-borne spores and mycelium but it also softens the pericarp, changes the structure of seed storage proteins, and kills the grain. These changes allow easier penetration of fungal hyphae and alter the availability of nutrients. Additionally, the contribution of grain respiration to total respiration cannot be measured.

1.5.2 Chemicals

a) **Fumigants** Fumigants used with grain include methyl bromide, phosphine, propylene oxide and ethylene oxide. These have mainly been used to kill insects and mites, but at high dose rates some may also kill microorganisms (Poisson & Cahagnier, 1988). The use of methyl bromide to treat foods with high lipid contents (e.g. nuts) is problematic as it is likely that residues of the chemical will persist. Also, the chemical is mutagenic and ozone depleting (Buffin, 1992) and all its uses, including grain post-harvest treatment and soil fumigation are being phased out.

b) **Surface sterilants** Early studies of respiration used sodium hypochlorite (NaOCl) (Larmour *et al.*, 1935) and carbon tetrachloride (CCl₄) (Hummel *et al.*, 1954) for surface sterilisation. However, NaOCl may decrease seed respiration by oxidising carbohydrate reserves in the seed and phenacridane chloride may be a more efficient sterilant (Woodstock & Combs, 1965). Surface sterilisation of wheat seeds with sodium hypochlorite, silver nitrate (AgNO₃), mercuric chloride (HgCl₂), and ethanol (C₂H₅OH) were compared by Speakman & Kruger (1983) who found that soaking seeds for 20 h in 10 ppm terramycin solution followed by 10 min in 0.1% AgNO₃ solution gave the best surface sterilisation without diminishing seed germination or subsequent development. NaOCl solutions may differ in their effectiveness, depending on pH but the method has been optimised by Sauer & Burroughs (1986). Solutions of 1-5% NaOCl completely killed spores of *Aspergillus* spp. in aqueous suspension but when grain was inoculated with *Aspergillus flavus* and *Eurotium* spp. (as *Aspergillus glaucus*),

air bubbles, cracks and debris prevented NaOCl wetting the entire grain surface. Decreasing the pH to 8.0 and rinsing seeds with ethanol before NaOCl treatment solved this problem. NaOCl was also found to give the best surface sterilisation by Ramakrishna *et al.* (1991) as changes to the seed were minimal. However, NaOCl does not penetrate the seed, leaving internal contamination viable. Some chemicals are selective in their action on grain fungi. For example, *A. flavus* spores were not completely killed by treatment with HgCl₂ and the use of NaOCl followed by HgCl₂ might be effective in controlling the wide range of fungi on the seed surface although a reduction in germination may occur (Ramakrishna *et al.*, 1991).

1.5.3 Gamma-irradiation

a) Effect on microorganisms Gamma-irradiation has long been used as a preservation method for food intended for human consumption (IAEA-FAO, 1978) but has been only slowly accepted, with doses of up to 10 kGy, equivalent to 1000 rad feasible (Board, 1983), and is not yet accepted in the UK (Goodburn, 1987). Irradiation splits water molecules into free radicals which damage DNA and are highly toxic to a wide range of organisms (Table 1.2). Hence, samples with a high water content will produce more free radicals and suffer more damage (Kiss & Farkas, 1977). Irradiation of grain to remove insect infestations was investigated by Freeman (1973) but costs were considered too high for large scale use.

The effects of up to 18 kGy on specific microbiological contamination of maize, rice, and barley grains (Cuero *et al.*, 1986, 1987a, 1987b; Ramakrishna *et al.*, 1991) and cereal meals (Hanis *et al.*, 1988) have been studied. Organisms differed in their sensitivity to gamma irradiation. On maize, *Penicillium* spp. and *Aspergillus* spp. were killed by doses of 0.3 kGy and 1.2 kGy respectively while 12 kGy was required to kill all microorganisms without loss of germination. This dose was used by Cuero *et al.* (1986, 1987a, 1987b, 1988) and Ramakrishna (1990) in their subsequent studies. Microbiologically, organisms were more susceptible at 22% water content than at 15% water content while dematiaceous fungi were more resistant than moniliaceous species when tested in aqueous suspension and on enriched agar (Saleh *et al.*, 1988). However,

smaller doses were required to kill fungi on grain than on agar (Table 1.2; Cuero *et al.*, 1986; Saleh *et al.*, 1988) perhaps because of differences in vigour and resilience between fungal isolates from different sources and between the water contents of the substrates. The growth of *Aspergillus flavus* on wheat grain was greater on grain treated with 40 kGy compared with grain that had been autoclaved (Niles, 1978).

b) Effect on grain respiration and seed vigour Seed vigour of wheat and barley and their sensitivity to ionising irradiation are largely dependent on the integrity of cell membranes in the seed with both characteristics (seed vigour was measured by emergence in field trials) negatively correlated (Sheppard *et al.*, 1989). It has also been suggested that losses of vigour in germinating seeds can result from changes in auxin metabolism or at the chromosome level (Woodstock & Justice, 1967). Surface sterilisation of maize grain with PAC or NaOCl followed by 0.8 kGy gamma irradiation inhibited seedling growth and respiration (Woodstock & Combs, 1965) detectable before radicle emergence. Further experimentation with wheat and maize at these early stages (Woodstock & Justice, 1967) showed that although 0.8 kGy alone inhibited respiration, 0.4 kGy slightly stimulated seedling growth and respiration. However, Cuero *et al.* (1986) found that percentage germination of maize was unaffected up to 12 kGy while doses of up to 0.6 kGy gamma irradiation stimulated germination of maize seeds, although emergence at the higher dose was less vigorous.

Table 1.2 Sensitivity of Organisms to Gamma Irradiation

Target Organism	Dose (kGy)	Ref
Lethal to humans	0.001-0.01	(1)
Sprouting of potatoes & carrots inhibited	0.01-0.1	(1)
Insects killed	0.01-1.0	(1)
Microorganisms killed:	0.1-100	(1)
<i>Bacillus</i> sp.	4.2-6.0	(2)
Bacterial endospores	30	(1)
Gram-negative bacteria	0.1-1.0	(1)
Fungi and yeasts	0.5	(1)
<i>Alternaria alternata</i>	11.5-13.9	(3)
<i>Aspergillus</i> sp.	0.3-1.2	(2)
	1.7-3.0	(3)
<i>Aspergillus ochraceus</i>	3.0	(4)
<i>Cladosporium cladosporioides</i>	6.0-6.5	(3)
<i>Curvularia</i> sp.	17.0-20.0	(3)
<i>Fusarium</i> sp.	6.0-12.0	(2)
	1.7-2.5	(3)
<i>Penicillium</i> sp.	<0.3	(2)
	1.7-2.5	(2)
<i>Hypopichia</i> sp.	4.2-6.0	(2)

(1) Board (1983)

(2) On maize grain at 15-22% water (Cuero *et al.*, 1986)

(3) On enriched agar (Saleh *et al.*, 1988)

(4) On agar (Paster *et al.*, 1985)

1.6 AIMS, OBJECTIVES AND SUMMARY OF THE RESEARCH

The risk of biodeterioration of grain by moulding is high after harvest until the grain has been dried to a safe level which depends on the period and method of storage. There is very little detailed information on mould development and respiration in stored grain, with previous work almost exclusively being based on the work of Kreyger (1972) who used a crude visible moulding index to assess fungal contamination. Research is currently underway at the Silsoe Research Institute involving computer modelling of near-ambient grain drying of which the biological component has, so far, been based on Kreyger's data. More precise data would enable grain safe storage periods to be refined through the production of predictive models of mould growth under a wide range of environmental conditions. There are equally limited data available on the effects of moulding on grain quality *before* it becomes visible and the relative contributions of grain and fungi separately to quality loss. Studies of grain respiration were conducted in this project to help improve the knowledge in this area. There are numerous reports in published literature on the difficulties of quantifying fungal biomass present on artificial media or in association with other living materials such as grain. It was hoped that these studies would help develop an alternative means of fungal quantification on grain by measuring their individual and combined respiratory activity.

The aims of this thesis were to address the following:

- a) Develop electrolytic and large scale respirometer systems suitable for use with grain under realistic storage conditions.
- b) Compare the respiration of a range of cereal grains and oilseeds.
- c) Determine the pattern of respiration with time in wheat grain.
- d) Evaluate the effects of water activity and temperature on stored wheat grain

respiration and consequent dry matter losses.

- e) Correlate dry matter loss with visible moulding and other more sensitive measures of quality in stored grain.
- f) Validate the data obtained in b) to e) by studying large grain samples.
- g) Investigate the relative contributions of fungi and the grain itself to total respiration, comparing wheat grain naturally contaminated with fungi with sterile grain re-inoculated with common storage fungi.

The research presented in this thesis has been divided into a number of sections with general methods used presented in Chapter 2 and the results and discussions presented in Chapters 3, 4 and 5:

Chapter 2: General Materials and Methods

The common methods used in this study are detailed, including the systems developed for the first time to study grain respiration.

Chapter 3: The effect of temperature and water activity on grain respiration measured with an electrolytic respirometer:

An automatic electrolytic respirometer was adapted for use in these investigations. This respirometer had many advantages over other systems used to date but it was originally developed to study microbial respiration in soils. This is generally much slower than respiration in grain, and normally needs to be studied over periods of months. Grain is usually dried to a safe water content within about a week of harvesting so that respiration data at faster rates than in soil were required over shorter time periods. Before attempting to measure grain respiration, therefore, it was first necessary to optimise the apparatus for its proposed use.

Experiments were then conducted to generate a large database on the respiration of

naturally contaminated grain under a wide range of conditions. Most of these studies utilised wheat grain, but barley, oilseed rape and linseed grains were also studied to provide comparisons. Many combinations of the water activities and temperatures likely to be encountered in stored grain were examined in 25 g (wet weight) samples.

The overall patterns of grain respiration with time and resulting respiration rates are described with reference to the attempts made by colleagues at Silsoe Research Institute to mathematically model these data. Respiration was compared to other parameters of seed quality loss (germination, mycotoxin and ergosterol production and dry matter loss) by the destructive sampling of respirometer experiments. These changes were then related to previously accepted safe storage periods.

Chapter 4: Large scale respiration studies:

After obtaining a wide range of respiration data from small samples (Chapter 3), respiration of 10 kg (wet weight) grain samples was studied to validate the earlier data. The environmental conditions and timescales encountered in grain stores and in the small scale experiments were maintained. A second respirometer apparatus was constructed which was insulated to be buffered from external temperature change and which allowed for the aeration of samples. CO₂ production in the exhaust air was then quantified. Aeration also prevented the accumulation of CO₂ (and its possible inhibition of respiration) giving conditions comparable to CO₂ absorption into NaOH in the electrolytic apparatus. The airflow was set at a rate low enough to prevent grain samples drying out and to allow the intergranular temperature to increase if appropriate.

Chapter 5: Respiration and dry matter loss in naturally contaminated and sterile-reinoculated wheat grain:

The small scale respirometer apparatus was used to evaluate the relative contributions of the grain itself and its resident fungi to total respiration. Sterilised grain was examined with and without inoculated species of storage fungi. The conventional system of inoculating sterile grain with aqueous suspensions of fungal spores may be misleading as the inoculum is briefly provided with unlimited water, stimulating spore

germination before addition to the reduced- a_w substrate. The early phases of fungal growth in progress when grain is *naturally* colonised by fungi was investigated using an alternative inoculation method.

Little is known of the response of wheat grain to γ -irradiation, so to determine these effects and assess the method's feasibility in fungal inoculation studies, wheat grain was extensively studied after γ -irradiation with doses up to 15 kGy.

Dry matter losses in 25 g grain samples stored in humid chambers were measured over 28 d periods in comparison with the method of DML calculation studied in Chapter 3. DML was then similarly measured in grain samples sterilised by autoclaving, inoculated with dry spores of storage fungi and incubated in humid chambers.

Chapter 6: General discussion

The results obtained in different aspects of the study are compared and discussed concluding with suggestions made for future areas of research.

CHAPTER 2
MATERIALS AND METHODS

2.1 GENERAL MATERIALS AND METHODS

2.1.1 Preparation and storage of grain

Wheat grain harvested in three seasons (1988-1990) was used in most experiments. The varieties are described where relevant. The source of 1988 wheat was not known but other harvests were obtained from Rothamsted Experimental Station Farm. All grain was cleaned by separation between 1.0 and 3.5 mm screens (Röber Mini Pektus winnowing machine) and was stored at 0-5°C with less than 14% (wet basis) water content after harvest so that fungal growth could not occur. All grain samples were handled carefully after incubation to minimise spore dispersal into the atmosphere and a respiratory protection mask was worn. Whenever possible samples were handled inside a negative pressure microbiological safety cabinet.

2.1.2 Determination of water content

Three published methods for the determination of water content by oven drying were compared. Wheat grain was hydrated to approximately 0.75, 0.80, 0.85, 0.90 and 0.95 a_w (Section 2.5) and six 10 g samples of each a_w were weighed into aluminium foil trays of known weight and dried at either: a) 105°C for 16 h (Pixton, 1982); b) 130°C for 2 h (BSI, 1987); c) 130°C for 20 h (Reed, 1987). Percentage water content (wb) was calculated using the formula:

$$\text{Water Content} = \frac{F - D}{F} \times 100$$

where: F = fresh weight (5 or 10 g), D = dry weight (g).

Samples dried at 105°C for 16 h lost less water than samples dried at 130°C, and drying at 130°C for 2 h removed less water than at the same temperature for 20 h (Table 2.1). Differences between the three oven drying methods were consistent across

Table 2.1 Effects of drying time and temperature on water content determination of wheat grain at different water activities (a_w).

Approx. a_w	Water Content (% wet basis) \pm SEM		
	using drying method:		
	105°C-16 h	130°C-2 h	130°C-20 h
0.75	14.29 \pm 0.02	16.07 \pm 0.11	17.82 \pm 0.09
0.80	14.91 \pm 0.03	16.68 \pm 0.07	18.51 \pm 0.05
0.85	16.46 \pm 0.02	18.18 \pm 0.07	19.96 \pm 0.05
0.90	20.03 \pm 0.03	21.48 \pm 0.13	23.21 \pm 0.13
0.95	25.60 \pm 0.05	27.27 \pm 0.08	28.70 \pm 0.07

the a_w range. So long as the a_w parameter is used to describe water availability and the drying method is standardised and routinely used (Pixton, 1982), any of the drying methods described are acceptable for determining the a_w /water content relationship. Drying at the lower temperature of 105°C for 16 h using 2-4 replicate samples of 5 or 10 g whole grains was selected for subsequent use.

2.1.3 Determination of water activity

Water activity was determined by placing 10 g grain sub-samples into a psychrometer chamber (Humidat IC II (Novasina AG, Switzerland)), equilibrating the sample at 25°C for approximately 1 h and measuring a_w .

2.1.4 Determination of adsorption isotherm

The a_w during adsorption of different amounts of water was determined for wheat grain cv. Avalon in order to determine the quantities of water necessary to hydrate grain for use in subsequent experiments. Sterile distilled deionised water (SDDW) was added in volumes of 0, 0.25, 0.5, 1.0, 2.0, 2.5, 3.0 or 5.0 ml to 10 g samples of grain from 1988 and 1989 harvests containing 14.9 and 13.8% water respectively. Two or three replicates were prepared of each treatment. After equilibration at 0-5°C for up to 3 d, the a_w of each sample was determined at 25°C and its water content was determined by oven drying at 105°C for 16 h. A_w was plotted against water content to give an adsorption isotherm for each year's grain (Figure 2.1) which was comparable to those published previously (Table 1.1). The water content required for a given a_w could then be determined from the fitted curve (Table 2.2).

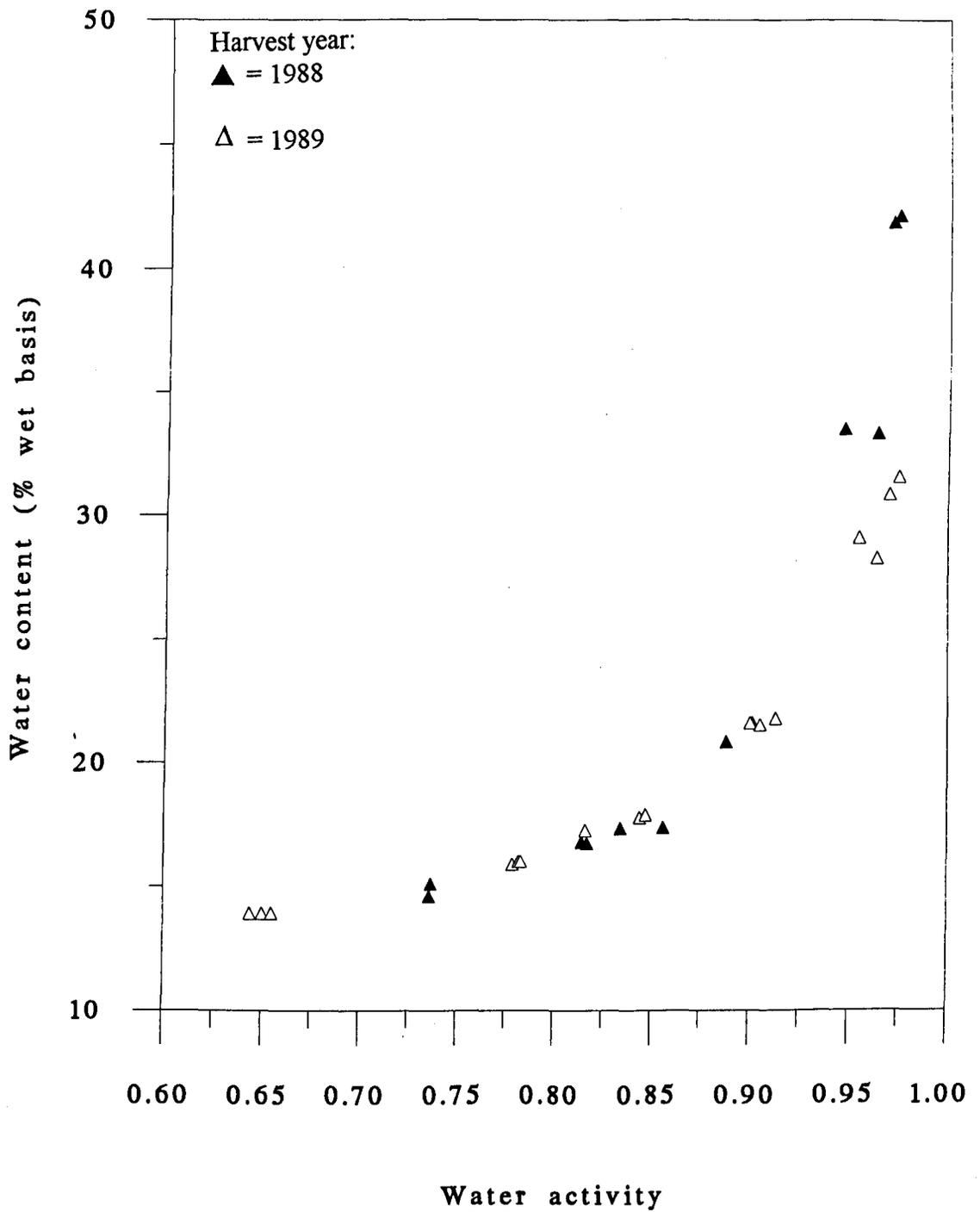


Figure 2.1 Water adsorption isotherm for wheat grain cv. Avalon harvested in 1988 and 1989.

Table 2.2 Mean water contents at different a_w at 25°C for wheat grain cv. Avalon (harvested in 1988 and 1989)

a_w	Water Content (% wet basis)
0.95	27.0
0.90	21.3
0.85	17.8
0.80	16.3
0.75	15.3
0.70	14.5

2.1.5 Hydration of grain samples

To determine how much water was needed to be added to grain samples for a given a_w (Section 2.4), the appropriate water contents were substituted into the formula:

$$SDDW \text{ required} = \frac{W_1 - W_2}{100 - W_1} \times F$$

where: SDDW = sterile distilled deionised water (ml); W_1 = required water content (%); W_2 = initial water content of grain (%), F = fresh weight of sample (g).

The calculated quantity of SDDW was then added to pre-weighed grain in conical flasks as they were shaken gently to ensure uniform incorporation. The flasks were sealed with foam rubber bungs and a layer of "Parafilm" before equilibration at 0-5°C overnight with thorough and regular mixing of flask contents. The water content of the hydrated samples was confirmed by oven drying (Section 2.1.2).

2.1.6 Culture and suspension media

Fungi were isolated, cultured and stored on agar media in 90 mm plastic Petri plates (containing 20 ml media) and in glass 1 oz Universal bottles (containing 5 or 10 ml agar slopes or 15 ml liquid media) of the following media. The type of medium used in each experiment is described where relevant.

0.1% agar diluent

DDW 1 l

Agar (Oxoid L13) 1 g

Autoclaved at 121°C for 15 min.

Czapek yeast liquid medium

DDW	1 l
Czapek-Dox liquid medium (Oxoid CM95)	33.4 g
Yeast Extract (Oxoid L21)	7 g

Autoclaved at 121°C for 15 min.

Malt extract agar (MEA)

DDW	1 l
Malt Extract (Oxoid L39)	20 g
Agar (Oxoid L13)	20 g
Pen/Strep Stock Solution (see below)	20 ml

Autoclaved at 121°C for 15 min.

Malt salt agar (MS) approximately 0.95 a_w , for xerophilic fungi.

DDW	1 l
Malt Extract (Oxoid L39)	20 g
Agar (Oxoid L13)	20 g
NaCl	100 g

Autoclaved at 121°C for 15 min.

Potato carrot agar (PCA) 5 ml slopes for storage under 12 ml paraffin oil.

DDW	1 l
Carrot	20 g
Potato	20 g
Agar	20 g

The carrot and potato were steamed for 1 h in 750 ml DDW and strained through muslin, added to other ingredients and made up to 1 l.

Autoclaved at 121°C for 15 min.

Wheat extract agar (WEA)

DDW	1 l
Wheat Grain (homogenised to a powder)	25 g
Agar (Oxoid L13)	20 g
Pen/Strep Stock Solution (see below)	20 ml

Grain powder was made up to 250 ml with DDW, autoclaved at 121°C for 1 h and then strained through single thickness coarse muslin. The resulting suspension was made up to 500 ml with DDW, then strained through double thickness muslin, and the remaining ingredients added. Autoclaved at 121°C for 15 min.

Pen/Strep stock solution

SDDW	1 l
Penicillin	0.625 g
Streptomycin	2.7 g

Filter sterilised and aseptically added to autoclaved media at <50°C.

2.1.7 Adjustment of water activity in media and incubation atmospheres***Media***

Glycerol solutions were used to adjust the a_w of MEA and WEA to 0.80-0.95 a_w (Table 2.3; Dallyn & Fox, 1980). Adjusted agars were named M95, W95 etc., depending on their a_w . Glycerol solutions were prepared according to the formula:

$$G = \frac{(MW \times MC \times S)}{1000}$$

where: G = weight of glycerol (g), MW = molecular weight of glycerol (92.09), MC = molal concentration of glycerol; S = weight of solvent (g).

Incubation atmospheres in humid chambers

Saturated salt solutions (200 ml) contained in glass dishes were placed in the centre of

Table 2.3 Molality of glycerol solutions used to control ERH in media (Dallyn & Fox, 1980).

A_w	Molality of Glycerol Solution
0.95	2.75
0.90	5.5
0.85	8.5
0.80	11.6

Table 2.4 Saturated salt solutions used to control relative humidity (Greenspan, 1977; Winston & Bates, 1960).

A_w	Temperature	
	20°C	25°C
0.90	MgSO ₄ .7H ₂ O	BaCl ₂
0.85	KCl	-

Table 2.5 Fungal isolates used to inoculate sterile grain.

Fungus	Authority	Code	Medium
<i>Eurotium amstelodami</i>	(Mangin) Thom & Church	2102	MS
<i>Penicillium aurantiogriseum</i>	Diercx	2682	MEA

incubation chambers (230 x 230 x 115 mm airtight polyethylene boxes) to control equilibrium relative humidity (ERH) at 20 and 25°C (Table 2.4; Winston & Bates, 1960; Greenspan, 1977). Samples of hydrated grain (25 g, wet weight) in oven-dried pre-weighed 50 ml Pyrex beakers were incubated in these humid chambers to measure dry matter loss over 28 d periods.

2.1.8 Culture maintenance and storage

Two species of field and storage fungi were used in inoculation studies (Table 2.5). Freeze-dried fungal cultures were revived by rehydration and plating onto MEA or MS agar as appropriate. They were then subcultured onto one PCA 5 ml slope and three MEA or MS 10 ml slopes. After incubation at 25°C for 7-14 d paraffin oil (12 ml) was added to each PCA slope. Slopes were then refrigerated at 0-5°C and MEA and MS cultures were transferred to fresh slopes every 6-12 months.

2.1.9 Assessment of seed quality

a) Visible moulding

Visible mould was classified using a scale divided as follows: 0; 1-25% grains moulded; 26-50% grains moulded; 51-75% grains moulded and >75% grains moulded.

b) Germination testing

The germination of seeds was assessed using methods approved by the International Seed Testing Association (ISTA, 1985). Humid chambers were prepared by placing 9 cm Whatman No. 1 filter papers inside inverted 9 cm Petri dish lids and then adding 1 ml SDDW. When grain samples were not hydrated, germination chambers were moistened with 1.5 ml SDDW. Ten grains were placed in each chamber and separated equally. The inverted Petri dish bases were replaced and they were incubated at 20°C in polyethylene bags for 8 d (wheat) or 7 d (all other seed types used in this research), before the number of germinated grains was counted. Three replicates of either 10, 50 or 100 grains were tested from each sample, the number of seeds per replicate depending on the replication of sample treatments (see relevant results sections). Measurements of seed vigour were sometimes made by measuring the length of each

wheat seedling's main germinating shoot at the end of the test.

c) Isolation and enumeration of microflora

Direct plating Ten grains taken directly from experimental samples were placed aseptically on each of three, five or ten agar plates. The agars used and incubation conditions are described where relevant. After 7-28 d incubation, the fungi isolated from each seed were classified as described below.

Dilution plating Samples of 5 g grain were soaked in 45 ml 0.1% agar for 20 min and then pounded for 10 min in a Colworth Stomacher (A. J. Seward Ltd., London). Subsamples were diluted 1:10 in 0.1% Agar to give serial dilutions. Plastic spreaders were used to spread 0.1 ml of each dilution on two to three replicate agar plates. All pipetting was carried out with 1 ml and 0.1 ml automatic pipettes (Gilson) with sterile plastic disposable tips. Plates were incubated at 25°C for 7-28 d. Fungi isolated were enumerated and identified as below.

Identification Fungi isolated were identified using macroscopic and microscopic characterisation. Some colonies growing poorly on low a_w media were subcultured onto MEA plates to aid identification by enhancing growth and sporulation. Sporulation was also induced in some fungi by incubation under periodic near ultra-violet radiation for (370 nm wavelength) for 8 h day⁻¹ (e.g. *Alternaria* spp.) or by aseptically scraping aerial mycelium away from the growth medium (e.g. *Epicoccum* spp.). Fungi were classified to genus level using standard texts (Domsch *et al.*, 1980; von Arx, 1970; Samson *et al.*, 1981) and to individual species of *Aspergillus*, *Eurotium* (Raper & Fennell, 1965; Kozakiewicz, 1971); *Fusarium* (Nelson *et al.*, 1983); and *Penicillium* (Pitt, 1988). Some of the *Penicillium* species isolated from wheat grain were kindly identified by the Commonwealth Agricultural Bureau International Mycological Institute.

2.1.10 Ergosterol assay

The ergosterol content of respirometer subsamples from a preliminary experiment incubated at 20-30°C and 0.6 - 0.945 a_w was determined by a method modified from Seitz *et al.* (1977). Samples of 5 g grain were ground twice for 60 sec in an analytical mill. The ground grain was added to 20 ml extraction solvent (80% HPLC grade methanol:20% HPLC grade hexane) and then shaken for 30 min on an orbital shaker. The extract was filtered through Whatman 541 filter paper and the filtrate made up to 30 ml with extraction solvent. The resulting solution was shaken with 3 g KOH until the KOH was fully dissolved, then placed in a 70°C water bath for 20 min. The mixture was cooled to room temperature and transferred to a separating funnel, 5 ml DDW were added and the mixture shaken vigorously for 10 - 15 sec. After separation, the upper hexane layer was collected and re-extracted twice more with 5 ml hexane to ensure all of the water fraction was removed. The final hexane extract was evaporated to dryness in a rotary evaporator and the residue dissolved in 1 ml HPLC grade methanol, which was filtered through a 0.2 μm filter (Acrodisc, LC13PVDF, Gelman Sciences). Samples (100 μl) were injected into an HPLC system (Varian LC 5000) set to the following conditions: Injection volume, 100 μl injection loop; Guard column, 50 mm x 4.6 mm Spherisorb C18 OD52 5 μm ; Main Column, 250 mm x 4.6 mm Spherisorb C18 OD52 5 μm ; Solvent, 100% HPLC grade methanol (degassed and filtered at 0.2 μm); Flow, 1.5 ml/min; Column temperature, constant 30°C; Column pressure, 30 atmospheres. Ergosterol content was calibrated against a standard curve prepared with pure ergosterol (Sigma Ltd.).

2.1.11 Monoclonal antibody-based competitive enzyme linked immunosorbent assay (ELISA) of aflatoxin B₁

To further examine the fungi isolated from wheat grain, a competitive ELISA employing monoclonal antibodies raised to aflatoxin B₁ (Candlish *et al.*, 1985) was used to detect AFB₁ in culture filtrates of five *Aspergillus flavus* isolates.

Isolates of *A. flavus* from grain incubated in respirometer experiments (Table 3.11) were sub-cultured on MEA plates at 30°C. An inoculating loop of conidia from each

culture was transferred to each of four Universal bottles of Czapek Yeast Liquid Medium (Section 2.1.6). Cultures were incubated for 14 d at 30°C then filtered through sterile 90 mm Whatman No. 41 filter papers. The filtrate was subdivided into 1 ml portions and stored in Eppendorf tubes. Unused 1 ml portions were immediately frozen at -40°C. One sample of each filtrate was diluted 1:10 with dilution buffer to remove optical interference by the coloured culture medium (pers. comm., Ramakrishna, 1991) and used in a direct competitive ELISA for AFB₁ described in detail by Ramakrishna *et al.* (1990). Antibody concentrations of 1:1,000, 1:2,000 and 1:4,000 were initially tested against standard solutions of AFB₁ (0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 ng ml⁻¹ toxin standard) to determine the optimal monoclonal antibody concentration for testing isolates (Figure 3.31).

2.2 MEASURING GRAIN RESPIRATION USING A SMALL SCALE RESPIROMETER SYSTEM

A respirometer system designed by Tribe & Maynard (1989) was used as follows:

2.2.1 Respirometer assembly

Grain samples hydrated to the required a_w (Section 2.1.5), were added to glass respirometer tubes sealed at the base with rubber bungs (Figure 2.2, A) and a Bijou tube (7 ml internal volume) containing 5 ml 2 M sodium hydroxide was placed on the surface of each sample. The glass tubes were each connected, through a U-tube, to a compensator unit (Figure 2.2, F) with approximately the same internal air volume. The U-tubes contained acid copper sulphate electrolyte, a platinum anode and a copper cathode; collectively known as the electrolysis unit. Joints made by rubber bungs with glass tubes and compensator bottles were sealed with silicone rubber compound (RS, Northants.) to make them both airtight and watertight. The types of grain used and summaries of treatments in each experiment are described, where relevant, in later chapters. Water content and percentage germination were determined for each sample before experiments. Treatments were randomised (Fisher & Yates, 1963) before the tubes were placed into stainless steel racks in groups of 16 and the electrodes connected to connector strips (Plate 2.1).

2.2.2 Electrolysis units for measurement of oxygen consumption

For every 16-20 respirometer cells, 100 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were dissolved in 160 ml 1 M H_2SO_4 at 70-75°C and about 8 ml were distributed into each electrolysis cell at 60-70°C. The cells were left to stand overnight until CuSO_4 recrystallised from the supersaturated solution. The complete electrolysis cell was assembled (Figure 2.2), and electrolyte levels were adjusted using a Pasteur pipette inserted through small holes in the cell cap (Figure 2.2, P), so that the platinum anode tip was positioned slightly above the electrolyte meniscus. The standard charge of electrolyte in each cell had the capacity to produce approximately 175 ml oxygen (dry, NTP) at 25°C.

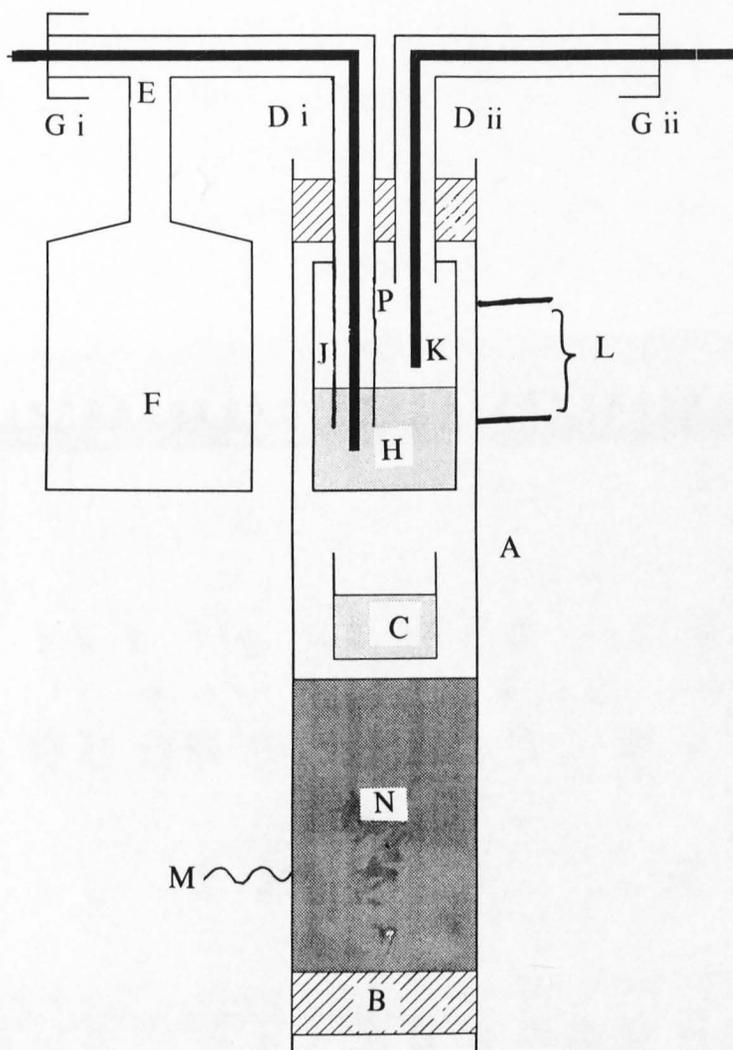
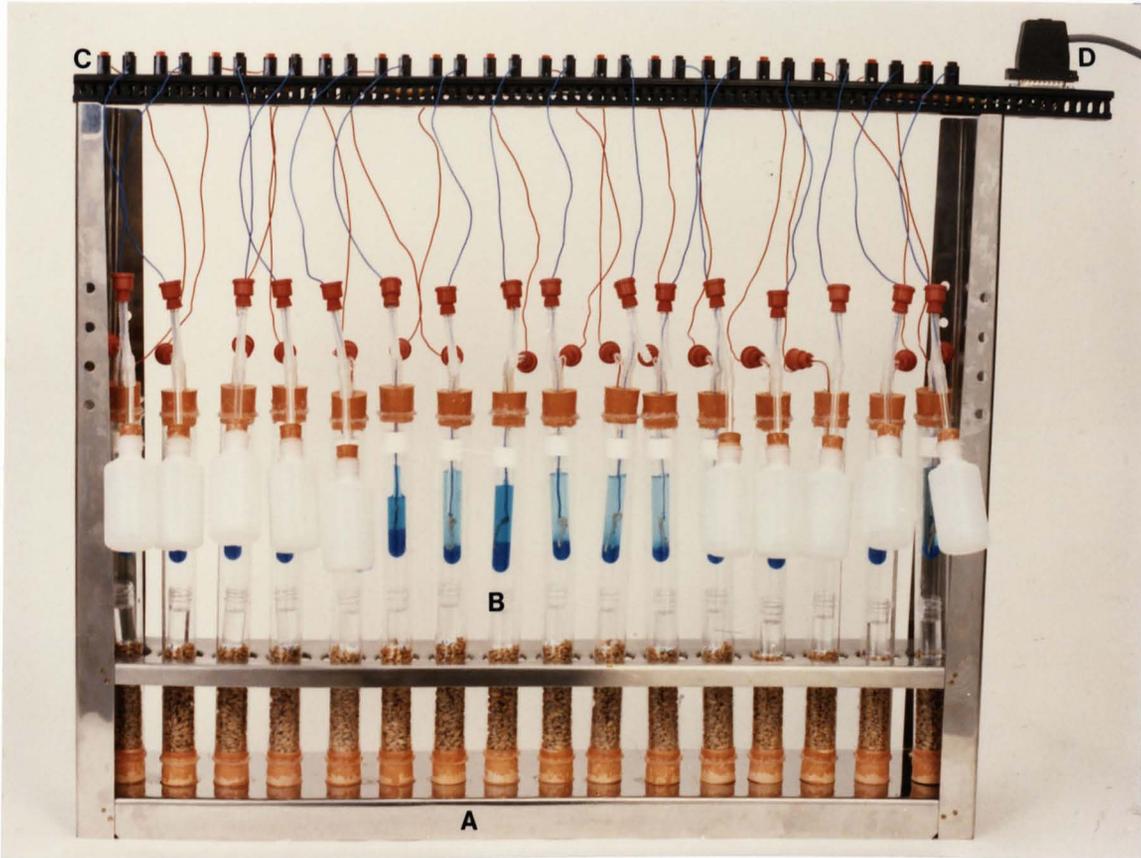


Figure 2.2 Schematic representation of one respirometer unit

A) glass leaching tube (280 mm x 27 mm); B) bottom bung and glass drainage tube, with nylon gauzes glued to the bungs; C) alkali vessel (Sterilin polystyrene bijou tube) resting on the sample; D) top bung fitted with two glass tubes: i) 110 mm glass tube, 10 mm above bung, 75 mm below bung, under electrolyte surface; ii) 50 mm glass tube, 20 mm above bung, projecting 20 mm outward; E) polypropylene Y-piece; F) compensator bottle; G_{i,ii}) suba seal caps through which the electrode wires are threaded; H) electrolysis cell (Sterilin polystyrene test tube) containing acidified CuSO₄ electrolyte; J) cathode of copper wire; K) anode of platinum wire; L) lead collar (for submergence of compensator bottle in water bath); M) position of wire gauze shelf for alternative positioning of CO₂ absorbent (see Section 3.1.3); N) grain sample; P) hole in cell cap.

2.2.3 Measurement of carbon dioxide production

The carbon dioxide evolved during respiration was absorbed into 5 ml aliquots of 2 M NaOH solution in the tubes, which were placed on the grain surface (Figure 2.23). Each tube had the capacity to absorb ca. 220 μl CO_2 (NTP). At least three 5 ml NaOH samples were prepared as controls for each experiment to allow correction for the absorption of atmospheric CO_2 into the NaOH during preparation.



connected to the electronic unit.

2.2.5 Data handling

Current flow to the electrolysis cells was measured by a four channel multiplexed ammeter inside the ECU and was recorded with a BBC Master Series microcomputer with dual disc drive and printer. Software written for the BBC Advanced Disk II was

Plate 2.1 Set of respirometer units

a) stainless steel rack; b) respirometer unit; c) connector strip; d) connector with lead to electronic control unit. (NB: The compensator bottles have been removed from 6 central tubes to show electrolysis cells more clearly).

2.2.3 Measurement of carbon dioxide production

The carbon dioxide evolved during respiration was absorbed into 5 ml aliquots of 2 M NaOH solution in the bijou tubes placed on the grain surface (Figure 2.2). Each tube had the capacity to absorb ca. 220 ml CO₂ (NTP). At least three 5 ml NaOH samples were prepared as controls for each experiment to allow correction for the absorption of atmospheric CO₂ into the NaOH during preparation.

2.2.4 Constant temperature environment

Glass tanks, (760 x 365 x 300 mm aquaria made of 5.5 mm glass and filled with deionised water; Plate 2.2, A) were used to maintain constant temperature environments over the temperature range of 15-35°C by heating with thermostatically controlled aquarium heaters (Visitherm). Heat loss was minimised by insulating the tanks with expanded polystyrene panels. The heaters were switched on at least one day before starting each experiment. Water baths were topped up with deionised water when evaporation uncovered the respirometer tubes or compensator bottles. Water temperatures were checked twice daily with thermometers, and they varied by < 1 degree. The temperatures of the tubes were equilibrated with that of the water bath by placing the racks in the water baths for 0.5-1 h before the Suba-seal caps were sealed and the experiment started: Up to two racks of 16 tubes were fitted into each water bath. Lead collars were placed around each unit to prevent the compensator bottles from floating, and the input sockets of the electronic control unit (ECU) were attached to the connector strips.

2.2.5 Data sampling

Current flow in the electrolysis cells was measured by a four channel multiplexed ammeter inside the ECU and was recorded with a BBC Master Series microcomputer with dual disc drive and monitor. Software written for the BBC Advanced Disk Filing System (ADFS) contained three programs to run the data sampler and print or plot data files. The program floppy disk and an empty, formatted floppy disk for data collection were inserted into the twin disk drives. When the Suba seal caps were closed, the ECU was switched on to supply current to each respirometer unit. The data sampling

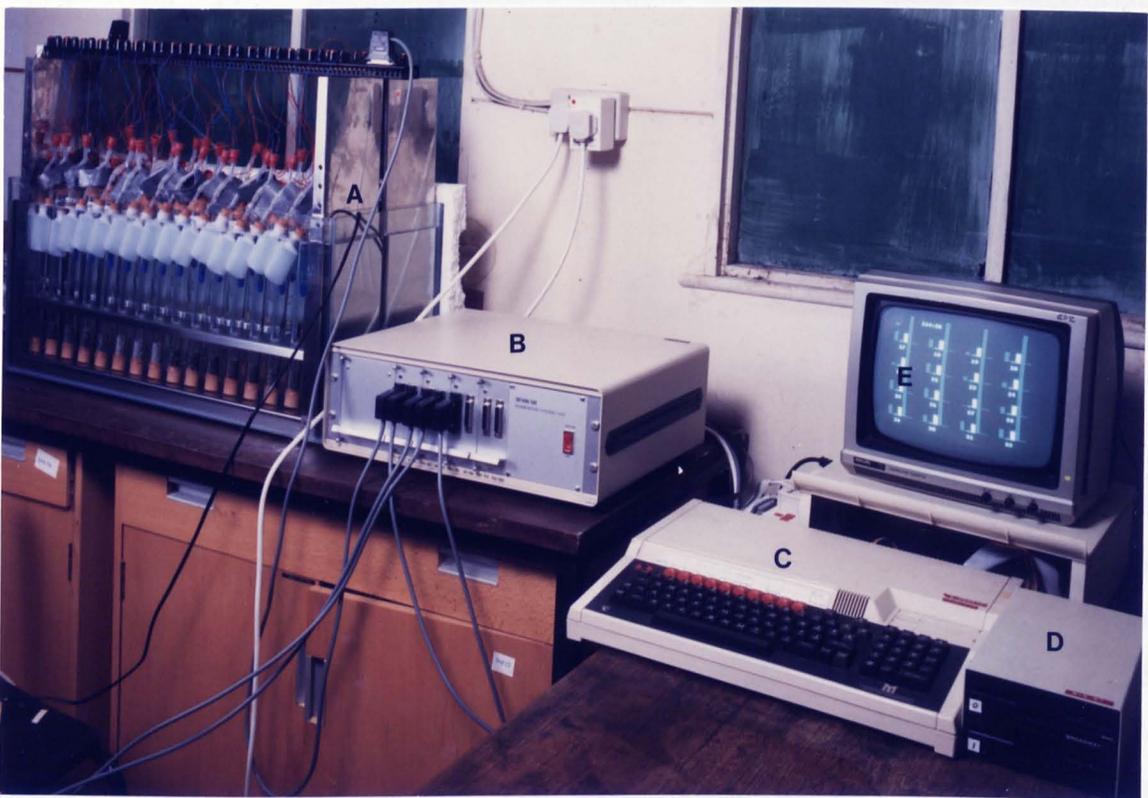


Plate 2.2 Respirometer hardware

A) water bath containing 32 respirometer units (insulation removed); B) electronic control unit; C) BBC Master Series microcomputer; D) dual disc drives; E) monitor displaying histograms from each unit.

program was run and data from each tube were sampled every 20 s (15 s in initial studies). Histograms were displayed on the monitor screen which showed the respiration characteristics of each group of 16 units at any one time. Data was recorded at 60 min intervals for 7-14 d.

