
Cardiff School of Health Sciences

# The Effect of Ozone and Open Air Factor on Environmental Microbial Isolates of Significance in the Food Industry.

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For the degree of Doctor of Philosophy

To my devoted husband, Mike and supportive parents.

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# PUBLICATIONS

## Paper

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## Talk

Brown, R. The Effect of Gaseous Ozone on the Survival of Surface Attached Environmental *Listeria monocytogenes serotype 1/2a.* Presented at IAFP conference, Gaylord Resort and Hotel, Grapevine, Texas, U.S.A. on 13th July 2009. (Appendix, Abstract, page 303)

## Poster presentations

Brown, R., Fielding, L., Griffith, C., and Tatham, A. 2008. The Effect of Gaseous Ozone on the Survival of Surface Attached Bacteria of Importance in the Food Industry. Presented by myself at Food Micro 2008 conference, Aberdeen Exhibition and Conference Centre, Aberdeen, UK. 1st-4th September, 2008. (Appendix, page 304)

# FIELDS OF STUDY

Food Microbiology, Food Science and Nutrition

# ABBREVIATIONS

- AcEW — Acidic Electrolysed Water
- ACH — Air Changes per Hour
- AD — Air Disinfection
- AFM — Atomic Force Microscopy
- AGI — All Glass Impingers
- AIEW — Alkaline Electrolysed Water
- AISI — American Iron and Steel Institute
- ANOVA — Analysis of Variance
- API — Advanced Pollution Instrument
- ATCC — American Type Culture Collection
- BC — Benzalkonium chloride
- BOD — Biological oxygen demand
- CAMR — Centre for Applied Microbiology and Research
- CCFRA — Campden and Chorleywood Food Research Association
- CFU — Colony Forming Units
- COD — Chemical oxygen demand
- CV — Crystal violet
- DPD — Diethyl-p-Phenylene Diamine
- ETU — Ethylenethiourea
- EO — Electrolysed Oxidising
- EPS — Extracellular polymeric substances
- ER — Electrolysed Reducing
- FA — Fatty acids
- FDA — Food, Drug and Administration

- FSIS — Food Safety and Inspection Service
- GMP — Good Manufacturing Practices
- HACCP — Hazard Analysis Critical Control Point system
- HEPA — High Efficiency Particulate Air
- IPA — Isopropyl Alcohol
- LLO — Listeriolysin O
- LPS — Lipopolysaccharide
- MAP — Modified Atmosphere Packaging
- MBC — Minimum bactericidal concentration
- MEL — Maximum exposure limits
- MIC — Minimum inhibitory concentration
- MNLR — Mean net log reduction (values)
- MRD — Maximum Recovery Diluent
- NB — Nutrient Broth
- NCIMB — National Collection of Industrial and Marine Bacteria
- NCTC — National Collection of Type Culture
- OAF — Open Air Factor
- OD — Optical Density
- OEL — Occupational Exposure Limit
- OES — Occupational Exposure Standard
- ORP — Oxidation Reduction Potential
- PAA — Peracetic acid
- PBS — Phosphate Buffered Saline
- PCTFE — Polychlorortrifluoroethylene
- PDFE — Polydichlorodifluoroethylene

- PID Photoisonisation Detector
- PLC Programme Logic Controller
- PP Polypropylene
- PPB Parts per billion
- PPM Parts per million
- PPO Polyphenol oxidases
- PTFE Polytetrafluoroethylene
- PVC Polyvinylchloride
- PVDF Polyvinylidenefluoride
- QAC Quaternary ammonium compounds
- RH Relative Humidity
- RPM Revolutions Per Minute
- SD standard deviation
- s.e.m standard error of the mean
- SEM Scanning electron microscopy
- SOD Superoxide Dismutase
- SPM Scanning probe microscopies
- SS Stainless steel
- STM Scanning tunnelling microscope
- THM Trihalomethanes
- TSA Tryptone Soya Agar
- TSB Tryptone Soya Broth
- TSC Technology Scientific Consultants
- USDA United States' Department of Agriculture
- US EPA United States' Environmental Protection Agency
- VOC Volatile Organic Compounds

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# ABSTRACT

Ozone and open air factor (OAF) have been reported in the research literature as being effective anti-microbial agents. The action of ozone on microbes is relatively well understood, but the action of OAF is not well characterised. Both ozone and OAF have relatively short half-lives and do not leave behind toxic residues. The advantages of using a gas instead of aerosols, or droplets produced by fogging to decontaminate surfaces, is that a gas will come into contact with the horizontal, vertical and inverted planes of the surfaces (by diffusion), whereas aerosols or droplets will be affected by gravity.

The effect of gaseous ozone, OAF and ozonated water with or without d-limonene emulsified in alcohol on surface attached and biofilm environmental *L. monocytogenes amd P. aeruginosa* were investigated. The interaction of each treatment with microorganisms was elucidated by determining microbial survival on different food contact surfaces, detecting cell injury by examining treated environmental *L. monocytogenes* cells using scanning electron and atomic force microscopy. All treatments were significantly more effective in eliminating the gram negative than the gram positive bacteria. This may be due to differences in cell wall structure and the cell's ability to produce extracellular polymeric substances. Scanning electron microscopy revealed that gaseous ozone caused the cells to bleb out their cellular contents, whereas for OAF treated cells, holes were apparent in the cell wall. The ozonated water treatments were more effective in stripping the biofilm away from the surface substrata. The atomic force microscope showed that OAF, ozonated water and terpene, and the terpene in water treatments caused visible cell surface property changes, compared to gaseous ozone and ozonated water treatment alone.

# Chapter 1. Introduction

## 1.1 Ozone

Ozone was first discovered by Christian Schönbein (1799–1868) in 1840 (Rubin, 2001; Kirschner, 2005). The German scientist determined that odour produced during sparking experiments was caused by a compound, which he called 'ozone'. 'Ozone' comes from the Greek word meaning 'to smell'. Schönbein noticed that it was the same smell that was produced after a lightning storm (Rubin, 2001). Ozone has long been used as an antimicrobial agent and has been used to disinfect drinking water since 1893 (Rice *et al*., 1981).

Ozone is a tri-atomic ($O_3$) allotrope of oxygen, having different chemical and toxicological properties to molecular oxygen (Anon, 2009 [online]; Grimes *et al*., 1983). Ozone is generated by an increase in the energy of oxygen molecules which causes them to dissociate into free oxygen atoms. These free oxygen atoms loosely attach to more unchanged bi-atomic oxygen molecules, thus creating unstable tri-atomic oxygen molecules known as ozone (Anon, 2005, [online]). The electronic structure of ozone resonates between two electronic states (Fig. 1.1) which accounts for its strong electrophilic nature (Kirschner, 2005).

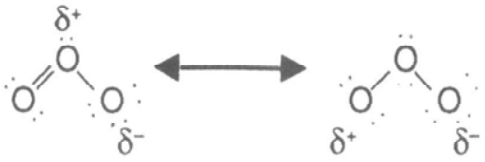


**Figure 1.1.** The resonant structure of an ozone molecule (Adapted from Langlais *et al*., 1991, cited in Kirschner, 2005).

Ozone's chemistry is based on its ability to attack double carbon bonds (ozonolysis). Ozone (CAS No. [100828-15-6]), has a relative molecular mass of 48. Pure ozone is a pale blue, toxic, water-soluble gas with a characteristic odour, described as "the smell after a lightning storm" (as it is produced by electrical discharges). It can condense to a dark bluish liquid at -112˚C and freezes at -193˚C (Rice *et al*., 1981; Shakhashiri, 2007 [online]). Scott and Lesher (1963) reported that ozone at concentrations of 0.02 to 0.04 ppm can be detected by humans as a sweet pleasant odour. Ozone exists in a gaseous state at room and refrigeration temperatures and has partial solubility in water (Adams, 1946). It is not flammable and has an oxidation-reduction potential of 2.07 volts (V), which makes it the strongest oxidant available for food applications, compared to hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl) and chlorine which have oxidation-reduction potentials of 1.78V, 1.49V and 1.36V, respectively (Gurley, 1985). The density of ozone gas is 2.144$gL^{-1}$ at 0˚C and 101.3 kPa, which makes it heavier than air (1.28$gL^{-1}$) under the same conditions and has a low vapour pressure. Ozone tends to sink to the floor and is not dispersed unless there is sufficient air circulation (Anon, 2009 [online]). Ozone has a high reactivity and penetrability, and is more effective in more humid environments (Foarde *et al*., 1997).

Ozone can be produced by photochemical reactions involving oxygen, oxides of nitrogen and hydrocarbons. Ozone is formed when a mixture of nitrogen dioxide ($NO_2$) and oxygen are exposed to bright light. Usually nitrogen and oxygen do not react together under normal temperatures. However, in hot reacting gases found in combustion engines, nitrogen and oxygen react with the heat to produce 2

molecules of nitric oxide (NO). Nitric oxide reacts spontaneously with oxygen in the air to form $NO_2$. $NO_2$ (red/brown gas) dissociates when it is irradiated with a bright light to give off NO and single oxygen atom. Oxygen atoms are extremely reactive and readily attach to oxygen to form ozone (Fig. 1.1) (Shakhashiri, 2007 [online]). Fig. 1.2 shows the photochemical reactions involving nitrogen in the formation of ozone. In 1970, it was shown that not only are nitrogen oxides involved in the formation of ozone but also in its decomposition (Kirschner, 2005).

$$N_2 + O_2 \longrightarrow 2NO$$

$$2NO + O_2 \longrightarrow 2NO_2$$

$$NO_2 \longrightarrow NO + O$$

$$O + O_2 \longrightarrow O_3$$

**Figure 1.2.** Photochemical Reactions: Formation of Ozone (Adapted from Shakhashiri, 2007 [online]).

Ozone can be produced by photocopying machines, laser printers, electrostatic air filters, and other electrical devices and these are the main sources of ozone emissions in indoor environments (Valuntaitė and Girgždienė, 2007).

### 1.1.1 Ozone solubility

The application of aqueous ozone as.a disinfectant is dependent on it's solubility in water. This is because the gas needs to be dissolved in the water to have an oxidative effect. In order to obtain maximum oxidative effect, optimal conditions need to be maintained to create the maximum transfer across the gas-water interface. This can be applied using a fine porous diffuser to create fine bubbles of ozone gas within a column of water, injecting ozone into water using jets, or violently mixing ozone with water in emulsion turbines (Graham, 1997b). Sonication can also be employed to create extremely fine bubbles, thus increasing

solubility in water by increasing the surface area (Burleson *et al*., 1975). Aqueous ozone remains a strong oxidising agent, compared to other currently used biocides and is three to four times more effective than chlorine (Greene *et al*., 1993; Smilanick, 2003). However, as shown in Table 1, its solubility is relatively low compared to many commercial sanitizers (Smilanick, 2003). Ozone is typically applied as a pre-disinfectant for the control of algae and the inactivation of bacteria and viruses in water by direct filtration processes. Ozone can be applied as a pre- and/or intermediate oxidant for the elimination of inorganic and organic matter, such as metal ions, levels of trihalomethanes (THM) and related organic precursors from water (Gottschalk *et al*., 2000; Franken, 2005), in order to eliminate taste, and odour and to reduce the turbidity of the water.

Ozone is soluble in many substances, forming stable or metastable solutions (Anon, 2005 [online]). Aqueous ozone is partially soluble in water, being about 12 times more soluble in water than oxygen (Graham, 1997b). Dissolved ozone has a low partial pressure and so it is difficult to obtain a high concentration of ozone in solution (for example, 0.00003 g of ozone per 100 ml of water can be dissolved at 20˚C). The solubility of ozone is influenced by several physical factors, including the presence of sensitizing impurities such as heavy metal cations or metal oxides, temperature and pressure (Kirschner, 2005). According to Henry's Law, ozone solubility in a liquid "is the amount of gas in solution at a given temperature, which is linearly proportional to the partial pressure of the gas" (Khadre *et al*., 2001). The most important parameter affecting the solubility of ozone is temperature. From Table 1, a 1.5% (by weight) ozone feed gas gives a maximum concentration of 6.43 ppm (mg/L) ozone concentration in water at a temperature of 20°C.

**Table 1.1.** The solubility of ozone in water at different temperatures (ppm). (Anon, 2009, [online]).

| $O_3$ Gas | 5°C | 10°C | 15°C | 20°C |
|---|---|---|---|---|
| **1.5%** | 11.09 | 9.75 | 8.40 | 6.43 |
| **2%** | 14.79 | 13.00 | 11.19 | 8.57 |
| **3%** | 22.18 | 19.50 | 16.79 | 12.86 |

As the water temperature decreases, the solubility of ozone increases (Graham, 1997b), but high pH values can also affect the solubility of ozone. When the pH of the solution increases, the rate of decomposition of molecular ozone into hydroxyl radicals increases (Glaze, 1986; Graham, 1997b).

### 1.1.2 Ozone stability

The stability of dissolved ozone is measured as 'half-life' and the half-life of ozone decreases as the pH of the medium and temperature increases (Graham, 1997a). Table 2.1 shows the half-life of gaseous ozone versus aqueous ozone at different temperatures. Ozone has a relatively long half-life in air at room temperature, approximately 12 hours in a closed room (Graham, 1997b). Its half-life increases with lower temperatures and lower humidity. In pure water at pH 7–8, ozone has a half-life between 20 and 30 minutes (Anon, 2009 [online]) (Table 1.2) depending on the temperature. This depends, however, entirely on the amount of ozone-demanding material in the water being ozonised. Table 1.2 shows the half-life of ozone in its two phases when compared to different temperature conditions.

**Table 1.2.** Typical half-life of gaseous and aqueous ozone when compared to different temperature conditions (Anon, 2009a [online]).

| Gaseous Ozone | | Aqueous Ozone | |
|---|---|---|---|
| **Temp (˚C)** | **Half-life (time)** | **Temp (°C)** | **Half-life (minutes)** |
| -50 | 3 months | 15 | 30 |
| -25 | 8 days | 20 | 20 |
| 20 | 3 days | 25 | 15 |
| 120 | 1.5 hours | 30 | 12 |
| 250 | 1.5 seconds | 35 | 8 |

Ozone has a half-life of 20 minutes in water at 20˚C (Table 2.1) (Suslow, 2001). Ozone is more stable in its gaseous state than aqueous state (Anon, 1998). The stability of ozone in water is greatly influenced by the presence of contaminants, especially metal ions (Glaze, 1986), and is dependent on the amount of ozone-demanding material (from food and soil) within the water (Smilanick, 2003), the pH, exposure to UV radiation, presence of radical scavengers, and temperature (Anon, 1998).

### 1.1.3 Ozone reactivity

Ozone reactions are limited to aromatic and aliphatic compounds and to specific functional groups (Kim *et al*., 2000; Khadre *et al*., 2001). Ozone slowly reacts with polysaccharides cleaving glycosidic bonds to form aliphatic acids and aldehydes. The reaction of ozone with primary and secondary aliphatic alcohols leads to the formation of hydroxyl-hydroperoxides (precursors to hydroxyl radicals) (Glaze,

1986). Hydroxyl radicals are more reactive than ozone and can react strongly with any organic substance (Glaze, 1986).

Ozone gas is very unstable and can decompose very quickly in air back into oxygen (Anon, 1998; Grimes *et al*., 1983). It has to be generated, therefore, at point of application (*in situ*), sparged in water and applied immediately to a closed system (Suslow, 2001). Ozone in water decomposes into molecules of oxygen and highly reactive free-radicals; hydroxyl radical ($HO^{\cdot}$), superoxide radical ($^{\cdot}O_2^-$), hydrogen peroxide ($H_2O_2$), and singlet oxygen ($^1O_2$), all of which react with a wide variety of organic groups such as those with double carbon-carbon bonds (Gurley, 1985). Minerals, metal ions, hydroxyl ions and halogens (chlorine, bromine, fluorine and iodine) catalyse ozone decomposition and increase the ozone demand of the medium. For example it is estimated that 1 molecule of chlorine can degrade one hundred thousand molecules of ozone (Kim *et al*., 2000; Sparling, 2003 [Online]). Reactions between ozone and inorganic compounds found in water follow first-order kinetics. For example, ozone oxidises ferrous ($Fe^{2+}$) into ferric ($Fe^{3+}$) species, which precipitates in water as ferric hydroxide ($Fe(OH)_3$). Ozone plays an important role in the removal of contaminants from drinking water (Glaze, 1986), including carcinogens and mutagens (Burleson *et al*., 1979). Thus ozone can act as a disinfectant in its gaseous state or dispersed in water. Gaseous ozone is an alternative cleansing agent for water-sensitive products such as porous surfaces (Majchrowicz, 1999).

### 1.1.4 Ozone generation

Ozone cannot be stored or transported in vessels as it decomposes spontaneously in the presence of any oxidizable impurities, humidity and solid

surfaces. Ozone has to be generated *in situ* as required and can be produced by various methods including photochemical, electrolytic, and radiochemical ozone generation (Franken, 2005). However, there are three commercially available techniques for producing ozone; corona discharge, UV radiation and electrolysis. Commercially large volumes of ozone are nearly always generated from atmospheric oxygen using a corona discharge, although sometimes these volumes are generated using ultraviolet irradiation of oxygen (photochemical). Low concentrations of ozone (approximately 0.03 ppm) can be produced by exposure of oxygen and/or air to radiation or high concentrations can be produced by using an electrical corona discharge method (so-called the 'silent electrical discharge' procedure), which involves high voltage current applied across a discharge gap, in the presence of oxygen (usually dried air mixture), causing an electron excitation. The corona discharge process is presently the most widely used method for ozone generation in aqueous and gaseous production (Kirschner, 2005). It is important that dry processed gas is applied to the corona discharge and nitric acid production is limited, in order to increase the efficiency of ozone generation and to protect the generator from corrosion (Kirschner, 2005). Ozone production depends upon the strength of the micro-discharges, which are influenced by the discharge gap width, gas pressure and rate, the voltage and its frequency, properties of the dielectric and metal electrodes, the power supply, concentration of oxygen and by the presence of moisture (Gurley, 1985; Kirschner, 2005). This can also be affected by the amount of organic material in the room (ozone-demand), and the concentration of outdoor ozone (in all circumstances) (Gurley, 1985; Kirschner, 2005).

Ozone generating devices (ozonators) are often sold as air cleaners, for remediation of bio-contaminated or smoke-damaged buildings (Boss and Day, 2003; Gurley, 1985; Foarde *et al*., 1997). Ozone generators are not effective at removing carbon monoxide (CO) or formaldehyde.. Ozone generators are not considered useful in removing odours from building ventilation systems and may not have any effect on biological contaminants that are embedded in porous materials such as duct lining or ceiling tiles (Boss and Day, 2003). Room and house-hold generators have been marketed in the United States for air purification to eliminate odours, volatile organic compounds (VOCs) and microorganisms including bacteria, moulds and spores (Foarde *et al*., 1997). There are numerous brands and different models of ozone generators on the market, which produce varying amounts of ozone (Kirschner, 2005).

### 1.1.5 Safety aspects of ozone

There are many safety aspects that need to be considered before and during the application of ozone. Ozone concentrations at levels of 1.0 ppm are toxic to man and animals (Pelleu *et al*., 1974; Anon, 1998), thus the application of ozone in food processing plants needs to be carefully planned, with the design of ozone systems being commissioned specifically for their purpose. The safety of food processing plant personnel and the safety of food products are paramount. Ozone is only applied, therefore, at levels necessary to attain the desired antimicrobial effect(s). There are permissible exposure limits (PEL) of ozone in ambient air, with an occupational exposure limit (OEL) of 0.1 ppm over 15 minutes (Anon, 2002 [online]). The tolerance condition for ozone in its aqueous phase is the level of residual ozone that will not allow the PEL in ambient air to be exceeded (exposing

plant workers to gaseous ozone) (Pryor and Rice, 2000 cited in Pascual *et al*., 2007).

### 1.1.6 Health impacts on personnel

Ozone's toxicity is largely related to its strong powerful oxidising properties and its toxicity is dependent on the concentration and length of exposure (Kirschner, 2005). The odour threshold (0.02 – 0.04 mg/m$^3$) concentration of 0.01 – 0.02 ppm can vary, and is dependent on different individual's detection, but levels can be detected at 0.01 – 0.04 ppm ozone in ambient air, which is below the limit for general comfort (Suslow, 2001; Kirschner, 2005). Ozone levels exceeding 0.15 ppm become intolerable to most people (Franken, 2005). Ozone is lethal to humans who have prolonged exposure to ozone at concentrations above 4 ppm (Suslow, 2001).

At low concentrations (0.1-1 ppm) of ozone exposure, symptoms include headaches, throat dryness, and irritation of the respiratory passages caused by lipidperoxidation of the membranes and burning of the eyes, caused by the formation of aldehydes. Exposure to ozone at concentrations above 1 ppm causes asthma-like symptoms, such as chest pain, coughing, shortness of breath, and also tiredness and lack of appetite (Chen *et al*., 2004; Suslow, 2001). Ozone exposure can also exacerbate chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD) and asthma, the thickness of lung lining fluid may be reduced. Healthy people as well as those with underlying respiratory conditions can experience breathing problems when exposed to ozone, as individuals will have variable susceptibility to ozone (Levy *et al*., 2001). Harmful effects can occur after short term exposure and at low concentration; however

more damaging effects and poorer recovery are at a higher concentration and longer exposure times (Boss & Day, 2003). As a consequence of these effects, several Federal agencies in United States have established health standards and recommendations to limit human exposure to ozone. These health effects have been summarized in Table 1.3.

**Table 1.3.** Health effects, risk factors and standards set up by government bodies to reduce ozone exposure (U.S EPA, 1999 cited in Franken, 2005).

| Health Effects | Risk Factors | Health Standards |
|---|---|---|
| Potential risks of exposure:<br>Decrease in lung function<br>Aggravation of asthma<br>Throat irritation and cough<br>Chest pain and shortness of breath<br>Inflammation of lung tissue<br>Susceptibility to respiratory infection | Factors that increase the risks and severity of health effects are:<br>Increase in ozone air concentration<br>Greater duration of exposure<br>Activities which raise respiratory rate (e.g. exercise)<br>Certain pre-existing lung diseases (e.g. COPD and asthma) | FDA requires output of indoor medical devices to be no more 0.05ppm.<br>Occupational Safety and Health Administration (OSHA) requires workers not be exposed to average concentration of <0.1ppm for 8 hrs and 0.3ppm for a 15 minute period.<br>National Institute of Occupational Safety and Health (NIOSH) recommends upper limit of 0.1ppm not to be exceeded at any time.<br>Environmental Protection Agency (EPA)'s national ambient air quality standard for ozone is an average outdoor concentration of 0.08ppm |

Chen *et al*. (2004) studied the effect of ozone exposure on the airway response of inhaled allergens in allergic asthmatic subjects. They discovered that exposure of ozone at relatively low-level concentration of ozone does not enhance late inflammatory or early bronco-constrictor responses to inhaled allergen in most allergic patients. The results seem to suggest that a subgroup of asthmatic patients may have increased sensitivity to aero-allergens after exposure to ozone.

### 1.1.7 Global Regulatory Status

Before June 2001, the use of ozone in food processing was regulated in the USA by a 1982 GRAS ruling (184.1 (b) (2)) on the use of ozone in bottled water, which required any other uses of ozone to be regulated by the direct food additive petition. Ozone was awarded "generally recognised as safe" GRAS status for broad use of ozone in food processing by an independent panel of experts in July 1997 from Electric Power Research Institute (EPRI) (Graham, 1997a, b; Federal Register 62, 1997 cited in Majchrowicz, 1999). The Federal Register of 13$^{th}$ September 2000 (65 FR 55264) the Food and Drug Administration (FDA) announced that a direct food additive petition (FAP 0A4721) had been filed. The petition proposed to amend the food additive regulations in part 173 (21 CFR part 173). In light of this petition, the FDA and United States Department of Agriculture (USDA)'s Food Safety and Inspection Service (FSIS) approved and amended section 21 CFR part 173, secondary direct food additives permitted in food for human consumption section, "for the safe use of ozone in gaseous and aqueous phases as an antimicrobial agent for the treatment, storage and processing of foods, including meat and poultry". This also included the use of ozone as an additive on raw agricultural commodities (RACs) in the preparing, packaging or

holding of such commodities for commercial purposes (Federal Register 66, 2001) and as a sanitizer for food contact surfaces, as well direct application on food products.

In Europe, the introduction of the Biocidal Products Directive (BPD) (Council Directive 98/8/EC) (European Union, 1998a) has meant that currently used and new biocides have to be registered with the BPD, which is costly. However, ozone (as a biocide) is economically more advantageous than many biocides because it has to be produced *in situ*, and is, therefore, exempt from the BPD (European Union, 1998a). Ozone generated *in situ* for use as a biocide and the placing on the market of equipment for generating ozone for subsequent use *in situ* does not fall under the scope of the BPD (European Commission, 2011).

## 1.2 Medium for ozone treatment

Ozone applied for the reuse of waste waters in the food industry, lowering the biological oxygen demand (BOD) and chemical oxygen demand (COD) of food plant waste, food storage applications, and in the sanitation of food plant equipment and food contact surfaces, as well as food surface hygiene (Güzel-Seydim *et al*., 2004b; Smilanick, 2003). It has been shown that the ozone concentration applied to food contact surfaces can decline rapidly to 4% of the original dosage in 100 cm distance, due to its high oxidative ability (Li and Wang, 2003). The ozone demand of the medium used greatly affects the concentration, the effective dose and the residual ozone that can be applied. Ozone demand is defined as “the quantity of ozone consumed by all reactions other than by disinfection” (Bancroft *et al*., 1984). Pure water has the least ozone-demanding

power. Impurities within water can react with ozone and can generate a small ozone demand. Some impurities such as glyoxylic acid and formic acid will initiate ozone decomposition (Bancroft *et al*., 1984). In aqueous solutions, the effectiveness of ozone depends on the ozone-demanding material present. The ozone demand of water and rate of chemical oxidation determines how much ozone is available for disinfection (Bancroft *et al*., 1984). Municipal water is the medium of choice for aqueous ozone applications, such as the removal of organics from waste waters, reduction of organic carbon present, while converting non-biodegradable organics into biodegradable organics by the process of oxidation and dissolution of sanitizers (European Standard EN1650:1997) (Bancroft *et al*., 1984; Gulyas *et al*., 1995). The reuse and recycling of industrial waste waters have been recommended for years by European Union (Council Directive 91/271/EEC) (European Union, 1991) and the USDA. However, potable water is required for use in the food industry under Council Directive 98/83/EC (European Union, 1998b).

Temperature, pH, relative humidity (RH), residual ozone, mixing degree and presence of ozone-demanding materials can all alter the activity and antimicrobial efficacy of ozone (Bancroft *et al*., 1984; Khadre *et al*., 2001; Kirschner, 2005; Li and Wang, 2003; Smilanick, 2003). As the temperature increases, ozone becomes less stable and less soluble, but the reaction of ozone with the substrate increases and this will affect the level of residual ozone available for disinfection (Khadre *et al*., 2001). There needs to be a distinction between the concentration of applied ozone and the residual ozone present for effective sanitization, and a clarification between the availability and the decay of ozone during the course of experiments in order to accurately estimate the actual effective dose applied.

## 1.3 Action of ozone treament

Ozone has high antimicrobial activity and is effective against a wide variety of target microorganisms (Kim *et al*., 1999b). Ozone is highly effective against microorganisms in pure culture; however, it is unlikely to be applied to processes where there is a high presence of ozone demand materials, such as organic debris on food contact surfaces or in food products. The action of ozone on microorganisms can be through direct reactions with molecular ozone, or with free-radical mediated destruction (Li and Wang, 2003). Ozone can be used to sanitise decontamination of packaging materials, equipment and food contact surfaces as a terminal disinfectant in the food industry (Moore *et al*., 2000), although the removal of biofilms requires a mechanical action as well during treatment (Kim *et al*., 2003). Ozone can be applied also as a postharvest storage treatment (Smilanick, 2003) and for the decontamination of 'uninhabited bioclean rooms' (Dyas *et al*., 1983).

### 1.3.1 Range of microorganisms

Ozone in gas and aqueous states, at a low concentration and with a short contact time is effective against numerous bacteria, moulds, fungi, protozoa, viruses and parasites (Kim *et al*., 1999b; Rodgers *et al*., 2004). The efficacy of ozone as a sanitizer depends on the target microorganism and the treatment conditions used. Different microorganisms will have inherently different sensitivity to ozone. Studies have shown that bacterial spores are more resistant whereas bacterial vegetative cells are most sensitive to ozone (Kim and Yousef, 2000). Therefore,

for effective destruction of bacterial spores, a greater ozone dose or longer contact time would be needed compared to their vegetative counterparts.

In nature, bacteria can survive for long periods in stationary phase. Changes in morphology and physiology occur in stationary phase bacteria; along with an established state of increased resistance against various stresses (Ishihama, 1997). Cells in the stationary phase are, therefore, more resistant to ozone than cells in the exponential phase of growth. Dormant cells from dry environments are extremely resistant to gaseous ozone (Kim and Yousef, 2000). The resistance of pathogens to ozone is greater in the presence of natural microflora on food, due to the ozone demand of the organic material present. Organic material (such as microflora on the surface of fruit and vegetables) will dimenish the action of ozone, and increase the rate of ozone decay (Kim *et al*., 1999b). Washing cells off the surface of food will decrease their tolerance to ozone by removing the ozone-demanding material from the food's surface (Smilanick, 2003). Colonies or clumps of bacteria on the surface of food/inert surfaces are harder to eliminate than individual cells, and are more likely to biofoul other surfaces (Dunne, 2002).

Ozone can inactivate numerous bacterial species including gram-positive and gram-negative bacteria and both vegetative cells and spores. The mechanisms involved in this are discussed in section 1.3.2. Spores of different species of *Bacillus* have varying susceptibility to ozone (Khadre and Yousef, 2001b). It has been observed that ozone combined with other factors increases the inactivation rates of bacterial spores. As the effectiveness of ozone varies with minor changes to the experimental variables, it is difficult to compare the sensitivity of bacteria

using results from different sources. However, studies have been carried out showing the effectiveness of ozone to a variety of bacterial species.

Ozone is also an effective viricidal agent (Kim *et al*., 1999b; Finch and Fairbairn, 1991). Relatively low concentrations and short contact times are needed to inactivate viruses. However, longer contact times and higher concentrations of ozone are required when inactivating viruses in wastewater than their inactivation in ozone demand-free systems, because of ozone demanding materials present in the medium (Graham, 1997b). Ozone inactivates viruses and bacteria faster than chlorine does (Garbon *et al*., 1983 cited in Herbold *et al*., 1989). Herbold *et al*. (1989) showed a 'clear-cut' difference in the resistance of viruses and bacteria to ozone. It was noted that ozone effectiveness diminished as the temperature increased. Hepatitis type A virus (HAV) was more resistant to ozone than Poliovirus type 1 (PV1), as HAV had greater stability to heat than PV1.

Protozoa such as *Giardia lamblia* and *Cryptosporidium parvum* in natural water are inactivated by ozone (Owens *et al*, 2000; Wickramanayake *et al*, 1984). Wickramanayake *et al*. (1984) reported the effect of the inactivation of cysts of *Naegleria gruberi* and *Giardia muris* using aqueous ozone. Researchers found that *N. gruberi* cysts were more resistant to ozone than *G. muris*. Korich and colleagues (1990) reported that *Cryptospordium parvum* oocysts are 30 times more resistant to ozone than *Giardia* cysts, and was supported by Owens and others (2000) who found that *Bacillus subtilis* endospores were more resistant to ozone than *Cryptospordium* oocysts which, in turn were more resistant than *Giardia* cysts and poliovirus. Finch *et al*. (1993a) compared two different species

of *Giardia* cysts and found that there was not a significant difference in the resistance of *Giardia lamblia* and *G. muris* cysts to ozone.

Ozone is an effective fungicidal agent. Yeasts appear to be more sensitive than moulds and fungal spores to ozone (Kim *et al*., 1999b). Li and Wang (2003) investigated the germicidal effect of ozone on microorganisms, and found that microorganism survival and ozone dosage (ozone concentration and exposure time) have an exponential relationship. The sensitivity of the organisms to ozone were from the most sensitive *E. coli*<yeast<*Penicillium citrinum*<spores of *Bacillus subtilis* to the most resistant. Ozone has been used to prevent post harvest decay from fungi and moulds of many fruit and vegetables, including strawberries (Keutgen and Pawelzik, 2008).

Ozone has been applied to reduce moulds in a cheese ripening room, as it is necessary to evaluate the overall ambient air in such areas in food processing plants. In 1965, Gammon and Kereluk reported work of Gibson *et al.* (cited in Serra *et al*., 2003), who used an atmosphere of 3-10 ppm ozone to prevent mould growth on cheese. The most frequent occurring genera of moulds were *Penicillium* and *Aspergillus*. Results indicated that mould spore load was reduced in the treated room without detrimental effects to the flavour of cheese. The ozone concentrations in this study seem high considering that generally accepted ozone levels are those not exceeding 0.1 ppm. Inactivation of spores with an application of 0.02 ppm of ozone at 25°C and RH of 80-85% increased the shelf life of the cheese product by several weeks and decreased odours, which were otherwise present in storage rooms. The results revealed that ozone reduced the overall airborne mould load, but did not affect the viable mould on the surface of cheese.

Only by wiping the surfaces of the cheese ripening room with a commercial sanitizer as well as ozonating the room was able to reduce viable mould counts on surfaces of cheese. However, to improve overall hygiene status in cheese ripening rooms, ozone decontamination needs to be applied in combination with stringent cleaning regimes. Moulds that develop in refrigerated storage are mainly *Penicillium* species which are well known to produce mycotoxins, including patulin, mycophenolic acid, ochratoxin A, citrinin and penicillin. *Penicillium* moulds are good indicators of food hygiene status (Serra *et al*., 2003).

### 1.3.2 Inactivation kinetics and mechanisms

Ozone is a strong broad spectrum antimicrobial agent that is active against a wide range of microorganisms, including bacteria, fungi, viruses, protozoa, bacterial and fungal spores, and also mycotoxins and other potential allergens (Beuchat *et al*., 1999; Broadwater *et al*., 1973; Burleson *et al*., 1975; da Silva *et al*., 1998; Finch *et al*., 1993a; Finch *et al*., 1993b; Foegeding, 1985; Kim *et al*., 1980; Kim and Yousef, 2000; Kim *et al*., 1999b; Korich *et al*., 1990; Murphy *et al*., 2006; Rickloff, 1987; Seymour and Appleton, 2001; Vaughn *et al*., 1987; Wickramanayake *et al*., 1984). Sensitivity of a single microorganism varies greatly depending on the species, strain, age of culture, the density of treated population, presence of attached cells, presence of ozone-demanding materials and/or compounds, method of applying ozone, RH, method of measuring the antimicrobial efficacy and accuracy of ozone measuring procedures and devices (Bancroft *et al*., 1984). The activity of ozone on microorganisms is a complex process and is probably linked to its molecular form as the main inactivator or its intermediate reactive oxidising species, for example hydroxyl radicals ($HO^{\cdot}$), superoxide radicals ($^{\cdot}O_2^-$), hydrogen

peroxide ($H_2O_2$), or singlet oxygen ($^1O_2$), as by-products of ozone decomposition (Korycka-Dahl and Richardson, 1980).

Ozone can eliminate bacteria within a few seconds by cell lysis. Ozone ruptures the cellular membranes, dispersing the cytoplasm, making reactivation impossible, which is why microorganisms are unable to develop ozone resistant strains (Pope *et al*. 1984). This eliminates the need to change biocides applied in food processing industry periodically, unlike traditional chemical sanitizers. Ozone causes damage to cell constituents such as proteins and unsaturated lipids (fatty acids) and respiratory enzymes in the cell membrane. The lipopolysaccharide layer of gram negative bacteria, peptidoglycan in the cell envelope in gram positive bacteria, other intracellular enzymes and nucleic acids in the cytoplasm are also damaged by ozone (Khadre *et al*., 2001). Pérez *et al.* (1995) showed that N-acetyl-glucosamine which is a component of peptidoglycan was resistant to the action of ozone in aqueous solution at pH 3–7. This is probably why gram positive bacteria have a higher resistance to ozone than gram negative bacteria, as they have a large amount of peptidogycan in their cell walls. Ozone can also react with cell membranes' glycoproteins, glycolipids or amino acids causing oxidation of lipids within the cell envelope. It can act on sulfhydryl groups on certain microbial enzymes causing the disruption of normal cellular activity (Yousef *et al*., 1999). Further oxidation may lead to cell leakage, damage to its genetic material and can eventually lead to cell death (Yousef *et al*., 1999). This may explain the rapid inactivation of bacteria and spores by ozone. Doroszkiewicz *et al*. (1994) studied the influence of ozone on complement-mediated killing of bacteria and found that ozone-treated cells were more susceptible to complement-mediated killing serum. The results suggested that ozone damages or changes the cell membrane leading

to more rapid penetration by the membrane attack complex of complement. Ozone reacts with cell dehydrogenases, DNA and RNA causing damage to cellular genetic material. Using electron microscopy, investigators have shown that ozone causes damage to cellular structures and this damage is more pronounced in gram-negative than gram-positive bacteria (Kim, 1998). Gram-positive bacteria lose only some mucoidal material outside the cell wall, whereas gram-negative bacteria tend to collapse and lose cellular components due to cell lysis, leading to rapid bacterial death. Ozone at low concentration damages the outer membrane of gram-negatives and can cause dramatic changes to the structure of gram-positive bacteria's cell wall, leading to intracellular damage of the cell (Yousef *et al*., 1999).

The mechanism of bacterial spore inactivation has been investigated. *Bacillus* spores were examined using Transmission Electron Microscopy (TEM), which revealed damage to the surface layer, known as the outer spore coat and also to the inner spore coat layer. This may have led to exposure of the cortex to the action of ozone (Khadre and Yousef, 2001b).

## 1.4 The Food Industry

Foodborne disease is a major cause of illness in the UK, which puts substantial burden on infected individuals, healthcare system and economy. The majority of foodborne disease is preventable and therefore there is scope to reduce levels (FSA, 2013). Contamination is a constant challenge for the food industry and can be caused by environmental microorganisms, including spoilage organisms contaminating food products. Foodborne bacteria have been found on a range of

equipment and different surfaces, including food contact surfaces but also cleaning cloths (Scott and Bloomfield, 1993). There has been an increase in the number of outbreaks associated with *Listeria monocytogenes* in people over 65 over recent years (Gillespie *et al*., 2006). The use of ozone and OAF against *Listeria monocytogenes* needs to be investigated to determine how each treatment attacks the bacteria. Therefore it is necessary to reduce microbial load from food contact surfaces in food processing environments in order to reduce the incidence of foodborne illnesses.

### 1.4.1 Ozone applications at different stages of food processing

Ozone has been used for many years and in many different types of applications, from the decontamination of bioclean rooms (Masaoka *et al*., 1982), decontamination of mould infected buildings, known as “sick building syndrome” (Kowalski *et al*., 2003), odour control to water purification (Güzel-Seydim *et al*., 2004b). Ozone can be applied in the food industry for the removal of pesticide residues (Wu *et al*., 2007), shelf-life extension of fruit and vegetables (Suslow, 2001 and 2004), the improvement of food plant effluents (wastewater), surface decontamination of whole fresh produce (Smilanick, 2003), equipment and food contact surface sterilization, used as a terminal disinfectant (Griffith *et al*., 2000).

There have been several recommendations for the use of ozone in an overall complementary sanitising regime for the cleaning and sanitization of beverage (Fielding *et al*., 2007) and other food processing facilities (Güzel-Seydim *et al*., 2004b), and as a standardised method for effective sanitising of raw fruit and vegetables (Beuchat *et al*., 2001). Ozone can be applied directly onto raw agricultural produce at all stages of food processing (pre-processing, during

processing or on the finished product) (Beuchat *et al*., 2001). This is more advantageous than applying ozone to the processed product as the sensory quality of some processed products, such as fruit juices, can be damaged by ozone (Williams *et al*., 2005). Some studies have used ozone to treat food ingredients before they are introduced into the food formulation. It has been suggested that ozone treatment of food ingredients prior to processing reduces ozone usage and minimises the damage caused to the sensory quality of the final product (Güzel-Seydim *et al*., 2004a). There is little evidence to suggest that ozone can accumulate in treated food products (Graham, 1997a). Consumers of organic foods are concerned by the presence of residues such as chlorinated organic compounds from disinfectants; mainly THMs from chlorine based and chlorinated disinfectants such as QACs. These THM compounds are potentially toxigenic and or carcinogenic. Ozonated water has the potential to be applied to fresh produce and food contact surfaces to reduce toxic residues left from disinfectants and pesticides (Wu *et al*., 2007) and to effluent wastewaters from food processing premises.

Treating fruit and vegetables with ozone can be achieved by adding gaseous ozone continuously or intermittently to the storage atmosphere throughout the storage period (Liew and Prange, 1994; Smilanick, 2003), or with ozonated water, involving washing or dipping procedures (Achen and Yousef, 2001). Ozone has been successfully applied in flume water in apple packing houses and is able to minimise chemical and microbial contamination of processed water from these post harvest handling facilities (Strasser and Tonjes, 1998). Processors have also applied gaseous ozone in storage rooms in order to aid the removal of ethylene (Skog and Chu, 2001). Gaseous ozone has been used to extend the shelf life of

fruit during storage in packinghouses (Smilanick, 2003), and ozone in combination with other oxidants such as hydrogen peroxide has been used to aid sanitisation of modified atmospheres inside packaging films (Daş *et al*., 2006).

Daş *et al.* (2006) analysed and compared the growth and survival characterisitics of *Salmonella enteritidis* during passive modified atmospheric packaging (MAP), controlled atmosphere and air storage of cherry tomatoes at 7 and 22°C with or without gaseous ozone treatment. Gaseous ozone treatment was applied to inoculated tomatoes before storage, to eliminate the risk of contamination. Gaseous ozone had a bactericidal effect on populations of *S. enteritidis* inoculated onto the surface of cherry tomatoes. There was, however, a surface colour change from red to yellow, but softening or any other texture change was not observed.

Novak and Yuan (2004a) exposed *Clostridia perfringens* spores to ozonated water and mild heat pre-treatment on beef surfaces placed under MAP. Resilient spores are known to survive temperatures of 100°C for up to 1 hour. Novak and Yuan (2003) (cited in Novak and Yuan, 2004b) noted that *C. perfringens* vegetative cells on beef surfaces were more susceptible to heat at 60°C following ozone treatment, compared to non ozone-treated cells. There was a synergistic relationship between heat and ozone treatment that was effective in reducing cells by 2.09 $\log_{10}$ $cfu^{-1}$ $g^{-1}$ and decreased spore counts by 1.24 $\log_{10}$ spores/g. Storage at 4°C following treatment with ozonated water (5 ppm ozone for 5 mins), heat (60°C for 30 mins) or both, and then vacuum packaging to 2 KPa for up to 10 days was better in preventing spore germination and growth compared with storage at 37°C or 25°C.

Decontamination of a multilaminated aseptic food packaging material and stainless steel by ozone to inactivate natural contaminants as well as bacterial biofilms and dried films of *Bacillus subtilis* spores has been investigated and found to be effective (Khadre and Yousef, 2001a). Ozone inactivated *Pseudomonas fluorescens* biofilm on stainless steel more effectively than on the multilaminated packaging material. They concluded that ozone is an effective sanitizer and has potential applications in the decontamination of packaging materials, equipment and food contact surfaces.

### 1.4.2 Cleaning of equipment and food contact surfaces

There are many food contact surfaces used throughout the food industry and any surface that is in contact with food, such as the surface of equipment, conveyor belts, kitchen utensils, food processing surface, chopping boards etc. (Norwood and Gilmour, 2001) can be contaminated. These food contact surfaces can be made from various materials including polished granite and marble, food grade polypropylene, plastics, rubber, but predominantly surfaces in the food industry are made food grade type AISI 304 or 316 with 2b finish (Anon, 1998; Van Houdt and Michiels, 2010). Stainless steel can have various different finishes coating the surface layer. Food grade 304 has a mixture of nickel and chromium and usually has a 2b finish (coating). Type 304 has better corrosion resistance to ozone than type 302 stainless steel. Stainless steel is corroded less by ozone than by chlorine (Greene *et al*., 1993). However, stainless steel will corrode with ozone at very high concentrations and at long periods of time (Van Houdt and Michiels, 2010). Ozone not only reacts with contaminants (microorganisms) found on food contact surfaces, the treatment medium, and the food product itself, but can also

interact with equipment. Areas on equipment that are prone to biofilm formation include dead ends, joints, valves and gaskets (Leriche and Carpentier, 2000; Norwood and Gilmour, 2001). Surfaces of equipment used for food handling, storage and processing can corrode with age and are major sources of contamination (Wang *et al*., 2003). The efficacy of ozone treatment may be influenced the type of materials used to manufacture equipment found in the food industry. Ozone is known to react explosively with oil and grease (Anon, 1998). Even in low concentrations, ozone has significant effects upon textiles, such as nylon, organic dyes, metals, some plastics, including polyamide, and paints (Gurley, 1985; Anon, 1998; Pascual *et al*., 2007). Other metals, such as aluminium, cast iron, zinc, magnesium, glavanized steel and steel (mild) will also corrode with prolonged exposure to ozone (Anon, 2013 [online]). Natural rubber is highly sensitive to ozone, and total disintegration occurs on exposure to ozone at low doses (Pascual *et al*., 2007). However, glass, Teflon®, titanium and stainless steel 316 and 304 finishes are resistant to the oxidising effects of ozone (Gurley, 1985; Pascual *et al*., 2007). Common plastics including polydichlorodifluoroethylene (PDFE), polytetrafluoroethylene (PTFE), polychlorortrifluoroethylene (PCTFE), which may be used instead of PTFE, polyvinylidenefluoride (PVDF), polyvinylchloride (PVC) are resistant to ozone (Anon, 1998). Silicone and polypropylene are resistant to ozone at short exposure times, but oxidises on extended exposure (Anon, 2013; Pascual *et al*., 2007).

Pathogens, such as *Salmonella enterica* and *Escherichia coli* O157:H7 have been found on hands of personnel, sponges, clothes, and utensils, and can survive on these surfaces for hours and days (Jiang and Doyle, 1999; Kusumaningrum *et al.*, 2002; Uradziński *et al*., 2005). Kusumaningrum *et al.* (2002) demonstrated that

antibacterial dishwashing liquid was effective in reducing pathogens in a water suspension, but not from used sponges. It has been noted that cleaning cloths impregnated with Quaternary Ammonium Compounds (QACs) give significant reductions in contamination of both surfaces and cloths, especially reductions in the numbers of *Enterobacteriaceae* and *Pseudomonads* (Scott and Bloomfield, 1993). Currency, and cleaning cloths, or sponges are potential vehicles for the transmission of foodborne pathogens, leading to cross contamination in the food industry as well as in retail, catering outlets and domestic kitchens.

### 1.4.3 Ozone as an alternative sanitizer

Chlorination is the commonly used disinfectant technology used throughout the food industry, but the build up of resistant strains of microorganisms has created the need to invest in alterative novel sanitizers. Ozone has been suggested as an alternative sanitizer for use in the food industry. With FDA approval as a sanitizer for food contact surfaces, ozone has been recommended as a good alternative. Ozone has the potential to be used as a terminal disinfectant (Bailey *et al*., 2007; Foarde *et al*., 1997; Moore *et al*., 2000). Ozone has many advantages over chlorine for use in the food industry. One important advantage over chlorine is that the food product can be still labelled as ‘organic’. An organic sanitizer has to be registered with the U.S. Environmental Protection Agency as a food contact sanitizer (Franken, 2005).

It has been reported that chlorine is ineffective against bacterial spores, some pathogenic microorganisms and viruses (Kim *et al*., 1999b). Some studies have suggested that chlorine applied at high concentrations cause only modest inactivation of pathogens on food. Chlorine causes microbial inactivation by

selectively destroying certain cellular enzyme systems. Chlorine treatment can lead to the formation of toxic or carcinogenic chlorinated organic compounds, such as trihalomethane (THM) compounds, in water, food or on food contact surfaces (Franken, 2005; Karaca and Velioglu, 2007). These carcinogenic compounds can cause kidney, bladder and colon cancers. Also chlorine degradation can result in the production of chloroform, carbon tetrachloride and chloromethane. Chlorine is a halogen-based chemical which is corrosive to stainless steel. Hydrogen peroxide, chlorine dioxide and peracetic acid (PAA) are alternatives to chlorine as sanitizers. Chlorine dioxide gas instead of chlorine has an oxidation capacity 2.5 times greater than chlorine itself. Chlorine dioxide is more stable over a broader pH range and is less corrosive to metal equipment. It is also less likely to form by-products than chlorine.

Ozone, on the other hand, can be used as an alternative to chlorine, due to its many advantages over traditionally used sanitizers. Ozone has a strong microbicidal action, a higher oxidation potential, and requires a shorter contact time than chlorine or hypochlorous acid (Bancroft *et al*., 1984; Kim *et al*., 1999b; Mari *et al*., 2003). Ozone causes microbial inactivation by the oxidation of the cell membrane and cellular components such as sulphydryl groups of bacterial enzymes, leading to rapid cell death of targeted microorganisms. The reaction of ozone with organic compounds does not produce toxic or carcinogenic compounds (Kim *et al*., 1999b). Ozone is unstable, so does not persist in the environment after use, by decomposing rapidly into oxygen, leaving no toxic residues. Ozone gas has to be generated on site and is inexpensive to produce in the long term, although the initial instalment of ozone generators can be costly. For example, in California, an ozone system cost $3.5 billion to replace a water

chlorination treatment system (Cutler, 2006 [Online]). The cost of ozone generation units and their maintenance are comparable to and can be even less than the cost of chlorine compounds. Another advantage of ozone over chlorine and other sanitizers is that ozone does not require heat. Ozone production can, therefore, save a company costs in terms of its power consumption (Ravishankar and Juneja, 1999).

### 1.4.4 Biocides

The term 'biocide' includes disinfectants, antiseptics and preservatives, but does not include antibiotics. They are widely used as antiseptics or disinfectants in hospitals, food industry and in domestic environments. Biocides are routinely and extensively used to preserve pharmaceuticals, food, cosmetics and other products (Boss and Day, 2003; Franken, 2005). Common sanitizers are biocides, including chlorine gas, chlorine dioxide ($ClO_2$), ozone, acidified sodium hypochlorite, quaternary ammonium compounds (QACs) such as peracetic acid (PAA) and are used for the disinfection of food contact surfaces and the production environment, as well as the prevention of postharvest decay (Mari *et al.*, 2003) of fresh fruit and vegetables. However, biocides do lack selective toxicity against different genera of microorganisms (Franken, 2005). Activity of antimicrobial agents is 'the minimum concentration required to inhibit growth of the target organism', known as the minimum growth-inhibitory concentration (MIC), or a concentration that leaves no detectable survivors after a specified contact time, which is known as minimum bactericidal concentration (MBC). Biocidal action involves a high level of target specificity, which facilitates a selective action against a specific cell target (Gilbert and McBain, 2003).

There are many factors that affect biocidal action including inoculum size, pH, concentration, temperature and organic load (Bancroft *et al*., 1984). Mafu *et al.* (1990) determined that common sanitizers need to be applied to *L. monocytogenes* on certain surfaces at concentrations 5-10 times greater than sanitizers need to be applied to stainless steel. Mechanisms of microbial inactivation by biocides predominantly act at the cell surface. Cell wall composition can affect biocidal uptake. Gram-positive bacteria consist of a peptidoglycan and teichoic acid in the cell wall, where as gram-negatives have an outer membrane that lies adjacent from the peptidoglycan layer, which contains no teichoic acid. Glutaraldehyde, formaldehyde, chlorhexidine, QACs, mercury, mercurials and phenols act on the cell wall. Biocidal inactivation mechanisms are also caused by: cellular effects caused by damaging one or more intracellular components, such as structural proteins, nucleic acid and enzymes. Such mechanisms include gross cytoplasmic membrane damage (causing leakage of intracellular components) (caused by phenolics, QACs and chlorhexidine) and electron transport (caused by parabens and isothiazolones), cytoplasmic coagulation (caused by phenols and cresols), protein coagulation (caused by biguanides and aldehydes), disrupt thiol groups in enzymes (mercury), nucleic acid (caused by glutaraldehyde and formaldehyde), and oxidation of thiol groups and amino groups in proteins and enzymes (caused by peroxygens and halogens) (Russell *et al*., 1997). Ozone has an oxidizing action and is a multiple-hit process. So a good target for ozone on bacteria would be cell wall/ membrane composition, structural proteins and/or enzymes. Biofilms act as another barrier affecting biocidal uptake.

Microorganisms have developed intrinsic defense mechanisms to environmental stresses that are encounter to confer tolerance to certain stresses (Fig. 1.3). Exposure to certain stresses can confer cross-protection against other stresses.

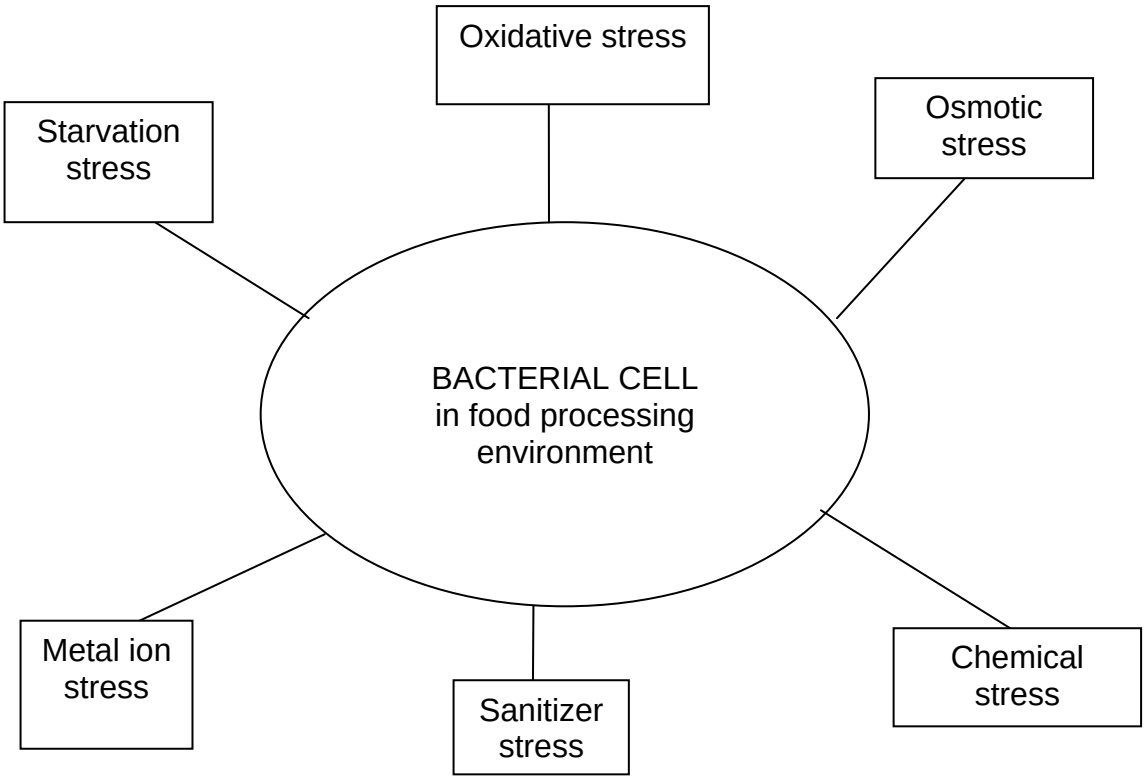


**Figure 1.3.** Various stresses encountered by bacteria.

General stress response systems are activated by several different stresses (heat, salt, bile, pH, starvation and oxidative stress) and can give rise to acquired cross-protection against multiple stresses with the exception of heat (Begley *et al*., 2002; Johnson, 2003; Wesche *et al*. 2009). Jenkins *et al*. (1988) noted that starvation or adaptive treatment with heat, hydrogen peroxide and ethanol protected *E .coli* against further oxidative damage. Sigma factor B ($\sigma^B$) is responsible for the general stress response in gram positive organisms, and sigma factor S ($\sigma^S$) is responsible for the general stress response in gram negative organisms (Mañas and Pagán, 2005). Genes induced by $\sigma^B$ or $\sigma^s$, include those genes encoding for

catalase, superoxide dismutase, and other enzymes used for DNA repair that are osmoprotectant. This suggests that the cell can prepare for oxidative and osmotic stresses at the same time (which tends to be seen in stationary phase cells). The activation of stress response leads to the reduction of growth rate and entry into stationary phase of growth (Mañas and Pagán, 2005). The regulation of the stress response is essential for synthesis of appropriate stress-related proteins (in order to protect the cell). General stress response induces multiple physiological changes in the cell, known as 'multiple stress resistance'. This type of stress response results in 'the accumulation of storage compounds, changes in cellular composition and induces altered morphology' (Yousef and Courtney, 2003).

The oxidative–stress response includes the production of neutralizing enzymes to prevent cellular damage such as superoxide dismutase (SOD), catalase, peroxidise, glutathioine reductase and other enzymes necessary for DNA repair (Mañas and Pagán, 2005). Genetic responses to oxidative stress occur in bacteria allowing them to better resist the damaging effects of a toxic/oxidising agent when they are first exposed to low doses. It is thought that there is a significant overlap between oxidative stress-induced proteins and those proteins induced by $\sigma^S$, suggesting that oxidative damage is significant in stationary phase and generally stressed cells (Mañas and Pagán, 2005; Wesche *et al*. 2009). There are many resistance genes involved in oxidative stress and other environmental stresses involved in stress hardening (adaptation). Cells that are subjected to oxidative stress (i.e. those that are phagocytosed) are able to adapt to this stress. Pretreatment of an oxidizing agent such as hydrogen peroxide with a subinhibitory dose can increase tolerance. Pretreatment induces a series of proteins (which are under control from a sensor/regulator protein (OxyR), including

catalase and other nonessential proteins that accumulate to protect the cell (McDonnell and Russell, 1999).

Resistance to a biocide is usually a natural property (intrinsic) of the microorganism, but can be acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or through transposons (chromosomal or plasmid integrating, transmissible DNA cassettes). Intrinisic resistance is a chromosomal controlled property that enables the cell to overcome the action of the biocide. Gram-negatives tend to be more resistant than gram-positives (McDonnell and Russell, 1999).

Acquired resistance arise by mutation or acquiring plasmids or transposons that carry genes conferring resistance to biocides, such as encode for enzymes that inactivate mercurial biocides, or for cationic biocides (QACs) encode proteins that actively pump (efflux pumps) the biocide out of the cell (Russell *et al*., 1997). As biocides act on multiple sites within the microorganism, resistance is often mediated by non-specific activity, such as efflux pumps, and cell-wall changes. Stressful environments cause changes in the protein folding within the bacterial membrane and changes in the fatty acid composition of gram positive bacteria (Gianotti *et al*., 2008; Giotis *et al*., 2007b; Mastronicolis *et al*., 2005). These cell-wall changes may play a role in cross-resistance between biocides and antibiotics, by reduce permeability of the cell wall (Fraise, 2002). There is a possible genetic linkage between genes for biocide resistance and those for antibiotic resistance. It is thought that therapeutic drug interactions occur due to the possibility that biocidal agents and antibiotics share similar target sites. Shared target sites, however, imply that genetic modifications selected by one agent, could lead to

changes in the susceptibility of another biocidal agent (Russell *et al*., 1997). The food industry, therefore, periodically changes of sanitizers are necessary in order to prevent resistance in microorganisms.

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Physiological (phenotypic) adaption as an intrinsic mechanism of resistance is biofilm production. Gilbert and McBain (2003) found that many microorganisms are intrinsically resistant to particular antimicrobial regimes due to their physiology and biochemical properties.

### 1.4.5 Biofilms

Food processing plants have abundant surfaces accessible to microorganisms, which, under suitable conditions, are able to adhere and form microcolonies, which lead to the formation of biofilms. These biofilms can break up and biofoul other areas, thus spreading the microbial load wider (Watnick and Kolter, 2000).

Biofilms are defined as “bacteria populations adhering to a surface, or to each other in aggregates, enclosed by a matrix of polysaccharides” (Ravishankar and Juneja, 2003). The purpose of a biofilm is to act as a protective barrier from the hostile environment and as a trap for nutrient acquisition. Water capillary channels have been observed in biofilms, which can distribute water and nutrients across the biofilm matrix and oxygen to the inner layers (Costerton *et al*., 1995). Biofilm formation occurs over various stages. Initially, bulk fluid containing microorganisms comes in contact with a surface, resulting in the adsorption of molecules to a surface, referred to as ‘surface conditioning’ (Hood and Zottola, 1995). This initial step involves bacterial attachment onto food contact surfaces, followed by micro colony formation. Marshall *et al*. (1971) proposed a two stage theory for biofilm formation. The initial stage known as the reversible stage involves bacterial cells being in proximity to the surface, held by electrostatic and hydrophobic interactions. The second stage is the irreversible stage, whereby cells attach to daughter cells and to the surface by producing extracellular polymeric substances (EPS) (often exopolysaccharides). Then the cells multiply, forming microcolonies and eventually form a biofilm.

Colorimetric assays for staining of EPS, including polysaccharides, aliginates, and various other proteins can be used to characterise biofilm cells and determine the quantity of protein and polysaccharide production. There are many fluorescent dyes that can be used to examine cell viability using epifluorescent microscopy, including *Bac*Light viability kit (L-7012; Molecular Probes, Eugene, OR, USA) consists of propidium iodide (PI) and SYTO9, and other dyes such as Rhodamine 123, 3′,6′-diacetyl fluorescein, 5-Cyano-2,3-di-4-tolyl-tetrazoliumchloride (CTC), acridine orange and 4’, 6’-diamidino-2-phenylindole (DAPI) (Brunius, 1980;

Davies, 1991;.Kaprelyants and Kell, 1992; Wirtanen *et al*., 1996). A differential fluorescent staining method for the quantitative analysis of surface hygiene of food debris and bacterial cells has been developed by Whitehead *et al*. (2009). The method comprised of dual fluorescent stains, at different concentrations and appropriate application method. The authors found that the best differential staining of bacterial cells and food soil was 0.1 mg/ml rhodamine B with 0.1 g/ml (100 mg/ml) DAPI. The staining of cells and soil worked regardless of application method used. Ahimou and others (2007) investigated the effect of protein, polysaccharide and dissolved oxygen concentrations on biofilm cohesiveness. They found that biofilm cohesion increased with depth, but not age. There was a strong correlation between the level of cohesive energy and polysaccharide concentration, which increased with depth of the biofilm. Dissolved oxygen also increased with depth and might be linked to polysaccharide production. Portein concentration did not however, influence biofilm cohesion.

A three stage process was proposed by Notermans *et al*. (1991), which included adsorption (bacteria adsorb to solid surfaces), consolidation (bacteria produce thin fibres and an extracellular slime layer) and colonisation. Biofilm architectures range from open structures containing channels and columns of bacteria, to densely packed regions of cells with no obvious pores. A mature biofilm consists of lots of cells, which are not actively growing cells. These cells are usually exposed to aerobic/anoxic zones within the biofilm depth (Bishop *et al*., 1995 cited in Ahimou *et al*., 2007).

Typical biofilms found on food contact surfaces can contain in excess of $10^7$ cells/$cm^2$ (Notemans *et al.,* 1991). Biofilms form at different interfaces: solid-liquid

(S-L) (most common type of biofilm, such as waste water pipes), solid-air (S-A) (food contact surfaces, including conveyor belts), solid-liquid-air (S-L-A) (found on open surfaces through intermittent contact with sprays and aerosols (fogging) formed at the meniscus), liquid-liquid (L-L) (in emulsions (oil:water)), and air-liquid (A-L) interfaces (Clarelli *et al*., 2013). The literature mainly focuses on S-L and A-L interfaces (where bacteria have access to both gaseous and liquid phases) (Constantin, 2009). Biofilm formations at air-liquid and solid-liquid interfaces have been studied in *Acinetobacter* spp. and revealed that 25°C is more important than 37°C in maintaining biofilm formation. At the solid-liquid interface, the biofilm was 3 times higher than other species, and at the air-liquid interface isolates were able to form a pellicle on top of the liquid media. It was also noted that the biofilm at the A-L interface, was 4 times higher for *Acinetobacter. baumannii* and *A*. G13TU, than for *A*. G3 (Marti *et al*., 2011). At the solid-liquid interfaces; static biofilms may be found in food production containers such as large vats and tanks, dynamic biofilms may be found in pipelines, floor drains or heat exchangers (Chmielewski and Frank, 2003), and impacted biofilms can be found in dead-ends, and in large surface defects such as factory floor surface and metal welds (Notemans *et al*., 1991).

Biofilms have been described as ‘self-regulating’, which due to biofouling can give rise to other biofilms (Notermans *et al*., 1991). Cell detachment as well as parts of the biofilm detaching from one another allows more cells to attach. The detachment of cells from biofilms is fundamentally important to the dissemination of bacterial contamination to other surfaces. The detachment of cells from biofilms is divided into two processes; erosion and sloughing. Erosion is the continual detachment of single cells and the removal of small amounts of biofilm, whereas

sloughing is a quick and large loss of biofilm. Detachment is influenced by nutrient limitations, growth phase, growth rate, the shape of the cells, and the nature of substrate (Stoodley *et al*., 2001). Poimenidou and others (2009) evaluated the attachment of *L. monocytogenes* to stainless steel coupons, followed by detachment and growth in foods under conditions simulating a dairy processing environment. There was a marked detachment of cells into yoghurt and custard media after initial attachment in milk. Biofilms have caused huge problems in the food industry, with biofouling being a major cause for concern.

The properties of attachment surfaces, such as surface roughness, cleanability, disinfectability, wettability (hydrophobicity) and vulnerability to wear are important factors that determine the ability of cells to adhere and thus affect the potential biofilm formation and the hygienic status of the food contact surface (Van Houdt and Michiels, 2010). In mixed biofilms, competition for nutrients and cooperation occurs with certain species, as well as coaggregation with different species. However, the accumulation of toxic by-products generated by primary colonisers can limit the species diversity within a biofilm. Biofilms composed of heterogeneous species (which occurs more likely in nature than single species) use metabolic by-products of one organism to support the growth of the other, whereas, adhesion of one organism can provide anchorage for others to attach (Dunne, 2002). Sasahara and Zottola (1993) found that *Listeria monocytogenes* alone was unable to attach to glass while *Pseudomonas fragi* could. When grown together in a mixed culture, *L. monocytogenes* was able to attach better to glass and form biofilms. Gram negative bacteria are able to attach to glass better than gram positives. Other researchers (Hood and Zottola, 1997b) have suggested that *L. monocytogenes* (non-exopolymer producer) needs an exopolymer producer

such as *P. fragi* for attachment and biofilm formation. This factor seems to be more important than other factors, such as hydrophobicity or flagella motility particularly for attachment to glass. Growth media and conditioning of contact surfaces are necessary for attachment of *P. fragi* and *L. monocytogenes* to stainless steel (Hood and Zottola, 1997b). Other factors, for example, temperature and pH affect attachment of *L. monocytogenes* to stainless steel and Buna-N rubber (Smoot and Pierson, 1998a). Microorganisms can attach to biotic surfaces, for example, fruits and vegetables, and if not completely removed by washing, can grow and form biofilms during storage (Smilanick, 2003). The close physical association of cells within biofilms leads to a structure with significant physical properties and changes in bacterial physiology compared with their free-living counterparts (Costerton *et al*., 1995). Rough morphology variants of microorganisms are better able to attach and form biofilms than variants with smooth morphology (Sasahara and Zottola, 1993; Dickson and Siragusa, 1994; Monk *et al*., 2004).

Bacteria become sessile within the biofilm as the matrix forms a protective barrier against the action of antimicrobial agents. Resistance is attributed to different mechanisms: a slow penetration of an antimicrobial, altered physiology of biofilm cells, and an expression of adaptive stress response by some cells and/or differentiation of small subpopulation of cells into persister cells (Van Houdt and Michiels, 2010). Biofilm (adhered) bacteria are more resistant to antimicrobials including disinfectants, antibiotics and antiseptics, such as hydrogen peroxide, than their free-living (planktonic) counterparts (Hood and Zottola, 1995). Biofilm bacteria are generally exposed to starvation, dehydration and oxidative stresses

and, as a result, biofilm bacteria can adapt to these stresses simultaneously by sending stress signals and cell-to-cell signals (quorum sensing) to each another.

