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Ozone inactivation of fungi associated with barley grain

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Ozone Inactivation of Fungi Associated with Barley Grain

by

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Abstract

The use of ozone as a fungicide for barley storage was studied. The effects of ozone on the inactivation of natural and inoculated fungi on barley were evaluated at different water activities and temperatures. Results indicated that higher ozone doses were better at inactivating the natural fungi on barley than lower ozone doses. An ozone dose of 0.98mg/g • min was able to reduce the natural fungi counts on the barley at 0.98a_w by over 97% after 45 minutes of ozone contact time. The inactivation of the natural fungi and *Aspergillus flavus* on the barley was favoured by higher temperatures. Ozone consumption of the barley was higher at higher temperatures. Tests on *A. flavus* spores inoculated on barley indicated that they were resistant to ozone doses up to 4.90mg/g while *Penicillium verrucosum* spores showed no resistance to any ozone doses that were tested. Inactivation of *A. flavus* (vegetative state) and *P. verrucosum* spores were favoured by higher water activities. The effect of ozone on barley germination was also examined. By 15 minutes of treatment at an ozone dose of 0.98mg/g • min, all the fungi tested were inactivated by over 90%, while germination was only reduced by 6%. Different water activities had no effect on the germination of barley. The findings show that ozone may be applied as a fumigant against fungi attacking high moisture content barley and provide the possibility of using it as an alternative to current chemicals for preserving stored barley.

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Chapter 1

1.1 Introduction

Cereal grains are a major source of food for the world. Losses of up to 50% are reported in some countries which puts pressure on food supplies (Jayas, 1999).

Wet harvests and warm temperatures often result in spoilage of stored grains. Fungi that grow on grains being stored can cause reductions in the quality (Hasan, 1999) and quantity of the grains. In addition, fungal species such as *Penicillium verrucosum* (*P.* verrucosum) and *Aspergillus flavus* (*A. flavus*) may be found during barley storage (Hill and Lacey, 1983; Mills and Wallace, 1979; Pettersson *et al.*, 1989) producing mycotoxins that are highly toxic to animals and humans.

To inhibit the growth of fungi, grains have been dried or treated with fungicides such as acids and commercially available pesticides. However, grain driers found on farms generally have inadequate capacity; they are inefficient and have inferior temperature controls and airflow (AACC, 1993). Also, treatment of grains with acids can result in reduced germination (McLelland, 1999), discoloration of grains and aflatoxin formation (Clevström *et al.*, 1989; Pettersson *et al.*, 1989). The public also holds negative views of pesticides being put on crops and often associate their uses with concerns about health (Miles and Frewer, 2001). With a world wide trend away from the above products, farmers are left defenceless against fungal attacks.

Ozone is described as being a powerful fungicidal agent and studies dealing with the microbiocidal properties on food have been widespread (Kim *et al.*, 1999; Barth *et al.*, 1995; Byun *et al.*, 1997; Zhao and Cranston, 1995; Sarig *et al.*, 1996; Greer and Jones, 1989). Ozone could be an attractive alternative to other chemicals being used because it is a non-residual fungicide and can be easily distributed throughout the grains. However, there is little information on the use of ozone as a fungicide for grain storage.

1.2 Statement of the problem

This study derived its significance from its potential for shedding light on a possible alternative to current chemical uses for storage of high moisture grains. Currently there is a world wide trend to eliminate the use of residual fungicides such as acids and methyl bromide. Under the terms of the Montreal Protocol, methyl bromide is scheduled to be completely eliminated by 2005 in developed countries and by 2015 in developing countries because of it's ozone depletion potential (Rice, 2001). Organic acids have had problems associated with reduced germination (McLelland, 1999), aflatoxin formation (Clevström *et al.*, 1989; Pettersson *et al.*, 1989) and a worldwide trend towards limiting their use on grains (Paster *et al.*, 1995). However, there have been no practical replacements for any of these residual type fungicides.

In addition, mycotoxin producing storage fungi such as *A. flavus* (Abdel-Hafez, 1984; Clevström *et al.*, 1989; Hill and Lacey, 1983; Mills and Wallace, 1979; Pettersson *et al.*, 1989; Sanchis *et al.*, 1993) and *P. verrucosum* (Hill and Lacey, 1983; Wallace and Mills, 1979) are associated with barley. These fungi are capable of producing carcinogenic mycotoxins which constitute a public health hazard if they enter the food supply. Formic acid has been prohibited for use as a preservative of high moisture grain in Sweden because of its association with aflatoxin production (Clevström *et al.*, 1989; Pettersson *et al.*, 1989). Therefore, it is important that the growth of these organisms is limited.

1.3 Purpose of the study

The purpose of the present study was to investigate the effects of ozone on both the vegetative and spore forms of fungi associated with barley grain. The objectives of the study were:

1.) to examine the effect of ozone dose on the inactivation of *A. flavus*, *P. verrucosum* and the naturally occurring fungi associated with the barley;

2.) to examine the effect of water activity on ozone inactivation of *A. flavus*, *P. verrucosum* and the naturally occurring fungi associated with barley;

3.) to examine the effect of temperature on ozone inactivation of *A. flavus* and the naturally occurring fungi;

4.) to determine the ozone consumption during the inactivation processes;

5.) to examine the effect of ozone on the germination of barley.

The present study will be a valuable addition to the current knowledge on the microbiocidal properties of ozone to a long standing food storage problem with grains.

1.4 Limitations and delimitations

Several limitations that were beyond the researchers control emerged in the study:

• Current microbiological procedures for counts are selective and will always underestimate actual counts. The culture media used in this study (potato dextrose agar) may pre-select organisms that favour the nutritional status of the agar. It is also assumed that each colony that formed on the culture media originated from a single fungus.

• Slight fluctuations in ozone dosage were present between experiments because of the difficulty in adjusting the ozone generator to an exact dose. Care was taken to minimize this variation by allowing the ozone monitor readings to stabilize and then taking the average of readings 10 minutes before and after the experiment. The average of these readings was deemed to be the ozone dose.

Several constraints were imposed on the project that should be taken into consideration when reviewing the results.

- One constraint was the small sample size of barley (50g) used in the study. Large bins of grain may have "dead" spots where ozone may not reach.
- Identification of the fungal microflora present on the barley were not done in this study. The wide variety of fungus found on barley may make it difficult to reproduce the results of this study dealing with the natural fungi.
- Fungi that were inoculated on the barley may not represent "real" conditions in storage, as many fungi grow internally in grains. Fungi that grow internally may be shielded from the action of ozone and therefore may exhibit inactivation rates different than that shown here.
- A hulled variety of barley was chosen for this study because it was thought it would be harder for the ozone to penetrate the hull to reduce internal fungi on the grain. Other hulless varieties may have different reactions with ozone.

Chapter 2 – Literature Review

2.1 Introduction

The articles included in the review were kept in the scope of three areas. The first area of the review deals with a brief look at the history and general information on ozone. The second section reviews the microbiological aspects of ozonation such as the effects of water activity and temperature. The scope of this section was focused on fungi, but a lack in research in this area required a look at papers dealing with other microorganisms. The last section of the review deals with the agricultural side of the paper. This part will include a look at the microbiology of barley with current/past concerns with barley.

2.2 History and general information on ozone

Disinfection of food (potable water) with ozone was first done in 1906 in Nice, France (Graham, 1997). Other countries, including the Netherlands, Germany, Austria and Switzerland soon incorporated ozone as a disinfectant into their public water supplies (Graham, 1997). In 1982 an expert panel in the United States granted ozone as a GRAS (generally recognized as safe) substance for use on food (Kim *et al.*, 1999; Graham, 1997). The new status of ozone has sparked interest among researchers and food processors.

Past research efforts on ozone have mainly been focused on water and wastewater applications. However, on June 26, 2001, the U.S.

Food and Drug Administration approved the use of ozone in gaseous and aqueous phases as an antimicrobial agent on food (Rice, 2001). The new status of ozone has sparked interest among researchers and food processors.

Ozone is a form of oxygen that has three atoms per molecule (O_3), clear to bluish in color, has a pungent odour and because of its unstable nature it decomposes fairly quickly into ordinary oxygen. Because of the strong oxidizing properties this gas possesses, it is a very effective disinfectant.

Ozone is generated on-site minimizing handling and transportation costs. There are currently several ways man can produce ozone. The first method is by subjecting oxygen to UV radiation of 185nm wavelength from high powered UV lamps (Kim *et al.*, 1999). Unfortunately, the UV method does not produce large quantities of ozone. The second and most popular method is the corona discharge method. This method is able to produce large amounts of ozone when a high voltage alternating current is applied across a discharge gap in the presence of air or oxygen (Kim *et al.*, 1999). This excites oxygen atoms and induces splitting. The split oxygen atoms then combine with other split oxygen atoms to produce ozone. Ozone may also be produced by chemical, thermal, chemonuclear and electrolytic methods (Kim *et al.*, 1999). Lynntech, Inc. has implemented a new way to produce ozone by an electrochemical method (McKenzie *et al.*, 1997; Kim *et al.*, 1999).

This process splits water into hydrogen and oxygen atoms by electrolysis. The hydrogen is then separated from the stream and the oxygen atoms combine to form ozone and diatomic oxygen (McKenzie *et al.*, 1997; Kim *et al.*, 1999). This method is able to produce up to 20% weight of ozone compared to the 4% weight of ozone with the corona discharge method (McKenzie *et al.*, 1997; Kim *et al.*, 1999).

Ozone concentrations can be determined by physical, physiochemical and chemical methods. Physical methods rely on measuring the direct absorption in the UV, visible or infrared region of the spectrum. Physiochemical methods measure effects such as heat or chemiluminescence caused by a reaction. Chemical methods measure the quantity of a product released when ozone reacts with a chemical reagent such as potassium iodide.

The indigo colorimetric method is a chemical method that is approved by the committee as a standard method for the examination of dissolved ozone (APHA, 1998). In this method ozone reacts additively with the carbon-carbon double bond of sulfonated indigo dye causing it to decolourize. The change in color can then be measured spectrophotometrically.

The iodometric method,is a standard method usually used for the examination of gaseous ozone (APHA, 1998). In this method, ozone is bubbled through a solution containing a known concentration of the

iodide ion. The ozone oxidizes the iodide ion, releasing iodine. The iodine is then titrated with sodium thiosulfate to a starch endpoint.

UV measurement of ozone is a physical method of determination. Gaseous ozone absorbs short-UV wavelengths with a maximum absorption at 253.7nm. Kim *et al.* (1999) indicated that for accurate determination of gaseous ozone, the UV spectrophotometric method should be used.

2.3 Microbial inactivation by ozone

Ozone is a broad spectrum antimicrobial agent that is active against bacteria, fungi, viruses, protozoa, bacterial spores and fungal spores. However, there is little agreement among researchers as to the sensitivity of different microorganisms to ozone. There is also variation between studies as to the sensitivity of single microorganisms to ozone. Comparisons between studies becomes difficult because of variations among the strain of the microorganisms, age of the culture, density of the treated population, presence of ozone demanding materials, method of applying ozone, accuracy of ozone measuring procedures and method of measuring antimicrobial efficacy (Khadre *et al.*, 2001).

It is difficult to assess how ozone inactivates microorganisms because of the numerous cellular constituents that are able to react with the gas. Some authors have suggested that ozone is the main inactivator of microorganisms while others have emphasized the importance of the

antimicrobial reactive by-products of ozone decomposition such as hydroperoxyl ($^{\cdot}HO_2$), hydroxyl ($^{\cdot}OH$) and superoxide (O_2^{-}) radicals (Kim *et al.*, 1999; Khadre *et al.*, 2001; Hunt and Mariñas, 1997).

It is generally agreed that fungal spores have greater resistance to ozone than that of fungi in their vegetative state. A study performed by Palou *et al.* (2001) used gaseous ozone on *Penicillium digitatum* and *Penicillium italicum* inoculated on Valencia oranges and lemons. Valencia oranges were exposed to continuous ozone at 0.3ppm for 4 weeks and lemons were intermittently exposed to 0.3ppm ozone for 9 weeks. Ozone exposure on both fruit and fungi delayed the incidence of disease by 1 week. Although infection developed more slowly under ozone, disease incidence at the end of the storage period was not reduced. Even though disease was not controlled, ozone exposure did inhibit the normal aerial growth of the mycelia and greatly reduced sporulation, until the product was removed from the ozone atmosphere. Also, inoculum density had no influence on the effect of ozone on both the incidence and severity of decay on the oranges and lemons. Barth *et al.* (1995) used 0.1 and 0.3ppm continuous ozone for up to 14 days on blackberries. No fungal decay was observed on ozone treated fruit after 12 days as compared to 20% of the control. Sarig *et al.* (1996) used ozone on table grapes to control fungi. The fungi were reduced very fast in the first five minutes of ozone treatment and then a slower more gradual decline in numbers were observed. Ozone was effective at

reducing *Rhizopus stolonifer* on grape cultivars Alphonse Lavallee and Thompson Seedless with no infected berries remaining after 5 minutes of treatment (Sarig *et al.*, 1996). However, Zeiny cultivar had infected berries remaining after a treatment time of 30 minutes (Sarig *et al.*, 1996). Caution in these results must be exercised because the experimenters only incubated inoculated Petri dishes for 24 hours at 28°C. This may underestimate the counts. A fibreglass duct liner, fiberboard duct and galvanized steel were inoculated with *Penicillium glabrum* spores and treated with an ozone dose of 9ppm (Foarde *et al.*, 1997). No differences were seen between the ozone treated materials and the control. Two ceiling tiles and a fibreglass duct liner were inoculated with *Penicillium chrysogenum* spores and treated with an ozone dose of 9ppm (Foarde *et al.*, 1997). No differences were seen between the control and ozone treated material. *Aspergillus niger* spores, *Candidas albicans, Zygosaccharomyces bailii* were exposed to 0.19ppm of ozone in deionized water (Restaino *et al.*, 1995). After five minutes of treatment, less than a one log reduction was achieved with *A. niger* spores while *Candidas albicans* and *Zygosaccharomyces bailii* were reduced by over 5 logs.