2.2.6 Dismantling the respirometer

At the end of each experiment, the respirometer tubes were removed from their water baths and the suba seal caps were opened carefully, in case pressures in the electrolysis cell and glass tube were unequal. Electrolysis cells were removed and the alkali vessels extracted and sealed. The tubes were then emptied in a microbiological safety cabinet and samples of the grain were assessed for visible moulding and germination (Section 2.1.9). The remainder of each sample was frozen at -40°C for later analysis of the microflora (Section 2.1.9). Some subsamples were assayed for ergosterol (Section 2.1.10) or mycotoxins (Section 2.1.11).

2.2.7 Carbon dioxide determination

Sodium hydroxide solutions were removed from bijou tubes and made up to 50 ml with DDW in volumetric flasks. 10 ml sub-samples of this were titrated with standardised 0.2 M HCl dispensed from a 50 ml automatic burette (Digitrate; Jencons, Leighton Buzzard) while stirring. The volume of acid required to adjust the solution to pH 8.3, indicated by a colour change of purple-red to colourless by the indicator (0.5% (w/v) phenolphthalein (BDH) in 95% ethanol) was recorded. The solution was then further titrated to pH 4.0, using a drop of screened methyl orange indicator (BDH '3046') which changed colour from green to blue-grey. The volume of carbon dioxide absorbed by the NaOH solution was then calculated using the formula described in Section 2.4.

2.3 MEASURING GRAIN RESPIRATION USING A LARGE SCALE RESPIROMETER SYSTEM

2.3.1 Sample chambers

Chambers were constructed from plastic cylindrical bins (31.3 litres internal volume) with lids secured by wing nuts and bolts and sealed with rubber flanges (Figure 2.3). The bins measured 350 mm diameter x 413.5 mm deep. Chambers were insulated by enclosure in fibreglass-lined hot water cylinder jackets. The grain samples were supported by a perforated metal shelf approximately 50 mm from the base of each bin.

A series of four Copper/Constantan thermocouples (T_1 to T_4) were rigidly arranged vertically through the centre of the sample chamber at heights of 25, 100, 175 and 250 mm above the metal shelf to monitor temperature change. The 10 kg (wet weight) grain sample was 250 - 300 mm deep so that thermocouple T_1 was situated just below the grain surface. The thermocouples were connected to a Campbell 21X datalogger which was programmed to sample data every 15 seconds and record hourly average temperatures of each thermocouple. The datalogger also recorded hourly mean temperatures for all the four grain thermocouples and ambient temperature entering the chamber.

2.3.2 Preparation of grain samples

Grain was equilibrated overnight to the required a_w at room temperature (Section 2.1.5) in plastic dustbins (90-100 litres internal volume) with sealing lids. The hydration bins could not be sterilised before hydration due to their material and the large sample size. The sample chambers were filled with 10 kg (wet weight) of wheat grain (cv. Avalon, harvested in 1990) at the required a_w .

2.3.3 Airflow

Experiments were conducted indoors at room temperature but the air drawn through the sealed chambers came from outside the building to minimise variations in atmospheric CO_2 concentration. On leaving the sample chamber, exhaust gases were passed through

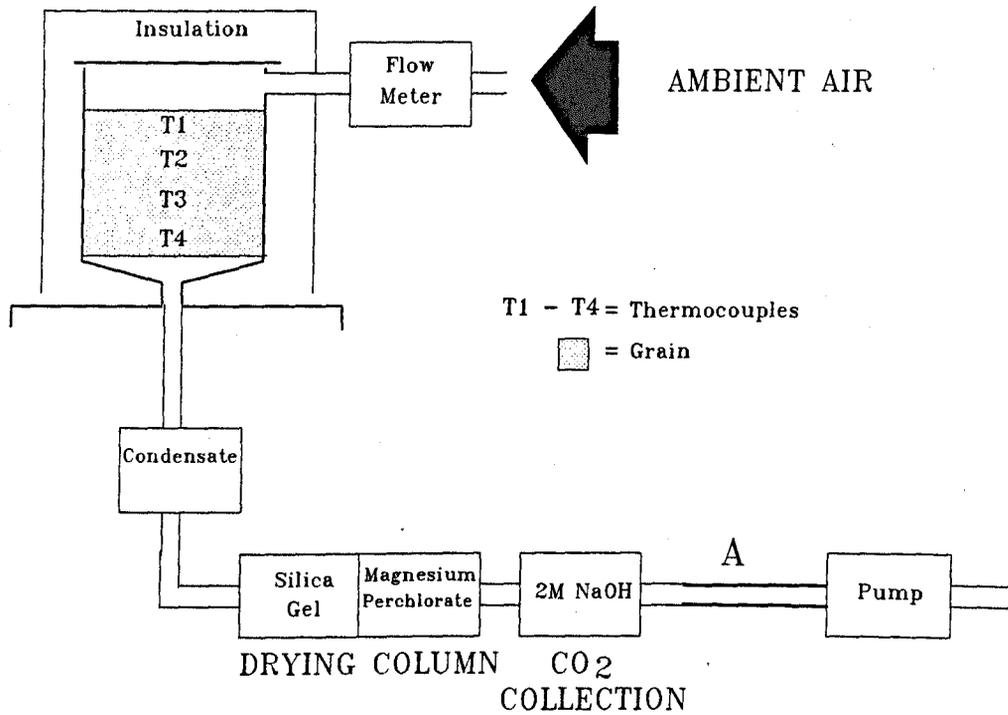


Figure 2.3 Schematic representation of the large scale respirometer apparatus developed and used in this study.

empty 500 ml conical flasks to collect any condensed free water, then through drying columns (395 x 60 mm open-ended plastic tubing) containing approximately two thirds (v/v) Blue Indicating 6-16 mesh Silica Gel (Aldrich) and one third Magnesium Perchlorate (Fisons), separated by glass wool (Figure 2.3). Vacuum pumps were situated at the end of the series to draw the air through the apparatus.

A 'MeteRaTe' flowmeter tube (Glass Precision Engineering Ltd, Hemel Hempstead, Herts) was connected at the air entry point on the outside of the chamber to monitor airflow rate. A flow rate of approximately 20 ± 5 ml air min^{-1} was maintained through the flow meter by adjusting the length of the rubber tubing 'A' (Figure 2.3) between the pump and sodium hydroxide container.

2.3.4 Carbon dioxide collection

Dried air was bubbled through 350 ml samples of 2 M NaOH solution in 0.5 l glass conical flasks to collect the CO_2 produced. NaOH solutions were freshly made and replaced daily. To quantify the CO_2 collected, subsamples of 2 M NaOH from each flask (two or three replicates of 5 ml) were made up to 50 ml with DDW in volumetric flasks, 10 ml of the resulting solution were transferred to beakers and titrated against standard hydrochloric acid as described in Section 2.2.7. The proportion of absorbed CO_2 in the whole sample was determined using the equation given in Section 2.4 and then multiplying the result V_{CO_2} by a factor of 70 to give the total volume CO_2 absorbed in 350 ml of NaOH.

2.4 TREATMENT OF RESULTS

Unless otherwise stated in the text the following analyses were conducted:

2.4.1 Oxygen consumption and carbon dioxide production

Oxygen consumption data was transferred from the floppy disk file to a VAX computer to allow statistical analysis at Silsoe Research Institute using the 'Genstat' package of programs (Payne *et al.*, 1987). The total volume of O₂ consumed by each sample was corrected to a dry matter basis using the formula:

$$V_{O_2} = \frac{40 X}{\left(1 - \frac{WC}{100}\right)}$$

where: V_{O₂} = total O₂ consumed kg⁻¹ dry grain; X = total O₂ consumed 25 g⁻¹ wet grain; WC = water content (% wb).

The volume of carbon dioxide produced by samples was calculated using the formula:

$$V_{CO_2} \text{ (dry, STP)} = (V_2 - V_1 - (C_2 - C_1)) \times 22.44$$

where: V_{CO₂} = volume (ml) of dry CO₂ absorbed at STP *; V₁ = volume (ml) HCl to pH 8.3 (sample); V₂ = volume (ml) HCl to pH 4.0 (sample); C₁ = volume (ml) HCl to pH 8.3 (control); C₂ = volume (ml) HCl to pH 4.0 (control).

* - Multiplied by 70 for large-scale respiration experiments.

2.4.2 Respiratory quotient

Respiratory quotients (RQ) were calculated using the formula:

$$RQ = \frac{V_{CO_2}}{V_{O_2}}$$

Where: V_{CO_2} = ml CO_2 produced by the sample; V_{O_2} = ml O_2 (dry, NTP) consumed by the sample

2.4.3 Dry matter loss

The formula used to calculate %DML from oxygen consumption data, assuming RQ=1.0, was as follows:

$$\%DML = R \times S \times T \times U$$

Where: R = l O_2 or CO_2 /kg dry grain; S = 44/22.44 (conversion from volume CO_2 to mass O_2); T = 0.682 (relates mass CO_2 produced to mass carbohydrate utilised; 1 g for 0.682 g respectively (Rees, 1982)); U = 100/1,000 (= 0.1) to convert weight of DML to percentage DML.

Dry matter losses were also calculated from direct weighings of samples stored in humid chambers and in this case dry matter was calculated as follows:

$$\%DML = 100 - \left(\frac{(DM_{at\ day\ 0} - DM_{of\ sample})}{(DM_{at\ day\ 0})} \times 100 \right)$$

Thus, the mean dry matter of unincubated hydrated samples at day 0 was always 100%

2.4.4 Germination data

Individual replicate germination percentages were converted to logits (Snedecor & Cochran, 1989) transforming data to a normal pattern of distribution. It was then possible to calculate mean values and perform statistical analyses on these data. Germination data are presented as mean logits with untransformed data given in the text for reference.

Fungal colonisation data were not subjected to transformation or any statistical tests because their collection was intended to provide a qualitative image of colonisation at specific environmental conditions. It was considered unwise to rely on the quantitative information obtained by direct plating, as its value has been widely doubted.

2.4.5 Significance of differences between means

Where treatments were compared against one another, potential significant differences were determined by comparison of the standard errors of means (SEM) represented on Figures by error bars. Where it was necessary to compare treatments with a single other treatment or to control values, T-tests were performed with differences deemed significant at the $p \leq 0.05$ level. Analyses were conducted using the "C-STAT" programs (Cherwell Scientific Publishing, Oxford). Significant differences between means at the $p \leq 0.05$ level are denoted on figures by the asterisk ('*') symbol and discussed in the text where appropriate.

2.4.6 Evidence of linearity

Graphs were plotted using the 'CricketGraph' software package (version 1.3, Microsoft Corporation Computer Associates, 1990) which conducts analyses of linear regression on data plotted. Data were assumed to conform to a linear relationship when the regression coefficient was $> 95\%$ ($R^2 > 0.95$).

CHAPTER 3

THE EFFECT OF TEMPERATURE AND WATER ACTIVITY ON GRAIN RESPIRATION MEASURED WITH AN ELECTROLYTIC RESPIROMETER

3.1 METHOD DEVELOPMENT

Several malfunctions in the respirometer hardware and software were identified during early experiments. This section describes measures developed to correct these, followed by an experiment devised to test the apparatus.

3.1.1 Failure to save data to disc caused by software malfunctions

In several preliminary experiments, the computer failed to save O₂ consumption data to disc, usually after 24-48 h incubation. This happened most often when using large numbers of samples or when they were hydrated to high a_w. The failure of the data sampler in these experiments was identified as a consequence of the apparatus being originally designed to study small amounts of respiration in soils. O₂ consumption in moderate to high a_w grain was much higher than in soil and it was necessary to modify the respirometer's data sampling programme to enable data to be saved satisfactorily. This was achieved by increasing the data sampling interval from 15 s to 20 s and by simplifying the histogram displays so that less time was required to write them to screen. These modifications allowed the data sampler 20 s rather than 15 s to display data to screen. The accuracy of the apparatus was not decreased as the interval at which data was saved remained constant (1 hour).

3.1.2 Background activity

Many samples from preliminary respirometer experiments showed an initial rapid rate of O₂ consumption which later declined to a slower steady rate, usually after up to 24 h (Figure 3.1). This background 'noise' appeared to relate to the apparatus itself rather than to the respiring sample. To diagnose its cause, respirometer tubes were set up with a range of different treatments.

A set of four tubes were first assembled containing 5 mm diameter glass beads as an inert substrate, instead of grain. These were similar to the size of wheat grains (Kent, 1978). These were incubated for 7 d at 20°C. Figure 3.2 shows that, even though the beads were inert, O₂ was still produced by the electrolyte, albeit very slowly compared

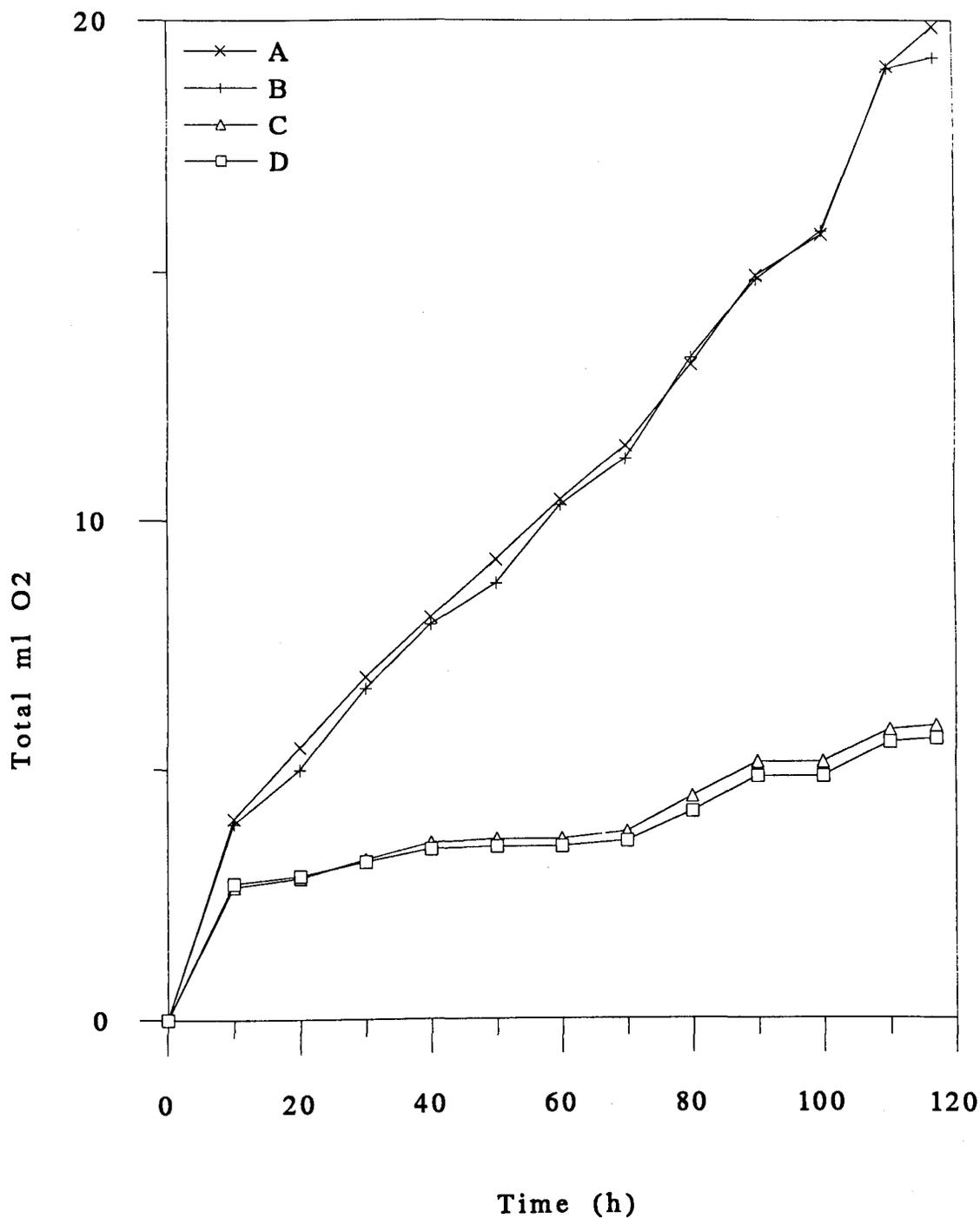


Figure 3.1 Preliminary data demonstrating the respiration of wheat grain samples (cv. Avalon) at 20°C with time showing the initial surge in oxygen consumption. Samples A, B = 0.92 a_w , samples C, D = 0.87 a_w .

to the respiration of grain samples (Figure 3.1 and Section 3.3). All four samples followed a similar initial trend with O₂ production by the electrolyte immediately at the start of the experiment to a maximum of 5 ml. After approximately 70 hours O₂ 'consumption' decreased to zero. However, the four samples described (A-D, Figure 3.2) also show that this phenomenon was not uniform. All the tubes had been assembled and treated in the same way so that identical results might have been expected. Thus the 'noise', described as apparatus-limited was not isolated or eliminated in this experiment, even though an inert substrate was used. Indeed, the results of samples C and D (Figure 3.2) may have been produced in other ways.

Other approaches were therefore necessary to determine the cause of this 'noise'. Close study of the respirometer units showed that when they were assembled with the anode of the electrolysis cell dipping under the electrolyte surface, O₂ was produced until the meniscus was depressed sufficiently to break the electrical circuit. When the platinum anode was placed just above, but not touching, the meniscus no such O₂ production occurred. This was confirmed by placing grain samples hydrated to 0.85 a_w in four units, two with the anode dipping into the electrolyte and two with it just above the meniscus, and incubating them at 20°C for 7 d. Figure 3.3 shows the rapid initial phase of 'respiration' during the first ten hours of the experiment, when the platinum anode was positioned under the meniscus (samples A and B) and its absence when the anode was placed above the meniscus (samples C and D). The apparent rapid O₂ consumption suggesting rapid respiration was thus false and curves C and D (Figure 3.3) show that respiration was undetectable until after 20-40 h incubation. As all four samples were randomised and incubated under the same conditions the cause of the 'noise' was positively identified as an artefact resulting from anode assembly. Subsequently, the anode tip was always carefully positioned above the electrolyte meniscus to prevent this artefact influencing later results.

3.1.3 The effect of collecting carbon dioxide below the grain sample

CO₂ is heavier than air and tends to sink to the bottom of grain stores (Sinha & Wallace, 1965). Therefore, CO₂ might be expected to settle in the bottom of the

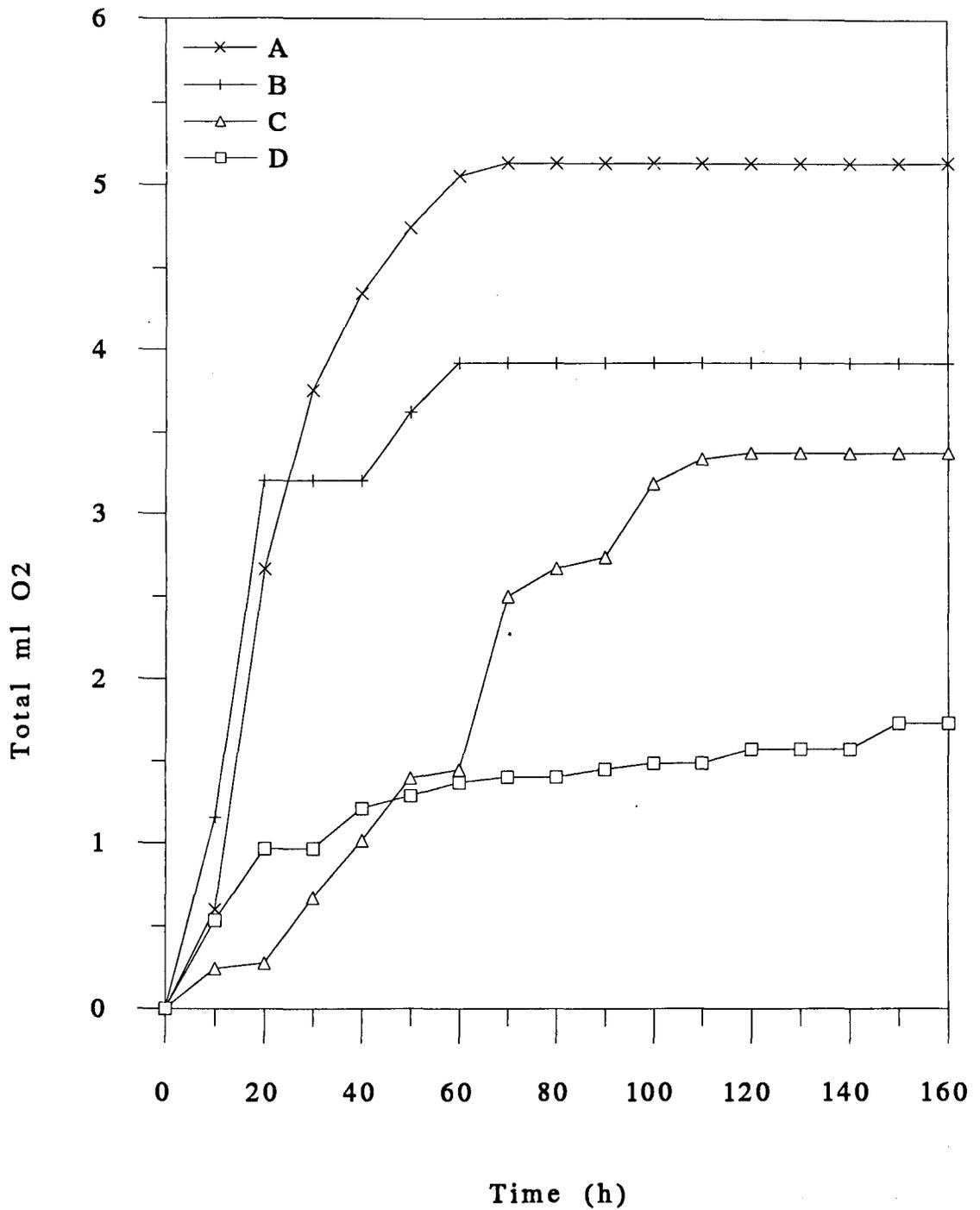


Figure 3.2 Oxygen production by the respirometer electrolyte with time when four identical samples of glass beads (5 mm diameter, Samples A to D) were substituted for grain samples.

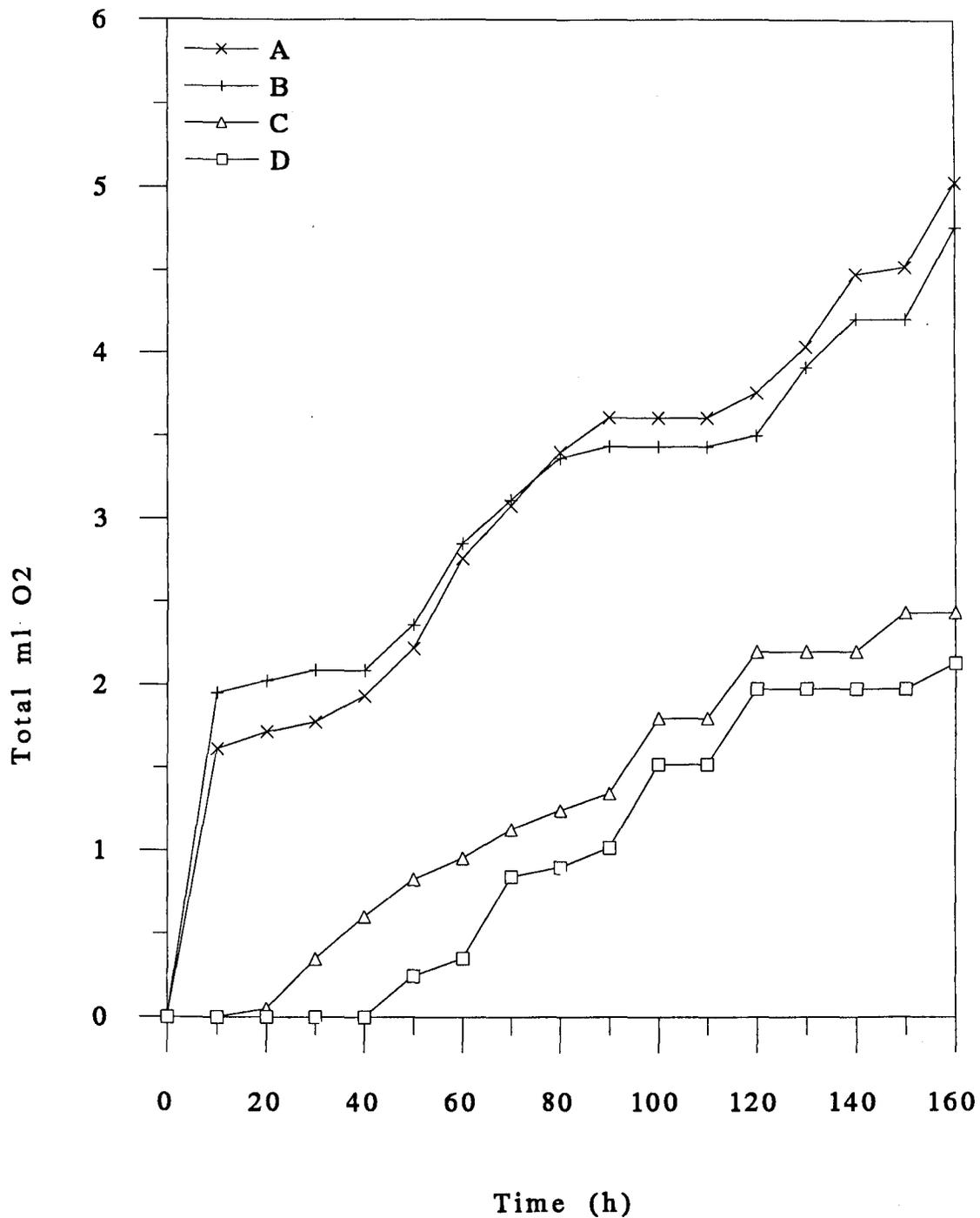


Figure 3.3 The effect of altering the initial anode position from below (samples A and B) to above (samples C and D) the electrolyte meniscus on the amount of O₂ consumption recorded by respiring wheat grain at 0.85 a_w and 20°C.

respirometer tubes so that absorption into NaOH solution above the grain (Section 2.2) could be incomplete. To determine whether tube position affected CO₂ absorption, perforated gauze shelves were placed in the tubes (Figure 2.2, Section 2.2) to allow the grain samples to be suspended over NaOH vessels, and to compare the results with the usual method of CO₂ collection (Section 2.2.3). Four replicates of 25 g wheat grain (cv. Avalon, 1989 harvest), hydrated to 0.90 and 0.95 a_w, were placed either above or below the NaOH vessel before the respirometer was assembled and run as described in Section 2.2. Figure 3.4 shows that O₂ consumption did not differ significantly with NaOH position at 0.90 and 0.95 a_w and 25°C. However, absorption of CO₂ into NaOH placed below the grain gave smaller differences between replicates, but because it was much easier to prepare tubes with alkali containers on the grain surface, it was concluded that this was adequate for all subsequent experiments.

3.2 COMPARISON OF THE RESPIRATION AND CONSEQUENT QUALITY CHANGES IN STORED CEREAL GRAINS AND OILSEEDS

3.2.1 Respiration

Grain respiration was first measured using cereal grains and oilseeds under standard a_w and temperature conditions (Table 3.1). Grain was harvested in 1990 from Rothamsted Experimental Station farm, except wheat cv. Rendezvous, which was harvested in 1989, and rapeseed which was harvested in 1990 in France. Grain was hydrated to 0.88-0.90 a_w and incubated at 25°C for 14 d using four replicates of each seed type.

Figure 3.5 shows the relationship between O₂ consumption of cereal grains and oilseeds under similar environmental conditions. Generally, O₂ consumption in both increased in a linear fashion with time (for linear regression analyses R²=0.9889 (barley), 0.9961 (wheat cv. Avalon), 0.9919 (wheat cv. Rendezvous), 0.9959 (wheat cv. Riband) and 0.9985 (rapeseed)). In contrast, linseed respired in a non-linear fashion with time, showing an initial slow phase followed by a more rapid increase with time towards the end of the experimental period (for linear regression R² = 0.8357 and to third order polynomial curve R² = 0.9945).

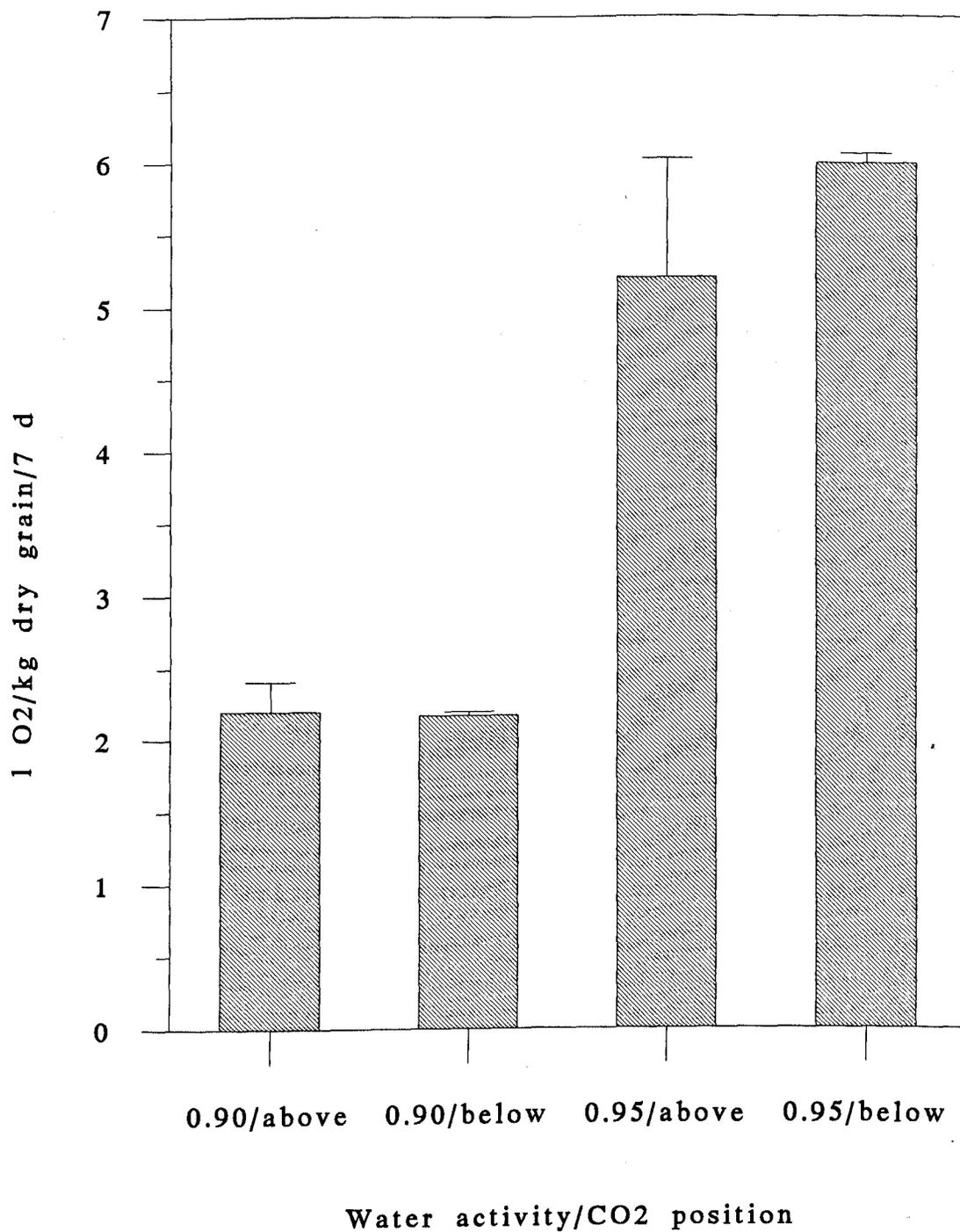


Figure 3.4 The effect of collecting CO₂ above or below the grain sample on the respiration of wheat grain cv. Avalon at 0.90-0.95 a_w and 20°C

Table 3.1 The varieties and a_w of grains used to compare the respiration and associated quality changes in cereal grains and oilseeds.

Crop	Variety	a_w^1	a_w^2	Reference
Barley (<i>Hordeum vulgare</i> L.)	Magie	0.90	0.90	Ramakrishna, 1990; Hunt & Pixton, 1974
Wheat (<i>Triticum aestivum</i> L.)	Avalon	-	0.90	
	Rendezvous	0.91	0.91	-
	Riband	0.92	0.92	
Oilseed Rape (<i>Brassica napus</i> L.)	Samourai	0.90	0.89	Pixton & Warburton, 1977
Linseed (<i>Linum usitatissimum</i> L.)	Antares	0.88	0.85	Hunt & Pixton, 1974

a_w^1 determined using a psychrometer (Section 2.2.3) using one or two replicates of at least 5 g hydrated grain (Section 2.2.5).

a_w^2 derived from the literature quoted, except for data for wheat grain which was determined from the sorption isotherm (Figure 2.1)

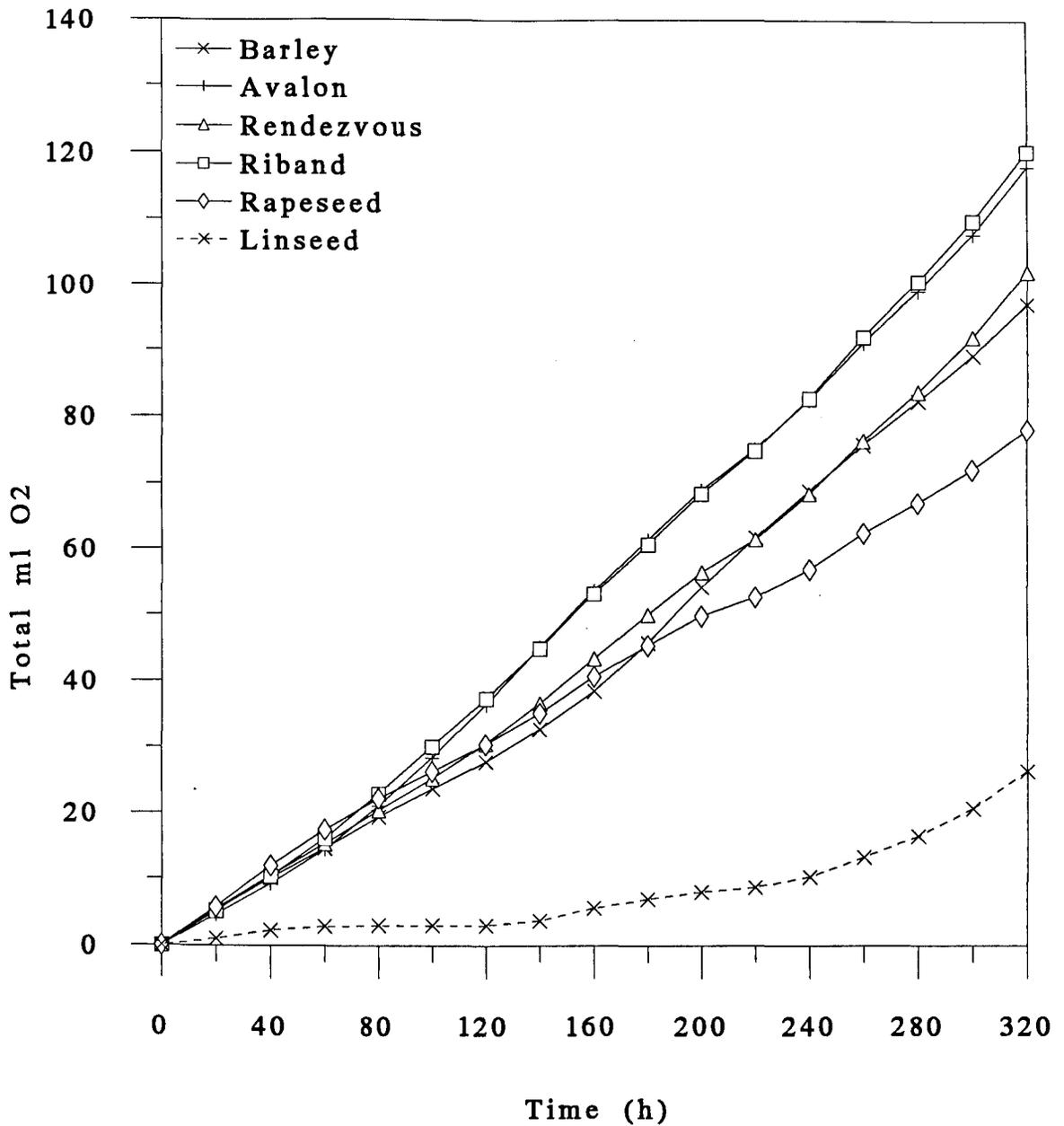


Figure 3.5 Patterns of O₂ consumption with time exhibited by barley, wheat (cvs. Avalon, Rendezvous and Riband), rapeseed and linseed grains at 0.88-0.90 a_w and 25°C measured using the electrolytic respirometer system.

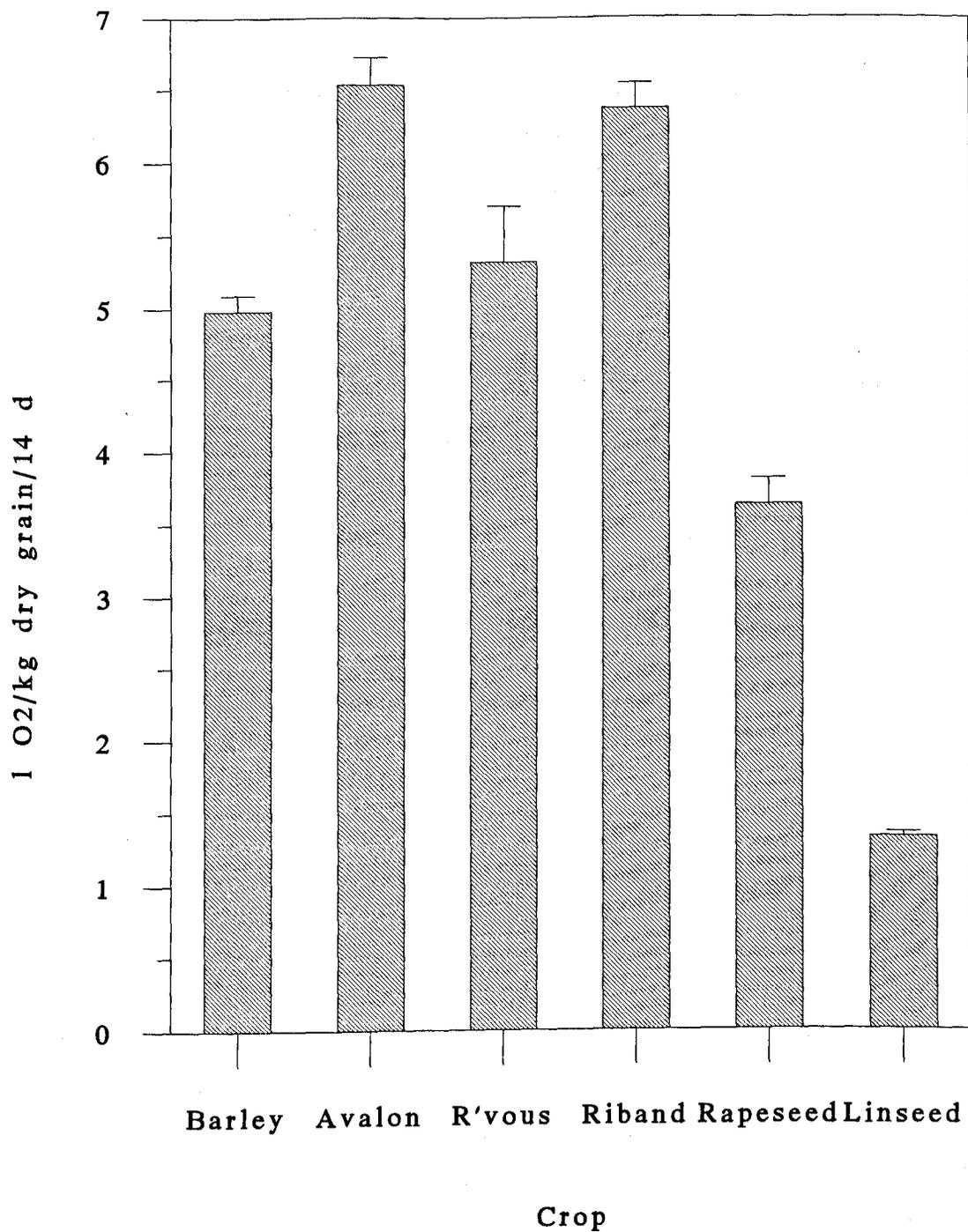


Figure 3.6 Total oxygen consumed over 14 days by barley, wheat (cvs. Avalon, Rendezvous and Riband), rapeseed and linseed grains at 0.88-0.90 a_w and 25°C.

Figure 3.6 shows the total respiration of the six grain types at 0.88-0.90 a_w and 25°C over a period of 14 days. Cvs. Avalon and Riband of wheat grain (both harvested in 1990) consumed O_2 at a similar rate over the experimental period, but that consumed by wheat cv. Rendezvous was significantly less ($p \leq 0.05$) than that of the other two. Similarly, barley grain cv. Magie consumed significantly less O_2 than wheat grain (cv. Avalon and Riband) but did not differ significantly from wheat grain cv. Rendezvous. The oilseeds respired significantly less than cereal grains with O_2 consumption by linseed the least of the six seed types studied.

Based on this data the DMLs calculated for the cereal grains (similar calculations were not made for oilseeds) were: Barley - 0.566%, wheat cv. Avalon - 0.743%, wheat cv. Rendezvous - 0.605% and wheat cv. Riband - 0.724%.

3.2.2 Changes in grain quality after the respirometer experiment

Figure 3.7 shows the changes in water content (% wet basis; Section 2.2.2) for all the seed types studied. Water contents increased significantly ($p \leq 0.05$) in all grains except barley and wheat cv. Avalon.

Three replicates of ten seeds were taken from each respirometer sample after the experiment and tested for germination (Section 2.2.9). Measured on a logit scale, this decreased significantly ($p \leq 0.05$) during the experiment in all six seed types studied (Figure 3.8) with the largest differences exhibited by cereal grains. In terms of untransformed data, the mean percentage germination of barley grain decreased from 95.3% to 74.4% and that of the wheat varieties decreased from 99.0% to 71.1% (cv. Avalon), 99.3% to 47.5% (cv. Riband) and 99.3% to 65.8% (cv. Rendezvous). Among the oilseeds, rapeseed germination decreased from 100% to 99.2% and linseed from 99.6% to 96.7%.