### 1.4.6 *Listeria monocytogenes*

*Listeria monocytogenes* is a gram positive, facultatively anaerobic, intracellular bacterium, widely distributed in nature (found in soil, vegetation, faecal matter, water and animal feed) and is frequently isolated from many materials in food processing environments (Beresford *et al*., 2001; Di Bonaventura *et al*., 2008). It has a psychrotrophic nature (being able to grow at refrigeration temperatures) (Norwood and Gilmour, 2001). Listeriosis causes enteric illness and severe non-enteric diseases such as meningitis and septicaemia in immunocompromised hosts and can cause abortions. Listeriosis has a mortality rate of between 20-30% (Rocourt, 1996).

There are 13 serovars of *L. monocytogenes*, but almost all human cases have been associated with serotypes 4b, 1/2a and 1/2b (Chae *et al*., 2006). Most outbreaks of listeriosis are caused by serotype 4b however serotype 1/2a is more frequently isolated from food and environmental samples (Borucki *et al*., 2003; Djordjevic *et al*., 2002). All strains have virulence associated genes carried on a pathogenicity island and sequences of many of these genes are conserved. Not all strains are, however, capable of causing disease. *Listeria* strains are divided into three distinct evolutionary groups: division I consists of serotypes 4b, 1/2b, 3c and 3b which are associated with listeriosis outbreaks; and division II which includes serotypes 1/2a, 1/2c and 3a which are occasionally associated with listeriosis outbreaks, and division III, which has recently been described,

containing serotypes 4a and 4c. (Borucki and Call, 2003; Di Bonaventura *et al*., 2008).

The highest incidence of *Listeria* spp. in food processing plants is associated with wet areas, which include floor, drains and conveyor belts, but also areas that are difficult to clean (including gaskets, joints of equipments) (Leriche and Carpentier, 2000; Norwood and Gilmour, 2001). *L. monocytogenes* can rapidly grow and attach to form biofilms on food contact surfaces, such as plastic, polypropylene, rubber, stainless steel, glass (Mafu *et al*., 1990; Hood and Zottola, 1997a) and can grow within mixed species biofilms at 10°C (Chae *et al*., 2006). The ability of *Listeria* to adhere to inert surfaces and form biofilms results in the cells being less susceptible to a particular cleaning process (Stopforth *et al*., 2002). *Listeria* strains are also known to persist for months or even years in food processing environments (Holah *et al*., 2004; Lundén *et al*., 2000, 2003). Lundén *et al*. (2000) demonstrated enhanced adherence to food contact surfaces with persistent strains after short contact times.

There is some controversy over the literature regarding *Listeria*. There is a disagreement between persistence of *Listeria* and its ability to form biofilms (Norwood and Gilmour, 1999; Djordjevic *et al*., 2002; Tresse *et al.*, 2006). Kalmokoff *et al*. (2001) simply argues that *L. monocytogenes* does not form a classic biofilm, but simply adheres to surfaces. Biofilm formation may enhance bacterial persistence in food processing environments, increasing the chances of contributing to post-processing contamination (Chae *et al*., 2006). Lin *et al*. (2006) investigated the cross contamination between processing equipment and deli meats by *L. monocytogenes.* It was found that environmental contamination

(mainly from processing equipment) was the most frequent source of *L. monocytogenes* and, therefore, a greater source of contamination of the final product than the raw materials. The degree of transfer correlated with the number of *Listeria* inoculated onto the processing equipment (slicer blade). Persistent strains could be specifically adapted to survive in biofilms and showed enhanced adherence to food contact surfaces (Holah *et al*., 2004; Norwood and Gilmour, 2001) and resistance to cadmium and the production of monocin (Harvey and Gilmour, 2001). It was reported that cadmium resistance and the production of type E monocin in strains of *L. monocytogenes* occured more frequently in recurrent than sporadic strains and may be important with regard to its ability to persist in food and food processing environments.

Borucki *et al*. (2003) investigated the variation of biofilm formation of *Listeria monocytogenes* strains. There were significant differences between phylogenetic divisions and also evidence for increased biofilm formation observed in division II strains. Persistent strains showed increased biofilm formation relative to non-persistent strains. No significant differences were seen between serotypes. Extracellular polymeric substances (EPS) production correlated with cell adherence for high biofilm-producing strains. Scanning Electron Microscopy (SEM) showed high biofilm-forming strains produced a dense, 3-D structure and had increased EPS. Low biofilm-forming strains produced thin, patchy biofilm and decreased EPS.

Strains from division I produce more biofilm than division II (Djordjevic *et al*., 2002) whereas others have shown that serotype 1/2c is better biofilm producer than 4b strains (Gandhi and Chikindas, 2007). However, Di Bonaventura *et al*. (2008)

demonstrated that there were no significant differences in biofilm formation between phylogenetic lineages, when tested on different surfaces or at different temperatures. Norwood and Gilmour (1999) found that serotype 1/2c displayed higher biofilm formation than 1/2a on stainless and, 1/2a and 4b on glass. Serotype 1/2c does not differ in hydrophobicity from other serotypes and factors such as electrostatic and exopolymer interactions could, therefore, lead to increased biofilm production. The cell surface plays a significant role in bacterial attachment to a surface. Although hydrophobicity and production of EPS play important roles in biofilm formation, there is a strong correlation between EPS produced and the 3-D biofilm matrix formation (Chae *et al*., 2006). Borucki *et al*. (2003) noticed that higher biofilm producers had more extracellular EPS present than weak biofilm producers. Hydrophobic cells attach more readily than hydrophilic cells to biotic or abiotic (inert) surfaces (Chae *et al*., 2006). Lower adhesion correlates with higher hydrophilic cell surface, making colonisation of hydrophobic surfaces almost impossible (Tresse *et al*., 2006). Increasing hydrophobicity of *P. aeruginosa* for instance, correlates with increased biofilm initiation (Chae *et al*., 2006). *Listeria monocytogenes* cell surfaces are generally negatively charged, regardless of pH status and tend to be hydrophilic (basic in character) (Giovannacci *et al*., 2000).

Chae *et al*. (2006) investigated the physicochemical surface characteristics of *L. monocytogenes* attachment to glass. They found out that in order for cells to attach to glass, the presence of EPS is an important factor for bacterial attachment to abiotic surfaces. Extracellular carbohydrates of attached cells at 3h incubation were higher than planktonc cells at 3h incubation period. It was also noted that strains that produced increased carbohydrates at 3h incubation in buffer may also

produce increase cell numbers after a 24h biofilm growth. Epidemic strains, such as those strains belonging to division I (serotypes 4b and 1/2b) were found to have significantly higher attachment than those that had originated from sporadic cases. Chae *et al*. (2006) concluded that the physiochemical cell surface properties, determined by electrophoretic mobility (EM), bacterial adherence to hydrocarbons (BATH) and adherence to polystyrene, and the cells ability to produce EPS are important factors in understanding the mechanism of *L. monocytogenes* biofilms.

It is indicated that surface composition, surface roughness, charge of the cells and substrata, as well as the environmental conditions (temperature and pH) influences the adhesion of *L. monocytogenes* to inert surfaces (Rodriguez *et al*., 2008: Tresse *et al*., 2007). Beresford *et al*. (2001) found that the substrata (surface) had a limited effect on adhesion. The intrinsic ability of *L. monocytogenes* to adhere to inert surfaces is stronger than the influence of the physiochemical surface properties. Bacterial attachment is influenced not only by physiochemical properties (temperature, pH and growth phase), surface properties (hydrophobicity), production of EPS but also flagellation and motility (Gorski *et al*., 2003; Di Bonaventura *et al*., 2008). Flagella expression is regulated by temperature (Di Bonaventura *et al*., 2008) and sodium chloride presence (Caly *et al*., 2009). Herald and Zottola (1988), using SEM, noted that *Listeria* cells produced EPS at 21°C, but not at 10 or 35°C. It is possible that EPS could also be produced at 18°C. Optimal adherence associated with 18°C could also be due to flagellular adherence. Vatanyoopaisarn *et al*. (2000) noted that flagella, not motility, were seen to facilitate early surface attachment of *L. monocytogenes* to stainless steel (SS) by acting as surface adhesins. Lemon *et al*., 2007 noted that flagella-mediated motility is critical for both adhesion and biofilm formation on inert

surfaces. Other studies suggest that flagella as adhesins facilitate initial attachment, but for further biofilm formation, motility involving rotary action does not show a positive correlation with biofilm formation, and may prevent long-term attachment (Herald and Zottola, 1989, cited in Di Bonaventura *et al*., 2008), suggesting that active functional flagella are not required for biofilm formation.

The adhesion and growth of *L. monocytogenes* on surfaces may be governed by the presence of other microorganisms (Sasahara and Zottola, 1993; Leriche and Carpentier, 2000; Carpentier and Chassaing, 2004). *Flavobacterium sp.* increased *L. monocytogenes* attachment (Carpentier and Chassaing, 2004). There is a correlation with increased presence of *Pseudomonas aeruginosa* biofilms and the increased colonisation of surfaces with *L. monocytogenes* and *L. innocua* in food processing plants. A study by Gandhi and Chikindas, (2007) revealed that a significantly higher proportion of cells attached to stainless steel in mixed species biofilms compared to single species biofilms. *L. monocytogenes* was able to survive for longer periods in mixed species biofilms than single species biofilms. *L. monocytogenes* gave higher cell counts in monoculture biofilms than in multispecies biofilms containing *S. xylosus* and *P. fragi* (Carpentier and Chassaing, 2004). It has been shown that *L. monocytogenes* is able to multiply and form microcolonies in the presence of *Pseudomonas spp.*, while alone grows sparsely to glass coverslips (Sasahara and Zottola, 1993; Chae *et al*., 2006). The attachment strength and transfer of *L. monocytogenes* cells in pure or mixed biofilms from an inert surface to food product is influenced by the detachment of cells. The attachment strength of *L. monocytogenes* depends on the non-Listerial organism with which it forms a biofilm (Midelet *et al*., 2006). It is known that other competing organisms, usually from different genera can inhibit

the growth of *L. monocytogenes* in biofilms. *Staphylococcus sciuri* was able to hinder the biofilm development of *L. monocytogenes* on stainless steel. This was due to the production of inhibitory substances and increased competition for attachment sites and nutrients (Leriche and Carpentier, 2000). Zhao *et al*. (2004) found 24 inhibitory isolates of *L. monocytogenes* in food processing plants, including *Enterococcus durans*, *Lactococcus lactis subsp. lactis* and *L. plantarum*. Both *E. durans* and *L. lactis* appear to be excellent competitive-exclusion candidates to control *L. monocytogenes* in biofilms at environmental temperatures of 4-37°C.

*Listeria monocytogenes* adapts to environmental stresses such as acid and low temperatures, by the uptake and accumulation of small molecules, called 'compatible solutes', which relieve the effects of the stress, by rehydrating the cell to maintain turgor (Bayles and Wilkinson, 2000). *L. monocytogenes* undergoes a process known as 'stress hardening'. Stress hardening is the exposure to sublethal stress which then leads to the protection of the cell to exposure from variety of normally lethal conditions. Stress hardening has been encouraged by food processing procedures put in place to control *L. monocytogenes* (Lou and Yousef, 1997).

*Listeria monocytogenes* frequently encounters acid and heat stress in food processing plants. Acidic or acidifying agents and thermal energy are important in eliminating microorganisms from food contact surfaces. *Listeria* cells can multiply at pH values below 5 and can survive pH values below 3 (Lundén *et al*., 2008). It is thought that differences in acid and heat tolerances may influence survival. However, persistent strains do not show higher tolerance to heat stress than non-

persistent strains. Most of the acid and heat sensitive strains were non-persistent and were easily eliminated during sanitation procedures. Persistent contamination is not, therefore, associated with heat tolerance (Lundén *et al*., 2008). The mechanism of heat resistance is not fully understood or known, but it is known that heat shock proteins contribute to heat resistance (Hanawa *et al*., 1995). Acid resistance is dependent on sigma factor B ($\sigma^B$) and acid tolerance response mechanisms are important in heat tolerance of exponential-phase cells (Lundén *et al*., 2008; Skandamis *et al*., 2008). It is believed that the mechanism for adaptation to heat and acid stresses is multifactorial.

With the potential of many bacteria including *L. monocytogenes* to produce biofilms on various surfaces, several studies have focused on increased resistance of sessile cells to increased concentration of disinfectants, for example, QACs and products containing chlorine or iodine, compared to their planktonic counterparts (Fatemi and Frank, 1999; Mereghetti *et al*., 2000; To *et al.*, 2002; Romanova *et al*., 2006). Taormina and Beuchat (2002) investigated the survival of *L. monocytogenes* in food processing equipment, cleaning solutions and its sensitivity to sanitizers and heat treatment. They determined that *L. monocytogenes* may survive exposure to high pH cleaners and be transferred to already sanitized surfaces. Chavant *et al*. (2004) investigated the antimicrobial effects of sanitizers against planktonic and sessile *L. monocytogenes* cells and found that renewing the media of *L. monocytogenes* biofilms had a positive impact on the adhesion, by increasing the rate of adhesion of *Listeria* cells to inert surfaces. Without renewing the growth media, cells adhered at a slower rate, and was concluded that this was due to the 'detachment phenomenon of cellular aggregates'. *Listeria* cells were also grown under various pH environments and it

was observed that by reducing the pH of the media with ethanoic acid to pH 5, biofilms without renewing media appeared to be more resistant than when media were renewed. Pre-acidification of the media caused a possible 'acid tolerance phenomenon'. It was noted that *Listeria* cells were very sensitive to alkaline treatment. A change in phenotype from smooth to rough colony morphology was observed in single strain of *L. monocytogenes.* Such changes in morphology causes enhanced biofilm capabilities and a decrease in virulence (Monk *et al*., 2004). Morpholological changes were seen, using SEM, in *Listeria monocytogenes* subjected to sublethal alkaline stress. Such changes involved single filamentous or elongated chain forms (Giotis *et al*., 2007a).

Responses to environmental stresses such as heat and hydrogen peroxide have been studied in *L. monocytogenes*, and many heat shock and oxidative stress proteins were induced. Of these proteins, 5 were common to both heat and oxidative stresses. Stress proteins known to be induced by environmental stresses were absent in intracellularly grown *L. monocytogenes* (Hanawa *et al*., 1995). This has been hypothesised due to the mechanism by which bacteria can rapidly escape from stressful environments, such as early stage of phagocytosis (possible due to *L. monocytogenes* secretion of listeriolysin O, and other enzymes and proteins).

### 1.5 Open Air Factor

The term, 'open air factor' (OAF) was first used in 1968. It refers to a phenomenon observed when ozone reacts with any compound containing unsaturated hydrocarbons (carbon-carbon double bond), known as olefins (May

and Druett, 1968). OAF is not a single molecule, but a collection of highly reactive chemical species (De Mik and De Groot, 1978). Research into OAF during the 1970s mainly involved defence programs, but very little data is in the public domain. OAF was first reported when experiments at the Microbiological Research Establishment (Porton Down, UK) indicated that bacterial survival in aerosolized particles was much greater in a closed vessel than in the open air at the same temperature and humidity (May and Druett, 1968). This phenomenon was observed only in external or outdoor environments. Since that time, it has been suggested that OAF might be formed from ozone-alkene complexes, similar to those found in external environments, such as photochemical smog (De Mik and De Groot., 1978) or in the presence of naturally occurring volatile plant compounds, such as terpenoids (isopreniols). Terpenoids are found in two classes; A which are caroterpenoid terpenoids (non-cyclic) and B which are non-caroterpenoids (cyclic). OAF can be produced artificially using non-cyclic or cyclic terpenes (Breitmaier, 2006). The production of OAF does fall under the scope of the BPD (European Commission, 2011).

The application of naturally occurring OAF is very limited. However, there is the potential to artificially produce OAF as an air-phase disinfectant and, more recently, as a surface disinfectant (Bailey *et al*., 2007). A recent study investigated the bactericidal effectiveness of gaseous ozone (2, 0.1 and 0.05 ppm) and OAF (derived from two monoterpenes (one cyclic terpene and one non-cyclic terpene)) against *Micrococcus luteus* (Bailey *et al*., 2007). OAF was delivered into ozonated air at concentrations of 2.0 $mgm^{-3}$ $h^{-1}$ (high), 0.75 $mgm^{-3}$ $h^{-1}$ (medium) and 0.3 $mgm^{-3}$ $h^{-1}$ (low). *M. luteus* was aerosolized into the air of the purpose built Bioaerosol Test chamber and the numbers of culturable survivors were

determined. Researchers noted that there was a significant reduction (up to 3 log), when exposed to ozone alone at all concentrations. There were no significant differences when bacteria were exposed to monoterpene or gaseous ozone alone. However, there was a significant difference observed after 20 min contact time when aerosolized bacteria were exposed to OAF (at high and medium concentrations in 0.1 ppm ozone).

The aim of this study was to investigate the effect of gaseous ozone and open air factor on environmental foodborne isolates. The objectives of this research were:

- To study the effect of gaseous ozone, open air factor and ozonated water with and without terpenes emulsified in alcohol on surface attached and biofilms of environmental isolates of *Listeria monocytogenes* (an important concern to the food industry) and *Pseudomonas aeruginosa* (biofilm producer).
- To study the interaction of biofilm environmental isolates on different food contact surfaces.
- To determine possible mode of action of gaseous ozone, OAF and ozonated water with and without terpenes emulsified in alcohol on biofilm environmental *L. monocytogenes* on food grade stainless steel.

# Chapter 2. Effect of gaseous ozone on surface attached foodborne isolates

## 2.1 Introduction

The Food Safety Act 1990, and subsequent Regulations in 2006, aim to ensure that food manufacturers and retailers provide food that is safe and wholesome (Taylor and Holah, 1996). Cleaning is important to all food processing industries, including caterers, retailers, manufacturers and processors. The choice of materials used for the food contact and non-food contact surfaces requires important consideration in terms of controlling product contamination, ensuring the function of equipment and preventing accidents.

Cleaning is necessary in order to remove undesirable materials and prevent cross-contamination, which is implicated in up to 30% of food poisoning cases. Cross-contamination is considered the transfer process by which surfaces referred to as 'soiled' (consisting of organic matter (food debris), microorganisms, and extraneous inorganic matter) contaminate other surfaces, including food produce, food contact surfaces, equipment parts and surfaces. Cleaning needs to be able to reduce the undesirable material to a level whereby residues that remain pose a minimal risk to the quality and safety of the food product (Holah, 1995a). Gibson *et al*. (1995) cited in Taylor and Holah (1996) found a variety of locations in food factories where bacteria had attached and grown to form biofilms. The majority of biofilms were found on non-food surfaces (associated with the presence of moisture and condensation), but were not found on food contact surfaces. The presence of biofilms on walls and floors is associated with indirect contamination.

Indirect contamination is the transfer of microorganisms from environmental surfaces, such as floors via air movement, cleaning activities, personnel handling the food product, utensils, and pests. Cleaning is not sufficient for the reduction of total microbial load, as surfaces that look 'clean' may still have large numbers of microbial flora present (Griffith *et al*., 2000).

The principle means of controlling surface contamination is by sanitation. Sanitation involves both cleaning and disinfection. After the cleaning process, disinfection has a crucial role in further reducing microbial load and viability of bacteria. Disinfection can be achieved by means of heat, steam or liquid chemicals such as chlorine. As well as being effective and non-toxic, disinfectants must be suitable for purpose and factory usage, non-tainting and safe for cleaning operatives (Holah, 1995b). The main disinfectants used in food production are chlorine-based, quaternary ammonium compounds (QACs) and amphoterics, such as peracetic acid. Chlorine-based disinfectants applied in food industry are a cause for concern, as they can produce carcinogenic by-products, known as trihalomethanes (THMs). Traditional biocides and sanitizers used in the food industry are mainly chlorine-based. Therefore alternative biocides need to be found (Russell *et al*., 1997). It is not only the type of disinfectant, but also the variety of cleaning methods used which leads to the effectiveness of a particular biocide. Traditional cleaning methods have included fogging systems. Fogging systems are used in the food industry to disperse a disinfectant as an aerosol, for the reduction in airborne microorganisms and general contamination, and for applying to difficult to reach overhead surfaces (Holah, 2003). For surface disinfection, conventional fogging is effective for upward facing surfaces, and only if sufficient chemical is able to deposit onto the surface, but is not effective on

vertical or downward facing surfaces. For vertical or downward facing surfaces, different application techniques, such as spraying or electrostatic fogging are necessary (Burfoot *et al*., 1999).

Gibson *et al*. (1999) studied the effectiveness of different cleaning methods on *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms. High pressure sprays and mechanical food scrubbers were the most effective in removing the biofilms from the food contact surfaces. The use of alkaline, acid, or neutral detergents prior to spraying with water at 17.2 bar pressure did not significantly increase the removal of *P. aeruginosa* and *S. aureus* biofilms. However, acidic and alkaline detergents significantly ($P \leq 0.05$) affected viability of biofilms and, therefore, minimised the potential for contamination.

On food contact surfaces, it is necessary to reduce the microbial load, and so additional measures are needed in order to reduce contamination. These additional measures involve heat or chemical disinfection and are performed after the initial cleaning process and are termed terminal disinfectants. Ozone has the potential to be used only as a terminal disinfectant on surfaces (Moore *et al*., 2000), due to the fact that organic debris on surfaces can interfere with its efficacy, causing inadequate cleaning. For ozone application in the food industry as a terminal sanitizer, good manufacturing practices need to be put in place. Ozone can react with many materials including textiles, organic dyes, metals, plastics and natural rubber, and can react explosively with grease and oil (Fielding and Bailey, 2005). Food-grade stainless steel is one of the most common food contact surface. A study by Holah (1990) showed that bacterial counts on stainless steel were ten times lower than on other materials used after only 5 seconds and there

were lower counts after 40 seconds of washing. These findings prove the relevance of using stainless steel for domestic sinks and the level of high-demanding stainless steel applications, such as in food and beverage industries, commercial catering, pharmaceuticals and hospitals.

The aim of this study was to determine the effect of gaseous ozone on environmental *Listeria monocytogenes* (known as L002), isolated from a food premise, surface attached or as a biofilm. The objectives were:

1. To isolate an environmental foodborne isolate from a food processing premise.
2. To identify strain and/or serotype.
3. To validate a sampling method (swab, beaker, or glass bead) for recovery of organisms from food-grade stainless steel food contact surface.
4. To determine the effect of gaseous ozone against environmental *Listeria monocytogenes* (L002) isolated from a food factory, compared to collection strains of *Micrococcus luteus, Pseudomonas aeruginosa* and *Listeria monocytogenes* surface attached to stainless steel (grade 304, type 2b finish)
5. To determine the effect of gaseous ozone against surface attached environmental *Listeria monocytogenes* to five food contact surfaces.
6. To determine the effect of gaseous ozone against environmental *Listeria monocytogenes and environmental Pseudomonas aeruginosa*.surface attached on food-grade stainless steel (grade 304, type 2B finish) and as single-species biofilms on food-grade stainless steel, polished granite, and food-grade polypropylene.

## 2.2 Materials and Methods

### 2.2.1 Validation of sampling method

The sampling methods evaluated were adapted from Bailey *et al*. (2007). In order to validate three appropriate sampling methods, reproducibility and sensitivity tests were performed. *Micrococcus luteus* NCTC 10083 was chosen as test organism, because *M. luteus* is a gram-positive, hazard group I microorganism and does not, therefore, pose any health and safety risks.

#### 2.2.1.1 Preparation of suspension culture.

A single cryobead (Technical Service Consultants (TSC), Lancashire, UK) of *M. luteus* NCTC 10083 was placed in 100ml nutrient broth (NB; Oxoid, Basingstoke, UK) and placed on shaking platform (Orbital shaker, Forma Scientific Inc., U.S.A) set at 250 rpm, at 30°C for 24 hours, in order to obtain stationary-phase cells (Beller *et al*., 2010). The cells were harvested by centrifugation at 3000 rpm (x 1068 g) in a refrigerated centrifuge (DuPont Sorvall Superspeed RC-5B refrigerated centrifuge, U.S.A; supplied by Thermo Fisher Scientific, UK) for 20 minutes at room temperature. The pellet was re-suspended in 20 ml of sterile phosphate buffered saline (PBS; Oxoid, UK) to give approximately 3.65 x $10^7$ CFU/ml in the suspension for the reproducibility test (determined as an average count of the three days; see below), and approximately 5.35 x $10^6$ CFU/ml for the sensitivity test (determined as an average count of the two methods; see below). Serial dilutions ($10^{-1}$ – $10^{-3}$) of the suspension for the sensitivity test were also performed. Serial decimal dilutions in MRD were carried out as necessary. 100 µl tryptone soya agar (TSA; Oxoid, UK) spread plates were inoculated in duplicate

and incubated at 30°C for 48 hours for enumeration. This was calculated using the following formula:

Equation: $N= C/v\ (n1+0.1_{n2})\cdot d$

C=number of colonies

V=volume applied to plates

$N_1$=number of replicates

d=dilution used

$N_2$=number of colonies of second dilution

For example, $56/0.1 \times (2+0) \times 10^{-5} = 5.6x10^{7}$

For the reproducibility test (inoculums):

Day 1 = $10^{-5}$ diilution, colony counts were 57, 55 giving $5.6x10^{7}$ cfu/ml.

Day 2, $10^{-4}$ diilution, colony counts were 49, 43 giving $4.6x10^{6}$ cfu/ml

Day 3, $10^{-5}$ diilution, colony counts were 33, 65 giving $4.9x10^{7}$ cfu/ml.

For the sensitivity test (inoculums):

- Swab method, $10^{-4}$ diilution, colony counts were 49, 53 giving $5.1x10^{6}$ cfu/ml
- Beaker method, $10^{-4}$ diilution, colony counts were 57, 55 giving $5.6x10^{6}$ cfu/ml

#### 2.2.1.2 Inoculation of stainless steel coupons.

For the reproducibility test, 10 µl of the culture was inoculated onto ten stainless steel grade 304 coupons (25 $cm^2$). The inoculum was spread over the entire surface using L-spreader and allowed to dry at ambient temperature for approximately 2 hours. Each method was performed in triplicate and all results are reported as log data of actual counts. For the sensitivity test, each dilution ($10^0$–$10^{-3}$) was inoculated onto five coupons. Both methods were performed in triplicate for each dilution and all results are reported as log data.

Three sampling methods were chosen and evaluated for the reproducibility test:

***Swab method.*** A sterile cotton tip swab (Fisher Scientific UK Ltd., UK) pre-moistened with maximum recovery diluent (MRD; Oxoid, UK) was swabbed over the entire surface of the coupons in a grid-like pattern. Each coupon was swabbed in 2 directions, the second direction being at right angles to the first. The swab was placed into 9 ml MRD and vortex mixed (Whirlimixer, Fisherbrand, Fisher Scientific UK Ltd., UK) for 30 seconds. Serial decimal dilutions in MRD were carried out as necessary. 100 µl tryptone soya agar (TSA; Oxoid, UK) spread plates were inoculated in duplicate and incubated at 30°C for 48 hours.

***Beaker method.*** Each coupon was aseptically inverted into a sterile 250 ml beaker containing 10 ml sterile PBS. Beakers were vortex mixed for 30 seconds. 100 µl TSA spread plates were carried out in duplicate and incubated at 30°C for 48 hours.

***Glass bead method.*** 10 g sterile glass beads (4 mm diameter; Fisher Scientific UK Ltd., UK) were placed into each sterile beaker containing 10 ml of sterile PBS. Coupons were then aseptically inverted into each beaker. Beakers

were vortex mixed for 30 seconds. 100 µl TSA spread plates were inoculated in duplicate and incubated at 30°C for 48 hours.

Following evaluation of the methods above, the two most appropriate were selected to carry out the sensitivity test. These were the swab method and the beaker method.

#### 2.2.1.3 Examination of coupons post sampling

An inverted microscope was used to see whether or not there was any organic material present on the coupon after each sampling method had been tested.

*Statistical Analysis.* Statistical analysis was carried out using Minitab version 14 (Minitab Ltd., Coventry, UK). Log data were analysed using one-way unstacked ANOVA with Tukey's comparison and a one-way ANOVA was performed between methods. Significant differences were reported where $P \leq 0.05$.

### 2.2.2 Gaseous ozone time curves

*Ozone production.* A corona discharge Aquamaid II model ozone generator (Model: AM3280, Ozone Industries Ltd., Farnborough, UK) was used to continuously supply gaseous ozone for 2 hours in a class 2 Bioaerosol Test chamber (Fig. 2.1a).

*Bioaerosol Test Chamber*. The 20 $m^3$ purpose built, safety level 2, bioaerosol test chamber used was clad internally with food-grade polypropylene. The internal dimensions of the chamber are 4.6 m long x 2.2 m high x 2.0 m wide = 20.24 $m^3$. The half-life of ozone inside the chamber was approximately 1.3 hours at 20˚C with 50±5% relative humidity (RH) (Beakers filled with hot water was placed in front of the internal mixing fans, to ensure constant humidity). The RH was measured using a portable RH meter using a handheld RH meter (RH-202; Omega Engineering Inc., UK) placed on the table inside the chamber.

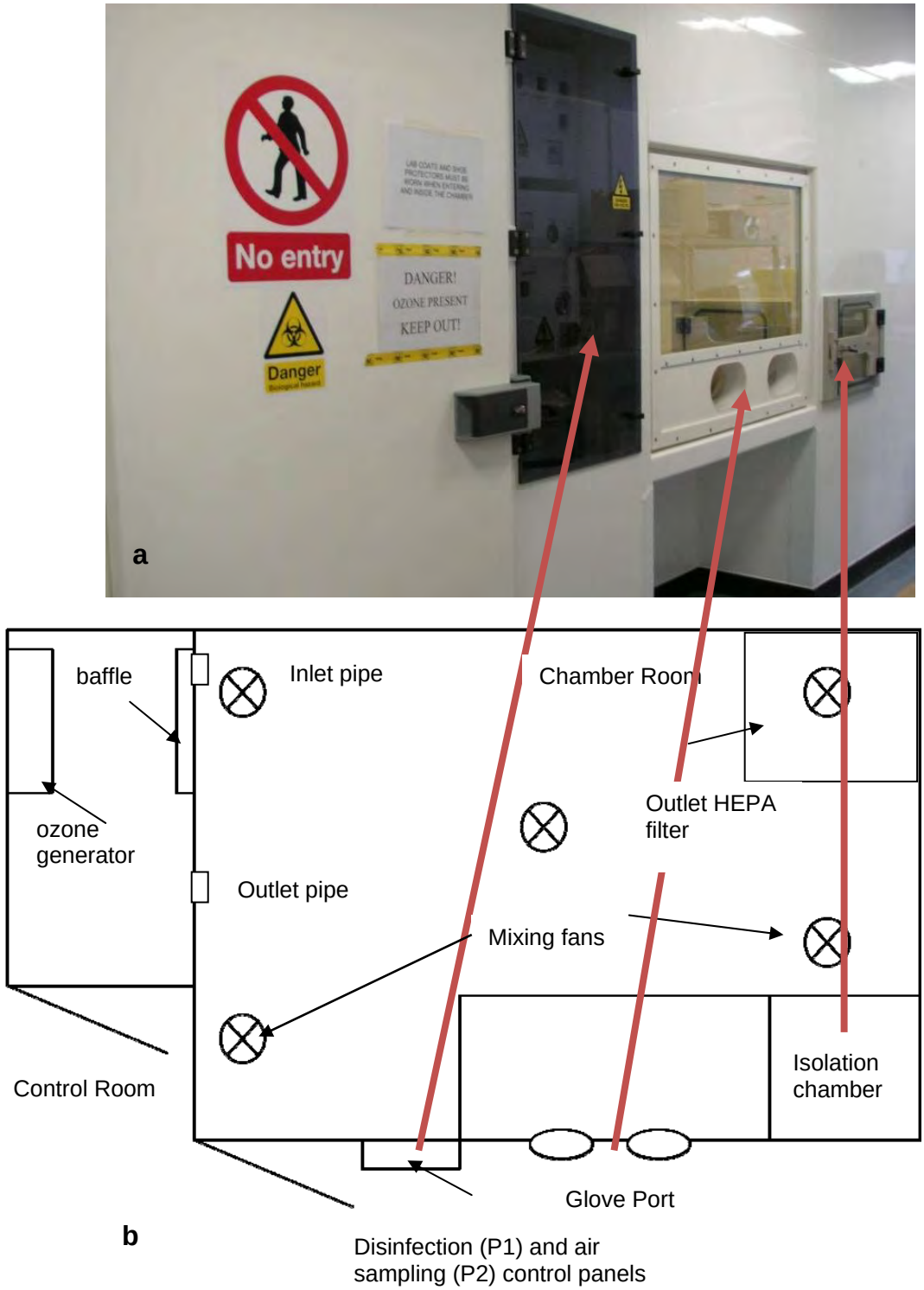



**Figure 2.1.** Fig. 2.1 a, the bioaerosol test chamber. The chamber room, houses the glove port and isolation hatch chamber. The control room adjacent to chamber room housed the ozone generator system. The disinfection control (P1) and air sampling control panels (P2) are situated adjacent to the door of the chamber (in a sealed cabinet on the outside of chamber). Fig. 2.1 b, Schematic diagram of the class 2 Bioaerosol Test Chamber (20 $m^3$) (adapted from diagram by Dr Andy Young).

Figure 2.1 b shows a schematic of the chamber, which consisted of a control room (adjacent to the chamber room). This housed the ozone generator system, desiccant tube, inlet filters/baffle and fan isolator, light and main ozone generator power switches). The ozone generator system was an Aquamaid II (corona discharge) ozone generator. The generator had a normal output of 0.9 g of ozone per hour. Dry air (<-15˚C dew point) from a molecular sieve dryer was pumped through the generator at a rate of 6 L/min. An inlet baffle was constructed between the ozone generator system and the chamber room.

The test area of the bioaerosol chamber housed a table (adjacent to the window) below the glove port, internal mixing fans and internal IP56 sockets. Five internal 120mm axial mixing fans were placed on the floor of the chamber. The main purpose of the mixing fans was to create sufficient mixing characteristics within the bioaerosol test chamber, thus allowing ozone and any other aerosols produced to be evenly distributed throughout the chamber. An outlet high efficiency particulate air (HEPA) filter and baffle were constructed to prevent the egress of test microorganisms into the ambient air. The outlet baffle was kept opened. An isolation chamber adjacent to the glove port was used for easy access from outside the chamber to samples inside the chamber.

*Monitoring ozone concentration.* The ozone concentration in the chamber was monitored for the duration of the treatment using an ultraviolet absorption Advanced Pollution Instrumentation (API) ozone monitor (model 450H, Single/Multi-Channel Ozone Analyzer, Advanced Pollution Instrumentation, Inc., supplied by Environment Technology Supplies PLC, UK), and a handheld ozone monitor (model OMC-1108, Ozone Solutions, Inc, Hull, UK, supplied by Ozone

Industries Ltd) was used to detect low levels of ozone present in the environment around the chamber.

The concentration of ozone produced by the generator was determined by monitoring ozone levels against time. The concentration was recorded every ten minutes for 2 hours. There were two variables (with and without increase in RH and/or monitoring levels with and without pvc tubing) that were applied in order to determine the best condition for gaseous ozone production and monitoring (single experiment). The RH inside the chamber was increased by filling beakers with hot water placed in front of the internal mixing fans, to ensure constant humidity in the chamber room, to a RH of 50±5%. The ozone levels were monitored either from the ozone inlet pipe positioned on the left chamber wall or by attaching pvc tubing to this inlet pipe to reach the centre of the chamber.

### 2.2.3 Isolation and identification of environmental isolates from a food premise

A high-care food factory was chosen and a range of environment swabs were taken in one specifc location within the food factory. This food factory had stringent cleaning protocols were in place.

*Preparation of swabs.* Sterile cotton-tip stick swabs (TSC: Lancashire) premoistened with MRD (4 replicates) were spread over a 10x10cm$^2$ area in 4 sampling points in one specific area of the factory.

Sampling points were as follows:

1. Bottom step
2. Waste hatch

3. Waste hatch floor/kerb
4. Corner of floor waste hatch in drain

The swabs were placed in 9 ml MRD and vortexed for 30 seconds ($10^{-1}$ dilution). 100 µl of $10^{-1}$ dilution was inoculated onto violet red bile glucose agar (VRBGA; Oxoid), *Pseudomonas* selective agar (PSA; Oxoid), Baird-Parker agar (BP) and plate count agar (PCA) spread plates, in duplicate. Plates were incubated at 37°C for 24 hours. 1ml of each $10^{-1}$ dilution was inoculated separately into 9 ml Listeria selective enrichment broth (LSB; Oxoid, UK), and incubated at 37°C for 24 hours. LSB were subcultured by inoculation of 100 µl into fresh LSB and incubated for a further 24 hours at 37°C. 100 µl of broth was inoculated onto Listeria selective agar (LSA; Oxoid, UK) in duplicate and incubated at 30°C for 48 hours.

Single colony picked and streaked onto nutrient agar (NA; Oxoid, UK) for Analytical Profile Index (API); BioMérieux, France) identification.

*Identification of isolates.* Catalase, oxidase tests, and API, followed according to manufacturer's instructions.

### 2.2.4 The effect of gaseous ozone on bacteria surface attached to food grade stainless steel 304 coupons

The following organisms were used for assessment of ozone sensitivity: environmental isolate *Listeria monocytogenes* (L002) (from UWIC's environmental stock cultures which had been isolated from a food premise, from specific area of the corner of floor waste hatch in drain), *Listeria monocytogenes* NCIMB 13451; environmental isolate *Pseudomonas aeruginosa* (C001) (was obtained from UWIC's environmental stock cultures (with permission from Dr. Andrew Hall),

which had been isolated from water pipes), *Pseudomonas aeruginosa* ATCC 15442, NCTC 10299 and *Micrococcus luteus* NCTC 10083. Data from the *Pseudomonas aeruginosa* ATCC 15442 experiment were taken from work performed by Bailey (2002) [data not published].

*Preparation of suspension culture.* The suspension cultures of all organisms were prepared as follows. A single cryobead (TSC, Lancashire, UK) of each organism was placed in 100ml nutrient broth (NB; Oxoid, Basingstoke, UK) in duplicate and placed on shaking platform (Orbital shaker, Forma Scientific Inc., USA) set at 250 rpm and at 37°C overnight (24 hours) giving an OD ($A_{600}$) 1.0, in order to obtain a stationary-phase culture (growth curves carried out) (Chavant *et al*., 2002), the cells were harvested by centrifugation at 3000 rpm (x 1068 g) in a refrigerated centrifuge (DuPont Sorvall Superspeed RC-5B refrigerated centrifuge, U.S.A; supplied by Thermo Fisher Scientific, UK) for 20 minutes at room temperature. The pellet was re-suspended into 20 ml of sterile phosphate buffered saline (PBS; Oxoid, UK) to give approximately 4 x $10^9$ CFU/ml. This was enumerated by $N= C/v\ (n1+0.1_{n2})\cdot d$

C=number of colonies

V=volume applied to plates

$N_1$=number of replicates

d=dilution used

$N_2$=number of colonies of second dilution

For example, $383/0.1 \times (2+0) \times 10^{-6} = 3.8 \times 10^9$

*Inoculation of stainless steel coupons.* 100 µl of each culture was inoculated separately onto 25 $cm^2$ stainless steel grade 304 coupons. The inoculum was spread using an L-shaped spreader over the entire surface and allowed to dry at ambient temperature for approximately 2 hours.

*Ozone production.* A corona discharge Aquamaid II model ozone generator (Ozone Industries Ltd., Farnborough, UK) was used to continuously supply gaseous ozone for 1 hour housed in a class 2 Bioaerosol Test chamber. The concentration of ozone produced by the generator which was available for treatment was monitored using API monitor and .

*Surfaces.* Food grade stainless steel (type AISI type 304, finish no. 2b, 0.1 cm thick; Fairwater Steelworks Company, Cardiff, UK) 5 x 5 $cm^2$ surfaces were used to determine the survival of surface attached bacteria. Before use, the surface of each coupon was disinfected with Pyroneg (Johnson Diversey) and rinsed with sterile deionized water, before autoclaving at 121°C for 15 mins.

*Ozonation of stainless steel coupons.* Five clamp stands containing three coupons were placed in different orientations in the centre of the chamber (Figs. 2.2 and 2.3) in front of the ozone outlet pipe. Fig. 2.2 shows a schematic diagram of the arrangement of the stands inside the chamber. The coupons were ozonated for an hour at varying concentrations (2, 5, 10, 45 ± 0.1 ppm) at RH of 50±5%.

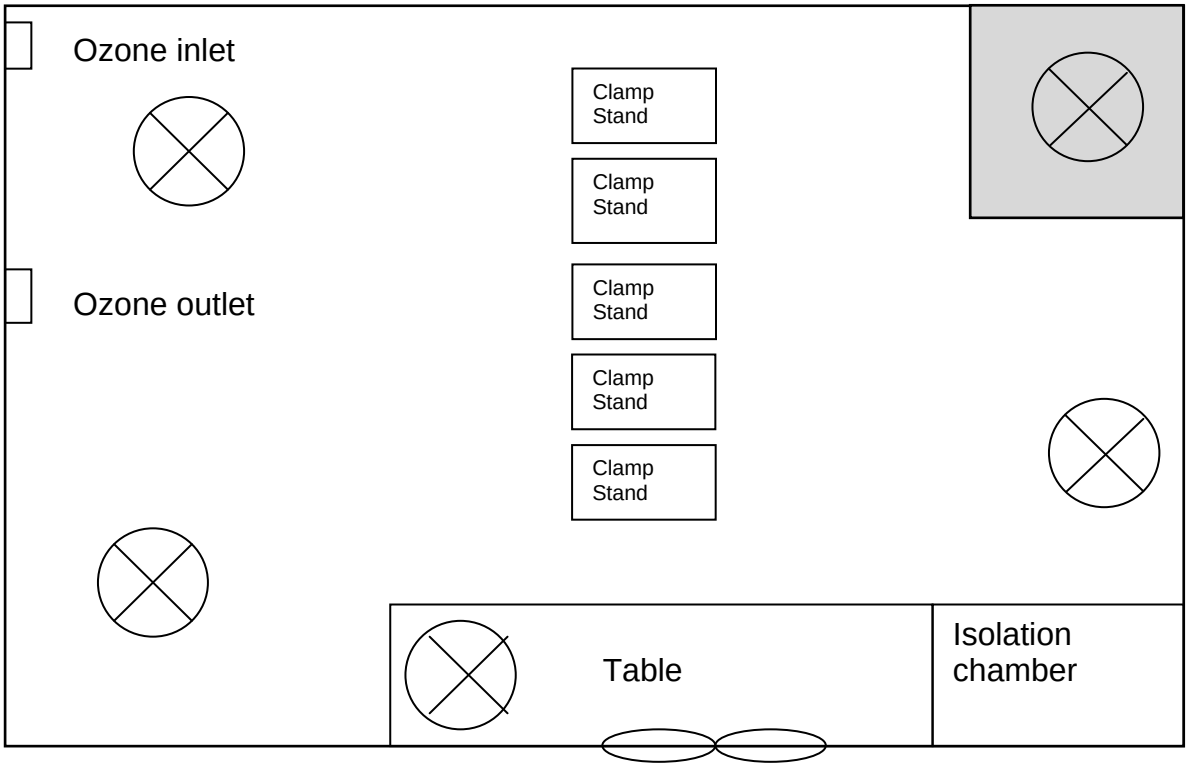



**Figure 2.2.** Schematic diagram of the arrangement of clamp stands inside the chamber. The clamp stands were situated facing the ozone outlet pipe and each stand had three clamps attached. The grey box in diagram,where the pump vent out of chamber with outlet HEPA filter.

The coupons were arranged in horizontal (H) (inoculum face upwards), vertical (V) (inoculum sideways facing) or inverted (I) (inoculum face downwards) orientations (Table 2.1 and Figure 2.3). The following combinations of coupon orientation and ozone concentrations were used:

**Table 2.1.** Illustration of the positioning of the coupons in different orientations on clamp stands.

| Position on stand | Stand Number | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Top coupon | H | I | V | H | V |
| Middle coupon | V | H | I | I | I |
| Bottom coupon | I | V | H | V | H |

**Figure 2.3.** Picture of clamp stand 1 to illustrate positioning of the coupons. The top coupon was inverted. The middle coupon was orientated vertically and the bottom coupon was orientated horizontally. The arrows point to the inoculated surface.

Subsequent experiments were performed on surface attached bacteria at various concentrations of gaseous ozone illustrated in the tabe below. Table 2.2 summarises the following combinations of ozone concentrations used for each microorganism.

**Table 2.2.** Ozone concentrations used for each of the test microorganisms.

| Microorganism | Strain | Ozone Concentrations (ppm) |
|---|---|---|
| *L. monocytogenes* | L002 | 2, 5, 10, 45 |
| | NCIMB 13451 | 45 |
| *P. aeruginosa* | ATCC 15442 | 0.05, 0.1, 2 |
| | NCTC 10299 | 45 |
| | C001 | 45 |
| *M. luteus* | NCTC 10083 | 45 |

*Sampling.* A sterile cotton tip swab pre-moistened in MRD was swabbed over the entire surface of the coupons in a two-directional, grid-like pattern as described in section 2.2.1.2. The swab was placed into 9 ml MRD and vortex mixed for 30 seconds. 100 µl TSA spread plates were inoculated in duplicate and incubated at 30°C for 48 hours.

*Enumeration of survivors.* Five coupons were for each different orientation (n=5) and for each concentration of ozone. Five coupons were untreated (control) (n=5). Each concentration was assessed in triplicate (n=15) and all results are reported as log data of actual counts. The effect of 45 ppm ozone against *Listeria monocytogenes* L002, *Pseudomonas aeruginosa* NCTC 10299 and *Micrococcus luteus* NCTC 10083 experiment and subsequent experiments only used the

horizontal orientation, as it had been determined that the reduction in survivors was concentration dependent and not dependent on surface orientation.

### 2.2.5 The effect of gaseous ozone on environmental *L. monocytogenes* L002 surface attached to five different surfaces: A comparison of adherence

The experiments performed in section 2.2.4 were repeated with different surfaces using *L. monocytogenes* L002: 25 $cm^2$ food grade stainless steel (type AISI 304; finish no. 2b, 0.1cm thick; Fairwater Steelworks Company, Cardiff, UK), 25 $cm^2$ food grade polypropylene, 25 $cm^2$ polished marble (Mandarin Stone, Cardiff, UK), 25 $cm^2$ polished granite (Mandarin Stone, Cardiff, UK) and glass microscope slides (76 x 26mm, 0.8 to 1.0mm thickness; Fisherbrand, Fisher Scientific UK Ltd, UK). All surfaces were disinfected with alcohol wipes (Fisher Scientific UK, UK), rinsed thoroughly with sterile deionized water, and air dried, with the exception of glass microscope slides which were alcohol flamed sterilised. The stainless steel and the polished granite coupons, once air dried were sterilized by autoclaving at 121°C for 15 minutes before use.

Only horizontal orientation was used in this study. The coupons were arranged centrally inside the bioaerosol test chamber (Fig. 2.2) in the same position. There were three coupons per surface (n=3) and the coupons were ozonated for 1 hour at 45 ppm. Each run was performed in triplicate (n=9).

### 2.2.6 The effect of gaseous ozone at 45 ppm on *L. monocytogenes* L002 and *P. aeruginosa* C001 isolates

Gaseous ozone at 45 ppm was applied to surface attached *L. monocytogenes* (L002) and *P. aeruginosa* (C001) to 25 $cm^2$ stainless steel (food grade 304) coupons, and on 72 h biofilms single-species biofilms of *L. monocytogenes* L002 and *P. aeruginosa* C001 adhering to 1 $cm^2$ food grade stainless steel 304, polished granite and food grade polypropylene coupons. *L. monocytogenes* L002 and *P. aeruginosa* C001 suspensions were prepared in the same way as in section 2.2.4. Only the horizontal orientation was used in this experiment, as it had been determined that there was no significant difference in coupons arranged in different orientations.

**Surface attached microorganisms.** For the surface attached work, the preparation of suspension cultures, inoculation of coupons, ozonation, sampling and enumeration of survivors were repeated as in section 2.2.1. There were five 25 $cm^2$ food grade (type AISI 304, finish no. 2b, 0.1 cm thick) stainless steel coupons per organism (n=5), and the coupons were ozonated for 1 hour at 45 ppm as before. Each run was performed in triplicate.

**Biofilm microorganisms.** The model biofilm method (adapted from Charaf *et al*., 1999) involved growing a biofilm of the chosen microorganism on inoculated filter paper (Whatman qualitative No. 2), which was placed on the surface of tryptone soy agar (TSA) (Oxoid, UK). 1 ml of *L. monocytogenes* L002, and *P. aeruginosa* C001 of separate overnight cultures were diluted (1/10) and pipetted onto the filter paper, so that the filter paper was evenly moistened. The filter paper was used in

order to reduce anoxic conditions underneath the coupons. Sterile coupons (food grade stainless steel, food grade polypropylene and polished granite) of approximately 1 $cm^2$ in size were placed on top of the inoculated filter paper and pressed down lightly in order to expel any air bubbles.

The biofilms were grown up on the underside of the coupons, illustrated in Figure 2.4 at 35±2°C.

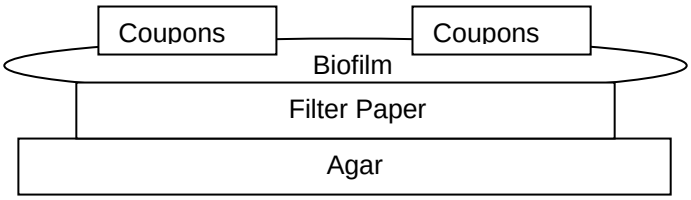



**Figure 2.4.** Schematic representative of the model biofilm setup (adapted from Charaf *et al.*, 1999).

The biofilm-covered coupons were harvested after 72 hours. Each of the coupons was carefully and aseptically removed from the surface of the filter paper using sterile forceps and placed with the biofilm facing upwards into sterile Petri dishes. In the study of Charaf *et al.* (1999), the coupons, once harvested, were either dried for 40 minutes at 35±2°C or used immediately. They noted that there was no significant difference between coupons used immediately after being harvested or those that had been dried. The biofilm-covered coupons were, therefore, examined immediately.

*Treatment of coupons.* Five of each (stainless steel, granite and polypropylene) of the 1 $cm^2$ coupons were placed inoculums side up in the centre of chamber on clamp stands as previously described. Once treated, the coupons were taken out of the chamber and sampled.

*Sampling.* A pre-moistened sterile cotton tip swab was swabbed over the entire surface of the coupons in a grid-like pattern. The swab was placed into 9 ml MRD and vortex mixed for 30 seconds. Serial dilutions were carried out as necessary.

*Enumeration of survivors.* The drop plate method was performed in order to enumerate the number of biofilm cells on the coupons (Herigstad *et al*., 2001). Serial dilutions were carried out as necessary. The bases of the agar plates were divided into quarters. Each serial dilution of each coupon occupied one quadrant of each plate. Each sample was vortexed for 8 seconds and then five evenly spaced 10 µl drops were pipetted onto the appropriate quadrant labelled for that particular dilution. These two steps were repeated for each dilution for each sample. All drops were allowed to soak into the medium before turning the plates over for incubation. TSA plates were used and incubated at 32±2°C and 35±2°C for 48 and 24 h, respectively. Once incubated, plates with dilutions containing 3-30 colonies per 10 µl drop were counted. Viable cell counts were expressed as CFU/surface area.

$Log_{10}$ ($CFU/cm^2$) was calculated according to the following formula by Lennox (2008) [online]: $Log_{10}$ ($CFU/cm^2$) = LOG [(average CFU/drop volume) x (Dilution counted) x (volume removed /surface area)].
For example, LOG [(16.6/0.01) x ($10^3$) x (10/1)] = 7.22 $cfu/cm^2$.

Five coupons were used for each surface (n=5). Each treatment was carried out in duplicate (n=10) and all results are reported as log data of actual counts.

*Statistical analysis.* Data analysis was performed using two-way ANOVA (Minitab version 15, Minitab Ltd, UK) with Tukey's comparison (n=10). Error bars are SD*1.96) on the effect of gaseous ozone on *L. monocytogenes* and *P. aeruginosa* (environmental) in single-species biofilms and surface attached cells to different food contact surfaces.

## 2.3 Results

### 2.3.1 Validation of sampling method

*Reproducibility test.* The results shown in Fig. 2.5 illustrate that all three methods are reproducible when carried out in triplicate (n=30, replicates 1, 2 and 3), as all results for each method gave approximately 2 log transforms. The glass bead method gave less reliable results, as illustrated by the large error bars. The error bars in Fig. 2.6 (and all subsequent graphs) denotes the confidence intervals (CI) with a confidence level of 95% (calculated by standard deviation x 1.96). This means that 95% of the distribution of the sample means, lies within 1.96 standard deviations (SD) of the population mean.

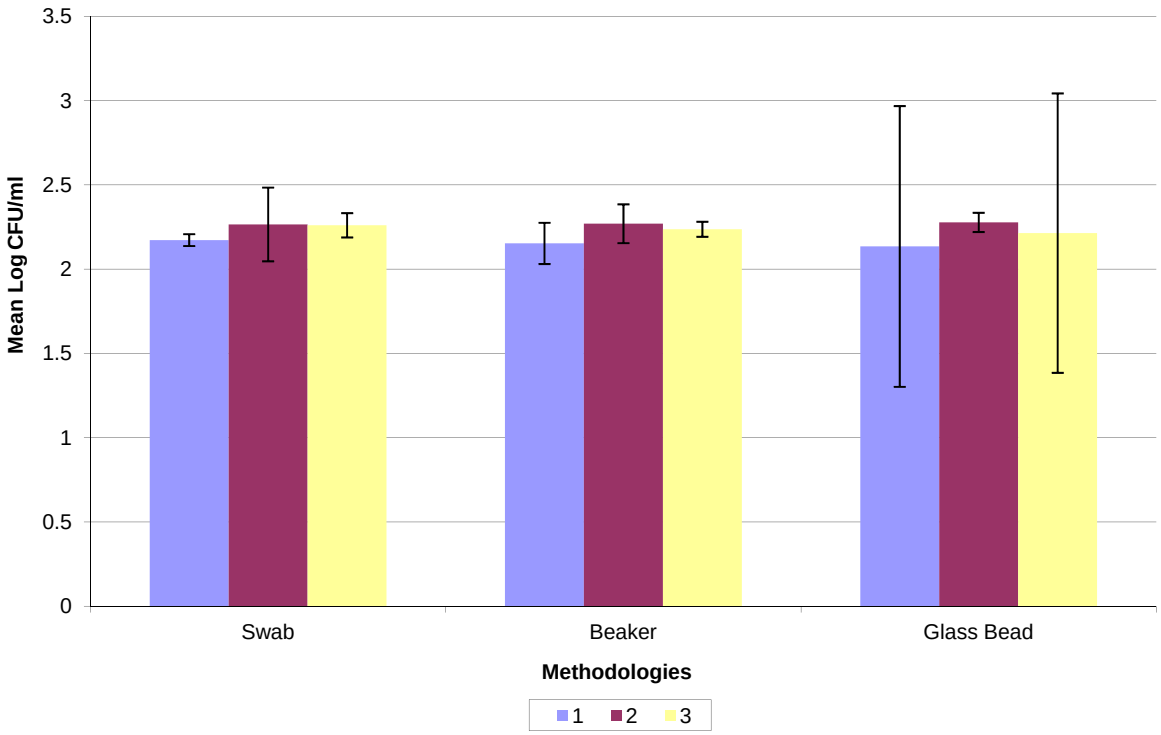



**Figure 2.5.** Reproducibility of the three sampling (swab/beaker/glass bead) methods. All data reported as mean $\log_{10}$ data and error bars denote confidence intervals (CI) with a confidence level of 95%. All three methods (swab, beaker and glass bead methods) were carried out in triplicate (n=30). Legend: 1-3 are individual replicates.

Generally, a gap between bars does not ensure significance, nor does overlap rule it out, it depends on the type of bar represented. Standard deviation (s.d) bars only reflect the mean of the data, and not the error in measurement. The standard error of the mean reflects the uncertainity in the mean and it dependent on sample size. However, confidence intervals (CI) are more intuitive measure of uncertainity, and indicate the reliability of a measurement. A specific confidence level (CI%)- i.e. the 95%CI is the most common to use. The bar captures the population mean CI% of a sample. The size of CI depends on n. In large samples, the standard error of the mean (s.e.m) bar can be interrupted as a CI with a confidence level of 67%. *P* = 0.05 value is not reached until s.e.m bars are separated by 1 s.e.m, but CI bars are more generous and can overlap by as much as 50% and still indicate a significant difference. When 95%CI bars just touch, the result is highly significant (*P* = 0.005) (Krzywinski and Altman, 2013).

Statistical analysis revealed that there was no significant difference between the different methods (P >0.05).

*Sensitivity test.* As shown in Figure 2.6, the swab and beaker methods gave a similar trend in results, with the swab method giving slightly lower recovery at the $10^{-1}$ and $10^{-3}$ dilutions.

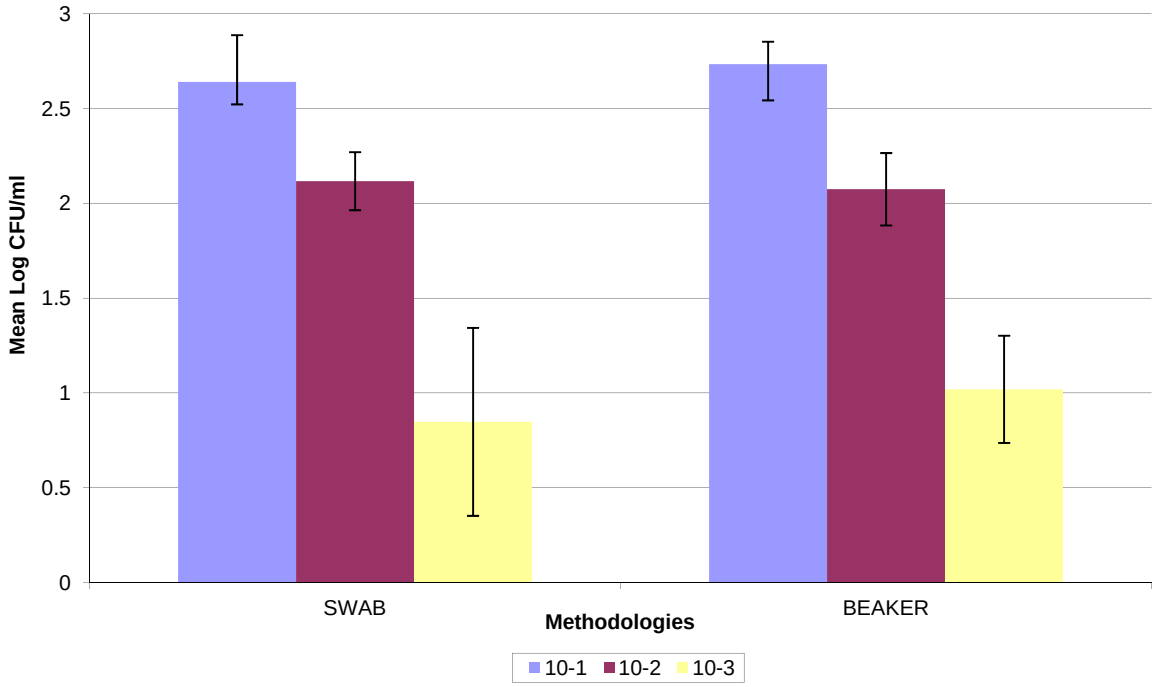



**Figure 2.6.** Sensitivity of the two sampling methods (swab and beaker). All data reported as mean $Log_{10}$ data, and error bars denote confidence intervals (CI) with a confidence level of 95%. Both methods were carried out in triplicate (n=15).

Statistical analysis revealed that there was no significant difference between the two methods (P >0.05).

Examination of the coupons after each sampling method using an inverted microscope revealed that there was little observable difference in organic debris left on the surface of each coupon. The unsampled coupon (control) showed more debris compared with the swab and beaker sampling methods.

### 2.3.2 Gaseous ozone time curves

Fig. 2.7 illustrates the gaseous ozone production inside the bioaerosol chamber, with no increase in RH (in duplicate). The levels of ozone were monitored from the ozone inlet pipe every ten minutes for 2 hours.

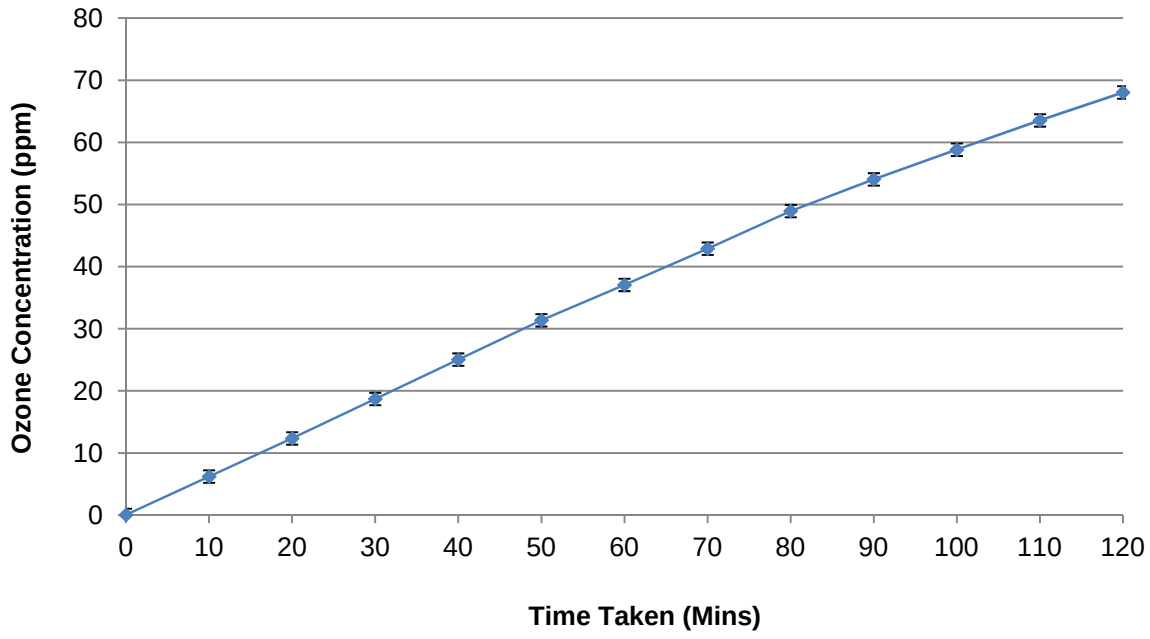



**Figure 2.7.** Gaseous ozone production time curve. There was no increase in RH and was monitored from the centre of chamber using ozone inlet pipe) in duplicate.

After 1 hour, the ozone concentration had increased to 37 ppm and to 68 ppm after 2 hours.

Fig. 2.8 illustrates the gaseous ozone time curves against different variables; with and without increase RH and with and without levels monitored from the centre of the chamber room using pvc tubing.

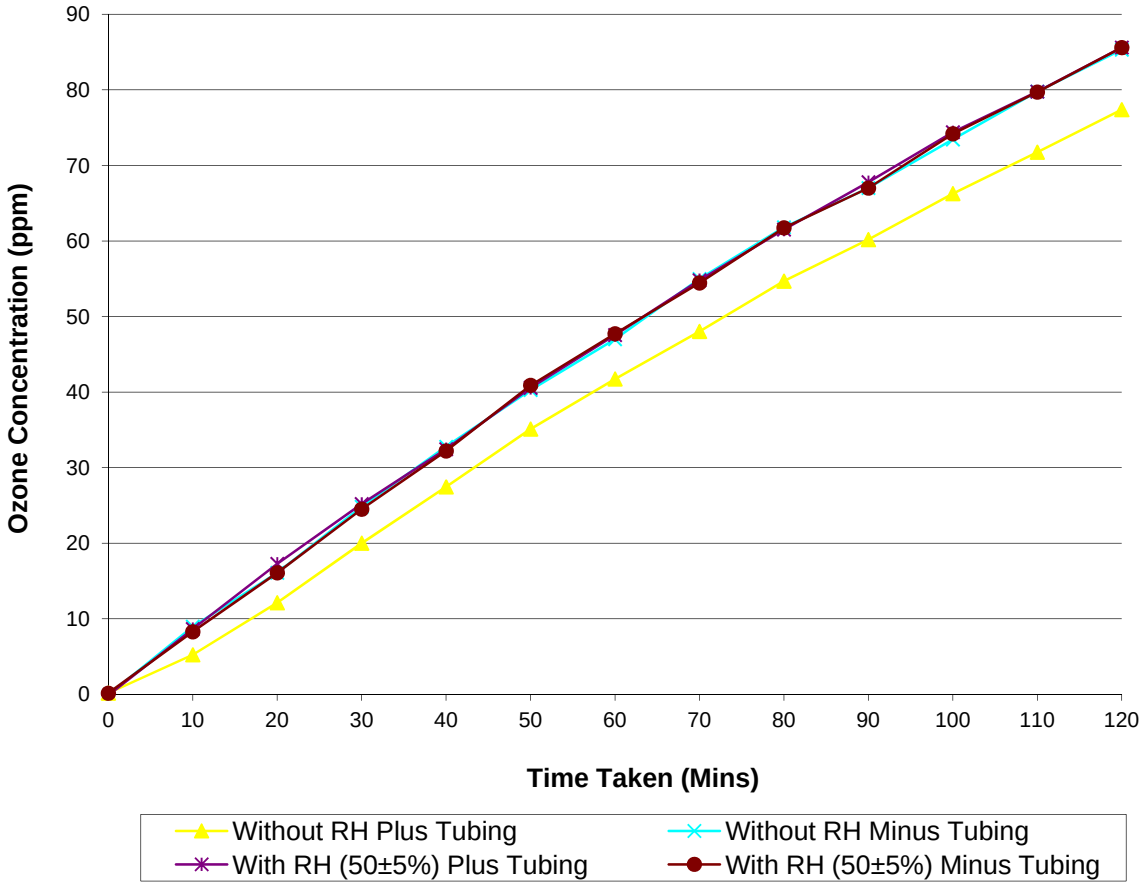



**Figure 2.8.** Gaseous ozone time curve with variables. Gaseous ozone concentration was monitored against four variables: with and without increased RH and with and without the levels being monitored from centre of chamber using pvc tubing (one experiment point).

It was concluded that it would be best suited if the ozone concentration was monitored from the centre of the chamber room where the samples would be positioned (using PVC tubing) and with increased RH of 50±5%. All subsequent gaseous ozone experiments were monitored using these parameters.

### 2.3.3 Isolation and identification of environmental isolates from a food premise

Table 2.3 illustrates the environmental swabs taken at the chosen areas within the food premise and any environmental isolates identified.

**Table 2.3.** Environmental swabs taken at 5 areas within one location in a food premise.

| Area | Colony count | Identification test |
|---|---|---|
| 1 | LSA - NBG<br>PSA - 3<br>VRGBA - 3<br>BP - 2. | <br>Oxidase neg, API 20NE *C. luteola* 99.9%<br>Catalase pos, API staph<br>*S. lentus* 99.9% |
| 2 | LSA -5<br>PSA - 2<br>VRGBA - 2<br><br><br>BP - 4 | API listeria, *L. welshimeri* 99.9%<br>Oxidase neg, API 20NE<br>*Aeromonas hydrophilia caviae 99.9%*<br>Oxidase neg, API 20E, *A. caviae* 56.2%<br>1. Catalase pos, API staph, *S. lentus* 99.9% |
| 3 | LSA - 3<br>PSA - 2<br>VRGBA - 6<br><br><br>BP - 2 | API listeria, *L. welshimeri* 99.9%<br>Oxidase neg, API 20NE<br>*Aeromonas hydrophilia caviae 99.8%*<br>Oxidase neg, API 20NE<br>*Aeromonas hydrophilia caviae 99.9%*<br>Catalase pos, API staph, *S. lentus* 99.9% |
| 4 | LSA - 1<br>PSA - 2<br>VRGBA - 2<br>BP - 2 | API listeria, *L. monocytogenes* 99.9%<br>Oxidase neg, API 20E *E. amnigenus* 63%<br>Oxidase neg, API 20E *E. coli* 54%<br>2. Catalase pos, API staph, *S. xylosus* 99.9% |

*L. monocytogenes* isolate, named L002 was identified by API again (API Listeria, 99.9%) before sending to the Health Protection Agency at Colindale, London, UK for serotyping. The isolate was serotyped as *Listeria monocytogenes* serotype 1/2a.

