LASER STERILIZATION OF MICROORGANISMS

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To Alex

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ABSTRACT

The effect of light from a variety of laser sources was investigated in a microbicidal capacity under a number of different conditions. Initially, *Escherichia coli, Serratia marcescens, Staphylococcus aureus, Deinococcus radiodurans, Micrococcus luteus, Bacillus subtilis, B. stearothermophilus, Candida albicans* and *Saccharomyces cerevisiae* were exposed to near infrared light, from a high-powered, 1064 nm, Neodymium:Yttrium Aluminium Garnet (Nd:YAG) laser, as lawned cultures on agar plates. The effect of the different Nd:YAG laser operation parameters (pulse repetition frequency, pulse energy, pulse width and beam diameter) were also investigated on lawned cultures of *E. coli*. A system of quantifying the effect of the laser light on microorganisms lawned on agar plates, which utilised the non-uniform energy distribution of the laser beam, was developed and the different organisms and laser parameters were compared.

Nd:YAG laser light killed all microorganisms mentioned above, producing clear areas within the microbial lawns. For each microbial species, a dose/response curve was obtained of area of clearing versus energy density (Jcm^{-2}). The energy density that produced an inactivated/sterilized area (IA) equal to 50 percent of the beam area was designated the IA_{50}-value and was plotted. Average IA_{50}-values were all within a three fold range and varied from 1768 Jcm^{-2} for *Serratia marcescens* to 4489 Jcm^{-2} for vegetative cells of *Bacillus stearothermophilus*. The differences in sensitivity could not be attributed to cell shape, size, pigmentation, or Gram reaction.

The PRF, pulse energy and beam diameter all affected the sterilization capability of Nd:YAG laser on a lawned culture of *E. coli*. The average IA_{50}-value at a PRF of 10 Hz was 2800 Jcm^{-2} which was decreased to 2200 Jcm^{-2} as the PRF was increased to 30 Hz. Similarly, an increase in the pulse energy from 10 J to 30 J enhanced the sterilization efficiency of the Nd:YAG laser on *E. coli* lawned on agar surfaces with a reduction in the average IA_{50}-value from 2700 Jcm^{-2} to 1850 Jcm^{-2} respectively. Variation in the beam diameter was shown to have a large effect on the threshold energy density (the lowest ED required to sterilize an area in the bacterial lawn). Laser light at a beam diameter of 2.75 cm had a threshold energy density of 1400 Jcm^{-2} which was reduced to 800 Jcm^{-2}, with a beam diameter of 1.25 cm. There was not such a notable effect on the IA_{50}-values with only 200 Jcm^{-2} separating both of

these beam diameters. The pulse width of Nd:YAG laser light did not affect the bactericidal ability of laser light.

At the lowest energy densities where sterilization was achieved for the majority of organisms (around 2000 Jcm^{-2}), no effect was observed on the nutrient agar surface, but as the energy density was increased, a depression in the agar surface was formed, followed by localised melting of the agar. Also around the periphery of the cleared areas microcolonies were frequently observed which indicated sub-lethal damage to the microbial cells. Preliminary investigations indicate that these colonies contain auxotrophic organisms.

E. coli in liquid suspension was also exposed to Nd:YAG laser light, to temperatures of 50°, 60° and 70°C. At 50°C, the viable count of a 1 x 10^4 cfu ml^{-1} *E. coli* suspension was reduced by around 3 D-values, whereas 10 min in a water-bath at 50°C, or rapidly heating in a PCR machine to 50°C, showed much less or no loss of viability. Increasing the cell concentration to 1 x 10^8 cfu ml^{-1} had little effect on the extent of killing with the laser. *E. coli* at 2 x 10^9 cells ml^{-1} was opaque yet some bacterial killing was still observed. The growth phase of the bacterial suspension did not appear to have any affect on its resistance to Nd:YAG laser light. RNA was released from the bacterial cells. In the samples heated to 60° and 70°C with laser light, SEM examination revealed changes in cell surfaces such that "blebbing" and the appearance of debris, possibly from ruptured cells, was observed. Differential scanning calorimetry produced different traces with bacteria heated to 50°C with a laser and in a 50°C waterbath. No major effects on the proteins within the *E. coli* cell were observed with SDS-polyacrylamide gel electrophoresis. Little difference was seen between the killing curves of *E. coli, S. aureus, L. monocytogenes* and *B. cereus*, however the SEM pictures show that there were differences between the different organisms which appeared to be related to the cell-wall construction. It was concluded that while some of the bactericidal effect of Nd:YAG laser light was due to a photothermal mechanism there was an additional killing mechanism, possibly photomechanical.

The effect of Nd:YAG laser light on bacteria seeded onto surfaces was investigated. It was not possible to kill bacteria on glass or plastic (nylon) surfaces without affecting the surface, however stainless steel was sterilized by Nd:YAG laser light in 6 sec at 30 Hz and 10 J. *S. aureus* was the most resistant to the laser in this environment as both a wet sample and a dry sample, though the resistance as a dry sample was due to the greater resistance to the drying procedure. Multifactorial analysis revealed that the ability of Nd:YAG laser light to kill bacteria was unaffected by growth at different NaCl concentrations and pH values. Increasing each individual laser parameter had a positive affects on laser killing, however few interactions were observed. Killing curves of bacteria on surfaces were modelled with three different systems to produce the best fit. The linear fit and the log-logistic methods were not suitable, however fitting the points to a quadratic function produced the closest fit of all.

CO_2 laser light was more effective in killing bacteria on surfaces than the Nd:YAG, sterilizing 1 cm^2 of plastic surfaces in 6 msec, glass surfaces in 40 msec and stainless steel in 200 msec. *S. aureus* was once again the most resistant to the laser light, which appeared to be related to the size of the initial inoculum. The CO_2 laser was also more effective in the sterilization of collagen sausage skin. Light from the Nd:YAG laser required 20 sec to sterilize an area of skin in comparison to the 2 msec required by the CO_2 laser.

E. coli as lawned cultures were exposed to seven different laser sources delivering a range of wavelengths from 355 nm to 118 μm. The effect of the CO_2 laser (MFKP) was compared to the Nd:YAG laser (MS830; 1064 nm) and the Q-switched frequency tripled Nd:YAG lasers (Surelight and Minilight; 355 nm). All of these lasers inhibited the growth of *E. coli* on nutrient agar plates. The most effective laser, however, was the CO_2 laser (1.06 μm) with a mean power of 600 W. The lasers which did not appear to inhibit the growth of *E. coli* were the far infra-red laser (118 μm), laser diode array (810 nm), argon ion laser (488 nm) and the Q-switched frequency doubled (532 nm) and standard frequency Nd:YAG lasers (1064 nm)

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LIST OF ABBREVIATIONS

µsec	microseconds
A_{max}	maximum absorbance
ArF	argon fluoride
CO_2	carbon dioxide
DSC	differential scanning calorimetry
GaAlAs	gallium aluminium arsenic
HCl	hydrochloric acid
HeNe	helium neon
hr	hours
ED	energy density
Hz	Hertz
IA_{50}	Inactivation/Sterilization area at 50% of the beam diameter
INT	iodo-nitro-tetrazolium violet
J	Joules
LAF	laminar air flow
min	minutes
mJ	millijoules
msec	milliseconds
MW	megawatts
mW	milliwatts
NaOH	sodium hydroxide
NaCl	sodium chloride
nsec	nanoseconds
Nd:YAG	neodymium:yttrium aluminium garnet
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRF	pulse repetition frequency
RPM	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
SEM	scanning electron microscope
SPM	scanning probe microscope
UV	ultra violet
UHT	ultra high temperature
W	Watts
XeCl	xenon chloride

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1. INTRODUCTION

1.1 PREFACE

The lethal interaction between laser light and microorganisms is of interest to a wide variety of disciplines, such as microbiology, dentistry, medicine, engineering and physics. However, published research into the microbicidal effects of laser light was slow to emerge since the first report (Saks and Roth, 1963), due in part to the initial outlay costs involved in acquiring a laser. Other problems included the physical size, availability and, for the bactericidal action itself, the lack of power of lasers in the early publications. In recent years, increased research into and use of the laser has reduced laser cost and physical size and increased availability and power. To date, the laser has many applications and is involved in areas relating to cellular interaction and used in medical operations, such as the removal of skin cancers and lung tumours (Deckelbaum, 1994). Lasers (CO_2 and Nd:YAG) are now used as "scalpels" in the operating theatre, and are reported to leave a dry sterile wound (Midda and Renton-Harper, 1991). The argon ion laser (488 nm), the third most common medical laser, is used routinely in ophthalmic surgery because light at this wavelength is absorbed by red pigments alone. The laser beam therefore passes through the aqueous and vitreous humour of the eye and is absorbed by the retina (Wilson, 1993).

Low power laser light was shown to accelerate biological regeneration, for example the acceleration of collagen production in a wound undergoing healing, by fibroblast stimulation (Kana *et al.*, 1981). However, whether this biostimulation is due to the specific properties of the laser light is still open to debate. Although biostimulation has been investigated over the past 15 years it has not yet become an established technique. Nevertheless, biostimulation in microbiological research was investigated with the effects of laser light on *E. coli* (Karu, 1987). With a variety of lasers (continuous wave argon ion laser (454 nm), continuous wave Helium Neon laser (632.8 nm), pulsed GaAlAs semiconductor laser (890 nm), pulsed super-luminous laser diode (950 nm), continuous wave laser diode (1300 nm) and various light sources), at low power, *E. coli* and other cell types were stimulated to grow (Karu *et al.*, 1990; Tiphlova *etal.*, 1991; Karu *et al.*, 1994).

Research in dentistry was the most active of all of the biological disciplines studying the microbicidal effects of laser light. Lasers were also used as drilling apparatus (Cobb *et al.* 1992) and the Nd:YAG laser has been used to treat dental hypersensitivity, over a period of weeks, and significantly increased the tolerance of teeth to cold (Renton-Harper and Midda, 1992). The authors suggested that the most probable reason for the decreased hypersensitivity was due to "obturation of the dental tubules."

The need for the introduction of new sterilization procedures was highlighted by the latest microbial contamination problems within the food and medical industries with the emergence of *E. coli* strain O157 and antibiotic resistant bacterial strains, respectively. There is a heightened awareness, and increased concern, over the ineffectiveness of current hygienic procedures.

In the literature there is little structured analysis of the biocidal effect of laser light on microorganisms. Current knowledge is the result of many small investigations into the microbicidal effect of lasers in applied situations, particularly in dentistry and medicine. Few of these reports analyse the mode of action of the microbicidal effects of the individual lasers investigated, and only report the direct effect of laser light on microbial counts. Lack of details of laser parameters and the variety of microbes in various applied situations make comparisons of previous publications difficult. However, the type of laser (i.e. the wavelength) and the output power appear to be of great importance and depend on the suspending medium, whether solid, liquid or gas.

Most of the early reports, in the 1960's, studied lasers operating at low power and therefore required long exposure times. In some cases dye was added to enhance and therefore change the microbicidal mechanism. Changes in the microbial physiology was observed by McGuff and Bell (1966) but no microbial lethality. The use of continuous wave CO_2 laser light proved to be very effective especially in dental applications where the relatively high power of 10 W could reduce bacterial contamination, and spores, within 3 sec (Hooks *et al.*, 1980).

Like CO_2 laser light, Nd:YAG laser light was effective but also possessed the ability to be transmitted through water or other translucent liquids, some plastics and glass. The reports varied between the use of pulsed and continuous wave laser light. The argon ion laser was another laser investigated and one report found it the most effective laser in sterilizing bacteria (Powell and Whisenant, 1991). The argon fluoride (ArF) and xenon chloride (XeCl) excimer lasers, operating at 193 and 309 nm respectively were also found to be microbicidal to yeasts and bacteria (Keates *et al.*, 1988; Frucht-Peri *et al.*, 1993; Stabholtz *et al.*, 1993). Most of the ubiquitous lasers and some relatively uncommon lasers have been investigated for their microbicidal qualities and in each case the mechanism of the bactericidal effect will be different but have not been discussed in many, if any, of the publications

1.2 INTRODUCTION TO TERMINOLOGY

1.2.1 STERILIZATION

Many terms are used to describe destruction of microorganisms, however for simplicity the term sterilization is used in this thesis. The term sterilization is defined as "the inability of an organism to reproduce". When this term is used in relation to microorganisms, it is taken as synonymous with death, because microbial activity is usually undetectable in the absence of multiplication (Schmidt, 1954). The most common method of microbial detection involves the ability of the organism to grow on or in a nutrient-rich medium, therefore relying on bacterial multiplication as the indicator of non-sterility. Sterilization is not scientifically accurate in this context, as there may be viable but non-culturable organisms remaining after treatment. However, no investigation in this thesis was carried out into the viability of organisms after laser light treatment so the term "sterilization" as used in this thesis describes the inability of the microorganisms to reproduce.

1.2.2 LASER OUTPUT

When describing electromagnetic waves produced by the laser the accepted terminology is radiation. Only electromagnetic waves in the visible range (about 400 to 800 nm) are referred to as light. However, under industrial advice it was decided that the term radiation would be omitted and exchanged for the term light, with concern for the public perception of radiation as necessarily having undesirable features.

1.2.3 KINETICS OF MICROBIAL KILLING

In 1897 Kronig and Paul demonstrated that a culture exposed to a biocidal agent died in an orderly sequence and not simultaneously. Madsen and Nyman (1907) submitted mathematical analysis to these results and found that the reaction velocity of killing was similar to that obtained in a unimolecular reaction (**Figure 1.1A**), i.e. the rate of microbial destruction was a first order reaction. In a first order reaction only one of the reactants is regarded as undergoing change where the rate of change is proportional to the concentration of the reactant as long as the temperature and other parameters remain constant according to the law of mass action, i.e.

$$v = k \cdot C$$

where v is the reaction velocity, C is the reactant concentration and k is a constant relating to the nature of the reactant (Wilson and Miles, 1964).

If a first order reaction is plotted on a linear-linear graph the shape of the resulting curve is exponential (**Figure 1.1A**), such that a plot of the same values on a log-linear graph produces a straight line (**Figure 1.1B**). This was found to be the case when Madsen and Nyman (1907) analysed the results of Kronig and Paul (1897); the plot of number of *B. anthracis* spores surviving $HgCl_2$ treatment against the exposure time was exponential and the resulting log survivors against time plot was a straight line. Since this work a number of authors (Eijkman, 1908; Henderson Smith 1921; Hobbs and Wilson 1942 and Withell, 1942) found that the logarithms of survivors did not yield straight lines but sigmoidal ones. Indeed, there appeared to be an initial

lag in many of the killing curves and in some cases a "tail" of survivors at the end were observed (**Figure 1.1C**). Rhan (1945), however expressed the view that almost all killing curves were basically exponential.

Reasons for sequential killing as opposed to simultaneous killing are not understood however, Chick (1910) postulated that the survival of any given bacterium was determined by statistical chance, with the presence of a biocidal agent at a constant level weighting the probability of survival by a constant amount. This is often termed the mechanistic theory and assumes that the rate of death is determined by chance interactions between the lethal agent and the target, i.e. the bacterial cell/spore, the DNA or other "sensitive" components. If the chance of survival for each bacterial cell is x and remains x over the whole of the experiment then the death rate will remain constant. This therefore will produce an exponential killing curve of the spores. Under the mechanistic theory any variability, from the straight line, in death curves are difficult to explain but some considerations of underlying causes have been reviewed by Cerf (1977), although, to date, no satisfactory explanation is available. The second theory commonly termed the vitalistic theory (Anderson *et al.* 1996) accepts that within a bacterial population there is a permanent range of heat resistances, producing shoulders and tails caused by cells or spores of varying resistance (Henderson Smith, 1920). The reasons for the variation in resistances throughout a population are not understood. Both of these theories are probably essentially correct and are amalgamated in a publication by Eddy (1953). He found no evidence of inherent resistance in any one bacterial cell after subculturing the survivors of a disinfection experiment and showed that the levels of resistance were similar to the original culture. The fact that there was invariably a lag before the logarithmic decline of the killing curve suggested to the author that certain events occurred in the cell before death and in some cells the events occurred more readily. The author postulated that the lag was due to bacterial resistance and the subsequent exponential decline, a mixture of the mechanistic theory and the vitalistic theory.

There are a number of reports on the reasons for the differing shapes of killing curves with a variety of theories reviewed by Sykes (1958), Wilson and Miles (1964) and Brown and Melling (1971).

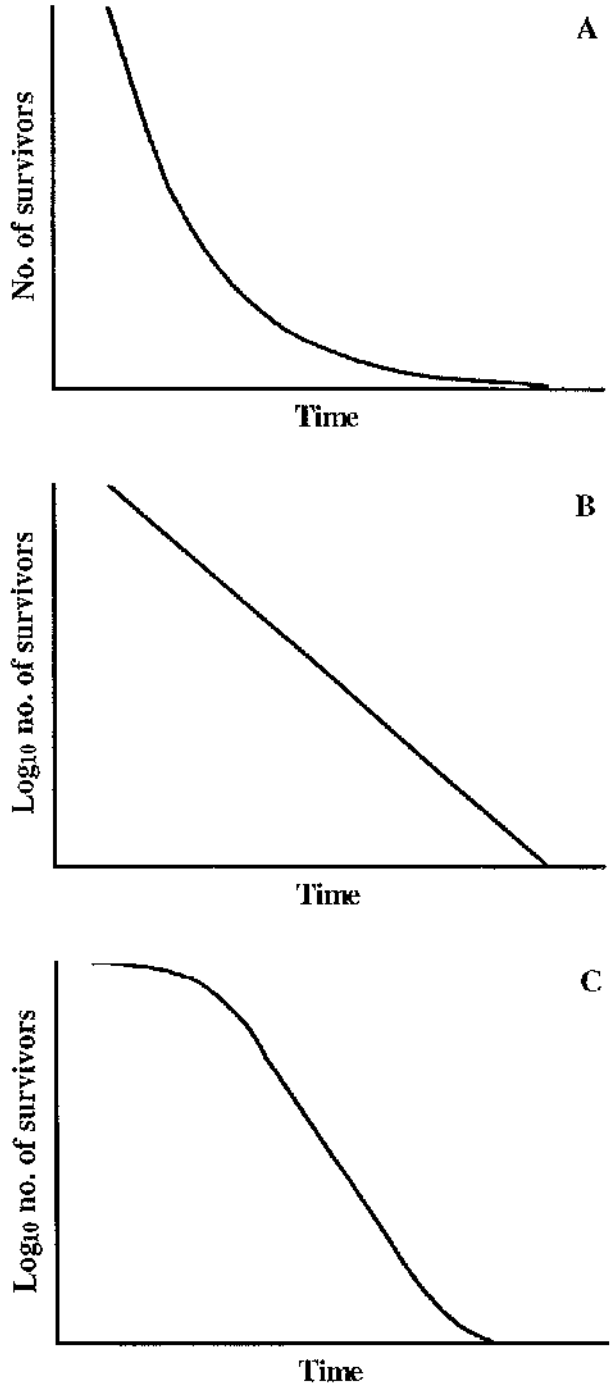


Figure 1.1 **The effect of exposure time to a lethal agent on the killing of vegetative bacterial cells and spores.** **A.** is the exponential curve associated with microbial killing; **B.** is the $\log_{10}$-linear fit of the curve in **A.** and **C.** shows one type of killing curve observed in practice.

1.2.4 D, z AND F VALUES

The killing of microorganisms is quantified by calculation of the decimal reduction time. This is defined as the D-value, i.e. the time taken to produce a 10-fold reduction in the viable count after exposure to a microbicidal agent (Stumbo *et al.*, 1950). A thermal death time curve is plotted with the D-value against temperature (in °C). The z-value is the temperature required to bring about a 10-fold change in D-value and is obtained from the negative reciprocal of the slope (Ball, 1943). Sterilization procedures can be compared with the thermal death point in relation to time (Bigelow, 1921) now recognised as the F-value. This represents the combination of all factors affecting the over-all sterilization process. The formula applied to determinate the F-value is shown below:-

$$F = D(\log n_1 - \log n_2)$$

where n_1 is the initial number of microorganisms, n_2 is the final number and D is the D-value (Sykes, 1958). Use of these values assumes that the killing curves for the particular microorganisms under the particular conditions are of an exponential nature.

1.3 MICROBIAL STERILIZATION

Following the investigations of Budd (1873), Baxter (1875) and Bucholtz (1875) into the effects of different disinfectants in combating infectious agents, Robert Koch (1881) initiated studies on the comparative germicidal effects of a series of disinfectants. These were the first studies of microbial sterilization.

1.3.1 HEAT STERILIZATION

1.3.1.1 Dry-heat

In 1881 Koch and Wolffhügel made initial investigations into the effect of heat on the vegetative bacterial cells noting that they were only sterilized at temperatures in excess of 100°C for 1.5 hours. Spores, however required 140°C for 3 hours. The increased resistance of spores was linked to the lower water content of the spores as published work indicated that desiccated proteins coagulate at higher time temperature limits (Hewlett, 1909). Described as high temperature damage (100°C+), Thomsen (1991) reviews the effects of dry-heat on the bacterial cell. Dry-heat was marked by the structural changes which occurred immediately after heating. Such structural changes included cell shrinkage, membrane rupture and cell lysis (Thomsen, 1991). This higher temperature damage is thought to be dominated by the internal vapourisation and release of water from the cells (Le Carpentier *et. al*, 1990). Any membrane damage to *E. coli* cells in these circumstances did not contribute to the death of the cells as cell leakage occurred after death (Russell & Harries 1967).

Dry-heat where high temperatures are reached rapidly is commonly called thermal ablation. The energy from the thermal heating process, in thermal ablation causes dissociation of molecular structures. Complex organic molecules break down into relatively inert carbon residues although the specific reactions are not well understood (Pearce 1990). Recognised thermal ablation mechanisms include, caramelisation, carbonisation, vaporisation, explosive fragment ejection, combustion, molecular disruption and plasma formation (MacKenzie, 1986; Partovi *et. al*, 1987; Rastegar *et. al*, 1988; Welch, 1984)

A variety of materials require dry-heat sterilization, such as solids, non-aqueous liquids, closed cavities and water-sensitive materials. Electrically heated ovens with mechanical air convection, reducing hot and cold spots in the oven, are the most common dry-heat sterilizing apparatus. Another method of dry-heat sterilization is radiant heat, produced by long wavelengths of electromagnetic radiation with low levels of radiant energy. Radiant heat processes include treatment with infra-red and microwave radiation. Unlike the shorter wavelength radiation (mentioned later in

this thesis) the sterilization mechanism depends completely on the conversion of radiant energy to heat when absorbed by solids or liquids (Shechmeister, 1983). However, authors have discussed the lethal mechanisms of microwave radiation on bacteria and have concluded the effect to be other than heat (Moore, 1966) and are reviewed by Fung and Cunningham (1980). Recent reports however, have refuted these ideas and have shown that the lethal mechanism for microwave radiation is purely thermal (Coote *et al* 1991; Welt *et al*, 1994).

The major disadvantage of dry-heat sterilization is that it requires extended operational times and higher temperatures than autoclaves. Penetration is slow and therefore there is an associated slow rise in temperature, and there may be oxidative damage to sterilized materials.

1.3.1.2 Moist-heat

Koch and Wolffhugel (1881) discovered that the germicidal action of moist-heat was more effective than dry-heat, in that the spores of *Bacillus anthracis* were sterilized in 10 min at 95°C. The authors were also the first to use water under pressure to raise the temperature of boiling. Koch *et al.* (1881) concluded that spores seldom survive the action of boiling water for more than a few minutes. However, this was disproven when Bigelow and Esty (1920) found that the spores of thermophilic bacteria survived boiling water for 1,320 min, almost a full day.

The mechanism of moist-heat sterilization was initially investigated by Chick (1910) who noted that the thermal death point of bacteria was determined by coagulation of proteins. This was a two stage process: denaturation, where water reacts with the protein molecule and agglutination, separation of the denatured protein. The coagulation of bacterial proteins was reported to be time-temperature critical. Thomsen (1991) reviewed the bactericidal mechanism of moist-heat, under the title of low temperature thermal damage (43°-150°C). The elevation of the growth temperature from 30°C to 44°C, caused *E. coli* to be defective in protein and RNA synthesis and postulated this was due to inactivation of a protein required for the initiation of protein synthesis. A temperature of 50°C caused cell membrane damage

in *E. coli* which preceded cell death (Russell & Harries, 1967). This damage caused leakage of cellular material as a direct effect of the heat and occurred in the early stages of viability loss. Based on 260 nm to 280 nm absorbancy ratios (A_{260} to A_{280}) and other tests the material appeared to be RNA-like (Russell & Harries, 1968). RNA breakdown occurred internally where ribosomal RNA was the RNA type most readily affected by heat. A loss of ribosomal material or disaggregation of the ribosome in *S. aureus* was also observed after heat treatment (Sogin & Ordal, 1962; Iandalo & Ordal, 1966; Allwood & Russell, 1968). This was considered to be a secondary phenomenon due to desorption of Mg^{2+} by Na^{+}. Furthermore, the thermal stability of ribosomes had a direct relationship with the maximal growth rate of the particular microorganism (Pace & Campbell, 1967). Moreover, high incubation temperatures weakened the cell walls of *E. coli* (Hoffman *et. al*, 1966) and death preceded lysis of the cells (Hagan *et. al*, 1964).

Steam sterilization in an autoclave is the universally applied method of microbial sterilization. Under increased pressure, water vapourises at temperatures higher than 100°C. This allows moist temperatures, restricted to 100°C at atmospheric pressure, to be increased to 121.1°C (at a pressure of 29.8 psi.) which inactivates temperature-resistant spores more readily than at 100°C (Bigelow and Esty, 1920). Microorganisms are more readily killed by moist-heat than dry-heat because they are in direct contact with the heat via airborne water particles (Pflug and Holcomb, 1983), and the temperature rises more rapidly because of increased mass heat transfer when the water condenses. However, there are inherent problems associated with autoclaving:-

(i) Perishable equipment can be destroyed and degradatory effects on the sensory characteristics and nutrient availability of food occurs (Henry, 1964; Stumbo, 1973).
(ii) Autoclaving is not considered a continuous process which require excessive time for heating and cooling cycles (Joslyn 1983), however continuous procedures are now established in the canning industry.
(iii) There is also the assumption that the material will heat rapidly but volume and material type must be taken into account. It is known that autoclaves will sterilize full loads with greater ease than small loads (Joslyn, 1978).

(iv) Large equipment has to be dismantled before sterilization and is therefore a time-consuming process.

1.3.2 RADIATION

At the time of the discovery of X-rays, by Roentgen in 1895, and radioactivity, by Becquerel in 1896, work began on the antimicrobial effects of radiation (reviewed by Goldblith, 1971). Definitive work (Gates, 1930), researched the effects of UV irradiation on bacteria and showed that lamps at wavelengths 260-270 nm were especially microbicidal. Subsequently there were numerous studies on the preservation of food and drugs with radiation (Goldblith, 1966). Inactivation of microorganisms by radiation has different mechanisms depending on the nature of the radiation.

1.3.2.1 Ionising radiation

Sterilization with ionising radiation is achieved by two methods: direct or indirect interactions. The more common method is by an indirect interaction which is the result of the ionisation of water in the surrounding medium and within the bacterial cell. Electrons are expelled from the water molecules and cause the breakage of chemical bonds. The products then recombine to form hydrogen, hydrogen peroxide, hydrogen radicals, hydroxyl radicals and hydroperoxyl radicals. These radicals are very short-lived (less than 10^{-5} sec) but still cause sufficient damage and destroy the bacterial cell. The damage to the cell is achieved with radical and ionic attack on the cell wall, membrane and on the cell metabolism. Later, the direct effect of the radiation, on the DNA molecule (Ginoza, 1967), becomes apparent when the helix fails to unwind and therefore the organism cannot reproduce. The damage to the DNA is due to the production of double-stranded breaks and occasionally thymine dimers (Arrage *et al.* 1993). Because ionising radiation relies upon the interaction of ions with the target, the smaller and simpler the organism, the higher the dose of radiation needed to destroy it; viruses tend to be most resistant.

Two types of ionising radiation are used in industry: γ-rays and high energy electrons (Wilson and Miles, 1964). Other radiations, such as α-particles, neutrons, protons and non-particulate X-rays are disregarded due to low penetration power, the possibilities of inducing radioactivity on the exposed sample and problems controlling the dosage. The sensitivity of the micro-organism to the attack by radiation is also measured on the D-value scale, in a dose-time relationship (Silverman, 1983). Ionising radiation will kill all types of micro-organism, however there are a number of disadvantages of ionising radiation in the sterilization of microorganisms:-

(i) Space requirements, safety precautions and costs are problems related to the installation of large automated sterilization facilities.
(ii) There are possible deleterious effects on the article or the packaging material.
(iii) Public concern about the use of ionising radiation.

1.3.2.2 Non-ionising radiation (UV radiation)

The region of maximum antimicrobial effect in the UV spectrum (15 nm to 350 nm) is the abiotic region (220 nm to 300 nm). In 1928, Gates noted that certain wavelengths of monochromatic UV radiation had antimicrobial properties. The action of radiation in the abiotic region was toward nucleic acids (Giese, 1964) and the major damage was against the more sensitive pyrimidine bases (McLaren and Shugar, 1964). The damage was primarily due to the production of linkages between successive pyrimidines on the DNA strand forming dimers, but a minor effect was due to double-strand breaks (Arrage *et al.* 1993). The cross-linking of the DNA and protein played a significant role in the killing of the cells (Smith, 1976)

The simplest method of producing UV radiation is by the passage of electric discharge through low pressure mercury vapour within a glass tube. These are known commercially as germicidal lamps and are commonly used for decontamination of air in hospitals and food production areas (Shechmeister, 1983). About 95% of the radiation released by germicidal lamps is at 253.7 nm (Shechmeister, 1983; Goldblith, 1971).

1.3.3 NOVEL METHODS OF MICROBIAL STERILIZATION

The processes described in **Table 1.1** have all been evaluated as sterilization treatments, with a sample of the publications describing the techniques:-

Treatment	Reference
Sonication	Sams and Feria, 1991; Lilliard, 1993
Microwave radiation	Fung and Kastner, 1982; Teotia and Millar 1975
UV radiation	Reagan *et al.*, 1973; Kaess and Weidemann, 1973
Electrical stimulation	Lin *et al.*, 1983; Mrigadat *et al.*, 1980
Pressure	Carlez *et al.*, 1993; Shoji and Saeki, 1989
Air ions	Mackey and Mead, 1990; Croegert *et al.*, 1986
Prerigor cooking	Abugroun *et al.*, 1993
Pulsed electric fields	Doevenspeck, 1960; Zimmerman, 1986
Visible light	Mertens and Knorr, 1992; Dunn *et al.*, 1993

Table 1.1. **A list of novel sterilization treatments and references describing the principles involved in them.**

Laser light is another possible addition to the above list, as a future sterilization procedure. This thesis hereon in concerns the application of laser light to bactericidal purposes.

1.4 INTRODUCTION TO LASERS

The introduction of the laser as a theoretical concept of light amplification was introduced by Einstein in 1917. He stipulated that if a photon was to interact with an excited atom a second photon with identical scalar and vector properties (i.e. frequency, phase, direction and polarisation) to the photon-inducing amplification would be released (Einstein, 1917). This remained theory for almost forty years before Townes discovered a medium which would allow such light amplification

(Gordon *et al.*, 1954). The medium was ammonia and the amplification was in the microwave region of the electromagnetic spectrum. The resultant device was termed the maser (microwave amplification by the stimulated emission of radiation). By the same principle Shawlow and Townes applied the maser theory to the optical region (Shawlow and Townes, 1958). Two years later, in 1960, Maiman of the Hughes Research Establishment (USA), with a synthetic ruby formed from aluminium oxide doped with chromium oxide, successfully demonstrated visible light amplification (694.3 nm), which heralded the first laser.

Since the introduction of the visible light laser, the range of lasers available can produce outputs from X-rays to microwaves, including ultraviolet, visible, near infrared and far infrared (Thyagarajan and Ghatak, 1981). This output is the unique property of the laser which provides the novel means for the sterilization of microorganisms. Appendix 1 has a more detailed review of the construction of the laser.

1.4.1 LASER OUTPUT

The laser output has characteristics which distinguish it from other light or radiation sources. All of these special properties can be associated with the difference between spontaneous and stimulated emission. Monochromacity describes the emission of light of a single wavelength, specific to the active medium and the resonator. However Doppler effects and pressure broadening causes very slight variations in wavelength leading to a finite line width. One particular application of highly chromatic light sources is in pure research to initiate changes in physical, chemical or biological systems, e.g. Raman Spectroscopy (Thyagarajan and Ghatak, 1981). Unidirectionality is one of the major advantages of the laser over other sources of light. It is the ability to point a beam at a distant object without significant loss of intensity. Although lasers are naturally divergent, they can be collimated to give a beam that is pseudo-parallel over a significant distance (Thyagarajan and Ghatak, 1981), e.g. Helium Neon lasers used as pointers for lectures. The high intensity or brightness of the beam has proven to be useful in drilling or cutting of metals and in laser surgery (Thyagarajan and Ghatak, 1981). Temporal coherence

describes the phenomenon whereby the phase difference of a propagating beam at a point in space and time (t) is equal to the difference at t = 0. This allows applications in laser interferometry to measure flatness, physical dimensions and distances between objects. By monitoring Doppler shifted interference patterns, velocities can be measured remotely, including fluid velocities, e.g. blood flow. Laser holography is another application that utilises the laser's coherence properties.

1.4.2 LASER PARAMETER UNITS

The physical parameters which have to be considered when operating the laser have been described below, showing the measurement units and the abbreviations for each:-

Energy	Joules	**J**
Pulse Repetition Frequency	Hertz	**Hz**
Power	Watts	**W**
Exposure time	Seconds	**sec**
Pulse width	Milliseconds	**msec**
Beam area	Square centimetres	**cm^2**
Energy density	Joules per square centimetre	**Jcm^{-2}**

Energy, by definition, is the capacity of a system to do work and with a laser the work or energy can be supplied in pulses or as a continuous wave (CW), measured in Joules.

When the laser system is pulsed there are extra parameters involved in the laser set up, namely the pulse repetition frequency (PRF), pulse energy and pulse width. The PRF is not to be confused with the frequency of the light itself, though wavelength is the term more commonly used in such contexts. The PRF is the rate of pulsing per second and is measured in Hertz (sec^{-1}). The higher the PRF the closer the laser is to continuous wave. The pulse energy is the energy delivered in a single pulse and the pulse width is the duration of the pulse.

Power, by definition, is the rate of doing work, or energy per unit time. With the laser the power is measured in Watts (J sec^{-1}). The power P of the pulsed laser beam is quantified by

$$P = E_p f$$

where f is the laser PRF in Hertz, and E_p is the pulse energy in Joules. The exposure time is the total time that the sample is exposed for. For a pulsed system, this is not strictly an accurate term. Between each pulse there are gaps where the laser is not operating, the length of which will depend on the PRF. But for simplicity the term will be used to describe the exposure time of the sample. The pulse width is the length of time each pulse is delivered over. The limits of the pulse width are controlled by both the PRF (e.g. at 10 Hz the pulse width must be less than 100 msec) and the peak power tolerance of the laser, e.g. can the laser deliver 10 J of energy in a pulse width of 100 nsec, therefore having a peak power of 100 MW. The laser beam is naturally divergent but it can be manipulated by lenses and therefore focused, defocused or collimated for specific applications.

The laser exposure received by a sample, with use of the parameters described above, can be quantified by defining the energy density, dose or fluence (as described in some reports), as the exposure per unit area and is calculated by

$$ED = \frac{Pt_e}{A}$$

t_e is the exposure time, A is the beam area in cm^2 and P is the mean laser power.

1.4.3 MICROBIOLOGICAL USES OF LASER LIGHT

1.4.3.1 Atomic Force Microscope (AFM)

This is a member of a relatively new class of microscope termed the scanning probe microscopes (SPM) which measure the properties of sample surfaces at nanometer

and sub-nanometer sizes (Roberts *et al* 1994). The AFM has a probe, mounted on a spring, which is in contact with the surface of the sample introducing a small interaction force. This probe is raster scanned across the sample surface. The deflections of the probe due to the changes in the sample topography are measured by alterations in a reflected laser beam (**see figure 1.2**).

1.4.3.2 Optical Tweezers

Lasers have been used to construct a variety of traps which hold bacteria while they are being viewed under an optical microscope (Chu, 1991). In addition, organelles within the cell can be manipulated without puncturing the cell membrane or wall. The use of lasers as optical tweezers was first designed by Ashkin *et al* (1987), and works on the principle that the strong electric field of a single focused laser beam induces a dipole moment on the object being trapped. The particle will then achieve a lower energy state by moving into the high intensity focal spot of the laser beam. With this theory, a system has been built whereby researchers can now use electromagnetic fields to remotely manipulate neutral particles with unprecedented control (Ashkin *et al.* 1987). One such example is the attachment of polystyrene spheres to each end of a lambda phage DNA molecule to measure the elastic properties of DNA by pulling the two polystyrene spheres apart along the surface of a microscope slide with two optical tweezers, one for each sphere (Chu and Kron, 1990).

1.4.3.3 Laser induced mass spectrometry

Analytical techniques for bacterial classification are always under investigation. One such method investigated was the laser-induced mass spectrometer. The machine vapourises the bacterial sample in the focal point of a short pulse frequency quadrupled Nd:YAG laser (wavelength 265 nm; power density = 10^{10} - 10^{11} Wcm^{-2}). The laser induced ion-formation produced ions of positive and negative charges and a time-of-flight spectrum of positive and negative ions were obtained by a single laser shot (Albrecht *et al* 1985).

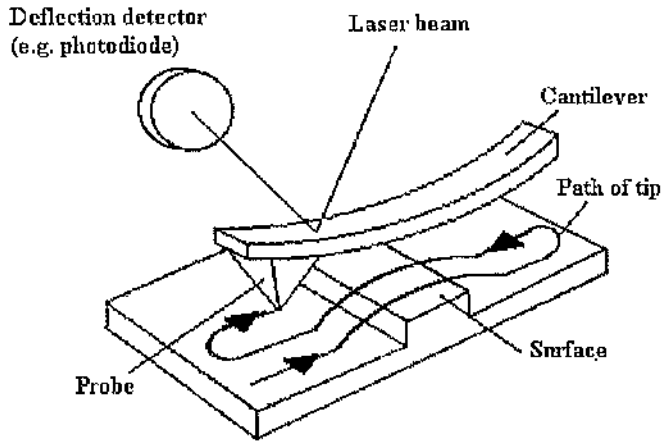


Figure 1.2 **Diagram of the atomic force microscope.** . The probe touches the sample surface and is raster scanned to produce a topographic map of the sample. Deflections in the probe height are measured with an optical lever, in the form of a laser (from Roberts et al, 1994).

1.5 LASER STERILIZATION OF MICROORGANISMS

The first publication describing the biocidal ability of laser light was produced three years after the discovery of the visible light laser (Saks and Roth, 1963). The authors demonstrated that the ruby laser (694.3 nm) had a significant biocidal effect on *Spirogyra*. It was a low power device, with an output of 20 mJ per pulse delivered over a pulse width of 500 µsec. Without the addition of methylene blue chloride, a dye which absorbs light at 694.3 nm, the beam was transmitted through the *Spirogyra* cell. The addition of the dye caused localised burning and puncturing of the cell-wall. If maximum power of the laser was used the *Spirogyra* filaments were disrupted as well as adjacent cells. The cells were lysed explosively and the cell constituents were ejected into the exogenous medium. Energies of 1.4 to 2.5 mJ per pulse produced 25 µm openings in the cell wall, damaging the cell and reducing the osmotic pressure within the cell. At a reduced energy, 0.16 mJ per pulse, pores of 15 to 25 µm were produced, but the cells appeared to be "normal" except for burned areas and pores produced by the laser beam. Although this investigation studied the effects of laser light on *Spirogyra*, a multi-cellular organism, for the specific application of microsurgery, it was the first report describing the biocidal effects of laser light. With a lack of laser parameters defined by the authors however, the energy density could not be calculated and therefore comparisons with other publications was difficult.

In 1965, Klein *et al.* reported the use of a low-power ruby laser to kill several different strains of microorganisms: *Serratia marcescens, Staphylococcus aureus, Pseudomonas*, *Pneumococcus* and *Aspergillus niger*. The species of *Pseudomonas* and *Pneumococcus* were not given. These microorganisms were exposed to laser light on solid and in liquid media at laser energies of 3 to 76 J, delivered over a pulse width of 1 msec for 5 to 15 min intervals as both focused and unfocused beams. Growth of the bacterial species was inhibited with the above laser parameters, but *Aspergillus niger* was resistant. *Pseudomonas* and *S. marcescens* showed a temporary decrease in pigment production but there was no apparent effect on the motility, colony formation or antibiotic resistance. This report sketched over many

of the results and left a short confusing overview of the microbicidal effects of the ruby laser on the different microbial species. Again, methylene blue was used in this investigation but the report was not clear as to which results were obtained with the dye. The author does state, however, that the methylene blue did increase organism sensitivity. As before, a lack of definition of the laser-parameter values, by the authors, meant that the energy density could not be calculated.

In 1966, McGuff and Bell carried out an investigation into the bactericidal effects of three lasers: Helium Neon (632.8 nm), Ruby (694.3 nm) and Neodymium:Glass (1,060 nm) on four different bacterial species: *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus* and *Bacillus subtilis*. There were three different ruby lasers, each with different pulse widths giving pulse powers of 125 kW, 10 MW and 40 MW. None of the lasers with the reported parameters were bactericidal, probably due to the low energy densities applied, however the authors noticed that the bacteria had exhibited other reactions to the laser light exposure. With the ruby laser at 10 MW, different growth patterns were observed. At 10 and 40 MW *P. aeruginosa* showed enhanced pigment production. The HeNe laser produced a diminished pigment production in *P. aeruginosa*. The Nd:Glass, on the other hand, produced a differential in pigment production from the centre of the laser exposed area, to the periphery. This tends to indicate that Nd:Glass laser light at 1060 nm affected the molecular structure of the bacterial cells. There was no indication of the energy density used in this publication and so it is not possible to compare this work with other reports or work in this thesis. The publication also produced many results indicating little or no effect of laser light on the bacterial viability and possibly left early readers with the assumption that the lack of biocidal effect did not warrant further research.

Whatever the reason, whether the lack of positive results produced by McGuff and Bell (1966), availability of lasers, the low powers of early lasers or a mixture of all three, there appears to be a 13 year gap in published research concerning the microbicidal capabilities of laser light. Adrian and Gross (1979), discovered the ability of the CO_2 laser to vaporise biological tissues and found it "appropriate to investigate the possibility of vaporising microorganisms from the surface of surgical

instruments to render them sterile." This led to the resurgence of interest in the microbicidal effects of laser light. The authors inoculated scalpel blades with *Bacillus subtilis* and *Clostridium sporogenes* spores and exposed them to continuous wave CO_2 laser light, at 10 W, for 1.5 to 2.0 min each. After 21 days of incubation in thioglycolate medium there was no evidence of bacterial growth from the treated scalpel blades. The CO_2 laser was effective in the decontamination of bacterial spores on metal surfaces, however, the authors concluded, "Excessive cost related to instrument purchase and operator time may not justify acquisition of the laser for sterilization only. However, those who possess or purchase a surgical laser should be aware of its ability to sterilize metal instruments or implants." This short publication showed the capabilities of the CO_2 laser in the sterilization of spores on metal surfaces, and indicated a possible application for the use of lasers, however as the authors intimated the cost of a CO_2 laser in 1979 was excessive. The authors unfortunately did not reveal enough parameters for the work to be repeated or compared.

A year later Hooks *et al.* (1980) with the same CO_2 laser investigated its bactericidal capacity on dental tools. Endodontic reamers were contaminated with *B. subtilis* and *B. stearothermophilus* spores at unknown concentrations. The continuous wave CO_2 laser was scanned along the surfaces of each reamer for 3 sec at 10 W and 100% of the bacterial spores were killed. It was interesting to note that the same laser was able to kill the spores 50 times faster than in the report by Adrian and Gross (1979). Neither of the authors divulged the beam diameters or the size of the instruments sterilized and a combination of these two parameters may be enough to account for the large difference in the exposure times.

In 1985, Mullarky *et al.* also published a report with a medical application. They investigated the possibility of sterilizing skin seeded with bacteria, with CO_2 laser light. The study was two-fold: first they examined bacterial survival on the skin surface and secondly within the plume emission, which is the vapourisation of a cell with the formation of pyrolysed cellular debris released as a puff of smoke. A mixed culture of *S. aureus* and *E. coli* was prepared containing 6×10^8 cells ml^{-1} of each organism. The seeded skin was exposed to 1.19 $MJcm^{-2}$, with a focused beam

delivered over 15 sec and 3540 Jcm^{-2}, with an unfocused beam delivered over 10 sec. The CO_2 laser was equally effective in its bactericidal action against both microorganisms. There was little bacterial activity in the plume released from the skin and around 6 D-values reduction in bacterial numbers was recorded after laser light exposure. There was no mention of the deleterious effect on the skin, especially after the application of 1.19 $MJcm^{-2}$ of CO_2 laser light. It was also confusing as to the reason why the authors used the higher energy with the focused beam and the lower energy with the unfocused beam. One possibility was that the exceptionally high energy density of 1.19 $MJcm^{-2}$ was to produce plume emissions.

The majority of research into laser sterilization of microorganisms, concentrated on dental applications. The use of Nd:YAG laser light (1064nm) on incipient caries was one of the first such report (Myres & Myres, 1985). At a pulse energy of 3.4 mJ, with a pulse width of 30 psec (30 x 10^{-12} sec), and a pulse repetition frequency of 1 Hz, 150 to 350 pulses (2.5 $MJcm^{-2}$ to 6.1 $MJcm^{-2}$) could remove caried debris from extracted human teeth. The peak power (i.e. the maximum power delivered in each pulse) was also extremely high, at 113 MW. The laser parameters used in this investigation produced very high peak powers and energy densities, purely because of the pulse width and the beam diameters respectively. The authors noted no damage to healthy areas of the teeth, only to the black caried region presumably due to the difference in colour and reflectivity of the two materials.

The bactericidal ability of the CO_2 laser was also investigated by Zakariasen *et al.* (1986), their investigation was designed to determine whether three different oral bacteria could be eliminated from the root canals of teeth. With glass slides, used as an *in vitro* model, supporting the bacterial samples, six different organisms were exposed to CO_2 laser light. **Table 1.2** shows the results.

There was a range of susceptibilities noted for each organism with *P. aeruginosa* being the most sensitive and *Streptococcus sanguis* the most resistant. However, the authors state that the difference between the most and least resistant was not significant. The application of these results to contaminated root canal models produced variable results. The main reason for the lack of consistency was put down

Bacterium	Exposure parameters		Energy density (Jcm^{-2})
	Power (W)	Time (s)	
Streptococcus sanguis	15	1	18750
Streptococcus mutans	5	1	6250
Actinomyces viscosus	5	1	6250
Bacillus cereus	5	1	6250
S. aureus	10	1	9370
P. aeruginosa	5	1	3250

Table 1.2 **Laser exposure required to reduce each bacterial sample, on glass slides, by 3 D-values.** (Zakariasen et al, 1986)

to the difficulty in the laser alignment. When the laser was well aligned >99.94% reduction in viability was observed. These results however did not take account of the adherent bacteria in the root canals which would account for a large part of root canal contamination.

Schultz *et al.* (1986), used a high-power Nd:YAG laser, with powers between 20 and 120 W for 10 sec, in the first scientific laboratory investigation of the effects of laser light on a variety of bacterial species. At the beam diameter of 6 mm, a range of energy densities (555 - 3333 Jcm^{-2}) brought about a reduction in the viable counts of aqueous suspensions of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* in wells of microtitre plates. A difference in the susceptibilities of each bacterial species was also observed, with *P. aeruginosa* the most sensitive and *E. coli* and *S. aureus* slightly more resistant. The authors also introduced two dyes, methylene blue and congo red, to the suspensions in an attempt to increase the effectiveness of Nd:YAG laser light on reducing the viability of the various microorganisms. Only the addition of the dyes to *P. aeruginosa* produced a significant increase in the bacterial susceptibility probably the result of the darker colour and therefore higher absorption.

The effect of an argon fluoride (ArF) excimer (excited dimer) laser operating at 193 nm, on seven microorganisms (*Serratia marcescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Haemophilus influenzae*, *Candida albicans* and *Aspergillus niger*), as a colonies on agar plates, was investigated by Keates *et al.* (1988). The response of the microbial species to the excimer laser was similar and the viability of all the microorganisms was destroyed. The laser parameters were 110-120 mJ per pulse, 10 Hz, with a pulse width of 15 nsec giving energy densities of 300 to 330 $mJcm^{-2}$. Any differences observed in the sensitivity of the different microbial species, were attributed to the size of the colonies and the depth to which the bacterial colonies had penetrated into the agar. The energy density required by the ArF excimer laser showed how effective this laser was on the decontamination of microorganisms. This laser did cause damage to the agar at these low energy density however the laser light could sterilize a whole colony without further growth after incubation and subculturing.

Miserendo *et al.* (1988a) used CO_2 laser light to remove three different bacterial cultures (organisms not named) from 70 - 90% of contaminated oral root apices. With further laser treatment the root apices were sterilized; no laser parameters were recorded. A further report by Miserendo *et al.* (1988b) showed that with a CO_2 laser delivering 1 to 4, 1 sec pulses at a power of 1 to 2 W and a 2.0 mm beam diameter, carious human dentine material was sterilized. The energy densities involved in these experiments ranged from 3200 to 25,450 Jcm^{-2}. For each energy density there were 17 samples and for energy densities above 6400 Jcm^{-2} all seventeen samples produced no further growth. Again the energy density required for the decontamination of the dentine material were very high. Both of these reports were abstracts and gave limited information: no laser parameters in Miserendo *et al.* (1988a) and no mention of the deleterious effects on the dentine material in Miserendo *et al.* (1988b).

Al-Qattan *et al.* (1989) compared the efficacy of CO_2 laser sterilization of rabbit wounds with that of iodine. A 15 Watt, continuous wave device with an exposure time of 5 min was used, at three different beam areas (dimensions not mentioned) on skin seeded with *P. aeruginosa*. Although the laser charred the skin surface it was more effective than iodine in the sterilization of *P. aeruginosa* in surgical wounds. Less than 10% of the laser treated wounds developed a *Pseudomonas* infection whereas nearly 40% of the iodine treated wounds remained infected. This publication was superseded by a report by Stranc and Yang (1992), comparing the efficacy of CO_2 laser sterilization of rabbit wounds with that of cautery. The authors used a 10 Watt, continuous wave device until the skin charred, at two different beam areas (dimensions not mentioned) on skin seeded with *P. aeruginosa*. In this instance the laser was not as effective as cauterisation. It is interesting to note that laser light was not as effective as cauterisation. However, CO_2 laser light is now utilised in hospitals as a procedure for removal of tumours and other skin disorders.

In 1990 a report was published on the bactericidal effect of CO_2 laser radiation on selected oral bacteria (Dederich *et al.* 1990). The oral bacteria were *Actinomyces viscosus*, two strains of *Streptococcus mitior*, *Strep. sanguis* and *Strep. mutans*. Two bacterial species not normally associated with the oral cavity, *P. aeruginosa* and

Staph. aureus were also used. Aliquots (1µl) of overnight bacterial cultures (between 9.8 x 10^7 and 1.3 x 10^9 cfu ml^{-1}) were dropped onto glass cover-slips and exposed to continuous wave CO_2 laser light at output powers of 1 to 10 W for 0.1 to 1 sec, and a beam diameter of 2 mm, energy densities of 200 Jcm^{-2} killed 99.5% of the bacteria. The authors concluded by noting that there were no differences in the susceptibility of the different bacteria and statistically, increasing the power or the exposure time increased the lethality of the CO_2 laser light.

Powell and Whisenant (1991) compared the biocidal effect of light from three different lasers: Nd:YAG, argon ion and CO_2 lasers, on contaminated endodontic reamers. Five different microorganisms: *P. aeruginosa*, *Streptococcus pyogenes*, *Candida albicans*, *Staphylococcus aureus* and *Bacillus subtilis* were used. At 1 Watt for 60 sec (no other parameters were noted), all three lasers were shown to be capable of disinfecting endodontic reamers. In this qualitative study no bacterial counts were given and the results were presented in a (±) configuration; (+) indicating non-sterile and (-) indicating sterile. They concluded that the argon ion laser was the most effective of the three lasers and additionally it could kill spores with 1 Watt for 120 sec. This report did not reveal enough detail about the laser parameters and thus energy densities could not be calculated. Therefore it is not known whether the authors were basing their argument as to the most effective laser on the energy density which is the only way that they can be readily compared.

Tseng *et al.* (1991) investigated the bactericidal effect of Nd:YAG laser light on periodontic bacteria (bacterial species were not revealed) with 0.7 to 1.5 W and 10 Hz delivered over 60 sec. After these exposures bacteria could still be seen under SEM, but no qualitative tests were carried out to see if the bacterial species were still active. When the parameters were increased to 1.75 W and 20 Hz for 30 seconds with a "contact hand-piece" virtually no bacteria were observed with the SEM. The method of microbial detection, scanning electron microscopy, was an unsatisfactory procedure as the viable state of the bacterial cells could not be determined. It would also be impossible to scan an area with the SEM to determine if the area was completely sterilized. The term "*virtually* no bacteria were observed" indicated that visibly there were bacteria within the exposed area.

White *et al* (1991) investigated the effect of Nd:YAG laser light on three different bacteria, *B. stearothermophilus*, *B. subtilis* and *E. coli*. The cultures inoculated onto standard dentine sections were in late stationary phase and were exposed to between 0.3 and 3.0 W for 20 sec to 2.0 min. *B. stearothermophilus* was the most resistant to Nd:YAG laser light and was not sterilized. At power levels greater than 0.5 W and for exposure times of 2 min the laser light produced a 6 D-value reduction in *E. coli* and *B. subtilis* viability.

The efficacy of sterilization of infected root canals, with Nd:YAG laser light and a fibre optic beam delivery system, was also investigated by Cobb *et al.* (1992), with and without root planing. The microorganisms used were the natural microflora found in infected teeth, but after exposure the presence of only three microbial pathogens, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia*, were analysed. The laser was pulsed at 20 Hz, 87.5 to 150 mJ $pulse^{-1}$, for 1 to 3 min. There was a decrease in bacterial counts and in some cases complete removal. SEM examination indicated that a vaporisation process only occurred when the optical fibre was in direct contact with the microbial deposit.

The bactericidal effect of a combination of wavelengths from an argon ion laser (458, 466, 476, 488, 496, 502 and 514 nm), on 21 different strains of bacteria, suspended in agar at concentrations of approximately 10^5 to 10^6 cfu ml^{-1}, was investigated (Yanagawa *et al.* 1992). Each organism was exposed to 20, 40 and 60 mW output power, delivered over 30 min, with a beam diameter of 8 mm and the results are shown in **Table 1.3**. The authors reported that the temperature rise in the agar was less than 1°C and concluded that the thermal effect was negligible, however the method of measuring the temperature was not mentioned in the report. The Gram +ve organisms were not sensitive to the laser light and therefore the authors concluded that the rigidity of the cell wall was playing an important part in the bacterial resistance. It is important to note that there were a number of resistant Gram -ve species. The authors ended the investigation by proposing photooxidation as the probable mechanism of argon ion laser light action. The method of determining the effect of argon ion laser light was not satisfactory. The fact that there was no observed reduction in the lawn does not indicate that the organisms

Organism	Sensitivity to laser light at energy density		
	72 Jcm^{-2}	144 Jcm^{-2}	216 Jcm^{-2}
G -ve aerobic rods			
Pseudomonas aeruginosa 1117	±	+	+
P. fluorescens K-5	±	+	+
P. fluorescens 2101	±	+	+
Acinetobacter calcoaceticus	-	-	-
G -ve facultatively anaerobic rods			
Escherichia coli 5208	-	±	+
E. coli 3280	-	±	+
E. coli 3284	-	-	±
Shigella sonnei	-	-	-
Salmonella typhimurium	-	-	-
Sal. enteritidis	-	-	±
Proteus mirabilis	-	-	±
Morganella morganii	±	+	+
Serratia marcescens IIa-1	-	-	-
S. marcescens Iva-1	-	-	-
Klebsiella pneumonia	-	-	-
Vibrio parahaemolyticus 8406-2	-	-	-
V. parahaemoliticus 8406-3	-	-	-
G +ve rods			
Bacillus cereus B6-ac	-	-	-
G +ve cocci			
Staphylococcus aureus 209P	-	-	-
St. aureus 100	-	-	-
St. aureus 196П	-	-	-

Table 1.3 **Sensitivity of various organisms to a multi-wavelength Argon ion laser light at 3 different energy densities.** If a cleared area with diameter > 6 mm was observed then the bacterial species was regarded as sensitive (+). For colonies observed growing in the exposed areas, but at a reduced number, the sensitivity was designated questionable (±). If no visible reduction in the colony formation was observed the bacterial species was regarded as insensitive (-) (Yanagawa et al. 1992).

were unaffected by the laser as there are around 10^5 organisms in the area exposed. Also where a reduction in the lawn was observed the result should not be classed as questionable but absolute; the laser light did reduce the bacterial viability. A more useful investigation would be to find the energy density required to produce a constant bactericidal level.

Frucht-Peri *et al.* (1993) investigated the effect of the ArF excimer laser (193 nm) on *C. albicans*. *C. albicans* colonies on Sabouraud agar were exposed to a variety of energy densities of hundreds of $mJcm^{-2}$ at 10 to 50 Hz. After exposure the colonies were scraped off the agar plate with a spatula and subcultured, and the cfu visible after 24 h were noted. *C. albicans* was sterilized at 300 $mJcm^{-2}$. Raising the pulse repetition frequency did not increase the sterilization of the plates, however an increase in the number of pulses (i.e. the exposure time) was directly proportional to the number of yeast eliminated. The technique used to examine the effect of excimer laser on *C. albicans* was, once again, unsatisfactory with exposure of yeast colonies and determination of remaining organisms. The number of organisms in a single colony will vary with the size and shape of the colony. The author used colonies between 1 and 1.5 mm in size for exposure so there would be a difference in the viable count of around 50% between the smallest and largest colonies. The colonies, with "superficial" agar were removed with a spatula until no bacteria were visible on the agar which has to be extremely inaccurate.

Stabholtz *et al.* (1993) investigated the use of light from an excimer laser to inactivate microorganisms. The bactericidal effects of xenon chloride (XeCl) excimer laser light (308 nm) on *Strep. mutans*, in liquid culture and on blood agar plates was investigated. For the liquid cultures the laser parameters were 0.7 Jcm^{-2}, with a 15 nsec pulse width and a 0.08 cm^2 beam area, delivered over 2, 4 and 8 sec (the pulse repetition frequency was not detailed). The 8 sec exposure produced a bacterial reduction of around 1 D-value; the exposure time was directly proportional to the bactericidal effect. The parameters used on the lawned cultures were similar to those required in liquid suspensions, apart from the exposure time which was kept constant at 4 sec with the pulse energy as the variable factor. The pulse repetition frequency was given as 5 Hz. When the lawned plate was exposed to laser light

there were inactivated areas on the plate after incubation, overnight and the area of the zones of inhibition increased as the energy density increased.

1.5.1 PHOTOSENSITISATION

All of the publications mentioned in section 1.5 consider the direct effect of laser light on a microbial subject. However an alternative method of laser sterilization was discovered and is currently topical when considering biocidal laser:cell interactions. Some bacteria contain endogenous compounds which absorb light of certain wavelengths and cause a secondary reaction in the cell, ultimately leading to death. Alternatively, exogenous photo-reactive chemicals can be applied to bacterial samples and after exposure to light of a specific wavelength to the photo-reactive chemical, with the same consequences as endogenous photosensitisers. This method of cell killing is termed photosensitisation and the photoreactive chemicals involved are photosensitisers. The discovery of photosensitisation was reported by Blum (1941) when he reported that paramoecia in a solution containing acridine dye were killed when exposed to sun-light, whereas they survived when no dye was added. Since this report a number of investigations (e.g. T'ung, 1938; Chick and Ellinger, 1941; David, 1946; Dobson and Wilson 1992) and practical uses (e.g. Lewis *et al.*, 1984; Marcus, 1992; Van Best *et al.*, 1993) were found for photosensitisation of biological systems, such that it now has a medical title of "Photodynamic Therapy".

The reaction which takes place in cells after exposure to light of the specific excitable wavelength of the photosensitiser, is first electron excitation of the photosensitising compound (i.e. light-induced promotion of electrons to higher energy levels). The excited particles may then react with an energy acceptor. A type II photo-process is where the energy acceptor is molecular oxygen (Mac Robert *et al.* 1989) which reacts to produce highly unstable singlet oxygen. A type I photo-process is where the acceptor is other than oxygen, reacting to produce hydroxyl and/or organic radicals. These reactive species within the cell cause damage to essential cellular components, such as the cytoplasmic membrane and may disrupt metabolic activities and often result in cell inactivation (Spikes and Jori 1987; Wilson 1993). There are a number of tried and tested photosensitisers available,

with different characteristics such as reactive wavelength specificity, absorbance maximum (A_{max}) and selectivity for target (Wilson *et al.*, 1992; Levi, 1995).

The majority of investigations into the microbicidal activity of photosensitisation have used polychromatic light sources, as there were no monochromatic light sources available prior to the introduction of the laser in 1960. The introduction of the laser gave a new dimension to photodynamic therapy. The beam could be directed at the target, it was more intense than polychromatic light and had a single wavelength which could be matched with the A_{max} of an exogenous or endogenous photosensitizer. Bactericidal action of helium neon (HeNe) laser light (632.8 nm) against *Sarcina lutea*, *E. coli* and *P. aeruginosa* sensitized with toluidine blue (A_{max} 632 nm) was first discovered by MacMillan *et al.* (1966). Takashi *et al.* (1975) found that with tuneable organic-dye laser light (590 nm) *E. coli* was not killed after being sensitized with toluidine blue (absorbs poorly at 590 nm). Martinetto *et al.* (1986) showed that *E. coli* and *S. aureus* were sensitized to HeNe laser light with haematoporphyrin. Bedwell *et al.* (1990) discovered the bactericidal effect of aluminium disulphonated phthalocyanine in combination with copper-vapour-pumped-dye laser light toward *Helicobacter pylori*. Okamoto *et al.* (1992), investigated the bactericidal effect of HeNe laser light in combination with 10 different dyes on *E. coli*, and *Strep. sobrinus*. Wilson and colleagues, have published reports on laser photosensitisation for dental applications. These reports concern the lethal photosensitisation of oral microorganisms (Wilson and Mia, 1993; Wilson *et al.*, 1992) under different conditions, e.g. in suspension (Wilson *et al.*, 1993a; Burns *et al.*, 1993), in biofilms (Dobson and Wilson, 1992) or in blood (Wilson *et al.*, 1993b), with a range of photosensitisers (Wilson *et al.* 1992). The results from each of these reports were variable with different combinations of parameters being more effective than others. This is the majority of publications concerning photoinactivation of microorganisms by photosensitisation in combination with laser light.

1.5.2 INITIAL EXPERIMENTATION

Preceding the initiation of this investigation preliminary experiments were carried out by Dr Ian Watson and Professor Duncan Stewart-Tull in the Departments of Mechanical Engineering and Microbiology (Latterly the Division of Infection and Immunity) for the purpose of securing finances to aid the development of this work.

They found that Nd:YAG laser light (1064 nm) had the ability to reduce the viability of *E. coli*, and *B. stearothermophilus* on spore strips. Laser light could sterilize *B. stearothermophilus* on moistened spore strips in under 10 sec - at 144 Jcm^{-2}. The effects of laser light on *E. coli* suspended in air as an aerosol were investigated by exposing a known concentration of bacteria sprayed into an aluminium tube aligned co-axially to the laser beam. After exposure the bacteria were harvested from the tube with the use of an air sampler. The laser showed a significant bactericidal effect but for geometrical reasons it was not possible to inactivate the bacterial content completely (the beam diameter was smaller than the internal diameter of the aluminium tube). Lawns of *E. coli* grown on MacConkey's agar were exposed to laser light with a range of parameters producing energy densities of ~2100 Jcm^{-2}. In all cases the lawned bacteria on the exposed agar surface were inactivated. The bacteria on the agar surface were also inactivated when light from the laser was fired through the plastic base and agar layer of the plate.

2. AIMS AND OBJECTIVES

The lethal effect of laser light on microorganisms may be governed by a number of different factors from the laser type to the bacterial growth conditions. The aim of this thesis is to identify a number of these variables and discover the influence they have on the microbicidal capacity of laser light. **Figure 2.1** highlights some of the parameters that may affect laser light sterilization and within this figure are the parameters investigated in this thesis. A number of lasers with wavelengths ranging from 355 nm to 118 μm will be investigated, but the major part of this thesis will centre around Nd:YAG laser light, with substantial investigation into the microbicidal capability of the CO_2 laser. A number of microorganisms will be treated with laser light and a range of sensitivities will be found. The effect of the age of the bacteria and the size of the initial inoculum on the laser-light sterilization of microorganisms will also be investigated. With a standard organism the effects of the different laser light settings will also be investigated. The thesis will also endeavour to find the effect of the medium (solid, semi-solid or liquid) on different bacteria and whether NaCl, pH or high or low water content will effect the lethality of laser light. In addition the thesis will investigate the mechanisms involved in the killing of bacteria in liquid suspensions.

In an attempt to minimise the experimental procedures and maximise the results, multifactorially-designed experiments will be used to determine the effects of growth parameters, such as NaCl concentration and pH, and laser parameters such as pulse frequency, pulse energy and exposure time on the bactericidal efficiency of Nd:YAG laser light. A method of modelling the killing curves of Nd:YAG laser light will be investigated for the purpose of developing predictive models for use in future studies.