When assessed by visual examination, more cereal grains than oilseeds had moulded (Table 3.2). The surfaces of three of the four barley samples were >51% moulded, with the fourth sample 26-50% moulded. All four wheat samples (cv. Avalon) were

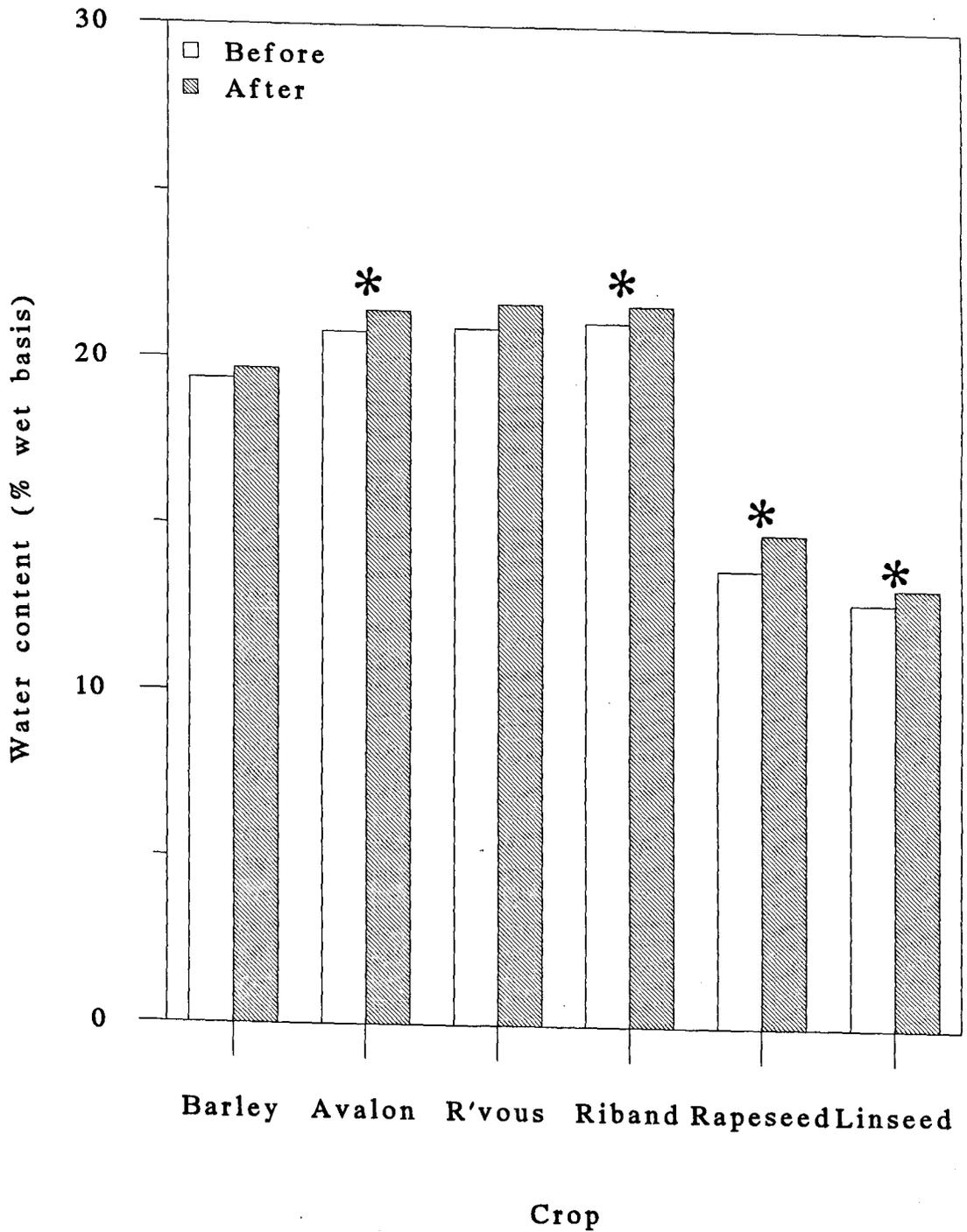


Figure 3.7 Changes in the water content of barley, wheat (cvs. Avalon, Rendezvous and Riband), rapeseed and linseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

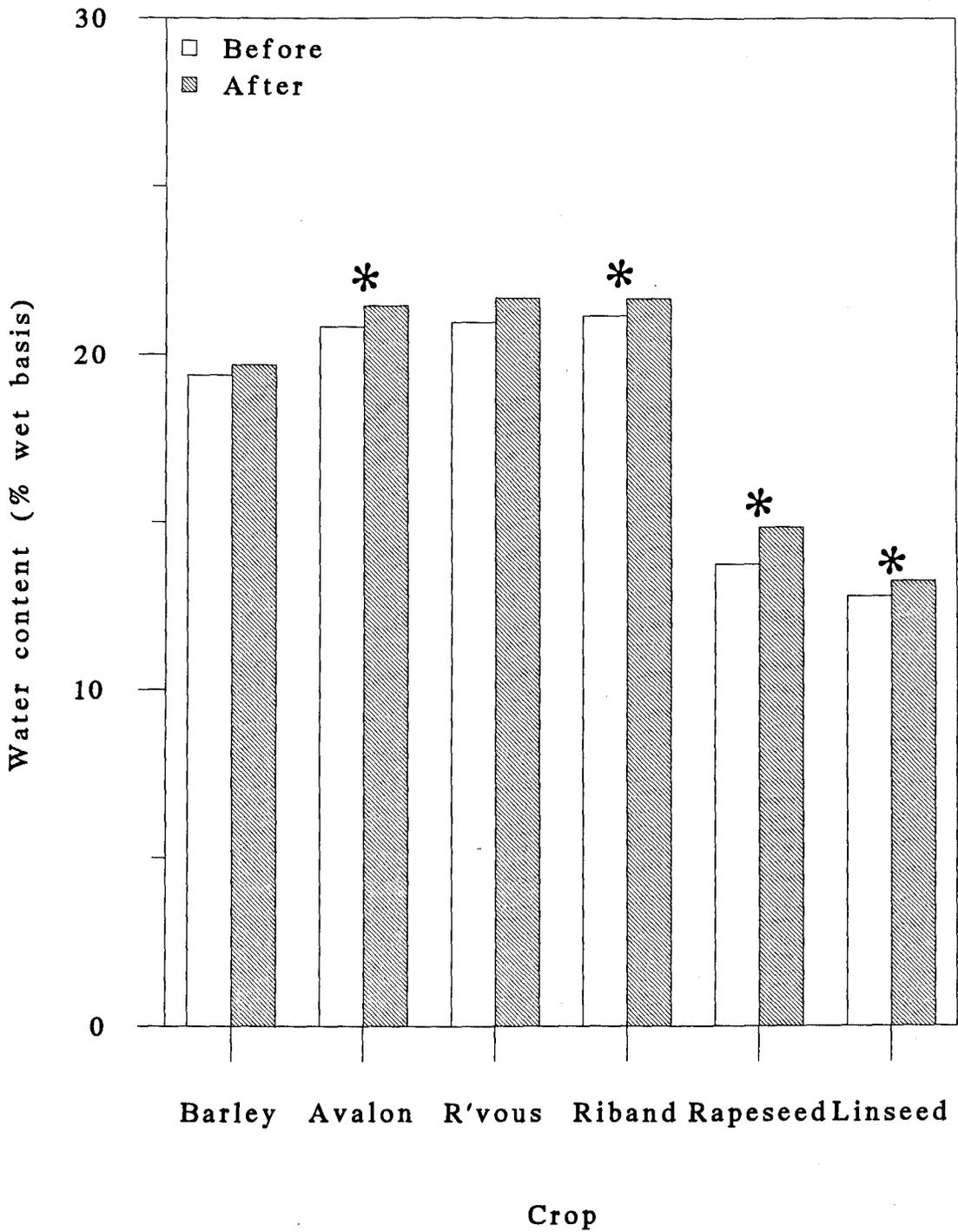


Figure 3.7 Changes in the water content of barley, wheat (cvs. Avalon, Rendezvous and Riband), rapeseed and linseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

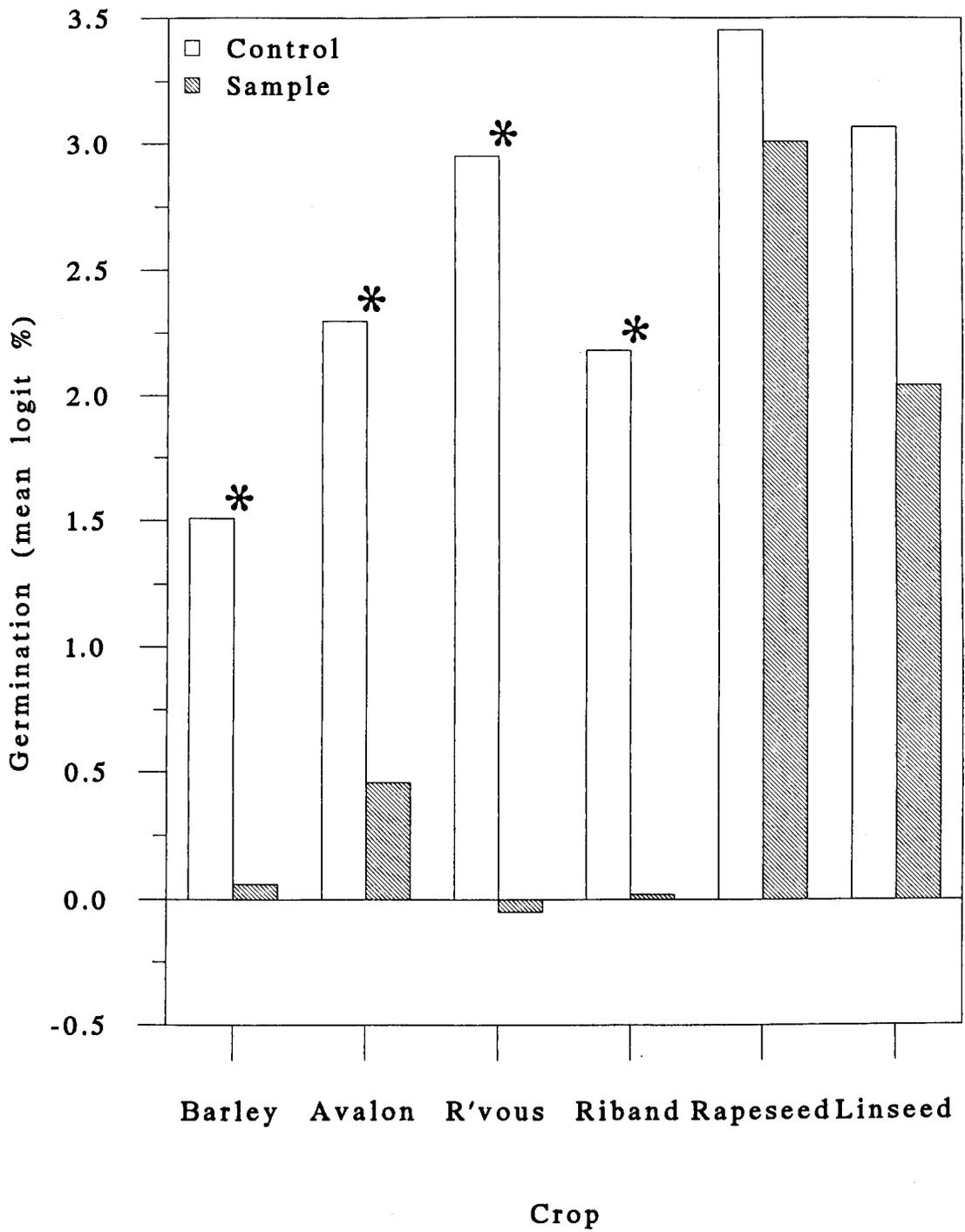


Figure 3.8 Germination in barley, wheat (cvs. Avalon, Rendezvous and Riband), rapeseed and linseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

Table 3.2 Visible moulding of cereal grains and oilseeds after incubation at 0.88-0.90 a_w and 25°C for 14 d.

Crop	Number of samples (out of 4) with the following percentages of grains visibly moulded:				
	0%	1-25%	26-50%	51-75%	76-100%
Barley	0	0	1	2	1
Wheat cv. Avalon	0	0	0	0	4
Wheat cv. Riband	0	0	3	1	0
Wheat cv. Rendezvous	0	0	3	1	0
Rapeseed	0	0	4	0	0
Linseed	0	0	4	0	0

over 75 % moulded and all samples of wheat cvs Rendezvous and Riband were between 26 and 75 % moulded. However, only 26-50% of all oilseed rape and linseed samples were contaminated with visible mould.

Three replicates of ten seeds from each of the four sample replicates were plated directly onto MEA and M90 media (Section 2.1.9) and incubated at 25°C for 7 days. Figures 3.9-3.13 show the relative incidence of fungi on each type of grain after incubation in the respirometer experiment, compared with controls (naturally contaminated grain). All cereal grains, both controls and samples, were colonised by fungi in these experiments but only 62 % and 24 %, respectively, of control rapeseed and linseed grains were colonised, although this increased to 100% of all samples after incubation in the respirometer.

Alternaria species were more commonly isolated from cereal grains than from oilseeds (Figure 3.9), but they never increased during incubation compared with control, unincubated grain. These species were isolated more commonly on MEA media than the lower a_w M90 agar. Conversely, *Cladosporium* species (Figure 3.11) were isolated most on M90 compared with MEA and only on control grain (except for wheat cv. Rendezvous samples where colonisation decreased compared with the control). More cereal grains than oilseeds usually carried *Cladosporium* species before and after incubation. *Aspergillus* species (excluding *A. glaucus* which is discussed here as *Eurotium* spp.) were isolated from control grains on MEA only from wheat cv. Riband (Figure 3.10). However, they grew from all controls of all seed types on M90 medium except for wheat cv. Avalon. *Aspergillus* spp. were isolated from all samples after incubation, more commonly on M90 agar. *Eurotium* spp. were isolated from all cereal grains on M90 medium before incubation, but in the oilseeds before incubation only a small number of linseed control grains yielded *Eurotium* spp. (Figure 3.12). After incubation almost all grains of all types tested were contaminated with species of *Eurotium* and more commonly on M90 medium. *Penicillium* species (Figure 3.13) were isolated from many grains before incubation, more commonly from M90 agar and from cereal grains, less from linseed and never from rapeseed. After incubation, all cereal

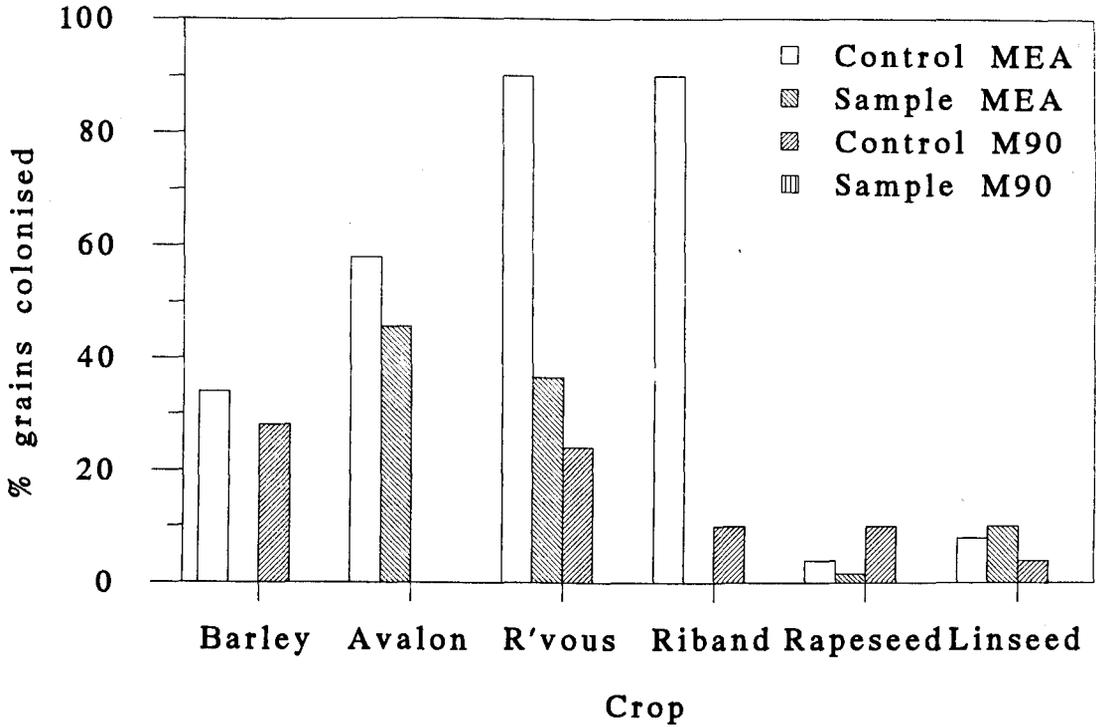


Figure 3.9 Incidence of *Alternaria* spp. on cereal and oilseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

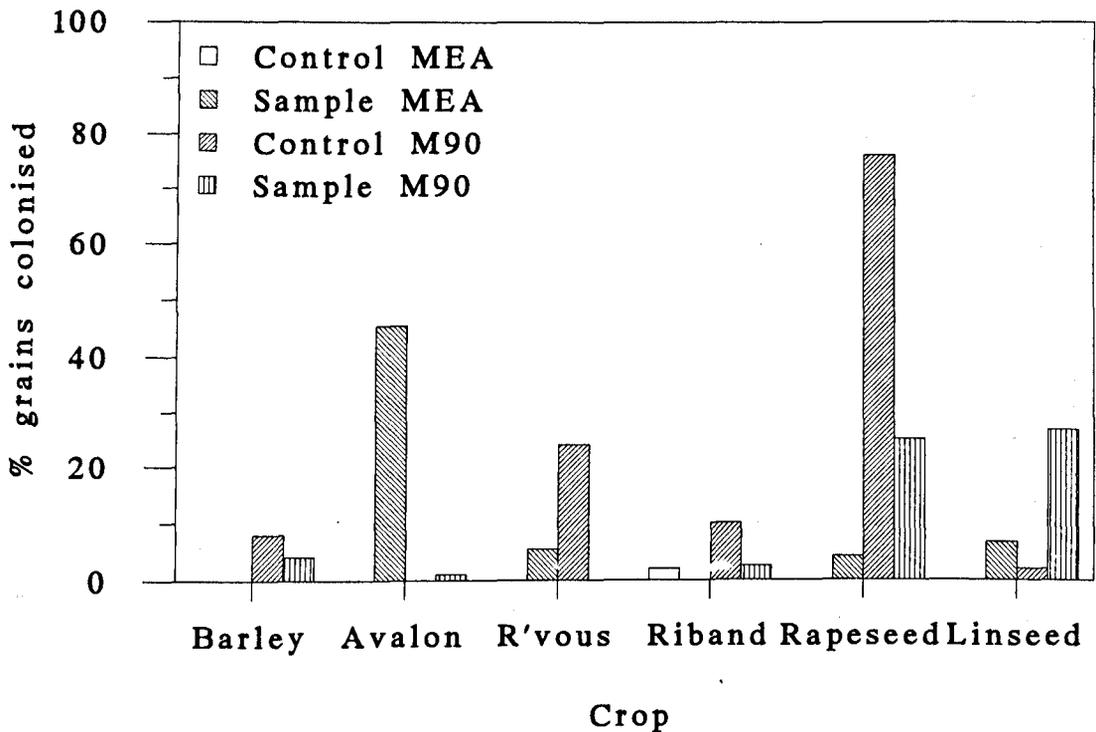


Figure 3.10 Incidence of *Aspergillus* spp. on cereal and oilseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

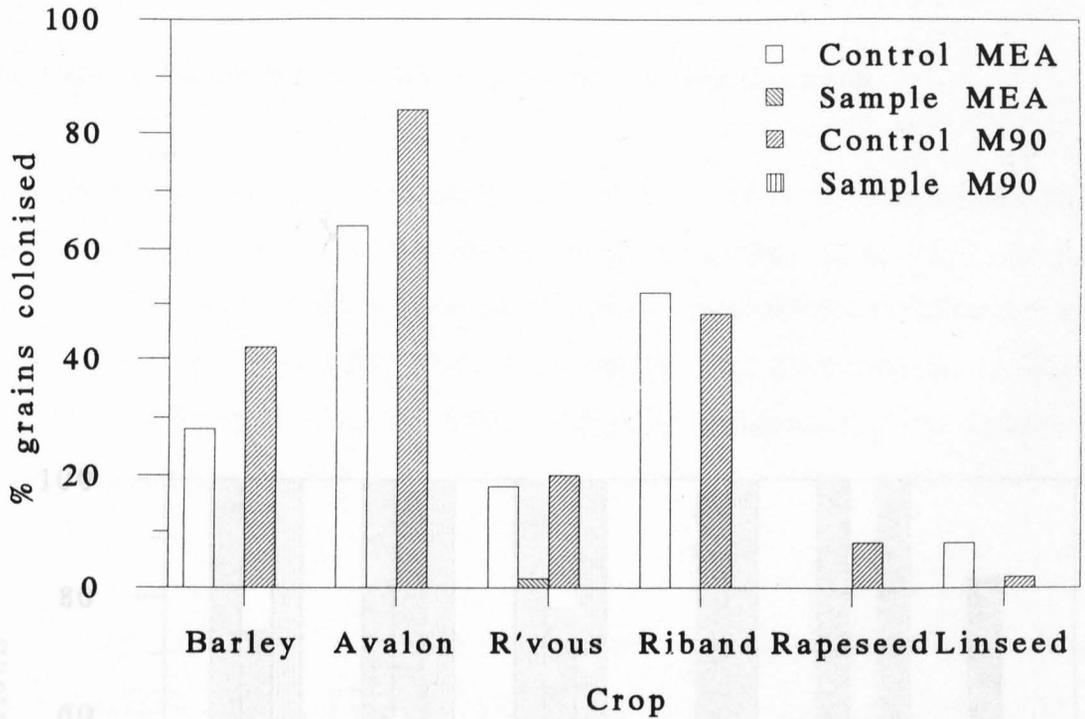


Figure 3.11 Incidence of *Cladosporium* spp. on cereal and oilseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

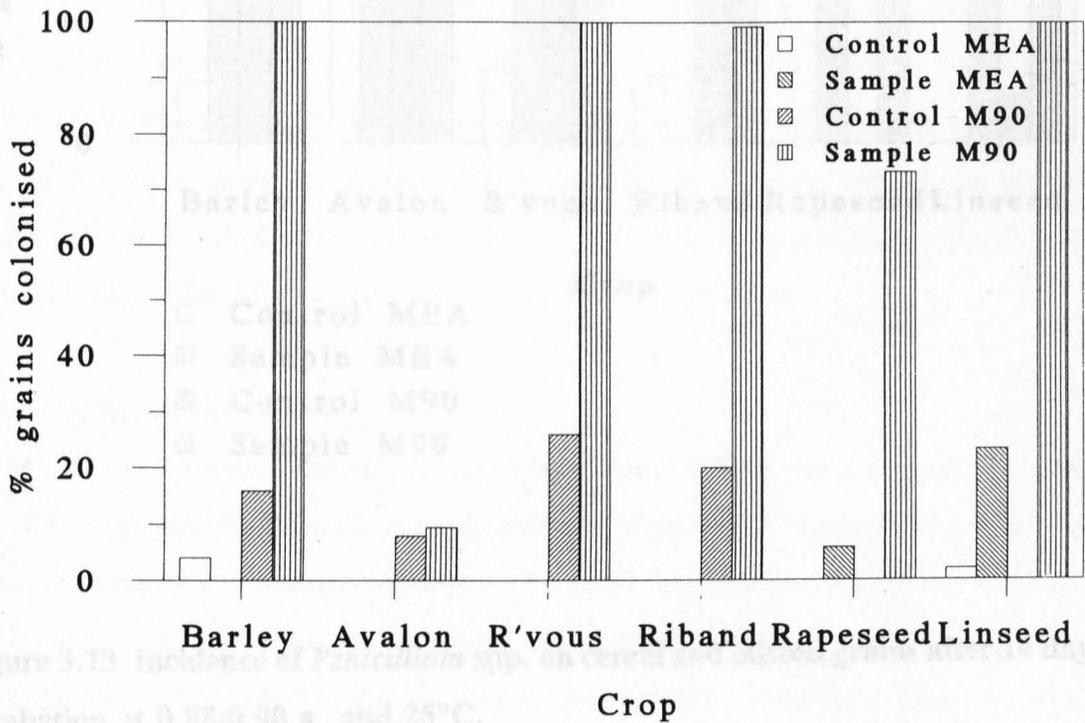


Figure 3.12 Incidence of *Eurotium* spp. on cereal and oilseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

grains and rapeseeds and most linseed seeds carried *Penicillium* spp.

Some other fungi were also occasionally isolated from one or two samples of control grain on MEA. For example, *Botrytis* spp. were isolated from 12%, 4%, and 4% of wheat cvs. Avalon and Rendezvous and barley grain, respectively, and *Epicoecium* spp. were isolated from 2% of barley grain. *Fusarium* spp. were more common, colonising 54%, 12%, 14% and 8% of barley, Avalon, Riband and Rendezvous grains respectively before

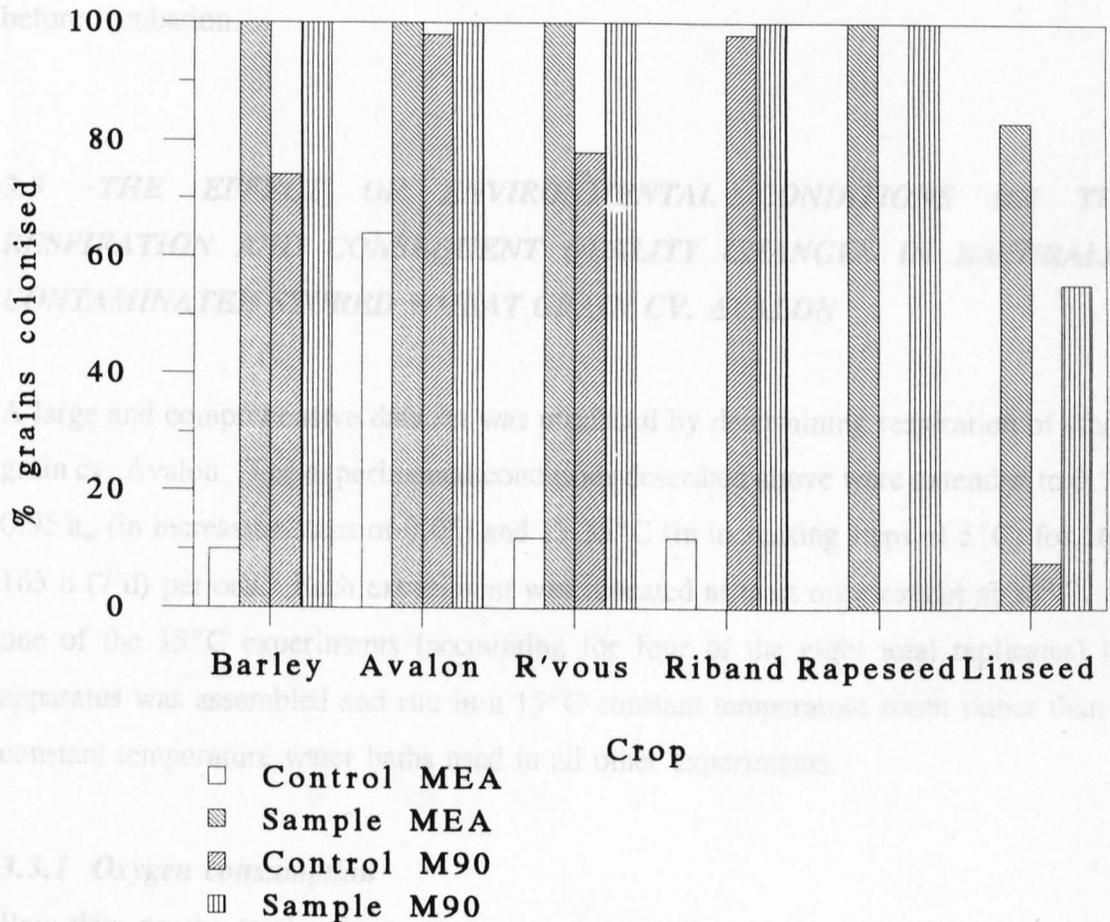


Figure 3.13 Incidence of *Penicillium* spp. on cereal and oilseed grains after 14 days incubation at 0.88-0.90 a_w and 25°C.

grains and rapeseeds and most linseed seeds carried *Penicillium* spp.

Some other fungi were also occasionally isolated from one or two samples of control grain on MEA. For example: *Botrytis* spp. were isolated from 12%, 4%, and 4% of wheat cvs. Avalon and Rendezvous and barley grain, respectively, and *Epicoccum* spp. were isolated from 2% of barley grain. *Fusarium* spp. were more common, colonising 54%, 12%, 14% and 8% of barley, Avalon, Riband and Rendezvous grains respectively before incubation.

3.3 THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE RESPIRATION AND CONSEQUENT QUALITY CHANGES IN NATURALLY CONTAMINATED STORED WHEAT GRAIN CV. AVALON

A large and comprehensive data set was produced by determining respiration of wheat grain cv. Avalon. The experimental conditions described above were extended to 0.70-0.95 a_w (in increasing steps of 0.05) and 15-35°C (in increasing steps of 5°C) for 160-165 h (7 d) periods. Each experiment was repeated at least once except at 35°C. In one of the 15°C experiments (accounting for four of the eight total replicates) the apparatus was assembled and run in a 15°C constant temperature room rather than in constant temperature water baths used in all other experiments.

3.3.1 Oxygen consumption

Raw data on the total volume of oxygen consumed in each replicate sample can be found in Appendix A. Overall, these experiments showed that O_2 consumption in wheat grain increased with increasing a_w (Figure 3.14) and temperature (Figure 3.15). Figure 3.14 shows that, at some temperatures, the overall pattern of respiration (i.e. O_2 consumption) with time changed with a_w as follows: At a_w less than 0.90, respiration increased in a non-linear fashion with time, with an initial lag phase followed by greater activity. However, at a_w greater than 0.90 respiration increased in a linear fashion with time. This phenomenon occurred at temperatures of 15-25°C.

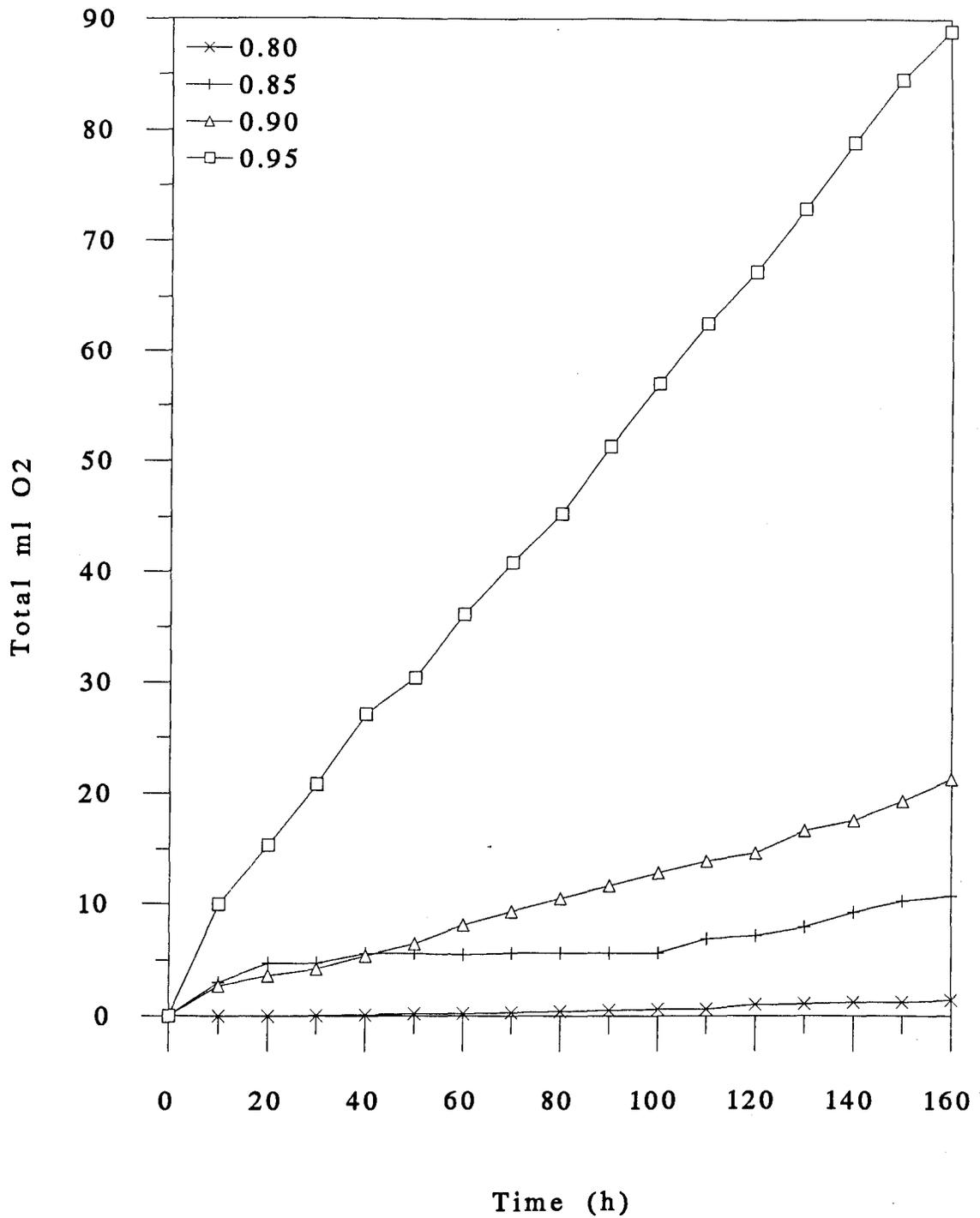


Figure 3.14 The effect of 0.80-0.95 a_w on the respiration of wheat grain cv. Avalon with time at 20°C.

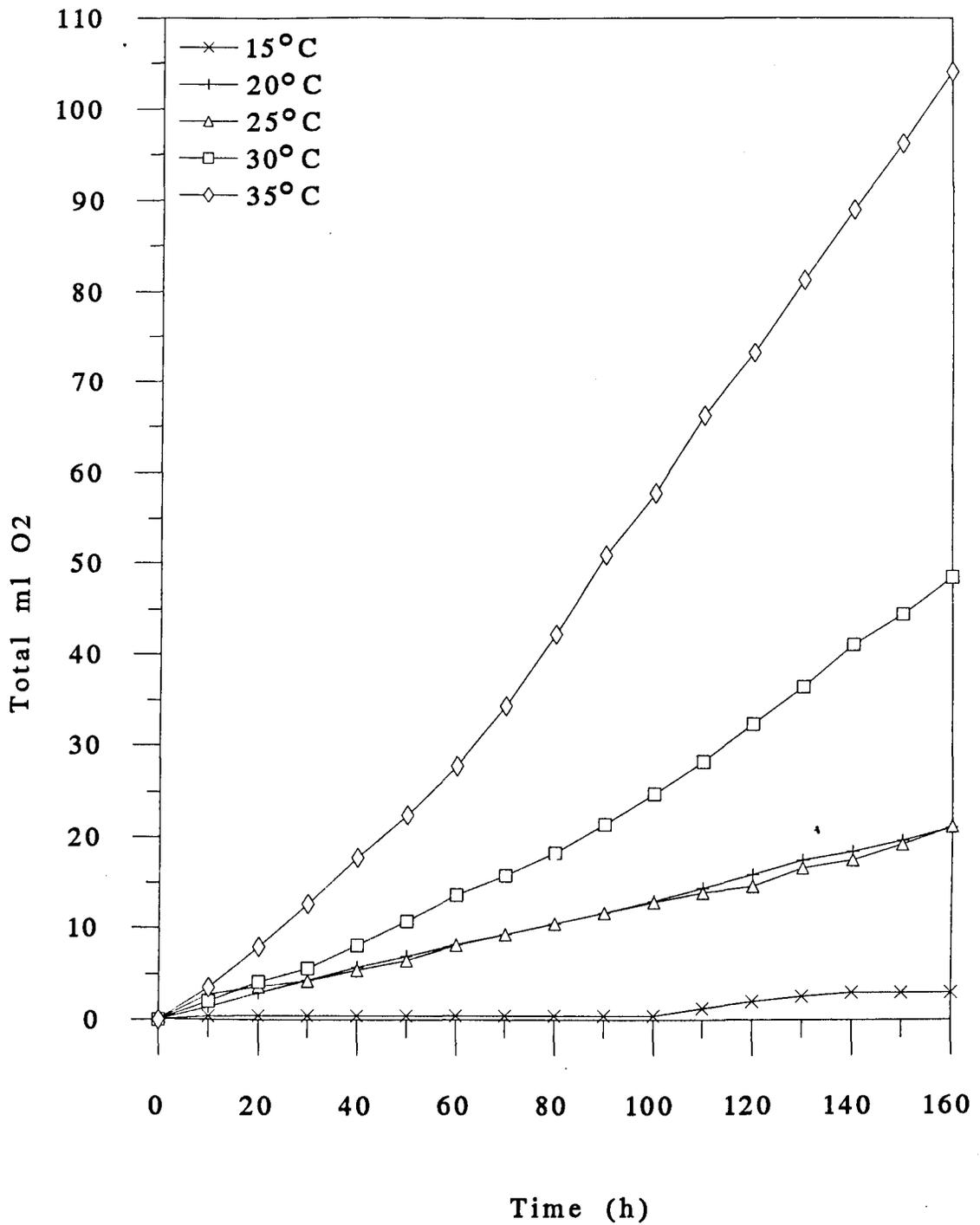


Figure 3.15 The effect of temperature (15-35°C) on the respiration of wheat grain cv. Avalon with time at 0.90 a_w .

These Figures also show that the *total* amount of O₂ consumed by wheat grain after 7 d incubation increased in a non-linear fashion with a_w and temperature. Respiration was most rapid at 0.95 a_w and 25-35°C within the experimental conditions tested. There was no significant statistical difference ($p \leq 0.05$) between results at these temperatures. Respiration was least in samples incubated at 0.80 a_w and 15°C.

The full data set was treated with a range of mathematical tests in attempts to produce mathematical models of the respiration of moulding wheat grain over a defined range of environmental conditions. These analyses were developed with generous help and advice from Prof. Martin E. Nellist and Mr. R. White of Silsoe Research Institute, and they assumed that respiration rate was linear with time under all conditions. The stages of the modelling process were as follows:

- a) a_w of grain samples was usually determined by extrapolation from percentage water content (wb) on the sorption isotherm curve derived at 25°C (see Figure 2.1). However, the relationship between a_w and water content is temperature dependent, so where experiments were incubated at temperatures other than 25°C, the actual a_w of individual samples may differ slightly from that extrapolated. Therefore, the a_w conditions discussed so far remain as working values referring to 25°C rather than the actual experimental a_w occurring in the sample. The following equation (Chen & Morey, 1989) was used to correct the 'working' a_w at 25°C to the 'predicted', temperature-dependent, a_w (Table 3.3):

$$a_w = 1 - \exp(-0.000043295 (\theta + 41.565) w^{2.111})$$

where: a_w = water activity (decimal; 0-1.0), θ = temperature (°C), w = water content (% db).

- b) Respiration data were then converted to mg O₂ day⁻¹ kg dry grain⁻¹, and then further divided by the incubation temperature (Schmidt and Jacobsen, 1982) to give the fraction, R/ θ , having units of mg O₂ day⁻¹ kg⁻¹ dry matter deg C⁻¹.

Table 3.3 Conversion of the experimental a_w determined at 25°C to the predicted temperature-specific a_w .

Experimental a_w	Specific a_w at °C, (Chen & Morey, 1989):				
	15	20	25	30	35
0.80	0.73	0.75	0.78	0.80	0.84
0.85	0.80	0.82	0.85	0.89	0.90
0.90	0.93	0.94	0.95	0.94	0.97
0.95	0.99	0.99	1.00	1.00	1.00

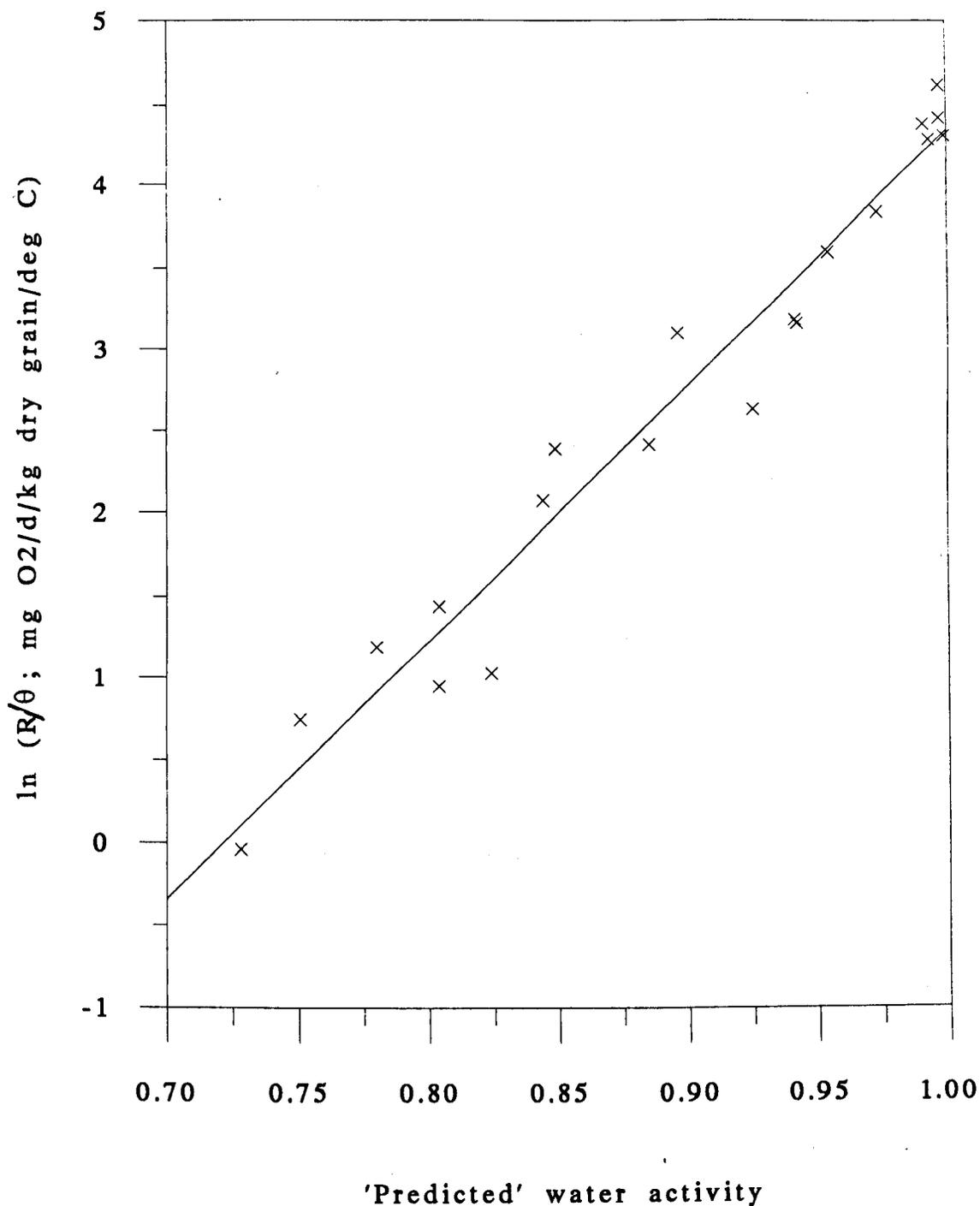


Figure 3.16 The effect of temperature-specific ('predicted') a_w on the ratio of O_2 consumption rate (R) to temperature (θ) in wheat grain (cv. Avalon) after 7 days incubation at 0.80-0.95 a_w and 15-35°C.

Figure 3.16 shows that $\ln(R/\Theta)$ increased in a linear fashion with increasing 'predicted' a_w ($R^2=0.9594$).

- c) Furthermore, the relationship could be expressed by the following equation (Nellist, pers comm.):

$$C = \frac{a_1 + a_2 t}{Y(1 + \text{EXP}(- (a_5 + a_6 t + a_7 \theta) (w - a_8)))}$$

where: C = Cumulative O₂ consumption (mg O₂/kg dry matter), t = time (h), w = water content (14.5 - 27.0% wb), Θ = temperature (15 - 35°C), $Y = (1 + \text{EXP}(a_3(a_4 - \Theta)))$, $a_1 = 345.83$, $a_2 = 125.2$, $a_3 = 0.1737$, $a_4 = 20.33$, $a_5 = 0.9143$, $a_6 = -0.001036$, $a_7 = -0.013634$, and $a_8 = 24.38$.

3.3.2 Carbon dioxide production and respiratory quotient

CO₂ and O₂ determined for wheat grain cv. Avalon incubated for 7 d were then used to calculate RQ (Section 2.4) over the range of conditions studied. Raw data on volumes of CO₂ produced in samples can be found in Appendix B. RQ values were generally in the range 0.5 to 1.5 (Table 3.4). However, RQ at 15°C were consistently higher than those at higher temperature, especially at low a_w . The mean RQ, calculated from all data in Table 3.4, is 1.11 (± 0.228). This agrees closely with published results indicating that the RQ of respiring wheat grain is close to 1.0 (Section 1.3.3), and a value of 1.0 was assumed in calculating dry matter loss from O₂ data (Table 3.5).

Dry matter losses (Table 3.5) mirrored the trends in respiration rate but in addition, allowed a description to be made of losses of grain material for comparison with previously accepted levels of dry matter loss, such as those given by Kreyger (1972). Greatest calculated losses of dry matter occurred in samples incubated at 0.95 a_w and 25-35°C. DML was least in samples incubated at 0.80 a_w and 15°C.

Table 3.4 The effect of a_w and temperature on respiratory quotients (RQ) calculated from the respiration of wheat grain cv. Avalon during 7 days incubation.

Mean respiratory quotient \pm SEM					
A_w	Temperature ($^{\circ}$ C)				
	15	20	25	30	35
0.80	5.13 \pm 0.91	0.68 \pm 0.13	0.45 \pm 0.04	0.61 \pm 0.04	0.84 \pm 0.04
0.85	2.50 \pm 0.70	0.34 \pm 0.07	0.54 \pm 0.21	0.87 \pm 0.07	0.67 \pm 0.11
0.90	1.81 \pm 0.39	0.75 \pm 0.11	0.54 \pm 0.17	0.90 \pm 0.01	0.82 \pm 0.03
0.95	1.18 \pm 0.24	0.73 \pm 0.02	0.59 \pm 0.09	0.90 \pm 0.07	1.02 \pm 0.09

Overall mean of data presented above = 1.11 \pm 0.228

Table 3.5 The effect of a_w and temperature on calculated dry matter loss in wheat grain cv. Avalon after incubation for 7 days.

Predicted % dry matter lost after 7 d:					
	Temperature (°C)				
A_w	15	20	25	30	35
0.80	0.007	0.02	0.039	0.061	0.133
0.85	0.018	0.027	0.130	0.161	0.372
0.90	0.085	0.226	0.436	0.547	0.774
0.95	0.517	0.762	1.21	1.187	1.239

Text in bold indicates that these samples were visibly mouldy at the end of the 7 d incubation period.