2.4 Water activity and humidity

Water content is a direct measurement of the amount of moisture in a particular food. This can be determined by the air oven method of the AOAC (1995) by drying a product in an oven at 130°C for 1 hour.

Water activity on the other hand is the ratio of water vapour pressure above any sample to the water vapour pressure of pure water at the same temperature (Jay, 1996). It can also be defined as 1/100 the equilibrium relative humidity (Jay, 1996).

Higher humidity is better at disinfection when using ozone. One proposed mechanism suggests that the penetrability of ozone increases as the environments moisture is increased (Ishizaki *et al.*, 1986). This would allow ozone greater contact with key enzymes and organelles causing greater damage. It is also thought that the presence of water accelerates reactions with organic substrates allowing ozone greater contact (Ishizaki *et al.*, 1986). It is also possible that higher moisture environments have a greater availability of water to react with ozone to produce reactive radicals.

The water activity (or moisture content) of foods affects the ability of ozone to inactivate microorganisms. Zhao and Cranston (1995) tested ground black pepper at different moisture contents (3.9%, 10.4% and 17.6%) and found that the inactivation of all the microoganisms tested favoured higher moisture contents than lower ones. Unpublished data reported by Kim *et al.* (1999) also established moisture contents role in the inactivation of microorganisms. They reported that when the water activity of a powder food product was $0.95a_w$, 10^2 - 10^5 CFU/g were inactivated with 200ppm of ozone. However, a similar ozone concentration has no effect on the microbial

load of the food with a water activity of 0.85. A study reviewed by Kim *et al.* (1999) showed that desiccated microorganisms were more resistant than hydrated cells to sterilization. Neither of the authors mentioned mechanisms that may have been responsible for seeing the differences in the microbiocidal activity of ozone to different moisture contents.

Several investigators have pointed to the role relative humidity plays in the deactivation of microorganisms with ozone. Ishizaki *et al.* (1986) demonstrated this when they tested the inactivation of *Bacillus subtillis* and *Bacillus cereus* spores being ozonated in atmospheres containing 95, 90, 80, 70 and 50% relative humidity. The investigators found that as the relative humidity increased so did the inactivation of the spores. Moore *et al.* (2000) found that the effect relative humidity had on the inactivation of microorganisms was dependent on the microorganisms being tested. The inactivation rates for *E. coli, S. liquefaciens* and *S. aureus* were not significantly different between the relative humidity's of 77% and 58%. However, the inactivation of *L. innocua* was significantly better with a relative humidity of 77% than at 58%. To achieve a three log reduction, Foarde (1997) found that *Penicillium* spores at 30% relative humidity needed 9ppm ozone while at 90% relative humidity they only needed 6ppm. This effect was even greater for *R. glutinis* when an ozone dose of 5.8ppm at 90% relative humidity achieved a 3 log reduction, while at 30% relative humidity less than a one log reduction occurred.

2.5 Temperature

Temperature affects the solubility, stability and reactivity of ozone affecting the way it interacts with a substrate. As temperature increases, the reaction rate with the substrate and ozone decomposition (reactive radicals) is increased (Achen and Yousef, 2001). However, as temperature decreases in an aqueous medium, the solubility of ozone increases (Kim *et al.*, 1999; Achen and Yousef, 2001).

The influence of temperature on ozone decay rates and ·OH concentrations was studied by Elovitz *et al.* (2000) who subjected lake water from Switzerland to ozone. As temperature was raised from 5 to 35°C, ozone decay rates increased more than an order in magnitude. The experimenters found that water temperature had no effect on the production of ·OH radicals. It was also found with increasing temperatures that O_3 exposure and therefore disinfection was significantly reduced.

Few studies have examined the effect of temperature on ozone disinfection on food. Of those studies that have been published, no firm conclusions can be made on whether a temperature increase is beneficial or detrimental to disinfection. Herbold *et al.* (1989) tested temperatures of 10°C and 20°C on the inactivation of *Hepatitis A virus, Poliovirus 1* and *E. coli* in flowing water. The experimenters found that the lower temperature (10°C) resulted in a faster inactivation of the *Hepatitis A virus, Poliovirus 1* and *E. coli* than the higher temperature (20°C). On

the contrary, Achen and Yousef (2001) treated *E. coli* contaminated apples by bubbling ozone in water at 4, 22, and 45°C, and observed that counts of the bacterium on the surface decreased 3.3, 3.7, and 3.4 logs, respectively. The differences among the treatment groups were not statistically significant. An experiment done by Hunt and Mariñas (1997) showed that higher temperatures led to faster inactivation of *E. coli* in a semi-batch reactor than did lower temperatures.

2.6 Physio-chemical changes in food treated with ozone

Ozone is a powerful oxidant that may oxidize many compounds, both beneficial and deleterious, when used on food. This property of ozone may contribute to food quality enhancement or loss depending on the compounds on the food being acted upon and therefore either strengthen or weaken the possibility of using it.

Many investigators have reported a range of possible deleterious and beneficial effects ozone has had on different foodstuffs (Barth *et al.*, 1995; Byun *et al.*, 1997; Greer and Jones, 1989; Kim *et al.*, 1999; Sarig *et al.*, 1996; Zhao *et al.*, 1995). Ozone treated aloe powders had changes in fatty acid compositions, increase in thiobarbituric acid values (associated with rancid flavours), decrease in barbaloin (laxative component) and decreases in Hunter colour parameters (Byun *et al.*, 1997). There were no changes detected in amino acids or elemental contents of the aloe products (Byun *et al.*, 1997). Ozone treatment of

whole peppercorns caused only slight changes to the volatile oil constituents and did not produce any detectable new components (Zhao and Cranston, 1995). When ground pepper was treated with ozone, there were noticeable changes in the volatile oil constituents with the destruction of 16 components and the production of 14 new components. This was deemed negative for the spice industry by the investigators. Blackberries being treated with ozone showed no significant changes in anthocyanin content (previously shown to be a response of plants to ozone) and remained significantly redder for a longer time in storage (Barth *al.*, 1995). POD (peroxidase) activity has been shown to increase in ozone stressed plant tissues but was shown to decrease in all ozone treated blackberries (Barth *et al.*, 1995). Beef carcasses treated with ozone showed significant shrinkage (Greer and Jones, 1989). The muscle colour of the carcasses was significantly darker in ozone treated meat, but fat colour and muscle shear value were not influenced by treatment (Greer and Jones, 1989).

2.7 Occurrence of fungi on barley

The microflora found on barley and other grains in storage is quite diverse and is dependent upon the microbial inoculum it carries and the conditions of storage (Hill and Lacey, 1983). The *Aspergillus* and *Penicillium* genus appear to be the most frequently isolated on barley (Abdel-Hafez, 1984; Hill and Lacey, 1983). However, the

proportion and species types are greatly dependent on climate. For example, *A. flavus* appears to be more of a problem in warmer tropical regions while *P.* verrucosum appears to be more of a temperate region problem (Marquardt, 1996). Hill and Lacey (1983) performed an extensive catalogue of the microorganisms isolated from stored barley grain in the UK and found over 80 taxa of which more than 40% were *Penicillium* spp and 15% *Aspergillus* spp. *A. flavus* and *P. verrucosum,* were found on the barley up to 7 logs and 5 logs per gram of barley, respectively, but both had a low frequency (<10% of samples) of isolation. The authors unfortunately did not include the conditions under which the fungi were isolated from. Mills and Wallace (1979) from Winnipeg, Canada, catalogued the fungus population of barley from the field to bins. *A. flavus* occurred from 0-34% of the fungal population while *P. verrucosum* occurred from 0-52%. Abdel-Hafez (1984) isolated fungus from stored barley in Saudia Arabia and found *Aspergillus spp.* comprising of 26% and *Penicillium spp.* comprising of 18% of the total fungal population. *A. flavus* comprised 6.9% of the total fungal population along with being present on every barley sample. *P. verrucosum* was not isolated from any sample.

2.8 Fungal toxins

P. verrucosum and *A. flavus* are storage fungi that are capable of producing mycotoxins that are highly carcinogenic to both man and

animals. It is this reason why the U.S. Food and Drug Administration has set an action limit on the mycotoxin produced by *A. flavus* called aflatoxins to 20ppb (Marquardt, 1996; Norred, 2000; Rustom, 1997). However, many animals are fed poorer quality grains which may contain aflatoxins. Animals such as dairy cattle that have been fed feed contaminated with aflatoxins may experience deleterious effects from the toxins and are also known to pass the toxin into their milk which in turn may affect humans.

Fungal toxins have also been shown to be detrimental to the quality of grains. Germination of seeds affected by fungal toxins was dependent upon the seeds being tested and the toxins the seeds were exposed to (Hasan, 1999). Barley was the most sensitive among Sorghum and wheat when treated with aflatoxins, diacytoxyscirpenol, kojic acid and tenuazonic acid (Hassan, 1999). A 50% reduction in seed viability in barley could be achieved with 0.83mg/L of aflatoxins demonstrating the toxicity of this compound to seedlings (Hassan, 1999).

McKenzie *et al.* (1997) and Maeba *et al.* (1988) have both demonstrated ozone's ability to detoxify aflatoxins. Aflatoxins AfB1 and AfG1 were rapidly degraded using 2% weight ozone while AfB2 and AfG2 required higher ozone doses (20% weight ozone) for rapid degredation (McKenzie *et al.*, 1997). Maeba *et al.* (1988) also found that AfB2 and AfG2 were more resistant than AfB1 and AfG1 to ozone.

Mycotoxins such as, CPA, FB1, OA, patulin, SAD and ZEN have been undetectable after treatment with ozone (McKenzie *et al.*, 1997).

The ability of ozone to degrade toxins on grains may prove to be a valuable asset to farmers and may prove to be both a reactive and proactive solution to problems with toxins and spoilage.

2.9 Current treatment for high moisture barley

Harvesting grains has always been difficult with respect to the moisture content of the grain. Grains usually reach maturity several days before harvest, but because these grains are wet, the risk of spoilage during storage is present. After reaching maturity, grains are left on the field for several more days to naturally reduce the moisture content. Leaving the crop on the field for longer periods of time increases the risk of insect and weather damage (hail and snow) (McLelland, 1999). Therefore, it is very important to harvest the crops as soon as possible to prevent losses. Several storage techniques have been developed to aid in storage of high moisture grains but each presents special considerations as discussed below.

Ensiling is a technique that promotes the growth of acid-producing bacteria in the absence of oxygen (McLelland, 1999). The bacteria ferment the sugars of the barley to produce acids to prevent further bacterial growth. This method will only work if the grain is placed in an air tight silo and if the barley is packed tightly. The best

moisture content for this method is 25 to 35%. However, eventually the high moisture barley will be exposed to oxygen in warm weather and will soon begin to spoil.

Organic acids have been successfully used when applied at the recommended concentrations to high moisture grains without spoilage. Currently there are several drawbacks that limit the use of organic acids which include germination is drastically reduced, elevators will not accept acid-treated grain, steel bins must be coated to prevent corrosion, the cost of preservation increases as the moisture content of the grain increases, special machinery is needed to uniformly disperse the acids all over the grain (McLelland, 1999).

Treatment of grains with formic and propionic acids in Sweden have been linked to aflatoxin production. Research done by Clevström (1989) demonstrated the ability of *A. flavus* to grow on high moisture barley treated with formic acid. A survey of farms in Sweden indicated that *A. flavus* could be isolated 62%, 31% and 17% of the time from barley treated with formic acid at 700 g/l, formic acid at 850 g/l and propionic acid, respectively (Pettersson, H. *et al.*, 1989). Aflatoxins were also isolated in 22%, 8% and 1% of the samples treated with formic acid at 700 g/l, formic acid at 850 g/l and propionic acid, respectively (Pettersson, H. *et al.*, 1989). Pettersson *et al.* (1989) came to the conclusion that inadequate treatment played a role in aflatoxin formation in formic acid treated grains in Sweden. Inadequate treatment

results from improper application rates of the acids or the inability to fully treat the whole bulk sample.

2.10 Benefits/ drawbacks of harvesting high moisture barley

There are several advantages to harvesting high moisture barley. The first advantage is that harvesting high moisture barley may result in a 12 day earlier harvest and would be extended further in wetter areas (McLelland, 1999). The earlier harvest would allow the crop to be taken off the field minimizing possible damage. Another advantage would be that yield per acre would increase 16.7% when compared to mature field-dried barley (McLelland, 1999). This may also increase because it would not be necessary to wait for maturing patches or second growth to ripen. Harvesting high moisture barley would make threshing is easier when the barley is between 25 to 40% moisture content and places that were seeded late in the spring can be taken off as high moisture barley (McLelland, 1999). Another advantage of harvesting high moisture barley is that stubble is left longer in and there is less volume to harvest (McLelland, 1999). Harvesting high moisture barley also aids in controlling weeds (McLelland, 1999). One last advantage is that harvesting can take place under adverse weather conditions, such as humid periods, when the crop is soaked with dew and during light showers (McLelland, 1999). This would allow farmers greater flexibility when they can harvest.