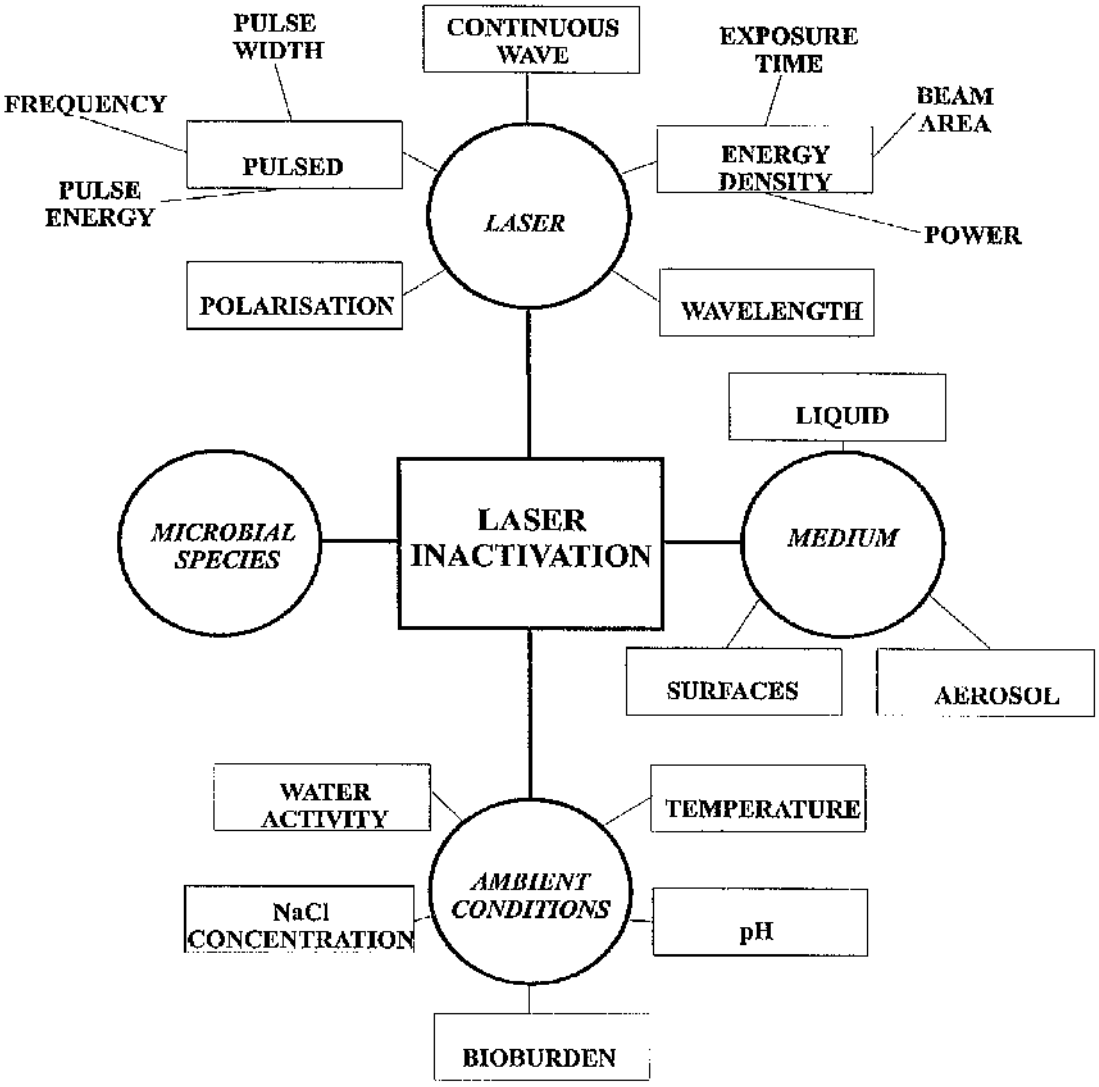


Figure 2.1 **Diagram of the major parameters with the potential to affect the microbicidal action of laser light.**

3. MATERIALS AND METHODS

3.1 PREPARATION

3.1.1 CULTURES

Seven bacterial and two yeast cultures (NCTC numbers provided) were used in these experiments: *Escherichia coli* B 10537, *Staphylococcus aureus* 6571 (Oxford strain), *Serratia marcescens* 2302, *Bacillus subtilis* 3610, *Bacillus cereus* 11145, *Bacillus stearothermophilus* 10339, *Micrococcus luteus* 2665, *Deinococcus radiodurans* 9279, *Listeria monocytogenes* strain Scott A, *Candida albicans* strain GDH2346 and *Saccharomyces cerevisiae* X2180-1Aa. The bacterial cultures were grown on nutrient agar (Oxoid) slopes at 37°C for 18 h, subsequently kept at 4°C and sub-cultured monthly. The yeast cultures were grown on nutrient agar slopes supplemented with 10% w/v sucrose.

3.1.2 GROWTH MEDIA

Each bacterial culture was grown in nutrient broth (10 ml) (Oxoid), and the yeast cultures in nutrient broth, with 10% w/v sucrose, and incubated (shaking for experiments requiring higher concentrations) for about 18 h at the following temperatures: *B. stearothermophilus* at 56°C; *E. coli, S. aureus, B. subtilis, B. cereus* and *C. albicans* at 37°C; *M. luteus, D. radiodurans, L. monocytogenes* and *S. cerevisiae* at 30°C and *S. marcescens* at 25°C. For vegetative cells of the *Bacillus* species, nutrient broth (10 ml) was heavily inoculated with the overnight culture (1 ml) and incubated for 5 h.

3.1.3 LAWNED PLATES

Aliquots (1.5 ml) of the overnight broth cultures were inoculated onto fresh nutrient agar plates, dried for 30 min in a class 100, laminar air flow cabinet (Flow Laboratories, Germany), and allowed to flood the surface. Several of the plates contained sucrose (10%w/v) in order to support the growth of *C. albicans* and *S. cerevisiae*. The excess liquid was decanted and the lawned plates were dried for 30 min in a Petric Class III microbiological safety cabinet. To keep the cells of the

Bacillus species in the vegetative state the lawns were made 30 min before laser exposure.

3.1.4 OPTICAL DENSITIES OF *E. coli* AT 1064 AND 600 nm

A shaken overnight *E. coli* culture was serially-diluted 2-fold in nutrient broth until the solution became translucent. Optical densities at 600 and 1064 nm, for each dilution, were read against a nutrient broth blank. The concentrated suspension was serially-diluted 10-fold in PBS and each dilution (50 µl) was plated on nutrient agar by the Miles and Misra method (Miles *et al.* 1938), incubated at 37°C overnight and the viable counts determined.

3.1.5 GROWTH MEDIA FOR MULTIFACTORIALLY-DESIGNED EXPERIMENTS

Prior to autoclaving, NaCl (Fisher Scientific International) was added to nutrient agar to produce a series of salt concentrations (0.5% to 15.5% w/v). The tolerance limits of each bacterial species tested were obtained and nutrient broth NaCl concentration was increased to these limits. These broth solutions were made to a range of pH values with the addition of NaOH and HCl (pH 5.5-pH 8). The tolerance limits, of the bacteria, to pH at the upper and lower NaCl concentrations were investigated. For the multifactorial investigation four nutrient broth solutions were prepared per bacterial species, made-up to the particular bacterial NaCl concentration and pH upper and lower tolerance limits. The bacterial species were grown as described in section 3.1.2

3.1.6 INOCULATION OF SURFACES

An aliquot (10 µl) of 0.1 % w/v bovine serum albumin (BSA; Sigma) in PBS was added to the centre of sterilized stainless steel, nylon plastic and borosilicate glass disks of dimensions: 13 mm diameter x 5 mm thickness, and dried in a laminar air-flow cabinet (LAF; Flow Laboratories, Germany) for 30 min. Aliquots (20 µl; 10 µl for experiments with CO_2 laser light) of 18 h cultures were added to the protein and

dried in a LAF for 30 min. The drying procedure was omitted for experiments which required a wet culture.

3.1.7 INOCULATION OF COLLAGEN FILM

Nutrient agar plates which contained iodo-nitro-tetrazolium violet (INT) were required for experiments on collagen film. INT dissolved in sterile distilled water was added to sterile molten agar to a concentration of 0.05% w/v before pouring into petri-dishes. Squares of ethylene oxide sterilized collagen film were reconstituted in sterile distilled water, placed in a petri-dish and dried for 10 min in a LAF. *S. aureus* suspensions (0.1 ml) were inoculated onto the skins and spread evenly over the surface. The collagen was dried for a further 10 min in a LAF cabinet.

3.2 LASER TYPES AND PARAMETERS

The lasers used in this thesis are detailed in **Table 3.1**.

3.3 LASER LIGHT EXPOSURE OF MICROORGANISMS

3.3.1 EXPOSURE OF LAWNED CULTURES

Individual lawned plates were mounted on a stand and a beam-absorbing brick was placed beneath the dish. Each petri-dish containing the lawned agar was placed above the absorbing brick and exposed six times, at rotational angles of approximately 60° to each other; each experiment was done in triplicate. The laser parameters are shown in **Table 3.2.**

Figure 3.1 illustrates the Nd:YAG laser geometry for the optical system utilised in the exposure of lawned cultures. A fibre optic beam delivery system was used which contained a double lens assembly. The first lens was a collimating lens which produced a parallel, or collimated, beam and the second lens focused the

Laser/ model	Manufacturer	Wave-length (μm)	Pulse Energy (J)	Pulse width (s)	PRF (Hz) /CW	Mean Power (W)	Peak power (W)
FIR/ FIRL100	Edinburgh Instruments, Edinburgh, UK	118	NA	NA	CW	0.150	0.150
CO_2/MFKP	Laser Ecosse, Dundee, UK	10.6	NA	NA	CW	600	600
Nd:YAG MS830	Lumonics, Rugby, UK	1.06	10	$8x10^{-3}$	20	200	$1.3x10^{3}$
Nd:YAG/ Minilite	Continuum, Santa Clara, CA, USA	1.06	$2.5x10^{-2}$	$5 x10^{-9}$	10	0.25	$5.0x10^{6}$
(Doubled)		0.532	$1 x10^{-2}$	$4 x10^{-9}$	10	0.10	$2x10^{6}$
(Tripled)		0.355	$4 x10^{-3}$	$4 x10^{-9}$	10	0.04	$1 x10^{6}$
Nd:YAG/ Surelite II-10		1.06	0.65	$6 x10^{-9}$	10	6.5	$1.08x10^{8}$
(Doubled)		0.532	0.3	$5 x10^{-9}$	10	3	$6.0x10^{7}$
(Tripled)		0.355	0.1	$5 x10^{-9}$	10	1	$2.0x10^{7}$
Laser Diode Array OPC-AO15	Opto Power Corporation, CA, USA	0.810	NA	NA	CW	15	15
Ar ion/ Beamlok 2060	Spectra Physics, Hemel Hempstead, UK	0.488	NA	NA	CW	2	2

NA = not applicable CW = continuous wave

Table 3.1 **Laser types, manufacturers and parameters.**

Parameter	Value
Pulse energy	10 J
Pulse Repetition Frequency	10 Hz
Pulse width	8 msec
Beam diameter	1.45 cm
Exposure time	variable

Table 3.2 **Laser parameters used for exposure of lawned agar plates.** These values were used in all experiments with the exception of situations where different laser parameters were varied

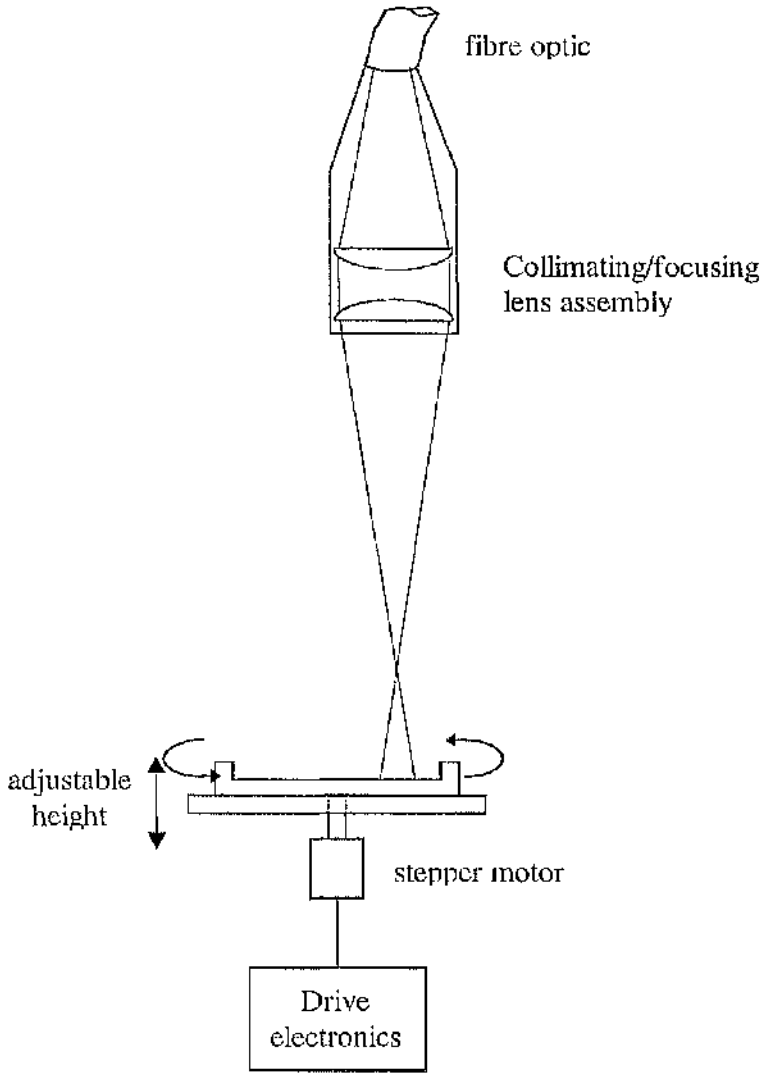


Figure 3.1 **Optical geometry of the Nd:YAG laser system, used in the exposure of microorganisms lawned on agar plates**

beam. Experiments were carried out with different beam diameters, by adjustment of the sample position relative to the focal point.

After 18 h incubation, at 37°C, plates were examined for growth and the areas of the clear zones were measured on a millimetre scale under a microscope with X4 objective, and expressed in cm^2. Sections from several cleared areas were removed and placed upside down on fresh nutrient agar plates and the plates incubated at 37°C for three days.

From the results with each culture, a graph of area of clearing on agar surfaces (cm^2) against energy density (Jcm^{-2}) was plotted, and the energy density of light needed to give an area of inactivation/sterilization (IA) equal to 50% of the beam area (IA_{50}-value), was determined. This was done with a statistics package (MINITAB), which used linear regression of the points in the approximately linear region of the curve and interpolation. A specially written macro was used to obtain the 95% confidence limits of the IA_{50}.

3.3.2 TEMPERATURE MEASUREMENTS IN AGAR

After agar plates were exposed to Nd:YAG laser light at the parameters shown in **Figure 3.1**, with the exception of the PRF which was varied between 10 and 40 Hz, the temperature of the cooling curves were recorded with a metal thermocouple probe, attached to an oscilloscope (LeCroy), inserted through the side of the dish into the exposed area. The cooling curves were extrapolated back to when the laser was switched off to find the maximum temperature the agar surface reached during exposure.

3.3.3 PROLONGED EXPOSURE OF *E. COLI* ON AGAR SURFACES

A nutrient agar plate lawned with *E. coli* was exposed to Nd:YAG laser light with the parameters shown in **Table 3.2** for 10 sec, corresponding to 300 pulses. The same agar plate was then exposed to Nd:YAG laser light at the same pulse energy,

pulse width, and beam diameter for 30 minutes delivering a pulse every 6 sec. The plates were incubated at 37 °C for 18 h.

3.3.4 EXPOSURE OF BACTERIAL SUSPENSIONS

The laser geometry in **Figure 3.1** was unsuitable for experiments on volumes of liquids and so a different laser assembly was required. The optical system illustrated in **Figure 3.2** produced a collimated beam so that a volume of liquid could be exposed. This assembly also allowed analysis of the beam during exposure by removal of a fraction of the beam energy with an anti-reflection coated wedge. The wedge was removed for all of the experiments detailed in this thesis.

The laser-exposure vessel (**Figure 3.3**) was a flat-bottomed, borosilicate glass tube of 13 mm internal diameter and a height of 45 mm. A small side tube, 12 mm from the base, housed a thermocouple, embedded in high temperature epoxy resin (Araldite, Ciba Geigy), which also sealed the vessel. The tip of the thermocouple was recessed slightly from the path of the laser beam, yet it was in contact with the 2.0 ml bacterial suspension placed in the vessel. The thermocouple was initially connected to a galvanometer but for the majority of the experimental work presented it was connected to a computer. The vessel containing the 2.0 ml aliquot of bacterial suspension, was placed in an aluminum holder and positioned vertically beneath the path of the beam. The sample was exposed to Nd:YAG laser light using the parameters described above. When the temperature of the thermocouple reached the required value the laser was switched off.

3.3.4.1 Exposure of dilute *E. coli* suspensions

An 18 h bacterial culture was diluted in PBS to approx. 1 x 10^4 cells ml^{-1} and 2.0 ml was exposed to Nd:YAG laser light at the parameters in **Table 3.3**, until 50°C was reached. This took about 25 sec. After each exposure, 0.2 ml of exposed sample and unexposed control were removed and diluted 10-fold in 1.8 ml PBS and 100 µl of sample and dilution were plated out on nutrient agar.

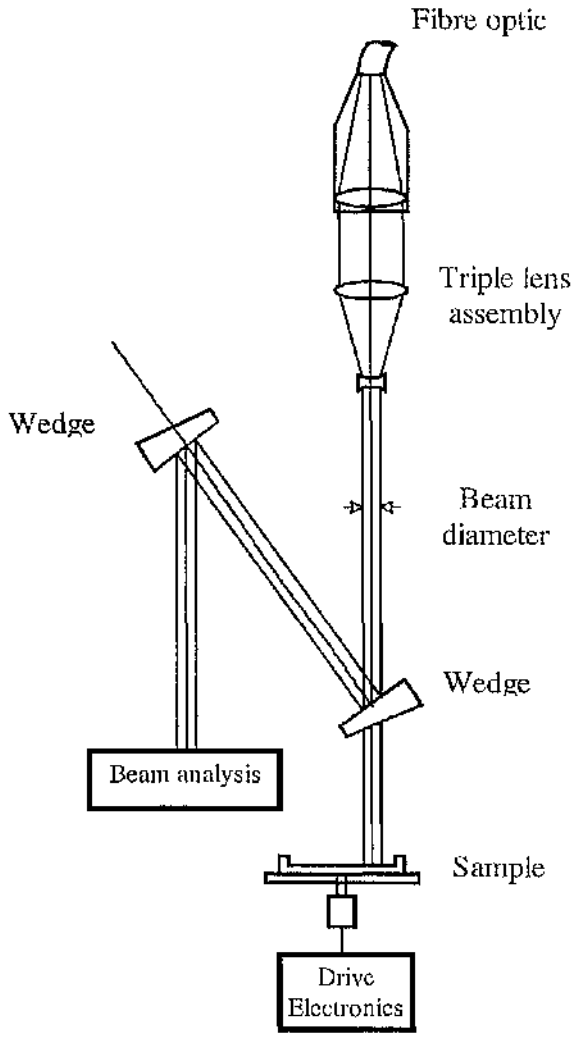


Figure 3.2 **Optical geometry of the Nd:YAG laser system, used in the exposure of microorganisms in liquid suspension**

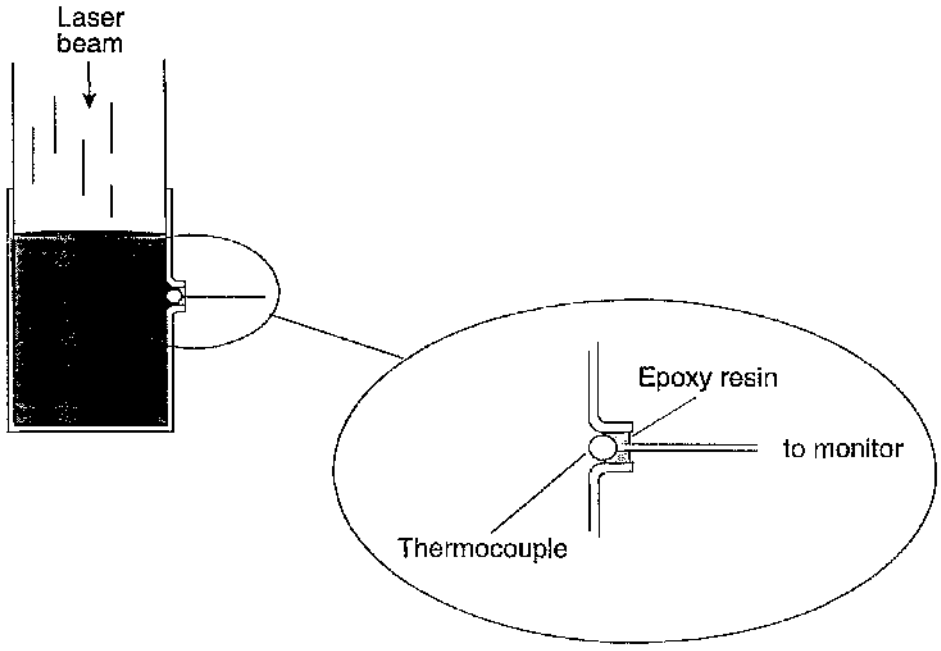


Figure 3.3 **Diagram of the glass vessel which contained the recessed thermocouple for laser exposure**

Parameter	Value
Pulse energy	10 J
Pulse Repetition Frequency	10 Hz
Pulse width	8 msec
Beam diameter	1.35 cm
Exposure time	variable

Table 3.3 **Laser parameters used for exposure of bacterial suspensions.**

For comparison with the laser heating, the bacterial suspension was heated in a water-bath at 50°C and in a PCR heating block (Techne PHC2, Cambridge, UK). Samples were removed for viability counting when the vessel contents reached 50°C in the waterbath and after 10 min at 50°C. For exposure in the PCR heating block, the dilute bacterial suspension (300 µl) was transferred to sterile Eppondorf tubes, placed in the PCR machine and heated to 50°C within 1 min. The samples were left at 50°C for 30 sec before removing and plating as before. All experiments were done in triplicate on three separate occasions.

3.3.4.2 Exposure of *E. coli, S. aureus, L. monocytogenes* and *B. cereus* suspensions

Cultures of *E. coli, S. aureus, L. monocytogenes* and *B. cereus* were diluted in PBS to approx. 1 x 10^6 cells ml^{-1} and 2.0 ml was exposed to Nd:YAG laser light, at the parameters shown in **Table 3.3**, to a range of temperatures. After each exposure, 0.2 ml of exposed sample and unexposed control were removed and diluted 10-fold in 1.8 ml PBS and 100 µl of sample and dilution were plated out on nutrient agar. The plates were incubated at 37°C for 18 h and the viable counts determined.

3.3.4.3 Exposure of *E. coli* at a range of concentrations

A shaken overnight *E. coli* suspension was serially diluted, ten-fold, in nutrient broth from approx. 1 x 10^9 to 1 x 10^4 cfu ml^{-1}. A 2.0 ml sample of each suspension was exposed to Nd:YAG laser light, at the parameters shown in **Table 3.3**, until 50°C was reached. Aliquots (0.2 ml) of exposed samples and unexposed controls were removed and 10-fold serial dilutions were made in 1.8 ml PBS. Aliquots (50 µl) of all dilutions were spotted on nutrient agar by the Miles and Misra method (Miles *et al.*, 1938), incubated at 37°C for 18 h and the viable counts determined.

3.3.4.4 Exposure of *E. coli* suspended in milk, soup or PBS

An 18 h bacterial culture was diluted in either lentil soup, UHT milk or PBS to approx. 1 x 10^4 cells ml^{-1} and 2.0 ml was exposed to Nd:YAG laser light, at the

parameters shown in **Table 3.3**, until 50°C was reached. After each exposure, 0.2 ml of the exposed sample and unexposed control were removed and diluted 10-fold in 1.8 ml PBS and 100 µl of sample and dilution were plated out on nutrient agar incubated at 37°C for 18 h and the viable counts determined.

3.3.4.5 Exposure of *E. coli* suspended in different protein concentrations

An aliquot (20 ml) of a shaking 18 h culture was centrifuged at 4000 x g (Megafuge, Hereaus Sepatech) for 10 min and the supernate discarded. The pellet was resuspended in distilled water (20 ml). This was repeated. Aliquots (0.1 ml) of the washed *E. coli* suspension were added to five different concentrations of ovalbumin dissolved in distilled water (0.001, 0.01, 0.1, 1 and 10% w/v) and one to distilled water as a control. A 2.0 ml sample of each *E. coli* suspension was exposed to Nd:YAG laser light, at the parameters shown in **Table 3.3**, for various exposure times (0, 4, 8 and 12 sec). After each exposure, 0.2 ml samples were removed and 10-fold serial dilutions were made in 1.8 ml PBS. Aliquots (0.05 ml) of all dilutions, for experiments and controls, were spotted on nutrient agar by the Miles and Misra method, incubated at 37°C overnight and the viable counts determined.

3.3.4.6 Exposure of *E. coli* at different growth phases

Sterile nutrient broth (50 ml) was inoculated with an 18 h culture of *E. coli* (1 ml) and the time noted. Around the time of inoculation a control sample was removed, diluted and plated out on nutrient agar. The samples were exposed to laser light, at the parameters shown in **Table 3.3**, for exposure times which raised the temperature until approximately 50°, 60° and 70°C was reached, and the samples were removed, diluted and plated on nutrient agar. This was repeated several times corresponding to different growth phases. All plates were incubated at 37°C for 18 h.

3.3.5 RELEASE OF RNA

A 20 ml aliquot of a shaken overnight culture was centrifuged at 4000 x g for 10 min and the supernate discarded. The cells were washed twice and resuspended in

distilled water to an OD_{600} of 0.5, equivalent to ~5 x 10^8 ml^{-1}. A 2.0 ml sample was exposed to Nd:YAG laser light until 50°C was reached, and with a fresh sample, until 60°C was reached. The exposed samples, a control (no exposure) and distilled water were centrifuged at 4000 x g for 10 min. The supernate was removed and analyzed for the presence of ribose by the method of Ceriotti (1955).

3.3.6 SCANNING ELECTRON MICROSCOPY

An overnight bacterial suspension was diluted in nutrient broth to an OD_{600} of 0.7 against a nutrient broth blank. Without exposure a sample was removed as a control. A 2.0 ml sample of the bacterial suspension was exposed to Nd:YAG laser light, at the parameters shown in **Table 3.3**, until 40°C was reached. This was repeated with fresh samples to final temperatures of 50°, 60° and 70°C. For comparison with the laser exposure, samples were heated in a water-bath at the same temperatures and at 100°C. Samples were pelleted by centrifugation (Biofuge 13, Heraeus Sepatech, Germany) at 7500 x g for 1 min and resuspended in 2.5% v/v glutaraldehyde (Sigma) in PBS for 1 h. Each sample was centrifuged, washed three times in PBS for 5 min and suspended in 1% w/v osmium tetroxide (OsO_4, TAAB Laboratory Equipment) in PBS for 1 h. The samples were pelleted again, washed three times in PBS for 10 min and suspended in 0.5% w/v aqueous uranyl acetate (Agar Scientific Int., UK) for 1 h in the dark. Each sample was centrifuged and resuspended in distilled water. The fixed cells were dehydrated in an acetone series (30, 50, 70, 90% v/v, absolute and twice with dried absolute acetone), critical point dried (Cowell, 1987), mounted on aluminum stubs and gold coated.

3.3.7 DIFFERENTIAL SCANNING CALORIMETRY.

An 18 h *E. coli* suspension was centrifuged at 4000 x g for 10 min and the supernate discarded. The culture resuspended in Tris buffer (pH 7.2; Boehringer Mannheim) to an OD_{600} of 0.7 against a Tris buffer blank. Without exposure a 15 ml sample was removed as a control. A 2.0 ml sample of the bacterial suspension was exposed to Nd:YAG laser light, at the parameters shown in **Table 3.3**, until 50°C was reached.

This was repeated with fresh samples until 16 ml of exposed sample was obtained. This procedure was repeated at temperatures of 60° and 70°C.

For comparison with laser exposure, the samples were heated in a water-bath. A 15 ml sample of the *E. coli* suspension (OD_{600} of 0.7 in Tris), in a test tube, was placed in the water-bath at 50°C. After shaking for 1 min the test tube was left in the waterbath for a further 5 min. This was repeated for temperatures of 60° and 70°C. The samples were prepared for DSC by centrifugation (4000 x g, 10 min) and resuspended to 10 mg *E. coli* cells ml^{-1}.

Thermal transitions in the *E. coli* suspension were obtained by differential scanning calorimetry (DSC) in a Microcal MC2-D instrument (Microcal Inc., Northampton, MA 01060-2327, USA) under standard operating conditions (Cooper and Johnson 1994a; Cooper and Johnson 1994b). Cell suspensions in Tris (pH 7.2; Boehringer Mannheim) were degassed briefly under moderate vacuum, with gentle stirring at room temperature, prior to loading into the DSC sample cell (active cell volume approximately 1.4 ml), and then scanned from 20 to 100°C at a nominal scan rate of 60 °C h^{-1} under 2-3 atm N_2 pressure (to inhibit further degassing and bubble formation). Occasional re-scans were performed after cooling the samples *in situ* in the DSC tantilum cell. The reference cell of the DSC contained degassed buffer alone, and instrumental baselines were obtained from repeat scans under identical conditions with buffer in both cells. Raw DSC data were scan-rate normalised and corrected by subtraction of control buffer baselines.

3.3.8 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

An 18 h *E. coli* suspension was centrifuged at 4000 x g for 10 min and the supernate discarded. The culture resuspended in PBS (pH 7.2) to an OD_{600} of 0.7 against a PBS blank. Without exposure a 2.0 ml sample was removed as a control. A 2.0 ml sample of the bacterial suspension was exposed to Nd:YAG laser light, at the parameters shown in **Table 3.3**, until 40°C was reached. This was repeated with fresh samples to temperatures of 50°, 60° and 70°C. For comparison with laser exposure, the samples were heated in a water-bath. Aliquots of *E. coli* suspended in

PBS (2.0 ml) at an OD_{600} of 0.7, were transferred to a test-tube and placed in a waterbath at 40°C. After shaking for 1 min the test-tube was left in the waterbath for a further 5 min. This was repeated for temperatures of 50°, 60°, 70° and 100°C.

SDS-PAGE was done by the method of Laemmli (1970) on 1.5 mm thick SDS-polyacrylamide gels with a vertical slab gel tank. The stock solutions and recipes for gel and buffer preparations are shown in Appendix 2. *E. coli* samples were solubilised in equal volumes of solubilising buffer and boiled in a waterbath at 100°C for 5 min. As molecular weight standards, SDS-6 and SDS-6H (Sigma) were run on either side of the gel. Samples were electrophoresed through the stacking gel and separating gel at 60 mA until the tracking dye reached the bottom of the gel. The gels were stained by the coomassie blue staining method of Weber and Osborne (1969).

3.3.9 ANALYSIS OF MICROCOLONIES

Fifty randomly selected *E. coli* microcolonies, from the periphery of the laser exposed areas, were subcultured onto minimal agar (recipe in Appendix 2) and nutrient agar. Colonies which grew on the nutrient agar but not on the minimal agar were further subcultured onto minimal agar containing amino acids, as detailed in Appendix 2.

3.3.10 EXPOSURE OF BACTERIA ON PLASTIC, GLASS AND STAINLESS STEEL SURFACES

Disks of plastic, glass and stainless steel (13 mm diameter) seeded with *E. coli, S. aureus, L. monocytogenes* and *B. cereus* as both wet and dry samples were placed in a holder similar to the diagram shown in **Figure 3.4**. The holder was situated so that the disks were centred directly beneath the laser beam and the laser was fired. The parameters for each experiment with Nd:YAG laser light, are shown in **Table 3.4, 3.5** and **3.6** which correspond to parameter requirements for the production killing curves, multifactorial experiments with variable laser parameters and multifactorial experiments with variable environmental parameters, respectively. **Table 3.7** details

the parameters required for the production of killing curves with CO_2 laser light. After exposure the disks - with controls - were transferred to 20 ml PBS (pH 7.2) and shaken at 200 rpm for 3 h. The PBS solution was diluted and plated by the method of Miles and Misra and incubated for 18 h. Each experiment was done several times for statistical purposes. Aliquots (10 ml) of the 20 ml PBS were filtered through 0.45 μm nitrocellulose filters (Whatman) and the filters were placed upright on nutrient agar plates and incubated for 3 h.

3.3.11 EXPOSURE OF *S. AUREUS* ON COLLAGEN SKIN

Collagen skins inoculated with *S. aureus*, as detailed in section 3.17, spread on petri-dishes were placed directly beneath the laser beam and exposed to laser light, at the parameters shown in **Table 3.4** for 10-30 sec (Nd:YAG) and **Table 3.5** for 1-8 msec (CO_2 laser). The collagen was removed from the petri-dishes and placed face-up on the nutrient agar plates, which contained 0.005% w/v INT, and incubated 37°C for 18 h.

Parameter	*E. coli*	*S. aureus*	*B. cereus*	*L. mono*
Pulse energy (J)	10	10	10	10
PRF (Hz)	30	30	30	30
Pulse width (msec)	8	8	8	8
Beam diam. (cm)	1.3	1.3	1.3	1.3
Exposure time (sec)	variable	variable	variable	variable

Table 3.4 **Nd:YAG laser parameters used for exposure of bacteria on surfaces to produce killing curves.**

Parameter		*E. coli*	*S. aureus*	*B. cereus*	*L. mono*
Pulse energy (J)		5&10	5&10	5&10	5&10
PRF (Hz)		20&30	20&30	20&30	20&30
Pulse width (msec)		8	8	8	8
Beam diam. (cm)		1.3	1.3	1.3	1.3
Exposure time (sec)	WET	0.5&1.0	1.0&2.0	0.5&1.5	0.5&1.0
	DRY	0.5&1.5	1.5&2.5	0.5&1.5	NA

Table 3.5 **Nd:YAG laser parameters used in multifactorial experiments on stainless steel surfaces.**

Parameter	*E. coli*	*S. aureus*	*B. cereus*	*L. mono*
Pulse energy (J)	10	10	10	10
PRF (Hz)	30	30	30	30
Pulse width (msec)	8	8	8	8
Beam diam. (cm)	1.3	1.3	1.3	1.3
Exposure	1.5	2.0	1.5	0.5

Table 3.6 **Nd:YAG laser parameters used in multifactorial experiments, varying the environmental parameters, on stainless steel surfaces.**

Parameter	*E. coli*	*S. aureus*	*B. cereus*	*L. mono*
Power (W)	800	800	800	800
Beam diam. (cm)	1.2	1.2	1.2	1.2
Exposure time (sec)	variable	variable	variable	variable

Table 3.7 **CO_2 laser parameters used for exposure of bacteria on surfaces to produce killing curves.**

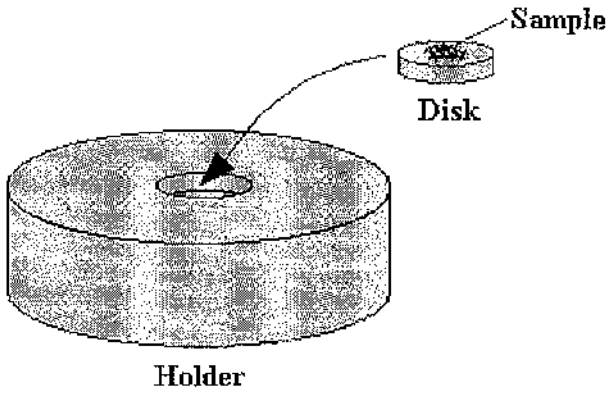


Figure 3.4 **Diagram showing the exposure set-up for metal, plastic and glass surfaces.**

4. RESULTS

4.1 THE EFFECT OF Nd:YAG LASER LIGHT ON MICROORGANISMS LAWNED ON AGAR SURFACES

4.1.1 DEVELOPMENT OF THE IA_{50}-VALUE

A quantitative method was developed to monitor microbial sterilization of an agar surface by Nd:YAG laser light and the non-uniformity of energy through the cross-sectional area of the beam was utilised. Although the light from the Nd:YAG laser was monochromatic and coherent, with a readily-measured energy in Joules (J) per pulse, the energy of the beam was not spatially uniform. **Figure 4.1** shows the beam profile of the Nd:YAG laser (Yeo, PhD thesis for submission in 1998). The profile was measured with a profilometer which measured a series of two dimensional sections through the beam producing a 3-dimensional profile. The base of the profile shows the circular cross-section through the laser beam, with flat areas outside the beam area. The energy, shown by the height of the peak indicates that the intensity increased towards the centre of the beam. As the Nd:YAG laser beam distribution was close to Gaussian and consequently non-uniform spatially, it was possible to exploit this fact to quantify the lethal effect of laser light on microorganisms lawned on agar surfaces. As the overall energy increased, the intensity throughout the beam area increased. When the intensity at the centre reached a value greater than that of the microbial resistance it cleared a circular area in the lawn of organisms. As the total energy was increased the intensity over a larger cross-sectional area of the beam was high enough to kill microorganisms in the lawn. With this knowledge it was possible to relate areas of microbial killing on agar surfaces to the laser light sensitivity of that organism. In an attempt to produce a procedure to compare the effects of laser light on the different organisms tested, several possible methods were tried. The method finally chosen was that of the IA_{50}-value, loosely based on the more common LD_{50}-values. The energy density at 0.825 cm^2 (one half the beam area of 1.65 cm^2) was noted and this was used as a standard intercept. It was proposed to denote the energy density at 50% beam area, the IA_{50}-value for each microorganism. The full definition of the IA_{50}-value is the energy density required to produce a inactivated/sterilized area equal to half of the beam area on a lawn of microorganisms on an agar plate. This procedure was then used in all of the

Figure 4.1 Energy profile through the cross-section of the Nd:YAG laser beam. The base shows the circular beam area, with the flat portion outside the beam area. The height of the Gaussian distribution describes the intensity of the beam.

experiments with measurable sterile areas on agar plates.

With lawn-inoculated nutrient agar culture plates, and the dimensions of the laser beam, it was convenient to have 6 exposure sites per plate, each site receiving a different dose of radiation in a graded series. The plates, after incubation, revealed zones of clearing where the microorganisms had been killed, against a background of a uniform lawn of growth (**Figure 4.2**).

Very little damage to the agar was observed at lower energy densities, where smaller sterile areas were produced. At the higher energy densities the agar within the sterile areas were slightly concave in shape until the highest energy densities were reached, where visible melting of the laser was observed. After exposure to lower energy densities of laser light, the number of viable bacteria within the exposed area was reduced, however no sterile area was produced.

4.1.2 LASER LIGHT EXPOSURE OF DIFFERENT MICROBIAL SPECIES

The microbicidal effect of the Nd:YAG laser was examined on a range of microorganisms selected for their diverse physiology and morphology. The range of seven bacteria and two yeasts are shown in **Table 4.1** alongside the reasons for choice of each organism. Three of the nine microorganisms were Gram +ve cocci, two were Gram +ve rods, two were G-ve rods and two were yeast. Three of the bacteria were pigmented, *Deinococcus radiodurans* was highly resistant to ionising radiation and *Bacillus stearothermophilus* was temperature resistant. Each of these microorganisms, lawned on nutrient agar was exposed to Nd:YAG laser light at increasing energy densities until a number of sterilized areas was produced. The cleared circles in the microbial lawns were measured and the areas determined. Killing curves of the nine microbial cultures, with replicate data points, for each energy density, were obtained (**Figure 4.3**).

For each species the results were similar. As the energy density was increased the area of the clearing also increased, in a uniform manner. For the range of energy

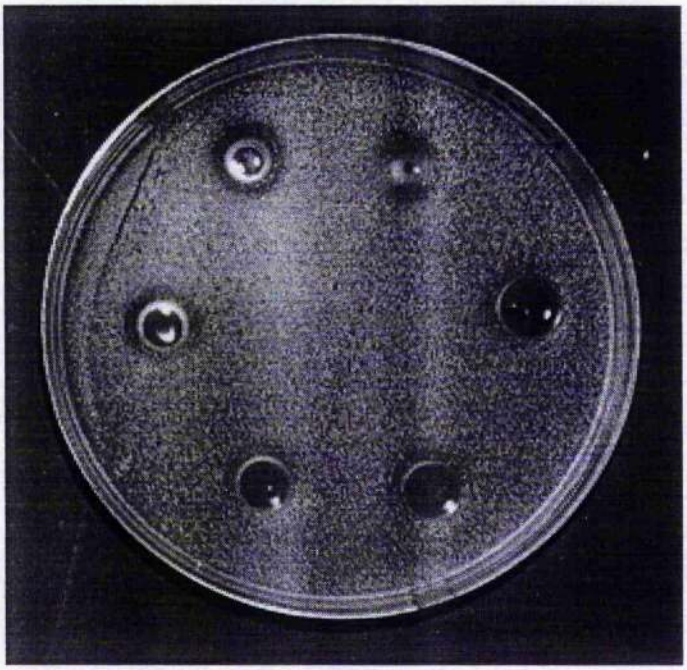


Figure 4.2 **Photograph of a lawn of *Micrococcus luteus* after Nd:YAG laser exposure and incubation**. The circles of clear agar are the sterile areas of the bacterial lawn. The figure shows increasing energy densities in an anti-clockwise direction from the uppermost clear areas.

Microorganism	Characteristics
Escherichia coli	Ubiquitous, common food pathogen, Gram -ve rod
Staphylococcus aureus	Ubiquitous, common food pathogen, Gram +ve cocci
Deinococcus radiodurans	Radiation resistant, orange pigment, Gram +ve cocci
Micrococcus luteus	Yellow pigment, small Gram +ve cocci
Serratia marcescens	Red pigment, small Gram -ve coccobacillus
Bacillus subtilis	Gram +ve rod
Bacillus stearothermophilus	Temperature resistant, Gram +ve rod
Candida albicans	Yeast
Saccharomyces cerevisiae	Yeast

Table 4.1. Table with seven bacteria and two yeasts chosen for experimentation with the specific characteristics determining their selection.

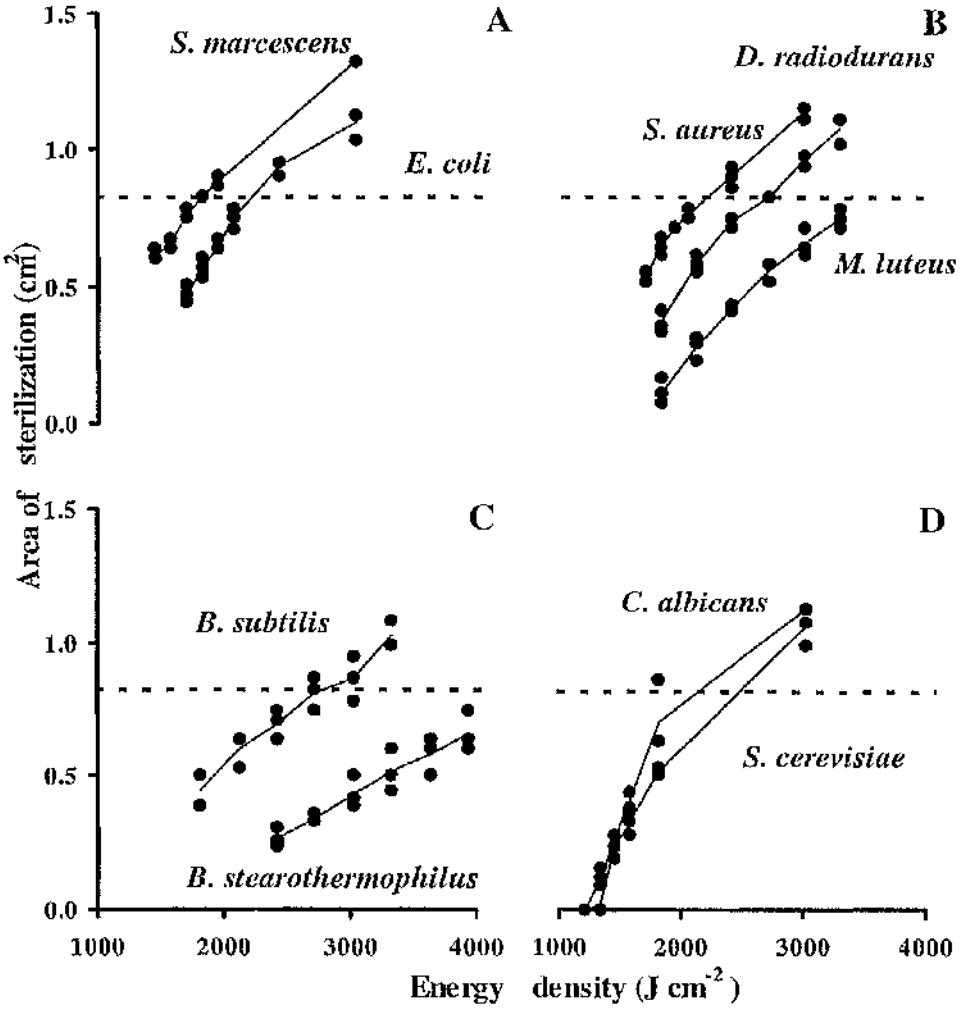


Figure 4.3 **Lethal effect of Nd:YAG laser light on lawned microbial cultures.** Four graphs showing the killing curves for the nine microbial cultures, obtained from measurement of the sterile areas after exposure to different laser energy densities. Each curve was plotted through the mean of triplicate experimental values as shown on each graph. The individual graphs show the killing curves for: **A.** Gram-negative rods; **B.** Gram-positive cocci; **C.** Gram-positive rods and **D.** yeasts. The dotted line is the intercept for the IA_{50}-values for each microorganism.

densities selected, the curves show a relatively straight line, with the only exceptions being the yeasts and *E. coli*. The higher energy density points for each of these three organisms were slightly deviated from the straight line. The yeasts appeared to be the most sensitive of the nine cultures tested, while the vegetative cells of *B. stearothermophilus* were the most resistant. For both of the yeast cultures the gradient of the killing curve appeared to be greater than the other organisms indicating that they could be killed more rapidly, however when the curve was extrapolated back to the x-axis the interception point was higher than that of the others. The final points, which overlap for both yeast cultures, were slightly offset from a straight line, thus indicating that the killing curves were approaching a maximum area of sterilization. Ideally there should have been an exposure at 2500 Jcm^{-2} which might have shown this more clearly. The Gram +ve cocci and the Gram -ve rods had very similar killing rates, indicated by the gradient of the straight line, the only difference being that there was a lateral shift with each bacterial species. Of these five bacteria *Micrococcus luteus* was the most resistant and *Serratia marcescens* the most susceptible. *Bacillus subtilis* and *B. stearothermophilus* were very different in their response to the Nd:YAG laser light, with the gradient of the *B. stearothermophilus* killing curve markedly lower than the other eight microorganisms. Statistical analysis of the data in the regions close to the IA_{50}-intercepts gave point estimates of the IA_{50}-values (with 95% confidence limits) in Jcm^{-2}. The approximate concentration of bacteria (excluding *D. radiodurans*) in the 0.825 cm^2 sterile area was 8 x 10^5 cfu, while *D. radiodurans* and the yeasts were at an approximate concentration of 4 x 10^4 cfu. On each graph there is a horizontal dashed line indicating the standard intercept for the calculation of the IA_{50}-value. The IA_{50}-values with 95% confidence limits for the laser sterilization of the seven bacterial and two yeast species, are summarised in **Figure 4.4.** The tests were made on two different occasions with triplicate plates per culture being exposed on each occasion. The figure includes, in summary form, the data represented in **Figure 4.3**, as individual data points. With each species, the IA_{50}-values were throughout somewhat higher on the second testing occasion, a difference which can be attributed to either a small alteration in the height of the stand on which the culture plates were positioned beneath the laser beam, or a difference in the ambient temperature which may have affected the laser and/or the microorganisms. However, both sets of IA_{50}-

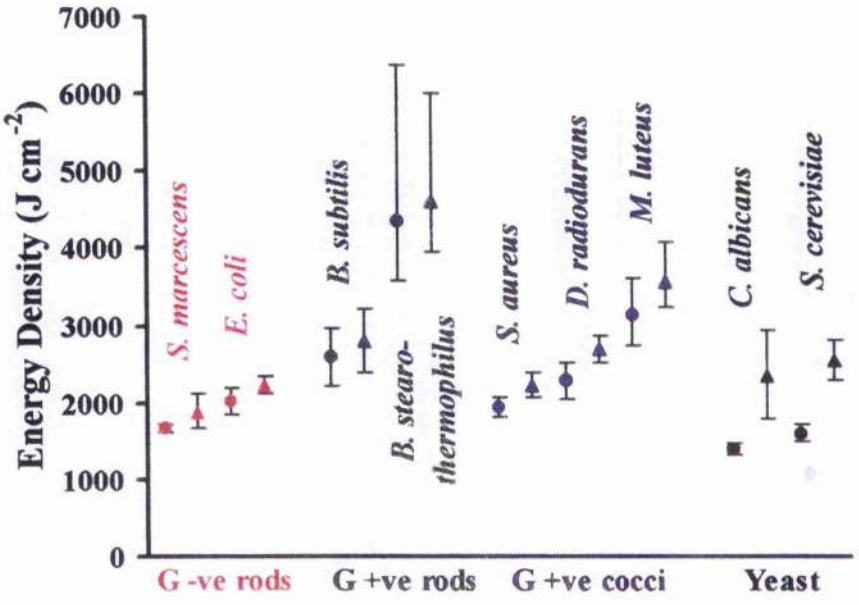


Figure 4.4 **Comparative sensitivities of seven bacterial and two yeast strains to Nd:YAG laser light (10 J, 8 msec, 10 Hz).** Each point is the energy density required to produce an IA of 0.825 cm^2. The errors are 95% confidence limits. The different symbols represent the two separate occasions, four months apart, when the experiments were done.

values for the bacterial cultures fell within the 95% confidence limits, though both yeast cultures showed distinct differences on each occasion, outside the confidence limits. These differences, however, did not obscure the consistent variation in sensitivity between some of the species within a relatively small overall range from most to least sensitive species.

The average IA_{50}-values from the two test occasions revealed, the most sensitive to the most resistant microbial strains (**Table 4.2**). *Ser. marcescens* appeared to be the most sensitive species, followed by the yeasts, *C. albicans* and *Sac. cerevisiae*. Both yeast cultures showed a big difference in their response to the laser light on the two testing occasions, making the averages larger than *Ser. marcescens*. However, the differences in the IA_{50}-values for *C. albicans, Sac. cerevisiae* and *Ser. marcescens* were not large. *St. aureus* and *E. coli* displayed a similar resistance, and the IA_{50}-values did not differ significantly from the yeasts. *D. radiodurans* and the vegetative cells of *B subtilis* also showed a similar resistance but slightly more tolerance than *St. aureus, E. coli, C. albicans, Sac. cerevisiae* and *Ser. marcescens*. *M. luteus* showed a very low susceptibility to the laser light requiring almost double that of *Ser. marcescens* to sterilize the same area, on the surface of the nutrient agar plate. Finally, vegetative cells of *B. stearothermophilus* were confirmed as the most resistant to laser killing with a high IA_{50}-value (over double that of the five most sensitive microorganisms). With the laser delivering 10 J pulses at 10 Hz, the exposure times required to produce the IA_{50}-values for *C. albicans* and vegetative *B. stearothermophilus* were 22.9 and 72.1 sec respectively. The difference in average IA_{50}-values between the least and most sensitive vegetative species was a factor of only 2.54. The laser exposed plates were incubated for up to fourteen days without evidence of growth in the cleared areas. Similarly, no growth occurred when sections of the laser exposed areas were removed and imprinted on fresh agar.

Limits to the length of exposure were imposed by the melting of the agar. An energy density of 2000 Jcm^{-2}, corresponding to an exposure of 33 sec, produced a slight concavity in the agar, while 5000 Jcm^{-2} melted the agar medium through about one-half of its 6 mm thickness. Apart from the areas of localised melting at high energy

Microorganism	Average IA_{50}-value
Ser. marcescens	1768
C. albicans	1869
Ssc. cerevisiae	2076
St. aureus	2086
E. coli	2123
D. radiodurans	2492
B. subtilis	2690
M. luteus	3347
B. stearothermophilus	4489

Table 4.2 **The average IA_{50}-values, for each of the microorganisms.** The values, obtained from sterilization of lawns of bacteria with Nd:YAG laser light, are presented in ascending order.

densities, the agar medium was not altered in its growth-supporting properties as revealed by incubating plates that were laser-treated before lawning.

4.1.3 EXPOSURE OF *E. coli* AT DIFFERENT LASER PARAMETERS

The Nd:YAG laser system in the investigation, delivered a pulsed output and therefore three parameters related to pulsing were further considered in exposing the microbial subject: the pulse repetition frequency (PRF) measured in Hertz, which describes the number of pulses delivered to the subject in one second, the pulse energy (Joules) describing the energy delivered to the subject in a single pulse and the pulse width (milliseconds) describing the duration of the pulse. As well as these three parameters, the effect of the beam diameter was investigated.

4.1.3.1 Pulse repetition frequency (PRF)

The laser had the capacity to produce PRF values of 1 to 200 Hz, but high PRF could only be accompanied with very low pulse energies, producing power levels well below the maximum of the machine. The pulse width affects the PRF such that the pulse width (in seconds) cannot be higher than the reciprocal of the frequency, i.e. a 100 msec (0.1 sec) pulse could not be accompanied with a PRF of 10 Hz, or it would be a continuous wave. To utilise the full power of the machine a range of PRF values was selected that could incorporate a pulse energy of 10 J. The PRF selected were: 5, 10, 15, 20, 25 and 30 Hz. The pulse width was 8 msec.

The effect of Nd:YAG laser light at different PRF on *E. coli* cultures lawned on agar plates was investigated with exposure to increasing energy densities to produce a number of sterile areas. Killing curves were plotted showing the area of bacterial sterilization against the energy density applied to the lawned culture on the agar plate, for each of the six PRF values. The energy densities in this experiment were varied with increasing exposure time and all other laser parameters were kept constant. Killing curves, obtained from the sterile areas of the lawn of *E. coli* at the different PRF, with replicate data points, for each energy density, are shown in **Figure 4.5**. Each curve shows a uniform rise in the area of sterilization of the

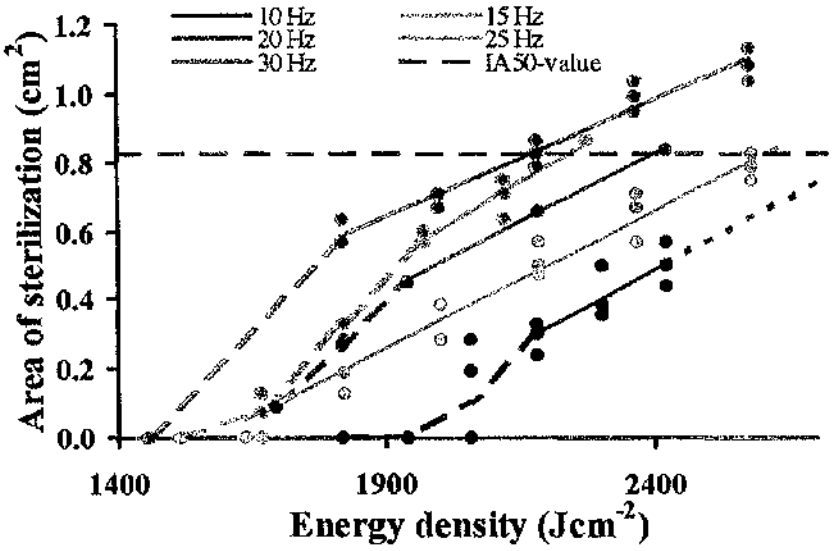


Figure 4.5 **Effect of PRF on laser light sterilization on *E. coli* lawned on nutrient agar plates.** Each curve is plotted through the mean of triplicate experimental values; all experimental values are shown. In each curve the solid line is the best fit through the points closest to an area of 0.825 cm^2. The horizontal dotted line shows intercept at the IA_{50}-value for each pulse repetition frequency.

bacterial lawn with an increase in applied energy density. The individual curves are approximately straight lines, as obtained previously (**Figure 4.3**). The solid line shows the best fit for the calculation of the IA_{50}-value, obtained from the point of interception with the black, horizontal dashed line. The dashed lines on each curve join the remaining points connected through the average areas of bacterial killing.

At the lowest frequency of 5 Hz, no sterilized areas were observed within the range of energy densities levels shown in **Figure 4.5** and therefore no curve for this PRF was presented. All curves intercept the x-axis where no sterilized areas were observed. It is apparent that the higher the PRF, the lower the energy density required to produce a sterilized area. At 10 Hz the lawn of *E. coli* required higher energy densities of laser light, to produce a sterilized area, than at a PRF of 30 Hz, indicating that 30 Hz was more effective in the decontamination of agar surfaces. For each killing curve the gradients were similar, but the smaller areas showed more inconsistency, possibly due to the methods of area measurement and manual timing of exposure. It can be seen that the curves were parallel but shifted laterally, but the gradients were very similar.

At the point of interception of each curve, and extrapolation of the curve at 10 Hz, with the horizontal dashed line in **Figure 4.5**, the IA_{50}-values were determined and are presented in **Figure 4.6**. This figure summarises the effect of varying the pulse frequency on *E. coli* lawned on an agar plate. At the lower PRF, the *E. coli* cultures lawned on the nutrient agar plates required 2800 Jcm^{-2} to clear an area in the lawn of 0.825 cm^2. At a PRF of 30 Hz an energy density of 2200 Jcm^{-2} produced the same effect. As the PRF was increased from 10 to 30 Hz there was a uniform decrease in the IA_{50}-value. For the first four frequencies (10, 15, 20 and 25 Hz) the decrease in the IA_{50}-value was linear, and the last point, at 30 Hz, was slightly offset. From these data a PRF of 5 Hz would be expected to have an IA_{50}-value of around 3000 Jcm^{-2}.

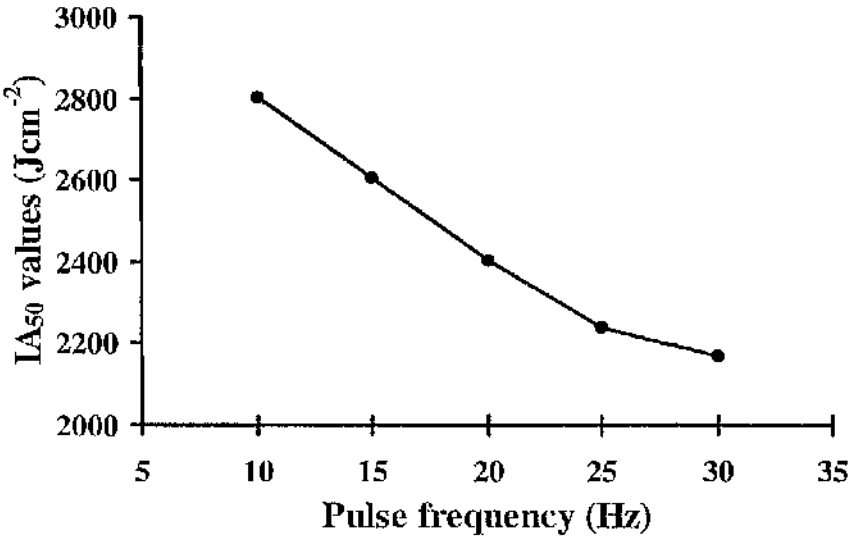


Figure 4.6 **Comparative sensitivities of *E. coli* to Nd:YAG laser light at different pulse repetition frequencies: 10, 15, 20, 25 and 30 Hz**. Each point is the energy density required to produce an IA_{50}-value. The values on this graph are obtained from the average of the results displayed in **Figure 4.5**.

4.1.3.2 Pulse energy

The pulse energy, by definition, is the energy delivered in one pulse. With the Nd:YAG laser used in this study, variation in pulse energy over the pulse length was relatively uniform as shown in **Figure 4.7**. At the start of the pulse, however, there was a small energy peak followed by uniform delivery of energy over the remainder of the pulse.

The Nd:YAG laser used here had a maximum pulse energy of 60 J, but the laser manual stated that above 55 J the laser lamps, within the cavity, could deteriorate rapidly and might be destroyed. The maximum pulse energy was also governed by the PRF, in that the total power (the product of energy and PRF) could not exceed the maximum power rating of the laser. The pulse width also dictated the maximum pulse energy available. Short pulse widths could not support high pulse energies.

The effect of increased pulse energy, on an *E. coli* suspension lawned on nutrient agar, was examined. To explore the power range of the machine a series of pulse energies was selected which could incorporate a PRF of 10 Hz. The pulse energy values selected were: 5, 10, 15, 20, 25, 30 and 40 J. The pulse width was 8 msec. *E. coli*, lawned on nutrient agar was exposed to Nd:YAG laser light at increasing energy densities until a number of cleared areas was produced. The sterilized circles in the microbial lawns were measured and the areas determined. Killing curves were plotted showing the area of sterilization against the energy density applied to the lawned culture on the agar plate, for each of the seven different pulse energies (**Figure 4.8**). The energy densities in this experiment were varied with increasing exposure time, but all other laser parameters remained constant.

Each curve shows a uniform rise in the sterile area in the bacterial lawn with the increased applied energy density. At the lowest pulse energy (5 J), no sterilized areas were observed within the range of energy densities in **Figure 4.8**. As the pulse energy was increased the energy density required to produce a sterilized area decreased. The gradients were similar, for each killing curve, but each was shifted

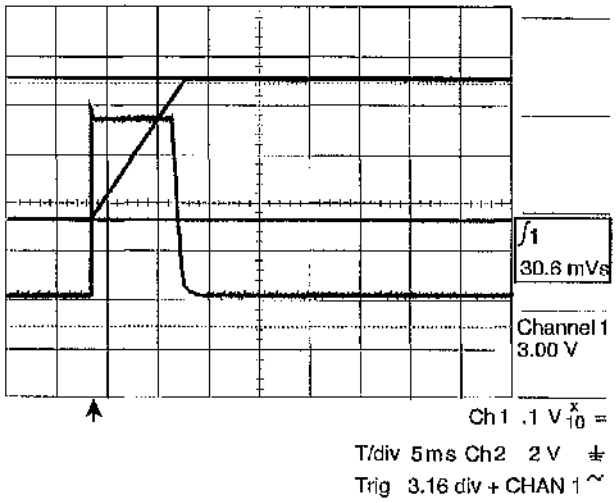


Figure 4.7 **The distribution of energy during an 8 msec pulse.**

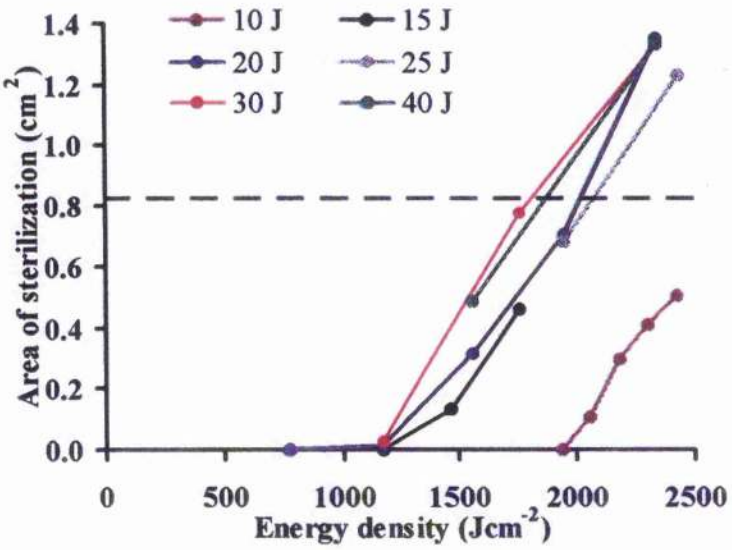


Figure 4.8 **Effect of pulse energy on the laser sterilization of *E. coli* lawned on nutrient agar.** Graph showing six killing curves of *E. coli* cultures obtained from the areas of sterilization on nutrient agar, against increasing energy densities at different pulse energies. Each curve is plotted through the mean of duplicate experimental values. The horizontal dotted line shows the intercept at the IA_{50}-value for each pulse energy.

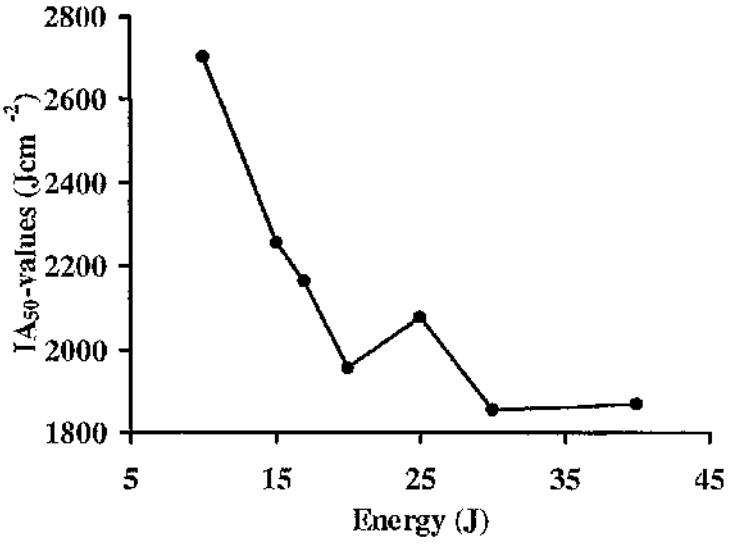


Figure 4.9 **Comparative sensitivities of *E. coli* to Nd:YAG laser light at different pulse energies, 10, 17, 15, 20, 25, 30 and 40 J.** Each point is the energy density required to produce an IA of 0.825 cm^2. The values on this graph are obtained from the average results displayed in the killing curves in **Figure 4.8.**

laterally as the pulse energy changed so that the laser, at an energy of 40 J, sterilized the lawn of *E. coli*, more readily than 10 J.

From the intercept at 10 and 15 J, with the horizontal dashed line in **Figure 4.8**, the IA_{50}-values were determined and are presented in **Figure 4.9**. This figure summarises the effect of varying the pulse energy on a lawn of *E. coli* on nutrient agar. At the lowest pulse energy (10 J), laser light at an energy density of 2700 Jcm^{-2} was required to sterilize 0.825 cm^2 of lawned *E. coli*. At pulse energies of 30 and 40 J, an energy density of around 1850 Jcm^{-2} had the same effect. Thus, increasing from 10 to 40 J produced a decrease, almost exponentially in the IA_{50}-value. The first four pulse energies (10, 15, 17 and 20 J) were linear, with the final two points, at 30 and 40 J, sharing similar IA_{50}-values. At 25 J a rise in the IA_{50}-value was observed. With these data it was estimated that the IA_{50}-value at 5 J would be 3000 J cm^{-2}.

4.1.3.3 Pulse width

The pulse width is the duration of a single pulse of laser light. This is distinct from the total exposure time which indicates the over-all time that the laser is fired. During the length of the pulse, the laser delivers energy to the subject. The length of a pulse is affected by the PRF (see section 4.1.3.1), and the pulse energy settings (see section 4.1.3.2). The laser used in this investigation has a minimum pulse width of 0.2 msec and maximum of 19.8 msec.

E. coli, lawned on nutrient agar was exposed to Nd:YAG laser light at increasing pulse widths which produced a number of sterile areas within the lawn. Killing curves were plotted showing the area of clearing of the *E. coli* lawn against the energy density applied to the lawned culture on the agar plate, for each of six different pulse widths: 4, 8, 10, 12, 16 and 19.8 msec. The energy densities in this experiment were varied with increasing, exposure time and all other laser parameters remained constant. Killing curves, obtained from the circular sterilization of the lawn of *E. coli* at the different pulse widths, with replicate data points, for each energy density, are shown in **Figure 4.10**.