Table 3.5 also summarises the conditions allowing visible moulding (1% or more of grains) during 7 d incubation in respirometer experiments. Visible moulding could occur with very little loss in dry matter, for instance 0.13% dry matter loss had occurred in visibly moulded grain at 0.85 a_w and 25°C. As temperature increased a wider range of a_w permitted visible moulding. Greatest dry matter loss occurred at the highest a_w (0.95) at each temperature and ranged from 0.517 to 1.239%, at 15 to 35°C respectively. A full description of visible moulding in these samples is presented later (Tables 3.6-3.10; Section 3.3.3).

Percentage water content remained unchanged in most samples after incubation but increased ($p \leq 0.05$) in 0.85 a_w /35°C and 0.95 a_w /15 & 25°C samples. Water content decreased ($p \leq 0.05$) after incubation in samples incubated at all a_w values studied at 30°C and 0.90 a_w /20°C (Figure 3.17).

3.3.3 Changes in grain quality in different environmental conditions during and after respirometer experiments

Seed germination

Three replicates of ten seeds from each replicate respirometer sample were assessed for germination after incubation and compared with grain that had been used as the starting material and stored with less than 0.65 a_w . Figures 3.18-3.22 show that seed germination decreased significantly during storage with $> 0.85 a_w$ at all temperatures except 30°C. The greatest decreases in germination over the control were observed in samples incubated at 0.95 a_w , the highest a_w tested, and 25°C. Under these conditions germination was significantly less ($p \leq 0.05$) than that at all other temperatures and 0.95 a_w . Germination was lower in 0.95 a_w samples compared to 0.90 a_w . Germination at 15°C and 0.80 and 0.85 a_w was not significantly different ($p \leq 0.05$) from the control at any temperature (Figures 3.18-3.22) but was decreased at 15-25°C in all samples over 0.85 a_w . Unusually, no samples incubated at 30°C showed any significant change in germination compared to the control, and at 35°C, germination was decreased significantly ($p \leq 0.05$; Figure 3.22) only in samples stored at 0.95 a_w . Germination

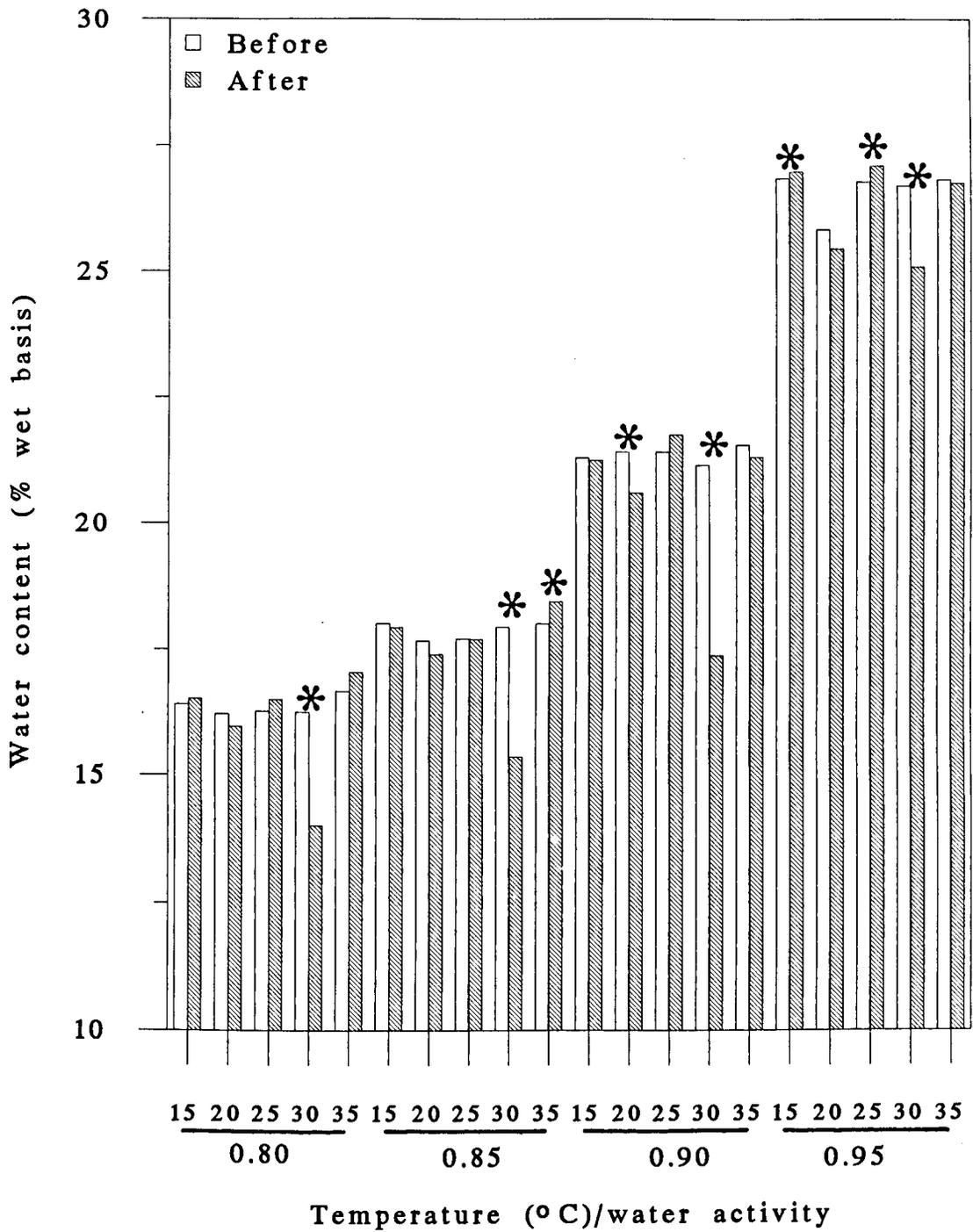


Figure 3.17 Changes in the water content (% wet basis) of wheat grain cv. Avalon during respirometer experiments at 0.80-0.95 a_w and 15-35°C.

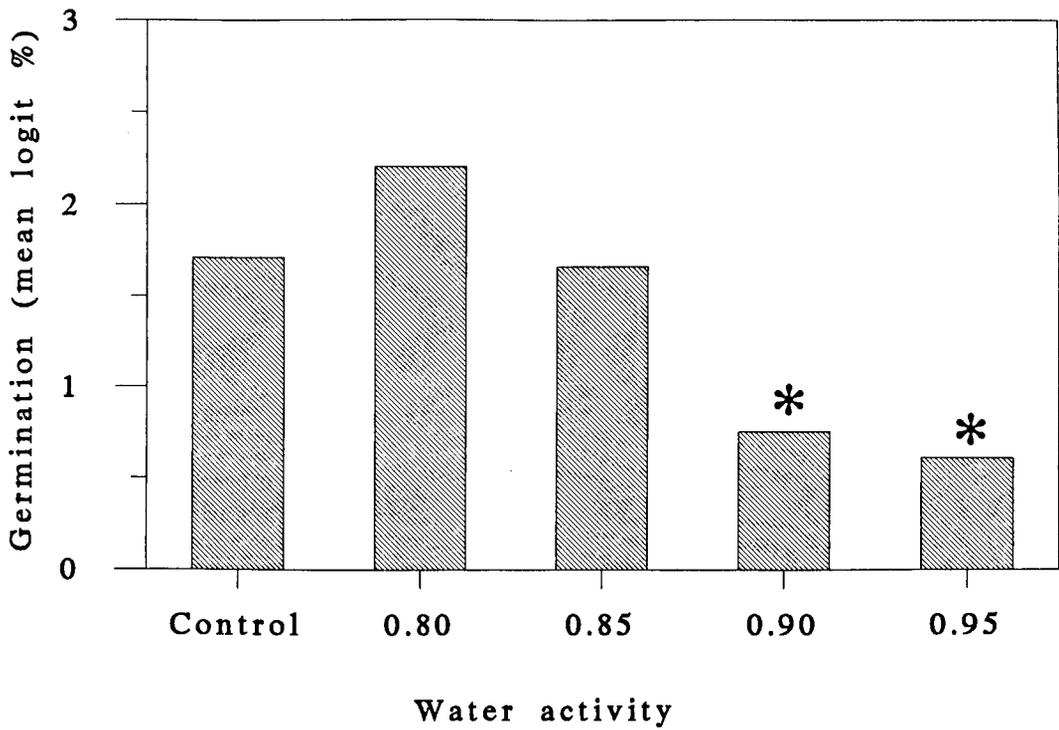


Figure 3.18 The effect of a_w on the germination of wheat grain cv. Avalon incubated for 7 days at 15°C.

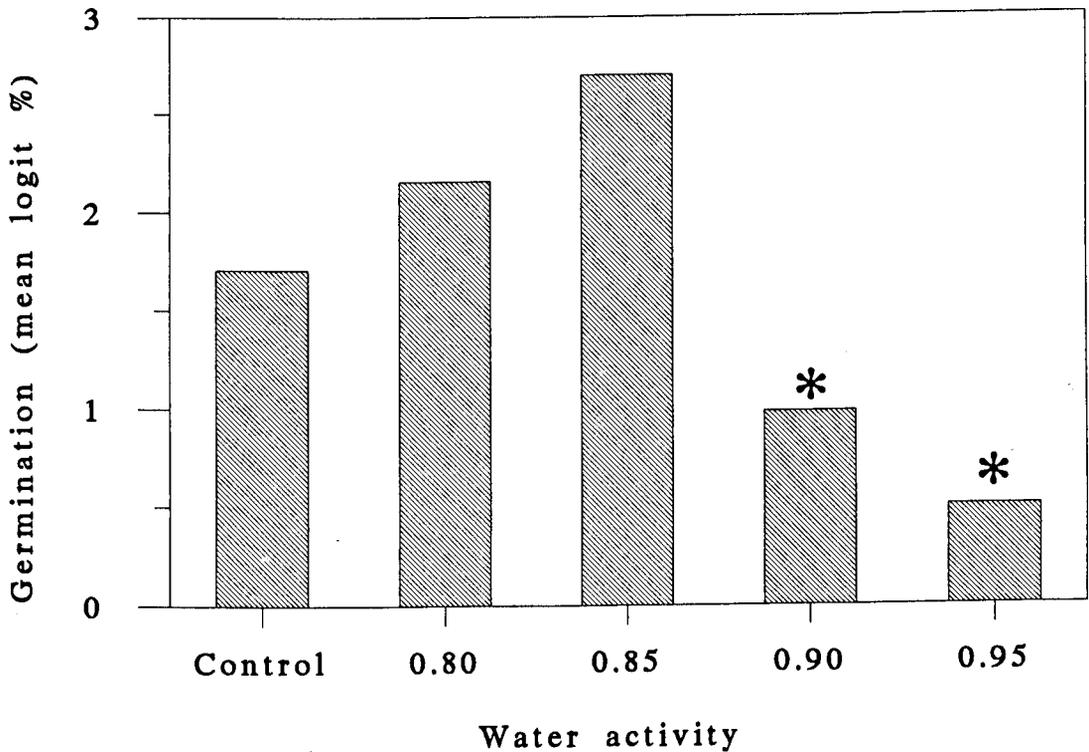


Figure 3.19 The effect of a_w on the germination of wheat grain cv. Avalon incubated for 7 days at 20°C.

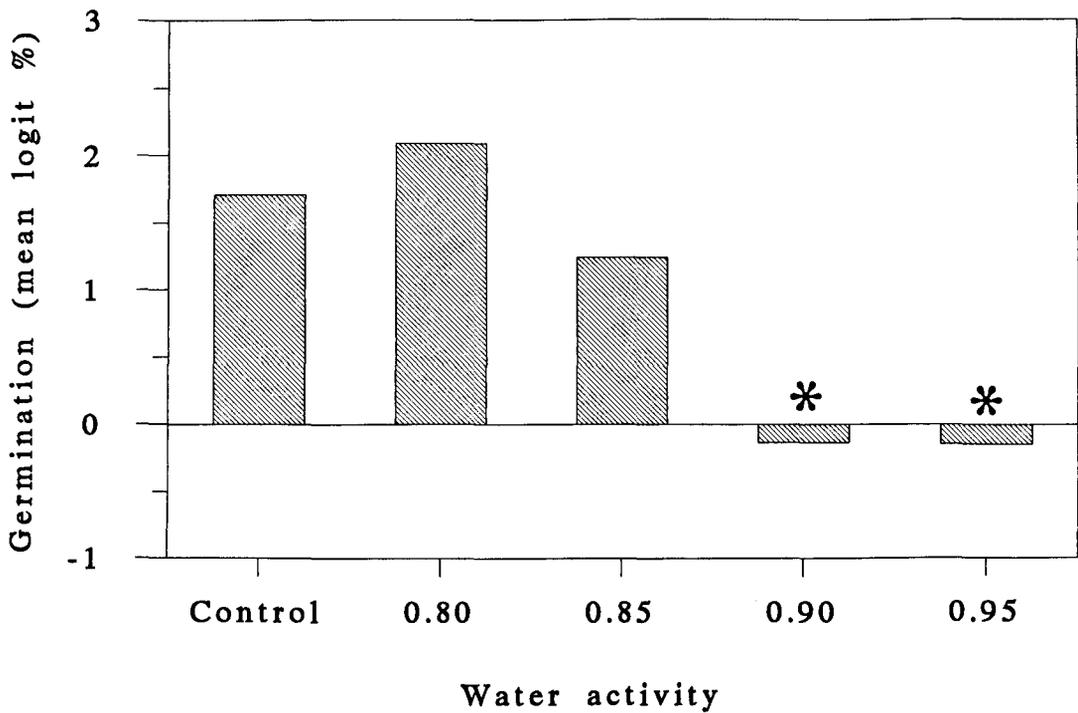


Figure 3.20 The effect of a_w on the germination of wheat grain cv. Avalon incubated for 7 days at 25°C.

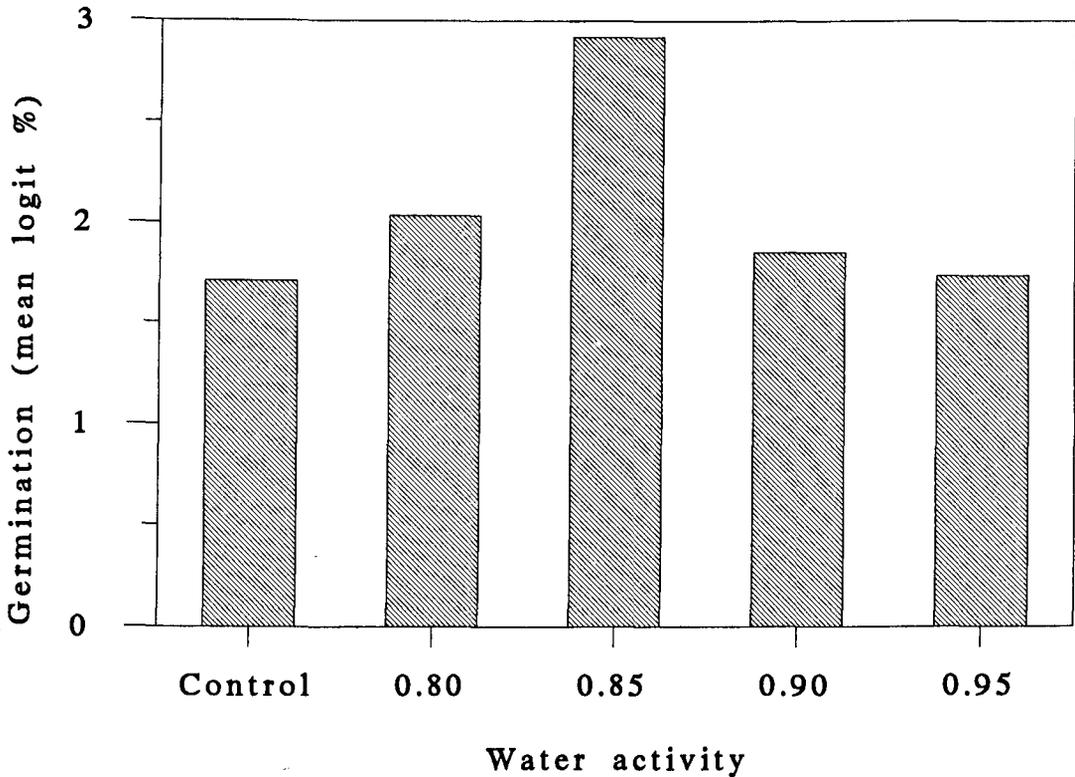


Figure 3.21 The effect of a_w on the germination of wheat grain cv. Avalon incubated for 7 days at 30°C.

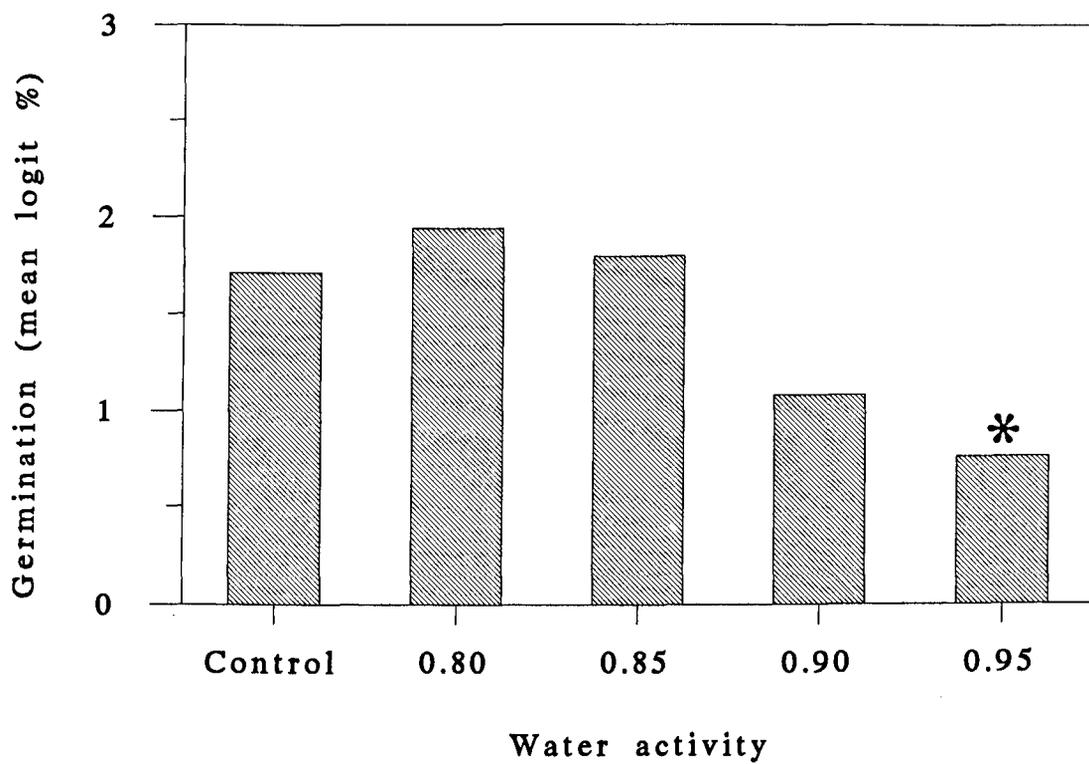


Figure 3.22 The effect of a_w on the germination of wheat grain cv. Avalon incubated for 7 days at 35°C.

was least in samples incubated at 0.95 a_w and 25°C (Figure 3.20) and highest in samples incubated at 0.85 a_w and 30°C (Figure 3.21).

Fungal colonisation

Visible moulding was recorded for each sample at the end of all respirometer experiments and, *where possible*, the fungi present were also identified to genus by eye and using a dissecting microscope (Tables 3.6-3.10). Below 0.85 a_w , only samples incubated at 35°C (Table 3.10) were visibly mouldy after 7 d incubation. All samples at 0.80 a_w were moulded, two with 1-25% of the seed surface covered and two with 51-75% of the seeds' surface covered. The lowest temperature at which visible moulding occurred at 0.85 a_w was 25°C with 1-25% of the surface area colonised by a mixture of *Eurotium* and/or *Aspergillus* species.

At 0.90 a_w , only samples incubated at 20°C or above were visibly moulded with fungal growth, with the area of seed surface colonised increasing as temperature increased. Greatest visible moulding occurred at 0.90 a_w /35°C where three samples (out of four) had 76-100% of their grains covered with visible mycelium. At 15°C, the only samples moulded were those incubated at the highest a_w (0.95 a_w) where half the samples had their grains covered with 1-25% visible mould (including *Fusarium* species). Five of the eight samples incubated at 0.95 a_w /20°C were affected in the same way as 0.95 a_w /15°C but the occurrence of *Penicillium* species was also noted in one sample. All samples incubated at 0.95 a_w /25°C were over 50% mouldy with species of *Penicillium* and *Eurotium* comprising the visible microflora. At 0.95 a_w /30°C and 0.95 a_w /35°C, 75% of grains in all samples were visibly moulded.

Three replicates of ten seeds from each replicate respirometer sample were plated onto agars at the same a_w and temperature as they had been incubated at in the respirometer study. Figures 3.23-3.29 show the occurrence of fungi isolated, compared with the fungal flora of clean, unhydrated (less than 0.65 a_w) grain. Control data in Figures

Table 3.6 The effect of a_w on the visible moulding of wheat grain cv. Avalon incubated for 7 days at 15°C.

A_w	Number of samples (out of 8) with the following percentage of grains visibly moulded:				
	0%	1-25%	26-50%	51-75%	76-100%
0.80	8	0	0	0	0
0.85	8	0	0	0	0
0.90	8	0	0	0	0
0.95	4	4 <i>F</i>	0	0	0

F = *Fusarium* spp.

Table 3.7 The effect of a_w on the visible moulding of wheat grain cv Avalon incubated for 7 days at 20°C.

A_w	Number of samples (out of 8) with the following percentage of grains visibly moulded:				
	0%	1-25%	26-50%	51-75%	76-100%
0.80	8	0	0	0	0
0.85	8	0	0	0	0
0.90	6	2	0	0	0
0.95	3	5 (1 <i>P</i> , 4 <i>F</i>)	0	0	0

F = *Fusarium* spp., *P* = *Penicillium* spp.

Table 3.8 The effect of a_w on the visible moulding of wheat grain cv Avalon incubated for 7 days at 25°C.

A_w	Number of samples (out of 8) with the following percentage of grains visibly moulded:				
	0%	1-25%	26-50%	51-75%	76-100%
0.80	8	0	0	0	0
0.85	0	6 (2E, 4A)	2 E	0	0
0.90	0	0	6 P, E, A	2 A, E	0
0.95	0	0	0	5 P, E	3 P, E

A = *Aspergillus* spp., *E* = *Eurotium* spp., *P* = *Penicillium* spp.

Table 3.9 The effect of a_w on the visible moulding of wheat grain cv Avalon incubated for 7 days at 30°C.

A_w	Number of samples (out of 4) with the following percentage of grains visibly moulded:				
	0%	1-25%	26-50%	51-75%	76-100%
0.80	4	0	0	0	0
0.85	0	0	0	4	0
0.90	0	0	0	4	0
0.95	0	0	0	0	4

Table 3.10 The effect of a_w on the visible moulding of wheat grain cv Avalon incubated for 7 days at 35°C.

A_w	Number of samples (out of 4) with the following percentage of grains visibly moulded:				
	0%	1-25%	26-50%	51-75%	76-100%
0.80	0	2	0	2	0
0.85	0	0	1	2	1
0.90	0	0	0	1	3
0.95	0	0	0	0	4

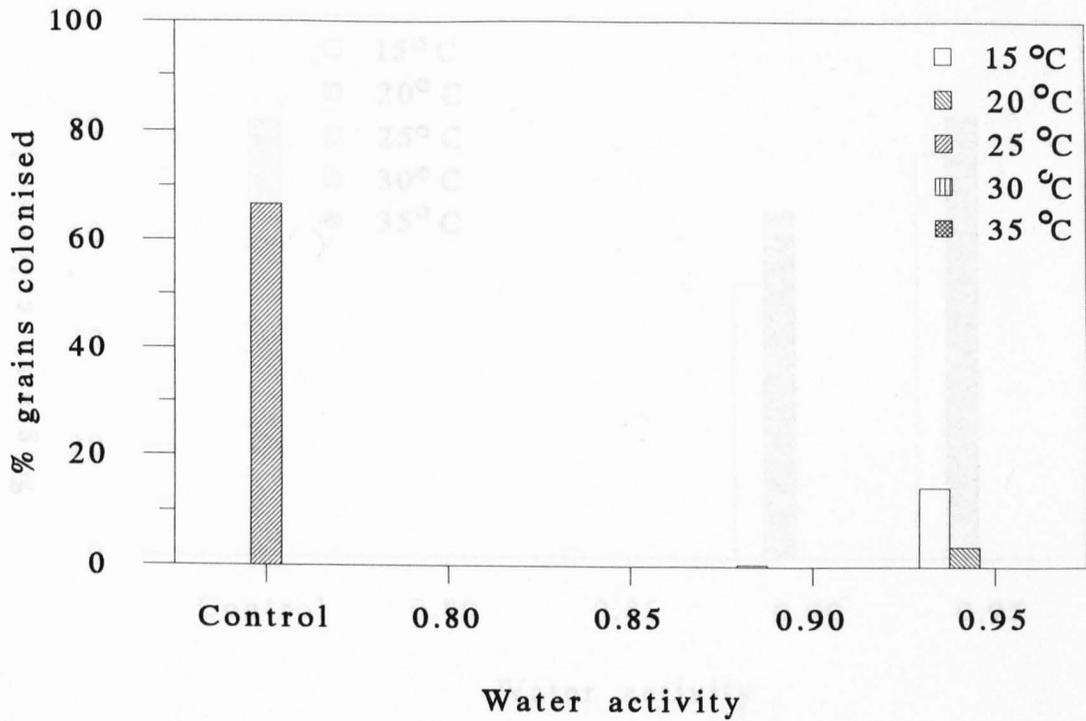


Figure 3.23 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Alternaria* spp. after 7 days incubation.

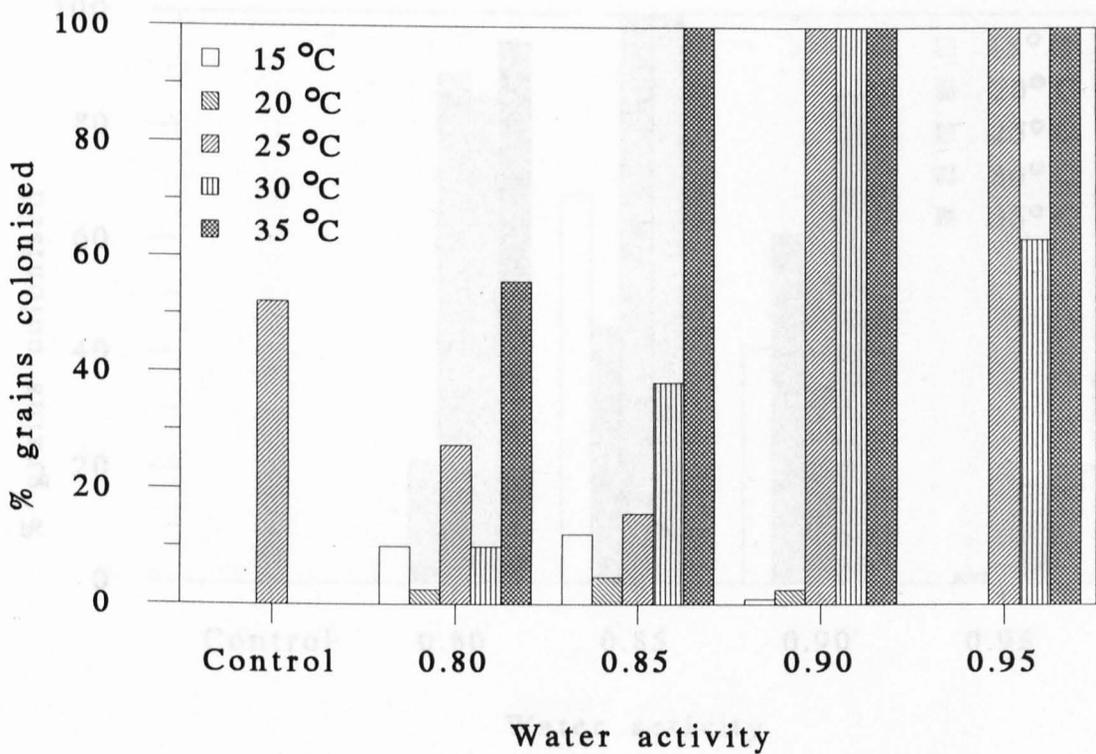


Figure 3.24 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Aspergillus* spp. after 7 days incubation.

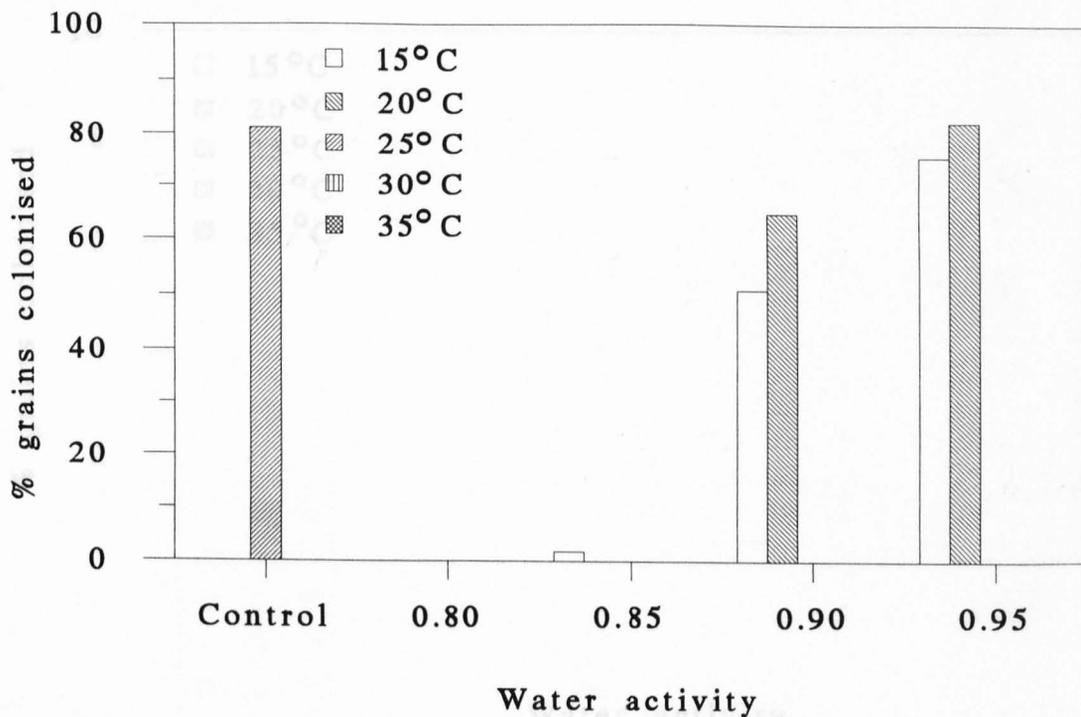


Figure 3.25 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Cladosporium* spp. after 7 days incubation.

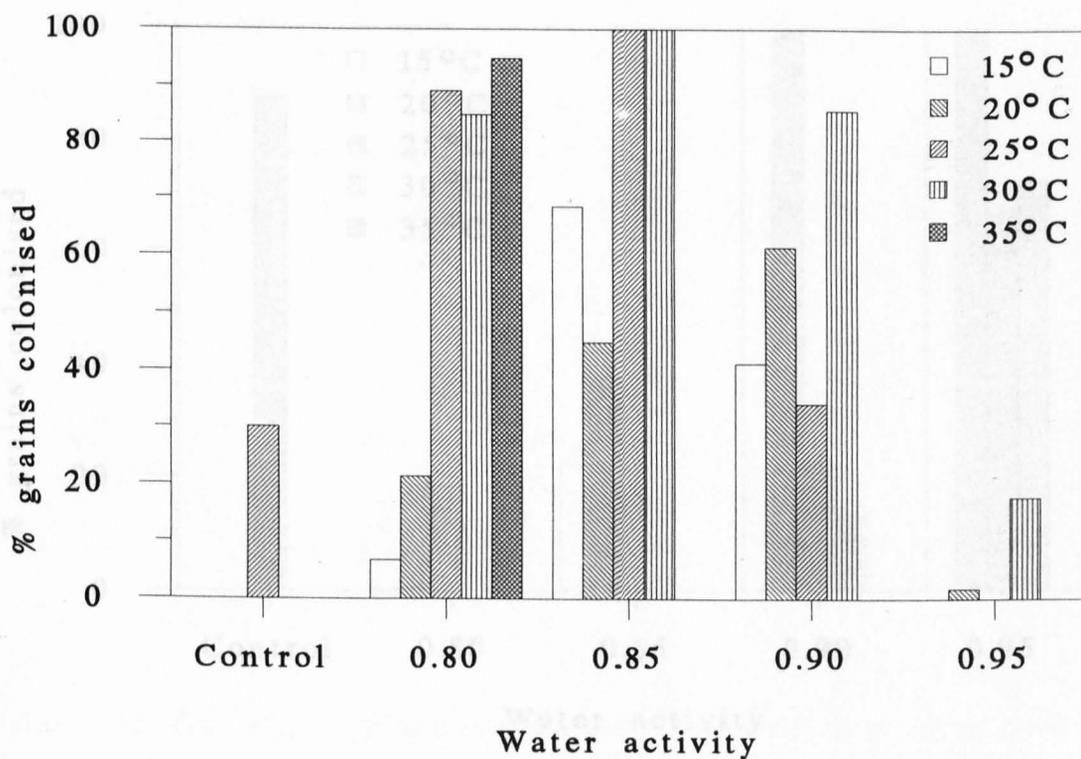


Figure 3.26 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Eurotium* spp. after 7 days incubation.

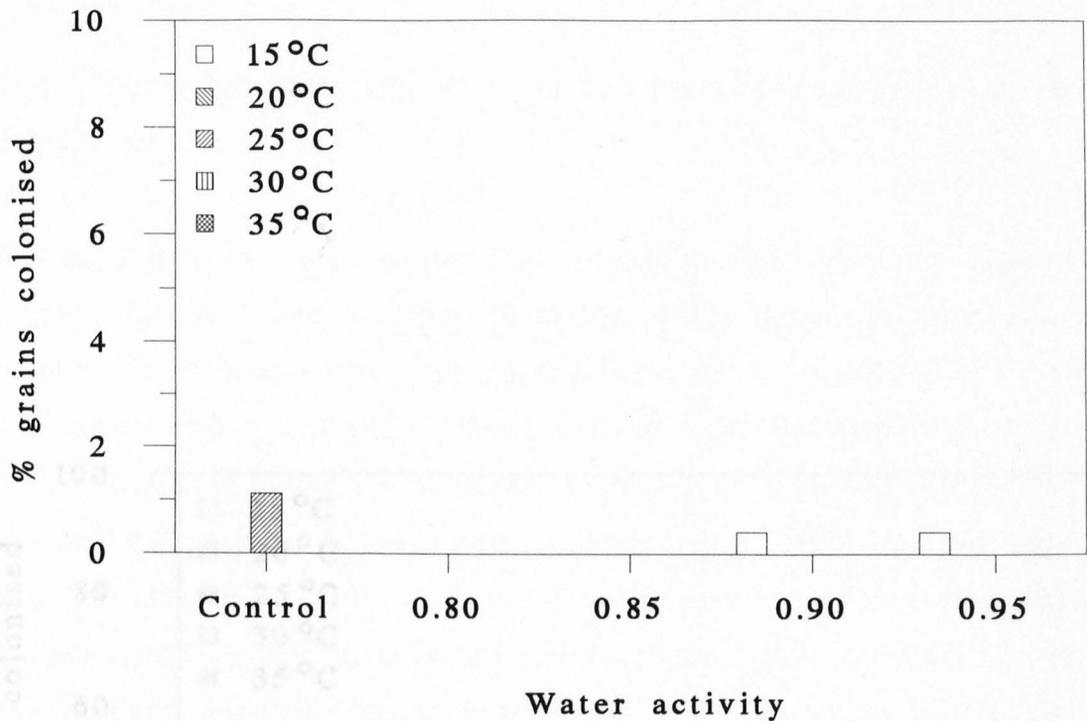


Figure 3.27 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Fusarium* spp. after 7 days incubation.

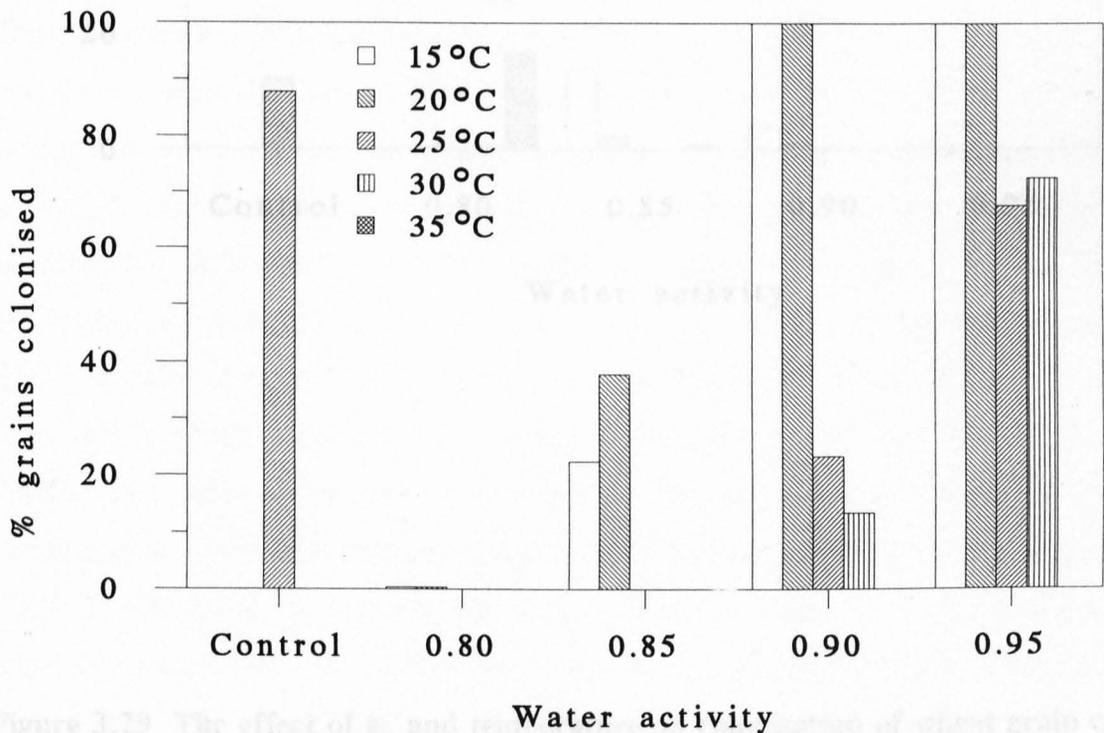


Figure 3.28 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Penicillium* spp. after 7 days incubation.

Figure 3.28 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Penicillium* spp. after 7 days incubation.

3.23-3.29 have been determined by taking the highest incidence from all media and temperatures used.

The most abundant fungi isolated after incubation were *Alternaria*, *Aspergillus*, *Cladosporium*, *Eurotium*, *Fusarium*, *Penicillium* and *Wallemia* spp. *Alternaria* spp. (Figure 3.23) were only isolated from 0.90 a_w /15°C and 0.95 a_w /15 and 30°C, having been isolated from control grain. Other field fungi in the *Cladosporium* genus (Figure

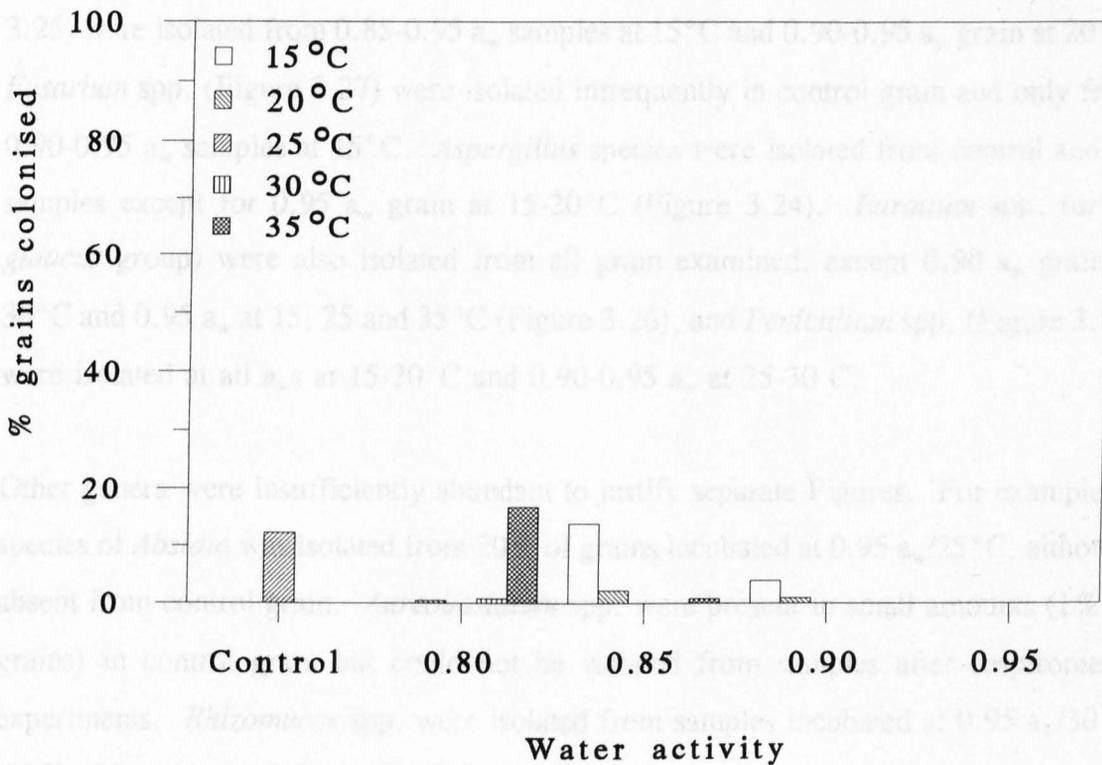


Figure 3.29 The effect of a_w and temperature on colonisation of wheat grain cv. Avalon by *Wallemia* spp. after 7 days incubation.

Figure 3.30 shows the species of fungi isolated at one set of environmental conditions, i.e. 0.90 a_w and 20°C. *Aspergillus* spp. comprised of *A. candidus*, *A. versicolor*

3.23-3.29 have been determined by taking the highest incidence from all media and temperatures used.

The most abundant fungi isolated after incubation were *Alternaria*, *Aspergillus*, *Cladosporium*, *Eurotium*, *Fusarium*, *Penicillium* and *Wallemia* spp. *Alternaria* spp. (Figure 3.23) were only isolated from 0.90 a_w /15°C and 0.95 a_w /15 and 20°C, having been isolated from control grain. Other field fungi in the *Cladosporium* genus (Figure 3.25) were isolated from 0.85-0.95 a_w samples at 15°C and 0.90-0.95 a_w grain at 20°C. *Fusarium* spp. (Figure 3.27) were isolated infrequently in control grain and only from 0.90-0.95 a_w samples at 15°C. *Aspergillus* species were isolated from control and all samples except for 0.95 a_w grain at 15-20°C (Figure 3.24). *Eurotium* spp. (or *A. glaucus* group) were also isolated from all grain examined, except 0.90 a_w grain at 30°C and 0.95 a_w at 15, 25 and 35°C (Figure 3.26), and *Penicillium* spp. (Figure 3.28) were isolated at all a_w s at 15-20°C and 0.90-0.95 a_w at 25-30 C.

Other genera were insufficiently abundant to justify separate Figures. For example, a species of *Absidia* was isolated from 20% of grains incubated at 0.95 a_w /25°C, although absent from control grain. *Aureobasidium* spp. were present in small amounts (1% of grains) in control grain but could not be isolated from samples after respirometer experiments. *Rhizomucor* spp. were isolated from samples incubated at 0.95 a_w /30°C (81% of grains) and 0.95 a_w /35°C (89% of grains) but were absent from control grain. The frequency of isolation of the pink yeast *Sporobolomyces roseus* decreased from about 1% in clean grain to 0.5% in samples incubated at 0.95 a_w /15°C, 20°C and 25°C. Two other species not isolated from clean grain but found in respirometer samples were *Syncephalastrum* spp. (10.8% of grains from 0.95 a_w /30°C samples) and *Trichoderma* spp. (6.7% of grains from 0.95 a_w /35°C). Finally, 3.3% of control grains were contaminated with *Ulocladium* spp. which increased to 17.9% of grains in samples incubated at 0.95 a_w and 20°C.

Figure 3.30 shows the species of fungi isolated at one set of environmental conditions, i.e. 0.90 a_w and 20°C. *Aspergillus* spp. comprised of *A. candidus*, *A. versicolor*.

