The Microbial Killing Effect of Airborne Ozone

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Abstract.

Microorganisms of the indoor air and surfaces represent a concern, mainly in the pharmaceutical industry, healthcare facilities and food industry. Microorganisms exist all around us, both in the surrounding air and on the surfaces. They may cause respiratory infections and other diseases, contaminate food, drugs, medical equipment and other products. Today we deal with this problem by disinfecting or sterilizing surfaces, to ventilate and by filtrating the indoor air but without total success. Ozone has since the beginning of the 20th century successfully been used to purify drinking water. The knowledge of the effect of ozone in air against microorganisms is poor. The purpose of this degree project was to get a deeper knowledge of the effect of airborne ozone during different conditions against microorganisms. The study has shown a good effect of airborne ozone and the importance of the air humidity. It shows that an almost total killing effect of some microorganisms in high humidity can be achieved already at low concentrations of ozone. The experiments also show the sanitizing effect of ozone of a heavily contaminated chamber. The overall conclusion of this work is that ozone is a potent decontaminating agent and has a good potential for use as an agent for disinfection and sterilization.

Sammanfattning

Mikroorganismer i vår inomhusmiljö kan vara ett problem. De finns i luften och på olika ytor i vår omgivning och kan orsaka infektioner och inflammationer hos människor, kontaminerar läkemedel, övrig medicinsk utrustning och förstöra våra livsmedel. Detta kan leda till både fysiskt och ekonomiskt lidande för individen som drabbas och för samhället. Det finns idag ingen väl fungerande lösning på detta problem. Idag försöker man använda sig av god ventilation och bra filter för att rena luften och på olika sätt desinficera och sterilisera ytor, men effekten är inte alltid tillräcklig. Ozon har sedan början av 1900-talet använts för desinfektion av dricksvatten och visat sig vara mycket effektiv. Ozonets verkan i luft och på ytor är mindre känd. Detta examensarbete har som syfte att bekräfta effekterna av ozon som gjorts i tidigare studier samt att med hjälp av nya försöksuppställningar erhålla och dokumentera effekten av ozon i luft i syfte att avdöda mikroorganismer. Denna studie visar att ozonet har en god effekt när det gäller att avdöda vissa bakterier och svampar. Experimenten utförda i detta arbete visar även att luftfuktigheten har stor betydelse på den avdödande effekten. Även låg koncentration av ozon kunde i vissa fall leda till total avdödning då luftfuktigheten var hög. Arbetet visar även att ozon i luft är ett effektivt medel för sanering av ett mycket kontaminerat rum. Efter 48 timmars behandling med ozon kunde inga levande mikroorganismer detekteras i luften. Detta visar att möjligheten att använda ozon för desinficering och sterilisering är mycket lovande och att det finns många möjliga applikations områden för användning av ozon.

Preface

This examination project work has been done during a period of 20 weeks at the Department of Ergonomics and Aerosol Technology at Lund Institute of Technology, LTH, in close collaboration with Ozone Tech Systems OTS AB, Malmö Sweden. The examination project work is a final part of a Master of Science in Biomedicine degree at the University of Kalmar, Department of Chemistry and Biomedical Sciences.

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1. INTRODUCTION

1.1. Background

This degree project was carried out at the Department of Ergonomics and Aerosol Technology at Lund Institute of Technology in close cooperation with Ozone Tech Systems, a company with their head office located in Stockholm and their research and development department in Malmö.
In the environment there are thousands of particles in different sizes floating around in the air. These particles can be everything from the smallest dust and hair or skin-depositions from humans or animals to particles of fabrics from clothes or furniture and different kinds of pollen and seeds from flowers and trees. There are many microorganisms such as different kinds of bacteria, fungi and viruses. Microorganisms in the indoor air and on surfaces represent a concern. In the medical sector and mainly for the pharmaceutical industry, packing of drugs and other sterile medical equipment, the absence of microorganisms and endotoxins are of a great importance. In the food industry different kinds of bacteria and fungi are the main reasons for the food spoilage and some microorganisms can also cause food poisoning. It can be a problem to store fruit, vegetables, meat and other food for a longer time. In some food processes e.g. souse vide production and other productions of "ready to eat" foods there is a demand of low microbial air contaminants to get safe products. Transmission of microorganisms that causes respiratory infections and other diseases can be a problem in indoor facilities where a large number of people are gathered, for example daycare places, schools and shopping centers. Healthcare facilities are problem areas for transmission of microorganisms causing infections due to the high number of infected people and immunocompromised people. It is estimated that 10 % of the patients in hospitals receives a nosocomial infection. Disease transmission often occurs by direct contact or indirect contact between people. Direct contact means that the microorganism is transmitted by physical contact between people while indirect contact is when microorganisms are transmitted by contact of a contaminated surface. Contamination of surfaces is often caused by microorganism admittance from the human body and clothes. It has been the opinion that airborne transmission of microorganisms is uncommon except for very close droplet contact between people and the transmittance of spores. Studies have now shown that the importance of airborne transmission of microorganisms may be underestimated and thus is subject to more investigations. Today the problem with microorganisms is handled by disinfecting surfaces, proper hand hygiene, good ventilation and filtration of the air of the different places. This is though not always satisfactory and new methods are needed. [1,2,3,4,5,6,7]

1.2. Purpose of this project

The purpose of this degree project was to investigate and get a deeper knowledge in a controlled way of how effective airborne ozone is at killing microorganisms in purpose to decontaminate, disinfect or even sterilize the air in indoor facilities. The good effect of ozone in water purification is well known and documented but the effect of ozone in air purification is not well known. This degree project looked primarily at how low concentrations of ozone in different humidity could affect the killing of different microorganisms, as bacteria and fungi. The aim would be to decrease or eliminate all harmful and pathogenic microorganisms in an environment with a concentration of ozone low enough not to be dangerous for humans to work in or live in. In the food sector a decrease of microorganisms in the air could lead to food with extended shelf life. The pharmaceutical industry often requires a total absence of microorganisms and endotoxins. If the effect of ozone in air can achieve a desired level of sanitation, can be used safely and is cost beneficial compared with other sanitizing agents, this would be an attractive alternative for many applications. [2,3,5,6]

2. OZONE

2.1. History

The presence of ozone is a necessity for all living organisms above water on this planet. The ozone-layer in the high atmosphere works as a sunscreen against the dangerous sun radiation. The knowledge of ozone can be traced back to the ancient Greece where the characteristic odour of ozone created during a thunderstorm was known but without understanding its true nature. In the Illiad and Odyssey the odour is referred as “sulphurous”. The discovery of ozone in modern time is credited to F.C.Schönbein who in 1840 was the first to equate the odour of the anodic gas created during the electrolysis of water with that observed during electric discharges such as lightning. He associated this characteristic odour with an unknown, strongly electronegative gaseous substance which he named ozone (after the Greek word όζειν, to smell). But it took five more years until it was stated that ozone was another form of oxygen by de la Rive and de Marignac. They also observed that ozone could be formed from pure oxygen, as the effect of electric discharge. In 1857 the first ozone generator, in form of a discharge tube, was made by W. von Siemens. The generator was for laboratory-scale generation of ozone, and the same principal is still used in modern ozone generators. 8 years later in 1865, J.L. Soret was the first to prove by indirect determination of its density that the ozone molecule consists of 3 oxygen atoms. The bactericidal effect of ozone was discovered by Ohlmüller in 1890 and during the following years ozone-generating equipment of larger capacity was made available by Siemens and Halske. Ozone for disinfection of drinking water has been in use since the beginning of the 20th century. The breakthrough for the use of ozone came in the 1950s mainly due to better generators. Since then ozone has been used in other applications such as cleaning the water in swimming-pools, in the paper-mass industry to bleach paper and for sanitation of bad odour, for example after a house fire to get rid of the smoke smell. Other promising areas of application in the future are in the food industry, pharmaceutical industry and in the laundry industry due to the microbial killing effect of ozone. [8,9]

2.2. Physical and chemical properties of ozone

Table 2.2.1

Chemical name	Ozone
Appearance	Colourless at room temperature
Smell	Sharp strong, electric odour
Molecule form	O_3
Molecule weight	48.0
Density in gaseous form (25ºC)	2.144 kg/m^3
Solubility in water	4.9ml/l (0 ºC)
Boiling point	-111.9 ºC
Melting point	-192.5 ºC

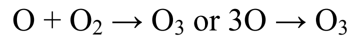
The two basic equations for the formation of ozone are:

$$3\ O_2 \leftrightarrow 2\ O_3 \text{ and } O_2 + O \leftrightarrow O_3.$$

Ozone is produced in the stratosphere as there is enough of UV-C radiation with a wavelength under 241 nm. The oxygen molecules which absorbs a photon from the sunlight with the wavelength of 241 nm or shorter receives enough energy to break up the oxygen molecule into atoms:

$$O_2 + \text{UV photon} \rightarrow 2O$$

When the oxygen molecule has been broken down to oxygen atoms the formation of ozone has begun:



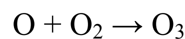
$$O + O_2 \rightarrow O_3 \text{ or } 3O \rightarrow O_3$$

Ozone can also appear closer to the ground and is then called ground-level ozone. There are different ways for ozone to be created close to ground:

- Electric discharges during thunderstorms
- Stratospheric ozone transported down by different kind of weather fronts
- Photochemical reactions from natural occurring substances like terpenes from coniferous forests
- Photochemical reactions by substances created by us humans like for example nitric oxides (NO_x), hydrocarbons (HC) and vaporous organic components (VOC):

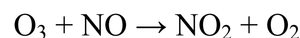
$$NO_2 + \text{UV-light} \rightarrow NO + O$$

The single oxygen atom can react with the surrounding oxygen molecules and create an ozone molecule:



$$O + O_2 \rightarrow O_3$$

The ozone molecule is quickly broken down again if there is enough nitric monoxide:



$$O_3 + NO \rightarrow NO_2 + O_2$$

Theoretically this would mean that no constant ozone production occurs due to the rapid decay of ozone by NO, but in reality NO reacts with free radicals in the air often created by the reaction between UV-light and VOC. These free radical products often occur where the temperature is over 20ºC. This leads to the ground-level production of ozone. A simplified reaction for the creation of ground-level ozone could look like this:

$$VOC + NO_x + \text{UV-light} \rightarrow O_3 + HNO_3 + \text{organic components}$$ [8,9]

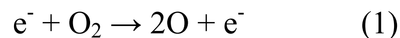
2.3. Ozone production

Ozone in a laboratory environment can be generated in many different ways, but they all require oxygen in some form:

- Photo chemical process
- Thermal process
- Electric discharge
- Electrolytic process
- Cryo chemical process
- Chemo nuclear process

The process of generating ozone by electric discharge is the most used and is described in more detail here:

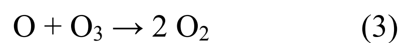
In a gaseous state the atoms of the different gas molecules are moving around in such a speed that there are always free electrons. When the gas moves between two electrodes with a different potential the free electrons accelerate towards the electrode with the highest potential. When an electron gains enough speed and collides with an atom an ion has been created:



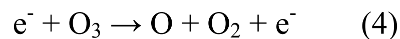
$$e^- + O_2 \rightarrow 2O + e^- \qquad (1)$$

$$O + O_2 \rightarrow O_3 \qquad (2)$$

But the ozone also decades:



$$O + O_3 \rightarrow 2\,O_2 \qquad (3)$$



$$e^- + O_3 \rightarrow O + O_2 + e^- \qquad (4)$$

There will always be an excess of ozone because the reaction (1) is faster then reaction (4). [8,9]

3. Microbial growth control

3.1. Methods for growth control of microorganisms

The control of microbial growth includes everything from growth inhibition, killing and removal of all viable organisms from a surface or product to antimicrobial agents like different kinds of antibiotics used *in vivo*. In general when we talk about microbial growth control we mean the control of microorganisms on surfaces, in products, in processes and in the air. Three key words often used are: [10,11]

