

Development of a novel strategy for the control of encrustation and blockage of Foley catheters

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ABSTRACT

Many patients undergoing long-term catheterisation experience encrustation of their catheters. The problem stems from infection by urease producing bacteria particularly *Proteus mirabilis*. *Pr. mirabilis* is a major pathogen of the catheterised urinary tract, and its presence is strongly correlated with catheter obstruction. This urease producing organism colonizes the catheter surface and produces biofilm communities embedded in a polysaccharide matrix. The urease enzyme hydrolyses urea to ammonia and carbon dioxide, elevating the urinary pH. Under these alkaline conditions crystals of magnesium and calcium phosphates form and become trapped in the organic matrix. The resulting crystalline biofilm can block the catheter lumen or eye-hole seriously compromising patients' health and welfare. Urinary incontinence due to urine bypassing the catheter can lead to breakdown of skin, bedsores and all consequent problems. Urinary retention is painful and if it goes unnoticed can lead ascending infection and pyelonephritis. Currently there are no effective procedures for controlling this problem.

Bacteria, once they are embedded in a biofilm, are very difficult to eliminate by antibacterial agents. Conceptually the simplest way to protect a medical device from colonization by biofilm would be to impregnate it with a broad range antimicrobial agent that eluted into the medium, and attacked bacteria before they attached to the surface and adopted the resistant biofilm phenotype. There have been two factors limiting the long-term effectiveness of antimicrobial coated and impregnated catheters. It has not been possible to load catheters with sufficient active agents or to control the release of the agent so that effective concentrations are released for the lifetime of the device. Exploitation of the catheter retention balloon as the reservoir for an active agent could solve both these problems. The balloon could be inflated with 10 ml of solutions containing high concentrations of an agent. The wall of the catheter balloon would provide a diffusion barrier, which could control the release of active agents into the bladder urine.

The general aim of this study was to develop a novel strategy for controlling this problem which specifically targeted *Pr. mirabilis*. The objectives were to: (a) establish whether *Pr. mirabilis* strains isolated from the catheterised urinary tract were fully sensitive to the biocide triclosan; (b) determine the minimal bactericidal concentrations for triclosan of clinical *Pr. mirabilis* isolates growing in urine; (c) investigate what concentrations of triclosan could be delivered through the balloons of catheters; (d) determine if inflating the catheter balloon with triclosan would delay or prevent catheter blockage by crystalline *Pr. mirabilis* biofilms; (e) ascertain what triclosan concentrations were required in the urine to eliminate established *Pr. mirabilis* biofilms; (f) investigate whether resistant cells emerged when populations of *Pr. mirabilis* were exposed to triclosan, and if so, whether the triclosan resistant cells exhibited cross-resistance to antibiotics; (g) examine how the triclosan strategy influenced the development of catheter biofilm communities by a variety of species that cause CA-UTI.

Isolates of *Pr. mirabilis* from encrusted catheters were found to be extremely sensitive to triclosan (minimum inhibitory concentration (MIC) in agar ranging from 0.1 – 0.3 µg/ml). At concentrations of 0.2 µg/ml in artificial urine, it prevented the rise in pH

that is normally produced by *Pr. mirabilis*. Concentrations of 100 µg/ml were required however to produce bactericidal effects.

A simple laboratory model of the catheterised bladder was used to examine whether triclosan could be delivered through the retention balloon. In experiments with all-silicone catheters, balloons were inflated with triclosan (10 mg/ml in 5% w/v PEG). High pressure liquid chromatography detected the presence of triclosan at 0.02 – 0.16 µg/ml in urine samples taken from the bladder chamber over a 48 h period. Sections (1 cm) taken from the catheters were placed onto agar plates that had been seeded with *Pr. mirabilis*. After incubation, clear zones of inhibition were visible around each of the sections on the bacterial lawn, indicating that the biocide had impregnated the silicone along the whole length of the catheter.

Experiments in laboratory models infected with *Pr. mirabilis* demonstrated that when the retention balloons of all-silicone, silicone-coated latex and hydrogel-coated latex catheters were inflated with water, the artificial urine in the bladder chamber became alkaline and the catheters blocked with crystalline biofilm after mean times of 26 h (all-silicone), 19 h (silicone-coated latex) and 23 h (hydrogel-coated latex). In contrast when the balloons were inflated with triclosan (10 mg/ml in 5% w/v PEG), the pH of the urine remained acid, all the catheters drained freely for the seven day experimental period and little sign of encrustation was found on the catheters. Subsequent experiments showed that concentrations of triclosan ranging from 0.5 – 10 mg/ml in a variety of solvents were effective in preventing catheter blockage. The strategy was also effective in models supplied with pooled human urine. In additional experiments it was established that the triclosan solution had no adverse effects on the physical properties of the balloons over an eight week test period.