*P aeruginosa* isolate (C001) was obtained from UWIC's environmental stock cultures (with permission from Dr. Andrew Hall), which had been isolated from water pipes. The isolate was identified by API 20NE, at 98.9%.

### 2.3.4 The effect of gaseous ozone on bacteria surface attached to food grade stainless steel 304 coupons

Figure 2.9 shows that, at 2 ppm ozone concentration, there was a 2.14–2.34 mean log reduction in the survival of *P. aeruginosa* ATCC 154421. Log reduction calculated by log transforms of treated coupons taken away from the log transforms of the untreated (control) coupons.

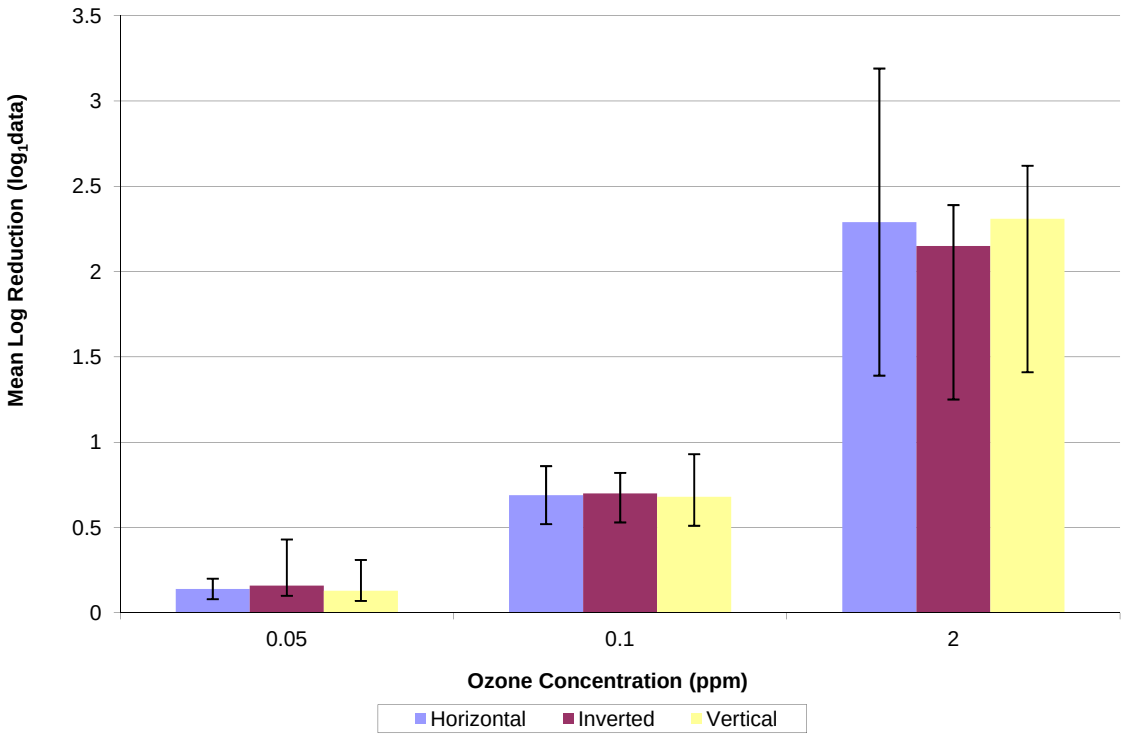



**Figure 2.9.** The effect of gaseous ozone on surface attached *P. aeruginosa* ATCC 15442 on stainless steel food grade (304, finish no. 2b) coupons. Log data denote as mean log reduction. Error bars denote confidence intervals (CI) with a confidence level of 95%. Data obtained from Bailey, 2002 [data not published].

At 0.1 ppm, this log reduction was 0.69–0.71 while at 0.05 ppm, the reduction was 0.14–0.18 log. There was no significant difference (P >0.05) between test and control at 0.05 ppm concentration. However, there were significant differences (P

≤0.05) between all three concentrations, with 2 ppm being significantly better than 0.1 ppm, and 0.1 ppm being significantly better than 0.05 ppm ozone concentration. Figure 2.10 illustrates the effect of gaseous ozone on surface attached environmental *L. monocytogenes* L002.

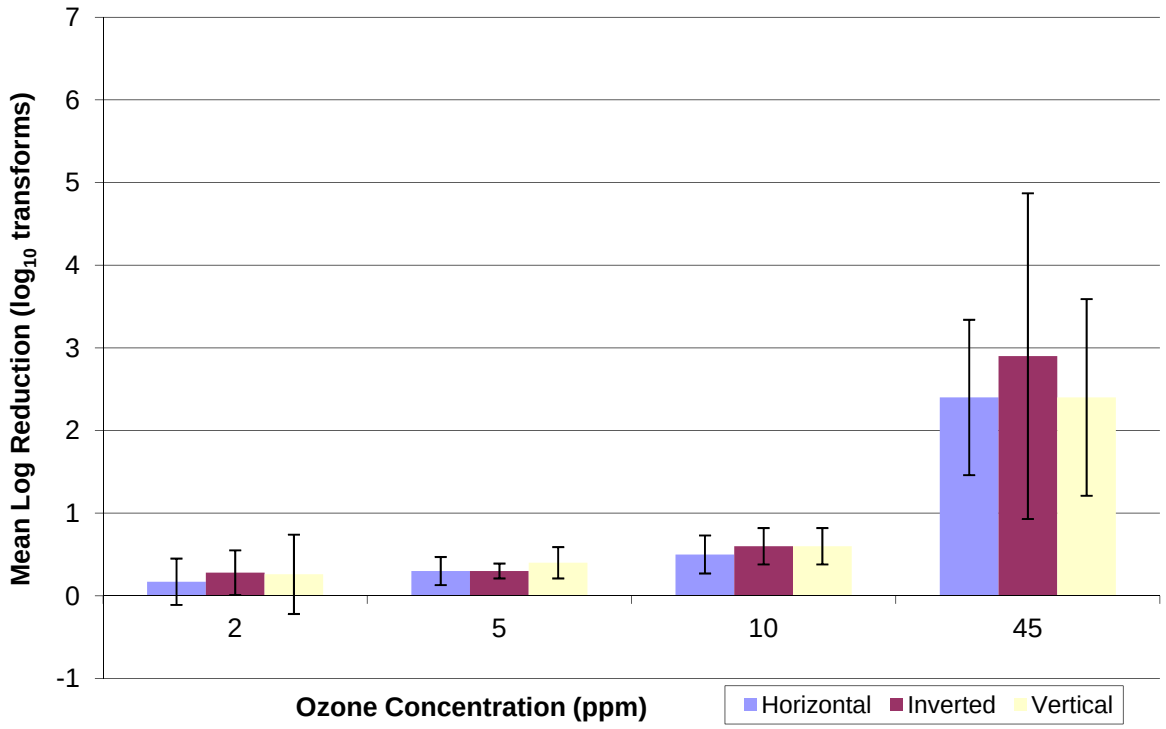



**Figure 2.10.** The effect of gaseous ozone on surface attached *L. monocytogenes* L002 onto food grade stainless steel food grade (304, finish no. 2b) coupons. Log data denotes mean log reduction. Error bars denote confidence intervals (CI) with a confidence level of 95%. Each orientation (n=5) (H, V and I) were performed for each concentration and each concentration was repeated in triplicate.

For the survival of *L. monocytogenes* L002 at 2 ppm (Fig. 2.10), the results showed a 0.17–0.28 log reduction compared to 2.14–2.34 log reduction for *P. aeruginosa* ATCC 15442. *L. monocytogenes* L002 was significantly more resistant (P <0.05) at 2 ppm ozone concentration than *P. aeruginosa* ATCC 15442. The concentration of ozone was, therefore, increased. Ozone concentrations of 5, 10 and 45 ppm were chosen in order to find a concentration that could give a similar log reduction as *P. aeruginosa*. An ozone concentration of 45 ppm for *L. monocytogenes* L002 gave a log reduction of 2.4–2.9, compared to 2.14–2.34 log for *P. aeruginosa* at 2 ppm. There was a significant difference (P

≤0.05) between test and control, and there was a significant difference (P ≤0.05) between the higher concentrations of 10 and 45 ppm for *L. monocytogenes*. Error bars (CI bars) for 45 ppm fall within negative range, as CI bars are more generous and can overlap by as much as 50% and still indicate a significant difference. There were no significant differences (P >0.05) between the different orientations of the coupons for either organism at any ozone concentration. The data suggest, therefore, that the reduction in survivors is concentration dependent and not dependent on surface orientation.

Figure 2.11 illustrates the effect of gaseous ozone at 45 ppm concentration on surface attached *L. monocytogenes* on stainless steel coupons.

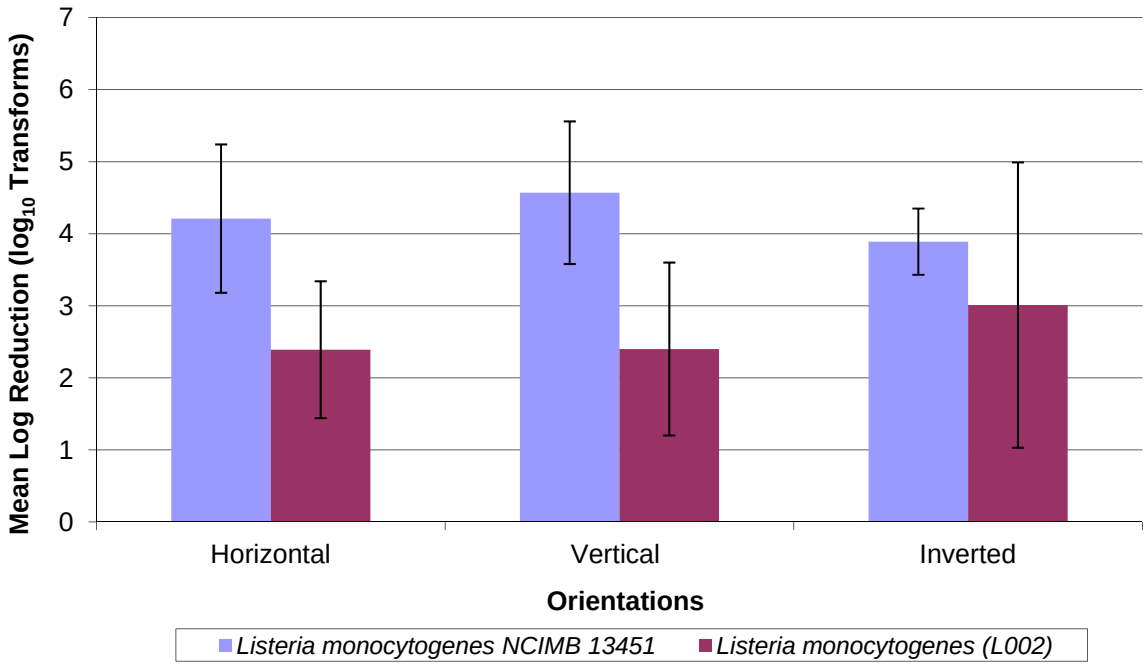



**Figure 2.11.** A comparison of *L. monocytogenes* NCIMB 13451 and environmental *L. monocytogenes* L002 at 45 ppm gaseous ozone, surface attached to stainless steel (304, finish no. 2b) coupons. Error bars denote confidence intervals (CI) with a confidence level of 95%.

The results (Fig. 2.11) showed that the culture collection strain, *L. monocytogenes* NCIMB 13451, gave a mean log reduction of approximately 4, whereas the environmental *L. monocytogenes* L002 gave a mean log reduction of

approximately 2.5. The environmental *L. monocytogenes* (L002) strain seems to be more resistant to 45 ppm gaseous ozone than the culture collection strain (NCIMB 13451). Two-way ANOVA (Minitab version 15, UK) showed that there was no significant difference in the orientation of the coupons ($P > 0.05$) but there was a significant difference between treatment and controls ($P \leq 0.05$). A one-way ANOVA (with Tukey's comparison) (Minitab Version 15, UK), however, showed that there were no significant differences between the two strains of *L. monocytogenes* ($P > 0.05$).

The results from Fig. 2.12 illustrate that there was a higher mean log reduction (3.2 log) for *M. luteus* NCTC 10083 than *L. monocytogenes* L002 (2.15 log) or *P. aeruginosa* NCTC 10299 (2.59 log) at 45 ppm ozone. This implies that *M. luteus* was more sensitive to gaseous ozone than *L. monocytogenes* and *P. aeruginosa*.

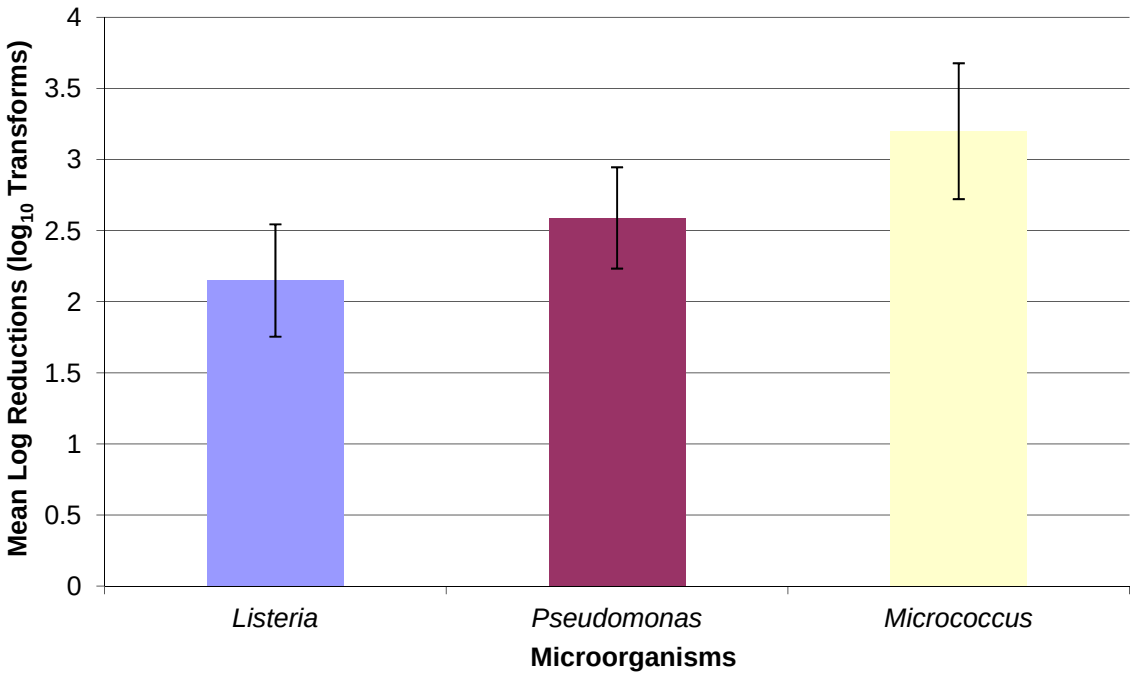



**Figure 2.12.** Mean log reductions for the three surface attached microorganisms to food grade stainless steel (304, finish 2b) coupons treated with 45 ppm gaseous ozone. Error bars denote confidence intervals (CI) with a confidence level of 95%.

A two-way ANOVA (Minitab version 15, Minitab Ltd., UK) showed that there was a significant difference between the three organisms (P ≤0.05) and there was a significant difference between treatment and controls (P ≤0.05).

### 2.3.5 The effect of gaseous ozone on environmental *L. monocytogenes* L002 surface attached to five different surfaces

Fig. 2.13 illustrates that *Listeria monocytogenes* L002 has different recovery rates from different food contact surfaces.

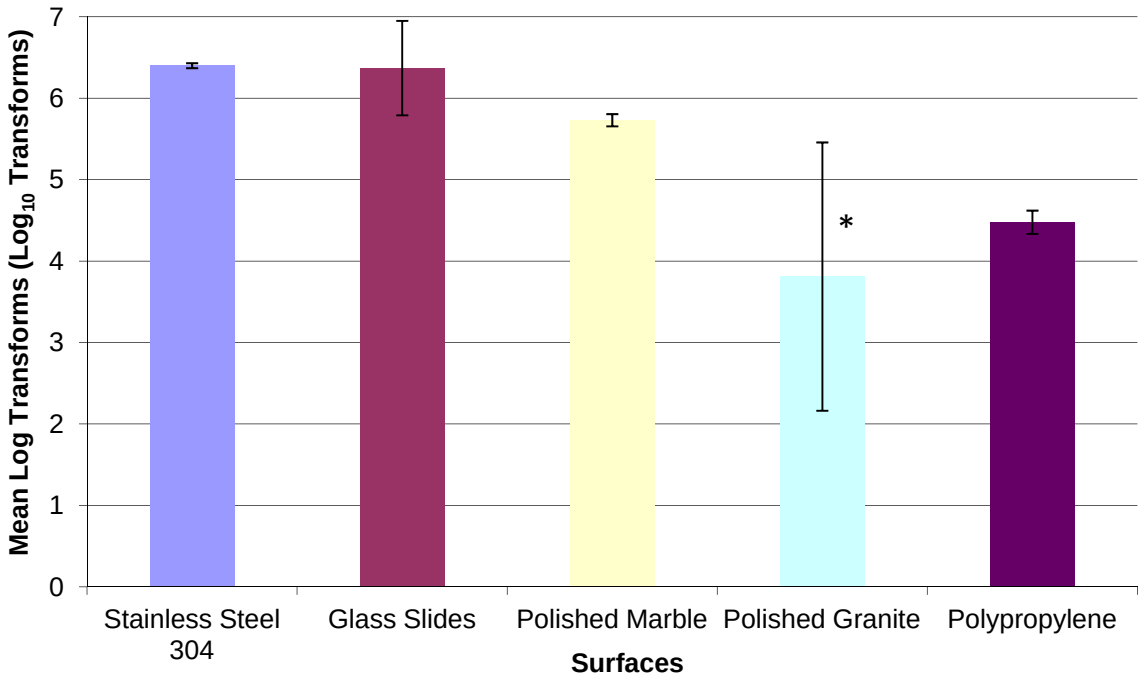



**Figure 2.13.** Mean log data of surface attached *L. monocytogenes* L002 recovered from five different surfaces (control surfaces only). n=15. The asterisk denotes where there was a significant difference. Error bars denote confidence intervals (CI) with a confidence level of 95%.

Fig. 2.13 illustrated that polished granite had fewer cells recovered; demonstrating possible stronger attachment to polished granite compared to the other surfaces and *Listeria monocytogenes* had a lower affinity for stainless steel and glass. Statistical analysis using one-way unstacked ANOVA (Minitab version 15, UK) with Tukey's comparison showed that there was a significant difference (P ≤0.05) between the polished granite surface and the other surfaces. There was no

significant difference (P >0.05) between stainless steel, polypropylene, marble and glass.

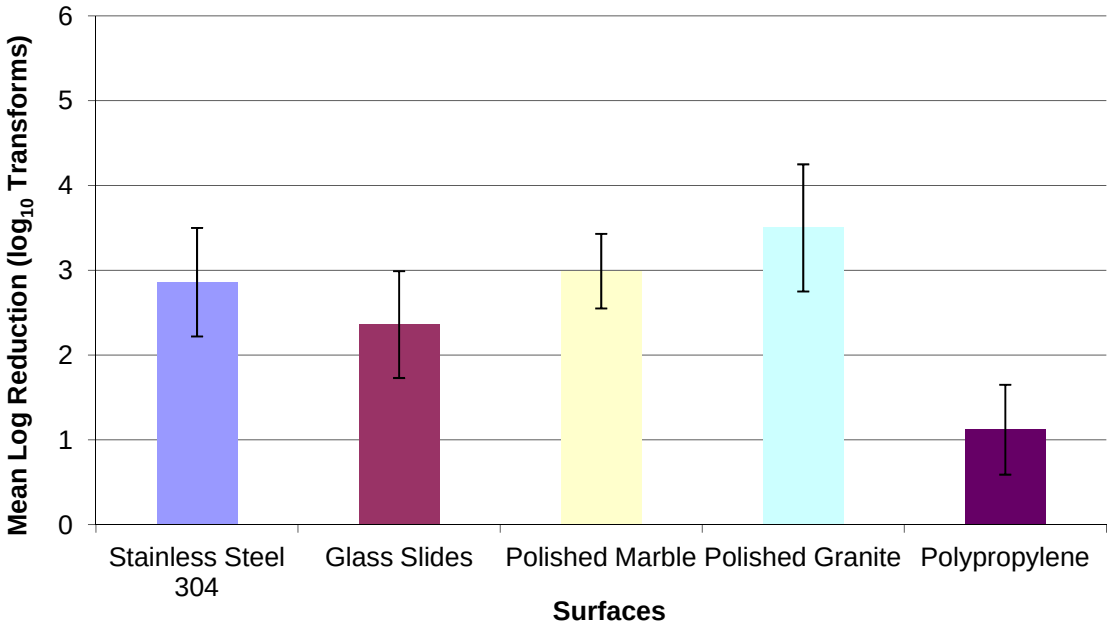



**Figure 2.14.** Mean log reduction of surface attached *L. monocytogenes* L002 on stainless steel (304, finish no. 2b) 25 $cm^2$ coupons, polished marble, polished granite, food grade polypropylene and glass microscope slides at 45 ppm gaseous ozone. Error bars denote confidence intervals (CI) with a confidence level of 95%.

The mean log reduction of L002 on five different surfaces treated with 45 ppm gaseous ozone (Fig. 2.14) revealed that treatment of *L. monocytogenes* on stainless steel, polished marble and polished granite resulted in approximately 2.9, 3 and over 3 log reductions, respectively, compared to polypropylene which gave 1 log reduction. The polypropylene and glass gave lower log reductions (between 1 and 2 $log_{10}$ data) suggesting that these surfaces provide some protection to the cells and that the cells were more resistant to gaseous ozone when on these surfaces. Statistical analysis using a two-way ANOVA (Minitab version 15, UK) with Tukey's comparison showed that there was a significant difference (P ≤0.05) between untreated and test, and between granite, polypropylene and stainless steel. However, there was no significant difference (P >0.05) between glass, polypropylene and marble.

### 2.3.6 The effect of gaseous ozone at 45 ppm on environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 isolates.

**Surface attached microorganisms.** Two microorganisms (*L. monocytogenes* L002 and *P. aeruginosa* C001) were surface attached to 25 $cm^2$ food grade stainless steel 304 coupons which were subjected to gaseous ozone at 45 ppm concentration. Fig. 2.15 illustrates the mean log reduction of environmental surface attached *L. monocytogenes* L002 and *P. aeruginosa* C001 isolates to food grade stainless steel.

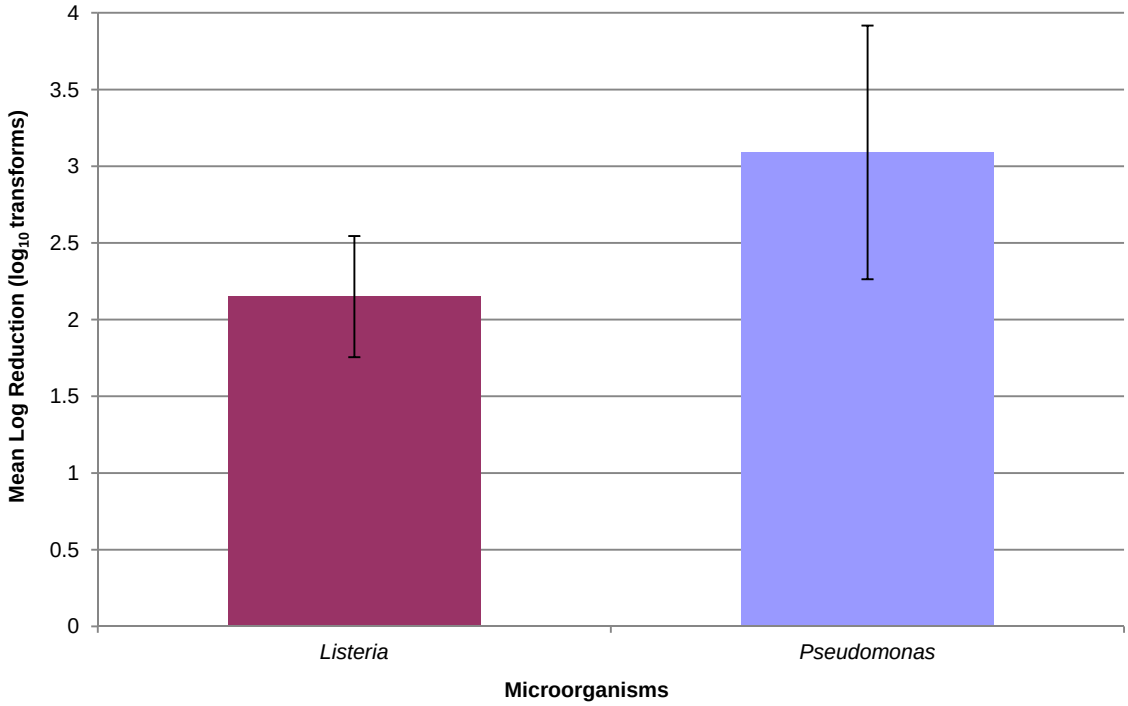



**Figure 2.15.** Mean log reduction of surface attached environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 on food grade stainless steel (304, finish no. 2b) 25 $cm^2$ coupons, at 45 ppm gaseous ozone. Error bars denote confidence intervals (CI) with a confidence level of 95%.

There was a significant difference of one log between the two organisms, with *Pseudomonas* being more sensitive to gaseous ozone than the *Listeria* isolate. Statistical analysis using two-way ANOVA (Minitab version 15, UK) showed that there was a significant difference between both organisms and between untreated (control) and test samples.

**Biofilm microorganisms.** Single-species biofilms of environmental strains *L. monocytogenes* L002 and *P. aeruginosa* C001 were grown on food grade stainless steel, food grade polypropylene and polished granite treated with 45 ppm gaseous ozone for 1 hour. Figure 2.16 illustrates the effect of 45 ppm gaseous ozone against *L. monocytogenes* and *P. aeruginosa* 72 h biofilms.

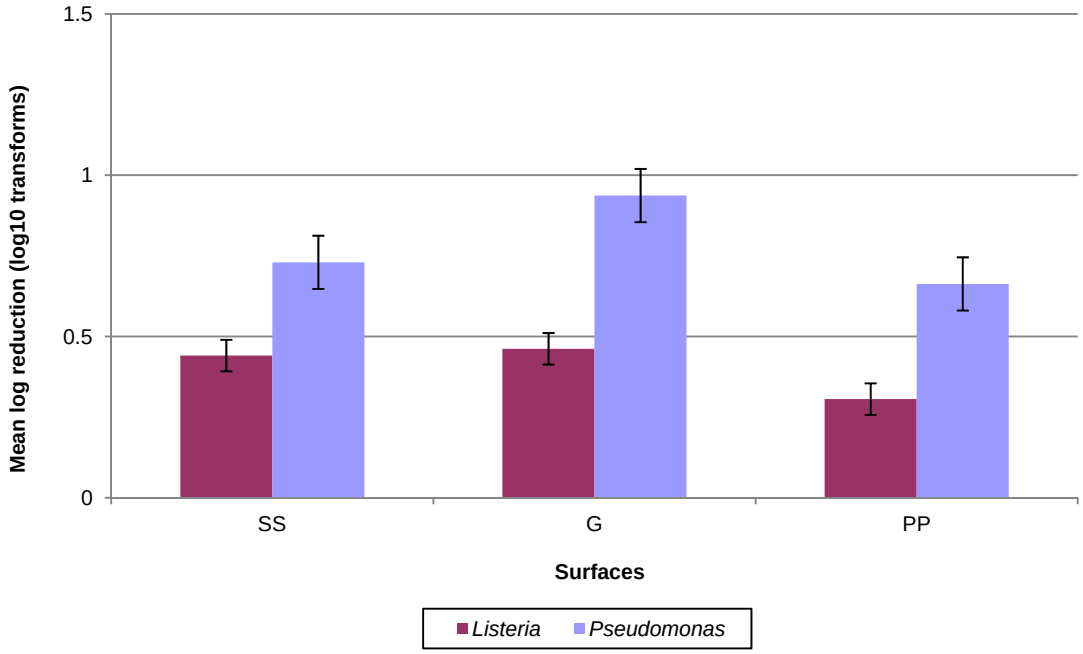



**Figure 2.16.** Mean log reduction of single-species biofilms of environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 on food grade stainless steel (304, finish no. 2b), polished granite and food grade polypropylene 1 $cm^2$ coupons, at 45 ppm gaseous ozone. n=10. *Key: SS – food-grade stainless steel type 304, G – polished granite, PP – food grade polypropylene.*

There was a difference of between 0.3-0.5 log reduction between the two organisms, with *Pseudomonas* being more sensitive to gaseous ozone than the *Listeria* isolate. There was a lower log reduction on the polypropylene surface compared to the other two surfaces, possibly due to the fact that fewer cells can be recovered from this surface. Statistical analysis using two-way ANOVA showed that there was a significant difference ($P \leq 0.05$) between the two organisms and between untreated and test, but there was no significant difference ($P > 0.05$) between surfaces. However, significant differences were found between

untreated and test samples, and there was a significiantly lower log reduction between biofilm and surface attached organisms.

## 2.4 Discussion

The results from the reproducibility test (section 2.2.1) show that all three sampling methods gave reproducible results. Statistical analysis revealed that there were significant differences between the swab and beaker methods, and between the beaker and glass bead methods, but there was no significant difference between the swab and glass bead methods. However, the swab and beaker methods gave more reproducible results than the glass bead method. This was shown by smaller error bars for swab and beaker methods, and larger error bars for glass bead method. The statistical analysis, applied Tukey's test, which is used to compare multiple comparisons. In order to determine which method would be best to use, a sensitivity test was conducted on the swab and beaker methods. From the results and statistical analysis, it was concluded that the swab method would be performed for subsequent experiments. There was little difference between the performances of the two methods, but the swab method was chosen for convenience over the beaker method. The swab method was cheaper, easier and quicker to perform.

Giaouris *et al*. (2005) investigated biofilm formation of *S. enterica* serovar *enteritidis* PT4 on stainless steel surfaces. The results indicated that traditional vortexing with beads did not remove biofilm cells completely from the stainless steel surface. Conductance measurements were performed which were capable of detecting down to a single viable cell and provided more sensitive testing.

These measurements were performed as an aid by indirectly measuring the attachment and biofilm formation of the organism to stainless steel by their metabolic activity. This method allowed the detection of cells that had remained on the surface of the stainless steel even after vortexing with beads. So it is necessary to validate the appropriate sampling method in order to reduce bias between recovered cells and those retend.

Time curves of gaseous ozone delivery were investigated (section 2.2.3) by measuring the time course of gaseous ozone production from the centre of the chamber using ozone inlet pipe. It revealed that, after 1 hour, the ozone concentration had increased to 32.4 ppm and by 2 hours it had increased to 58 ppm. The time course of gaseous ozone production under certain parameters was studied and revealed that the best parameters to use would be high RH of >50±5% and monitoring the ozone concentration of the centre of the chamber, where the samples would be positioned, instead of monitoring the main chamber air from the ozone inlet pipe. In order to maintain 45 ppm for one hour, the ozone generator was switched on and left to get up to concentration, with the baffle door open. Once up to concentration level, the baffle door was closed, and the concentration was monitored using API and handheld monitors throughout the time period.

The effect of gaseous ozone on *L. monocytogenes* L002 and *P. aeruginosa* ATCC 15442, surface attached to food grade stainless steel was investigated (section 2.2.4). The data were dependent on ozone concentration and not dependent on surface orientation. The results from this study suggest that *P. aeruginosa* (gram negative) is more sensitive to ozone than *L. monocytogenes* (gram positive). This

could be due to differences in cell wall structure and morphology, which could be important factors linked to the resistance of microorganisms to ozone. The fact that the *Listeria* isolate was an environmental strain, whereas the *Pseudomonas* isolate was a culture collection strain (ATCC 15442) could be another reason why *Listeria* appears to be more resistant to ozone than *Pseudomonas*.

The effect of treating *L. monocytogenes* NCIMB 13451 and *L. monocytogenes* L002 with 45 ppm gaseous ozone concur with the results of treating *P. aeruginosa*, *M. luteus* and *L. monocytogenes* isolates, as the environmental *L. monocytogenes* L002, serotype 1/2a isolate was significantly more resistant than the culture collection *L. monocytogenes* NCIMB 13451 strain to gaseous ozone.. The differences between environmental and collection strains in terms of their genetic characteristics could be the reason why the environmental *L. monocytogenes* isolate L002 serotype 1/2a, appeared to be more resistant to ozone than the culture collection strain of *L. monocytogenes* NCIMB 13451.

The effect of treating *P. aeruginosa*, *M. luteus* and *L. monocytogenes* with 45 ppm gaseous ozone for 1 hour was investigated (section 2.2.4). The results showed that *M. luteus* was significantly more sensitive to ozone at 45 ppm than *L. monocytogenes* and *P. aeruginosa,* whereas *P. aeruginosa* was significantly more sensitive than *L. monocytogenes*. The results from this study suggest that morphology (cocci being more sensitive than rods) as well as cell wall structure may affect the ability of microorganisms to resist gaseous ozone treatment. These results support work by Russell (2003b). Neither the fact that environmental nor collection strains were chosen affected the sensitivity of the organisms to ozone, but the nature of the organisms i.e. the chemical composition of outer cellular

layers seemed to play an important factor, with less susceptibility to bacterial spores>gram-positives>fungi>gram-negatives>cocci>lipid enveloped viruses being more susceptible. Restaino *et al*. (1995) investigated the efficacy of ozonated water against four gram-positive and four gram-negative, including *P. aeruginosa* ATCC 15442, *E. coli* ATCC 25922, *S. aureus* ATCC 6538, and *L. monocytogenes* 4b, as well as *Candida albicans* ATCC 22572 and *Aspergillus niger*. They observed more than 5 log reduction in the survival of *S. typhimurium* and *E. coli* at 20 ppm ozone concentration. *Listeria monocytogenes* was significantly more sensitive to ozonated water treatment in contrast to *S. aureus* or *Enterococcus faecalis*.

The effect of *L. monocytogenes* L002 surface attached to different surfaces and treated with 45 ppm gaseous ozone for one hour was investigated (section 2.2.5). *L. monocytogenes* has different adhesion characteristics when attached to different surfaces (Borucki *et al*., 2003; Frank and Koffi, 1990; Lee and Frank, 1991). There was a higher recovery of surface attached cells from stainless steel, glass and polished marble (between 5-6 $\log_{10}$ data), compared to approximately 4 $\log_{10}$ data of cells recovered from the granite and polypropylene surfaces. *Listeria monocytogenes* seems to show a higher affinity towards stainless steel, polished marble and glass. Silva and others (2008) reported that *L. monocytogenes* attached to all surfaces studied, but to different extents. The organism adhered more tightly to granite and marble represented by higher log numbers (6-6.5 log data), followed by stainless steel and glass (5.5-6 log data). The surfaces with the lowest number of adhered cells had a higher percentage of viable cells. In contrast, Saá *et al*. (2009) reported *Listeria* adhesion was stronger to PP than to SS. Previous authors have suggested that *L. monocytogenes* does not grow and

form biofilms well on glass surfaces (Sasahara and Zottola, 1993), however its ability to grow well on stainless steel surfaces compared to other surfaces is well documented (Beresford *et al*., 2001; Herald and Zottola, 1988; Hood and Zottola, 1997a, b; Kim and Frank, 1994; Mafu *et al*., 1990a; Mai *et al*., 2006; Norwood and Gilmour, 1999; Poimenidou *et al*., 2009; Rodriguez *et al*., 2008; Silva *et al*., 2008; Smoot and Pierson, 1998a, b; Teixeira *et al*., 2008; Tresse *et al*., 2007). There was a significant difference between granite and the other surfaces. When treated with 45 ppm ozone, more cells were recovered from stainless steel, polished marble and polished granite, giving 2.9, 3 and more than 3 log reductions, respectively. The results suggest that the cells were less protected or adhered more strongly to these three surfaces. This is due to differences in surface charge, hydrophobicity and roughness (Szlavik *et al*., 2012).

The effect of gaseous ozone at 45 ppm on surface attached environmental isolates of *L. monocytogenes* L002 and *P. aeruginosa* C001 on 25 $cm^2$ food grade stainless steel coupons illustrated (section 2.2.6) that *P. aeruginosa* was significantly more sensitive than *L. monocytogenes* by one log reduction. A two-way ANOVA revealed that there was a significant difference between the two organisms and between test and untreated samples. Biofilm microorganisms (single-species, 72h static biofilms) on food grade stainless steel, polished granite and food grade polypropylene revealed that *P. aeruginosa* was more sensitive than *L. monocytogenes* isolate to the gaseous ozone treatment by between 0.3-0.5 log reductions depending on the surface to which they were adhered. There was a higher log reduction from both stainless steel and granite surfaces compared to food grade polypropylene. Mafu and others (1990b) however, reported that *L. monocytogenes* was more resistant to sanitizing agents when

attached to PP and rubber than to SS or glass. In 1991, Mafu and others revealed that polypropylene and rubber surfaces have lower energy surfaces than glass or stainless steel. A two-way ANOVA revealed there was a significant difference between the two organisms and between untreated and test samples, but there was no significant difference between surfaces. There was a marked difference between surface attached and biofilm adhered organisms, with surface attached organisms being more sensitive with an increased log reduction, compared to the biofilm adhered organisms. The protective ability of biofilms and the production of EPS aid the bacteria's ability to survive (McSwain *et al*., 2005).

It has been hypothesised that the mechanism of *L. monocytogenes* L002 isolate's resistance to gaseous ozone treatment could be due to the fact that the strain studied was isolated from a high-care food processing plant, where stringent validated cleaning protocols, based on oxidising agents (such as hydrogen peroxide, peracetic acid (PAA) and chlorine dioxide), had been put in place. This could have potentially led to its apparent enhanced resistance (high tolerance) against gaseous ozone at such a high concentration (Russell, 2003b). The reduction in levels of surface attached *L. monocytogenes* and *P. aeruginosa* by gaseous ozone is concentration dependent and not dependent on surface orientation. This is advantageous over traditional fogging methods that are used in the food industry, where vertical and inverted surfaces are less exposed to the fogging agent (Burfoot *et al*., 1999; Pascual *et al*., 2007). Gaseous ozone has shown its potential usage as a terminal sanitizer for environmental surfaces in the food industry (Moore *et al*., 2000).

# Chapter 3. Biofilm formation

## 3.1 Introduction

Bacteria are able to attach to a variety of surfaces in natural, industrial and medical environments and can develop into biofilms (Donlan, 2002). Biofilms consist of a complex consortium of microorganisms within an extracellular matrix, constructed mainly of water and various polymers (polysaccharides and glycoproteins) (Christensen and Characklis, 1990). Biofilms have a heterogeneous structure consisting of microcolonies (Costerton *et al*., 1995).

Biofilms in the food processing environment can result in contamination of food by spoilage organisms or pathogens. The food processing environment provides a variety of factors that encourage the formation of biofilms (Hood and Zottola, 1995), such as the presence of food debris as a nutrient substrate, surfaces that facilitate attachment and the presence of moisture (Holah *et al*., 2004). Adhered bacteria are known to be more resistant to disinfection and biocides than their planktonic counterparts (Chavant *et al*., 2004). Biofilms are also highly tolerant to biocides (Costerton *et al*., 1995; Daş *et al*., 1998) and have proved to be difficult for the food industry to control and remove them from food contact surfaces. The consequences of biofilm communities developing in food processing environments are significant (Notermans *et al*., 1991; Hood and Zottola, 1995). Many bacteria are not necessarily in direct contact with the food product, but some bacteria will thrive on food processing equipment, leading to biofouling of those surfaces, which can lead to detachment of portions of the biofilm into food products causing contamination and spoilage problems. There are many sources of contamination

in the food processing environment, including water, personnel, pests and airborne microflora from the food processing plant. The accumulation of food residues on inert surfaces, such as floors, ceilings, walls, drains and conveyor belts (Carpentier and Cerf, 1993) are sources of continuous contamination. Stringent cleaning regimes are a necessity and good manufacturing practices (GMP) and Hazard Analysis Critical Control Point (HACCP) systems must be in place to ensure the quality and safety of the food product (Tuompo *et al*., 1999).

*L. monocytogenes* is ubiquitous in the environment and can be found on many environmental surfaces. This organism is able to grow at refrigeration temperatures, which means its presence in food premises becomes a major concern for food manufacturers, especially in high-care facilities where ready-to-eat foods are produced. Outbreaks of listeriosis usually originate from the consumption of contaminated food products. There has been an increase in the incidence of listeriosis in the over 65 age group in recent years reported in England and Wales (Gillespie *et al*., 2006; Cairns and Payne, 2009). This bacterium can form microcolonies on equipment surfaces or other areas of the processing plant, which in turn form biofilms. *Listeria monocytogenes* can adhere rapidly to many inert surfaces found in the food industry such as polypropylene (PP) and stainless steel (SS) (Frank and Koffi, 1990).

The mechanisms by which food pathogens (including *L. monocytogenes*) can survive these extreme conditions of physical, chemical and oxidative stress are not fully understood (Hellion *et al*., 2003). It is known however, that adherence to surfaces (Lee and Frank, 1991) or forming biofilms (Norwood and Gilmour, 2000) confers protection against these stresses. Although ensuring effective measures

for controlling *Listeria monocytogenes* has been a constant challenge for the food industry, current cleaning and disinfection practices and personnel hygiene have been able to sufficiently maintain *L. monocytogenes* at low levels (Holah *et al*., 2004). Persistent strains have, however, been found in high-care production areas and are known to remain there over a considerable period of time (sometimes years) (Costerton *et al*., 1999). Some researchers have considered the relationship between persistent strains with enhanced adherence and biofilm formation (Norwood and Gilmour, 1999; Lundén *et al*., 2000; Borucki *et al*., 2003). Djordjevic *et al*. (2002), however, found that there was no relationship between environmental persistence and their ability to form biofilms. The presence of *Pseudomonas* spp., in food processing environments is said to be indicative of the presence of biofilms and that food pathogens such as *Bacillus cereus*, *L. monocytogenes*, *Escherichia coli* O157, *Salmonella* spp., and *Staphylococcus aureus* may be present (Peters *et al*., 1999).

Coaggregation is the process whereby genetically distinct bacteria are able to attach to one another *via* specific molecules (Rickard *et al*., 2003a). This process can be observed among bacteria found in the human urogenital tract, human oral cavity, mammalian gut, and potable water supply systems (Handley *et al*., 2001 cited in Rickard *et al*., 2003a). Coaggregation is a widespread phenomenon that plays an important role in the development of multi-species biofilms. Multi-species biofilms have complex physiology and metabolism (Gilbert *et al*., 2002) and it is postulated that such biofilms develop on surfaces through a series of steps of adhesion and multiplication. The first organisms are known as primary colonizers such as *P. aeruginosa* and primary colonisation is initiated through specific and non-specific physico-chemical interactions (Dang and Lovell, 2000; van

Loosdrecht *et al*., 1990). These primary colonizers then grow on the substratum to form microcolonies. As the biofilm matures, it is subjected to environmental changes, allowing secondary colonizers such as poor biofilm formers to attach to the primary colonizers, forming a multi-species community of different bacteria. Within this process of coaggregation, planktonic cells are also able to specifically adhere to biofilm cells by a process of co-adhesion (Busscher *et al*., 1995). There is little evidence, however, that the presence of biofilms in high-care food processing operations causes any risk to the consumer (unless food pathogen is present), but as a reminder that the food industry needs to impose constant cleaning measures.

The viability of bacterial cells can be assessed using LIVE/DEAD *Bac*Light bacterial viability kits. These provide sensitive, single-step, fluorescence-based assays, which can be viewed using a fluorescent microscope or for use in quantitative assays involving a fluorescence microplate reader, flow cytometer or fluorometer. The bacterial viability kits employ two nucleic acid dyes which differ in their ability to penetrate healthy bacterial cells – the green fluorescence SYTO 9® stain and propidium iodide that fluoresces red. When SYTO 9® stain is used alone, both live and dead cells are labelled. However, when both stains are used together, the propidium iodide stain will only penetrate bacterial cells with damaged membranes and will therefore reduce SYTO 9® fluorescence. So, live bacteria with intact membranes fluoresces green, while dead cells with damaged mambranes will fluoresce red. Also an intermediate state can be observed (Berney *et al*., 2007). The kits versatility means that live and dead bacteria cells in mixed populations can be distinguished and viewed either separately or

simultaneously by fluorescence microscopy (Molecular Probes, Inc. 2004. MP07007).

The aim of the study was to investigate coaggregation and biofilm formation capabilities of environmental isolates of *P. aeruginosa* and *L. monocytogenes* compared with collection strains and to examine the state of the bacteria *in situ*. The objectives were:

1. To perform a coaggregation assay and microtitre plate biofilm formation assays in order to determine whether or not the chosen environmental isolates would form a biofilm together.
2. To perform *Bac*light Live/dead staining in order to examine the state of the bacteria *in situ*, before and after treatment with gaseous ozone.

## 3.2 Materials and Methods

*Bacterial strains and culture preparation.* The four organisms studied were assigned a code letter; *L. monocytogenes* NCIMB 13451 (A), *L. monocytogenes* L002 (B), *P. aeruginosa* NCTC 10299 (C) and *P. aeruginosa* C001 (isolated from water pipework) (D). Stock cultures were stored on cryobeads (TSC, UK) at -80°C, and were taken out and used when needed. Combinations of the four microorganisms (AB, AC, AD, BC, BD and CD) were designed to give dual biofilms. Overnight cultures were prepared by inoculating single cryobeads in 10 ml tryptone soya broth (TSB; Oxoid, UK) and were incubated at 37°C without shaking.

### 3.2.1 Visual coaggregation assay

The method was taken from Cisar *et al*., (1979), which had been followed by Rickard *et al*. (2003b). The four organisms were grown in a complex medium as described by Maryanski and Wittenberg (1975) consisting of a mixture of tryptone, yeast extract, Tween 80 and glucose (0.2 %) buffered to pH 7.5 with $K_2HPO_4$. Cryobeads of each organism were inoculated separately into 100 ml of complex media. Cultures were incubated at 37°C without shaking and the cells were harvested at mid-exponential growth phase (which is equivalent to an OD of 1.0 at 660 nm). Growth curves of each microorganism at certain time points were taken in order to estimate mid-exponential phase (above OD 1.0) of growth. This optical density resulted in approximately $10^7$ – $10^8$ CFU/ml, (100 µl of each microorganism were inoculated onto TSA spread plates and incubated at 37°C for 48 h for total cell counts (CFU/ml) of each bacterial suspension for OD 1.0 and OD 2.0), and was determined with a spectrophotometer (CECIL model 1011, CECIL Instruments, Cambridge, UK). Harvested cells were prepared for coaggregation assays by three cycles of centrifugation (at 10, 000 x *g* for 10 mins at 4°C) (DuPont Sorvall Superspeed RC-5B refrigerated centrifuge, USA; supplied by Thermo Fisher Scientific, UK). The pellet was suspended in coaggregation buffer (calcium chloride (1 x $10^{-4}$ M), magnesium chloride (1 x $10^{-4}$ M), and sodium chloride (0.15 M) (Fisher Scientific UK, UK) dissolved in 0.001 M of Tris (hydroxymethyl) aminomethane (Fisher Scientific UK, UK) and adjusted to pH 8.0. Cells were stored in the buffer and used the following day. Bacterial suspensions were adjusted to a turbidity of approximately 1.9 OD (approximately 260 Klett units) at 660 nm and contained $10^9$ - $10^{10}$ CFU/ml.

The coaggregation reactions were performed in sterile Khan tubes (Fisherbrand, Fisher Scientific UK, UK). Equal volumes (0.2 ml) of each suspension were mixed for 10 sec using a vortex mixer, and allowed to stand for 2 h, mixed again and scored for the degree of visual coaggregation using the criteria of Cisar *et al*. (1979) (Table 3). The Khan tubes were allowed to stand overnight at room temperature. The tubes were mixed the next day for 10 sec and were scored again. Tubes containing each cell suspension (0.2 ml) and 0.2 ml of buffer were set up as controls.

Scores for degree of coaggregation ranged from 0 – 4+ following a certain set of criteria; shown in Table 3.1.

**Table 3.1.** Criteria for scoring the degree of coaggregation in visual coaggregation assay (Cisar *et al*., 1979).

| Degree of coaggregation | Scores |
|---|---|
| no visible aggregates in the cell suspension | 0 |
| small uniform coaggregates in suspension | 1+ |
| definite coaggregates easily seen but suspension remains turbid without immediate settling of coaggregates | 2+ |
| large coaggregates which settle rapidly leaving some turbidity in supernatant | 3+ |
| clear supernatant and large coaggregates which settle immediately | 4+ |

Self-aggregation or autoaggregation was assessed by preparing Khan tubes with each isolate on its own. If autoaggregation occurred, it was scored using the same criteria and this score was then deducted from the coaggregation score at 24 h (Rickard *et al*., 2004).

### 3.2.2 Microtitre plate biofilm formation assay

The microtitre plate biofilm production assay first described by Christensen *et al*. (1985); modified by O'Toole and Kolter (1998) and others (Djordjevic *et al*., 2002; Moltz and Martin, 2005) was further adapted from the original method. 100 µl of each of the overnight cultures (two *Listeria* strains and two *Pseudomonas* strains) prepared in 10 ml TSB at 37°C without any shaking, were transferred to 10 ml of fresh TSB (1/100 dilution), mixed and then transferred (100 µl per well, approximately $10^8$ CFU/ml) to 96-well polyvinyl chloride (PVC) microtitre well plates (Nunclon™ surface, Nunc Inc, USA; supplied by Fisher Scientific, UK, Leicestershire, UK). For the single biofilms, eight wells (in duplicate) contained sterile TSB as negative controls, and eight wells for each culture (in duplicate on each plate) were prepared. Duplicate plates were incubated at 32°C (24 h and 48 h) and 37°C (48 h and 72 h). For the dual-species biofilms, duplicate plates of eight wells containing sterile TSB as controls and eight wells for each culture were prepared and incubated as above.

After incubation, the supernatant was removed and 200 µl of 2% glutaraldehyde (Sigma Aldrich, UK) / 0.1 M phosphate buffer (Fisher Scientific UK, UK) was added to fix the cells for 5 mins. This was followed by 2 x 200 µl washes with phosphate buffer saline (PBS) (Oxoid, UK) to remove loosely attached cells. The wells were then stained with 200 µl of 1% crystal violet (CV) (Prolab, UK) for 5

mins. Staining was followed by a second series of five 200 µl PBS washes. After washing, the plates were then air dried and then 200 µl of 95% ethanol was added to each well for de-staining for 5 mins. The concentration of CV was determined by measuring the optical density at 570 nm (CV-$OD_{570}$ value) using a Dynex plate reader (MRX$^{e}$ Revelations, Dynex Technologies Ltd, West Sussex, UK). Each organism was repeated in duplicate on each plate and each plate was carried out in duplicate. Standard deviations were calculated using Excel (Microsoft) and statistical analysis using one-way and two-way ANOVA were performed using Minitab version 15 (Minitab Ltd., Coventry, UK).

### 3.2.3 LIVE/DEAD *Bac*light viability staining kit

LIVE/DEAD® *Bac*Light™ Kit (L7012) (Molecular Probes; Invitrogen) was used to stain live and dead cells. The solution was prepared according to manufacturer's instructions. (by combining equal volumes of Component A and Component B in a microfuge tube and mixed thoroughly. A 3 µl volume of the dye mixture (i.e. A + B) was then added to 1 ml of an undiluted overnight bacterial suspension (*L. monocytogenes* L002), which was thoroughly mixed and incubated at room temperature in the dark for 15 minutes. Bacterial staining was assessed by trapping 5 µl of the stained bacterial suspension between a slide and a 22 x 22 mm coverslip. The slides were immediately observed under an epifluorescence microscope (Olympus BX40 and a Retiga 1300 camera (Qimaging)). The live cells fluoresced green at 510 – 540 nm wavelength and the dead cells fluoresced red at 620 – 650 nm wavelength, and both could be viewed at the same time.

This method was adapted in order to treat surface attached *L. monocytogenes* L002 to glass microscope slides (76 x 26 mm, 1.2 mm thickness; Menzel-Gläser®,

Thermoscientific, UK) with gaseous ozone at 10 ppm. 100µl of an overnight suspension was inoculated onto five glass microscope slides (untreated) and five slides which were ozonated with gaseous ozone at 10 ppm for 1 hr (treated). 5 µl of dye mixture was placed on top of inoculum and covered immediately with a coverslip (24 x 40 mm, 0.5 mm thickness: Menzel-Gläser®, Thermoscientific, UK), incubated in a dark 20°C incubator for 15 minutes and viewed in the same way.

## 3.3 Results

### 3.3.1 Visual coaggregation assay

Growth curves of the four microorganisms in order to determine mid-exoponential growth phase, which was equilivalent to OD 0.6 at absorbance wavelength of 600nm.

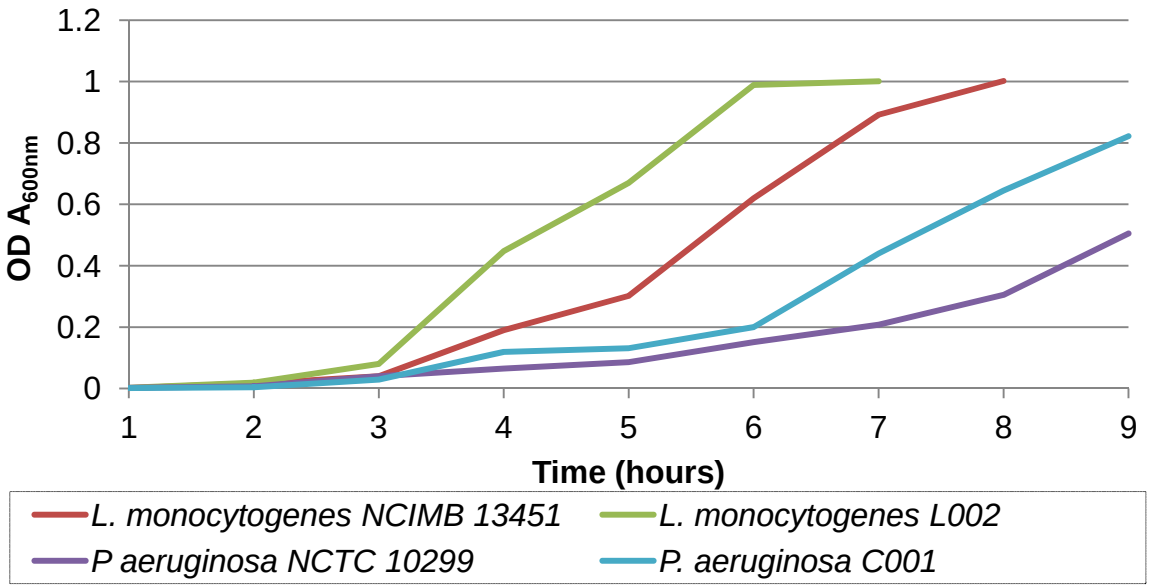



**Figure 3.1.** Growth curves of the four microorganisms.

There were six test combinations consisting of; AB, AC, AD, BC, BD, and CD, and four controls (containing the single organisms only). The scores were taken after 2h and again after 24 h and are shown in figure 3.2. As there was no

coaggregation after 2 h, the tubes were left at room temperature overnight and scored at 24 h. A final reading was taken after 48 h.

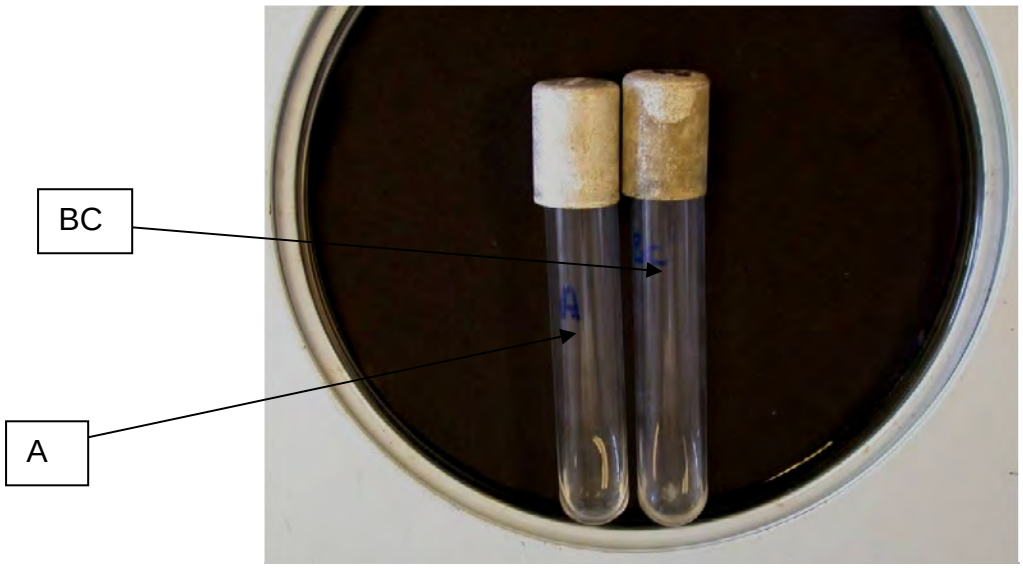



**Figure 3.2.** Visual coaggregation assay. Tube labelled A is the control tube and tube labelled BC is the test tube showing large coaggregates settling at the bottom of the tube.
*Key: A = L. monocytogenes NCIMB 13451 only, and B = L. monocytogenes L002 and C = Pseudomonas aeruginosa NCTC 10299.*

**Table 3.2.** Visual coaggregation assay scores. Final coaggregation scores read at 48 h. Auto-aggregation scores were deducted from coaggregation scores after 24 h.

| Microorganisms | Scores of Coaggregation | |
|---|---|---|
| | 24 h | 48 h |
| *L. monocytogenes* NCIMB 13451 (A) | 0 | 0 |
| *L. monocytogenes* L002 (B) | 1 | 1 |
| *P. aeruginosa* NCTC 10299 (C) | 1 | 1 |
| *P. aeruginosa* C001 (D) | 1 | 1 |
| **AB** | **1** | **0** |
| **BC** | **4** | **2** |
| **BD** | **4** | **2** |
| **AC** | **4** | **3** |
| **AD** | **4** | **3** |
| **CD** | **3** | **1** |

*Key: AB = L. monocytogenes NCIMB 13451 with L. monocytogenes L002, CD = P. aeruginosa NCTC 10299 with P. aeruginosa C001, BC = L. monocytogenes L002 with P. aeruginosa NCTC 10299, BD = L. monocytogenes L002 with P. aeruginosa C001, AC = L. monocytogenes NCIMB 13451 with P. aeruginosa NCTC 10299 and AD = L. monocytogenes NCIMB 13451with P. aeruginosa C001.*

There was self-aggregation (autoaggregation) with organisms B, C and D, but not with organism A. The scores from the autoaggregation were deducted from the coaggregation scores (after 24 h) resulting in final coaggregation scores (after 48 h) (Table3.2).

From Table 3.2, the environmental *Listeria* (L002) isolate with both *Pseudomonas* strains produced lower visual coaggregation scores of 2, compared to the collection *Listeria* NCIMB 13451 isolate which produced visual coaggregation scores of 3. All the combinations of *Listeria* and *Pseudomonas* strains produced higher visual coaggregation scores compared with *Listeria* alone (coaggregation score of 0).

### 3.3.2 Microtitre plate biofilm formation assay.

*Single species biofilm formation assay.* The results showed that at 37°C (48 h or 72 h), the environmental isolates of both *Listeria* spp. and *Pseudomonas* spp. produced more biofilm than their culture collection strain counterparts. It was also clear that with increased time all four organisms produced more biofilm. The same was also true for 32°C (24 h and 48 h), but to a lesser extent. It was noted that *P. aeruginosa* C001 (environmental) at 37°C at both 48 h and 72 h, produced a green pigment (Fig. 3.3 a and b). *P. aeruginosa* C001 (environmental isolate) did not, however, produce a green pigment at 32°C.

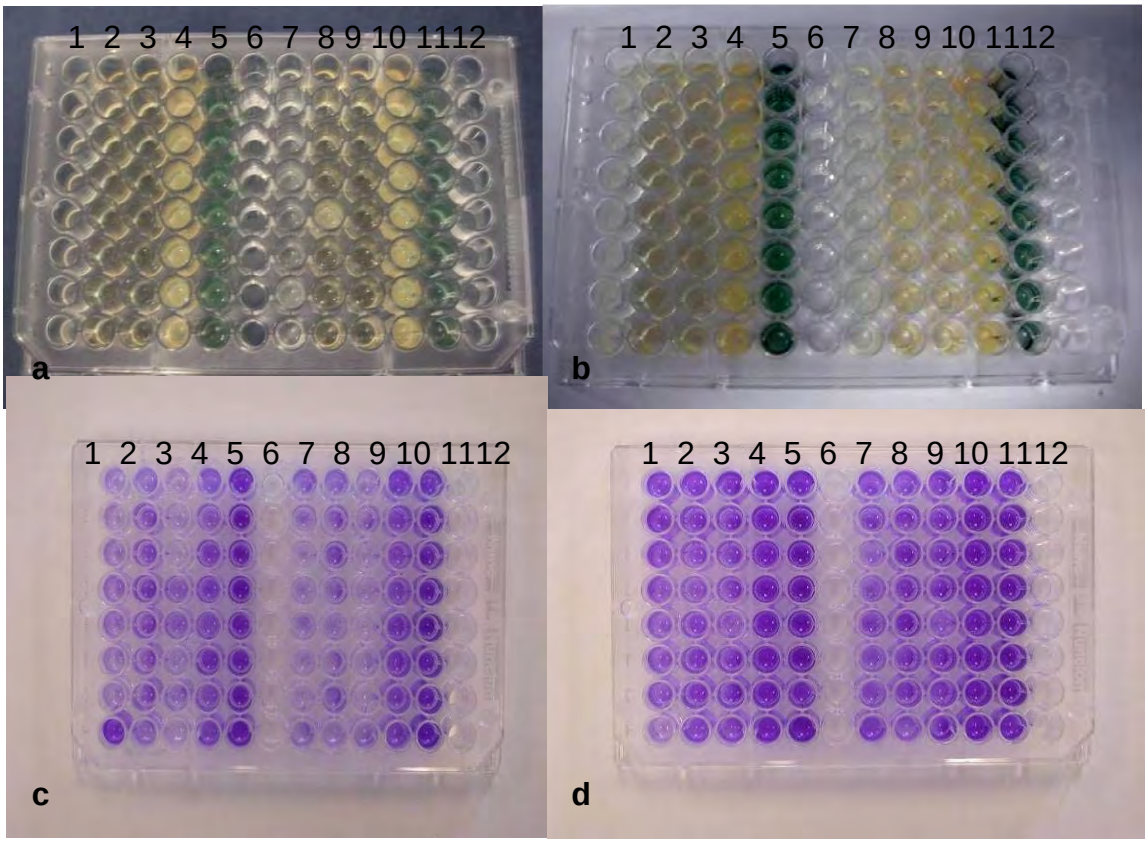



**Figure 3.3.** Microtitre plate (single biofilm formation assay). Fig. 3.3 a, 48 h and Fig. 3.3 b, 72 h at 37°C. Fig. 3.3 c, 48 h, and Fig. 3.3 d, at 72 h stained with 1% crystal violet. Rows 1 and 7 were controls, rows 2 and 8 were organism A, rows 3 and 9 were organism B, rows 4 and 10 were organism C and rows 5 and 11 were organism D. Only organism D in rows 5 and 11 produced a green pigmentation in Figs. A and b. Fig. 3.3 d produced darker stain and higher absorbance values compared with Fig. 3.3 c.

*Key: L. monocytogenes NCIMB 13451 (A), L. monocytogenes L002 (B), P. aeruginosa NCTC 10299 (C), P. aeruginosa C001 (D), and Control (sterile TSB only) (CT).*

There was more intense staining with crystal violet in Fig. 3.3 d (72 h plate) compared with Fig. 3.3 c (48 h plate). This demonstrated the ability of the four microorganisms in a single biofilm formation assay to produce biofilms. There was more staining for both *P. aeruginosa* strains compared to the *L. monocytogenes* isolates, which is equivalent to higher absorbance values, indicating more biofilm production.

Statistical analysis for the single biofilm formation assays revealed that, comparing the two incubation temperatures (32°C and 37°C) at 48 h, using a two-way

ANOVA, there was a significant difference (P ≤0.05) between organisms and temperature and in their interactions. A one-way ANOVA revealed that, for either temperature, there were significant differences between organisms, with a significant difference between *P. aeruginosa* C001 (D) and the other organisms (A, B and C) (plus control). There were no significant differences between *L. monocytogenes* NCIMB 13451 (A), *L. monocytogenes* L002 (B), or *P. aeruginosa* NCTC 10299 (C).

*Dual species biofilm formation assay.* The dual biofilms were performed at 32°C and 37°C for 24 h, 48 h and 72 h. Statistical analysis using a two-way ANOVA revealed that there was a significant difference (P ≤0.05) between the different strains of microorganisms and combinations with the incubation times. At 24 h and 48 h incubation period (at 32°C), the *Pseudomonas* strains did not produce any pigments; however, at 72 h, as with the single biofilm assay, organism D (*P. aeruginosa* C001), the environmental isolate produced a green pigment at 32°C, illustrated in Fig. 3.4.

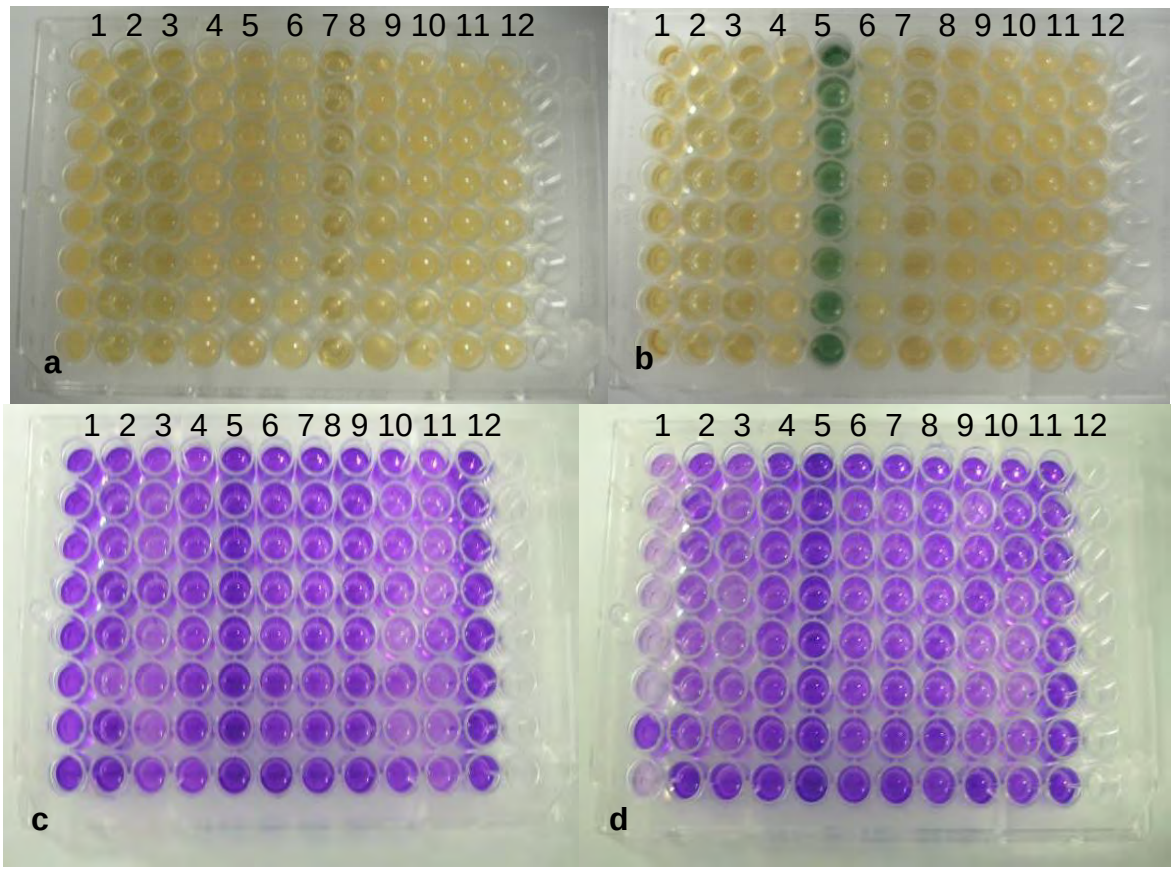



**Figure 3.4.** Microtitre plate (dual biofilm) assay. Fig. 3.4 a, 48 h and Fig. 3.4 b, 72 h at 32°C. Fig. 3.4 c, 48h, and Fig. 3.4 d, 72 h at 32°C stained with 1% crystal violet. Row 1 was control (sterile TSB), row 2 was organism A, row 3 was organism B, row 4 was organism C, row 5 was organism D, combinations of the two different microorganisms; row 6 was combination AC, row 7 was combination AD, row 8 was combination BC, row 9 was combination BD, row 10 was combination AB and row 11 was combination CD.