There are several drawbacks to harvesting high moisture barley. The main weakness in harvesting high moisture barley is that the barley must be treated and stored immediately after harvesting (McLelland, 1999). Storage is usually done by using air tight silos or treating the grain with organic acids. The disadvantage with using air tight silos is that the barley needs to be rolled and hammered to exclude oxygen which could create delays at harvest time (McLelland, 1999). The disadvantage with using organic acids to treat barley include elevators not accepting the barley; the barley must be stored in bins that are corrosion resistant; and care must be taken when handling acids and acid treated materials (McLelland, 1999). Another disadvantage is that high moisture barley is heavier than dry barley which may place a strain on equipment (McLelland, 1999). Harvesting high moisture barley may also require the combine speed to be 25% less than dry barley reducing the speed, slowing the harvest down (McLelland, 1999). The final disadvantage of harvesting high moisture barley is that some bridging may occur in the combine and truck boxes if the barley is greater than 24% moisture content (McLelland, 1999).

2.11 Summary

Ozone may prove to be an attractive fumigant for grains lessening post-harvest losses, but little research has been done on this area. Current theory shows that the conditions resulting in spoilage of

crops are the same conditions that favour ozone application. Research done by Zhao and Cranston (1995) showed that higher moisture content black pepper led to a greater reduction in the microbial load of the spice. Foarde *et al.* (1997) also showed that higher relative humidity's were better at reducing fungal spores. However, the mechanism behind ozone being a better disinfectant in high moisture environments is not known. Few studies have been performed that have shown temperature effects on ozone disinfection. Of those studies done, little agreement is made as to the effect temperature has on the disinfection capabilities of ozone. Several studies have been published pointing to the fungicidal powers of ozone on both vegetative and spore stages, but none could be found that dealt with *A. flavus* and *P. verrucosum* specifically. This paper will add and help strengthen current theory and knowledge on using ozone as a fumigant on foodstuffs.

Chapter 3 – Experimental

3.1 Materials and source of barley grain

Barley, cultivar Excel, used throughout the experiments was obtained from The Pas, Manitoba. Initial moisture content of the two barley batches was 9.5% and 12.5%.

Potato dextrose agar (PDA) (Becton Dickinson), Petri dishes and gas washing bottles were purchased from VWR Canlab (Mississauga, ON). All other chemicals used were reagent grade and obtained from Sigma-Aldrich (Toronto, ON).

3.2 Preparation of PDA and phosphate buffer

The PDA was prepared according to the manufacturers directions by adding 16g of the dehydrated PDA to 400ml of distilled water. The culture media was then mixed and autoclaved at 121°C for 15 minutes. For experiments with excessive bacterial contamination, an antibiotic was prepared by dissolving 1g of tetracycline in 100ml of sterile distilled water and filtering the fluid through a 0.45μm membrane for a concentration of 10 000μg/ml. 1.6ml of the stock antibiotic solution was added to the 400ml of the culture media to achieve a 40μg/ml medium. Approximately, 20ml of the culture media was poured into each Petri dish. The plates were then placed in the incubator at 25°C for approximately 2 days and then used.

Butterfield's phosphate buffer was prepared according to the FDA (1984) by dissolving 34g of KH_2PO_4 in 500ml of distilled water. The stock solution was adjusted to a pH of 7.2 with 1N NaOH and brought to a volume of 1 litre. The solution was then sterilized at 121°C for 15 minutes and stored in the refrigerator. When the phosphate buffer was needed, 1.25ml of the stock solution was taken and diluted to 1 litre with distilled water for a working solution of 0.6mM. The buffer was then sterilized for 15 minutes at 121°C.

3.3 Water activity adjustments

The water activity of the barley was increased by adding calculated amounts of water using the equation $v = [w(a - b)/(100 - a) - I$, where v = volume of water to be added (ml), w = weight of grain to be treated (g), a = the water content required in the grain (%), b = initial water content of the grain (%) and I = the volume of water to be added later with the inoculum (Ramakrishna *et al.*, 1993; Ramakrishna *et al.*, 1996a; and Ramakrishna *et al.*, 1996b). An example of the calculation can be seen in Appendix A. Water activity was determined before each test by the average of five measurements using an AquaLab CX-2 water activity meter (Decagon Devices Inc., Pullman, Washington USA). The AquaLab CX-2 was standardized before each test as directed by the manufacturer using a saturated potassium sulphate solution. The machine was warmed up until the standard read 0.973±0.005.

Water content was determined by weighing and placing approximately 3 to 5g of barley in a 130°C oven for 1 hour (AOAC, 1995). The sample was then covered and transferred to a desiccator, where upon reaching room temperature it was weighed again (AOAC, 1999). The difference in the weight before and after the oven was the moisture content. The average of two tests was deemed to be the moisture content of the barley. This was correlated to the water activity by using the moisture absorption curve prepared by Hill and Lacey (1983) in Figure 3.1. According to the moisture absorption curve for barley obtained by Hill and Lacey (1983) experimentally, water activities of 0.98, 0.95, 0.93 and 0.88 were 30%, 24%, 22% and 19% moisture content, respectively (Figure 3.1).

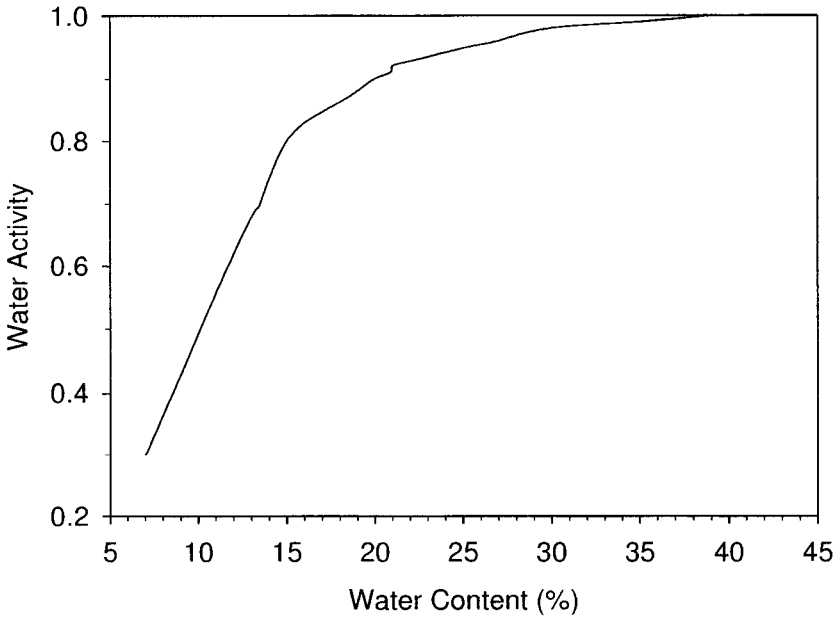

Figure 3.2. Relationship between moisture content and water activity of barley (Hill and Lacey, 1983).

3.4 Inoculum preparation

A. flavus (UAMH 4336) and *P. verrucosum* (UAMH 7557) were purchased from the University of Alberta's Microfungus Collection and Herbarium. Both fungi were grown on PDA for at least 14 days at 25°C prior to harvesting the spores. The spores were washed off using a 0.05%Tween 20 solution (Ramakrishna *et al.* 1993, Ramakrishna *et al.*, 1996a; Ramakrishna *et al.*, 1996b). The suspension was analyzed in a Beckman DU 650 spectrophotometer at a wavelength of 640nm (Sietz, L.M. *et al.*, 1982). *A. flavus* was adjusted to a light transmittance of 1.60% while *P. verrucosum* was adjusted to a transmittance of 5.0%, which after inoculation with appropriate volumes resulted in approximately 2.0×10^4CFU/g. New spore suspensions were prepared for each experiment.

3.5 Phase I - Natural fungi

The batch of barley used for the vegetative fungi test (first batch) had little natural fungi or bacterial contamination present with an initial water content of 9.5%. This sample was incubated for three days at 25°C at a water activity of 0.98 to allow growth of the fungi and therefore the sample consisted of both vegetative forms and spore forms of fungi. A water activity of 0.98 was maintained using an autoclaved metal tray covered with a wet towel (hung dry for one minute to stop dripping on the barley) and aluminium foil. Tape was placed across the metal tray to ensure that the towel did not come into contact with the

barley. The water activity of the barley within the tray remained constant throughout the incubation time as tests indicated. The wet towel was replaced daily to ensure the high water activity was maintained.

The batch of barley (second batch) used for spore inactivation tests contained higher counts of natural fungi and the initial water content was 12.5%. Barley samples with appropriate volumes of water added were kept at 1-2°C for five days to allow equilibration. A low temperature was used to ensure that no spores would germinate in the five days to allow for equilibration. The samples were examined under the microscope to ensure that spores did not germinate.

3.6 Phase II – *A. flavus* and *P. verrucosum* spores

Tests on ozone's effect on spores were done with both *A. flavus* and *P. verrucosum.* The microflora of the barley was eliminated by autoclaving the barley at 121°C for 30 minutes. One hundred grams of the barley with a specific a_w was inoculated with 0.75ml of the spore suspension. The barley was then kept at 1-2°C for 12 hours to allow the water added with the inoculum to come into equilibrium. A low temperature was used to ensure the spores would not germinate. The barley was shaken regularly.

3.7 Phase II – *A. flavus* (vegetative state)

The microflora of the barley was eliminated by autoclaving the barley at 121°C for 30 minutes. Different water activities (a_w) required different inoculum volumes as outlined in Table 3.1 to be added to achieve approximately $2.0x10^4$CFU/g of *A. flavus* on the barley. The barley was shaken vigorously to distribute the spores. Barley with water activities of 0.95, 0.93 and 0.88 were placed in desiccators with glycerol water solutions of corresponding relative humidity's (Ramakrishna *et al.*, 1993; Ramakrishna *et al.*, 1996a; and Ramakrishna *et al.*, 1996b). Glycerol/water solutions of corresponding relative humidity's (Figure 3.2) were found by allowing the barley to come into equilibrium with the desiccator atmosphere for five days and then analyzing for the moisture content. With the moisture absorption curve developed by Hill and Lacey (1983) (Figure 3.1) the moisture content was matched up with the water activity which in turn was correlated with the relative humidity. A water activity of 0.98 was maintained using a metal tray covered with a wet towel and aluminium foil (hung dry for one minute to stop dripping on the barley). The water activity of the barley and humidity within the

Table 3.1. Inoculation volumes of spore suspension for *A. flavus*.

a_w	Inoculum Volume (ml)
0.98	1.40
0.95	1.40
0.93	2.00
0.88	3.75

tray remained constant throughout the incubation times. An incubation time of 6 hours at 30°C was used to allow all the spores at $0.98a_w$ and $0.95a_w$ to germinate (Ramakrishna, N. *et al.*, 1993). For water activities of 0.93 and 0.88 the desiccators were placed in the incubator at 30°C for 8 hours and 14 hours, respectively to allow germination of the spores.

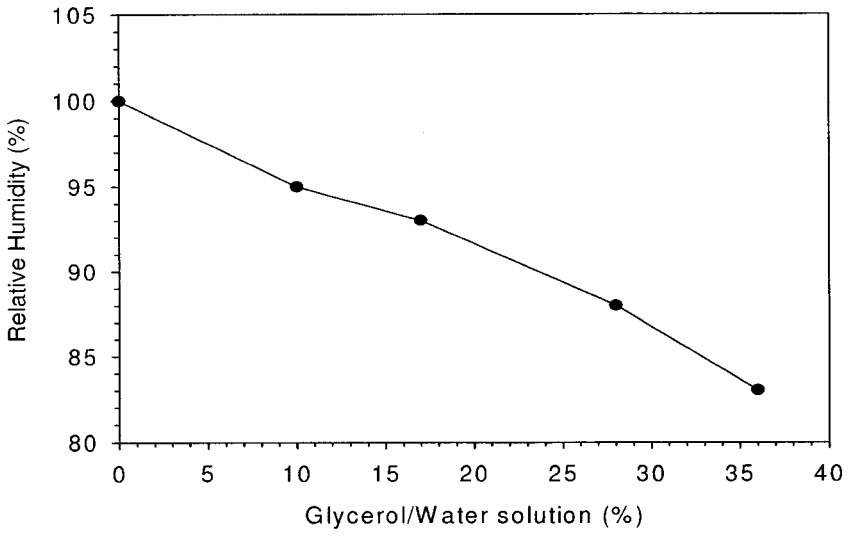

Figure 3.2. Glycerol/water solutions to produce corresponding relative humidity's in a desiccator.

3.8 Enumeration of fungi

PDA was used for all experiments (FDA, 1984). The PDA for the second barley batch received was amended with 40ppm of tetracycline to reduce bacterial contamination. A barley suspension was prepared by taking a 50g barley sample and diluting it with 500ml of Butterfields phosphate buffer. The sample was blended in an Osterizer 16 speed blender at the lowest speed for 1 minute. Serial dilutions were made by transferring 1ml of the barley homogenate to 9ml's of

phosphate buffer. Five plates were prepared for each dilution using the spread plate technique. Plates were then incubated at 25°C and counted on day 5 (FDA, 1984). Plates that contained 10-150 colonies were counted (FDA, 1984). Experiments were duplicated and the average of these two tests was deemed to be the number of fungi.