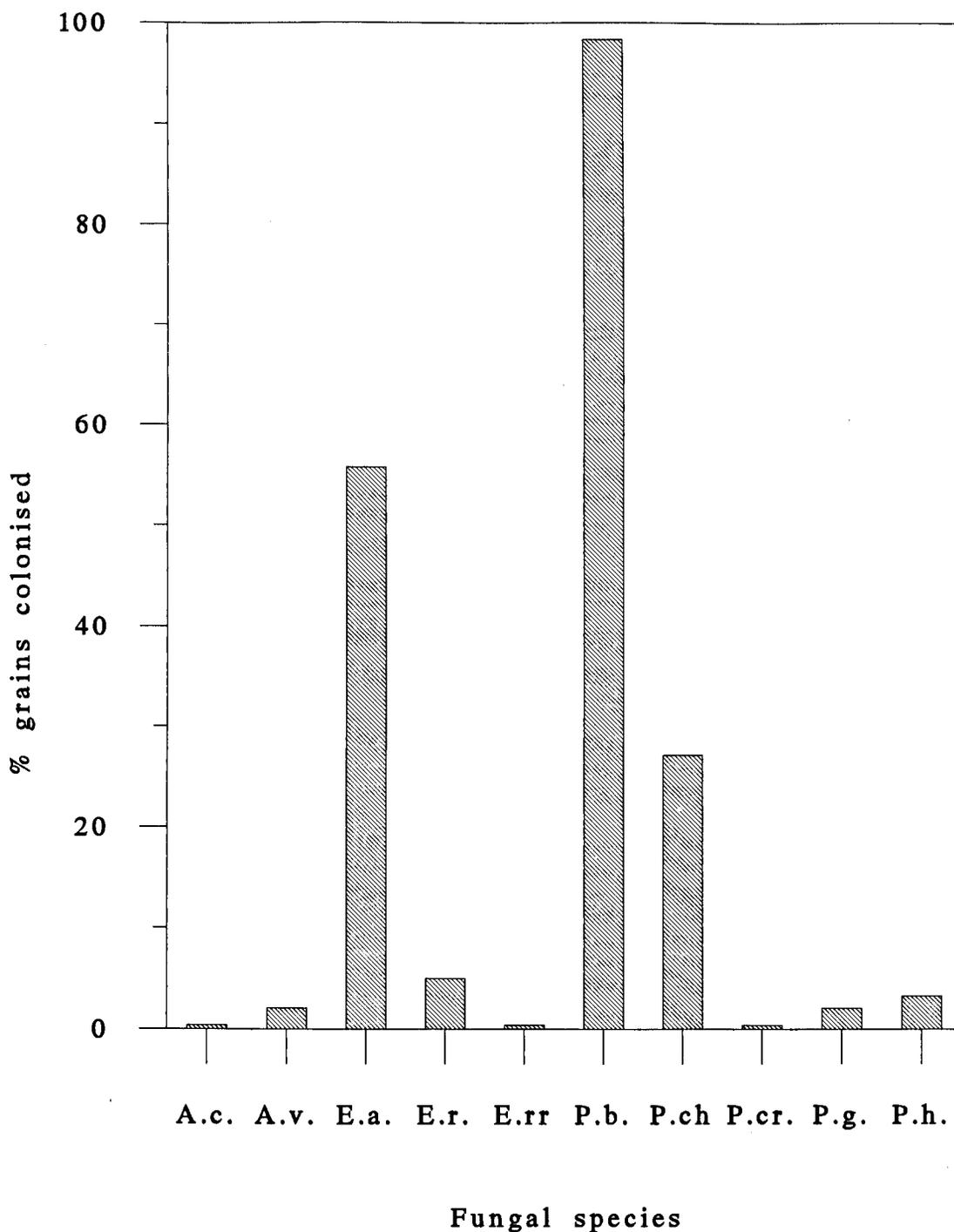


Figure 3.30 The colonisation of wheat grain cv. Avalon by different species of storage fungi at 0.90 a_w and 20°C. Abbreviations represent the following species: A.c. - *Aspergillus candidus*, A.v. - *Aspergillus versicolor*, E.a. - *Eurotium amstelodami*, E.r. - *Eurotium repens*, E.rr. - *Eurotium rubrum*, P.b. - *Penicillium brevicompactum*, P.ch. - *Penicillium chrysogenum*, P.cr. - *Penicillium crustosum*, P.g. - *Penicillium granulatum* and P.h. - *Penicillium hordei*.

Eurotium amstelodami formed most of the *Eurotium* spp. isolated, with *E. repens* and *E. rubrum* being less common. The identity of the *Penicillium* species recorded were verified by the International Mycological Institute and of these *P. brevicompactum* Dierckx, (IMI 344561) was most common with the presence of *P. chrysogenum* Thom (IMI 344560), *P. crustosum* Thom (IMI 344563), *P. granulatum* Bainier (IMI 344562) and *P. hordei* Stolk (IMI 344568) also noted.

Ergosterol as an indicator of fungal biomass

Grain samples from a preliminary respirometer experiment (Section 3.1.2) were assayed for ergosterol using an HPLC technique (Section 2.1.10). Figure 3.31 shows that ergosterol content in grain ($\mu\text{g g}^{-1}$ dry matter) increased from $3.7 \mu\text{g g}^{-1}$ to $10 \mu\text{g g}^{-1}$ dry grain as a_w increased from 0.6 - 0.95 a_w .

AFB₁ content of culture filtrates of A. flavus isolated from respirometer experiments

Four cultures of *Aspergillus flavus* were isolated from treatments incubated at 25°C and 0.90-0.95 a_w and a fifth was isolated from unhydrated wheat grain. Four replicate cultures of each of the five isolates (described in Table 3.11) were tested for the presence of AFB₁ using an ELISA (Section 2.1.11). A preliminary test to determine the optimum antibody concentration was first conducted using concentrations of 1:1,000, 1:2,000 and 1:4,000. An antibody concentration of 1:2,000 gave the best results (Figure 3.32) and was used to derive a standard curve for determining AFB₁ concentration in culture filtrates (Figure 3.33). The limits of detection were absorbances in the range of 0.232-1.774 at 450 nm, corresponding to a toxin concentration of 0-20 ng AFB₁ ml⁻¹, but none of the three replicate wells assayed per sample gave an absorbance value within these limits.

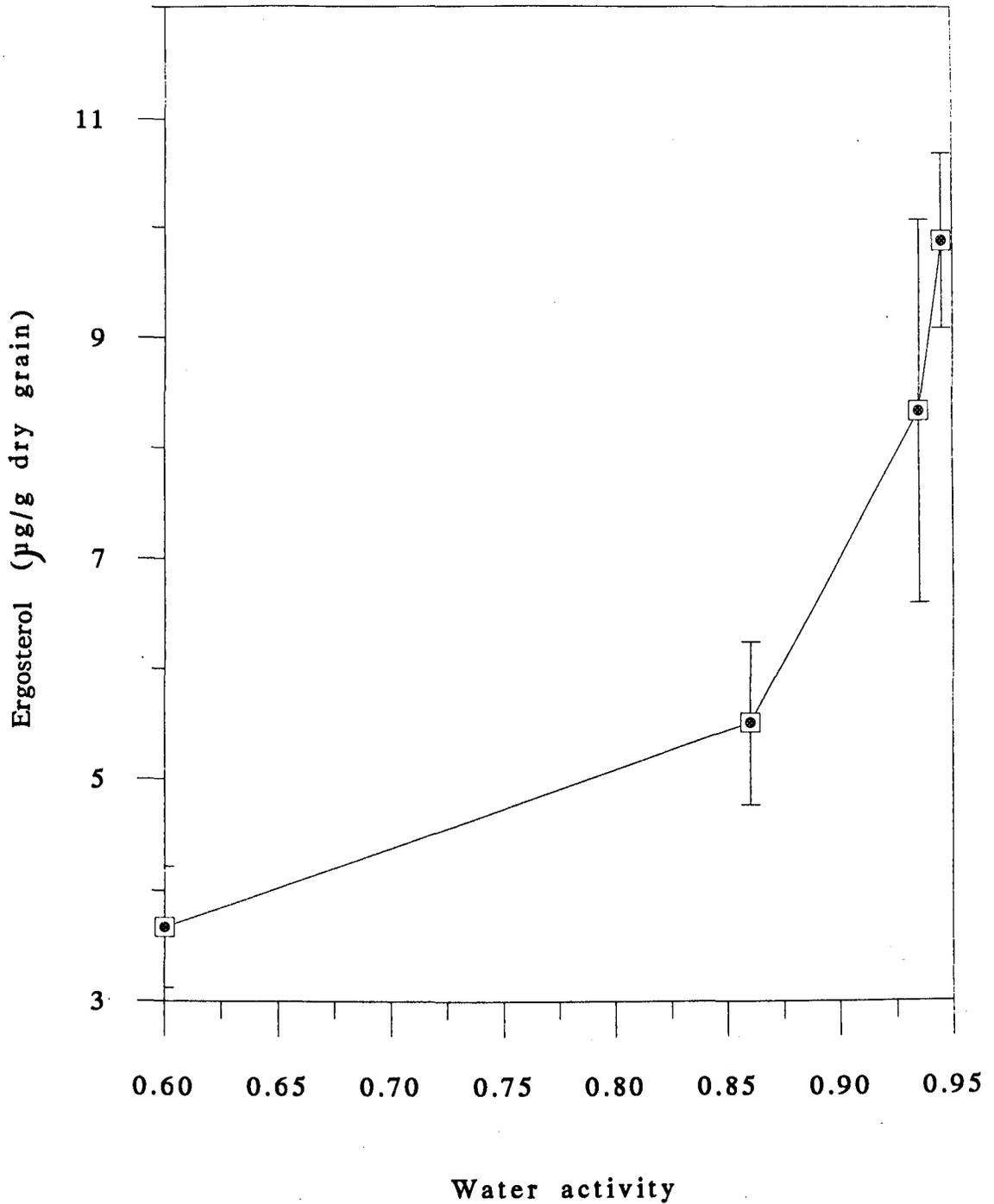


Figure 3.31 The effect of a_w and temperature on the ergosterol content of wheat grain cv. Avalon incubated at 20-30°C for 7 days.

Table 3.11 The source of *Aspergillus flavus* isolates screened for aflatoxin B₁ production.

Isolate Number	Source of isolate (length, temperature and a _w of incubation)	Mean absorbance ± SEM (n=12)	ng AFB ₁ g ⁻¹ sample (Figure 3.33)
1	Fresh wheat cv. Avalon (1989)	1.9422 ± 0.0352	0
2	7 d, 25°C, 0.90 a _w	1.7690 ± 0.0698	0
3	7 d, 25°C, 0.95 a _w	1.8777 ± 0.389	0
4	7 d, 25°C, 0.90 a _w	1.8847 ± 0.0408	0
5	7 d, 25°C, 0.95 a _w	2.8835 ± 0.9871	0

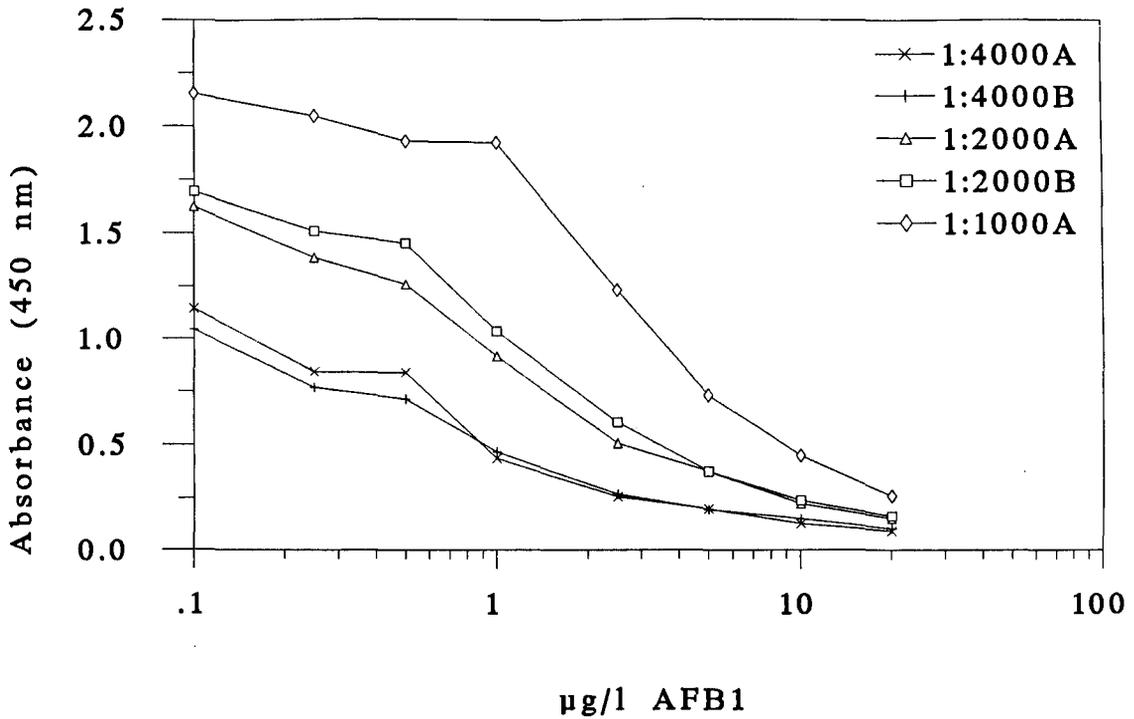


Figure 3.32 Preliminary standard curves for aflatoxin B₁ screen of *Aspergillus flavus* isolates.

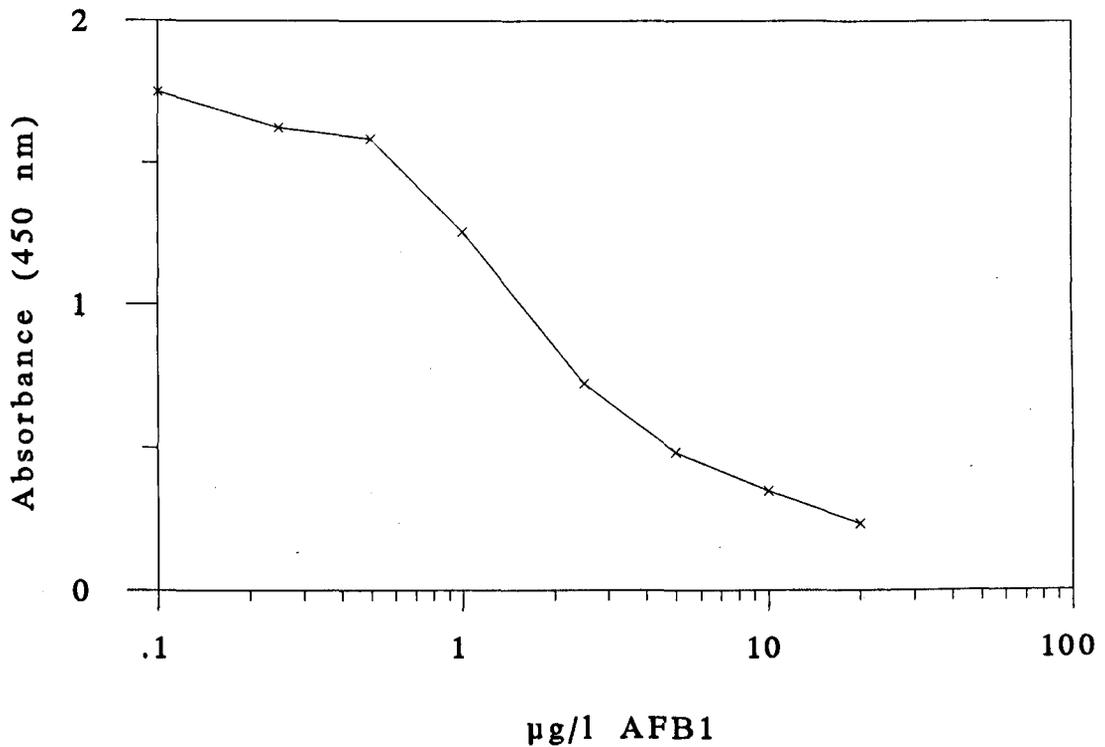


Figure 3.33 Standard curve used in aflatoxin B₁ screen of *Aspergillus flavus* isolates (antibody concentration 1:2,000).

3.4 DISCUSSION

3.4.1 *The electrolytic respirometer*

Numerous problems were encountered during the first 6-9 months of research (Section 3.1) that needed to be overcome before the electrolytic respirometer could be used efficiently with stored grain. Modifications to its data sampling and recording programs, along with other apparatus-limited features, had lost or masked results and had to be resolved. Eventually the system was successfully optimised for use in preliminary experiments, and this enabled a very large data set to be obtained.

Respirometer systems used in the past have required changes in the levels of liquids in manometer arms to be measured to indicate O₂ uptake, and have frequently resulted in errors (Peterson *et al.*, 1956; Scherer *et al.*, 1980) and required periodic attention throughout the experiment. Similarly subject to errors are more sophisticated systems used to measure respiration in grain, soil and fungal cultures which absorb CO₂ into alkali (Beare *et al.*, 1990; Pazout & Pazoutova, 1989; Bailey, 1940; Steele *et al.*, 1969). Many early studies (for example Christensen *et al.*, 1949) used the method of Milner & Geddes (1945a) which utilised a Haldane gas analyser to determine CO₂ concentration. This allowed simultaneous monitoring of six samples, more than in earlier systems, but the apparatus had to be disconnected for 30 minutes during each sampling period to allow for gas analysis.

It is now shown that the electrolytic respirometer system used in the current studies can efficiently and accurately measure the respiration of multiple grain samples in a single experiment, with minimal human input and minimal destructive sampling required during the monitoring period. The apparatus was designed to sample data from 168 samples at once (Tribe & Maynard, 1989), but practically, it was found that only 32 samples per experiment could be set up by one person. However, even this number of samples was greater than in earlier respirometers and allowed more treatments and replicates to be studied.

In view of the range of microbial species growing on grains and their irregular distribution within grain bulks (Lacey *et al.*, 1991) data on representative sample sizes are essential to accurately assess respiration in grain. For this reason, data were obtained on respiring wheat from two replicate experiments at each set of environmental conditions (except at 35°C). Similarly it was important that the sample size itself was large enough as the sample size used of 25 g (wet weight) was limited by the size of the apparatus. Samples weighing 25 g (wet weight) were considered acceptable in these experiments as validating experiments are described later using even larger samples. Thus it is likely that the Gilson differential respirometers used by Kittock & Law (1968) and Woodstock & Justice (1967), which utilised single samples of 25 grains, would not give reproducible results in studies of microbial colonisation.

A big advantage of the electrolytic system was that it allowed measurement of both O₂ consumption and CO₂ production in the respiring samples, although CO₂, unlike O₂, could only be measured continuously in a single sample. Respiratory quotients could then be calculated from these data (discussed later in more detail). Instant response of the electrolysis cell to pressure changes in the respirometer unit was important because more CO₂ is produced in grain when shorter sampling intervals are used (Bailey, 1940; Larmour *et al.*, 1935). This relates to the removal of CO₂, because otherwise it may have replaced O₂ consumed and accumulated to inhibitory levels (Pomeranz, 1974).

Experiments reported in Chapter 3 met the proposed objective of describing, for the first time, the respiration of stored wheat grain, of known variety and quality, over the wide range of environmental conditions likely to be encountered by grain between crop harvest and the end of drying. Four samples of each treatment were usually examined in each of two replicate experiments, making the data statistically valid and suitable for incorporation into mathematical models.

3.4.2 Comparison of cereals and oilseeds

Examination of the six different grain types at comparable environmental conditions (0.88-0.90 a_w and 20°C) showed that oilseeds consumed significantly less O₂ than cereal grains despite the fact that oilseeds, having a higher surface area:volume ratio, allow more rapid gas exchange with the atmosphere. This agrees with the findings of Bailey (1940) who suggested that respiration was proportional to kernel size.

Table 3.12 compares the findings in these studies on respiration and quality losses in the six cereal and oilseed grains. Consumption of O₂ with time was at 0.90 a_w /20°C linear in all grains except for linseed, which appeared to have a longer lag phase before linear growth started. It was suggested that linseed could be stored with less loss in germinability and fungus activity than mustard seeds due to the lower activity of the former (Mondal *et al.*, 1981). However, linseed respiration has not been measured before. Linseed grains consumed significantly less O₂ than rapeseed samples, but only 62% of rapeseeds and 24% of linseed seeds were colonised by fungi before incubation. Thus, a lower initial inoculum on these seeds may have contributed to their slower respiration and longer safe storage than in comparison to cereal grains which were 100% colonised at the beginning of experiments. Whether this phenomenon indicated that linseed could be stored with less deterioration than rapeseed or that its lower a_w (0.88 compared with rapeseed at 0.90 a_w) was responsible, would require further investigation. Also cereals and oilseeds are very different in their composition, biochemistry and bio-degradation (Section 1.3.3), hence it is important that inappropriate comparisons are not made between the two groups.

Wheat cv. Rendezvous was harvested and placed in storage one year before the other varieties of wheat and it consumed less O₂ over 14 d than the other two varieties. Its extra year in storage is reflected by its carrying more *Eurotium* spp. before and after incubation than cvs. Avalon and Riband. The presence of more *Eurotium* spp. combined with losses in seed activity during the one years' storage were considered responsible for the lower respiration of wheat cv. Rendezvous, as Kittock & Law (1968) showed that 17% less respiration was recorded from wheat grain that had been

Table 3.12 Summary of data on the respiration and quality losses in cereal grains and oilseeds (presented in full in Section 3.3) ** = most; * = least; Y = present; N = absent; L = linear; C = non-linear; P, P2 = 100% or 50-99% grains colonised by *Penicillium* spp.; E, E2 = 100% or 50-99% grains colonised by *Eurotium* spp.**

Parameter	Grain type					
	Wheat, Avalon	Wheat, Riband	Wheat, R'vous	Barley	Rapeseed	Linseed
Year of harvest	1990	1990	1989	1990	1990	1990
a_w	0.90	0.92	0.91	0.90	0.90	0.88
Respiration	****	****	***	***	**	*
H ₂ O increase?	Y	Y	N	N	Y	Y
Germination decrease?	Y	Y	Y	Y	N	N
Visible moulding	****	**	**	***	*	*
% grains colonised at start	100	100	100	100	62	24
Main fungi after incubn.	P -	P E2	P E	P E	P E2	P2 E
Pattern of O ₂ use with time	L	L	L	L	L	C

stored for two years after harvest, compared with that in storage for only one year.

Highest levels of respiration in wheat grain did not relate to the initial a_w of samples because cv. Avalon respired most and started at 0.90 a_w . Cv. Riband respired similarly but its initial a_w was 0.92, while cv. Rendezvous had an initial a_w of 0.91 and respired least of the wheat varieties. Additionally, therefore, there may be differences in the respiratory activity of different wheat varieties. Varietal differences in susceptibility to moulding have been reported in maize (Cantone *et al.*, 1983) but such properties could not be detected in rice (Ilag & Juliano, 1982).

Percentage DML resulting from respiration of the four cereal grains after 14 d incubation ranged from 0.283% (barley) to 0.371% (wheat cv. Avalon). Table 3.12 compares respiration (and hence DML) in the six grain types and summarises measured losses in quality in the same samples. High levels of respiration occurred where there were high levels of visible moulding and often significant decreases in germination, for example in wheat cv. Riband. It is not *directly* evident from these results whether decreasing germination occurred in cereals as a result of fungal colonisation, as only crude assessments of fungi could be made, *but* since decreased germination correlated with increasing respiration, fungi must have contributed most to this respiration as they colonised internal parts of the grain including the embryo.

Approximately 3.8 litres O_2 were consumed over 14 d by rapeseeds which was close to that measured by Magan (1991) and Schmidt & Jacobsen (1982) (Nellist, 1993). Kreyger (1972) considered these conditions acceptable for safe storage but small amounts of visible mould growth were observed on samples, hence this is debatable. Germination remained unaffected in the oilseeds after incubation and they were least moulded of the six after the experiment; perhaps because they have melanin-rich testae to be penetrated and are richer in fatty acids than carbohydrates, which are less readily metabolised.

There are little previously published data on barley grain respiration but these data

agree with the conclusions of Kreyger (1972) *in principle* in that less DML occurs in barley grain compared with wheat. Hence barley can be stored longer than wheat before similar losses in germination or visible moulding occur. The lower respiration rates in barley grain may also indicate that it is less easily penetrated by fungi, because of the protection afforded by the physical structure of the lemma and palea surrounding the seed which are not found in wheat grain. These two components comprise 13% of the barley seed and, in addition, barley grain has a thicker aleurone layer than wheat grain (Kent, 1978).

Kreyger (1972) considered a maximum of 2% DML and no visible moulding to be acceptable in barley grain. Grain stored at 23% water content (equivalent to 0.90 a_w) and 15°C was visibly mouldy after only 2.5 weeks, during which period 0.46% dry matter was lost. In the current study, less than 0.283% dry matter (i.e. less than 0.46%) was lost after two weeks storage at 0.90 a_w and 25°C, although a greater DML might have been expected at the higher temperature. Additionally, these samples were heavily moulded and germination had decreased significantly. The extensive study of Ramakrishna (1990) showed that barley grain inoculated with mycotoxigenic strains of fungi contained unacceptable levels of aflatoxin B₁, T-2 toxin and ochratoxin A after 14 d at 0.90 a_w /20°C, the actual levels depending on interactions between the different species present. Therefore, the extensive use of Kreyger's (1972) predictions is of concern as it has been found practically that barley grain may currently be considered to be 'safely stored' when it clearly is not. Further discussion below shows that wheat grain has been subject to the same problems.

In these studies storage fungi were isolated with greater frequencies on M90 agar than MEA. This suggests that M90 would be a more appropriate medium to use in future experiments. All cereal grains of all types were contaminated with *Penicillium* spp., with *Eurotium* spp. isolated less commonly, especially in the seeds that respired most i.e. wheat cv. Avalon and cv. Riband. 100% of the cereal seeds with lower respiration (cvs. Riband and barley) carried *Eurotium* spp. It therefore appears that *Eurotium* spp. were out-competed by *Penicillium* spp. in rapidly respiring grain even though

environmental conditions were similar.

Among the cereals, high respiration occurred with significant increases in water content and moderate *Eurotium* colonisation (wheat cvs. Avalon and Riband). Lower respiration correlated with no significant increase in water content and 100% *Eurotium* colonisation (wheat cv. Rendezvous and barley).

No oilseeds showed significant decreases in germination but water content increased significantly. The highest respiration of the two (rapeseed) occurred where there was 100% *Penicillium* colonisation and moderate *Eurotium* colonisation. Conversely, in linseed there was 100% *Eurotium* and less *Penicillium*. This may be because linseed seeds were incubated at a slightly lower a_w , favoured by *Eurotium* spp., than rapeseed.

3.4.3 Respiration of wheat grain

The type of respiration curve produced by samples with $a_w < 0.90$ at some temperatures (15-25°C) gave a curve with a similar basic shape to that described by others (Peterson *et al.*, 1956; Fernandez *et al.*, 1985) and with the exponential parts of the curves similar to those described in wheat (Bailey, 1940; Srour, 1988). The graphs at 15-35°C and high a_w and 30-35°C with all a_w values were linear and probably corresponded to the linear part of curved graphs, with the initial slower phase having taken place rapidly because of the elevated a_w or temperature; possibly even during refrigerated equilibration. Seitz *et al.* (1982b) also found a linear relationship between DML and time over 14 d storage at 24.9°C and 23.9% water content for fungus-free maize grain and for naturally contaminated grain, but when the grain had been surface sterilised, re-inoculated with *Aspergillus flavus* and stored at the same conditions, a pattern of respiration occurred which was similar to the pattern seen in these studies at low a_w and temperature.

Total respiration data were examined in relation to previously published data in which CO₂ production had been measured with many types of apparatus, sampling methods and varieties of grain. Figure 3.34 compares some of this published data with relevant

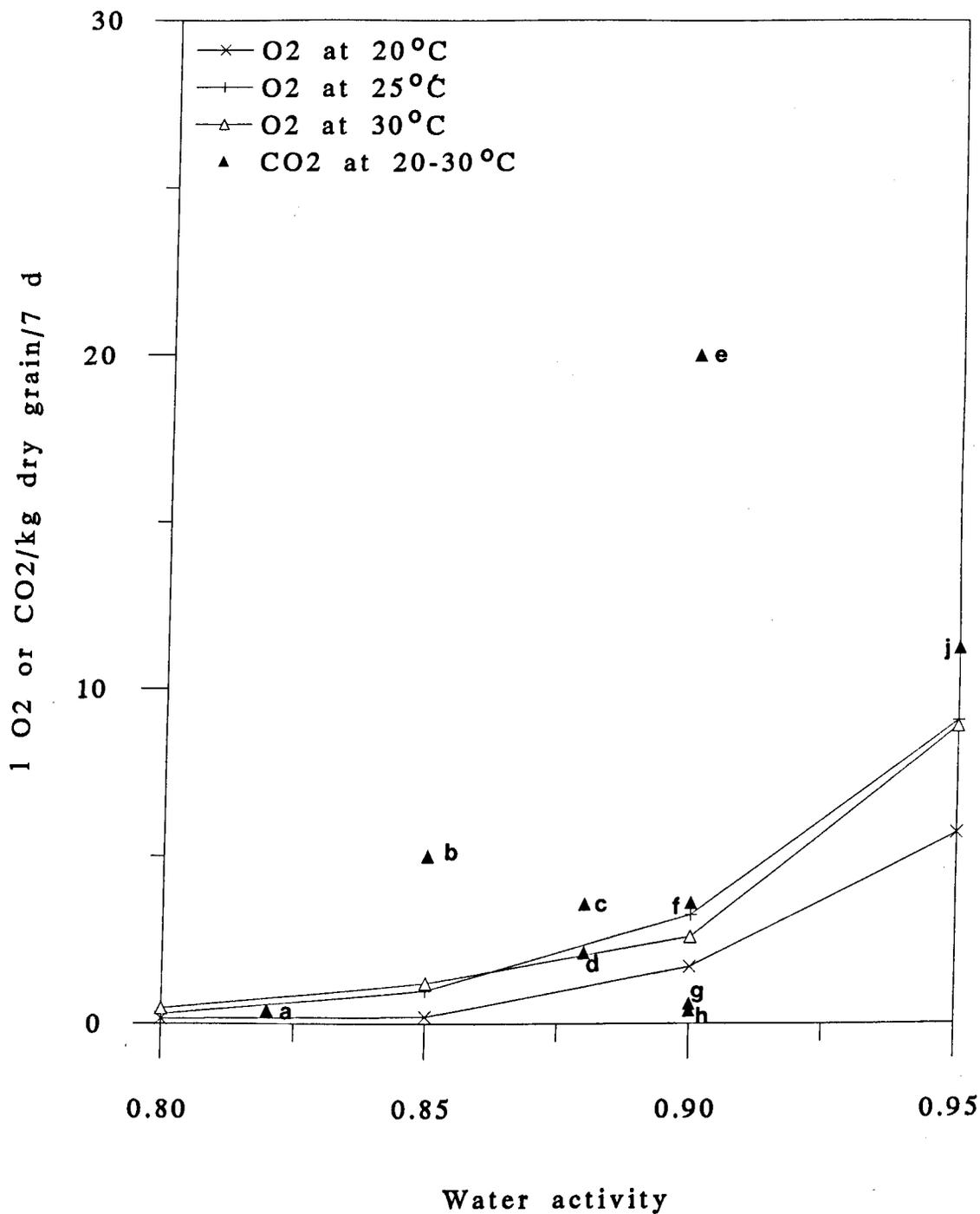


Figure 3.34 The respiration of wheat grain cv. Avalon measured in the electrolytic respirometer over 7 d compared with previously published data on wheat grain respiration. Data points represent the following published findings: a) Bailey (1940); b), e) Scholz (1962); c), f) Milner *et al.* (1947a); d) Larmour *et al.* (1935); g), h) White *et al.* (1982a); j) Woodstock & Justice (1967).

examples of O₂ consumption data from these experiments. Respiration rates described by Scholz (1962) and Milner *et al.* (1947b) were much faster than in these experiments and out of the range of the other data in Figure 3.34. Faster respiration was also found by Kittock & Law (1968) but their studies measured respiration of the seed during seedling emergence which may be higher than that attributable to the grain and its resident microflora during storage. Data of White *et al.* (1982a) are rather lower than those determined with the electrolytic respirometer. They used an accurate method of CO₂ detection (gas chromatography) and large sample sizes, but respiration vessels were only sampled three times weekly at 20°C and five times weekly at 30°C. This may have caused CO₂ levels to accumulate to levels inhibitory to respiration in the 2-3 d periods between sampling, and to an uneven extent between samples at different temperatures. With the exception of the results of the above and those of Scholz (1962), which will be discussed later in relation to acceptable levels of safe storage, previously published data on the respiration of wheat grain under a limited number of sets of environmental conditions are in agreement with the detailed new findings from studies with the electrolytic respirometer, bearing in mind the differences in experimental methods used.

For wheat grain cv. Avalon, a linear relationship was formed between ln(modified respiration:temperature) with increasing 'predicted' a_w. This agreed with the findings of Schmidt & Jacobsen (1982) who showed a similar relationship with respiring rapeseed. The O₂ consumption data had been transformed mathematically to take account of the effect of incubation temperature on a_w, but as well as transforming a_w at 15-20°C and 30-35°C, the calculation also modified the 'predicted' a_w at 25°C from its verified empirical value at 25°C. The linear relationship suggested that it would be feasible to develop a mathematical model of total respiration over a known timescale under defined conditions, but the observed modification of the a_w values at 25°C illustrates how models can sacrifice accuracy in some areas when standardising the whole data set.

Muck *et al.* (1991) proposed a two-segment linear model of fungal growth on silage.

They standardised microbial growth rates from other published material (e.g. Magan & Lacey, 1986; Hocking & Pitt, 1979) and showed there was a linear increase in the growth rate of yeasts and fungi below 0.99 a_w , and that from 0.99-1.00 a_w growth rates increased more slowly.

The more detailed relationship between respiration, environmental conditions and time proposed in Section 3.3.1 c) will hopefully be used to predict how much O_2 would be consumed by samples at a given time during storage. The validity of this model is under investigation along with its potential for incorporation into a strategy for the simulation and optimisation of near-ambient grain drying. This project is still in progress and the validation of this or another model is enthusiastically awaited.

3.4.4 Respiratory quotient in wheat grain

The respiratory quotients (RQ) calculated for most wheat grain (cv. Avalon) samples were generally < 1.0 . They were higher and closer to 1.0 at 0.95 a_w compared with 0.80 a_w , at all temperatures $> 15^\circ C$. However, at $15^\circ C$, RQ decreased from 5.13 to 1.18 with increasing a_w . Anaerobic respiration may be indicated where RQ was > 1.0 in $15^\circ C/0.80-0.85 a_w$ samples, but this would have been impossible as O_2 was supplied to and consumed in each sample, and a_w conditions were too low to allow anaerobic conditions to occur.

Practical experience with the collection of CO_2 in alkali and its subsequent analysis suggest that future investigations would require a more accurate method of CO_2 quantification to be conducted immediately after experiments. Gas chromatography (White *et al.*, 1982a) or infra-red gas analysis (Kaspersson, 1986) would be appropriate but these methods are incompatible with the electrolytic detection of O_2 used in the respirometer. It was ensured in large scale respiration experiments (Chapter 4) that NaOH was analysed immediately for absorbed CO_2 . Magan (1993) suggested that, in view of the findings of White *et al.* (1982a), the use of RQs of 1.0 may not be accurate enough for use in predictions of safe storage periods.

Possible explanations of $RQs < 1.0$ are that the methods of detecting CO_2 or O_2 were inaccurate, that samples were metabolising compounds other than carbohydrates, or that more O_2 was being utilised by fungi than CO_2 released. These will be discussed in turn:

- a) The RQ is the ratio of CO_2 produced: O_2 consumed, so with an $RQ < 1.0$ there may have been an overestimation in O_2 consumption, thus altering the ratio. As the apparatus was designed for its accuracy in O_2 detection, this is unlikely. However, if O_2 was overestimated, the DML calculated will also have been overestimated and corrections would need to be made to take this into account; for example, visible moulding with no significant loss in germination occurring in samples at $0.85 a_w$ and $25^\circ C$ with *less than* 0.13% DML. However, the effects of treatment conditions on levels of visible moulding, fungal colonisation and germination are real and valid.
- b) The $CO_2:O_2$ ratio may also have been slightly altered by an underestimation of CO_2 production. This may have happened because the titration procedures used to determine CO_2 depended on two visual assessments of colour change in chemical indicators, clearly very subjective. Secondly, titration volumes were subjected to a correction factor, so the incorrect addition of a single 0.01 ml titrated acid (the lowest limit of accuracy of the burette used) would alter the apparent volume of CO_2 produced by $\pm 20\%$ of its real value. Even if the RQ was < 1.0 due to the underestimation of CO_2 , it remains acceptable to consider the DML calculations proposed because they were based on O_2 consumption data.
- c) Lipid and protein metabolism give $RQ < 1.0$, so respiration at temperatures above $15^\circ C$ may have involved the metabolism of substrates other than carbohydrates. If this had occurred, a different biochemical pathway would have been involved instead of the conventional respiration equation and DML calculations may need to be corrected. Increases in fat acidity denote

degradation of lipids and form the basis of a grain quality test (Zeleny & Coleman, 1938), but the study did not compare increases with the appearance of microscopic or visible moulding (Magan, 1993) so it is uncertain whether low RQ in visible-mould free grain in these experiments may be due to lipid degradation. Fungi utilising seed components led to an increase in protein content in some studies (Cross & Thompson, 1971; Farag *et al.*, 1985) but its source was attributed to the fungal protein. Dry wheat grain contains 80% carbohydrate, 16% protein and 4% lipid (White *et al.*, 1982a) so that it is likely that lipids and proteins would be utilised after prolonged storage and at higher a_w and temperatures when the carbohydrate supply was depleted rather than at lower a_w and temperatures as the RQ data suggest.

- d) The first barrier encountered by the growing fungus is the pericarp which contains 93% of the cellulose in the seed (Kent, 1978) and only 30% of grain fungi may be able to digest carboxy-methyl cellulose (Flannigan 1970). At low a_w fungi could only penetrate the seed coat slowly. Their RQ could indicate species' relative ability to metabolise cellulose before they penetrate to more readily available carbohydrate components. At high a_w , the seed coat would be softened by added water allowing faster hyphal penetration and more rapid utilisation of carbohydrate in the endosperm.
- e) If the ratio of $\text{CO}_2:\text{O}_2$ is less than 1.0 and is valid, it may suggest that less CO_2 is being produced by grain and its fungi than might be expected from the amount of O_2 consumed. Respiration is not 100% efficient in grain or in any other biological system (Deacon, 1984), so that assuming that $\text{RQ} = 1.0$ may simplify what happens in nature. Fungi consume O_2 and use it with energy produced from respiration to manufacture cellular materials and increase their biomass. Cell walls which are 80-90% chitin (a polysaccharide, Bartnicki-Garcia (1968)) and secondary metabolites (such as mycotoxins and volatile compounds) all require carbon for their assembly. However, Muck *et al.* (1991) assumed that substrate was completely respired to CO_2 and water in their

modelling of microbial growth in silage.

- f) Fungi grow more slowly at low a_w than higher a_w and different species are present under different a_w conditions. Therefore, different enzyme systems may be involved and the RQ may also reflect the different fungal populations on grain samples under different conditions.
- g) A flush of CO_2 evolution accompanies the addition of water to grain (Yamamoto & Mitsuda, 1980) but it is unlikely that this would have occurred in these experiments because samples were weighed and placed into respirometer tubes after overnight equilibration and any CO_2 flushed from the sample would have been lost to the atmosphere during respirometer tube preparation (White *et al.*, 1982a).

It is assumed that the O_2 data are more accurate because the pattern of RQ values changes across the temperature ranges. It is therefore valid to draw conclusions on the basis of the O_2 data rather than in the CO_2 data although, for completeness, these are compared in Chapter 6.

Published studies most often give $\text{RQ} \geq 1.0$ even though markedly different detection methods have been used (White *et al.*, 1982a; Woodstock & Justice, 1967). It will therefore be of interest to investigate thoroughly the factors causing RQ to differ from 1.0.

3.4.5 Dry matter loss in wheat grain

Table 3.13 summarises the changes in grain quality in wheat grain cv. Avalon samples with a_w and temperature.

This study showed for the first time that, using this method, levels of DML occurring with visible moulding were less than many published accepted values for safe storage (Section 1.3.4). Results obtained here showed that, for example, at 0.90 a_w and 15°C,

Table 3.13 Summary of patterns in respiration, dry matter loss and quality parameters in wheat grain (cv. Avalon) stored at 0.80-0.95 a_w and 15-35°C for 7 d. L = linear respiration with time; V = at least one sample with any visible mould; G = germination significantly decreased compared with control; A, E, P = 100% grains contaminated with *Aspergillus*, *Eurotium* and *Penicillium* spp.; A2, E2, P2 = 50-99% grains contaminated with *Aspergillus*, *Eurotium* or *Penicillium* spp.

	Temperature (°C)														
	15			20			25			30			35		
0.80	-	-	-	-	-	-	-	-	-	L	-	-	L	V	-
	-	-	-	-	-	-	-	E2	-	-	-	E2	-	A2	E2
0.85	-	-	-	-	-	-	-	V	-	L	V	-	L	V	-
	-	E2	-	-	-	-	-	E	-	-	-	E	A	-	-
0.90	L	-	G	L	V	G	L	V	G	L	V	-	L	V	-
	-	-	P	-	E2	P	A	-	-	A	E2	-	A	-	-
0.95	L	V	G	L	V	G	L	V	G	L	V	-	L	V	G
	-	-	P	-	-	P	A	-	P2	A2	-	P2	A	-	-

0.085% DML occurred after 7 d along with a decrease in seed germination and no visible moulding. Thus storage under these conditions is unacceptable because the loss in germination indicated that fungi had invaded grains. Kreyger (1972) stated that wheat grain can be stored with no loss in germination for up to three weeks under these conditions, and for up to 1.5 weeks before the onset of visible moulding (where 0.27% DML would have occurred). It is unlikely that the 0.085% DML measured in the current samples would have increased to 0.27% DML after a further 0.5 week incubation, so these results show that *less than* 0.27% DML occurs after 1.5 weeks at 0.90 a_w /15°C but samples have suffered losses in germination before then, rather than afterwards as suggested by Kreyger (1972).

In earlier 14 d experiments conducted here, 0.371% DML occurred in samples at 0.90 a_w and 25°C with a decrease in germination and heavy moulding. Kreyger (1972), considered these conditions to be the borderline for acceptable storage for 14 d, although storage for a shorter period at low temperature or a_w would be acceptable. Again, this contrasts with these findings because at 0.85 a_w and after only 7 d, samples which had lost as little as 0.130% dry matter were visibly mouldy. Furthermore, Kreyger (1972) suggested that 1 to 2.4% DML would occur in wheat grain stored at 0.90 a_w and 25°C for 14 d. These figures are significantly greater than the 0.371% DML calculated in the present study.

Kreyger used an overall value of 1.0% DML as acceptable for 'safe storage' of wheat. Even the refined 'conservative' level of 0.5% DML widely used for safe storage of maize grain (Seitz *et al.*, 1982a) appears unacceptable for wheat in view of the above discussion. Indeed 0.5% DML is nearly four times greater than the lowest level of DML in visibly moulded grain in these experiments (0.130% DML at 0.85 a_w /25°C after 7 d). However, if losses in germination are used to indicate unacceptable levels of fungal invasion of grain in the pre-visible stages, a limit of 0.085% DML would seem more realistic from these new experiments. This agrees exactly with predictions made by Brook (1987) who showed that 0.085% DML in wheat was equivalent to 0.5% DML in maize, and should be the limit of acceptability. White *et al.* (1982a) suggested

a level even lower than that found here as the boundary of acceptability during wheat grain storage, i.e. 0.04% DML. However, respiration could have been underestimated (see above) so that the limit of acceptable DML could be only 0.04%. Only one treatment in these experiments gave a DML over 0.04% without visible moulding or decreased germination (0.80 a_w and 30°C). It is important that acceptable levels of DML are relevant to the experimental conditions used in their derivation. 0.5% DML could occur during prolonged storage at low a_w and low temperature through endogenous seed respiration where fungi do not develop which would not detrimentally affect seed quality, only its value.

These experiments show clearly that Kreyger's (1972) predictions do not represent what might happen in practice. Firstly in their use of visible moulding as a criterion to mark the end of 'safe storage', because germination may indicate fungal attack before moulding is visible, and secondly through the overestimation of DML in other studies. The respiration data of Scholz (1962), on which Kreyger's calculations were largely based, were approximately 11 to 18 times greater than data from comparable storage conditions in the present study (Figure 3.34). This could have led Kreyger (1972) to overestimate DML. Other studies may have used different levels of visible moulding as baseline figures; but in a 'worst case' example at 0.85 a_w /25°C; 25-50% grains in a quarter of samples were visibly moulded, and in the remainder 1-25% grains were moulded. In large grain stores during inspection, and certainly in the laboratory, such moulding would be detected easily by eye and scored as positive.