- Decontamination - treatment that renders an object or inanimate surface clean to handle. This is something we all do in our home when we for example are wiping off the table and washing the dishes after a meal. This removes potential microbial nutrients and contaminating microbes, preventing growth of microorganisms like bacteria or fungi.
- Disinfection - the process of eliminating nearly all pathogens, but not all microorganisms, from inanimate objects or surfaces. This is done with chemical or physical agents with the aim of destroying microorganisms.
- Sterilization - the elimination or destroying of all living microorganisms and viruses. Microorganisms are not killed instantly when exposed to a lethal agent. The population decreases in a logarithmic way, which means that in theory an object never can be sterile. A microorganism is considered dead when it is unable to grow in conditions that would normally support its growth. The definition of a sterile product in the pharmaceutical industry is that the probability of a microorganism surviving should be less than one in a million. [10]

In killing or elimination of microorganisms several factors most be considered:

- It must be known what the material that is undergoing the treatment is made of. Can it resist a high temperature or a certain chemical agent?
- The size of the population, larger populations take longer time then smaller population.
- Population composition is an important factor due to the difference in sensitivity to the killing agent between different kinds of microorganisms.
- The concentration of the specific agent can affect the efficiency in killing the microorganisms.
- Local environment-environmental factors, such as pH, viscosity and the concentration of organic matter can also affect the efficiency of the specific agent. [10,12]

There is different ways to disinfect or sterilize a surface or a product. These can be divided into two groups:

- Physical methods to kill or eliminate microorganisms:

    A. Heat – is an efficient way to sterilize a product. Boiling water is effective in killing vegetative cells and eukaryote spores. But due to the presence of bacterial spores a product needs a higher temperature to be sterilized and this is done in a process called autoclaving in pressure. The temperature is in most applications 121°C and the time is calculated according to volume and the amount of microorganisms. Moist-sensitive products, like for example powders, can be sterilized in dry heat and require a temperature of 160-170°C during 2-3 hours. To sterilize a heat-sensitive product a

process called Tyndallization is used. This means that first a killing of vegetative cells with an elevated temperature and then incubated at an appropriate temperature, to let spores germinate to form new vegetative cells. At new exposure for elevated temperature new vegetative cells are killed. Pasteurization is also a common way of controlling the growth of microorganisms mostly in dairy products. This is often done by exposing the product, milk for example, for a temperature of 71°C during 15 seconds. This process reduces the amount of microorganisms in the product.

B. Filtration – is used to sterilize heat-sensitive liquids or gases by removing microorganisms. A depth filter which is a fibrous sheet that can consist of a random array of paper. Depth filter is often used as a prefilter due to its capacity to remove larger particles. Membrane filter are thin filter with a defined pore-size and allows relatively high fluid flow rate due to the fact that 80-85 % of the surface area of the membrane consists of open pores. To reach sterilization high-efficiency particulate air (HEPA) filters or nucleopore filters are used for areas with the highest demand but this is also not effective against viruses.
C. Radiation – is an effective way to kill microorganisms. Ultraviolet (UV) radiation causes damage to the DNA, but its use is limited to surface and air sterilization because low or limited penetration of glass, dirt films, water and other substances. Ionizing radiation like x-rays and γ-rays are efficient at penetrating the material and causes damage to both the DNA and the proteins. In Sweden ionized radiation is only allowed for sterilization of medical- and laboratory-equipment and some types of spices. [10,11,12]

- Chemical agents for killing and elimination of microorganisms:

A. Alcohols – widely used disinfectants and antiseptics that act by denaturing proteins and possibly by dissolving membrane lipids. Does not kill endospores.
B. Phenols – used in laboratory and hospitals to disinfect. Works by denaturing proteins. Two types of phenols are hexachlorophene and chlorhexidine.
C. Halogens – are common used disinfectants and antiseptics. Iodine acts by oxidizing cell constituents and iodinating cell proteins. Chlorine acts primarily by oxidizing cell constituents.
D. Gases – are used when the product is heat-sensitive or if it is of great volume. The products are sterilized in a closed chamber with a gas for example ethylenoxide. The gas penetrates paper, some plastic and reaches everywhere. It works by denaturing proteins and destroying DNA within 4-18 hours. Ethylenoxide is a hazard for humans and all gas must first be eliminated before the product can be used. Therefore the process is very time demanding and slow. Other gases can be betapropiolactone, propylenoxide and ozone.
E. Oxidizing substances – these substances work by oxidizing substances on or in the cell so that the cell gets killed. The most common one used is hydrogen peroxide which is used for both disinfection and sterilization depending on what concentration you use and for how long time. Peracetic acid is also an oxidizing agent. Ozone also belongs to this group and is mostly used for disinfection of drinking water at the moment. [10,11,12]

3.2. Microbial killing with ozone

Ozone is the 4th most oxidizing agent known. The oxidizing potential for ozone is 2.07 mV compared with ethylene oxide 0.699 mV and chlorine gas 1.36 mV. Ozone is a strong, fast and broad-spectrum antimicrobial agent that works effectively against bacteria, bacterial spores, virus, fungi, fungal spores and protozoa. Unlike many other sterilizing agents ozone is easy and fast to remove after the process and does not leave any remaining chemicals, odour or taste. Inactivation of bacteria is thought to occur by ozone oxidizing the fatty acids in the cell-membrane and macromolecules, like proteins and DNA. The damage caused by ozone is irreversible and causes lyses of the cell wall and the death of the bacteria. It also kills spores and viruses as it oxidises DNA and proteins in the spore as well as in viruses. The effect of ozone in water is well known and seems to be more effective than ozone in air. It has also been indications that ozone needs a higher humidity in the air to be really effective. Some of the studies made with airborne ozone and ozone in water can be seen in *table 3.2.1, 3.2.2, 3.2.3* and *3.2.4*: [8,12,13]

Table 3.2.1The effect ozon has on bacteria and fungi in air with high humidity. No exact data of the humidity was mentioned [14]

Organism	Ozone concentration (% by weight)	Initial Population (organisms/ml)	Time to 100% kill (minutes)
Streptococcus pyrogeneses	2.02	$5.4*10^8$	10
Staphylococcus aureus	2.09	$1.0*10^5$	10
Escerichia coli	3.96	$1.6*10^7$	5
Clostridium sporogenes	7.5	$4.0*10^5$	30
Bacillus subtilis	8.0	$9.7*10^6$	21
B. subtilis	9.5	$9.2*10^4$	10
Aspergillus niger	9.5	$2.0*10^5$	10
Candida albicans	9.5	$5.0*10^6$	10
Proteus vulgaris	4.2	$3.3*10^6$	3
S. aureus	10.0	$5.8*10^5$	1
Mycobacterium tuberculosis	10.0	$3.3*10^5$	15
B. stearothermophilus	10.0	$10*10^5$	25
B. globigii	10.0	$10*10^5$	20

Table 3.2.2 Summary of airborne ozone results on bacteria and fungi [15]

Test Microbe	Ozone (PPM)	RH (%)	Time (min)	Survival (%)	Ref.
Staphylococcus aureus	0.3-0.9	No data	240	0.5	Dyas et al.
Pseudomonas aeruginosa	0.3-0.9	n.d.	240	31	Dyas et al.
Serratia spp.	0.3-0.9	n.d.	240	3.2	Dyas et al.
Proteus	0.3-0.9	n.d.	240	0.9	Dyas et al.
Aspergillus fumigatus	0.3-0.9	n.d.	240	8	Dyas et al.
Streptococcus salivarus	0.6	60-75	100	2	Elford & van de Eude
Bacillus cereus	3	95	60	0.013	Ishizaki et al.
Fusarium oxysporum	0.1	35-75	240	2	Hibben & Stotzky
Aspergillus niger	0.1	35-76	240	84	Hibben & Stotzky
Rhizopus stolonifer	0.1	35-77	240	43	Hibben & Stotzky
Penicillium chrysogenum	3-9	90	1380	0.1	Foarde et al.

Table 3.2.3 Results made of ozonation in water on bacteria [13]

Test organism	Ozone (PPM)	Time (sec)	Survival (%)	Ref.
B. subtilis	2.2	90	0.01	Botzenhart et al.
Escerichia coli	1.3	10	0.003	Katzenelson & Shuval
Salmonella. typhimurium	0.36	36	0.0002	Farooq et al.
E. coli	0.81	30	0.00003	Finch et al.
E. coli	12	62	0.00015	Bunning & Hempel
E. coli	2	15	0	Burleson et al.
S. aureus	2	15	0	Burleson et al.

Table 3.2.4 Summary of results performed with airborne ozone on E.coli and S.aureus [13,15]

Test organism	Ozone (PPM)	RH (%)	Time (min)	Control (CFU/ml)	Survival (%)
E. coli	5.7	18-22	60	$4.08*10^{11}$	0.10
	0.3	18-22	60	$1.02*10^{11}$	58.0
	1.4	18-22	60	$3.89*10^{9}$	0.010
	1.2	18-22	60	$8.6*10^{9}$	0.156
	300	No data	0.25	$1.80*10^{5}$	0.056
	327	n.d.	0.25	$4.01*10^{5}$	0.005
	389	n.d.	0.25	$7.17*10^{5}$	0.021
	631	n.d.	0.25	$1.80*10^{5}$	0.007
S. aureus	300	n.d.	0.25	$1.88*10^{5}$	0.004
	389	n.d.	0.25	$1.95*10^{6}$	0.004
	631	n.d.	0.25	$1.88*10^{5}$	0.005
	1500	n.d.	0.25	$4.15*10^{5}$	0.003

The studies in *table 3.2.2, 3.2.3 and 3.2.4* shows a great difference between the effect of ozone in water and airborne ozone. This indicates the importance of humidity for the effect of ozone in air to kill or inhibit the growth of microorganisms. [12,13,15,16,17,18]

3.3. Physiological effects of ozone on humans

Ozone is an effective oxidizing agent destroying microorganisms and it is also a hazard for humans. The National Board of Occupational Safety and Health in Sweden has established a time-weighted average permissible exposure level of ozone for workers for an eight-hour day of 0.10 ppm and a short-term exposure limit of 0.30 ppm for an exposure less than 15 minutes. Research on the effects of ozone on humans and animals has been done over the years but they are not conclusive. On humans there have been many experimental studies on short-term exposure to ozone at low concentrations, at which reversible subjective irritative symptoms from the respiratory tract and subclinical effects such as increased airway resistance have been recorded. In studies with animals pulmonary edema has been observed but there are only few reports of pulmonary edema in man. These reports are old and the circumstances of exposure are not fully known. The persons affected have been welders that have been exposed to ozone at rather low concentration and to other dangerous gases at a higher concentration. One important thing when exposed to ozone seems to be the amount of physical activity, or rather how intense the breathing is. A person that does not exercise during ozone exposure seems to get fewer symptoms than the one that exercise. It seems like the studies made shows that exposure to ozone is a great hazard but the results are inconclusive at what exposure concentration and time the ozone becomes dangerous. [19,20]

3.4. Effects on material after exposure to ozone

A problem often associated with sterilizing agents is the effect of the chemical on the material exposed. Ozone is a strong oxidizing agent and may cause oxidation of material exposed. There are both studies on the effects on material exposed to ozone and charts of ozone compatible material done. No specific data seems to exist on exactly how well different material handles the exposure to ozone. Therefore a material to be disinfected or sterilizated with ozone should always be fully investigated in how well it resists oxidation of ozone. [14,21]

4. Equations

The theoretical calculation of the concentration of ozone generated by the ICT-5 generator: At an oxygen flow rate of 1 l/min the ICT-5 generator generates about 6 g ozone/hour, and that gives:

Ozone concentration:	1.0 ppm = 2.0 mg/m^3
Oxygen flow:	1 l/min
Ozone generator at full effect:	6 g/h O_3
Amount ozone in the oxygen flow after the generator:	0.1 g/l O_3 = 100 mg/l O_3
Concentration of O_3 after 5 minutes in the chamber (5 dm^3):	100000 mg/m^3 = 50000 ppm (1)

5. Material and methods

5.1. Airborne ozonation in a small pressure chamber

5.1.1 Material

- Special made cylindrical pressure test chamber made of stainless steel with a volume of approximately 5 dm^3, *see picture 5.1.1.1.*

Picture 5.1.1.1. Small pressure chamber made of stainless steel

- Ozone sensors:
  - Dasibi ozone sensor 1008-AH, optimal working range of 0-1.0 ppm but can be used up to 50 ppm with good accuracy.
  - Thermo Environmental Instruments U.V. Photometric Ambient O_3 Analyzer/Calibrator, optimal working range of 0-1.0 ppm but can be used up to 40 ppm with good accuracy.
- Airflow regulators that regulates the airflow between 0-2.5 l/min and 0-10 l/min.
- Needle airflow regulator.
- Pressure regulator, regulating the pressure between 0-4 bars.
- Catalytic material consisting of Carulite, composed of magnesium dioxide, copper oxide and aluminium oxide, for the destruction of ozone.
- Temperature probe.
- Ozone generators:
  - Ozone Tech System special made ACT-3000 model with only one ceramic plate.
  - Ozone Tech System ICT-5 model.
- Sterile filtered compressed air used for the AM-3000 ozone generator.
- Pure oxygen on tube used for the ICT-5 ozone generator.
- Tryptone Soya Agar 90 mm, 12002, BIOTRACE FRED BAKER LIMITED
- Sanitiser Neutralising Agar 55 mm, 11005, BIOTRACE FRED BAKER LIMITED.
- Different unknown bacterias isolated on Tryptone Soya Agar 90 mm and Sanitiser Neutralising Agar 55 mm at a temperature of 22°C from the surrounding environment air and surfaces in Ozone Tech Systems facilities. The agar plates were then incubated at 37°C for 24-48 hours. Named bacteria #1 – bacteria #4.
- Stainless steel plates, 70*70 mm^2 and approximately 2 mm thick.