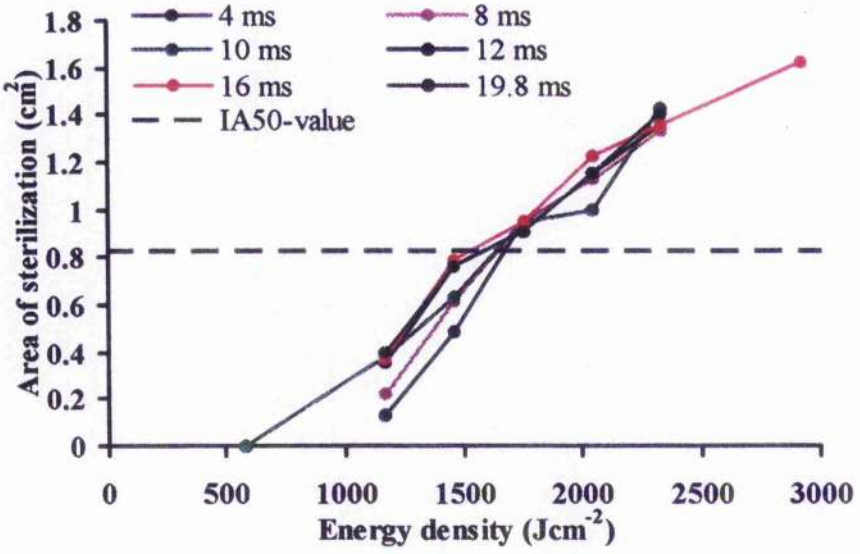


Figure 4.10 **Effect of pulse width on the laser sterilization of *E. coli* lawned on nutrient agar.** Graph showing six killing curves of *E. coli* cultures obtained from the areas of inactivation on nutrient agar, against increasing energy densities, at different pulse widths. Each curve is plotted through the mean of triplicate experimental values. At each pulse width a line best fit was drawn through the points and the IA_{50}-value was measured at 0.825 cm^2. The horizontal dotted line shows intercept at the IA_{50}-value for each pulse width.

As previously seen with the bacterial lawns treated with laser light, there was a uniform rise in the area of bacterial clearing as the energy density was increased. Each curve in **Figure 4.10** was a relatively straight line which intercepted the black horizontal dashed line which gave the IA_{50}-values. The results show that varying the pulse width, within the capability of this machine (0.2 msec to 19.8 msec), had little or no effect on the killing curves. All of the curves representing the different pulse widths were intertwined around each other, such that at around 1800 Jcm^{-2} the areas of sterilization for each curve were very similar. At the lower energy densities there was greater variation in the sterilized areas, indicating inherent inaccuracies brought about by the method of measuring the exposure time and sterile area. The points of intercept with the horizontal dashed line gave IA_{50}-values for each pulse width and are shown in **Figure 4.11.**

Little or no effect on the IA_{50}-value was observed, over the range of pulse widths investigated. The laser could not produce 10 J pulses at pulse widths lower than 4 msec and therefore this was accepted as the lower parameter value. The figure shows that there were negligible differences in the sterilization efficiency of the Nd:YAG laser as the pulse width was increased or decreased, over this range of pulse widths.

4.1.3.4 Beam diameter

Increasing the beam diameter has the effect of expanding the Gaussian distribution shown in **Figure 4.1**, without varying the overall energy of the beam. This is important when an efficient method to sterilize a large area is contemplated; the laser beam could be raster scanned over the area as either a focused beam or an unfocused beam with variable diameters. Alternatively, the beam diameter could be increased to the size of the area to be decontaminated, depending upon the dimensions of that area. An important aspect to be considered for these purposes, therefore, was the most effective beam diameter and therefore the most effective scanning speed.

With a focused beam and variable platform height, the lethal effect of Nd:YAG lasers at variable laser beam diameters was investigated, on *E. coli* cultures lawned

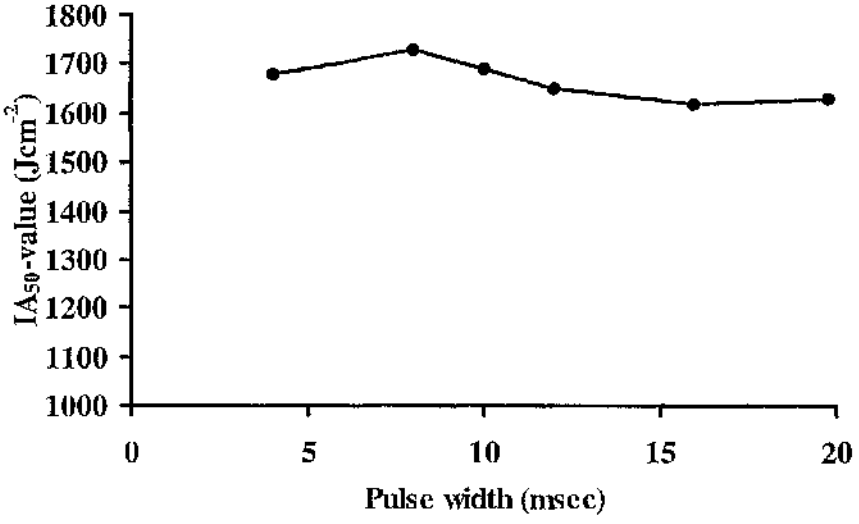


Figure 4.11 **Comparative sensitivities of *E. coli* to Nd:YAG laser light at different pulse widths: 4, 8, 10, 12, 16 and 19.8 msec.** Each point is the energy density required to produce an IA of 0.825 cm^2. The values on this graph were obtained from the average results shown in **Figure 4.10**.

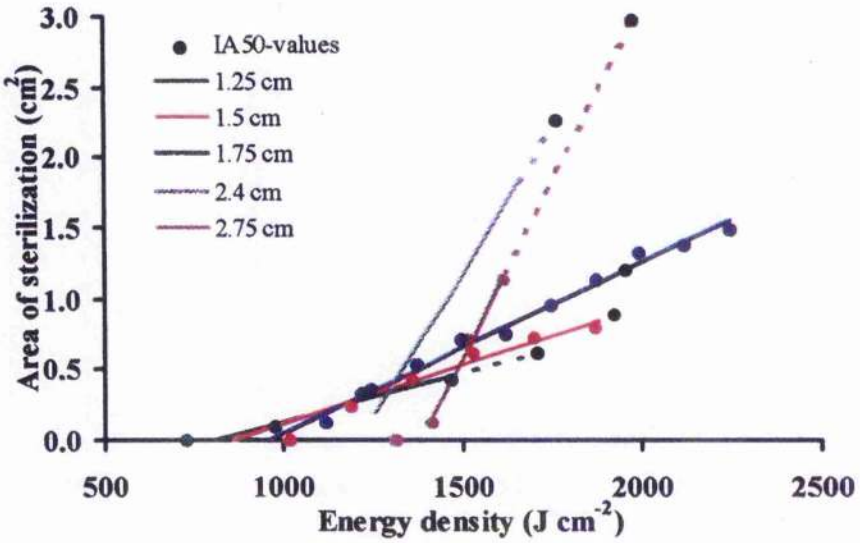


Figure 4.12 **Effect of beam diameter on the laser sterilization of *E. coli* lawned on nutrient agar.** Graph showing five killing curves *of E. coli* cultures obtained from the areas of inactivation on nutrient agar, against increasing energy densities at different beam diameters. Each curve is plotted through the mean of duplicate experimental values. At each pulse width a line best fit was drawn through the points and the IA_{50}-value was measured at 50% of the beam area. The black circles show the IA_{50}-values for each beam diameter. The dotted lines are the extrapolation of the killing curves to the IA_{50}-values.

on nutrient agar plates. Killing curves were plotted showing the area of bacterial clearing against the energy density applied to the lawned agar plate, for each of five different beam diameters: 1.25, 1.5, 1.75, 2.4 and 2.75 cm. The energy density in this experiment was varied by increasing the exposure time. Killing curves of *E. coli* at five different beam diameters, plotted as averages of two points, for each energy density, are shown in **Figure 4.12.** As the energy densities increased so too did the areas of clearing, most notably at larger beam diameters. The larger beam diameters had the steepest gradients. In comparison with smaller beam diameters, higher energy densities were required to produce any cleared areas in the *E. coli* lawn with larger beam diameters (i.e. the larger beam diameters took longer to have an effect on the bacterial lawns). When lethal energy densities were reached, the area of bacterial sterilization increased more rapidly. The threshold energy density describes the intercept of each killing curve with the x-axis as the energy density above which a sterilized area was produced.

Figure 4.13 shows the increase in threshold energy density, as the beam diameter was increased, as a straight line. There was a large difference between the effect of laser light at smaller beam diameters and at larger beam diameters on the *E. coli* lawns. At a beam diameter of 1.25 cm the threshold energy density was ~800 Jcm^{-2}, with almost double this value required at a beam diameter of 2.75 cm. Extrapolation of this line (dotted line) to the y-axis shows that as the beam diameter approached zero the threshold energy density approached 295 Jcm^{-2}, which was the lowest predicted threshold energy density, with the parameters used.

Unlike previous graphs where the IA_{50}-values were calculated from the energy density required to produce a sterilized area of 0.825 cm^2, the diameter used to obtain IA_{50}-values varied. The killing curves were produced by laser beams of different diameters and therefore different beam areas. The sterilized area (50% beam area), for calculation of the IA_{50}-value, varied also. The black circles (**Figure 4.12**) indicate the point on each curve where the IA_{50}-value was calculated. The solid lines on each curve show the best fit used to obtain the IA_{50}-values and the dotted lines are the extrapolation of the curves to calculate the IA_{50}-values.

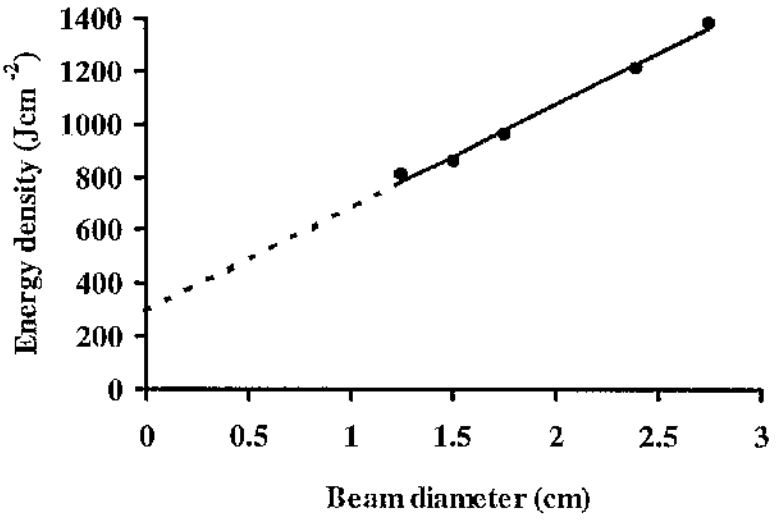


Figure 4.13 **Effect of beam diameter of Nd:YAG laser light on the threshold energy density.** The values on this graph are obtained from the average results displayed in **Figure 4.12**, and has been extrapolated back to the Y-axis (dotted line).

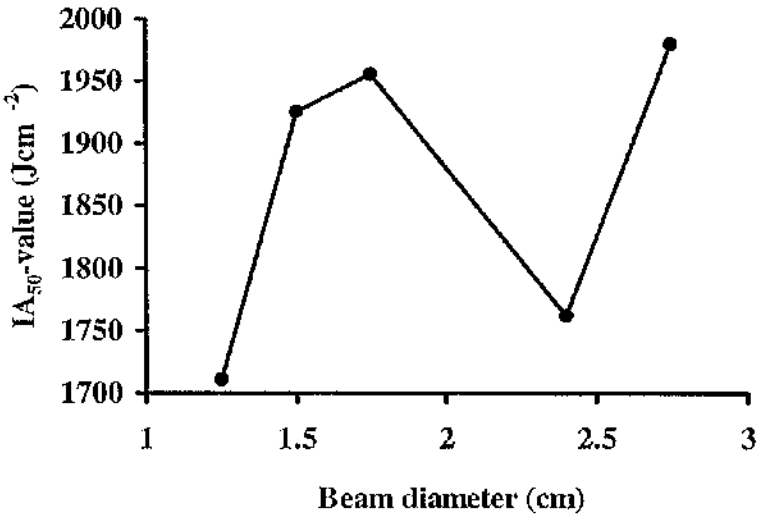


Figure 4.14 **Effect of beam diameter of Nd:YAG laser light on the IA_{50}-value.** The values on this graph are obtained from the average results displayed in **Figure 4.12**, and are connected by the bold line.

Figure 4.14 however, shows the effect of increasing the beam diameter on the IA_{50}-value. This figure indicates that as the beam diameter was increased the IA_{50}-values increased, until a plateau was reached. The IA_{50}-value at a beam diameter of 2.4 cm shows a deviation from this possible plateau. However, the difference between the highest IA_{50}-value and the lowest IA_{50}-value, which incidentally are the highest and lowest beam diameters, respectively, is only a matter of ~250 Jcm^{-2}, in contrast to the difference of~600 Jcm^{-2}, seen with the threshold energy densities.

4.1.4 TEMPERATURE OF AGAR EXPOSED TO LASER LIGHT

At the lowest lethal energy densities (~1500 J cm^{-2}) of laser light on *E. coli* lawned on nutrient agar there was little or no effect on the agar. As the energy densities were increased to 2000 Jcm^{-2} it was noticed that a slight concavity appeared in the agar, and at 5000 Jcm^{-2} the laser light melted the agar through half of its thickness. It was apparent that a temperature effect was occurring within the cleared area and so a method of measuring the temperature of the post-exposed area was investigated. The method chosen after much discussion was to insert a thermocouple into the agar after exposure and record the cooling temperature. The cooling curves were extrapolated back to the time of laser switch off and the maximum temperature thus calculated. The cooling curves obtained in the investigation are shown in Appendix 3. Inserting the thermocouple directly into the agar during exposure caused erroneous temperature measurements.

The final temperatures from the extrapolation of the cooling curves are shown in **Figure 4.15**. The graph shows the increase in the temperature of the exposed area with increasing energy densities for four different PRF. As the PRF increased, the gradient of the line, and therefore rate of heating increased. When the temperature reached around 75° - 80°C there was, in general, a decrease in the heating rate at 10, 20 and 30 Hz. The dashed line indicates the temperature at the threshold energy densities. Indeed, as the PRF decreased there was an increase in the temperature at the threshold energy density. At 30 Hz the value appears to be around 50°C as opposed to 55°C at 10 Hz.

4.1.5 PROLONGED EXPOSURE OF *E. COLI* LAWNS

The effect of possible accumulation of toxic elements within the *E. coli* cell, as a lawn on nutrient agar, during exposure to the Nd:YAG laser was investigated with prolonged exposure. A lawned plate was split into two and on one half an area of *E. coli* was exposed to 300 pulses of laser light at 30 Hz and 10 J; this took 10 sec. The other half was exposed to the same number of pulses but delivered evenly over 30 min (**Figure 4.16**). The area exposed to the 10 J pulses of light at 10 Hz were sterile, whereas 300, 10 J pulses delivered over 30 min had no visible effect.

4.1.6 INVESTIGATION OF MICROCOLONIES OF *E. COLI* AFTER LASER LIGHT EXPOSURE

When lawned bacteria were exposed to Nd:YAG laser light, a circle of clearing was left in the incubated lawn (**Figure 4.2**). These areas appeared to have a distinct border however, around the perimeter of the cleared areas, miniature colonies were frequently observed. It was possible that in these exposed areas the cells were exposed to sub-lethal laser energy densities, because of the non-uniform energy distribution of the laser beam. An example of these microcolonies is shown in **Figure 4.17**. This phenomenon appeared to be most prominent with lower energy densities, especially with cells which were exposed to energy densities slightly higher than the threshold energy density. Random samples of these microcolonies were replica plated onto *E. coli* minimal media and nutrient agar and incubated overnight at 37°C. The result showed that eight of the 50 randomly selected colonies grew on nutrient agar but not on the minimal agar. When the sub-lethally exposed cells, growing only on the nutrient agar, were sub-cultured onto minimal agar containing different combinations of amino acids and minimal agar itself they grew normally. No further work has been done to examine this phenomenon and to elucidate the damage undergone by these bacterial cells

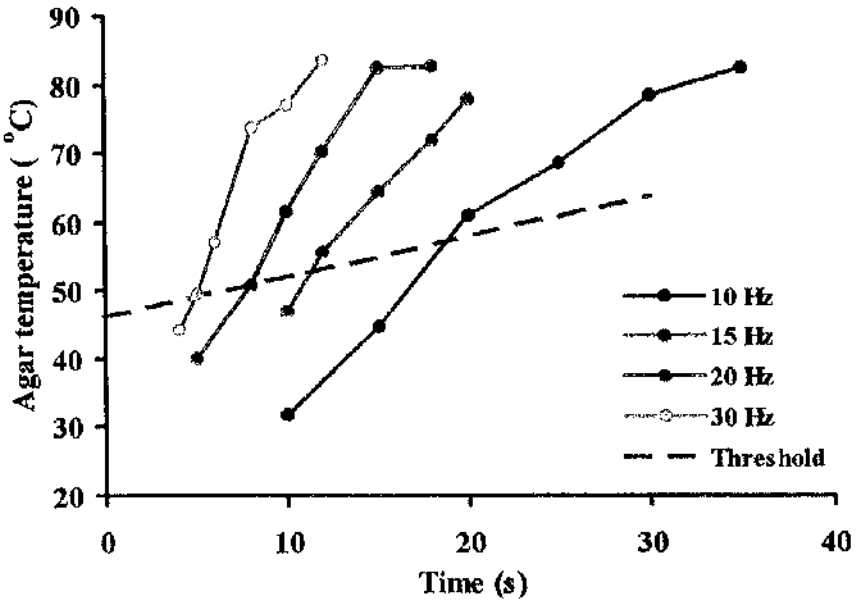


Figure 4.15 **Effect of Nd:YAG laser light on the temperature rise of the agar within the sterilized area at increasing PRF.** The black dashed line indicates the threshold energy densities required for the inactivation of *E. coli*.

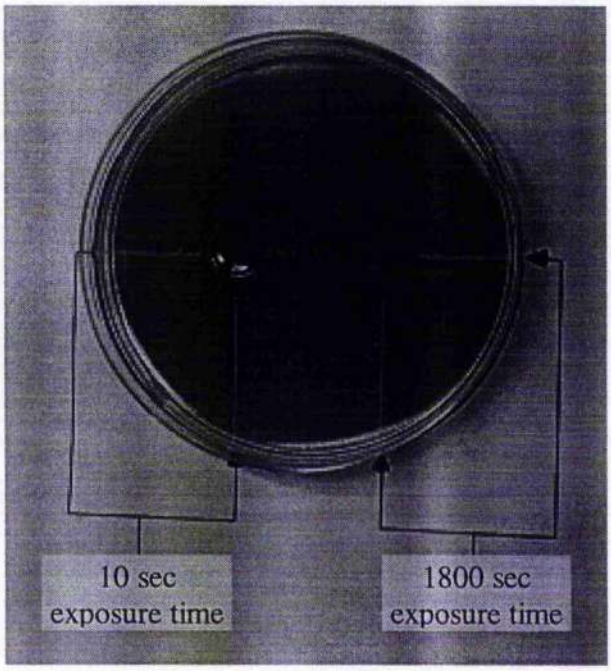


Figure 4.16 **Photograph of the exposure of an *E. coli* lawned plate on nutrient agar to an energy density of 1818 J cm^{-2}.** On the left is the area of clearing produced by 300 pulses at 30 Hz in 10 sec and the right shows the area of the lawn exposed to 300 pulses delivered over 30 minutes. The other laser parameters were 10 J, delivered over 8 msec at a beam diameter of 1.45 cm.

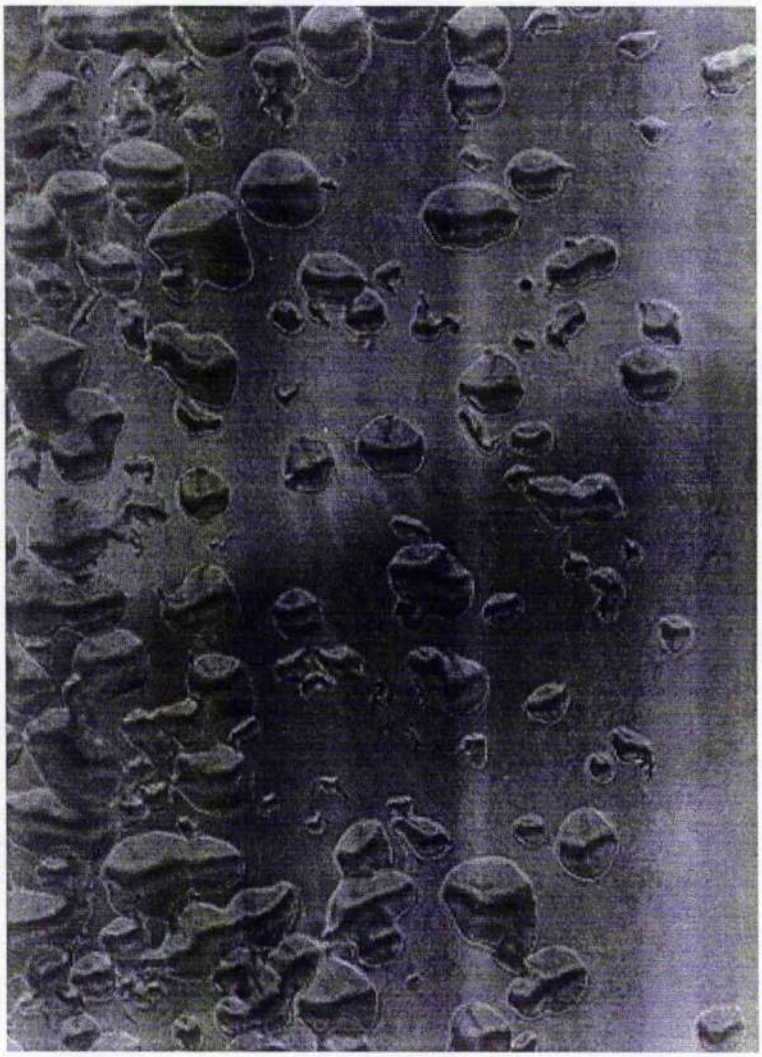


Figure 4.17 **Photograph of the microcolonies found around the periphery of the areas exposed to laser light.** To the left of the picture is the lawn of *E. coli* and to the right, the laser exposed area. The lawn of E. coli was exposed to 10 J, delivered over 8 msec at 10 Hz for 33 sec.

4.2 THE EFFECT OF Nd:YAG LASER LIGHT ON MICROORGANISMS IN LIQUID SUSPENSION

4.2.1 ELECTROMAGNETIC ABSORBANCE SPECTRA

Figure 4.18 shows the absorbance spectrum of water over a range of 700 nm, through the visible spectrum into the near infra-red (NIR) at 1300 nm. Throughout the visible spectrum (about 400 nm to 800 nm), water absorbed little or no light, hence its characteristic colourless property. The absorbance spectrum of water, in the near infra-red (NIR) from 900 nm to 1360 nm, showed increased light absorbance at 974 nm and 1205 nm. Between these two absorption peaks there was a trough of low absorbance, where the wavelength of the Nd:YAG laser is located, indicated by the arrow in **Figure 4.18**. Beyond these peaks was an increase in absorbancy until virtually no significant penetration into the water by infra-red light was observed. There are however, several small windows of reduced absorbancy throughout the infra-red region. The absorbancy at 1064 nm was 0.08 cm^{-1}, which meant that most of the incident light was transmitted through water.

Figure 4.19 shows the absorbance spectrum of *E. coli* suspended in distilled water, at 1 x 10^9 cells ml^{-1}, against a reference cell containing distilled water. It is a misnomer to term this absorbancy as much of the light was scattered rather than absorbed, hence the reason for the common use of the term "optical density". At either end of the visible spectrum there were areas showing high absorbance. Between 400 nm and 1200 nm there was a decrease in the "absorbance", most of which could be attributed to reduction in the scattering of the light, as blue light is scattered to a greater extent than red.

Simple experiments were also done to find the absorbancy of cell constituents over the visible and near-infra red regions of the electromagnetic spectrum. **Figure 4.20** shows the spectrum from 400 nm to 1400 nm of the absorbance of sonicated cells. The sonicated suspension was still visibly cloudy indicating that there would be a high ratio of scattering to absorbancy within the suspension. The absorbance at 400 nm dropped from over 1.0 with the whole cells (**Figure 4.19**) to 0.4. The curve in

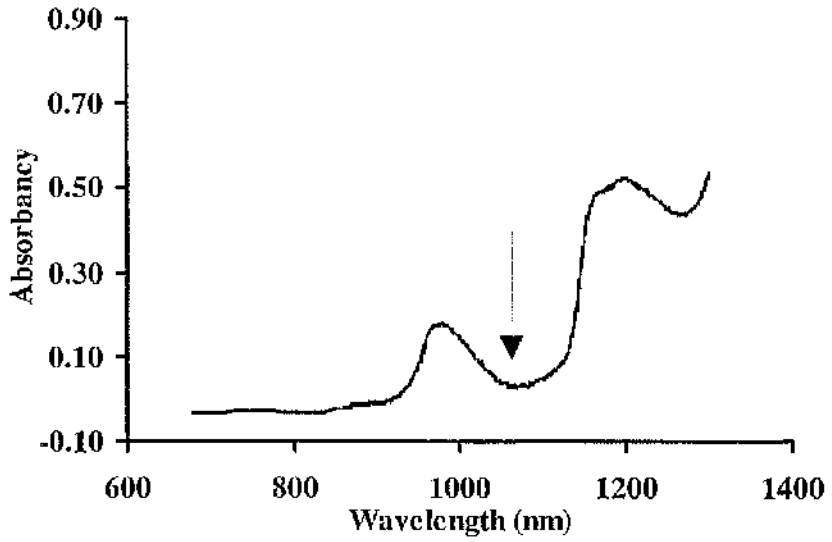


Figure 4.18 **The absorbance spectrum for distilled water.** The wavelength of Nd:YAG laser light is shown by the arrow.

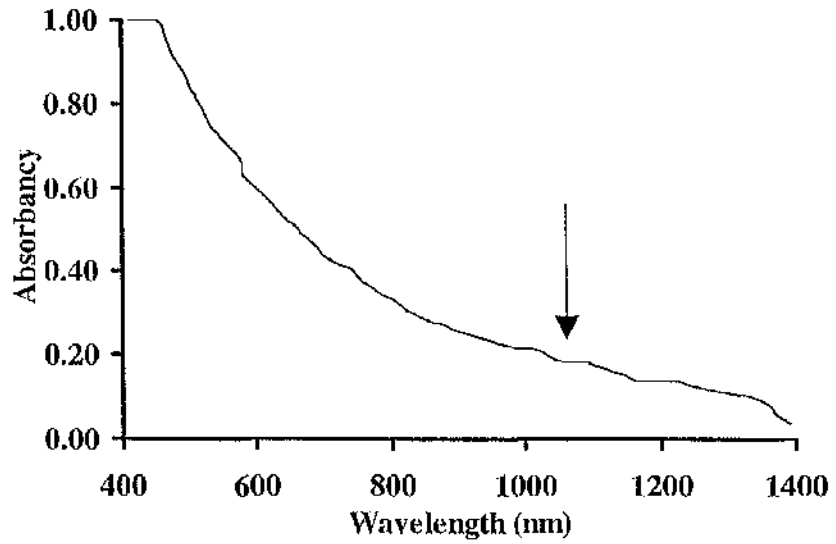


Figure 4.19 **The absorbance spectrum of *E. coli* whole cells**

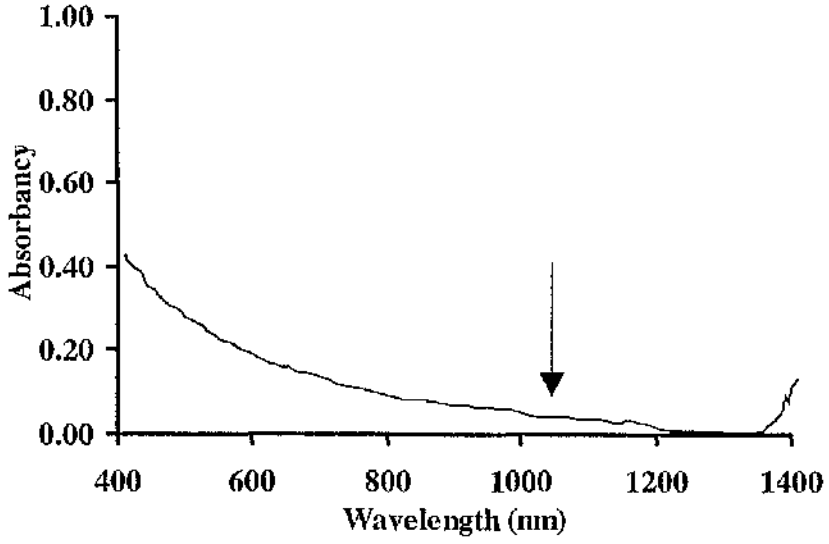


Figure 4.20 The absorbance spectrum of a sonicated suspension of *E. coli*.

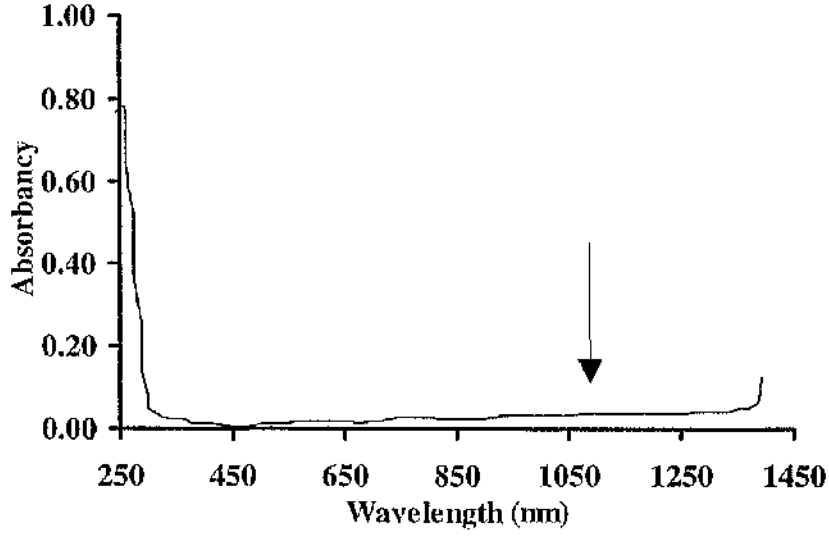


Figure 4.21 The absorbance spectrum of a sonicated suspension of *E. coli* after centrifugation

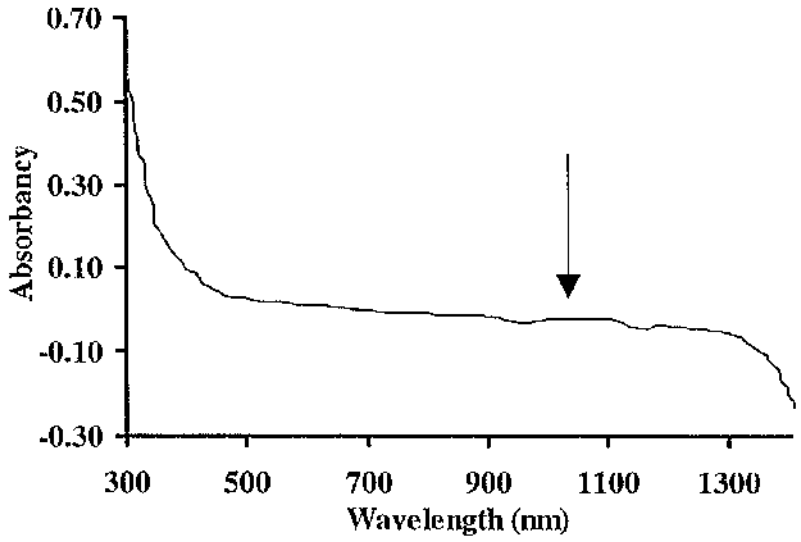


Figure 4.22 **The absorbance spectrum of 10% w/v BSA**

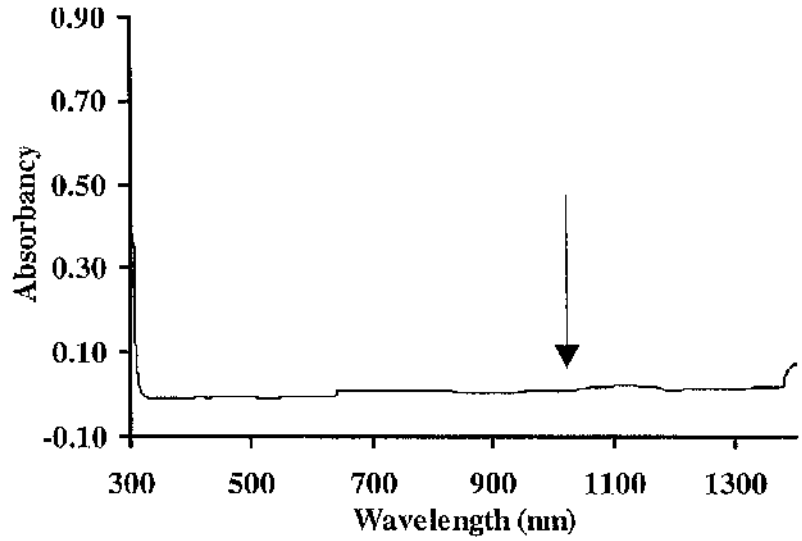


Figure 4.23 **The absorbance spectrum of 0.001% w/v DNA**

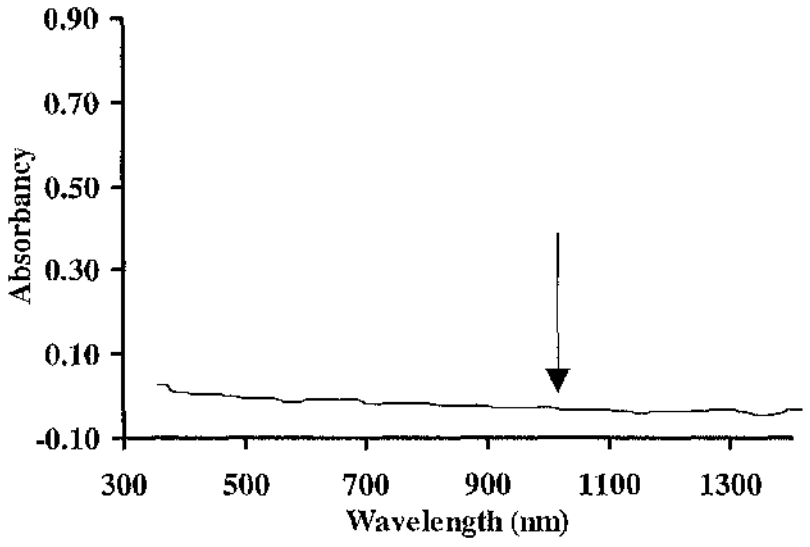


Figure 4.24 **The absorbance spectrum of 0.1% w/v RNA**

Figure 4.20 had a similar shape to that of *E. coli* whole cells, only there was not such a steep decrease in absorbance when the wavelength was increased. The sonicated cells would have released cell constituents but the presence of turbid cell-wall residues, in the suspension, would mask any absorbancy due to other intracellular components released from the disrupted cells. To free the suspension of the cell wall debris, it was spun down in a centrifuge and the absorbance spectrum of the supernate was recorded (**Figure 4.21**). The solution remaining after centrifugation of the sonicated suspension was visibly transparent but a peak at 250 nm was observed. The spectrum showed that no intracellular components, released from sonicated *E. coli* cells, absorbed any light at wavelengths between 400 nm and 1400 nm. Like the absorbance spectrum of the sonicated cells (**Figure 4.20**) there was a rise in absorbance at ~1400 nm. As additional experiments the absorbance spectrum of BSA, DNA and RNA (**Figures 4.22, 4.23** and **4.24** respectively) were determined. At a high protein concentration of 10% w/v, there was little or no absorbance in the range of around 600 nm to 1200 nm, there was an increased absorbancy below 400 nm, and a decreased absorbancy above 1300 nm. With DNA at a concentration of 0.001% w/v (**Figure 4.23**), and RNA at a concentration 0.1% w/v (**Figure 4.24**) there was no absorbancy between 400 nm and 1400 nm.

4.2.2 TEMPERATURE OF LIQUIDS EXPOSED TO LASER LIGHT

When a volume of water was exposed to Nd:YAG laser light a significant temperature rise, within the volume was observed (See section 4.1.4). As there was some absorption at 1064 nm by water (**Figure 4.18**) and the energy applied to the subject was relatively high (100 W in the majority of cases) a temperature rise was expected. Unlike the heating mechanism seen with standard methods, such as a waterbath, steamer, autoclave or microwave, the application of energy by laser light to the volume of liquid was different and therefore a method of measuring the temperature within the liquid volume was required.

In preliminary experiments on the temperature rise, after laser light exposure, in a volume of liquid, the temperature measurement was with a mercury-in-glass thermometer inserted after exposure similar to the method used to measure the

temperature in agar (section 4.1.4). This method was too inaccurate for the exact temperature measurement of the liquid volume. A method of measuring the liquid temperature during exposure was more desirable, and a thermocouple embedded in the wall of a vessel was adopted (**Figure 3.3**).

4.2.2.1 Gradients within the thermocouple vessel

In all cases the water was exposed via the open top of the vessel, and through the 1.5 cm depth of liquid. As the laser light penetrated the liquid a small percentage of the beam was absorbed by the sample. By the time the laser beam completely passed through the volume of water there was a slight reduction in the beam energy. The amount of energy in the beam at the surface was higher than at the bottom of the vessel and thus the amount of energy absorbed by the volume of liquid decreased from the top to the bottom of the vessel. Therefore, a longitudinal temperature gradient was present throughout the depth of the vessel. In addition the profile of the laser beam (**Figure 4.1**), demonstrated that the centre of the beam was the most intense with decreasing energy radially outwards. The consequence of this energy distribution was that the temperature at the centre of the vessel was higher than at the outside. Unfortunately the design of the thermocouple vessel was such that the temperature could only be recorded at the perimeter of the beam where the temperature rise was slowest. A mathematical model of this temperature differential within the sample was constructed by Wang *et al* (1997) and is shown in **Figure 4.25**. **A**, **B**, **C** and **D** illustrate the calculated transient temperature profiles on the cross-sectional plane through the central axis after 1, 10, 21.6 and 35 sec Nd:YAG laser exposure respectively. In the model the laser was turned off at 21.6 sec. The temperature at the centre of the vessel rises most rapidly especially immediately below the liquid surface, reaching a theoretical peak of 90.5°C, 0.3 cm below the surface. At 35 sec, 13.4 sec after the laser was switched off, the temperature 0.3 cm from the surface, at the centre, had decreased to 80.3°C.

In addition to the longitudinal temperature distribution through the sample there was the planar cross-sectional temperature differential. The extent of the cross-sectional temperature differential within the vessel was also modelled by Wang *et al.* (1997)

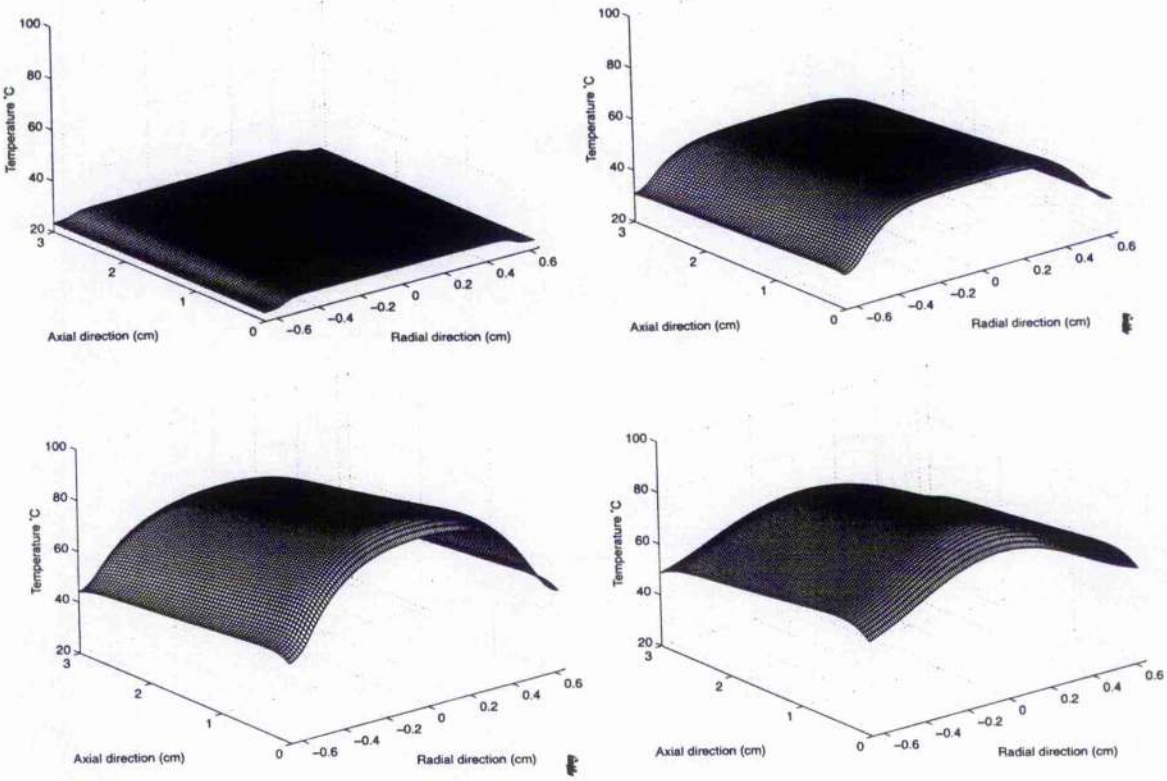


Figure 4.25 **Modelled transient temperature profiles of the longitudinal plane, through the central axis of the thermocouple vessel.** Measurements were taken at A. 1 sec, B. 10 sec, C. 21.6 sec and D. 35 sec. The laser in the model was switched off after 21.6 sec after raising the temperature of the fluid at the vessel wall to 50°C

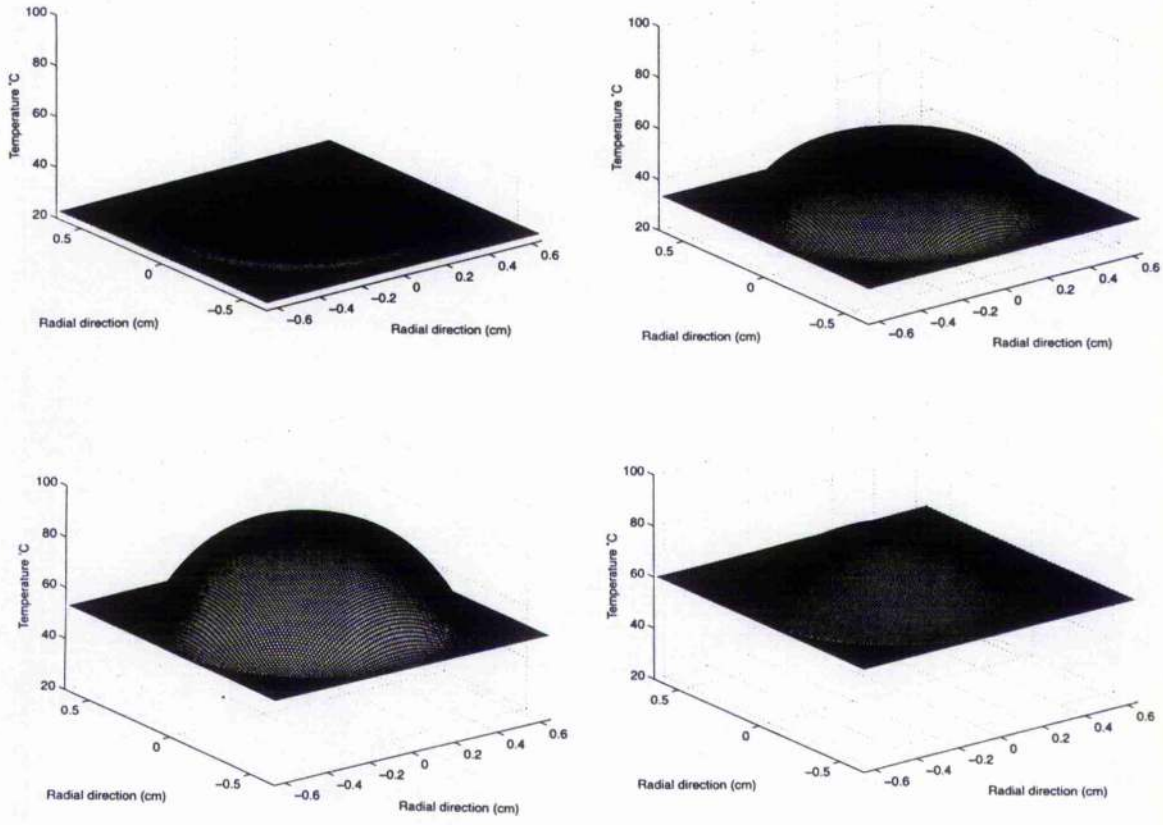


Figure 4.26 **Modelled transient temperature profiles of the cross-sectional plane, perpendicular to the central axis of the thermocouple vessel.** Measurements were taken at A. 1 sec, B. 10 sec, C. 21.6 sec and D. 35 sec. The measurements took place 0.3 cm below the liquid surface. The laser in the model was switched off after 21.6 sec after raising the temperature of the fluid at the vessel wall to 50°C

and is shown in **Figure 4.26**. **A, B, C** and **D** show the temperature profiles on the cross-sectional plane, perpendicular to the central axis and 0.3 cm below the surface, for each of the times given in **Figure 4.25**. The laser in the model was once again switched off after 21.6 sec. Due to absorption of laser energy, and conduction of heat, the temperature at any point in the vessel increased with exposure time. In **A** the temperature profile throughout the exposed region was relatively flat though slightly higher than the ambient temperature. With increasing exposure, the temperature continued to rise and the distribution through-out the exposed area became more rounded. After 21.6 sec the temperature in the centre of the exposed region had reached the maximum value of 90.5°C. After exposure the temperature in the centre of the volume of liquid began to fall and the temperature at the vessel wall began to fall. **Figures 4.25** and **4.26** show that there is a temperature gradient within the thermocouple vessel, however the effects of Brownian motion, eddy currents and turbulence or the glass vessel-walls and aluminium holder were not taken into account when the theoretical model was prepared.

4.2.2.2 Temperature rise of bacterial suspensions exposed to laser light

As the volume of water in the laser exposure vessel was exposed to Nd:YAG laser light there was a rise in overall temperature. This was monitored to allow the temperature of the liquid sample to be raised to a specific value. **Figure 4.27** shows three profiles of the temperature rise of bacterial suspensions, exposed to Nd:YAG laser light (10 J and 10 Hz), containing different concentrations of *E. coli* cells. This investigation studied the effect of decreased light penetration on the temperature rise within the thermocouple vessel and the subsequent effect on bacterial killing.

Figure 4.27 details the effect of increasing energy densities of laser light on the temperature of a 2 ml volume of bacterial suspensions during exposure. The bacterial sample containing 1 x 10^4 cells ml^{-1} was essentially similar to the exposure of water alone, in terms of the absorbancy and the temperature rise. The first 10 sec of the temperature profile showed a relatively slow initial temperature rise which increased to a maximum rate, indicated by the gradient, after 12 sec. At 50°C when the laser was turned off, it can be seen that the temperature slowly crept up to ~52 °C

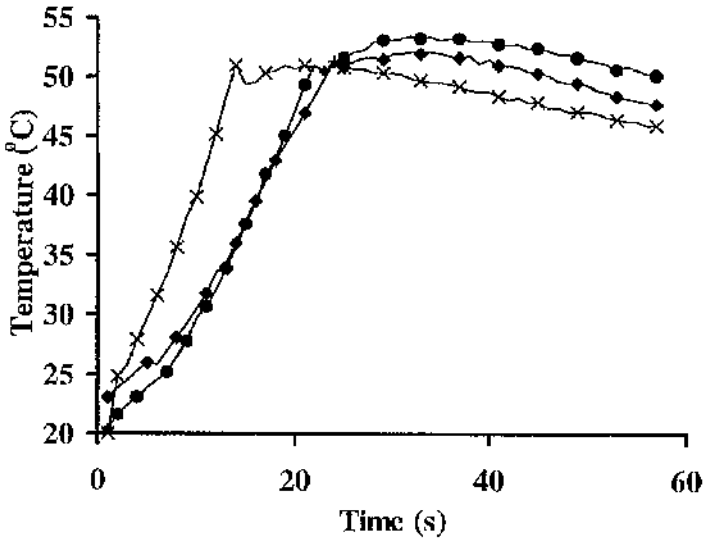


Figure 4.27 **Effect of bacterial cell concentration on the rate of heating by laser light.** The graph shows the temperature profiles of three *E. coli* cultures at different cell concentrations (1 x 10^4 [♦], 1 x 10^8 [•] and 2x10^9 [x] cfu ml^{-1}). Each curve is a trace of the rise in temperature recorded every second but, to avoid crowding, only every third point is represented. The laser parameters were: 10 J pulses at a frequency of 10 Hz, with an 8 msec pulse width and a beam diameter of 1.35 cm.

and after about 10 sec later, cooling began. The figure also shows the temperature rise of two other *E. coli* concentrations 1 x 10^8 and 2 x 10^9 cfu ml^{-1}. Other concentrations were examined (1 x 10^5, 1 x 10^6 and 1 x 10^7 *E. coli* ml^{-1}) and the temperature profiles, similar to those obtained with 1 x 10^4 and 1 x 10^8 cells ml^{-1}, could not be superimposed on top of these two concentrations, without loss of clarity, hence these other data were not presented. At 1 x 10^8 cfu ml^{-1} the *E. coli* suspension had a higher OD_{600} (0.15) than the cell concentration of 1 x 10^4 *E. coli* cells ml^{-1} but the temperature rise was very similar, albeit marginally steeper. At the highest concentration (2 x 10^9 cfu ml^{-1}) the sample had a high OD_{600} (~1.5). The high opacity therefore meant that the light was obstructed in its passage through the volume of liquid. This indicates that the bacteria in the suspension, at the base of the vessel, received less laser light than those nearer the surface of the liquid volume. The temperature rise in the culture containing 2 x 10^9 cfu ml^{-1} was more rapid than all of the other concentrations taking almost half the exposure time to reach 50°C than the other five suspensions. As the laser was turned off a rapid fall in the temperature of the most concentrated cell suspension was observed, possibly due to rapid dissipation of the heat through the liquid volume.

4.2.3 OPTICAL DENSITIES OF *E. COLI* AT 600 AND 1064 nm.

The turbidity of a liquid sample increased with the concentration of bacteria. Therefore the optical densities, at 600 nm and 1064 nm, of the increasing *E. coli* concentrations, in nutrient broth, measured against a sterile nutrient broth blank increased also (**Figure 4.28**). The figure shows that as the concentration of bacteria in the suspension increased, the OD at both 600 nm and 1064 nm increased. The rise in OD at 1064 nm was half the increase at 600 nm, indicating that the light at the operating wavelength of the Nd:YAG laser was more transmissive than at the visible light wavelength, through increasing concentrations of bacterial suspensions. Therefore, exposure of higher concentrations of *E. coli* by Nd:YAG laser light, in comparison to visible light was possible. Below 1 x 10^7 cfu ml^{-1}, there was no turbidity visible in the suspension, and it therefore looked transparent. Because of this, the optical densities of cell suspensions below this concentration were not investigated.

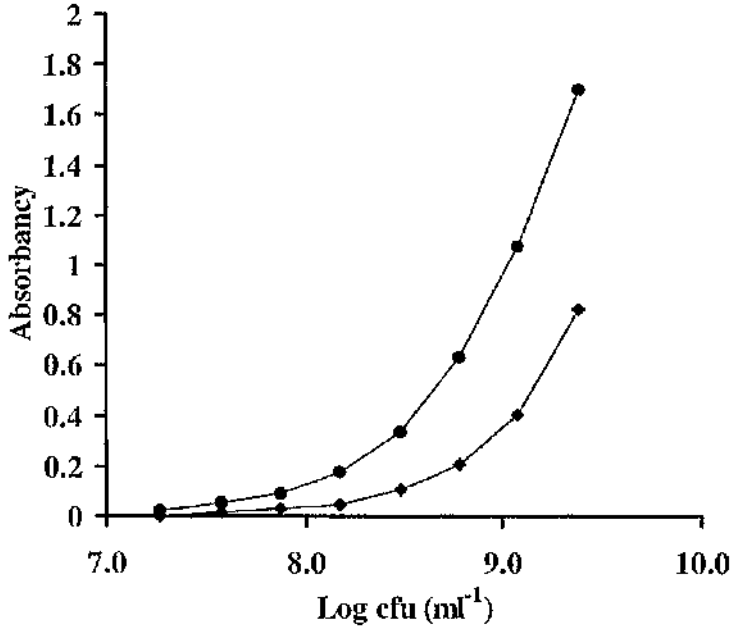


Figure 4.28 **Change in optical density of *E. coli* cultures at different cell concentrations, measured at both 1064 nm [♦] and 600 nm [•].** The cultures were in nutrient broth and measured against a nutrient broth blank.

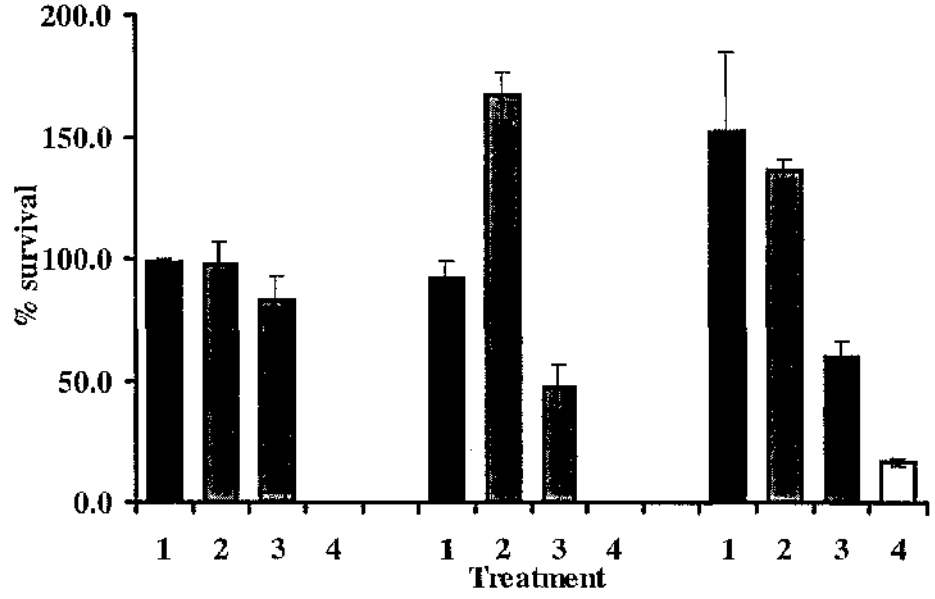


Figure 4.29 **The effect on the viability of dilute *E. coli* suspensions in PBS when exposed rapid heating in a polymerase chain reaction (PCR) machine up to 50°C (1), a water-bath up to 50°C (2) and at 50°C for 10 min (3), and to laser light up to 50°C (4).** The x-axis shows the three heat-treatments, each carried out on separate occasions and the y-axis describes the average percentage survival from triplicate experiments. Each error bar is the SEM for triplicate tests.

4.2.4 HEATING *E. COLI* SUSPENSIONS TO 50°C WITH LASER LIGHT

Due to the low absorbance of light at 1064 nm, complete exposure of a volume of liquid was possible and the effect of Nd:YAG laser light on *E. coli* suspended at low concentrations (~1 x 10^4 cfu ml^{-1}) in phosphate buffered saline (PBS) was examined. The effect of the laser exposure of the *E. coli* suspension, to 50°C, is shown in **Figure 4.29**. The x-axis describes the type of heat treatment for three experiments, each carried out on separate occasions with error bars representing the standard errors for triplicate experiments and the y-axis shows the percentage survival after each exposure. On the x-axis there are two alternative heating methods to the laser: in a PCR machine (**1**) and a waterbath (**2**) and (**3**). When the bacterial suspension was heated to 50°C with Nd:YAG laser light there were no viable cells detected, in two of the three experiments, after fourteen days of incubation at 37°C. In the third experiment there was a low number of survivors, attributed to misalignment of the laser beam with the suspension. The initial counts for the bacterial suspensions were around 1 x 10^4 cfu ml^{-1}. The lower detection limit of the experimental system was 10 cfu ml^{-1} (100 µl samples plated out). In the first two experiments with the laser, there was a reduction in the viable counts to 0, i.e. around three D-values. Controls to the laser-induced temperature rise were heating to 50°C in a water-bath and removing a sample immediately, leaving the suspension at 50°C for a further 10 min before removing a second and heating in a PCR machine. The results showed that heating the cells to 50°C in a water-bath, which took around 3 min, had little or no detrimental effect on the viability of *E. coli*. In fact, most of the data from these experiments showed an increase in cell viability after raising the temperature to 50°C. Leaving the suspension in the water-bath at 50°C for a further 10 min after reaching 50°C produced around 50% reduction in viability. The D-value for *E. coli* at 50°C, in a water-bath was measured to be around 20 min (data not shown). This experiment showed however, that a standard waterbath temperature rise to 50°C was not as effective as the bactericidal action of the Nd:YAG laser, at the same temperature.

Because the laser can heat the bacterial suspension to 50°C in 20 sec, but the waterbath required a total of 3 min to achieve the same effect, an alternative method of control with a faster temperature rise was necessary to compare with the laser light. When the *E. coli* suspension was placed in the PCR machine, the time to reach 50°C was only 1.0 min, but the temperature rise had little or no effect on the viability of the *E. coli* cells. In experiment 3 the first count was much greater than the control with 50% more viability after exposure.

When a similar energy density of Nd:YAG laser light, as was used to raise an *E. coli* suspension from room temperature to 50°C, was applied to an *E. coli* suspension, cooled to 0°C, the temperature rose to ~30°C. The temperature of the *E. coli* suspension was reduced to 0°C with the addition of ice. Little or no bactericidal effect of the Nd:YAG laser light upon *E. coli*, under these conditions, was observed.

4.2.5 EXPOSURE OF HIGH CONCENTRATION *E. COLI* SUSPENSIONS

Section 4.2.2.2 reported on the effect of varying the concentration of bacteria on the temperature rise within the thermocouple vessel, but revealed nothing about the effect of Nd:YAG laser light on the viability of different bacterial concentrations. The effect of laser light on *E. coli* suspensions with increasing cell concentrations, is shown in **Figure 4.30**. The x-axis describes the log of the initial viable numbers within the cell suspensions exposed to the laser light. Each individual point is a fresh suspension diluted from a common stock. The y-axis is a log scale describing the reduction in viable counts obtained at each individual cell concentration. This graph is a combination of two experiments, carried out on separate occasions. After each occasion the viable reductions were slightly different at higher concentrations (10^7 to 10^8) with 3 to 4 D-values observed on one occasion and 4 to 5 on the other, as seen by the variation in the reduction of viability at these initial concentrations. As the concentration of *E. coli* was increased from 1×10^4 to 5×10^8 cfu ml^{-1}, where the temperature profiles were very similar (**Figure 4.27**), there was a large reduction in viability of *E. coli*, from around 2.2 to an average of 4.5 D-values, for each concentration. When the concentration of the suspension was increased further, to 2

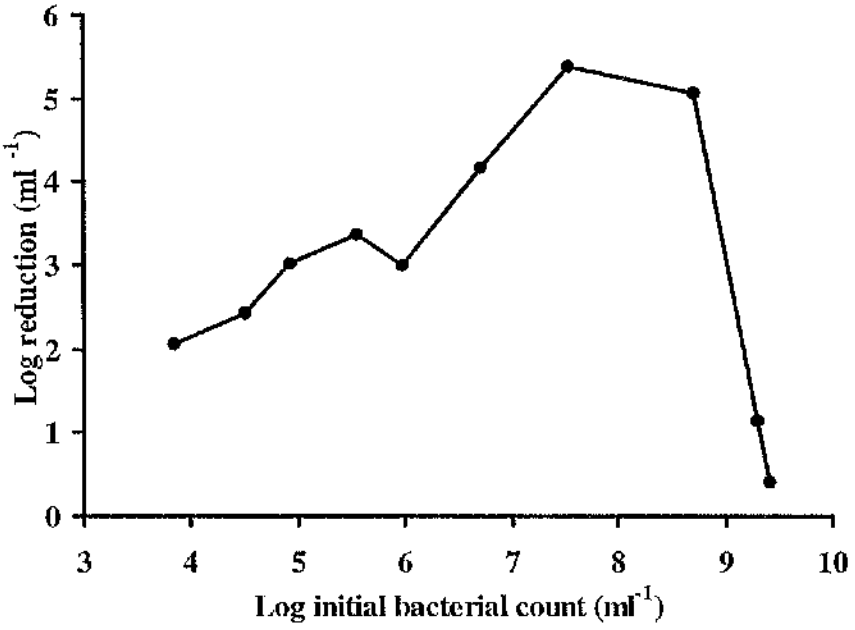


Figure 4.30 **The effect of culture concentration on the bactericidal action of Nd:YAG laser light.** The graph shows the logarithmic cell reduction, after laser exposure, for a series of different initial *E. coli* concentrations described on the x-axis. The graph is the combination of two experimental results. Each suspension was exposed to laser light at the same parameters until 50°C was reached and the laser was switched off. The exposure times varied; see **Figure 4.27.**

x 10^9 *E. coli* ml^{-1}, equivalent to an OD_{1064} of 0.8 and an OD_{600} of 1.7, the reduction in viable count decreased considerably to less than one D-value. There was a definite point at which the reduction in viability dramatically decreased with this higher concentration indicating low penetration of the laser light through the dense bacterial suspension.

4.2.6 EXPOSURE OF *E. COLI* SUSPENDED IN SOUP, MILK OR PBS.

As previously reported, the effect of Nd:YAG laser light penetration of opaque suspensions is an important consideration in the determination of bactericidal efficiency of laser light. Therefore, the effect of other opaque suspensions on the efficacy of laser killing on bacteria was examined with *E. coli* suspended in lentil soup or milk and compared with bacteria suspended in PBS. The effect of Nd:YAG laser light on the viability of *E. coli* suspended in soup (diluted 2-fold to reduce thickness for easier pipetting), milk (UHT for sterility) and PBS after heating to 50°C is shown in **Figure 4.31**. The x-axis in this figure describes the suspension exposed to the laser, for three experiments, and the y-axis, the percentage of viable count remaining compared to a control. For every exposure there was a separate control, receiving no exposure; all controls contained ~1 x 10^4 cfu ml^{-1}. Not detailed in the figure was the fact that on average the opaque suspensions reached 50°C quicker than the PBS; 16 sec for soup, 15 sec for milk and 19 sec for PBS. After each exposure the PBS contained no viable cells as obtained in **Figure 4.29**, with a loss of over 99% viability. *E. coli* suspended in lentil soup lost on average 40% cell viability and suspended in milk, 20% viability. The results obtained with the milk suspension were very erratic and in one experiment there were more bacteria in the exposed milk than in the control.

4.2.7 EXPOSURE OF *E. COLI* SUSPENDED IN SOLUTIONS OF PROTEIN

The addition of exogenous protein to the PBS containing *E. coli* was examined to investigate its effect on the bactericidal action of the Nd:YAG laser. The cells were resuspended in solutions of sterile ovalbumin ranging in concentrations from 0.001% to 10% w/v. When the bacterial suspensions which contained different

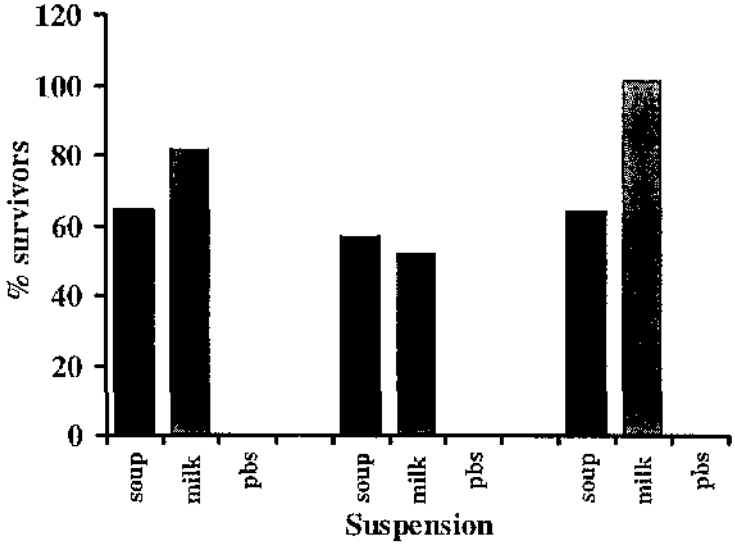


Figure 4.31 **The effect of medium opacity on the killing of *E. coli* in suspension.** The suspending media used were soup, milk or PBS, after exposure to Nd:YAG laser light, raising the temperature to 50°C. The initial counts were ~1 x 10^4 cfu ml^{-1}.

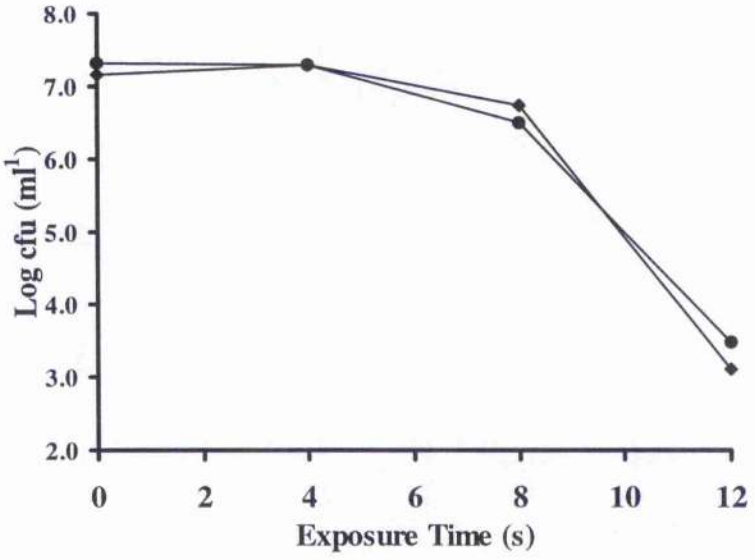


Figure 4.32 **Effect of [•] 10% w/v ovalbumin compared to a control [♦] containing no exogenous protein on the bactericidal action of Nd:YAG laser light toward *E. coli*.** The laser parameters were 10 J pulses at a frequency of 10 Hz, with an 8 msec length of pulse. Exposure took place below the focus of the laser beam.