Experiments investigating the concentration of triclosan required in the artificial urine supplying the bladder models to inhibit further development of an established biofilm revealed that concentrations of 10 µg/ml triclosan or higher were needed. Introducing triclosan into the balloon of catheters where encrustation by *Pr. mirabilis* had already begun had no significant effect on delaying catheter blockage. This suggested that if the strategy is to be effective in patients who are “blockers”, it will be necessary to change catheters and then inflate the new catheter with the triclosan solution.

Inflating the catheter balloon with triclosan significantly reduced the numbers of viable cells in the biofilm on catheters in models inoculated with *Pr. mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Providencia stuartii*, *Serratia marcescens*, *Staphylococcus aureus* and *Enterococcus faecalis* but not *Pseudomonas aeruginosa*. Despite being capable of producing the urease enzyme, the pH of control and test models inoculated with *Ser. marcescens*, *S. aureus*, *K. pneumoniae*, *Ps. aeruginosa* and *M. morganii* remained acidic over the 48 h test period.

Under laboratory conditions, it was possible to select for *Pr. mirabilis* mutants with increased resistance to triclosan, however no cross-resistance to antibiotics was observed. The triclosan strategy was ineffective at controlling catheter encrustation in models inoculated with mutants with MICs of 40 µg/ml. Therefore should clinical trials based in this strategy go ahead, the sensitivities of urinary tract organisms to triclosan should be monitored carefully.

This strategy does not disrupt the integrity of the closed drainage system, and delivering the antibacterial agent straight to the bladder could avoid the selection of antibacterial-resistant gut flora caused by oral drug administration. The results from this study suggest that if this novel strategy for controlling catheter encrustation could be transferred successfully from the laboratory to the clinic, it could improve the quality of life and well-being of many elderly and disabled people who are currently enduring the complications associated with long-term indwelling bladder catheterisation.

Publications.

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1. INTRODUCTION

1.1. Managing bladder dysfunction

The physiology underlying the functioning of a healthy bladder is a complex integrated process relying on muscular, neurological and psychological responses (Sarkar and Ritch, 2000). When the bladder fills, it expands spontaneously until the volume of urine reaches close to its maximum capacity, at which point healthy individuals can empty their bladders at will. Most of us take the mechanisms governing this process for granted, but for a variety of different reasons drainage of urine from the bladder often requires medical intervention.

Failure to pass urine results in painful bladder distension which if not dealt with can be life threatening. Urinary incontinence causes social and hygiene problems and in immobile patients can lead to bedsores, skin infections and septicaemia (Kunin, 1987c). Patients who have undergone prostatectomies, other lower urinary tract operations and some gynaecological operations often require bladder management for a few days to facilitate surgical repair (Slade and Gillespie, 1985). Others patients requiring bladder management include those with temporary anatomical or physiological bladder obstructions or those who are severely ill or comatosed (Kunin, 1987c). Long-term incontinence in the elderly, in patients who have suffered strokes and in patients with spinal cord injuries or neurological diseases also necessitates the use of a bladder drainage system.

A report by the Royal College of Physicians (1995) estimated that 25% of patients in residential homes and 40% of patients in nursing homes suffer urinary incontinence. It was also estimated that 10 - 20% of women and 7 - 10% of men over the age of 65 living at home had urinary incontinence. A literature review by Minassian *et al.*

(2003) of 35 studies revealed that the median prevalence of urinary incontinence, ranging from mild to severe was 27.6% in females and 10.5% in males. Many incontinent people are ashamed of their lack of bladder control, and this in turn affects their psychological health and social interaction (Busby-Whitehead and Johnson, 1999). The severity of the problems associated with incontinence is underlined by the fact that elderly people consider urinary incontinence to be one of the most embarrassing disabilities (Wilde, 2003; Sarkar and Ritch, 2000).