5.1.2 Method

At the initial part of the project the building of the small chamber and the whole test equipment took place. As soon as the test equipment was ready to use, the installation of the equipment began. The initial purpose of the project was to test a low ozone concentration, 0.1 ppm. At first only one stream of gas containing oxygen passed through the ICT-5 ozone generator but the concentration of ozone became too high. At a theoretic calculation a flow of 1 l/min of oxygen through the ICT-5 generator would generate an ozone concentration of approximately 50000 ppm in the test chamber after 5 minutes which is much higher then the desired concentration of 0.1 ppm O_3, *see equation chapter 4*. Even if the effect of the ozone generator would be turned down to 1 % of the maximum effect, the concentration would still be 500 ppm in the chamber. The problem with changing the effect is that the ozone generator does not produce 1% of the original amount continuously but instead produces ozone at full effect for 0.1 seconds and then shuts down the production during 9.9 seconds which results in a huge difference of the concentration at different times. A different way of ozonating the gas flow into the chamber was done by using sterile filtered compressor air instead of oxygen, because it gives a lesser production of ozone because air only contains 21 % of oxygen. The use of compressor air through the ICT-5 generator did not lower the concentration enough and it was still too high. The generating of ozone changed from the ICT-5 generator to a smaller one called ACT-3000. The ACT-3000 produces only 200 mg/h but it is built for the use in a room of 12 m^3 or bigger and it is fed with air by a vent built in the construction. After rebuilding the ACT-3000 generator so that it only has one ceramic plate instead of a whole package, the ceramic plate was enclosed into a metal chamber so that the air flow through the generator was done by sterile filtered compressor air, instead of a vent feeding it with normal air of the room. This had to be done so that the amount of air passing through the generator could be controlled. To reach the concentration desired the need for mixing the ozonated air with clean compressor air became necessary. The ACT-3000 required an air-flow of at least 1 l/min because of the cooling action the airflow contributes with, and therefore a needle regulator in stainless steel was put in after the ozone generator to separate some of the ozonated air. A mass flow regulator would have been better and more accurate but the cost of a mass flow regulator that could handle ozone made this impossible. Another thing that could be a problem was that the Dasibi ozone sensor needs a gas flow of 1.5 l/min and therefore the ozonated air flow into the chamber had to be at least more than this. A simplified sketch of the arrangement of the experimental equipment can be seen in *fig.5.1.2.1.*

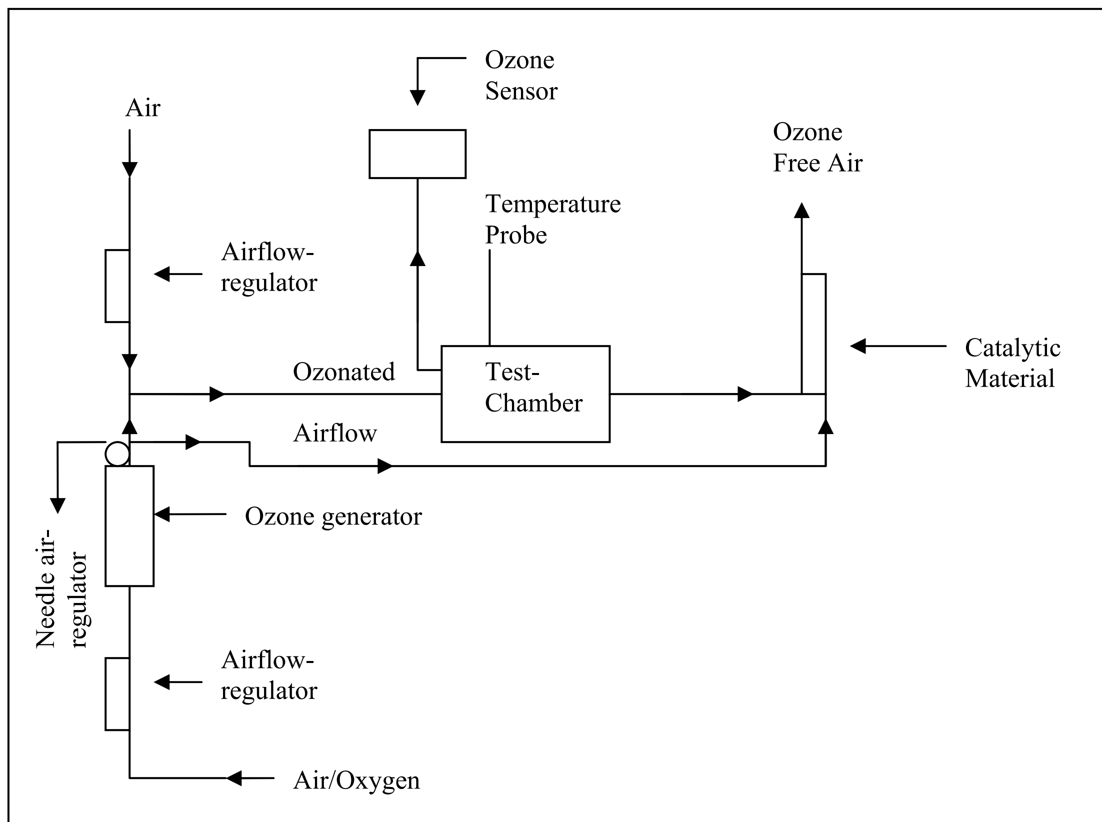


Fig.5.1.2.1. Schematic sketch of the airborne ozonation of microorganisms in the small chamber

The purpose of the first part of the project was to investigate the killing effect of ozone on microorganisms. The microorganisms for the experiments were collected from the surrounding environment air and surfaces, and this was done by placing petri dishes with agar on different spots of the Ozone Tech Systems facility overnight. Microorganisms from the facility were also collected by contact plates with agar. After incubation at 37°C for 24-48 hours bacteria from different colonies were suspended in sterile water. The suspensions were then diluted to a concentration that the colony forming units of the microorganism could be counted when growing on an agar plate. This was done by performing dilution series where a suspension from each step of the series was poured on a petri dish with agar medium. After incubated at 37°C for 24-48 hours the colony forming units were counted to determine the most appropriate concentration to use of the microorganism. On each petri dish 500 µl of a selected suspension was poured and dried for an hour before placed in the test chamber at different ozone concentrations and exposure times. The plates were then incubated at 37°C during 16-48 hours depending on microorganism. The temperature was chosen after the temperature of the human body and not for what might have been the optimal growth temperature of the microorganisms. Due to ozone being a strong oxidizing agent the possibility for the ozone to oxidize the medium which might give a false result because if it was consumed or essential nutrient components was destroyed. An experiment was therefore designed with microorganisms on a stainless steel plate. The contact agar plate might not give a correct result, as all the microorganisms on the stainless steel plate might not stick to the agar. But it gives an indication of how the microorganisms survive on the steel plate and the results could be compared. To each stainless steel plate 20 µl of a suspension with the appropriate concentration of microorganisms were added and allowed to air-dry for half an hour. The steel plates was then inserted into the test chamber and exposed to different concentrations of ozone during different length of times. A Sanitiser Neutralising Agar was then pressed on the steel plate and the agar plate was incubated at 37°C for 16-48 hours.

5.2. Airborne ozonation of microorganisms in various humidity in a cleanroom chamber

5.2.1. Material

- Cleanroom chamber of approximately 20 m^3 made of stainless steel with the possibility of controlling both the humidity and temperature, at Lunds Tekniska Högskola
- Ozone generator:
    - Ozone Tech Systems ACT-6000 with appropriate channel fan
- Heat fan
- 4 Fans
- Hotplate
- Saucepan of approximately 20 dm^3
- Cooling element for drying the moist air
- Humidity sensor
- Temperature probe
- *Aspergillus niger* ATCC 16404, a fungi that produces spores. Incubated at a temperature of 25°C for about 5 days. The *Aspergillus* family contains the most common human pathogenic moulds. *Aspergillus* can be found almost everywhere and is an opportunist pathogen. This pathogen can cause aspergillosis to immunocompromised people. *Aspergillus niger* was chosen because it represents a fungi and produces spores. It has previously shown great resistance against ozone. [7,15,22,23]
- Yeast Extract Glucose Chloramfenicol Agar both as 90 mm agar plates and as 55 mm contact plates was used for *A.niger*
- *Pseudomonas aeruginosa* ATCC 10145, bacteria – a gram negative rod. Incubated at a temperature of 25°C for 1-2 days. *Pseudomonas aeruginosa* is an opportunistic pathogen that can cause a variety of infections like eye infections, lung infections in cystic fibrosis patients and nosocomial lung and wound infections. *P.aeruginosa* is also resistant to many antibiotics. It was chosen because it is a gram negative bacteria and has previously shown some resistance against ozone. [15,22,24]
- Cetrimide Nalidixine agar plates as 90 mm agar plates and as 55 mm contact plates was used for *P.aeruginosa*
- *Staphylococcus aureus* ATCC 29737, bacteria – a gram positive cocci. Incubation at 35°C for 1-2 days. *Staphylococcus aureus* exist in air, dust, sewage, water, milk and food or on food equipment, environmental surfaces, humans and animals. Humans and animals are the primary reservoirs. Staphylococci can cause a number of infections, mostly wound infections, and staphylococci can also produce enterotoxins that cause illness in humans. *Staphylococcus aureus* was chosen because it represents gram positive bacterias and has been used in previous studies with ozone. [13,25,26]
- Baird Parker Medium as 90 mm agar plates and as 55 mm contact plates was used for *S. aureus*
- Unknown bacteria collected from the surrounding environment. Incubated at a temperature of 25°C for 2-3 days.
- Tryptone Soya Agar 90 mm, 12002, BIOTRACE FRED BAKER LIMITED was used for the unknown bacteria and for *S. aureus*
- Sanitiser Neutralising Agar 55 mm, 11005, BIOTRACE FRED BAKER LIMITED was used for the unknown bacteria and for *S. aureus*

5.2.2. Methods

The cleanroom with an aggregate that controls humidity and temperature was installed. Because of the low airflow reaching the cleanroom chamber from the aggregate caused by the HEPA-filter inside the pipeline between the aggregate and the cleanroom chamber the humidity of the air never reached the desired level. This was solved by putting in a hot plate in the cleanroom with a large saucepan filled with water. When the desired humidity of the air was achieved the humidity was maintained by turning the hot plate on and off from outside the cleanroom. Due to the boiling of the water the temperature in the chamber went up to 27°C. To get the same temperature during the experiments with dry air a heat fan was used. Another problem appeared when the ozonated air with high humidity was passed through the ozone sensor. The ozone sensors that were used in these experiments can not be used for air with humidity over 90 %. In the Dasibi 1008-AH there is a small ozone destructor consisting of carulite to calibrate the ingoing gas to 0 ppm ozone. When air with high humidity enters the destructor the water condensates on the walls of the destructor and the water binds to the carulite which results in that the ozone can pass through the destructor and therefore gives a wrong zero point. This was solved by building a cooling element reaching a temperature of nearly 0°C. By putting the cooling element between the chamber and the ozone sensor the moist air could flow through the cooling element and a great part of the humidity could condensate on the walls of the cooling element. In this study 500 µl of a suspension containing a single kind of microorganism was poured on the agar plate and 20 µl to the stainless steel plates. After being exposed by ozonated air during 60, 120 or 180 minutes the agar plates were incubated at the appropriate temperature for the specific microorganism in question for the specific time acquired. The experiment was to investigate how different concentrations of ozone during different humidity of the air would affect the killing of the microorganisms. At the initial stage the concentration of the ozone began at 0.3 ppm and the aim was to try the effect of a low concentration of ozone. The experiments were conducted in 15-25 %, 50-60 % and 85-95% humidity. The ozone was generated by the fan blowing the air inside the chamber through the ceramic plate package at a flow rate of 150 m^3/h through the ozone generator. The generator could be controlled from the outside of the chamber by a controlling device. There were an additional of 4 more fans to circulate the air inside the chamber to get a good mix of air and ozone.