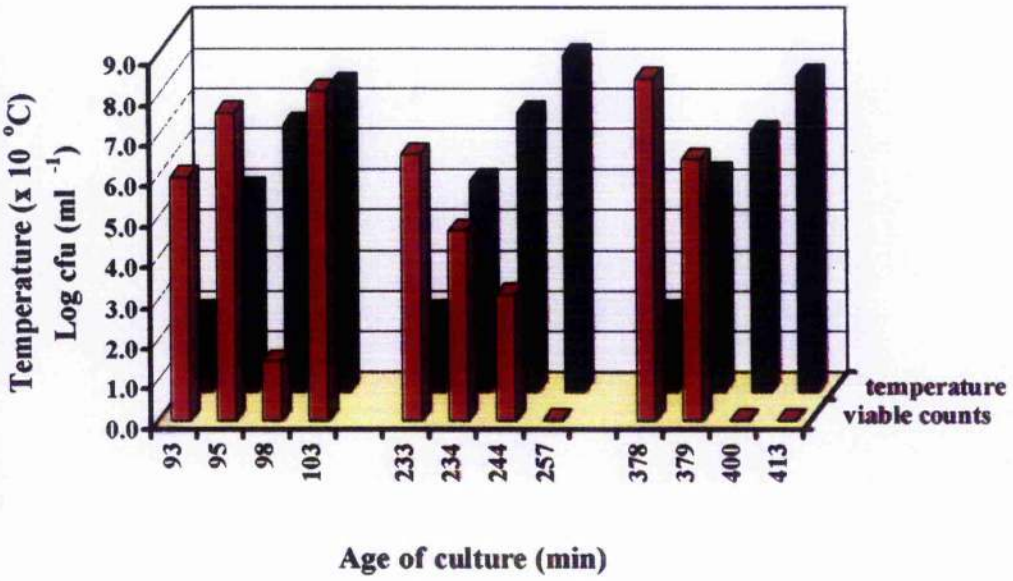
concentrations of ovalbumin, and a control with no exogenous protein, were exposed to different energy densities of laser light the results shown in **Figure 4.32** were obtained. The graph describes the killing of *E. coli* in 10% w/v ovalbumin compared to a control containing no ovalbumin, after exposure to Nd:YAG laser light (10 J and 10 Hz). This experiment was carried out before the introduction of the thermocouple. This meant that the temperature of the suspensions during and after exposure were not obtained. For clarity only the curves for the extreme protein concentrations (10% w/v and control) were presented as representatives of the other four protein concentrations exposed to Nd:YAG laser light (0.001, 0.01, 0.1 and 1% w/v) showed similar tendencies. After 8 sec of laser exposure, a reduction in both the 10% w/v protein and the control of around 1.0 D-value was observed and after exposure of 12 sec produced over 3 D-values of killing. The fact that there was a large lethal effect after 12 sec was by virtue of the fact that the beam diameter was reduced to 1.3 mm (slightly larger than the diameter of the vessel), and therefore the energy density increased, at the liquid surface (beam was diverging beyond focus) and a smaller volume (1 ml) was used. There was also no apparent pattern in the difference between the control containing no protein and the presence of 10% w/v ovalbumin. After 12 sec there was a small amount of coagulation of the 10% w/v ovalbumin.

4.2.8 EXPOSURE OF *E. COLI* AT DIFFERENT GROWTH PHASES

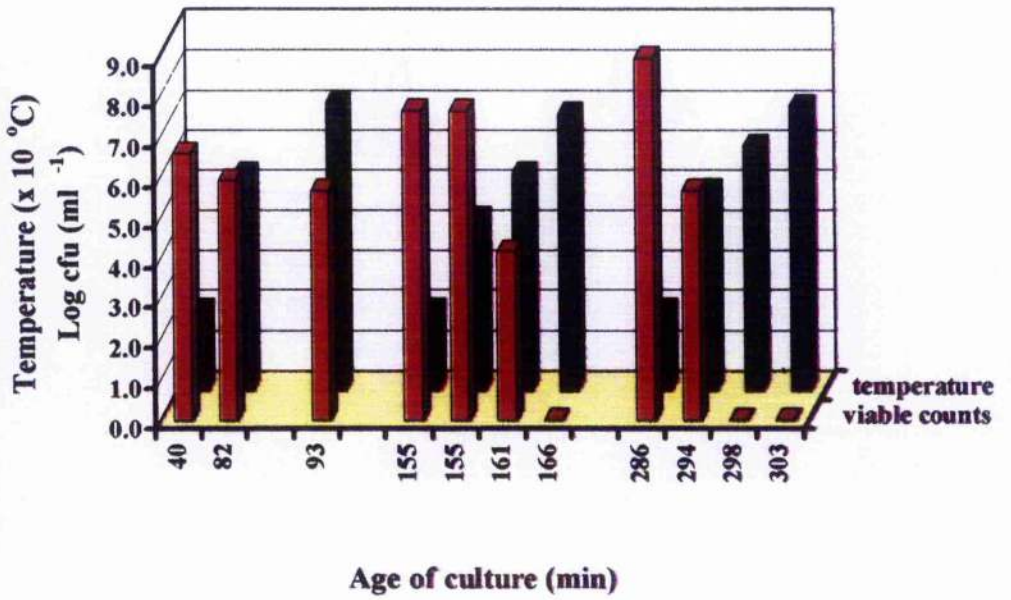
The response of bacteria to lethal substances may be affected by the growth phase of the bacteria. To investigate this the bacteria were exposed to laser light at various stages of the *E. coli* growth cycle. The results are shown in **Figure 4.33**. Both **Figure 4.33A** and **Figure 4.33B** show the results of an investigation into the effect of the growth phase on the tolerance of *E. coli* to Nd:YAG laser light at three energy densities. Each of the two experiments was done in three parts: during the lag phase, during the log phase, and finally, during the early stationary phase of growth. The first readings, in both experiments, took place when the cells were in the lag phase of the growth cycle at 90 and 40 min, respectively. The initial inoculum was such that the control counts were 1×10^6 and 4×10^6 cells ml^{-1}, respectively. The following energy densities of Nd:YAG laser light were used in each figure (698, 1048 and 1397

Figure 4.33 **Effect of the growth phase on the resistance of *E. coli* to Nd:YAG laser light.** Two graphs, separate experiments carried out on separate occasions (**A** and **B**), show the temperature values in green associated with log viable counts displayed in red. The x-axis describes the time of sampling and are displayed in groups of four. The first of the groups are controls (no exposure) and the other three values have received exposures at 698, 1048 and 1397 Jcm^{-2} for **A** and 1118, 1537 and 2096 Jcm^{-2} for **B**. The laser parameters were 10 J, 10 Hz delivered over 8 msec.

A


Temperature (x 10 °C)
Log cfu (ml -1)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
93
95
98
103
233
234
244
257
378
379
400
413
temperature
viable counts
Age of culture (min)

B


Temperature (x 10 °C)
Log cfu (ml -1)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
40
82
93
155
155
161
166
286
294
298
303
temperature
viable counts
Age of culture (min)

Jcm^{-2} for **Figure 4.33A** and 1118, 1537 and 2096 Jcm^{-2} for **Figure 4.33B**) were chosen for the purpose of raising the temperatures to approximately 50°, 60° and 70°C, but the final temperatures were variable with some values at ±10°C. There was little or no effect of Nd:YAG laser light on the viability of *E. coli* in the lag phase of growth, even after raising the temperature to 70°C. In **A** the viable count for two of the exposure temperatures (50° and 70°C) were higher than the control. The second phase of the bacterial growth cycle represented in the figures was in the log phase of the growth cycle, as shown by the increase in the control counts. The energy density required to raise the temperature to 50°C reduced the viable count in **A** by 2 D-values but produced no change in **B**, where the temperature only reached 40°C. Unlike the results in the lag phase, in both the experiments the viable counts were reduced to zero with the highest energy density. In **A**, however, the temperature within the thermocouple vessel was 80°C. The third set of laser exposures was as the organisms were entering early stationary phase. The controls showed that the viable counts had reached ~1 x 10^9 cfu ml^{-1}. After the application of an energy density, which raised the temperature to 50°C, the viable count was reduced by 2 to 3 D-values, and at 60° and 70°C the viable counts were reduced further, to zero, in both experiments.

To achieve a more accurate record of the effect of the growth phase the experiment was repeated with more intervals within the growth cycle for exposure of the *E. coli* suspensions. Also, instead of the application of three standard energy densities with the temperature rise recorded, the temperature was taken to a standard value of 50°C irrespective of the applied energy density. The results are shown in **Figure 4.34**. From the control counts the growth curve for the *E. coli* culture is evident. The cell inoculum was 1 x 10^5 cfu ml^{-1} and remained at this level throughout the lag phase to 160 min. By 230 min the bacteria were well into log phase and dividing rapidly. At 317 min the bacterial growth had slowed down in late log, and early stationary phase began after 385 min. The cells in this experiment were not aerated during growth to limit the final culture concentration, and therefore turbidity at stationary phase (~3 x 10^8 cfu ml^{-1}). In a shaking culture, *E. coli* will grow to around 2 x 10^9 cells ml^{-1} which would reduce laser light penetration. **Figure 4.30** indicated that a cell

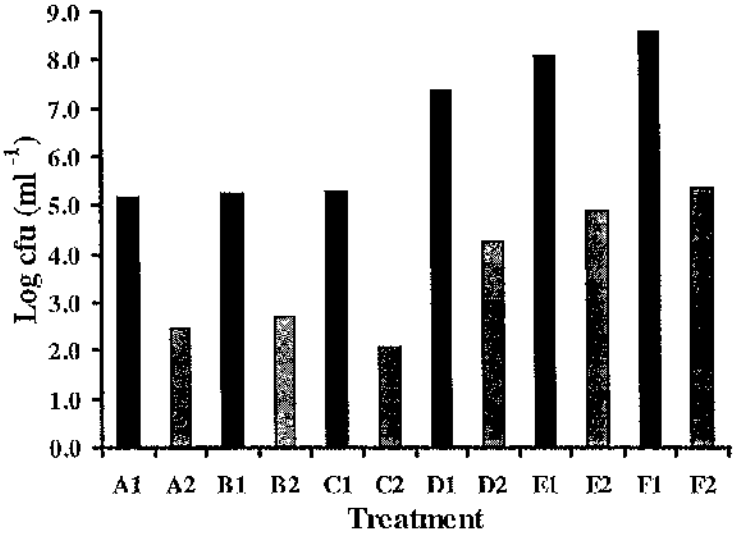


Figure 4.34 **Effect of Nd:YAG laser light on *E. coli* during different growth phases.** The laser parameters were 10 Hz, 10 J with 8 msec pulses. The temperature was raised to 50°C with each exposure. **1** - indicates the control counts, before the bacterial suspension is exposed. **2** - indicates the counts after exposure. Each sample was removed at A - 10 min, B - 83 min, C - 160 min, D - 230 min, E - 317 min and F - 385 min.

concentration of 2 x 10^9 *E. coli* ml^{-1} did not allow the laser beam to penetrate fully into the suspension and so the viability of post-exposure suspensions was higher. At each of the phases of growth described above, the samples were exposed to pulsed Nd:YAG laser light at 10 Hz and 10 J. The pulse width was 8 msec with a beam diameter of 1.35 cm. With each laser exposure the bacterial suspensions were raised to 50°C and the laser was immediately switched off. The suspensions began to cool almost immediately and within 30 sec the samples were removed. From the results it can be seen that after each exposure reduced the bacterial numbers by ~3 D-values during all phases of growth. The largest viable reduction was after 160 min (late lag phase) with a value of 3.3 D-values and the least reduction in viability was after 83 min (mid-lag phase) with a value of 2.5 D-values.

4.2.9 RELEASE OF RNA BY *E. COLI* AFTER LASER EXPOSURE

After exposure to laser light, at 50°C and 60°C, the PBS medium with 5 x 10^8 cfu ml^{-1}, was examined for increased RNA content. **Table 4.3** shows that there was over double the RNA concentration, after heating to 50°C with Nd:YAG laser light with a value of 1.52 $\mu g ml^{-1}$. When the temperature was increased a further 10°C to 60°C the RNA concentration increased to 4 $\mu g ml^{-1}$ - six times the value of the untreated *E. coli* suspension.

4.2.10 EXPOSURE OF DIFFERENT BACTERIA IN LIQUID SUSPENSION

The bactericidal effect of Nd:YAG laser light on bacteria in liquid culture was investigated under a number of conditions. Killing curves were obtained with the procedure as used in previous experiments; the temperature of the bacterial suspensions (~5 x 10^6 cfu ml^{-1}) was raised to 45°, 50°, 55°, 60°, 65° and 70°C, with Nd:YAG laser light. At each temperature the laser was switched off and a sample was taken within 30 sec; during which time, cooling took place. **Figure 4.35** shows the killing curves for *St. aureus, E. coli, B. cereus* and *L. monocytogenes*, when exposed to Nd:YAG laser light. The graphs are plots of the final temperature of the bacterial suspension immediately after exposure to the laser light against the log viable cells remaining. The lower detection limit was 7 cfu ml^{-1} (triplicate results

with 50 µl samples). Also with the y axis as a log scale the zero values are given a value of zero (corresponding to a count of 1). Each graph was separated so that the individual curves could be seen clearly, as they are all superimposable one upon another. In all cases the initial counts appeared to be relatively consistent, between 2 x 10^6 and 8 x 10^6 cfu ml^{-1}. At 45°C there was little effect on all of the suspensions, but once 50°C was reached killing was realised. For *E. coli*, *B. cereus* and *L. monocytogenes* the reduction at this temperature was approximately 10% to 30% of cells, whereas *St. aureus* lost over a D-value. At 55°C the curves show a 2 to 3 D-value reduction in counts. At 70°C the curves show that the laser completely killed all the bacterial cells, with the exception of *B. cereus*. At ~500 *B. cereus* cells ml^{-1} the curve tailed off and temperature of up to 70°C has no further lethal effect on this organism. The standard errors on these graphs show the consistent results obtained during the course of the experiment. The thermocouple vessel assembly, however, was slightly different to previous experiments in that the aluminium holder which encased the vessel was geometrically different. The base was bulkier with higher sides. This may have affected the temperature distribution within the thermocouple vessel, and therefore may be the reason for the reduction in effect at 50°C as seen in previous experiments.

4.3 MECHANISM OF Nd:YAG LASER KILLING OF BACTERIA IN LIQUIDS SUSPENSIONS

4.3.1 SCANNING ELECTRON MICROSCOPY

SEM was introduced as a method of analysing the effects of Nd:YAG laser on the surfaces of different bacterial species. With use of this technique it was possible to compare the physical effects of laser exposure, on the cell surface of *E. coli*, *St. aureus* and *L. monocytogenes* with exposure to waterbath heat treatments, as a control.

Fluid	Temperature (°C)	Supernate RNA absorbancy	Supernate RNA conc. ($\mu g\ ml^{-1}$)
Distilled water	25	0	0
E. coli suspension	25	0.036	0.74
E. coli suspension	50	0.074	1.52
E. coli suspension	60	0.195	4.00

Table 4.3 **Concentration of RNA, in the distilled water supernatant fluid of washed *E. coli* cells (5×10^8 ml^{-1} ;OD_{600} 0.532) compared to a control of distilled water, both without exposure (control) and after exposure to Nd:YAG laser light to heat the suspensions to 50° and 60°C.** The method used in the determination of the RNA concentration was absorption at 675 nm after reaction with orcinol reagent. The laser parameters were 10 J pulses at a frequency of 10 Hz, with an 8 msec length of pulse and a beam diameter of 1.35 cm.

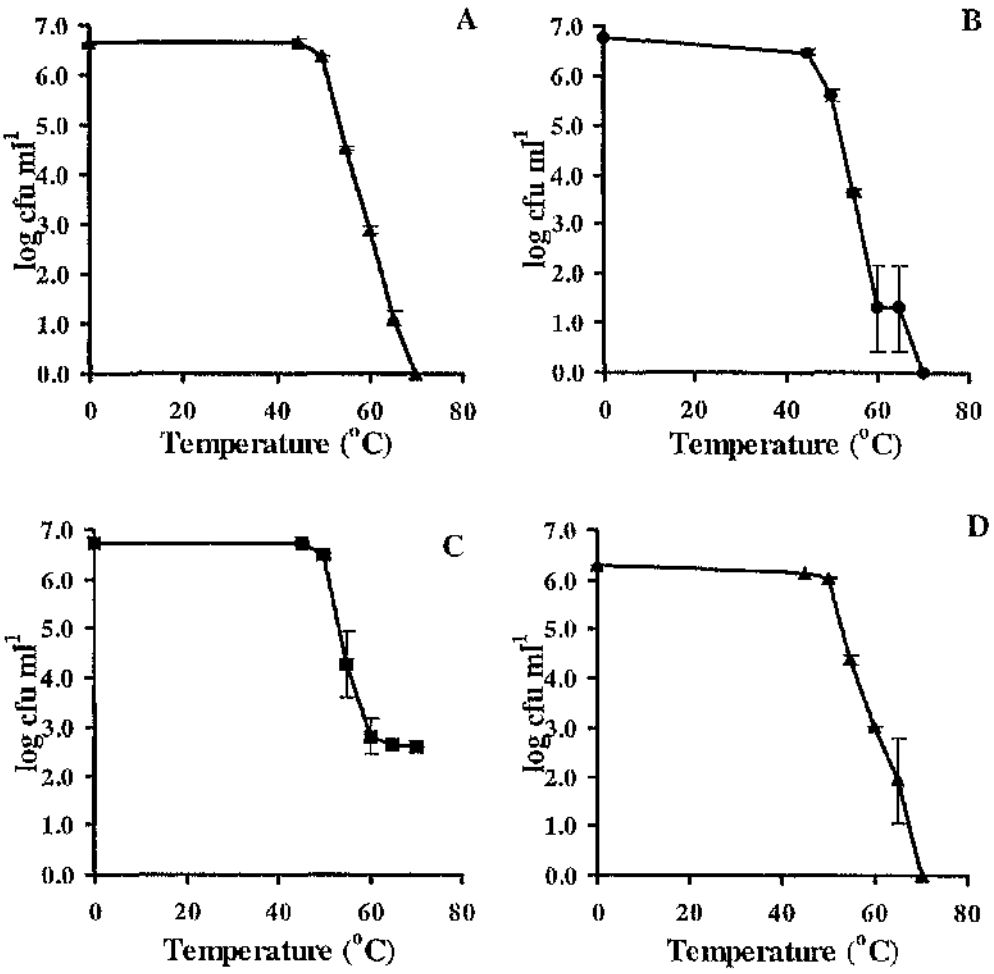


Figure 4.35 **Killing curves for four bacteria when exposed to Nd:YAG laser light at 10 J and 10 Hz until specific temperatures were reached. A:** *E. coli*, **B:** *St. aureus*, **C:** *B. cereus* and **D:** *L. monocytogenes*. Each point is a separate exposure of a fresh solution, raising the temperature from room temperature to the required temperature. The error bars represent the SEM for triplicate results.

The cells in the control *E. coli* sample (**Figure 4.36A** and **B**) had no exposure to laser light or any other heat source. The cell surfaces appeared relatively smooth and unblemished, and the cell shape was regular and the cells were a uniform size. Bacteria within this sample were actively dividing. **Figure 4.37A** and **B** show the effects of laser treatment to 40°C. As with the controls these bacteria were actively dividing with no apparent damage to the cell. Pieces of debris were observed around some of the bacterial cells (**Figure 4.37A**) but otherwise the cells looked smooth and unblemished. After heating to 50°C (**Figure 4.38A** and **B**) with Nd:YAG laser light the cell surfaces became slightly less uniform, more clearly shown in the higher magnification picture. There were fewer bacteria seen under the microscope which could be the result of the filtration or fixation procedure. At 60°C (**4.39A** and **B**) there was evidence of damage to the bacterial cell surface. The cells were not uniform in shape and had undulating surfaces. More prominent in this sample was the presence of a large amount of debris. This debris appeared almost explosive in shape with some pieces attached to cells. The damage to the cells was more prominent after exposure to laser light raising the temperature to 70°C (**Figure 4.40A** and **B**). As well as the presence of debris similar to that observed in the 60°C sample there was more visible damage to the bacterial surface. At the higher magnification (**Figure 4.40B**) the effect the laser light had on the bacterial cell was clear. The surface showed high levels of blebbing where the cell surface began to bubble. In **Figure 4.40A** there was evidence of several elongated cells which appeared to have lost the ability to divide.

When this damage was compared to the effect of plunging a 2 ml of *E. coli* culture into a water-bath, at different temperatures, for five min, it was clear that the laser had a different effect. At 40°C in the waterbath (**Figure 4.41**) again the cell surfaces appeared relatively smooth, and not too dissimilar to the 40°C laser-treated cells in **Figure 4.37A**. There was also evidence of dividing bacteria within the sample. At 50°C in the waterbath the surfaces once again became slightly damaged as shown in **Figure 4.42**. The cells looked as though they were beginning to shrivel up. At 60° and 70°C (**Figure 4.43** and **3.44** respectively) the cells were very badly damaged and had shrivelled. At 70°C the cells began to disintegrate. To further investigate the

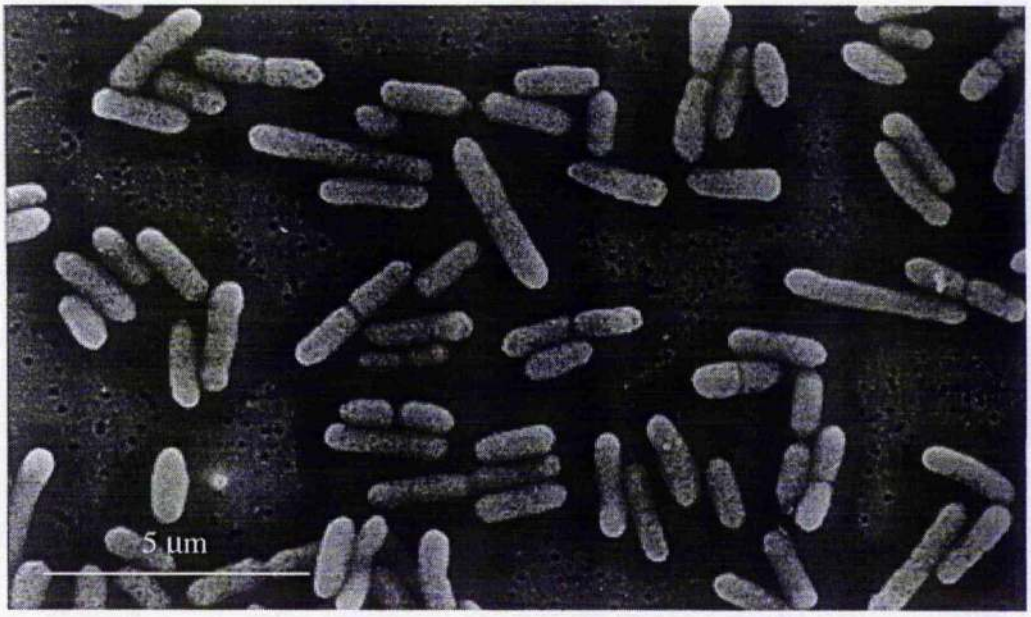


Figure 4.36A **Scanning electron micrograph at a low magnification (dimensions shown) of an *E. coli* control having had no laser or heat exposure.**

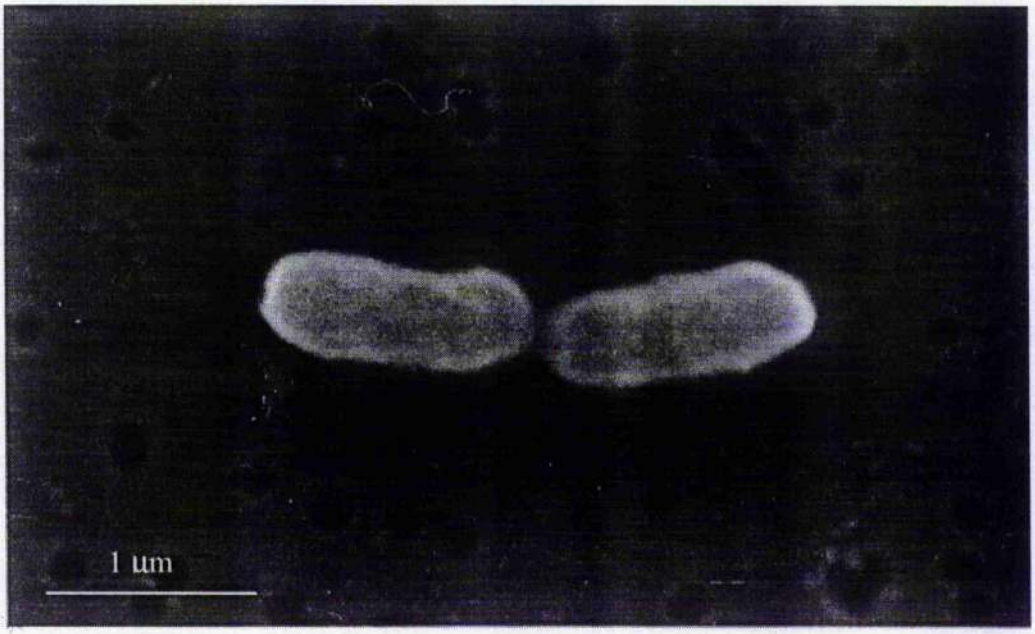


Figure 4.36B **Scanning electron micrograph at a high magnification (dimensions shown) of an *E. coli* control having had no laser or heat exposure.**

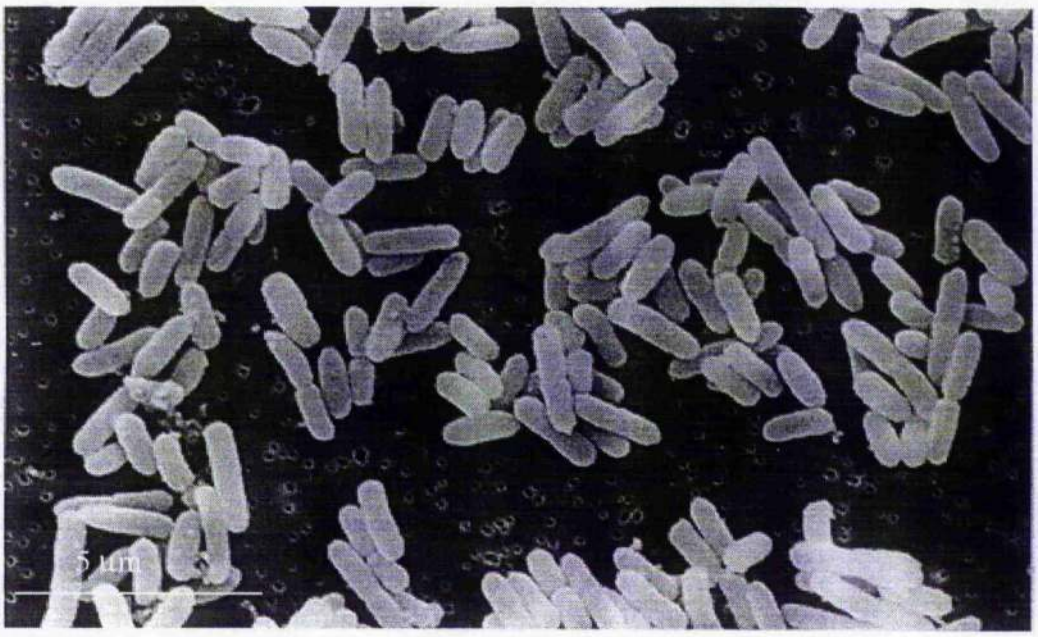


Figure 4.37A **Scanning electron micrograph at a low magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to**

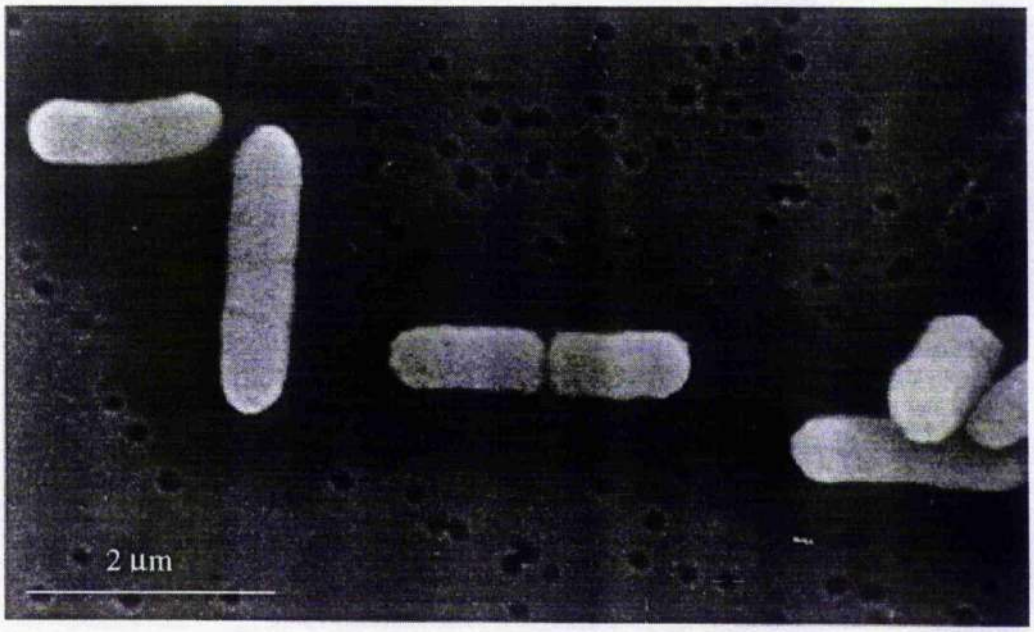


Figure 4.37B **Scanning electron micrograph at a low magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to**

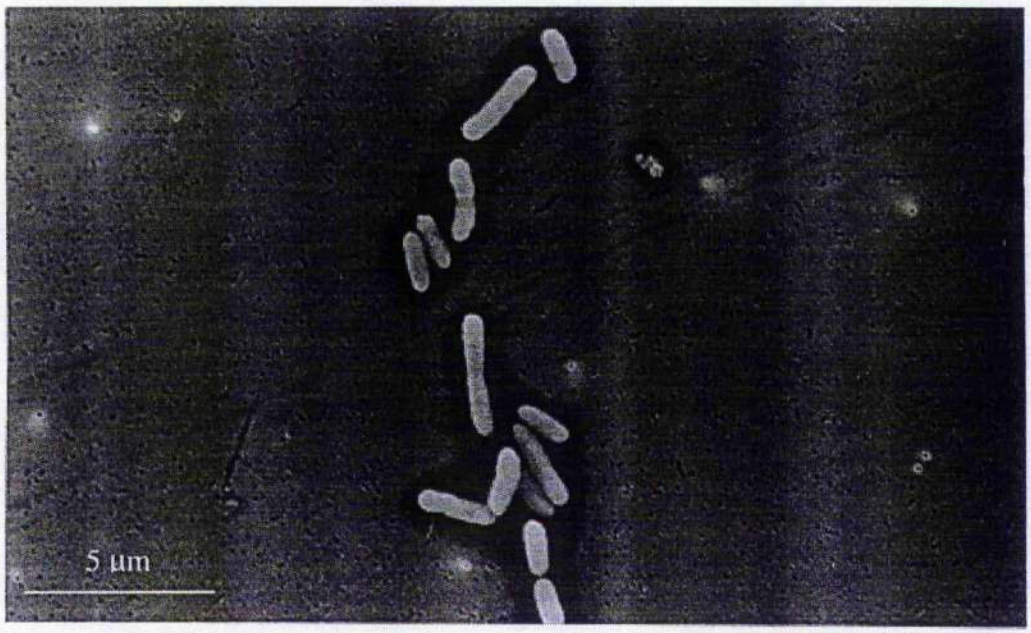


Figure 4.38A **Scanning electron micrograph at a low magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to 50°C.**

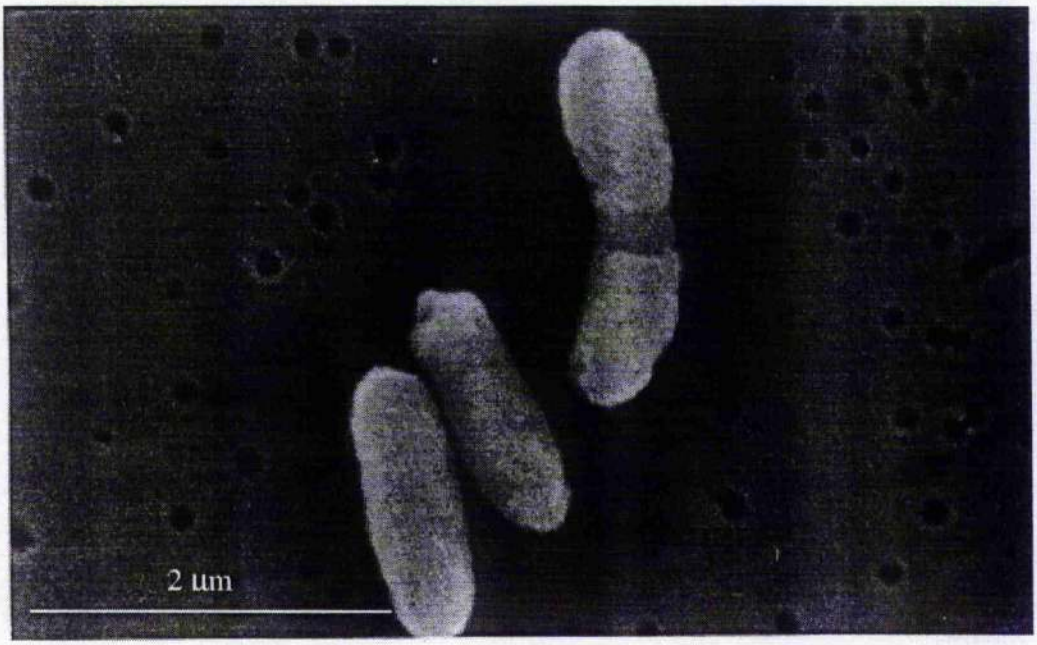


Figure 4.38B **Scanning electron micrograph at a high magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to 50°C.**

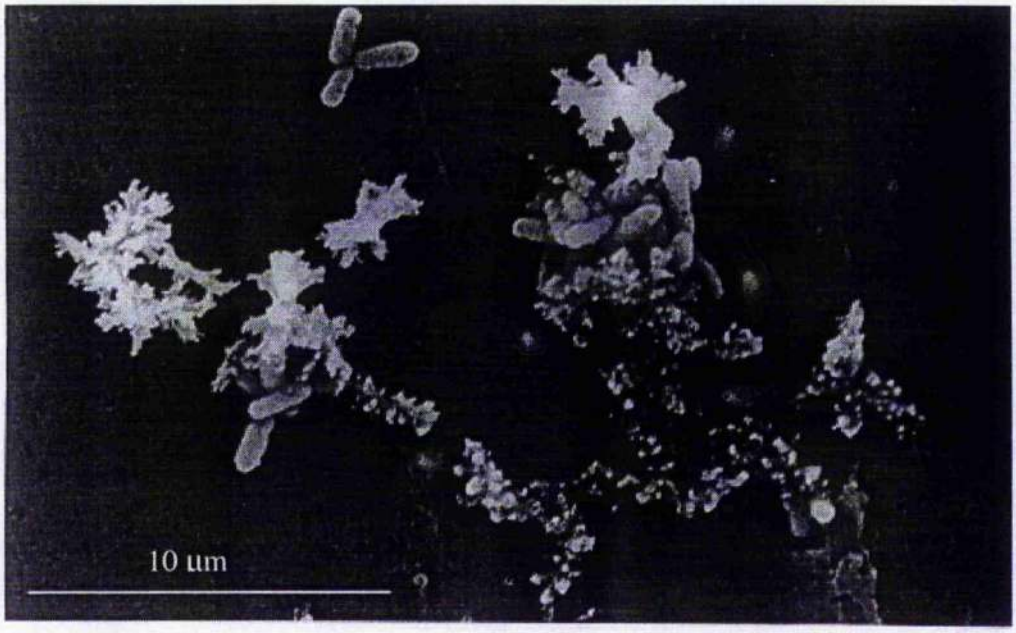


Figure 4.39A **Scanning electron micrograph at low magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to 60°C.**

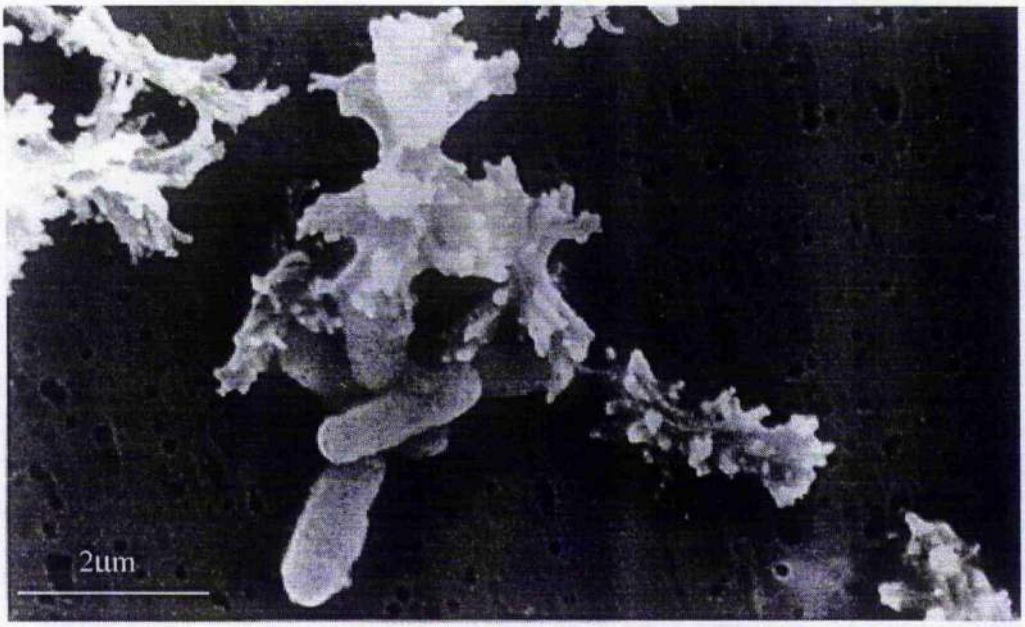


Figure 4.39B **Scanning electron micrograph at high magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to 60°C.**

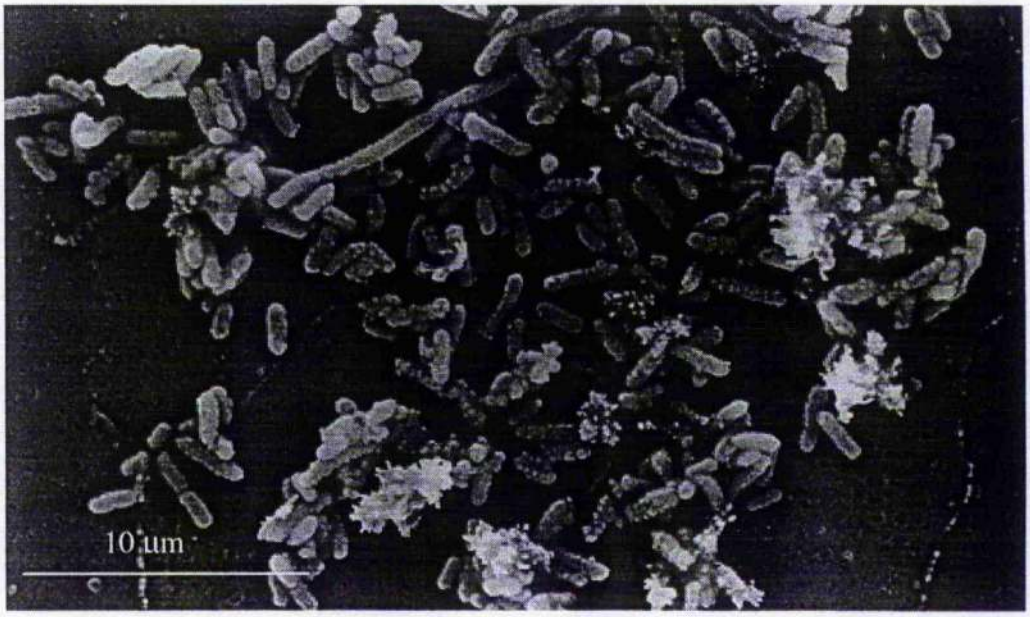


Figure 4.40A **Scanning electron micrograph at low magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to 70°C**

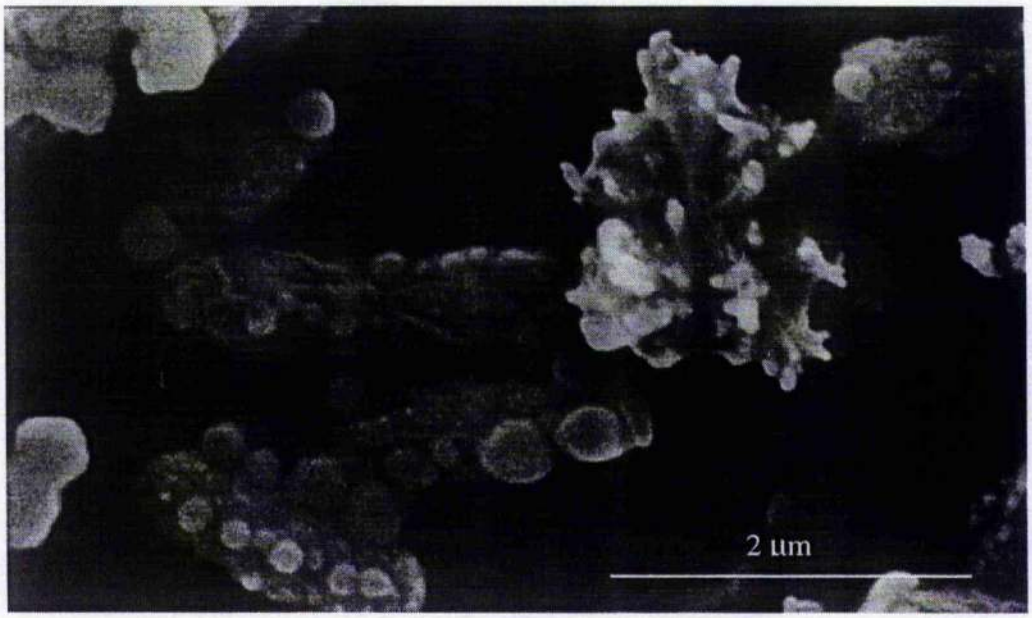


Figure 4.40B **Scanning electron micrograph at high magnification (dimensions shown) of *E. coli* after laser exposure raising the temperature to 70°C.**

effect of the waterbath treatments SEM examination of cells exposed to 100°C in the waterbath for 5 min were also carried out (**Figure 4.45A** and **B**). Damage was obvious in the higher magnification picture (**Figure 4.45A**) but there was no evidence of any debris associated with waterbath heat-treated cell suspensions, as seen in the laser-treated samples. The damage to the cells was also very different (**Figure 4.45B**). Whereas the bacterial cell exposed to the laser light (**Figure 4.40B**) appeared to be rupturing, the waterbath heat-treated cells appeared to be collapsing. After treatment with laser light such that the temperature was raised to 50°, 60° and 70°C, the optical densities decreased to 0.68, 0.58 and 0.59, respectively, from a control absorbancy of 0.74.

The effects of laser light on *E. coli* was compared with the effects on *L. monocytogenes*, an organism with a similar morphology but a different cell wall structure. The effect of both laser treatment, raising the temperature to 70°C and with waterbath treatment, raising the temperature to 100°C was investigated. The results show that there was little, if any, visible damage to the *L. monocytogenes* cell-wall with all of the different treatments. **Figure 4.47** shows the effect of laser light after raising the temperature to 70°C. Compared to the control in **Figure 4.46** there was very little difference in the cell wall integrity. This was also compared to the effect of heating a sample of *L. monocytogenes* to 100°C in a waterbath for 5 min (**Figure 4.48**). Again, as seen in this figure, there was little apparent effect caused by the heat treatment. No visible cellular damage was observed in any of the samples however, all of the samples contained coccoidal bacterial forms.

The effects of laser light on *E. coli* was also compared with the effect on *St. aureus*, an organism with very different morphology to *E. coli* and a different cell wall construction. **Figure 4.50** shows the effect of laser light, raising the temperature of a suspension of *St. aureus* to 70°C. Compared to the control in **Figure 4.49** there was very little, if any, change in the cell wall integrity. The effect of laser light was also compared to heating a sample of *St. aureus* to 100°C in a waterbath for 5 min (**Figure 4.51**). Again, as obtained with *L. monocytogenes* there was little visible damage due to either treatment.

Figure 4.41 **Scanning electron micrograph (dimensions shown) of *E. coli* after exposure to a temperature of 40°C in a waterbath.**

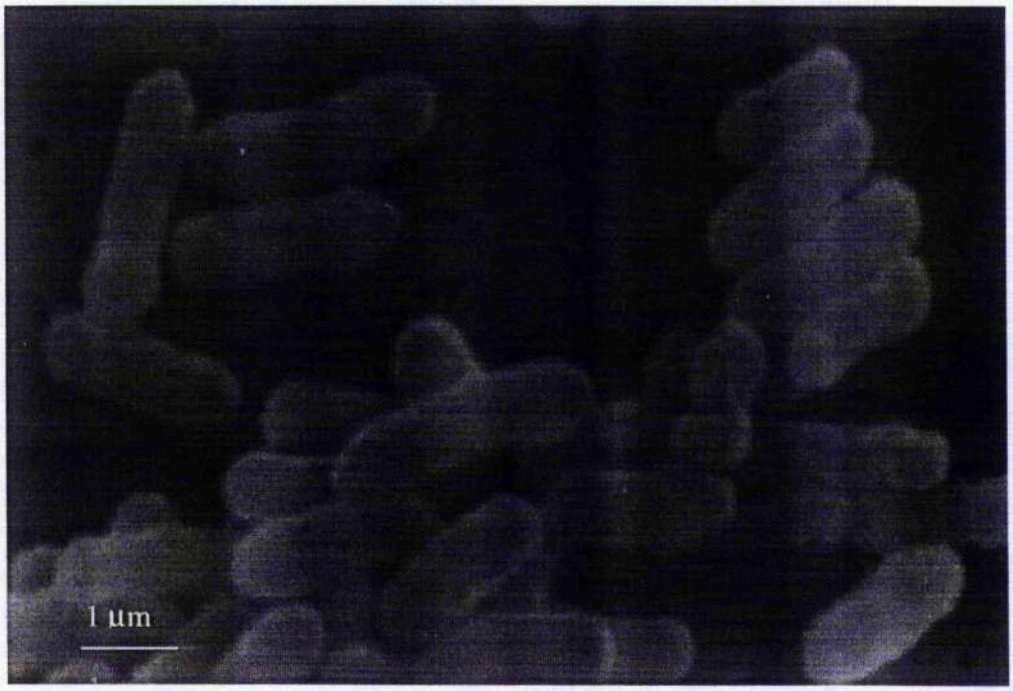


Figure 4.42 **Scanning electron micrograph (dimensions shown) of *E. coli* after exposure to a temperature to 50°C in a waterbath.**

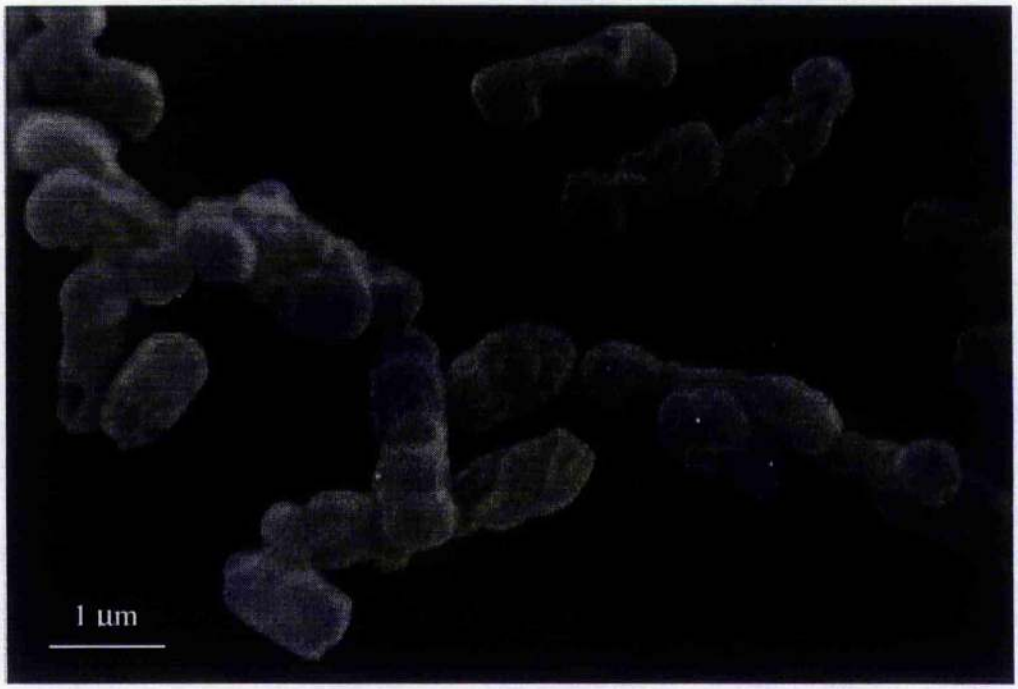


Figure 4.43 **Scanning electron micrograph (dimensions shown) of *E. coli* after exposure to a temperature to 60°C in a waterbath.**

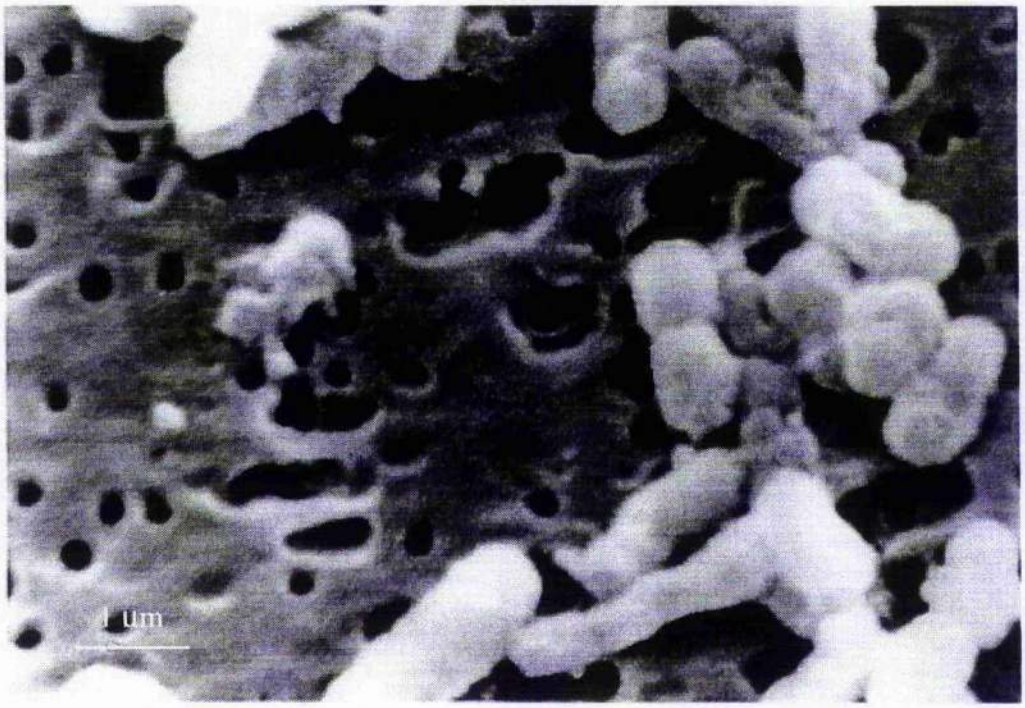


Figure 4.44 **Scanning electron micrograph (dimensions shown) of *E. coli* after exposure to a temperature of 70°C in a waterbath.**

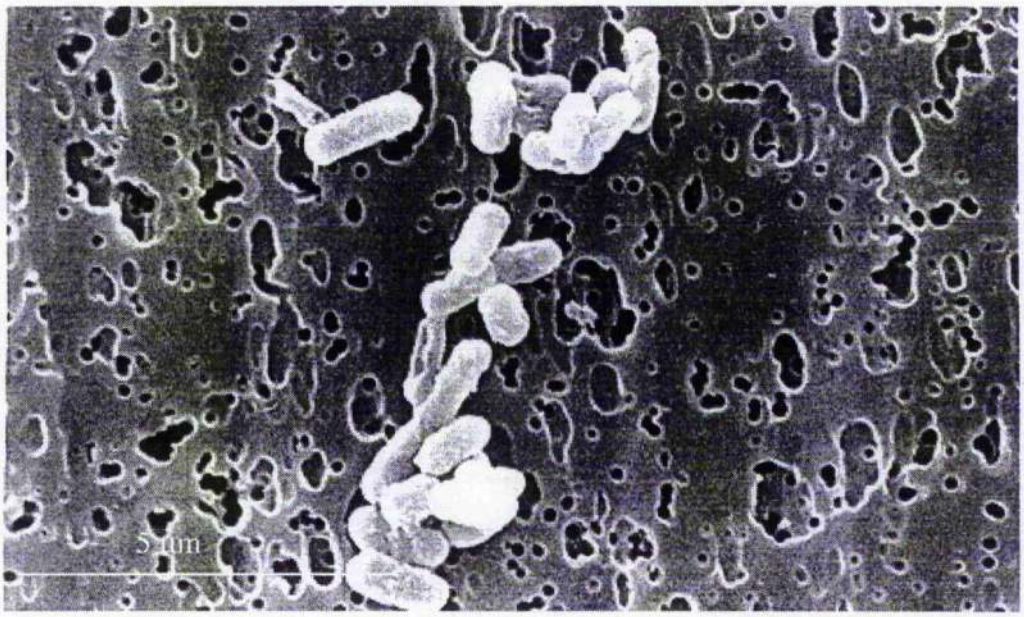


Figure 4.45A **Scanning electron micrograph at a low magnification (dimensions shown) of *E. coli* after exposure to a temperature to 100°C in a waterbath.**

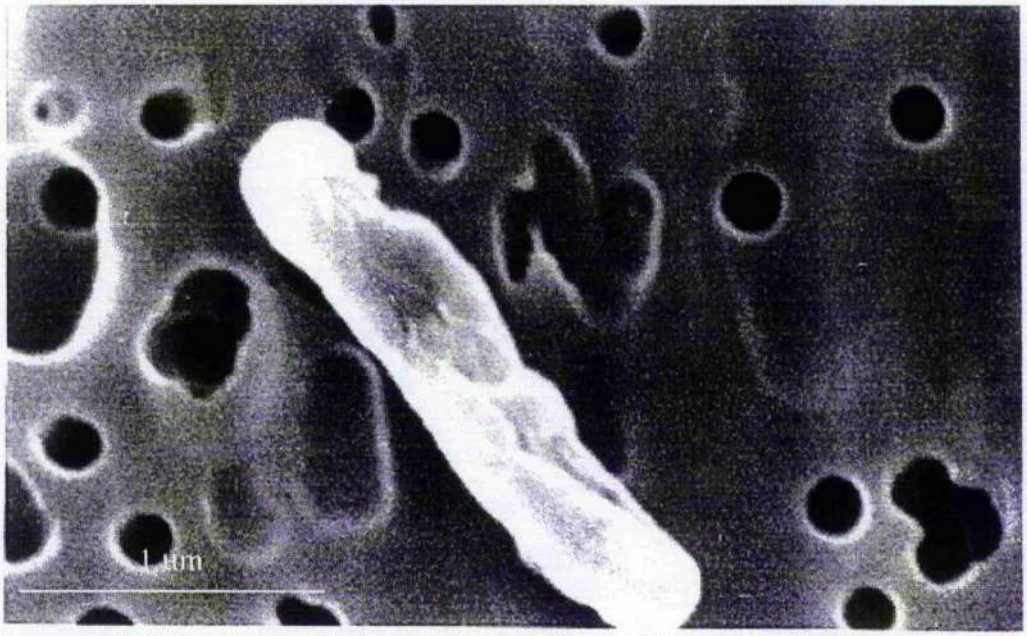


Figure 4.45B **Scanning electron micrograph at a high magnification (dimensions shown) of *E. coli* after exposure to a temperature of 100°C in a waterbath.**

4.3.2 DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry was applied to laser-treated and waterbath-treated bacterial cells to investigate the denaturation of proteins and nucleic acids. It was found that bacterial components denature in a sequential manner with time and temperature. Because of this the bacterial components produce irreversible endothermic peaks in the scans at particular temperatures which allows the calculation of the approximate internal cellular temperature.

Initially, the idea was to attempt to reproduce results obtained by Mackey *et al* (1991) on untreated bacterial cells. The first scan and re-scan is shown in **Figure 4.52**. The scan shows a large peak at 70°C which followed an area of endothermic and exothermic noise. On the re-scan the noise disappeared and the peak at 70°C was reduced considerably. However, irrespective of the number of times this experiment was repeated the result shown in **Figure 4.52** could not be reproduced. The scans produced by the repeated experiments are shown in Appendix 4. The problem appeared to be the use of inappropriate buffer (20mM Glutamate buffer; pH 7.2). The most successful buffer was 20mM Tris (pH 7.2). The effect of laser light on *E. coli* in liquid suspension was compared with 100°C waterbath treatment for five min by differential scanning calorimetry (**Figures 4.53** and **4.54**, respectively). In each figure there were four scans each representing a control, the heated samples to 50°, 60° and 70°C with either laser light (**Figure 4.53**) or for 5 min in a waterbath (**Figure 4.54**). A trough in a thermograph scan shows exothermic activity within the bacterial suspension, whereas a peak shows endothermic activity. For both of the control scans (**1**), where the bacterial suspensions (10 mg ml^{-1} wet weight) were not exposed to any thermal pre-treatment there were similar scan shapes. The scans both showed increased exothermic activity between 20° and 43°C, where a sharp cut off of exothermic activity was observed. Both scans showed a shoulder at 45°C which ran into an endothermic peak, with a maximum at 60°C. In **Figure 4.54** the scans were a little noisier than in **Figure 4.53** caused by the DSC machine. Occasionally the machine, as it is sensitive, reacts to moisture on the outside of the tantalum cells

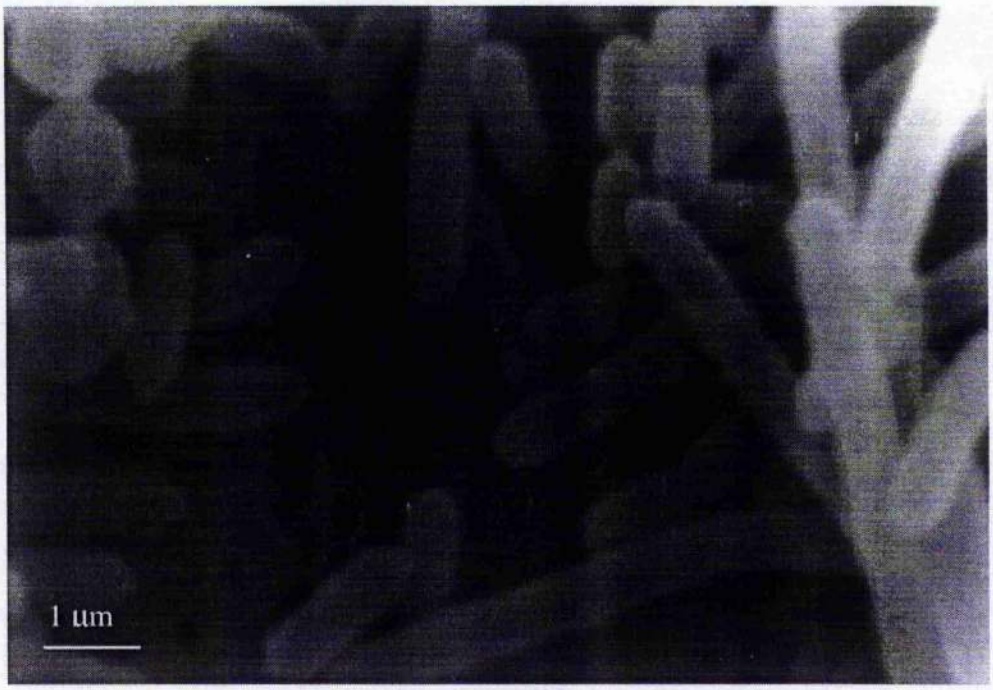


Figure 4.46 **Scanning electron micrograph (dimensions shown) of *L. monocytogenes* after no laser or heat exposure.**

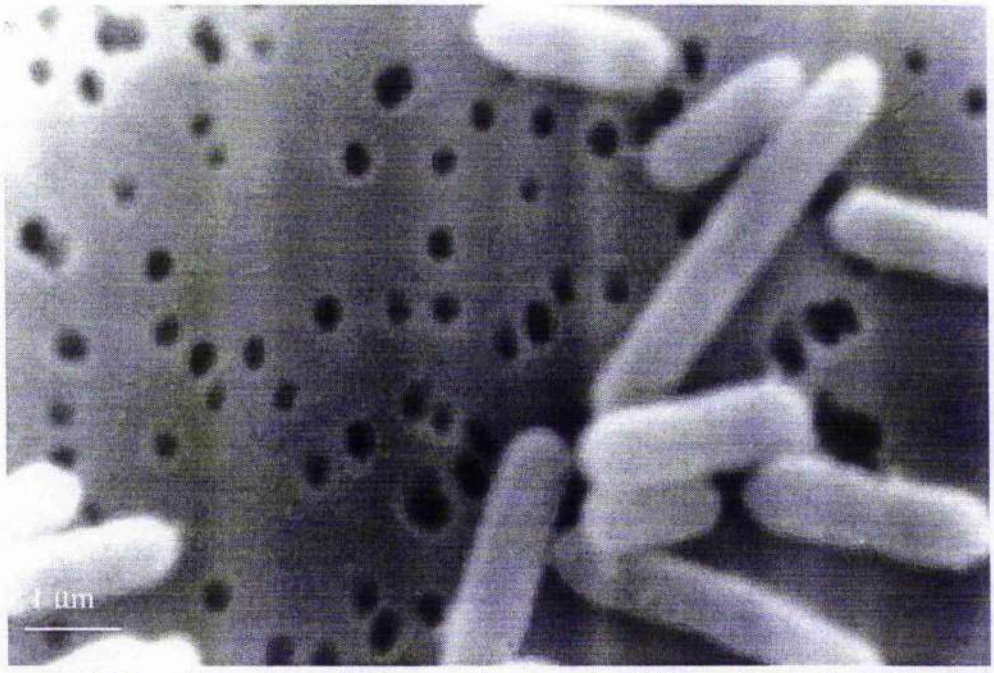


Figure 4.47 **Scanning electron micrograph (dimensions shown) of *L. monocytogenes* after laser exposure raising the temperature to 70°C.**

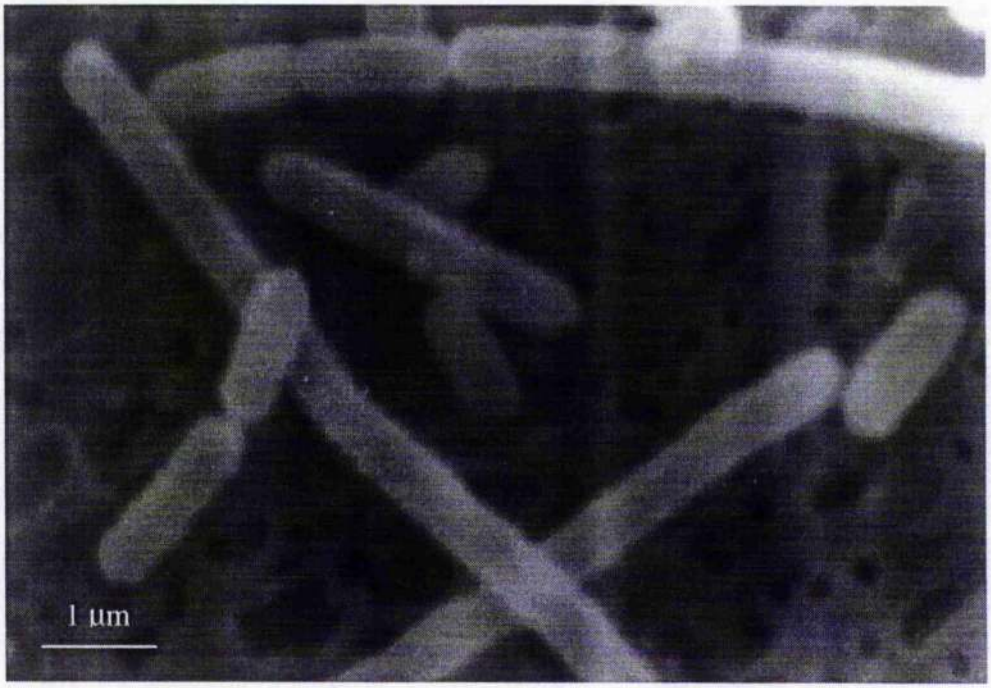


Figure 4.48 **Scanning electron micrograph (dimensions shown) of *L. monocytogenes* after exposure to a temperature of 100°C in a waterbath.**

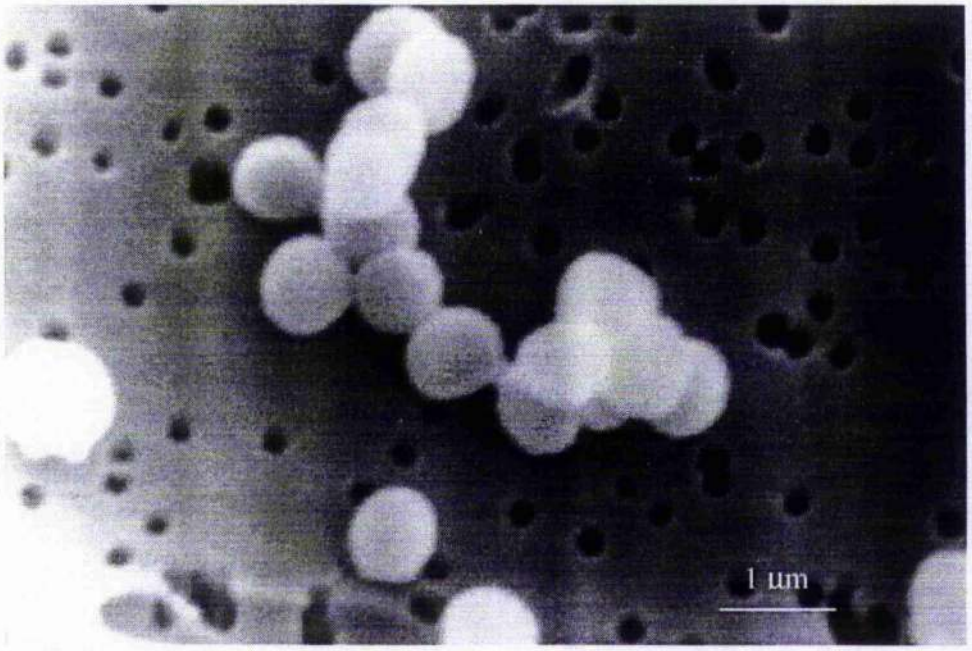


Figure 4.49 **Scanning electron micrograph (dimensions shown) of *S. aureus* without laser or heat exposure.**

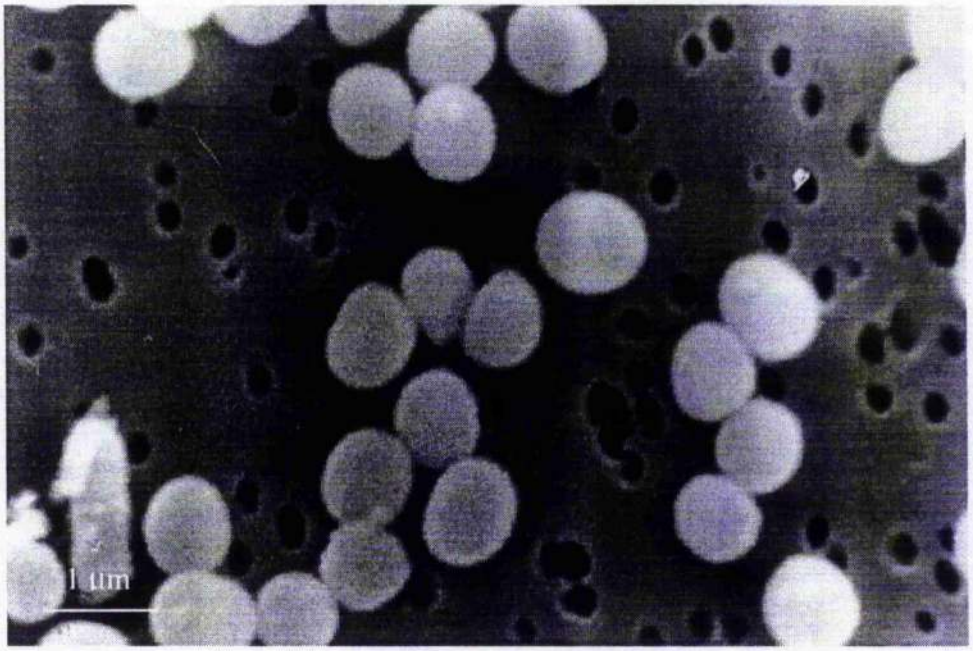


Figure 4.50 **Scanning electron micrograph (dimensions shown) of *S. aureus* after laser exposure raising the temperature to 70°C.**

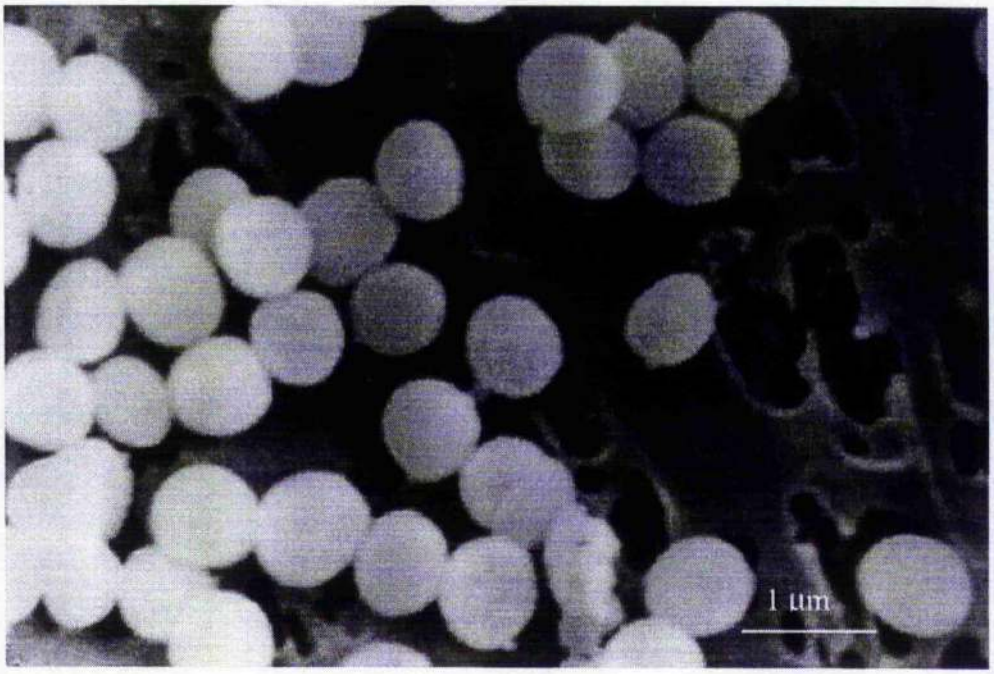


Figure 4.51 **Scanning electron micrograph (dimensions shown) of *St. aureus* after exposure to a temperature of 100°C.**

and produces noise. In **Figure 4.53** the scan of the 50°C laser pre-treatment (2) had a very similar shape to that of both control scans, except that the exothermic maximum shifted 5°C to a higher temperature of 48°C. The scan as before lost the exothermic activity sharply but ran straight into the endothermic peak at 60°C, without the shoulder previously seen in the controls. For the bacterial suspensions treated in the waterbath there was no exothermic activity at the temperatures up to 45°C as with both controls and 50°C laser pre-treatment scans. The 50°C scan kinks at 30°C but shows no exothermic activity. At 60°C this scan, as with the previously described scans, had an endothermic peak. The remaining scans from samples pre-treated to 60° and 70°C with laser light or for 5 min in a waterbath, showed no exothermic or endothermic activity. The vertical separation between each scan has been attributed to differences in either the ambient temperature at the time of scanning or the concentration of organic material in the samples.