3.9 Ozone treatments

Ozone was generated from pure oxygen with a Model GL-1 ozone generator (PCI-WEDECO Environmental Technologies, West Caldwell, NJ). Inlet and exit gas ozone was determined by an ozone monitor (Model HC-400, PCI-WEDECO Environmental Technologies, West Caldwell, NJ) that compares the absorption of ozone at 254nm with the absorption of pure oxygen. The difference between the levels of inlet and exit gas ozone was calculated and defined as the amount of ozone the barley had consumed. Control measurements, without barley, indicated that there was no loss of ozone in the system. Excess ozone leaving the reactor was destroyed by a catalytic ozone-destruct unit filled with Carulite catalyst (Carus Chemical Company, Peru, IL). Details of the setup are shown in Figure 3.3.

The reactor used was a Pyrex 350ml gas washing bottle with a porous disc sealed into the bottom of the bottle. The reactor was washed out for 2 minutes at ~2.0% weight ozone before the barley was placed in. This was to ensure that any ozone consuming material in the bottle

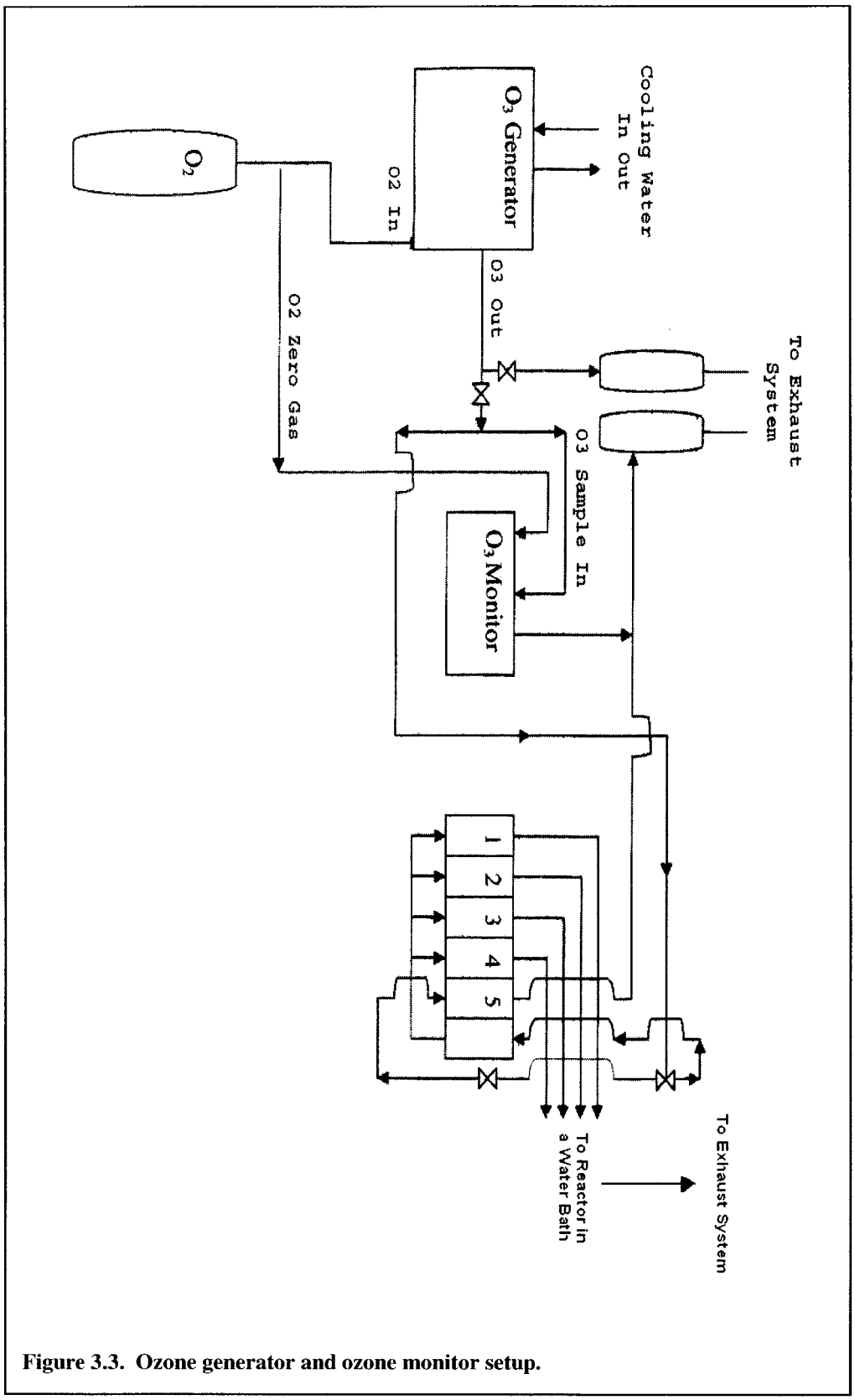

Figure 3.3. Ozone generator and ozone monitor setup.

was oxidized. A 50g sample of barley with a specific a_w was then aseptically transferred to the reactor by using a sterile metal scoop. The ozone and oxygen mixture was fed into the reactor at 1.26 SLPM (standard liter per minute) (sample calculation in appendix B). The temperature of the reactor was controlled using a constant temperature circulating bath that maintained the temperature ±0.1°C (Model 1187, VWR Canlab, Mississauga, ON). The ozonation period was started when the ozone was sparged into the reactor. After the ozonation, the reactor was washed out with oxygen to remove any remaining ozone. Ozone dosages were controlled by either turning up the power on the ozone generator or by extending the ozonation time.

3.10 Germination assessment

Germination was assessed according to the International Seed Testing Association (1985). Seeds, at the required water activity, were placed in the refrigerator at 7°C for seven days to aid in breaking suspected dormancy. Seeds were then spread out on an autoclaved metal tray to be randomly selected by making a selection from each corner of the metal tray and the center. The tray was then remixed and the process repeated until 200 seeds were selected. From these 200 seeds the process was repeated so that two 100 seed samples were

selected where one would be used as the control and the other as the treatment. The seeds were transferred to the reactor to ozonate. Once the ozonation was completed, the treated seeds and the control seeds were transferred to a wet towel and distributed evenly to not interfere with each other in the germination process. The seeds were then covered with another wet towel and placed in a sealed bag where they were placed in an upright position. Counts were done on day 5. If seeds appeared to be growing slow, they were left until day 7. The seeds were assessed according the International Seed Testing Association (1985) (Appendix C). Germination assessments were repeated four times.

Chapter 4 - Results and Discussion

Results of this study are reported below, in the order in which they were arranged in the two phases presented in Chapter 2.

4.1 Phase I Trial

4.1.1 Inactivation of natural spores and vegetative fungi (1st batch)

With the length of ozonation time kept at 15 minutes, ozone doses of 2.40 (0.16mg/g barley • min), 7.50 (0.50mg/g barley • min) and 14.70mg/g barley (0.98mg/g barley • min) were all effective in reducing the natural fungus on the first batch of barley at 0.98a_w (Figure 4.1). As ozone dose increased, the number of fungi remaining on the barley decreased with the largest reduction of 88.7% occurring with a final

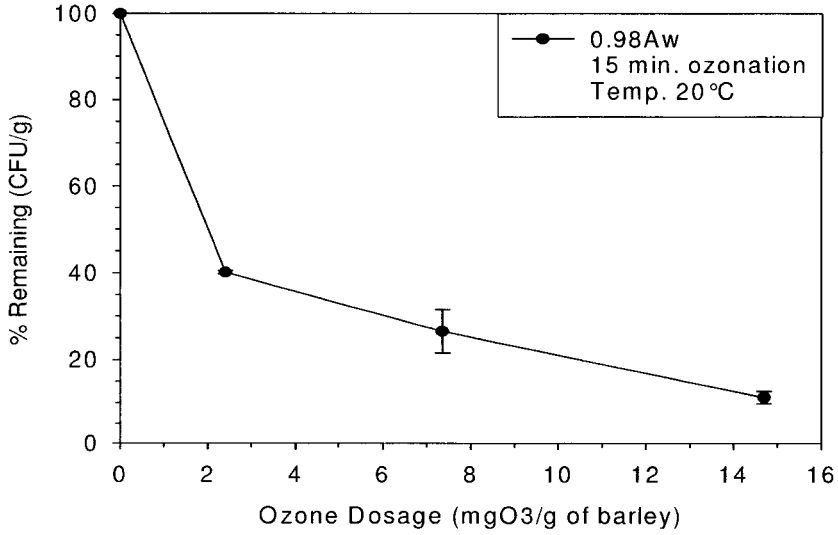

Figure 4.1. The effect of ozone dosage on the natural vegetative fungi and fungal spores.

dose of 14.70mg/g barley. Since this ozone dose provided the largest reduction of fungi, it was used as a benchmark to evaluate other fungi on.

To help explain the difference seen in the inactivation of fungi at different ozone doses, the consumed ozone by the barley was calculated. The consumed ozone is defined as the amount of ozone lost in the reactor calculated from the differences between the exit and inlet gas ozone streams. Since control tests determined that there was no ozone loss when the reactor was empty, it was deemed that the ozone lost in the reactor in the presence of barley had been consumed by the barley. The largest ozone dose corresponded with the largest ozone consumption by the barley (Figure 4.2). At 15 minutes of treatment over 3.8 times more ozone was being consumed by the barley with a

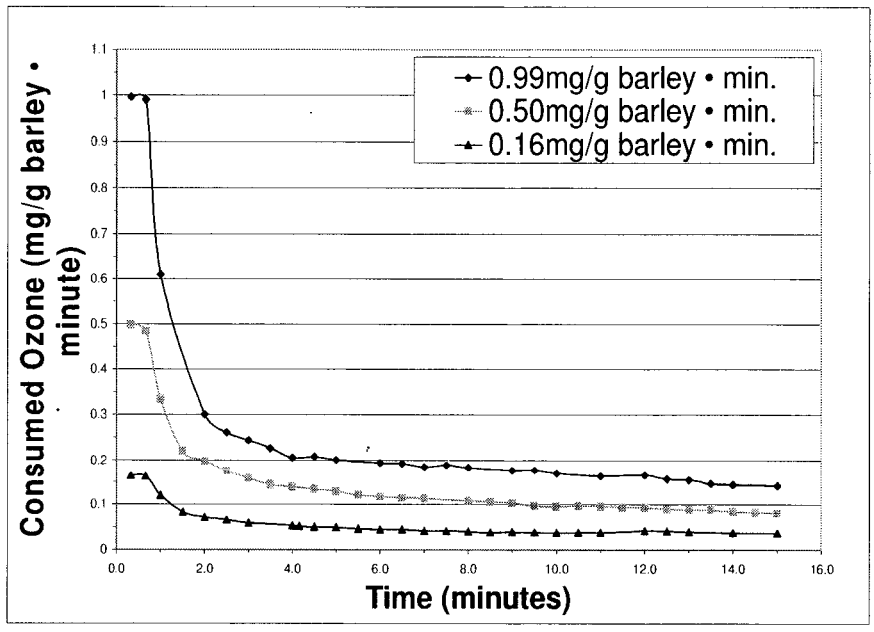

Figure 4.2. Consumed ozone at 0.98aw after treatment with different ozone doses.

treatment of 0.99mg/g barley • min (ozone dose 14.70mg/g barley) than with a treatment of 0.16mg/g barley • min (ozone dose 2.40mg/g barley) even though the dose was 6 times greater. The extra ozone consumption of the larger ozone dose reduced the fungi by an additional 30% when compared to the lowest ozone dose.

To enhance the inactivation power of ozone on fungi while making it more economical a 15 minute continuous supply of ozone at 0.98mg/g barley • min was followed by a 30 minute holding period for a total ozone exposure time of 45 minutes (Figure 4.3). The greatest reduction in fungus population occurred in the first 5 minutes of ozone exposure with 75.4% of the natural fungi being reduced on the barley. After this first large initial decline a tailing effect was observed with a final reduction of 97.2% of the population of fungi. The largest reduction in the first five minutes (75.4%) only required an ozone input of 4.90mg/g barley while the ozonation period from 5 - 15 minutes (double the time) required an ozone input of 9.80mg/g barley (double the dose) while only achieving an additional reduction of 13.4%. The tailing effect seen in Figure 4.3 may represent a proportion of fungi that might be resistant to ozone or shielded from the ozone due to grain aggregation. By holding the ozone in the reaction chamber required no additional ozone input (no extra costs) and reduced the fungus population by an additional 8.5%.