5.3. Ozone treatment of the air in a cleanroom chamber filled with raw meat

5.3.1. Material

- Microbiological air sampler, SAS, Super 100 Air Sampler
- Sanitiser Neutralising Agar 55 mm, 11005, BIOTRACE FRED BAKER LIMITED for the Microbiological air sampler
- Particle counter in particle sizes of 0.5 μm and larger and 5 μm and larger, HACH ULTRA ANALYTICS
- 30 kg of raw fresh meat from Atria Lithells AB

5.3.2. Methods

The aim was to let the meat begin to rotten to get an increase of airborne microorganisms to a level that could be confirmed by microbial air sampling. After about 4 days airborne microorganisms was detected. The aim was to start at a low concentration of ozone and increase the concentration to get a desired effect. The ozone was generated by the ACT-6000 with the air inside the chamber. This was done so that no entering of fresh air from outside could disturb the experiment and so that no air would escape from the chamber. There was also an addition of 4 fans in the chamber to circulate the air inside. After starting at a concentration of 0.3 ppm for 4 hours the ozone generator broke down. Instead ozonated air was blown into the chamber from the outside. The test was first performed with air without ozone and a measurement was done to get a value of the microbial growth and the amount of particles in the chamber when blowing in clean air from the outside. By the time the experiments with ozonated air were about to begin the appearance of both flies and larvae was observed inside the chamber. Due to the unpleasant feeling and odour when entering the chamber for measurements, the initial plan of this study was cancelled and instead changed into a sanitizing plan. The first 24 hours the concentration of ozone inside the chamber was 2.5 ppm by the ACT-6000 ozone generator until the ICT-5 generator was installed. With the ICT-5 generator the concentration of ozone was raised to 30 ppm and kept there during 24 hours. After this new measurements of the air were conducted.
An experiment was also performed to study the difference in the decrease of a high ozone concentration in an empty cleanroom chamber compared with a chamber filled with rotten meat.

5.4. The effect of high concentration of ozone in air against microorganisms during short time exposure

5.4.1. Material

- A suspension containing different kind of microorganisms collected from the experiments made in *5.2* and *5.3*.
- Tryptone Soya Agar 90 mm, 12002, BIOTRACE FRED BAKER LIMITED

5.4.2. Methods

The aim of this experiment was to see how a high concentration of ozone during a short exposure time would effect the growth of microorganisms. The concentration of ozone could only be calculated at a theoretical basis due to the fact that an ozone sensor in that range was not available, *see equations chapter 4*. The concentration was calculated to be approximately 5000 ppm ozone as 1 l/min of a 50000 ppm concentration of ozone in oxygen was mixed with 9 l/min of compressor air which should result in a 1:10 dilution of the zone concentration. The solutions containing microorganisms were poured on agar plates and were allowed to dry for approximately one hour in air. The agar plate was then put in the test chamber and was the exposed for the high concentration of ozone in air during a predetermined time. The agar plate was then incubated for the appropriate temperature and time. In this experiment we used a higher concentration of microorganisms on the plates exposed to ozone. To be able to estimate the total number of colony forming units on the control plates we had to dilute the solution to a concentration which could be counted.

6. Results

6.1. Airborne ozonation of a small pressure chamber

As an initial part of the experimental part of the project many experiments were conducted with an ozone concentration of 0.1 ppm. The exposure time was from 20 minutes up to several days with different kind of microorganisms collected from the surrounding environment air and surfaces. The tests did not show any significant effect at all at this concentration of ozone. In the tests performed longer than 24 hours there were problems with dehydration of the agar plates. With an increase of the ozone concentration the killing effect increased, see *fig. 6.1.1*. Less then 90 % of the bacteria survived 90 minutes of 1.0 ppm of ozone and 120 minutes of 0.5 ppm of ozone.

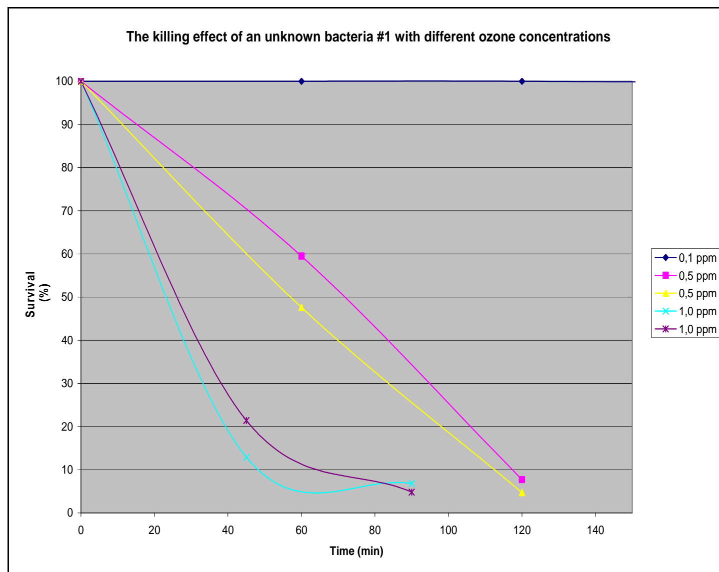


Figure 6.1.1. Reduction of the unknown bacteria #1 at different ozone concentrations

Tests were also conducted with the unknown bacteria #1 a 50 ppm ozone concentration for 20 minutes, *see fig 6.1.2.* Less than 5 % of the bacterias survived this treatment.

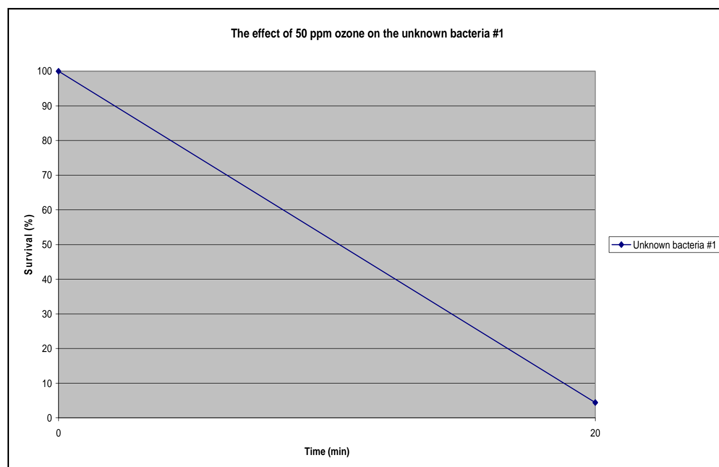


Figure 6.1.2. The effect of 50 ppm ozone on the unknown bacteria #1

No significant difference in effect could be seen between the microorganisms on agar and on the stainless steel plates, see *fig. 6.1.3.*

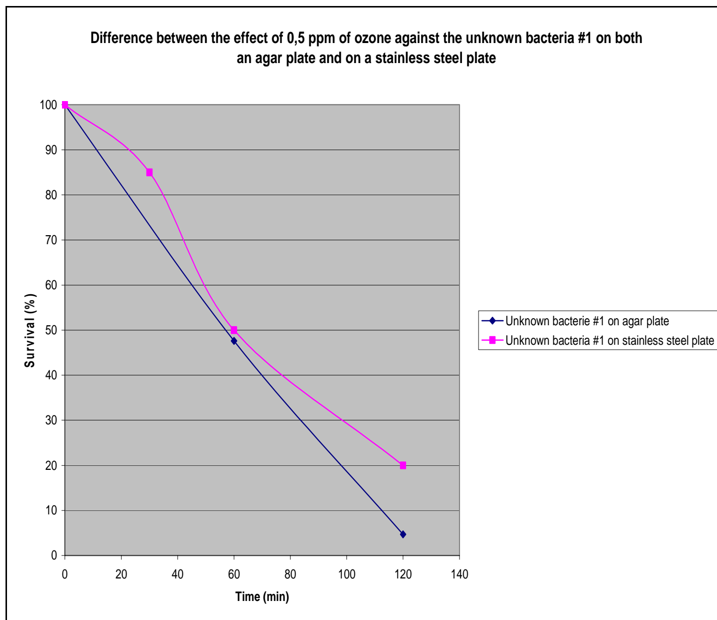


Figure 6.1.3. Difference between the effect of 0.5 ppm of ozone against the unknown bacteria #1 on both an agar plate and on a stainless steel plate

The difference in sensitivity to ozone of different concentrations of the isolated microorganism is shown in fig *6.1.4.* The exposure time was 20 minutes. The isolate #4 was much more tolerant than the other isolates, at 2.5 ppm 180 minutes exposure time was needed to reduce the survival to 10 %, see fig *6.1.5.*

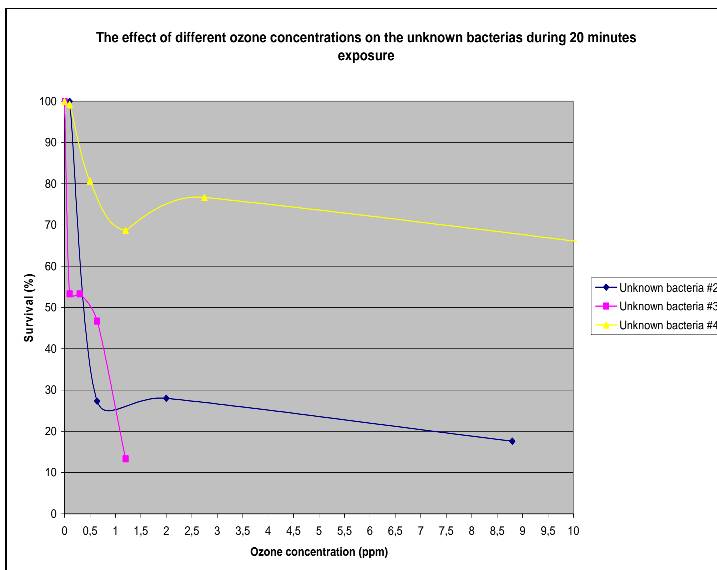


Figure 6.1.4. The effect of different ozone concentrations on the unknown bacterias during 20 minutes.

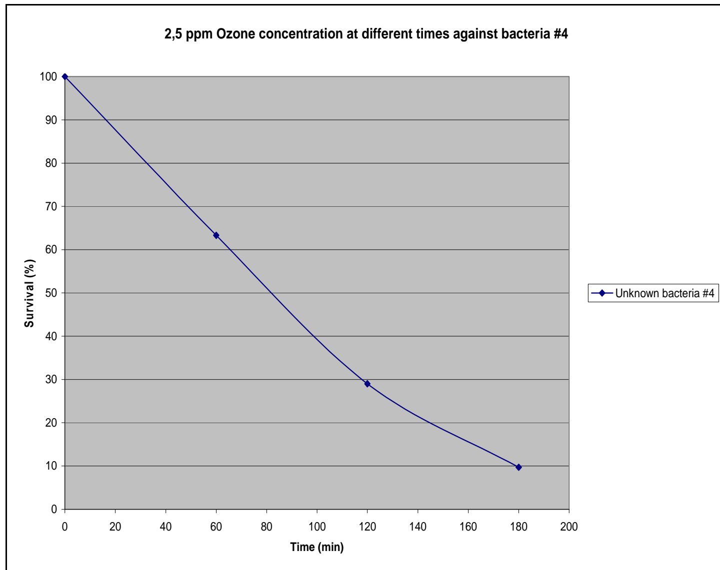


Figure 6.1.5. The effect of different exposure times at 2.5 ppm ozone on the unknown bacteria #4.