There are essentially four choices of bladder drainage systems (Slade and Gillespie, 1985):

1. Management of incontinence by external devices. This involves the use of absorbent pads and pants, or condom sheaths that empty into a drainage tube that can manage incontinence in men.
2. Suprapubic catheterisation. This involves draining urine from the bladder by inserting a catheter directly through the anterior abdominal wall into the bladder. Although suprapubic catheterisation has its advocates, and is preferred as an alternative to indwelling urethral catheters for patients with urethral stricture or fistula, it is not as popular as other methods of bladder management (Stickler and Zimakoff, 1994).
3. Intermittent or self-intermittent urethral catheterisation. These bladder management techniques involve using urethral catheters intermittently. At intervals the catheter is inserted into the bladder, the urine drained and the catheter removed. People have been using intermittent catheterisation to drain urine from the bladder for centuries. Their earliest use was recorded in the third millennium BC, when catheters made from different metals were passed into the bladder

intermittently via the urethra to drain urine (Pomfret, 2000). The same principle is still in use today, and some patients can easily be taught to perform this procedure. If a patient is unable to self-catheterise, a nurse or carer can catheterise the patients at regular intervals. In a retrospective study of 75 patients who performed intermittent self-catheterisation for a mean of seven years, 92% of patients with neuropathic bladder dysfunction were continent (Wyndaele and Maes, 1990).

4. Continuous urethral catheterisation. Urethral catheterisation involves inserting a catheter into the bladder via the urethra (Figure 1). By leaving the catheter in place, urine is continually drained from the bladder. Indwelling urethral catheters (IUCs) used in this way are an essential part of modern medical care, and are used extensively in hospitals, care facilities and by patients at home.

A prevalence study of catheterised patients in Maryland nursing homes revealed that 0.4% used intermittent catheterisation, 0.6% used a suprapubic catheter, 1.3% used external collection devices and that IUCs were used by 8.7% of patients (Warren *et al.*, 1989). Nine per cent of residents in nursing homes in the UK have urinary catheters, with an equal male: female split (McNulty *et al.* 2003). Zimakoff *et al.* (1993) reported that in Denmark 13.2% of hospitalised patients, 4.9% of nursing home residents and 3.9% of patients receiving home care had IUCs. About 10% of all hospitalised patients will receive an IUC, making urinary catheters the most commonly implanted medical devices (Kunin, 1987c; Darouiche, 2001)

The bladder remains collapsed with long-term indwelling catheter drainage, and as a result of chronic infection often becomes contracted with unhealthy mucosa and phosphatic debris capable of causing catheter blockage (Slade and Gillespie, 1985).