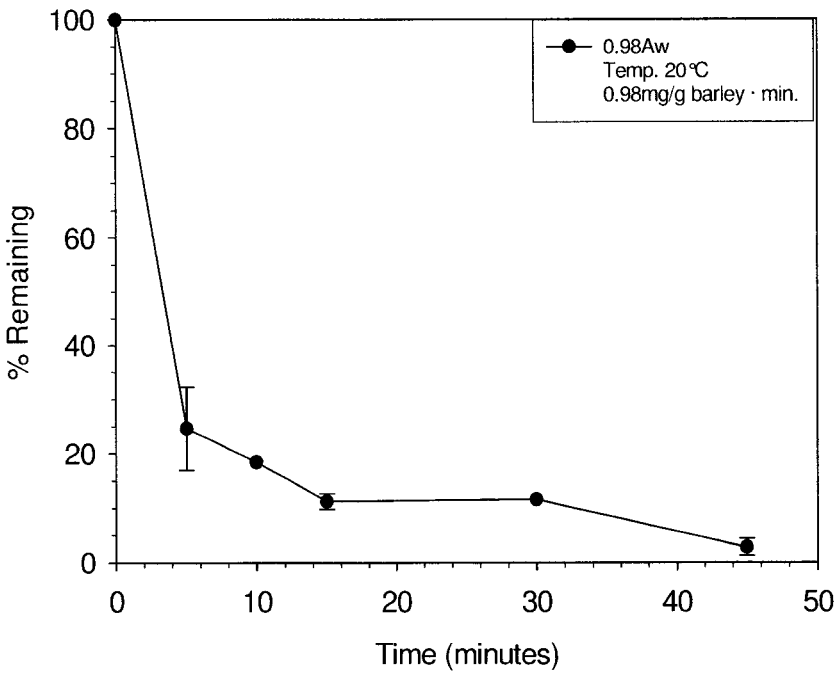

Figure 4.3. Inactivation of the natural fungi on the barley. Ozone contact times of 30 and 45 minutes represent a 15 minute continuous ozone supply followed by a 15 and 30 minute holding period, respectively.

4.1.2 The effect of ozone dose on the natural fungal spores (2nd batch)

The effect of ozone dosage on the natural fungal spores was tested by using various ozone doses for five minutes of treatment. The results are presented in Figure 4.4. As ozone dose increased, so did the inactivation of the fungal spores. A dose of 0.80mg/g barley was sufficient to eliminate 95.0% of the natural fungi on the barley. However, ozone doses greater than 0.80mg/g barley on the 2nd batch of barley did not result in further inactivation of the natural fungi. This may indicate that the remaining fungal population on the barley was resistant or shielded from the ozone. The minimum ozone

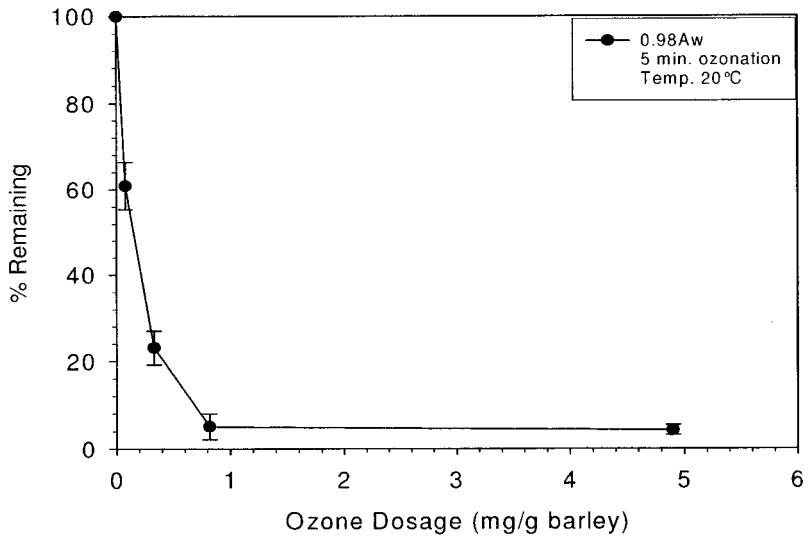

Figure 4.4. The effect of ozone dosage on spores associated with the second batch of barley.

dose to reduce 90% of the population for this batch of barley was found to exist between 0.80mg/g barley and 0.33mg/g barley. This dose was far less than that needed for the first batch of barley where a dose of 14.70mg/g barley was needed to eliminate 88.7% of the natural fungi. Since there appeared to be such large variations in fungal resistance to ozone and no quick way for farmers to decide how resistant the fungi on their crops are to ozone, a larger ozone dose of 0.98mg/g barley • min was used as a benchmark for the inoculated fungi. This way it is ensured that a 90% disinfection level is achieved in natural conditions.

Figure 4.5 presents the consumption of ozone for the ozone doses tested in Figure 4.4. An ozone dose at 0.98mg/g barley • min consumed 4.1 times more ozone as that of a treatment of 0.16mg/g barley • minute, but did not result in a larger inactivation. The remaining fungal

population may represent a proportion of fungi that might be resistant from ozone or shielded from the ozone due to grain aggregation.

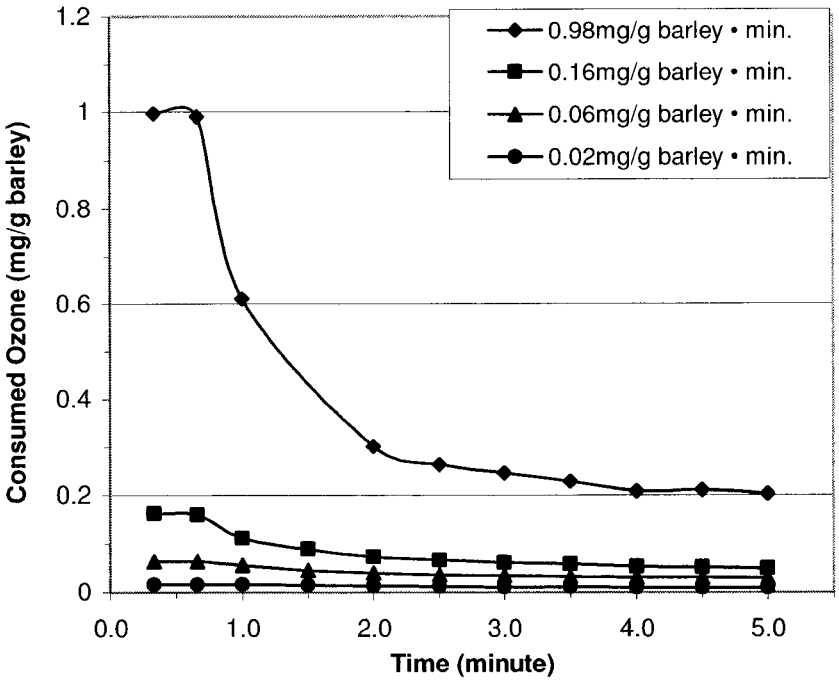

Figure 4.5. Consumed ozone for the second batch of barley with different ozone doses at 0.98a_w.

4.1.3 The effect of a_w on the natural fungal spores (2nd batch)

Figure 4.6 shows the effect that ozone had on the natural fungal spores after ozone contact times of 5 and 45 minutes at different water activities. The natural fungal spores on the second batch of barley achieved similar results as that of the first batch of barley at 0.98a_w. An ozone dose of 0.98mg/g barley • min for 15 minutes with a 30 minute holding period reduced the spore population by over 99%. However, as the water activity decreased from 0.98 to 0.88a_w, the overall

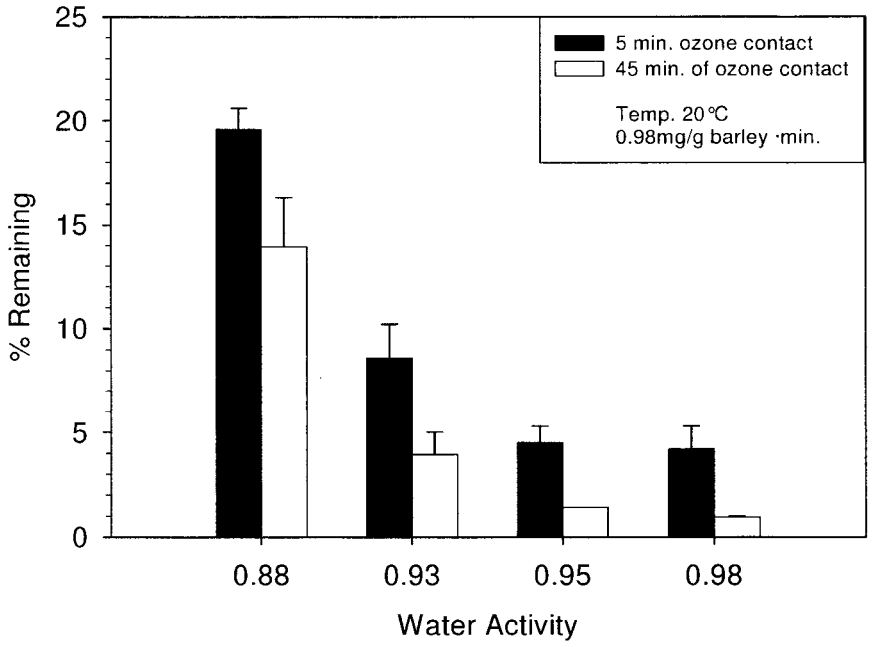

Figure 4.6. The effect of water activity on the natural fungal spores on barley. An ozone contact time of 45 minutes represents a 15 minute continuous ozone supply followed by a 30 minute holding period.

effectiveness of ozone against the fungal spores diminished. At 0.98, 0.95, 0.93 and 0.88a_w the reduction in population was 99.1%, 98.6%, 96.1% and 86.1%, respectively after 45 minutes of ozone contact time. After five minutes of exposure at 0.98, 0.95, 0.93 and 0.88a_w, the reduction in fungus population was 95.8%, 95.5%, 91.4% and 80.4%, respectively. At 0.98, 0.95, 0.93 and 0.88a_w the additional 40 minutes of ozone contact time only provided an additional reduction of fungal spores of 3.3%, 3.1%, 4.7% and 5.7%, respectively, even though the ozone contact time was 9 times longer. Even with an ozone contact time of 45 minutes and the barley at 0.88a_w, the population of fungi was still

greater than at water activities of 0.98, 0.95 and 0.93 after only five minutes of ozone exposure highlighting the resistance of the fungi to ozone inactivation at lower water activities.

No large differences were detected for the consumed ozone between the different water activities tested, except for at $0.88a_w$ where a small difference was observed (Figure 4.7). At $0.88a_w$ less ozone was being consumed by the barley than at the other water activities. It is not known whether this small difference seen in ozone consumption between the water activities of 0.98 and 0.88 was enough to cause the difference seen in the fungi inactivation (13% less fungi were inactivated after being treated at $0.88a_w$ than being treated with $0.98a_w$).

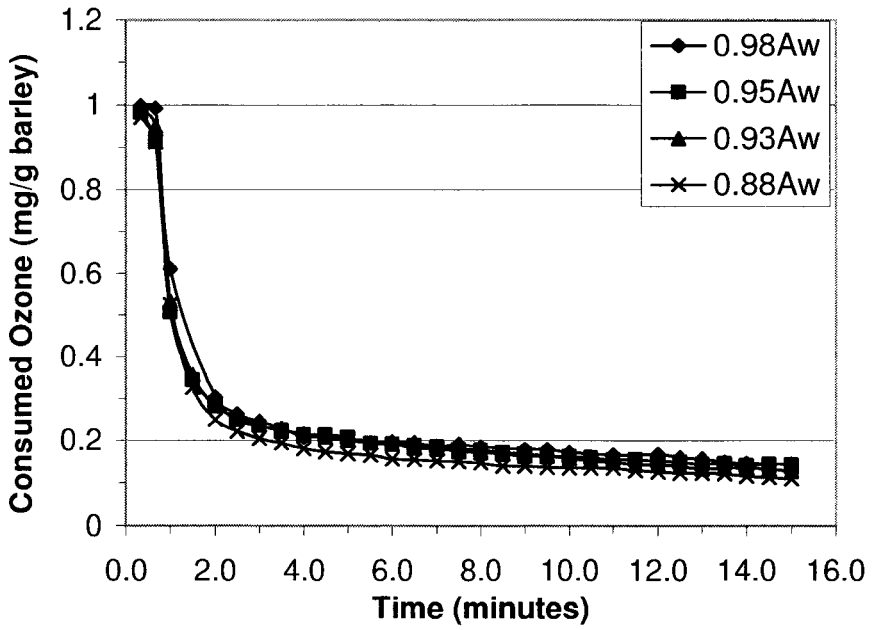

Figure 4.7. Ozone consumption of the second batch of barley with different water activities and a treatment of 0.98mg/g barley · min.

4.1.4 The effect of temperature on the natural fungal spores (2nd batch)

The effect of temperature was also tested on the natural fungal spores on the second batch of barley at 0.88a_w. The results are presented in Figure 4.8. Higher temperatures led to a greater inactivation of the natural fungal spore population. As temperature increased from 0°C to 20°C an additional 22.2% of the fungi were reduced, but as the temperature increased from 20°C to 40°C only an additional 9.8% of the fungi were reduced. At temperatures of 0 and 20°C the ozone dose used was unable to achieve the target 90% reduction. When the temperature was raised to 40°C, 95.8% of the population was eliminated from the barley achieving the target 90% reduction.