6.2. Airborne ozonation of microorganisms in various humidity in a cleanroom chamber

6.2.1. *Aspergillus niger*

6.2.1.1. 0.3 ppm and 0.6 ppm

0.3 ppm ozone 0.3 ppm had no effect on spores of *Aspergillus niger*. No significant effect was conceived with 0.6 ppm ozone. The only effect seen was a small one in 85-95 % humidity and for an exposure time of at least 2 hours.

6.2.1.2. 1.2 ppm

The result of the experiment can be seen in *table 6.2.1.2.1.* On the plates of stainless steel with *Aspergillus niger* there were no effect of the ozone except in a humidity of 50-60%. On the agar plates it was possible to see an effect which shows that ozone may have a killing effect on *Aspergillus niger* at this concentration. But as can be seen in the *fig.6.2.2.1,* the difference in humidity does not seem to have any effect.

Table 6.2.1.2.1. The survival rate of Aspergillus niger in an ozone concentration of 1.2 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	15-25	10	100
Stainless steel plates	120	1.2	15-25	10	100
Stainless steel plates	180	1.2	15-25	10	100
Agar plates	60	1.2	15-25	96	92
Agar plates	120	1.2	15-25	96	70
Agar plates	180	1.2	15-25	96	65
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	50-60	14	100
Stainless steel plates	120	1.2	50-60	14	100
Stainless steel plates	180	1.2	50-60	14	86
Agar plates	60	1.2	50-60	71	100
Agar plates	120	1.2	50-60	71	83
Agar plates	180	1.2	50-60	71	73
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	85-95	7	100
Stainless steel plates	120	1.2	85-95	7	100
Stainless steel plates	180	1.2	85-95	7	100
Agar plates	60	1.2	85-95	90	77
Agar plates	120	1.2	85-95	90	72
Agar plates	180	1.2	85-95	90	84

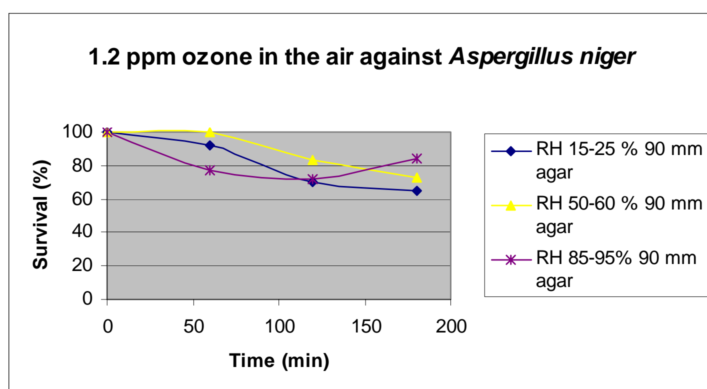


Fig. 6.2.2.1. The survival of Aspergillus niger after exposure to 1.2 ppm ozone

6.2.2. *Pseudomonas aeruginosa*

6.2.2.1. 0.3 ppm

Table *6.2.2.1.1* and fig *6.2.2.1.1* shows that ozone is effective at 0.3 ppm and the effect increases with increasing humidity. The effect was greater when the bacteria was placed on stainless steel than on agar.

Table 6.2.2.1.1. The survival rate of Pseudomonas aeruginosa in an ozone concentration of 0.3 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	15-25	73	93
Stainless steel plates	120	0.3	15-25	73	86
Stainless steel plates	180	0.3	15-25	73	67
Agar plates	60	0.3	15-25	45	71
Agar plates	120	0.3	15-25	45	40
Agar plates	180	0.3	15-25	45	24
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	50-60	54	26
Stainless steel plates	120	0.3	50-60	54	0
Stainless steel plates	180	0.3	50-60	54	0
Agar plates	60	0.3	50-60	109	97
Agar plates	120	0.3	50-60	109	42
Agar plates	180	0.3	50-60	109	26
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	85-95	130	86
Stainless steel plates	120	0.3	85-95	130	0
Stainless steel plates	180	0.3	85-95	130	0
Agar plates	60	0.3	85-95	123	98
Agar plates	120	0.3	85-95	123	23
Agar plates	180	0.3	85-95	123	7

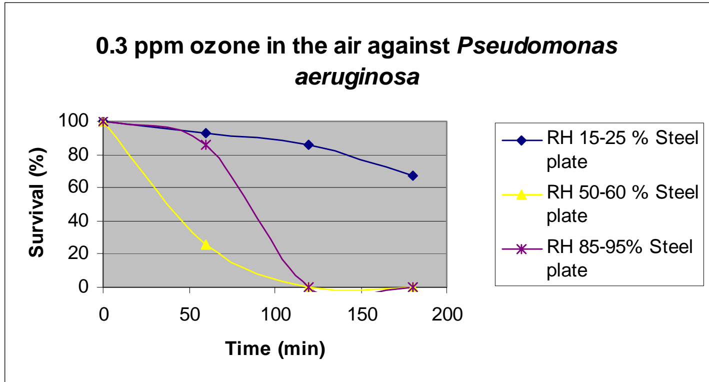


Fig. 6.2.2.1.1. The survival of P.aeruginosa on stainless steel after exposure to 0.3 ppm ozone

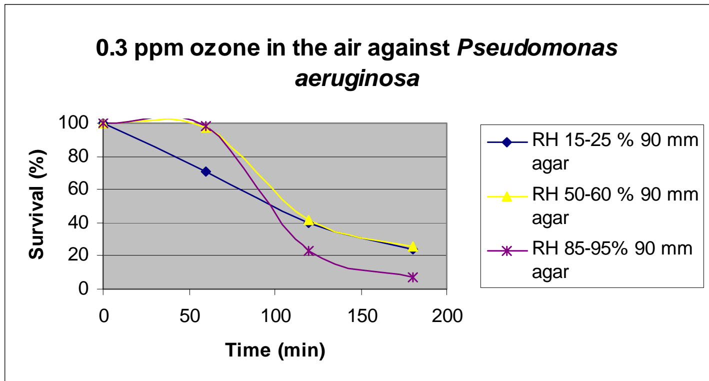


Fig. 6.2.2.1.2. The survival of P.aeruginosa on agar after exposure to 0.3 ppm ozone

6.2.2.2. 0.6 ppm

Increasing ozone concentration and humidity are more effective against *P.aeruginosa* as shown in table *6.2.2.2.1* and fig *6.2.2.2.1*. The effect is also greater here on the stainless plate in comparison to the agar plate.

Table 6.2.2.2.1. The survival rate of Pseudomonas aeruginosa in an ozone concentration of 0.6 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.6	15-25	126	71
Stainless steel plates	120	0.6	15-25	126	0
Stainless steel plates	180	0.6	15-25	126	0
Agar plates	60	0.6	15-25	63	70
Agar plates	120	0.6	15-25	63	62
Agar plates	180	0.6	15-25	63	46
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.6	50-60	153	70
Stainless steel plates	120	0.6	50-60	153	1
Stainless steel plates	180	0.6	50-60	153	0
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.6	85-95	49	8
Stainless steel plates	120	0.6	85-95	49	0
Stainless steel plates	180	0.6	85-95	49	0
Agar plates	60	0.6	85-95	500	53
Agar plates	120	0.6	85-95	500	10
Agar plates	180	0.6	85-95	500	0

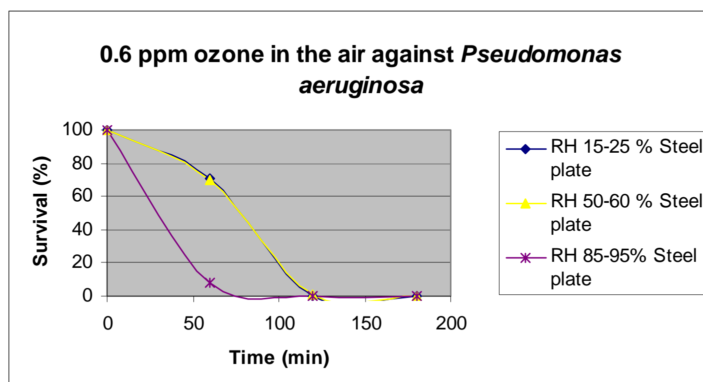


Fig. 6.2.2.2.1. The survival of P.aeruginosa on stainless steel after exposure to 0.6 ppm ozone

6.2.2.3. 1.2 ppm

1.2 ppm ozone is very effective against *Pseudomonas aeruginosa* as shown in table *6.2.2.3.1*.

Table 6.2.2.3.1. The survival rate of Pseudomonas aeruginosa in an ozone concentration of 0.6 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	15-25	48	54
Stainless steel plates	120	1.2	15-25	48	0
Stainless steel plates	180	1.2	15-25	48	0
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	50-60	69	13
Stainless steel plates	120	1.2	50-60	69	0
Stainless steel plates	180	1.2	50-60	69	0
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	85-95	57	0
Stainless steel plates	120	1.2	85-95	57	0
Stainless steel plates	180	1.2	85-95	57	0

6.2.3. *Staphylococcus aureus*

6.2.3.1. 0.3 ppm

The effect of 0.3 ppm ozone is shown in *table 6.2.3.1.1*. The effect increases with higher humidity. As there was a low control CFU count on the plates exposed to higher humidity compared with the test with low humidity the figures could be higher or lower. In the *fig.6.2.3.1.1.* a diagram of the effect of ozone on the bacteria is shown.

Table 6.2.3.1.1. The survival rate of Staphylococcus aureus in an ozone concentration of 0.3 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	15-25	94	92
Stainless steel plates	120	0.3	15-25	94	84
Stainless steel plates	180	0.3	15-25	94	65
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	50-60	8	75
Stainless steel plates	120	0.3	50-60	8	63
Stainless steel plates	180	0.3	50-60	8	50
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	85-95	10	0
Stainless steel plates	120	0.3	85-95	10	0
Stainless steel plates	180	0.3	85-95	10	0

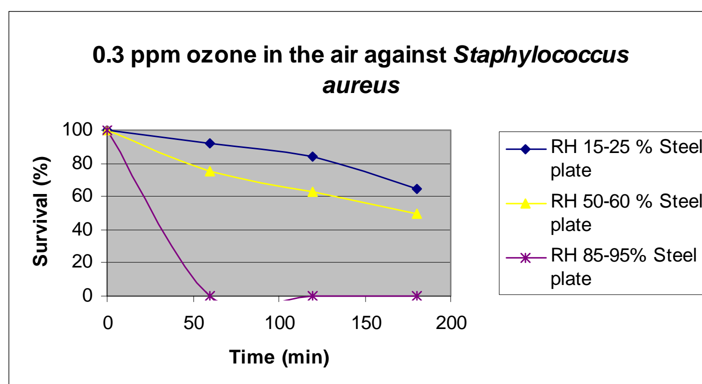


Fig. 6.2.3.1.1. The survival of S.aureus on stainless steel after exposure to 0.3 ppm ozone.

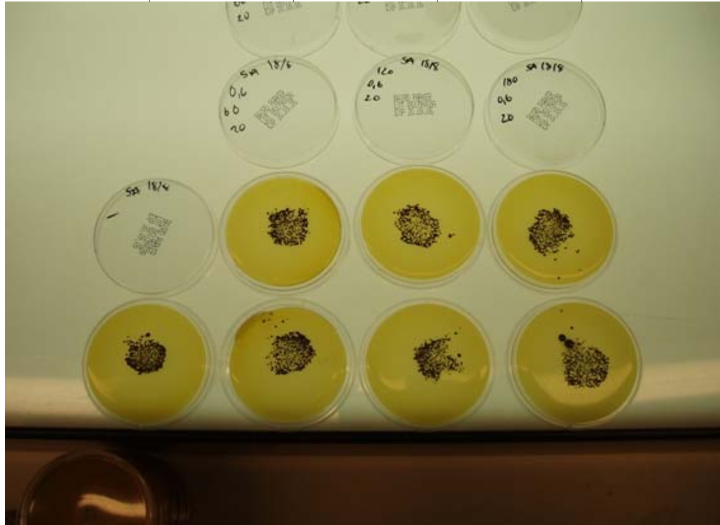
6.2.3.2. 0.6 ppm

In *picture 6.2.3.2.1* it can be seen that 0.6 ppm ozone in 15-25 % humidity has hardly any killing effect but in the picture *6.2.3.2.2.* it can be seen that exposure to 0,6 ppm ozone in 85-95 % humidity kills 100 % of the bacteria after 2 hours exposure.