Kreyger (1972) did not consider the occurrence of mycotoxins. In UK conditions, mycotoxins are rarely formed in wheat grain (Lacey, 1989). *Aspergillus flavus* was sometimes isolated during the present study but cultures were not toxigenic. However, other mycotoxins may have or could have been formed; for example ochratoxin A by the commonly isolated species *Penicillium viridicatum* and *Aspergillus ochraceus*. Germination sometimes decreased in grain samples with no visible moulding (e.g. at 0.90 a_w and 15°C), and conversely, visible moulding can be detected without a decrease in germination capacity, (e.g. at 0.85 a_w and 25-35°C). It may follow that mycotoxins

are produced in seed that is apparently sound but is internally colonised. If visible moulding is seen, the seed handler may be alerted to potential problems, but more rigorous testing of apparently clean grain is necessary to ensure that grain used for animal feed or even baking is safe when the germination is too low for seed quality.

It is important that accurate methods are used to detect fungal growth on grain before it is visible. Many compounds have been investigated as potential indicators including chitin, ergosterol and numerous enzymes (Magan, 1993). The ergosterol content of grain increased with a_w at 20-30°C although the quantity of ergosterol in the 0.60 a_w samples after 7 d incubation was similar to that found in freshly harvested grain at 0.65 a_w (Cahagnier *et al.*, 1991). Tothill *et al.*, (1992) and Magan (1993) have suggested that ergosterol is a useful experimental tool for quantifying fungal growth, and that it also may be a useful indicator of early fungal activity in grain.

3.4.6 Effects of incubation on fungal growth

As discussed, visible moulding was usually present where germination was significantly decreased. Where respiration was highest (0.95 a_w and 25-35°C) *Aspergillus* spp. were most abundant, whereas *Penicillium* spp. were most commonly isolated from 0.90-0.95 a_w samples at 15-20°C, where germination was more often decreased. This suggests that *Penicillium* spp. may be more invasive than *Aspergillus* spp. and the total respiration where *Aspergillus* spp. were most common, especially at 30-35°C, could have been divided more equally between the grain itself and its resident fungi. At 0.95 a_w and 25, 30 and 35°C there was no significant difference between total respiration after 7 d and the dominant fungal species comprised mainly of *Aspergillus* with some *Penicillium* spp. favouring the lower of the two temperatures. Levels of *Eurotium* spp. were moderate where germination was decreased suggesting overall that *Penicillium* spp. were the most competitive of the storage fungi isolated and over 7 d were capable of removing most germinability. Magan & Lacey (1984a) also showed that *Penicillium* spp. were most competitive of grain fungi.

The species of fungi isolated from wheat and barley grains were representative of those

isolated from other sources (Magan & Lacey, 1988; Hill & Lacey, 1984; Lacey, 1989). The International Mycological Institute made the following comments about the *Penicillium* spp. isolated: "*P. chrysogenum* is a ubiquitous fungus, and being xerophilic grows at as low as 0.78 a_w ; *P. brevicompactum* is also known to grow as low as 0.78 a_w and *P. hordei* is very common on cereals". Thus, the grain used was contaminated with a representative cross-section of species and extrapolation of the results to other UK sources of wheat grain would be acceptable.

In these studies the field fungi, *Alternaria* and *Cladosporium* spp., decreased during the respirometer experiments and were only isolated after incubation at 15-20°C and 0.90-0.95 a_w suggesting that any incidence in samples probably related to the survival of their spores rather than mycelial growth. Although wheat grain cv. Rendezvous carried similar populations of *Alternaria* spp. to other grains studied, the low occurrence of *Cladosporium* spp. in comparison with other grains suggests the decline of this field fungus during cv. Rendezvous' extra year in storage. Magan & Lacey (1984a) showed that field fungi were uncompetitive with storage spp. on wheat grain.

CHAPTER 4
LARGE SCALE RESPIRATION
STUDIES

4.1 EXPERIMENTAL DETAILS

The development of accurate mathematical models that would allow practical application required that results from small scale respirometer studies (Chapter 3) were validated. Larger samples were studied under similar environmental conditions and timescales encountered in grain stores and as used in the small scale experiments.

The apparatus described in Section 2.3 was developed and used to measure the respiration of three 10 kg (wet weight) samples of wheat grain (cv Avalon, harvested in 1990) in two experiments. The first (experiment A) monitored one sample for 5 d at 0.90 a_w and the second (experiment B) monitored two samples for 12 d at 0.91 and 0.93 a_w . The experiments were conducted indoors but subject to diurnal fluctuations in ambient temperature.

At the end of experiment A, four grain samples were taken adjacent to the thermocouples T_1 to T_4 (Section 2.3.1). These samples are described from now on as 'top', 'upper-middle', 'lower-middle' and 'bottom' respectively. A fifth sample (referred to as 'core') was also taken with a sampling spear, bulking the grain at all depths. The five samples were then subdivided and tested for water content, germination and fungal colonisation (Section 2.1.9).

4.2 TEMPERATURE CHANGES

Representative data from thermocouples T_1 , T_2 , T_3 and T_4 on temperature change in grain samples (Section 2.3.1) were taken at 10 hour intervals, and are presented in Figures 4.1 and 4.2. Hourly mean temperatures for the four thermocouples (T_{Ave}) and ambient temperatures (T_{Amb}) were also recorded. These data show that the ambient temperatures encountered in experiment A (17.5-21.5°C) were generally higher than those in experiment B (10-20°C) due to seasonal differences in the times when the two experiments were conducted.

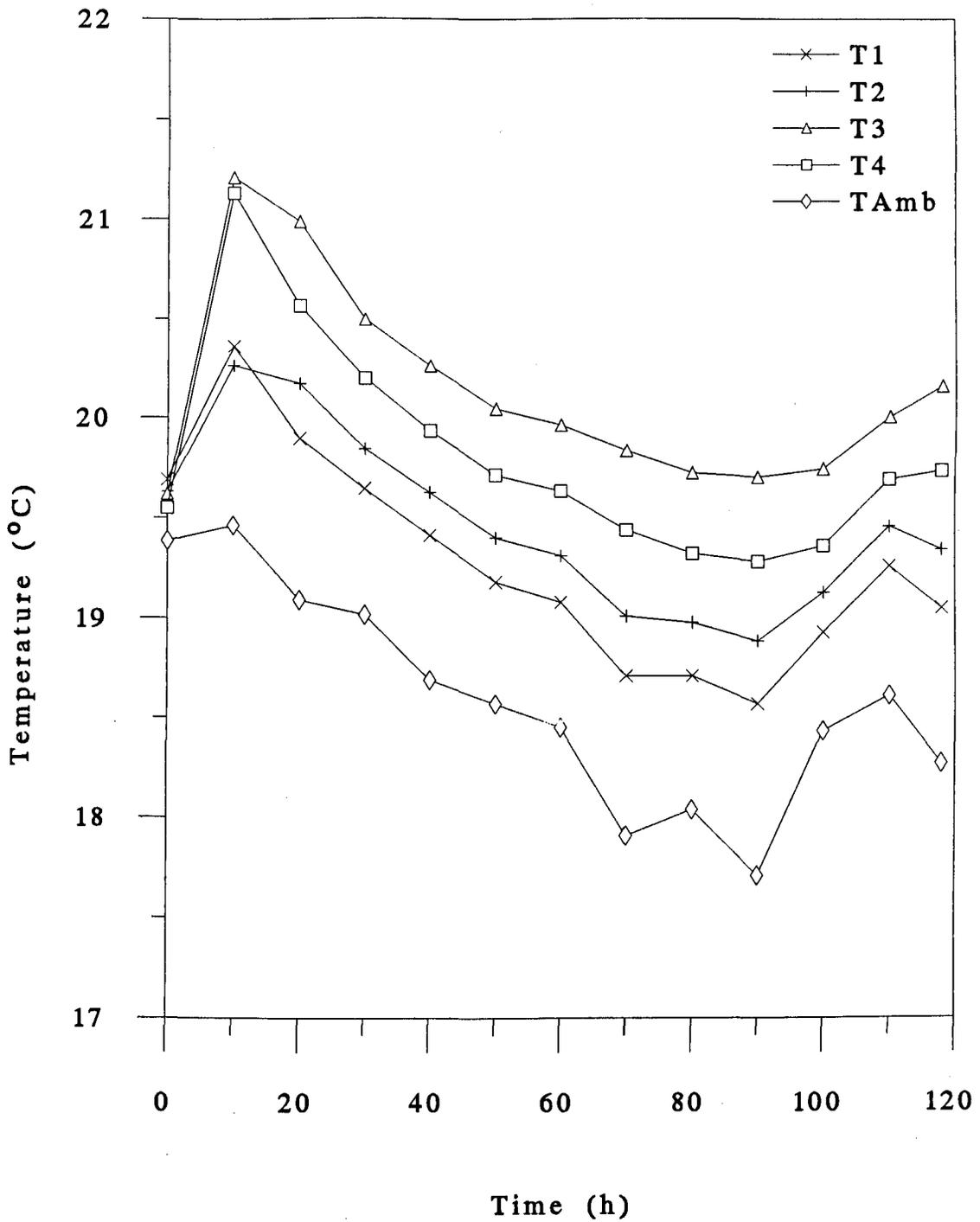


Figure 4.1 Temperature changes in a 0.90 a_w wheat grain bulk (10 kg) incubated for 5 d in the large scale respirometer (Expt A). T_1 - T_4 are temperature thermocouples within the sample chamber.

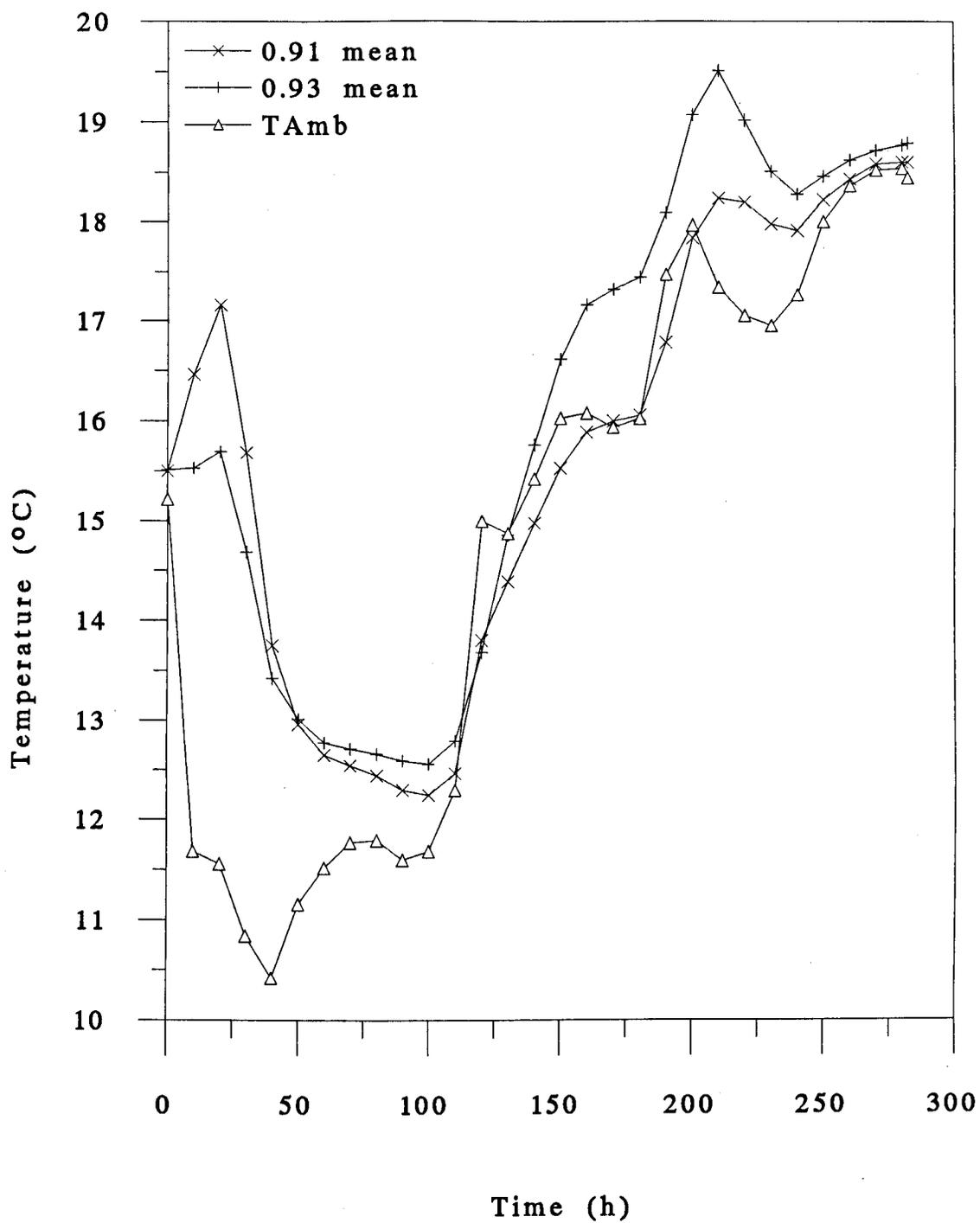


Figure 4.2 Temperature changes in 0.91 and 0.93 a_w wheat grain bulks (10 kg) incubated for 11 d (Expt B) in the large scale respirometer.

In experiment A, temperatures within the grain bulk were 0.5 to 1.5 degrees higher than ambient temperatures, but echoed ambient fluctuations (Figure 4.1). Temperatures within the grain were most different from ambient temperature in the deeper grain. For example, the top thermocouple read about 0.5 degrees higher than ambient temperature, while that at the lower-middle position was hottest (about 1.5 degrees above ambient). The bottom thermocouple registered the next highest temperature within the grain. There was an overall decrease in ambient temperature during the 5 d experiment which was evident at all monitoring levels in the chamber. Between about 70 and 100 h erratic changes in the ambient temperature were recorded but the grain was buffered against this, increasingly so moving downwards through the bulk.

Grain in experiment B was exposed to more variable ambient temperatures than in experiment A (Figure 4.2). The ambient temperature decreased from 15.5°C to 10.5°C during the first 50 h of the experiment, but mean grain temperatures in both samples resisted this change, dropping only to about 12.5°C. Ambient temperature then increased steadily to a maximum of 18.5°C after 282 h. Grain temperatures echoed this increase with slightly higher temperatures than ambient being reached in the 0.91 a_w sample. The grain temperatures were also slightly increased in the 0.93 a_w sample and were markedly different from the ambient temperatures between 0 to 100 h and 150 to 250 h. The highest temperature reached in experiment B grain was 19.5°C in the 0.93 a_w sample after 210 h.

The behaviour of individual thermocouples within the layers for the two experiment B samples is presented in Appendix C.

4.3 CARBON DIOXIDE PRODUCTION

Figure 4.3 shows that CO₂ production in wheat grain in both experiments increased linearly with time ($R^2=0.9757$ (0.90 a_w), 0.9963 (0.91 a_w) and 0.9904 (0.93 a_w)). Up to 2.021 l CO₂ were produced by grain stored at 0.93 a_w for 12 days. After 5 d (the

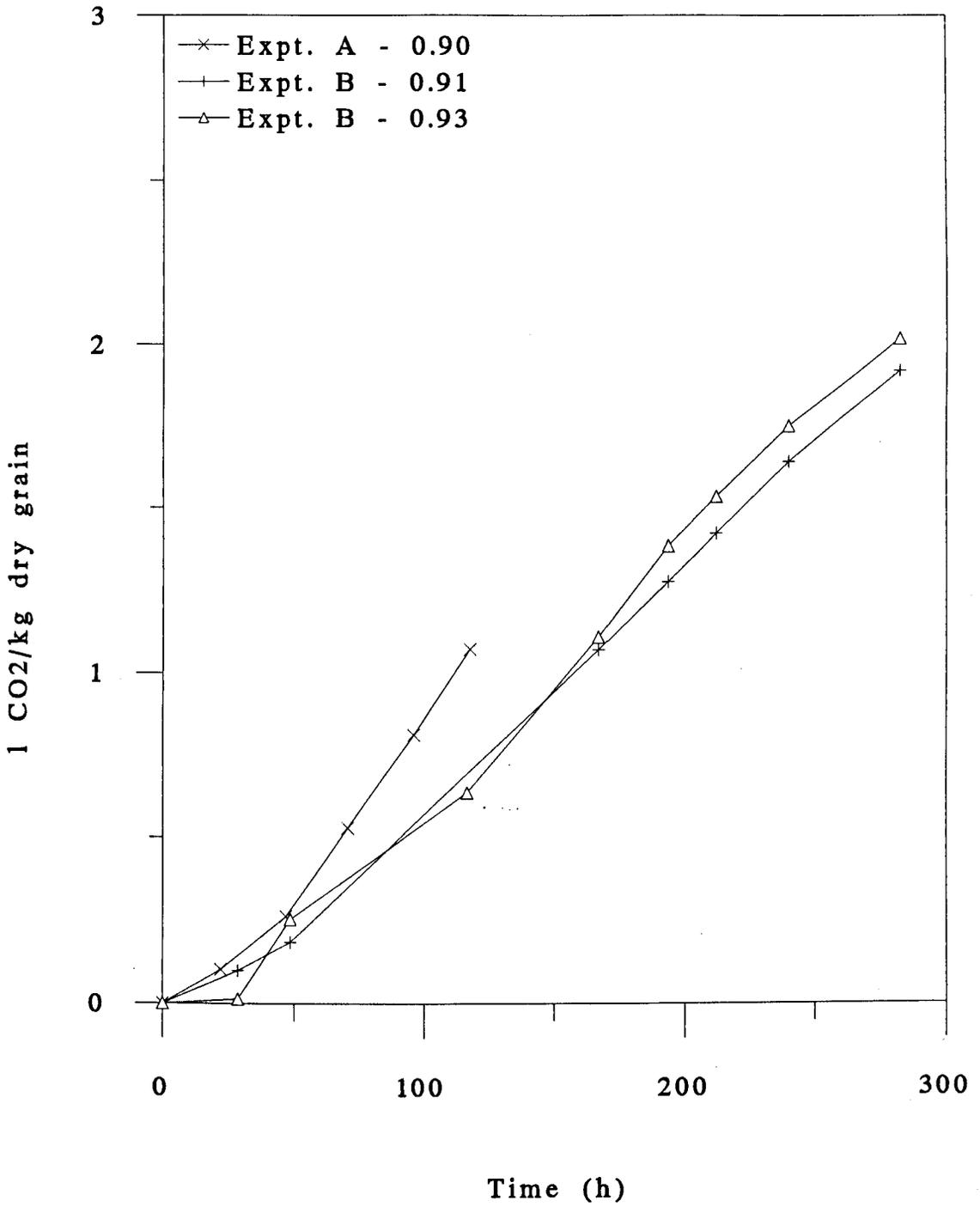


Figure 4.3 CO₂ production in samples of wheat grain cv. Avalon incubated for 5-11 d at 0.90-0.93 a_w and ambient temperatures in the large scale respirometer.

length of experiment A) all three treatments in both experiments had produced at least 0.6 l CO₂ kg⁻¹ dry grain. After 5 d, the 0.90 a_w sample (experiment A) had produced more CO₂ than the higher a_w samples in experiment B, but ambient temperatures were higher in the later experiment. Grain stored at 0.93 a_w in experiment B respired slightly faster over 12 d (producing 2.02 l CO₂ kg dry grain⁻¹) than that stored at 0.91 a_w (producing 1.92 l CO₂ kg dry grain⁻¹).

4.4 WATER CONTENT (Experiment A only)

Figure 4.4 shows that the water content of grain samples from experiment A differed with position within the grain bulk. Water content increased significantly ($p \leq 0.05$) in the 'upper middle' and 'lower middle' positions during the experiment but water contents at the top and bottom of the grain bed remained similar to those at the start of the experiment. In neither of the experiments was free water found to have accumulated in the condensate collection flask.

In experiment A, the drying column in the exhaust air stream had increased in weight by 84.4 g after 5 d, indicating a total loss of 84.4 ml water from the respiring grain. At the end of experiment B, the drying columns in the airstreams from the 0.91 and 0.93 a_w treatments had increased in weight by 17.6 g and 177.7 g respectively.

4.5 CHANGES IN GRAIN QUALITY (Experiment A only)

Seed germination

Percentage germination was assessed in ten replicates of ten seeds from each sample. The germination of all samples decreased significantly ($p \leq 0.05$) in all grain samples in experiment A, except in that from the bottom of the chamber (Figure 4.5). 97% of control grains germinated but only 72-87% of grain from the experiment germinated, depending on sampling position (untransformed data).

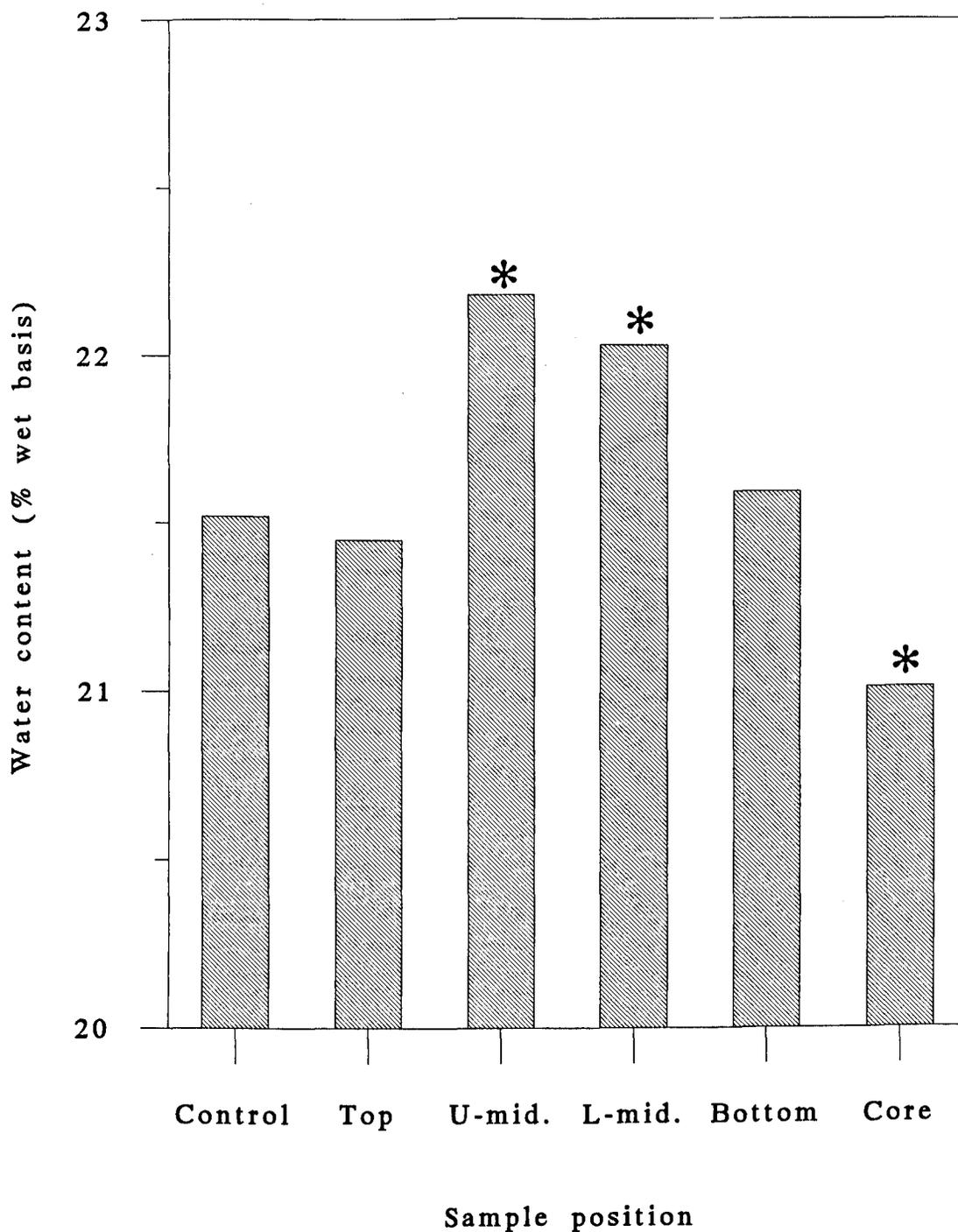


Figure 4.4 Changes in water content of wheat grain cv. Avalon incubated at 0.90 a_w for 5 d at ambient temperatures in the large scale respirometer. The abbreviations 'U-mid.' and 'L-mid.' represent upper-middle and lower middle positions respectively.

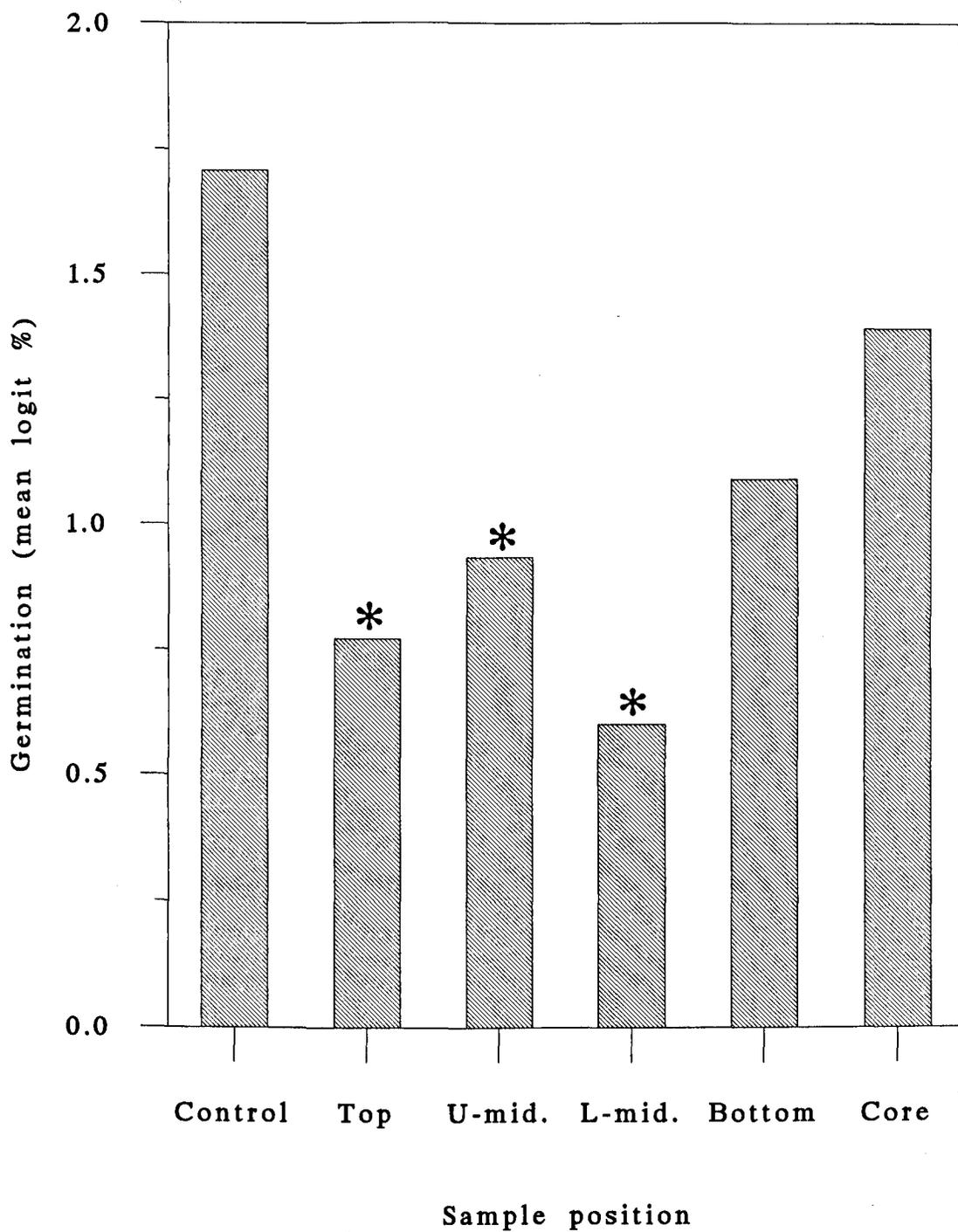


Figure 4.5 Germination in wheat grain cv. Avalon incubated at 0.90 a_w for 5 d at ambient temperatures in the large scale respirometer. The abbreviations 'U-mid.' and 'L-mid.' represent upper-middle and lower middle positions respectively.

Fungal colonization

Apart from the 'upper middle' sample, all samples in experiment A were visibly moulded with 1-25% of grain surfaces covered with mycelium.

Five sub-samples of ten grains from each sample were examined for fungal colonisation by direct plating onto M90 agar and incubating at 25°C (see Section 2.1.9). Clean, unhydrated samples of grain were examined for comparison. Figure 4.6 shows that *Penicillium* spp. dominated both clean grain and all samples after the experiment with all grains carrying one or more species. *Eurotium* spp. were also isolated from all samples, contaminating more grains after the experiment than initially. About twice as many grains carried *E. amstelodami* or *E. repens* as carried *E. rubrum*. *Cladosporium* spp. were isolated from clean grains (on MEA) but were not present after plating any experimental samples. Unusually, *Fusarium poae* was isolated from the bulk sample and clean grains but was not isolated from any of the 'layer' samples. The only other fungal taxon isolated in this experiment was *Aspergillus versicolor*, present on only 12% of clean grains.

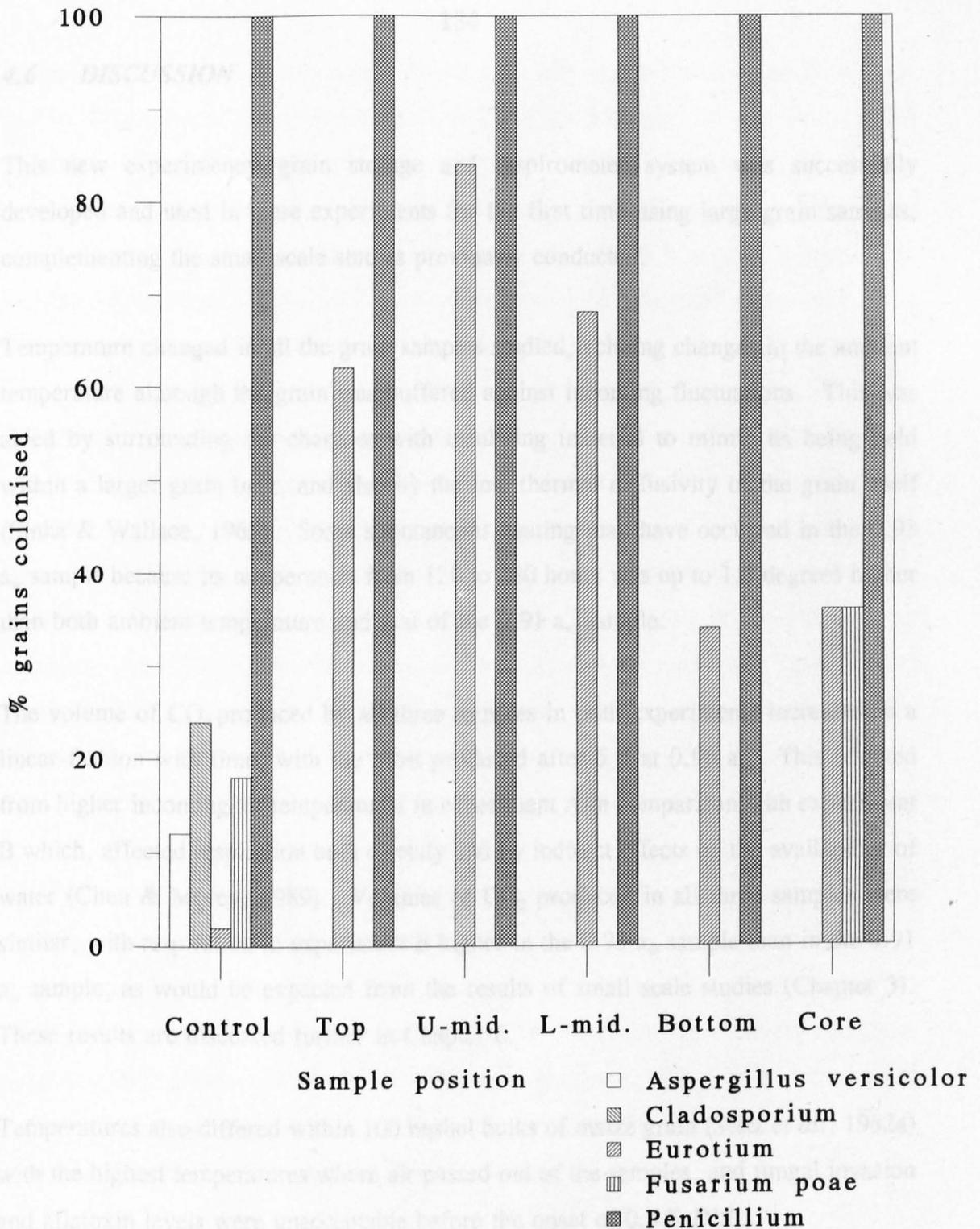


Figure 4.6 Relative colonisation of wheat grain cv. Avalon incubated at 0.90-0.93 a_w for 5-11 d in the large scale respirometer. The abbreviations 'U-mid.' and 'L-mid.' represent upper-middle and lower middle positions respectively.

4.6 DISCUSSION

This new experimental grain storage and respirometer system was successfully developed and used in these experiments for the first time using large grain samples, complementing the small scale studies previously conducted.

Temperature changed in all the grain samples studied, echoing changes in the ambient temperature although the grain was buffered against incoming fluctuations. This was aided by surrounding the chamber with insulating material to mimic its being held within a larger grain bulk, and also by the low thermal diffusivity of the grain itself (Sinha & Wallace, 1965). Some spontaneous heating may have occurred in the 0.93 a_w sample because its temperature from 120 to 280 hours was up to 1.5 degrees higher than both ambient temperature and that of the 0.91 a_w sample.

The volume of CO_2 produced by all three samples in both experiments increased in a linear fashion with time, with the most produced after 5 d at 0.90 a_w . This resulted from higher incoming air temperatures in experiment A in comparison with experiment B which, affected respiration both directly and by indirect effects on the availability of water (Chen & Morey, 1989). Volumes of CO_2 produced in all three samples were similar, with respiration in experiment B higher in the 0.93 a_w sample than in the 0.91 a_w sample, as would be expected from the results of small scale studies (Chapter 3). These results are discussed further in Chapter 6.

Temperatures also differed within 100 bushel bulks of maize grain (Seitz *et al.*, 1982a) with the highest temperatures where air passed out of the samples, and fungal invasion and aflatoxin levels were unacceptable before the onset of 0.5% DML.

In experiment B the 0.93 a_w sample lost ten times more water to the drying column than that lost from the 0.91 a_w sample. This removal of water from the grain may have influenced subsequent respiration. To stimulate conditions in a grain bulk, it would be necessary to minimise water loss so that any water produced by respiration is retained

in the sample to be utilised by fungi. Water loss may be minimised by decreasing the rate of airflow through samples and by humidifying the incoming air with saturated salt solutions so that its vapour pressure would be in equilibrium with the sample. Alternatively, higher air flow rates could be used in studies to mimic large scale aeration and drying of stored grain by dissipating respiratory heat and moisture. For example, Kaspersson (1986) used an air flow rate of 200 ml min⁻¹ to maintain large barley grain samples at a constant temperature of 24.9°C.

Water content increased significantly in the centre of the grain bulk in experiment A, remained unchanged in the 'top' and 'bottom' samples and decreased in the 'core' sample. The 'core' result is at odds with the pattern of increase in the samples from layers within the bin and may indicate that the 'core' sample dried out between sampling and weighing to determine its water content. Respiration produces CO₂, water and heat. Raised temperatures can move water in convection and down temperature gradients, but assuming that water content *had* increased in the centre of the grain bulk, and considering the observed patterns of temperature change in the layers, it appears that respiration was greatest in the centre of the sample.

Respiration was solely attributable to the grain and its microflora, since no insects were observed during the handling of samples and no mites were detected during stereomicroscopy of grain before and after plating for fungal colonisation. Paster *et al.* (1990) ensured insects and mites were removed from experimentally stored grain samples by fumigation with phosphine. However, phosphine treatment has been shown to decrease fungal populations in grain (Poisson & Cahagnier, 1988) making it unsuitable for experiments studying fungal growth.

The occurrence of greater activity in the centre of the sample in comparison with the upper and lower areas is further supported by the decrease in percentage germination (a symptom of fungal colonisation inside grains) in the 'top', 'upper-middle' and 'lower-middle' samples compared with the control. 'Hot spots' are localised regions of high temperature in large volumes of stored grain (Sinha, 1973). The trends noted

in experiment A suggest that such an area could form in 10 kg grain samples, and that this sized sample behaved similarly to grain stored on a larger scale.

The incidence of fungi in samples from different layers was uniform with species of *Eurotium* and *Penicillium* found in all levels. Additionally, the 'core' sample contained *Fusarium poae*, otherwise absent from other samples after the experiment. Its incidence in this one sample demonstrates the difficulty in obtaining a clear picture of fungal colonisation. *F. poae* is an important pathogen of cereals in the field and its survival during storage under damp conditions further emphasises the need for careful drying of stored cereal grain, especially when it is to be used for seed.

Discussion so far has centred on the physical components of the respiration process i.e. carbohydrate (DML), O₂, CO₂ and water. The remaining part of the equation, i.e. the release of heat energy, has not been used here to make quantitative measurements of respiration. However, a known increase in the temperature of stored grain can be used to quantify the amount of respiration that has taken place (Toledo *et al.*, 1969). This approach has been utilised in modelling all parts of the stored grain ecosystem, including the contributions made by insects and mites. In practice, the easiest two components of respiration to be measured by grain store managers are temperature and water content in a grain bulk, so that accurate predictive models could show that conditions were approaching danger levels for unacceptable quality loss, and preventative action could rapidly be taken to aerate or dry grain and reduce the risk. Srour (1988), for example, showed that the following formula could be used in practice to predict DML from respiratory measurements, including heat:

$$q = 1.07 d$$

Where: q = quantity of heat produced 1000 kg⁻¹ dry grain h⁻¹, d = release of CO₂ (mg 100 kg dry grain⁻¹ 24 h⁻¹)

The chambers used in these experiments for the first time are ideal for further study of grain under both steady and changing environmental conditions because:

- a) Samples are large enough to mimic large scale storage because localised micro-environments can be formed.
- b) The apparatus allows for temperature to be monitored in these localised areas.
- c) Many components of respiration can be measured accurately:
 - i) Water content before and after storage and water lost from samples.
 - ii) CO₂ produced by samples, especially if infra-red gas analysis equipment were used.
 - iii) Temperature of air entering, leaving and within grain samples.
- d) The aeration rate can be modified to allow spontaneous heating at low levels, simulation of drying at high levels and of modified atmosphere storage in sealed chambers.
- e) The system has potential for further sophistication and possible automation, if gas analyzers and electronic ERH detectors are used and their data are stored in the datalogger along with existing temperature records.

CHAPTER 5
RESPIRATION AND DRY MATTER
LOSS IN NATURALLY
CONTAMINATED AND STERILE
RE-INOCULATED WHEAT GRAIN

5.1 SAMPLE PREPARATION

5.1.1 Naturally contaminated grain

Naturally contaminated wheat grain (cv. Rendezvous, harvested in 1989) was hydrated to 0.85 or 0.90 a_w and stored at 20°C in beakers in chambers humidified to the corresponding ERH (Table 2.4). Five replicate beakers were oven-dried at 7 d intervals (Section 2.1.2) to measure dry matter loss, calculated as described in Section 2.4.3.

5.1.2 Irradiation-sterilised grain

Three sealed bags, each containing 60 g wheat grain (cv. Avalon, harvested in 1988 and 1989 respectively), were γ -irradiated for two experiments at a range of doses (Isotron, Swindon) using a ^{60}Co source. In experiment A, grain at 13.7 % water content was γ -irradiated with 1.0, 2.0, 2.5, 4.3 and 5.6 kGy and in experiment B grain at 13.8 % water content was irradiated with 4.0, 6.0, 8.0, 10.0, 12.0 and 15.0 kGy. Treated grain was subsequently tested for fungal colonisation, percentage germination and seedling vigour (Section 2.1). Grain treated in experiment B was measured for respiration (Section 2.2). Grain was handled aseptically after irradiation and all equipment was sterilised either by autoclaving or with methanol.

5.1.3 Sterile re-inoculated grain

Wheat grain was hydrated to 0.85 and 0.90 a_w and autoclaved at 121°C for 1 hour. When cool and equilibrated, pre-weighed beakers were aseptically filled with 24.98 g sterile grain and were inoculated using the novel technique as described below:

Streak cultures from loops of *Eurotium amstelodami* and *Penicillium aurantiogriseum* conidia were grown for 7 d on WEA plates before 115 autoclaved dry seeds were placed on the agar surface of each plate and shaken by hand for 1 min in a negative pressure microbiological safety cabinet. Two of the dusted grains were then added, approximately 20 mm apart, to the surface of each grain sample (either in pre-weighed beakers or respirometer tubes) to make each sample up to 25 g total fresh weight of grain. Samples were then incubated in humid chambers (Section 2.1.7) at 20°C. Five

replicate beakers were oven-dried at 7 d intervals for 28 d and DML was calculated.

Six grains from each inoculum batch were also added to samples of autoclaved grain (two grains to each of three 5 g samples) and used to estimate total CFU in the inoculum using the dilution plating method described in Section 2.1.9.

Samples treated in the same way were added to methanol sterilised respirometer tubes (three per treatment) to determine O_2 consumption at 0.85 and 0.90 a_w and 20°C over 14 d (Section 2.1.2). A mixed inoculum of the two fungi, with one dusted grain of each spore type added to respirometer tubes, was used as one treatment. Each *E. amstelodami*-dusted inoculum grain carried 9.3×10^2 spores and each *P. aurantiogriseum*-dusted grain carried 3.8×10^4 spores.

5.2 RESPIRATION

In experiment B the respiration of four replicate 25 g sub-samples (one from each of two treated bags and two from the third bag) was measured at 25°C for 7 days. As different irradiation treatments were hydrated separately, there were unavoidable differences in the a_w of samples used, but they were always in the range of 0.89 to 0.90 (Table 5.1).

Figure 5.1 shows examples of the respiration of irradiated samples of wheat grain in experiment B with time on a wet weight basis. O_2 consumption increased in a linear fashion with time at 25°C and all treatment levels, although the coefficient of linear regression for 6 kGy-irradiated grain respiration was slightly below 0.95. For 0 kGy, 4 kGy, 6 kGy, 8 kGy, 10 kGy, 12 kGy and 15 kGy treatments respectively; $R^2 = 0.9960, 0.9780, 0.9436, 0.9728, 0.9659, 0.9819$ and 0.9711 .