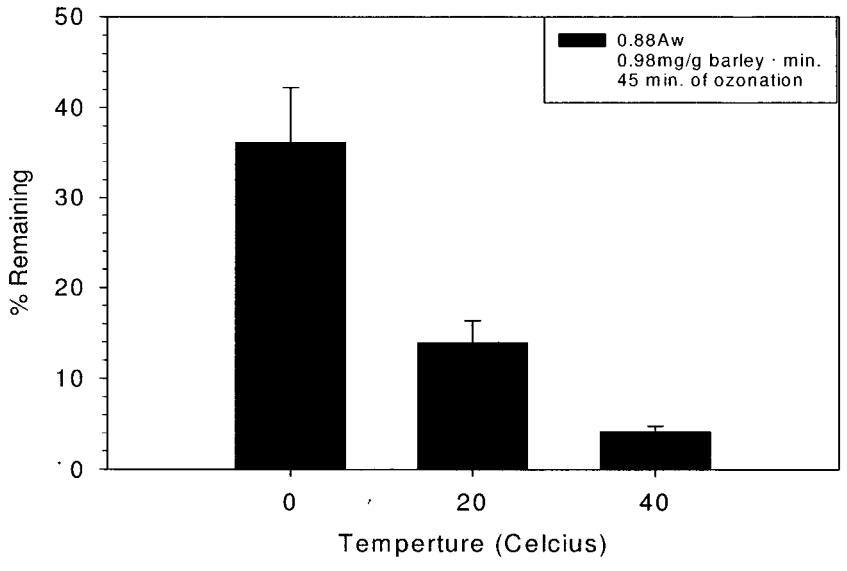

Figure 4.8. The effect of temperature on the inactivation of the natural fungal spores on the second barley batch. 45 minutes of ozone contact time includes a 15 minutes of continuous supply of ozone with a 30 minute holding period.

The ozone consumption of the barley presented in Figure 4.9 helps to explain why higher temperatures were better at reducing the spore population than lower temperatures. Higher temperatures resulted in more ozone being consumed by the barley than lower temperatures. This may be caused by increased reaction rates on the barley and fungi at higher temperatures. As temperature increased from 0°C to 20°C the ozone consumption of the barley at 15 minutes of treatment increased 1.8 times (spores were reduced an additional 22.2%), while an increase in temperature from 20°C to 40°C resulted in an ozone consumption increase of only 1.2 times (spores were reduced an additional 9.8%).

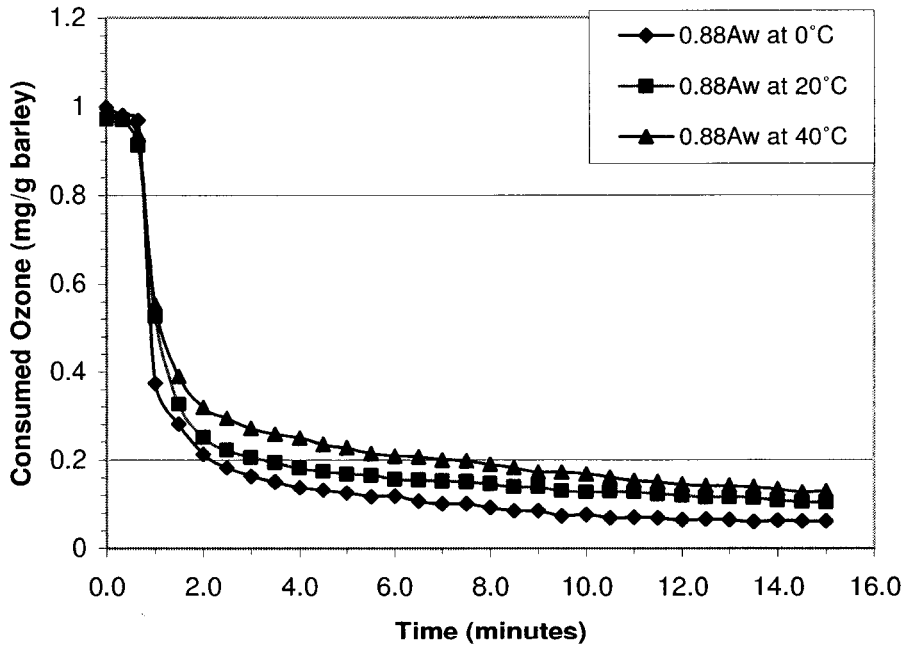

Figure 4.9. Consumed ozone for the second batch of barley with different temperatures at 0.88a_w.

4.2 Phase II Trial

4.2.1 Inactivation of *A. flavus* spores

Spores of *A. flavus* were tested as to their resistance to ozone. *A. flavus* spores were resistant to an ozone dosage of 4.90mg/g barley resulting in a negligible difference between control and test samples. Ozone doses of 9.80mg/g barley had a small effect on *A. flavus* spore numbers (20.9% reduction) and not much more of a decline after a dose of 19.60mg/g barley (25.6% reduction) (Figure 4.10).

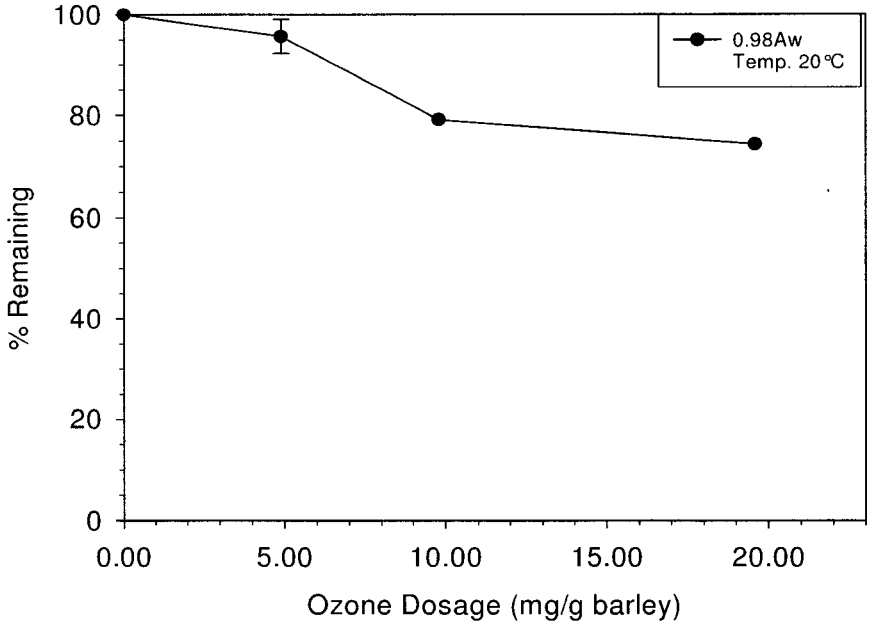

Figure 4.10. The effect of ozone dose on *A. flavus* spore survival.

4.2.2 Inactivation of the vegetative form of *A. flavus*

Figure 4.11 shows the inactivation kinetics of *A. flavus* on barley at $0.98a_w$ with an ozone dose of 0.16mg/g barley • min. The largest

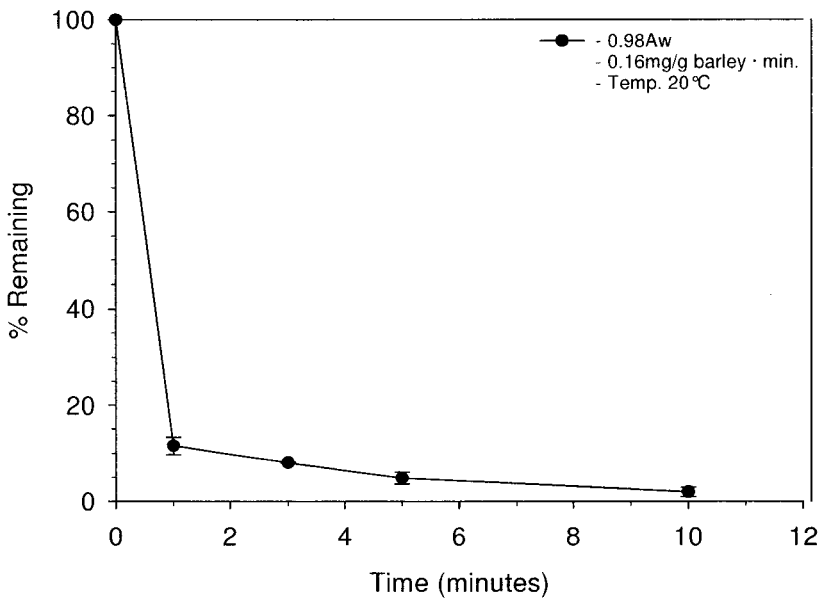

Figure 4.11. The effect of ozone on the inactivation of *A. flavus* (vegetative state).

reduction in the *A. flavus* population occurred in the first minute of treatment where over 88.5% of the population was reduced with an ozone dose of 0.16mg/g barley. With an extra 9 minutes of treatment only an additional reduction of 9.6% was achieved with an extra ozone dose of 1.44mg/g barley. The first large initial decline in the *A. flavus* population may be a proportion of the population that is easily accessible to the ozone. The slower decline in population after one minute of exposure may be a proportion of the population that may have been shielded from the ozone by grain aggregation.

4.2.3 The effect of a_w on the vegetative form of *A. flavus*

The effect of water activity on the inactivation of the vegetative form of *A. flavus* with ozone was tested. An ozone dose of 4.90mg/g barley was sufficient to reduce the fungus population at all water activities by over 97%. Since tests on spores of *A.* flavus were resistant to this ozone treatment, it was deemed that majority (>97%) of the spores had germinated within the incubation times.

An ozone dose of 0.16mg/g barley • min for 10 minutes was conducted at different water activities to show any effects that water activity had on the reducing power of ozone (Figure 4.12). Ozone at this treatment dose and time was effective at water activities of 0.98, 0.95 and 0.93 with only 2.0%, 1.7% and 4.8% of the *A. flavus* population

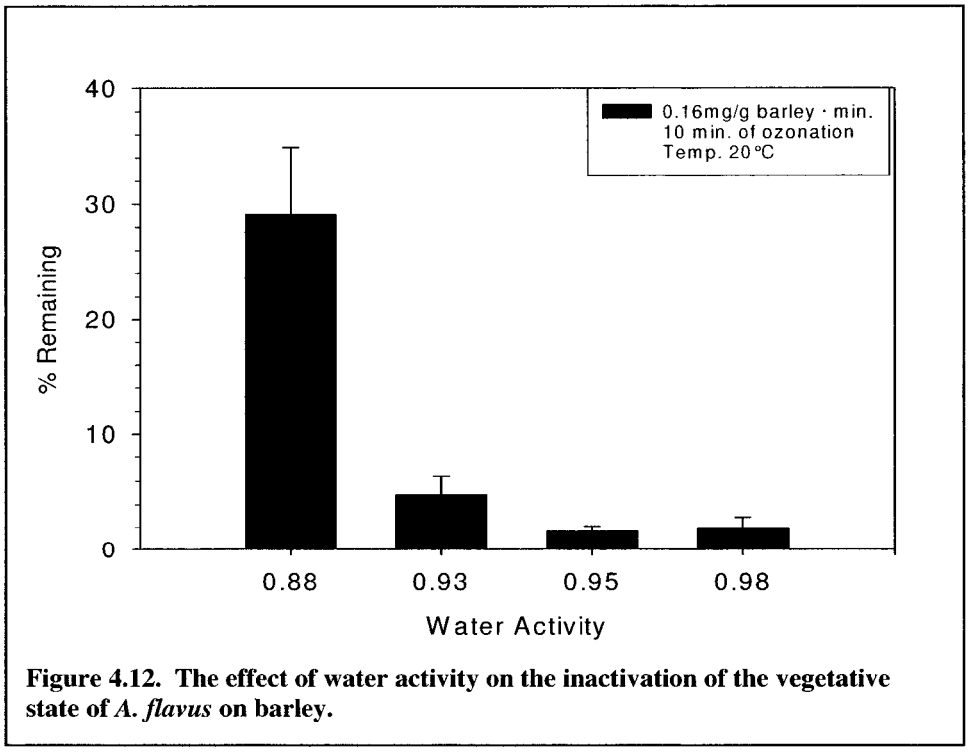

Figure 4.12. The effect of water activity on the inactivation of the vegetative state of *A. flavus* on barley.

remaining, respectively. At $0.88a_w$, 29.1% of the fungi remained on the barley after 10 minutes of treatment. Since the lower ozone dose (0.16mg/g barley • min. for 10 minutes) was not effective at $0.88a_w$, an increase in the dose to 0.98mg/g barley • min. for 10 minutes of treatment was tested. This resulted in greater than 97% of the fungus being removed at $0.88a_w$.

Figure 4.13 shows the exit gas ozone concentrations between different water activities after treatment with 0.16mg/g barley • min. and 10 minutes of treatment. No differences were detected in the ozone consumptions between all the water activities. This indicates that the

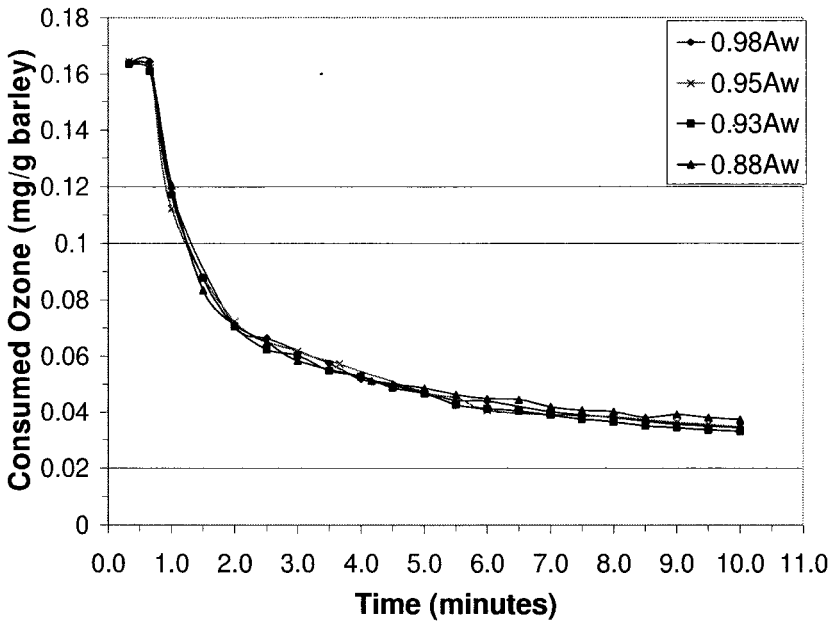

Figure 4.13. Ozone consumption at different water activities after treatment with 0.16mg/g barley · min.

different fungicidal power of ozone on *A. flavus* that was observed among the different water activities might not be from different ozone

consumptions but from the way ozone interacts with the different water activities.