Table 6.2.3.2.1. The survival rate of Staphylococcus aureus in an ozone concentration of 0.6 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

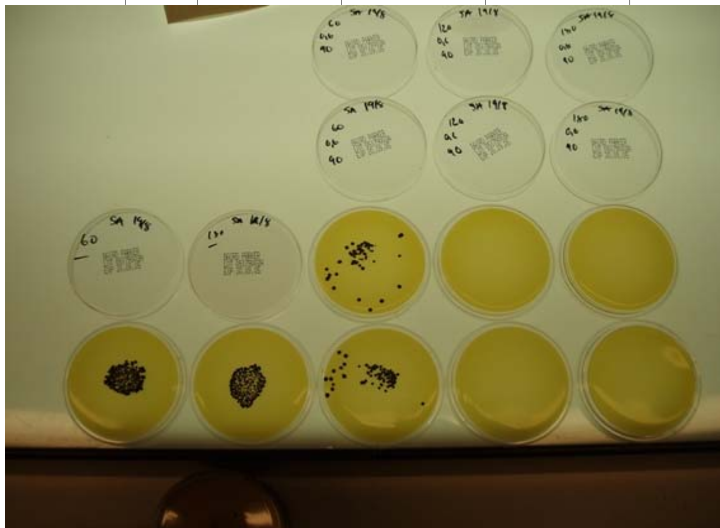
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.6	15-25	n.d.	
Stainless steel plates	120	0.6	15-25	n.d.	*See picture 6.2.3.2.1.*
Stainless steel plates	180	0.6	15-25	n.d.	
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	*Survival after exposure to ozone (%)*
Stainless steel plates	60	0.6	50-60	12	*0*
Stainless steel plates	120	0.6	50-60	12	*0*
Stainless steel plates	180	0.6	50-60	12	*0*
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	*Survival after exposure to ozone (%)*
Stainless steel plates	60	0.6	85-95	n.d.	*See picture 6.2.3.2.2*
Stainless steel plates	120	0.6	85-95	n.d.	*0*
Stainless steel plates	180	0.6	85-95	n.d.	*0*
Agar plates	60	0.6	85-95	n.d.	*See picture 6.2.3.2.3*
Agar plates	180	0.6	85-95	n.d.	

0h exposure | 1h exposure | 2h exposure | 3h exposure



Picture 6.2.3.2.1. The effect 0.6 ppm ozone in air with a humidity of 15-25% has on Staphylococcus aureus on a stainless steel plate. The plate furthest to the left has not been exposed to ozone. The plates on the second row from the left have been exposed to ozone during 1 hour. In the third row from the left the plates are after 2 hours of exposure to ozone and the plates on the last row are after 3 hours of ozone exposure.

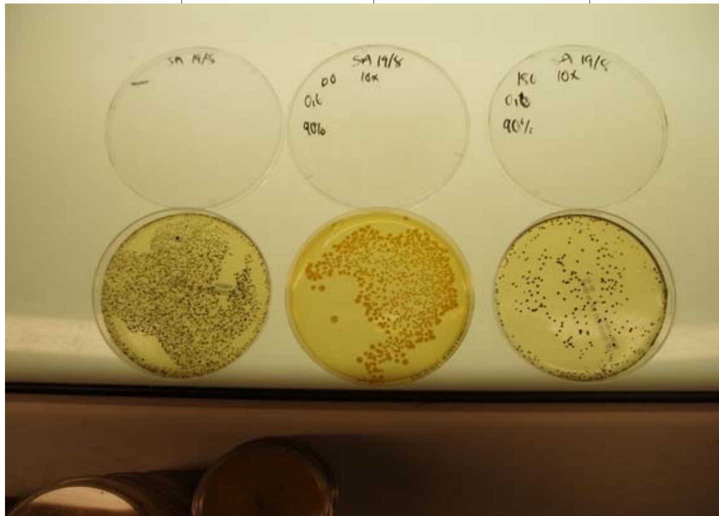
0h exposure | 1h exposure | 2h exposure | 3h exposure



Picture 6.2.3.2.2. The effect 0.6 ppm ozone in air with a humidity of 85-95% has on Staphylococcus aureus on a stainless steel plate. The two plates furthest to the left have not been exposed to ozone. The plates on the third row from the left have been exposed to ozone during 1 hour. In the fourth row from the left the plates are after 2 hours of exposure to ozone and the plates on the last row are after 3 hours of ozone exposure.

In the *picture 6.2.3.2.3.* the effect of ozone treatment of air with high humidity has on *Staphylococcus aureus* on agar plates can be seen.

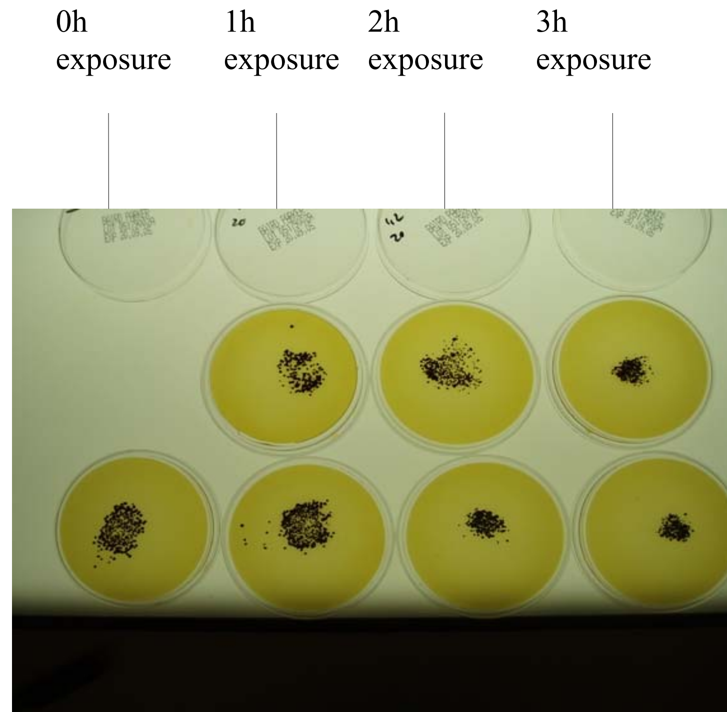
0h exposure 1h exposure 3h exposure



Picture 6.2.3.2.3. The effect 0.6 ppm ozone in air with a humidity of 85-95% has on Staphylococcus aureus on an agar plate. The petri dish furthest to the left has not been exposed to ozone, the plate in the middle has been exposed to ozone for 60 minutes and the plate to the right has been exposed for 3 hours.

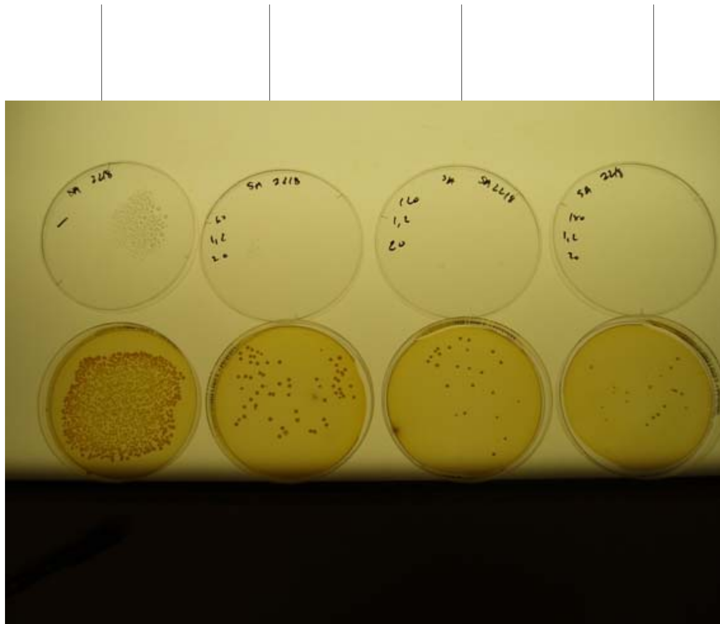
6.2.3.3. 1.2 ppm

In the *picture 6.2.3.3.1.* it can be seen that an ozone concentration of 1.2 ppm in dry air is much less efficient compared to 0.6 ppm in high humidity against this bacteria. The *picture 6.2.3.3.2.* shows that ozone in dry air is more effective against *Staphylococcus aureus* on agar than on a steel plate.



Picture 6.2.3.3.1. The effect 1.2 ppm ozone in air with a humidity of 15-25% has on Staphylococcus aureus on a stainless steel plate. The plate furthest to the left has not been exposed to ozone. The plates on the second row from the left have been exposed to ozone during 1 hour. In the third row from the left the plates are after 2 hours of exposure to ozone and the plates on the last row are after 3 hours of ozone exposure.

0h exposure 1h exposure 2h exposure 3h exposure



Picture 6.2.3.3.2. The effect 1.2 ppm ozone in air with a humidity of 15-25% has on Staphylococcus aureus on an agar plate. The petri dish furthest to the left has not been exposed to ozone. The second plate from the left has been exposed to ozone for 1 hour, the third for 2 hours and the last one has been exposed for 3 hours to ozone.

6.2.4. Unknown bacteria

An unknown bacteria was collected from the surrounding environment air or surface.

6.2.4.1. 0.3 ppm

In the experiment with ozonated air against an unknown bacteria-strain the effect of ozone in combination with high humidity is good, even at a low concentration of 0.3 ppm. The effect of ozone against the bacteria on a stainless steel plate can be seen in *fig 6.2.4.1.1.* and the result of ozone against the bacteria in agar in *picture 6.2.4.1.1.*

Table 6.2.4.1.1. The survival rate of an unknown bacteria in an ozone concentration of 0.3 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	15-25	27	100
Stainless steel plates	120	0.3	15-25	27	100
Stainless steel plates	180	0.3	15-25	27	100
Agar plates	60	0.3	15-25	153	100
Agar plates	120	0.3	15-25	153	100
Agar plates	180	0.3	15-25	153	100
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	50-60	42	100
Stainless steel plates	120	0.3	50-60	42	98
Stainless steel plates	180	0.3	50-60	42	86
Agar plates	60	0.3	50-60	127	100
Agar plates	120	0.3	50-60	127	95
Agar plates	180	0.3	50-60	127	84
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.3	85-95	32	50
Stainless steel plates	120	0.3	85-95	32	0
Stainless steel plates	180	0.3	85-95	32	0
Agar plates	60	0.3	85-95		
Agar plates	120	0.3	85-95		*see picture*
Agar plates	180	0.3	85-95		*6.2.3.1.1.*

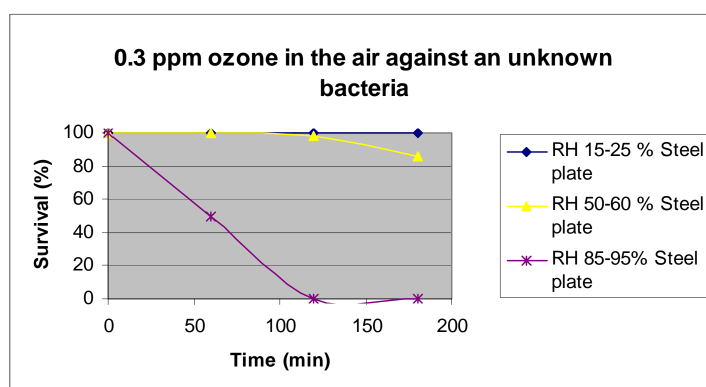
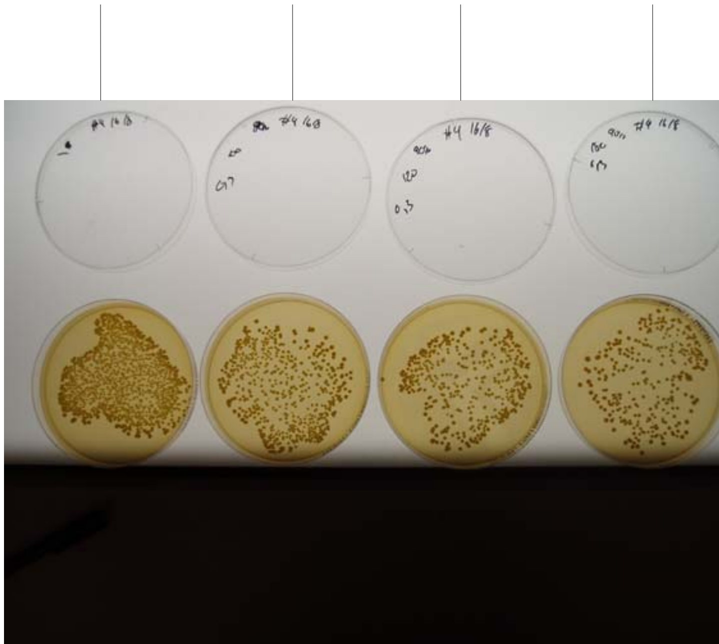


Figure 6.2.4.1.1. The survival of an unknown bacteria on stainless steel after exposure of 0.3 ppm ozone

0h exposure 1h exposure 2h exposure 3h exposure



Picture 6.2.4.1.1. The effect 0.3 ppm ozone in air with a humidity of 85-95% has on the unknown bacteria on an agar plate. The petri dish furthest to the left has not been exposed to ozone. The second plate from the left has been exposed to ozone for 1 hour, the third for 2 hours and the last one has been exposed for 3 hours to ozone.