*Key: L. monocytogenes NCIMB 13451 (A), L. monocytogenes L002 (B), P. aeruginosa NCTC 10299 (C), P. aeruginosa C001 (D). AB = L. monocytogenes NCIMB 13451 with L. monocytogenes L002, CD = P. aeruginosa NCTC 10299 with P. aeruginosa C001, BC = L. monocytogenes L002 with P. aeruginosa NCTC 10299, BD = L. monocytogenes L002 with P. aeruginosa C001, AC = L. monocytogenes NCIMB 13451 with P. aeruginosa NCTC 10299 and AD = L. monocytogenes NCIMB 13451with P. aeruginosa C001.*

The mean absorbance ($A_{570}$ nm) readings for single species and dual species biofilm formation assays at two chosen temperature conditions and two different incubation periods of four microorganisms are shown in Table 3.3.

**Table 3.3.** Mean absorbance readings of single species and dual species biofilms at two different incubation temperatures and periods. Standard deviation is denoted in brackets.

| Microorganisms | Mean Absorbances (570 nm) Single species biofilms | | | | | |
|---|---|---|---|---|---|---|
| | 32°C | | | 37°C | | |
| | 24h | 48h | 72h | 24h | 48h | 72h |
| *Listeria monocytogenes* NCIMB 13451 (A) | 1.21 (0.21) | 1.98 (0.31) | 0.99 (0.06) | 1.74 (0.03) | 1.82 (0.38) | 1.43 (0.29) |
| *Listeria monocytogenes* L002 (B) | 1.19 (0.32) | 0.81 (0.07) | 0.85 (0.03) | 2.27 (0.51) | 1.87 (0.25) | 0.99 (0.01) |
| *Pseudomonas aeruginosa* NCTC 10299 (C) | 2.00 (0.15) | 1.26 (0.02) | 1.35 (0.01) | 2.88 (0.22) | 3.73 (0.02) | 3.48 (0.11) |
| *Pseudomonas aeruginosa* C001 (D) | 2.07 (0.34) | 3.13 (0.05) | 2.37 (0.06) | 3.39 (0.123) | 3.58 (0.06) | 3.35 (0) |
| **Dual species biofilms** | | | | | | |
| **Microorganisms** | **24h** | **48h** | **72h** | **24h** | **48h** | **72h** |
| AC | 1.77 (0.36) | 1.89 (0.03) | 1.06 (0.04) | 2.73 (0.12) | 3.12 (0.17) | 2.68 (0.04) |
| AD | 1.21 (0.24) | 2.09 (0.06) | 1.39 (0.03) | 1.01 (0.15) | 0.82 (0.07) | 0.60 (0.06)( |
| BC | 2.24 (0.36) | 2.18 (0.14) | 1.21 (0.04) | 2.95 (0.22) | 3.46 (0.11) | 3.34 (0.25) |
| BD | 1.15 (0.36) | 0.74 (0.19) | 1.05 (0.06) | 2.56 (0.26) | 2.07 (0.34) | 1.77 (0.28) |
| AB | 0.56 (0.12) | 0.59 (0.07) | 0.74 (0.16) | 2.86 (0.12) | 2.38 (0.39) | 2.16 (0.30) |
| CD | 1.29 (0.03) | 2.10 (0.05) | 2.12 (0.45) | 3.31 (0.21) | 3.34 (0.28) | 3.83 (0.24) |

The mean absorbance of the single biofilm formation assays illustrate the fact that *L. monocytogenes* produced more biofilm at 37°C than at 32°C, even though its optimal growth temperature is closer to 32°C. *P. aeruginosa* produced more biofilm at 37°C than at 32°C, which is close to its optimum temperature. Furthermore, increasing the incubation period increases the microorganisms' capabilities to produce more biofilm. The *P. aeruginosa* strains (C and D) produced more biofilm (higher absorbance values) at the longer incubation periods (72 h) at 32°C. The production of a green pigment was only produced by organism D, and was seen in the single biofilm assay (Fig. 3.3 b, and Fig. 3.4 b) at both temperatures. The environmental isolates of both microorganisms (in single biofilm formation assay) produced more biofilm than their respective collection strain at both temperatures and incubation periods.

The single microorganisms (A and D), (at 32°C) produced more biofilm (higher absorbance values) at the extend incubation time of 48 h compared to 24 h. Microorganism C however, produced more biofilm at 24 h then at 48 h or 72 h. Combinations of AC, AD and BC gave higher mean absorbance readings at 48 h compared to 72 h at 32°C, whereas BD, AB and CD, produced higher readings (more biofilm production) at 72 h compared to 48 h (at 32°C). Microorganisms C and D (at 37°C) produced more biofilm at 48 h than at 24 h, whereas A and B produced more biofilm at 24 h compared to 48 h and 72 h. All microorganisms produced less biofilm at 72 h than at 48 h, with the exception of CD. As seen with the coaggregation experiment (Table 3.2), it was expected that for the dual biofilm formation assay, the mean absorbance readings for combination AB (*Listeria* isolates only) would be low compared to combination CD (*Pseudomonas* isolates only). This was confirmed, the mean absorbance readings for *Pseduomonas* CD

was greater as a result of *Pseudomonas*' ability to form biofilms, It was also noted that the mean absorbance readings for combination AB (*Listeria* alone) was lower than combinations with the two *Pseudomonas* strains (AC, AD, BC and BD). This is evidence to support the fact that *L. monocytogenes* forms more effective biofilms in the presence of *Pseudomonas* spp. in the food processing environment.

### 3.3.3 LIVE/DEAD *Bac*Light viability staining kit

*L. monocytogenes* L002 surface attached to glass microscope slide was stained using LIVE/DEAD *Bac*Light staining kit (L7012; Molecular probes, Invitrogen). The untreated slide (Fig. 3.5 a) showed that live cells fluoresced green, while the dead (damaged) cells fluoresced red on the treated slide (Fig. 3.5 b). An intermediate state of bacterial cells was also observed in the untreated cells (Fig. 3.5 a), which fluoresced yellow/orange in colour. The treated sample revealed the majority of dead cells with approximately 5 cells in each view that fluoresced green, indicating the cells were still alive.

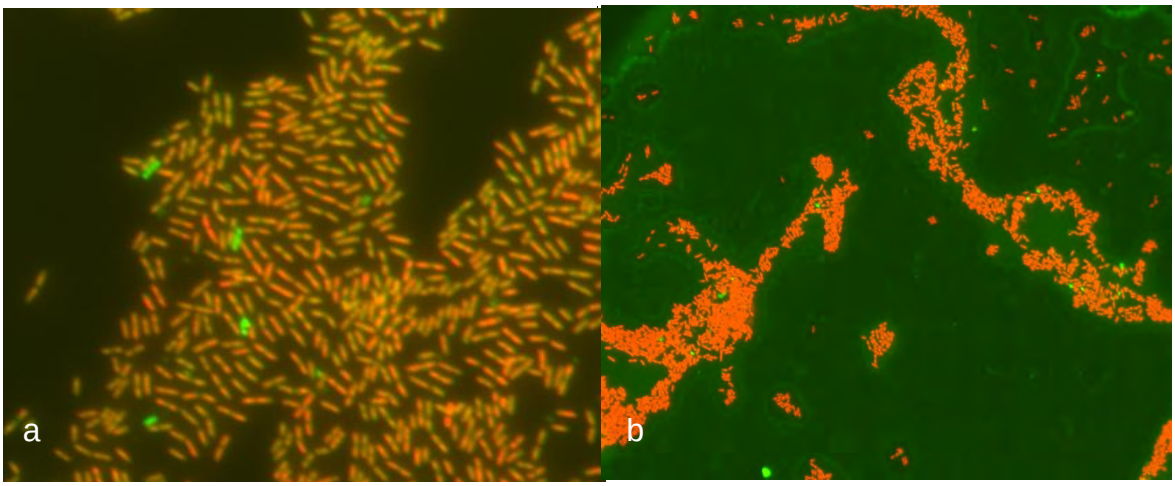



**Figure 3.5.** LIVE/DEAD *Bac*Light stain. Fig. 3.5 a, *L. monocytogenes* L002 untreated (control), and Fig. 3.5 b, *L. monocytogenes* L002 treated with gaseous ozone at 10 ppm.

Excessive green background fluorescence was observed in many slides, which caused difficulties in visualising the cells. This complication was probably caused by the modifications in the protocol, and so this experiment was not continued.

## 3.4 Discussion

The microtitre plate biofilm formation assays demonstrated the ability of the four chosen microorganisms to produce biofilms, whether as single-species or dual-species biofilms. The single species biofilm formation assay was conducted under different incubation temperatures and different incubation periods, which resulted in varying results compared with the dual-species biofilms. The results, however, demonstrate the ability of *P. aeruginosa* to promote biofilm formation and its encouragement of other pathogens to form biofilms in its presence. All strains were able to produce more biofilm with an increasing incubation period. Holah *et al.* (2004) and Chae *et al.* (2006) suggested that biofilm formation by *L. monocytogenes* on inert surfaces within the food processing environment is an important factor in their survival and strains differ in their adherence to these surfaces and their biofilm forming ability (Norwood and Gilmour, 1999; Lundén *et al*., 2000; Borucki *et al*., 2003). Some researchers have determined a link with higher production of biofilm and certain lineages of *L. monocytogenes* (Djordjevic *et al*., 2002; Borucki *et al*., 2003; Chae *et al*., 2006), but Takahashi *et al*. (2009) conducted a microtitre biofilm formation plate assay on ready-to-eat seafood *L. monocytogenes* isolates and found that biofilm formation by isolates from lineage I produced significantly greater biofilms than by isolates from lineage II. Other studies have found no relationship between adherence and serotype of *L. monocytogenes* (Kalmokoff *et al*., 2001). Studies have also reported that biofilm formation from *L. monocytogenes* isolates of clonal lineage is affected positively or negatively by environmental factors such as neighbouring microorganisms (Carpentier and Chassaing, 2004).

The coaggregation assay demonstrated that both environmental and collection strains of *L. monocytogenes* and *P. aeruginosa* coaggregated. All organisms with the exception of *L. monocytogenes* NCIMB 13451 self-aggregated. The autocoaggregation scores were deducted from the coaggregation scores giving final coaggregation scores. There were 4 coaggregation pairs with 2 coaggregation bacterial pairs involving *L. monocytogenes* NCIMB 13451 and both strains of *P. aeruginosa* giving a maximum visual coaggregation score of 3+. The environmental *Listeria monocytogenes* L002 isolate and its coaggregation partnerships of *P. aeruginosa* NCTC 10299 and the environmental isolate C001 produced visual coaggregation scores of 2+. This was a higher coaggregation score compared to *Listeria* isolates alone (coaggregation score of 0). The two different strains of *L. monocytogenes* together did not coaggregate (intraspecies coaggregation), whereas the 2 strains of *P. aeruginosa* did coaggregate. This seems to suggest that the ability of *Pseudomonas* spp. to promote biofilm formation is necessary for coaggregation with other species. Rickard *et al*. (2003b) reported that intraspecies coaggregation from freshwater biofilm bacteria compared with their planktonic counterparts, was less common and only occurred between strains isolated from a biofilm. McIntire *et al*. (1978) found that the coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34 required calcium and was dextran and pH dependent. The electron microscopic studies suggested that fibrils on *A. viscosus* may be involved.

The coaggregation results were supported by the dual biofilm formation assay results. The results from the dual biofilm formation assay revealed that both *Listeria* strains accompanied by the *Pseudomonas* strains form better biofilms at 37°C (at both incubation periods of 48h and 72 h) with mean absorbance values of 1.82 to 3.35 than alone. Jensen *et al*. (2007) investigated the adherence and

aggregation capabilities of different strains of *L. monocytogenes* isolated from a fish processing plant. The researchers also studied the invasiveness of these strains. The results revealed that sodium chloride enhances adherence and aggregation, which aids in the persistence of certain strains, while strain variation influences invasiveness of *L. monocytogenes* strains.

The LIVE/DEAD *Bac*Light bacterial viability kit revealed that *L. monocytogenes* L002 cells that had been left untreated had a yellow/orange fluorescence. This meant that most cells were in an intermediate state. There were a few cells that appeared to be live with fully intacted membranes. These cells fluoresced green. Most of the treated cells appeared dead with damaged membranes. These cells had a red fluorescence. There are limitations with this kit and these include: the fact that the manufacturer claims there is membrane impermeability of propidium iodide. It is known that some bacterial strains possess efflux pumps that can actively remove propidium iodide from the cell (Stocks, 2004).

As *L. monocytogenes* and *P. aeruginosa* together as environmental isolates or collection strains can coaggregate and form biofilms, this work has led to further investigations to study the effects of treating environmental isolates together and alone, surface attached or as mature static 72h biofilms on food grade 304 stainless steel coupons with ozonated water (and terpenes), gaseous ozone and OAF.

# Chapter 4. The effect of ozonated water and terpenes on *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* C001

## 4.1 Introduction

Ozone has been applied to the disinfection of drinking water (Hirotsuji *et al*., 1996; Jackson and Overbeck, 1997; Rice, 1999 cited in Smilanick, 2003), as well to municipal industrial wastewaters (Gulyas *et al*., 1995; Stopka, 1997; Arana *et al*., 1999). As previously mentioned in section 1.2, municipal water (microfiltrated, ultrafiltrated) is the medium of choice for ozone applications. However, potable water is required for use in the food industry. The units that express the concentration of ozone in air and water are parts per million or ppm. In water, ppm is a unit of weight/volume (µg/ml), whereas, in air, ppm is a unit of volume/volume (µl/l) (Smilanick, 2003). Ozone in water at a concentration above 1 ppm can be liberated into the surrounding air (ozone off-gassing) at levels that exceed the occupational exposure level (OEL) (Smilanick, 2003).

Ozone in water (ozonated water) is often used as an alternative to hypochlorite, which is used as a disinfectant or sanitizer (Smilanick *et al*., 1999) in the food industry. Ozone has been applied to flume water in apple processing (Achen and Yousef, 2001; EPRI, 1998) and in wash waters of other fruit and vegetables to remove pesticides used to control postharvest diseases (Hwang *et al*., 2001; Ong *et al*., 1996; Wu *et al*., 2007). Hwang *et al*. (2002) studied the effectiveness of various wash treatments on the removal of Mancozeb (a carbamate fungicide) and ethylene-thiourea (ETU) (a degradation product of ethylenebisdithio carbamate fungicides) on/in fresh and processed apples. An ozone wash at 3 ppm and

chlorine wash at 500 ppm were determined as the most effective treatments for Mancozeb and ETU removal.

Enzymatic browning causes a colour change in fruit and vegetables, especially lettuce (Koseki and Isobe, 2006), as a result of a group of enzymes known as polyphenol oxidases (PPO) that are found in all plants (Karaca and Velioglu, 2007). Zhang *et al*. (2005) studied the inhibitory effect of ozonated water treatment on PPO activity in fresh-cut celery and reported that the PPO activity and the respiration rate of fresh-cut celery was inhibited by the ozonated water treatment and the sensory quality of the treated celery was better than non-treated celery.

Ozone treatment systems have been installed to supply the flume water in apple processed plants. Gaseous ozone is pumped into the stream of water at a concentration of 0.05 to 0.15 ppm, meaning that the flume water does not need to be replaced daily (EPRI, 1998). Another application is the use of low concentration ozone in the air of storage and packaging houses to reduce yeast and mould counts on the surface of apples and in the plant environment. It also allows for better control of the ripening process by oxidising ethylene generated by the apples. Other fruit processors have had good success in sanitizing fresh fruit and have been able to extend shelf life by spraying fruit with super-saturated ozonated water at 1 - 4 ppm ozone concentration (Strasser and Tonjes, 1998).

Ozonated water can be applied as a sanitizer to the surface of fruit and vegetables (Bialka and Demirci, 2007; Koseki and Isobe, 2006; Rodgers *et al*., 2004). The effect of immersing pre-cut green peppers (*Capsicum annuum* L.) in ozonated water with a range of concentrations and contact times was investigated by

Ketteringham *et al*. (2006). They determined that ozonated water was not significantly more effective than washing with non-ozonated water and, therefore, aqueous ozone application on pre-cut green peppers was found not to be commercially viable.

Baur *et al*. (2004) studied the effects of chlorinated, ozonated and tap water (control) in different washing procedures of shredded iceberg lettuce (*Lactuca sativa* L.) on the microbiological and sensory quality during storage. The authors determined that pre-washing trimmed lettuce heads (to minimize cross-contamination between less contaminated inner layers and highly contaminated outer leaves during cutting) gave approximately 2 log reduction in the initial microbial population. Chlorine was found to be the most effective treatment in maintaining the best sensory and microbiological properties. It was not, however, effective in extending the product shelf-life. Selma *et al.* (2007) investigated the ability of ozone to inactivate *Shigella sonnei* from shredded lettuce and in water. Treatments with ozone at 1.6 and 2.2 ppm for 1 minute decreased *S. sonnei* population in water by 3.7 and 5.6 log cfu $ml^{-1}$, respectively. After 5 minute exposure at 2 ppm ozone with or without UV-C activation, *S. sonnei* counts were reduced by 0.9 and 1.4 log units in shredded lettuce. Furthermore counts were reduced by 1.8 log units after 5 ppm for 5 minutes. Koseki and Isobe (2006) investigated the effect of ozonated water on the microbial load and browning effect of Iceberg lettuce (*Lactuca sativa* L.). Fresh cut lettuce was washed in aqueous ozone (ozonated water) at 3, 5 and 10 ppm for 5 mins at room temperature. The native bacterial count declined in response to an increase in ozone concentration. There was no further reduction (1.4 log cfu/g) above 5 ppm ozone. The phenylalanine lyase activity increased, whereas the ascorbic acid content was not

affected. The Iceberg lettuce browning ability increased dramatically with 10 ppm ozone.

Yuk *et al*. (2006) investigated the effects of combined ozone and organic acid treatments on the control of *Listeria monocytogenes* and *Escherichia coli* O157:H7 on lettuce. They found that ozone treatment (5 ppm for 5 mins) alone was ineffective in reducing *E. coli* O157:H7 and *L. monocytogenes.* However, treatment of ozone (3 ppm) combined with 1% citric acid for 1 min gave significant ($P < 0.05$) log reductions of 2.31 and 1.84, respectively. This combined treatment during long storage (at 15°C for 10d), did not have any antimicrobial effect. The combined ozone-organic acid treatment was more effective in reducing these pathogens on Iceberg lettuce (*Lactuca sativa* var. *capita*) than the individual treatments. This study also suggested that the action of ozone on the bacteria on lettuce was most probably due to the bubbling action disrupting the cell clusters of bacteria on the surface of lettuce, thus increasing the exposure of single bacterial cells' surface to ozone and leading to greater log reductions of pathogens. Alternatively, it could have been from the physical forces of these delivery methods increasing the detachment of cells from lettuce surfaces with no antimicrobial effectiveness from the ozone.

The disinfection of onions, carrots, escarole lettuce and spinach wash waters for the fresh-cut industry using ozone, ultraviolet-C (UV-C) and their combination for reducing microflora has been investigated (Selma *et al.,* 2008c). They found that all three treatments were effective in the disinfection of vegetable wash waters, with ozone-UV-C giving the maximum microbial reduction of 6.6 log CFU $ml^{-1}$. Ozone and ozone plus UV-C treatments were more effective in reducing the turbidity of wash water. UV-C had no effect on the physicochemical properties of

the wash waters. These treatments provide evidence as alternatives to chlorinated agents and illustrate that treated wash water would require less frequent changing, providing a more cost-effective measure.

Ozonated water has been applied for the sanitation of soiled food contact surfaces, such as stainless steel (Güzel-Seydim *et al*., 2000). Greene *et al*. (1993) investigated the effects of ozonated water and chlorinated sanitizers against biofilms of milk spoilage bacteria on stainless steel. Both treatments were found to be effective in reducing bacterial populations on stainless steel surfaces. Fielding *et al*. (2007) evaluated the effect of ozonated water as an alternative to chemical cleaning and sanitation of beer lines. Ozonated water gave a significant reduction compared with chemical cleaner in biofilm formation of brewery microorganisms of 3 and 2.7 logs, respectively.

Terpenes or terpenoids are active against bacteria, fungi, viruses and protozoa (Cowan, 1999). Aureli *et al*. (1992) found that terpenoids present in plant essential oils were effective in controlling *L. monocytogenes*. Terpenes are a class of naturally occurring compounds found mainly in plants as constituents of essential oils. Terpenes are mostly hydrocarbons, alcohols and their glycosides, ethers, aldehydes, ketones, carboxylic acids and esters. Their basic structure follows a general principle consisting of *2*-methylbutane residues. Generally they are referred to by the number of *isoprene* units, (C5)n. Terpenes have cyclic or acyclic, saturated or unsaturated structures. There are different classes of terpenes which depend on the number of 2-methylbutane (isoprene) subunits; *hemi*- (C5), *mono*- (C10), *sesqui*- (C15), *di*- (C20), *sester*- (C25), *tri*- (C30), *tetraterpenes* (C40) and *polyterpenes* (C5)n. Monoterpenes include camphor, limonene, neral (citral and geraniol) and alpha-terpinene (Breitmaier, 2006;

Cowan, 1999). Dietary monoterpenes including limonene and geraniol have anti-carcinogenic properties (Crowell, 1999). The rationale for choosing monoterpenes; d-limonene, geraniol and alpha-terpinene is that, although they have the same number of carbon atoms (C10), they also have the same number of double carbon bonds but are arranged in different positions. It has been hypothesised that they may react differently to ozone.

Fig. 4.1 illustrates the chemical structure of monoterpenes, including limonene, geraniol and alpha-terpinene. Limonene and alpha-terpinene are cyclic while geraniol is acyclic in structure.

**Limonene** **Geraniol** **Alpha-terpinene**

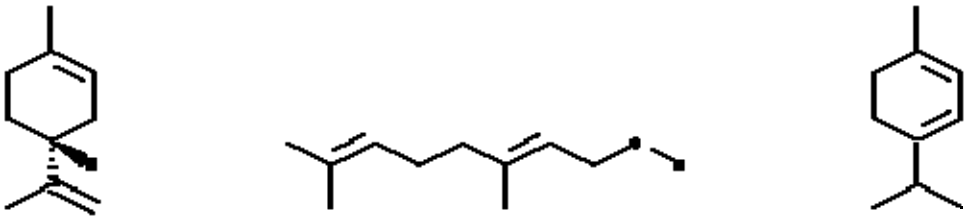


**Figure 4.1.** The chemical structures of limonene, geraniol and alpha-terpinene (Fisher Scientific Ltd. UK [online]).

Limonene is a cyclic terpene and has two forms; l-limonene which has a turpentine 'piney' odour and d-limonene which has a citrus 'orange or lemon' odour. D-limonene also has anti-carcinogenic activity in rat mammary and other tumour development. Limonene is found in orange and other citrus peel oils (Tsuda *et al*., 2004). Geraniol is an acyclic terpene and has a rose-like odour. It is the chief constituent of many essential oils including oil of rose, ylang-ylang, lemongrass, geranium and lavender oils. Alpha-terpinene is a constituent of cardamom and marjoram oils and plants such as *Melaleuca alternifolia* (tea tree) (Bretimaier, 2006).

In the literature, there is mention of essential oils (terpenes or terpenoids) and ozone in the disinfection of certain microorganisms on fresh produce (Singh *et al*., 2002a). However, there is no mention of the use of terpenes and ozone together for the sanitation of food contact surfaces. The aim of this experiment, therefore, was to determine the effect of ozonated water and terpene treatment in different combinations and concentrations on surface attached *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* NCTC 10299 to stainless steel food grade 304 coupons, and note any synergistic effects that occurred between ozone and terpene treatments.

The aim of this chapter was to determine the effect of ozonated water and terpene against surface attached and biofilm (single and dual) of environmental isolates *L. monocytogenes* L002 and *P. aeruginosa* C001. The objectives were:

1. To determine if there was any off-gassing produced at different concentrations of ozonated water.
2. To determine ozone decay in water, and the effect of organic load on ozone activity in water (in combinations of ozonated water and terpenes (d-limonene, geraniol and alpha-terpinene) treatments at different concentrations of ozone).
3. To determine the effect of ozonated water and terpene (d-limonene) on environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 surface attached on food grade stainless steel and in biofilms (single and dual) to various food contact surfaces.

## 4.2 Materials and Methods

*Ozone production.* An ozonated water device (Elix 3, Millipore; Fig. 4.2 a, b) was used to continuously supply gaseous ozone into water inside a class 2 Bioaerosol Test chamber (Chapter 2), by bubbling ozone through a diffuser into water.


**Figure 4.2.** Ozonated water generator. Fig. 4.2 a, ozonated water device connected to class 2 Bioaerosol Test chamber, Fig. 4.2 b, close up of ozonated water device, and Fig. 4.2 c, panel of dials on the front of the ozone generator. The Variac dial controlled the concentration of ozone produced by the device. The flow rate was maintained at 1 L/ min oxygen. The ozone output light illuminated when the variac dial was turned above zero.

The ozonated water generator was connected to the chamber from outside. The flow rate of the device was maintained at 1 L/min oxygen (chapter 7, section 7.2.3).

*Monitoring ozone concentration.* The ozone concentration in the chamber was controlled and monitored for the duration of the treatment using an ultraviolet absorption API ozone monitor, by measuring the concentration of off-gassing from the API monitor's inlet on the chamber wall and from about 15 cm above the water line. The concentration of ozone which was available for treatment, produced by the ozonated water device, was determined by measuring the concentration of ozone in the water using Palintest Ozone 1000 meter. A single DPD (diethyl-p-phenylene diamine) no. 4 tablet (Palintest Ltd, UK) was crushed in the bottom of a glass cuvette into which 10 ml of treated water was syringed. The contents of the cuvette were mixed and placed in the Palintest meter (a calibrated device from Ozone Industries Ltd, UK), and the reading was recorded.

*Preparation of ozonated water.* Four litres of water were poured into a sterile plastic container and placed on top of a magnetic stirrer on the table inside the chamber. A diffuser (allow the gas bubbles into the water) connected to the ozonated water device tubing was placed into the 4 litres of water adjacent to the magnetic stirrer (to allow sufficient mixing of gas and water (plus terpene)). Tap and deionised water were compared for the rate of degradation and off-gassing experiments, whereas tap water alone was used to prepare ozonated water for the ozonated water and terpene experiments.

### 4.2.1 Off-gas production

Off-gases produced were determined by monitoring the production of off-gases produced from the surface of the ozonated water in duplicate. The medium of the water was tap water. The API ozone monitor was set up to sample the ozone concentration 15 cm above the water line. This was to ensure accurate monitoring of any off-gases from the ozonated water itself. Any negative values are reported where there was a lag in sampling from the API monitor.

### 4.2.2 Ozone decay

Ozone decay in ozonated water was determined by setting the ozonated water device to a set arbitrary unit (60, 70) on the device, and measuring the ozone concentration in the water after 30 mins using the Palintest Ozone 1000 meter. The ozonated water device was then switched off and the concentration of ozone was measured at 10 min intervals using the Pailntest. Throughout the single experiment the API ozone monitor continuely measured the levels of off-gases present. The experiments were concentration and time dependent.

### 4.2.3 The effect of organic load on the activity of ozone in water

The combinations of ozonated water and terpenes (geraniol, alpha-terpinene and limonene) were:

A) ozonated water (4 ppm) plus alcohol (methanol) (250 ppm),

B) ozonated water (4 ppm) plus alcohol (methanol) (50 ppm),

C) ozonated water (4 ppm) plus alcohol (methanol) (50 ppm) / terpene (25 ppm),

D) water plus alcohol (methanol) (50 ppm) / terpene (25 ppm), then the addition of ozone (4 ppm).

All experiments were carried out in triplicate.

The terpenes are immiscible in water, so in order to disperse them in the ozonated water, 0.1 ml of each terpene was dissolved in 0.2 ml alcohol (methanol), before adding to the ozonated water or tap water before ozonation.

Alcohol and/or terpenes were added to ozonated water see if there was any effect on ozone decay. The ozone concentration in ozonated tap water was measured every 15 mins for 30 mins. After 30 mins, A) 1 ml alcohol (methanol) (250 ppm), or B) 0.2 ml alcohol (50 ppm), or C) 0.2 ml alcohol (50 ppm) / 0.1 ml terpene (25 ppm) was added to the water. The ozone was switched off 10 mins later (at 40 mins). The ozone concentration was measured 10 mins later, and then every 10 mins for another 30 mins until the ozone concentration approached zero (60 mins).

The alcohol and terpene were added to the tap water (D) and then the water was ozonated (4 ppm) to see if there was any effect on ozone decay. The ozone concentration in tap water was measured every 15 mins. 0.2 ml alcohol (50 ppm) / 0.1 ml terpene (25 ppm) was added to 4 L tap water and then ozone was bubbled in, to a concentration of approximately 4 ppm. After 30 mins, the ozone was switched off and the ozone concentration in the water was measured every 10 mins for another 20 mins.

### 4.2.4 The effect of ozonated water and terpene on environmental *L. monocytogenes* and *P. aeruginosa*

Four treatments were performed on *L. monocytogenes* (L002) and *P. aeruginosa* (C001) surface attached on 25 $cm^2$ stainless steel (food grade 304) coupons. Treatments were performed in 4 L of tap water only:

1) terpene (d-limonene) (5 or 25 ppm) (A),

2) ozonated water (0.1 ppm) (B),

3) ozonated water (0.1 ppm) and terpene (d-limonene) (5 or 25 ppm) (C) and

4) ozonated water (4 ppm) (D).

All experiments were carried out in triplicate.

Treatments 1, 2 and 3 were also performed on three 72 h biofilms (single-species biofilms consisting of *L. monocytogenes* L002 and *P. aeruginosa* C001 and a dual-species biofilm of both *L. monocytogenes* L002 and *P. aeruginosa* C001; grown together) adhering to 1 $cm^2$ food grade stainless steel 304, polished granite and food grade polypropylene coupons as in section 2.2.6. Limonene (Acros Organics, Fisher Scientific UK, UK), was the chosen terpene and was used for all further experiments.

**Surface attached microorganisms.**

*Preparation of suspension culture.* The preparation of the suspension cultures of environmental isolates *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* C001 were repeated as in section 2.2.4. The resulting suspensions gave approximately 2 x $10^9$ CFU/ml. For example, 152.5/0.1 x (2+0) x $10^{-6}$ = 1.5X$10^9$.

*Inoculation of stainless steel coupons.* 100 µl of each culture was inoculated separately onto five 25 $cm^2$ food grade stainless steel 304 coupons. The inoculum was spread evenly over the surface and allowed to dry at ambient temperature.

*Ozonation of inoculated stainless steel coupons.* Five 25 $cm^2$ inoculated stainless steel coupons were placed faced up on a sterile rack which was placed into 4 litres of tap water (plus treatments) with a contact time of 5 mins. Once treated with the various treatments (A-D), the coupons on the rack were taken out of the treatment solution and sampled. Five inoculated 25 $cm^2$ stainless steel coupons in sterile Petri dishes were placed faced upwards into sterile PBS with a contact time of 5 mins (untreated).

*Sampling of ozonated stainless steel coupons.* A pre-moistened sterile cotton tip swab was swabbed over the entire surface of the coupons in a 2-directional pattern. The swab was placed into 9 ml maximum recovery diluent (MRD) (Oxoid, UK) and vortex mixed for 30 seconds. Serial dilutions were carried out as necessary. 100 µl tryptone soya agar (TSA) (Oxoid, UK) spread plates were plated in duplicate and incubated at 30°C for 48 hours.

*Enumeration of survivors.* Five coupons were used for the different combination of treatments (n=5). Each treatment carried out in triplicate (n=15) and all results are reported as $\log_{10}$ transforms of actual counts.

**Biofilm microorganisms.** As in Section 2.2.6.

*Treatment of coupons.* Five of each (stainless steel, granite and polypropylene) 1 $cm^2$ coupons were placed faced up on a sterile rack which was

placed into 4 litres of tap water (plus treatments) with a contact time of 5 mins. Once treated with the various treatments (A-D), the coupons on the rack were taken out of the treatment solution and sampled. Five of each (stainless steel, granite and polypropylene) 1 $cm^2$ inoculated coupons in sterile Petri dishes were placed faced up into sterile PBS with a contact time of 5 mins (untreated).

*Sampling.* As in Section 2.2.6.

*Enumeration of survivors.* As in Section 2.2.6.

For *Listeria* L002 and *Pseudomonas* C001 biofilms only, however, R2A plates were used and incubated at 32±2°C and 35±2°C for 48 and 24 h, respectively. For the dual-species biofilms, Listeria selective agar (LSA; Oxoid, UK) for L002 and Pseudomonas selective agar base with C-N supplement (PSA; Oxoid, UK) for C001 isolate were used. LSA plates were incubated at 32±2°C for 48 h and the PSA plates were incubated at 35±2°C for 24 h. Once incubated, plates with dilution that contains 3-30 colonies per 10 µl drop were counted. Viable cell counts were expressed as CFU/surface area (section 2.2.6).

Five coupons were used for each surface treated with the different combination of treatments (n=5). Each treatment carried out in triplicate (n=15) and all results are reported as $\log_{10}$ transforms of actual counts.

*Statistical analysis.* Data analysis was performed using one-way ANOVA and two-way ANOVA (Minitab version 15, Minitab Ltd, UK) with Tukey's comparison on the effect of ozonated water and terpene on *L. monocytogenes* and *P. aeruginosa*

(environmental) in single-species and dual-species biofilms and surface attached cells to different food contact surfaces.

### 4.3 Results

The design of the ozonated water device meant that the amount of ozone produced was controlled using arbitrary units (Fig. 4.2 c). This meant that the arbitrary units had to be validated and monitored each time in order to determine the safe level of ozone to work at, which had to be below the OEL of 0.2 ppm for 15 minutes. Therefore, in order to use the device, the API monitor was used constantly throughout the experiments in order to determine the level of off-gassing given off from the ozonated water treatment.

### 4.3.1 Off-gas production

To determine the potential levels of off-gases generated by the ozonated water treatment, the levels of off-gases were monitored using the API ozone monitor and hand-held ozone monitor in order to carry out a full risk assessment and safety measures of the treatment method used. It was determined that when the device was set to an arbitrary unit of 60, the production of ozone off-gases produced from the ozonated tap water were more than the OEL (0.2 ppm over a 15 min period) of ozone (Fig. 4.3) permitted. These were carried out in duplicate.

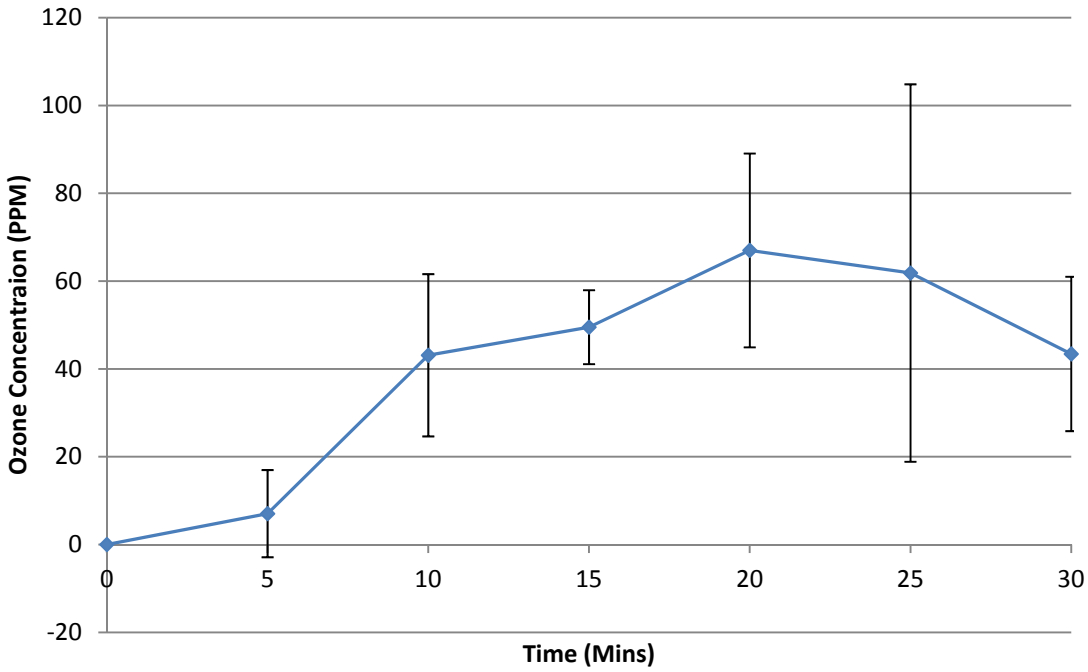



**Figure 4.3.** The production of ozone off-gas levels of ozonated tap water, monitored from above the water line of the container. It was noted that the level of off-gas exceeded the OEL of 0.2 ppm over a 15 min period (in duplicate).

After 15 mins, the concentration of ozone off-gases had reached above 50 ppm ozone.

The decision was taken, therefore, to use a lower arbitrary setting on the device to ensure a low level of ozone off-gas was produced. In order to decrease the production of ozone and, therefore, the production of any potential off-gases (Fig. 4.4), a lower arbitrary unit was used. It was noted that turning down to 55 and 58 reduced the level of off-gases to below 0.1 ppm throughout the 30 min period. The negative value was recorded due to a predicted inherent overshoot error caused by the API ozone monitor.

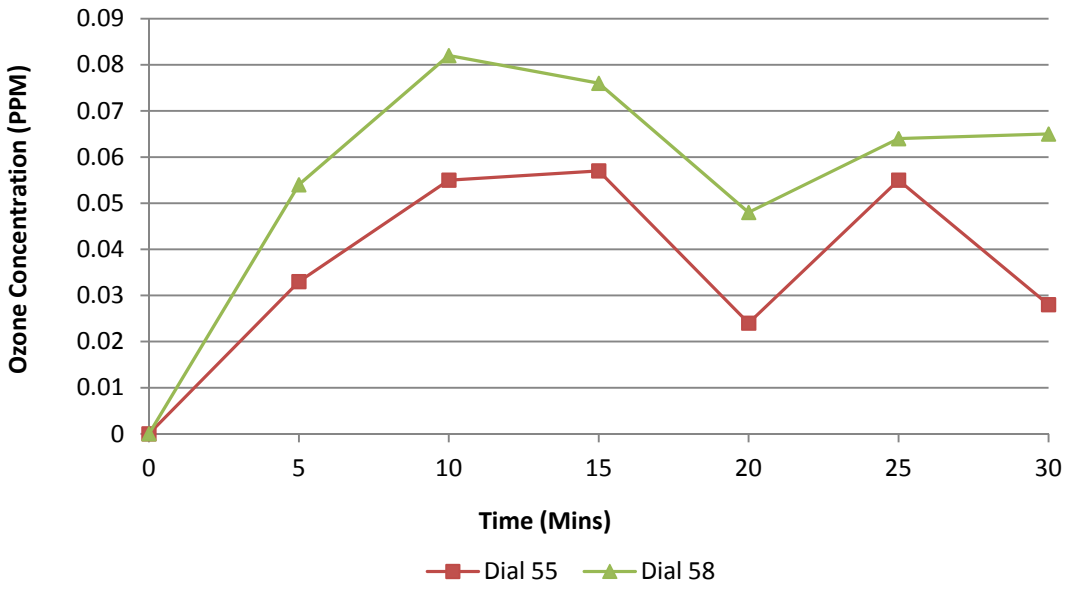



**Figure 4.4.** The production of off-gas levels monitored from above the water line of the container, when ozonated water device was set at 55 and 58. It was noted that the levels of off-gas fell below the OEL of 0.2 ppm over a 15 min period (one experiment point).

The food industry requires potable water to be used in food premises, whereas municipal water is the medium of choice for ozonated water applications. Therefore, the levels of off-gas in both deionised water and tap water was determined (Fig. 4.5). The off-gas from tap water reached a maximum level of 140 ppm compared to a maximum of 62 ppm for the deionised water. There was more ozone off-gas produced when tap water was used instead of deionised water. The levels of off-gassing in both types of water were unacceptable, when set at 60, so a decision was taken to use a setting at 58 in order to minimise off-gassing.

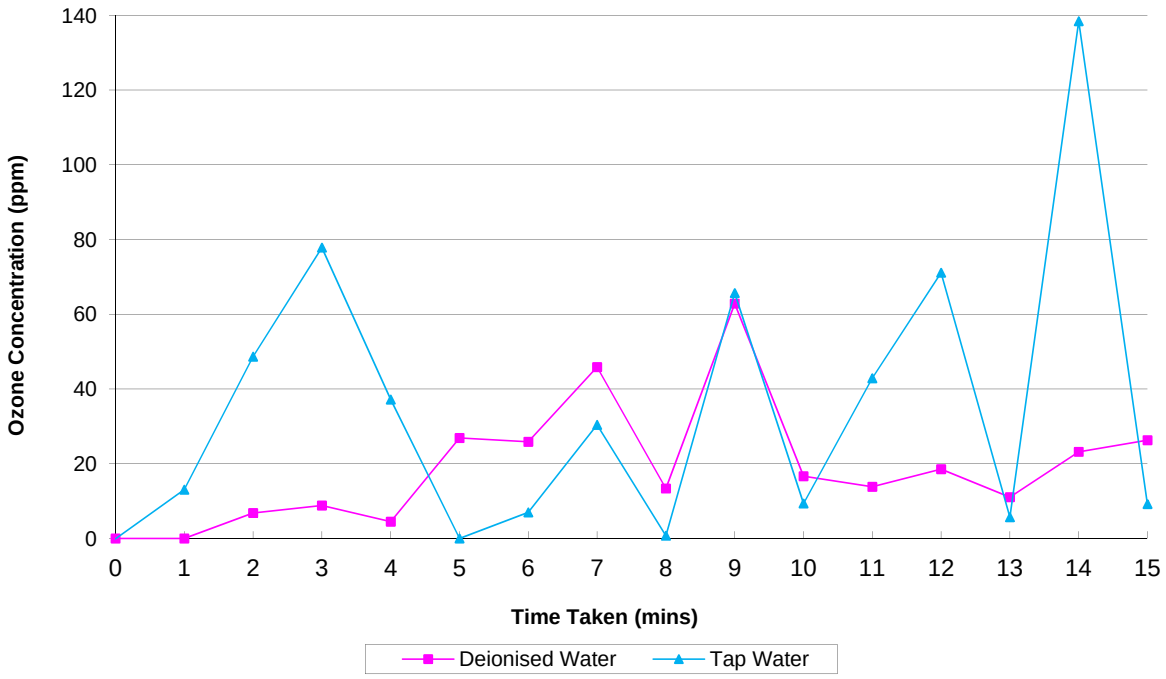



**Figure 4.5.** The production of off-gas monitored from above the water line, using deionised and tap water.

### 4.3.2 Ozone decay

Ozone decay was determined by measuring the ozone concentration in the water after 30 mins. The ozonated water device was then switched off and level of ozone was measured at 10 min intervals. After 30 mins, the concentration of ozone reached just below 2 ppm (Fig. 4.6) and when the device had been switched off, the ozone concentration fell to below 0.5 ppm over a 20 min period.

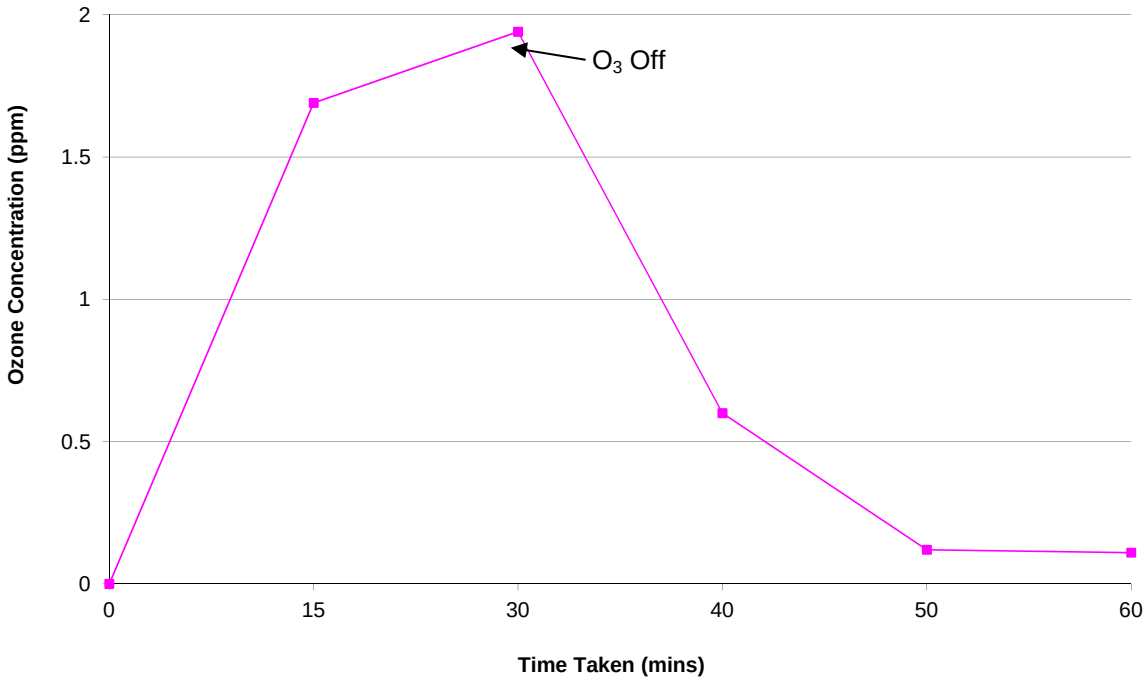



**Figure 4.6.** Ozone decay of ozonated tap water. The ozonated water device was switched off after 30 mins.

The ozone concentration exceeded 2.5 ppm after 15 mins. Once the ozonated water device was switched off, the ozone concentration decreased to 0.5 ppm after 15 mins (Fig. 4.7).

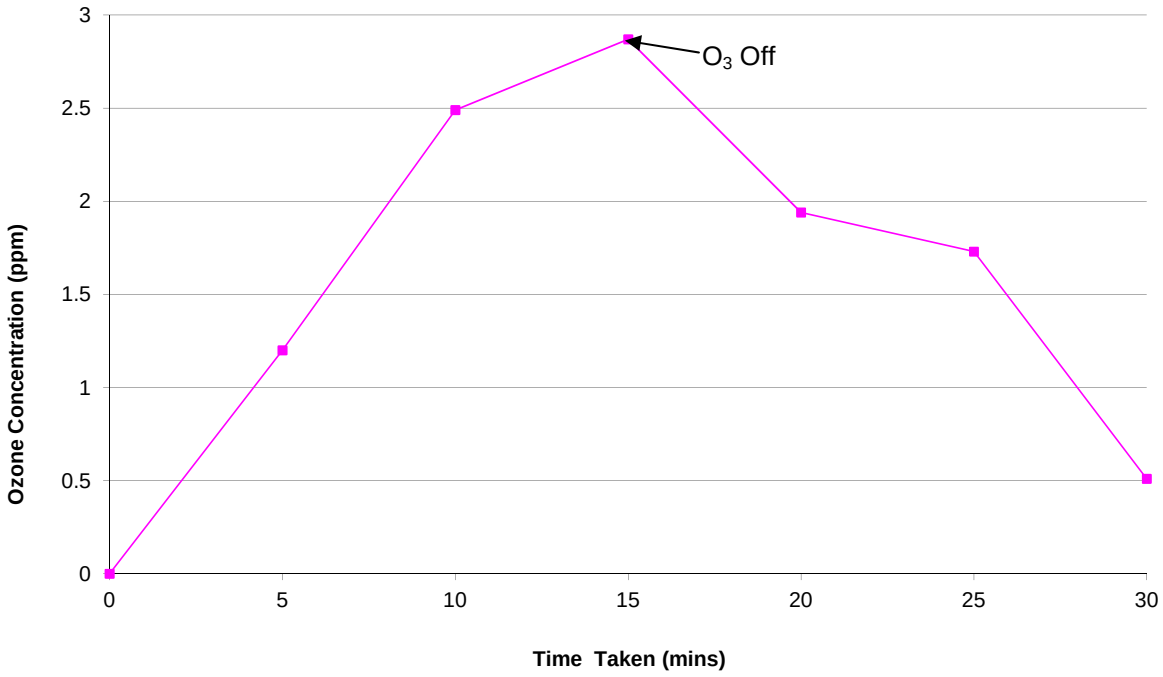



**Figure 4.7.** Ozone decay of ozonated tap water. The ozonated water device was switched off after 15 mins

It was determined that after approximately 15 mins from ozonated water device being switched off, the concentration of ozone in water would decrease to below 0.5 ppm.

Ozone decay in deionised water gave a sharp decline followed by slow decay compared to the tap water (Fig. 4.8).

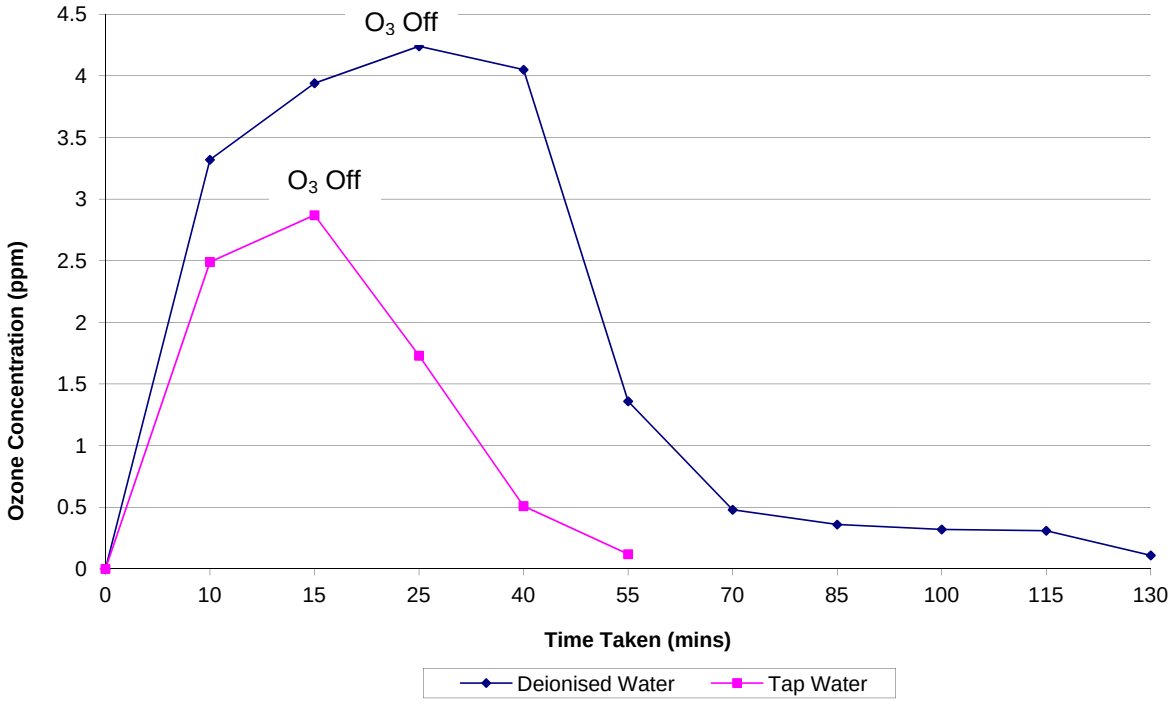



**Figure 4.8.** Ozone decay in deionised and tap water.

### 4.3.3 The effect of organic load on the activity of ozone in water

The effect of organic matter (in the form of alcohol) on the efficacy of ozonated water was investigated. Figure 4.9 illustrates the effect of alcohol (combination A and B) on the degradation of ozone in tap water. The ozonated water device was switched on and run for 30 mins before the organic load (alcohol) was added. It was noted that there was a slight decrease of 0.01 ppm ozone. The ozonated water device was switched off 10 mins after the alcohol had been added. There was a sharp decrease in ozone over 10 mins, once the ozonated water device had been switched off. The ozone concentration in the water declined from 3.46 to 0.26 ppm in 20 mins. When the concentration of alcohol decreased from 250 to 50 ppm a more gradual decrease in ozone concentration from about 3.5 ppm to 0.2 ppm over a 30 min period was observed.

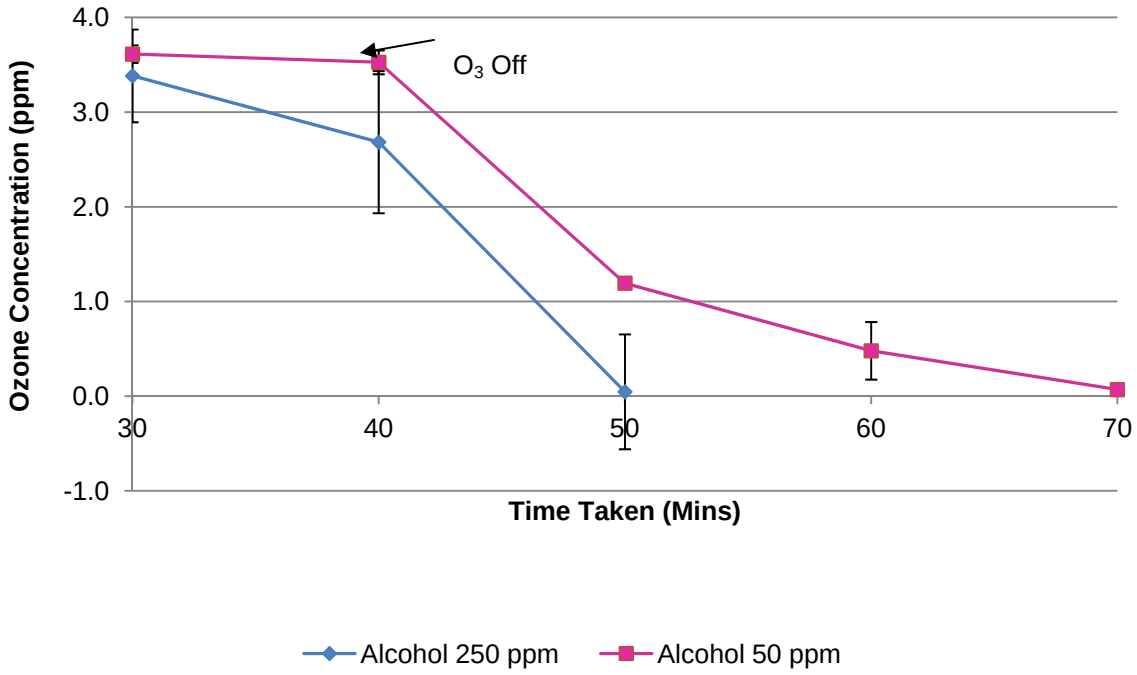



**Figure 4.9.** Ozonated water and alcohol (250 ppm and 50ppm). Ozonated water (tap water) was generated and once it had reached a concentration of approximately 3.5 ppm (at 30 mins), the alcohol was added. At 40 mins (10 mins later) the ozonated water device was switched off. Error bars denote confidence intervals (CI) with a confidence level of 95%. (Data in triplicate).

Figure 4.10 illustrates the effect each of three terpenes (limonene, geraniol and alpha-terpinene) in ozonated water (combination C) had on the ozone demand. Each terpene produced a slightly different reaction. When limonene was added the level of ozone decreased from about 4 ppm to about 0.5 ppm over 20 mins. With geraniol the concentration of ozone, dropped from 2.5-3.5 ppm to below 0.5 ppm showing a gentle decline in the level of ozone. The addition of alpha-terpinene to ozonated water caused a gentler decline in ozone concentration. There was a decrease of ozone concentration from nearly 4 ppm to less than 1ppm over a 20 min period (Fig. 4.10).

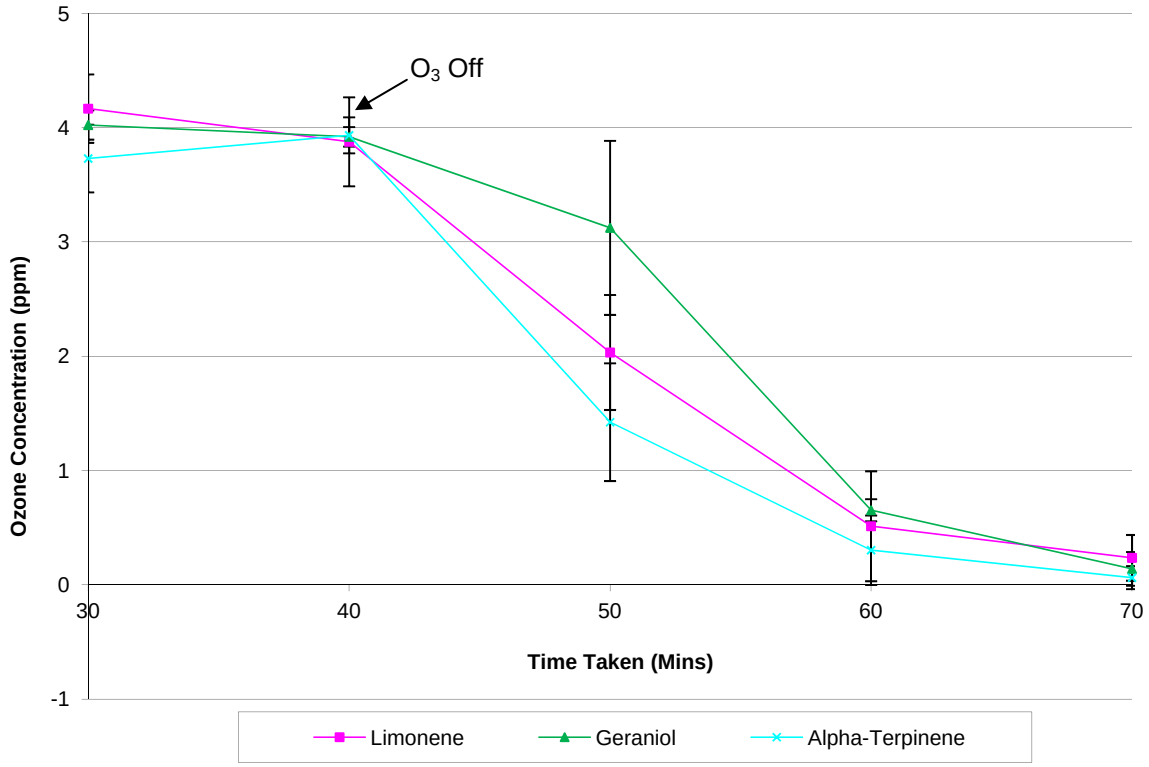



**Figure 4.10.** Ozonated water plus alcohol/terpenes (combination C). Ozonated water (tap water) was generated and once it had reached a concentration of approximately 4 ppm (at 30 mins), the terpene in alcohol was added to the ozonated water (combination C). 10 mins later (40 mins from start) the ozonated water device was switched off. Error bars denote confidence intervals (CI) with a confidence level of 95%. (data in triplicate).

When terpenes (limonene, geraniol and alpha-terpinene) at a concentration of 25 ppm dispersed in 50 ppm alcohol were added to tap water in various combinations (combinations C and D), there were apparent differences in the reactions with ozone that occurred. The reaction was determined by an observation of vapour or odour produced. Combination C (ozonated water plus alcohol (50 ppm)/terpene (25 ppm)) was the most reactive (producing an increased vapour production from surface of water) of the three combinations with combination D (tap water only plus alcohol (50 ppm)/terpene (25 ppm)) being the least reactive. Limonene produced a strong citrus lemon odour, geraniol gave a slight piney odour, and alpha-terpinene gave a sweet odour.

When the terpenes dispersed in alcohol were added to tap water (before the ozone was added) (combination D) (Fig. 4.11), a very small amount of vapour was produced from the surface of the water initially. An emulsion of terpene in the water was also observed. After half an hour, the ozonated water device was switched off, and over a 20 min period, the ozone concentration decreased. This decrease was more rapid and steeper compared with addition of the terpene to pre-ozonated water (combination C). Figure 4.11 illustrates the average results for Combination E (tap water and alcohol/terpene, plus ozone (then ozonated)) for all three terpenes. There was a sharper decline in ozone concentration when using limonene from 4 ppm to 0.2 ppm, compared to geraniol and alpha-terpinene.

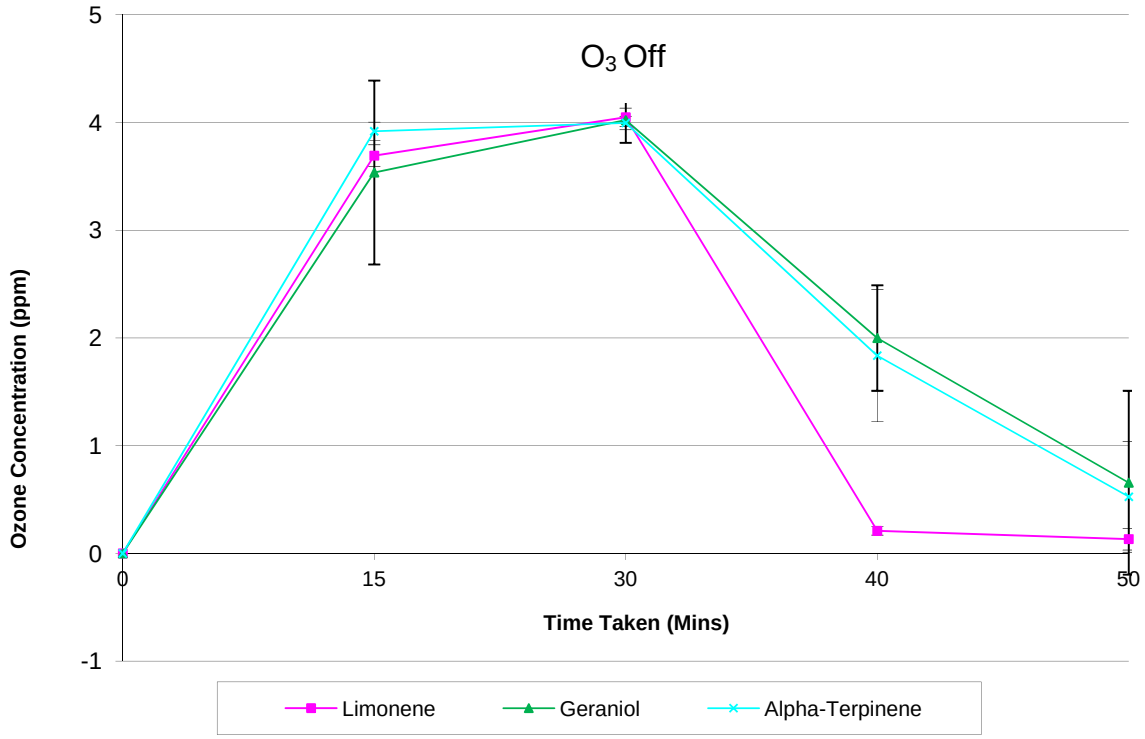



**Figure 4.11.** Tap water and alcohol/terpenes, plus ozone (Combination D). At time zero, ozone was added to the water with the terpene in alcohol. At 30 mins, ozonated water device was switched off. Error bars denote confidence intervals (CI) with a confidence level of 95%.

There were no differences seen in the reactivities of the three terpenes with ozone.

### 4.3.4 The effect of ozonated water and terpene on environmental *L. monocytogenes* and *P. aeruginosa*

**Surface attached microorganisms.** Two microorganisms (*L. monocytogenes* L002 and *P. aeruginosa* C001) were surface attached to food grade stainless steel 304 coupons which were subjected to various treatments. Treatments were performed in 4 L of tap water:

1) Terpene (d-limonene) (5 and 25 ppm) (A),

2) Ozonated water at 0.1 ppm (B),

3) Ozonated water and terpene (d-limonene) (5 and 25 ppm) (C) and,

4) Ozonated water at 4 ppm (D).

It has been hypothesised that a combination of ozonated water and terpene could have a strong effect on log reduction of foodborne pathogens, including *L. monocytogenes*. In order to see the effect of the action of ozonated water and terpene, compared with using ozonated water (0.1 ppm), or terpene treatments individually, the experiment was repeated but the concentration of terpene (limonene) was increased from 5 ppm (20 µl) to 25 ppm (100 µl).

Figure 4.12 illustrates the mean log reduction of *L. monocytogenes* L002 and *P. aeruginosa* C001 surface attached to stainless steel coupons treated with four different treatments, but at a concentration of terpene of 5 ppm. The ozonated water (~4ppm) (D) gave approximately 2.5 logs for both organisms, ozonated water and terpene (C) resulted in the highest log reduction of 2.7 and 3.5 log reduction, ozonated water (B) gave a 2.2 and 2.25 log reduction, whereas limonene treatment (A) resulted in a log reduction of approximately 1.75 and 1.26 logs for *Listeria* and *Pseudomonas*, respectively (Fig. 4.13). There were

significant differences (P≤0.05) between tests and controls, but there were no significant differences between the four treatments against *Listeria*, but a significant difference (P≤0.05) between each treatment tested against *Pseudomonas*.

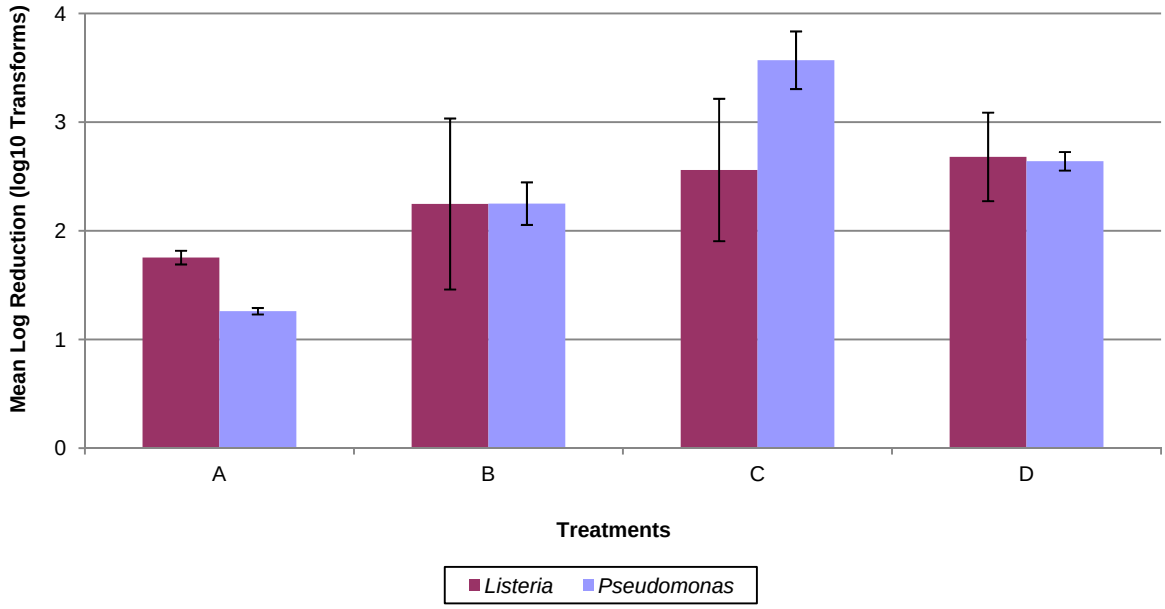



**Figure 4.12.** Mean log reduction of surface attached *L. monocytogenes* L002 and *P. aeruginosa* C001 on food grade 304 stainless steel coupons treated with different combination of ozonated water and terpene (limonene) (5 ppm). Error bars denote confidence intervals (CI) with a confidence level of 95%.