4.2.4 The effect of temperature on the vegetative form of *A. flavus*

Temperature had an impact on the fungicidal properties of ozone against the vegetative form of *A. flavus* (Figure 4.14). Higher temperatures had a greater effect on reducing the *A. flavus* population than lower temperatures at 0.88a_w and 0.16mg/g barley • min for 10 minutes of treatment. At 40°C, 20°C and 0°C the *A. flavus* population reduced on the barley was 87.8%, 70.9% and 53.9%, respectively.

As temperature increased the ozone consumption of the barley also increased (Figure 4.15). As temperature increased from 0°C to

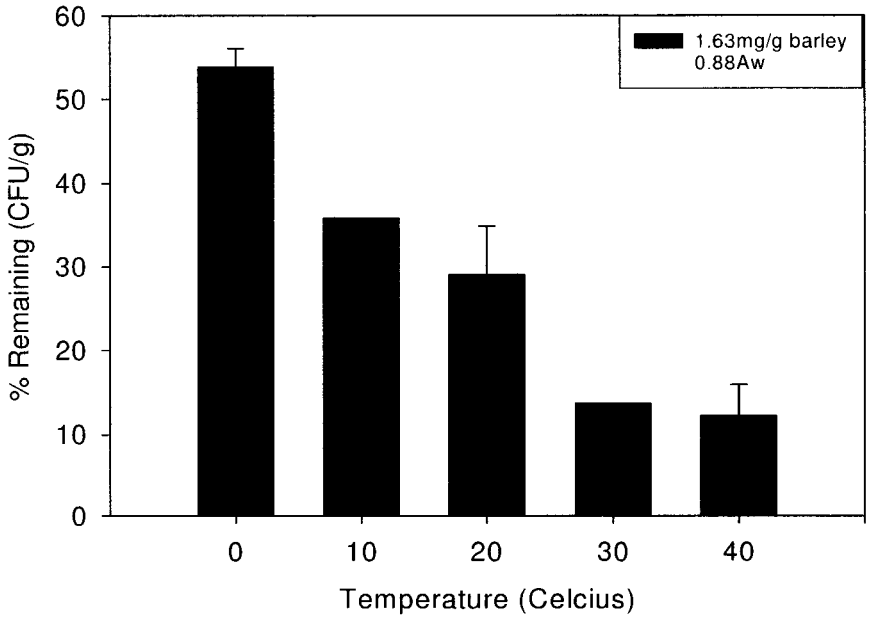

Figure 4.14. The effect of temperature on the inactivation of the vegetative state of *A. flavus*.

20°C the ozone consumption of the barley at 10 minutes of treatment increased 2 times (spores were reduced an additional 24.8%), while an increase in temperature from 20°C to 40°C resulted in an ozone consumption increase of only 2.5 times (spores were reduced an additional 16.9%).

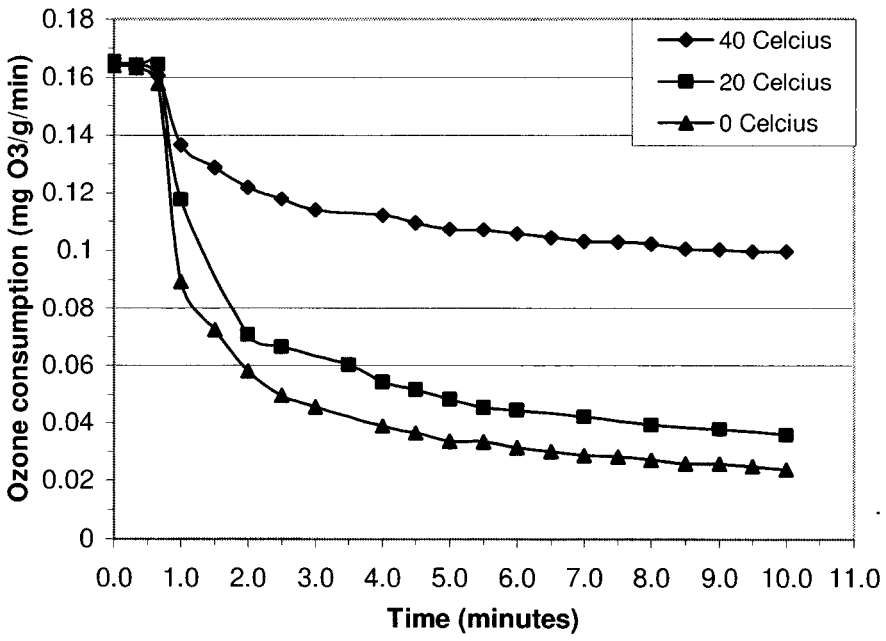

Figure 4.15. Ozone consumption of the barley at 0.88a_w after treatment with different temperatures at 0.16mg/g · minute.

4.2.5 The effect of ozone dosage on the vegetative form of *A. flavus*

The effect of ozone dosage on the vegetative form of *A.* flavus is presented in Figure 4.16. The minimum ozone dose necessary to reduce the fungi by 90% at 0.98a_w was found to be 0.33mg/g barley. Additional ozone beyond 0.33mg/g barley only produced a negligible reduction on the *A. flavus* population. It is possible that a small proportion of the *A.*

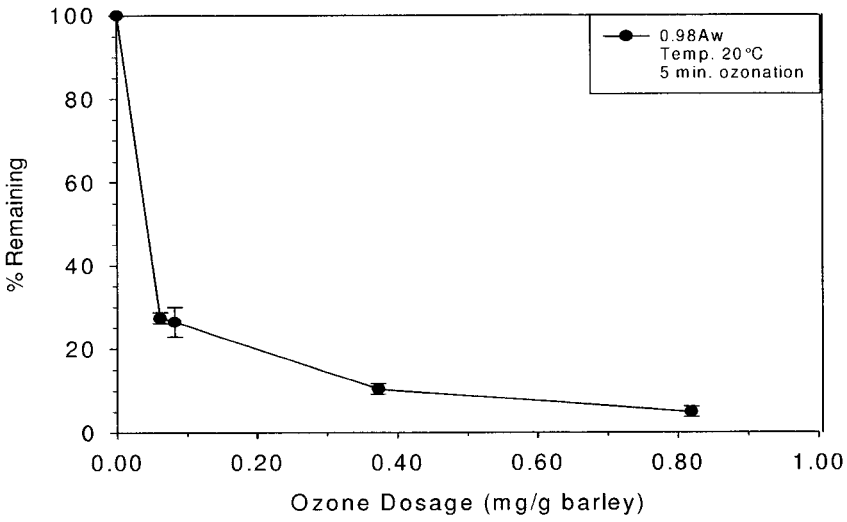

Figure 4.16. The effect of ozone dosage on *A. flavus* (vegetative state).

flavus population was still in the resistant spore stage and therefore additional ozone would not reduce the population any further.

4.2.6 The effect of a_w on *P. verrucosum* spores

The kinetics curve for the inactivation of *P. verrucosum* spores are shown in Figure 4.17. After one minute of treatment with 0.16mg/g barley at 0.98, 0.95 and 0.93a_w, more than 94.0% of the spores were eliminated. However, after one minute of treatment at 0.88a_w only 71.1% of the spores were reduced. The more resistant nature of the lower water activity (0.88a_w) to ozone was observed up until five minutes of treatment where the inactivation of *P. verrucosum* followed the higher water activities. All water activities by 10 minutes of treatment removed over 97.0% of the *P. verrucosum* population. For 0.98, 0.95 and 0.93a_w after one minute and for 0.88a_w after five minutes

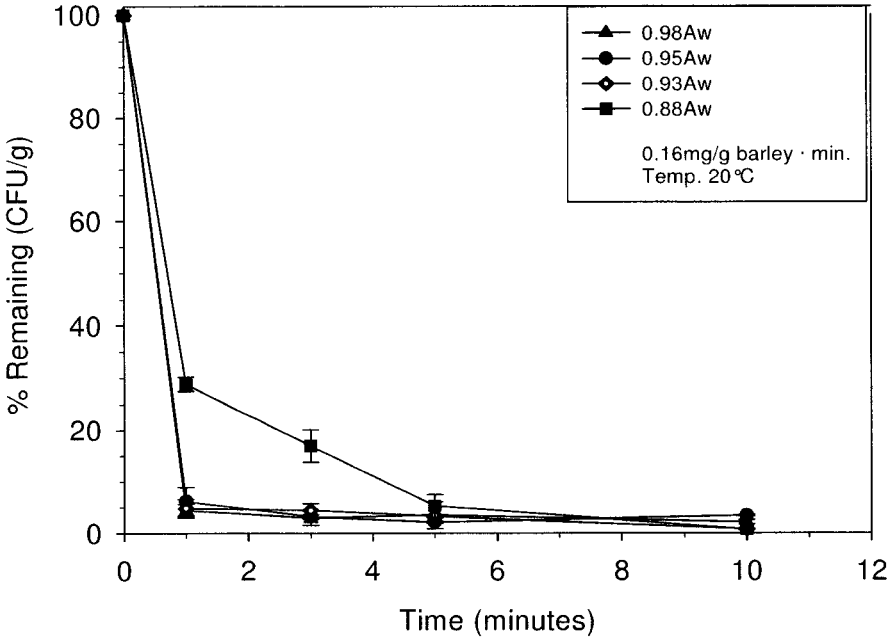

Figure 4.17. ***P. verrucosum*** **spore inactivation at different water activities.**

a tailing effect was observed. The tailing effect may have been a proportion of the spore population shielded from the ozone.

Other researchers have also confirmed that higher water activities are more effective at ozone disinfection than lower ones (Kim *et al.*, 1999; Zhao and Cranston, 1995). However, the exact mechanism this is used by is still unknown. One method suggests that the penetrability of ozone increases as the environments moisture is increased (Ishizaki *et al.*, 1986) allowing ozone greater contact with key enzymes and organelles causing greater damage. It is also thought that the presence of water accelerates reactions with organic substrates allowing ozone greater contact (Ishizaki *et al.*, 1986). These mechanisms are still unconfirmed.

4.2.7 The effect of ozone dosage on *P. verrucosum* spores

P. verrucosum spores were the least resistant fungi (spores and vegetative state) tested in this study to ozone. *P. verrucosum* and *A. flavus* spores represented the two extremes in sensitivity to ozone among the fungi being tested. After an ozone dose of 9.80mg O_3/g at all water activities *P. verrucosum* was undetectable (<10CFU/g, the detection limit), while at this dose 79.1% of the *A. flavus* spores remained viable at 0.98a_w.

P. verrucosum spores were sensitive to the lowest ozone dose tested at 0.05mg O_3/g of barley (Figure 4.18). At this dose at 0.98a_w, only 32.8% of the *P. verrucosum* spores were viable on the barley, but 85.8% remained viable at 0.88aw. As ozone dose increased, so did the inactivation of *P. verrucosum*. An ozone dose of 0.07mgO3/g at 0.98aw was able to eliminate over 99% of the spores.

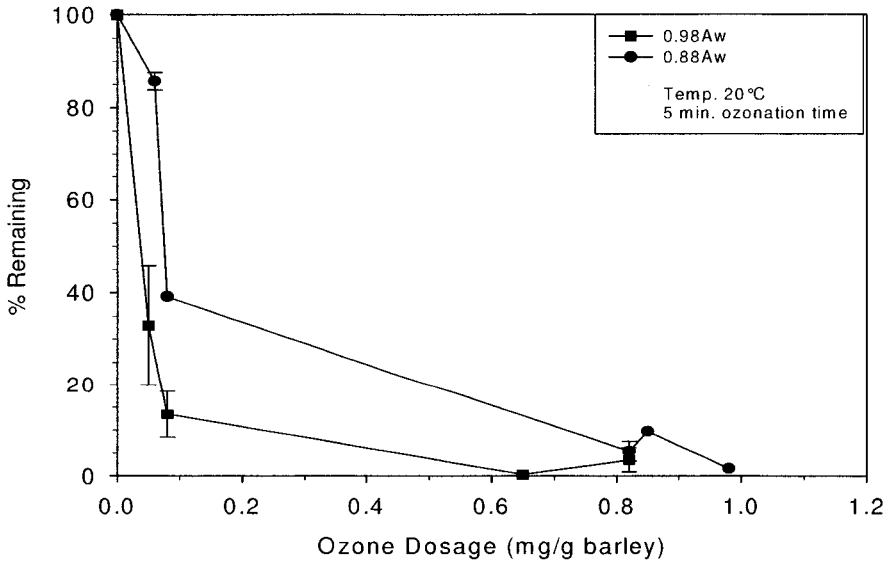

Figure 4.18. The effect of ozone dosage on *P. verrucosum* spores.