6.2.4.2. 0.6 ppm

The effect of ozone in air with a high humidity increases and can be seen in *table 6.2.4.2.1*. After 2 hours in a humidity of 85-95 % there is no survival of the bacteria on the stainless steel plate, *see fig 6.2.4.2.1.* The effect on bacteria on a 90 mm agar plate can be seen in the *picture 6.2.4.2.1.*

Table 6.2.4.2.1. The survival rate of an unknown bacteria in an ozone concentration of 0.6 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.6	15-25	24	100
Stainless steel plates	120	0.6	15-25	24	*100*
Stainless steel plates	180	0.6	15-25	24	96
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.6	50-60	36	19
Stainless steel plates	120	0.6	50-60	36	22
Stainless steel plates	180	0.6	50-60	36	28
Agar plates	60	0.6	50-60		
Agar plates	120	0.6	50-60		*see picture*
Agar plates	180	0.6	50-60		*6.2.4.2.1.*
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	0.6	85-95	15	20
Stainless steel plates	120	0.6	85-95	15	0
Stainless steel plates	180	0.6	85-95	15	0
Agar plates	60	0.6	85-95	1120	44
Agar plates	120	0.6	85-95	1120	19
Agar plates	180	0.6	85-95	1120	17

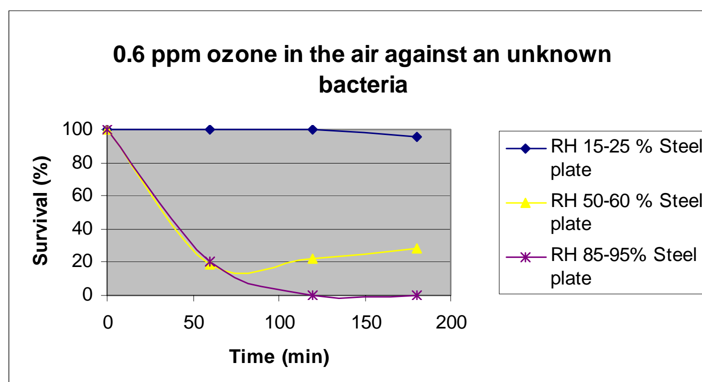
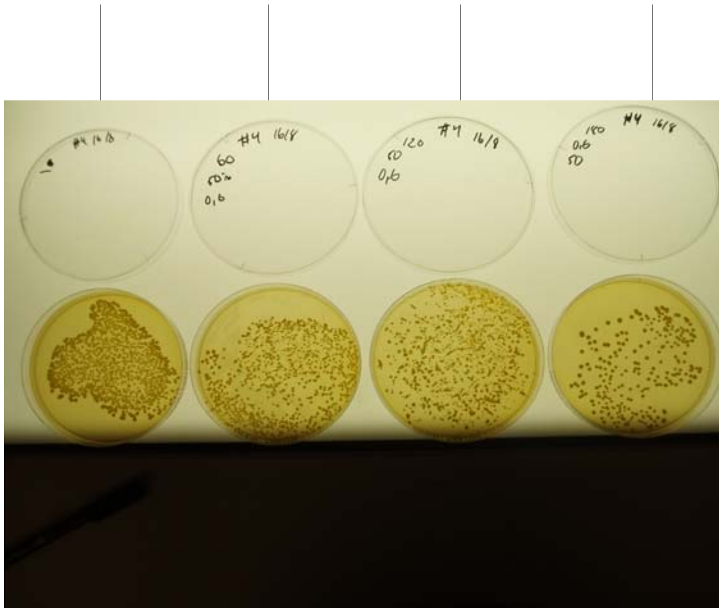


Figure 6.2.4.2.1. The survival of an unknown bacteria on stainless steel after exposure to 0.6 ppm ozone

0h exposure 1h exposure 2h exposure 3h exposure



Picture 6.2.4.2.1. The effect 0.6 ppm ozone in air with a humidity of 50-60% has on the unknown bacteria on an agar plate. The petri dish furthest to the left has not been exposed to ozone. The second plate from the left has been exposed to ozone for 1 hour, the third for 2 hours and the last one has been exposed for 3 hours to ozone.

6.2.4.3. 1.2 ppm

The increasing effect of ozone in high humidity can be seen in *table 6.2.4.3.1*. The results can also be seen in *fig.6.2.4.3.1.* and *fig.6.2.4.3.2.*

Table 6.2.4.3.1. The survival rate of an unknown bacteria in an ozone concentration of 1.2 ppm. The control CFU is the lowest number of microorganisms that could form a colony (CFU) counted on a plate not exposed to ozone. The survival after exposure shows the highest percentage of surviving microorganisms that could form a colony (CFU) on a plate exposed to ozone compared with the plate not exposed to ozone.

Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	15-25	16	94
Stainless steel plates	120	1.2	15-25	16	*75*
Stainless steel plates	180	1.2	15-25	16	81
Agar plates	60	1.2	15-25	77	42
Agar plates	120	1.2	15-25	77	*29*
Agar plates	180	1.2	15-25	77	8
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	50-60	95	1
Stainless steel plates	120	1.2	50-60	95	1
Stainless steel plates	180	1.2	50-60	95	3
Agar plates	60	1.2	50-60	132	3
Agar plates	120	1.2	50-60	132	0
Agar plates	180	1.2	50-60	132	0
Type	Exposure time (min)	Conc.ozone (ppm)	RH (%)	Control CFU	Survival after exposure to ozone (%)
Stainless steel plates	60	1.2	85-95	23	4
Stainless steel plates	120	1.2	85-95	23	0
Stainless steel plates	180	1.2	85-95	23	0
Agar plates	60	1.2	85-95	325	9
Agar plates	120	1.2	85-95	325	*2*
Agar plates	180	1.2	85-95	325	0

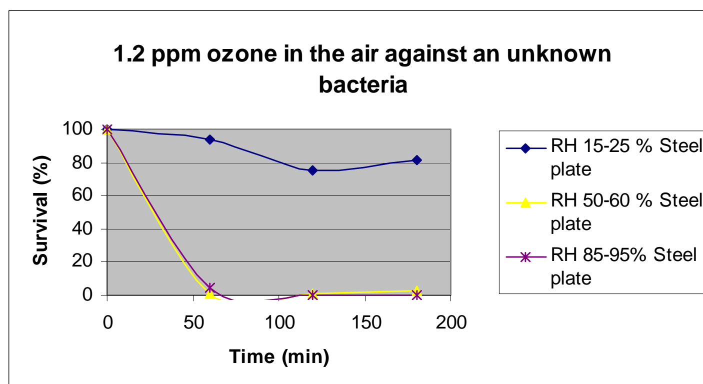


Figure 6.2.4.3.1. The survival of an unknown bacteria on stainless steel after exposure to 1.2 ppm ozone

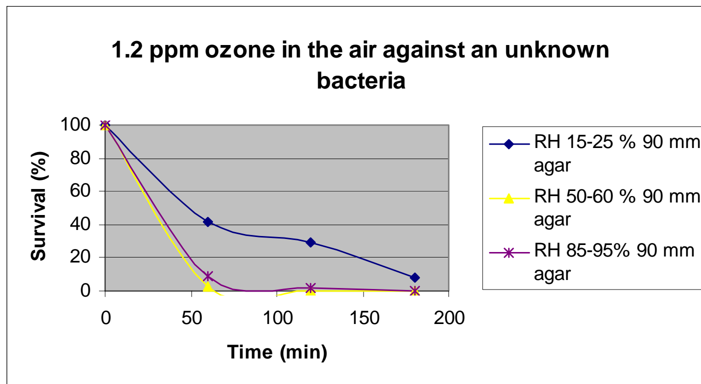


Figure 6.2.4.3.2. The survival of an unknown bacteria on an agar plate after exposure to 1.2 ppm ozone

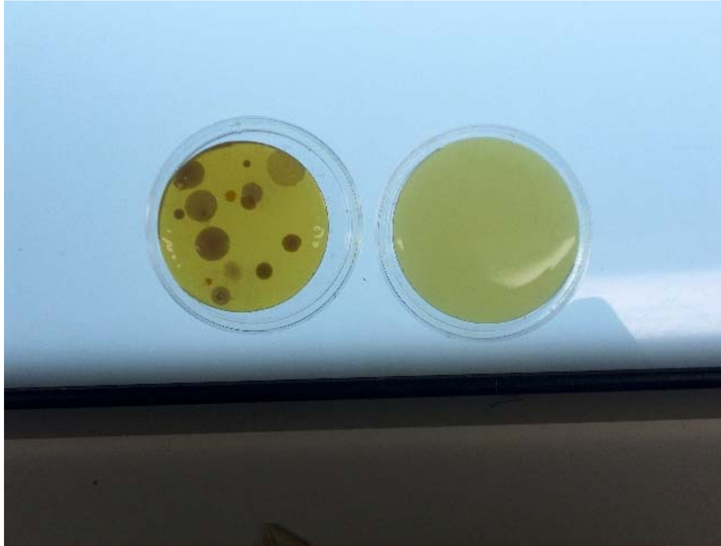
6.3. Ozone treatment of the air in a cleanroom chamber filled with raw rotten meat

6.3.1. Raw meat

Table 6.3.1.1. The effect of ozone as a sanitizing agent in a cleanroom chamber containing 30 kg of rotten meat

Ozone conc. (ppm)	Time (hours)	Particles 0.5 µm and larger	Particles 5.0 µm and larger	Microbial control of the air (CFU)	Flies and larvae
0	-	22456	4	16	Only flies
0.3	4	67032	4	15	Flies and larvae
0	24	34768	6	-	Flies and larvae
2.4	24	247549	3	-	Only larvae
30	24	958695	2	0	No life

During the experiment with the raw meat many difficulties appeared. The ozone generator broke down twice and it ended with a rapid change of the experiment. As can be seen in *table 6.3.1.1.,* the effect of 0.3 ppm ozone during 4 hours did not make any difference on the amount of bigger particles and living microorganisms in the air. But a slightly higher amount of small particles could be because the ozone may have an effect on breaking down particles into smaller ones. At this concentration no significant effect could be seen on the flies and the larvae in the chamber. After being exposed for an ozone concentration of 2.4 ppm during 24 hours an increase in small particles could be observed and that all the flies were dead but almost all larvae were still alive. The reason for not making any microbial tests was the unpleasant feeling of entering the chamber with the microbial air sampler. 24 hours later after being exposed to 30 ppm ozone the particle amount increased even more and all the larvae were also dead. The amount of microorganisms that could form a colony on agar in the air was also gone, *see picture* 6.3.1.1, and the terrible smell before the sanitation was heavily reduced.