4.3.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

The effect of the laser was further investigated with SDS-PAGE to analyse any gross effects on the proteins within the bacterial cells. The cells were exposed to laser light, raising the temperature to 40°, 50°, 60° and 70°C and 5 min waterbath treatments at 40°, 50°, 60°, 70° and 100°C. Along with a control, the treated cells were digested and prepared for SDS-PAGE. The results in **Figure 4.55** showed the consistency in the bands between all of the cell digests. The only notable difference between any of the lanes was that the bands in the 100°C water bath treatment were fainter than the other treatments and control, which may be the result of a lack of suspension volume or bacterial numbers initially in the wells.

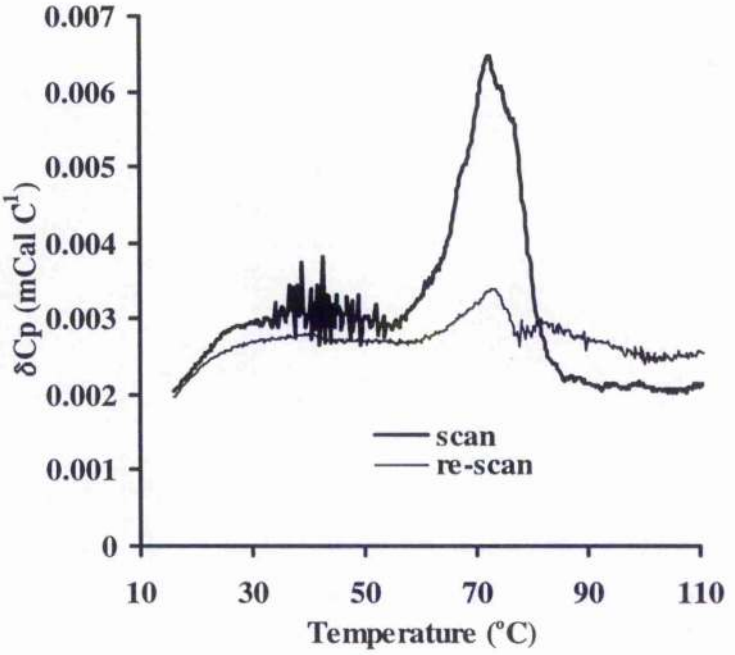


Figure 4.52 **Differential scanning calorimeter scan and re-scan of *E. coli* (10 μgml^{-1}) suspended in glutamate buffer against a glutamate buffer reference and a nominal scan rate of 60°C h^{-1}.**

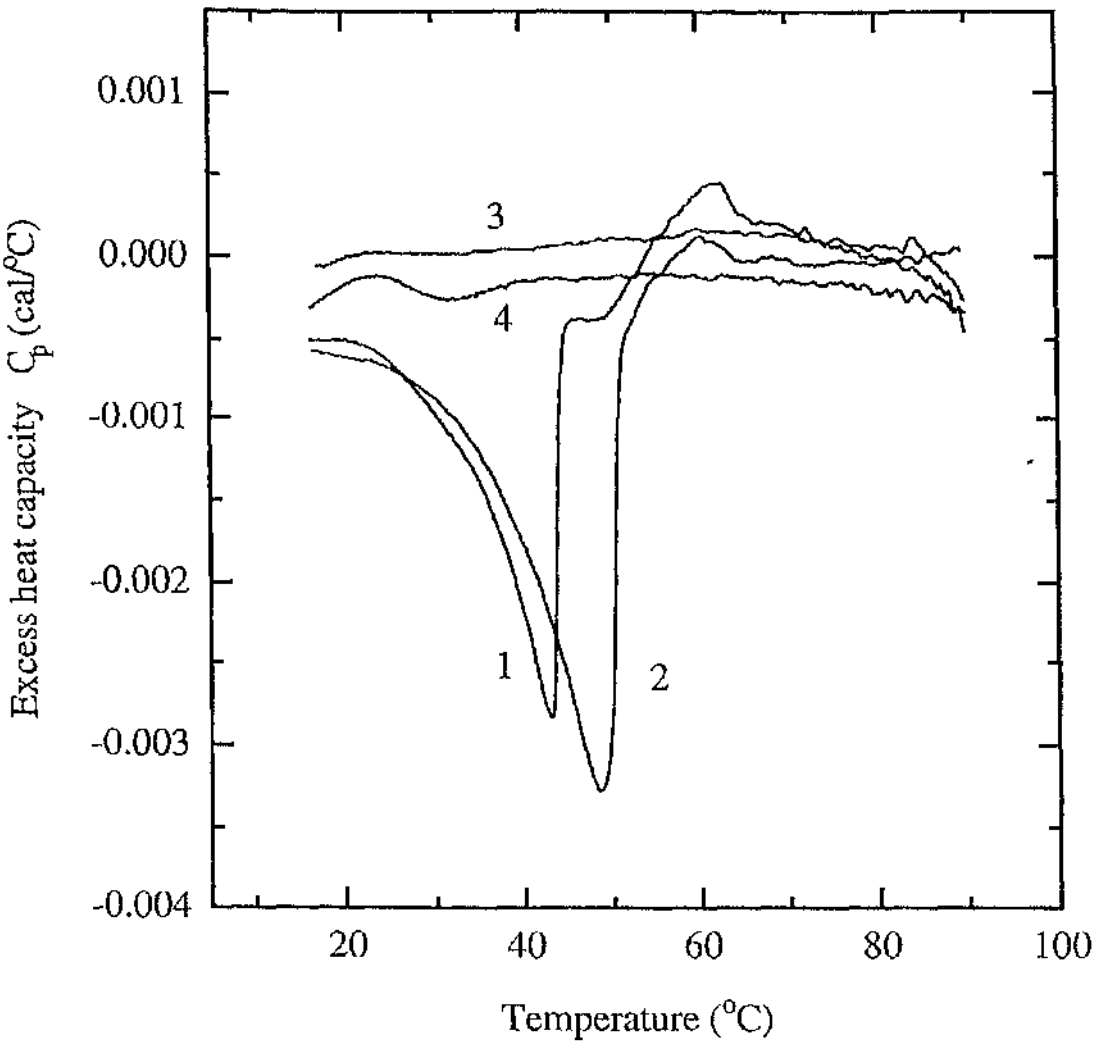


Figure 4.53 **Differential scanning calorimeter thermogram of *E. coli* (10 mgml^{-1}) suspended in 20mM Tris buffer (pH 7.2) against a Tris buffer reference (1).** The *E. coli* was pre-treated with laser light to 50° (2), 60° (3) and 70°C (4) prior to scanning.

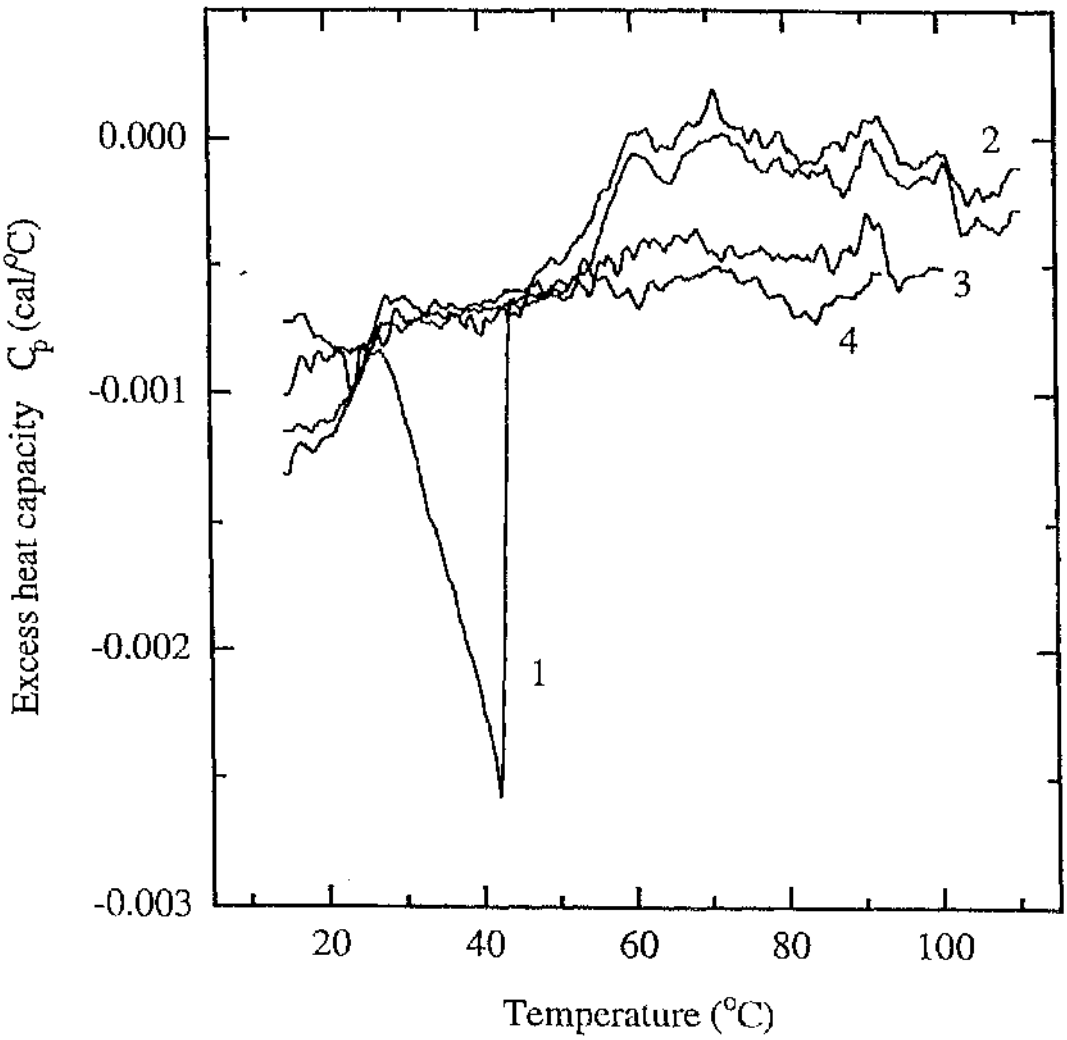


Figure 4.54 **Differential scanning calorimeter thermogram of *E. coli* (10 mgml^{-1}) suspended in 20mM Tris buffer (pH 7.2) against a Tris buffer reference(1).** The *E. coli* was pre-treated in a waterbath to 50° (2), 60° (3) and 70°C (4).

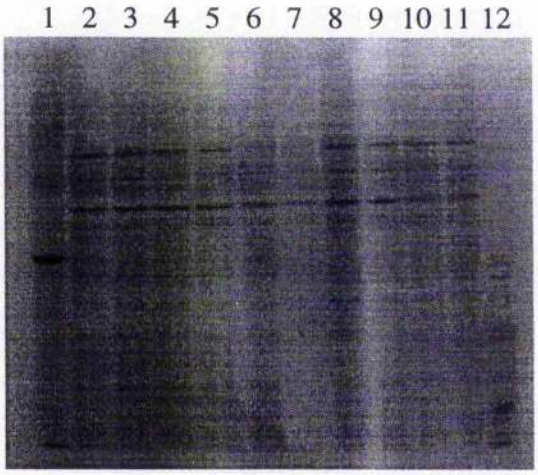


Figure 4.55 **SDS-polyacrylamide gel electrophoresis of whole cell digests of *E. coli* after laser and heat pre-treatments.** Heat pre-treated samples were exposed to specific temperatures, in a waterbath, for 5 min and laser pre-treated samples were raised to specified temperature and removed. Aliquots of 20µl of all control and laser pre-treatment samples were added to the wells

Lane
1. SDS 6H standard (5 µl)
2. *E. coli* - no pre-treatment (20 µl)
3. *E. coli* - 40°C heat pre-treatment (20 µl)
4. *E. coli* - 50°C
5. *E. coli* - 60°C
6. *E. coli* - 70°C
7. *E. coli* - 100°C
8. *E. coli* - 40°C laser pre-treatment (20 µl)
9. *E. coli* - 50°C
10. *E. coli* - 60°C
11. *E. coli* - 70°C
12. SDS 6 standard (5 µl)

4.4 KILLING OF BACTERIA ON METAL, PLASTIC AND GLASS SURFACES WITH Nd:YAG LASER LIGHT

The following section investigates the effect of Nd:YAG laser light on bacteria coated onto different solid surfaces. Three surfaces (stainless steel, glass and plastic) and four bacteria (*E. coli*, *St. aureus*, *L. monocytogenes* and *B. cereus*) were investigated.

4.4.3 DRYING BACTERIA ONTO SURFACES

On a solid surface it is possible for some bacterial contaminants to exist in a dry state (with reduced extraneous water), or alternatively in various states of hydration. To examine the effect of laser light on bacteria, coated onto surfaces, the state of hydration of the sample had to be investigated. For simplicity two extremes were used, i.e. the bacterial culture either as a wet suspension on a surface or as an air-dried film. Before the investigation could begin the effect of drying the selected bacteria, onto the surfaces, was examined. The bacteria chosen in this study, *E. coli*, *St. aureus*, *B. cereus* and *L. monocytogenes* were tested for viability during the drying procedure on a stainless steel surface. **Figure 4.56** is the plot of the drying time, in a laminar air-flow cabinet, against the viable count. The bacteria were from an overnight culture and aliquots (20 µl) were dropped onto the centre of stainless steel disks, 13 mm in diameter.

The effect of the drying procedure on the organisms after 1 h of drying in a laminar air-flow (LAF) cabinet is shown in **Figure 4.56**. The number of organisms in the overnight cultures varied between the bacterial species. *L. monocytogenes*' initial viable count was the lowest and *St. aureus* was the highest. *E. coli* and *B. cereus* had a similar overnight bacterial concentration. For the first 5 min of drying, little or no reduction in viability was observed on the surface of the stainless steel disk. The samples were visibly dry after 20 min in the laminar air flow cabinet. The samples remained in the cabinet for a further 40 min over which time the cell viability was monitored.

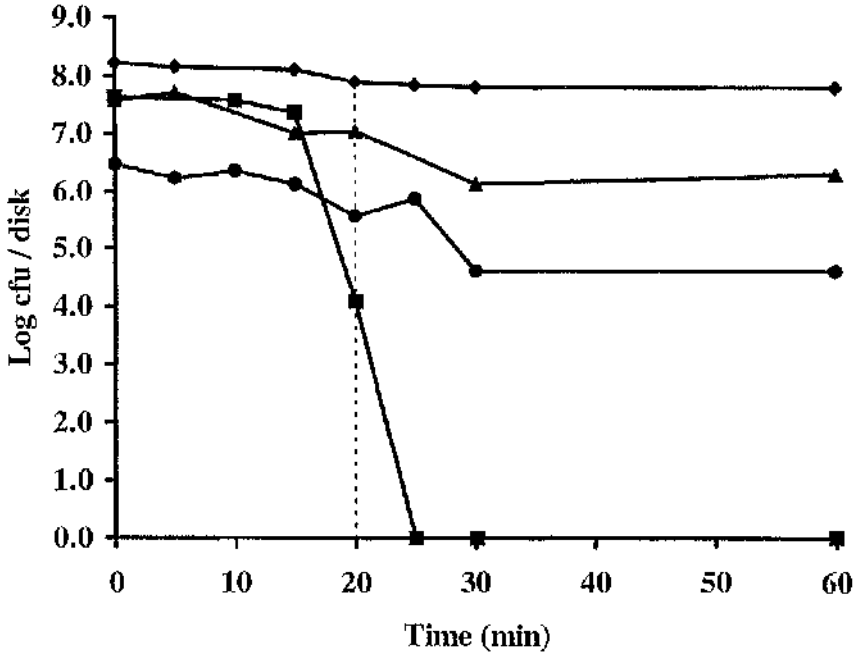


Figure 4.56 **Effect of drying 20 µl of overnight [◆] *St. aureus,* [■] *B. cereus,* [▲] *E. coli* and [●] *L. monocytogenes* suspensions, on stainless steel surfaces, in a laminar air flow cabinet.** At 20 min the surface of the disks appeared dry; this is indicated by the dotted line.

After removing the organisms from the disks by shaking for 3 h in PBS there was little change in the viable counts for *St. aureus* over 60 min, except for a slight decrease between 15 and 25 min which corresponded to the time taken for the disks to dry. The total loss in viability was around 0.5 D-values. Indeed, as this organism had the highest initial viable counts and as little reduction in viable cfu ml^{-1} was observed over 60 min of drying, *St. aureus* was an ideal microorganism for investigation of the effects of Nd:YAG laser light on bacteria coated onto surfaces. *E. coli* and *L. monocytogenes* had a similar response to drying onto a stainless steel surface. Approximately two D-values were lost after 1 h of drying in the LAF cabinet with the most notable decrease again seen around the time that the samples began to visibly dry. The only difference between these two organisms was the initial viable counts. The viability of vegetative cells of *B. cereus* over the first 15 min was relatively constant. As the disks dried, the viability of the samples reduced dramatically. Between 15 and 25 min the viability dropped to "zero", however, in this experiment the lower detection limit was 400 organisms ml^{-1}. *B. cereus* could therefore not be utilised as dried samples on solid surfaces.

Stainless steel was used as the standard surface to compare the viabilities of four bacterial species after drying in the LAF cabinet. The effect of the surface material was also examined, **Figure 4.57** shows the results of drying an overnight *E. coli* culture on stainless steel and nylon plastic surfaces. On both disk types the initial culture concentrations were comparable. The disks were dry at the same time (i.e. after 20 min) indicated by the dotted line. As shown in **Figure 4.56**, the viability on stainless steel was similar in this experiment. In addition the result shown in **Figure 4.57** revealed that the nylon plastic showed no significant difference to that of stainless steel; the viable cell number was reduced by 2.0 D-values after 60 min of drying.

4.4.4 EXPOSURE OF BACTERIA ON DIFERENT SURFACES

The effect of Nd:YAG laser light on *E. coli*, *St. aureus*, *L. monocytogenes* and *B. cereus* was examined, both as a wet film and a dry film, on three different surfaces (stainless steel, plastic and glass)

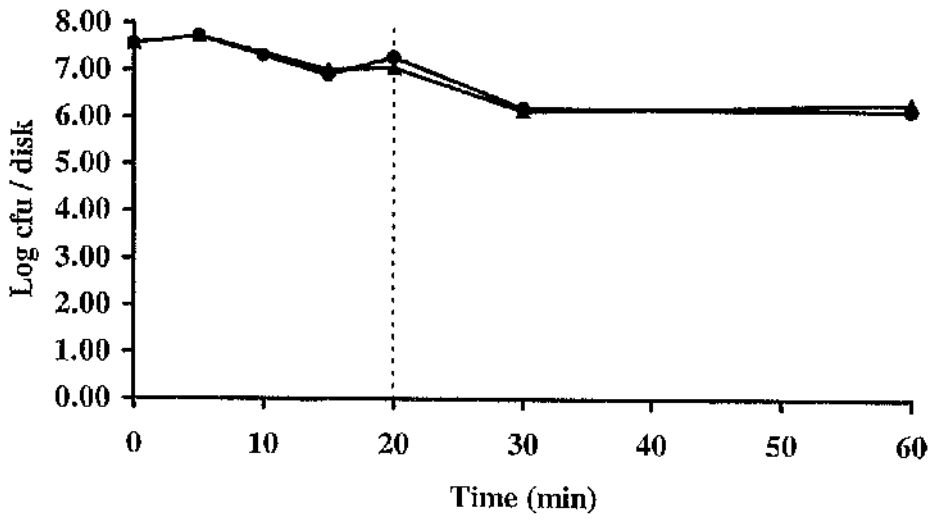


Figure 4.57 **Effect of drying 20 µl of an overnight *E. coli* suspension, on [▲] stainless steel and [●] nylon plastic surfaces, in a laminar air flow cabinet.** The dotted vertical line at ~20 min indicates the time at which the surfaces of the disks looked dry.

The four bacterial species were exposed to laser light in the presence of excess liquid on stainless steel disks. **Figure 4.58** shows the killing curves for *E. coli*, *St. aureus*, *L. monocytogenes* and *B. cereus* treated as a wet film of overnight culture on stainless steel disks. The results presented in **Figure 4.58** show the viable counts (log_{10}) for the four bacterial species after exposure to increasing energy densities of Nd:YAG laser light. Each point is a separate exposure of bacteria seeded on to separate disks; the results show the average values for two experiments. *L. monocytogenes*, *E. coli* and *B. cereus* all had a similar initial bioburden and showed comparable cell reduction at exposures up to 100 Jcm^{-2}. Viability of both *E. coli* and *B. cereus* continued to fall but *L. monocytogenes* levelled off at 1 x 10^5 cfu ml^{-1} before a final drop in viability at 400 Jcm^{-2}. *E. coli* also appeared to level off at around 10 bacteria / disk, and zero was reached after 500 Jcm^{-2}. The lower detection limit for this graph is 2 bacteria / disk. *B. cereus* showed a straight line killing curve. The most resistant organism to laser light was a wet film of *St. aureus*. It survived a higher ED of Nd:YAG laser light than the other three microorganisms, before reaching zero at 800 Jcm^{-2}. The order of resistance appeared to be *St. aureus* > *L. monocytogenes* > *E. coli* > *B. cereus*.

The results presented in **Figure 4.59** show the viable counts (log_{10}) for *E. coli*, *L. monocytogenes* and *St. aureus* seeded onto stainless steel disks after exposure to increasing energy densities of Nd:YAG laser light. The initial bioburden for each bacterial species was different; *St. aureus* had the highest count and *E. coli*, the lowest. It was apparent that the effect of the laser on each of the organisms was uniform in that the rate of killing was similar with all three. *L. monocytogenes* was almost completely killed at 630 Jcm^{-2}, followed by *E. coli* at 790 Jcm^{-2} and *St. aureus* at 900 Jcm^{-2}. The lower detection limit for this experiment was 2 cfu / disk.

When different surfaces were contaminated with bacteria the effect of decontaminating with laser light differed. Three surfaces, stainless steel, nylon plastic and glass were contaminated with an aliquot (20 µl) of overnight *St. aureus* culture and exposed to different energy densities of Nd:YAG laser light. The results in **Figure 4.60** show the viable counts (log_{10}) after treatment with increasing energy

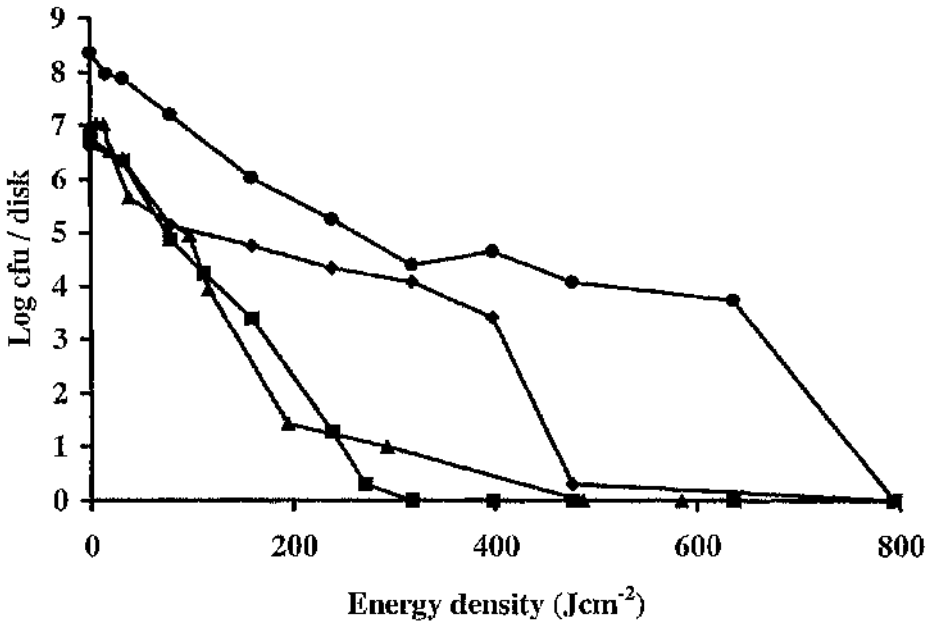


Figure 4.58 **Killing curves for [◆] *L. monocytogenes*, [■] *B. cereus*, [▲] *E. coli* and [●] *St. aureus* as a wet film of overnight culture on the surface of stainless steel disks.** Each point is a separate exposure on different disks. The results are the average of two separate experiments.

densities. Within the range of energy densities used in this experiment the Nd:YAG laser could only be used to decontaminate the stainless steel however, the dried bacterial suspensions on the surfaces began to char a little, though no damage to the surface was seen. Little or no reduction in the bacteria viability on the glass or plastic surfaces was observed within the range of energy densities used. The highest energy densities of Nd:YAG laser light, used on the plastic and the glass disks, damaged the surfaces.

4.4.5 MULTIFACTORIALLY-DESIGNED EXPERIMENTS

Several types of parameters potentially influence laser sterilization systems as mentioned in appendix 5. The parameters examined in these experiments fell into three broad categories: growth (NaCl concentrations and pH values), exposure (water content) and laser parameters (PRF, pulse energy and exposure time).

To determine the maximum and minimum parameter values tolerable to bacterial growth for use in a multifactorially-designed experiment a range of NaCl concentrations and pH values were investigated with each bacterial species. The effect of varying NaCl concentration on the growth of the four different bacterial species is summarised in **Table 4.4**. As *St. aureus* is halotolerant NaCl at 10% w/v was used as the maximum concentration. The other three bacterial species had NaCl tolerances between 2.5 and 4.0% w/v. To keep values constant, *E. coli, B. cereus,* and *L. monocytogenes* were given the same upper limit of 2.5% w/v. For each species the lower limit was 0.5% w/v NaCl, the normal concentration in nutrient agar.

The effect of different pH values at the limits of NaCl concentration for each species is summarised in **Table 4.5**. The pH values were obtained with the addition of HCl or NaOH to the broth before autoclaving, but the pH value changed very little during the sterilization process. The broth at pH 5.5, at most increased by a value of 0.2 and

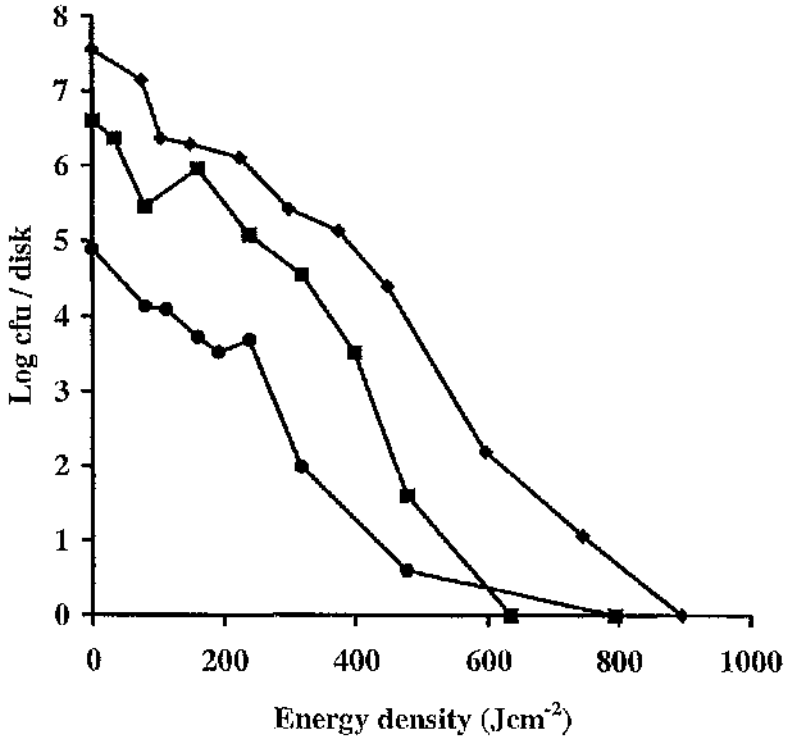


Figure 4.59 **Killing curves for [●] *E. coli*, [◆] *St. aureus* and [■] *L. monocytogenes* as a dried film of overnight culture on the surface of stainless steel disks.** Each point is a separate exposure on different disks. The results are the average of two separate experiments.

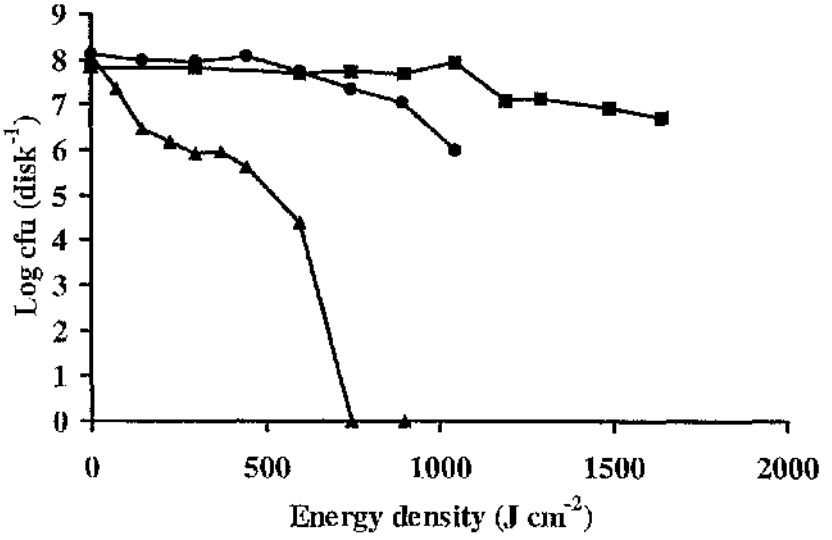


Figure 4.60 **Killing curves for overnight cultures of *St. aureus* as dry films on three different surfaces**: stainless steel [▲], Nylon plastic [■] and glass [●]. Each point is a separate exposure on different disks. The results are the average of two separate experiments.

those at pH 8, decreased by 0.2, i.e. the cultures were between pH 5.5 and 5.7 and pH 7.8 and 8. With the pH values and at the two NaCl concentrations shown the different bacteria grew but beyond these limits there was some variation in the ability of the organisms to grow. To maintain consistency, pH values of 5.5 and 8 were chosen for all species.

For simplicity, the most extreme limits of water content, for a bacterial sample was a drop of overnight culture on the surface (wet suspension) and a bacterial sample dried onto a surface (dry suspension). The effect of drying the different bacterial species onto surfaces has been previously discussed. Because all viability was lost with *B. cereus* when dried down it could not be used as a dried sample.

Table 4.6 shows the range of parameters used in the multifactorial analysis. The exposure times were variable as different microorganisms require different values according to their sensitivity as a dry film or a wet film as shown in **Figures 4.58** and **4.59** respectively. The pulse energy and the PRF were kept constant at 5 and 10 J and 20 and 30 Hz.

For simplicity, each individual bacterial species was considered separately. Only stainless steel was examined, because plastic and glass were not decontaminated with Nd:YAG laser light. The parameters were split into two groups, for further simplification: the laser parameters (pulse energy, PRF and exposure time) and the other parameters, termed environmental for this study (NaCl concentration, pH and water content). By this method 8 experiments per analysis and only 8 analyses were required. These were repeated several times for statistical purposes.

4.4.5.1 Exposure of bacterial species at different laser parameters

Each set of analyses are presented as a series of twelve graphs accounting for three laser variables at both limits of water content. For each bacterial species the variable values were the same with the exception of the exposure time as previously discussed. Each analysis requires two graphs and therefore in each figure **A** and **B**, **C** and **D**, and **E** and **F** are representative of all of the three different parameter

Organisms	NaCl concentrations (% w/v)													
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	9.5	11.5	13.5
St. aureus	++	ND	ND	ND	++	ND	ND	ND	ND	ND	++	++	+/-	-
E. coli	++	++	++	++	++	+	+/-	-	-	ND	ND	ND	ND	ND
B. cereus	++	++	++	++	++	++	+	+/-	-	-	ND	ND	ND	ND
L. monoc	++	++	++	++	++	++	++	++	+	+/-	ND	ND	ND	ND

++	Good growth	+	Growth
+/-	Slight growth	-	No growth
ND	Not done		

Table 4.4 **Effect of NaCl concentration on the growth of the four bacterial species to find limits of NaCl tolerance**

Organisms	pH values at NaCl concentrations (% w/v)											
	5.5		6.0		6.5		7.0		7.5		8.0	
	0.5	2.5/10	0.5	2.5/10	0.5	2.5/10	0.5	2.5/10	0.5	2.5/10	0.5	2.5/10
St. aureus	++	+	++	++	++	+	ND	ND	++	++	++	++
E. coli	++	++	++	++	++	++	ND	ND	++	++	++	++
B. cereus	++	++	++	++	ND	ND	ND	ND	++	++	++	++
L. monoc	++	+	++	++	++	++	++	++	ND	ND	++	++

++	Good growth	+	Growth
ND	Not done		

Table 4.5 **The effect of pH on the growth of the four bacterial species to find their limits of pH tolerance**

Parameters	Organisms			
	E. coli	*St. aureus*	*L. monocytog*	*B. cereus*
NaCl (% w/v)	0.5 - 2.5	0.5 - 10.0	0.5 - 2.5	0.5 - 2.5
pH	5.5 - 8.0	5.5 - 8.0	5.5 - 8.0	5.5 - 8.0
A_w	wet and dry	wet and dry	wet and dry	wet only
Pulse energy (J)	5 - 10	5 - 10	5 - 10	5 - 10
PRF (Hz)	20 - 30	20 - 30	20 - 30	20 - 30
Exposure time (s)	variable	variable	variable	variable

Table 4.6 **Table summarising the parameter limits for each bacterial species and the different variables**

combinations. The two levels of the individual parameters are represented as follows: one parameter as different points on the x-axis, the second as the different lines and the third as different graphs. Additionally there are tables of statistical analysis, produced on MINITAB (Minitab Inc., USA) with the general linear model, of the data represented in each figure. The direct output of the computer program are shown in the tables and the important columns are the mean square, *F*-ratio and the probability. The mean square is the division of the sum of squares by the degrees of freedom. The *F*-ratio is the ratio of the experimental mean square to the error mean square. From the *F*-ratio the 95% probability of proving the null hypothesis is determined. Each table describes the significance of a change in each parameter or an interaction between two parameters on the bactericidal capabilities of Nd:YAG laser light.

From the graphs in **Figure 4.61** the effect of an increase in the laser parameter values on the killing of *St. aureus*, as a dried sample on stainless steel is clearly visible. A range of killing from 0.1 to 3.8 D-values between the low and high parameters was observed. The largest effect on laser light killing was when all parameters were at the higher setting. Significant effects with all of the individual parameters on the bactericidal ability of Nd:YAG laser light were seen (**Table 4.7**). From the figure it is apparent that there was little effect when only one parameter was increased to the upper value. The presence of interactions between the different parameters is indicated by the deviation of the lines from parallelism. It appears that the in all of the graphs there was a deviation from parallelism but the statistics in **Table 4.7** show that only when one variable was low and the other two were varied, was the interaction between the two high variables, significant. It is worth noting that the error mean square (MS) with the non-significant interactions were around ten times higher than the error MS of the insignificant interactions.

As a wet sample on stainless steel, the effects of the different laser parameters on *St. aureus* were not as significant as with the dried sample (**Figure 4.62**). There appeared to be less separation between the lines and less interaction between the parameters. A range of 0.2 to 2.6 D-values was obtained with the parameter values indicated. The significance of the effects produced by each individual parameter

were variable (**Table 4.8**); the majority of values however, were significant, with exceptions being increased pulse energy at 30 Hz, PRF at 1 sec and PRF at 10 J. All of these insignificant parameters were linked with the PRF. Only one of the interactions was significant, the PRF and the exposure time at a pulse energy of 5 J.

The effect of the Nd:YAG laser parameters on the bactericidal ability of laser light on a dried film of *E. coli* is shown in **Figure 4.63**. The range of values obtained with the upper and lower PRF and pulse energies with an increase in exposure time of 0.5 and 1.5 sec exposure was 1.0 to 3.8 D-values, compared with 0.1 and 3.8 with *St. aureus* with the longer exposure times of 1.5 and 2.5 sec. The results suggest that an increase in each parameter value caused increased bacterial reduction however most of the lines in each graph were parallel to one another suggesting a lack of interactions. The statistics in **Table 4.9** shows that only one of these parameters was significant, i.e. at 5 J an increase in the exposure time from 0.5-1.5 sec significantly affected the results. All other parameters including interactions were insignificant, however there were relatively large errors in this experiment.

Figure 4.64 shows the effect of the different parameters on the bactericidal ability of Nd:YAG laser light toward *E. coli* as a wet sample. When one parameter was low there was very little influence from the other two parameters, and there does not appear to be any interaction between the different parameters. The exact opposite can be said when one parameter was kept high. The level of killing after 1.0 sec was extremely high, at 7 D-values. From the statistics in **Table 4.10** all of the parameters were significant with the exception of the increase in exposure time at a pulse energy of 5 J. Indeed this is the exact opposite result to that obtained with the dried samples. All of the interactions with the parameters at the higher value were significant and at the lower values insignificant.

From the graphs in **Figure 4.65** the effects of increased parameter values of Nd:YAG laser light, on *L. monocytogenes* as a dried sample on stainless steel is clearly visible. A range of killing from 0.3 to 3.4 D-values was observed with the different parameter combinations and the combination of the three highest values again producing the highest effect on killing. The effect of varying individual parameters

on the bactericidal ability of Nd:YAG laser light were highly variable with insignificant effects with PRF (**Table 4.11**). From the figure it appears that in the majority of the graphs there was a deviation from parallelism but the statistics presented in **Table 4.11** show that none of the interactions were significant.

The effect of the different laser parameters on the bactericidal capacity of Nd:YAG laser light towards *L. monocytogenes* as a wet sample on the surface of stainless steel was examined (**Figure 4.66**). Laser light with the lower parameter values, in some cases, produced negative results indicating that laser light caused a slight increase in viability. This may be due to experimental errors or the fact that the cells have been heated up to such an extent that some cell doubling was seen, but the fact is that the increase in viability was very small. Only two of the individual parameters were not significant (**Table 4.12**), namely increased exposure time at 20 Hz and at 5 J. It can be seen in the figure that all of the values of cell reduction were low and the error produced by the results was relatively large, therefore resulting in insignificant effects on the bactericidal ability of laser light on *L. monocytogenes*. The results of the interactions as a wet sample were all insignificant, as obtained with *L. monocytogenes* as a dry film on stainless steel.

B. cereus was only analysed as a wet sample on the surface of stainless steel because of the biocidal effect of drying on the organism. In **Figure 4.67** it is obvious that Nd:YAG laser light had the greatest effect on this organism, which concurs with results previously obtained (**Figure 4.13**). The higher exposure times (1.0 sec) produced a log cell reduction of 7.0. Each of the different parameters under each combination were highly significant. When one variable was kept high (**Figures 4.67B, D** and **F**) the interactions between the other two were significant.

4.4.5.2 Exposure of bacterial species under different environmental stresses

These results consist of three different parameters which affect either the growth of the bacterial species or the state of the bacteria when exposed. There was originally a fourth parameter, the ambient temperature, however keeping the temperature at the higher value constant was difficult and the results proved to be highly inconsistent.

Figure 4.61 **Effect of Nd:YAG laser light on *St. aureus* on stainless steel surfaces.** Multifactorially-designed experiment investigating the effect of different laser parameters on the efficiency of killing of *St. aureus*, as a dry film, by laser light. Each graph shows the reduction in viability of *St. aureus* at a **A.** PRF of 20 Hz, **B.** PRF of 30 Hz, **C.** exposure time of 1.5 sec, **D.** exposure time of 2.5 sec, **E.** pulse energy of 5 J and **F.** pulse energy of 10 J. For **A.** to **D.** the curves represent [●] 5 J and [■] 10 J and in **E.** and **F.** [●] 20 Hz and [■] 30 Hz.

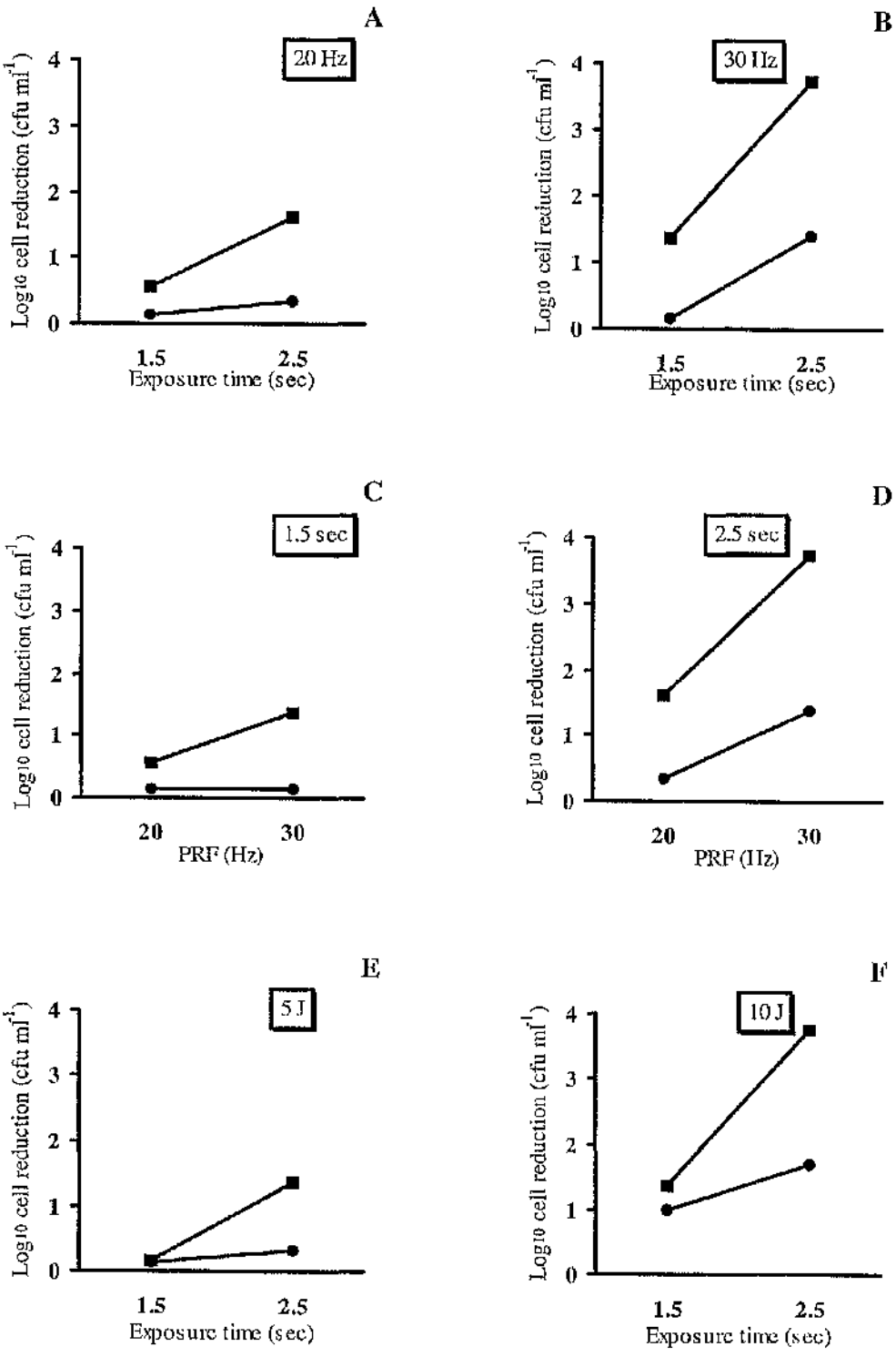

A
20 Hz
Log10 cell reduction (cfu ml-1)
4
3
2
1
0
1.5
2.5
Exposure time (sec)
B
30 Hz
Log10 cell reduction (cfu ml-1)
4
3
2
1
0
1.5
2.5
Exposure time (sec)
C
1.5 sec
Log10 cell reduction (cfu ml-1)
4
3
2
1
0
20
30
PRF (Hz)
D
2.5 sec
Log10 cell reduction (cfu ml-1)
4
3
2
1
0
20
30
PRF (Hz)
E
5 J
Log10 cell reduction (cfu ml-1)
4
3
2
1
0
1.5
2.5
Exposure time (sec)
F
10 J
Log10 cell reduction (cfu ml-1)
4
3
2
1
0
1.5
2.5
Exposure time (sec)

Table 4.7 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.61*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.61.*** Each table shows the ***F*** ratio and the probability for the parameters and interactions shown in column 1

A. PRF 20 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	2.1590	2.1590	2.1590	60.11	0.000
Time	1	1.1719	1.1719	1.1719	32.63	0.000
Energy*Time	1	0.5677	0.5677	0.5677	15.81	0.004
Error	8	0.2873	0.2873	0.0359		
Total	11	4.1859				

B. PRF 30 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	9.5944	9.5944	9.5944	22.39	0.000
Time	1	9.7741	9.7741	9.7741	22.81	0.000
Energy*Time	1	0.9919	0.9919	0.9919	2.32	0.167
Error	8	3.4275	3.4275	0.4284		
Total	11	23.7878				

C. Exposure time 1.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	1.98453	1.98453	1.98453	57.19	0.000
Freq	1	0.51253	0.51253	0.51253	14.77	0.005
Energy*Freq	1	0.48000	0.48000	0.48000	13.83	0.006
Error	8	0.27760	0.27760	0.03470		
Total	11	3.25467				

D. Exposure time 2.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	9.9736	9.9736	9.9736	23.21	0.000
Freq	1	7.6161	7.6161	7.6161	17.73	0.003
Energy*Freq	1	0.8748	0.8748	0.8748	2.04	0.191
Error	8	3.4372	3.4372	0.4296		
Total	11	21.9018				

E. Pulse energy 5 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	0.8533	0.8533	0.8533	6.25	0.037
Time	1	1.5123	1.5123	1.5123	11.07	0.010
Freq*Time	1	0.8112	0.8112	0.8112	5.94	0.041
Error	8	1.0925	1.0925	0.1366		
Total	11	4.2694				

F. Pulse energy 10 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	6.5121	6.5121	6.5121	19.87	0.002
Time	1	8.8752	8.8752	8.8752	27.08	0.000
Freq*Time	1	1.3068	1.3068	1.3068	3.99	0.081
Error	8	2.6223	2.6223	0.3278		
Total	11	19.3164				

Figure 4.62 **Effect of Nd:YAG laser light on *St. aureus* on stainless steel disks.** Multifactorially-designed experiment investigating the effect of different laser parameters on the efficiency of killing of *St. aureus*, as a wet film, by laser light. Each graph shows the reduction in viability of *St. aureus* at a **A.** PRF of 20 Hz, **B.** PRF of 30 Hz, **C.** exposure time of 1.0 sec, **D.** exposure time of 2.0 sec, **E.** pulse energy of 5 J and **F.** pulse energy of 10 J. For **A.** to **D.** the curves represent [●] 5 J and [■] 10 J and in **E.** and **F.** [●] 20 Hz and [■] 30 Hz.

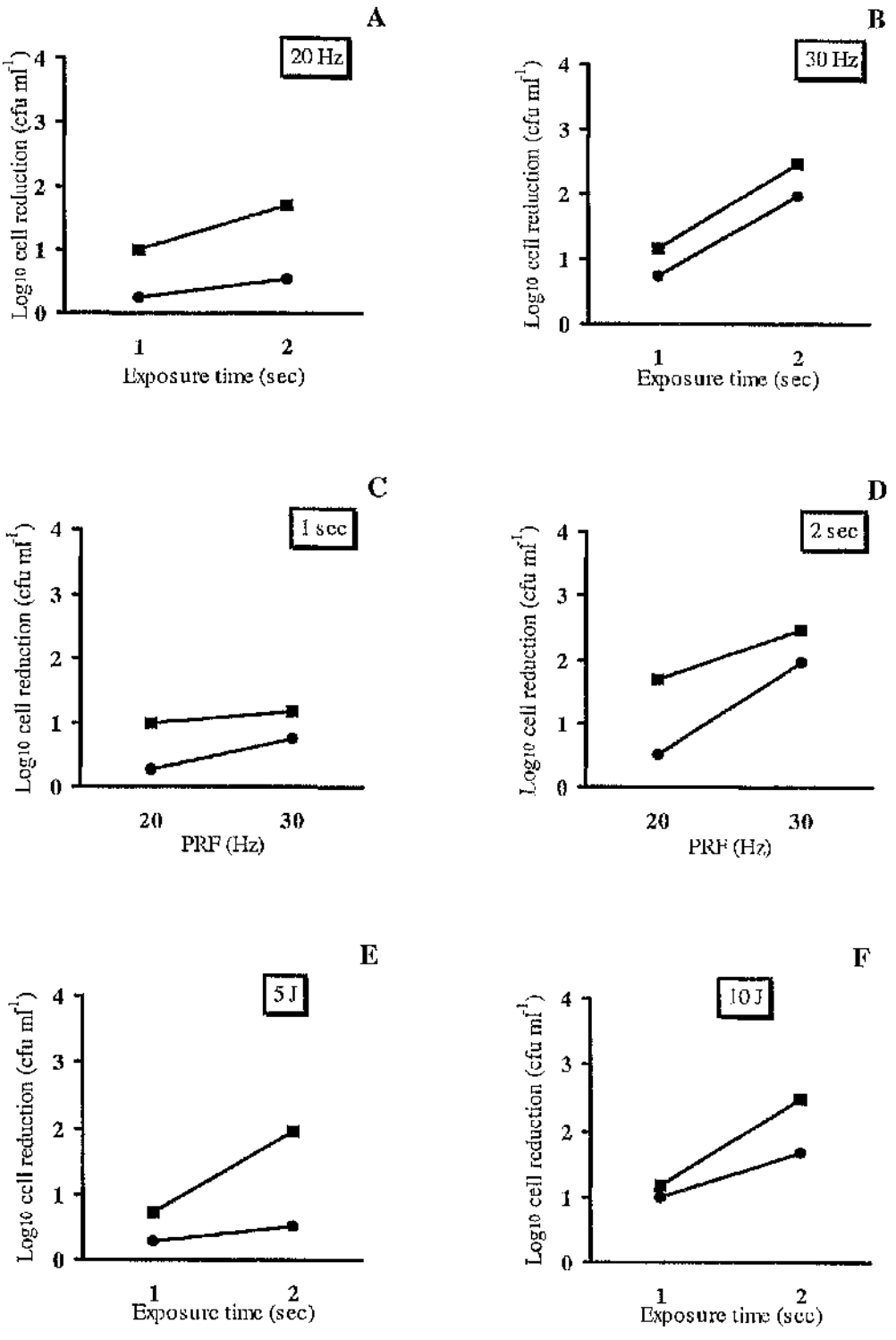

A
20 Hz
Log10 cell reduction (cfu ml-1)
Exposure time (sec)
B
30 Hz
Log10 cell reduction (cfu ml-1)
Exposure time (sec)
C
1 sec
Log10 cell reduction (cfu ml-1)
PRF (Hz)
D
2 sec
Log10 cell reduction (cfu ml-1)
PRF (Hz)
E
5 J
Log10 cell reduction (cfu ml-1)
Exposure time (sec)
F
10 J
Log10 cell reduction (cfu ml-1)
Exposure time (sec)

Table 4.8 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.62*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.62.*** Each table shows the F ratio and the probability for the parameters and interactions shown in column 1

A. PRF 20 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	1.75781	1.75781	1.75781	80.96	0.001
Time	1	0.42781	0.42781	0.42781	19.70	0.011
Energy*Time	1	0.09901	0.09901	0.09901	4.56	0.100
Error	4	0.08685	0.08685	0.02171		
Total	7	2.37149				

B. PRF 30 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	0.4465	0.4465	0.4465	4.92	0.091
Time	1	3.2131	3.2131	3.2131	35.44	0.004
Energy*Time	1	0.0045	0.0045	0.0045	0.05	0.834
Error	4	0.3627	0.3627	0.0907		
Total	7	4.0268				

C. Exposure time 1.0 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	0.64980	0.64980	0.64980	8.89	0.041
Freq	1	0.19845	0.19845	0.19845	2.72	0.175
Energy*Freq	1	0.04205	0.04205	0.04205	0.58	0.490
Error	4	0.29230	0.29230	0.07307		
Total	7	1.18260				

D. Exposure time 2.0 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	1.4112	1.4112	1.4112	35.91	0.004
Freq	1	2.5088	2.5088	2.5088	63.84	0.001
Energy*Freq	1	0.2048	0.2048	0.2048	5.21	0.085
Error	4	0.1572	0.1572	0.0393		
Total	7	4.2820				

E. Pulse energy 5 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	1.8050	1.8050	1.8050	39.15	0.003
Time	1	1.0658	1.0658	1.0658	23.12	0.009
Freq*Time	1	0.4802	0.4802	0.4802	10.42	0.032
Error	4	0.1844	0.1844	0.0461		
Total	7	3.5354				

F. Pulse energy 10 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	0.47045	0.47045	0.47045	7.10	0.056
Time	1	2.00000	2.00000	2.00000	30.18	0.005
Freq*Time	1	0.19845	0.19845	0.19845	2.99	0.159
Error	4	0.26510	0.26510	0.06628		
Total	7	2.93400				

Figure 4.63 **Effect of Nd:YAG laser light on *E. coli* on stainless steel surfaces.** Multifactorially-designed experiment investigating the effect of different laser parameters on the efficiency of killing of *E. coli*, as a dry sample, by laser light. Each graph shows the reduction in viability of *E. coli* at a **A.** PRF of 20 Hz, **B.** PRF of 30 Hz, **C.** exposure time of 0.5 sec, **D.** exposure time of 1.0 sec, **E.** pulse energy of 5 J and **F.** pulse energy of 10 J. For **A.** to **D.** the curves represent [●] 5 J and [■] 10 J and in **E.** and **F.** [●] 20 Hz and [■] 30 Hz.

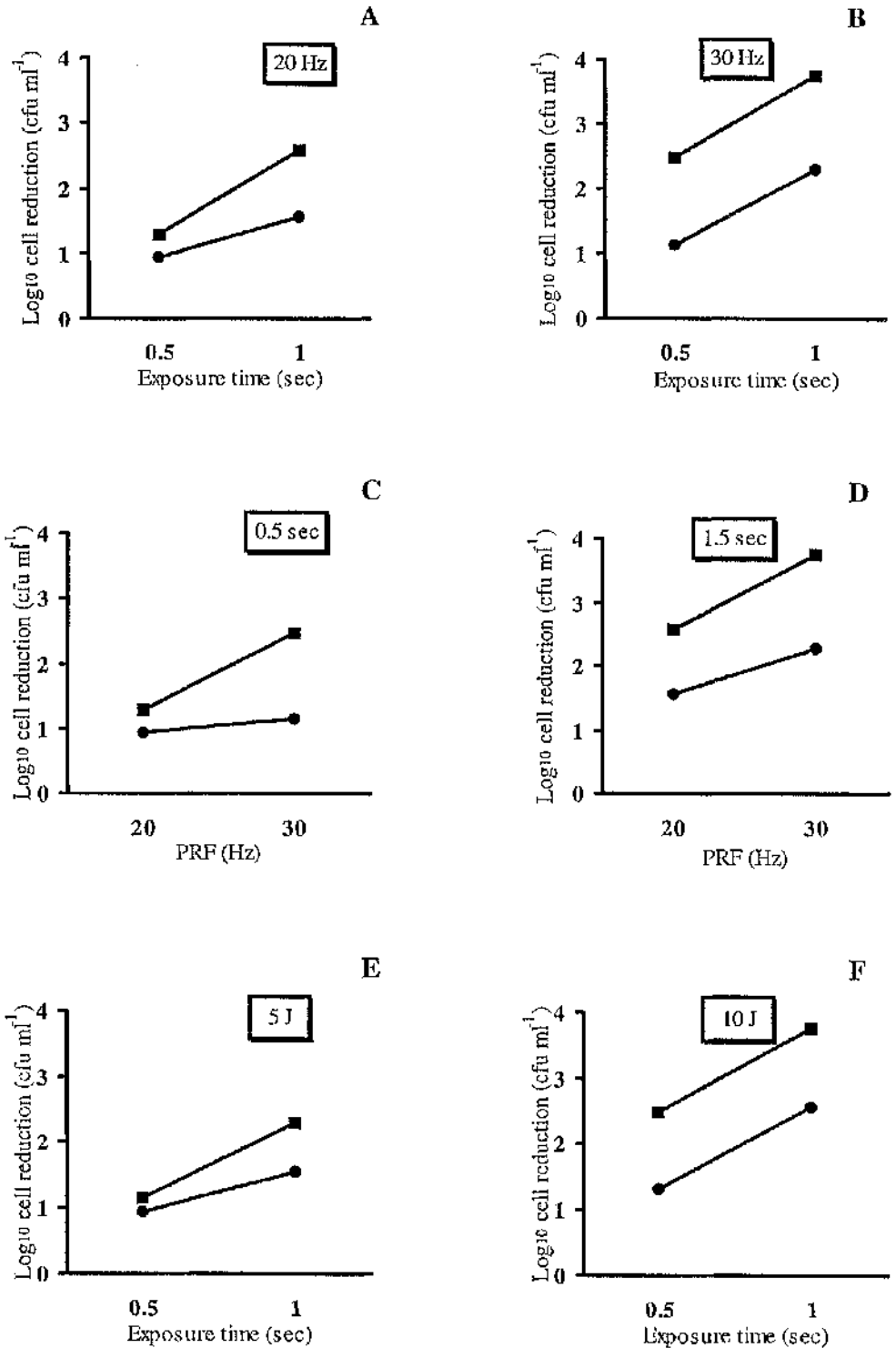

A
20 Hz
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
1
Exposure time (sec)
B
30 Hz
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
1
Exposure time (sec)
C
0.5 sec
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
20
30
PRF (Hz)
D
1.5 sec
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
20
30
PRF (Hz)
E
5 J
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
1
Exposure time (sec)
F
10 J
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
1
Exposure time (sec)

Table 4.9 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.63*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.63.*** Each table shows the F ratio and the probability for the parameters and interactions shown in column 1

A. PRF 20 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	1.4145	1.4145	1.4145	2.51	0.152
Time	1	2.6696	2.6696	2.6696	4.73	0.061
Energy*Time	1	0.3072	0.3072	0.3072	0.54	0.482
Error	8	4.5158	4.5158	0.5645		
Total	11	8.9072				

B. PRF 30 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	5.908	5.908	5.908	4.94	0.057
Time	1	4.368	4.368	4.368	3.65	0.092
Energy*Time	1	0.013	0.013	0.013	0.01	0.919
Error	8	9.577	9.577	1.197		
Total	11	19.866				

C. Exposure time 0.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	2.1760	2.1760	2.1760	3.10	0.116
Freq	1	1.4491	1.4491	1.4491	2.07	0.189
Energy*Freq	1	0.7057	0.7057	0.7057	1.01	0.345
Error	8	5.6135	5.6135	0.7017		
Total	11	9.9443				

D. Exposure time 1.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	5.267	5.267	5.267	4.27	0.073
Freq	1	2.279	2.279	2.279	1.85	0.211
Energy*Freq	1	0.304	0.304	0.304	0.25	0.633
Error	8	9.859	9.859	1.232		
Total	11	17.709				

E. Pulse energy 5 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	0.6580	0.6580	0.6580	1.59	0.244
Time	1	2.3320	2.3320	2.3320	5.62	0.045
Freq*Time	1	0.2002	0.2002	0.2002	0.48	0.507
Error	8	3.3209	3.3209	0.4151		
Total	11	6.5111				

F. Pulse energy 10 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	4.213	4.213	4.213	3.13	0.115
Time	1	4.826	4.826	4.826	3.58	0.095
Freq*Time	1	0.000	0.000	0.000	0.00	0.994
Error	8	10.772	10.772	1.346		
Total	11	19.810				

Figure 4.64 **Effect of Nd:YAG laser light on *E. coli* on stainless steel disks.** Multifactorially-designed experiment investigating the effect of different laser parameters on the efficiency of killing of *E. coli*, as a wet sample, by laser light. Each graph shows the reduction in viability of *E. coli* at a **A.** PRF of 20 Hz, **B.** PRF of 30 Hz, **C.** exposure time of 0.5 sec, **D.** exposure time of 1.5 sec, **E.** pulse energy of 5 J and F. pulse energy of 10 J. For **A.** to **D.** the curves represent [●] 5 J and [■] 10 J and in **E.** and **F.** [●] 20 Hz and [■] 30 Hz.