4.3 The effect of ozone on the germination of barley

The effect of ozone on the germination of barley was determined. The ability of a seed to germinate determines the field planting value of a seed (I.S.T.A., 1985).

The effect of 0.98mg/g barley • min for 15 minutes with a 30 minute holding period on the germination of barley is shown in Figure 4.19. Germination of the barley was not adversely affected with a treatment time of up to 10 minutes. At this point in treatment *P. verrucosum* (spores) and *A. flavus* (vegetative form) were completely inactivated. Also, at 5 minutes of treatment the natural fungal spores on the 2nd batch of barley were deactivated by over 95%. The spores and vegetative fungi on the 1st batch of barley were more resistant than the 2nd batch and therefore needed 15 minutes of treatment to achieve a 90%

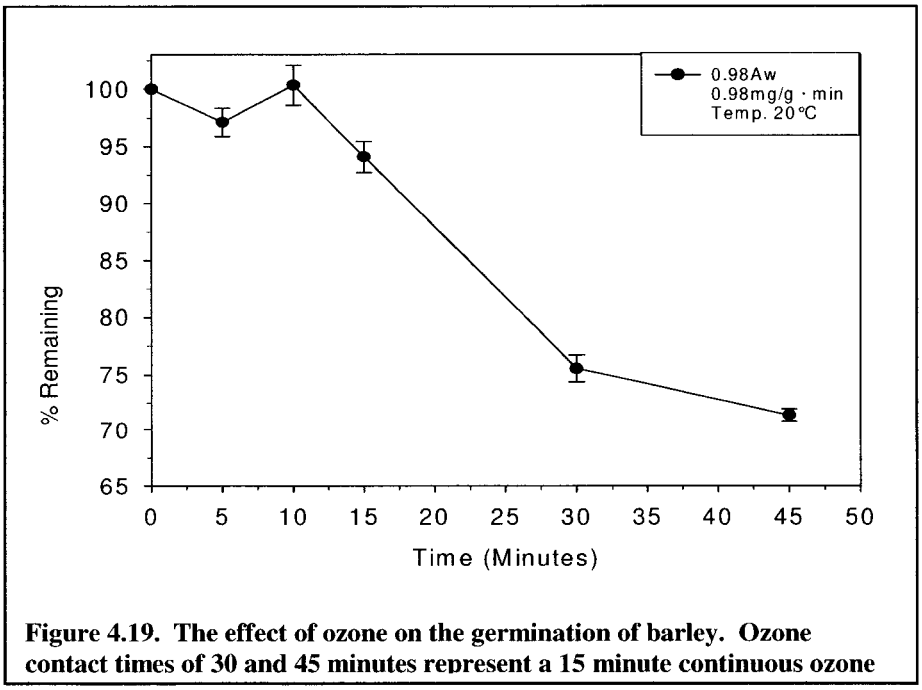

Figure 4.19. The effect of ozone on the germination of barley. Ozone contact times of 30 and 45 minutes represent a 15 minute continuous ozone

reduction. At this point the germination was only reduced by 5.9%. A full 45 minutes of treatment reduced the germination by 28.5%, but this lengthy ozone contact time was not needed to make significant changes in the fungus population at 0.98aw. At lower water activities such as 0.88aw, a full 45 minutes of ozone contact time was needed to reduce the fungus population on the second batch of barley by 86.1% and as such reduced the germination by 26%.

The losses in germination rates between 0.98 and $0.88a_w$ with $0.98mgO_3/g \cdot min$ for 15 minutes with a 30 minute holding period were 28% and 26%, respectively. This possibly indicates that the water activity of the barley may not play a large role in reducing germination.

As ozone dosage increased, the ability of the barley to germinate was reduced. After treatment with 0.16, 0.49 and $0.98mg\ O_3/g \cdot min$ for 15 minutes with a 30 minute holding period, germination was reduced 0.3%, 14.5% and 28.5%, respectively.

4.4 Summary

Current world wide trends away from residual fungicides and inferior storage techniques prompted this study to investigate the effects of ozone on both the vegetative and spore forms of fungi associated with barley grain. With estimated cereal losses of 50% reported in some countries, the need to reduce losses is of importance.

The literature review conducted in Chapter 2 revealed that little research has been done using ozone on grains. It is agreed that ozone is a powerful microbiocidal agent, but there is little agreement among researchers as to the sensitivity of different microorganisms to ozone. Because of complicating factors such as of variation among the strain of the microorganisms, age of the culture, density of the treated population, presence of ozone demanding material, method of applying ozone, accuracy of ozone measuring procedures and method of measuring antimicrobial efficacy (Khadre *et al.*, 2001) comparisons among studies are difficult.

The present study was a quantitative examination of the fungicidal effects ozone has on the natural fungi, *P. verrucosum* and *A. flavus*. It was undertaken to provide defensible data that would offer a basis for using ozone as an alternative treatment method for high moisture content grains to reduce *A. flavus, P. verrucosum* and the natural fungi on high moisture barley (>0.93a_w) by over 90% at temperatures of 20°C or greater without reducing germination by more than 10%.

The following are the major, relevant findings that resulted from the present study, listed in relation to the two phases of experiments talked about in the first chapter:

Phase I. The natural fungal population on the first batch of barley (consisted of vegetative and spore forms) received was more

difficult to inactivate with ozone than the second batch of barley (consisted of spores) and therefore was used as a benchmark against all other fungi. Ozone doses of 0.98mg/g barley • min were used to reduce the natural fungi on the barley by over 90%. When the water activity of the second batch of barley was at 0.88 the ozone became less effective against the natural fungi and was unable to reduce the population by 90%. This could not be explained by different ozone consumptions. However, when the temperature was increased to 40°C the natural fungi were reduced by over 90%. This was shown to be from higher ozone consumptions at higher temperatures.

Phase II. Spores of *A. flavus* were shown to be resistant to ozonation while *P. verrucosum* spores showed little or no resistance. Both the vegetative form of *A. flavus* and the spore form of *P. verrucosum* were reduced by over 90% at all water activities. However, at lower water activities (0.88aw) resistance to ozone became apparent, although ozone consumptions were the same as those of higher water activities. Higher temperatures appeared to be better at reducing the vegetative state of *A. flavus* than lower temperatures.

As ozone dose increased the rate of germination decreased. The maximum rate of germination loss to achieve a 90% reduction with all the fungi tested in this study was 6%. No differences were observed among water activities.

4.5 Implications

This study revealed that warm wet conditions that lead to spoilage of stored crops are the exact conditions that favour ozone disinfection of barley. Currently, farmers have to increase the input of preservatives (organic acids) as the water activity of the barley increases (McLelland, 1999). This results in increased costs for the farmer. However, as the water activity of the barley increases (increased risk of spoilage), the better ozone works. Since no additional costs are necessary for the treatment of barley with a high water activity than that of barley with a lower water activity, the farmer may reap the benefits of harvesting high moisture barley (outlined in Chapter 2). The findings of this study show that ozone may be applied as a fumigant against fungi attacking stored high moisture content barley and provide the possibility of using it as an alternative to current chemicals for preserving stored barley.

Chapter 5 - Conclusions and Recommendations

Within this chapter, various defensible conclusions are offered. With these in mind, several recommendations for further study were offered in a concise form, in the interest of providing meaningful directions for subsequent researchers in the area.

5.1 Conclusions

The foremost conclusion drawn from this study is that ozone may be used to reduce *A. flavus, P. verrucosum* and the natural fungi on high moisture barley (>0.93a_w) by over 90% at temperatures of 20°C or greater without reducing germination by more than 10%.

Another conclusion is that ozone dosage played a role in the inactivation of fungi. The results presented in this paper showed that as ozone dose increased, the inactivation of fungi also increased. Presented in Table 5.1 is an overview of the effect ozone dose had on the fungal populations encountered in this study. The sensitivity of the fungi tested to ozone, from the lowest sensitivity to highest was *A. flavus* (spores), the fungi on the first batch of barley, the fungi on the second batch of barley, *A. flavus* (vegetative state) and *P. verrucosum* (spores).

Table 5.1. The effect ozone dosage has on the inactivation of fungi.

Fungal population	Dosage (mgO_3/g)	Inactivation (%)
The vegetative and spore forms of fungi on the 1st batch of barley	14.70	88.7%
The fungal spores on the 2nd batch of barley (spores)	0.80 – 0.33	95.0 – 76.4%
A. flavus (spores)	19.60	25.6%
P. verrucosum (spores)	0.08	86.6%
A. flavus (vegetative)	0.33	89.6%

The current study indicated that barley with higher water activities is easier to disinfect than barley of lower water activities. In all experiments conducted on the effect of water activity, it was shown that resistance to ozonation was greatest at $0.88a_w$ than that of all other water activities.

It was also shown in this study that higher temperatures made ozone a better disinfectant.

5.2 Recommendations for further study

1.) A similar study should be conducted on whether any toxic metabolites are formed when the ozone reacts with the barley or any other compound in a storage unit.

2.) A study should be conducted with ozone on different grains to determine its effectiveness.

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Appendices

Appendix A
Sample calculation for raising a_w

Equation

v = volume of water to be added (ml)
w = weight of grain to be treated (g)
a = the water content required in the grain (%)
b = initial water content of the grain (%)
I = the volume of water added later with the inoculum

$v = [w(a - b)/(100 - a)] - I$

Sample Calculation

Assumptions:
Weight of grain – 50g
Initial water content – 9.5%
Inoculum volume – 1.4ml
Water content required – 30%

$v = [w(a - b)/(100 - a)] - I$
$= [50g(30\% - 9.5\%)/100 - 30\%] - 1.4ml$
$= [50g(20.5)/70] - 1.4ml$
$= [1025/70] - 1.4ml$
$= 14.6 - 1.4$
$= 13.2ml$

Appendix B
Sample calculation of ozone dose

Equations

1. Flow meter correction

P = pressure in psi

$$= 1\text{L/min} * \frac{\sqrt{(14.7 + P)}}{14.7} * 0.95$$

= standard litre per minute (SLPM)

2. O_3 dosage

$$= \text{SLPM} * \text{density of } O_2\text{(L/min)} * \frac{\text{weight \% of } O_3}{\text{weight of grain}} * \frac{1000\text{mg of } O_3}{1\text{g of grain}} * \frac{1}{\text{min}}$$

$$= \frac{\text{mg of } O_3}{\text{g of grain} \cdot \text{min}}$$

Sample Calculation

Assumptions:
O2 density – 1.3g/L
Pressure – 11psi
Weight of grain – 50g
Average O_3 weight percentage – 3.0%

1. Flow meter correction

$$= 1\text{L/min} * \frac{\sqrt{(14.7 + 11\text{psi})}}{14.7} * 0.95$$

= 1.26 SLPM

2. O_3 dosage

$$= 1.26\text{L/min} * 1.3\text{g/L} * \frac{0.03}{50\text{g}} * \frac{1000\text{mg of } O_3}{1\text{g of grain}} * \frac{1}{\text{min}}$$

= 0.98mg/g barley · minute

Appendix C
The Germination Test (ISTA, 1985)

Germination of a seed in a laboratory test, is the emergence and development of the seedlings essential structures so that it is able to develop further into a satisfactory plant under favourable conditions in soil.

Normal seedlings show the potential for continued development into satisfactory plants when under favourable conditions. It must conform with one of the following categories:

1. Intact seedlings – seedlings with all their essential structures well developed, complete, in proportion and healthy.
    - several seminal roots
    - a well developed, straight coleoptile, containing a green leaf extending to the tip and eventually emerging through it.
2. Seedlings with slight defects – seedlings showing certain slight defects of their essential structures, provided they show an otherwise satisfactory and balanced development comparable to that of intact seedlings of the same test.
    - only two seminal roots
    - coleoptile with limited damage
    - coleoptile with a split from the tip extending downward not more than on third of the length
    - coleoptile loosely twisted or forming a loop
    - coleoptile with a green leaf not extending to the tip but reaching at least half-way up the coleoptile
3. Seedlings with secondary infection – seedlings which it is evident would have conformed with the above, but which have been affected by fungi or bacteria from sources other than the parent seed.
    - seedlings which are seriously decayed by fungi or bacteria are classified as normal, if it is evident that the parent seed is not the source of infection, and if it can be determined that all the essential structures were present.

Abnormal seedlings will not grown into satisfactory plants when under favourable conditions. These seeds were not included in counts.

1. The primary root:
    - stunted
    - stubby
    - retarded
    - missing
    - broken
    - split from the tip

- constricted
- spindly
- trapped in the seed coat
- with negative geotropism
- glassy
- decayed as a result of primary infection

The seminal roots:

- only one or none

Note:

Seminal roots showing one or more of the above defects are abnormal and cannot replace an abnormal primary root in cases where the presence of several secondary roots or at least two seminal roots determine the value of a seed.

2. The coleoptile
   - deformed
   - damaged
   - missing
   - with the tip damaged or missing
   - strongly bent over
   - forming a loop or spiral
   - twisted slightly
   - split for more than one-third of the length from the tip
   - split at the base
   - spindly
   - decayed as a result of primary infection

   The first leaf:

   - extending less than half-way up the coleoptile
   - missing
   - shredded or otherwise deformed

3. The seedling as whole:
   - deformed
   - fractured
   - two fused together
   - yellow or white
   - spindly
   - glassy
   - decayed as a result of primary infection