Picture 6.3.1.1. The difference in the amount of living microorganisms in the air between the one on the left side which has not been exposed to ozone and the one to the right which has been exposed to 30 ppm ozone during 24 hours.

6.3.2. The decrease of the ozone concentration during different conditions

In *fig. 6.3.2.1.* the difference in the decrease of the ozone concentrations can be seen.

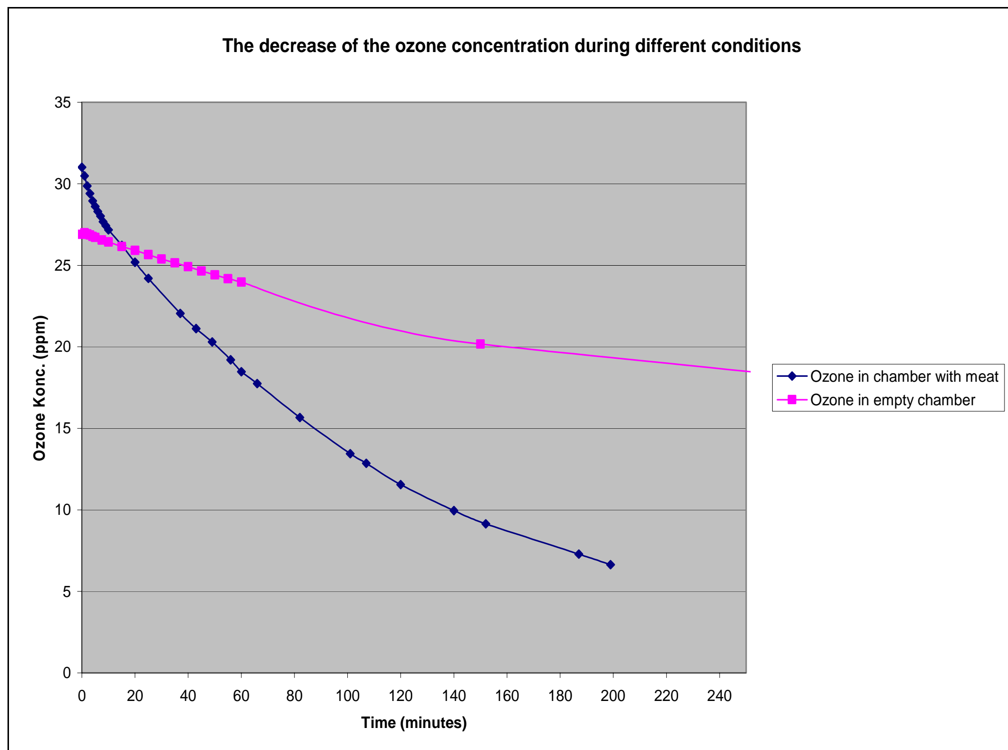


Figure 6.3.2.1. The decrease of the ozone concentration in an empty cleanroom chamber compared with when the chamber contains 30 kg of raw meat.

6.4. The effect of a high concentration of ozone in air against microorganisms during a shorter time exposure

The effect of 5000 ppm ozone can be seen in *table 6.4.1.*

Table 6.4.1. The killing effect of a high ozone concentration during shorter exposure times against different kind of microorganisms

Ozone conc. (ppm)	Exposure time (minutes)	Microorganism (name)	Control (CFU)	Survival (%)
5000	0.33	*Aspergillus niger*	$3*10^4$	Not possible to count
5000	5		$3*10^4$	0.27
5000	20		$3*10^4$	0.19
5000	0.33	*Pseudomonas aeruginosa*	$1*10^6$	0.004
5000	5		$1*10^6$	0.0004
5000	20		$1*10^6$	0
5000	0.33	Mix of microorganisms	$5*10^5$	Not possible to count
5000	5		$5*10^5$	0.0018
5000	20		$5*10^5$	0.0016

7. Discussion and conclusion

The purpose of this examination project work was to investigate the potential of using airborne ozone to kill microorganisms. In this study different aspects of the use of ozone in air, such as concentration, humidity and time has been considered. The results reached during these experiments shows that ozone may work as a sanitizing agent for disinfection and maybe even for sterilization, although a higher ozone concentration was needed to get a major killing effect on *Aspergillus niger*. At a very low concentration of ozone such as 0.1 ppm the effect of eliminating microorganisms was almost none but it must be taken into consideration that this was performed with dry compressor air. During the experiments the very important role of the humidity on the effect that ozone has on microorganisms has been showed and particularly against different bacteria. Already at a low concentration such as 0.3 ppm ozone in air with a humidity of 85-95 % the effect was noticeable within 2-3 hours, where a total inhibition of the microbial growth in some cases was achieved. The study has shown that a lower concentration of ozone with a higher humidity can be more effective than a higher concentration of ozone in dry air at killing the bacteria in this study. The assumption that ozone had a different killing effect when the bacteria were poured on agar than on stainless steel plates were in some cases confirmed. The increasing killing effect that could in some cases be observed with bacteria on steel plates with increasing humidity could not be observed in the same way with bacteria on agar medium. This may suggest that the agar medium which consists of water increases the killing effect of ozone at a lower humidity and the increase of the killing effect high humidity is smaller than on a stainless steel plate. Other reasons for the difference in the killing effect could be that ozone also might affect the agar medium. This could also be the reason why there in some cases in this study has been a higher survival rate of the bacteria poured on agar medium at a higher humidity. An experiment to see the effect of 5000 ppm ozone has on a high concentration of microorganism in a short time was also performed, and the killing effect was high. In just 5 minutes almost all of the tested microorganisms including the very tolerant *Aspergillus niger* were killed. The experiment performed to see how well ozone at a lower concentration could be used for eliminating airborne microorganisms in a chamber full of rotten meat failed due to problems with the equipment. The conclusion of this was that the generator and more exactly the ceramic plate package could not handle the amount of organic particles in the air of the chamber. A HEPA-filter should have been installed before the fan. But then the flow rate maybe would have been to low for the generator to handle. But on the other hand it showed that ozone works very well as a sanitizing agent. During 48 hours with a concentration of 2.4 ppm under the first 24 hours and 30 ppm during the final 24 hours a total elimination of the microorganisms in the air in the cleanroom chamber could be achieved despite the heavy contamination of 30 kg rotten meat. Not only the microorganisms got affected, but also the flies that died within the first 24 hours and the durable larvae within 48 hours. The difference in the decrease of a high concentration of ozone in an empty cleanroom chamber with a chamber containing 30 kg of rotten meat was also investigated and a big difference could be seen. The overall conclusion of this study is that it has been showed that ozone works well as a decontaminating agent at a low concentration against the bacterias used in this study, but a higher concentration was needed for the killing of the tolerant *Aspergillus niger*. Much more research is needed to do be done in the area of ozone treated air for killing and eliminating microorganisms.

8. Suggestions for future research

- It needs to be determined at exactly what concentration of ozone and exposure time and in what humidity of the air it eliminates all microorganisms and spores.
- Research must be conducted to determine why a high humidity increases the efficiency of ozone. Is it because ozone reacts with water and other reactive substances are created or because water in it self helps ozone to oxidize the microorganism?
- Some microorganisms are airborne and therefore there should be research performed on airborne ozone against airborne microorganisms.
- Investigation of how ozone works at a low concentration for a long exposure time. The effect the ozone has on the food it self is also an important part to consider.
- Research on which effects ozone in different concentration and exposure time has on animals and on humans. This is an important part to investigate due to the fact that the research that has been conducted in the past are of the most part inconclusive and can not give any concrete answers to what levels of ozone is dangerous for humans to be in. [19]
- Tests to determine what effect the ozone has on different kind of materials both under shorter and longer exposure times. It is of most importance to know what materials can be used in industries, ventilation systems, in buildings, for food storage and other indoor facilities that requires or desires a decrease of microorganisms.

References

1. Matts Ramstorp: Renhetsteknik och rena rum – en introduktion, Kristianstads Boktryckeri AB, 2001
2. W.Kowalski, W.Bahnfleth: Airborne respiratory diseases and mechanical systems for control of microbes, The Pennsylvania State University, Department of Architectural Engineering, University Park, 2005
3. C.B.Beggs: The Airborne Transmission of Infection in Hospital Buildings: Fact or Fiction?, Indoor and Built Environment, Vol. 12, No. 1-2, 9-18, 2003
4. Airborne transmission worse than thought - *Call to sanitize air in airplanes, schools, and hospitals,* Harvard University Gazette, 2004
5. Source of cold infections, The Pennsylvania State University, Department of Architectural Engineering, University Park, 2005
6. Epidemiology for engineers, The Pennsylvania State University, Department of Architectural Engineering, University Park, 2005
7. Centers for Disease Control and Prevention Healthcare Infection Control Practices Advisory Committee (HICPAC): Draft Guideline for Environmental Infection Control in Healthcare Facilities, 2001
8. M.Horváth, L.Bilitzky, J.Hüttner: Ozone. Oxygen and Dissousgas Company, Elsevier & Akademiai Kiado, 1985
9. Peter Göransson, Håkan Svensson: Applikationer för ozon. Examensarbete på Lunds Tekniska Högskola, 1998
10. L.Prescott, J.Harley, D.Klein: Prescott's Microbiology 4th Edition. 1999
11. M.T.Madigan, J.M.Martinko, J.Parker: Brock Biology of Microorganisms Int. Edition. Pearson Education, Inc. 2003
12. Anna Blücher: Metoder för sterilisering/kontroll av bakterienivå på ytor. Litteraturstudie för Ozone Tech Systems OTS AB, 2004
13. W.Kowalski, W.Bahnfleth, T.Whitam: Bactericidal Effects of High Airborne Ozone Concentrations on *Escherichia coli* and *Staphylococcus aureus*. Ozone Science and Engineering 20:205-221, 1998
14. E.L.Karlson: Ozone sterilization. J-Health-Mater-Manage, 7(5):43-5, 1989
15. W.Kowalski, W.Bahnfleth, T.Whitam, B.Striebig: Demonstration of Hermetic Airborne Ozone Disinfection System: Studies on E.coli. AIHA Journal 64:222-227, 2003
16. J.Kim, A.Yousef: Inactivation Kinetics of Foodborne Spoilage and Pathogenic Bacteria by Ozone. Journal of Food Science 65(3): 521-528, 2000
17. J.Kim, A.Yousef, A.Khadre: Ozone and its Current and Future Application in the Food Industry. Advances in Food and Nutrition Research, 2002
18. K.Ishizaki, N.Shinriki, H.Matsuyama: Inactivation of *Bacillus spores* by gaseous ozone. J. Appl. Bact. 60:67-72, 1986
19. B.Grenqvis-Nordén: 70. Ozone. Nordiska Expertgruppen För Gränsvärdesdokumentation, Arbete och Hälsa 1986:28, Helsingfors, 1998
20. Marianne Walding, Ozonhalter vid aktiv hantering, Rapport 2000:1, Arbetsskyddsstyrelsen, The National Board of Occupational Safety and Health and the Labour Inspectorate
21. http://www.ozoneapplications.com/info/ozone_compatible_materials.htm, 2005-10-05
22. Ozone Technology AB, Report and Recommendations on the application of gaseous ozone for sanitization of ambient air and for surface sterilization, including transcripts of original research undertaken by TNO Nutrition & Food Research Institute for the benefit of Van Gool Mileutechniek and Ozone Technology AB, Ozone Technology Systems AB, Malmö, Sweden

23. L.Edebo, H. Hallander, B.Petrini: Svampinfektioner, Infektionsdiagnostik, Referensmetodik för laboratoriediagnostik vid kliniskt mikrobiologiska laboratorier, Smittskyddsinstitutet, 2005-10-05
24. A.A.Salyers, D.D.Whitt: Bacterial Pathogenesis, A molecular Approach, 2nd Edition, ASM Press, American Society for Microbiology, 2002
25. *Staphylococcus aureus*, Foodborne Pathogenic Microorganisms and Natural Toxins Handbook, Center for Food Safety & Applied Nutrition, U.S. Food & Drug Administration, 2005-10-05
26. http://www.smittskyddsinstitutet.se, 2005-10-05