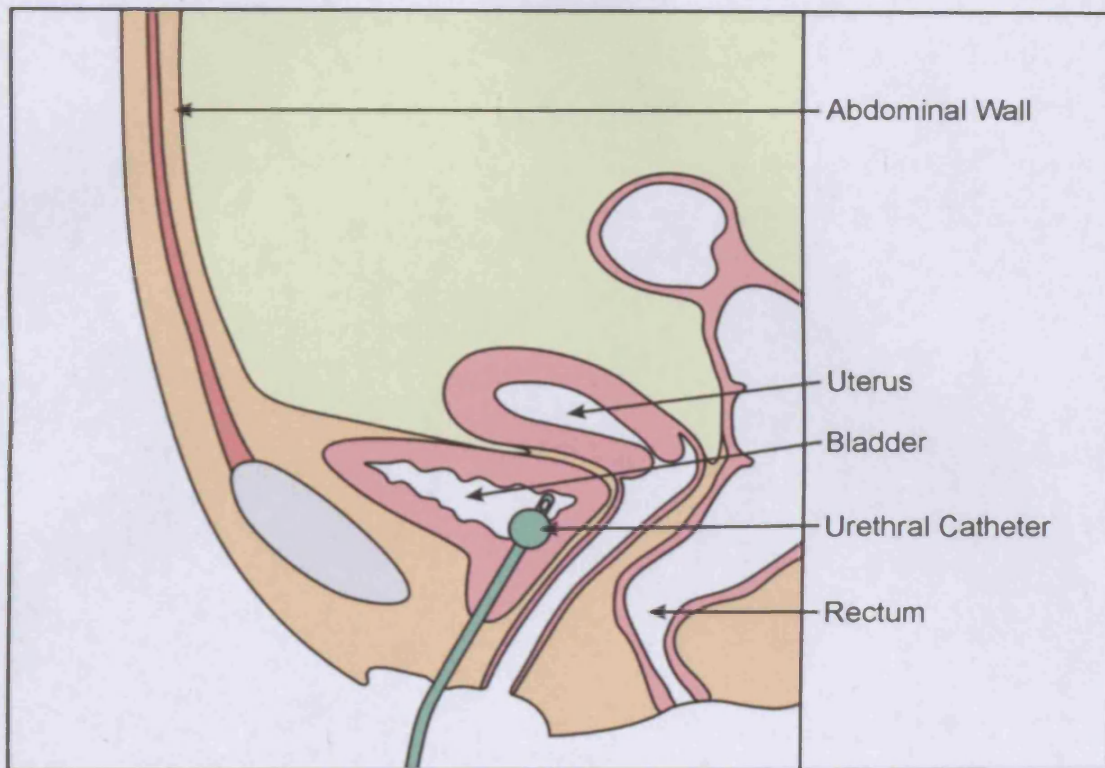


Figure 1: Diagram of the catheterised urinary tract.

These complications can be overcome using intermittent catheterisation or intermittent drainage with a catheter valve, both of which keep the bladder functioning normally. A multi-centre clinical evaluation of the performance of seven different catheter valves revealed that using the most expensive valve, 100% of patients reported the valve as good or acceptable (Fader *et al.*, 1997). Woods (1999) discovered that there was a 50/50 split in the number of patients who liked and disliked the valve as an alternative to conventional drainage. From their study they conclude that instead of trying to find which drainage system is 'best', it would be more beneficial to encourage patients to use both methods for them to decide which best suits them. Self-intermittent catheterisation and catheter valves rely on patients having sufficient manual dexterity to be able to insert the catheter or open and close the valves. Since catheter drainage bags do not have to be used with either of these methods, they are more discreet and can dramatically improve certain patients' quality of life.

Many different urethral catheters are available varying in length, balloon size, base material and surface coating. Catheters can be made of all-silicone, polyurethane, polyvinyl chloride, latex or latex coated with silicone, teflon, hydrogel or silver hydrogel. The Foley catheter designed by Dr. Frederick Foley in 1934 is the most commonly used IUC and its basic design has remained unchanged for seventy years. This catheter features a balloon that is inflated with sterile water to secure the catheter in the bladder. The standard Foley catheter therefore has two interior channels, one for draining the urine and the other for inflating the retention balloon.

The suggested catheter balloon size is 5 - 10 ml, the use of larger balloons is discouraged because they can cause bladder spasm and more trauma to the urethral

mucosa during catheter removal (Slade and Gillespie, 1985; Woollons, 1996; Pomfret, 2000). The external diameter of a catheter is measured in Charrier (Ch) or French gauge (F) units, which are equivalent to 0.33 mm. Sizes ranging from six to 24 are available. These measurements give no information about the diameter of the internal drainage lumen, which can vary enormously (Morris *et al.*, 1997), depending on catheter design, material, and coatings. It is recommended that the smallest diameter catheter that provides adequate drainage be used (usually 12 – 14 Ch) to minimise urethral irritation and trauma (Pomfret, 2000; Woollons, 1996). Foley catheters are available in three lengths, male (standard), female and paediatric. Female patients are often catheterised with male catheters, when shorter catheters would be less conspicuous and reduce drainage problems associated with catheter kinking and looping (Pomfret, 2000; Woollons, 1996).

1.2. Infections associated with the use of IUCs

Although IUCs constitute a convenient way of draining urine from the dysfunctional bladder, they can actually be hazardous to the very people that they are designed to treat. A Foley catheter *in situ* in the bladder provides a convenient bridge for bacteria to move from the outside world into the sterile bladder. In recent years it has also become apparent that the catheter itself constitutes an attractive site for urinary tract pathogens to colonize and proliferate.

Bacterial attachment to bladder cells causes an apoptotic-like innate host defence mechanism that results in exfoliation of colonized host cells (Mulvey *et al.* 1998). In contrast, catheter materials have no inherent defence mechanisms. In healthy individuals, the periodic emptying of the bladder also flushes out small numbers of

organisms colonizing the urinary tract, preventing their entry into the bladder. The use of IUCs undermines this process. Small residual volumes of urine collect in the bladder below the level of the catheter eye-holes due to their positioning above the catheter balloon (Slade and Gillespie, 1985). This urinary stasis enables any bacteria that enter the catheterised urinary tract to thrive. Stark and Maki (1984) demonstrated that isolation of any microorganisms from an intraluminal specimen, even when microbial counts were initially in the lowest detectable range almost invariably increased to more than 10^5 cfu/ml within three days.