Figure 5.2 shows total O_2 consumption per kg dry matter for each irradiation treatment. Respiration at all γ -irradiation doses was significantly less than that of control grain ($p \leq 0.05$) and, although a gradual decrease in O_2 consumption was noted with

Table 5.1 Initial water contents and a_w of hydrated irradiated wheat grains (cv Avalon) before respirometer experiment B

kGy	% water content	a_w
Control	20.4	0.89
4.0	20.5	0.89 - 0.90
6.0	21.2	0.90
8.0	21.3	0.90
10.0	21.2	0.90
12.0	21.1	0.90
15.0	21.0	0.90

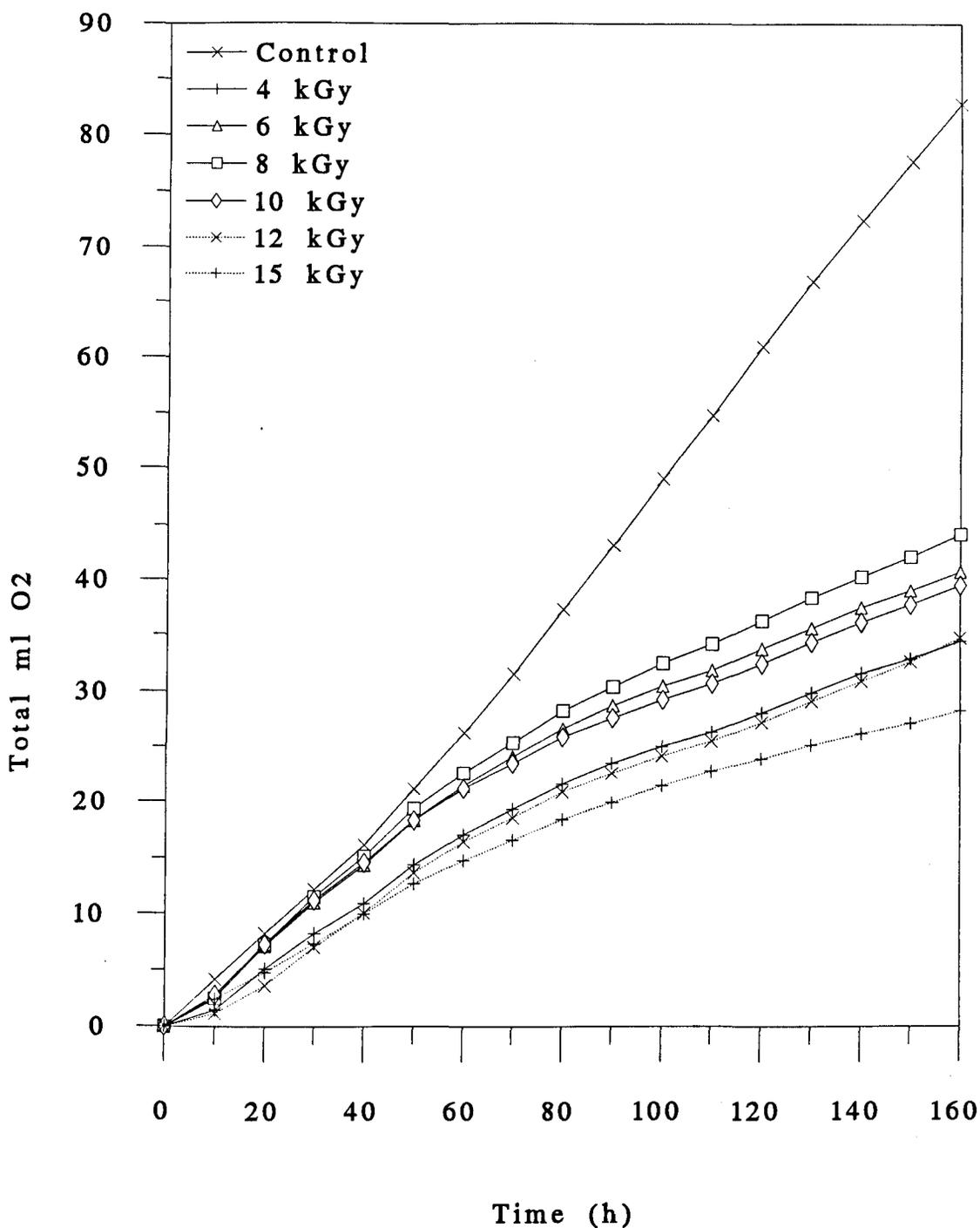


Figure 5.1 Examples of the pattern of respiration in wheat grain samples gamma-irradiated at 0 - 15 kGy with time at 25°C.

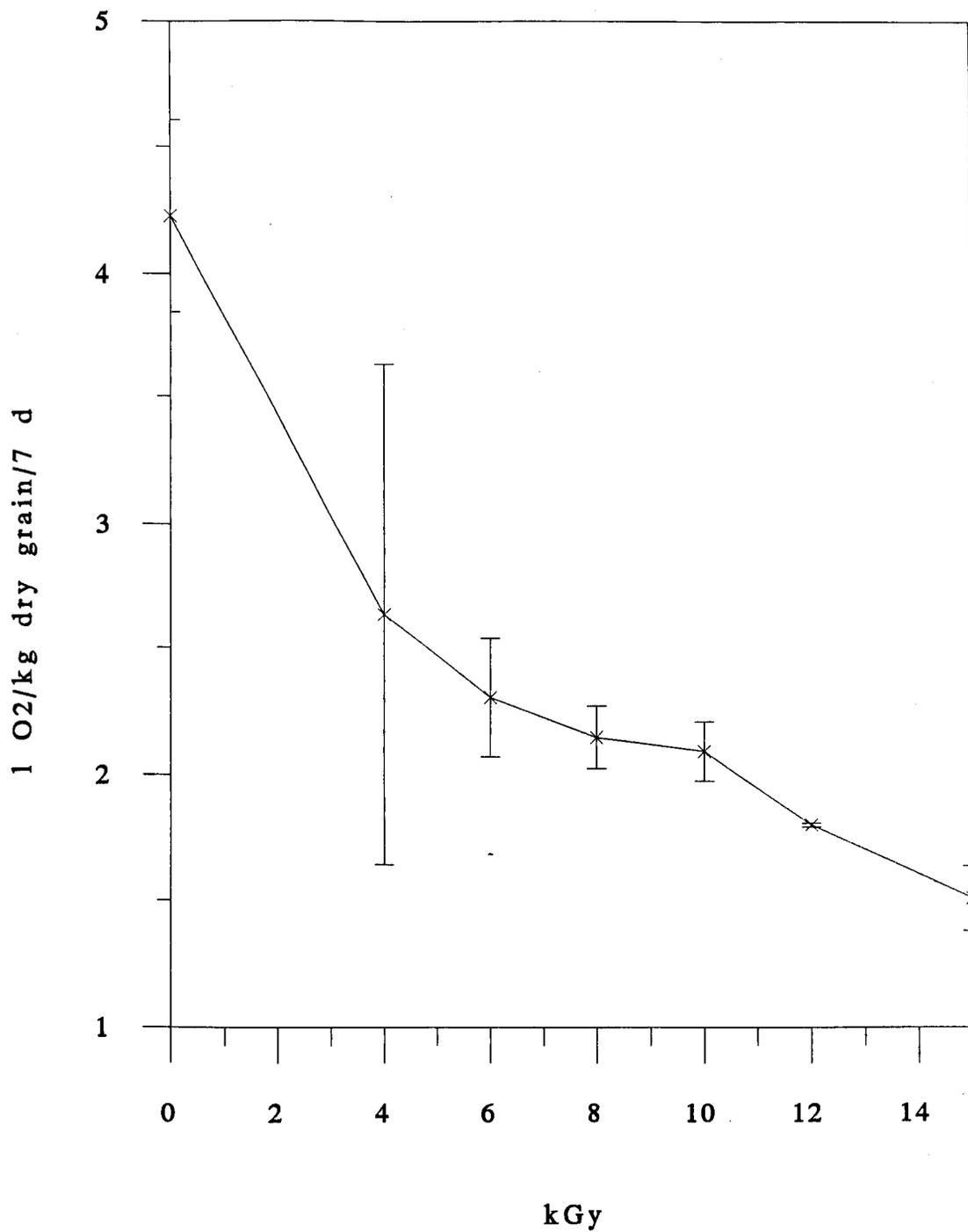


Figure 5.2 The effect of 0 - 15 kGy γ -irradiation on total O₂ consumption in wheat grain stored at 0.89 - 0.90 a_w with 25°C.

increasing irradiation dose, these were not all significantly different from each other. There were no differences between treatments at 4-10 kGy, but respiration at 12 kGy was significantly less than at 6-10 kGy, and that at 15 kGy was significantly less than at 12 kGy.

Figure 5.3 shows examples of the respiration of sterile re-inoculated wheat grain. The pattern of O₂ consumption with time appears to follow a non-linear pattern in most samples, with a slow rate of respiration initially increasing with time and levelling off in some samples towards the end of the experiment. The total volumes of O₂ consumed on a dry weight basis at 7 and 14 d are compared for each treatment in Figure 5.4. *Eurotium amstelodami* at 0.90 a_w showed least activity at 14 d (although statistical comparisons could not be made as data from only one replicate sample was saved). The mixed culture at 0.90 a_w consumed most O₂ although this was not significantly greater than respiration of *Penicillium aurantiogriseum*-inoculated grain at 0.90 a_w. At 0.85 a_w there was similar respiration in the mixture of the two species compared with *P. aurantiogriseum*. Respiration of samples at 0.90 a_w was greater than at 0.85 for the mixed inoculum samples and *P. aurantiogriseum* but less than for *E. amstelodami*.

Respiratory quotients (RQ) for inoculated grain were near to 1.0 with large SEMs (Table 5.2). The lowest RQ was 0.75 (*P. aurantiogriseum* at 0.85 a_w) and the highest was 0.89 (mixture of both species at 0.85 a_w). Unfortunately RQ could not be calculated for *E. amstelodami* at 0.90 a_w as the NaOH vessel was lost from the one sample remaining after incubation. Percentage DML calculated from the O₂ consumption data at 14 d were: 0.122 & 0.061 (*E. amstelodami* at 0.85 & 0.90 a_w), 0.199 & 0.450 (*P. aurantiogriseum* at 0.85 & 0.90 a_w), and 0.177 & 0.797 (mixture of both species at 0.85 & 0.90 a_w).

5.3 DRY MATTER LOSS AND VISIBLE MOULDING

Table 5.3 compares DML in naturally contaminated grain stored in humid chambers with that in inoculated grain incubated in the same way. After incubation of naturally

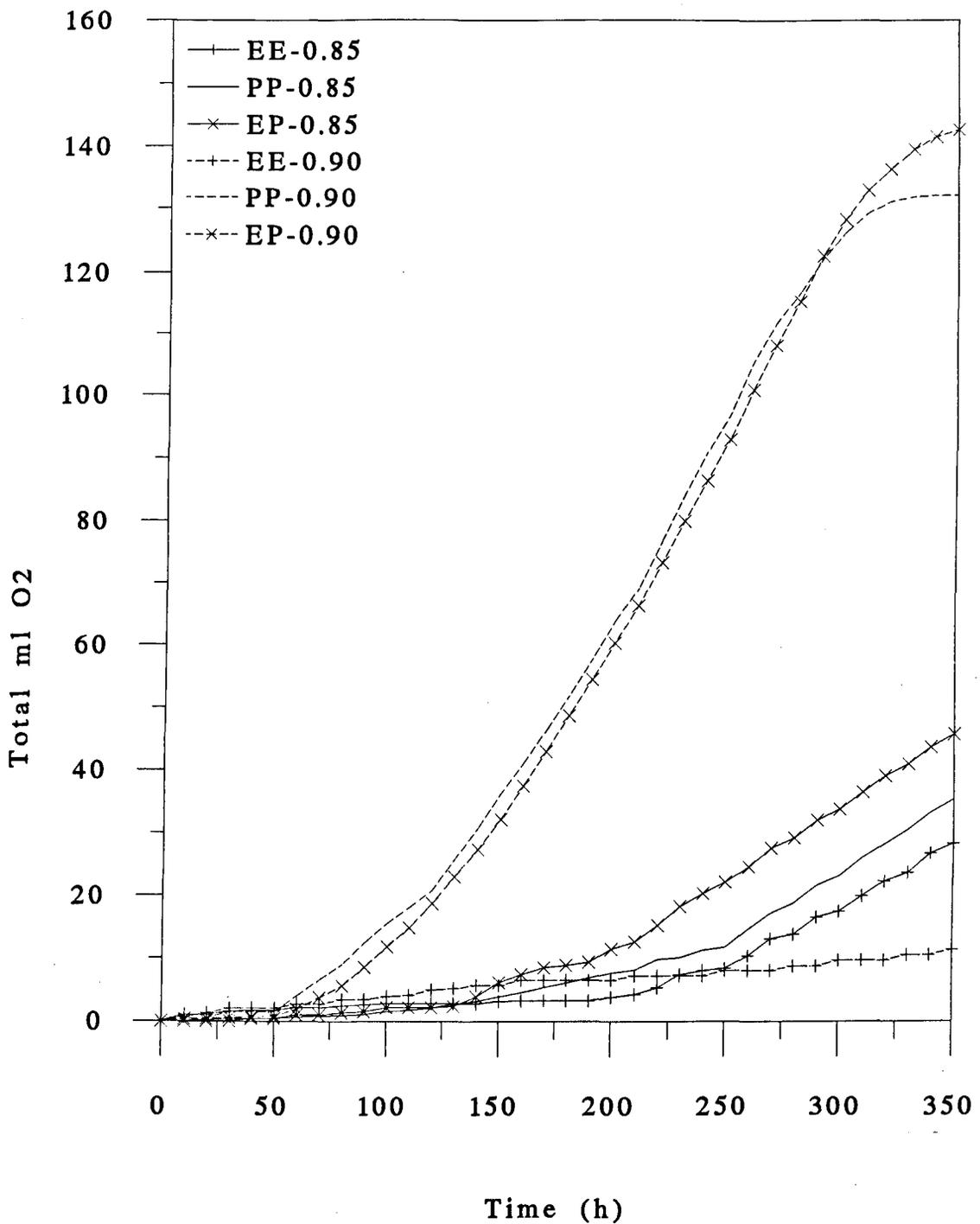


Figure 5.3 Patterns in the respiration of wheat grain over 14 d after autoclaving and re-inoculation with *Eurotium amstelodami* (EE), *Penicillium aurantiogriseum* (PP) and a mixture of both species (EP) at 0.85 and 0.90 a_w and 20°C.

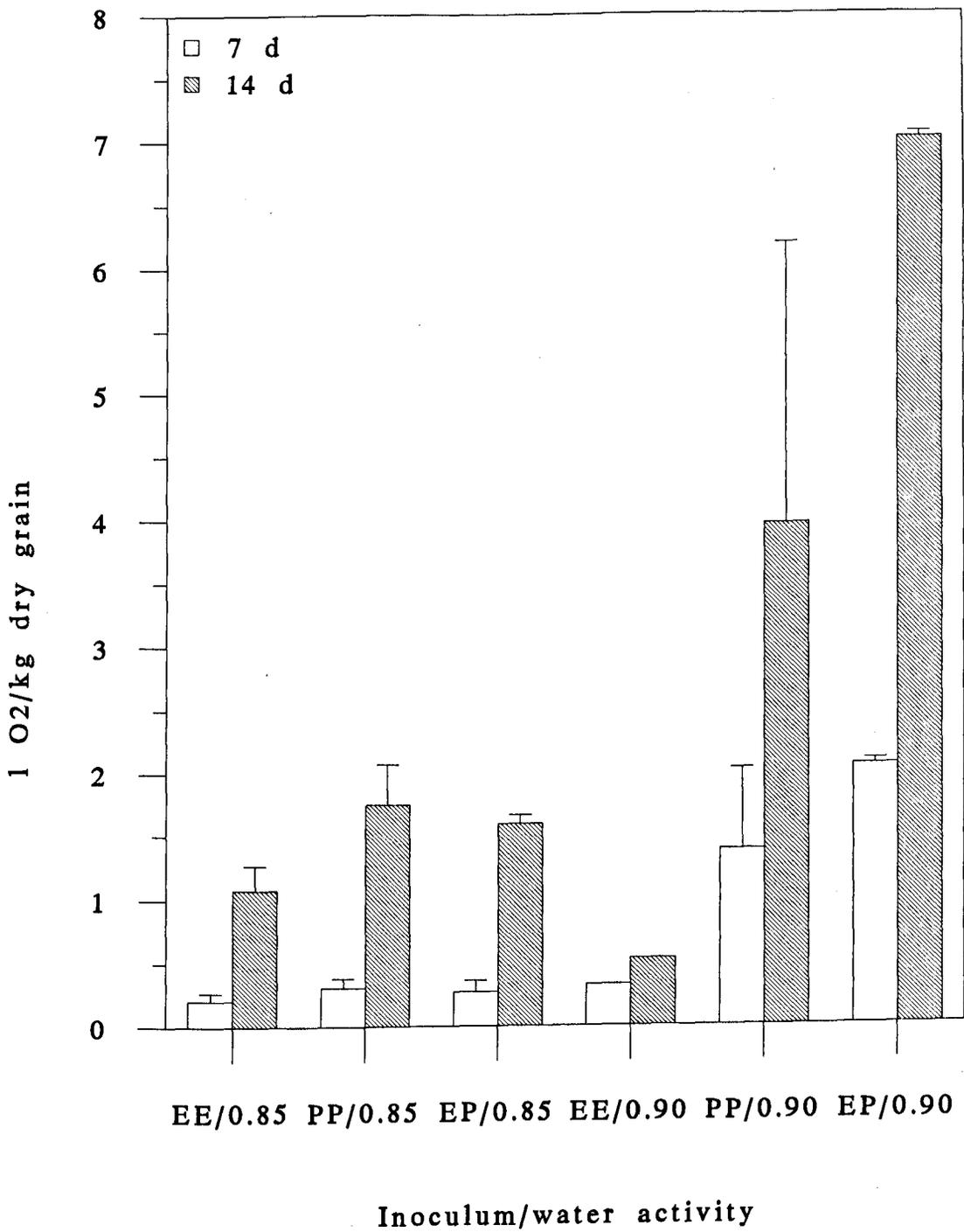


Figure 5.4 Total respiration of wheat grain after autoclaving and re-inoculation with *Eurotium amstelodami* (EE) and *Penicillium aurantiogriseum* (PP) and a mixture of both species (EP) at 0.85 and 0.90 a_w and 20°C for 14 d.

Table 5.2 Respiratory quotients for inoculated grain incubated in the respirometer for 14 d at 0.85 and 0.90 a_w and 20°C

Inoculum	a_w	mean RQ \pm SEM
<i>E. amstelodami</i>	0.85	0.765 \pm 0.207
<i>E. amstelodami</i>	0.90	nd
<i>P. aurantiogriseum</i>	0.85	0.747 \pm 0.043
<i>P. aurantiogriseum</i>	0.90	0.879 \pm 0.337
Both species	0.85	0.889 \pm 0.296
Both species	0.90	0.778 \pm 0.012

nd - not determined

Table 5.3 Dry matter losses in wheat grain (cv. Rendezvous, harvested in 1989) autoclaved and reinoculated or naturally contaminated (cv. Avalon, 1989) and incubated for 28 d at 0.85 and 0.90 a_w and 20°C.

Time (d)	% Dry matter \pm SEM					
	Naturally contaminated grain		<i>E. amstelodami</i> -inoculated		<i>P. aurantiogriseum</i> -inoculated	
	0.85 a_w	0.90 a_w	0.85 a_w	0.90 a_w	0.85 a_w	0.90 a_w
Estimated No. spores per dusted inoculum grain	-		2.2×10^3		4.7×10^4	
0	100	100	100	100	100	100
7	99.904	100.010 V	100.181	100.013 V	99.718	101.093 *
14	99.627 V	99.508 V *	99.833 V	99.902 V	99.483	100.894 *
21	99.746 V	99.047 V *	100.022 V	99.620 V *	99.791 V *	98.957 *
28	99.657 V	98.893 V *	99.853 V	99.288 V *	-	-

V = samples were visibly moulded; * = significantly different ($p \leq 0.05$) from day 0.

contaminated grain for 7 d, all samples at 0.90 a_w and 20°C were visibly moulded (0 to 24% of grains). After 14 d visible moulding had increased to 50 - 74% of grains and after 21 d to 74 - 100%. At 0.85 a_w however, there was no visible growth until 14 d when 50 - 75 % of grains in all samples were moulded. From 21 d onwards 75 - 100% of grains in all samples were moulded as in the 0.90 a_w grain at the same temperature. Percentage dry matter generally decreased in naturally contaminated grain at both a_w , but this was not statistically significant at 0.85 a_w . After 14 d incubation at 0.90 a_w DML had decreased significantly ($p \leq 0.05$) compared with the starting material.

The data showing changes in dry matter for *P. aurantiogriseum*-inoculated grain (Table 5.3) show a significant increase over the first 7 d ($p \leq 0.05$), when compared with the starting material. Dry matter then declined to less than 100% at 21 d ($p \leq 0.05$). None of the samples examined were visibly moulded during this part of the experiment. At 0.85 a_w *P. aurantiogriseum*-inoculated samples became visibly moulded at 21 d which coincided with a significant decrease in % dry matter compared with the starting material. In *E. amstelodami*-inoculated grain dry matter remained unaltered at 0.85 a_w , although visible moulding was detected from 14 d onwards; and decreased significantly after 21 d incubation at 0.90 a_w , 14 d after the first incidence of visible mould.

5.4 MICROBIAL COLONISATION OF γ -IRRADIATION STERILISED GRAIN

To quantify the efficacy of γ -irradiation as a sterilisation technique, 50-grain samples from each irradiated bag were direct plated onto MEA and MS media (Section 2.1.9). In contrast to previous studies of fungal colonisation, these samples were plated after irradiation but not after incubation in the respirometer.

Figures 5.5 and 5.6 show the percentage of seeds colonised with the most commonly isolated fungi in experiments A and B respectively. The greatest incidence of fungi on the two media (MEA and MS) has been presented. Percentage colonisation by fungi was decreased by irradiation doses as low as 1.0 kGy (Figure 5.5), but the effects on

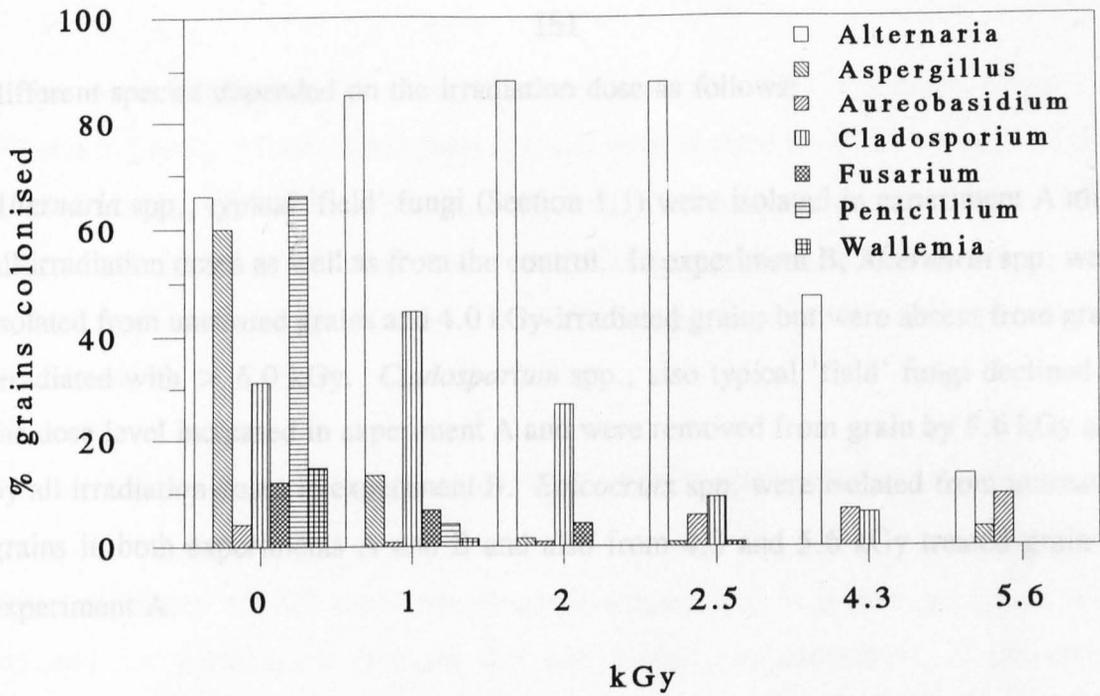


Figure 5.5 The effect of 0 - 5.6 kGy γ -irradiation on fungal colonisation of wheat grain.

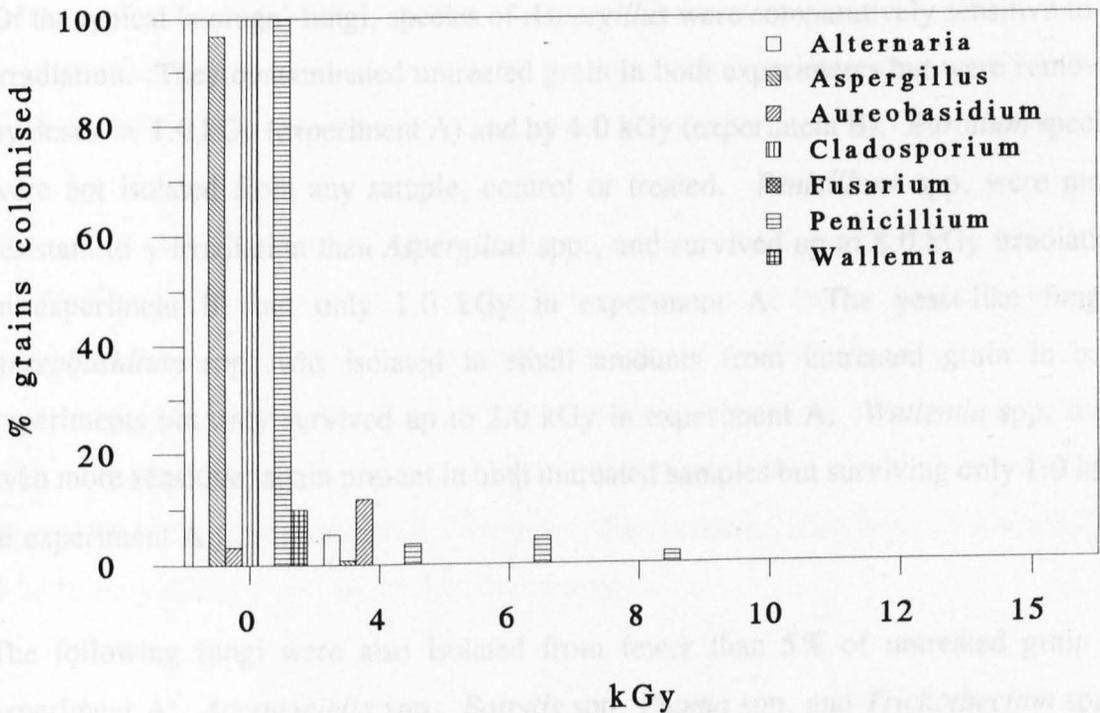


Figure 5.6 The effect of 0 - 15 kGy γ -irradiation on fungal colonisation of wheat grain.

different species depended on the irradiation dose as follows:

Alternaria spp., typical 'field' fungi (Section 1.1) were isolated in experiment A after all irradiation doses as well as from the control. In experiment B, *Alternaria* spp. were isolated from untreated grains and 4.0 kGy-irradiated grains but were absent from grain irradiated with > 6.0 kGy. *Cladosporium* spp., also typical 'field' fungi declined as the dose level increased in experiment A and were removed from grain by 5.6 kGy and by all irradiation doses in experiment B. *Epicoccum* spp. were isolated from untreated grains in both experiments A and B and also from 4.3 and 5.6 kGy treated grain in experiment A.

Fusarium spp. were common in untreated grain in experiment A, in contrast with experiment B, and in irradiated grain receiving > 4.3 kGy, whereas none were detected in experiment B.

Of the typical 'storage' fungi, species of *Aspergillus* were comparatively sensitive to γ -irradiation. They contaminated untreated grain in both experiments but were removed by doses > 1.0 kGy (experiment A) and by 4.0 kGy (experiment B). *Eurotium* species were not isolated from any sample, control or treated. *Penicillium* spp. were more resistant to γ -irradiation than *Aspergillus* spp., and survived up to 8.0 kGy irradiation in experiment B, but only 1.0 kGy in experiment A. The yeast-like fungus *Aureobasidium* spp. was isolated in small amounts from untreated grain in both experiments but only survived up to 2.0 kGy in experiment A. *Wallemia* spp. were even more sensitive, again present in both untreated samples but surviving only 1.0 kGy in experiment A.

The following fungi were also isolated from fewer than 5% of untreated grain in experiment A: *Acremoniella* spp., *Botrytis* spp., *Phoma* spp. and *Trichothecium* spp., with *Acremonium* spp. colonising less than 18% of untreated grain. *Arthrinium* spp. were isolated from less than 5% of untreated grain in both experiments A and B. None of these species were isolated from treated grain in either experiment.

Yeasts were often the only micro-organisms to survive high irradiation doses. As Figures 5.7 and 5.8 show, some yeast colonies were isolated after irradiation at all dose levels, except 2.0 kGy (experiment A) and 12.0 kGy (experiment B). More importantly, however, yeast colonies were isolated from untreated grain and from the highest irradiation levels (i.e. at 5.6 kGy in experiment A and at 15.0 kGy in experiment B).

5.5 GERMINATION OF γ -IRRADIATION-STERILISED GRAIN

Three replicates of 100 seeds from each irradiated bag in both experiments were assessed for germination (Figures 5.9 and 5.10). In experiment A percentage germination increased following 1.0, 2.0 and 2.5 kGy γ -irradiation from 73.5% to 86.0, 90.0 and 86.0% respectively (untransformed data; $p \leq 0.05$ for transformed data). By contrast, 4.3 and 5.6 kGy had no effect on percentage germination (untransformed data were 76.0 and 80.3% respectively). The findings in experiment B (Figure 5.10) were similar with 4.0 kGy, which increased germination from 96.7% to 99.7% (untransformed data), the only irradiation dose to affect percentage germination. With 6.0 to 15.0 kGy, 89.3 to 98.3 % of the grains germinated.

The length of the shoot of germinating seeds decreased visibly with increasing irradiation dose in experiment A, but measurements were not made. However, this was quantified in the experiment B where measurements of seedling vigour (Section 2.1.9) confirmed that seedling shoot length decreased with increasing dose of γ -irradiation (Figure 5.11). The mean shoot length in untreated grain was about 20 mm after 8 d incubation in the germination test. However, this decreased with increasing irradiation dose to only about 1 mm in the 15 kGy treatment.

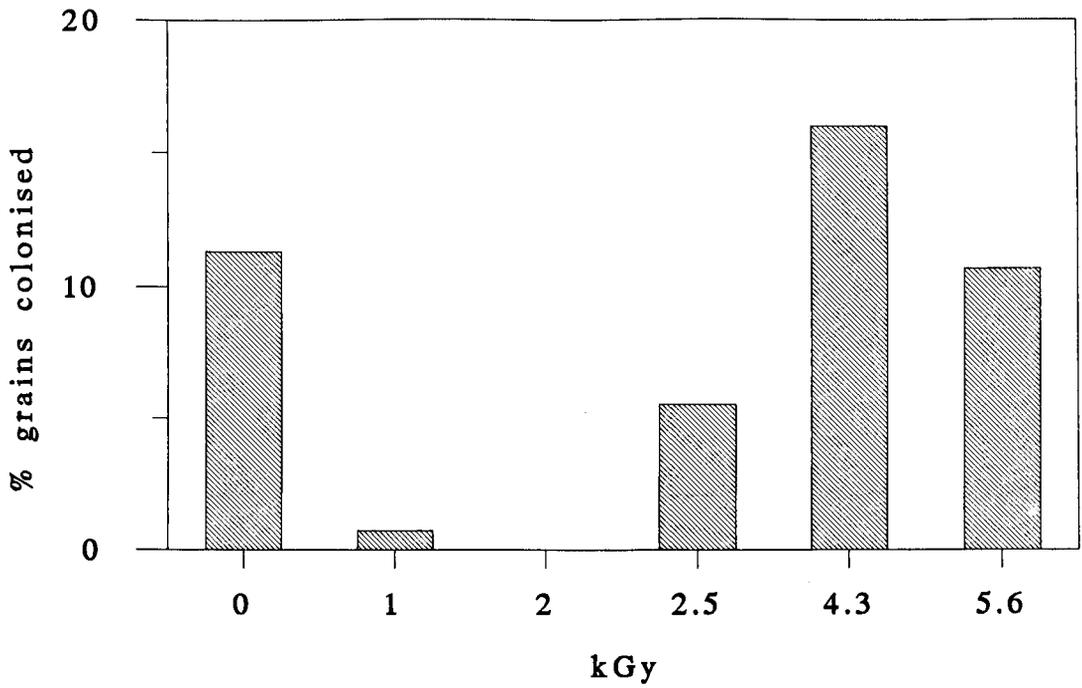


Figure 5.7 The effect of 0 - 5.6 kGy γ -irradiation on yeast colonisation of wheat grain.

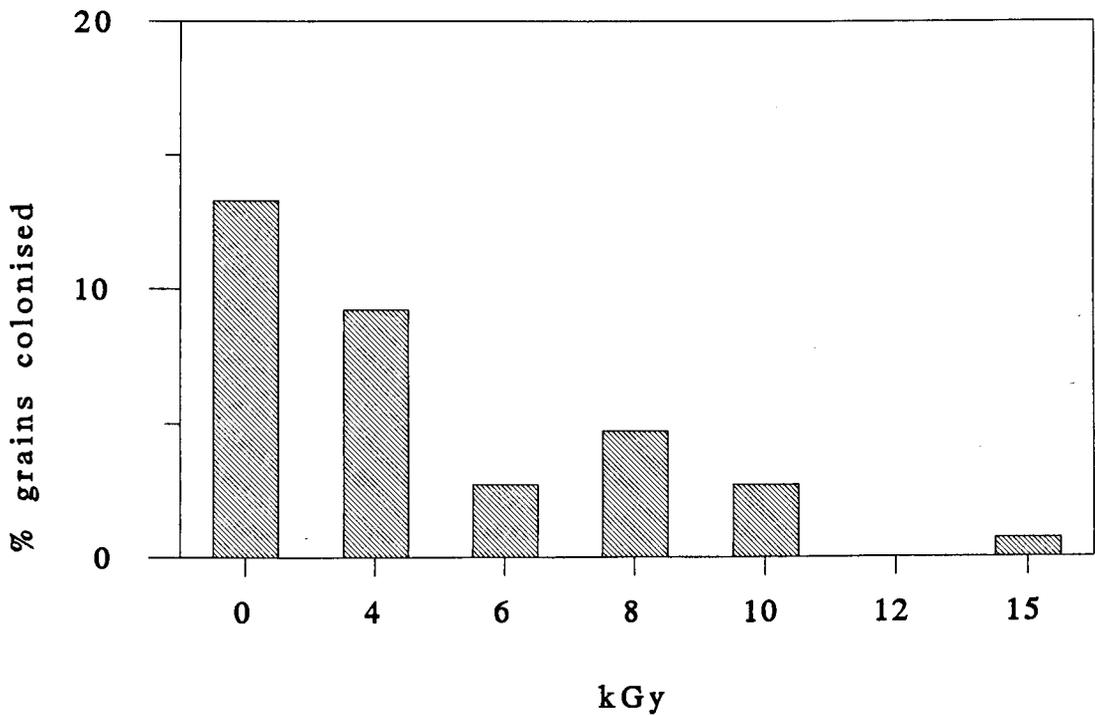


Figure 5.8 The effect of 0 - 15 kGy γ -irradiation on yeast colonisation of wheat grain.

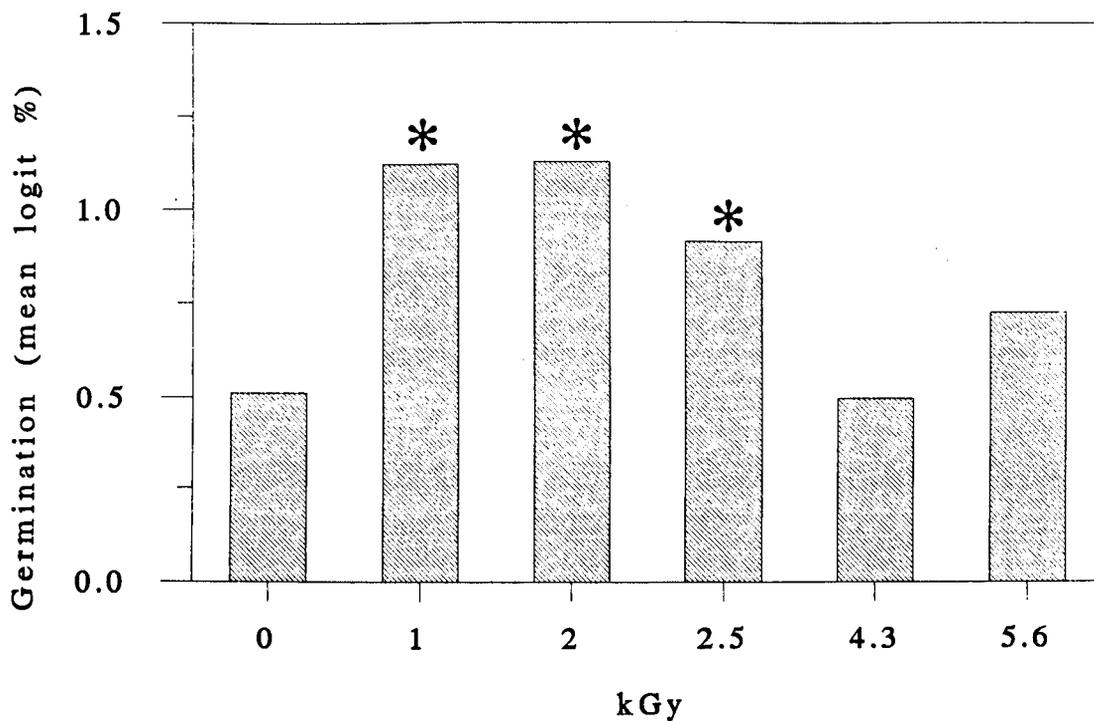


Figure 5.9 The effect of 0 - 5.6 kGy γ -irradiation on wheat grain germination.

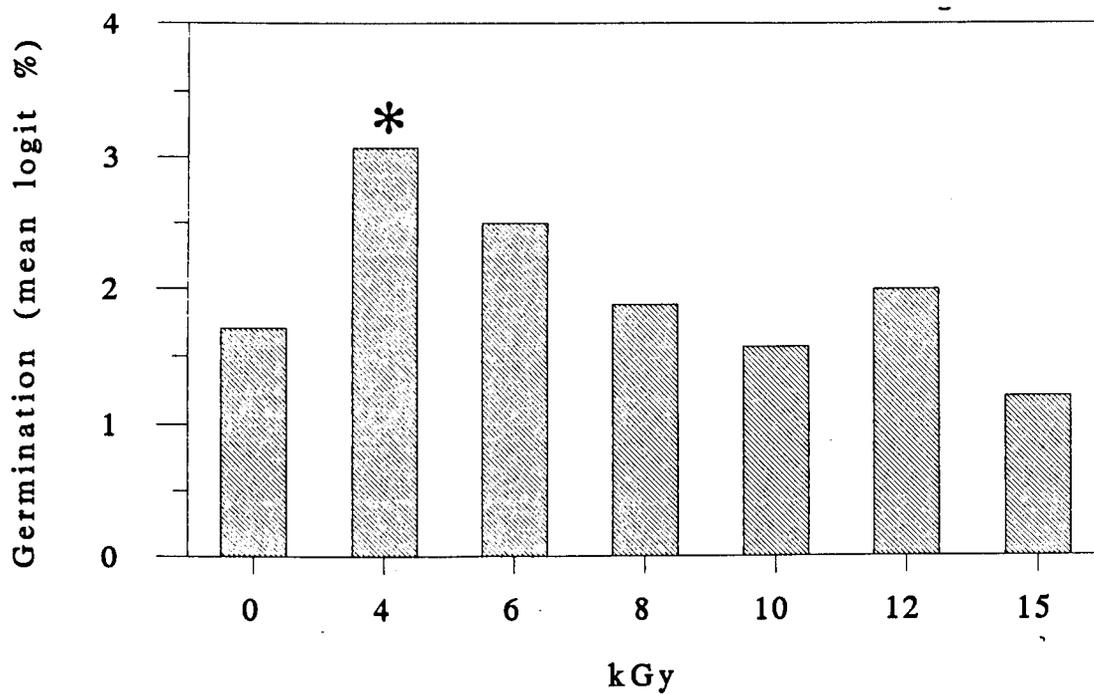


Figure 5.10 The effect of 0 - 15 kGy γ -irradiation on wheat grain germination.

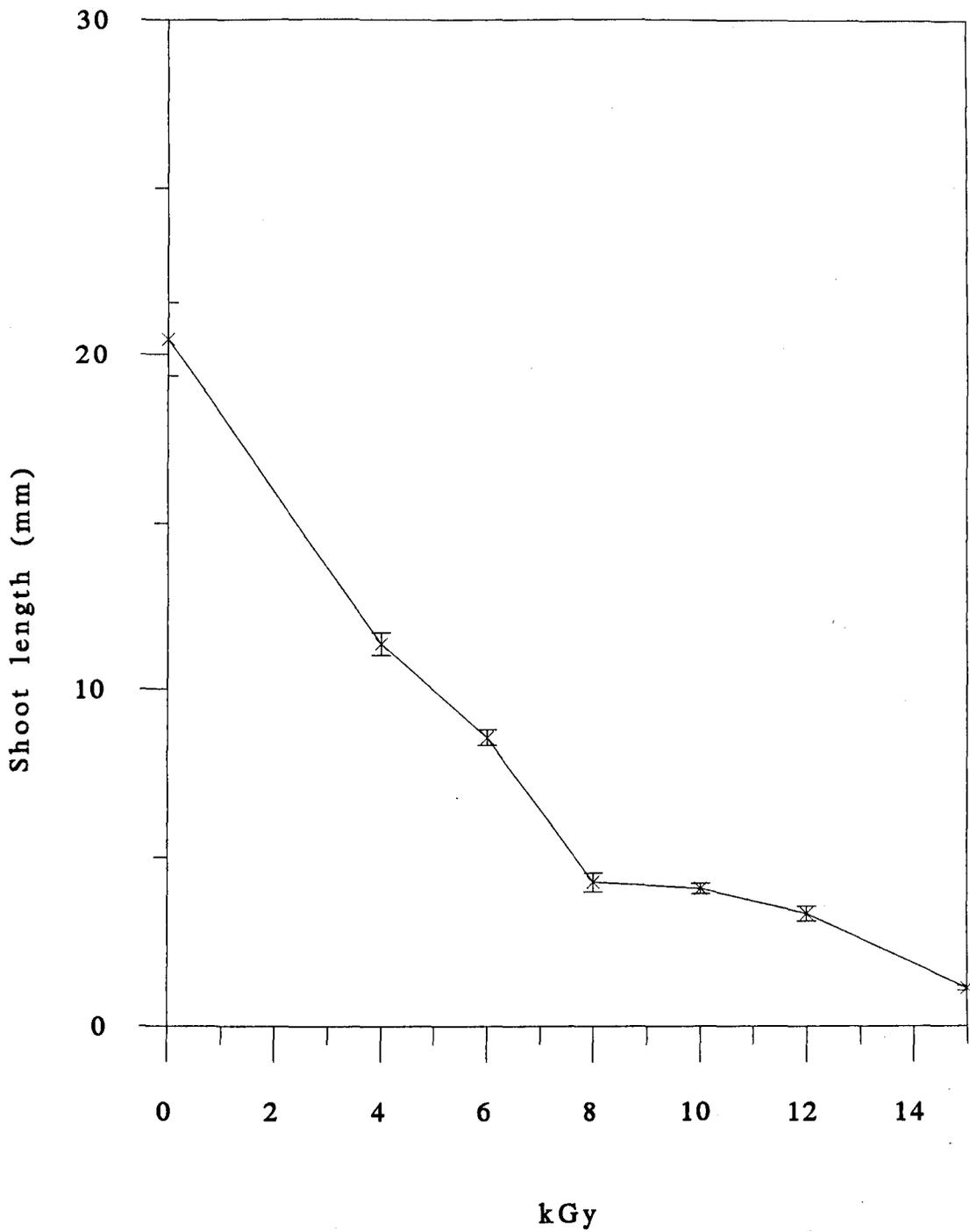


Figure 5.11 The effect of 0 - 15 kGy γ -irradiation on seed vigour in wheat grain.

5.6 DISCUSSION

5.6.1 Inoculation method

In previous respiration experiments, wheat grains that were known to have *Aspergillus flavus* growing on them were used to inoculate maize grain (Seitz *et al.*, 1982b) and sterile barley was inoculated with mouldy grains colonised by storage fungi (Kaspersson, 1986). Thus, fungi were already actively growing on the inoculum grain at the start of experiments modifying the initial pattern of respiration. In the new method used in these experiments, the inoculum grain was used as a carrier for *dry* spores, giving a situation comparable with that which occurs naturally and allowing more stages of fungal growth to be detected than in previous work. This new approach of inoculation succeeded because a lag phase of fungal respiration was measured at the start of the experiments, that had not been detected in earlier studies using a similar inoculation method.

Spore inocula were prepared twice using this new method, the first attempt (for the respiration study) gave 9.3×10^2 *E. amstelodami* spores per inoculum grain and the second study used 2.2×10^3 spores per grain. For *P. aurantiogriseum*, the first attempt gave 3.8×10^4 spores per inoculated grain and the second resulted in 4.7×10^4 spores per grain. This was acceptably uniform for preliminary experiments but, because inoculum concentration is an important factor in determining grain deterioration (Cahagnier *et al.*, 1991), further experimentation should ensure similar spore concentrations are obtained repeatedly. In these experiments, up to 40 times more *P. aurantiogriseum* than *E. amstelodami* conidia were added, so the use of younger colonies of *Penicillium* spp. and/or older colonies of *Eurotium* spp. for inoculum preparation may enable a similar number of spores of each fungus to be inoculated.