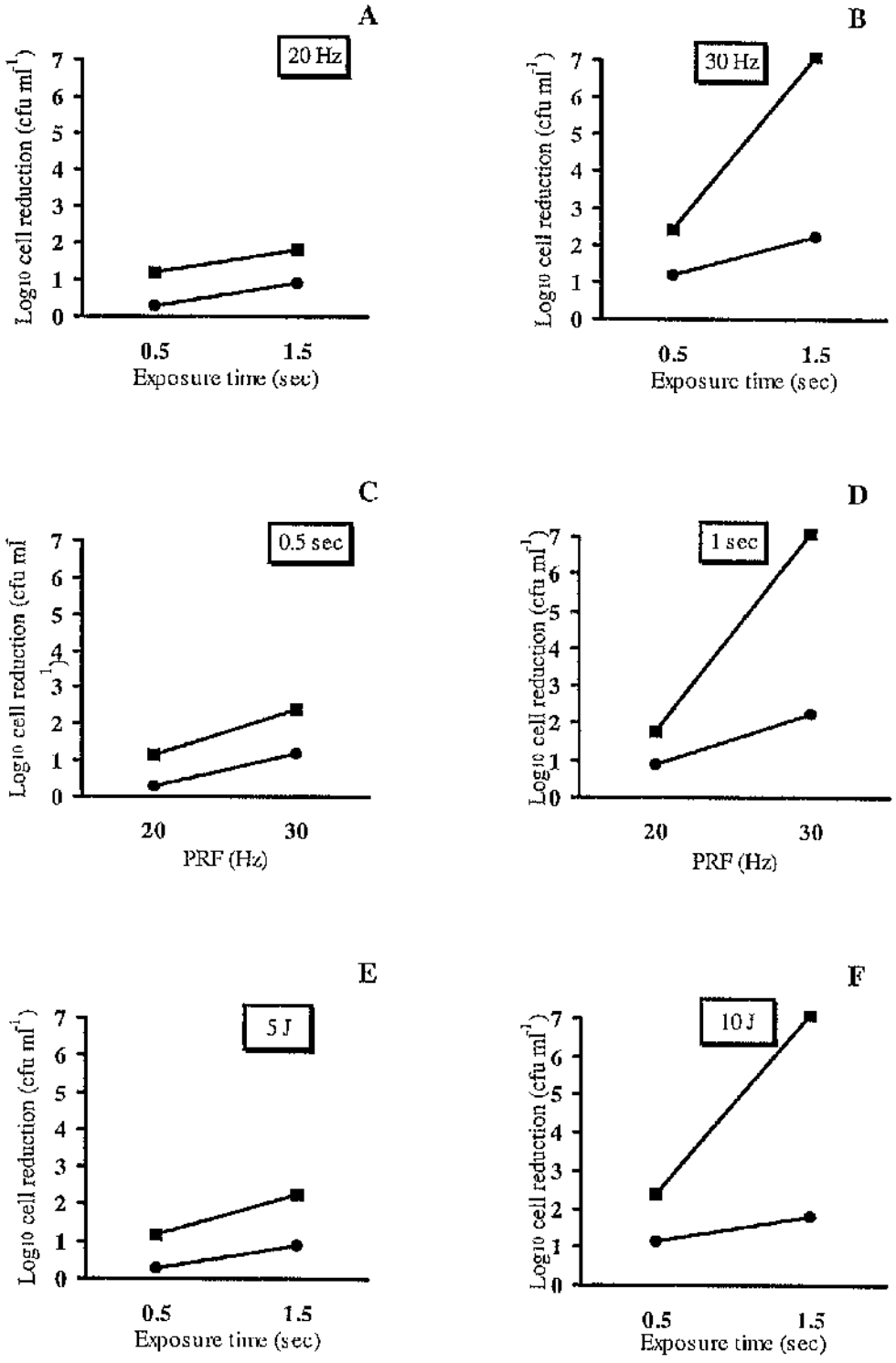

A
20 Hz
Log10 cell reduction (cfu ml-1)
0 1 2 3 4 5 6 7
0.5 1.5
Exposure time (sec)
B
30 Hz
Log10 cell reduction (cfu ml-1)
0 1 2 3 4 5 6 7
0.5 1.5
Exposure time (sec)
C
0.5 sec
Log10 cell reduction (cfu ml-1)
0 1 2 3 4 5 6 7
20 30
PRF (Hz)
D
1 sec
Log10 cell reduction (cfu ml-1)
0 1 2 3 4 5 6 7
20 30
PRF (Hz)
E
5 J
Log10 cell reduction (cfu ml-1)
0 1 2 3 4 5 6 7
0.5 1.5
Exposure time (sec)
F
10 J
Log10 cell reduction (cfu ml-1)
0 1 2 3 4 5 6 7
0.5 1.5
Exposure time (sec)

Table 4.10 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.64*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.64***. Each table shows the ***F*** ratio and the probability for the parameters and interactions shown in column 1

A. PRF 20 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	3.1418	3.1418	3.1418	10.66	0.007
Time	1	1.5563	1.5563	1.5563	5.28	0.040
Energy*Time	1	0.0001	0.0001	0.0001	0.00	0.989
Error	12	3.5360	3.5360	0.2947		
Total	15	8.2340				

B. PRF 30 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	36.997	36.997	36.997	32.62	0.000
Time	1	32.976	32.976	32.976	29.07	0.000
Energy*Time	1	13.195	13.195	13.195	11.63	0.005
Error	12	13.611	13.611	1.134		
Total	15	96.779				

C. Exposure time 0.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	4.4416	4.4416	4.4416	13.02	0.004
Freq	1	4.5689	4.5689	4.5689	13.39	0.003
Energy*Freq	1	0.1173	0.1173	0.1173	0.34	0.569
Error	12	4.0945	4.0945	0.3412		
Total	15	13.2223				

D. Exposure time 1.0 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	33.034	33.034	33.034	30.37	0.000
Freq	1	43.990	43.990	43.990	40.44	0.000
Energy*Freq	1	15.741	15.741	15.741	14.47	0.003
Error	12	13.052	13.052	1.088		
Total	15	105.817				

E. Pulse energy 5 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	4.9729	4.9729	4.9729	6.62	0.024
Time	1	2.8056	2.8056	2.8056	3.74	0.077
Freq*Time	1	0.1892	0.1892	0.1892	0.25	0.625
Error	12	9.0090	9.0090	0.7507		
Total	15	16.9767				

F. Pulse energy 10 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	42.772	42.772	42.772	63.07	0.000
Time	1	28.249	28.249	28.249	41.66	0.000
Freq*Time	1	16.484	16.484	16.484	24.31	0.000
Error	12	8.138	8.138	0.678		
Total	15	95.642				

Figure 4.65 **Effect of Nd:YAG laser light on *L .monocytogenes* on stainless steel disks.** Multifactorially-designed experiment investigating the effect of different laser parameters on the efficiency of killing of *L. monocytogenes*, as a dry sample, by laser light. Each graph shows the reduction in viability of *L. monocytogenes* at a **A.** PRF of 20 Hz, **B.** PRF of 30 Hz, **C.** exposure time of 1.5 sec, **D.** exposure time of 2.5 sec, **E.** pulse energy of 5 J and **F.** pulse energy of 10 J. For **A.** to **D.** the curves represent [●] 5 J and [■] 10 J and in **E.** and **F.** [●] 20 Hz and [■] 30 Hz.

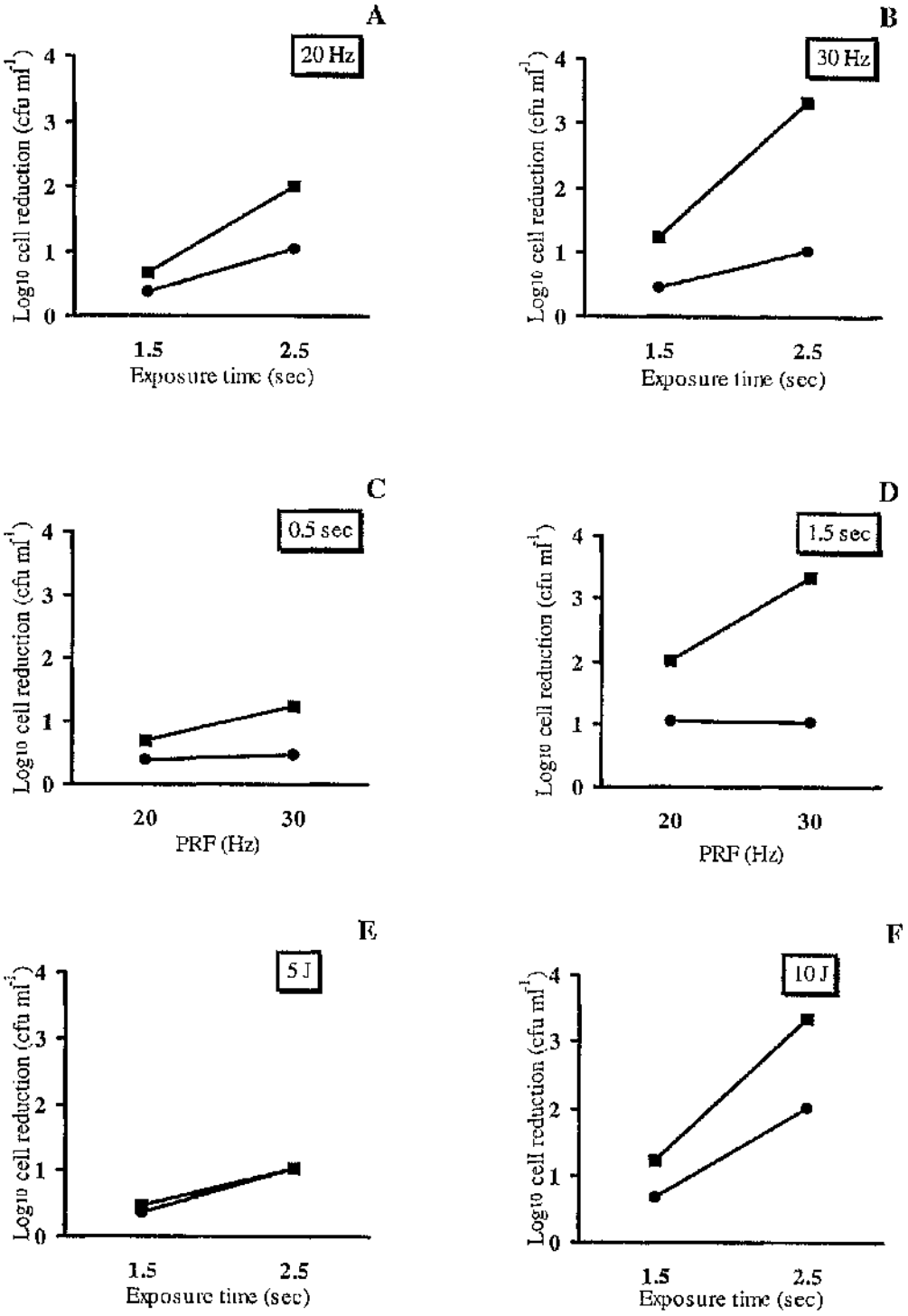

A
20 Hz
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
1.5
2.5
Exposure time (sec)
B
30 Hz
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
1.5
2.5
Exposure time (sec)
C
0.5 sec
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
20
30
PRF (Hz)
D
1.5 sec
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
20
30
PRF (Hz)
E
5 J
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
1.5
2.5
Exposure time (sec)
F
10 J
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
1.5
2.5
Exposure time (sec)

Table 4.11 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.65*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.65.*** Each table shows the ***F*** ratio and the probability for the parameters and interactions shown in column 1

A. PRF 20 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	1.1844	1.1844	1.1844	2.07	0.188
Time	1	2.9502	2.9502	2.9502	5.16	0.053
Energy*Time	1	0.3300	0.3300	0.3300	0.58	0.469
Error	8	4.5776	4.5776	0.5722		
Total	11	9.0422				

B. PRF 30 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	7.0380	7.0380	7.0380	16.69	0.004
Time	1	5.4002	5.4002	5.4002	12.81	0.007
Energy*Time	1	1.7864	1.7864	1.7864	4.24	0.074
Error	8	3.3727	3.3727	0.4216		
Total	11	17.5973				

C. Exposure time 0.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	0.8374	0.8374	0.8374	6.97	0.027
Freq	1	0.2914	0.2914	0.2914	2.42	0.154
Energy*Freq	1	0.1610	0.1610	0.1610	1.40	0.271
Error	9	1.0818	1.0818	0.1202		
Total	11	2.2106				

D. Exposure time 1.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	7.9870	7.9870	7.9870	8.57	0.017
Freq	1	1.3134	1.3134	1.3134	1.41	0.265
Energy*Freq	1	1.3534	1.3534	1.3534	1.54	0.250
Error	9	8.3829	8.3829	0.9314		
Total	11	17.6833				

E. Pulse energy 5 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	0.0037	0.0037	0.0037	0.03	0.859
Time	1	1.1347	1.1347	1.1347	10.43	0.012
Freq*Time	1	0.0061	0.0061	0.0061	0.06	0.819
Error	8	0.8703	0.8703	0.1088		
Total	11	2.0147				

F. Pulse energy 10 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	2.7170	2.7170	2.7170	2.87	0.128
Time	1	8.9960	8.9960	8.9960	9.52	0.015
Freq*Time	1	0.5852	0.5852	0.5852	0.62	0.454
Error	8	7.5619	7.5619	0.9452		
Total	11	19.8601				

Figure 4.66 **Effect of Nd:YAG laser light on *L. monocytogenes* on stainless steel disks.** Multifactorially-designed experiment investigating the effect of different laser parameters on the efficiency of killing of *L. monocytogenes*, as a wet film, by laser light. Each graph shows the reduction in viability of *L. monocytogenes* at a **A.** PRF of 20 Hz, **B.** PRF of 30 Hz, **C.** exposure time of 0.5 sec, **D.** exposure time of 1.5 sec, **E.** pulse energy of 5 J and **F.** pulse energy of 10 J. For **A.** to **D.** the curves represent [●] 5 J and [■] 10 J and in **E.** and **F.** [●] 20 Hz and [■] 30 Hz.

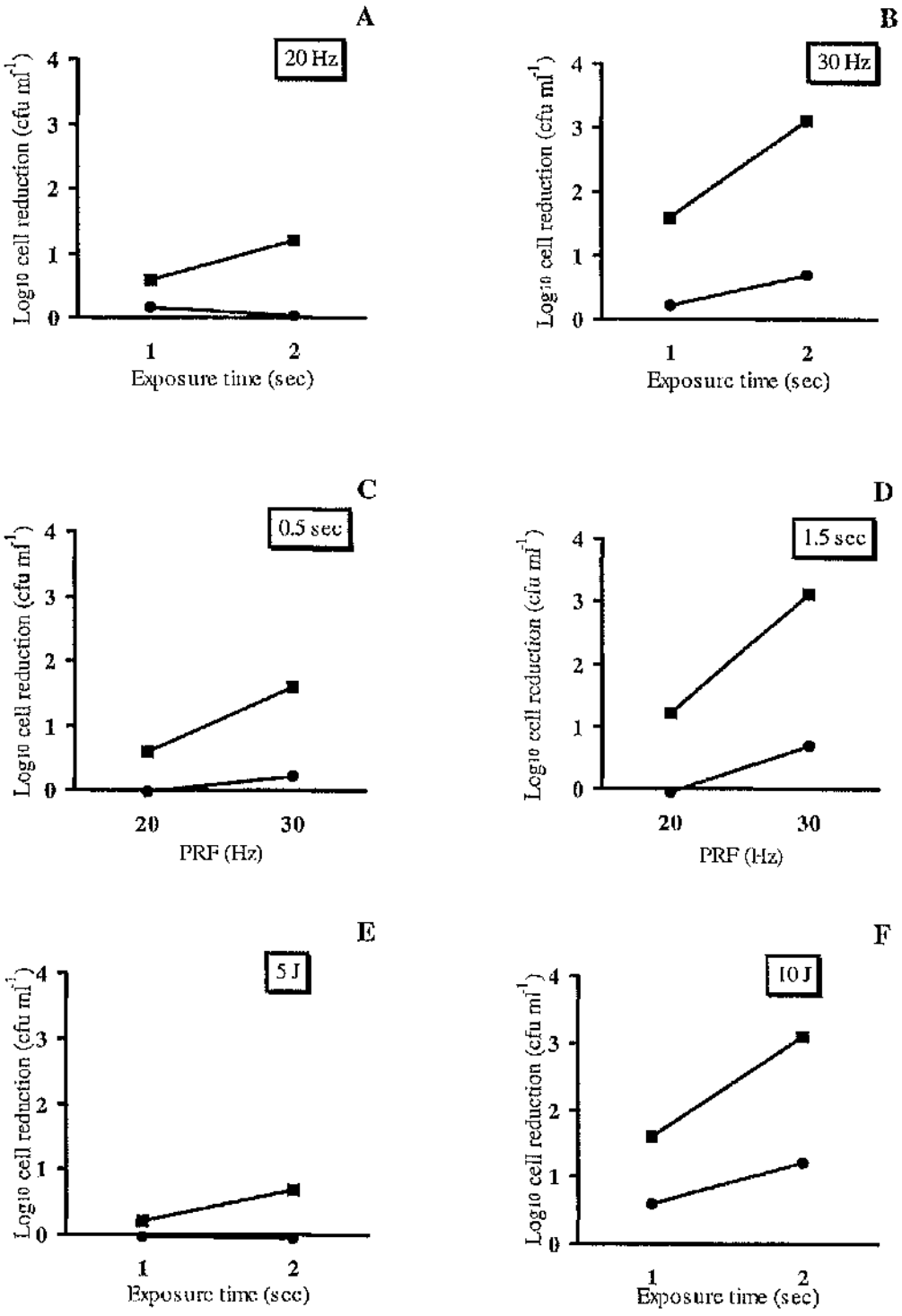

A
20 Hz
Log10 cell reduction (cfu ml-1)
Exposure time (sec)
B
30 Hz
Log10 cell reduction (cfu ml-1)
Exposure time (sec)
C
0.5 sec
Log10 cell reduction (cfu ml-1)
PRF (Hz)
D
1.5 sec
Log10 cell reduction (cfu ml-1)
PRF (Hz)
E
5 J
Log10 cell reduction (cfu ml-1)
Exposure time (sec)
F
10 J
Log10 cell reduction (cfu ml-1)
Exposure time (sec)

Table 4.12 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.66*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.66.*** Each table shows the *F* ratio and the probability for the parameters and interactions shown in column 1

A. PRF 20 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	3.5438	3.5438	3.5438	35.16	0.000
Time	1	0.3630	0.3630	0.3630	3.60	0.082
Energy*Time	1	0.4001	0.4001	0.4001	3.97	0.070
Error	12	1.2093	1.2093	0.1008		
Total	15	5.5162				

B. PRF 30 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	27.905	27.905	27.905	11.10	0.006
Time	1	12.058	12.058	12.058	4.80	0.049
Energy*Time	1	6.313	6.313	6.313	2.51	0.139
Error	12	30.171	30.171	2.514		
Total	15	76.447				

C. Exposure time 0.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	4.0401	4.0401	4.0401	12.43	0.004
Freq	1	1.5876	1.5876	1.5876	4.89	0.046
Energy*Freq	1	0.5776	0.5776	0.5776	1.90	0.193
Error	13	4.2245	4.2245	0.3250		
Total	15	9.8522				

D. Exposure time 1.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	26.574	26.574	26.574	9.95	0.008
Freq	1	17.057	17.057	17.057	6.39	0.025
Energy*Freq	1	6.970	6.970	6.970	3.02	0.108
Error	13	34.703	34.703	2.669		
Total	15	78.334				

E. Pulse energy 5 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	0.9900	0.9900	0.9900	9.27	0.010
Time	1	0.2162	0.2162	0.2162	2.02	0.180
Freq*Time	1	0.2450	0.2450	0.2450	2.29	0.156
Error	12	1.2819	1.2819	0.1068		
Total	15	2.7332				

F. Pulse energy 10 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	13.104	13.104	13.104	11.89	0.005
Time	1	6.579	6.579	6.579	5.97	0.031
Freq*Time	1	1.638	1.638	1.638	1.49	0.246
Error	12	13.227	13.227	1.102		
Total	15	34.549				

Figure 4.67 **Effect of Nd:YAG laser light on *B. cereus* on stainless steel disks.** Multifactorially-designed experiment investigating the effect of different laser parameters on the efficiency of killing of *B. cereus*, as a wet film, by laser light. Each graph shows the reduction in viability of *B. cereus* at a **A.** PRF of 20 Hz, **B.** PRF of 30 Hz, **C.** exposure time of 0.5 sec, **D.** exposure time of 1.0 sec, **E.** pulse energy of 5 J and **F.** pulse energy of 10 J. For **A.** to **D.** the curves represent [●] 5 J and [■] 10 J and in **E.** and **F.** [●] 20 Hz and [■] 30 Hz.

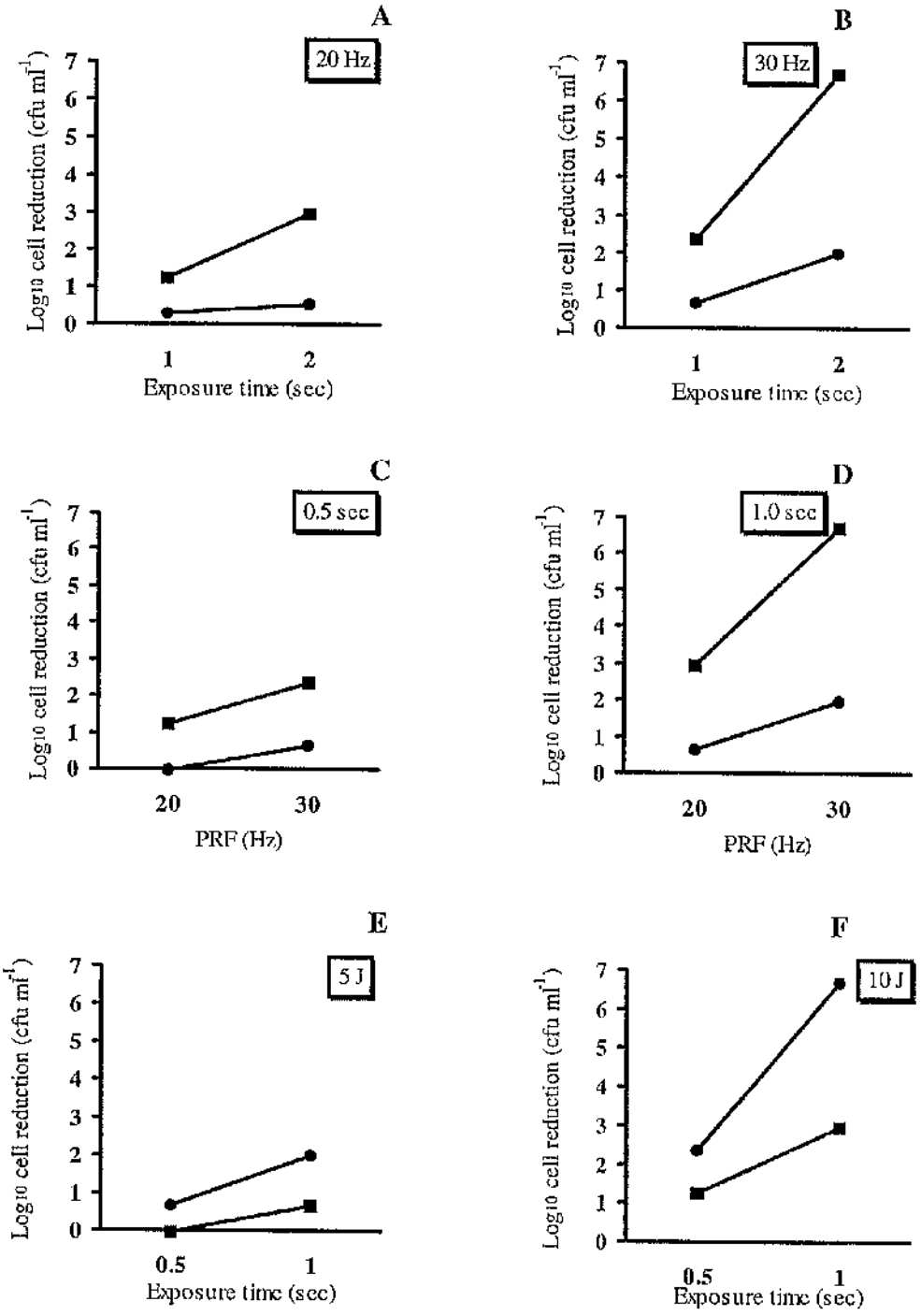

A
20 Hz
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
6
7
1
2
Exposure time (sec)
B
30 Hz
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
6
7
1
2
Exposure time (sec)
C
0.5 sec
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
6
7
20
30
PRF (Hz)
D
1.0 sec
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
6
7
20
30
PRF (Hz)
E
5 J
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
6
7
0.5
1
Exposure time (sec)
F
10 J
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
6
7
0.5
1
Exposure time (sec)

Table 4.13 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.67*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.67.*** Each table shows the ***F*** ratio and the probability for the parameters and interactions shown in column 1

A. PRF 20 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	12.7806	12.7806	12.7806	23.05	0.000
Time	1	5.5932	5.5932	5.5932	10.09	0.008
Energy*Time	1	1.0100	1.0100	1.0100	1.82	0.202
Error	12	6.6541	6.6541	0.5545		
Total	15	26.0380				

B. PRF 30 Hz

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	40.992	40.992	40.992	70.59	0.000
Time	1	32.234	32.234	32.234	55.51	0.000
Energy*Time	1	9.166	9.166	9.166	15.78	0.002
Error	12	6.968	6.968	0.581		
Total	15	89.360				

C. Exposure time 0.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	8.8358	8.8358	8.8358	26.47	0.000
Freq	1	3.1952	3.1952	3.1952	9.57	0.009
Energy*Freq	1	0.1620	0.1620	0.1620	0.49	0.499
Error	12	4.0055	4.0055	0.3338		
Total	15	16.1984				

D. Exposure time 1.5 sec

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Energy	1	49.070	49.070	49.070	61.23	0.000
Freq	1	26.010	26.010	26.010	32.45	0.000
Energy*Freq	1	5.881	5.881	5.881	7.34	0.019
Error	12	9.617	9.617	0.801		
Total	15	90.578				

E. Pulse energy 5 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	4.1209	4.1209	4.1209	33.99	0.000
Time	1	4.0200	4.0200	4.0200	33.15	0.000
Freq*Time	1	0.4160	0.4160	0.4160	3.43	0.089
Error	12	1.4550	1.4550	0.1213		
Total	15	10.0120				

F. Pulse energy 10 J

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Freq	1	23.814	23.814	23.814	23.46	0.000
Time	1	36.724	36.724	36.724	36.18	0.000
Freq*Time	1	6.996	6.996	6.996	6.89	0.022
Error	12	12.180	12.180	1.015		
Total	15	79.714				

Measurement of the temperature of the disks, with an optical pyrometer, prior to exposure showed that the temperature fell considerably such that there was only 1-2°C between the upper and lower value.

With the halotolerant *St. aureus*, the upper salt concentration was 10% w/v NaCl and the lower was 0.5% w/v (the standard concentration found in nutrient agar). The effect of each of these parameters on the ability of Nd:YAG laser light to reduce the viability of *St. aureus* is shown in **Figure 4.68.** The effect of each of the different parameters on the reduction of viable counts with the exception of the water content was small; the effect of water content is shown by the separation between the different lines in **Figure 4.68A, B, C** and **D**. The other effects appear to be negligible. From the statistics in **Table 4.14** it can be seen that there was only one significant effect, the water content at 10% w/v NaCl. None of the interactions were significant. It is worth noting that the errors in this experiment were very large, almost five times the size of the larger errors associated with the laser parameters.

The results for the effect of the environmental parameters on *E. coli* shown in **Figure 4.69** appear to be a little more erratic that those for *St. aureus* (**Figure 4.68**). At the lower pH value and salt concentration the difference between the wet and dry films, shown by the separation between the lines appeared, once again, to be at its greatest. The statistics in **Table 4.15** show that the effects on *E. coli* observed in the graphs were, for the majority, insignificant however, the increase in the NaCl concentration at pH 8 and as a wet film showed a 4% probability that the null hypothesis was correct and therefore is significant, which can also be seen by the gradient of the curves in both of the graphs (**Figure 4.69B** and **E**). Again none of the interactions appeared to be significant. As with the previous results with *St. aureus* the errors shown in the statistics were relatively high.

The results of varying the environmental parameters on the bactericidal capacity of Nd:YAG laser light against *L. monocytogenes* are shown (**Figure 4.70**). The results appear to be extremely similar with each data points within one D-value. The statistics in **Table 4.16** indicate that no parameters or interactions between

Figure 4.68 **Effect of Nd:YAG laser light on *St. aureus* on stainless steel surfaces.** Multifactorially-designed experiment investigating the effect of different environmental parameters on the efficiency of killing of *St. aureus* by laser light. For **A.** to **D.** the curves represent [●] wet sample and [■] dry sample and in **E.** and **F.** [●] pH 5.5 and [■] pH 8.

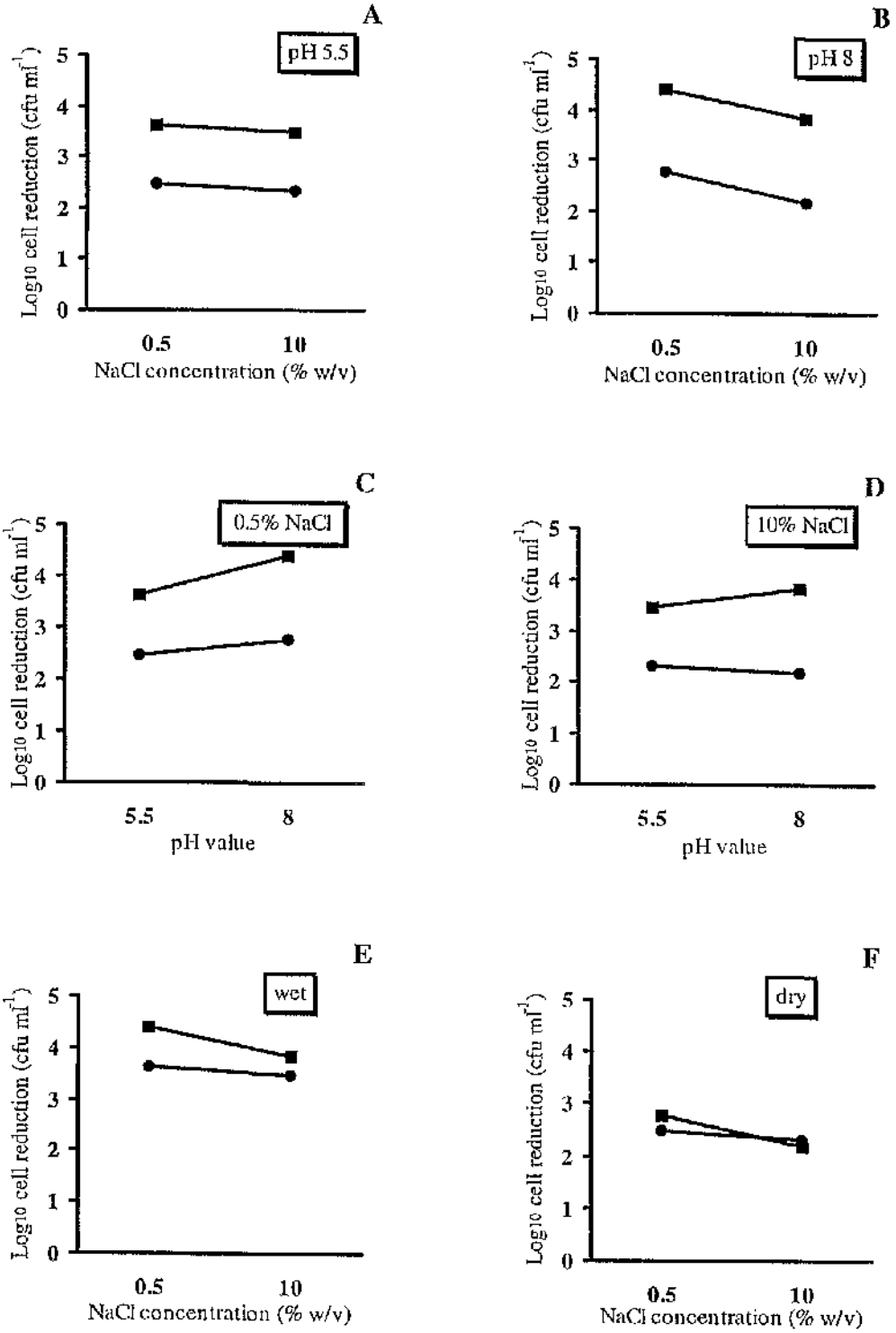

A
pH 5.5
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
0.5
10
NaCl concentration (% w/v)
B
pH 8
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
0.5
10
NaCl concentration (% w/v)
C
0.5% NaCl
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
5.5
8
pH value
D
10% NaCl
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
5.5
8
pH value
E
wet
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
0.5
10
NaCl concentration (% w/v)
F
dry
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5
0.5
10
NaCl concentration (% w/v)

Table 4.14 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.68*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 68.*** Each table shows the ***F*** ratio and the probability for the parameters and interactions shown in column 1

A. pH 5.5

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.0690	0.0690	0.0690	0.07	0.796
Aw	1	3.9560	3.9560	3.9560	4.10	0.077
NaCl*Aw	1	0.0002	0.0002	0.0002	0.00	0.989
Error	8	7.7112	7.7112	0.9639		
Total	11	11.7364				

B. pH 8

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	1.038	1.038	1.038	0.66	0.440
Aw	1	7.954	7.954	7.954	5.05	0.055
NaCl*Aw	1	0.001	0.001	0.001	0.00	0.980
Error	8	12.590	12.590	1.574		
Total	11	21.583				

C. 0.5% w/v NaCl

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0.864	0.864	0.864	0.50	0.500
Aw	1	5.741	5.741	5.741	3.32	0.106
pH*Aw	1	0.154	0.154	0.154	0.09	0.773
Error	8	13.844	13.844	1.731		
Total	11	20.603				

D. 10% w/v NaCl

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0.0300	0.0300	0.0300	0.04	0.852
Aw	1	5.8241	5.8241	5.8241	7.22	0.028
pH*Aw	1	0.1925	0.1925	0.1925	0.24	0.638
Error	8	6.4566	6.4566	0.8071		
Total	11	12.5033				

E. Dried sample

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.422	0.422	0.422	0.30	0.602
pH	1	0.018	0.018	0.018	0.01	0.912
NaCl*pH	1	0.161	0.161	0.161	0.11	0.746
Error	8	11.439	11.439	1.430		
Total	11	12.040				

F. Wet sample

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.400	0.400	0.400	0.36	0.565
pH	1	0.935	0.935	0.935	0.84	0.385
NaCl*pH	1	0.126	0.126	0.126	0.11	0.745
Error	8	8.862	8.862	1.108		
Total	11	10.323				

Figure 4.69 **Effect of Nd:YAG laser light on *E. coli* on stainless steel disks.** Multifactorially-designed experiment investigating the effect of different environmental parameters on the efficiency of killing of *E. coli* by laser light. For **A.** to **D.** the curves represent [●] wet sample and [■] dry sample and in **E.** and **F.** [●] pH 5.5 and [■] pH 8.

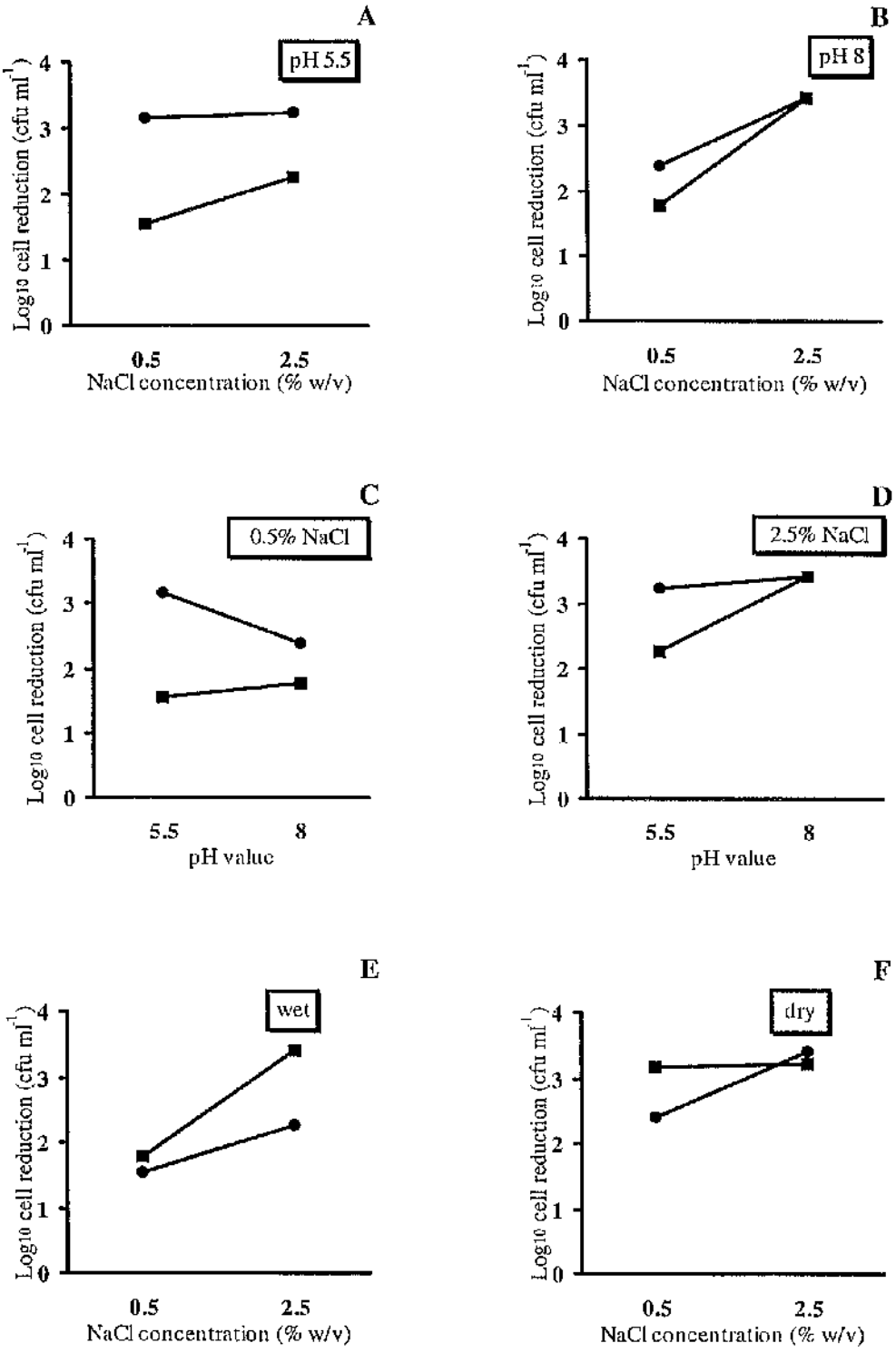

A
pH 5.5
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)
B
pH 8
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)
C
0.5% NaCl
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5.5
8
pH value
D
2.5% NaCl
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5.5
8
pH value
E
wet
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)
F
dry
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)

Table 4.15 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.69*.** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.69.*** Each table shows the F ratio and the probability for the parameters and interactions shown in column 1

A. pH 5.5

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.624	0.624	0.624	0.31	0.585
Aw	1	6.631	6.631	6.631	3.34	0.093
NaCl*Aw	1	0.410	0.410	0.410	0.21	0.658
Error	12	23.822	23.822	1.985		
Total	15	31.486				

B. pH 8

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	6.996	6.996	6.996	5.31	0.040
Aw	1	0.384	0.384	0.384	0.29	0.599
NaCl*Aw	1	0.360	0.360	0.360	0.27	0.611
Error	12	15.814	15.814	1.318		
Total	15	23.554				

C. 0.5% NaCl

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0.289	0.289	0.289	0.21	0.654
Aw	1	4.917	4.917	4.917	3.59	0.083
pH*Aw	1	0.995	0.995	0.995	0.73	0.411
Error	12	16.453	16.453	1.371		
Total	15	22.655				

D. 2.5% NaCl

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	1.736	1.736	1.736	0.90	0.362
Aw	1	0.956	0.956	0.956	0.49	0.495
pH*Aw	1	0.917	0.917	0.917	0.47	0.504
Error	12	23.183	23.183	1.932		
Total	15	26.791				

E. Dry film

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	1.205	1.205	1.205	0.53	0.481
pH	1	0.345	0.345	0.345	0.15	0.704
NaCl*pH	1	0.898	0.898	0.898	0.39	0.542
Error	12	27.313	27.313	2.276		
Total	15	29.761				

F. Wet film

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	5.464	5.464	5.464	5.32	0.040
pH	1	1.870	1.870	1.870	1.82	0.202
NaCl*pH	1	0.824	0.824	0.824	0.80	0.388
Error	12	12.323	12.323	1.027		
Total	15	20.480				

Figure 4.70 **Effect of Nd:YAG laser light on *L. monocytogenes* on stainless steel surfaces.** Multifactorially-designed experiment investigating the effect of different environmental parameters on the efficiency of killing of *L. monocytogenes* by laser light. For **A.** to **D.** the curves represent [●] dry sample and [■] wet sample and in **E.** and **F.** [●] pH 5.5 and [■] pH 8.

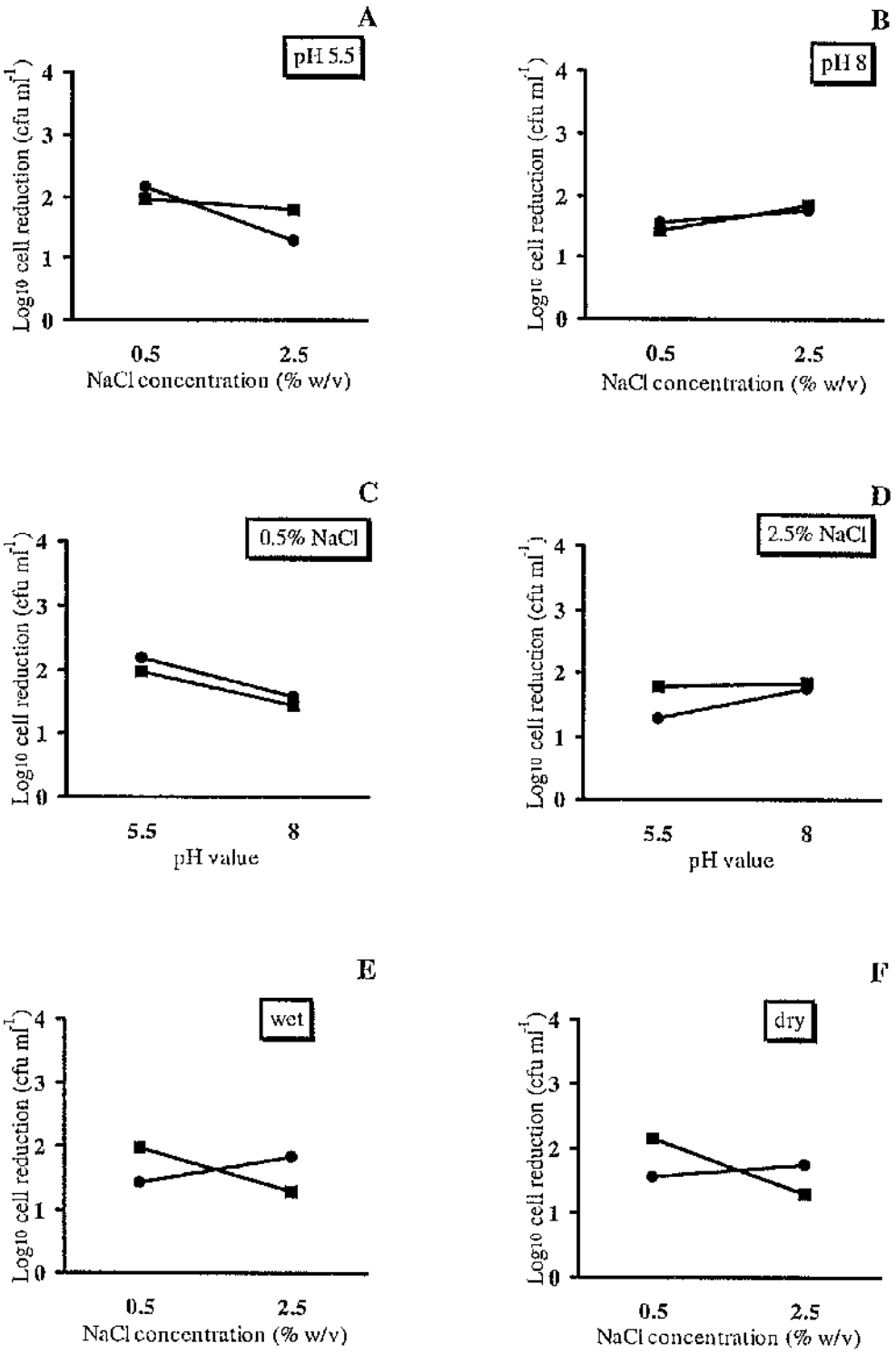

A
pH 5.5
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)
B
pH 8
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)
C
0.5% NaCl
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5.5
8
pH value
D
2.5% NaCl
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
5.5
8
pH value
E
wet
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)
F
dry
Log10 cell reduction (cfu ml-1)
0
1
2
3
4
0.5
2.5
NaCl concentration (% w/v)

Table 4.16 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.70.*** Each table, **A.** to **F.** corresponds to the graph of the same letter in ***Figure 4.70.*** Each table shows the F ratio and the probability for the parameters and interactions shown in column 1

A. pH 5.5

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	1.108	1.108	1.108	0.74	0.405
Aw	1	0.080	0.080	0.080	0.05	0.821
NaCl*Aw	1	0.466	0.466	0.466	0.31	0.586
Error	12	17.863	17.863	1.489		
Total	15	19.517				

B. pH 8

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.3630	0.3630	0.3630	1.50	0.244
Aw	1	0.0033	0.0033	0.0033	0.01	0.909
NaCl*Aw	1	0.0431	0.0431	0.0431	0.18	0.681
Error	12	2.9022	2.9022	0.2419		
Total	15	3.3116				

C. 5% NaCl

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	1.271	1.271	1.271	0.89	0.364
Aw	1	0.111	0.111	0.111	0.08	0.786
pH*Aw	1	0.005	0.005	0.005	0.00	0.956
Error	12	17.161	17.161	1.430		
Total	15	18.547				

D. 2.5% NaCl

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	1	0.2783	0.2783	0.2783	0.93	0.355
Aw	1	0.3108	0.3108	0.3108	1.03	0.329
pH*Aw	1	0.1661	0.1661	0.1661	0.55	0.471
Error	12	3.6046	3.6046	0.3004		
Total	15	4.3597				

E. Dry film

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.449	0.449	0.449	0.36	0.559
pH	1	0.017	0.017	0.017	0.01	0.909
NaCl*pH	1	1.134	1.134	1.134	0.91	0.358
Error	12	14.924	14.924	1.244		
Total	15	16.524				

F. Wet film

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.0484	0.0484	0.0484	0.10	0.758
pH	1	0.2209	0.2209	0.2209	0.45	0.513
NaCl*pH	1	0.3481	0.3481	0.3481	0.72	0.414
Error	12	5.8411	5.8411	0.4868		
Total	15	6.4585				

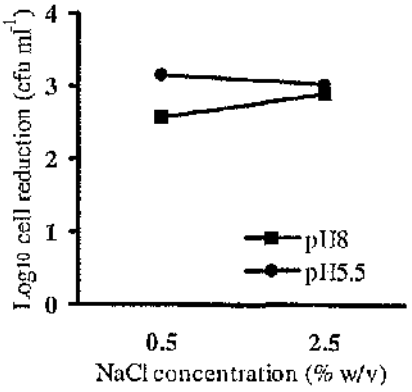


Figure 4.71 **Effect of Nd:YAG laser light on *B. cereus* on stainless steel surfaces.** Multifactorially-designed experiment investigating the effect of different environmental parameters on the efficiency of killing of *B. cereus* by laser light. The curves represent [●] pH 5.5and [■] pH 8.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NaCl	1	0.0136	0.0136	0.0136	0.03	0.865
pH	1	0.2485	0.2485	0.2485	0.60	0.483
NaCl*pH	1	0.0990	0.0990	0.0990	0.24	0.652
Error	4	1.6687	1.6687	0.4172		
Total	7	2.0298				

Table 4.17 **Analysis of variance, with the General Linear Model, of the results shown in *Figure 4.71*.** The table shows the *F* ratio and the probability for the parameters and interactions in column 1

the parameters had any significant effect on the killing of *L. monocytogenes* on stainless steel disks.

The environmental parameters studied with *B. cereus* were reduced to two, as only a wet film was used. This allowed all of the results to be displayed in a single graph. It is obvious in **Figure 4.71** that the effect appears to be negligible. Indeed the statistics in **Table 4.17** indicated that the results of this experiment showed no significance either the individual parameters or the interactions.

4.4.6 DEVELOPMENT OF RESULTS FROM MULTIFACTORIAL EXPERIMENTS

One major disadvantage with the 2^N multifactorial experiment is the assumption that the measurable quantity within the experiment varies linearly with the change in the variable parameter between the limits. Unfortunately, this is not always the case so when the major parameters affecting a system are found, a detailed investigation of these parameters can then be done. The multifactorial investigations of the effects of the salt concentration and the pH were used for this particular purpose. Although the results showed that there was little effect from each of these parameters the effect over a range of values was studied. By introducing a larger number of values for each parameter their influence could be studied in greater detail and substantiate the results from the multifactorial experiments.

The effect of increased NaCl concentration, in the growth medium, from 0.5 to 10.0% w/v, on the susceptibility of *St. aureus* as a dry film on stainless steel surfaces is shown in **Figure 4.72.** It appeared that there was little difference in the susceptibility of the organism after growth in nutrient broth which contained different concentrations of NaCl. The data points in the curve were not uniform possibly due to the subtle differences between the surfaces of the metal disks, or minor effects due to drying. **Figure 4.73** shows the effect of increasing the pH of the growth medium from pH 6.0 to 8.0, on the susceptibility of *St. aureus* as a dry film on stainless steel surfaces. From the graph it appeared, once again, that there was

little difference in the susceptibility of the organism after growth at the range of pH values.

Because of the nature of the killing curves a method of applying best-fits to compare rates of killing was pursued. Three methods were attempted and the results are presented in **Figures 4.74** and **4.75**. Each of these fits were done with the highest and lowest values for each parameter for ease of comparison. The initial fits (**Figures 4.74A** and **B** and **4.75A** and **B**) were log-linear but it can be clearly seen from **Figure 4.74** that the fit for the NaCl concentrations was not accurate, however with the killing curves at the different pH values the fit was relatively good. It was also decided to model the killing curves with a system initially utilised by Cole *et al.* (1993) where by the results were plotted on a log-log scale (Appendix 7). This was initially designed to take account of the initial lag and the tail observed with many heat killing curves (section 1.2.3). It can be clearly seen that this method of modelling the killing curves was inaccurate. There was minimal lag at the start of the curve, especially in the experiment with the different pH values, and there appeared to be no tail at the end of the experimental procedure. The third method of analysing the killing curves was with the use of a quadratic function which took into account increased or decreased killing rates over the course of the experiment. This method appeared to give the closest fit to the point on the curve, indicating that the rate of killing in the experimental curves increased with an increase in the ED applied to the disks.

One of the purposes of this investigation was that the results obtained could be used to produce a predictive model so that a formula would be obtained to fit the curve allowing the prediction of the log count from the laser parameters and the salt concentration or pH value, in the case of these experiments, at time *t*.

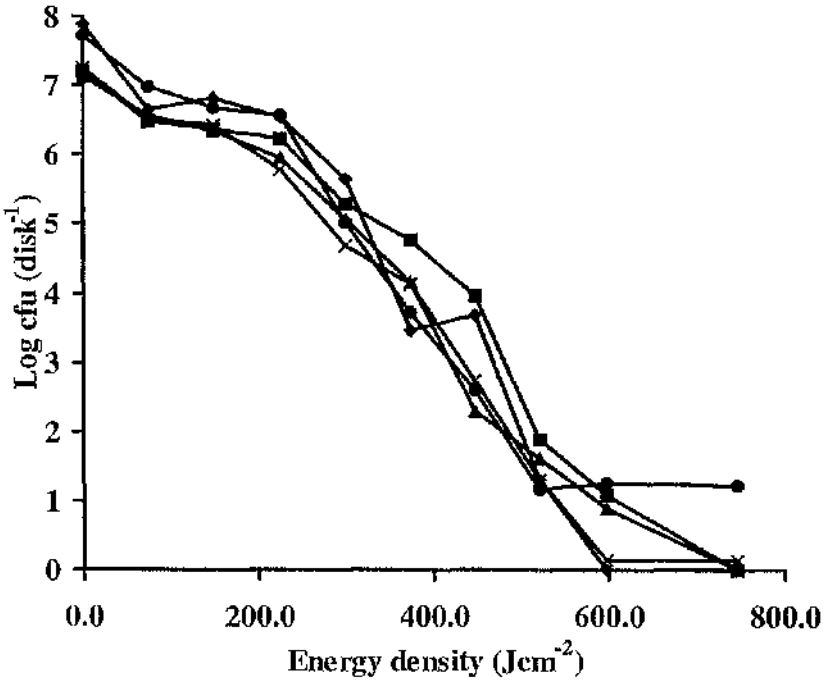


Figure 4.72 **Effect of growth at different NaCl concentrations on the susceptibility of *St. aureus* to Nd:YAG laser light.** Each killing curve was a dried overnight culture on the surface of stainless steel disk and each point is a separate exposure on different disks. The results are the average of two separate experiments.

[◆] 0.5 % w/v, [●] 2.5 % w/v, [▲] 5.0 % w/v, [O] 7.5 % w/v, [■] 10.0 % w/v

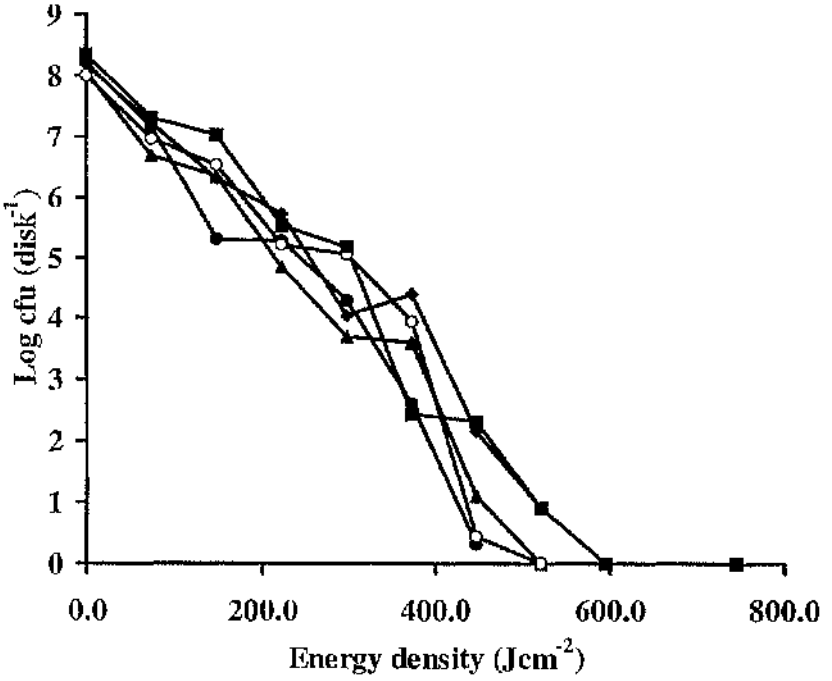
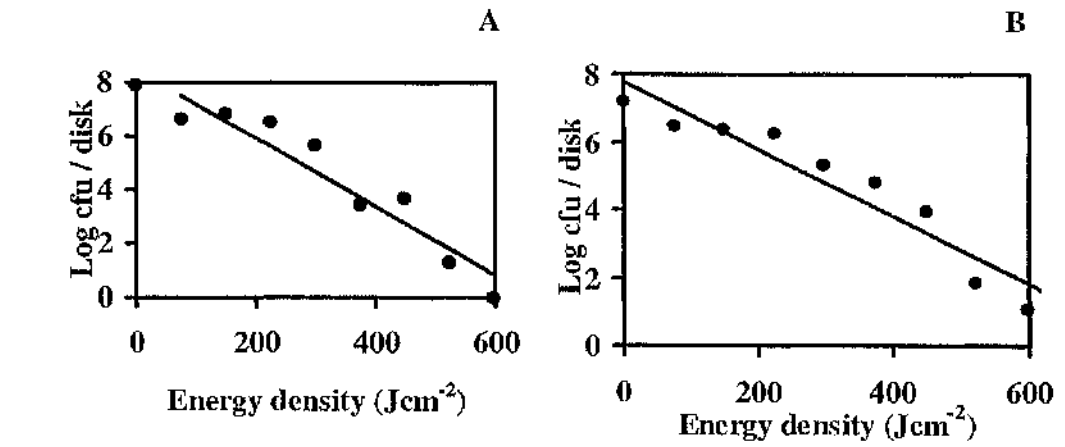
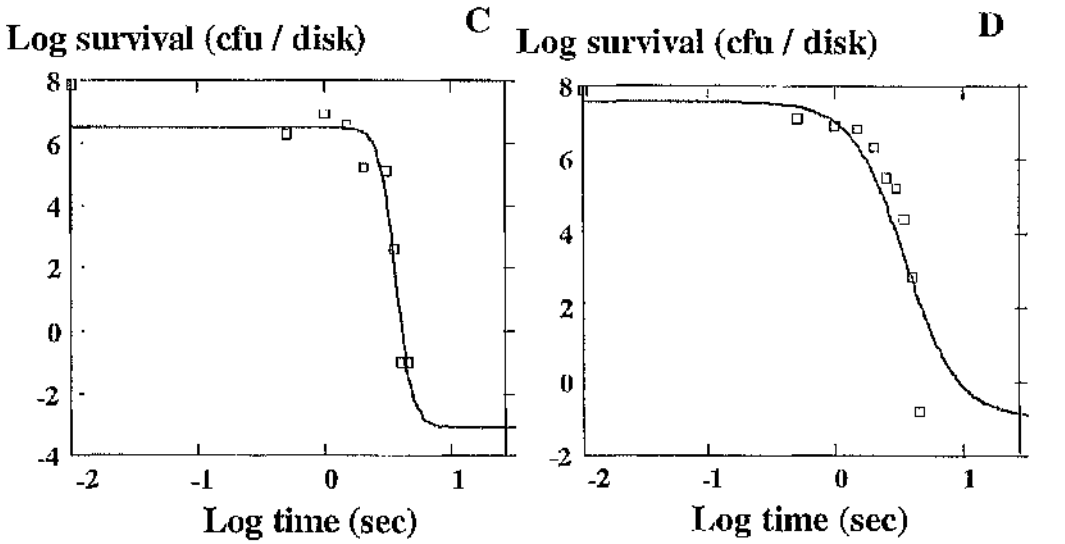


Figure 4.73 **Effect of growth at different pH values on the susceptibility of *St. aureus* to Nd:YAG laser light.** Each killing curve was a dried overnight culture on the surface of stainless steel disk and each point is a separate exposure on different disks. The results are the average of two separate experiments.
[◆] pH 6, [●] pH 6.5, [▲] pH 7, [○] pH 7.5, [■] pH 8

Figure 4.74 **Different types of best fit lines for two sets of data obtained from Figure 4.72.** **A.** is the linear best fit for the killing curve of *St. aureus* grown in 0.5% w/v NaCl on a log-linear scale, **B.** is the linear best fit for the killing curve of *St. aureus* grown in 10.0% w/v NaCl on a log-linear scale, **C.** is the log-logistic best fit for the data in **A** on a log-log scale, **D.** is the log-logistic best fit for the data in **B** on a log-log scale, **E.** is the quadratic best fit for the data in **A** on a log-linear scale, **F.** is the quadratic best fit for the data in **B** on a log-linear scale.


A
B
Log cfu / disk
Energy density (Jcm-2)
0
200
400
600
0
2
4
6
8


C
D
Log survival (cfu / disk)
Log survival (cfu / disk)
Log time (sec)
Log time (sec)
-4
-2
0
2
4
6
8
-2
-1
0
1

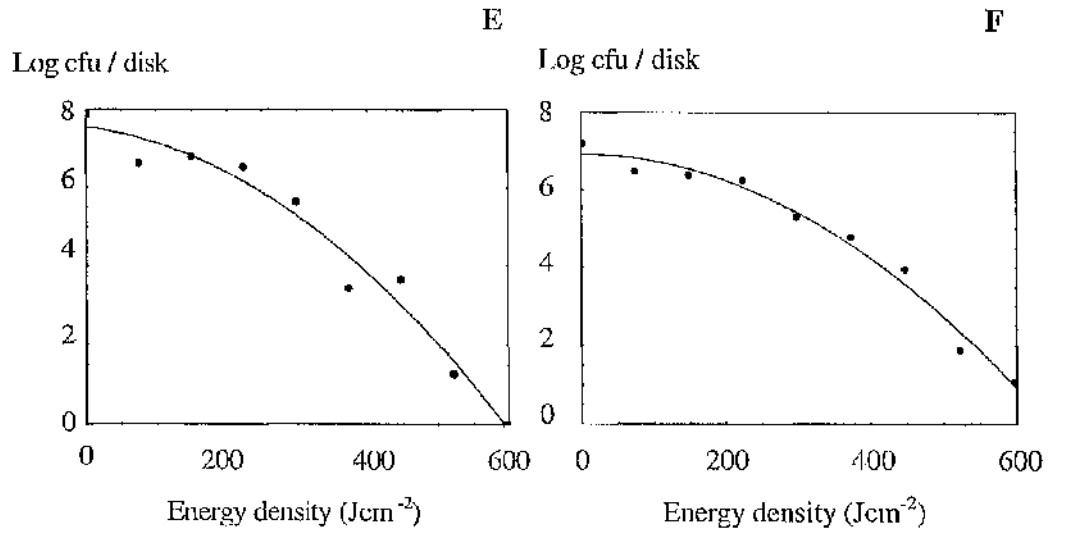
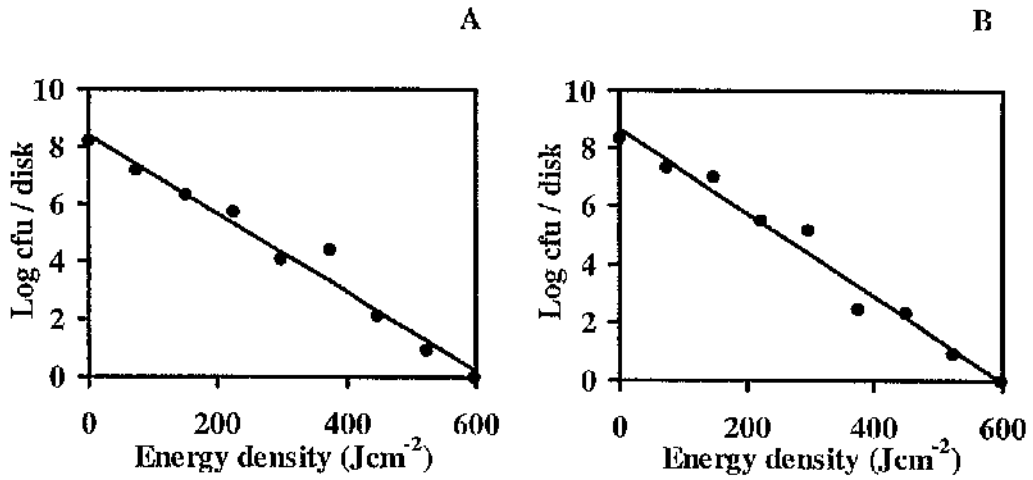
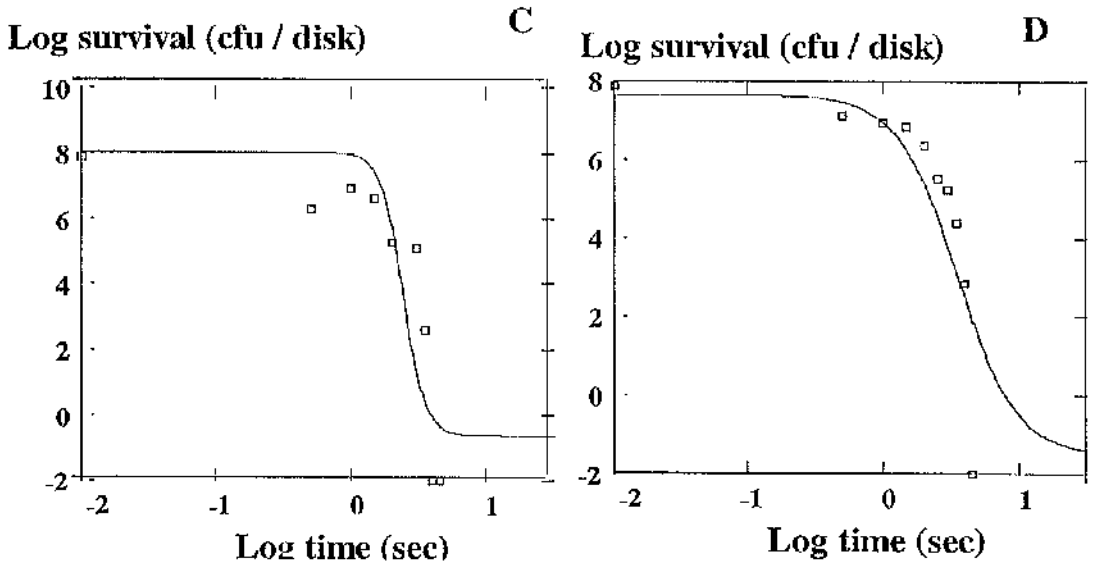
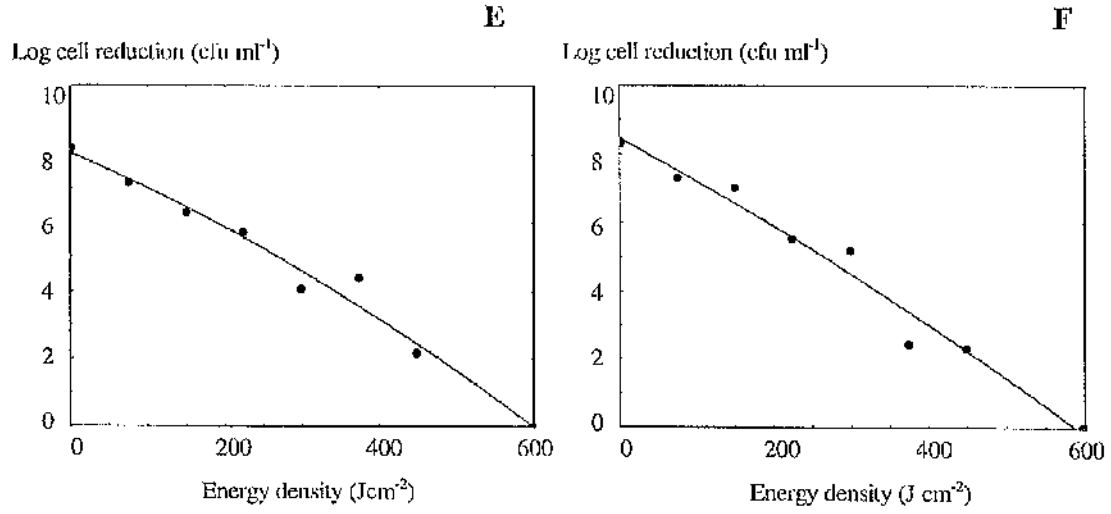

E
F
Log cfu / disk
Log cfu / disk
Energy density (Jcm-2)
Energy density (Jcm-2)
0
2
4
6
8
0
200
400
600

Figure 4.75 **Different types of best fit lines for two sets of data obtained from Figure 4.73.** **A.** is the linear best fit for the killing curve of *St. aureus* pH 6 on a log-linear scale, **B.** is the linear best fit for the killing curve of *St. aureus* grown at pH 8 on a log-linear scale, **C.** is the log-logistic best fit for the data in **A** on a log-log scale, **D.** is the log-logistic best fit for the data in **B** on a log-log scale, **E.** is the quadratic best fit for the data in **A** on a log-linear scale, **F.** is the quadratic best fit for the data in **B** on a log-linear scale.