*Key: A – Terpene (limonene) at 5 ppm, B – Ozonated water at 0.1 ppm concentration, C – Ozonated water (0.1 ppm) and limonene at 5 ppm and D – Ozonated water (4 ppm) (in triplicate).*

In order to prove that the action of the terpene and ozonated water applied together is synergistic, the effect (X) must be greater than the additional effects of terpene (5 or 25 ppm) (A) alone and ozonated water at 0.1 ppm (B) alone.

**A + B = X.**

Where:

A = average $\log_{10}$ transform for (A) terpene only

B = average $\log_{10}$ transform for (B) ozonated water (0.1 ppm) only

C = average $\log_{10}$ transform for (C) ozonated water/terpene results.

The result X was then compared with the sum of average $\log_{10}$ transform for the combined treatment of ozonated water and terpene (C). If X was larger than C, then no synergy was involved. However, if sum of average $\log_{10}$ transform for the combined treatment of ozonated water and terpene (C) was larger than X, synergy was involved. Synergistic action is proved when **C>X**.

There was no synergistic action seen between ozonated water (0.1 ppm) and terpene, compared to treating the coupons with the terpene and ozonated water (0.1 ppm) alone (X vs C = 3.95 vs 2.6) against *Listeria*. There was a significant synergistic action seen between ozonated water (0.1 ppm) and terpene, compared to treating the coupons with the terpene and ozonated water (0.1 ppm) alone (X vs C = 3.51 vs 3.57) against *Pseudomonas* isolate. .

Figure 4.13 illustrates the mean log reduction of surface attached *L. monocytogenes* on food grade stainless steel coupons, when treated with four different treatments, but at a concentration of 25 ppm terpene.

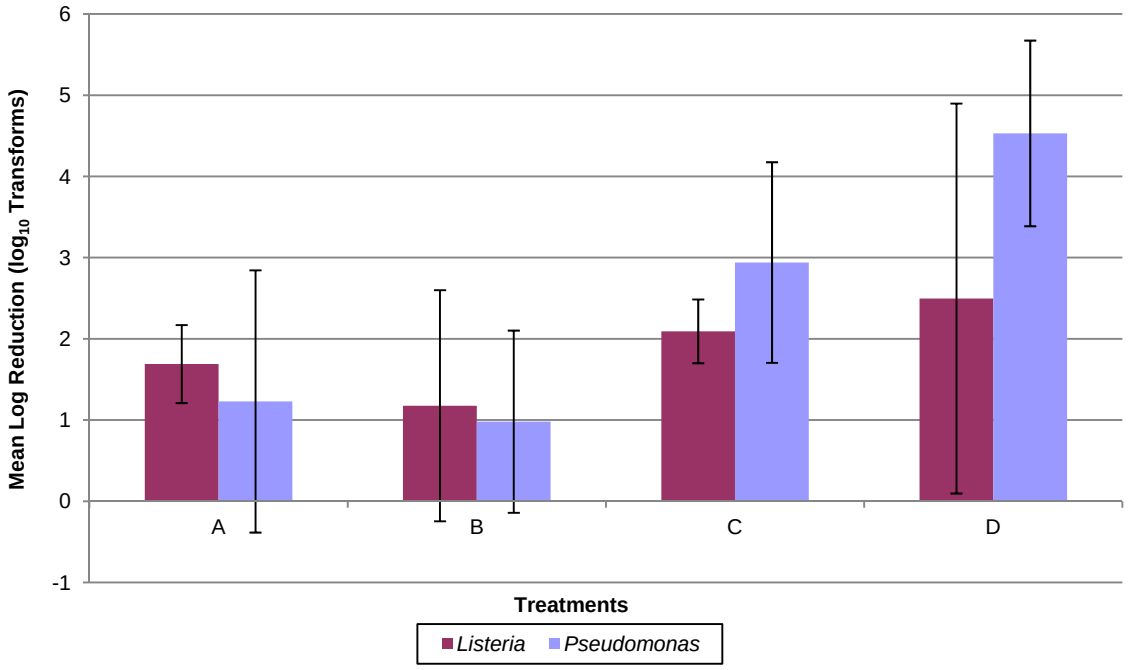



**Figure 4.13.** Mean log reduction of surface attached *L. monocytogenes* L002 and *P .aeruginosa* C001 on 25 $cm^2$ stainless steel (food grade 304) coupons treated with different combination of ozonated water and terpene (limonene) (25 ppm). Error bars denote confidence intervals (CI) with a confidence level of 95%.

*Key: A – Limonene at 25 ppm, B – Ozonated water at 0.1 ppm concentration, C – Ozonated water (0.1 ppm) and limonene at 25 ppm and D – Ozonated water (4 ppm) (in triplicate).*

Ozonated water at 4 ppm gave the highest log reduction of 4.5 and 2.5 log reduction for *P. aeruginosa* C001 and *L. monocytogenes* L002, respectively, whereas ozonated water at 0.1 ppm gave the lowest log reduction of about 1 log reduction for both organisms. Treatment C gave a log reduction of 3 and 2 log reduction for *P. aeruginosa* C001 and *L. monocytogenes* L002, respectively, compared to 1.2 and 1.7 log reduction for limonene only. Statistical analysis using one-way ANOVA revealed that there was significant difference ($P \leq 0.05$) between untreated and test, but there was no significant difference between treatments for *L. monocytogenes*. There was significant difference ($P \leq 0.05$) between certain

treatments (between treatments B and C) and also between C and D). There was no significant difference between A and B. There was a large significant difference between treatments C and D against surface attached *P. aeruginosa*, compared to the same treatments against *L. monocytogenes* (Fig. 4.13).

There was no synergistic action when surface attached *L. monocytogenes* was treated with ozonated water (0.1 ppm) and a terpene (limonene) (25 ppm), compared to just treating with ozonated water (0.1 ppm) and terpene (25 ppm) separately (X vs C = 2.7 vs 2). However, there was significant synergy when surface attached *P. aeruginosa* was treated with ozonated water (0.1 ppm) and terpene (limonene) (25 ppm) compared to just treating with ozonated water (0.1 ppm) and terpene (25 ppm) separately (X vs C = 2.2 vs 3).

**Biofilm microorganisms.** Single-species biofilms of the environmental strains *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* C001 were grown on food grade stainless steel, food grade polypropylene and polished granite and treated with ozonated water, ozonated water plus terpene (limonene, 25 ppm) and terpene only (limonene, 25 ppm) for 5 mins. Ozonated water at 4ppm (D) was not used, as this treatment produced unacceptable off-gas levels. Figure 4.14 illustrates the effect of ozonated water, ozonated water plus terpene and terpene against single-species biofilms of *L. monocytogenes* and *P. aeruginosa*.

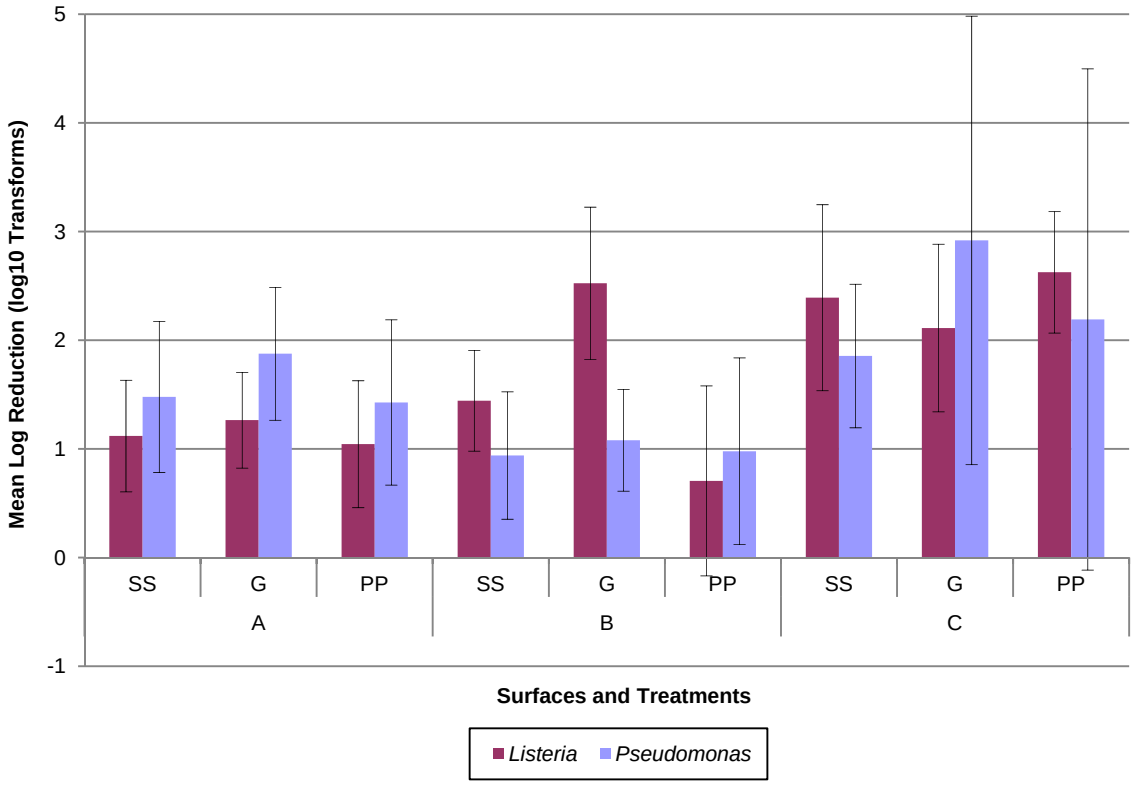



**Figure 4.14.** Mean log reduction of single-species biofilm of *L. monocytogenes* and *P. aeruginosa* on three surfaces treated with ozonated water (B) and terpene alone (A), and ozonated water plus terpene (limonene) (C). Error bars denote confidence intervals (CI) with a confidence level of 95%.

*Key: SS – food-grade stainless steel type 304, G – polished granite, PP – food grade polypropylene. A = Terpene, B = (~0.1 ppm) – ozonated water at 0.1 ppm, and C – ozonated water plus terpene.*

On stainless steel only, ozonated water treatment (B) gave 1.5 and 0.96 log reductions and the terpene treatment (A) gave 1.1 and 1.5 log reductions for *Listeria* and *Pseudomonas*, respectively, compared to ozonated water plus terpene (C) which gave 2.3 and 1.8 log reductions for *Listeria* and *Pseudomonas*, respectively. Statistical analysis using two-way ANOVA revealed there was a significant difference between test and untreated samples. There was no significant difference between surfaces or treatments for *Listeria*. However, there is a significant difference ($P \leq 0.05$) between treatments but not between surfaces, and/or their interaction for *Pseudomonas*. A one-way ANOVA revealed that there was a significant difference ($P \leq 0.05$) in log reduction between ozonated water and ozonated water plus terpene treatments; and between terpene and ozonated water plus terpene treatments.

There was no synergistic action seen between ozonated water (0.1 ppm) and terpene, compared to treating the coupons with the terpene or ozonated water (0.1 ppm) alone against *Listeria* and *Pseudomonas* biofilms. Table 4.1 illustrates the non synergistic action of combined ozonated water and terpene treatment, compared to using the treatment separately on *Listeria* and *Pseudomonas* biofilms.

**Table 4.1.** Table to illustrate the non synergistic action of using combined water and terpene treatment, compared to using the treatment separately on *Listeria* and *Pseudomonas* biofilms

| Surfaces | *Listeria* X vs C | *Pseudomonas* X vs C |
|---|---|---|
| SS | 2.563 vs 2.392 | 2.419 vs 1.856 |
| G | 3.79 vs 2.113 | 2.956 vs 2.92 |
| PP | 3.67 vs 2.626 | 2.407 vs 2.192 |

A dual-species biofilm of the two organisms grown together on food grade stainless steel, food grade polypropylene and polished granite was treated with ozonated water, ozonated water plus terpene (limonene, 25 ppm) and terpene only (limonene, 25 ppm). Figure 4.15 illustrates the effect of ozonated water, ozonated water plus terpene and terpene against dual-species biofilm (72 h).

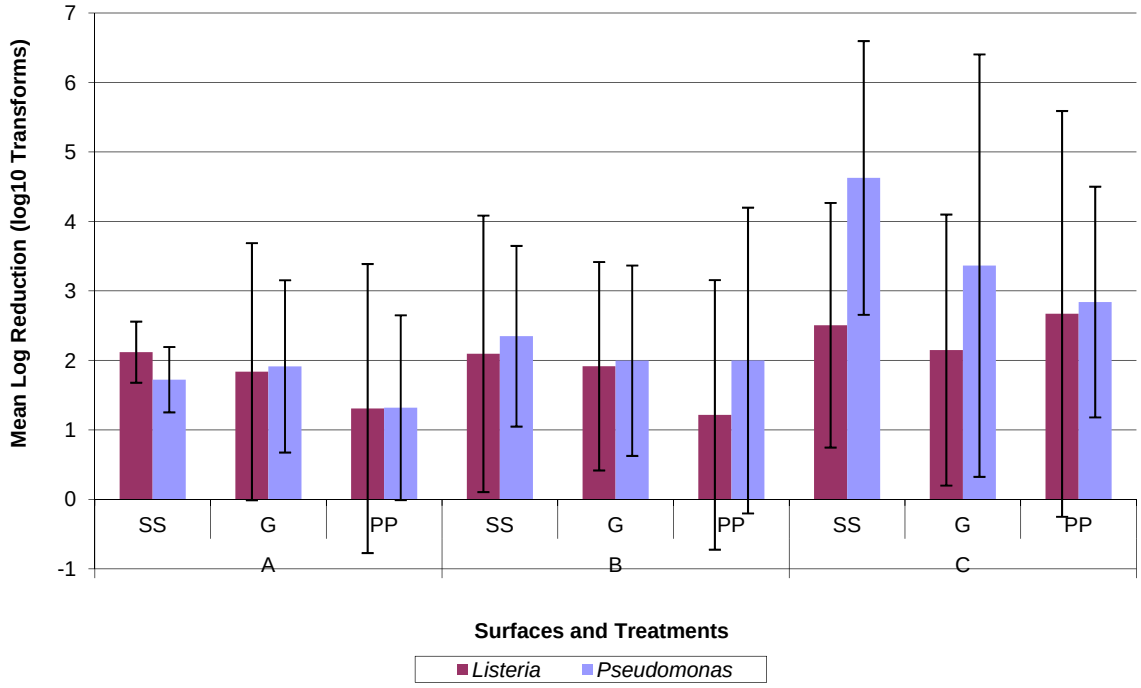



**Figure 4.15.** Mean log reduction of dual-species biofilm of *L. monocytogenes* L002 and *P. aeruginosa* C001 on three surfaces treated with ozonated water (B) and terpene alone (A), and ozonated water plus terpene (limonene) (C). Error bars denote confidence intervals (CI) with a confidence level of 95%.

*Key: SS – food-grade stainless steel type 304, G – polished granite, PP – food grade polypropylene. A = Terpene , B = (~0.1 ppm) – ozonated water at 0.1 ppm, and C – ozonated water plus terpene.*

On stainless steel only, ozonated water treatment (B) gave 2 and 2.3 log reductions and the terpene treatment (A) gave 2.1 and 1.7 log reductions against *L. monocytogenes* and *P. aeruginosa*, respectively, compared to ozonated water plus terpene (C) which gave 2.44 and 3.61 log reductions against *L. monocytogenes* and *P. aeruginosa*, respectively (Fig. 4.15). Statistical analysis using two-way ANOVA revealed a significant difference ($P \leq 0.05$) between treatments (between ozonated water and ozonated water plus terpene treatments; and between terpene and ozonated water plus terpene treatments) in the log reduction of *Pseudomonas* C001, but there were no significant differences between the three surfaces. A two-way ANOVA revealed that there was no significant difference in log reduction between treatments and/or surfaces with *Listeria* L002.

There was no synergistic action for the single-species biofilms (*Listeria* or *Pseudomonas*) on all three surfaces (G, SS or PP). However, there was a synergistic action seen with the dual-species biofilm. There was no synergy seen with the *Listeria* L002 isolate, but there was a synergistic action seen with the *Pseudomonas* C001 isolate on SS and PP surfaces only (X vs C = for SS – 4.071 vs 4.626 and PP – 2.627 vs 3.364).

### 4.4 Discussion

In order to determine the potential levels of off-gassing produced if any, from the ozonated water treatments, a series of experiments was designed from each combination of treatments against the two chosen microorganisms. Deionised water and potable tap water from the laboratory were initially used as the media of choice, but in subsequent experiments, tap water was selected as the appropriate medium for the generation of ozonated water treatments, because food processing facilities would typically have access to tap water. The ozonated water generator device had set arbitruary units. Setting 70 was selected at first and ozone was bubbled through deionised and tap water, the levels of off-gas produced from both types of water were at unacceptable levels. Setting 60 was then selected, as a setting needed in order to produce a concentration of 0.1 ppm ozone in water and produce as little as 0.1 ppm off-gases (section 4.3.1). However, at this setting, the ozone concentration steadily increased from zero to above 60 ppm after 25 minutes of being switched on. Thus, it was decided, therefore, to reduce the dial setting to below 60 to minimise the level of ozone off-gas produced. When the dial was set to 58, the level of ozone gas produced never reached more than 0.1 ppm,

and was thus selected for all subsequent experiments to give an ozone concentration of 0.1 ppm.

Ozone degradation was also measured in order to determine the activity and stability of ozone in the water (ozonated water treatment). When the device was set at 70, the level of ozone in the water in tap water, reached nearly 3 ppm, after 15 mins and took 20 mins to fall to 0.5 ppm, compared to when the device was set at 60, the level of ozone in the water gave a reading of approximately 2 ppm and took 15 mins to fall to below 0.5 ppm. Ozone decay when prepared in deionised water was slower and took 45 minutes to fall to below 0.5 ppm from a level of 4 ppm. The tap water, however had a faster rate of decay, from a concentration of 3 ppm, it took 25 mins to fall to 0.5 ppm. This could be due to the biological and chemical ozone demand (BOD & COD) of different types of water, as deionised water is filtered and so microbial cells, impurities and excess ions are removed. This would cause a lower BOD & COD than tap water. The tap water, however, had a faster rate of decay as the ozone was being consumed, resulting from a higher BOD & COD. Güyem-Seydim *et al*. (2000) reported that cold (at 4°C) ozonated water (in deionised water) had a significantly lower COD compared to warm (at 40°C) ozonated water (in deionised water).

The effect of organic matter on the activity of ozone was also determined. 250 ppm alcohol (methanol) was used to determine the degradation of ozone in the water. Ozone decay in the water fell from 3.46 to 0.26 ppm in 20 minutes, from the point when the alcohol was added. The ozonated water device was switched off 10 minutes after the alcohol had been added. When the concentration of alcohol was decreased to 50 ppm, a gradual decline in ozone was seen, followed by a slower decay compared to using 250 ppm alcohol, from 3.5 to 0.2 ppm in 40

mins (from when the alcohol was added). The ozonated water device was again switched off 10 mins after the alcohol had been added. The higher the level of alcohol (organic matter), the faster the rate of decay of ozone, which is due to the ozone demand of the organic load. The organic matter (alcohol) absorbs the ozone present (giving the water a high ozone demand) in the water. The chosen alcohol, methanol could suppress the activity of hydroxyl radicals. The antimicrobial efficacy of ozonated water (with and without added organic matter) has been investigated previously (Restaino *et al*., 1995). The study looked at the antimicrobial effects of the treatment in a re-circulating concurrent reactor against four gram-positive and four gram-negative bacteria, and yeasts and spores of *Aspergillus niger*. Ozone can be unstable in water, whereas re-circulating water through an ozonator allowed for better maintenance of a sufficient ozone concentration. Researchers found that among gram-positives, *L. monocytogenes* 4b was significantly ($P \leq 0.05$) more sensitive than either *S. aureus* or *E. faecalis* to ozonated water treatment. The organic matter (bovine serum albumin; BSA) affected the efficacy of ozonated water treatment by significantly reducing the ozone output levels to 1.49 mg/ml. However, water with soluble starch did not significantly reduce (1.98 mg/ml) the ozone output levels compared to 0.188 mg/ml with deionised water.

Ozonated water and three selected terpene (geraniol, limonene and alpha-terpinene) treatments were performed in order to determine the level of reaction between ozone and terpene and ozone decay in response to the addition of the terpene in 2 different combinations. The terpenes (25 ppm) were added to 50 ppm alcohol (methanol) in order to dissolve the terpenes and allow the terpenes to be evenly dispersed in the water. The terpenes were added in various combinations (the addition of terpene to ozonated water (combination C), or water and terpene

and then the addition of ozone (combination E) or water and terpene only (combination D)) to determine the desired combination of ozone and terpene for later experiments. A production of a vapour and/or odour was observed and suggested an indication of a reaction between ozone and terpene had occured. Combination C was the most reactive, with combination D being the least reactive. Combination C (for all terpenes) showed a slower decay (from when the ozone was switched off) of 30 minutes, from 4 ppm to zero. Combination E (for all three terpenes) showed a faster decay of 20 mins (from when the ozone was switched off), from 4 ppm to zero. The main decline was seen after the first 10 mins and was only observed with the limonene. Combination C and the terpene, limonene was chosen for subsequent experiments.

Ozonated water treatment alone is well known to be effective in removing biofilms (Fielding *et al*., 2007), and terpenes (essential oils from plants) are known to have strong antimicrobial activity (Singh *et al*., 2002b). The effect of ozonated water and/or terpene (limonene) on surface attached and single- and multi-species biofilms of environmental isolates, *L. monocytogenes* and *P. aeruginosa* was investigated. Initially, surface attached single-species were treated with ozonated water at 0.1 ppm (treatment B), water and terpene only (treatment A), ozonated water and terpene at 0.1 ppm (treatment C) and ozonated water at 4 ppm (treatment D). The ozonated water (~4ppm) (D) gave approximately 2.5 logs for both organisms. The ozonated water and terpene (C) resulted in the highest log reduction of 2.7 and 3.5, compared to ozonated water (B) and limonene treatment (A) used individually for *Listeria* and *Pseudomonas*, respectively. There were significant differences ($P \leq 0.05$) between test and untreated samples, but there were no significant differences between the four treatments against *Listeria*, but a significant difference ($P \leq 0.05$) between each treatment tested against

*Pseudomonas,* with the exception between the terpene only (treatment A) and ozonated water at 0.1 ppm (treatment B) treatments. For the *P. aeruginosa* C001 isolate, there was a synergistic effect with the combined treatment, compared to the treatments alone. However, there was no synergy using the combined treatment compared to the individual treatments seen with the *L. monocytogenes* L002 isolate. The decision was taken to increase the concentration of terpene added from 5 to 25 ppm, in order to see if there was a clearer synergistic effect. Again, using the higher concentration of terpene, ozonated water and terpene (C) was more effective, giving 2 and 3 log reduction for environmental *L. monocytogenes* and *P. aeruginosa*, respectively, compared to ozonated water at 0.1 ppm (B), and the terpene (treatment A). The ozonated water at 4 ppm (D) treatment gave higher log reduction compared to treatment C. Robbins and others (2005) reported that a concentration of 4 ppm ozonated potassium phosphate buffer (PPB) was necessary in order to significantly reduce attached *L. monocytogenes* to SS. The log reduction of all four treatments at the lower terpene concentration against the *L. monocytogenes* L002 isolate gave surprisingly higher log reductions than with the higher concentration of terpene. This synergistic action was again significantly more noticeable with the surface attached *P. aeruginosa*, but not *L. monocytogenes*, and there was no marked difference in synergy when using an increased concentration of terpene.

For the biofilms, the higher concentration of terpene was chosen in order to see a larger effect and only three of the treatments were used. Ozonated water (treatment D) was not used, as this produced unacceptable off-gas levels. Single-species 72 h static biofilms of both organisms were grown on three surfaces (food grade stainless steel, type AISI 304, with 2b finish, polished granite and food grade polypropylene). There was a significant difference between test and

untreated. For the *L. monocytogenes* L002 isolate, there were no significant differences between surfaces or treatments. For the *P. aeruginosa* C001 isolate, however, statistical analysis using a two-way ANOVA revealed a significant difference between treatments, but not between the surfaces. The combined treatment (C) gave higher log reductions than using ozonated water and terpene individually. The dual-species 72 h static biofilm of the two organisms was grown up on the same three surfaces and treated against the same three treatments, and again a similar pattern was noticed. There was no synergistic action seen when using the ozonated water and terpene treatment (treatment C) compared to using the treatments alone with the *Listeria* isolate in the dual-species biofilm, but there was a synergistic action seen with the *P. aeruginosa* C001 isolate in the dual-species biofilm, but only on the stainless steel and polypropylene.

It has been noticed that essential oils were found to be more active against Gram positives than Gram negatives (Cowan, 1999; Demirci *et al*., 2008). This partially supports the findings of this experiment. *L. monocytogenes* had higher log reductions for the terpene only treatment compared to *P. aeruginosa* surface attached or in dual-species biofilm on SS and PP.

Ozonated water and terpene (C) treatment was more effective than using the individual treatments and had a synergistic effect compared to the individual treatments against *Pseudomonas* only. All treatments used, with the exception of terpene only against *P. aeruginosa* C001 isolate, either surface attached or in single and dual-species biofilms, gave higher log reductions compared to *L. monocytogenes* L002 isolate. This can be explained partially by differences in cell wall structure, as *L. monocytogenes* is gram positive and *P. aeruginosa* is gram negative. Gram negatives can withstand the action of essential oils and their

components, because of the lipopolysaccharide present in outer membrane (Oussalah *et al*., 2007). However, the nature of the gram negative outer membrane makes the bacterium more susceptible to other antimicrobials, such as oxidising agents. It consists of phospholipids, lipoproteins, lipopolysaccharides and contains porins (channels) which makes the membrane semi permeable to antimicrobial agents. Gram positives have a much thicker peptidoglycan layer which can more easily withstand antimicrobial agents, such as terpenes, disinfectants and biocides (Schleifer and Kandler, 1972).

# Chapter 5. Open Air Factor (OAF)

## 5.1 Introduction

Open air factor (OAF) was discovered in 1968 and is a highly reactive chemical species, formed when ozone reacts with any compound containing unsaturated hydrocarbons (carbon-carbon double bond), known as olefins or alkenes (May and Druett, 1968). Natural olefins are released by plants and flowers (terpenes), but many olefins originate in the atmosphere from petroleum products (Dark and Nash, 1970). OAF is not a single molecule, but a collection of chemical species that vary in nature (De Mik and De Groot, 1978). In order to determine the activity of ozone and terpene as 'a form of open air factor', but in water, terpenes that are natural olefins (that contain carbon-carbon double bond) were emulisified in alcohol before adding to ozonated water (chapter 4).

There are a few air disinfection devices on the market that have been manufactured for commercial and public sector areas. These devices were designed for air quality and chemical disinfection. Professor Ellwood from Porton Down and David Macdonald from Inov8 Science Ltd. (Milton Keynes, UK) characterised together the chemical nature of open air factor and developed an effective, affordable and safe system of air disinfection. Aerte Ltd. (UK) manufacture and design air disinfection (AD) devices for air disinfection in enclosed indoor spaces. The AD device is a floor standing unit which is designed to be left switched on in a 300 $m^3$ enclosed indoor space with continuous personnel movement, to provide precise levels of ozone gas and terpene. The AD (Aerte Ltd., UK) works on the principle of taking in ordinary air, incorporating ozone and within the confines of the machine, quenches the ozone with the olefin

(d-limonene) to generate hydroxyl radicals and produce a faint pleasant odour. Hydroxyl radicals are emitted while avoiding the release of ozone (Aerte Ltd., 2010 [online]). The AD device has been designed to be used in busy indoor environments, such as homes, offices, schools and hospitals, with continuous personnel movement, as well as quiet areas with a few people passing through it, and can be found as wall mounted or as floor standing units (Figure 5.1). The AD device operates continuously 24 hours a day to provide maximum benefits and is guaranteed for one year, although the life of the device is suspected to last several years (Aerte Ltd., 2010 [online]).

**Figure 5.1.** Aerte Ltd. Air disinfection (floor standing) device.

(http://www.inov8science.com/) [Online] [Accessed on 07.06.2010] (with permission from Aerte Ltd).

The AD device is made from an aluminium casing cover and requires a safe electrical low voltage (SELV) of 12V AC and 1.5 amps to power the device.

Table 5.1 below describes all the technical specifications for the AD device. The device has an operating noise level of 38 db. The external ozone level generated by the device is ≤ 0.2 ppm, which is below the safe exposure limit for the UK. The

device has undergone rigorous testing and has EU approval and meets all necessary safety regulations (Inov8 Science Ltd., 2010 [online]).

**Table 5.1.** The specifications for the AD device. (Aerte Ltd. [Online] http://www.inov8science.com/ [Accessed on 07.06.2010]).

| | |
|---|---|
| Electrical Supply | 12V AC 1.5A @ 50/60 Hz |
| External Ozone Level | ≤ 0.2 ppm |
| Olefin Consumption | 1g per day (approx) (or 1-2ml) |
| Weight | 4.5 Kg (approx) |
| Consumable bottle capacity | 180 ml |
| Minimum life of consumable | 90 days |
| Temperature | 15°C, 35°C 60% RH |
| Dimensions | Height 420 (mm) x Diameter 200 (mm) |
| Treatment area | 30 $m^3$ to 300 $m^3$ |

The device requires refillable cartridges containing distilled extract of plants and flowers (d-limonene) which need to be replaced every 90 days. A warning sounds if the cartridge has not been replaced and when the olefin is exhausted, the machine will switch itself off. The refill cartridges are specially designed to house precise mixes of ozone and olefins (d-limonene) in order to produce a hydroxyl radical cascade (Aerte Ltd. [online]).

Limonene is a natural constituent of many citrus oils including orange, lemon, mandarin, grapefruit and lime and many other plant species. Limonene can be found in the form of l-limonene or d-limonene. L-limonene smells of pines (like turpentine) whereas d-limonene has a citrus odour. D-limonene (l-methyl-4-isopropenyl-l-cyclohexane) is found as a liquid and is a by-product of orange juice manufacture (Best, 1990). Limonene is commonly used in food and cosmetics

(fragrances and perfumes) industries and also in air fresheners (Tsuda *et al*., 2004). The Swedish government has set a safe exposure limit for limonene of 150 mg/m$^3$ (27 ppm) over an 8 h period and 300 mg/m$^3$ exposure limit for short term contact time of 15 mins (Inov8 Science Ltd [online]). Limonene in high concentrations is a potential irritant. Few people develop sensitivities to perfumes and fragrances but it is these people that may acquire an allergic reaction to limonene. However, this sensitivity is extremely rare (EPA, 1994).

The AD device is safe for humans, but can kill airborne bacteria, yeasts, and viruses found in indoor rooms of approximately 300 m$^3$ (ideal for offices). The technology relies on the production of a hydroxyl radical cascade which means that the AD can produce rapid air disinfection without the need of air circulation or filtration (Aerte Ltd. [online]). The hydroxyl radical is a reactive oxygen species, highly reactive, unstable and has a half life of $10^{-6}$ seconds. They react with a wide range of organic molecules, particularly attacking carbon-carbon double bonds. The reaction of a hydroxyl radical with a double carbon bond results in the production of other radicals in a cascade reaction. This cascade reaction continues until all the chemicals available to react have reacted and the concentration of radicals decreases rapidly (Aerte Ltd. [online]). Hydroxyl radicals are known as “nature’s disinfectant” and the activity of hydroxyl radicals is essential to life. The hydroxyl radical (OH•) is not usually present in enclosed spaces. Natural systems found in the atmosphere and in the human body rely on the hydroxyl radical. It is a key component of the macrophage defence mechanism in combating invading pathogens, by reacting with proteins on the surface of pathogens, destroying the cell wall (Hood, 1974).

Research by the Health Protection Agency (HPA) into the AD device identified the rate of disinfection to be in the order of a 5 log reduction in less than one hour against gram positive and gram negative bacteria and viruses. Further research has been carried out at Leeds University into the effect of the AD device on the rate of survival of aerosolized clinical isolates of *Staphylococcus aureus* and *Clostridium difficile.* There was a < 1.5 % survival rate for both organisms. Similar tests for *Pseudomonas spp*., *Mycobacteria spp*., *Burkholderia spp.*, and *Acetinobacter spp*. showed similar results. The AD device is unique as it releases a continuous stream of hydroxyl radicals into the indoor air without creating any additional ozone levels into the room (Aerte Ltd. [online]).

The research into the AD device has led to the demand for units to be supplied to many UK hospitals in Sunderland, Hereford, Manchester, Wigan, Cumbria and Kent, and many hospitals in the USA (Anon, 2008). A Burns' ITU at St. Andrew's Centre for Plastic Surgery and Burns carried out a study into the effects of AD device in the Burns' ITU. Environmental colony counts in settle plates were significantly higher in rooms where the patient was residing. The AD device significantly reduced the environmental colony counts from the air. Air disinfection units have played an important role in infection prevention within the Burns' ITU (Hafeez *et al*., 2009).

OAF is a potentially effective antibacterial agent and has the potential to reduce the microbial load of the air. This study investigated the effectiveness of Open Air Factor using an Air Disinfection device (manufactured and supplied by Aerte Ltd., Milton Keynes, UK) in the form of d-limonene at a concentration of ≤ 0.2 ppm against environmental *Listeria monocytogenes* L002 and *Pseudomonas*

*aeruginosa* C001 surface attached and in single- and multi-species biofilms for the purpose of surface disinfection.

The aim of this study was to investigate the effect of OAF on surface attach and biofilm environmental *Listeria monocytogenes* (L002) and *Pseudomonas aeruginosa* (C001) on different surfaces. The objectives were:

1. To study the effect of OAF on surface attached *L. monocytogenes* L002 and *P. aeruginosa* C001 on food grade stainless steel coupons at different orientations treated with OAF for 1 hour.

2. To study the effect of OAF on 72 h single-species biofilms of *L. monocytogenes* L002, *P. aeruginosa* C001 and dual-species biofilm.

## 5.2 Materials and Methods

*Preparation of suspension culture.*

**Surface attached microorganisms.** The preparation of the suspension cultures of environmental isolates *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* C001 were repeated as in section 2.2.4. The resulting suspensions gave approximately 2 x $10^9$ CFU/ml. For example, 152.5/0.1 x (2+0) x $10^{-6}$ = 1.5x$10^9$. Inoculation and orientation (horizontal, vertical and inverted) of stainless steel coupons was reproduced as in section 2.2.4.

**Biofilm microorganisms.** Biofilms were prepared as in section 2.2.6 on 1 $cm^2$ food grade stainless steel, polished granite and food grade polypropylene.

*Treatment of coupons.* Open Air Factor was generated using an Air Disinfection (AD) device (manufactured and supplied by Aerte Ltd., Milton Keynes, UK).

The floor standing AD unit was situated 50 cm away from the positioning of the samples and 40 cm high, surrounded by the mixing fans in the chamber. The AD unit was left switched on in a well mixed 100 $m^3$ class 2 Bioaerosol test chamber, and operated according to manufacturer's instructions. The AD device was switched on permanently throughout and between the test runs and operated at all times inside the chamber. The OAF was generated by the delivery of d-limonene. The AD device is designed to house disposable cartridges that deliver d-limonene at < 1 ppm (0.0167 ppm) per day over a 90 day period. Based on 0.1g d-limonene per day consumption 4.167mg/hour were consumed (Aerte Ltd).

A study carried out by Dr Jackson for Inov8 Science Ltd (now Aerte Ltd) investigated the maximum concentrations of d-limonene delivered by the AD device when operated in a 60$m^3$ room with one air change per hour. The concentration of d-limonene in a 60 $m^3$ room over an hour was calculated:

$4.167 = C \times 60$

Where C is the d-limonene concentration in $mg/m^3$.

$C = 4.167 / 60 = 0.0694$ $mg/m^3$.

Assuming that 1 $mg/m^3$ = 0.180 ppm.

= 0.0694 x 0.180 ppm

= 0.0125 ppm (12.5 ppb) in a 60$m^3$ room.

In the 20 $m^3$ Bioaerosol Test Chamber, using the same calculation, 0.0375 ppm (37.5 ppb) of terpene could be delivered into the Bioaerosol chamber.

Surface attached coupons were placed face up on clamp stands. Bioflm inoculated coupons were placed face up in sterile Petri dishes on clamp stands,

which were placed in the centre of the chamber approximately 50 cm away from the AD device. The coupons were exposed to OAF for a contact time of one hour. Once treated, the coupons were removed from the chamber. All untreated (control) coupons were inoculated and left untreated for 1 hour at room temperature before being sampled.

*Sampling.* As in Section 2.2.1.

*Enumeration of survivors.* The drop plate method was performed, as in section 4.3.1, into order to enumerate number of biofilm cells (Herigstad *et al*., 2001). Serial dilutions were carried out as necessary. Five coupons were used for each surface (stainless steel, food grade polypropylene, polished granite) (n=5). Each test run was carried out in triplicate (n=15) and all results are reported as $\log_{10}$ transforms of actual counts.

*Statistical analysis.* Data analysis was performed using one-way and two-way ANOVA (Minitab version 15, Minitab Ltd, UK) on the effect of OAF (d-limonene) using an AD device on environmental *L. monocytogenes* and *P. aeruginosa* isolates in single-species and dual-species biofilms and surface attached to different food contact surfaces.

## 5.3 Results

**Surface attached microorganisms.** *L. monocytogenes* L002 and *P. aeruginosa* C001 were surface attached to food grade stainless steel 304 coupons and treated with OAF in the form of d-limonene, produced by AD device (Inov8 Science Ltd., UK).

Figure 5.2 illustrates the effect of OAF against surface attached environmental *L. monocytogenes* L002 to food grade stainless steel 304 coupons.

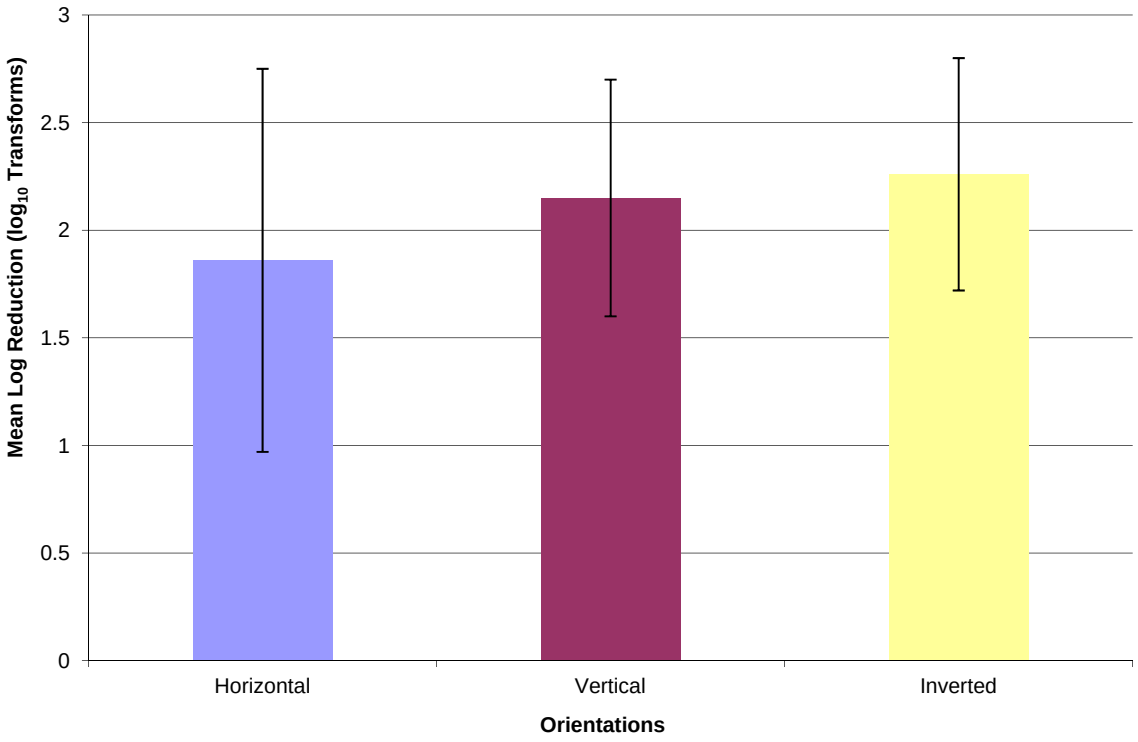



**Figure 5.2.** Mean Log Reduction of surface attached *L. monocytogenes* L002 to food grade stainless steel coupons in different orientations treated with OAF for 1 h. Error bars denote confidence intervals (CI) with a confidence level of 95%.

OAF in the form of d-limonene against surface attached *L. monocytogenes* cells to food grade 304 stainless steel coupons gave approximately 2 log reduction. Statistical analysis using a two-way ANOVA revealed that there was no significant difference between orientations of coupons, but there was a significant difference

(P ≤0.05) between untreated (control) and test samples. The untreated coupons were sampled and enumerated the same as test coupons, see section 5.2).

Figure 5.3 illustrates the effect of OAF against surface attached environmental *P. aeruginosa* C001 to food grade stainless steel 304 coupons.

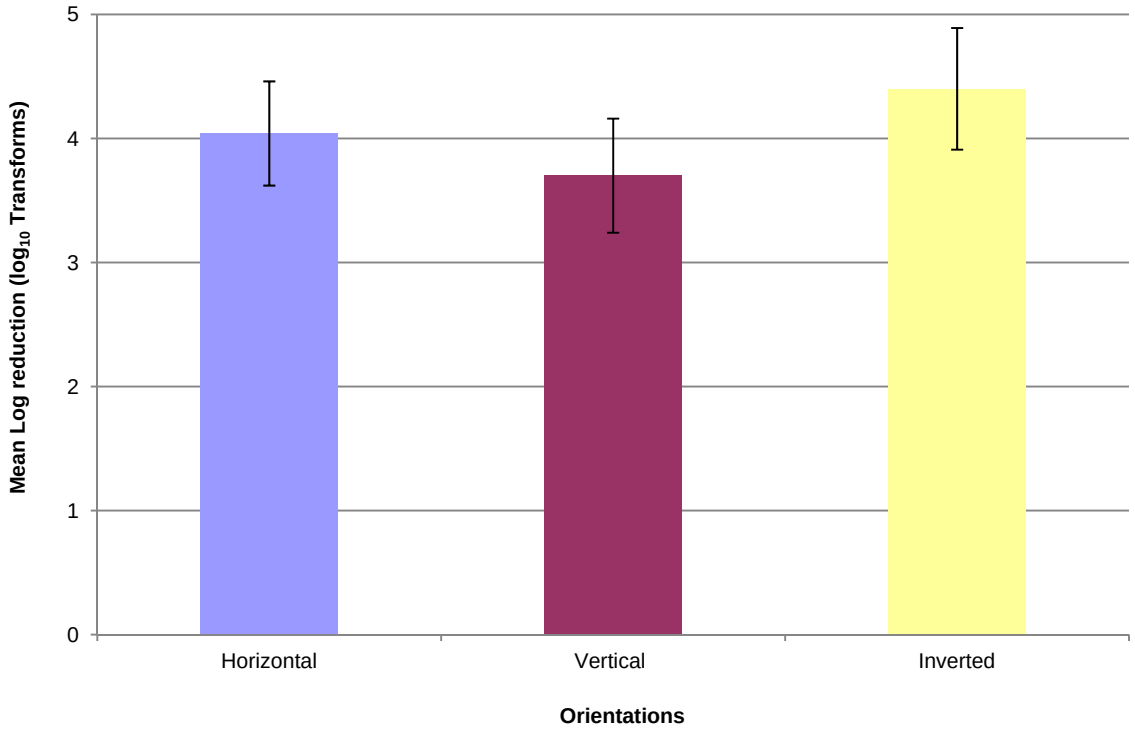



**Figure 5.3.** Mean Log Reduction of Surface Attached *P. aeruginosa* C001 to food grade stainless steel coupons in different orientations treated with OAF for 1 h. Error bars denote confidence intervals (CI) with a confidence level of 95%.

OAF in the form of d-limonene against surface attached *P. aeruginosa* cells to food grade 304 stainless steel coupons gave approximately 4 log reduction. Statistical analysis using a two-way ANOVA revealed that there was no significant difference between orientations of coupons, but there was a significant difference (P ≤0.05) between untreated and test samples. Statistical analysis revealed that there was a significant difference (P ≤0.05) between the two surface attached organisms. Statistical analysis also revealed that there was a significant difference between gaseous ozone (chapter 2) and OAF treatments on surface attached *L. monocytogenes* L002 to food grade SS.

**Biofilm microorganisms.** Single- and dual- species biofilms of environmental *Listeria monocytogenes* and *Pseudomonas aeruginosa* on food grade stainless steel, food grade polypropylene and polished granite were treated with OAF in the form of d-limonene (< 0.2 ppm). Figure 5.4 illustrates the effect of OAF against environmental *L. monocytogenes* L002 biofilm on food grade stainless steel 304, food grade polypropylene and polished granite coupons.

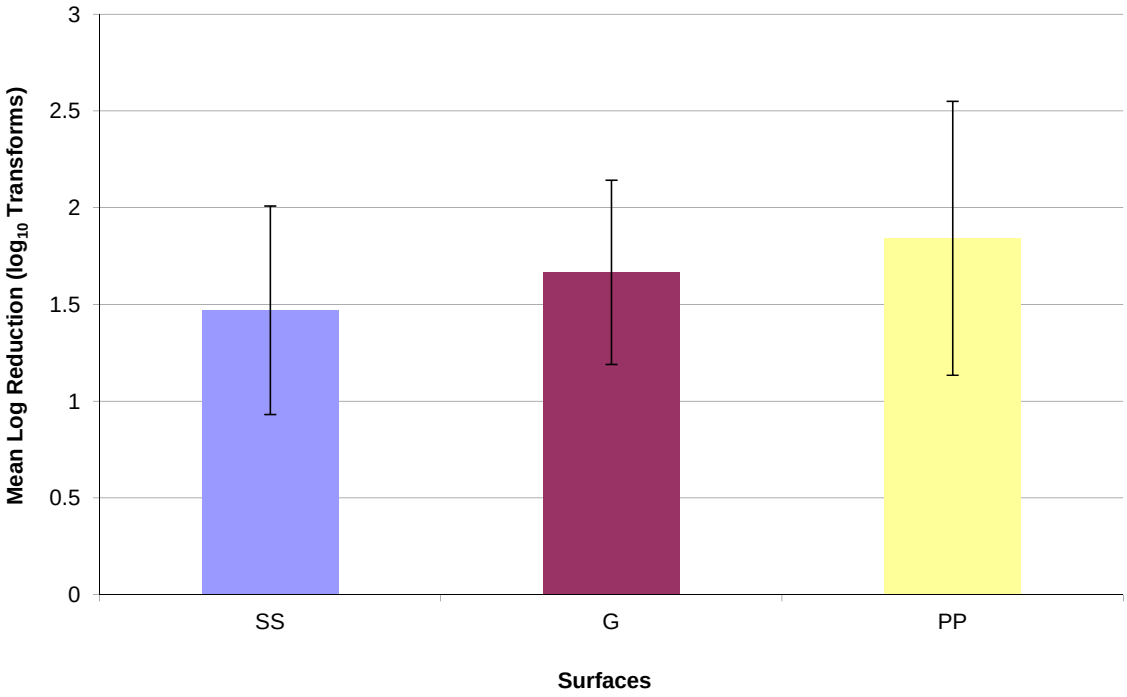



**Figure 5.4.** Mean Log Reduction of *Listeria monocytogenes* L002 biofilm on food grade stainless steel, food grade polypropylene and polished granite coupons treated with OAF for 1 h. Error bars denote confidence intervals (CI) with a confidence level of 95%.

Key: SS – stainless steel, G – granite and PP – polypropylene

OAF in the form of d-limonene against *L. monocytogenes* biofilm on various surfaces gave approximately 1.5 log reduction. Statistical analysis using a two-way ANOVA on log transforms revealed that there was a significant difference ($P \leq 0.05$) between stainless steel and polypropylene and granite and polypropylene coupons, but there was no significant difference between stainless steel and granite. There was also a significant difference ($P \leq 0.05$) between untreated (control) and test (SS, G, and PP) samples.

Figure 5.5 illustrates the effect of OAF on environmental *Pseudomonas aeruginosa* biofilms on food grade stainless steel, food grade polypropylene and polished granite coupons.

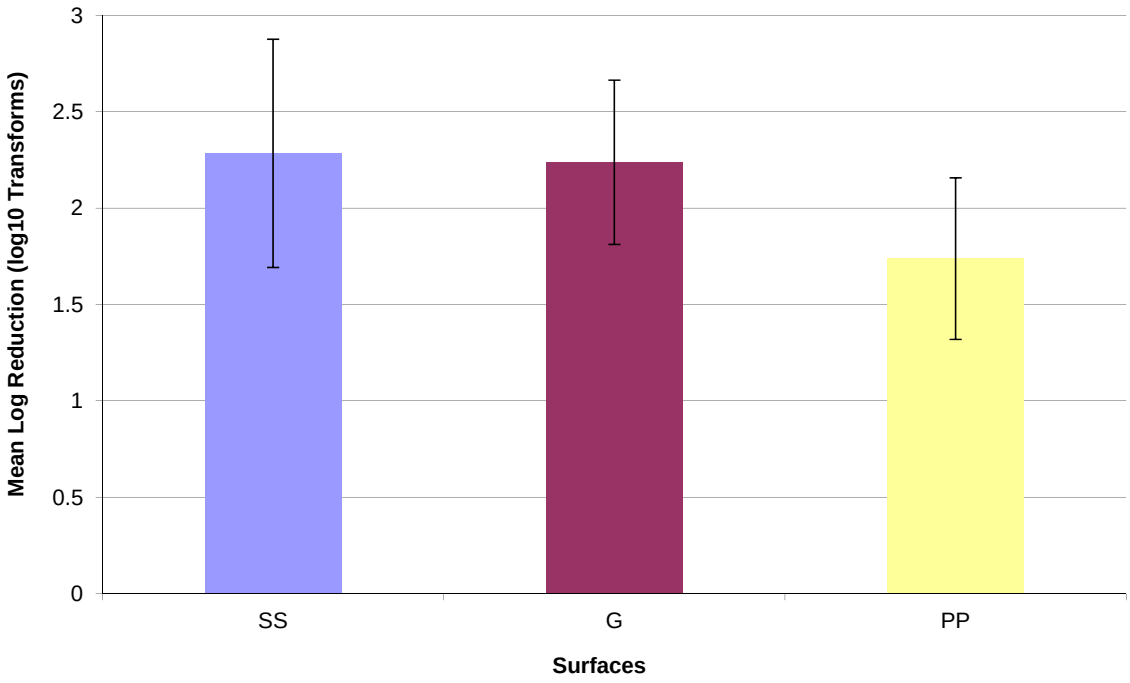



**Figure 5.5.** Mean Log Reduction of *Pseudomonas aeruginosa* C001 biofilm on food grade stainless steel, food grade polypropylene and polished granite coupons treated with OAF for 1 h. Error bars denote confidence intervals (CI) with a confidence level of 95%.

Key: SS – stainless steel, G – granite and PP – polypropylene

OAF (d-limonene) against *P. aeruginosa* biofilm on various surfaces gave approximately 2 log reduction. Statistical analysis using a two-way ANOVA on log transforms revealed that there was a significant difference ($P \leq 0.05$) between stainless steel and polypropylene and granite and polypropylene coupons, but there was no significant difference between stainless steel and granite. There was also a significant difference ($P \leq 0.05$) between untreated and test samples. There was a significant difference ($P \leq 0.05$) between surface attached and biofilm organisms on the stainless steel surface.

Statisitcal analysis using a two-way ANOVA with Tukey's comparison revealed that there were significant differences between surface attached and biofilm Listeria.

Figure 5.6 illustrates the effect of OAF on dual-species biofilm on food grade stainless steel, food grade polypropylene and polished granite coupons.

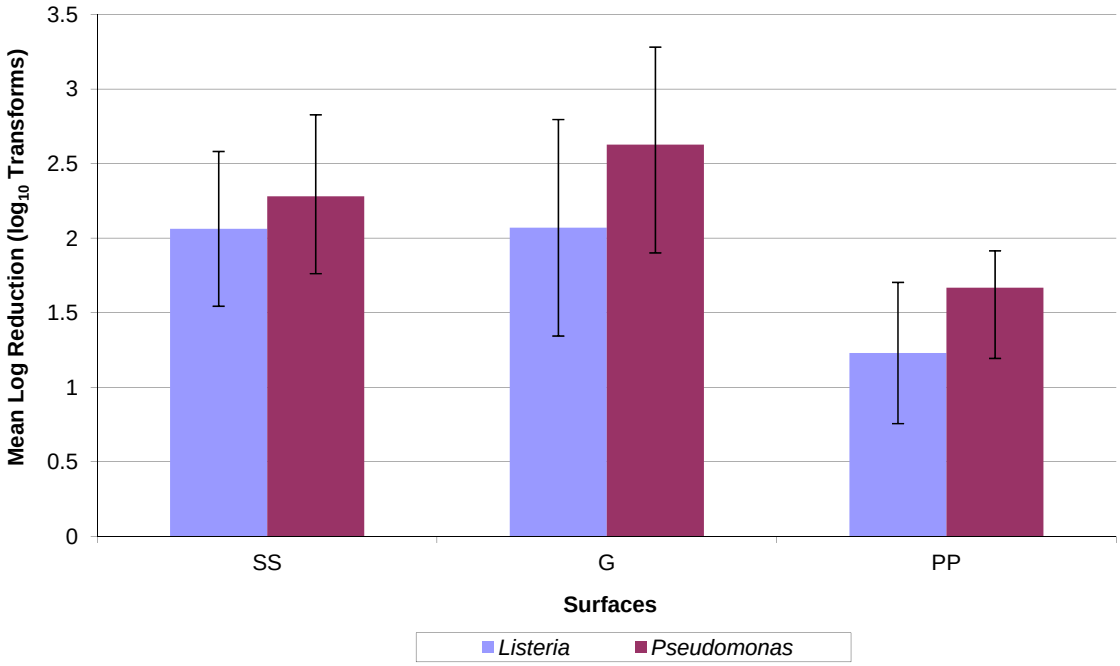



**Figure 5.6.** Mean Log Reduction of dual-species biofilm on food grade stainless steel, food grade polypropylene and polished granite coupons treated with OAF in the form of d-limonene for 1 hour produced from AD device (Inov8 Science Ltd., UK). Error bars denote confidence intervals (CI) with a confidence level of 95%.

Key: SS – stainless steel, G – granite and PP – polypropylene

OAF (d-limonene) against dual-species biofilm to various surfaces gave for *Listeria* L002 and *Pseudomonas* C001 approximately 1.8 and 2.2 log reductions, respectively. Statistical analysis using a two-way ANOVA revealed that there was a significant difference ($P \leq 0.05$) between stainless steel and polypropylene and between granite and polypropylene coupons, but there was no significant difference between stainless steel and granite. There was also a significant difference ($P \leq 0.05$) between the two organisms.

Table 5.2 shows a synopsis of mean log reduction (CFU $cm^{-2}$) and standard deviation of surface attached and biofilm *L. monocytogenes* on stainless steel (SS), granite (G) and polypropylene (PP) coupons after 1 hour treatment with ozone and open air factor.

**Table 5.2.** Effect of ozone and open air factor (OAF) treatment on the survival of *Listeria monocytogenes* on stainless steel (SS), granite (G) and polypropylene (PP) coupons after 1 hour. (Nicholas *et al*., 2013).

| | | Mean log reduction (CFU $cm^{-2}$) | | |
|---|---|---|---|---|
| Inoculum | Treatment | SS | G | PP |
| Surface attached | Ozone (45 ppm) | 3.41 (1.48)$^{Aa}$ | 3.42 (0.98)$^{Aa}$ | 1.11 (0.53)$^{Ab}$ |
| | OAF | 1.86 (0.51)$^{B}$ | ND | ND |
| Biofilm | Ozone (45 ppm) | 0.56 (0.45)$^{Ba}$ | -0.20 (0.45)$^{Bab}$ | 0.90 (1.71)$^{Ab}$ |
| | OAF | 1.47 (0.71)$^{Ba}$ | 1.67 (0.60)$^{Ba}$ | 1.84 (0.49)$^{Aa}$ |

Values are mean (standard deviation). Within columns, means followed by different capital letters are significantly different. Wi8thin rows, means followed by different lowercase letters are significantly different.

A two-way ANOVA determined that there are significant differences ($P \leq 0.05$) overall between surface attached and biofilm organisms, but not between ozone and OAF.

Data from this chapter, chapter 2 (gaseous ozone) and chapter 6 were published in paper (Nicholas *et al*., 2013) (see Appendix, pages 293-302).

## 5.4 Discussion

Open air factor (OAF) as an air-phase and surface disinfectant has only recently been considered. Bailey *et al*. (2007) investigated the bactericidal effectiveness of gaseous ozone and OAF (in the form of two monoterpenes at high, medium and low concentrations in 0.1 ppm ozone) against aerosolised *Micrococcus luteus*. Their findings revealed that there was a greater significant difference when aerosolised *M. luteus* was exposed to OAF (at high and medium concentrations in ozonated air) after 20 min exposure than with ozone at 0.1 ppm alone.

This study concentrated on the potential of OAF as a surface disinfectant. The investigation involved the terpene, d-limonene delivered by an AD device supplied by Inov8 Science Ltd., against surface attached and biofilm environmental bacteria (*L. monocytogenes* L002 and *P. aeruginosa* C001), isolated from a high-care food processing facility. There was a significant 2 log reduction of surface attached *L. monocytogenes* treated with OAF. There was no significant difference between the orientations (H, V and I) of the food grade stainless steel (SS) coupons. Surface attached *P. aeruginosa* C001 to SS gave a significant 4 log reduction. There was again no significant difference between orientations of coupons. There was a significant difference between the two organisms.

Single-species biofilm of *L. monocytogenes* L002 grown for 72 h on food grade SS, food grade polypropylene (PP) and polished granite, revealed a 1.5 log reduction on the three surfaces. Food grade PP gave the highest log reduction compared to food grade SS, which gave the lowest log reduction. Mafu and others (1990b) found that *L. monocytogenes* was more resistant to sanitizing

agents (sodium hypochlorite, quaternary ammonium, iodophor A and B) when attached to PP and rubber, than to SS and glass. This was not true for OAF as no significant differences were found between surfaces. There was a significant difference between SS and PP, and between granite and PP, but not between SS and granite. The ability of *L. monocytogenes* to adhere to different food contact surfaces with different affinities (Silva *et al*., 2008; Mafu *et al*., 1990a). Adhesion kinetic studies have revealed that adhesion of *L. monocytogenes* cells was higher to PP than to SS (Saá *et al*., 2009). Bacterial attachment on abiotic surfaces is influenced by presence of surface properties and the presence of surface appendages, such as flagella. Lemon *et al*. (2007) reported that flagellum-mediated motility is essential for adhesion and biofilm formation. It has been noted that strains with poor adherence lack the presence of surface fibrils (Kalmokoff *et al*., 2001). The effect of OAF on single-species biofilm of *P. aeruginosa* biofilm grown for 72 h was studied and again, there was a significant log reduction of 2 logs. There was a significant difference between SS and PP, and between granite and PP, but not between SS and granite.

The dual-species biofilm showed 1.8 and 2.2 log reductions for *L. monocytogenes* and *P. aeruginosa*, respectively. There were the same significant differences between surfaces, and the two organisms gave similar pattern of log reductions in the dual-species biofilm, than in single-species biofilms, apart from on PP surface. In a dual-species biofilm, *Listeria* was more protected (lower log reduction) by *Pseudomonas* on PP than as a single biofilm. It is well known that *L. monocytogenes* is a poor biofilm former and requires a primary coloniser such as *P. aeruginosa*. *Listeria monocytogenes* has been known to only form biofilms on surfaces when part of a consortium of species (Sashara and Zottola, 1993).

Previous research has shown that using air disinfection devices can kill methicillin-resistant *Staphylococcus aureus* (MRSA) in one hour on glass and metal surfaces (Tri-Air Developments Ltd., 2008 [online]). Hood in 2009 found that unidentified open-air factors can adversely affect the survival of microorganisms, such as *Francisella tularensis* present on microthreads. This study was shown to be more effective against the environmental gram-negative organism than the environmental gram-positive organism; both surface attached or in biofilms (single- or dual-species). OAF was not as effective against the *L. monocytogenes* and *P. aeruginosa* in biofilms (approximately 1.5 and 2 log reduction) compared to 2 and 4 log reductions for surface attached *L. monocytogenes* and *P. aeruginosa* bacteria. This is because a biofilm is an effective defence mechanism in protecting cells against environmental stresses including antimicrobial agents such as biocides. The inherent nature of a biofilm allows for poor absorption of such chemicals, thus the prevalence of persistent strains with greater adherence capabilities within food processing premises (Norwood and Gilmour, 1999).

Comparing ozone and OAF for surface attached organisms, there was a significant difference ($P \leq 0.05$) with ozone at 45 ppm, giving a better log reduction that OAF, whereas OAF was significantly better than ozone at reducing biofilm organisms (Nicholas *et al*., 2013). OAF was slightly less effective against biofilm compared with surface attached *L. monocytogenes* cells. The potential application of OAF has demonstrated its application as an alternative biocide, to ozone, as it can be use while personnel are present. Further studies are necessary to determine its potential use as a biocide to control other environmental contaminants in food processing premises. When comparing gaseous ozone and OAF, for surface attached Listeria cells on SS, there was a significant difference, with ozone at 45 ppm giving better log reductions that OAF. OAF was significantly

better than ozone at reducing the Listeria biofilm population. *Listeria monocytogenes* demonstrated different resistance to ozone and OAF, suggesting different mechanism of action. Further studies are necessary to determine the precise mechanisms of action of these two biocides.

# Chapter 6. Scanning Electron Microscopy and Atomic Force Microscopy on *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* C001 Biofilms

## 6.1 Introduction

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) have been standard tools used to examine microbiological specimens including bacterial cells for signs of damage, including disruption to cellular membranes. Each technique is designed to provide information on surface structure, but the image information mechanisms for these techniques are quite different providing different types of information about surface structure from differences observed in friction, adhesion, elasticity, hardness, electric fields, magnetic fields, spreading resistance and conductivity, as well as cell morphological changes (Hannig *et al*., 2010). It is now common practice for SEM to be performed alongside AFM in laboratories.

The scanning electron microscope was invented in 1938 by von Ardenne by using a rastering electron beam of a transmission electron microscope (TEM) to form a scanning transmission electron microscope (STEM). In 1942, the first scanning electron microscope (SEM) was built by Zworkin *et al*. and in 1965, Cambridge Scientific Instruments produced the first commercial SEM. Since then the resolution has increased from 50 nm in 1942 to approximately 0.7 nm. SEM was developed for morphological imaging in order to detect signals used to determine compositional information. SEM is an electron microscope used to take images of the sample surface by scanning it with a high-energy beam of electrons in a

scatter pattern. The electrons are able to interact with the atoms that make up the sample surface by producing signals showing the surface topography, composition and other properties. There are different types of signals produced such as secondary electrons, back scattered electrons (BSE), characteristic x-rays, light (cathodeluminescence) and transmitted electrons. The most common mode is secondary electron imaging (SEI). Secondary electrons are low energy electrons (< 50 eV) produced as a result of interactions between beam electrons and weakly bound electrons found in the conduction band of the sample. The SEM image is formed as a result of the intensity of secondary electron emission from the sample at each x and y data point during rastering of the electron beam across the surface. The scanning electron microscope can produce very high-resolution images of a specimen's surface at about 1 to 5 nm in size (Russell *et al*., 2008).

The field of scanning probe microscopies (SPM) is a new era in a family of techniques involving scanning a sharp tip across a sample surface as well as monitoring the tip-sample interaction to form a high resolution image. Atomic Force Microscopy (AFM) was preceded by the development of scanning tunnelling microscope (STM) in 1981 by Binnig and Rohrer. This gave the inventors the ability to view the atomic lattice of a sample surface and earned them the Nobel Prize in Physics in 1986. Although STM provided subangstrom resolution in all dimensions, it was limited to conductive and semi-conductive samples. This led, therefore, to the development of AFM in 1986 by Binnig, Quate and Gerber. Commercial microscopes were produced in 1989 by Digital Instruments USA (now Veeco Instruments, USA) (Russell *et al*., 2008).

AFM provides three-dimensional surface topography at nanometer lateral and subangstrom vertical resolution on insulators and conductors. AFM is the most

commonly used form of SPM. The AFM consists of a microscale, flexible cantilever typically made of silicon or silicon nitride, which has a sharp tip at the end, known as a probe. The tip usually has a radius of 2 nm to 20 nm. This probe is used to scan the surface of the sample while maintaining a small constant force between the tip and the sample surface, by employing a feedback mechanism. When the tip is brought into proximity with the sample surface, the forces between the two, leads to a deflection of the cantilever, according to Hooke's Law. The scanning motion is created by the piezoelectric scanner which scans the tip in a raster pattern with respect to the surface. The tip-sample interaction (feedback mechanism) is monitored by reflecting a laser beam off the back of the cantilever into a split photodiode detector. The detection of different output voltages in the photodetector, changes in the cantilever deflection and oscillation amplitude can be determined (Russell *et al*., 2008). There are two common modes used to operate the AFM; contact mode AFM and tapping mode AFM, which can used in air and liquid environments. Contact mode AFM works by raster-scanning the probe while monitoring the change in cantilever deflection. Tapping mode AFM consists of oscillating the cantilever at the resonance frequency and light tapping of the tip on the surface during scanning (Beech *et al*., 2002; Dufrêne, 2002; Nagao and Dvorak, 1998).

Since its invention, AFM technology has been applied to all aspects of science, including food science and technology. The technology was introduced in 1993 to provide information with regards to monitoring changes to food proteins. In 1994, Thomson and others used real-time imaging of enzymatic degradation of starch granules using AFM. This technique can be used to qualitatively analyse the structure of food macromolecules, including proteins, lipids, and polysaccharides (Thomson *et al*., 1994). In 2005 to 2006 the new imaging modes were developed

which allowed the technique to be applied to biological and material science (Yang *el al*., 2007).

The application of AFM for visualising biofilms has been studied to aid our understanding how biofilms are involved in biodeterioration of materials. Prior to this, previous investigations involved AFM imaging of bacterial cells that were artifially immobilised to the substratum, without studying the bacteria sessile nature. However, AFM used to visualise biofilms on a particular substrata can now be studied using the Tapping mode (Beech *et al*., 2002). AFM can be used as a tool for distinguishing biological components of bacterial cells and biofilms, such as the production of EPS. EPS is made up of macromolecules including proteins, polysaccharides, nucleic acids, and lipids, and often referred to as the glycocalyx or slime layer, which aids bacterial attachment. The visualisation of EPS by AFM has illustrated the observation of the independent distribution of EPS within the biofilm matrix (Beech *et al*., 2002).

The aim of this investigation was to examine the effect of treatments on surface attached and biofilm (single- or dual-species) environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 isolates on food grade stainless steel coupons using SEM and AFM techniques.

## 6.2 Materials and Methods

The model biofilm method (adapted from Charaf *et al*., 1999) was set up as described in Section 2.2.6. Untreated coupons (control) were kept covered with 0.1M phosphate buffer for ozonated water treatments or left in air covered by lid of Petri dish or microtitre plate for gaseous ozone and OAF treatments. Treated (test) coupons were exposed to ozonated water (0.1 ppm), ozonated water plus terpene or terpene (limonene) only, for 5 minutes contact time. For gaseous ozone (45 ppm) and OAF treatment, the contact time was 1 hour (for SEM and AFM).