5.6.2 Sterile re-inoculated grain

The shape of the O₂ consumption curve with time was non-linear in re-inoculated grain, hence different to that found in samples of naturally contaminated grain with > 0.90 a_w. It was similar to that found in naturally contaminated grain with < 0.90 a_w (further

discussed in Chapter 6). A lag period occurred before linear growth started and, in some samples, respiration declined towards the end of the experiment. This pattern resembles those found during growth of fungi in batch cultures, as the number of cells increases with time (Deacon, 1984) and during mycelial growth of some fungi on surfaces, as hyphae branch and their numbers double with time. Growth of *Aspergillus oryzae* (a storage fungus of rice) and *Penicillium cyclopium* (a storage fungus of cereals) has been estimated by measuring CO₂ production of cultures on, respectively, solid media and the surface of liquid media (Sugama & Okazaki, 1979; Pazout & Pazoutova, 1989). *A. oryzae* respiration increased logarithmically with time, over 32 hours, on sterile rice grains with other work showing that glucosamine content (from chitin) of *A. oryzae* growing on rice and wheat bran over a similar time period increased in a linear fashion (Sakurai *et al.*, 1977). The pattern of respiration of *P. cyclopium* cultures (Pazout & Pazoutova, 1989) was more erratic with O₂ consumption fluctuating in cycles, even though dry matter content of the culture increased logarithmically with time. Trinci (1983) also showed that when *Aspergillus niger*, another fungus common on cereal grains, was grown as filamentous mycelium in shake-flask culture the biomass concentration increased with characteristic lag, exponential and deceleration phases.

Cereal grains are colonised by a mixture of spores and mycelium of many different fungal species and their interactions are complex with up to six reaction types described between fungi meeting (Magan & Lacey, 1984a; Ramakrishna *et al.*, 1993). Furthermore, numerical 'Indices of Dominance' varied with a_w , temperature and substrate. Most field fungi were uncompetitive and *Penicillium* spp. were more competitive than any other storage species. Modelling this complex ecosystem by studying single and paired species is over-simplistic but essential to attempt understand processes occurring in their simplest form.

In these experiments, it is likely that the lag phase indicates the time taken for dry spores to germinate and produce germ tubes on grain and the time taken for hyphae to gain access to the nutritional reserve within the grains. The rapid linear phase of growth that followed probably reflected digestion of available substrates within the

endosperm, and the decline phase may denote either that readily-available substrates had been utilised or that the fungi were limited in space. The decline could not have been a consequence of limitation of O₂ from the electrolyte or saturation of the NaOH solution with CO₂, because their respective capacities of 175 and 220 ml (Tribe & Maynard, 1989) were never exceeded. Had the total capacity of the electrolysis cell and NaOH been reached, they could have been replenished during the experiment.

The respiration of soil fungi on agar was studied using the same type of electrolytic respirometer used in these experiments (Tribe & Maynard, 1989). Less O₂ was consumed by *Fusarium sambucinum*, *Gliocladium roseum* and *Trichoderma viride* on agar over a total period of 19 weeks (< 80 ml) than was consumed here by *E. amstelodami* and *P. aurantiogriseum* over two weeks. During the first two weeks of Tribe & Maynard's (1989) study up to 30 ml O₂ (depending on the fungus) were rapidly consumed in a linear fashion with time, but, afterwards respiration decelerated, suggesting the three fungi had differing abilities to metabolise nutrients in the medium. No initial lag phase in respiration was detected by Tribe & Maynard (1989), possibly perhaps because fungi were grown on a high-a_w/high nutrient agar medium and the lag was undetectably short. Muck *et al.* (1991) showed that on silage, growth of fungi (*Penicillium expansum* and *Penicillium oxalicum*) and yeasts (*Hyphopichia burtonii*) conformed to a simple two-segment linear model of increasing growth rate with increasing a_w.

As there was a non-linear pattern of growth of *E. amstelodami* and *P. aurantiogriseum* on grain, total respiration more than doubled between 7 and 14 d. It initially appeared that *P. aurantiogriseum* respired more than *E. amstelodami*, but forty times more inoculum was added on the *Penicillium*-dusted seeds, so the correct interpretation may be that, at 0.85-0.90 a_w and 20°C, *E. amstelodami* may have performed best of the two species. The behaviour of the mixture of the two species depended on a_w; at 0.85 a_w respiration was approximately equal to that which would be expected from a sum of the two species, but at 0.90 a_w, respiration of the mixed inoculum may have exceeded the sum of the two parts. Conversely, Magan (1992) showed that there were antagonistic

interactions between *Eurotium amstelodami* and *Aspergillus versicolor* on respiring rapeseed resulting in a less than additive increase in respiration.

RQs calculated from inoculated autoclaved grain samples were approximately 1.0 when the large standard errors were taken into account. This contrasts with the results of Tribe & Maynard (1989) who measured RQs of 1.13-1.27 in respiring cultures of fungi on an agar medium.

DML calculated from O₂ consumption after 14 d incubation ranged from 0.122% (*E. amstelodami* at 0.85 a_w) to 0.797% (both species at 0.90 a_w). Kaspersson (1986), showed that after 15 d at 0.94 a_w and 24°C, *E. amstelodami*-inoculated barley had lost 0.5% dry matter and *P. aurantiogriseum*-inoculated barley under similar conditions had lost 0.26% dry matter. In studies here, losses from *E. amstelodami* and *P. aurantiogriseum*-inoculated grain after 14 d were, respectively, 0.061% and 0.450%. However, the two experiments are not directly comparable because Kaspersson (1986) used barley rather than wheat, at higher a_w (0.94) than studied here. Also, the inoculum was approximately 27 times smaller and in an initially less active state for *E. amstelodami* in the electrolytic respirometer study than used by Kaspersson (1986). The size of the *Penicillium* inoculum was similar in both studies so that the higher DML measured using the electrolytic respirometer were probably due to differences in metabolism of the different substrates under different conditions in the two experiments.

Ghosh & Nandi (1986) used a colorimetric biochemical method to detect DML in wheat grains and found that, after 15 d at 0.85 a_w and 30°C, '*Eurotium*'-inoculated wheat grain had lost 0.64% dry matter and *Penicillium jensenii*-inoculated grain 0.83% dry matter. Grains had been inoculated with more spores (2.5 x 10⁵ g⁻¹ grain) than either in the respirometer study or in Kaspersson's (1986). It is valid to compare the DMLs for *Eurotium* spp. with respirometer data for *E. amstelodami* as they may have been the same species. However, almost five times more DML was recorded by Ghosh & Nandi (1986) in *Eurotium*-inoculated grain but with nearly 7 x 10³ more conidia g⁻¹ added than in the respirometer experiment and spores added in liquid suspension and grain

incubated at a higher temperature (spores were also mixed into clean grain samples rather than added to the surface of the sterile substrate). The fungus was therefore provided with a head start over respirometer samples. More than 150 times more *P. jenseni* spores were added to each sample compared with *P. aurantiogriseum* spores added in the respirometer study, and the DML from each experiment were 0.83% and 0.199% respectively. This may indicate differences in the metabolic abilities in different *Penicillium* species, because *P. jenseni* were favoured by environmental conditions and inoculum size but still resulted in less DML.

Differences between fungi in their contributions to respiration and DML of autoclaved grain may depend on the abilities of these species to digest different forms of carbohydrate (Kaspersson, 1986). Wheat grain is composed of 2.5% glucose and 64% starch (Kent, 1978). If the two fungi possess different α -amylase activity then the starchy component may be preferentially utilised by one of the species. Foster, Jenkins & Magan (unpublished) showed that a marked increase in total α -amylases occurred during incubation of 0.95 a_w /25°C in wheat grain cv. Avalon. Response of four cvs. of wheat grain in their expression of α -amylase activity differed with the variety and conditions of storage (Kiss & Farkas, 1977). Ghosh & Nandi (1986) found that *Aspergillus flavus* produced more α -amylase than *Aspergillus restrictus*.

5.6.3 Dry matter losses measured in humid chambers

DML, measured by weighing dried grain samples after storage in beakers in humid chambers, increased after 7 and 14 d incubation at 0.90 a_w in *P. aurantiogriseum*-inoculated wheat grain even though the grains were not visibly moulded. A possible explanation is that increases in *P. aurantiogriseum* biomass could have caused a net increase in dry matter. Tables 5.4 and 5.5 compare the DML data from these experiments with those calculated under similar conditions in the electrolytic respirometer and it is evident that the DML estimated by respirometry are much lower. Hence, the sensitivity afforded by this method is less than could accurately be used to detect fungal activity in stored grain.

Table 5.4 Dry matter losses measured by incubation in humid chambers and in the electrolytic respirometer after incubation at 0.85 a_w and 20°C

Method		% dry matter loss with treatment:			
Time (d)		Naturally contaminated	Eurotium-inoculated	Penicillium-inoculated	Inoculated with both species
'D'	7	0.096	+0.181	0.282	nd
	14	0.373	0.167	0.517	nd
'R'	7	0.027 a	0.024	0.036	0.031
	14	nd	0.122	0.199	0.177

'D' - direct weighing of moulding samples, 'R' - calculated from respirometer data, a - determined in Chapter 3, nd - not determined.

Table 5.5 Dry matter losses measured by incubation in humid chambers and in the electrolytic respirometer after incubation at 0.90 a_w and 20°C

Method		% dry matter loss with treatment:			
Time (d)		Naturally contaminated	Eurotium-inoculated	Penicillium-inoculated	Inoculated with both species
'D'	7	+0.010	+0.013	+1.093 *	nd
	14	0.492	0.098	0.894	nd
'R'	7	0.226 a	0.038	0.157	0.234
	14	0.743 a	0.061	0.451	0.797

* - significantly greater than control (day 0 dry matter content), 'D' - direct weighing of moulding samples, 'R' - calculated from respirometer data, a - determined in Chapter 3, nd - not determined.

Autoclaving has been examined as a method of grain sterilisation in many published studies (Kaspersson, 1986; Niles, 1978) because it kills the grain, thus removing its background respiration, but is a more realistic natural substrate than agar media. It also removes all contaminating microorganisms allowing inoculation with pure cultures of fungi to be conducted. However, it may be difficult to compare autoclaved grain with grain in its natural form because the autoclaving process affects the physical structure of the seed coat by softening it which may allow fungi more rapid access than would normally occur. The high temperature and pressure of autoclaving also cause chemical changes in the grains. Proteins, especially glutenins, are damaged by high temperature (Every, 1987) and starch is 'gelatinized' as water is absorbed; seeds may even burst (Kent, 1978). Thus, the fungi inoculated onto autoclaved grain encounter a softer, more easily degraded substrate than those inoculated onto naturally moistened grain. There is a need for a method of grain sterilisation that could be used *alongside* autoclaving to more accurately represent realistic grain composition during inoculation studies. Gamma-irradiation offers such a possibility (see below).

5.6.4 Gamma-irradiated grain

Irradiation experiment B indicated that a dose of 10 kGy was sufficient to kill all viable fungi on the grain with minimal effects on respiration, seed germination and vigour. No fungi were isolated on agar media from samples treated at 10-15 kGy, but yeasts were isolated from a few grains at 10 and 15 kGy. Interestingly, Niles (1978) found that *Aspergillus flavus* grew faster on irradiated wheat grain than on autoclaved grain. This may have been because irradiation increases the solubility of starch (McArthur & D'Appolonia, 1984) and hence increased the susceptibility of grains to α - and β -amylase hydrolysis by fungi (Bhatty & McGregor, 1988). Many fungi possess α -amylase activity (Deacon, 1984) with the level of activity depending on the species and incubation conditions (Kaspersson, 1986).

Ramakrishna *et al.* (1990) and Cuero *et al.* (1986) selected 12 kGy for their inoculation studies on barley and maize. A lower dose may be sufficient for wheat because it is smaller than maize grain and lacks the protective lemma and palea of barley seeds

Fungi differed in their sensitivity with *Aspergillus* and *Penicillium* spp. eliminated from maize by 1.2 kGy and Phycomycetes by 2.4 kGy while *Fusarium* spp. and yeasts required 12 kGy (Cuero *et al.*, 1986). 10 kGy may have been suitable for irradiation-sterilisation of maize, but only levels in the 0.3-6.0 and 12-18 kGy ranges were tested by Cuero *et al.* (1986). Alam *et al.* (1992) have recently shown that stored spices need to be treated with 10 kGy γ -irradiation to kill the microbial population but 5 kGy was recommended as a practical level for use since fungi and coliforms had been removed. Ground spices may require less irradiation to remove fungal contamination than grains due to their finely divided structure allowing easier penetration of rays.

Aspergillus and *Penicillium* species were generally removed by smaller doses of irradiation (<2.5 and <1.0 kGy respectively in experiment A (although *Penicillium* spp. survived 8 kGy in experiment B)). Other fungi, e.g. *Alternaria* and *Cladosporium* species survived, respectively, ≤ 5.6 and ≤ 4.3 kGy. Possibly, the spores of these field fungi are more resistant to irradiation as they are protected by melanin pigmentation in the spore wall which is absent from *Aspergillus* and *Penicillium* spores. This is consistent with the findings of Saleh *et al.* (1988) who also found that Moniliaceous fungi were less tolerant to γ -irradiation than Dematiaceous fungi. *Fusarium* spp., however, were isolated after relatively large doses of radiation (only in experiment A), although from fewer kernels than *Alternaria* and *Cladosporium* species. The apparent increase in *Aureobasidium* infection with increasing dose in experiment A probably resulted from the removal of other competing fungi, also noted by Ramakrishna *et al.* (1990). If organisms are resistant to the sterilising treatment, growth will continue unhindered during incubation giving false results, so it is important to ensure that irradiated grain to be used in inoculation studies is free from fungi before inoculation. Similarly, fungal inocula may respire faster on sterile grain in the absence of other species interacting and competing for substrates. The water sorption isotherm for rapeseeds was slightly changed by irradiation from its natural state (Magan, personal communication) so it is essential this is checked for other grains.

Deviations from and within published data on irradiation doses required to kill different

species of fungi may result from differences in the physical shape, density and water content of the substrate. More active free radicals, which cause irreparable damage to DNA and subsequent death of the organism, are produced as water content increases (Goodburn, 1987). Only large molecules like DNA and polysaccharides are usually affected by γ -irradiation. Fats may be turned rancid when in large quantities, for example in prepared foods, but their content in cereal meals and grains is too small for spoilage to occur with up to 15 kGy, although amino acids may be affected by doses of up to 25 kGy (Hanis *et al.*, 1988).

Percentage germination was stimulated in experiment A at 1.0-2.5 kGy inclusive, agreeing with previously published data including that for barley (Cuero *et al.*, 1986) and maize grains (Woodstock and Combs, 1965; Woodstock & Justice, 1967). However, percentage germination of unirradiated grain was low in experiment A indicating that it was initially of poor quality. At 4.3 - 5.6 kGy there was no stimulation in germination so the optimum level must have been exceeded. In experiment B percentage germination was stimulated at 4 kGy suggesting that the cut-off point for stimulation of germination in wheat grain was between 4 and 4.3 kGy. Grain to be used in inoculation studies should not, therefore, be irradiated below 5.6 - 6.0 kGy as increased germination, although associated with decreased respiration, may mask results. Germination may have been stimulated by removing the fungi that would normally invade and destroy the embryo during germination tests. Additionally, there is evidence that irradiation levels stimulating germination also stimulate biochemical processes within the seed, i.e the activities of catalase and peroxidase and auxin metabolism (Woodstock & Justice, 1982) which may be expressions of damage at the chromosome level (Goodburn, 1987).

The effect of γ -irradiation on seedling vigour, measured by the length of seedling shoots was investigated in detail in experiment B. Although percentage germination was increased by 4.0 kGy, the mean shoot length of the germinating seed was decreased compared with the control. The mean shoot length decreased from 21 mm in the untreated control to about 4 mm after irradiation at 8 kGy. This was shorter than the

mean shoot lengths recorded by Woodstock & Justice (1967) who found, using a different variety of wheat, that grain treated with the same level of γ -irradiation gave rise to mean shoot lengths of 29 mm (control) decreasing to 13 mm (8.0 kGy irradiated). The length lost from shoots in each study are similar, but their relative decreases are not (81% and 55% loss of shoot length respectively). This may have been because the water content of wheat grain used in the current study was 1.5% higher during irradiation than in the samples treated earlier, and that there may have been differences in susceptibility between the wheat varieties used. At 4.0 kGy respiration was inhibited, contrasting with the stimulated germination rate at this dose. This suggests that fungi had been killed sufficiently for more grain to germinate but radiation-induced changes had affected the development of the germinated shoot. These findings agree with those of Woodstock and Justice (1967) who showed that levels of irradiation sufficient to stimulate percentage germination caused inhibition of respiration and seedling vigour. They compared measures of seedling length, root length and seedling fresh weight finding that all three parameters reflected increased activity equally well in maize, wheat, sorghum and radish seeds.

There are many theories on the relative contributions of grain and its microbial population to total respiration. On soybean, micro-organisms were considered to be the major contributors to respiration at 18.5% water content (Ramstad & Geddes, 1942). In one study, with maize at 17-26% water content, there was no difference between treated grain and that treated with a fungicide (Ragai & Loomis, 1953). In another, respiration rate was doubled in sodium hypochlorite-sterilised seeds compared with that in naturally colonised grain and the time to 0.5% DML with 'fungus-free' maize was 4.5 d earlier than in naturally contaminated grain (Seitz *et al.*, 1982b). Woodstock & Combs (1965) considered that sodium hypochlorite inhibited seed activity and decreased germination at the high levels examined by Ramakrishna *et al.* (1991). The reverse was true for wheat grain at 16-24% water content. Milner *et al.* (1947b) showed that treatment with eight sterilant chemicals decreased respiration by at least 20% at water contents above 16%, but no indication was given of how effective these chemicals had been at reducing or removing fungal contamination.

Sterilising grain to measure respiration in the absence of fungal activity is complex, as there is a fine balance between the separate contributions of grain and its microflora and, as discussed, the sterilising process may itself interfere with the seed's own biochemistry. Removing fungi from the seed will itself stimulate germination for a number of reasons: Competition for the endosperm substrate, which growing fungi and the germinating grain utilise, will be eliminated; fungi will not grow into and destroy the embryo; and mycelial growth over the seed will not inhibit gas and water vapour exchange between the grain and the atmosphere. For this and reasons described above, respiration attributable to sterile grain or fungi on autoclaved grain, when added together may be expected to be greater than naturally contaminated grain under identical conditions. This aspect is discussed further in Chapter 6 in comparison with the respiration of naturally contaminated grain.

CHAPTER 6
GENERAL DISCUSSION

6.1 METHOD DEVELOPMENT

The two systems developed and used in this study for measuring respiration in stored grain were successful and had considerable potential for future use. Calculations made using respirometer data represented accurate losses in grain dry matter. This method is likely to be more precise than calculating DML from changes in the weight of incubated grain since it is not complicated by increasing fungal biomass. Consequently, the technique of incubating samples in humid chambers was considered unsuitable for use in future studies. Additionally, the large scale apparatus allowed the results to be interpreted in terms of energy flow.

RQ calculated from the use of the electrolytic respirometer were generally < 1.0 which was different from some other published determinations. For example, White *et al.*, (1982a) found RQ closer to 1.0. It would be of further interest to investigate RQ changes with different CO_2 detection methods. Other factors which may affect the RQ include: spore germination and germ tube development, penetration of the seed coat containing complex compounds, invasion of the carbohydrate-rich endosperm and growth through the embryo, death of the seed, utilisation of carbohydrate remaining in the non-viable grain and utilisation of lipids and proteins during or after carbohydrate depletion.

Studies of γ -irradiated grain showed a dose of 10 kGy to be sufficient to remove fungal contamination from grains with minimal effect on seed germination and vigour. This means of sterilisation may be used in future studies of fungal colonisation of sterile grain, but it should be combined with studies on autoclaved grain because fungi may colonise irradiated grain faster than autoclaved and naturally contaminated grains (Niles, 1978).

Figure 6.1 compares DML calculated in large scale respiration studies, where only CO_2 was measured, with those from the electrolytic respirometer data, calculated from both O_2 and CO_2 data, over the range of temperatures found in the large scale experiments.

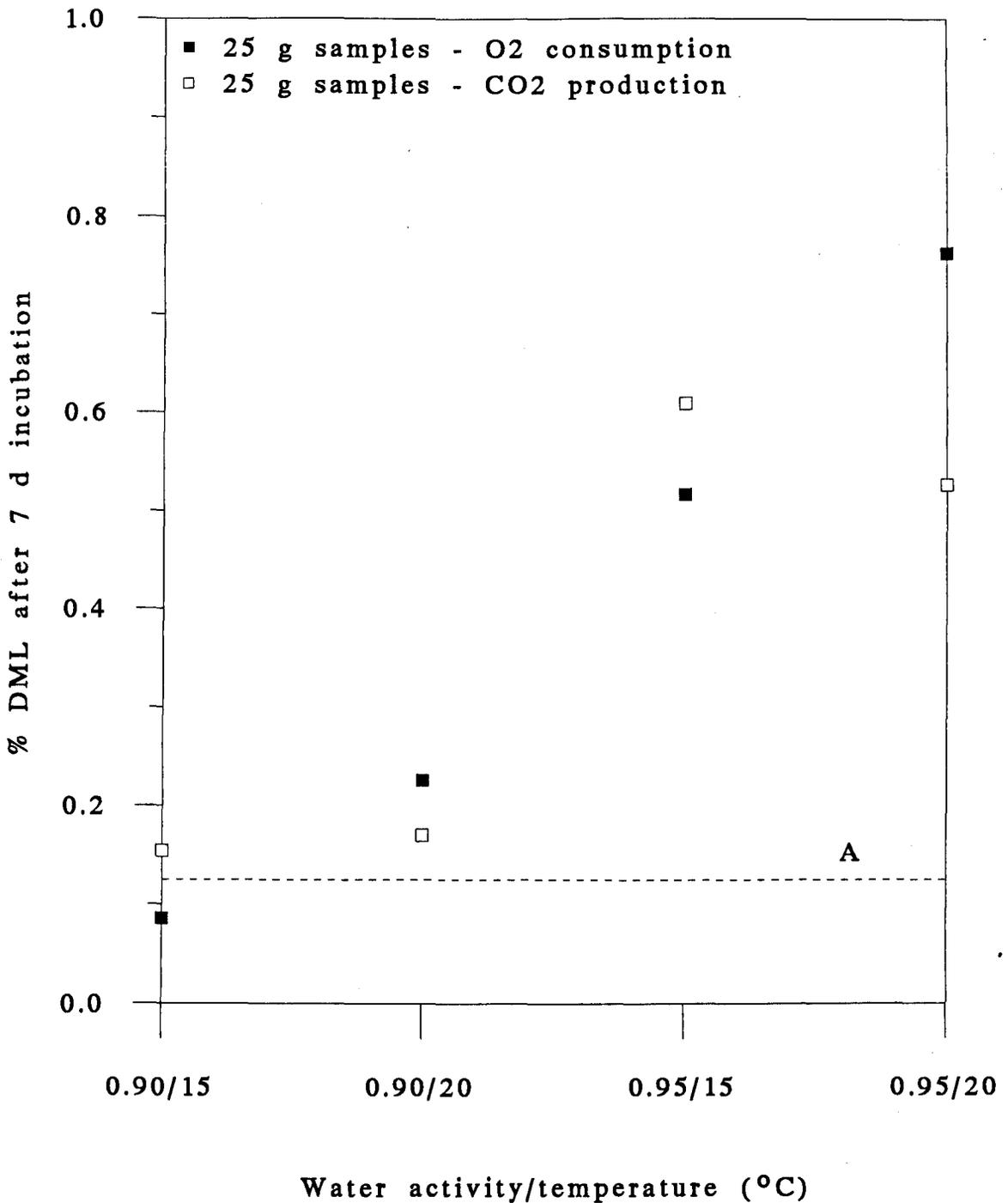


Figure 6.1 Comparison of determinations of % dry matter loss calculated for wheat grain cv. Avalon during storage at 0.90-0.95 a_w /15-20°C (using 25 g wet weight samples in the electrolytic respirometer) with respiration 0.90-0.93 a_w /12-20 or 18-21.5°C (using 10 kg wet weight samples in large scale respiration chambers, DML in all three samples represented by horizontal line A).

DML at 15°C calculated from O₂ consumption data were smaller than those calculated from CO₂ production at 15°C, but at 20°C, DML calculated from O₂ consumption were greater than from CO₂ production. DML from 0.90 a_w/15°C and 0.90 a_w/20°C in the respirometer were closest to DML at 0.90 a_w/18-21.5°C over 5 d and 0.91-93 a_w/12-20°C over 7 d (Figure 6.1).

6.2 PATTERNS OF GRAIN RESPIRATION AND DRY MATTER LOSS

A wealth of data have been obtained for the first time on grain respiration under steady state conditions which have enabled mathematical modelling. The pattern of O₂ consumption in wheat grain with time depended on its a_w and temperature during incubation. The difference observed between 'low' environmental conditions (0.80-0.85 a_w and 15-25°C) and 'higher' conditions (0.90-0.95 a_w and 15-25°C and 0.80-0.95 a_w and 30-35°C) was as follows:

A non-linear pattern of respiration was observed in the following samples:

- a) Wheat grain cv. Avalon at 0.80-0.85 a_w and 15-25°C.
- b) Autoclaved wheat samples that had been re-inoculated with dry fungal spores.
- c) Linseed where total respiration was low and only 24% of the seeds carried fungi at the start of the respiration experiment.

Growth curves such as these are characteristic of fungi and exhibit a lag phase indicative of spore germination and the beginning of hyphal penetration of seeds to reach substrates. The non-linear pattern of respiration was always associated with conditions suitable for slow fungal growth on grain as a result of low a_w, low temperature and/or poor initial inoculum.

At low a_w, the respiration of naturally contaminated wheat grain increased slowly with time. No fungal growth was visible and germination was not inhibited. Therefore the inputs from grain and fungi to total respiration were steady during 7 d incubation.

At 'high' a_w and temperature, fungal growth disrupted the grain's own respiration by suffocating and penetrating the seed, and decreasing germination as grains were killed. Fungal respiration was then contributing by increasingly greater amounts than grain respiration. A linear pattern of respiration was observed in the following samples:

- a) Wheat cv. Avalon at 0.90-0.95 a_w /15-25°C and 0.80-0.95 a_w /30-35°C.
- b) Barley, rapeseed and wheat cvs. Rendezvous and Riband at 0.90 a_w /20°C.
- c) Grain irradiated with up to 15 kGy incubated at 0.90 a_w /25°C.

The absence of a lag phase during incubation of such naturally contaminated samples could suggest that fungi were active and their spores had germinated before incubation. This might have occurred during hydration and overnight refrigerated equilibration or while samples were held at room temperature to be placed in respirometer units. This pattern of respiration could thus have indicated fungal activity already in the linear phase of the characteristic growth curve. The lag phase may also have been concealed by respiration of the grain itself which is linear, as described above, and may have been less masked by the hydration period than the fungal component. When mycelium penetrates grains and inhibits seed respiration, the grain component of respiration decreases, allowing the fungi to utilise all remaining substrates. Some samples decreased in germination rate without visible moulding, so it can be assumed that losses in germination occur before visible moulding and sporulation. Had experiments been allowed to continue for longer than 7 d, respiration would have declined as all available substrates were utilised by the fungi.

Respiration increased with a_w and temperature, both in naturally contaminated grain and in autoclaved grain that had been inoculated with representative storage fungi. Respiration of wheat grain increased in a non-linear fashion with increasing a_w and temperature, possibly because the sum of grain and fungal respiration was measured. By contrast, Muck *et al.* (1991) proposed a two-segment linear model for fungal growth rate in silage, where fungal growth rate (of some species common on stored grains) increased linearly with $a_w \leq 0.99$. RQ also increased with a_w , probably indicating that

the efficiency of respiration, i.e. the ratio of CO₂ produced:O₂ consumed, increased with increasing a_w and temperature. The increase in fungal biomass on grain means that not all CO₂ will be released into the atmosphere. Lower a_w favour different fungi with different enzymes that contribute to respiration. Also, where larger RQ were noted, fungi and grain may have utilised predominantly starch and glucose, while at low a_w fungi utilised resources to penetrate the seed coat with cellulolytic enzymes.

The interdependence of fungal with grain respiration as time progresses makes it difficult to quantify their relative contributions by measuring respiration. In practical grain storage systems, it is more important that overall respiration and dry matter loss are known, and then, if necessary, fungi can be quantified using more rapid biochemical tests.

Studies have confirmed that certain environmental conditions are more conducive to fungal growth and grain quality losses than suggested by earlier studies and also that acceptable DML for safe storage may be less than thought acceptable, although earlier assessments of quality loss have depended on the highly subjective criterion of presence or absence of visible mould. Significantly decreased germination accompanied by DML as low as 0.085% or less may render grain unfit for use, while mycotoxins may occur with even smaller DML.

6.3 *STERILE RE-INOCULATED GRAIN*

Table 6.1 shows the respiration of naturally contaminated grain, γ -irradiation-sterilised grain and fungi inoculated onto autoclaved grain under different conditions. Samples inoculated with a mixture of *Penicillium aurantiogriseum* and *Eurotium amstelodami* at two points respired faster than naturally contaminated grain samples, in which 100% of the grains carried fungi. This shows that it would be unwise to attempt to calculate the relative contributions of grain and fungi to total respiration by simple subtraction. Similarly, at 25°C, naturally contaminated grain utilised 1.0 l O₂ kg⁻¹ d⁻¹ more than sterile (γ -irradiated) grain. For the reasons discussed above fungi may have utilised

more than 1.0 l O₂ because there would be a net increase in the fungal contribution and a net decrease in the grain's contribution to total respiration during storage and colonisation. However, it is evident that the endogenous respiration of grain under these conditions may be important in its contribution to DML at the beginning of storage before fungi develop. This being the case, the use of fungicides to inhibit fungi for safe storage would only delay, rather than arrest, quality losses and for safe, clean storage of grain there would be no better alternative to drying. Table 6.2 compares the RQ calculated from respiration of naturally contaminated and inoculated wheat grain showing that the biochemical processes in metabolism of grain's rich nutrient source may differ between the grain and fungal component of respiration and at different environmental conditions.

The population of microbial species on grain is subject to antagonistic, additive, synergistic interactions (Magan & Lacey, 1984a; Ramakrishna *et al.*, 1993) and the study of individual components of the complex ecosystem by respiratory measurement may help towards developing a new method for quantifying fungal material on grain, perhaps even other substrates.

Varietal differences in respiration during storage of wheat grain cultivars could be important because the recommended varieties alter annually (NIAB, 1991) with new varieties introduced to meet disease resistance and improved yield and baking quality demands. Unfortunately, wheat cultivars are not selected for their resilience to DML in store, but knowledge of a range of possible varieties is necessary to set base-line figures.

Table 6.1 Differences in the respiration of naturally contaminated, sterile and re-inoculated wheat grain.

Temperature (°C)	l O ₂ consumed kg dry grain ⁻¹ 7 d ⁻¹				
	Naturally contaminated	10 kGy γ - irradiated	Inoculated with:		
			EE	PP	EP
20°C	1.7	nd	0.3	1.4	2.1
25°C	3.3	2.3	nd	nd	nd

nd - not determined

EE - *Eurotium amstelodami* inoculated

PP - *Penicillium aurantiogriseum* inoculated

EP - Inoculated with both species

Table 6.2 Comparison of RQ determined for naturally contaminated and re-inoculated wheat grain at 20°C.

Grain treatment:	0.85 a _w	0.90 a _w
Naturally contaminated	0.34	0.75
<i>Eurotium</i> -inoculated	0.77	nd
<i>Penicillium</i> -inoculated	0.75	0.88
Inoculated with both species	0.89	0.78

6.4 *CONCLUSIONS*

1. Respirometry gave more sensitive estimates of DML than direct weighing in wheat grain under a wide range of environmental conditions.
2. Visible moulding occurs in wheat grain with as little as 0.130% DML and losses in germination occur with only 0.085% DML. Thus previously accepted safe storage periods must be refined.
3. Wheat grain respire more rapidly than barley grain, rapeseed and linseed because of structural and biochemical differences in their composition.
4. RQ in wheat grain differed with storage conditions for many complex reasons.

6.5 *SUGGESTIONS FOR FUTURE WORK*

1. Observation of the changes in RQ during different stages of seed colonisation by fungi and the effects of prior irradiation using destructive sampling of respirometer experiments.
2. Comparison of fluctuating and steady state environmental conditions to determine the effects of changing temperature on a_w , and hence respiration of fungi and grain, in respirometers and large scale chambers.
3. Modelling of the interactions between different fungal species and insects, to include temperature changes representative of hot spots.
4. Investigation of DML with different interacting fungi and the effect of field fungi on subsequent fungal colonisation during storage.

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APPENDICES

APPENDIX A

RAW OXYGEN CONSUMPTION DATA FROM THE ELECTROLYTIC RESPIROMETER - WHEAT GRAIN CV. AVALON INCUBATED FOR 7 D.

Temperature (°C)	Expt rep.	Total ml oxygen consumed per 25 g (wet weight) at water activity:			
		0.80	0.85	0.90	0.95
15	A	1.318	1.869	7.849	63.208
		1.280	1.625	10.178	61.935
		1.278	2.351	12.233	nd
		1.073	4.049	9.741	63.164
15	B	1.013	2.630	21.565	79.262
		1.120	2.920	nd	75.866
		1.076	4.607	20.232	77.210
		0.445	2.565	21.188	74.880
20	A	1.671	2.962	nd	56.231
		3.516	4.872	19.004	56.426
		1.568	2.756	20.916	53.967
		nd	5.070	21.687	57.918
20	B	3.842	1.876	25.543	92.754
		4.619	5.548	16.534	94.486
		2.408	8.229	28.371	97.570
		4.310	3.122	22.417	92.246

25	A	9.560	19.733	69.702	153.629
		8.080	16.533	77.288	167.808
		7.785	21.975	76.286	169.415
		7.319	21.583	79.778	170.375
	B	4.569	12.108	52.176	173.932
		4.696	13.903	52.384	166.833
		2.966	11.365	51.992	164.162
		nd	11.670	nd	158.726
30	A	nd	27.880	nd	170.284
		nd	29.099	nd	nd
		nd	nd	nd	129.371
		nd	nd	nd	130.641
30	B	10.566	26.074	54.232	172.164
		10.189	21.448	nd	168.694
		8.962	24.197	51.096	145.420
		8.188	27.092	50.079	163.934
35	A	15.583	51.334	107.666	184.318
		25.659	48.392	121.941	181.907
		24.416	71.213	107.096	151.791
		17.276	nd	117.172	157.919

nd - not determined (e.g. samples lost)

All data files showing oxygen consumption with time in the above samples have been lodged on floppy disk with Dr N Magan of the Biotechnology Centre of Cranfield University.

APPENDIX B

RAW CARBON DIOXIDE PRODUCTION DATA FROM THE ELECTROLYTIC RESPIROMETER - WHEAT GRAIN CV. AVALON INCUBATED FOR 7 D.

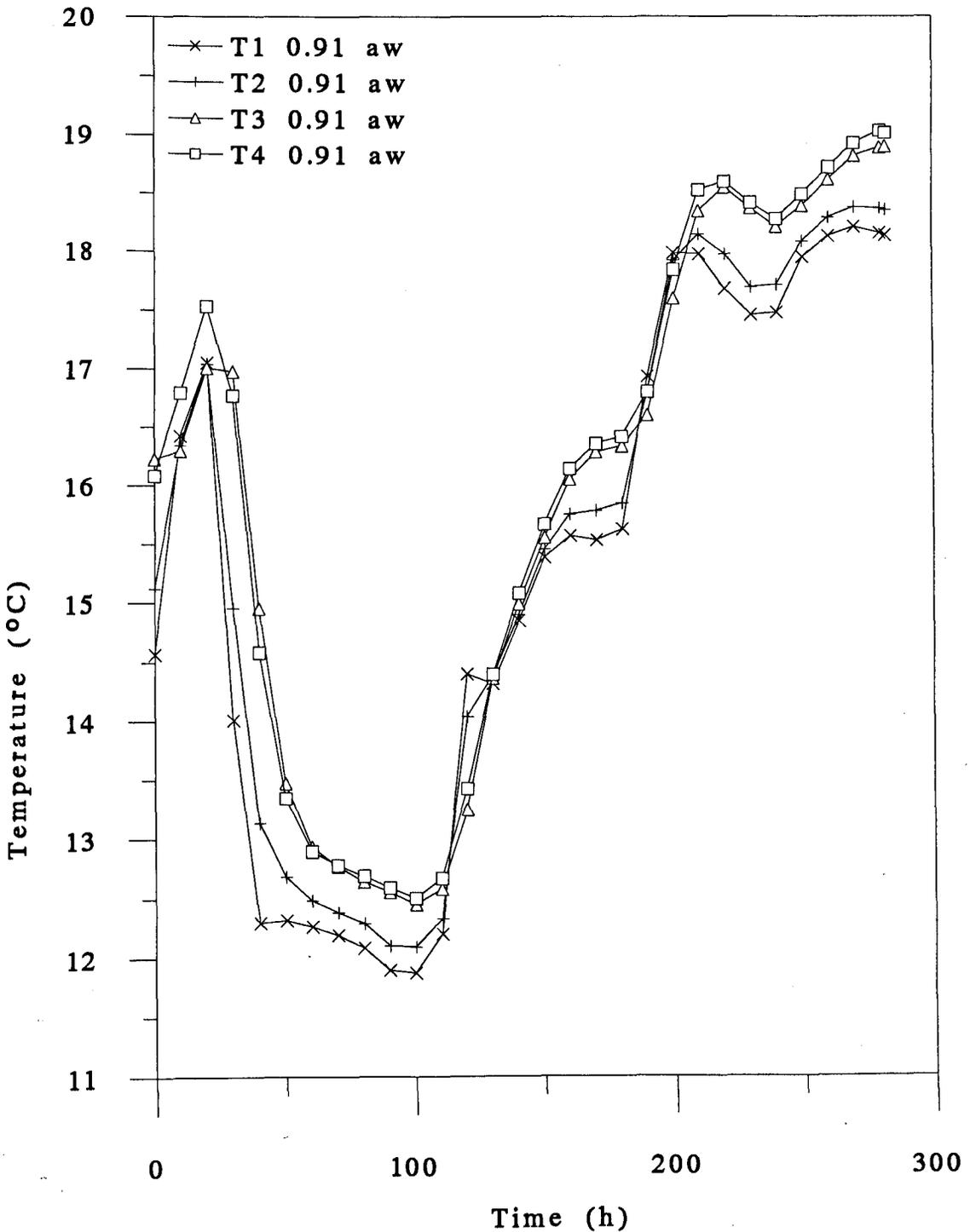
Temperature (°C)	Expt rep.	Total ml carbon dioxide produced per 25 g (wet weight) at water activity:			
		0.80	0.85	0.90	0.95
15	A	nd	2.08	11.73	44.61
		-ve	1.86	8.00	33.65
		2.082	1.64	8.00	nd
		-ve	2.30	nd	43.29
15	B	5.64	nd	55.25	nd
		7.89	14.66	nd	127.41
		5.64	14.66	2.73	136.43
		5.64	12.40	2.61	125.15
20	A	-ve	-ve	nd	37.96
		-ve	0	8.93	40.19
		-ve	-ve	11.17	35.73
		-ve	-ve	11.17	42.43
20	B	2.23	0	20.10	71.46
		-ve	2.23	20.10	69.22
		2.23	2.23	20.10	75.92
		2.23	0	22.33	73.69

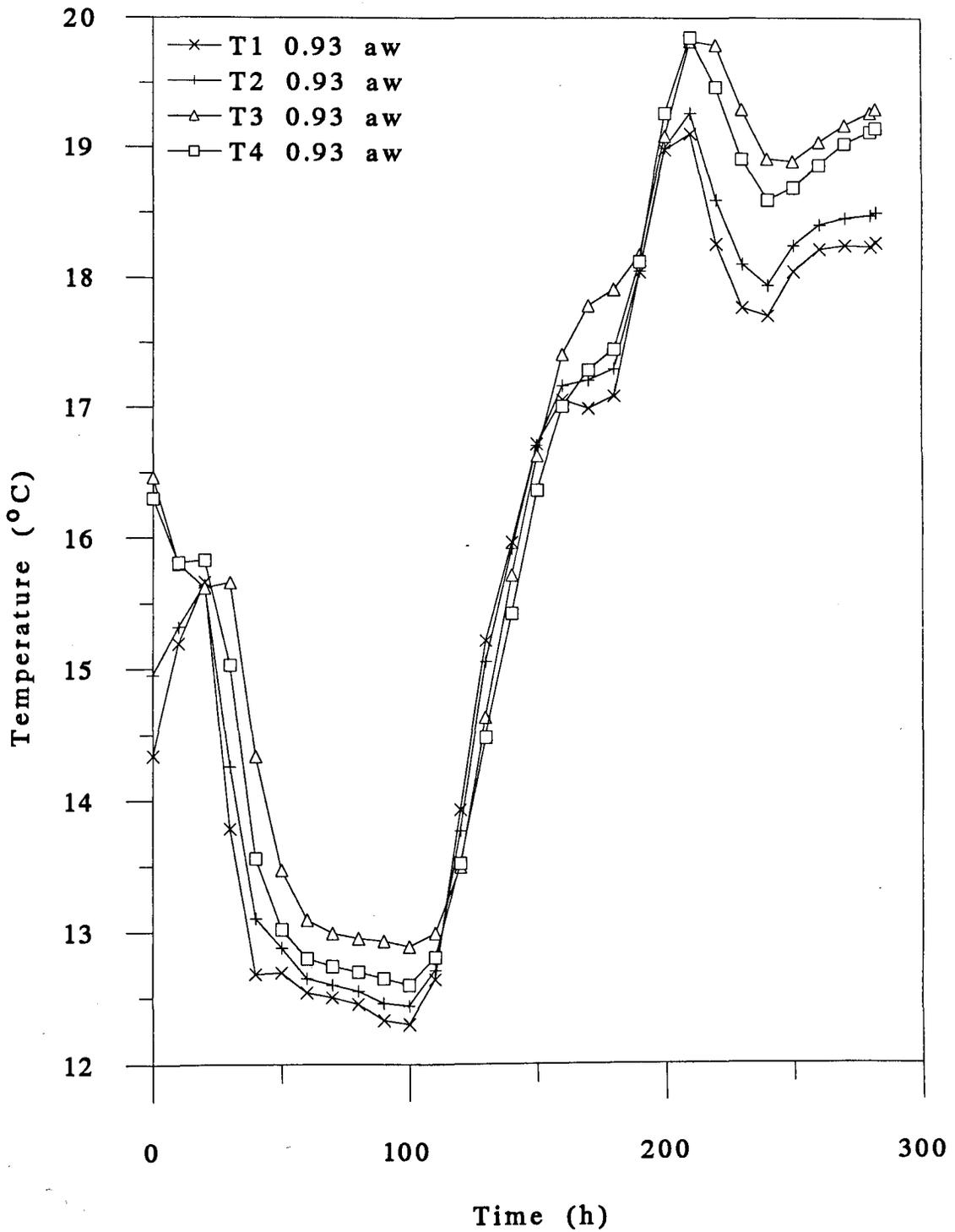
25	A	1.58	1.58	17.36	62.46
		-ve	1.58	19.62	60.21
		-ve	1.58	15.11	60.21
		-ve	1.58	15.11	60.21
25	B	2.26	20.30	41.72	134.17
		2.26	7.89	37.21	138.68
		1.13	7.89	71.03	138.68
		nd	12.40	nd	128.54
30	A	nd	23.83	nd	156.00
		nd	20.57	nd	154.74
		nd	nd	nd	120.64
		nd	nd	nd	117.12
30	B	6.73	22.05	50.91	152.14
		5.84	21.82	35.59	150.58
		4.73	19.60	43.14	nd
		5.62	30.48	44.47	146.81
35	A	11.39	38.92	93.08	219.18
		21.82	38.47	93.75	nd
		21.60	31.81	89.29	145.48
		15.61	nd	nd	142.15

-ve - negative values were calculated from titration results so these results samples were not utilised further.

nd - not determined (e.g. samples lost)

APPENDIX C

INDIVIDUAL DATA FROM THERMOCOUPLES T₁ TO T₄ IN BOTH SAMPLES STUDIED IN LARGE SCALE RESPIRATION EXPERIMENT BFigure A. 0.91 a_w sample,

Figure B. 0.93 a_w sample

APPENDIX D**PUBLICATIONS RESULTING FROM THE RESEARCH**

The following publications are available, having resulted from the research presented in this thesis:

1. HAMER, A., LACEY, J. AND MAGAN, N. (1991). Use of an automatic electrolytic respirometer to measure the respiration of stored grain. In *Proceedings of the 5th International Working Conference on Stored Product Protection*. Edited by F. Fleurat-Lessard.
2. LACEY, J., RAMAKRISHNA, N., HAMER, A., MAGAN, N. AND MARFLEET, I. (1991). Grain Fungi. In *Handbook of Applied Mycology*, Vol. 3. Edited by D.K. Arora, K.G. Mukerji & E.H. Marth, pp. 121-177. New York, Marcel Dekker.
3. HAMER, A., LACEY, J. & MAGAN, N. (1992). Respiration and fungal deterioration of stored grains. In *Extended Abstracts, International Symposium on Stored Grain Ecosystems*. Edited by G.S. Jayas, N.D.G. White, W.E. Muir & R.N. Sinha. University of Manitoba, pp 65-6. ISBN 0-9696254-0-5.