There are three main ways bacteria can enter the catheterised bladder: (1) during catheter insertion, (2) through the catheter's internal lumen, or (3) along the catheter-urethral mucosal interface (Warren, 1991). Although bacteria can be pushed into the bladder during catheter insertion, when strict sterile techniques are employed, this mechanism does not account for much more than approximately 2% of catheter-associated urinary tract infections (CA-UTI) (Nickel, 1991).

Dukes (1928) was concerned that patients on IUCs after excision of the rectum for cancer inevitably and rapidly developed UTIs. It was suspected that infection rates would decrease if urine draining through the catheter emptied into a closed system instead of an open container. This was proven when a closed drainage system prevented infection in virtually all patients during the postoperative period. Surprisingly the significance of this finding went unnoticed for over 30 years. Miller *et al.* (1960a) showed that in patients undergoing short-term catheterisation for acute retention of urine, the incidence of UTI was much higher with open catheter drainage (73%) than with closed aseptic drainage (10%). Use of the closed drainage system

with disinfection of the urethra and urethral instruments had similar results in preventing urinary infection after prostatectomies, they dropped from 83% to 11% (Miller *et al.* 1960b). The use of disposable, plastic, sterile closed-drainage systems in 676 hospitalised patients that were catheterised for one to 39 days kept patients' urine sterile from the time of catheter insertion until the time of catheter removal, or until the patients were discharged from hospital in 77% of patients (Kunin and McCormack, 1966).

The advent of the closed drainage system is said to be the most important development in preventing CA-UTIs, and has greatly limited the intraluminal entry of organisms (Warren, 1991). However, opening the closed system either by disconnecting the catheter and drainage bag, or whilst emptying the drainage bag can introduce bacteria into the catheterised urinary tract. These organisms can ascend intraluminally from both these sites into the bladder.

Even with a strict closed catheter regime, bacteria can move from the highly contaminated urethral meatus by migration towards the bladder along the outside of the catheter, or by capillary action in the thin mucosal film coating the external catheter surface (Maki and Tambyah, 2001). This is known as the peri-urethral route, and is the predominant entry point when strict closed drainage systems are maintained. Kass and Schneiderman (1957) inoculated the peri-urethral epithelium of three catheterised patients with *Serratia marcescens*, and within three days *Ser. marcescens* was recovered from the urine of all these patients. Garibaldi *et al.* (1980) showed that patients with positive meatal cultures were significantly more likely to acquire bacteriuria than patients with negative ones. The percentage of positive meatal

cultures was 72% for female patients compared with 30% of male patients. In patients with positive meatal cultures who acquired bacteriuria, the causative organism in 85% of cases was the same bacterial species previously isolated from the urethral meatus.

The role of prior rectal or urethral bacterial colonization in the pathogenesis of CA-UTI was investigated in a prospective study by Daifuku and Stamm (1984). In patients catheterised for at least three days, urethral colonization with the same infecting strain preceding bacteriuria was observed in 12 of 18 infections in women and five of 17 in men, whilst rectal colonization with the same infecting strain isolated prior to bacteriuria occurred in 14 of 18 infections in women and five of 17 in men. This work confirmed that entry of bacteria into the catheterised urinary tract often occurs via the peri-urethral route, and more so in female patients due to the close proximity of the urethra to the rectum. Garibaldi *et al.* (1980) noted that catheterised patients who are; female, over 50 years old, not receiving antibiotics and who have conditions not treated by surgery have an increased risk of acquiring bacteriuria.

Using an animal model to ascertain the relative importance of the intraluminal versus the extraluminal route of CA-UTI, Nickel *et al.* (1985b) reported that contamination of the drainage spout or disconnection of the drainage system resulted in bacteriuria within two days. If however the closed drainage system was maintained, the extraluminal route assumed more importance, although this pathway was much slower. The study established that bacteria moved through the catheterised urinary tract by forming thick biofilms that extend proximally from the drainage bag to the bladder.

It is widely recognised that CA-UTI represent a significant proportion of all hospital and community acquired infections. UTIs account for 14.5% of all community-acquired infections and 30.3% of all nosocomial infections, making them the most prevalent hospital-acquired infections (Meers *et al.*, 1981). This survey by Meers *et al.* (1981) found that 8.6% of hospitalised patients were catheterised, and that over one in five of these patients were infected. Warren *et al.* (1989) studied the prevalence of urethral catheterisation in Maryland nursing homes, and combined this information with data obtained from studies on the incidence of bacteriuria in long term catheterised patients. They concluded that bacteriuria associated with the use of IUCs was the most common infection in American health care facilities. Even with the strictest implementation of the closed drainage system and the best catheter care, Garibaldi *et al.* (1974) found there to be an 8.1% cumulative daily risk of acquiring bacteriuria whilst an IUC was in place, and that after 10 days half of all catheterised patients were bacteriuric. All long-term catheterised patients (over 28 days) inevitably develop bacteriuria (Slade and Gillespie, 1985; Stickler and Zimakoff, 1994).