### 6.2.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopies were operated by Dr. Antony Hann from Cardiff University and by Dr. Jan Hobot (Medical Microscopy Sciences, University Hospital Wales, Cardiff, UK).

#### 6.2.1.1 Biofilm *L. monocytogenes* L002 on food grade stainless steel coupons treated with gaseous ozone at 45 ppm from Cardiff University

Dr Antony Hann from Cardiff University operated the SEM Philips XL30 ESEM-FEG microscope for the preliminary work on surface attached *L. monocytogenes*. Surface attached cells were prepared as before (section 2.2.4), and inoculated onto round coverslips no.1 (diameter of 16 mm) (Fisher Scientific UK Ltd., UK). Once dry the coverslips were treated with gaseous ozone at a concentration of 45 ppm for 1 hour. After treatment, untreated (control) and treated (test) coverslips were each placed separately into tissue culture plate wells and prepared for SEM examination by pipetting 2% glutaraldehyde in 0.1M phosphate buffer (Sigma

Aldrich, UK) down the side of each well for overnight fixation. The fixative was then removed and replaced with 0.1M phosphate buffer (Oxoid, UK) (2 x 10 min). This was then followed by a dehydration step (50% ethanol (5 min), 70% ethanol (5 min), 80% ethanol (5 min), 90% ethanol (5 min), 100% ethanol (3 x 5 min)). This involved replacing water in the cells with an organic solvent (ethanol). The reagents were pipetted down the side of each well. The samples were then dried in a critical point dryer (CPD) using $CO_2$ (Sarndri-780, Tousimis, Maryland, USA) and sputter coated with gold (Gold sputter coater, EITI scope, UK). Samples were viewed using Philips XL30 ESEM-FEG microscope at 20 kV.

#### 6.2.1.2 Preliminary samples - Biofilm *L. monocytogenes* L002 on glass coverslips treated with gaseous ozone at 45 ppm from University Hospital Wales

Dr Jan A. Hobot at Medical Microscopy Sciences, University Hospital Wales, Cardiff, UK imaged all samples prepared for scanning electron microscopy (SEM). The protocol was modified due to experimental issues with image quality, quality of the bacterial cells and biofilms in general, and the operation of a different microscope.

Preliminary samples were necessary in order to check the effect of fixative on the condition of the cells. Biofilms (72 h) of *Listeria monocytogenes* L002 were cultured on round glass cover slips (16 mm diameter) (Fisher Scientific UK Ltd., UK). Untreated samples were placed into 0.1M phosphate buffer whilst the test samples were treated with 45 ppm gaseous ozone for 1 h. Coverslips were placed in 12-well tissue culture plate (Greiner® Bio-One Ltd., Stonehouse, UK) and placed in various fixative concentrations either overnight or for 3 h before processing.

Coverslips not placed in fixative overnight were placed in 0.1M phosphate buffer overnight instead, which was removed before the addition of the fixative.

The reagents used for SEM preparation were fixative – final concentration 2% v/v or 2.5% v/v glutaraldehyde in 0.1M phosphate buffer, pH 7.4; (stock sol. 50% glutaraldehyde; Sigma Aldrich, UK), ethanol A.R. (50%, 70%, 90% and 100%) and hexamethyldisilazane A.R (Sigma Aldrich, UK). Hexamthyldisilazane solvent was found to be suitable for SEM examination, instead of critical point drying, in order to remove liquids from microbiological samples (Araujo *et al*., 2003).

*SEM preparation.* After the samples had been fixed in glutaraldehyde, they were washed using double distilled water (2 x 5 min) followed by a dehydration step (50% ethanol (5 min), 70% ethanol (5 min), 90% ethanol (5 min), 100% ethanol (5 min)), which involved replacing water in the cells with an organic solvent (ethanol). Hexamethyldisilazane (3 x 5 min) was applied. The reagents were added by pipetting them down the side of each well. The samples were then air dried and mounted on a specimen stub using electrically-conductive double-sided adhesive tape. The samples were finally sputter coated with gold (Sarndri 780, Tousimis Research Corporation, Maryland, U.S.A.) for 2.5 min before examination in the microscope. SEM preparations were observed under JEOL 840A scanning electron microscope operating at 5-10 kV and images were recorded on SIS imaging software.

#### 6.2.1.3 Gaseous ozone at 45 ppm

Biofilms (72 h) of *Listeria monocytogenes* L002 were cultured on food-grade 1 $cm^2$ stainless steel coupons for 72 hours. After biofilms had been established, the samples were treated (section 4.2.1). After treatment, samples were placed in

overnight fixative (2.5% v/v glutaraldehyde in 0.1M phosphate buffer), before SEM preparation. Untreated samples were placed in 0.1M phosphate buffer during the 1 h test period, which was removed before the addition of the fixative. SEM preparation was performed and observed as before (section 6.2.1.3) and observed under JEOL 840A scanning electron microscope operating at 10 kV.

#### 6.2.1.4 Ozonated water treatments

Single species and dual-species biofilms of environmental *L. monocytogenes* L002 and *Pseudomonas aeruginosa* C001 were cultured on food-grade 1 $cm^2$ stainless steel coupons for 72 h. After biofilms had been established, samples were subjected to different treatments (section 4.2.1). Untreated samples were placed in 0.1M phosphate buffer during the test period, which was removed before the addition of the fixative. After treatment, the samples were placed in fixative (2.5% v/v glutaraldehyde in 0.1M phosphate buffer) overnight before SEM preparation. SEM preparation was performed and observed as before (section 6.2.1.3) and observed under JEOL 840A scanning electron microscope operating at 10 kV.

#### 6.2.1.5 OAF

Single species and dual-species biofilms of environmental *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* C001 were cultured on food-grade 1 $cm^2$ stainless steel coupons for 72 hours. After biofilms had been established, the samples were treated (section 5.2). Untreated samples were placed in 0.1M phosphate buffer during the 1 h test period, which was removed before the addition of the fixative. After treatment, samples were placed in overnight fixative (2.5% v/v glutaraldehyde in 0.1M phosphate buffer), before SEM preparation. SEM preparation was performed as before (section 6.3.1.3) and observed under JEOL 840A scanning electron microscope operating at 5 kV.

#### 6.2.1.6 Uninoculated surfaces, untreated and treated with ozonated water, ozonated water and terpene, and terpene

As the images from 6.3.1.5 showed a deposit on the stainless steel surface, uninoculated stainless steel coupons were treated with each treatment as before (section 4.2.1), in order to determine whether any of the treatments caused surface artefacts. An untreated surface was also set up which was uninoculated and untreated. SEM preparation was performed as before (section 6.3.1.4) and observed under JEOL 840A scanning electron microscope operating at 5 kV.

### 6.2.2 Atomic Force Microscopy (AFM)

Preliminary experiments were designed in order to determine the mechanism of action of each treatment used against surface attached *L. monocytogenes* L002 to food grade stainless steel (AISI type 304; finish 2b) coupons (1 $cm^2$ in size). Stainless steel coupons were cut to size and polished by colleagues in Bristol University's workshops (Bristol University, Bristol, UK). Bacterial suspension preparation was repeated as before in section 2.2.4 and section 6.2.1.

Imaging was performed by Dr. Peter Dunton from Bristol University. All imaging was carried out on a PicoScan I Atomic Force Microscope (Molecular Imaging), with a separate acoustic modulation module (Molecular Instruments (MI), Tempe, USA), housed in an acoustic-isolation chamber. Imaging software used to analyse the images was Picoscan 5.3.3 (MI, Tempe, USA). The cantilevers used were Tap-150-G tapping mode cantilevers (BudgetSensors, Sophia, Bulgaria). The average cell dimensions were calculated from a representative sample (n=10).

## 6.3 Results

### 6.3.1 SEM images

#### 6.3.1.1 Biofilm *L. monocytogenes* L002 on food grade stainless steel coupons treated with gaseous ozone at 45 ppm from Cardiff University.

Figure 6.1 (a, b) illustrates a static 72 h biofilms of *L. monocytogenes* on food grade stainless steel coupons. The untreated sample (control) had formed a dense biofilm structure (Fig. 6.1 a). The treated sample (Fig. 6.1 b) illustrated that many cells appeared to have blebbed their cellular contents into the surrounding environment. The micrograph is representative of 15 fields of observation.

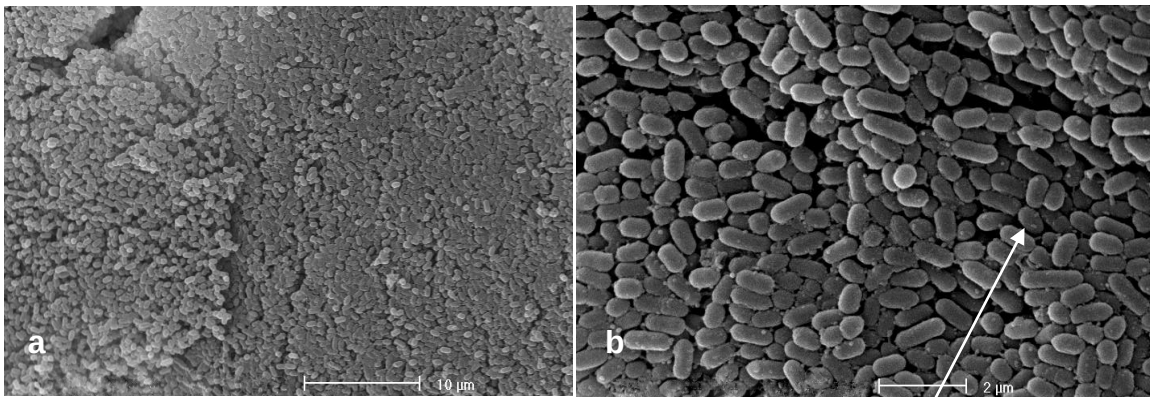
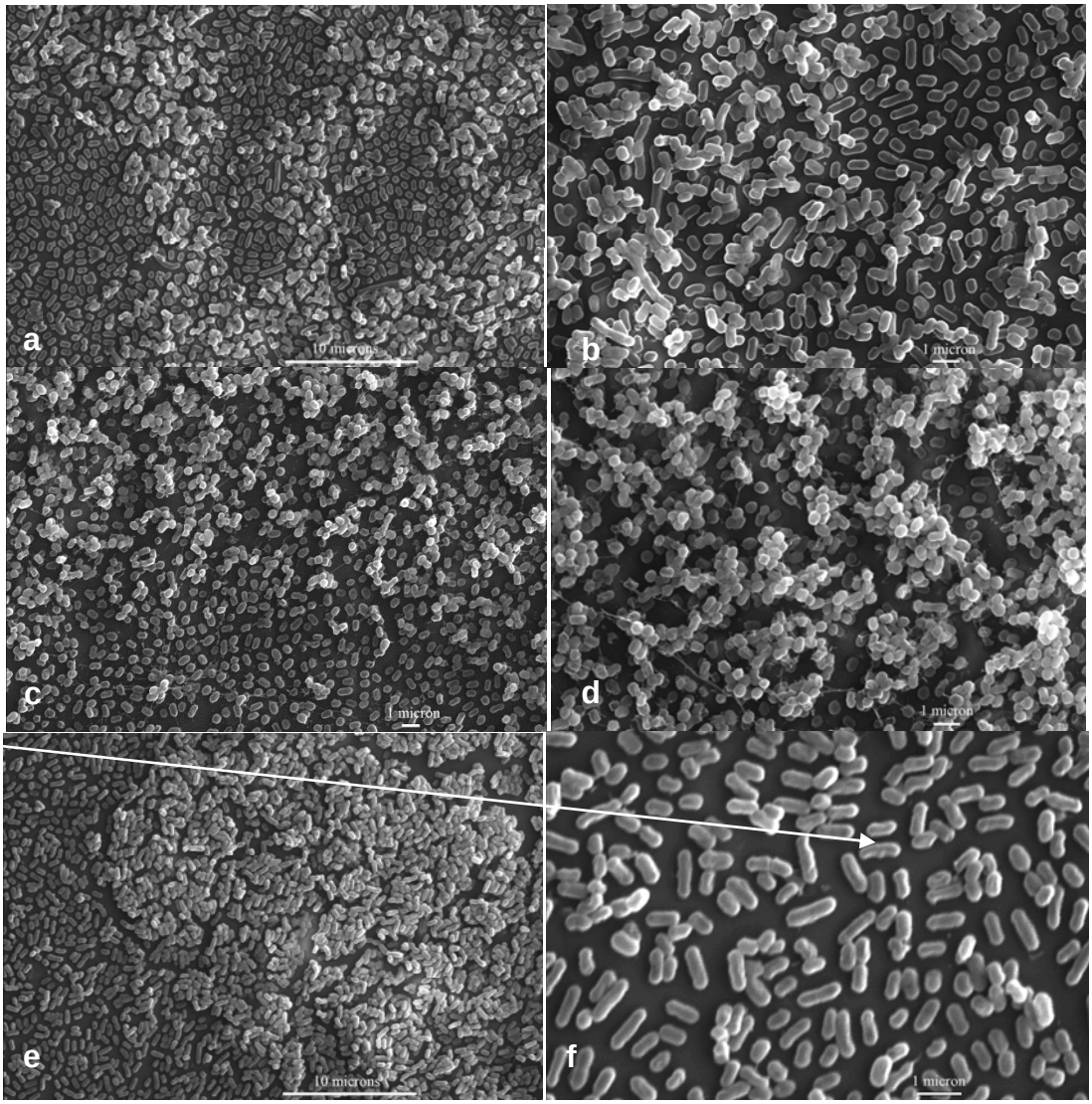



**Figure 6.1.** SEM images of 72 h biofilm of *L. monocytogenes* L002 on food grade stainless steel coupons exposed *in situ* to gaseous ozone at 45 ppm. Fig. 6.2 a, untreated sample, bar = 10 µm and Fig. 6.2 b, test sample, bar = 2 µm.

#### 6.3.1.2 Preliminary samples - Biofilm *L. monocytogenes* L002 on glass coverslips treated with gaseous ozone at 45 ppm from University Hospital Wales.

In order to determine the optimum protocol for biofilm formation and the necessary fixation steps, preliminary samples were prepared of *L. monocytogenes* cells surface attached to glass cover slips. The protocol for the SEM fixation step was slightly modified as stated above in the method (section 6.2.1.2). The JEOL 840A microscope was operated at 10 kV by Dr. Hobot.

**Biofilm Formation.** Figure 6.2 illustrates the preliminary study into the different fixation methods used. The untreated samples (control) (Figs. 6.2 a, c, and e) have retained their turgid shape. There were no obvious signs of blebbing or holes in the cell membranes in any of the controls when compared to the test samples. The treated cells (Figs. 6.2 b, d and f) blebbed their contents into the surrounding environment.




Blebbing

**Figure 6.2.** SEM images of *L. monocytogenes* L002 biofilm on glass cover slips (16 mm diameter) exposed to 45 ppm gaseous ozone. Fig. 6.2 a, untreated sample and Fig. 6.2 b, test sample fixed overnight in 2.5% v/v glutaraldehyde in 0.1M phosphate buffer.. Fig 6.2 c, untreated sample and Fig. 6.2 d, test sample fixed for 3 h in 2.5% v/v glutaraldehyde in 0.1M phosphate buffer. Fig 6.2 e, untreated sample and Fig. 6.2 f, test sample fixed overnight in 2% v/v glutaraldehyde in 0.1M phosphate buffer. Bar marker is 1 micron (viewed at x 6,000 magnification).

As there was little difference in the three samples tested with different fixation methods, 2.5% v/v glutaraldehyde in 0.1M phosphate buffer was chosen for subsequent experiments.

Figure 6.3 a, b, c, d illustrates the different areas of the static 72 h biofilm of untreated *Listeria monocytogenes* cells. Single layers of cells show round circle where cells could have aligned around them to form nutrient channels or were attracted to particular surface charge (Fig. 6.3 a). Some had begun to form unorganised layers (Fig. 6.3 b). Figure 6.3 c, d shows dense biofilm consisting of organised layers of cells, with apparent cracks.

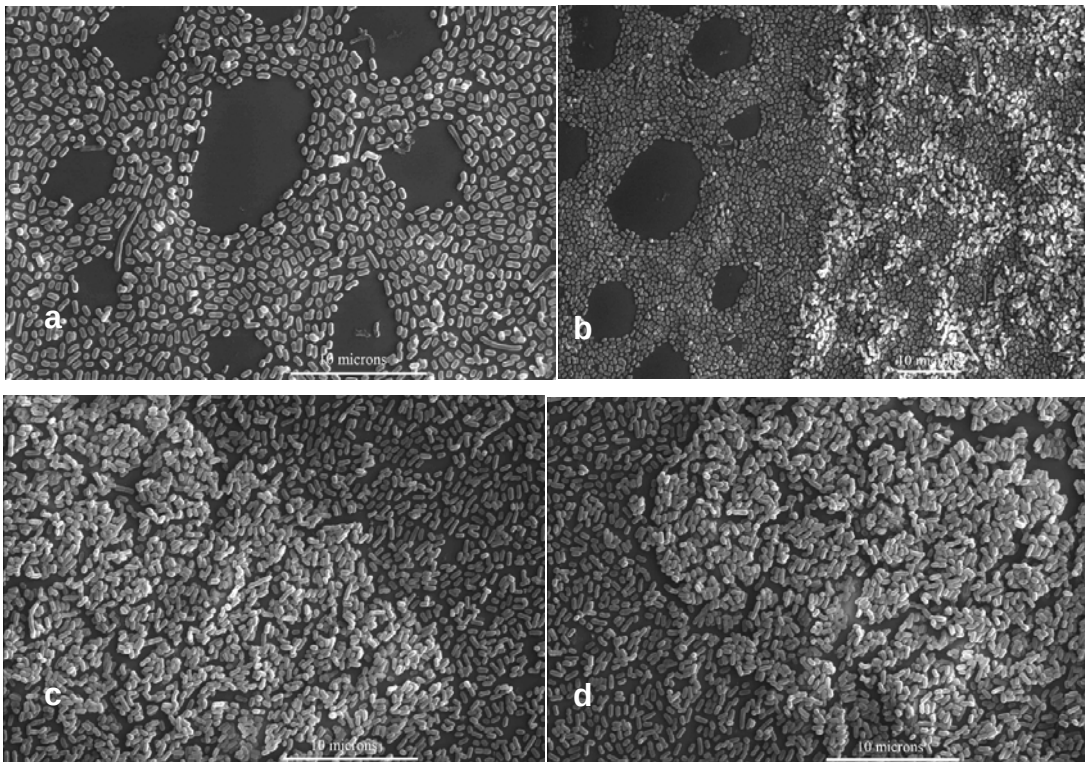



**Figure 6.3.** SEM images of biofilm formation of untreated *L. monocytogenes* L002 72 h biofilm on glass cover slips (16 mm diameter). Fig. 6.3 a, b, c, d, showing different areas of the biofilm at 72 h. Bar markers are 10 microns (x 2,000 magnification).

It is known from the literature that *Listeria* does not attach well to glass surfaces (Chae *et al*., 2006; Mafu *et al*., 1990), therefore, for the continuation of the study, food grade stainless steel coupons were used to grow up the static biofilms.

### 6.3.1.3 Gaseous ozone at 45 ppm

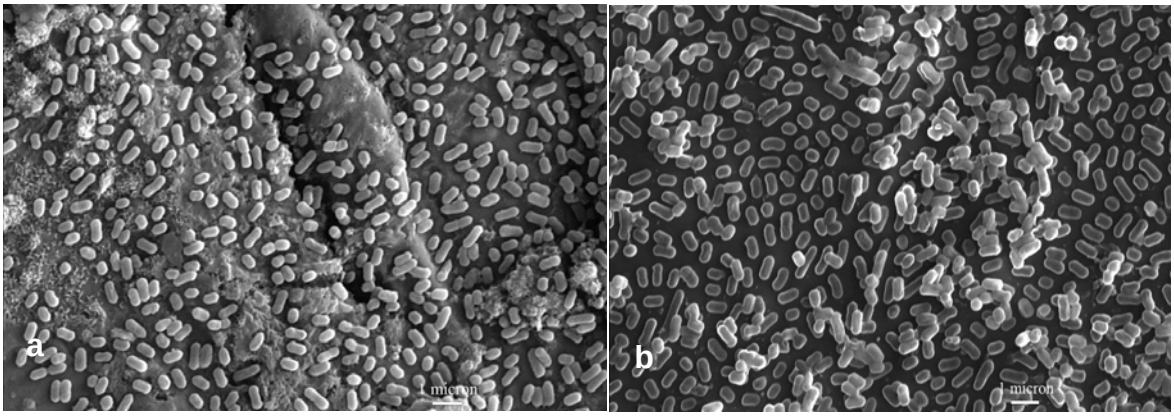



**Figure 6.4.** SEM images of *L. monocytogenes* L002 biofilm on food grade stainless steel coupons exposed *in situ* to gaseous ozone at 45 ppm. Fig. 6.4 a, untreated sample. Fig. 6.4 b, treated sample. Samples fixed overnight with 2.5% v/v glutaraldehyde in 0.1M phosphate buffer. Bar marker is 1 micron (x 6,000 magnification).

Some cells treated with gaseous ozone at 45 ppm seem to have failed to divide (Fig. 6.5).

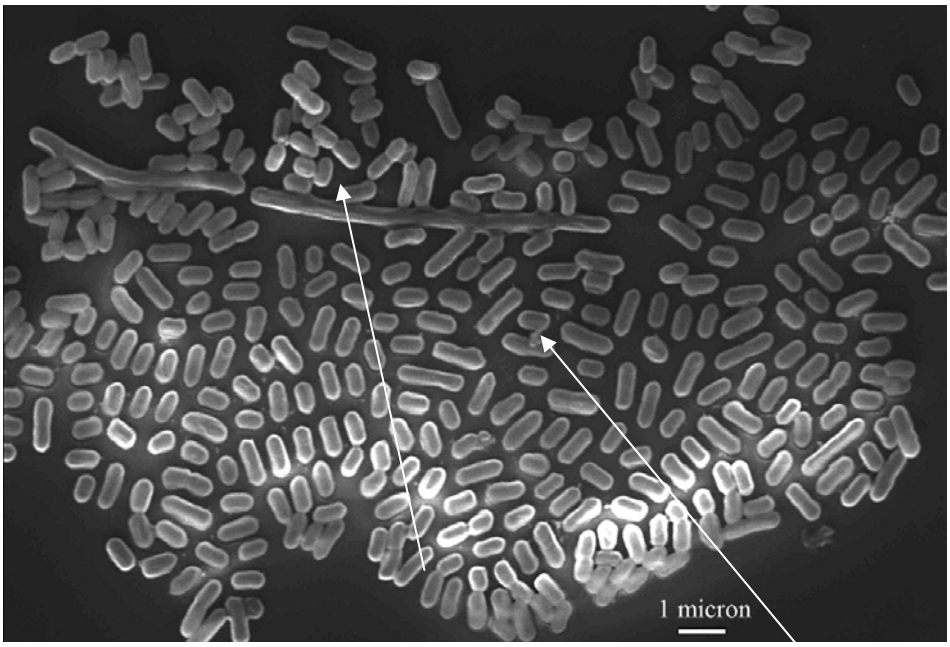



**Figure 6.5.** SEM images of *L. monocytogenes* L002 biofilm on glass cover slip (16 mm diameter) exposed *in situ* to gaseous ozone at 45 ppm. Samples fixed overnight with 2.5% v/v glutaraldehyde in 0.1M phosphate buffer. Bar marker is 1 micron (x 6,000 magnification).

#### 6.3.1.4 Ozonated water treatments

The JEOL 840A SEM microscope was operated at 10 kV.

In the untreated samples, the cells appeared turgid with no obvious malformation of the cell membrane (Fig. 6.6 a, b,). Cracking was seen on a few coupons (Fig. 6.6 b), which could be due to crevices and scratches on the surface of the SS. All three treatments (ozonated water, ozonated water and terpene, and terpene only) stripped the biofilm from the surface of the stainless steel coupons (Fig. 6.6 c, e, f, h). The ozonated water treatment at 0.1 ppm concentration (Fig. 6.6 c, d) caused the cells to bleb and holes were evident, whereas the ozonated water (0.1 ppm) and terpene (25 ppm) (Fig. 6.6 e, f), and the terpene in water treatments (Fig. 6.6 g and h) holes were evident in cell wall.

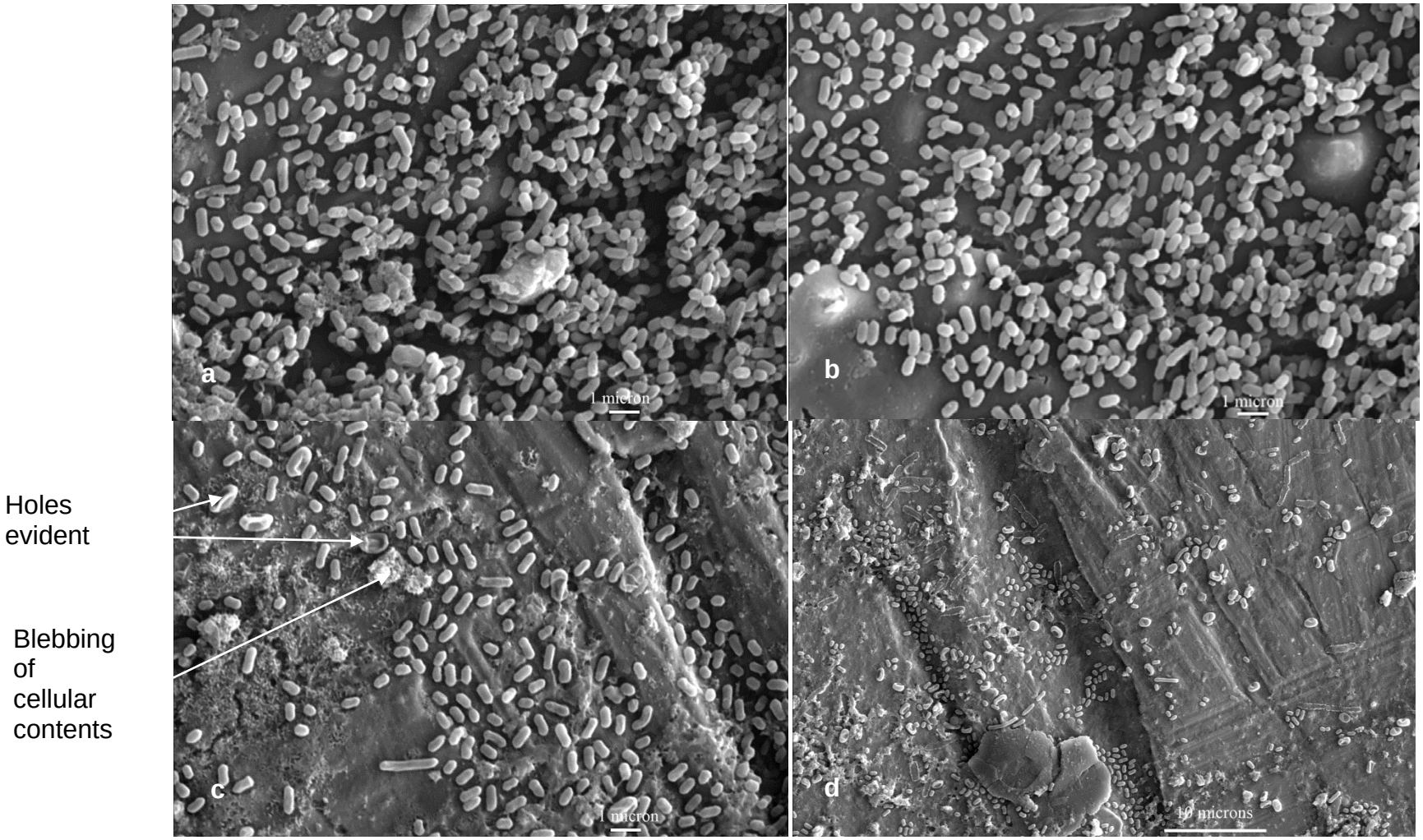



**Figure 6.6.** SEM images of *L. monocytogenes* L002 72 h biofilm. Fig. 6.6 a, b, untreated samples. Fig. 6.6 c, d, test samples exposed to ozonated water (0.1 ppm). Bar markers are 10 microns (x 2,000 magnification) and 1 micron (x 6,000 magnification).

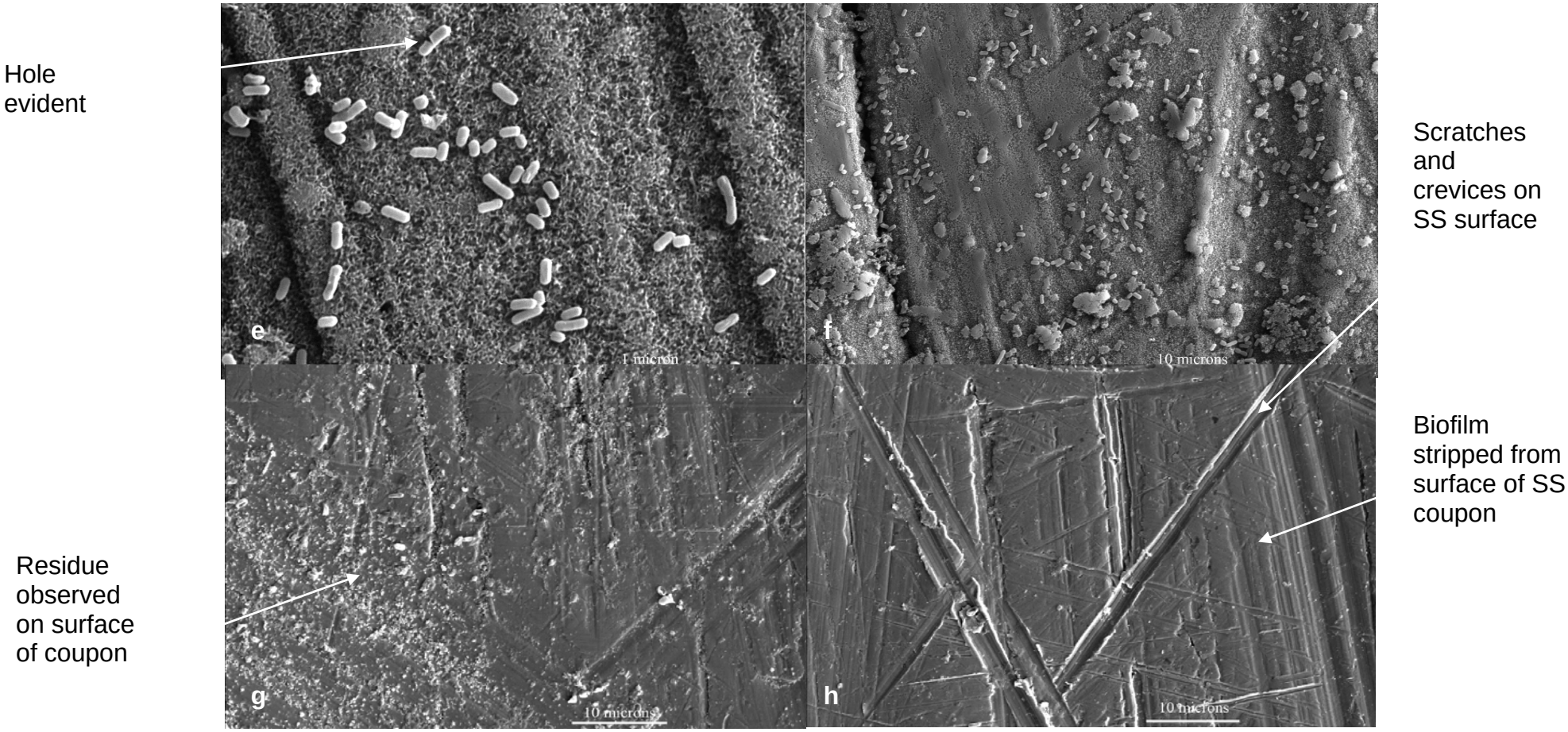



**Figure 6.6.** SEM images of *L. monocytogenes* L002 72 h biofilm. Fig. 6.6 e, f, exposed to ozonated water (0.1 ppm) and terpene (limonene) (25 ppm). Fig. 6.6 g, h, terpene (limonene). Bar markers are 10 microns (x 2,000 magnification) and 1 micron (x 6,000 magnification).

Figure 6.7 a, b, c, d illustrates the effect of the three treatments on *P. aeruginosa* C001 72 h biofilm. Fig. 6.7 a, illustrated the biofilm formation of untreated *P. aeruginosa* on food grade stainless steel coupons. Fig. 6.7 b, c, d, illustrated the effect of the three treatments on *P. aeruginosa* C001 biofilm. The ozonated water treatment (Fig. 6.7 b) caused the *P. aeruginosa* cells to dehydrate and appeared flattened and shrivelled, compared to the blebbed *L. monocytogenes* L002 cells exposed to ozonated water treatment. There was an apparent exudate (extracellular polymeric substance (EPS)) present, produced by *P. aeruginosa* C001 (Fig. 6.7 d).

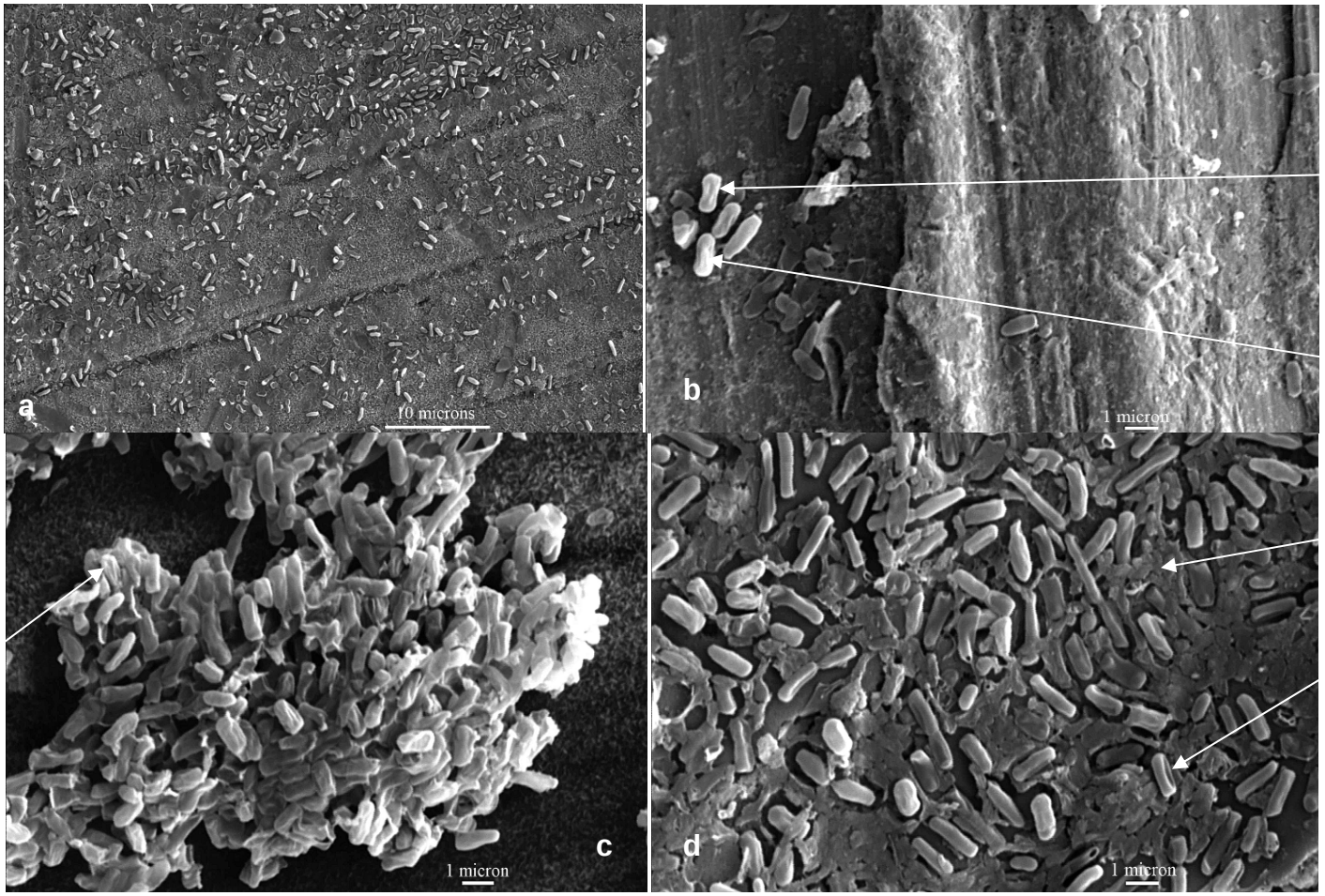



**Figure 6.7.** SEM images of *P. aeruginosa* C001 72h biofilm. Fig. 6.7 a, untreated sample. Bar is 10 microns (viewed at x 2,000 magnification). Fig. 6.7 b, ozonated water (0.1 ppm). Fig. 6.7 c, ozonated water (0.1 ppm) and terpene (limonene) (25 ppm), and Fig. 6.7 d, terpene (limonene). Bar markers are 10 microns (x 2,000 magnification) and 1 micron (x 6,000 magnification).

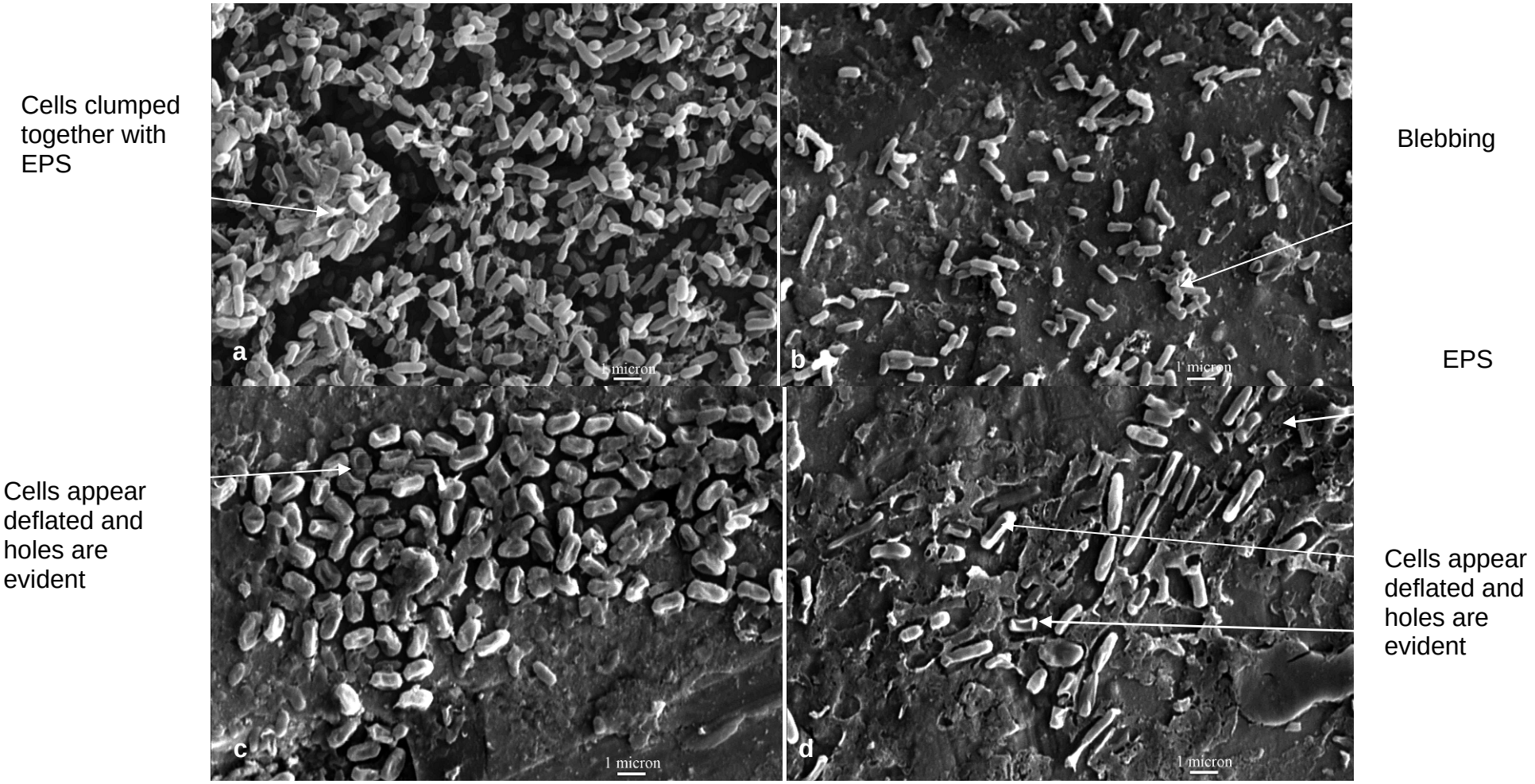



**Figure 6.8.** SEM images of dual-species biofilm 72 h. Fig. 6.8 a, untreated sample. Fig. 6.8 b, ozonated water (0.1 ppm). Fig. 6.8 c, ozonated water (0.1 ppm) and terpene (limonene) (25 ppm), and Fig. 6.8 d, terpene (limonene). Bar markers are 1 micron (x 6,000 magnification).

The same effect can be seen for the dual-species biofilm exposed *in situ* to the three treatments (Fig. 6.8 a, b, c, d). Cells appeared to be dehydrated, with a shrivelled and flattened appearance, compared to the untreated samples. An exudate of EPS surrounding the cells was also present.

There seems to be an apparent mode of action when both organisms either as single- or dual-species biofilms are exposed to the presence of terpene (limonene), as all cells appeared to have holes punched out of the cell membranes.

#### 6.3.1.5 Uninoculated surfaces treated with ozonated water, ozonated water and terpene and terpene only

The SEM JEOL 840A microscope was operated at 5 kV.

On all the surfaces treated with the terpene in water and the ozonated water and terpene treatment that had been inoculated, a deposit or residue was observed on the surface of these coupons and so coupons uninoculated were examined. Figure 6.8 a, b illustrates uninoculated, untreated surface of SS coupon. Lines of scratches and crevices were evident on the surface of the stainless steel. There was no deposit seen on any surfaces treated with ozonated water (Fig. 6.9 c, d), but there was a deposit seen on the terpene only treatment (Fig. 6.9 g, h), and to a lesser extent on the surface treated with ozonated water and terpene (Fig. 6.9 e, f).

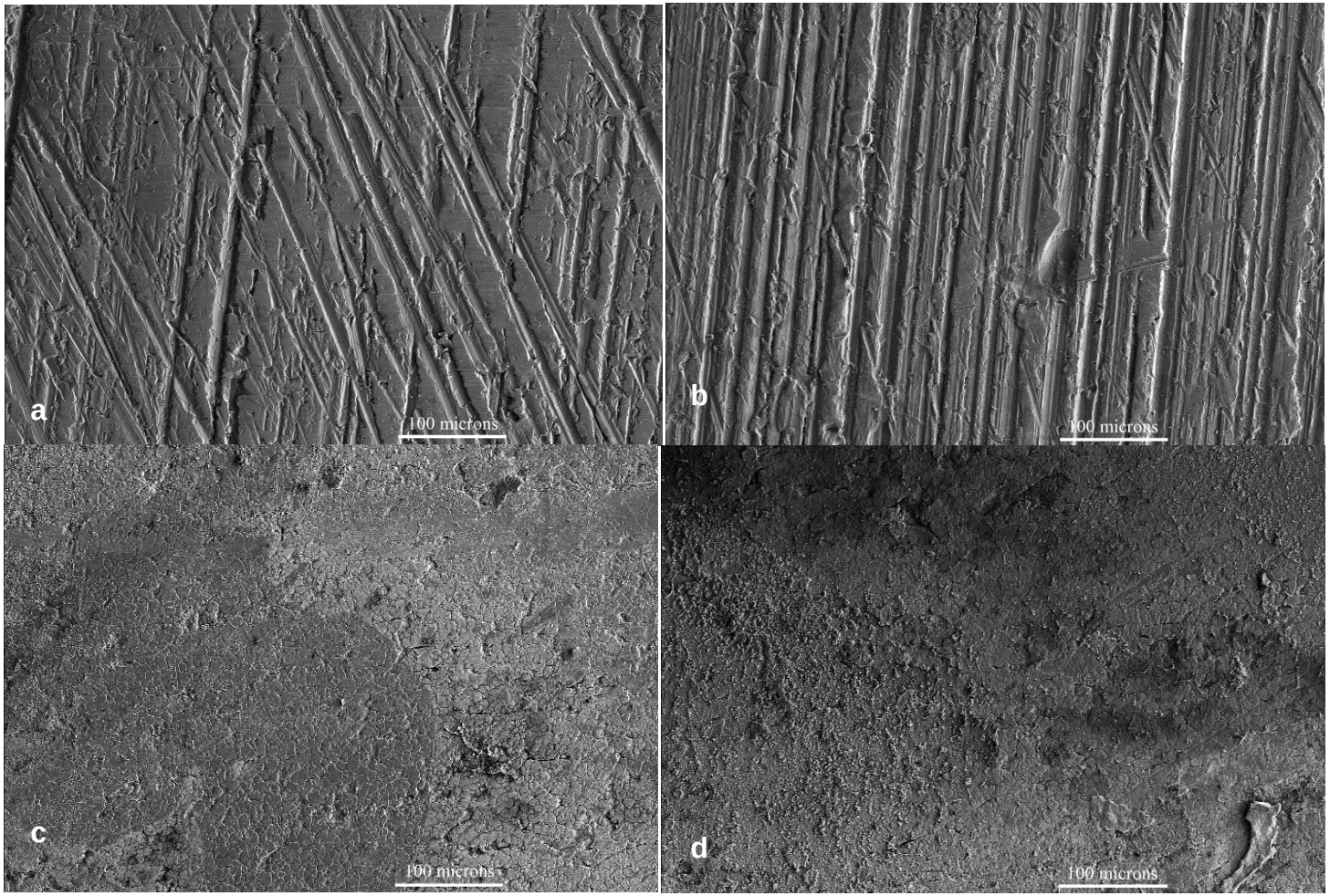
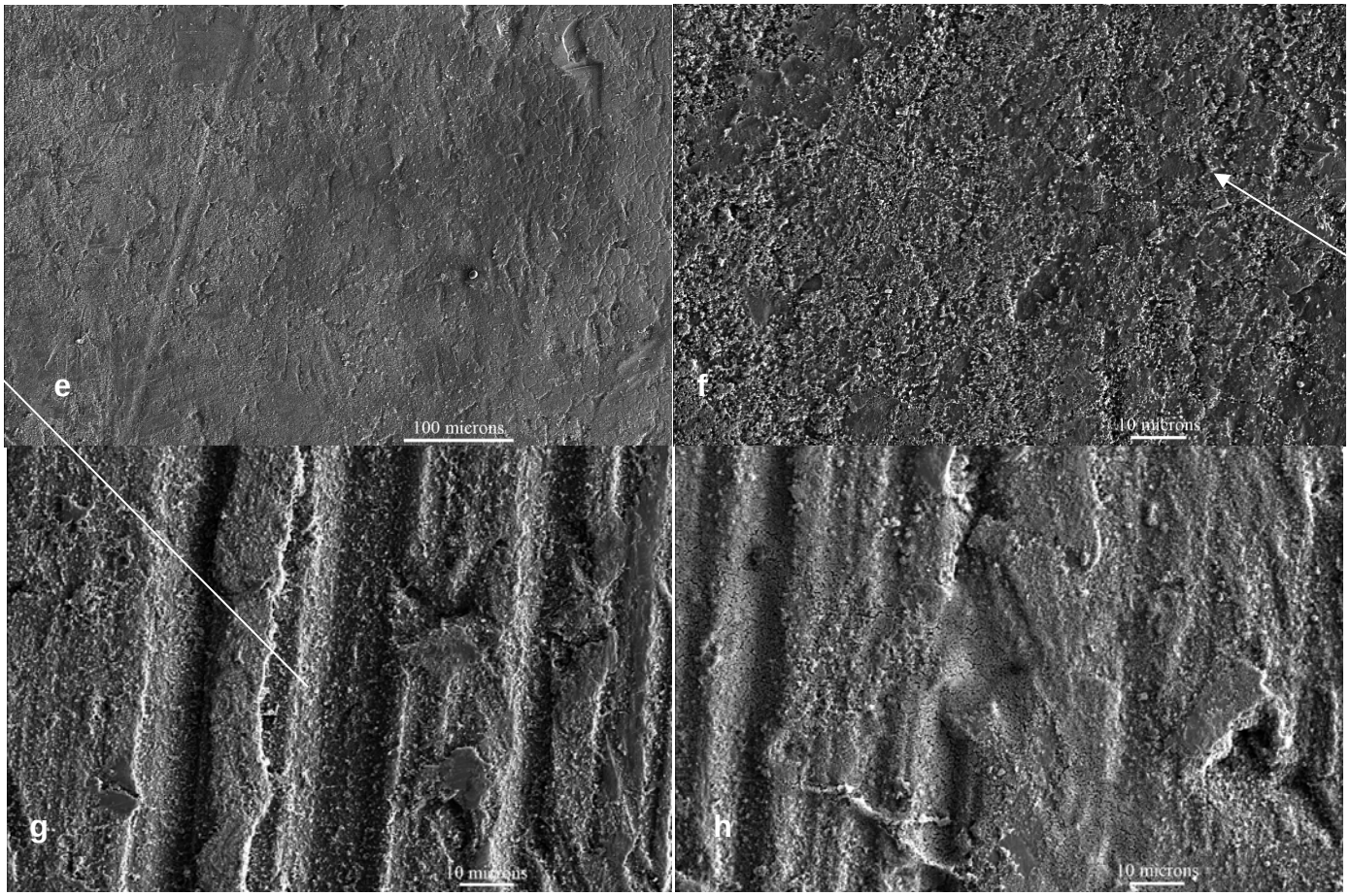



**Figure 6.9.** SEM images of uninoculated, clean food grade stainless steel surfaces. Fig. 6.9 a, b, untreated, and Fig. 6.9 c, d ozonated water. Bar markers are 100 microns (x 1,000 magnification).




Artefacts from treatment (terpene residue)

**Figure 6.9.** SEM images of uninoculated, clean food grade stainless steel surfaces. Fig. 6.9 e, f, ozonated water and terpene and Fig. 6.9 g, h, terpene. Bar markers are 10 microns and 100 microns (x 200 and x 1,000 magnification).

#### 6.3.1.6 OAF

The SEM JEOL 840A microscope was operated at 5 kV.

Figure 6.10 a, b, c illustrates the effect of OAF (in the form of d-limonene) exposure on *L. monocytogenes* L002 72 h biofilm on food grade stainless steel. The cells appeared deflated and holes are evident in cell wall (Fig. 6.10 c), compared to the untreated sample (control) (Fig. 6.10 a). The biofilm was substantial and dense, and EPS was observed more clearly in the test samples (Fig. 6.10 b, c), although not as much as the *P. aeruginosa* isolate. This production of EPS from *L. monocytogenes* has been observed in various degrees in all SEM images.

Figure 6.11 a, b, c illustrates the effect of OAF (in the form of d-limonene) exposure on *P. aeruginosa* C001 72 h biofilm on food grade stainless steel. The same mode of action can be seen; cells appear flattened with visible holes in the cell wall (Fig. 6.11 b, c). This action was evident in the dual-species biofilm (Fig. 6.12 b, c). However, the dual-species biofilm did appeared denser (Fig. 6.12 c) than the single-species biofilms (Fig. 6.10 b and Fig. 6.11 b).

There was also the same deposit seen as before on the surfaces of stainless steel coupons treated with OAF, which appeared on the ozonated water and terpene and the terpene only treatments. The terpene seemed to produce this deposit (residue) with all treatments and was seen on all SEM images.

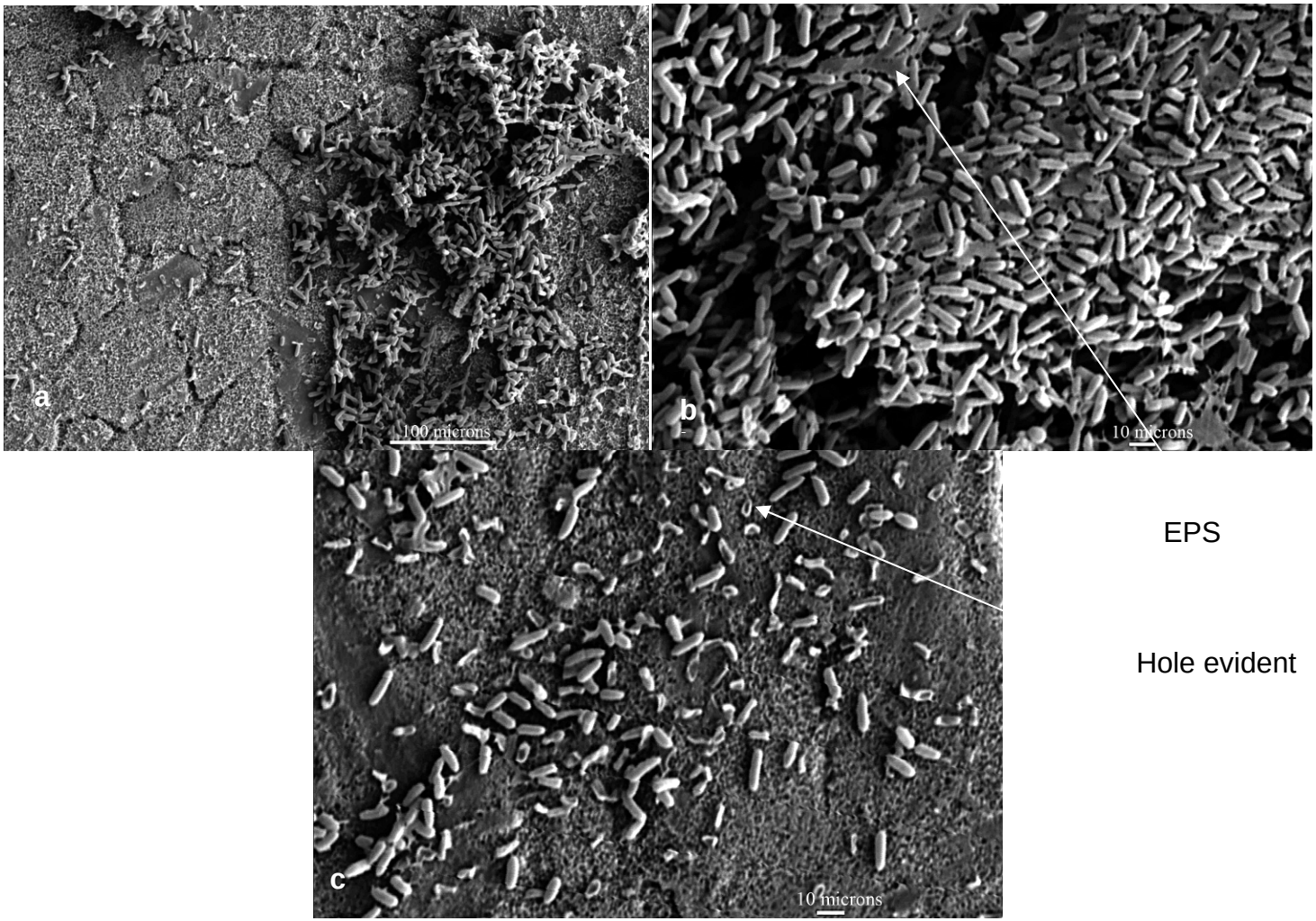



**Figure 6.10.** SEM images of *L. monocytogenes* L002 72 h biofilm exposed *in situ* to d-limonene (OAF). Fig. 6.10 a, untreated sample. Bar marker is 100 microns (x 200 magnification). Fig. 6.10 b, c, treated samples. Bar marker is 10 microns (x 1,000 magnification).

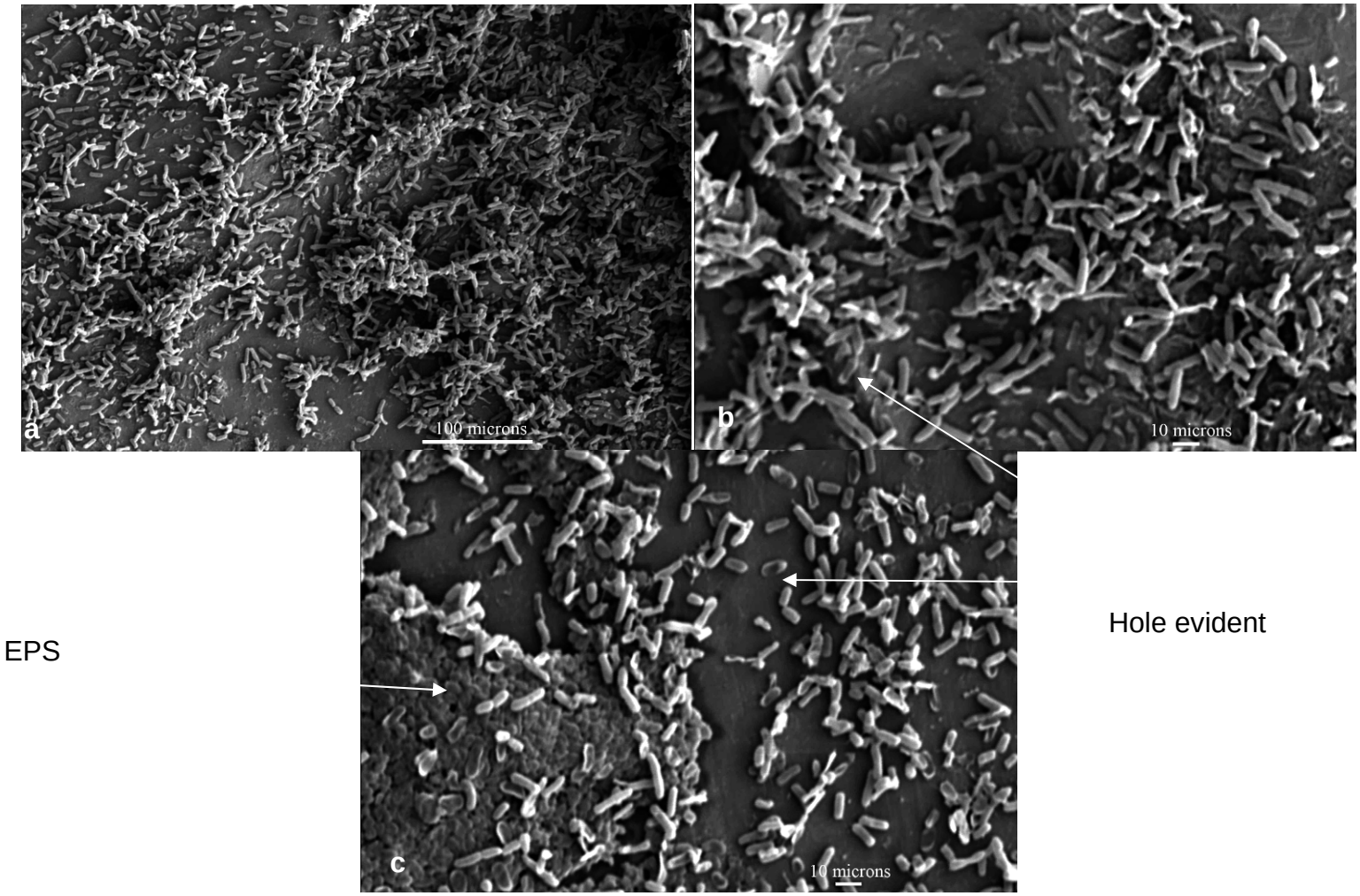



**Figure 6.11.** SEM images of *P. aeruginosa* C001 72 h biofilm exposed *in situ* to d-limonene (OAF). Fig. 6.11 a, untreated sample. Bar marker is 100 microns (x 200 magnification). Fig. 6.11 b, c, treated samples. Bar marker is 10 microns (x 1,000 magnification).

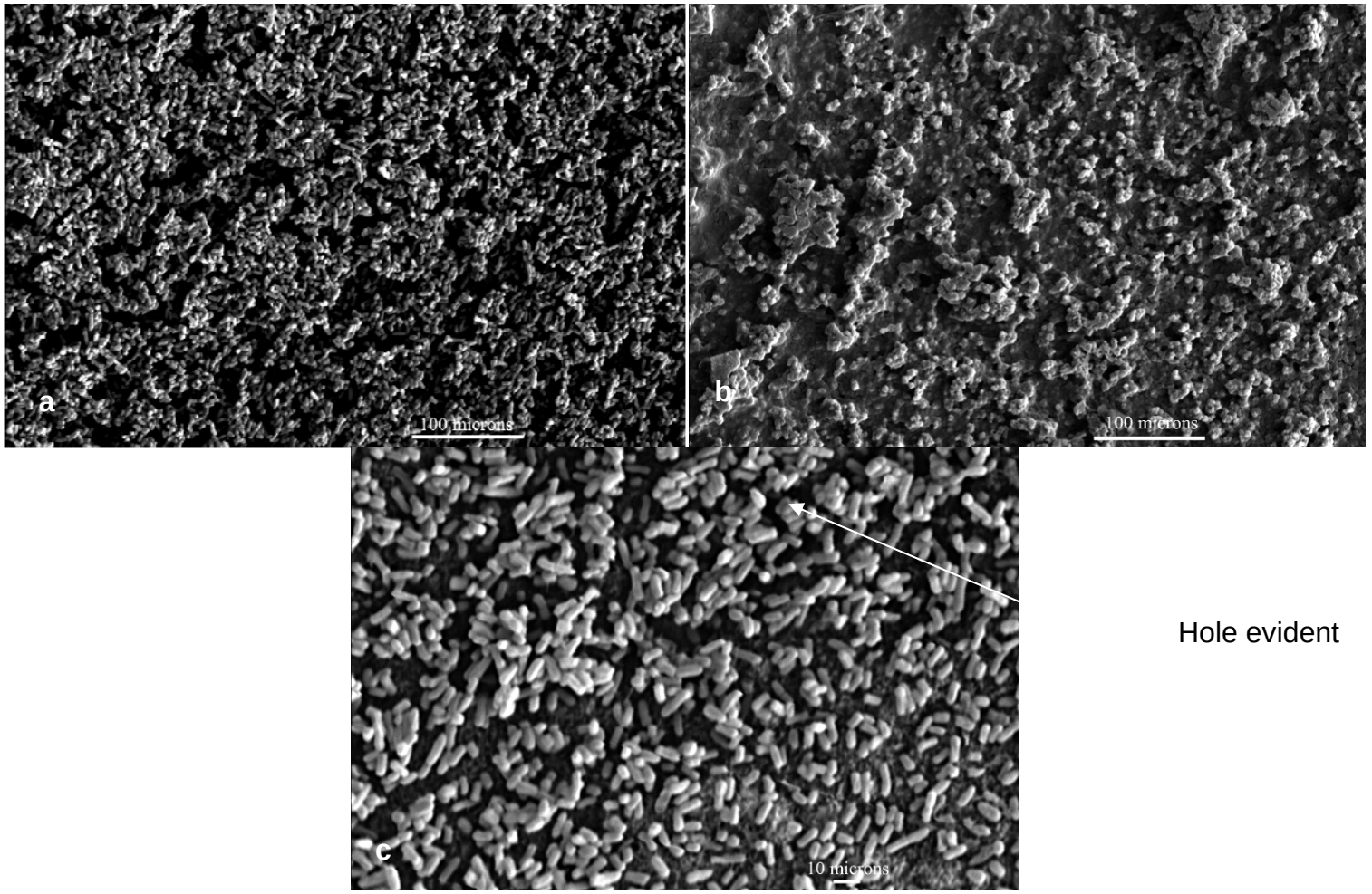



**Figure 6.12.** SEM images of dual-species biofilm 72 h exposed *in situ* to d-limonene (OAF). Fig. 6.12 a, untreated sample. Bar marker is 100 microns (x 200 magnification). Fig. 6.12 b, c, treated samples. Bar markers are at 100 microns (x 200 magnification), and at 10 microns (x 1,000 magnification).

### 6.3.2 AFM images

All imaging was carried out on cut and polished stainless steel coupons (AISI type 304). Tapping mode AFM was used to operate the microscope. The nominal spring constant, k, was 5 N/m and resonant frequency, f, was 137 kHz. Imaging the blank (plain surface) cut and polished stainless steel coupon surfaces were sufficiently unstable as to render imaging them impossible.

#### 6.3.2.1 Untreated (gaseous ozone and OAF control)

Fig. 6.13 illustrates the untreated cells (control–Listeria 1) for both gaseous ozone and OAF treatments. Cells appear collapsed, instead of being smooth and turgid. This could be due to dehydration, as samples were left to desiccate before viewing.

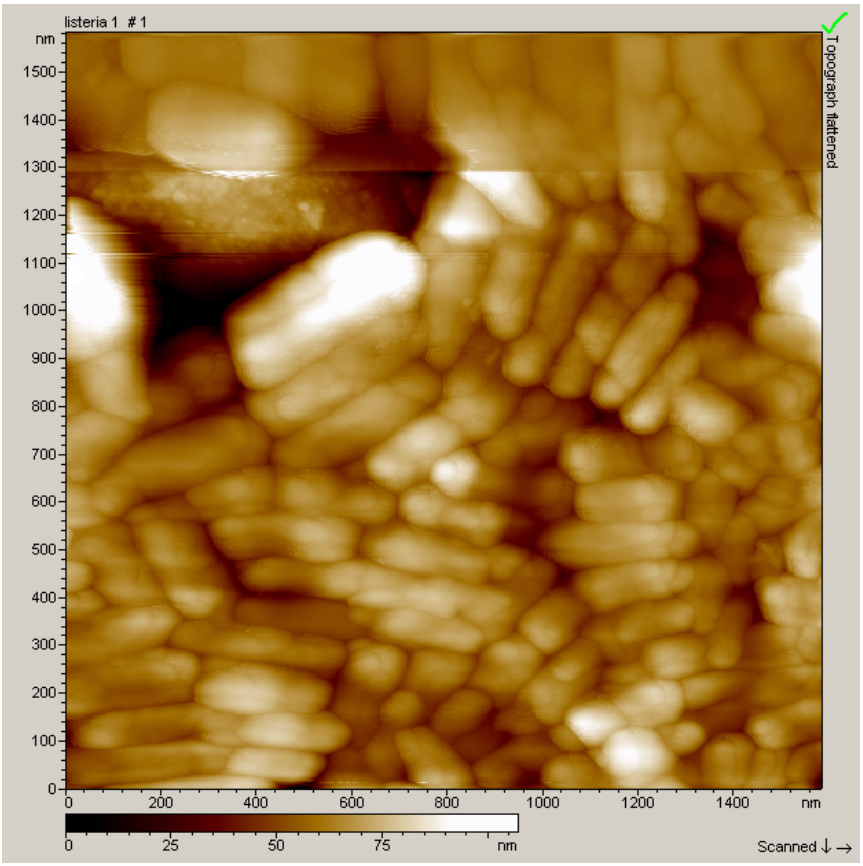



**Figure 6.13.** 1.5 micron tapping mode scan height image (3rd order fattened) showing a regular layer of untreated (control) *L. monocytogenes* cells.

Table 6.1 illustrates the average cell dimensions for untreated cells (number 1, control).