A
B
Log cfu / disk
Energy density (Jcm-2)
0
200
400
600
0
2
4
6
8
10


C
D
Log survival (cfu / disk)
Log survival (cfu / disk)
Log time (sec)
Log time (sec)
-2
-1
0
1
2
4
6
8
10


E
F
Log cell reduction (cfu ml-1)
Log cell reduction (cfu ml-1)
Energy density (Jcm-2)
Energy density (J cm-2)
0
200
400
600
0
2
4
6
8
10

4.5 KILLING OF BACTERIA ON METAL, PLASTIC AND GLASS SURFACES WITH CO_2 LASER LIGHT

The CO_2 laser beam profile was not Gaussian; it was almost oval with a small area, within the profile, of low energy. This was essentially an irreparable artefact related to the optical cavity within the laser, mainly due to the age of the machine. However this did not interfere with the results presented in this thesis. There were two power settings on this laser, low power and high power with outputs of 500 and 800 W, respectively.

E. coli, *St. aureus*, *L. monocytogenes* and *B. cereus* were exposed to laser light in the presence of excess liquid on stainless steel disks, but the liquid sample splashed over the surface of the disk due to the rapidity of the temperature rise within the sample and its rapid expansion. Because of the risk of recontamination of the surface and the surrounding area and hence the threat of cross-contamination of following disks it was not possible to examine the effects of CO_2 laser light on liquid suspensions of bacteria.

Figure 4.76 shows the killing curves for *E. coli*, *St. aureus* and *L. monocytogenes* as a dry film of an overnight culture on nylon disks. First, it was apparent that each of the bacterial cultures started at a different inoculum size, shown by the first point. With each organism the first exposure, corresponding to a 1 msec exposure time, produced no killing and a slight rise in viable counts for *E. coli*. The following exposure times, which increased in increments of 1 msec, showed consistently greater reductions in viable counts. However, the rate of reduction, irrespective of the initial inoculum size was constant with the three microorganisms. The final point for *St. aureus* showed a very steep drop in the number of viable organisms. In none of the cases were the log counts reduced to zero as the exposure times were not sufficiently high, however it was possible to completely sterilize *St. aureus* from the plastic surface as shown in **Figure 4.77**.

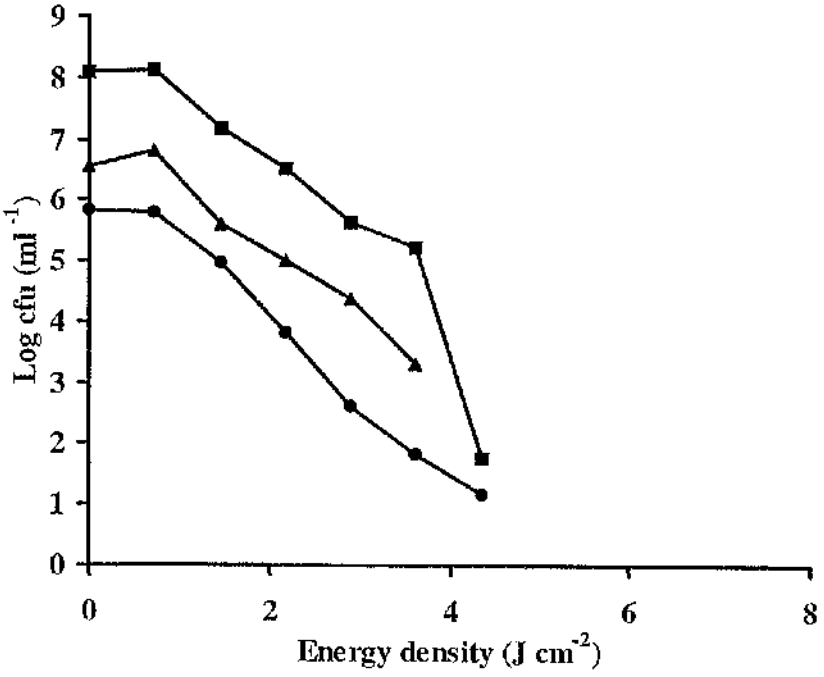


Figure 4.76 **Killing curves for overnight cultures of *St. aureus* [■], *E. coli* [▲] and *L. monocytogenes* [●] as dry films on nylon plastic surfaces.** Each point is a separate exposure on different disks. The results are the average of two separate experiments.

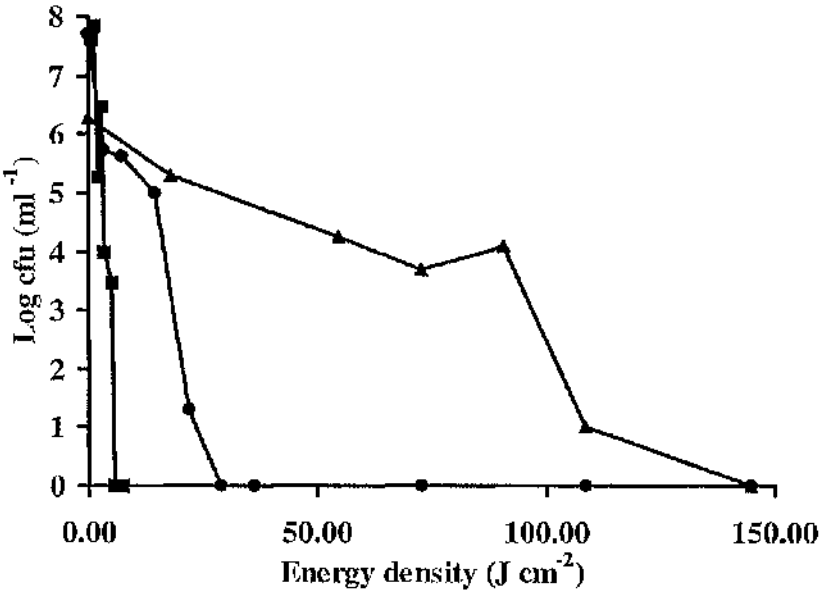


Figure 4.77 **Killing curves for overnight cultures of St. aureus as dry films on three different surfaces**: stainless steel [▲], nylon plastic [■] and glass [●] with high power CO_2 laser light (820 W). Each point is a separate exposure on different disks. The results are the average of two separate experiments.

The type of surface that was inoculated had an effect on the ability of the CO_2 laser to reduce the viability of bacteria. In **Figure 4.77** CO_2 laser light was used to decontaminate three different surfaces: stainless steel, glass and nylon. Stainless steel required the longest exposure times, and the highest ED (even with a lower initial inoculum concentration) to effect the viability of the microorganisms. On this surface the area which contained the dried bacterial suspension started to burn around the edges after about 50 msec (36 Jcm^{-2}) but still the organisms were not completely sterilized. A large difference was found between these results and the energy densities required to reduce the viability of *St. aureus* on glass surfaces. It took ~40 msec to reach zero viability on these surfaces. The lower detection limit was 2 organisms / disk because the solutions were filtered. After the time interval of decontamination of glass (40 msec) only one D-value of reduction in bacterial viability was obtained on stainless steel compared to almost 8 D-values on the glass surface. The easiest surface to decontaminate was the plastic. In around 6 msec, *St. aureus* on the plastic surface was completely killed - a reduction in bacterial viability of 8 D-values compared to 2 D-values at this ED with the glass surface, and no measurable effect on the stainless steel. Within the range of energy densities required to sterilize the different materials, there was no visible damage to the surfaces.

4.6 EFFECT OF LASER LIGHT ON *ST. AUREUS* ON COLLAGEN SKIN SURFACES

The decontamination of sausage skins made from collagen was investigated by laser treatment. The exact nature of the production of the collagen could not be disclosed by the producers but this was not pertinent to the problems of contamination. Light from the Nd:YAG laser, with a generally high penetrability, was used to decontaminate *St. aureus* from the surface.

After reconstituting the collagen in distilled water to make it a more pliable surface, it was seeded with *St. aureus*, and placed face up on a nutrient agar plate. Plates were incubated for two days but growth was difficult to visibly determine. The

addition of iodo-nitro-tetrazolium violet (INT) to the nutrient agar overcame the problem as the viable bacteria absorbed the dye and were coloured red due to the production of formazan crystals within viable bacterial cells. With this method, any areas where the bacteria were killed would be clear within the lawn of red microbial growth. **Figure 4.78** shows the effect INT had on bacteria seeded on the collagen skin. The petri-dish on the right contained a square of skin, without any inoculum, placed face-up on nutrient agar containing INT, there was no coloration of the skin. However, the dish on the left contained the control collagen seeded with bacteria, clearly, the growth was coloured over the whole area of the skin.

Killing of St. aureus on collagen skin with Nd:YAG laser light

The results of exposure of collagen seeded with *St. aureus* to Nd:YAG laser light are presented in **Figure 4.79**. At a PRF of 10 Hz there were no visible cleared areas in the bacterial lawn therefore no effect on either the skin or the bacteria was observed. Also, with a single pulse of 50 J, there was no visible effect (data not shown). However, at 30 Hz for exposures of 20 and 30 seconds a clear circle was evident in the bacterial lawn, indicated bacterial sterilization, additionally there was no evidence of damage to the skin. The non-uniformity of the lawns of bacteria were due to bubbles between the skin and the agar surface.

Killing of St. aureus on collagen skin with CO_2 laser light

The effect of CO_2 laser light on contaminated collagen skin are shown in **Figures 4.80A, B** and **C**. After as little as 1 msec the CO_2 laser at the high-power setting of 800 W sterilized the bacterial contaminant within a circular area on the surface of the collagen film (**Figure 4.81A**). At 450 W there was a definite clear area after 4 msec (**Figure 4.81B**). The damage threshold of the collagen skin was around 8 msec at 800 W. One concern was whether or not the lack of penetrability of the CO_2 laser would allow the laser light to sterilize the lawn through the skin. **Figure 4.82** shows a sterile area produced after 3 msec exposure to CO_2 laser light at 800 W, and therefore below the damage threshold of the collagen film.

CONTROLS

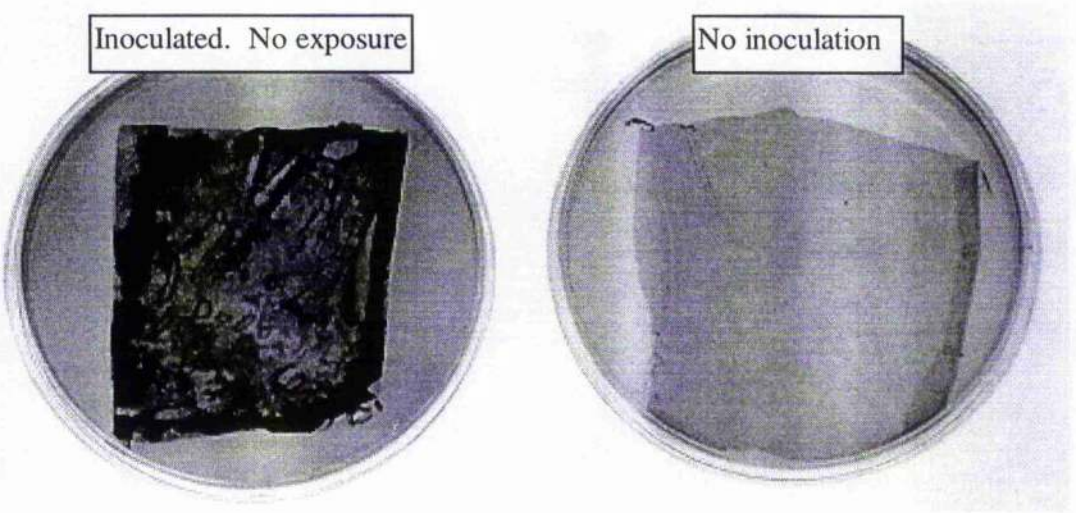


Figure 4.78 **Collagen film controls with and without *St. aureus* inoculation, spread over the surface of nutrient agar plates which contain INT.** The plate on the left contains reconstituted collagen film seeded with *St. aureus* prior to spreading on the agar surface, whereas the plate on the right contains collagen film with no prior inoculation

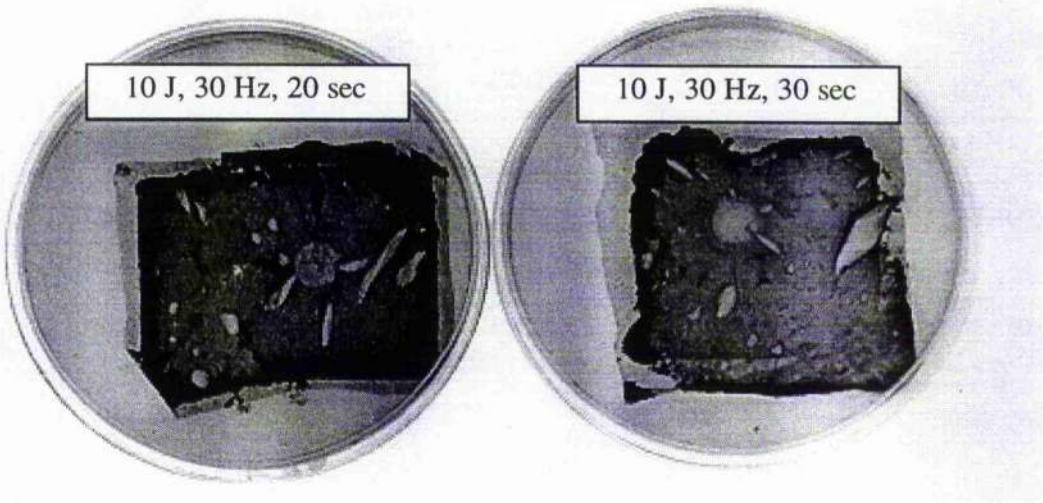


Figure 4.79 **Collagen film, inoculated with *St. aureus*, spread over the surface of nutrient agar plates which contained INT.** Each film was reconstituted with sterile distilled water and dried slightly before inoculating with 100 µl overnight *St. aureus* culture. The films were exposed to Nd:YAG laser light at the parameters shown in the figure.

Figure 4.80 **Photograph showing collagen film spread on the surface of INT-nutrient agar plates.** Each film was reconstituted with sterile distilled water and dried slightly before inoculating with 100 µl overnight *St. aureus* culture. The films were exposed to high-power laser light (1 kW) for the exposure times shown in the figure.

Figure 4.81 **Photograph showing collagen film spread on the surface of INT-nutrient agar plates.** Each film was reconstituted with sterile distilled water and dried slightly before inoculating with 100 µl overnight *St. aureus* culture. The films were exposed to low-power laser light (500 W) for the exposure times shown in the figure

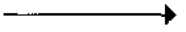
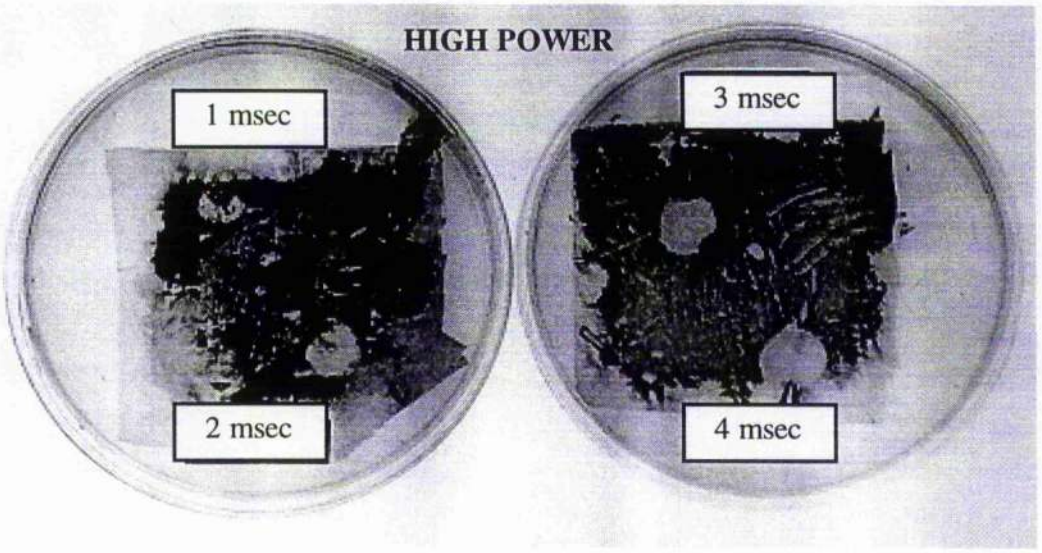
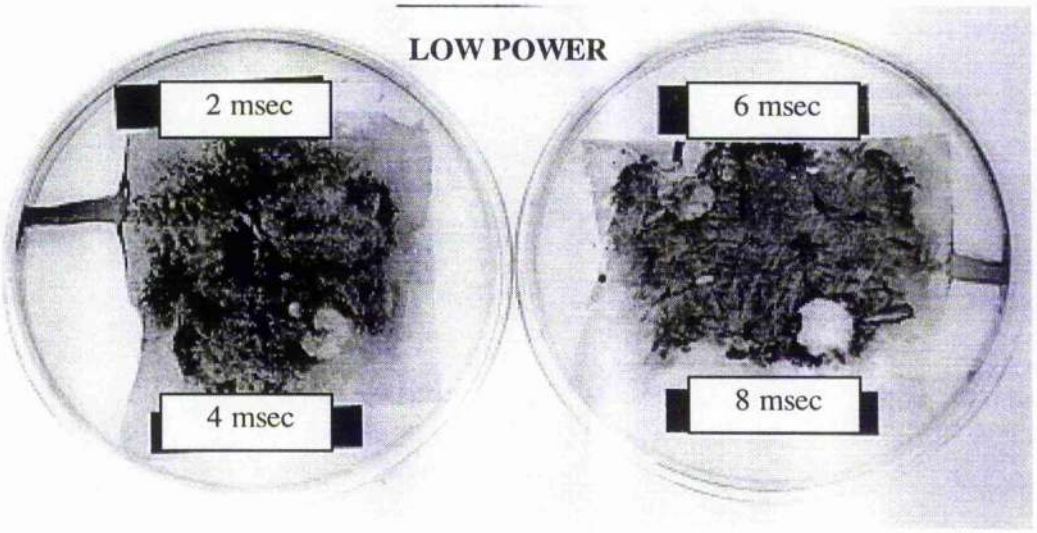
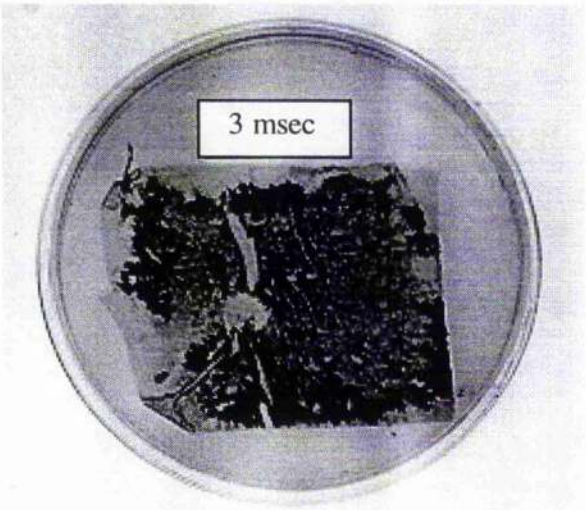


Figure 4.82 **Photograph showing collagen film spread on the surface of INT-nutrient agar plates.** Each film was reconstituted with sterile distilled water and dried slightly before inoculating with 100 µl overnight *St. aureus* culture. The films were exposed to high-power laser light (1 kW), for 3 msec, however the laser was fired at the bacteria through underside of the collagen film.


HIGH POWER
1 msec
2 msec
3 msec
4 msec


LOW POWER
2 msec
4 msec
6 msec
8 msec


3 msec

4.7 THE EFFECT OF LASER LIGHT AT DIFFERENT WAVELENGTHS ON *E. COLI* LAWNED ON NUTRIENT AGAR

The biocidal effect of light from seven different lasers, on *E. coli*, lawned on nutrient agar, was examined from the area of microbial sterilization after incubation at 37°C overnight. The results were quantified by determining the ratio of the area of clearing to the laser beam diameter. The beam diameters, mean power, exposure times and ED applied to the lawn of *E. coli*, with the areas of sterilization, for each laser under investigation, are shown in **Table 4.18**.

The longest exposure time and the maximum ED applied to the lawn of bacteria was 13,754 J cm^{-2} with the laser diode array. The beam diameters for the argon ion laser varied between 1.5 and 40 mm. In the investigation, only the Nd:YAG lasers were pulsed and pulse energies varied from 4 mJ to 10 J, at frequencies of 10 Hz, apart from the Nd:YAG MS830 (with the highest pulse energy) at a frequency of 20 Hz. Mean power in all cases with the exception of the CO_2 and the Nd:YAG (MS830) lasers were very small, some in the order of fractions of Watts. **Table 4.18** shows, where applicable, the areas of *E. coli* sterilization for the different lasers. Of the seven lasers tested only four wavelengths in combination with the specified parameter settings caused sterilization, namely: CO_2, Nd:YAG (MS830), Minilite frequency-tripled Nd:YAG and Surelight frequency-tripled Nd:YAG. For the Lumonics Nd:YAG MS830 laser, the ED had to be higher than 1200 Jcm^{-2} to produce a noticeable decrease in the thickness of the bacterial lawn. With the CO_2 laser and the Lumonics Nd:YAG laser an increase in the applied ED produced a significant increase in the area of *E. coli* sterilization. Very little damage to the agar was observed with frequency-tripled Nd:YAG laser light, but the CO_2 laser and the Lumonics Nd:YAG laser (at higher energy densities) produced surface damage to the agar. No bactericidal action was observed with light from the following lasers: FIR, Q-switched Nd:YAG (at 1064 nm and 532 nm), laser diode array and Argon ion, with the combination of parameters used. The peak powers of the Q-switched lasers (1064 nm) were 1000-fold higher than the Lumonics laser (1064 nm), but no visible

effect was observed on the bacterial lawn. These peak powers were however, sufficiently high to melt and burn the plastic of the petri dish

Figure 4.83 compares the performance of each laser investigated with the observed response plotted, with different symbols, against the applied ED (Jcm^{-2}). There are three different responses: no observed effect, sterilization of an area less than 15% of the beam area and sterilization of an area greater than 15% of the beam area. It can be seen that light from the minilite frequency tripled Nd:YAG laser produced areas of sterilization less than 15% of the beam area. Nd:YAG laser light (MS830) sterilized areas greater than 15% beam area for energy densities greater than 1460 Jcm^{-2} and the CO_2, greater than 15% above 2.63 J cm^{-2}.

The results in **Figure 4.84** show the areas of sterilization as a function of the applied ED for each of the four lasers which visibly affected the lawn of *E. coli*. The areas of sterilization produced by the light from the Lumonics Nd:YAG laser required an ED 1000-fold higher than that required to produce the same area of sterilization. For example, light from the CO_2 laser at energy densities of 2.63 and 7.88 Jcm^{-2} produced cleared zones of 0.66 and 1.215 cm^2 respectively, whereas light from the Lumonics Nd:YAG laser, to produce a sterilized area of 0.715 cm^2 required 1940 Jcm^{-2}. For the frequency-tripled laser the areas did not vary a great deal, although the areas produced by the light from the Surelite II-10 were larger than those produced by the Minilite. Both of these lasers had a similar beam diameter (as quoted by the manufacturer) but the pulse energy of the Surelite was higher. A single pulse from the frequency-tripled Surelite laser reduced the lawn of *E. coli* but was not quantifiable.

Figure 4.85 plots the fractional area sterilized per unit ED against the exposure time on a log-log scale. This figure normalises the zones of inhibition to the average applied ED and beam area as a function of the exposure time. Light from the CO_2 laser provided the most rapid sterilization, with zones of clearing of 1.2 cm^2 in 30 msec, as opposed to the Lumonics Nd:YAG laser, which required 16 sec exposure to produce an area of 0.715 cm^2. Both of the frequency-tripled Nd:YAG lasers were more efficient than the Lumonics Nd:YAG.

Laser (model)	beam area (cm^2)	mean power (W)	exposure time (sec)	energy density ($J\ cm^{-2}$)	area of inactivation (cm^2)
[a]FIR (FIRL 100)	0.385	0.15	60	7.96	0
CO_2 (MFKP)	2.30	600	0.005	1.131	0.160
			0.010	2.63	0.660
			0.020	5.26	1.04
			0.030	7.88	1.21
Nd:YAG (MS830)	1.65	200	9	1090	0
			10	1210	0.038
			12	1460	0.31
			14	1700	0.54
			16	1940	0.715
[a]Nd:YAG (minilite 10)	0.283	0.25	10	8.84	[c]0
[a]doubled Nd:YAG (minilite 10)	0.283	0.1	15	5.31	[c]0
tripled Nd:YAG (minilite 10)	0.283	0.04	5	0.71	0.0107
			15	2.12	0.0178
			20	2.82	0.0277
			30	4.22	0.0362
			60	8.49	0.0365
[a]Nd:YAG (surelite II-10)	0.283	6.5	3	69	[d]0
[nb]doubled Nd:YAG (surelite II-10)	0.283	3.0	3	31.8	[c]0
tripled Nd:YAG (surelite II-10)	0.283	1.0	1	3.54	0.121
			2	7.07	0.112
			3	10.6	0.123
[a]laser diode array (OPCAO15)	0.196	15	120	17,350	0
[a]argon ion (beamlok 2060)	[e]12.56	0.65	60	2210	0

Table 4.18 Bactericidal effect of light from seven different lasers against E. coli lawned on nutrient agar. The table shows the common parameter, for comparison and the areas of sterilization for each energy density.

[a] No bactericidal effect observed with shorter times and smaller energy densities

[b] A single shot reduced the lawn thickness but was not quantifiable

[c] The plastic petri dish, beneath the agar was burnt after exposure

[d] The plastic petri dish, beneath the agar was melted after exposure

[e] The minimum beam diameter tested was 0.15 cm

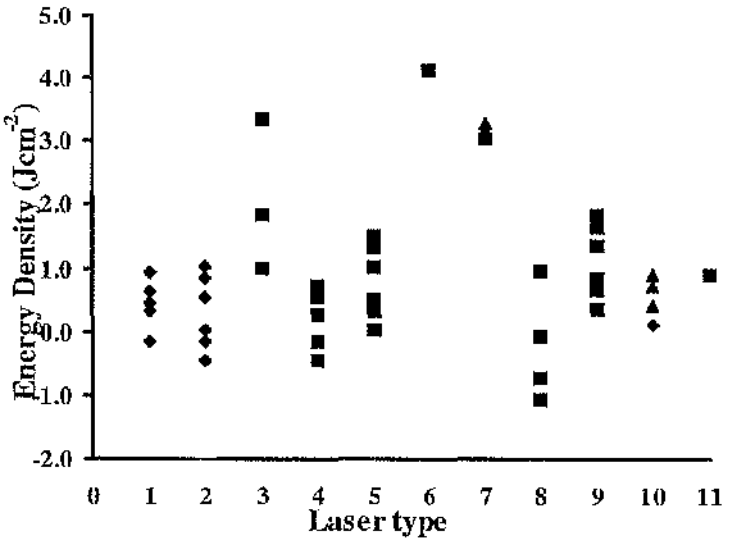


Figure 4.83 **The bactericidal effect of 11 different lasers on *E. coli* lawned on nutrient agar plates.** For each laser a response is given for a number of energy densities: no sterile area [■], sterile area <15% beam area [◆] and sterile area >15% beam area [▲].

Lasers:-

1. Minilite (355 nm)
2. Surelite (355 nm)
3. Argon ion (488 nm)
4. Minilite (532 nm)
5. Surelite (532 nm)
6. Laser diode array (810 nm)
7. Nd:YAG MS830 (1064 nm)
8. Minilite (1064 nm)
9. Surelite (1064 nm)
10. CO_2 laser (10.6 μm)
11. FIR (118 μm)

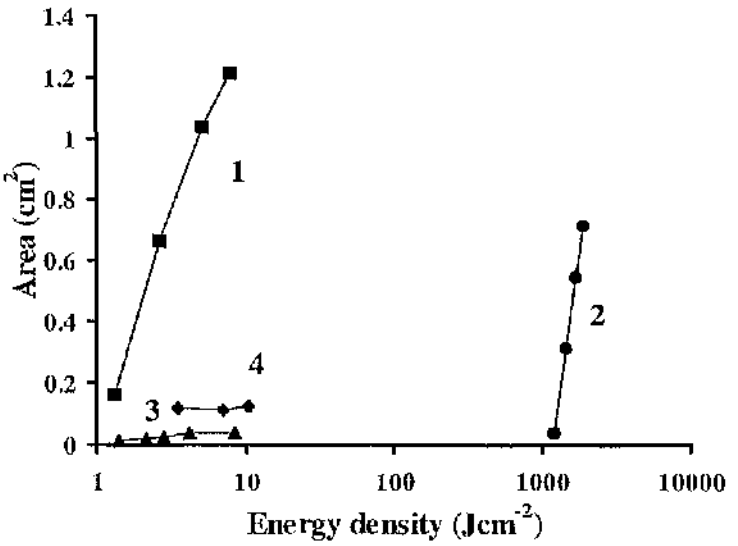


Figure 4.84 **Zones of clearing as a function of the applied energy density, on a log scale, for each laser that demonstrated a bactericidal capacity against *E. coli*, grown on nutrient agar.**

1 CO_2 laser

2 Nd:YAG MS830

3 Nd:YAG minilite (tripled)

4 Nd:YAG surelite (tripled)

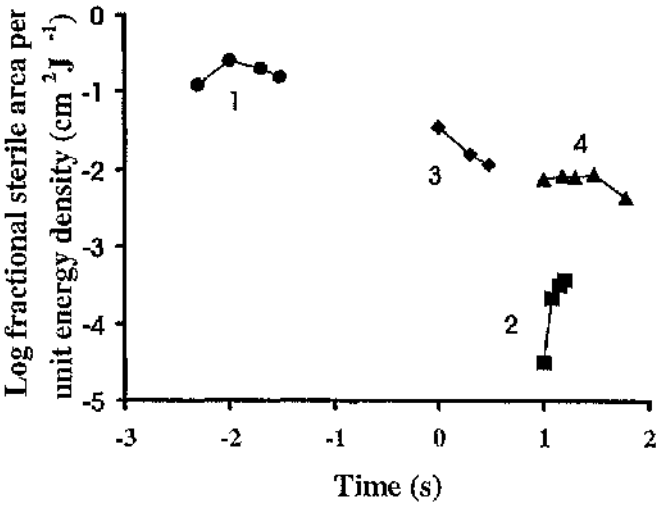


Figure 4.85 **Exposure times to generate zones of clearing normalised to the laser beam area and applied energy density, for each laser that demonstrated a bactericidal capacity against *E. coli* grown on nutrient agar**

1 CO_2 laser

2 Nd:YAG MS830

3 Nd:YAG surelite (tripled)

4 Nd:YAG minilite (tripled)

5. DISCUSSION

In the 33 years of laser technology, preceding this project there have been around 20 publications discussing the bactericidal effects of the direct use of laser light. Only nine of these publications gave enough parameter information to allow the calculation of the different laser energy densities. This presented problems when comparing the efficacy of laser light at a variety of wavelengths and other parameters on different microorganisms under a range of environmental conditions. The majority of research presented in these reports were applied, predominantly to dental applications with very few purely scientific investigations. It is however, obvious that two lasers predominate in published work, namely the CO_2 and Nd:YAG lasers, as effective systems for sterilizing microorganisms. Both of these lasers were compared to the argon ion (Powell and Whisenant, 1991) and were found to be less effective in sterilizing. From the results in this thesis, the argon ion was ineffective at comparable energy densities in the sterilization of a lawn of *E. coli*, contradicting the results of Powell and Whisenant. The authors did not reveal the beam diameters in their investigation and so the energy densities can not be calculated and therefore a useful comparison to the work presented in this thesis was not possible.

Although there is substantial literature on the bactericidal activity of light from different laser sources, there have been few studies in which different laser wavelengths were compared for activity in relation to energy density with standardised microbial targets. A variety of lasers were studied throughout the course of this thesis with more detailed investigations of the microbicidal abilities of Nd:YAG and CO_2 laser light.

5.1 MICROBIAL STERILIZATION ON AGAR SURFACES AND IN LIQUIDS WITH Nd:YAG LASER LIGHT

Nd:YAG laser light provided adaptability to study the lethal action of the laser light on microorganisms both on surfaces and in liquids. Initial experiments were to investigate the effects of laser light on microorganisms lawned on nutrient agar surfaces. This method of exposure was initially designed to be a simple qualitative exercise to provide information about the ability of Nd:YAG laser light to sterilize

bacteria and yeasts, and allow monitoring of damage to the agar surface. However, it was observed that increased energy densities of laser light produced increased areas of microbial sterilization, thereby giving a novel method of quantitatively monitoring killing of microorganisms on agar surfaces. A killing curve was produced by plotting the areas of clearing against the applied energy density. From these curves the energy density required to sterilize an area of microbiological lawn one half the area of the beam was denoted the IA_{50}-value and therefore provided means of comparing different parameters.

The first investigation incorporating this procedure examined the susceptibility of a variety of microorganisms to Nd:YAG laser light. A standard set of parameters were used in the experiments on agar surfaces varying only the exposure time within acceptable limits for accurate manual control. The laser control method was upgraded to control with a computer during the course of the project.

To ensure that the areas produced resulted from the direct action of laser light on the microorganisms, the agar was lawned after laser exposure and the growth supporting ability of the agar was unaffected. Also some areas of clearing after exposure were incubated for 14 days without further growth. Other areas were removed from the lawned agar and imprinted onto fresh nutrient agar and there was no further growth in 14 days of incubation. These results help to show that the damage to the microorganisms was long-term and most probably lethal.

The results in the first experiment, exposing different microbial cultures, suggested that there was variability in the sensitivity of bacteria or yeasts to killing by 1064 nm Nd:YAG laser light. The most resistant organism to the laser light was the vegetative cells of the thermophilic *B. stearothermophilus* whose cell components are known to be relatively heat-resistant. The most sensitive organism was *Ser. marcescens* which are unclustered, pigmented, Gram-negative rods. The sensitivity of *Ser. marcescens* was not much different from *E. coli*, which is unpigmented and of similar size and shape, *St. aureus*, which is a clustering organism, pigmented, and spherical, or from the two yeast species with at least 300-times the cell volume and no pigment. *D. radiodurans* which is highly resistant to ionizing radiation, because

of efficient DNA-repair mechanisms, was not particularly resistant to the laser, but had a slightly higher IA_{50}-value. *M. luteus* was surprisingly resistant to the laser light with an IA_{50}-value double that of *Ser. marcescens*. It was, however possible to sterilize all of these organisms with little or no damage to the agar surface in around 20 sec, at 10 Hz, 10 J and a beam diameter of 1.45 cm.

These results suggest that the lethal mechanism was not primarily determined by cell size, shape or clustering, or by Gram-staining characteristics. Because this selection of Gram-positive and Gram-negative bacteria, with cells that were rod-shaped or spherical, clustered or individual, pigmented or colourless, together with two yeasts species, were all killed within a relatively narrow range of energy densities, with the thermophile *B. stearothermophilus* as the most resistant, the application of thermal energy may account for the majority of the lethal mechanism.

Different microbial species was only one of the many variable parameters affecting the efficiency of Nd:YAG laser light to sterilize microorganisms (see **Figure 2.1**). There were a variety of laser parameters to be investigated. For simplicity *E. coli* was used as the standard microorganism for the investigation of the optimum laser parameters for microbial sterilization. *E. coli* was chosen because it is a ubiquitous, well studied organism and would allow assessment of the effects of the laser parameters on the other microorganisms.

The results indicate that the PRF influenced the microbicidal action of Nd:YAG laser light. This was summarised with the IA_{50}-values (**Figure 4.7**). As the PRF was increased the IA_{50}-value decreased such that the laser operating at 30 Hz required 21.5% less energy density to produce the 50% cleared area. The reduction in the IA_{50}-values over the range of PRF was relatively uniform. These results were at a pulse energy of 10 J, pulse width of 8 msec and a beam diameter of 1.45 cm, and different combinations of these parameters may further influence the microbicidal action of the Nd:YAG laser.

The pulse energy also had an effect on the ability of Nd:YAG laser light to clear an area in the lawn of *E. coli*. Increasing the pulse energy from 10 to 20 J decreased the

IA_{50}-value by almost 30%. The effect of increasing the pulse energy beyond 20 J at a standard PRF of 10 Hz was variable but showed little enhancement of the Nd:YAG laser light sterilization efficiency. As these results were produced at a relatively low PRF of 10 Hz and a pulse width of 8 msec, the gap between each pulse was almost 10 times the pulse width. Increasing the peak energy will affect the length of time that the lawned bacterial system takes to equilibrate to the new energy state after exposure. There will be a peak energy above which the bacterial system will not have sufficient time in the 92 msec gap between the pulses to equilibrate to the new energy state of the system. This may cause the change in gradient of between the pulse energies above 20 J. Though the results shown in **Figure 4.9** are from triplicate results there are some curves with only two values, due to lower energy densities producing no sterile areas. The IA_{50}-values for each of these curves (25 and 40 J) will therefore be slightly inaccurate. The IA_{50}-value at 25 J was slightly out of line compared to the other values and this can therefore be put down to inaccuracies brought about by the lack of results in the killing curves at this pulse energy. At 10 Hz, a pulse energy of 20 J appears to be close to optimum.

The range of pulse widths used in this investigation was limited. Present day laser systems have capabilities of producing pulse widths of femtoseconds (10^{-15} sec) producing very high intensity pulses. The range of pulse powers was from 2500 W (4 msec) to 505 W (19.8 msec) for a 10 J pulse. The results showed that the varying the pulse width had little or no effect on the laser sterilization efficiency. It is worth noting that in section 4.7 the use of a Q-switched Nd:YAG laser (Surelite and Minilite) with pulse width of 6×10^{-9} and 5×10^{-9} sec respectively did not visibly affect the lawn of *E. coli*. The decrease in the pulse width which produced pulse powers of 108 MW and 5 MW respectively was ineffective, however the pulse energy was very small. If the pulse energy was able to have been increased to 10 J it may be possible to produce the same cleared area as the Nd:YAG laser MS830. Varying the pulse width of the Nd:YAG laser produce no enhancement of the bactericidal capacity of the Nd:YAG laser.

Whilst the previous three variables discussed were parameters associated with pulsed laser systems alone, the beam diameter is a universal parameter. Varying the beam

diameters has wide ranging implications, from an engineering perspective, and is instrumental in the design of effective laser sterilization systems. The beam diameter is a necessary variable for calculating the most effective scanning system to sterilize larger areas. The results show that smaller beam diameters appear to be the most effective in decontaminating the surface of the agar plate requiring the lowest energy density to reach the sterilization threshold of *E. coli*. The smallest beam diameter utilised in this investigation was 1.25 cm, but theoretically the diameter could have been reduced much further. Practically however, this was a problem, firstly in achieving measurable areas of sterilization on the surface of the agar and accurately timing the shorter exposure times required for smaller beam diameters. The results suggest that a reduction in the threshold energy density so that the smallest possible beam diameter would be most efficient. In practice however, there will be a limit to the size of the most effective beam diameter because of the increase in the rate of diffusion of heat, or other toxic products, away from the exposed areas. Once the threshold energy densities were reached with the larger beam diameters the increase in the area of sterilization was more rapid and the areas were larger. These were both put down to the fact that the areas of lawned bacteria exposed to the laser light were larger and the cross-sectional energy distribution of the laser was flatter. The difference in the IA_{50}-values over the range of beam diameters was only 250 Jcm^{-2} but appeared to show a regular shape. Between the beam diameters of 1.25 and 1.5 cm there was a 10% increase in the IA_{50}-value, levelling off at larger beam diameters. It would have been interesting to find out if the IA_{50}-value decreases further if the beam diameter was decreased.

In all of these experiments it was interesting to note that little or no damage was observed on the exposed agar surface at the lower bactericidal energy densities. The temperature of the agar surface after exposure was duly recorded to assess the thermal effects of this laser on the bacteria. The results showed that the rate of the temperature rise increased, but the temperature at the threshold energy density decreased from 58° to 50°C, with an increase in the PRF from 10 to 40 Hz. These temperatures were rather low for the level of sterilization produced by laser light in the short times of exposure (around 20 sec) if the mechanism was thermal. The method used to monitor the temperature was not ideal. Firstly, it relied on retracing

the cooling curve back to the time that the laser was switched off and invariably produced inaccuracies. The thermocouple used in the experiment was metallic and before insertion into the agar it was at room temperature, therefore heat transfer would have taken place with the result of the area appearing cooler than it was. The temperature was also recorded below the surface of the agar and not on the surface where the bacteria were in direct contact with the agar. However, this was the most feasible method of monitoring the temperature of the exposed areas giving an idea of the approximate temperature ranges. This particular investigation showed that the agar temperatures, post exposure, were at lethal levels for *E. coli* but under normal heating conditions would require longer exposure times.

With the different microbial strains exposed to the laser light there were clearly damaged cells at the periphery of the exposed areas, similar observations were previously reported by Schultz *et al.* (1986) after exposure to liquid suspensions of bacteria. The authors concluded that the cells had undergone sub-lethal damage. Some simple investigations concerning the microcolonies of *E. coli* discovered around the periphery of the exposed areas concluded that the colonies contained auxotrophic organisms. The exact nature of the microcolonies were not further elucidated.

Schultz (1986) also noted that the laser appeared to be producing significant killing at around 50°C in liquid suspensions. The authors used a similar method of temperature measurement as used with agar surfaces in this investigation, whereby inserting a thermocouple after laser light exposure and recording the temperature. Due to the comparable temperatures between the results obtained by Schultz *et al.* (1986) and those observed after laser exposure of agar surfaces it was a natural progression to attempt to further elucidate the lethal mechanism within a liquid environment. A thermocouple vessel was designed to allow instantaneous measurement of the temperature during exposure with reduction of the error due to localised cooling, when the thermocouple was inserted into the laser exposed medium and the time interval between stopping the laser and taking a temperature measurement.

As water absorbed a small amount of the laser light it was not surprising that the volume of distilled water within the thermocouple vessel heated up to temperatures which were lethal to bacteria. Because the volume of water had depth the intensity of incident laser light was greater than the transmitted laser light due to absorption and to a lesser extent, scattering. This therefore caused the surface temperature to rise at a quicker rate than the temperature at the base of the liquid volume leaving a temperature gradient in the vessel. There was also a gradient caused by the Gaussian distribution of energy through the beam cross-section producing a lateral temperature gradient in the liquid volume. The results shown in **Figures 4.25 and 4.26** are computer-modelled predictions of these gradients and not 3-dimensional experimental results and so subtle effects, such as water movement due to uneven heating producing currents or Brownian motion, reflection off micro-air bubbles as the water is degassed during heating, reflection off the base of the vessel and the walls, conduction of heat by the glass vessel and the metal holder or reflection off and refraction through the meniscus at the liquid surface have not been taken into account. These models were designed to indicate the presence of the temperature gradients within the volume of water.

When lower bacterial concentrations were heated to 50°C with laser light, there was a slight temperature rise after switching the laser off. In the most extreme cases this temperature rise was between 2° and 3°C. This phenomenon was put down to dissipation of the heat from the centre of the suspension in the thermocouple vessel to the periphery and from the surface to the base. The rise in temperature after 23 sec, (exposure time required to raise *E. coli* at 1 x 10^4 cfu ml^{-1} to 50°C) can be calculated theoretically with

$$E = mc\Delta T$$

where E is the absorbed energy, m is the mass of the water, c in the specific heat capacity of water and ΔT is the change in temperature of water, therefore

$$\Delta T = \frac{E}{mc}$$

$$\Delta T = \frac{240}{2.0 \times 4.18}$$

$$\Delta T = 28.7°C$$

assuming the absorption coefficient for water is 8% and the energy was delivered over 20 sec.

This value is very similar to the experimental value of approximately 27°C, where the temperature was raised to 50°C, from room temperature, however there are differences to the modelled peak temperatures. Although this calculation was simplified and did not take account of the increase in the specific heat capacity as the temperature increased (Yeo *et al.* 1996) or reflections and scattering within the thermocouple vessel, it is still very much lower than the highest temperature in the modelled temperature profiles.

When more concentrated bacterial cultures were exposed to laser light the temperature at the position of the thermocouple rose faster than the dilute cultures, reaching the cut-off temperature of 50°C in almost half the time. With an OD at 1064 nm of 0.8 the amount of light scattered and absorbed is calculated as follows:-

$$A = log\left(\frac{I_o}{I_o - a}\right)$$

where A is the absorbancy, I_o is the percentage of incident light and a is the percentage of light either scattered or absorbed, thus:-

$$a = I_o - 10^{(log I_o - A)}$$

$$a = 100 - 10^{(\log 100 - 0.8)} = 84.2\%$$

Therefore 84% of the laser light is either scattered or absorbed in the first centimetre of bacterial suspension. It has to be remembered that the OD_{1064} measurement of 0.8 was against a reference of distilled water and so therefore the bacterial suspension absorbed or scattered 84% more laser light than water, and so the gradient between the surface of the *E. coli* suspension at 2 x 10^9 cells ml^{-1}, will also be much greater than water. The bacteria at the suspension surface would receive the full 100 W of laser light but the bacteria at the bottom of the 2 ml suspension, with a path length of 1.5 cm, would only receive 9 W. This helps to establish why such a large reduction in the amount of killing of *E. coli* at higher optical densities was observed. Concentrations up to 1 x 10^8 cells ml^{-1} had little effect on the temperature rise of the suspension. The OD_{1064} of an *E. coli* at 1 x 10^8 was around 0.04, thus only 8.8% of the Nd:YAG laser light was absorbed in one centimetre of suspension. The bacteria at the base of the 2 ml suspension volume would receive 87 W. It can therefore be concluded that the lack of light reaching the base of the suspension was the reason for the reduction in the lethal effect of Nd:YAG laser light on *E. coli* at high cell concentrations.

Raising the temperatures of the bacterial cultures with different heating sources gave a useful indication of the rapidity of the laser temperature rise in comparison to two other methods. The temperature reached in 23 sec (50°C) with laser light took over 1 min with the PCR machine and around 2.5 min in a waterbath at 50°C. The effect on the bacterial viability after heating the *E. coli* suspension with laser light was also greater than the other thermal sources. All of the bacteria in the vessel were killed by Nd:YAG laser light which amounted to a viable reduction of around 3 D-values. Even after 10 min in the waterbath at 50°C, a 50% reduction was the most observed. The fact that in the laser treated samples there was the presence of temperature gradients will no doubt affect the viability of the bacteria within the suspensions. In the centre, 0.3 cm below the surface of the liquid suspension the temperature was predicted to be at its maximum. Evaporation losses at the surface keep the surface slightly cooler. At the periphery at the base of the vessel, the temperature of the

suspension was coolest. This presents a multitude of problems in analysing the effects of the laser induced temperature on the bacterial suspensions. However at a temperature of 50°C, measured at a position 5 mm from the volume surface at the vessel wall, the Nd:YAG laser reduced the viability of a suspension of *E. coli* less than or equal to 1 x 10^8 cfu ml^{-1} by over 3 D-values.

As the penetration power of Nd:YAG laser light was dependent on the sample, with high transmission through glass, clear plastics, proteins, nucleic acids and water and low transmission through high concentrations of bacteria, it was logical to assess the effect of the laser light on *E. coli* suspended in opaque media. Lentil soup and milk were chosen for this study with possible laser sterilization applications and both suspensions were ideal for supporting bacteria. For easy pipetting the soup was diluted 2-fold. Bacterial survival was determined, after raising the temperature to 50°C, in both opaque suspensions and in PBS, with laser light. The results show that the laser light has a low penetrability to both soup and milk with only 80% reduction in viability, on average, in milk and 60% in soup. Reducing the depth of the suspensions, and therefore the path length through which the laser light has to penetrate, would produce further reductions in the bacterial viability after laser light exposure. Penetrability of laser light through opaque suspensions was very low.

The addition of ovalbumin to the suspension of *E. coli* at a range of concentrations was carried out initially as a control to experiments involving exposure of liquid suspensions. Reports suggest that the presence of organic material in suspending media act as inhibitors to heat sterilization of microorganisms (Fay, 1934). After bacterial growth in protein rich nutrient broth it would be foolish to assume that there would be no protein from the media, carried over into the resuspending fluid. It was prudent therefore, to examine the effect of protein on the sterilization efficiency of laser light. From the results it is clear that the presence of exogenous protein did not affect the reduction in viability of *E. coli* after exposure to Nd:YAG laser light.

A further consideration is the effect of the age of the bacterial cells on laser light sterilization efficiency. Indeed it is known that young bacteria tend to be more susceptible to heat and chemical disinfectants (Wilson and Miles, 1964). The initial

results presented in **Figures 4.33A** and **B** indicate that the bacterial susceptibility to laser light was influenced by the age of the cells. It appeared that older cells were more susceptible to the laser light. In **A** a laser induced temperature of ~67°C produced a reduction in viability of over three D-values compared to a reduction of eight D-values at 62°C with early stationary phase cells. It is also apparent that these two samples had been raised to different temperatures after application of the same ED of laser light. Due to this problem it was decided that an experiment designed to raise the temperature to a standard value irrespective of the energy density, with three further sampling times, would be more accurate. It can be seen that the first three samples were removed during the lag phase and the last sample at the beginning of the stationary phase of a static bacterial culture. It can be seen that the results show that the phase of bacterial growth does not appear to affect the susceptibility of *E. coli* to laser light. The results show that there were 3 D-values of viable cell reduction with *E. coli* at all phases of growth.

The first thing to note about the killing curves of all four bacteria in liquid is their similarity, except for *B. cereus* which showed a resistant faction of about 5×10^2 cfu ml^{-1}. This was most probably due to the presence of spores in the bacterial cultures, which showed resistance to the laser up to at least 70°C. The second thing to note is the fact that raising the temperature to 50°C did not reduce the bacterial numbers by more than one D-value. These are recent results and the exact reason for the apparent increase in resistance has not been elucidated, as yet. The most probable reason is due to a change in the geometry of the holder which supports the vessel. The new design encased the lower half of the vessel with aluminium securing it to an aluminium block. The aluminium block may act as a heat sink and more likely, the aluminium casing may be causing internal reflections both of which affect the interaction of laser light with the vessel contents. The original design however, allowed the vessel to stand freely with a securing ring at the top and the bottom. The securing ring at the top may also be an influencing factor. There have been no further alterations to the experimental set-up.

It was very difficult to compare these killing curves with standard killing curves as these show a plot of temperature, as opposed to exposure time, against the viable counts. Exposure time was plotted however this again was not comparable due to the presence of an increasing temperature component, in contrast to the normal constant temperature. The cell reduction was not first order with respect to the temperature or exposure time as there was a very long lag phase before killing begins at around 45°C. The rate of killing, after the lethal temperature is reached does appear to be first order and unlike many killing curves a tail of resistant organisms was not observed.

Bactericidal Mechanism

The bactericidal effect of Nd:YAG laser light has been known for over ten years, but the mechanism involved is not fully understood. Almost all of the published work concerns only the application of laser sterilization without an indication of the mechanism involved. There is, however, considerable analyses of the pathological mechanisms involved in laser-cell interactions in tissues reviewed by Thomsen (1991) who postulated three types of laser:cell interactions: photochemical, photothermal and photomechanical.

The bactericidal mechanism of Nd:YAG laser light does not appear to be a photochemical process, which relies on the absorption of the laser light by an intracellular phytochrome, initiating chemical reactions and causing cell death from free radical or other toxic by-product formation, known as photosensitisation. The important factor involved in a photochemical mechanism is the energy density applied. When an *E. coli* suspension was reduced to 0°C and the same lethal energy density used in experiments at 50°C, was applied, there was little or no reduction in viable counts. If the mechanism was photochemical, the applied energy density should have a similar effect, irrespective of the temperature reached. In the experiment where a similar energy density of laser light was applied to a lawn of *E. coli* over two different exposure times there was a marked difference in the lethal effect. Little or no bactericidal action was observed with the longer exposure time. This therefore suggests that the accumulation of toxic products does not take place

and therefore the exposure time is an important consideration with respect to the energy density.

The results of this investigation indicate that bactericidal mechanism of Nd:YAG laser light is most likely to be predominately photothermal. Photothermal effects can be further categorised into: bulk temperature, peak temperature and intracellular temperature. An *E. coli* cell will withstand 50°C for a relatively long period; one D-value being approximately 20 min. In the 25 sec to reach 50°C with laser light, a reduction of over 3 D-values was observed. Results of waterbath heat treatments showed increased viable counts when samples were removed immediately. This was possibly due to the cells approaching the end of a cell division cycle. The large reduction after exposure to laser light, however, can not be accounted for by a bulk temperature rise to 50°C. The thermal diffusion equation was solved by Wang *et al.*(1997) to find the temperature distribution of the suspension during Nd:YAG laser exposure. The temperature gradients that exist in the vessel were found and are shown in **Figures 4.25** and **4.26**. The fact that there were temperature differentials within the vessel makes the analysis of the effects of the temperature more complex.

The peak, or instantaneous temperature, is the temperature that the culture reaches during the 8 msec pulse. After each pulse there was a 92 msec gap before the next pulse. During this period there would be some dissipation of heat throughout the suspension volume. The overall peak temperature reached by the *E. coli* suspension after each pulse are not fully elucidated, but calculations show that the peak temperatures are not very large, and again will vary spatially. Temperature fluctuations caused by pulsing however, may adversely effect the *E. coli* cells in the suspension.

The third possible effect is the rise in temperature of the internal environment of the bacterial cell. If a photon of light was to enter the bacterial cell at a particular angle there could be total internal reflections from the inside of the cell membrane producing abnormally high intracellular temperatures through an increased absorption. In addition, a small percentage of water inside a bacterial cell is chemically bound and the degree of binding may affect the absorption coefficient of

water in the bacterial cell. Little information on the microscopic optical properties of bacteria exists, producing further complications when analysing the lethal mechanism. However, the DSC results tend to refute the theory of the internal temperature rise. The scans in **Figures 4.56** and **4.57** show that there is a peak at 60°C for both of the control samples and the samples with the 50°C laser and waterbath pre-treatment. Mackey *et al.* (1991) showed that the DSC scan of a bacterial suspension gave a number of peaks due to the sublimation of bacterial components. The report showed that at around 60°C the ribosomal sub-units in *E. coli* cells sublime irreversibly so that on a re-scan, when the initial scanning temperature was taken beyond 60°C, the endothermic peak disappeared. If the internal components of the *E. coli* cells, after laser light exposure to 50°C, were heated to over 60°C then there would be little or no peak. The fact that there was a peak at 60°C suggests that the internal temperature was lower than 60°C and therefore probably has little or no effect on the bactericidal action.

A fourth possibility could be the rate of the temperature rise with Nd:YAG laser light. Although this rate of temperature rise was not matched, the PCR machine increased the temperature to 50°C in 1 min. This, however, still did not equal the rapidity of the laser, and was not pulsed like the laser, and hence the possible reason for the difference in effect.

After laser exposure to temperatures of 40°, 50°, 60° and 70°C, SEM analysis revealed that the laser caused extensive surface damage to the Gram-negative cell wall. There was also evidence of extracellular RNA after laser exposure to 50° and above (**Table 4.3**). This has a number of implications which could help to identify the bactericidal mechanism. It is known that at around 45°C the cytoplasmic membrane starts melting (Mackey *et al.*, 1991). Moreover, higher incubation temperatures weakened the cell walls of *E. coli* (Hoffman *et al.*, 1966) and Hagan *et. al* (1964) showed that death preceded lysis. With a weakened membrane, leakage of intracellular components from the cell would be expected. With the increase in temperature RNA leakage increased and was another possible reason for cell death. The SEM pictures indicated that at the higher energy densities the cells were

damaged to the extent that "blebbing" was observed on the cell surface. This is comparable to the effect of penicillin on the Gram-positive cells which is therefore consistent with cell wall and membrane damage and the increase in the intracellular pressure whether by osmosis, or the rapid increase in temperature. This plasmolysis was not observed with the waterbath temperature rises to 60°, 70° or 100°C. In these cases the opposite, plasmopthesis, occurred. The cells seemed to shrink as the cell wall disintegrated. In the laser treated samples there was also evidence of cellular debris scattered throughout the 60° and 70°C laser-treated samples, consistent with ruptured cells. There is also a reduction in the optical densities of the 50°, 60° and 70°C laser-treated samples. Thomsen (1991) observed an effect in tissue caused by the production of vacuoles of super-heated steam. As exposure continued the vacuoles enlarged and eventually ruptured explosively; termed the "popcorn" effect. With the laser-bacteria interaction the weakened cell wall due to the temperature increase would be more susceptible to an increase in internal pressure making it more likely to rupture.

Photomechanical stresses occur through the rapid delivery of laser energy via high intensity laser pulses (generally less than 10^{-6} sec). This laser energy leads to rapid heating and expansion of the target, causing ablation. Secondary to the stresses of explosion expulsion of the ablation products will be the stresses associated with elastic recoil (Thomsen, 1991). In this investigation the pulse widths were not particularly short and therefore the peak powers were relatively low. Results in section 4.7 showed that nanosecond pulses of Nd:YAG laser light, which produced peak powers in the order of megawatts, had no visible effect on a lawn of *E. coli* spread on nutrient agar plates. However the results shown in the SEM micrographs indicate that the biocidal effects of the Nd:YAG laser on *E. coli* were not explained by a thermal mechanism. The possibility exists that the effect on *E. coli* cells, observed in this investigation, were the result of combination of photothermal effects and photomechanical stresses, even though photomechanical stresses were reported as the result of short, high intensity pulses ($<10^{-6}$ sec). The micrographs of *St. aureus* and *L. monocytogenes* show that these explosive effects were confined to *E. coli*. The G +ve wall of the other two bacterial species appeared to be resistant to the mechanical disruption observed after exposure to Nd:YAG laser light. In none of the

SEM micrographs was there any change in the cell shape or size after exposure to laser light. It appears that the nature of the cell wall was the factor involved in the photomechanical effects observed after exposure of the *E. coli* to the Nd:YAG laser, however the killing curves for each bacterial species were shown to be similar. An interesting idea would be to observe the effects of the laser on the cell interior with transmission electron microscopy. In the time permissible for this project the use of the DSC was not able to be applied to either *St. aureus* or *L. monocytogenes*.

Differential scanning calorimetry (DSC) of material detects endothermic and exothermic processes within the samples after heat-treatment and records them as a time-temperature sequence. With bacteria these include the denaturation of membranes, proteins and DNA. DSC was used on bacterial cells for the further elucidation of the effects of raised temperatures on cellular components (Miles *et. al* 1986; Mackey *et. al* 1991). The results revealed the major events which occurred in heat-treated cells however, the authors stressed that the events that they described were their own analysis and are likely to be more complex. From 20 to 40°C there was an endothermic peak probably relating to a conformational change in the membrane lipids, caused by melting, and possible conformational changes in the cell wall peptidoglycan. At around 47°C the first denaturation process took place which was thought to be due to the melting of the 30S ribosomal subunit. Between 47 and 85°C there are three endothermic peaks. These are also thought to be further associated with the melting of ribosomes. The subunits S30, S50 and S70 all melt sequentially but overlap. It was during these peaks that Stephens and Jones (1993) showed that the death of the cell takes place. This depended upon the duration of the exposure to the lethal temperature. Ribosomal melting involved denaturation and/or loss of ribosomal protein, denaturation and/or loss of ribosomal RNA, loss of the higher order structure and melting of the helical regions of rRNA. Between 75 and 76°C an endothermic peak was found and attributed to tRNA denaturation. At 95°C melting of a portion of the cellular DNA occurred along with an unknown portion of the cell wall (thought to be linked with protein). At 105°C further melting of the cellular DNA occurred as did more of the unknown cell-wall material. Finally,

between 118 and 125°C denaturation of thermostable envelope associated proteins were thought to occur as well as the possible denaturation of nucleic acids.

The peak of interest in this investigation was the suspected ribosomal-melting peak at 60°C. This peak was shown to be irreversible after a bacterial sample was heated beyond 60°C and then re-scanned to reveal little or no peak (Mackey *et al.* 1991). This was therefore a valuable indicator of whether the contents of the bacterial cells were raised to temperatures beyond 60°C. In direct comparison with the DSC scans obtained be Mackey *et al.* (1991) the peaks were not as clear, but after personal communication with Dr Bernard Mackey it was revealed that the differential scanning calorimeter in Glasgow University Chemistry Department was more sensitive. However, the scans showed that the 60°C peak was visible and could therefore be used. The DSC scans of *E. coli* in **Figure 4.53** and **4.54** showed differing responses to both laser light and waterbath pre-treatments when the temperature was raised to 50°C. For the laser pre-treatment the scan shape was similar to the control, apart from the shift in the temperature at the maximum exothermic activity. This exothermic activity which increased between 20 and around 45°C in all of the scans was probably due to enzymatic and other metabolic activity within the bacterial cell. These cells were degassed and in a nitrogen environment with no exogenous metabolites, therefore all of the activity seen in the 50°C laser pre-treatment samples and controls appear to be through metabolism of endogenous supplies (Dawes and Ribbon, 1964).

The 50°C waterbath pre-treatment scan showed that there was no exothermic activity in the bacterial suspensions. At 50°C, 5 min in a waterbath had little effect on the viability of the *E. coli* cells. The lack of activity observed in the DSC thermogram has, however, been attributed to the utilisation of the endogenous metabolites in the 5 min of pre-heating at 50°C. Therefore, on the run up to 45°C in the DSC, the bacteria utilised their endogenous metabolite supply and little or no exothermic activity was seen.

Microcolonies were observed with laser-exposed cells in liquid culture, as reported with the lawned bacteria, indicating sub-lethal damage. The microcolonies associated with the bacterial lawns were thought to contain auxotrophic organisms. If this is the case then the lethal mechanism of Nd:YAG laser light on bacteria becomes more complex. During the log phase bacterial cells replicate at a maximum rate under optimum conditions, thus the *E. coli* DNA strand fully replicates every 20 min. It would only take one molecule of water bound to the DNA molecule to absorb some of the laser energy during the replication procedure causing excitation of the water molecules which may subsequently introduce a mismatch or break in the DNA strand affecting the growth of the bacterial species.

The investigation shows that there is a thermal requirement for Nd:YAG laser sterilization but also shows that an alternative mechanism exists. The most likely secondary effect is a photomechanical one but further investigation is required.

5.2 MICROBIAL STERILIZATION ON SOLID SURFACES

5.2.1 Nd:YAG LASER

A number of parameters are potentially influential towards the bactericidal capacity of laser light, as shown in **Figure 2.1**, therefore to reduce the time involved in the study of these parameters, multifactorially-designed experiments were done. The limits enforced by bacterial tolerance to both laser and environmental parameters were variable and very narrow which complicated the experimental procedure; the combination of these parameters enhanced the problem. In advance of the multifactorial experiments however, killing curves for the range of four bacteria, on the surfaces were determined, with and without exogenous liquid.

Initially the effect of the drying procedure on different bacterial species on surfaces was investigated. The idea was to find a procedure which caused as little reduction in viability as possible. The most successful method was drying the disks in a laminar air flow cabinet, which took a little over 20 minutes. Pre-treatment of the disks by drying 0.1% w/v bovine serum albumin on to the surface appeared to aid the

survival of the bacterial cells but was introduced initially for the purpose of evenly distributing bacterial cells as they dried and containing suspensions within a small area in the centre of the disk.

It was found that the vegetative cells of *B. cereus* were susceptible to the drying procedure and therefore could not be used in the experiments which required dried cultures. *St. aureus* was the most resistant to the drying procedure which was not surprising as the organism is often found on the surface of skin where it is exposed to continual drying procedures. The disks material had no effect on the viability of cells during the drying procedure.

The exposure set-up for the disks was for the purpose of mimicking a larger surface area with a smaller, removable and recyclable part for experimentation. The set-up had to have a larger mass than a disk alone to act as a heat sink, as would be present in applied situations, however the small disk was required for ease of removing viable organisms for analysis. For exposures on glass, it was decided that the desired effect would be obtained with perspex which, like glass, is transparent to Nd:YAG laser light, and both are thermal insulators.

The killing curves on the stainless steel surface suggest that, with exogenous moisture, *St. aureus* was the most resistant to the laser beam, with over double the energy density of laser light required to sterilize *B. cereus* and *E. coli*. These three bacterial species appear to be similar to a first order killing curve. They showed no evidence of a lag phase and any tail of resistant species was not determined. The non-uniformity shown in the curves were the result of each individual exposure taking place on separate disks where slight variations within each disk, such as tarnished surfaces leading to higher light absorption, will produce slight errors during exposure. The shape of the curve for *L. monocytogenes* was however, unusual with a slow reduction in viability to 400 Jcm^{-2} followed by a sudden drop at 480 Jcm^{-2} to almost zero. These results were duplicate experiments and this phenomenon was observed in both cases. All of the disks inoculated with a wet film were observed to dry after around 400 Jcm^{-2} and this may provide one explanation for the observed effect on *L. monocytogenes* and combined with laser light, such that the sudden drop

in viability occurred once the film dried. A second possibility was the effect of the presence of the coccoidal *L. monocytogenes* in the sample, which may be slightly more resistant than the rod-shaped organisms. These results suggest that the inoculum level and the bacterial species, and their tolerance to rapid drying, were the major influential factors in the bactericidal action of Nd:YAG laser light.

In direct contrast to the killing curves for the wet films of bacteria, the dry films were relatively straight forward. All three of the bacterial species had similar shaped killing curves. These results suggested that the major parameter affecting the killing of the different bacterial species dried on the stainless steel surface was the initial inoculum concentration. This therefore reinforces the theory that the effect of drying of the liquid suspensions on the stainless steel disks was an important part of the mechanism of killing on surfaces. *St. aureus* which was the most tolerant to drying was least affected by the laser light when associated with exogenous moisture. As dried suspensions, the greater tolerance of *St. aureus* was not apparent, and the differences between the bacterial species was the result of the size of the initial inoculum.