**Table 6.1.** Average cell dimensions (n=10) for surface attached *L. monocytogenes* cells to food grade stainless steel (AISI 304; finish 2b). Untreated sample (1).

| Dimensions | nm (± sd) |
|---|---|
| Length | 347.6 (± 10.2) |
| Width | 97.0 (± 54.2) |
| Height | 311.2 (± 76.0) |

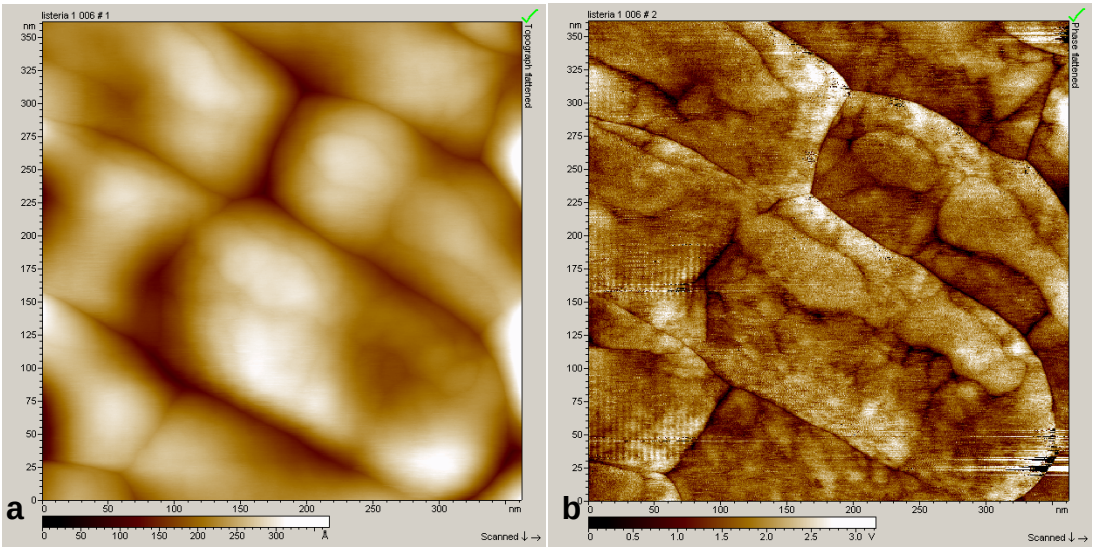



**Figure 6.14.** The a) height and b) phase images of untreated cells. The cell surface does not appear smooth and turgid, but are collapsed and structured.

The phase image suggests some crumpling (dehydration) areas, but no significant areas of altered material properties. Stripes are artefacts left by cantilever excessively oscillating (Fig. 6.14).

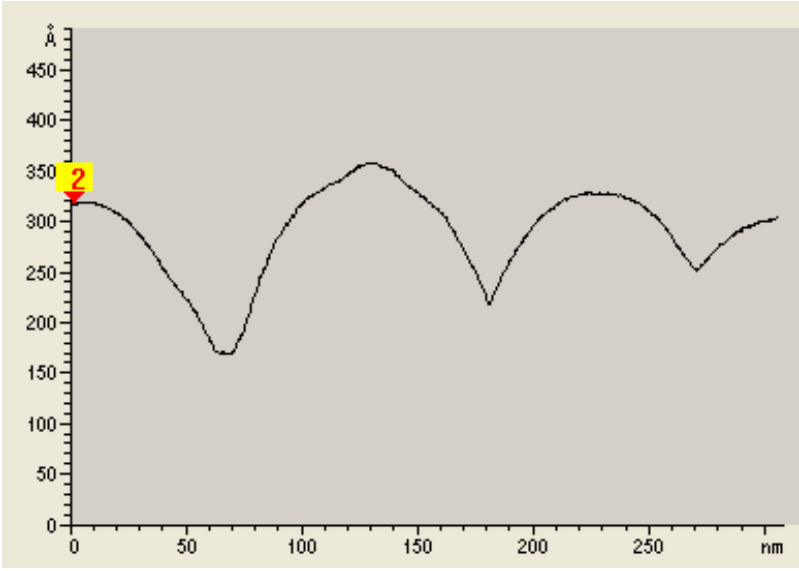



**Figure 6.15.** Cross-sectional view of two untreated *L. monocytogenes* cells. Cells have a rounded appearance.

#### 6.3.2.2 Gaseous ozone

The effect of gaseous ozone treatment on surface attached *L. monocytogenes* L002 to food grade stainless steel was investigated. Fig. 6.16 illustrates *L. monocytogenes* L002 exposed *in situ* to gaseous ozone at 45 ppm for 1 h.

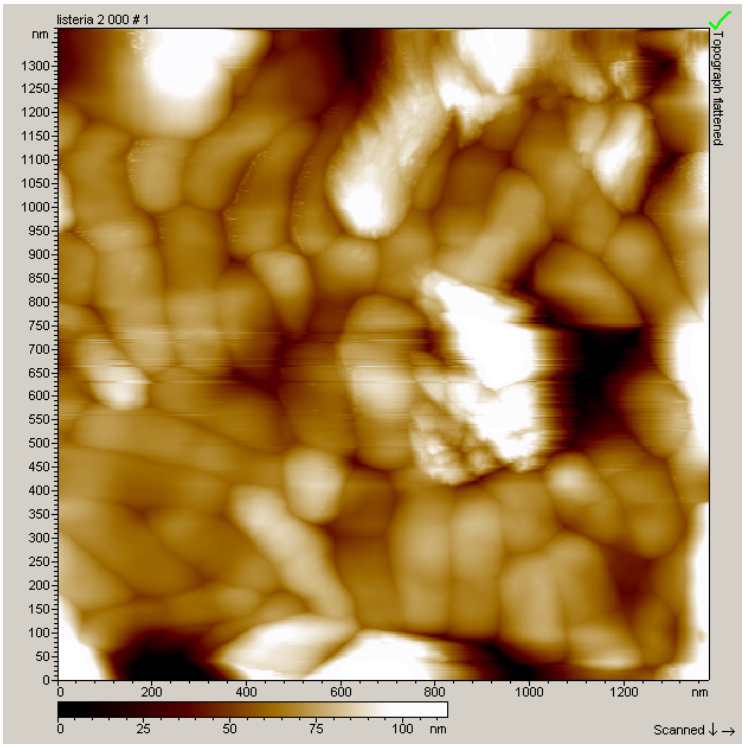



**Figure 6.16.** 1380 nm tapping mode scan height image (3rd order fattened) showing the edge of a regular layer of cells.

The average cell dimensions (table 6.2) for the treated cells are shorter and wider compared to theuntreated cells (table 6.1).

**Table 6.2.** Average cell dimensions (n=10) for surface attached *L. monocytogenes* cells to food grade stainless steel (AISI 304; finish 2b). Test sample-gaseous ozone treated cells (2).

| Dimensions | nm (± sd) |
|---|---|
| Length | 332.8 (± 70.6) |
| Width | 114.7 (± 11.0) |
| Height | 361.2 (± 66.2) |

The surface of these cells did show significant surface property changes as shown in the phase image below (Fig. 6.17 b).

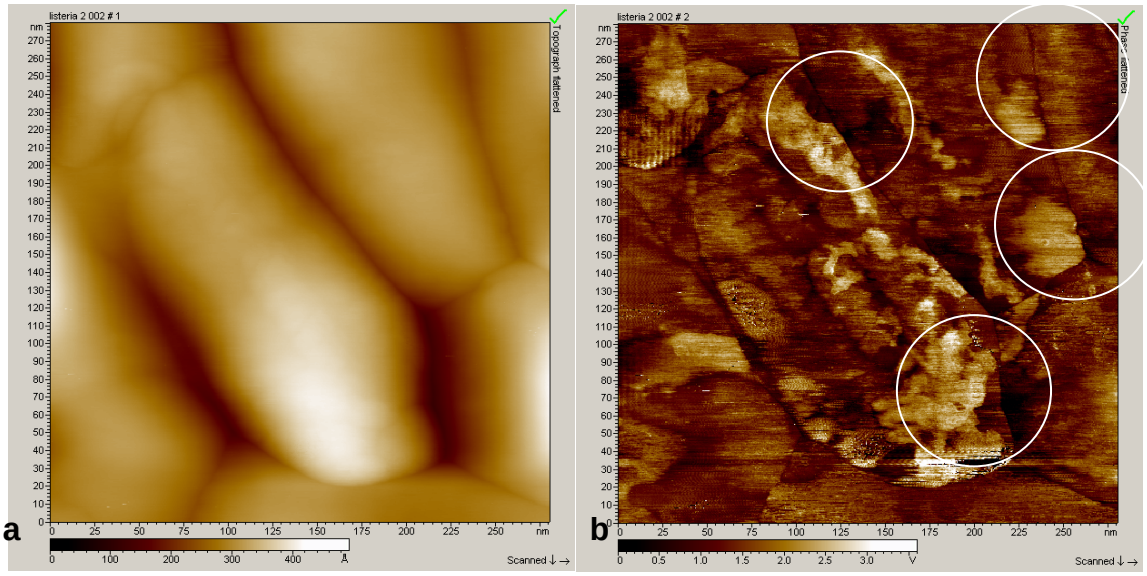



**Figure 6.17.** The a) height and b) phase images of treated cells. Some of the areas of altered cell properties are circled in white.

Phase images give information on the surface 'stickiness' or elasticity. While not quantitative, they can pick out areas of differing material properties. The cell shapes and surface features in Fig. 6.17 a, are similar to the untreated sample (Figs. 6.13 and 6.14), but this phase image, Fig. 6.17 b, has shown considerable

areas of changed material properties. Changes of around 1 V can be seen in the phase image, while the background variation is around 0.5 V. The cross sectional view (Fig. 6.18) revealed that the cells have lost their turgid shape and are deflated.

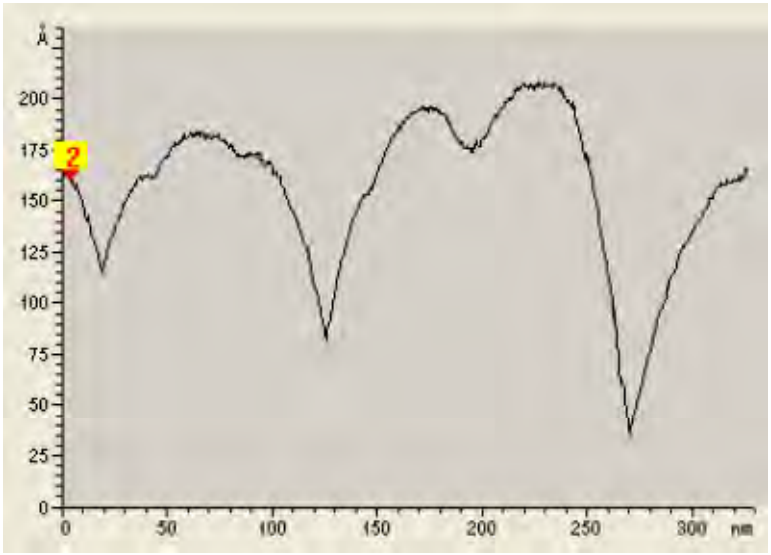



**Figure 6.18.** Cross-sectional view of two *L. monocytogenes* cells treated with gaseous ozone. The cells have an indented surface. Other areas show larger depressions, which could be due to dehydration of cells.

The cross-sectional view revealed untreated cells were more rounded (Fig. 6.15) compared to the treated cells (Fig. 6.18) which have an indented shape.

#### 6.3.2.3 Ozonated water treatments

The effect of ozonated water treatments on *L. monocytogenes* L002 surface attached to food grade stainless steel coupons was investigated. Fig. 6.20 shows an AFM micrograph of untreated *L. monocytogenes* L002 surface attached cells (control-D2). The cells were imaged after storage for 20 h at 4˚C. Cells were smooth and turgid (Fig. 6.19).

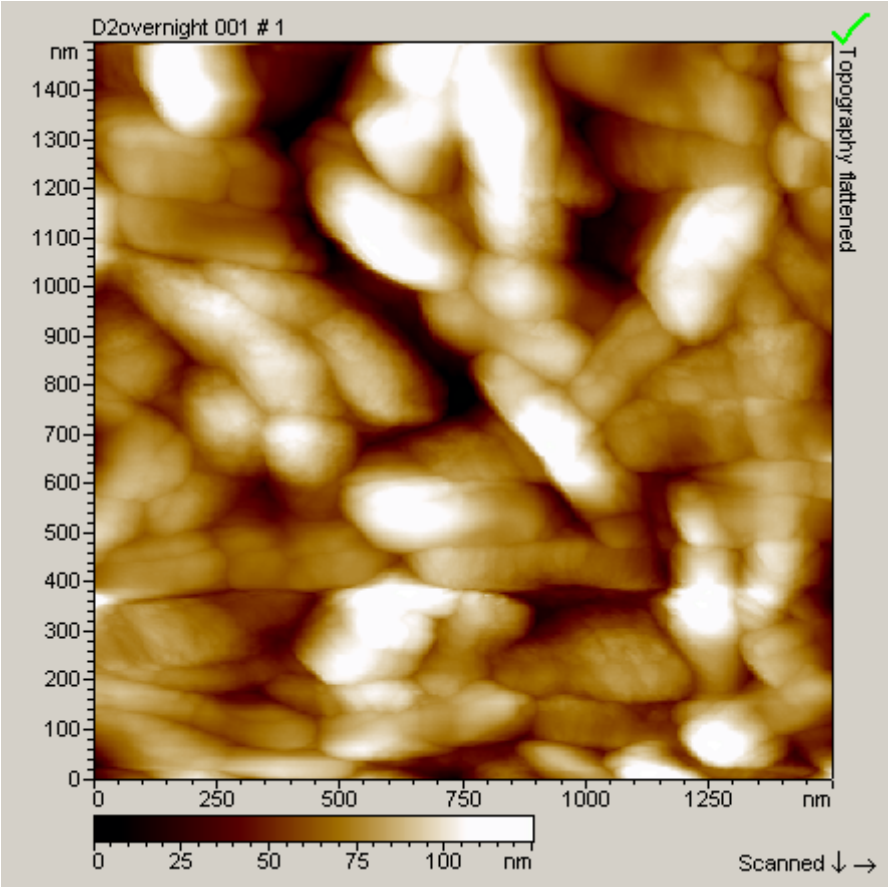



**Figure 6.19.** 1.5 micron scan of untreated *L. monocytogenes* surface attached to food grade stainless steel.

Table 6.3 illustrates the average cell dimensions for untreated cells. The cells have similar length and height compared to the untreated cells for the gaseous ozone and OAF treatments. However, these cells appeared wider.

**Table 6.3.** Average cell dimensions (n=10) for surface attached *L. monocytogenes* cells to food grade stainless steel (AISI 304; finish 2b). Control sample-untreated cells (D2).

| Dimensions | nm (± sd) |
|---|---|
| Length | 365.0 (± 143.9) |
| Width | 132.9 (± 13.5) |
| Height | 391.8 (± 78.4) |

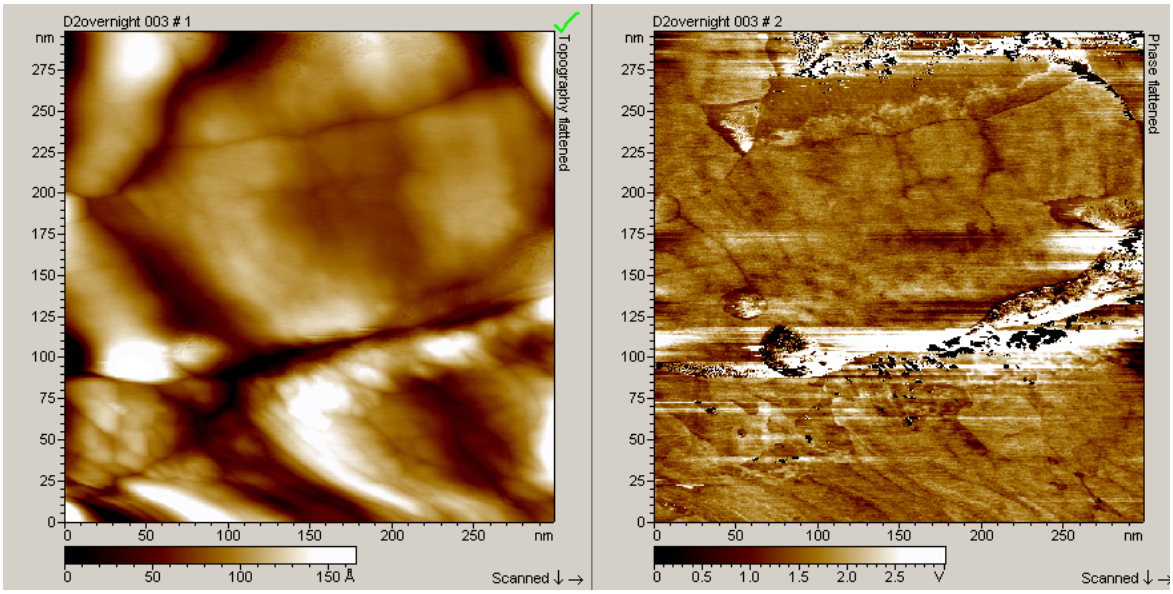



**Figure 6.20.** Surface detail of *L. monocytogenes* (untreated) cells with phase image along side. While there are some white/black regions of instability artefacts, and there are also some darker regions across the cell wall.

The cross section (Fig. 6.21) of three untreated *L. monocytogenes* cells, show the cells have a rounded appearance, similar to the untreated *L. monocytogenes* cells for the gaseous ozone and OAF experiments (Fig. 6.15).

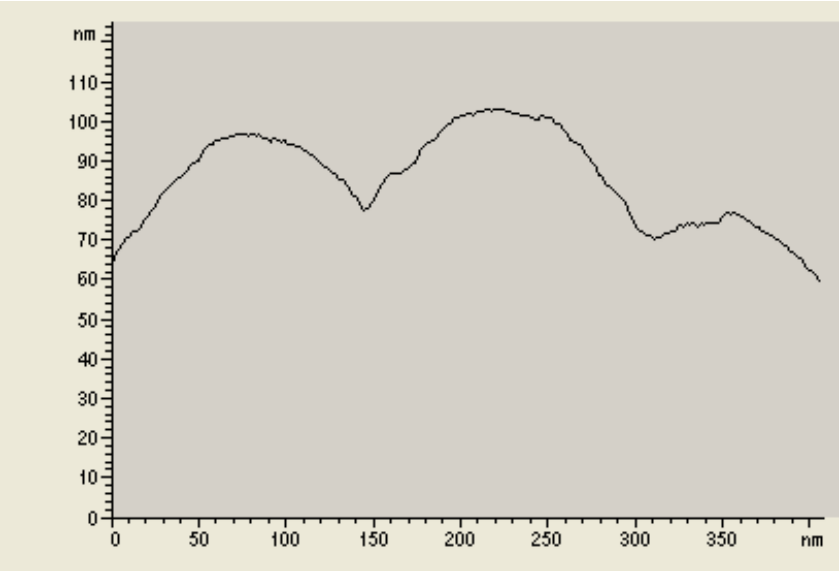



**Figure 6.21.** Cross-sectional view of three untreated *L. monocytogenes* cells which have rounded appearances.

Fig. 6.22 illustrates the effect of ozonated water (0.1 ppm) treatment (test sample-B1) on *L. monocytogenes* L002 surface attached to food grade stainless steel

coupons. *L. monocytogenes* L002 was exposed *in situ* to the treatment for 5 mins. Cells were imaged after 20 h and stored at 4˚C.

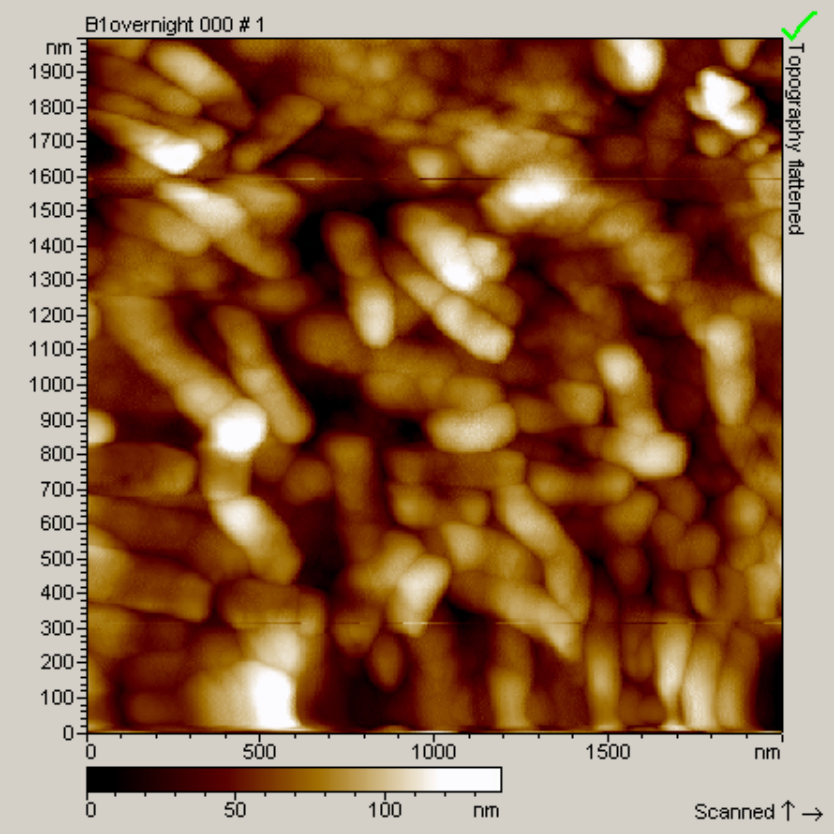



**Figure 6.22.** Two micron tapping mode scan height image (3rd order fattened) showing an irregular layer of cells

Table 6.4 illustrates the average cell dimensions for treated (ozonated water) cells. The cells are shorter and wider than untreated (table 6.3).

**Table 6.4.** Average cell dimensions (n=10) for surface attached *L. monocytogenes* cells to food grade stainless steel (AISI 304; finish 2b). Test sample-ozonated water treated cells (B1).

| Dimensions | nm (± sd) |
|---|---|
| Length | 266.7 (± 38.8) |
| Width | 133.4 (± 12.6) |
| Height | 451.6 (± 120.3) |

The cell surface is does not appear smooth or turgid, but has collapsed. The cell surface appears structured, showing various layers, for example, cell wall, cell membrane, cytoplasm or presence of pili (Fig. 6.23).

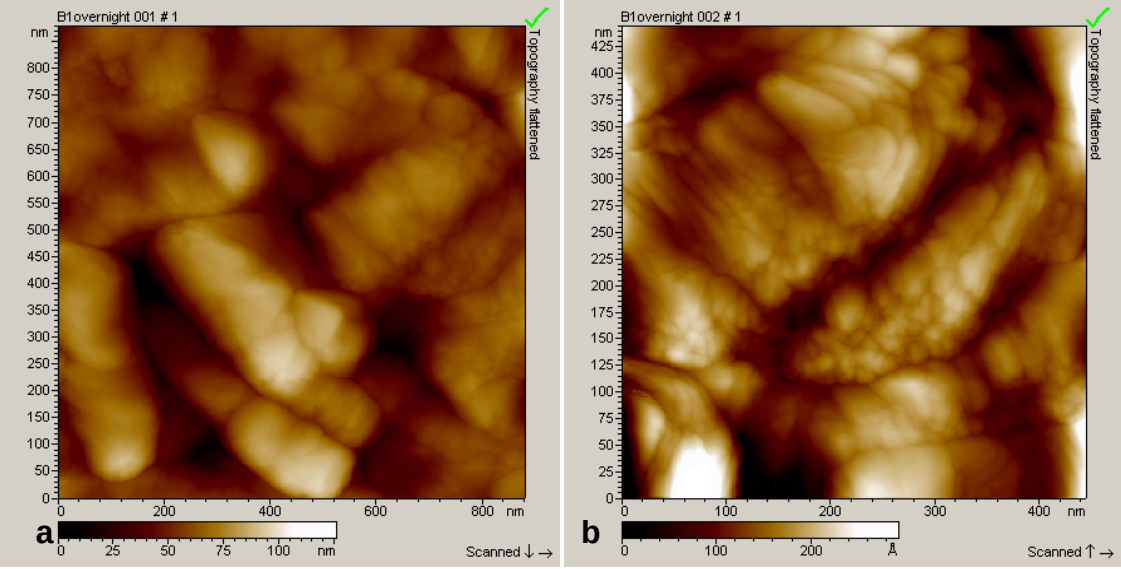



**Figure 6.23.** Higher magnification images of *L. monocytogenes* (B1) cells, showing that the cell surface does not appear smooth and turgid, instead the cells have collapsed and appear structured (Fig. 6.23 b).

Fig. 6.24 illustrates some small darker regions (circled) on the treated cells that suggest changes in the cell surface. The speckled regions are artefacts.

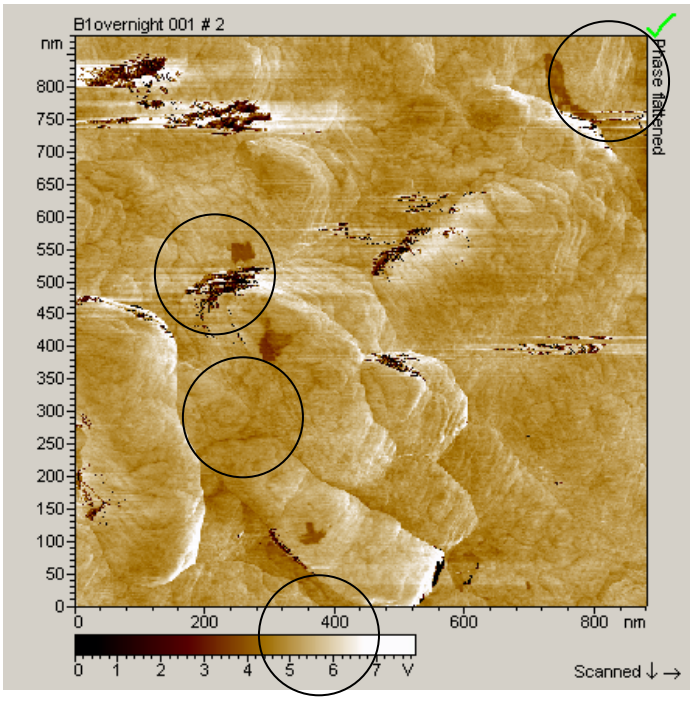



**Figure 6.24.** A phase image of B1 cell surface.

A cross-sectional view (Fig. 6.25) illustrates ozonated water (0.1 ppm) treated cells appeared round in shape to the untreated (Fig. 6.21).

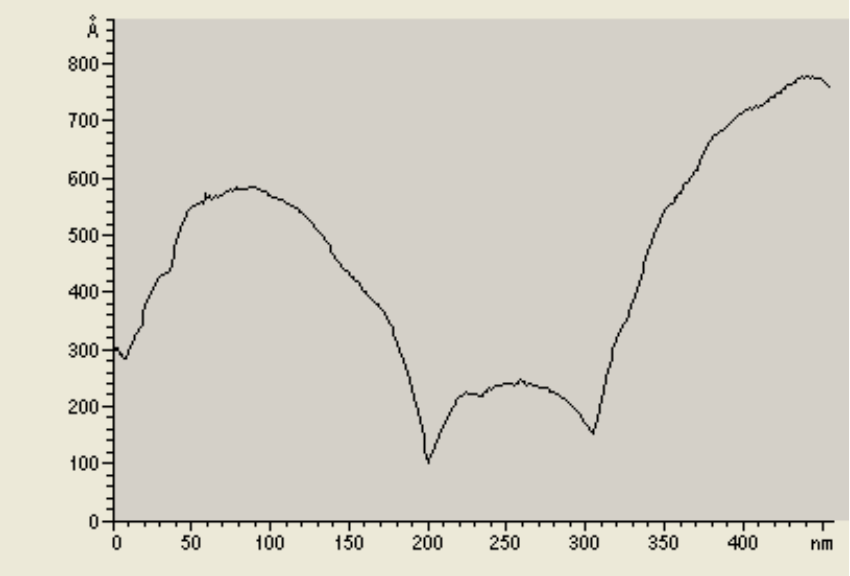



**Figure 6.25.** Cross-sectional view of three B1 cells showing a rounded appearance.

Fig. 6.26 illustrates the effect of ozonated water (0.1 ppm) and terpene, limonene (25 ppm) treatment (test sample-C1) on *L. monocytogenes* L002 surface attached to food grade stainless steel coupons. *L. monocytogenes* L002 (C1) cells were exposed *in situ* to the treatment for 5 mins. Cells imaged after 20 h storage at 4˚C.

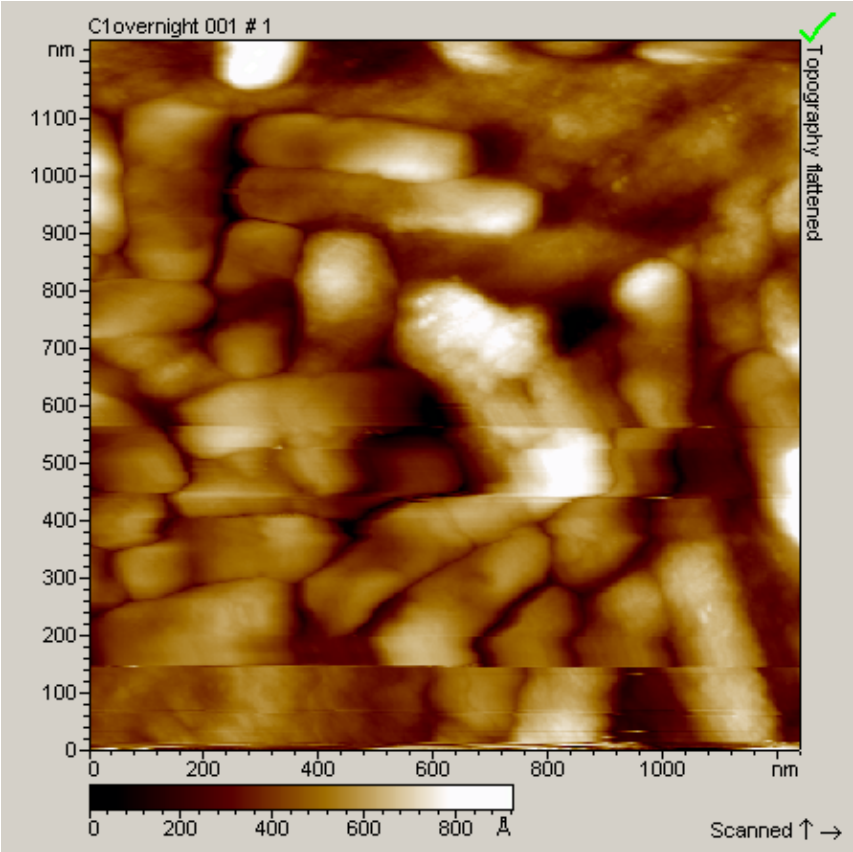



**Figure 6.26.** 1238 nm tapping mode scan height image (3rd order fattened) showing the edge of an irregular layer of cells.

Table 6.5 shows the average cell dimensions of ozonated water and terpene treated cells. The cells appear to be longer and narrower than untreated cells (table 6.3) and ozonated water treated cells (table 6.4).

**Table 6.5.** Average cell dimensions (n=10) for surface attached *L. monocytogenes* cells to food grade stainless steel (AISI 304; finish 2b). Test sample-ozonated water and terpene treated cells (C1).

| Dimensions | nm (± sd) |
|---|---|
| Length | 367.4 (± 85.4) |
| Width | 124.1 (± 24.1) |
| Height | 399.8 (± 91.9) |

There were some interesting structures in the cell surface of ozonated water and terpene treated cells, including ribbon-like features (Fig. 6.27 b, c).

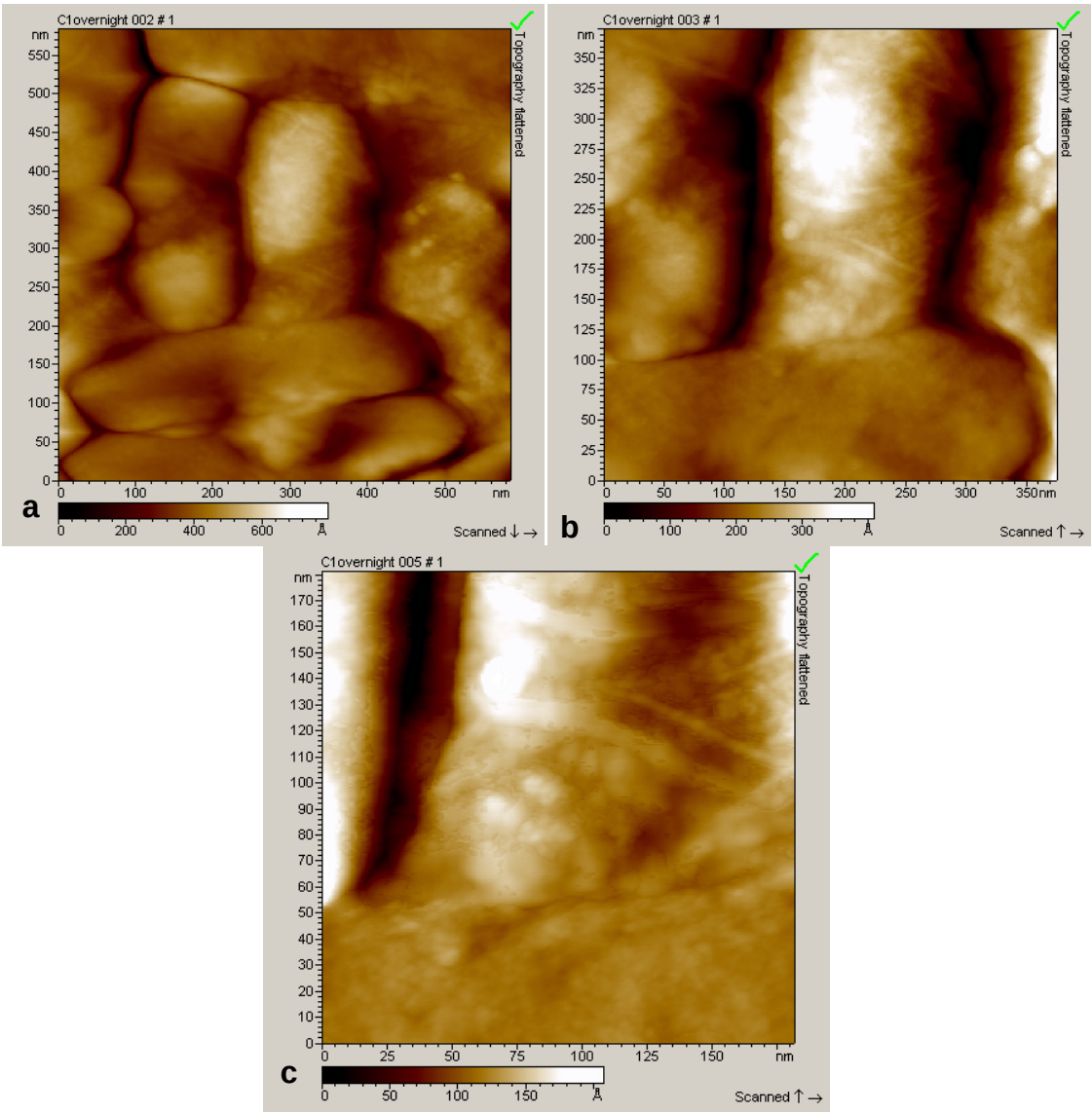



**Figure 6.27.** A series of increasing magnification images of bacterial cell surface structure. Fig. 6.27a, is at the lower magnification, whereas Fig. 6.27 c, is at the higher magnification.

The phase image of these cells (Fig. 6.28) was unstable in a large part, but some cells were visible, though none showed the dark patches seen in ozonated water B1 treatment (Fig. 6.27).

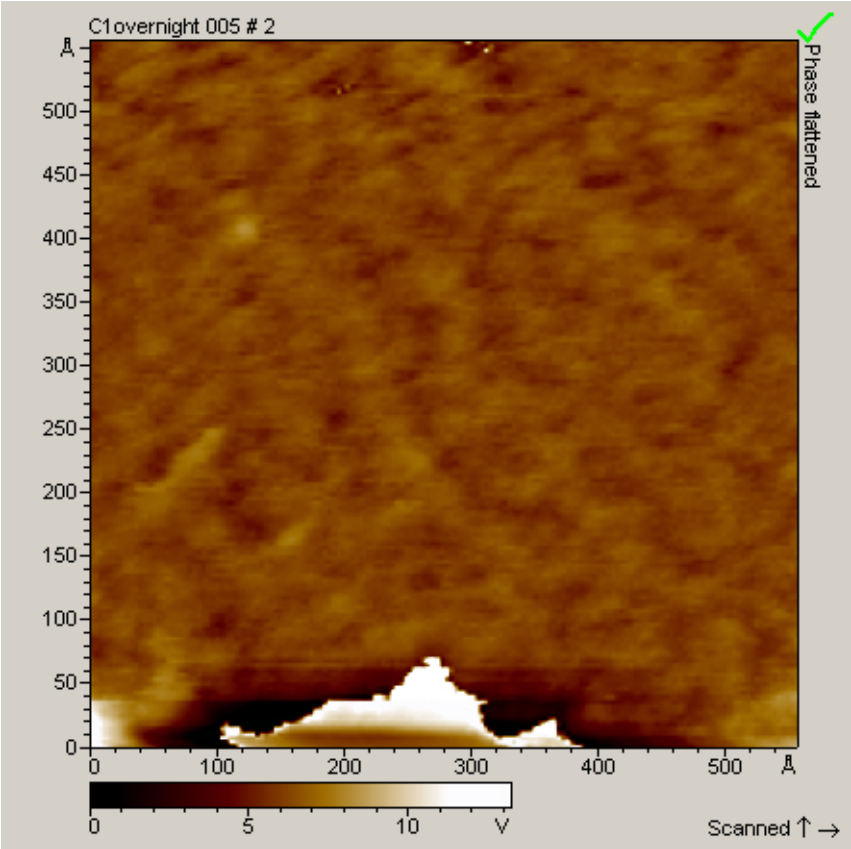



**Figure 6.28.** A 55nm phase image of a single C1 cell surface. A textured surface can be seen. The white region at the bottom centre is an artefact.

A cross-sectional view (Fig. 6.29) illustrates three cells treated with ozonated water and terpene. Cells appeared flattened and had collapsed, compared to the ozonated water treated cells (Fig. 6.25).

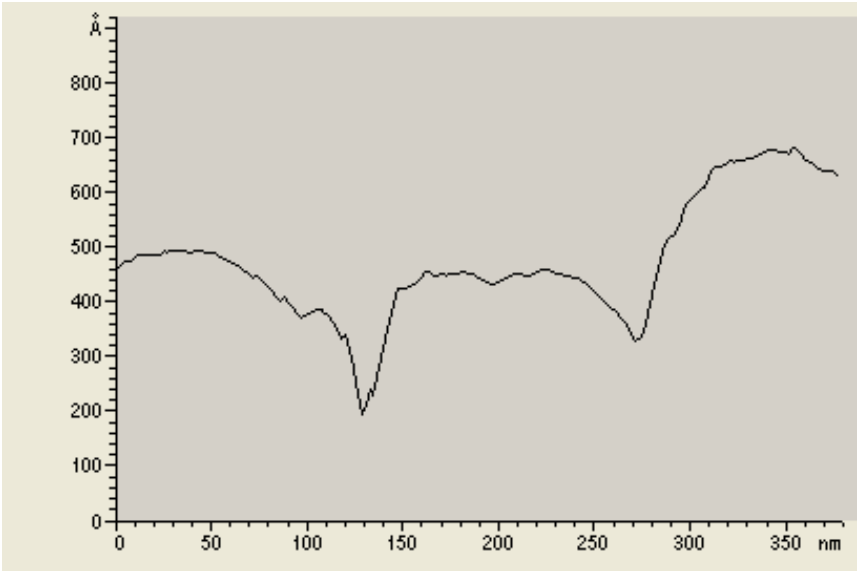



**Figure 6.29.** Cross-sectional view of three C1 cells, which show a flattened appearance. Each cell is approximately 150 nm in width.

Fig. 6.30 illustrates the effect of terpene (limonene) only treatment (test sample-D1) on *L. monocytogenes* L002 surface attached to food grade stainless steel coupons. *L. monocytogenes* L002 was exposed *in situ* to the treatment for 5 mins. Cells were imaged after 20 h storage at 4˚C. Two sets of cells were measured from both days of microscopy (n=7 in both cases). The images (Fig. 6.30 a, b) clearly shows that the D1 (terpene only) treated cells were able to grow and divide when stored at 4˚C for 20 h.

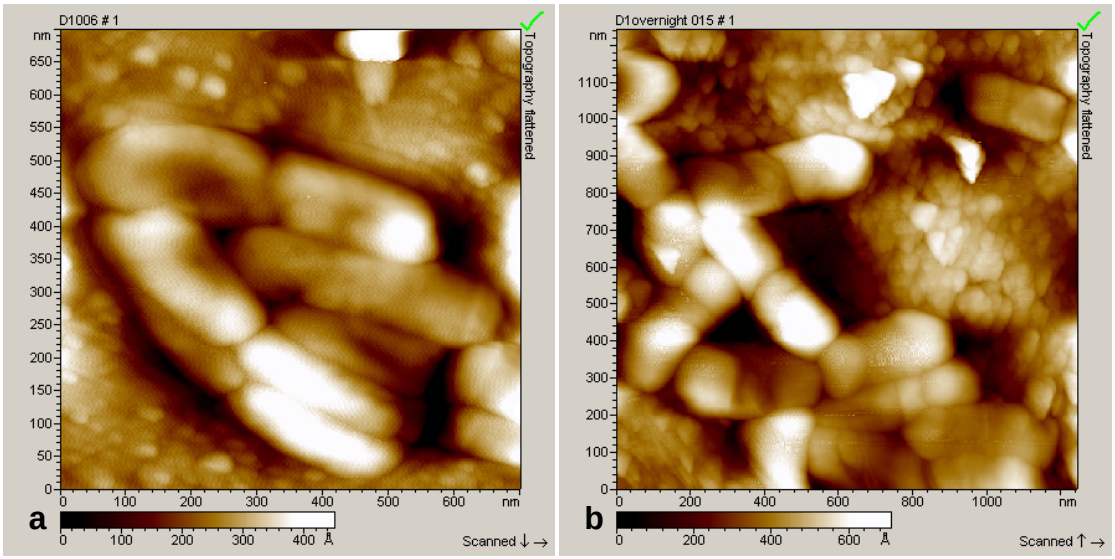



**Figure 6.30.** Comparison of *L. monocytogenes* cells (D1) treated with terpene only, Fig. 6.30 a, on day of treatment and Fig. 6.30 b, after 20 h storage at 4˚C. While the scales are not the same, there is a greater degree of cell division evident in the older sample stored for 20 h at 4˚C.

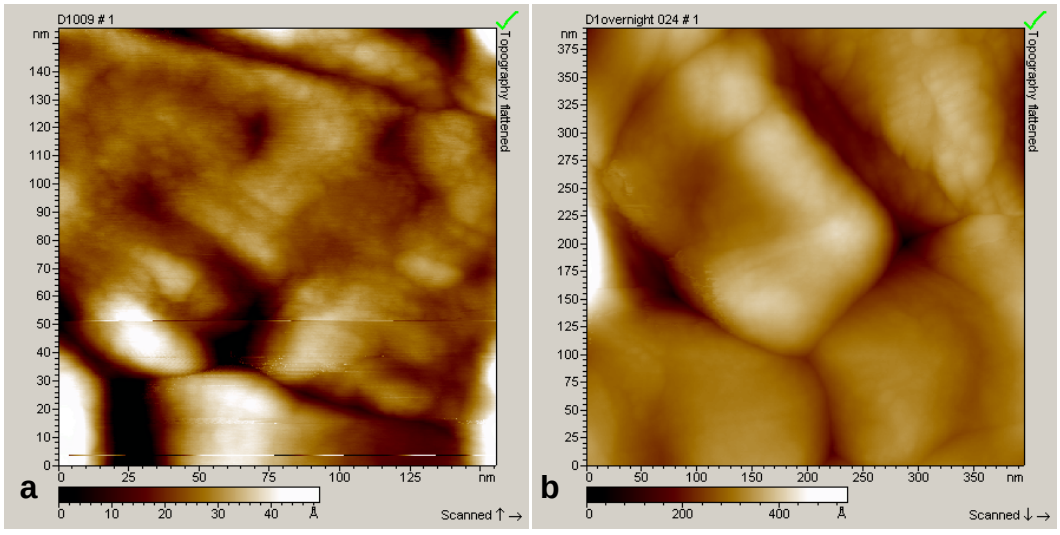



**Figure 6.31.** Surface detail of *L. monocytogenes* cells (D1) treated with terpene only. Fig. 6.31 a, on the day of treatment and Fig. 6.31 b, after 20 h storage at 4˚C.

Table 6.6 illustrates the average cell dimensions of treated cells with terpene in water only. The cells combined were shorter and wider compared to untreated cells. By measuring the cells 20 hrs later stored at 4°C, it was apparent that the cells were able to grow. The cells were longer and wider compared to cells examined on the day. The cells (on day of treatment) were shorter and narrower than untreated cells.

**Table 6.6.** Average cell dimensions of surface attached *L. monocytogenes* (D1) cells to food grade stainless steel (AISI 304; finish 2b). Test sample-terpene only treated cells (D1).

| | Dimensions | nm (± sd) |
|---|---|---|
| On the day | Length | 297.2 (± 65.3) |
| n=7 | Width | 103.2 (± 25.5) |
| | Height | 202.9 (± 45.8) |
| 20 hrs later | Length | 314.1 (± 82.6) |
| n=7 | Width | 165.5 (± 14.7) |
| | Height | 339.5 (± 95.2) |
| Combined | Length | 305.7 (± 72.1) |
| n=14 | Width | 134.4 (± 38.0) |
| | Height | 271.3 (±100.9) |

The phase image showed some distinct dark regions on several cells, suggesting changes in the properties of the cell walls (Fig. 6.32 and Fig. 6.33).

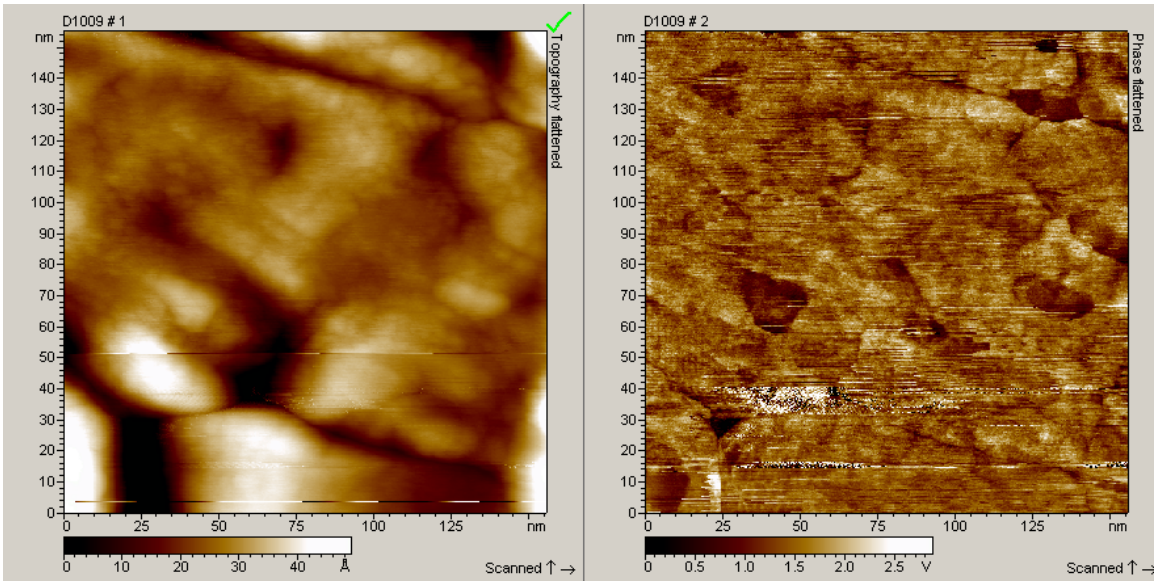



**Figure 6.32.** Height and phase image of *L. monocytogenes* cells (D1) treated with terpene only on the day of treatment. Darker regions are present in the phase image.

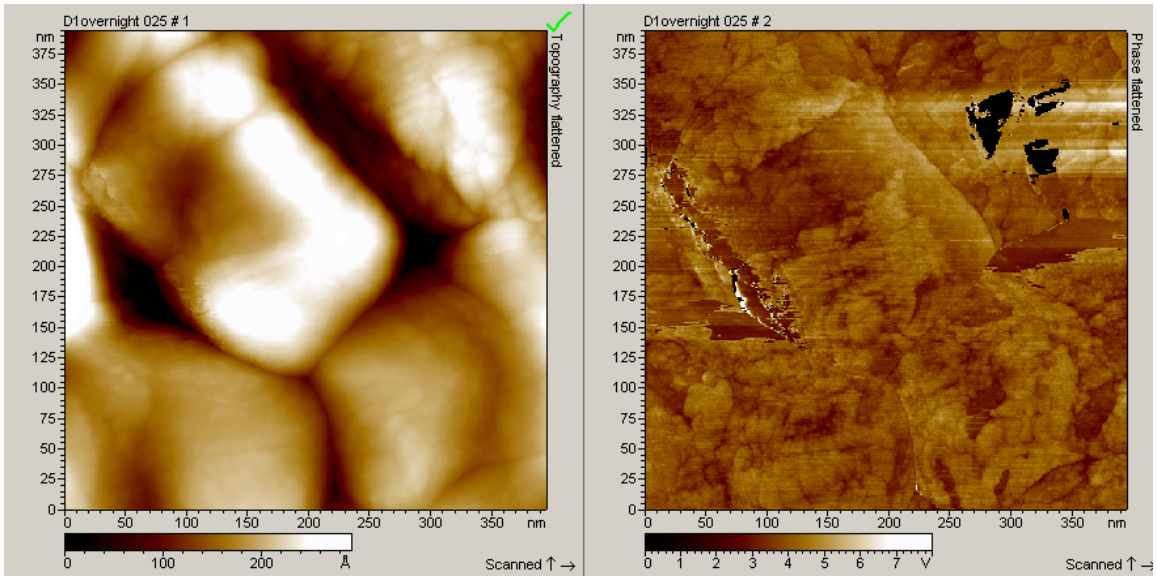



**Figure 6.33.** Height and phase images of *L. monocytogenes* (D1) cells after 20h storage at 4˚C. Darker regions are seen in the phase image.

A cross-sectional view (Fig. 6.34) illustrates four cells treated with terpene in water treatment. The cells are flattened and have collapsed, similar to the ozonated water and terpene treated cells (Fig. 6.28).

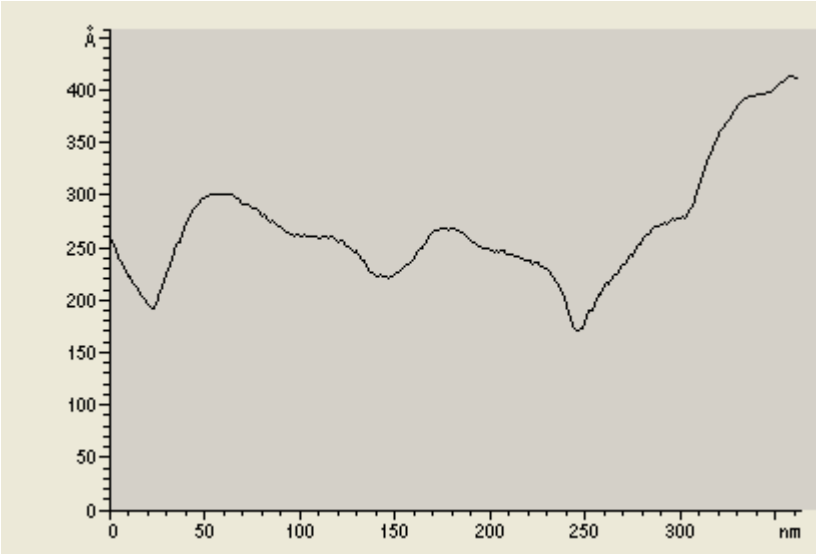



**Figure 6.34.** Cross-sectional view of four *L. monocytogenes* (D1) cells, showing flattened, collapsed shapes.

#### 6.3.2.4 OAF

The effect of OAF on surface attached *L. monocytogenes* L002 cells to food grade stainless steel coupons was investigated. *L. monocytogenes* L002 was exposed *in situ* to the treatment for 1 h. Fig. 6.35 illustrates *L. monocytogenes* cells treated with OAF for 1 h.

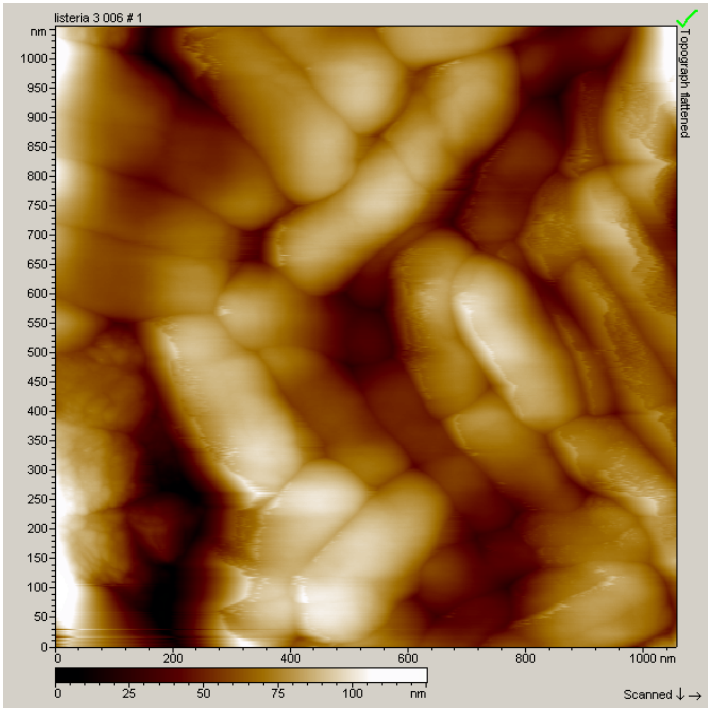



**Figure 6.35.** A 1055 nm tapping mode scan height image (3rd order fattened) of an irregular layer of OAF treated cells. Some artefacts due to noise in the scan signal are seen on some of the edges of the cells

Table 6.7 illustrates the average cell dimensions of treated cells. The cells appear to be shorter and wider compared to untreated cells (table 6.1).

**Table 6.7.** Average cell dimensions of surface attached *L. monocytogenes* cells to food grade stainless steel (AISI 304; finish 2b). Test sample-OAF treated *L. monocytogenes* cells (3).

| Dimensions | nm (± sd) |
|---|---|
| Length | 293.8 (± 40.3) |
| Width | 133.2 (± 20.3) |
| Height | 449.3 (± 132.6) |

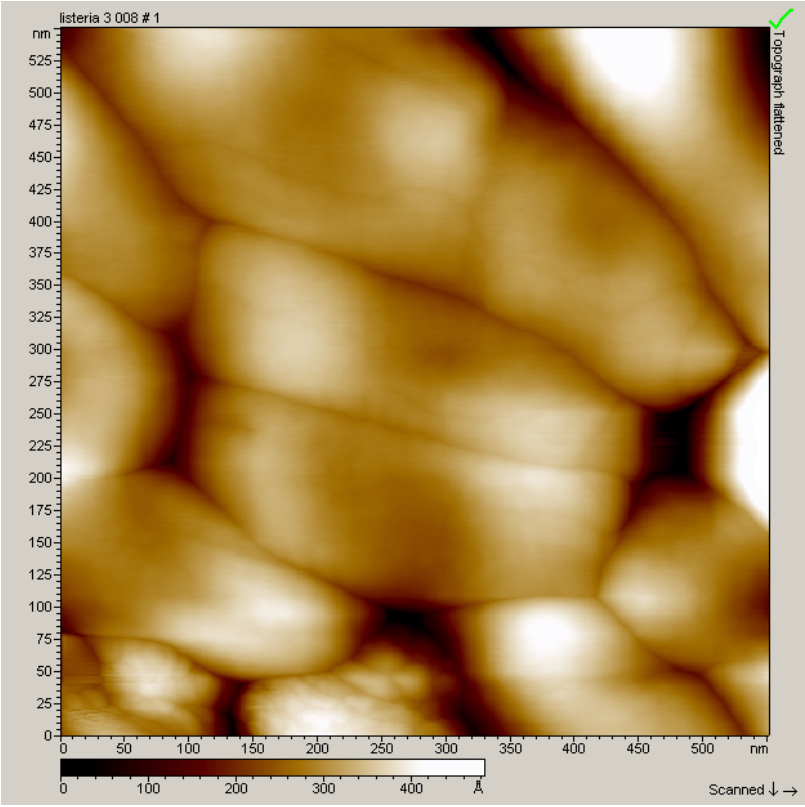



**Figure 6.36.** Surface detail from a 550 nm scan of *L. monocytogenes* L002 cells treated with OAF. There is some crumpling and concave areas within the cells (Fig. 6.39).

The phase image (Fig. 6.37) illustrates small regions of material property modification in the cell walls, as seen in Fig. 6.32 and Fig. 6.33. It is nearly as widespread as that observed in *L. monocytogenes* cells treated with gaseous ozone (Fig. 6.16), and of a similar magnitude in size (2V against a background of 1V variation, so a 50% increase as before, as observed in gaseous ozone treated cells).

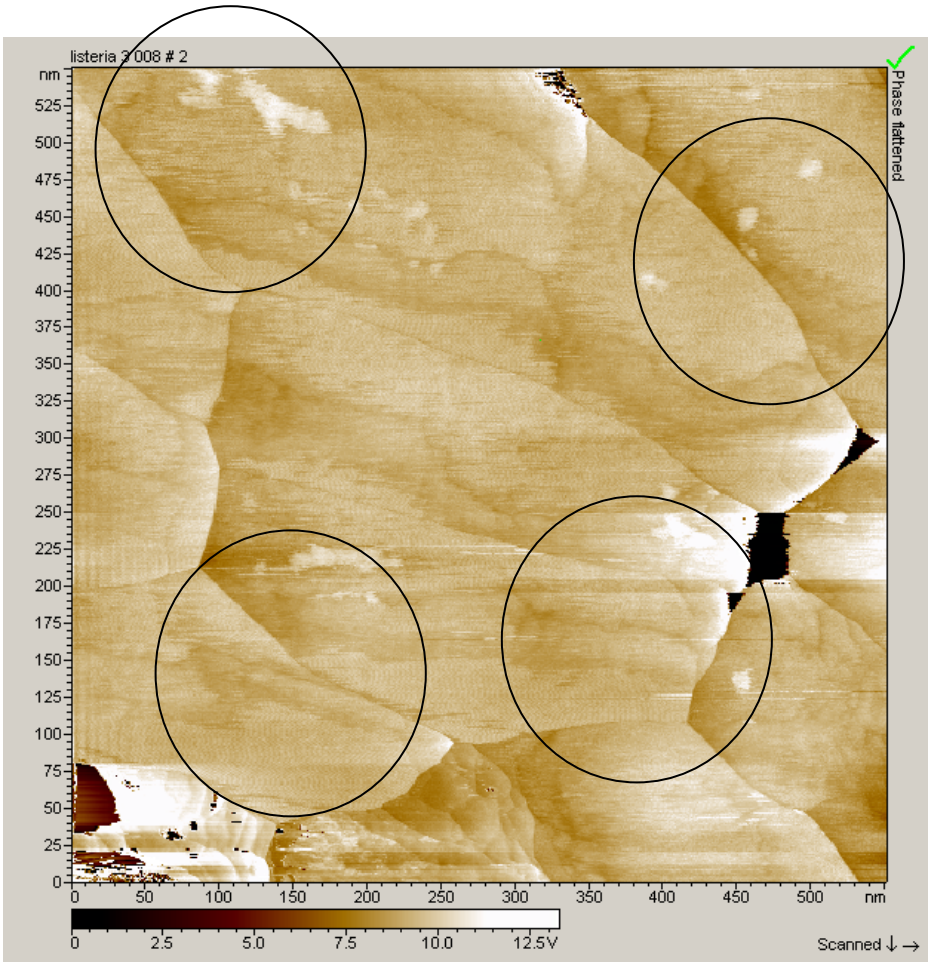



**Figure 6.37.** Phase image of *L. monocytogenes* L002 cells treated with OAF. Black regions are artefacts, but of interest are the lighter regions which are ringed, showing material property modifications.

A cross-sectional view (Fig. 6.38) illustrates two cells treated with OAF. Cells are slightly flattened and crumpling areas.

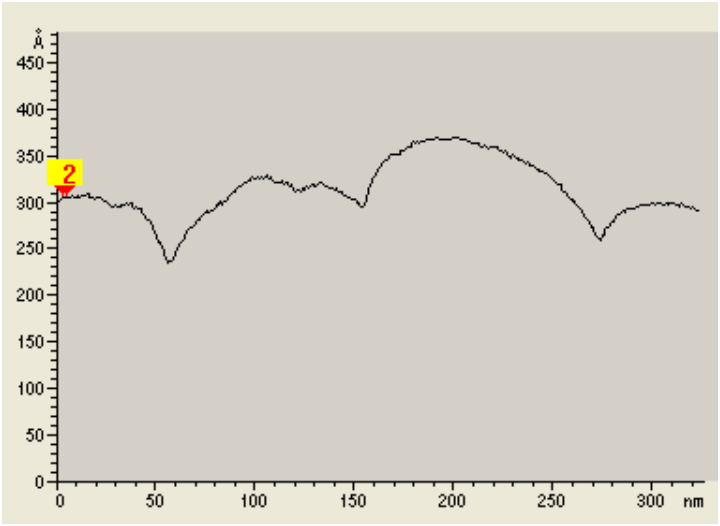



**Figure 6.38.** Cross-sectional view of two complete *L. monocytogenes* cells treated with OAF, showing only slight flattening and crumpling.

Table 6.8 illustrates the average cell dimensions for surface attached *L. monocytogenes* cells to food-grade stainless steel coupons (Nicholas *et al*., 2013).

**Table 6.8.** Average cell dimensions (n = 10) for surface attached *Listeria monocytogenes* cells to food-grade sainless steel (AISI 304; finish 2b) (Nicholas *et al.,* 2013).

| Dimension<br>nm (± SD) | Untreated | Ozone treated | Open air factor treated |
|---|---|---|---|
| Length | 347.6 (±54.2)[a] | 332.8 (±70.6)[a] | 293.8 (±40.3)[a] |
| Width | 97.0 (±10.2)[a] | 114.7 (±11.0)[b] | 133.2 (±20.3)[c] |
| Height | 311.2 (±76.0)[a] | 361.21 (±66.2)[ab] | 449.3 (±132.6)[b] |

Within rows, means followed by different letters are significantly different.

The ozone-treated cells were significantly wider compared to the untreated sample with the OAF-treated cells showing significant differences with width and height compared with the untreated cells. They were also significantly wider than the ozone-treated cells.

This data from this chapter has been published in a paper (Nicholas *et al*., 2013) (see Appendix, pages 293-302).

## 6.4 Discussion

The scanning electron microscope has been extensively used to study the characteristics of surface structure of biomaterials such as bacterial cell surface and to investigate biological responses such as cell attachment and changes in morphology. Kenzaka *et al*. (2005) recently developed a new scanning electron microscopic method for gaining both the combination of phylogenetic information as well as morphological structure about target microbes, by using *in situ* hybridization with oligonucleotide probes (SEM-ISH) (Kenzaka *et al*., 2009). In order to further our understanding of the mechanism involved in bacterial resistance of biocidal treatments, this new method might further assist in our knowledge of *Listeria monocytogenes*' behaviour when present in dual-species biofilms.

The SEM imaging taken by Dr. Hann, on surface attached *L. monocytogenes* L002 to glass cover slips exposed to gaseous ozone at 45 ppm did not show any major differences compared to untreated cells in cell appearance, apart from a few blebbed cells in the test sample. The static 72 h biofilm on food grade stainless steel (SS) coupons however revealed many blebbed cells when treated with gaseous ozone. SEM imaging by Dr. Hobot was performed using a different fixation protocol, due to issues with image quality and issues with biofilms. An experiment was designed, therefore, to determine the optimal sample fixation method. The appropriate three methods were chosen but there was no difference was found between them. The chosen method for subsequent experiments was 2.5% v/v glutaraldehyde in 0.1M phosphate buffer. Hexamethyldisilazane was

used instead of critical point drying, due to issues with using a different microscope. The solvent was found not to cause any cellular or structural disruption (Araujo *et al*., 2003; Braet *et al*., 1997). There was an apparent elongation of some cells treated with gaseous ozone where the cells seemed to have failed to divide. Reiu *et al*. (2008) revealed that morphology of *L. monocytogenes* was affected by growth conditions. Short rods were typical of static conditions, but under dynamic conditions, produced a network of long knitted chains.

SEM and AFM techniques were able to demonstrate the mode of action for the killing of surface attached and biofilm environmental bacterial isolates (*L. monocytogenes* and *P. aeruginosa*) exposed to various novel biocidal treatments. SEM demonstrated that gaseous ozone and OAF's mechanisms of action are quite different; with gaseous ozone causing blebbing of the cellular contents, while OAF punched holes in the cell membrane. The ozonated water treatments gave similar results to gaseous ozone and OAF and demonstrated that the terpene (limonene; in the form of d-limonene) alone or in ozonated water, caused the cell membrane to rupture with large holes, compared to ozonated water alone, which caused blebbing of the cellular contents. In comparison with the gaseous ozone treatment, the aqueous based ozone treatments were more effective in eliminating biofilm formation by stripping the biofilm off the surface of food grade stainless steel coupons. Fielding *et al*. (2007) evaluated ozonated water treatment as an alternative to chemical cleaning and sanitisation of beer lines. The results revealed a greater reduction in number of bacteria present after ozonated water treatment, compared to the chemical cleaner. After two cleaning cycles with ozonated water, there was a significantly lower level of contamination in the

dispensed medium from the beer lines. The study demonstrated the ability of ozone in cleaning beer lines by reducing the contamination and increasing the time period between cleaning cycles.

The ozonated water and terpene experiments revealed differences in cellular changes and the appearance and state of the biofilms treated. The untreated samples (which had been placed in 0.1 M phosphate buffer over the test period, in order to prevent the cells from drying out), illustrated healthy and non-stressed cells that had begun to a line themselves in and along scratches and imperfections on the surface of SS coupons. Whitehead and Verran (2006) reviewed the effects of surface topography on the retention of microorganisms. The authors discussed the differences in microbial retention on surfaces. Whitehead *et al*. (2005) studied the retention of cells in substratum surface features. Their findings supported data from Medilanski *et al*. 2002 (cited in the review by Whitehead and Verran, 2006) that many bacteria; particularly rod-shaped bacteria will orientate themselves into scratches on the surface substratum along their length. The ozonated water treatments revealed signs of dehydration as cells appeared shrivelled and flattened in appearance. This could be due to osmosis occurring. The ozonated water treatment caused the cells to bleb, whereas the biofilm cells treated with ozonated water and terpene, and terpene in water treatments showed holes had been punched through cell membranes. The same action could be seen for the *P. aeruginosa* C001 and dual-species biofilms, however, with these two biofilms, EPS was present surrounding the cells. There was a deposit left on the surface of the SS coupons by ozonated water and terpene, and terpene in water treatments. The OAF treatment caused the cells to appear flattened with holes punched through their membranes. The dual-species biofilm appeared thicker than the

single-species biofilms. The same deposit present on the coupons treated with ozonated water and terpene, and terpene in water was seen on the OAF treated coupons.

AFM was performed on *L. monocytogenes* L002 only as there is more interest and concern over this organism in the food industry. The AFM was able to distinguish surface property changes in treated cells (gaseous ozone and OAF treatments), compared to the untreated (control) cells. The untreated cells did however; show that desiccation had occurred, as the cells were collapsed but had a structured appearance with some crumpling areas. Takahashi *et al*. (2011) investigated desiccation survival of *L. monocytogenes* and other foodborne pathogens on SS over a 60 day storage period and found that *L. monocytogenes* had the highest survival. They noted that gram positives have a thicker peptidoglycan layer than gram negatives, and are thus able to resist dryness, which might result in higher survival rates when desiccated. Compared to the untreated cells, the gaseous ozone treated cells were shorter and wider and had lost turgid shape and were deflated. There was also evidence of altered cell surface properties. Similarly, following OAF treatment, the cells were shorter and wider than untreated cells and again there was evidence of cell surface property modifications. However, cells did appear flattened and had crumpled, concaved areas.

The ozonated water treatments revealed slight differences in cell appearance. The ozonated water treated cells appeared shorter and wider compared to the untreated cells. The treated cells were not smooth or turgid, but had collapsed. Their cell walls were highly structured, such as plasma membrane and cell wall and round in shape. There were significant surface property changes. The

ozonated water and terpene treated cells appeared longer and narrower. The cells had a flattened and collapsed appearance and there were some interesting ribbon-like features in the cell walls of some cells. The terpene in water treatment caused distinct changes in surface property and the cells had collapsed and flattened. The cells were also shorter and wider compared to the untreated cells. There was evidence that the cells could grow at 4°C for 20 h storage, cells were longer and wider compared to the sample imaged on the day of treatment.

Although all treatments used in this study shows evidence of altered appearance in the treated cells (by SEM); there was also evidence of changes to cell surface property (by AFM) in terms of possible mechanism of action. This was more apparent in the treatments associated with the terpene (OAF, ozonated water and terpene and terpene in water treatments). The synergy of using terpene with ozone either in its gaseous or aqueous phase seems to have a positive effect in controlling *Listeria monocytogenes* in the food industry. It would be interesting to investigate whether there was a genetic reason for the cells response to the environmental stresses place on them and the cause of the physical changes seen in the cell, as this would give a better understanding to the whole picture of microbial adaptation to environmental stress.

## General discussion and future work

This investigation has studied the effect of decontaminating food processing premises using ozone and other novel biocides. This involved studying the efficacy of the treatments applied and the possible mechanism involved in their action. This investigation was designed to look at the effects of ozone and other novel biocidal treatments (including ozonated water and/or terpene and OAF) applied to foodborne pathogens of concern to the food industry. The research concentrated on the foodborne pathogen *Listeria monocytogenes*, of major importance in the food industry. This organism is well known for its presence in the food processing plants and can persist there in biofilms for months or even years on a range of surfaces (Holah *et al*., 2004). *L. monocytogenes* is well adapted to living in such environments, and its adaptation to sub-lethal stresses has been demonstrated to protect the pathogen from the exposure to a variety of normally lethal conditions that are present in certain niches, for examples in foods or in its surrounding environment (Lou and Yousef, 1997). This bacterium can be disseminated by aerosolisation, can survive in aerosols and contaminate food products and food contact surfaces (Spurlock and Zottola, 1991). When *L. monocytogenes* forms biofilms it has enhanced resistance to sanitizers (Frank and Koffi, 1990). The isolate studied (named L002) was isolated from a high-care premise, where stringent cleaning protocols and regimes, based upon oxidising chemicals were in place.

Chapter 2 focused on the effect of gaseous ozone on environmental isolates, *Listeria monocytogenes* L002 and *Pseudomonas aeruginosa* C001, both surface

attached and as a biofilm adhered to food grade stainless steel (type 304) and other surfaces. A swab method was designed and validated as the sampling method for all subsequent experiments. This method was validated by determining reproducibility and sensitivity (section 2.2.1). Time curves of gaseous ozone production were determined. Production of ozone was monitored under these parameters; with and without high RH and with and without monitoring samples from the centre of chamber using pvc tubing. It was determined that the best parameters for the production and monitoring of ozone would be a high RH of >50% and monitoring the ozone concentration from the centre of the chamber, where the samples would be positioned, instead of monitoring the main chamber air from the ozone inlet pipe.

The initial study investigated the effect of gaseous ozone on surface attached *Listeria monocytogenes* L002 at 2, 5 10 and 45 ppm ozone for 1 hour on food grade stainless steel coupons in different orientations, compared to data from Bailey (2002) who studied the effect of gaseous ozone on surface attached *Pseudomonas aeruginosa* ATCC 15442 isolate at 0.05, 0.1 and 2 ppm ozone. The data revealed that *P. aeruginosa* was more sensitive than *L. monocytogenes* to gaseous ozone. The results suggested that the reduction of survivors is concentration dependent and not dependent on surface orientation.

When comparing different microorganisms' ability to survive gaseous ozone at 45 ppm for 1 hour (section 2.2.3), *M. luteus* was significantly more sensitive than both *L. monocytogenes* and *P. aeruginosa,* whereas the *P. aeruginosa* was significantly more sensitive than *L. monocytogenes***.** The results seemed to suggest that cell morphology and structure is linked to their ability to resist treatment. A

comparison between culture collection strains and environmental isolates, revealed that the environmental *L. monocytogenes* L002 was significantly more resistant than its collection strain counterpart, *L. monocytogenes* NCIMB 13451, possibly suggest that genetic characteristics could be involved in protecting the bacteria from the effects of ozone.

*L. monocytogenes* has different adhesion characteristics on different surfaces (Borucki *et al*., 2003; Frank and Koffi, 1990; Lee and Frank, 1991). There was a higher recovery of surface attached *L. monocytogenes* L002 cells from stainless steel, glass and polished marble (between 5-6 $\log_{10}$ data), compared to approximately 4 $\log_{10}$ transforms from the granite and polypropylene surfaces (section 2.2.5). When treated with 45 ppm ozone, more cells were recovered from stainless steel, polished marble and polished granite, giving 2.9, 3 and more than 3 log reductions, respectively compared to polypropylene and glass which gave lower reductions (between 1 and 2 $\log_{10}$ data), suggesting that these surfaces provided some protection as more cells were retained more strongly on these surfaces. Maximum attachment depends on high free surface energy or wettability of a surface. Surfaces with a high free surface energy, such as stainless steel and glass are more hydrophilic, and allow greater attachment and biofilm formation than hydrophobic surfaces, such as Teflon, nylon, and Buna-N rubber (Chmielewski and Frank, 2003). In subsequent experiments, glass slides were excluded as a surface, as glass is not permitted in the food industry and is not, therefore, a representative surface to test.

The effect of gaseous ozone at 45 ppm on surface attached and single species biofilms of environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 was

investigated. Surface attached *P. aeruginosa* C001 was significantly more sensitive than *L. monocytogenes* L002 by 1 log and both surface attached organisms gave higher log reductions compared to the single-species biofilms (for stainless steel). The single-species biofilms revealed that there was a significant difference between the two organisms and between untreated and test samples, with *P. aeruginosa* C001 being more sensitive than *L. monocytogenes* L002.

Chapter 3 described the ability of four chosen isolates of *Listeria monocytogenes* and *Pseudomonas aeruginosa* to form biofilms. One culture collection and one environmental isolate were chosen for each bacterium. The microtitre plate biofilm formation assays demonstrated the ability of the four chosen microorganisms to produce biofilms; whether as single or dual-species biofilms. The co-aggregation assay demonstrated that both environmental and collection strains of *L. monocytogenes* and *P. aeruginosa* co-aggregated. All organisms with the exception of *L. monocytogenes* NCIMB 13451 self-aggregated. These results suggest that the ability of *Pseudomonas* spp. to promote biofilm formation and produce copious amounts of extracellular polymeric substance (EPS) is necessary for co-aggregation with other species. LIVE/DEAD *Bac*Light staining assay revealed untreated cells fluoresced green which indicated the cells were alive with intacted cell walls, whereas the treated cells fluoresced red showing the cells were damaged.

The effect of ozonated water and/or presence of terpene (d-limonene) on surface attached, single- and multi-species biofilms environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 was investigated. Again the data revealed that *P. aeruginosa* C001 was significantly more sensitive to the treatments than *L.*

*monocytogenes* L002. The ozonated water and/or terpene treatments, especially ozonated water at 4 ppm, were more effective than gaseous ozone treatment in both removing surface attached and biofilm bacteria. Ozonated water and terpene provided a synergistic action against *P. aeruginosa* (surface attached or in biofilms on certain surfaces) compared with using ozonated water or terpene alone, but there was no synergistic action seen when used against the *L. monocytogenes* L002 isolate.

The effect of Open Air Factor (OAF) on surface attached, single- and multi-species biofilms of environmental *L. monocytogenes* L002 and *P. aeruginosa* C001 was investigated (chapter 5). OAF (0.1 ppm ozone) was significantly more effective in reducing survivors compared with gaseous ozone treatment (45 ppm) for both organisms either surface attached or in biofilms. Again, the *P. aeruginosa* C001 isolate was more sensitive to the treatment than the *L. monocytogenes* L002 isolate. OAF was slightly less effective against biofilm *L. monocytogenes* compared with surface attached cells. Pan *et al.* (2006) demonstrated differences in the resistance of *L. monocytogenes* biofilms to sanitizers between different surfaces, with biofilms on stainless steel being more sensitive than those on Teflon. In contrast, Deza *et al.* (2005) found no significant differences in survival of a range of organisms on glass or stainless steel.

There was a tenfold difference in inoculums size for surface attached and biofilm cells; with biofilm cells having a lower inoculums size. It would be expected that increased inoculums would reduce antimicrobial activity. The results from this study are contrary to that and reveal that the biofilm effect, rather than inoculums size is responsible for the results observed.

SEM and AFM analysis revealed comparable differences in the appearance and nature of cells (surface attached or in biofilms) on food grade stainless steel. The effect of gaseous ozone on both organisms in biofilms revealed blebbing on the surface of the bacteria, whereas with the open air factor treatment caused holes to be punched through the cell wall. The ozonated water treatments revealed signs of dehydration as cells appeared shrivelled and flattened in appearance. The cells blebbed their cellular contents when treated with ozonated water, whereas the ozonated water and terpene, and the terpene in water treatments had holes punched out of their cell walls. There was evidence of altered cell surface properties, which was more apparent in the treatments associated with the terpene (OAF, ozonated water and terpene and terpene in water treatments). The synergy of using terpene with ozone either in its gaseous or aqueous phase seems to have a positive effect in controlling *Listeria monocytogenes* in the food industry.

Environmental *P. aeruginosa* C001 throughout all treatments (gaseous ozone, ozonated water and/or terpene treatments and OAF) used against it, was significantly more sensitive than *L. monocytogenes* L002, either surface attached or in biofilms. It is thought the differences in sensitivity to treatments are linked to cell morphology and structure of cell wall. Differences in cell wall may account for differences in sensitivity to ozone as cell walls of gram positive bacteria consist of layers of peptidoglycans which form a rigid structure, whereas gram negative bacterial cell walls consist of an outer membrane containing lipoproteins, lipopolysaccharides with a few layers of peptidoglycans underneath (Thanomsub *et al*., 2002). This theory supports the study by Komanapalli and Lau (1998) suggesting that ozone reacts more readily with proteins than lipids (fats). The

production of EPS and the formation of biofilms play another important role aiding the bacteria's ability to survive harsh conditions. The genetic characteristics of *L. monocytogenes* may also play a protective role in combating certain environmental stresses.

There are many different serotypes of *L. monocytogenes* and the various serotypes belong to different to lineages (I to III). Serotypes 1/2a and 1/2b lack galactose or glucose from teichoic acid in the cell wall and have instead N-acetylglucosamine and rhamnose. Teichoic acid-associated N-acetylglucosamine and rhamnose (linked to clpC gene involved in stress response and virulence (Rouquette *et al*., 1996)) have been shown act as phage receptors in *L. monocytogenes* serotype 1/2a. Transposon-induced mutations in two loci in serotype 1/2a results in phage resistance and causes a lack of N-acetylglucosamine in the teichoic acid of the cell wall (Tran *et al*., 1999).

Future work. To increase our understanding as to why the L002 isolate is more tolerant (resistant) to ozone treatments applied in this study future work would be to:

1. Complete the microbial counts of dual-species biofilm of *L. monocytogenes* L002 and *P. aeruginosa* C001 on food grade stainless steel, polished granite and food grade polypropylene treated with 45 ppm gaseous ozone
2. Determine the possible mechanism of action using SEM on *P. aeruginosa* and dual-species biofilms treated with 45 ppm gaseous ozone, and using AFM on surface attached *P. aeruginosa* treated with all treatments.

It would be interesting to investigate whether cell wall differences affects the sensitivity of the bacteria to ozone. Lipids are a major target during oxidative

stress. Free radicals such as superoxide anion radical ($O_2^-$) and hydroxyl radicals ($^{\bullet}OH$) which are generated during oxidation, can attack polyunsaturated fatty acids in membranes and initiate lipid peroxidation. Lipid peroxidation causes changes to membrane permeability and can alter membrane-bound proteins. Researchers have looked at lipopolysaccharide (LPS) profiles in gram negative bacteria to identify any changes that occur when bacteria are exposed to oxidative stress and have examined fatty acid profile in gram positive bacteria such as *Listeria monocytogenes* (Gianotti *et al*., 2008; Giotis *et al*., 2007b; Mastronicolis *et al*., 2005). Cell fatty acid (FA) composition and lipid metabolism are involved as part of an adhesion mechanism. Attachment to surfaces and the formation of biofilms provide nutrition and protection against sanitation procedures, which is an adaptive advantage for attached microorganisms (Harvey *et al*., 2007). Membrane phospholipid composition and their modulation can affect surface properties, as well as other factors, such as change in gene expression profiles in response to belonging to a biofilm community (Harvey *et al*., 2007; Juneja and Davidson., 1993). Cell fatty acid (FA) composition of adhered and planktonic cells of *L. monocytogenes* has been selected on the basis of biofilm forming ability of 2 strains. Cells (adhered and planktonic) exposed to acid stress had an increase in individual and total branched FAs in the floating cells, whereas in the adhered cells of the strain with a lower biofilm forming ability, there was a relevant intracellular accumulation of straight, medium chain FAs (Gianotti *et al*., 2008). Membrane lipid fatty acids are also involved in cold adaptation (Mastronicolis *et al*., 2005) and pH stress tolerance (Giotis *et al*., 2007b). The addition of fatty acids to the growth medium can alter the lipid composition of the membrane and has been linked to susceptibility to antimicrobials (Juneja and Davidson, 1993).

Microarrays and PCR techniques would be also used in future work to determine possible genes that are up or down regulated as a general stress response to the treatments used in this study. There are certain areas or parts of equipment in food processing premises that are inaccessible for cleaning. These areas are more vulnerable to the formation of biofilms and such bacteria are exposed to sub-lethal doses of sanitizers and other disinfection treatments. Once bacterial cells are exposed to a mild stress, they are able to tolerate other more severe stresses/treatments later on. This ability is the stress adaptive response; also known as stress hardening. Stress hardening is the exposure to sub-lethal stress which then leads to the protection to exposure to variety of normally lethal conditions. Stress hardening has been introduced during food processing and has prevented the control of *Listeria monocytogenes* (Lou and Yousef, 1997). There are many resistance genes involved with oxidative stress and other stresses which are involved with stress hardening (adaptation). Cells that are subjected to oxidative stress (such as phagocytes) are able to adapt to this stress. Responses to the environmental stresses of heat and hydrogen peroxide have been studied in *L. monocytogenes*, and many heat shock and oxidative stress proteins were induced. Of these proteins, 5 were common to both heat and oxidative stresses. Stress proteins known to be induced by environmental stresses were absent in intracellularly grown *L. monocytogenes* This has been hypothesised as the mechanism by which bacteria can rapidly escape from stressful environments at early stage of phagocytosis (possibly due to the fact that *L. monocytogenes* secretes listeriolysin O, and other enzymes such as ClpC ATPase (Rouquette *et al*., 1998), superoxide dismutase (SOD), and catalase and proteins (Hanawa *et al*., 1995)).

*L. monocytogenes* can survive many environmental stresses; including acid, alkaline, salt, antibiotic, high pressure, osmotic, cold, starvation, oxidative, metal ion and sanitizer stress (Dhaliwal *et al*., 1992; Lou and Yousef, 1997; Ren and Frank, 1993; Russell and Day, 1996). It has also been found to be able survive in lubricants used in the food industry (Aarnisalo *et al*., 2007). Resistance to certain stresses can lead to adaptation to these stresses and cross-protection against other stresses (Johnson, 2003; Wesche *et al*., 2009). For example, resistance to metal ions is related to antibiotic resistance and certain plasmids seem to be involved. It is known that the resistance can be either intrinsic or acquired (Russell, 2003). Antibiotic resistance acquisition through a plasmid can alter the cell membrane composition and cause changes in its susceptibility to biocidal treatments, and can confer some cross protection between antibiotics and biocidal action (Russell and Day, 1996). The surface substrata and limited nutrient availability can affect the resistance of *L. monocytogenes* to certain sanitizers (Dhaliwal *et al*., 1992; Ren and Frank, 1993). The ability of microorganisms, including *L. monocytogenes*, to adapt to various stresses for example, acid, ethanol (alcohol), hydrogen peroxide, heat or sodium chloride enhances their survival in higher concentrations of hydrogen peroxide (Lou and Yousef, 1997).

General stress response systems are activated by several different stresses and can give acquired cross protection against multiple stresses (Yousef and Courtney, 2003). Sigma factor B ($\sigma^B$) is responsible for general stress response in gram positive organisms, and Sigma factor S ($\sigma^S$) is responsible for general stress response in gram negative organisms. The modifications of the sigma factor ($\sigma$) which is bound to RNA polymerase, gives rise to alternative sigma factors, such as $\sigma^S$ and $\sigma^B$. Sigma factor S ($\sigma^S$), *sig*S in gram negative bacteria regulates the

transcription of more than 50 genes involved in resistance to osmotic, heat, oxidative and acid stress. Its induction occurs in response to starvation and entry into stationary growth phase, or when exponential growth cells are exposed to stresses other than starvation (Herbert and Foster, 2001). Alternative $\sigma^B$, *sig*B has the equivalent physiological functions that *sig*S has in gram negative bacteria. Both types of bacteria, therefore, have a parallel mechanism for acquisition of multiple stress resistance (Ferriera *et al*., 2001; Mañas and Pagán, 2005). $\sigma^B$ regulates the transcription of over 40 genes (Wesche *et al*. 2009) and has a significant role in detergent stress response in *Listeria monocytogenes* (Ryan *et al*., 2008). The disruption of $\sigma^B$ in *B. subtilis* increases its sensitivity to oxidative stress, whereas in *L. monocytogenes*, its disruption causes a decreased resistance to acid and osmotic stress (Wesche *et al*. 2009). Genes induced by $\sigma^B$ or $\sigma^S$ include those genes encoding for catalase, for enzymes used in DNA repair and for osmoprotectants. This suggests that the cell prepares for oxidative and osmotic stresses. It is thought that there is a significant overlap between oxidative stress-induced proteins and those proteins induced by $\sigma^S$, suggesting that oxidative damage is also significant in stationary phase and generally stressed cells.

The genes that would be studied in greater detail would be those involved in protection against oxidative stress. *L. monocytogenes* uses mechanisms to protect against oxidative stress, with enzymes such as catalase (CA) and superoxide dismutase (SOD), and produce proteins such as thioredoxin and glutaredoxin. Oxidative stress in bacteria is regulated by Oxy R system for hydrogen peroxide and Sox RS system for superoxide (Farr and Kogoma, 1991). Oxidative stress activates OxyR by formation of a disulfide bond. OxyR triggers

the expression of reductive activities such as enzymes that degrade the oxidant and reduce disulfide bonds (Cabiscol *et al*., 2000). SOD and CA antioxidant enzymes protect bacteria against superoxides and reactive oxygen species (ROS). SOD activity in bacteria, especially *L. monocytogenes* is inactivated by heat (Dallmier and Martin, 1988), and is more heat labile than catalase (CA). The presence of acid can affect enzyme activity of both CA and SOD and listeriolysin O (LLO) (which is required for virulence) (Dimmig *et al*., 1994). The effects of iron and selenium on the production of CA, SOD and LLO in *L. monocytogenes* were investigated. The addition of either iron or selenium increased the production of CA, SOD and LLO. Selenium had no increase in LLO production, but gave a slight decrease in its activity (Fisher and Martin, 1999).

Superoxide dismutase (SOD, EC1.15.1.1) is the key defence weapon against the superoxide anion ($O_2^-$) radical (Hassett *et al*., 1995). SODs catalyse the conversion of superoxide anion radical to hydrogen peroxide, and thus prevent the formation of the hydroxyl ($^{\cdot}OH$) radical. The $O_2^-$ radical is seen as toxic to bacteria deficient in SOD. Nearly all bacteria that utilise oxygen generate different types of SODs (such as MnSOD, FeSOD), as a response to oxidative stress and to be able to eliminate toxic oxygen by-products of metabolism. Many bacterial species possess two distinct types of SOD. In *L. monocytogenes*, a single SOD gene encodes for a functional manganese-SOD (MnSOD) (Vasconcelos and Deneer, 1994). MnSOD activity has been found to be down-regulated by serine/threonine phosphorylation during the stationary phase of growth (Archambaud *et al*., 2006). The presence of NaCl can increase or decrease the expression of SOD in *L. monocytogenes*, depending on the concentration (Dallmier and Martin, 1990). Catalase (EC 1.11.1.6) converts hydrogen peroxide into oxygen and water.

Higher expression of catalase can be correlated with resistance to killing by phagocytic cells such as macrophages, and the ability of *L. monocytogenes* to survive intracellularly (Bortolussi *et al*., 1987). *Listeria monocytogenes* strains that are catalase-negative possess a two-fold greater SOD activity, compared to catalase-positive strains (Welch *et al*., 1979). However, increased SOD activity does not correlate with increased virulence, and strain-to-strain differences in total SOD activities have also been seen. Different isolates of *L. monocytogenes* produced equivalent levels of SOD however different species of *Listeria* produced greater differences in SOD expression (Vasconcelos and Deneer, 1994). Fisher *et al*. (2000) investigated the effect of catalase and superoxide dismutase on ozone inactivation of *L. monocytogenes.* Strains were all inactivated upon exposure to ozone. However, catalase and superoxide dismutase were found to protect the cells from ozone attack, with SOD being more important than catalase for this protection.

The HtrA gene encodes for Htr A enzyme, which is a new class of oligomeric serine protease (Clausen *et al*., 2002). It is thought that HtrA protease is involved in the degradation of mis-folded proteins that accumulate under stress conditions, in particular in resistance to oxidative damage caused by hydrogen peroxide (Wonderling *et al*., 2004). Since this, other studies have shown that the HtrA protease is involved in the resistance of *L. monocytogenes* to oxidative stress caused by superoxide radicals generated by redox-cycling agents, such as paraquat (oxidizing reagents/disinfectant), is involved in virulence and used for growth in biofilms at high temperatures (Wilson *et al*., 2006), which is an important pathogenic trait. The study by Wilson *et al.* (2006) also suggested htrA as a potential target for antibacterial therapeutics for gram-positive bacteria.

Quaternary ammonium compounds (QACs) are used widely in the food industry as disinfectants for processing lines and surfaces, and used in antiseptics in human medicine. Strains of *L. monocytogenes* have possibly acquired resistance to these disinfectants. However, no mechanism of resistance to QACs has been fully investigated. It has been suggested that a mechanism found in *Staphylococcus aureus* involves a multidrug efflux pump system encoded by *qacA* and *smr* genes. The multidrug efflux pump is encoded by the *mdr*L gene, and another gene, *orf*A a putative transcriptional repressor of *mdr*L. Such pumps can be associated with multiple drug resistance (Mereghetti *et al*., 2000). Mereghetti *et al*. (2000) noted that low sensitivity of *L. monocytogenes* strains to QACs was not purely down to resistant genes carried on plasmids, but the results suggested the resistance was due to an intrinsic resistance due to modifications in the cell wall. The mdrL (encoded by lmo 1409) efflux pump can pump out heavy metals, antimicrobials (macrolides and cefotaxime) and ethidium bromide (EtBr). There has also been another efflux pump discovered; known as *Lde*, which is associated with fluoroquinolone resistance and partly with resistance to acridine orange and EtBr (Romanova *et al*., 2006). Romanova *et al*. (2006) discovered that the efflux pump was partly responsible for *L. monocytogenes* strains adaptation to benzalkonium chloride (BC). Previous studies have showed that *L. monocytogenes* exhibits morphological and physicochemical changes to the bacterial cell surface following adaptation to BC (To *et al*., 2002). A study performed by Kastbjerg *et al.* (2010) determined the expression of virulence genes when *L. monocytogenes* strains were exposed to sub-lethal concentrations of common disinfectants. They determined that peroxy- and chlorine based compounds had a reduced expression of virulence genes, compared to QACs that induced virulence expression.

Northern blot analysis confirmed that a peroxy compound induced a down-regulation of *prf*A and *Inl*A expression, whereas QACs induced an up-regulation of these genes in *L. monocytogenes* EGD-e strain.

Current sanitation technologies are crucial in maintaining the quality and safety of fresh commodities and food products. It is necessary that effective, reliable, economical and industrially relevant alternative sanitization methods are developed for the food processing industry to combat persistent isolates, like *L. monocytogenes* L002 serotype 1/2a isolate. Ozone seems to be a promising candidate as an alternative terminal sanitizing agent (Moore *et al.,* 2000) for use in the food processing industry, but it is not feasible to apply to large scale, due to toxicity levels. The potential application of OAF as an alternative biocide has been demonstrated, as it can be used in the presence of personnel. Its application, as well the application of ozonated water and/or terpene needs to be further investigated as a tool for combating environmental contaminants in food processing premises.

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## Appendix

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ORIGINAL ARTICLE

# The effect of ozone and open air factor on surface-attached and biofilm environmental *Listeria monocytogenes*

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## Abstract

Aim: The effects of gaseous ozone and open air factor (OAF) on environmental *Listeria monocytogenes* attached to three common food contact surfaces were investigated.

Methods and Results: *Listeria monocytogenes* on different food contact surfaces was treated with ozone and OAF. Microbiological counts, scanning electron microscopy (SEM) and atomic force microscopy (AFM) were performed. Ozone at 10 ppm gave <1-log reduction when *L. monocytogenes* was attached to stainless steel, while 45 ppm gave a log reduction of 3·41. OAF gave better log reductions than 10 ppm ozone, but lower log reductions than 45 ppm. Significant differences were found between surfaces. Biofilm organisms were significantly more resistant than those surface attached on stainless steel. SEM and AFM demonstrated different membrane and cell surface modifications following ozone or OAF treatment.

Conclusions: The strain used demonstrated higher resistance to ozone than previous studies. This may be due to the fact that it was isolated from a food manufacturing premises that used oxidizing disinfectants. OAF was more effective at reducing the levels of the organism than an ozone concentration of 10 ppm.

Significance and Impact of the Study: Pathogen management strategies must account for resistance of environmental strains when validating cleaning and disinfection. OAF has shown potential for surface decontamination compared with ozone. SEM and AFM are valuable tools for determining mechanisms of action of antimicrobial agents.

## Introduction

*Listeria monocytogenes* has frequently been isolated from food-processing environments (Di Bonaventura *et al.* 2008) and is a cause for concern in the chilled food industry as it has a psychrotrophic nature (Norwood and Gilmour 2001). Cases of listeriosis doubled between 2000 and 2007, and it is the leading cause of death from food-borne disease (Food Standards Agency 2013). Almost all human cases have been associated with serotypes 4b, 1/2a and 1/2b. Most outbreaks of listeriosis are caused by serotype 4b, but serotype 1/2a is more frequently isolated from food and environmental samples (Djordjevic *et al.* 2002; Borucki *et al.* 2003). All strains have virulence-associated genes carried on a pathogenicity island, and sequences of many genes are conserved. *Listeria monocytogenes* can rapidly form biofilms on food contact surfaces, such as plastic, polypropylene, rubber, stainless steel and glass (Mafu *et al.* 1990; Hood and Zottola 1997), and can grow within mixed-species biofilms at 10°C (Chae *et al.* 2006). The ability of *Listeria* to adhere to inert surfaces and form biofilms results in them being less susceptible to cleaning operations (Stopforth *et al.* 2002).

Ozone is a colourless gas with a distinctive odour. It has a very high oxidation potential and leaves no toxic residues because it decomposes into oxygen. Ozone has a

short half-life and does not accumulate on surfaces (Graham 1997). It has powerful antimicrobial properties (Gurley 1985) and reacts with cells by attacking cell membranes resulting in lysis of carbon-carbon double bonds in the membrane, causing cell lysis and death. Ozone targets membrane glycoproteins, glycolipids or amino acids and acts on the sulfhydryl groups of certain enzymes, disrupting normal cellular activity (Greene *et al.* 1993; Moore *et al.* 2000). Ozone has many advantages over traditional sanitizers, as it is currently exempt from the Biocidal Products Directive (BPD) 98/8/EC, as ozone has to be produced at the point of use (Anon 1998). The occupational exposure standard (OES) for ozone in United Kingdom is 0·2 ppm averaged over a 15-min period (Anon 1998). Due to the short half-life and lack of toxic residues, ozone has the potential to be used as a terminal sanitizer (Moore *et al.* 2000).

'Open Air Factor (OAF)' was discovered in 1968 and refers to a phenomenon observed when ozone reacts with any compound containing unsaturated hydrocarbons (May and Druett 1968). OAF is not a single molecule, but a collection of highly reactive chemical species (De Mik and De Groot 1978). OAF was first reported when experiments indicated that bacterial survival in aerosolized particles was much greater in a closed vessel than in the open air at the same temperature and humidity (May and Druett 1968). It has been suggested that OAF might be formed from ozone-alkene complexes, similar to those found in external environments, such as photochemical smog (De Mik and De Groot 1978), or naturally occurring volatile plant compounds, such as terpenoids. OAF can be produced using caroterpenoid (noncyclic) or non-caroterpenoid (cyclic) terpenoids (Breitmaier 2006). The production of OAF does fall under the scope of the BPD (European Commission 2011). The application of naturally occurring OAF is very limited. There is, however, the potential to artificially produce OAF as an air-phase disinfectant and, more recently, as a surface disinfectant (Bailey *et al.* 2007). The bactericidal effectiveness of gaseous ozone and OAF derived from two monoterpenes (one cyclic and one noncyclic) against aerosolized *Micrococcus luteus* was investigated. There were significant log reductions when the organism was exposed to ozone alone and to OAF at levels similar to those used in this study (Bailey *et al.* 2007).

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) are standard tools used to examine bacterial cells for signs of damage, including disruption to cellular membranes. Each technique is designed to provide information on surface structure, but different data related to friction, adhesion, elasticity, hardness, electric fields, magnetic fields, spreading resistance, conductivity and cell morphological changes are generated (Hannig *et al.* 2010). SEM takes images of the sample surface by scanning it with a high-energy beam of electrons in a scatter pattern. The electrons interact with the atoms that make up the sample surface by producing signals showing the topography of the surface, composition and other properties (Russell *et al.* 2008).

Atomic force microscopy provides three-dimensional surface topography at nanometre lateral and subangstrom vertical resolution. The AFM consists of a microscale, flexible cantilever, which has a sharp tip at the end, known as a probe. This probe is used to scan the surface of the sample while maintaining a small constant force between the tip and the sample surface by employing a feedback mechanism. When the tip is brought into proximity with the sample surface, the forces between the two lead to a deflection of the cantilever. The tip–sample interaction is monitored by reflecting a laser beam off the back of the cantilever into a split photodiode detector. The detection of different output voltages in the photodetector, changes in the cantilever deflection and oscillation amplitude can be determined (Russell *et al.* 2008). This study employed tapping mode AFM, which consists of oscillating the cantilever at the resonance frequency and light tapping of the tip on the surface during scanning (Nagao and Dvorak 1998).

This study investigates the comparative effects of gaseous ozone and OAF, produced from D-limonene, on the survival of surface-attached *L. monocytogenes*.

## Materials and methods

### Inoculum and coupon preparation – surface-attached cells

The environmental strain of *L. monocytogenes* (L002) was isolated from the drain of a high-care food-processing premise where stringent validated cleaning protocols, based on oxidizing disinfectants, were in place. *Listeria monocytogenes* L002 was serotyped as *L. monocytogenes* serotype 1/2a. Following initial isolation, the colonies were transferred to cryobeads (Technical Service Consultants, Lancashire, UK) and stored at −80°C. The working culture was produced by aseptically placing a single cryobead into each of two 250 ml Erlenmeyer flasks containing 100 ml of nutrient broth (Oxoid, UK). The flasks were incubated on a shaking platform (Orbital Shaker, Forma Scientific Inc., Marietta, OH, USA) at 250 rev $min^{-1}$, at 35°C for 24 h to obtain stationary-phase cells. The cells were harvested by centrifugation at 1068 *g* (DuPont Sorvall Superspeed RC-5B; Wilmington, DE, USA) for 20 min. The pellet was resuspended in 20 ml of sterile phosphate-buffered saline (Oxoid) to give approximately $10^9$ CFU $ml^{-1}$ (adapted from Bailey *et al.* 2007).

One hundred microlitres of this suspension was inoculated onto five 5 × 5 cm food-grade surfaces: stainless steel (304, 2b finish); polypropylene (retail food container) and polished granite (Mandarin Stone, Cardiff, UK) to give an initial cell density of approximately $2 \times 10^6$ CFU $cm^{-2}$. All surfaces were previously disinfected with alcohol wipes, rinsed thoroughly with sterile deionized water and air-dried. The stainless steel and the polished granite coupons were additionally sterilized by autoclaving at 121°C for 15 min before use (the polypropylene coupons could not be autoclaved due to degradation problems). The coupons were placed in individual, sterile Petri dishes and inoculated by evenly spreading the inoculum over the surface using a sterile plastic spreader (Fisher Scientific, Loughborough, UK). Inoculated coupons were allowed to air-dry at ambient temperature. Coupons were attached to clamp stands and placed inside a bioaerosol test chamber, which was maintained at a temperature of 20°C (±2°C) and a relative humidity of 50% (±5%).

### Inoculum and coupon preparation – biofilm

A static biofilm of the chosen micro-organism was grown on inoculated filter paper (Whatman qualitative No. 2) placed on the surface of a TSA plate (adapted from Charaf *et al.* 1999). One millilitre of a $10^{-1}$ dilution of *L. monocytogenes* L002 was pipetted onto the filter paper, ensuring even coverage. The filter paper was used to reduce anoxic conditions underneath the coupons. One centimetre squared sterile stainless steel, polypropylene and polished granite coupons were placed on top of the inoculated filter paper and pressed down lightly to expel any air bubbles. The biofilms were grown on the underside of the coupons for 72 h to give an initial cell density of approximately $2 \times 10^8$ CFU $cm^{-2}$. The cell density of the biofilm was less than that of the surface-attached cells due to the concentration of the latter by centrifugation, compared with the formation of the biofilm structure on the glass slide (which could not be concentrated in the same way). Five replicates were prepared. Each of the coupons was aseptically removed from the surface of the filter paper using sterile forceps, placed face-up in sterile Petri dishes and positioned in the centre of the bioaerosol test chamber. Biofilm coupons were not clamped so as to maintain the integrity of the biofilm.

### Ozone generation

Ozone was generated throughout the 1-h test period, and internal mixing fans were operated at all times. Ozone was generated using an Aquamaid II generator (Ozone Industries Ltd., Hampshire, UK). Ozone concentrations in the chamber were monitored with an Advanced Pollution Instrumentation ozone monitor (Model 450; Advanced Pollution Instrumentation Inc., San Diego, CA, USA). The surface-attached bacteria were subjected to ozone concentrations of 2, 5, 10 (stainless steel only) and 45 ppm (all surfaces) for 1 h. The biofilms were exposed to 45 ppm for 1 h.

### Open air factor generation

Open air factor was generated using an Air Disinfection (AD) device (Aerie Ltd., Milton Keynes, UK) which takes in ambient air, incorporates ozone and then quenches the ozone with D-limonene (0·0375 ppm) to generate hydroxyl radicals. Hydroxyl radicals are emitted while avoiding the release of ozone (external ozone levels ≤0·2 ppm). All samples were exposed to OAF for 1 h. The AD device was allowed to operate in the bioaerosol chamber, for a minimum of 1 h under ambient conditions, prior to exposure of the samples.

### Enumeration of survivors

The coupons were removed, and a premoistened sterile cotton tip swab was used to swab the entire surface of the coupons in two directions, with the second swabbing being performed at right angles to the first. Serial dilutions were performed as necessary and spread plated (0·1 ml) in duplicate on TSA plates and incubated at 30°C for 48 h.

### Statistical analysis

All ozone and OAF treatments were conducted in triplicate using five replicates ($n = 15$). Log transforms were performed on plate counts, and results were imported into Minitab version 15 (Minitab Ltd., Coventry, UK). Data were analysed using one- and two-way analysis of variance (ANOVA) with Tukey's pairwise comparison as appropriate. Significant differences were reported where $P \leq 0{\cdot}05$.

### Scanning electron microscopy imaging

Biofilms of *L. monocytogenes* were required as SEM was not possible on the surface-attached cells due to problems with visualization. Additionally, all microscopy was undertaken on stainless steel coupons. Treated coupons were placed in 24-well tissue culture plates (Cellstar; Greiner Bio-One Ltd., Gloucestershire, UK). 2·5% v/v glutaraldehyde in 0·1 mol $l^{-1}$ phosphate buffer for overnight fixation was added to all samples. Untreated biofilm coupons were placed in 0·1 mol $l^{-1}$ phosphate buffer during the 1-h test period. Washing using double-distilled water (twice for 5 min) followed by dehydration [50% ethanol

(5 min), 70% ethanol (5 min), 90% ethanol (5 min), 100% ethanol (5 min)] were performed. Hexamethyldisilazane (thrice for 5 min) was used instead of critical point drying (Araujo *et al.* 2003). The samples were air-dried and mounted on a specimen stub using an electrically conductive double-sided adhesive tape and sputter-coated with gold (Sandri 780; Tousimis Research Corporation, Rockville, MD, USA) for 2·5 min. SEM preparations were observed under a JEOL 840A scanning electron microscope (JEOL Ltd., Welwyn Garden City, UK), and images were recorded on SIS imaging software.

### Atomic force microscopy

Biofilm coupons were prepared as for SEM above. Untreated biofilm coupons were also prepared. All imaging was carried out on a PicoScan I atomic force microscope (Molecular Imaging Inc., Ann Arbor, MI, USA), with a separate acoustic modulation module (Molecular Instruments, Tempe, AZ, USA), housed in an acoustic isolation chamber. The imaging software used was Picoscan 5.3.3 (Tempe, MI, USA). The cantilevers used were Tap-150-G tapping mode cantilevers (BudgetSensors, Sophia, Bulgaria). The average cell dimensions were calculated from a representative sample ($n = 10$).

## Results

### Effect of ozone and OAF on survival of *Listeria monocytogenes*

Table 1 provides a synopsis of all the microbiological counts performed. A two-way ANOVA with Tukey's comparison determines that there are statistically significant differences ($P \leq 0{\cdot}05$) overall between surface-attached and biofilm organisms, but not between ozone and OAF. However, significant differences were found within individual experiments, and these are discussed below.

Ozone concentrations of 2, 5 and 10 ppm resulted in log reductions in 0·24, 0·33 and 0·57, respectively, of cells surface attached on stainless steel. The organism demonstrated different survival rates on different food contact surfaces when exposed to 45 ppm gaseous ozone (Table 1). The mean log reduction in *L. monocytogenes* treated with 45 ppm gaseous ozone revealed that treatment on stainless steel and granite resulted in 3·41 and 3·42 $\log_{10}$ reductions, respectively, compared with polypropylene which gave a lower log reduction (1·11 $\log_{10}$), suggesting that this surface provided some protection to the cells. A two-way ANOVA with Tukey's comparison showed that there were significant differences ($P \leq 0{\cdot}05$) between untreated and test and between granite or stainless steel and polypropylene. There was no significant difference between granite and stainless steel. Table 1 also shows that there is a significantly ($P \leq 0{\cdot}05$) lower log reduction in biofilm organisms compared with surface attached when treated with ozone alone. A two-way ANOVA with Tukey's comparison showed that there were significant differences ($P \leq 0{\cdot}05$) between granite and polypropylene. There was no significant difference between granite and stainless steel or stainless steel and polypropylene.

Open air factor reduced the number of surface-attached *L. monocytogenes* on stainless steel by 1·86 $\log_{10}$ ($P \leq 0{\cdot}05$). There was also a significant ($P \leq 0{\cdot}05$) difference between surface-attached and biofilm organisms on stainless steel. A two-way ANOVA with Tukey's comparison revealed that there was no significant difference ($P > 0{\cdot}05$) between stainless steel, granite and polypropylene when biofilm *L. monocytogenes* were treated with OAF. There was a significant difference ($P \leq 0{\cdot}05$) between untreated and test samples, with test samples showing reduction in numbers recovered in all cases.

When comparing ozone and OAF for surface-attached cells, there was a significant difference ($P \leq 0{\cdot}05$) with ozone at 45 ppm giving a better log reduction than OAF. Conversely, OAF was significantly better than ozone at reducing the number of biofilm organisms.

### SEM Imaging of *Listeria monocytogenes* biofilm

Figure 1 illustrates the effect of gaseous ozone treatment on *L. monocytogenes* on stainless steel coupons. Figure 1a,

**Table 1** Effect of ozone and open air factor (OAF) treatment on the survival of surface-attached and biofilm *Listeria monocytogenes* on stainless steel (SS), granite (G) and polypropylene (PP) after 1 h

| | | Mean log reduction (CFU cm$^{-2}$) | | |
|---|---|---|---|---|
| Inoculum | Treatment | SS | G | PP |
| Surface attached | Ozone (45 ppm) | 3·41 (1·48)$^{Aa}$ | 3·42 (0·98)$^{Aa}$ | 1·11 (0·53)$^{Ab}$ |
| | OAF | 1·86 (0·51)$^{B}$ | ND | ND |
| Biofilm | Ozone (45 ppm) | 0·56 (0·45)$^{Ba}$ | −0·20 (0·45)$^{Bab}$ | 0·90 (1·71)$^{Ab}$ |
| | OAF | 1·47 (0·71)$^{Ba}$ | 1·67 (0·60)$^{Ba}$ | 1·84 (0·49)$^{Aa}$ |

Values are mean (standard deviation). Within columns, means followed by different capital letters are significantly different. Within rows, means followed by different lowercase letters are significantly different.

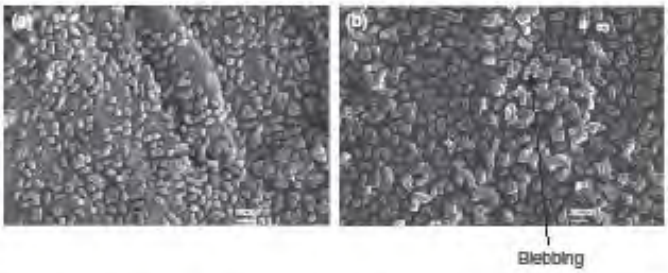


**Figure 1** Scanning electron microscopy micrographs of *Listeria monocytogenes* 72 h biofilm treated with (a) untreated and (b) gaseous ozone at 45 ppm at 1 h contact time. Magnifications and bar markers are × 6000, 1 μm.

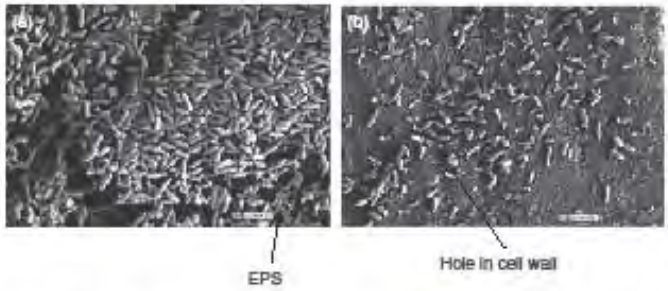


**Figure 2** Scanning electron microscopy images of *Listeria monocytogenes* L002 72 h biofilm exposed *in situ* to D-limonene (open air factor). (a, b) Test samples. Bar is 10 μm (viewed at ×1000 magnification).

a micrograph of the untreated *L. monocytogenes* biofilm, reveals that all cells appear healthy and undamaged. Figure 1b shows the biofilm treated with 45 ppm gaseous ozone, and the cells appear to have sustained damage, displayed as blebbing. There were also marked differences seen in the ozone-treated biofilms, in terms of the amount of glycocalyx present, compared with the untreated biofilm. Figure 2a, b illustrates the effect of OAF on *L. monocytogenes* biofilm on stainless steel. The cells appeared flattened and have visible holes in the cell walls (Fig. 2b). The biofilm was substantial and dense, and extracellular polymeric substance (EPS) was observed clearly in the test samples.

### Atomic force microscopy imaging of *Listeria monocytogenes* biofilm

All imaging was carried out on stainless steel coupons. The nominal spring constant, k, was 5 N $m^{-1}$ and resonant frequency, f, was 137 kHz. Figure 3 illustrates the untreated sample. Figure 3a shows that the cells appeared to have a smooth cell surface but are showing some signs of collapse. This could be due to dehydration of the cells, as samples were left to dry out before viewing. Figure 3b is a phase image of the untreated sample and suggests some crumpling of the surface (dehydration), but no significant patches of variation in material properties. Phase images give information on the surface 'stickiness' or elasticity. The stripes on the left are artefacts caused by the excessive oscillation of the cantilever.

Figure 4 illustrates *L. monocytogenes* L002 exposed to gaseous ozone at 45 ppm for 1 h. These cells show surface property changes as shown in the phase image (Fig. 4b). While not quantitative, they highlight areas of differing material properties. The cell shapes and surface features in Fig. 4a are similar to the untreated sample (Fig. 3a), but this phase image, Fig. 4b, shows considerable areas of changed material properties. Changes of around 1 V can be seen in the phase image, while the background variation is around 0·5 V. Figure 5 shows the effects of OAF on the cells. There are small regions of material property modification in the cell walls. These are of a similar magnitude in size (2 V against a background of 1 V variation) as observed in gaseous ozone-treated cells.

The average cell dimensions of the untreated, ozone and OAF-treated cells are shown in Table 2. The ozone-treated cells were significantly wider compared with the untreated sample with the OAF-treated cells showing significant differences in width and height compared with the untreated cells. They were also significantly wider than the ozone-treated cells. A cross-sectional view of the surface topography of the cells (Fig. 6) showed that the untreated cells (Fig. 6a) were more rounded compared with the treated cells. The cross-sectional view revealed that ozone-treated cells had lost their turgid shape and were deflated (Fig. 6b), while the OAF-treated cells were flattened and crumpled (Fig. 6c).

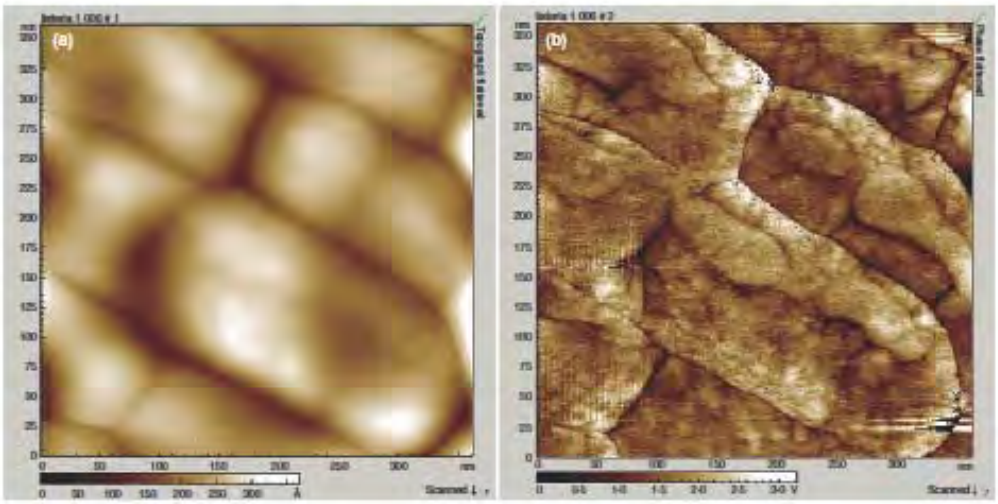


**Figure 3** (a) A 1·5 μm tapping mode scan height image (3rd order flattened) showing a regular layer of untreated *Listeria monocytogenes* cells. (b) A phase image of *L. monocytogenes* L002 cell surface (untreated).

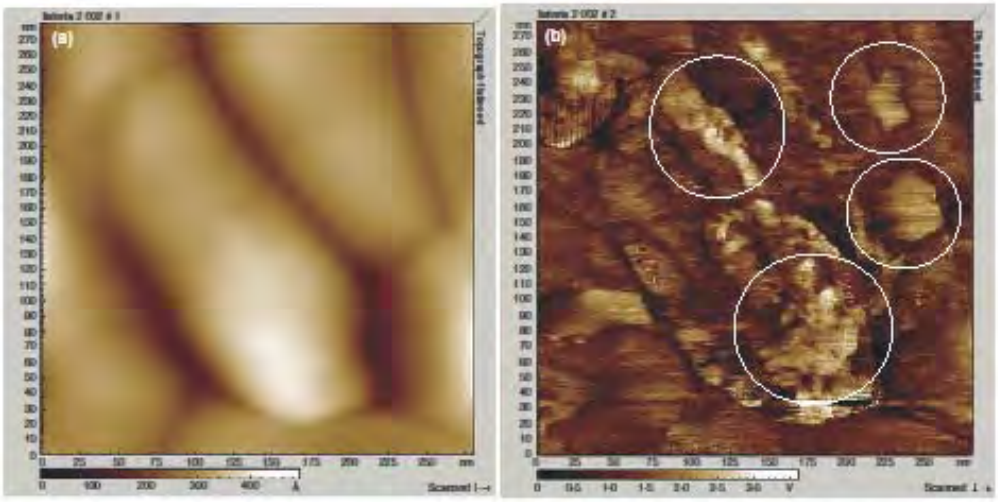


**Figure 4** (a) A 1380 nm tapping mode scan height image (3rd order flattened) showing the edge of a regular layer of cells treated with ozone at 45 ppm for 1 h. (b) A phase image of *Listeria monocytogenes* L002 cell surface after treatment with ozone. Altered cell surface properties are shown by the ringed areas.

## Discussion

This study demonstrated that the *L. monocytogenes* 1/2a, obtained from a high-care food premises, was relatively resistant to ozone. Moore *et al.* (2000) reported a 1-log reduction in *Listeria innocua* under similar conditions after exposure to 5 ppm ozone for 1 h. This study demonstrated that 10 ppm ozone gave a similar log

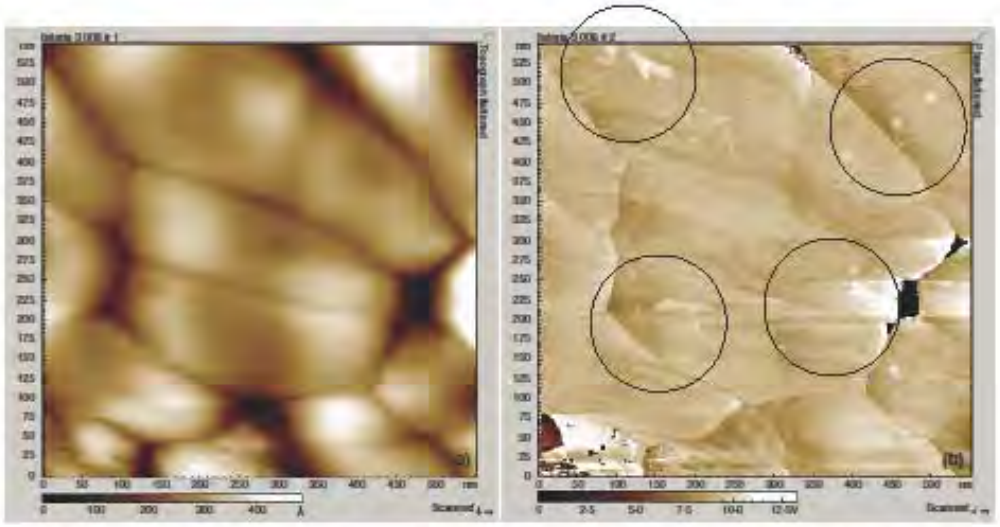

**Figure 5** (a) Five hundred and fifty nanometre tapping mode scan height image (3rd order flattened) showing the edge of a regular layer of cells treated with open air factor (OAF) for 1 h. (b) A phase image of *Listeria monocytogenes* L002 cell surface after treatment with OAF. Altered cell surface properties are shown by the ringed areas.

**Table 2** Average cell dimensions ($n = 10$) for surface-attached *Listeria monocytogenes* cells to food-grade stainless steel (AISI 304; finish 2b)

| Dimension nm (±SD) | Untreated | Ozone treated | Open air factor treated |
|---|---|---|---|
| Length | 347·6 (±54·2)$^a$ | 332·8 (±70·6)$^a$ | 293·8 (±40·3)$^a$ |
| Width | 97·0 (±10·2)$^a$ | 114·7 (±11·0)$^b$ | 133·2 (±20·3)$^c$ |
| Height | 311·2 (±76·0)$^a$ | 361·21 (±66·2)$^{ab}$ | 449·3 (±132·6)$^b$ |

Within rows only, means followed by different letters are significantly different.

reduction, while 45 ppm ozone was needed to effect a 3-log reduction. This increased resistance could be due to the fact that this strain was isolated from a high-care food-processing plant, where stringent, validated cleaning protocols based on oxidizing disinfectants were practiced and were therefore better able to survive the treatment or were retained more strongly on the surfaces. When treated with ozone, fewer cells were recovered from stainless steel or granite, suggesting that fewer cells survived the treatment or adhered more strongly to these three surfaces. The application of live-dead stains was unable to elucidate this as the technique was unable to visualize any treated cells (data not shown). Mafu *et al.* (1990) found that *L. monocytogenes* was more resistant to sanitizing agents when attached to polypropylene and rubber, compared with stainless steel. This was not true for OAF in this study as no significant differences were found between surfaces. A significantly better log reduction was observed on polypropylene compared with granite and stainless steel when treated with ozone. This may be due to the ability of *L. monocytogenes* to adhere to different food contact surfaces at different affinities (Mafu *et al.* 1990; Silva *et al.* 2008). Adhesion kinetic studies have revealed that adhesion of *L. monocytogenes* cells was higher to polypropylene than to stainless steel (Saá *et al.* 2009). These differences in survival or recovery from the surface are due to surface charge, hydrophobicity and roughness (Sizlavik *et al.* 2012). The findings of this study concur with those of Bailey *et al.* (2007) who found that OAF significantly reduced the numbers of *M. luteus* in an identical indoor system. Hood (2009) determined that unidentified OAFs can adversely affect the survival of micro-organisms, including *Francisella tularensis* present on microthreads.

Open air factor was slightly less effective against biofilm *L. monocytogenes* compared with surface-attached cells. Pan *et al.* (2006) demonstrated differences in the resistance of *L. monocytogenes* biofilms to sanitizers between different surfaces, with biofilms on stainless steel being more sensitive than those on Teflon. In contrast, Deza *et al.* (2005) found no significant differences in survival of a range of organisms on glass or stainless steel.

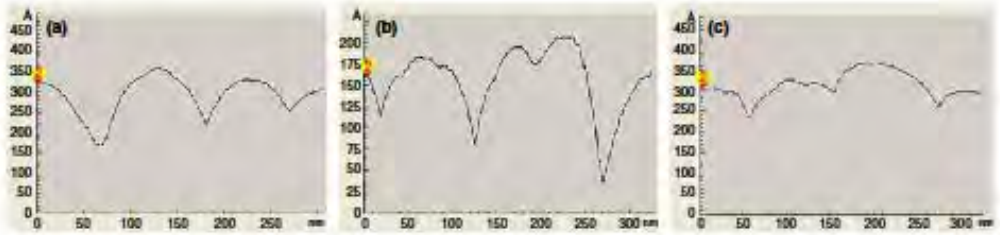


**Figure 6** Cross-sectional view of two *L. monocytogenes* L002 cells; a) control sample, where the cells have a rounded appearance; b) Ozone treated cells, where the cells have an indented surface. Other areas show larger depressions, which could be due to dehydration of the cells; c) OAF-treated cells, showing flattening and crumpling.

There was a tenfold difference in the inoculum size for surface-attached and biofilm cells, with the latter being the lower. Previous work is contradictory regarding the effect of inoculum size on the efficacy of antibiotics (Morrissey and George 1999). It would be expected that an increased inoculum size would reduce the antimicrobial activity. However, the results presented here are contrary to that, suggesting that, it is the biofilm effect rather than the inoculum size that is responsible for the results observed. A biofilm is an effective defence mechanism in protecting cells against environmental stresses including antimicrobial agents such as biocides. The inherent nature of a biofilm allows for poor absorption of such chemicals, thus the prevalence of persistent strains with greater adherence capabilities within food-processing premises (Norwood and Gilmour 1999).

Scanning electron micrographs of the treated *L. monocytogenes* biofilms revealed a possible mechanism of action. The treated cells appeared to have blebbed their cell contents, indicating changes in the cytoplasmic membrane as a result of ozone treatment. When treated with OAF, there was material present around the cells that may be increased EPS production or blebbed cell contents. There were also apparent holes in the cells. The AFM was able to distinguish surface property changes in ozone and OAF-treated cells. Treated cells showed desiccation, resulting in the cells having a deflated appearance. There was also evidence of altered cell surface properties. Changes in phase are related to changes in material property of the surface as well as containing topographic information. Material properties such as viscoelasticity and adhesion (Tamayo and Garcia 1996) delay or enhance the oscillation of the cantilever away from its original set point. Although it can only be used relatively, the areas of altered phase in the AFM images suggest changes in the cell surface imaged, in keeping with chemical modification of the cell wall. Further studies are necessary to determine the precise mechanistic differences between the action of the two biocides.

The biocidal effects of ozone have been studied in a number of gram-positive and gram-negative bacteria (Restaino *et al.* 1995). Inactivation involves ozone acting on cell membrane components, affecting membrane-bound enzymes and oxidizing unsaturated fatty acids, resulting in cell lysis (Guzel-Seydim *et al.* 2004). It is known that ozone and free radicals produced by ozone decomposition are involved in inactivation; however, the species responsible for this activity have not been identified. Ozone decomposition leads to the formation of hydroxyl, superoxide and hydroperoxide radicals. Some researchers suggest that reaction with molecular ozone is the predominant mechanism for inactivation (Hunt and Mariñas 1997), others that reaction with radicals is the predominant process (Bancroft *et al.* 1984). OAF, produced by the reaction of ozone and terpenes (limonene in this study), has been extensively studied due to their importance in indoor pollution, and hydroxyl radicals are the main products formed by the reaction (Aschmann *et al.* 2002). The results presented here suggest that the mechanism of action of ozone is via molecular ozone as *L. monocytogenes* demonstrated different resistance to ozone and OAF, suggesting different mechanisms of action.

Areas that are inaccessible for cleaning are more vulnerable to the formation of biofilms, and such bacteria are exposed to sublethal doses of disinfection treatments. Once bacterial cells are exposed to a mild stress, they are subsequently able to tolerate other more severe stresses. Responses to environmental stresses of heat and hydrogen peroxide have been studied in *L. monocytogenes*, and many heat shock and oxidative stress proteins were induced. General stress response systems are activated by several different stresses and can give acquired cross-protection against multiple stresses (Yousef and Courtney 2003). Sigma factor B ($\sigma^B$) is responsible for general stress response in gram-positive organisms, including *L. monocytogenes*, and regulates the transcription of over 40 genes (Ryan *et al.* 2008; Wesche *et al.* 2009). The

disruption of $\sigma^B$ in *L. monocytogenes* causes a decreased resistance to acid and osmotic stress (Wesche *et al.* 2009). Genes induced by $\sigma^B$ include those genes encoding for catalase, for enzymes used in DNA repair and osmoprotectants. This suggests that the cell has the ability to prepare for oxidative and osmotic stresses.

## Conclusion

While gaseous ozone treatment (45 ppm) is effective for surface disinfection, it is not feasible to apply this on a large scale due to toxicity effects. This study has demonstrated the potential application of OAF as an alternative technology as it can be used while personnel are present. Its potential use as a tool in pathogen management strategies to control environmental contaminants such as *L. monocytogenes* should be further investigated.

## Acknowledgements

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# IAFP 2009 POSTER/TECHNICAL ABSTRACT

## The Effect of Gaseous Ozone on the Survival of Environmental Surface Attached *Listeria monocytogenes* serotype 1/2a

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*Introduction:* Gaseous ozone has considerable potential for use as a terminal sanitizer in the food industry. Traditionally used antimicrobials and fumigants have led to antimicrobial resistance and potential adverse health effects. Ozone leaves no toxic residues and has GRAS status for food processing. Listeriosis is an increasing problem in the over-60 age group and *Listeria monocytogenes* can be found on a range of surfaces within food processing plants and can form biofilms. Adhered bacteria can be more resistant to disinfectants and sanitizers than planktonic organisms.

*Purpose:* To determine the effect of gaseous ozone on survival of surface attached *Listeria monocytogenes* isolated from the floor drains of a chilled ready to eat food processing plant.

*Methods:* Stainless steel coupons (25cm$^2$) were inoculated with 0.1 ml of a suspension containing $10^9$ cfu/ml of the environmental isolate. The inoculum was spread over the entire surface and allowed to dry. The coupons were exposed to gaseous ozone (2-45 ppm) for 1 hour. Coupons were oriented horizontally, vertically and inverted. Surviving organisms were recovered by swabbing and spread-plating on tryptone soya agar. Data were analyzed using a two-way ANOVA in Minitab 15.

*Results:* At lower ozone concentrations (2-5 ppm) there was no significant difference in survival of the organism between test and control coupons (0.4 log reduction). The results showed a significant difference ($P \leq 0.05$) between control and test coupons at both 10 and 45 ppm (up to 2.9 log reduction). There were no significant differences between different orientations of coupons treated (horizontal, vertical or inverted). The environmental isolate displayed increased resistance compared to culture collection strains of *Listeria* spp. (4 log reduction when exposed to 5 ppm for 1 hour).

*Significance:* The reduction in survivors of surface attached *L. monocytogenes* by gaseous ozone is concentration dependent. It is not dependent on surface orientation. This is advantageous over traditional fogging, where vertical and inverted surfaces are less exposed to the fogging agent. It is hypothesized that the mechanism of resistance of this strain could be due to the fact that this strain was isolated from a high-care food processing plant, where stringent validated cleaning protocols were practiced, potentially leading to enhanced resistance to antimicrobials.

# The Effect of Gaseous Ozone on the Survival of Surface Attached Bacteria of Importance in the Food Industry

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**Introduction**
Evaluating the efficacy of novel decontaminants is increasingly important in the food industry. Traditionally used antimicrobials and fumigants have demonstrated microbial resistance and adverse health effects. Ozone leaves no toxic residues as it rapidly decomposes back into oxygen. Ozone was granted GRAS status for food processing by EPRI and was approved by the US FDA and the USDA's FSIS. In Europe, the Biocidal Products Directive (BPD) 98/8/EC's introduction has meant the cost of registering new and existing biocides has increased. However, as ozone needs to be produced *in situ,* it is exempt from the BPD, giving ozone an economic advantage over currently used biocides. In the United Kingdom, the current occupational exposure standard (OES) is 0.2 ppm, averaged over a 15 minute period.

*Listeria monocytogenes* and *Pseudomonas aeruginosa* can be found adhered to a range of surfaces within food processing and are known to form biofilms. Adhered bacteria appear to be more resistant to disinfectants and sanitizers than their planktonic counterparts.

**Aim**
The aim of this study was to investigate the effect of gaseous ozone on the survival of surface attached bacteria commonly isolated from food processing plants.

**Methods**
**Ozone Generation**
Gaseous ozone was generated at concentrations of 0.05, 0.1, 2, 5, 10, and 45 ppm, using a corona discharge ozone generator and levels were monitored using an API ozone monitor.

**Bacterial Suspension Preparation**
A single cryobead of either an environmental isolate *L. monocytogenes* or *P. aeruginosa* ATCC 15442 was placed into a 250 ml conical Erhlenmeyer flask containing 100 ml nutrient broth (NB) (Oxoid, UK), in duplicate. Broths were placed onto a shaking platform (Orbital Shaker) at 250 rpm at 35°C for 24 ± 1 hours. The broths were then centrifuged at 3000 rpm (1,068 X g) for 20 minutes. The pellets were resuspended in 20 ml sterile phosphate buffer saline (PBS) (Oxoid, UK).

**Surface Attached Bacteria**
Food grade type 304 stainless steel coupons (25cm$^2$) were inoculated with 0.1 ml of the suspension culture and were allowed to air dry at ambient temperature (20°C). Five coupons of each of three orientations (horizontal (H), vertical (V) and inverted (I)) were placed inside the Bioaerosol Test chamber (figure 1), and exposed to test conditions for 1 hour. Five coupons were prepared and not exposed to ozone (control conditions). Enumeration of survivors was carried out using a conventional swab technique. The swab was placed into 9 ml aliquots of sterile maximum recovery diluent (MRD) (Oxoid, UK). Serial dilutions were carried out as necessary and tryptone soya agar (TSA) (Oxoid, UK) spread plates prepared in duplicate. The plates were incubated at 30°C for 48 hours. Each experiment was repeated three times.

Results were reported as mean log reduction, calculated from log transforms of actual counts. Data were analysed using a two-way ANOVA. Significant interactions that had been identified were more closely analysed using a unstacked one-way ANOVA with Tukey's comparisons. Significant differences are reported where P ≤ 0.05 (Minitab version 14).


**Figure 1.** The Bioaerosol Test Chamber

**Acknowledgements**
Authors would like to thank Dr. Andy Young, Ozone Industries Ltd for his technical support and advice in this investigation and Roger Bailey for the Pseudomonas data.

**Results**
Figures 2 and 3 show the effect of gaseous ozone on surface attached *L. monocytogenes* and *P. aeruginosa*, respectively.

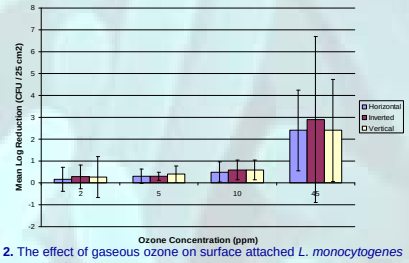


**Figure 2.** The effect of gaseous ozone on surface attached *L. monocytogenes* (environmental isolate)

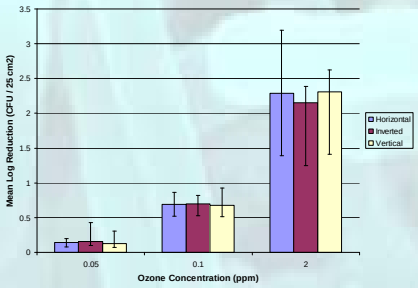


**Figure 3.** The effect of gaseous ozone on surface attached *P. aeruginosa* ATCC 15442

**Discussion and Conclusions**
The results showed that 2 ppm ozone concentration gave a 2.14 – 2.34 log reduction in the survival of *P. aeruginosa* compared to the control. At 0.1 ppm this reduction was 0.69 – 0.71 log reduction while at 0.05 the reduction was 0.14 – 0.18. There was no significant difference between 0.05 ppm and control. There were significant differences between all three concentrations, with 2 ppm being significantly better than 0.1 ppm, and 0.1 ppm being significantly better than 0.05 ppm.

However, for the survival of *L. monocytogenes* at 2 ppm, the results showed a 0.17 – 0.28 log reduction compared to the control. An ozone concentration of 45 ppm gave a log reduction of 2.4 – 2.9 compared to the control. The results showed a significant difference P ≤ 0.05 between control and test results, with a significant difference (P ≤ 0.05) at the higher ozone concentrations between 10 ppm and 45 ppm ozone. *Listeria monocytogenes* (environmental isolate) was significantly more resistant (P ≤ 0.05) at 2 ppm ozone concentration than the *Pseudomonas aeruginosa* ATCC 15442. There were no significant differences between the different orientations of the surface being treated for either organism.

These results suggest that surface attached environmental isolate *L. monocytogenes* is more resistant than the surface attached culture collection strain *P. aeruginosa* ATCC 15442. We have hypothesised that the mechanism of resistance of the Listeria strain could be due to the fact that Listeria environmental strain had been isolated from a high-risk food processing plant, which practised stringent validated cleaning protocols, potentially leading to enhanced resistance against antimicrobial treatments.

The reduction in levels of surface attached *P. aeruginosa* and *L. monocytogenes* by gaseous ozone is concentration dependent and not dependent of surface orientation. This is advantageous over traditional fogging methods used in the food industry, where vertical and inverted surfaces are less exposed to the fogging agent. Gaseous ozone has shown potential for use as a terminal sanitizer for environmental surfaces in the food industry.

**Further Work**
Further work on looking at surface attached *L. monocytogenes* (environmental isolate) on different surfaces and orientations and its related mechanism of resistance.

**References**
A number of references were used, which are on a separate sheet.