The surface material was very influential in the ability of Nd:YAG laser light to reduce the viability of *St. aureus* as dried samples. Little effect on *St. aureus* was observed after exposure to Nd:YAG laser light when dried onto plastic or glass surfaces prior to surface damage; only stainless steel was sterilized by Nd:YAG laser light. The most likely reason for this was that the stainless steel conducted the heat away from the surface more rapidly than the two insulating surfaces reducing the damage to the surface and additionally the reflectivity of the surfaces will vary and therefore so will the rates of heating. The fact that Nd:YAG laser light was only effective on stainless steel resulted in the use of stainless steel as the surface for multifactorially-designed experiments with this laser.

The design of the multifactorial experiments was difficult to keep constant due to the use of different metal disks for each exposure and very narrow limits of tolerance of each bacterial species to a number of hostile conditions, with the result that in many of the experiments the errors were relatively large. The initial type of experimental

procedure involved the analysis of the effect of different Nd:YAG laser parameters: pulse energy, PRF and exposure time. The values for the exposure times were varied between the different bacterial species in all of the experiments and *St. aureus* required the longest which was consistent with the results shown in **Figures 4.58** and **4.59**. Due to the fact that there were a large number of results obtained in the different multifactorial experiments they were grouped and compared. As a dried sample the effect of Nd:YAG laser light was examined on *St. aureus, E. coli* and *L. monocytogenes* with each of the three different laser parameters. The reduction in viability of all three bacterial species was affected by variation of the individual different laser parameters, most especially in the graphs with at least one high parameter value however, only *St. aureus* showed consistent statistical significance, within 5% probability, for all laser parameters. In addition, interactions between the parameters with at least one lower parameter value were significant for *St. aureus.* The parameter with the greatest significance on the effect of laser light with all three organisms was the exposure time. Increases in the parameter values for Nd:YAG laser light exposure of *E. coli* were not significant but the large errors in these experiments, the highest for all three organisms, was concluded to be the major reason. It appears strange that the effect of increasing the different laser parameters in some cases did not increase the killing of the *E. coli* or *L. monocytogenes* but the reason could be attributed to the use of different surfaces per exposure or the narrow values utilised to obtain a range of bacterial viabilities.

As a wet film there were four bacterial species examined and the results showed that the introduction of liquid to the system changed the outcome of the results. This was observed in previous killing curves of bacteria as wet samples where *St. aureus* was the most difficult to reduce in viability. Once again it was easier to kill *St. aureus* as a dried sample than as a wet sample however, the opposite was observed the *E. coli* and *L. monocytogenes.* As a wet sample the majority of the individual parameters had significant effects on the bactericidal efficiency of Nd:YAG laser light on each bacterial species. *B. cereus* and *E. coli* showed that an increase in the value of each parameter produced an increase in the probability of significant interactions. Most of the parameters, individually, had significant and positive effects on the reduction of viability of each the bacterial species. Therefore, it does appear that the introduction

of moisture into the system enhances the ability of Nd:YAG laser light to sterilize surfaces and in some situations, increased input from interactions between the parameters was observed.

The final group of experiments involved the introduction of the growth parameters, i.e. NaCl concentration and the pH. From all of the figures there was little evidence of enhancing effects supplied by the introduction of NaCl or HCl/NaOH and only the presence or absence of water appeared to have any affect on the reduction in viability of *St. aureus* and to a slightly lesser extent *E. coli*. In fact in one of the figures (**Figure 4.68 D**) for *St. aureus* the effect of the water content was statistically significant. It would be safe to conclude that, if the NaCl concentration or pH had any effect on the bactericidal ability of Nd:YAG laser light, it would be minimal.

As the 2^N multifactorial analysis requires only two values for each parameter there is the assumption that linearity exists between the two values but this is not always the case. For this reason, parameters found to have an effect on the system would therefore have to be further examined. For the continuation of this system of analysis it was convenient to further examine the effects of NaCl concentration and the pH with a range of values for each. Even though these parameters had little apparent effect on the bactericidal effect of Nd:YAG laser light it was worthwhile testing the results obtained in the multifactorial experiments. *St. aureus* was used in this investigation as it had the highest tolerance to the drying procedure and NaCl concentrations (a larger range of NaCl concentrations was possible). The results of the investigation showed that the killing curves of *St. aureus* at each NaCl concentration and pH were very similar. The shape of the killing curves for the NaCl concentrations and the pH values were not first order as there was evidence of an increased rate of killing as the energy density was increased. This was clearer when a log-linear fit was plotted through the results of the highest and lowest NaCl concentrations. The log-linear best fits through the plots of the pH values appeared a little more accurate.

An alternative method to compare the bacterial killing curves was sought and log-logistic analysis was attempted. Although the curves were not essentially shaped to

fit a log-logistic function, with no tail of resistant organisms observed at the end of the curve, there was a slight lag at the beginning of the killing curves. It is however, obvious that the fits to the log-logistic function were not ideal; the rate of killing of the bacterial samples increased as the energy density was increased. Unlike the work of Cole *et al* (1993) these experiments did not have a constant temperature in the system but an increasing one and therefore a fit was required which would take account of the resultant increase in the rate of killing.

The third type of fit investigated was a quadratic function of the form $y = ax^2 + bx + c$ which provided an increasing gradient and therefore an increasing killing rate. The value for the rate of killing can be calculated by differentiating the quadratic which gives a value for comparison of different curves. This quadratic best-fit was the most accurate. The future possibilities from this discovery is the production of predictive models to anticipate the effect of each parameter under investigation on the reduction in bacterial viability with laser light. The quadratic function should be applicable to most laser sources and situations because of the nature of laser light, but much experimental work is still required to test these hypotheses.

5.2.2 CO_2 LASER

As the CO_2 laser wavelength does not penetrate aqueous solutions it was only utilised in experiments on sterilization of bacteria on surfaces. These experiments were initially attempted on both wet and dry films of bacteria on different surfaces but the excess liquid was splashed over the surface outside the laser beam when the sample was exposed to laser light. This caused contamination problems over the surrounding areas and was therefore not a viable option for investigation. With dry films of *St. aureus*, the results showed that the surface material, as with the Nd:YAG laser light, affected the efficiency of sterilization with CO_2 laser light. However, unlike Nd:YAG laser light the CO_2 laser could sterilize all three surfaces. Indeed, light from the CO_2 laser was able to sterilize all of the surfaces within 150 Jcm^{-2} which corresponds to a 200 msec exposure time. These results suggested that the two insulating surfaces, glass and plastic, were sterilized more readily than the

stainless steel, which is the opposite case for Nd:YAG laser. The glass surface was sterilized in 40 msec and the nylon in 6 msec. There is a large difference in the rate of microbial destruction on the three different surfaces and by the two different lasers. It was suggested this was once again due to the absorption, reflection and conduction properties of each surface at 10.6 μm.

The numbers of viable bacteria, *E. coli*, *L. monocytogenes* and *St. aureus*, in the inoculum dried onto nylon plastic disks, were all reduced at the same rate. The only difference between each of the bacterial species appeared to be the initial inoculum level. *St. aureus* with the highest tolerance to drying and therefore the highest bioburden appeared to be the most difficult to kill. Indeed, this phenomenon was seen with the use of Nd:YAG laser light on the three bacterial species dried onto stainless steel surfaces.

The natural progression from here would be to continue with multifactorial experiments to investigate the effects of different parameters which potentially affect the sterilization efficiency of CO_2 laser light. The number of parameters was less than the Nd:YAG laser as the beam was continuous and therefore there was not a PRF or pulse energy to consider. The energy delivered was constant and therefore the exposure time was the only variable factor of the three. The water content presented a complex situation and may cause problems by aerosolising bacteria if excess liquid is present on the surfaces during exposure. Other conditions have been tried and will be reported in the final year MAFF report. These conditions include the NaCl concentration and the pH.

5.3 LASER STERILIZATION ON COLLAGEN FILM

5.3.1 Nd:YAG LASER

The results show that the Nd:YAG laser was capable of killing *St. aureus* on the surface of collagen film. *St. aureus* being the most resistant of the four species on surfaces to the laser and to the drying procedure was used in these experiments. The exposure time required by Nd:YAG laser light, to sterilize a $1cm^2$ area on surface of

the collagen film, was 20 sec: this was excessive and not likely to be applicable to sterilization systems. The most likely reason for the lengthy exposure times was that much of the light was transmitted through the film and the bacteria because of a low level of absorption. However, it is worth noting that the collagen was not affected by the laser light within the exposure time required to sterilize the 1 cm^2 circular area of *St. aureus* on the collagen surface.

5.3.2 CO_2 LASER

This laser was more efficient than the Nd:YAG laser in the sterilization of the surface of the collagen. The CO_2 laser used was 3 times more powerful than the Nd:YAG laser delivering ~1 kW of light at 10,600 nm but sterilized the surface 10,000 times faster than Nd:YAG laser light. After 1 msec at 800 W and 4 msec at 500 W clear areas were seen in the lawn of bacteria on the collagen surface. The majority of CO_2 laser light was absorbed by the collagen and so it was interesting to note that the laser sterilized *St. aureus* on the surface when exposed through the collagen in a matter of only 3 msec. The fact that the laser can kill *St. aureus* through the collagen film with only a slight reduction in efficiency indicates a possible combination of two effects: a direct interaction between the laser and the bacteria, and the an effect on either the collagen, the perspex base supporting the collagen or both. After exposure of *St. aureus* on collagen, laid on different surfaces and with no backing, it was found that the bactericidal ability of CO_2 laser light was unaffected. Over the duration of this project Talebzadeh *et al.* (1994) investigated the effect of the CO_2 laser on mono-layers of bacterial cells: *E. coli*, *Streptococcus mutans* and *Bacillus stearothermophilus* on the surface of a collagen membrane filter. The filter was used for the purpose of minimising extraneous water. The cells were exposed to power densities of between 0 and 600 Wcm^{-2} (power = 0 to 8.0 Watts, beam diameter = 1.3 mm) delivered over a 0.1 second pulse, equivalent to 0 to 60 Jcm^{-2}.

From these results, shown in **Table 5.1** the author concluded that the CO_2 laser was bactericidal and that there was no statistical difference in the susceptibility of the three different bacterial species tested. For commercial reason the characteristics of

the collagen film used in this thesis were not divulged and therefore direct comparisons between the results detailed in this thesis and those in the publication described was difficult, however it was interesting to note that the differences between the different species examined were negligible, even though *B. stearothermophilus* was thermophilic. One other notable comparison was the fact that Talebzadeh *et al.* (1994) required 15-30 Jcm^{-2} to have any effect on the bacteria on the collagen, compared to 0.8 Jcm^{-2} observed in the investigation detailed in this thesis. This difference is very large and may be put down to the nature of the collagen, the beam diameters of the lasers, the power of the laser, the exposure time or any interactions between these.

Energy Density	Percentage surviving cells compared to control		
Jcm^{-2}	*B. stearotherm*	*Strep. mutans*	*E. coli*
0	100	100	100
4.2	100	150	58
15.0	17	26	11
30.0	6.1	27	1.9
45.0	3.4	7.5	1.7
60.0	1.2	3.5	0.28

Table 5.1 **Average percentage of cell colony counts after CO_2 laser exposure compared to a control** modified from Talebzadeh *et al.* (1994)

5.4 BACTERICIDAL EFFECTS AT DIFFERENT WAVELENGTHS

Of the lasers tested, significant ability to kill *Escherichia coli* was observed with light from the CO_2 (600 W), Minilite and Surelite frequency-tripled Nd:YAG (1 and 0.04 W) and Lumonics Nd:YAG (200 W) lasers. Growth inhibition was observed after exposure to laser radiation at 355 nm. No bactericidal capacity was observed with the FIR device, Q-switched lasers operating at 1.06 μm and 532 nm, the argon ion laser, or the laser diode array.

The results suggest that the most effective laser source for the sterilization of *E. coli* lawned on agar plates was the 10.6 μm light delivered by the CO_2 laser. This instrument, operating with a beam area of 2.3 cm^2, was able to sterilize a circular 1.21 cm^2 area of *E. coli* lawn in 30 msec, an energy density of 7.8 Jcm^{-2}. The sterilization mechanism will differ for lasers operating in the UV and IR. Interestingly, sterilization was not observed for the Q-switched lasers operating at 1.06 and 0.532 μm, where the intensities were above the damage threshold of the plastic petri-dishes but below that required to kill the bacteria. Because high peak powers of light at 1064 nm showed no bactericidal effect and high mean powers at the same wavelength did, it is apparent that the sterilization mechanism at this wavelength was thermally dominated, as was discussed previously. At 355 nm a single pulse of 4 nsec killed *E. coli* within the lawn. No publications have been found which describe laser sterilization of microorganisms at 355 nm however, the observed sterilization may be due to fluorescence occurring at wavelengths within the abiotic range of wavelengths, 240 to 300 nm, which may be a result of a bi-photonic process; this hypothesis was not further elucidated.

The rate of exposure from the frequency-tripled Nd:YAG lasers was limited because the highest pulse repetition frequency was only 10 Hz. In practice, the 3 s exposure was the result of 30 pulses, each 5 nsec long, totalling an exposure time of 150 nsec. This represents an extremely fast and efficient sterilization system which may have useful implications for sterilization practices in general, providing the frequencies

can be increased. The argon ion laser at comparable energy densities to both the CO_2 and Nd:YAG lasers, was not lethal to the lawn of *E. coli*.

This investigation was in a small sector of two large fields of current research, namely investigation of novel methods of microbial sterilization and the development of new laser applications. Although laser light as an antimicrobial agent has been has been known for over 34 years it has not yet been implemented in this capacity. To date there are a number of laser sources available which produce light at a range of wavelengths from X-rays to microwaves. Of the more common lasers utilised today, three were investigated in this thesis, namely the argon ion, Nd:YAG and CO_2 lasers.

It can be seen that the most influential laser parameter in the sterilization of microorganisms is the wavelength. This is an important consideration, with regard to penetration into and absorption of laser light by suspending media and/or microbial species, when the material to be decontaminated is investigated. In this investigation only Nd:YAG laser light was studied in liquids however other lasers may be more effective. CO_2 laser light could not penetrate liquids but was found to be effective on surfaces, in particular insulating surfaces, such as plastics and glass. On plastics it was shown that CO_2 laser could sterilize approximately 1.0 cm^2 in around 5 msec. In comparison to many conventional techniques this is extremely rapid and the fact that the beam is directional equipment can be sterilized in place, therefore saving time in the dismantling procedure.

Laser sterilization has therefore much potential as an antimicrobial agent but the biggest hurdle which has to be overcome before industry will be convinced of the benefits is the cost of acquiring, operating and maintaining a laser. The cost of laser systems is continually reducing, as is the physical size of the laser, whilst maintaining the same output power levels. With these advances, and the apparent increase in scientific awareness of the benefits of laser sterilization, the development of operational systems may have future potential.

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7. APPENDICES

7.1 APPENDIX 1

7.1.1 LASER CONSTRUCTION

The laser is constructed of three main sections as detailed in **Figure 7.1**. The laser input energy is supplied by a variety of means; the most common is the flash lamp. The flash lamp, or lamps depending on the laser medium, supply photonic energy to the active centre which initiates laser action. This principle is termed optical pumping, Other methods of pumping include magnetic fields and electric pulses. (Thorp, 1967)

7.1.2 THE ACTIVE CENTRE OF A LASER

In order for a laser to operate the system must satisfy the following three conditions (O'Shea *et al.*, 1977):-

(i) There must be an active medium containing photo-reactive molecules, e.g. the ruby rod containing Cr^{2+} ions. The medium can be gaseous, liquid or crystalline.

(ii) The condition known as a population inversion must exist in the medium. This is an abnormal condition and relies upon the excitation process of optical pumping.

(iii) There must be an optical feedback system associated with the active medium.

7.1.2.1 Quantum mechanics of light amplification within the active centre

The theory behind laser operation is fundamentally governed by the following facts:-

(i) All atoms and molecules have individual energy levels relating to their specific motion at any one time.

(ii) At any one time an electron will occupy an energy level whether it be ground state (lowest energy state of an electron) or an excited state (a higher state, generally short lived).

(iii) In a solid, liquid or a high pressure gas the atoms or molecules constantly collide with one another causing each other to "jump" from one energy level to another.

(iv) Often a particle will change energy level by a transition involving the absorption or emission of a photon according to the resonance condition, $\Delta E = h\nu$ where ΔE is the change in energy after absorption or emission of a photon, h is Planck's constant and ν is the frequency of electromagnetic light (Einstein, 1917). The emission of photons from an atom or molecule is the result of one of two emission processes, first described by Einstein in 1917.

Spontaneous emission

The relaxation of an electron, from a higher excited state to a lower one, causes the spontaneous emission of a photon with conservation of the total energy. In laser technology the spontaneously emitted photon will have a random phase, propagation, direction and polarisation, but often, if not nearly always, it will initiate stimulated emission.

Stimulated emission

A photon of resonance frequency, $h\nu$, perturbs the excited species causing it to relax from its higher energy level and fall to a lower one, emitting a photon of exactly the same frequency and phase, travelling in the same direction. This causes the amplification of the input radiation, and is the type of emission central to the functioning of a laser.

7.1.2.2 Absorption and spontaneous and stimulated emission: Simple kinetics

With a two energy level system, a simplified model of the quantum procedure in a laser can be described:-

1.....lower energy level
2.....upper energy level
A_{21}.....Einstein coefficient for the spontaneous emission probability
B_{12}.....Einstein coefficient for the absorption probability
B_{21}.....Einstein coefficient for the stimulated emission probability
N_1 & N_2.....Populations of the respective energy levels.

Under normal thermodynamic conditions, $N_1 > N_2$. As a resonant photon passes through a volume of these particles with two energy levels, they may interact with one in level 1. In this situation the photon will be annihilated in an absorption process leaving the particle in the excited position of energy level 2. ***Probability*** = $B_{12} \times N_1$

This same resonant photon could alternatively pass through this volume of particles and interact with one in energy level 2. Here, the result is the return of an electron to the lower level generating a second photon with identical scalar and vector properties, to the incident photon. ***Probability*** = $B_{21} \times N_2$

Unfortunately N_2 is short-lived and spontaneous decay takes place, before interaction with a photon. This results in the emission of a photon in any direction, but more often than not causes stimulated emission elsewhere. Spontaneous emission depletes N_2 at a rate proportional to A_{21}. Therefore it can be seen that before we can have any possible amplification N_2 has to be greater than N_1. This situation is called a population inversion. Because of losses, like spontaneous emission, associated with lasers there has to be a minimum value of N_2 - N_1. This is called the threshold inversion (Thyagarajan and Ghatak, 1981).

In a two level system an enormous amount of energy is required to excite the majority of electrons into the upper state. These pumping powers would be prohibitive to the functioning of the laser. As two level systems are not found in laser media, this does not present a problem. To explain population inversion the presence of a third energy level is necessary, as shown in **Figure 7.2**. Electrons in some of the particles are excited by an energy source (e.g. a lamp) from level 1 to 3. Level 3 is metastable (i.e. long-lived), which allows the active excited medium to store up energy. Naturally, there are few electrons in energy level 2 as they are normally in level 1. Thus a population inversion exists between energy levels 3 and 2. If a photon of frequency $(E_3 - E_2)/h$, where E is the energy for the levels and h is planck's constant, passes through this medium of particles then stimulated

emission results. Electrons can decay from level 2 to level 1 by spontaneous emission or non-radiative transitions (Lamb and Rutherford, 1950).

7.1.3 RESONANT CAVITY

An oscillator of any sort (a laser falls into this category) has to have two distinct parts:-

1. An amplifier. (Already discussed in section 7.1.2)
2. Positive feed-back (from an optical resonator in the case of a laser).

7.1.3.1 Positive Feedback

The sustenance of laser action requires a volume of specific excited material in an optical cavity or resonator; the most common resonator system being between two mirrors (Thyagarajan and Ghatak, 1981). A feed-back system between two plane mirrors is called a Fabry-Perot cavity, where one of the mirrors is partially transparent and partially reflective (O'Shea *et al.*, 1977). The released photons are continually reflected through the lasing medium, greatly increasing the probability of stimulated emission. The type of material used to make a mirror is dependent on the wavelength of the laser. The simplest resonator configuration is the planar system (**Figure 7.3A**), consisting of two completely flat mirrors aligned on either side of the lasing medium. The problem with this is that they must be perfectly aligned and flat. With a confocal design (**Figure 7.3B**) the mirrors are slightly concave to the extent that the radius of curvature of the mirrors equals the distance between them. The mirrors of the concentric system (**Figure 7.3C**) are much more concave in shape such that the mirror's radius of curvature equals half of the distance between the mirrors. Often a combination of planar and confocal are used. One such system is the hemispherical resonator.

The gain (or amplification) per pass between the mirrors varies immensely. For example, a HeNe (Helium Neon) laser has around 10% gain per pass and so 99% (1% transparent) and 99.9% reflective mirrors can be used. In direct contrast, high gain lasers in theory do not actually need mirrors. Also lasers that produce multiple

wavelengths require optimal mirrors or intra-cavity gratings to produce a single wavelength output.

Figure 7.1 **Diagram of the basic construct of a laser comprised of three main sections**

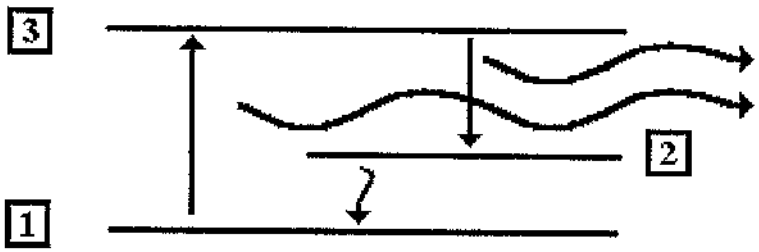


Figure 7.2. **A simple illustration of a three energy level system to explain population inversion.** 1. Ground state, 2. Lower energy level and 3. Upper energy level

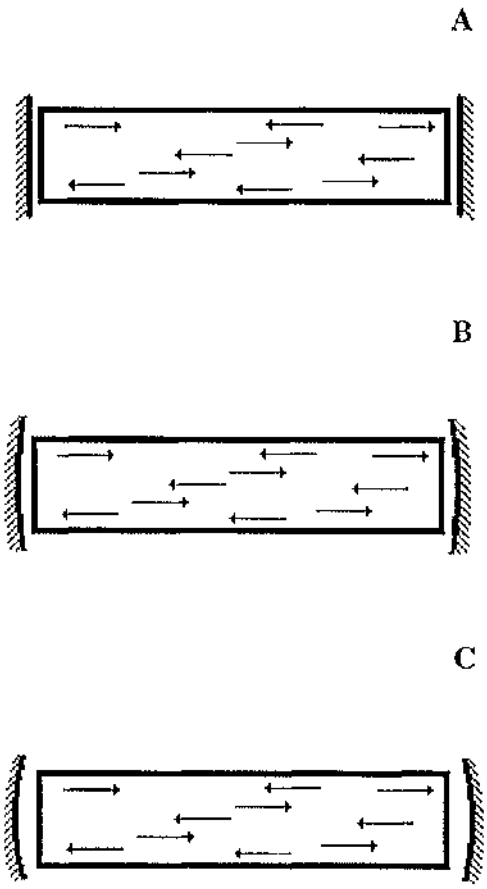


Figure 7.3 **Simple diagrams to illustrate laser resonant cavities with three different mirror configurations: A** Planar, **B** Confocal, **C** Concentric. The arrows indicate the passage of light between the mirrors and the box is the active medium

7.2 APPENDIX 2

Separating Gel (12 %)

30% Acrylamide / Bis	6 ml
Distilled Water	4.475 ml
1.5M Tris (pH 8)	3.75 ml
20% Sodium dodecyl sulphate	75 µl
TEMED	7.5µl
10% Ammonium persulphate	150 µl

Stacking Gel

30% Acrylamide / Bis	2 ml
Distilled Water	5.345 ml
0.5M Tris (pH 6.8)	2.5 ml
TEMED	5µl
10% Ammonium persulphate	100 µl

5 x Sample Buffer

Glycerol	5 ml
20% SDS	2.5 ml
2-mercaptoethanol	0.5 ml
0.5M Tris (pH 6.8)	2.5 ml

Add a few grains of bromophenol blue

10 x Electrode Buffer (500 ml)

Tris	15 g
Glycine	72 g
SDS	5 g

make to pH 8

Stain

Methanol	80 ml
Glacial acetic acid	20 ml
Coomassie blue	0.1 g
Distilled water	100 ml

Destain

Methanol	200 ml
Glacial acetic acid	50 ml
Distilled water	250 ml

E. coli Minimal Agar

NH_4Cl	5.0 gl^{-1}
NH_4NO_3	1.0
Na_2SO_4	2.0
K_2HPO_4	3.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.1
Agar (Oxoid L28)	15.0
10% Glucose	20 ml

Filter sterilized glucose was added to the media after autoclaving.

Amino acids were added to the media at 20 µg ml^{-1} with the following combinations.

	1	2	3	4	5
6	Adenine	phe	ala	arg	leu
7	Cytosine	ser	cysteine	ornithine	gly
8	Guanine	trp	thr	asp	ileu
9	Thymine	tyr	meth	pro	his
10	Uracil	cystine	lys	glu	val

All 10 combinations were inoculated.

7.3 APPENDIX 3.

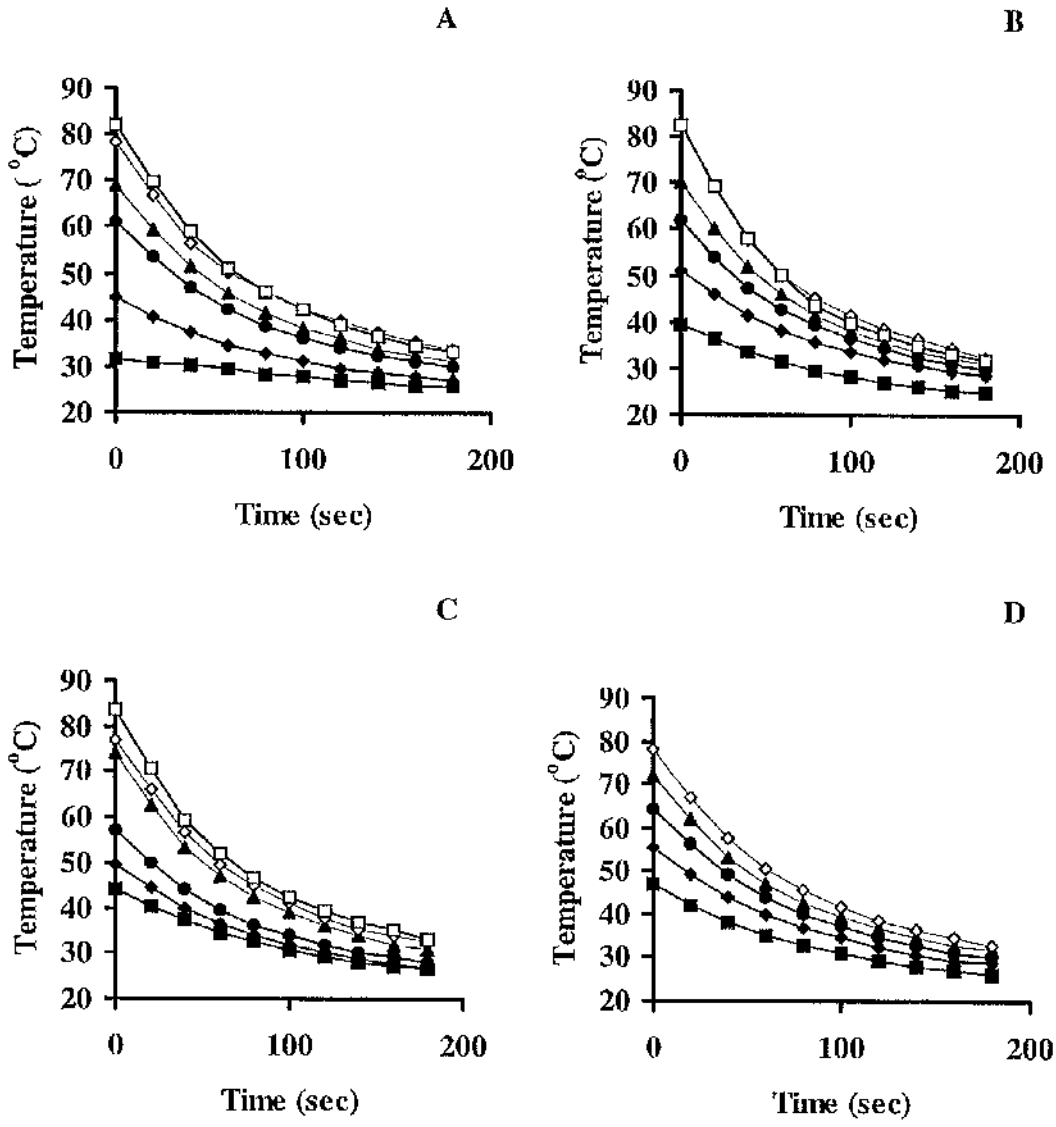
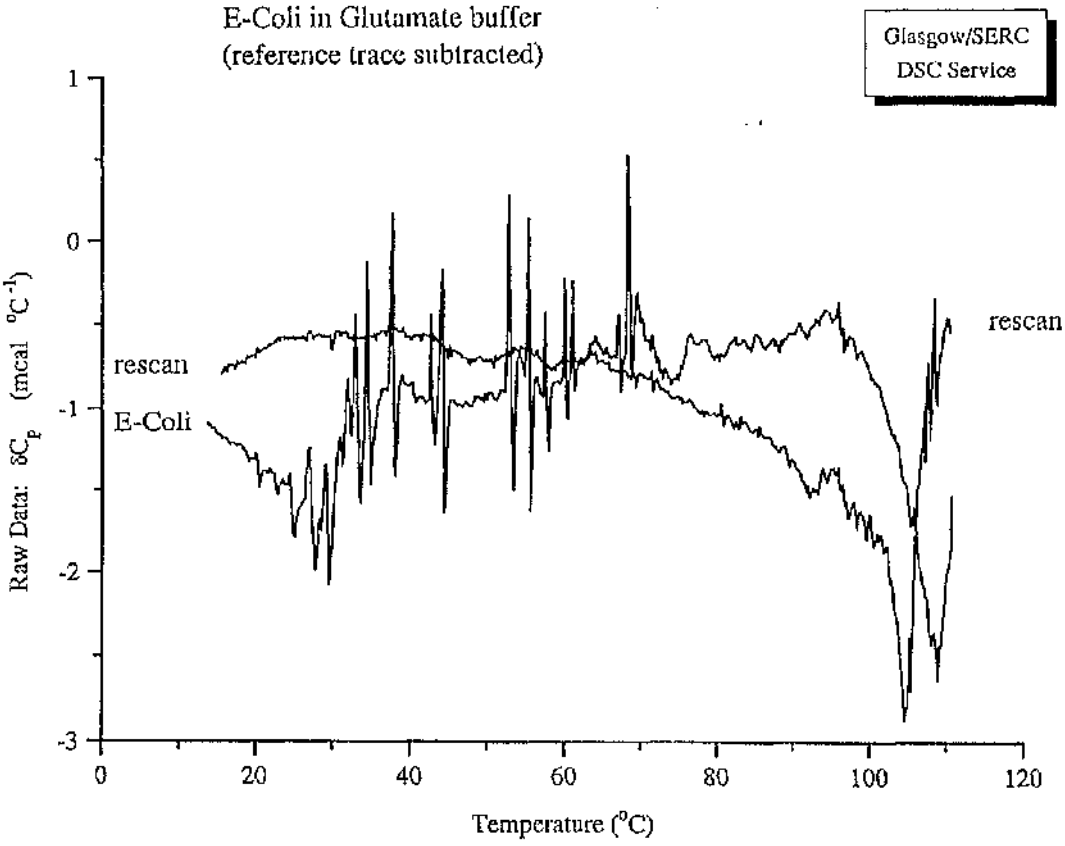
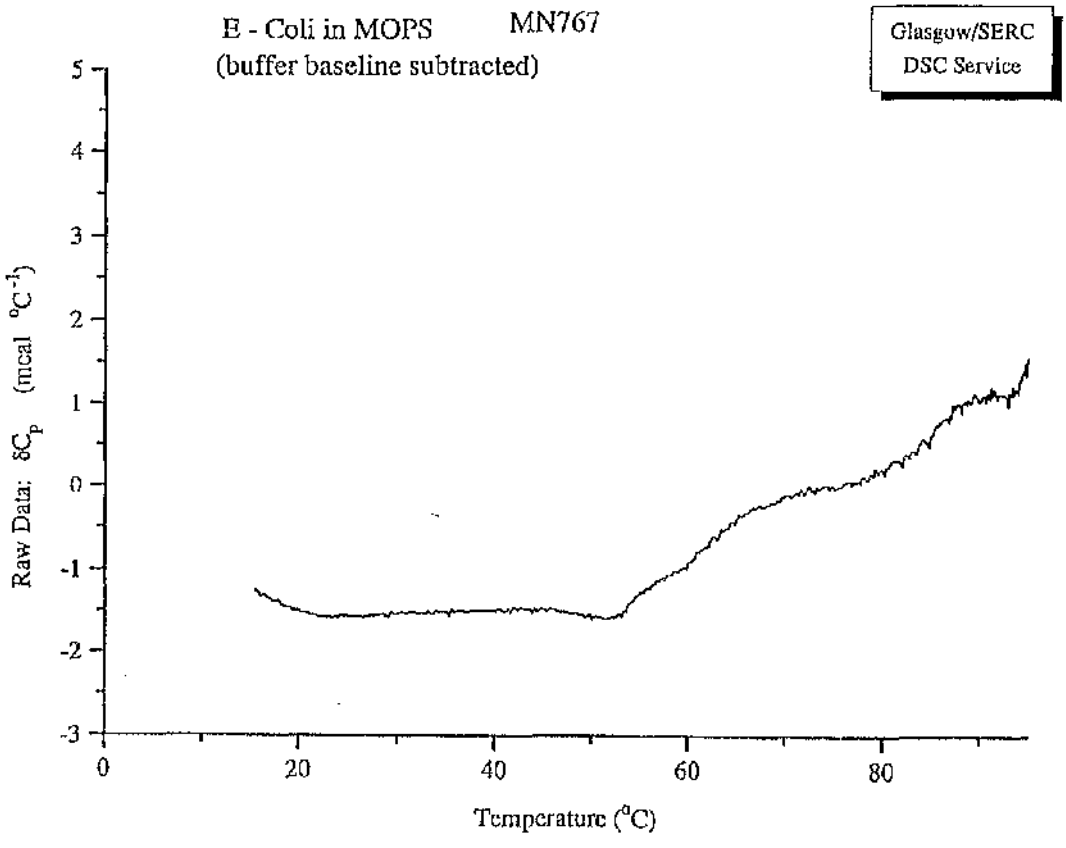


Figure 7.4 **Data used to calculate the temperatures of the agar media after Nd:YAG laser light exposure.** Each graph shows temperature cooling curves for a range of initial temperatures, indicated by the different symbols, after exposure to Nd:YAG laser light. Each exposure was at different PRF (**A.** 30 Hz, **B.** 15 Hz, **C.** 10 Hz and **D.** 20 Hz) and the extrapolation to the Y-axis, for the approximate temperature at the time the laser was switched off. The extrapolation was achieved by logging the Y-axis to produce linear fits for each cooling curve.

7.4 APPENDIX 4





7.5 APPENDIX 5

7.5.1 MULTIFACTORIAL ANALYSIS

7.5.1.1 Introduction

The combination of lasers and microorganisms introduces a large number of parameters which may play a part in increasing the efficiency of laser inactivation or alternatively inhibit the sterilizing mechanism. The parameters selected for this investigation were categorised as follows:-

Laser parameters

The laser parameters are those that govern the nature of the laser output and therefore the combination of certain parameters may be more effective than others. The parameters varied in this investigation were the pulse repetition frequency, the pulse energy and the exposure time.

Growth parameters

The state of the bacteria, prior to exposure, is an important consideration as in a practical situation the growth conditions vary considerably. However, in the laboratory the bacteria are normally grown under optimal conditions. The parameters considered for variation were the pH and sodium chloride concentrations.

Exposure parameters

This is another set of important parameters which will govern the response of the bacteria to the laser light. The condition of the bacteria during exposure will play a major part in the determination of the efficacy of laser light inactivation. Such conditions are the environmental water content or the ambient temperature of the surrounding environment.

Bacterial type

The effect of laser light inactivation may be more efficient with one bacterial species or even one strain than another. Because there are so many different bacteria it is impossible to test all bacteria under all combinations of parameters or, for that matter, under one combination. Four bacteria were chosen to represent the major classes of bacteria, namely *Staphylococcus aureus* (Gram +ve cocci), *Escherichia coli* (Gram -ve rod), *Listeria monocytogenes* (Gram +ve rods and cocci) and *Bacillus cereus* (Gram +ve sporulating rods).

Supporting material

The effect of laser light on the bacteria may also be governed by the material supporting the bacteria. There are a number of possibilities but those chosen in this category were stainless steel, plastic (nylon) and glass. In other areas of this investigation other media have been used, such as agar and a variety of liquids.

If all combinations of the 14 parameters were to be varied using a one- parameter-at-a-time method, with only two values for each parameter, the number of individual experiments required would be $\mathbf{2^{14} = 16384}$

The number of experiments can be greatly reduced using the multifactorial model which has the ability to assess a number of different parameters at one time. The advantages are that the amount of time spent experimenting is reduced greatly and therefore so is the cost. The multifactorially-designed experiments can also show the effect of interactions between particular parameters, which cannot be done using the one-at-a-time experimental method. The disadvantages of doing multifactorial investigations are that the results are not precise and only show a trend, although a trend can highlight the more important parameters warranting further investigation. Also, multifactorial analyses in microbiological contexts are made difficult by slow, even inadequate bacteriological methods. "The number of combinations of conditions which can be studied is limited largely by practical problems such as the lack of simple automated methods for enumeration of microorganisms." (Roberts 1990).

The multifactorial method used in this investigation is the 2^N (or two level) method. This method determines how the output of an experimental system depends on two or more external factors which may influence it simultaneously.

7.5.1.2 The 2^N multifactorial method

In the 2^N multifactorial system each parameter, or factor, is limited to two values, or levels - level 0 (lower value) and level 1 (higher value). The application of a set of parameters is a treatment combination. **Figure 7.5** is a diagram of a system governed by three parameters: A, B and C. Each of these either has a high or a low value. Monitoring of the system is achieved using an output (M).

To provide an overall picture of the influence of each factor, all combinations of the three external factors must be applied and the value for M recorded. **Table 7.1** shows the method used to achieve a full set of data for a multifactorial experiment.

	PARAMETER			
EXPERIMENT	A	B	C	RESULT
1	0	0	0	M_{000}
2	0	0	1	M_{001}
3	0	1	0	M_{010}
4	0	1	1	M_{011}
5	1	0	0	M_{100}
6	1	0	1	M_{101}
7	1	1	0	M_{110}
8	1	1	1	M_{111}

Table 7.1 **Arrangement of variables in the multifactorial experiments showing the number of experiments alongside each combination.**

As can be seen therefore, if there are N parameters in the treatment combination then there are 2^N combinations. For the system described in **Figure 7.5**, 8 experiments must be performed and therefore 8 results are obtained.

7.5.1.3 Analysis of multifactorial results

With the two level system, with three variable parameters described in **Figure 7.5**, there are 8 results per experiment. There are several methods of analysing the results:-

Graphical

This was the method adopted in this thesis, purely for the ease of analysing the results and interactions between the different parameters. See results section 4.4.5.

Mathematical

Each of the three parameters has to be taken individually and assessed in conjunction with each of the other two. Taking parameter A firstly, four of eight results are high values and four are low. The main effect for factor A, E_A is defined as:-

$$E_A = \frac{\left(M_{111} + M_{110} + M_{101} + M_{100}\right)}{4} - \frac{\left(M_{011} + M_{010} + M_{001} + M_{000}\right)}{4}$$

This equation represents the average value of the high level M values minus the average value of the low level M values for factor A. E_B and E_C can be calculated by the same method.

One of the reasons that the multifactorial technique is advantageous over the one-at-a-time method is the ability to evaluate interactions between each parameter.

The interaction between parameters A and B in the system described in **Figure 7.5**, F_{AB} is defined as:-

$$F_{AB} = \frac{\left(\left(M_{111} + M_{110}\right) - \left(M_{011} + M_{010}\right)\right)}{4} - \frac{\left(\left(M_{101} + M_{100}\right) - \left(M_{001} + M_{000}\right)\right)}{4}$$

The first term is the measurements for which factor B is high and the second term is measurement when factor B is low. Within these terms there is a calculation to find the key influence for factor A. This is achieved by the subtraction of the average value of M for the low factor A from the average value of M for the high factor A, when factor B is high in the first term and low in the second term. In the calculation of E or F the value may be positive or negative depending on whether the particular parameter influences or inhibits laser inactivation.

The problem with the 2^N multifactorial system is, as said previously, that the result will only show a trend and leads to an assumption that the measurable quantity varies linearly between the high and low levels within each factor. Therefore the analysis is useful as a tool to find the main influencing parameters in each experiment and will allow the disregard of those with no effect. Some the results were analysed with this method and are presented in Appendix 6. Other methods of experimentation and analysis are possible, for example partial multifactorials.

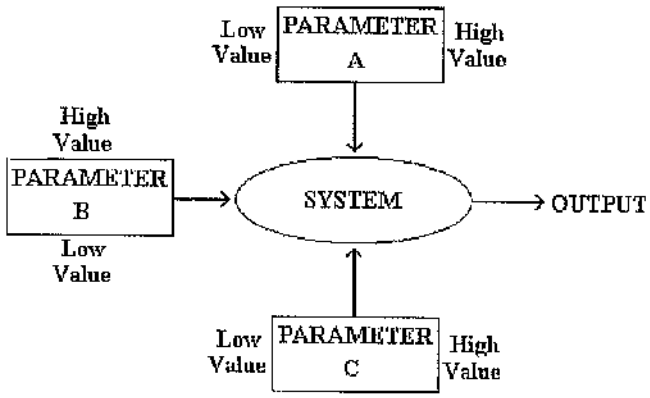
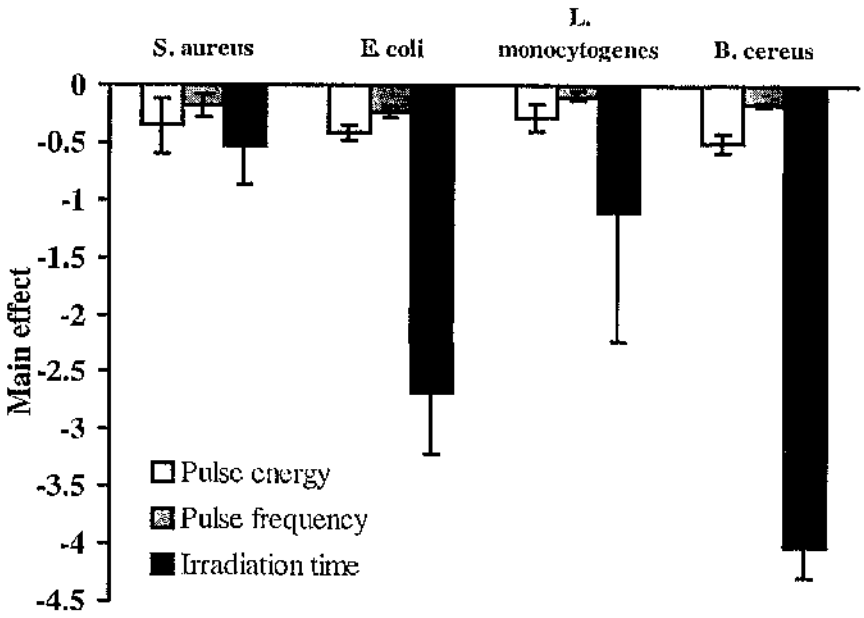


Figure 7.5 **A 2^N experimental system influenced by three parameters each of which contains a higher and a lower value**

7.6 APPENDIX 6

Figure 7.6 **Multifactorial analysis of the effect of the Nd:YAG laser parameters: pulse energy, PRF and exposure time on *E. coli*, *S. aureus*, *L. monocytogenes* and *B. cereus* as wet films of overnight culture on stainless steel surfaces.** The effect is proportional to the final bacterial counts after exposure to the laser light at the specified parameter values.

Figure 7.7 **Multifactorial analysis of the effect of the Nd:YAG laser parameters: pulse energy, PRF and exposure time on *E. coli*, *S. aureus* and *L. monocytogenes* as dry films of overnight culture on stainless steel surfaces.** The effect is proportional to the final bacterial counts after exposure to the laser light at the specified parameter values.


S. aureus
E. coli
L. monocytogenes
B. cereus
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
Main effect
Pulse energy
Pulse frequency
Irradiation time

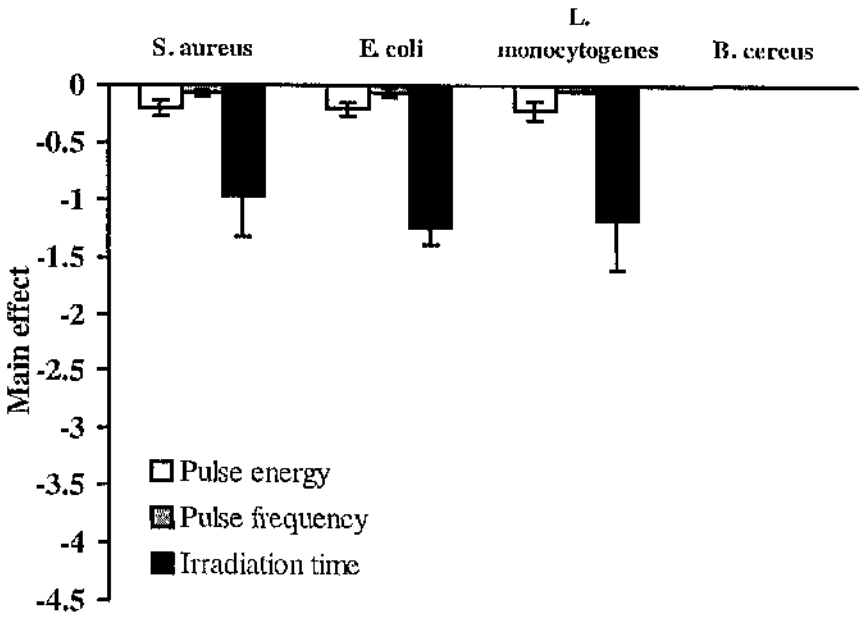
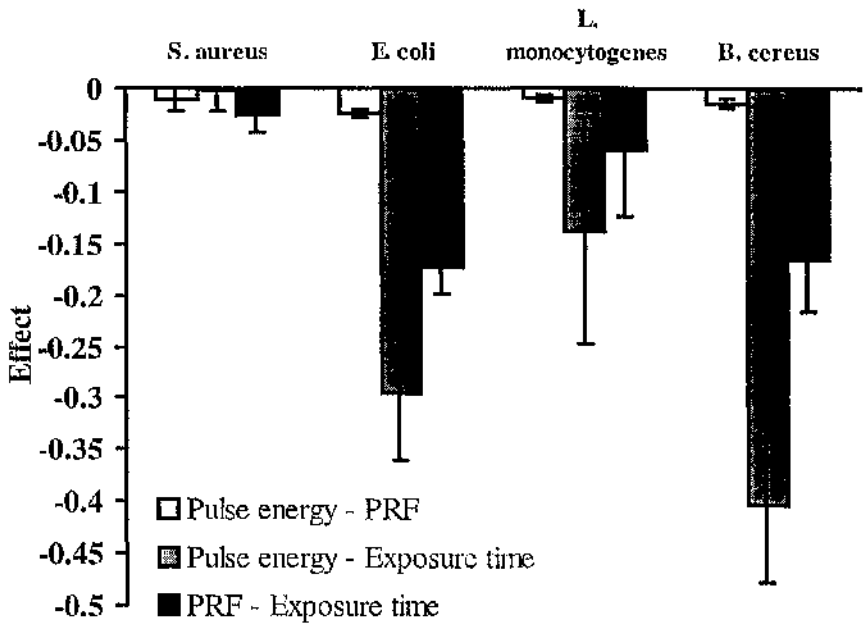

S. aureus
E. coli
L. monocytogenes
B. cereus
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
Main effect
Pulse energy
Pulse frequency
Irradiation time

Figure 7.8 **Multifactorial analysis of the effect of the interactions between the different Nd:YAG laser parameters: pulse energy, PRF and exposure time on *E. coli*, *S. aureus*, *L. monocytogenes* and *B. cereus* as wet films of overnight culture on stainless steel surfaces.**

Figure 7.9 **Multifactorial analysis of the effect of the interactions between the different Nd:YAG laser parameters: pulse energy, PRF and exposure time on *E. coli*, *S. aureus* and *L. monocytogenes* as dry films of overnight culture on stainless steel surfaces.**


S. aureus
E. coli
L. monocytogenes
B. cereus
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.35
-0.4
-0.45
-0.5
Effect
Pulse energy - PRF
Pulse energy - Exposure time
PRF - Exposure time

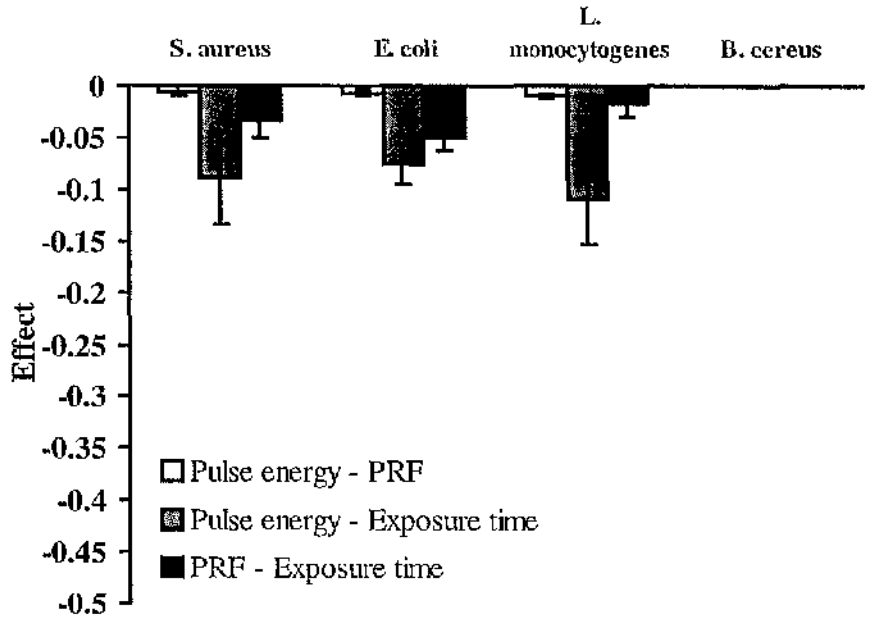
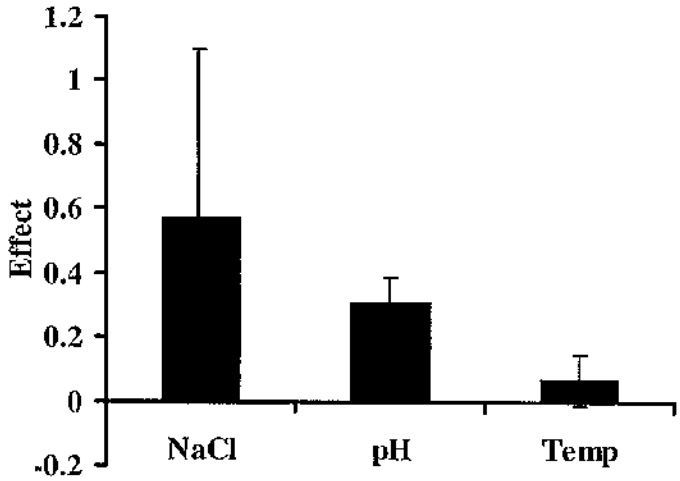

S. aureus
E. coli
L. monocytogenes
B. cereus
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
-0.35
-0.4
-0.45
-0.5
Effect
Pulse energy - PRF
Pulse energy - Exposure time
PRF - Exposure time

Figure 7.10 **Multifactorial analysis of the effect of the Nd:YAG laser light at 30 Hz and 10 J on *E. coli* as wet films of overnight culture, grown under different salt concentrations and pH, on stainless steel surfaces, at two different temperatures.** The parameter values were 0.5 and 2.5 % w/v NaCl, pH 5.5 and 8.0 and 20° and 37°C The effect was proportional to the reduction in counts after exposure to the laser light at the specified parameter values.

Figure 7.11 **Multifactorial analysis of the effect of the interactions between the environmental parameters (NaCl concentration, pH and ambient temperature) on *E. coli* exposed to Nd:YAG laser light (30 Hz and 10 J) as wet films of overnight cultures on stainless steel surfaces.** The parameter values were 0.5 and 2.5 % w/v NaCl, pH 5.5 and 8.0 and 20° and 37°C.


1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Effect
NaCl
pH
Temp

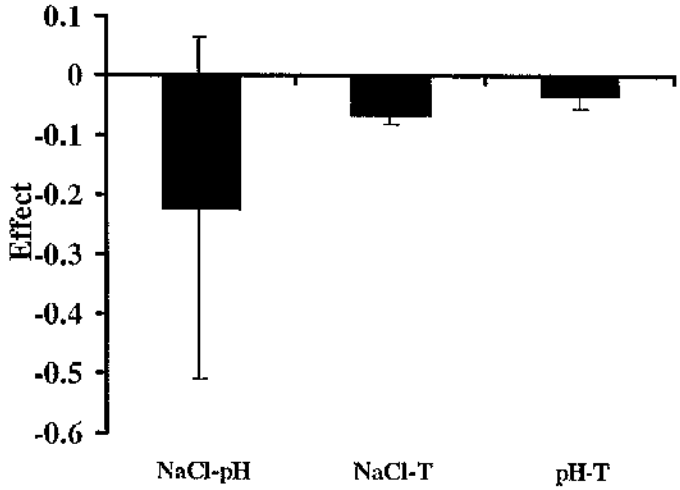
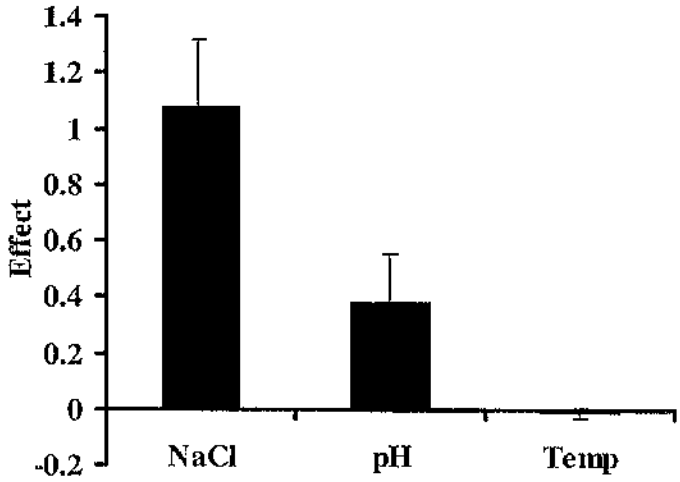

0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
Effect
NaCl-pH
NaCl-T
pH-T

Figure 7.12 **Multifactorial analysis of the effect of the Nd:YAG laser light at 30 Hz and 10 J on *E. coli* as dry films of overnight culture, grown under different salt concentrations and pH, on stainless steel surfaces, at two different temperatures.** The parameter values were 0.5 and 2.5 % w/v NaCl, pH 5.5 and 8.0, and 20° and 37°C The effect was proportional to the reduction in counts after exposure to the laser light at the specified parameter values.

Figure 7.13 **Multifactorial analysis of the effect of the interactions between the environmental parameters (NaCl concentration, pH and ambient temperature) on *E. coli* exposed to Nd:YAG laser light (30 Hz and 10 J) as dry films of overnight cultures on stainless steel surfaces.** The parameter values were 0.5 and 2.5 % w/v NaCl, pH 5.5 and 8.0, and 20° and 37°C.


1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Effect
NaCl
pH
Temp

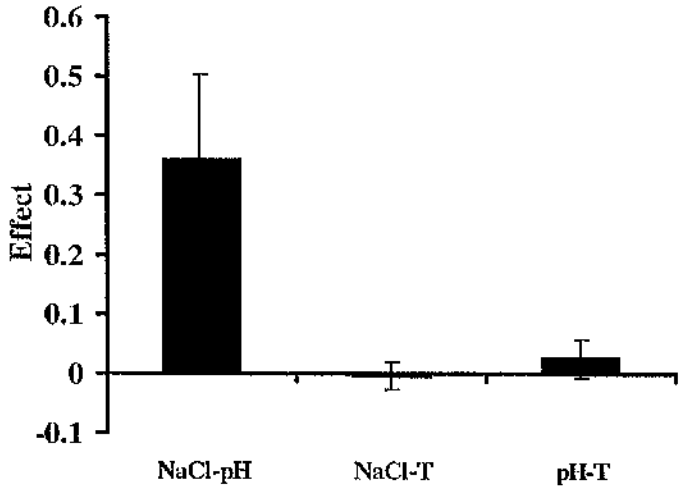
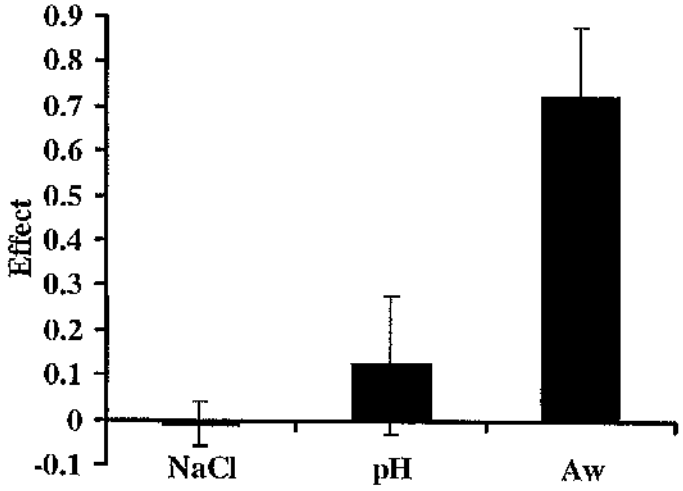

0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Effect
NaCl-pH
NaCl-T
pH-T

Figure 7.14 **Multifactorial analysis of the effect of the Nd:YAG laser light at 30 Hz and 10 J *on S. aureus* as both wet and dry films of overnight culture, grown under different salt concentrations and pH, on stainless steel surfaces.** The parameter values were 0.5 and 10 % w/v NaCl, and pH 5.5 and 8.0. The effect shown on the y-axis, was proportional to the reduction in counts after exposure to the laser light at the specified parameter values.

Figure 7.15 **Multifactorial analysis of the effect of the interactions between the environmental parameters (NaCl concentration, pH and water activity) on *S. aureus* on stainless steel surfaces, exposed to Nd:YAG laser light (30 Hz and 10 J).**


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Effect
NaCl
pH
Aw

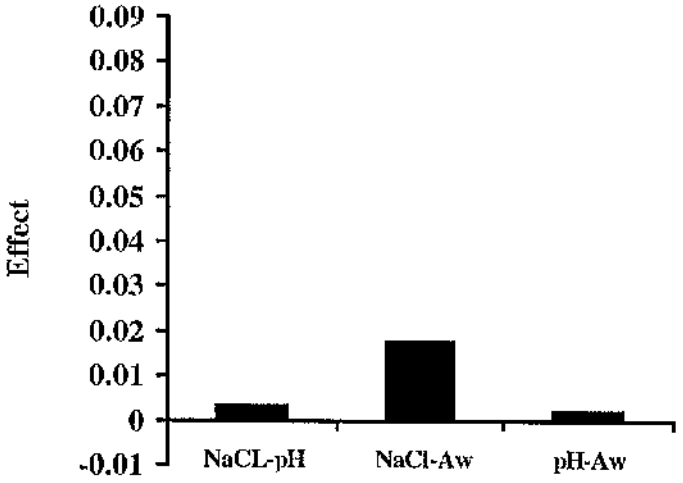
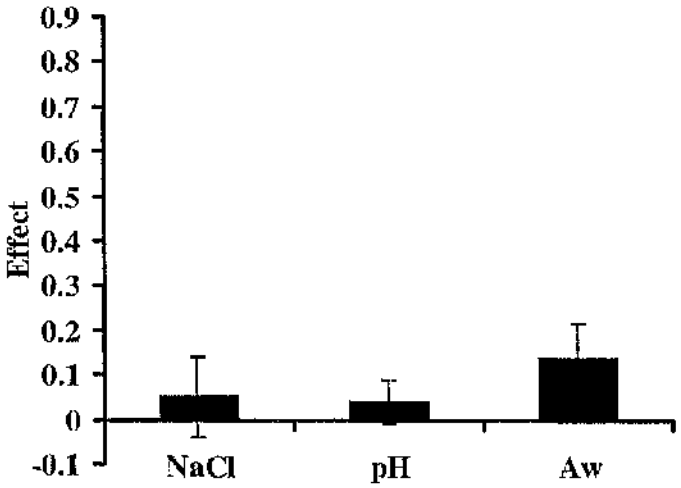
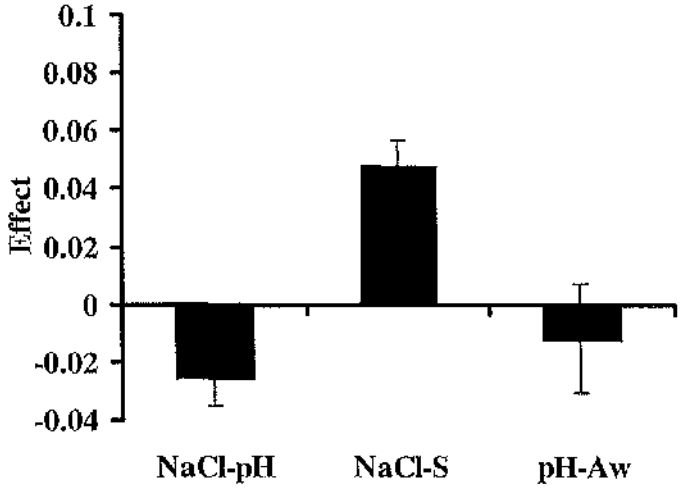

0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
Effect
NaCL-pH
NaCl-Aw
pH-Aw

Figure 7.16 **Multifactorial analysis of the effect of the Nd:YAG laser light at 30 Hz and 10 J on *L. monocytogenes* as both wet and dry films of overnight culture, grown under different salt concentrations and pH, on stainless steel surfaces.** The parameter values were 0.5 and 2.5 % w/v NaCl, and pH 5.5 and 8.0. The effect, shown on the y-axis was proportional to the reduction in counts after exposure to the laser light at the specified parameter values.

Figure 7.17 **Multifactorial analysis of the effect of the interactions between the environmental parameters (NaCl concentration, pH and water activity) on *L. monocytogenes* on stainless steel surfaces, exposed to Nd:YAG laser light (30 Hz and 10 J).**


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Effect
NaCl
pH
Aw


0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
Effect
NaCl-pH
NaCl-S
pH-Aw

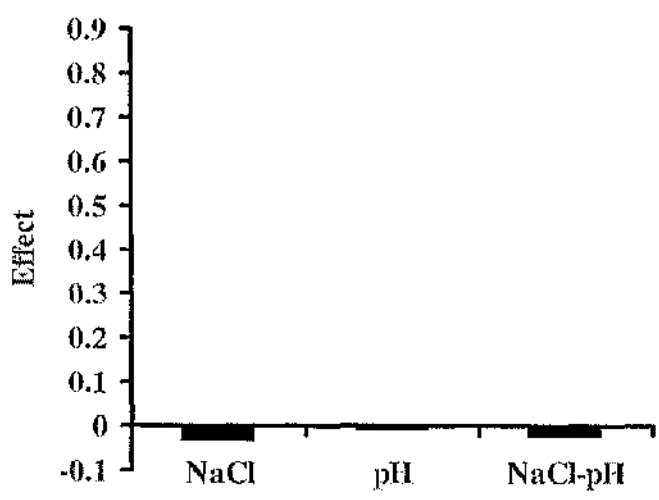


Figure 7.18 **Multifactorial analysis of both the main and interaction effects of Nd:YAG laser light at 30 Hz and 10 J on *B. cereus* as wet films of overnight culture, grown under different salt concentrations and pH's spread onto stainless steel surfaces.** The parameter values were 0.5 and 2.5 % w/v NaCl and pH 5.5 and 8.0. The effects of both the NaCl concentrations and the pH values are recorded along with the interaction between both parameters.

7.7 APPENDIX 7

7.7.1 LOG-LOGISTIC MODELLING

7.7.1.1 Introduction

Cole *et al.* (1993) used the log-logistic model, as a procedure to introduce the vitalistic approach to death curve data interpretation. Instead of a log-linear plot of the viable count against time a log-log plot was created. A four parameter equation was used to fit these points as follows:-

$$y = \alpha + \frac{\beta}{1 + e^{\lambda - \delta . x}}$$

The equation represents a curve with symmetry about the point $x = \frac{\lambda}{\delta}$ where the slope has a maximum $\frac{\beta\delta}{4}$ and two horizontal asymptotes given by $x = \alpha$ and $x = \alpha + \beta$ the equation was written in the following form:-

$$y = \alpha + \frac{\omega - \alpha}{1 + e^{4\sigma(\tau - x)/(\omega - \alpha)}}$$

where α is the upper asymptote, ω is the lower asymptote, σ is the maximum slope and τ is the position of the maximum slope. **Figure 7.19** shows these values schematically. The effect of different conditions were analysed from the above model by comparing the values for τ and σ which indicate the position of the killing curve and the rate of killing, respectively. τ and σ are therefore indications of the efficiency of killing.

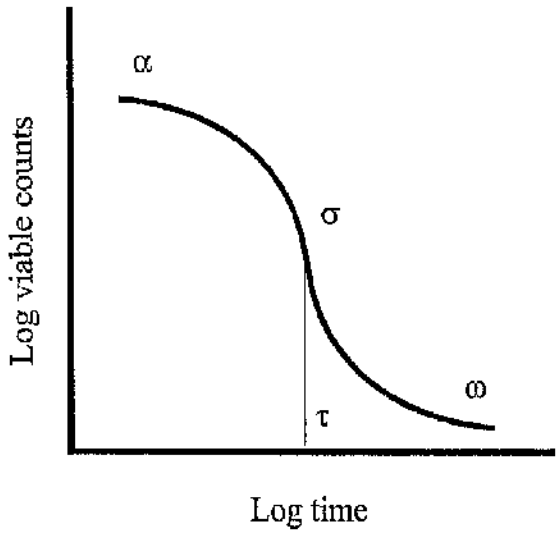


Figure 7.19 Diagram to illustrate the four parameters, α - the upper asymptote, ω - the lower asymptote, σ - the maximum slope and τ - the position of the maximum slope