In the first week of catheterisation infection is usually due to a single bacterial species, including *Escherichia coli* which accounts for a quarter of all nosocomial UTIs, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, coagulase negative staphylococci, enterococci or *Candida* species (Warren, 2001; Jarvis and Martone, 1992; Stark and Maki, 1984). There is an increased likelihood of the patient being colonized by multiple bacterial species' the longer the catheter remains in place. A prospective study of patients with chronic IUCs revealed that bacteriuria was polymicrobial with up to five different bacterial species in 77% of all weekly urine specimens (Warren *et al.*, 1982). Clayton *et al.* (1982) found that in long-term

catheterised patients, the mean number of organisms cultured from each urine sample was 3.6, and that single organisms were rarely isolated.

1.3. Complications associated with catheter associated urinary tract infection

Bacteriuria in catheterised patients is generally asymptomatic. Compared to non-catheterised individuals however, these patients are at a higher risk of developing a range of other complications. A one-year prospective study by Kunin *et al.* (1992) of 1540 nursing home patients demonstrated a stepwise increase in mortality with duration of catheterisation. Patients who were catheterised for over 76% of their days in the nursing home were three times more likely to die within an year than matched non-catheterised patients. The frequency and duration of hospitalisation, as well as antibiotic usage was also three times higher for catheterised patients. This is consistent with a previous prospective study of a similar size among hospitalised patients, which concluded that acquiring a UTI whilst an IUC was in place was associated with approximately a three fold increase in mortality (Platt *et al.*, 1982).

A variety of different complications can afflict catheterised patients. Complications associated with bacteriuria in catheterised patients fall into two categories, the first affects short- and long-term catheterised patients and includes symptomatic UTIs i.e. fever, acute pyelonephritis and bacteraemia. The second more frequently afflicts long-term catheterised patients and symptoms include: catheter obstruction, urinary tract stones, local periurinary infections, chronic pyelonephritis and for patients catheterised for extensive periods of time, bladder cancer (Warren, 1996; Stickler and Zimakoff, 1994).

1.3.1. Bacteraemia

Bacteraemia is the presence of bacteria in the blood, and can occur in patients with infected urine after catheter insertion, removal or manipulation. *E. coli* is significantly more likely than other organisms to cause bacteraemia, although even other ‘non-uropathogens’ such as *Providencia stuartii* and *Morganella morganii* can do so as well (Warren, 1996). Only a small number of patients with bacteraemia suffer frank, clinically evident septicaemia (Slade and Gillespie, 1985). CA-UTIs are responsible for 8% and 10.6% of all hospital-acquired bacteraemia in teaching and non-teaching hospitals respectively (Coello *et al.*, 2003). Siegman-Irga *et al.* (1994) showed that patients with IUCs were twice as likely to have polymicrobial rather than monomicrobial episodes of urinary tract associated bacteraemia. A study in a veterans extended care facility showed that the urinary tract was the source of over half the incidences of bacteraemic illnesses (Rudman *et al.*, 1988). The overall mortality rate was 21%, increasing to 35% for polymicrobial bacteraemias. The study also highlighted that although chronically catheterised patients only represented 5% of the patient population, they accounted for 40% of the Gram-negative bacteraemic illnesses, with the majority of catheterised men suffering from one or more each year. Since 90% of the patients had fevers with their episodes of bacteraemic illnesses, Rudman *et al.* (1988) suggest that patients in high risk catheterised groups should have their temperature monitored daily to lower the case fatality rate. Sir Andrew Clark in 1883 used the term “catheter fever” to describe the bouts of febrile illness in men that were healthy prior to the use of catheters to alleviate prostatic obstruction. The prognosis for the patients suffering from “catheter fever” was not good, and invariably resulted in the death of the afflicted patients.

A study of catheterised men in the community revealed that nearly all patients had infected urine after four weeks, and that 20 out of 197 catheter changes resulted in bacteraemia (Jewes *et al.*, 1988). Most patients, however, developed no clinical symptoms. These authors conclude that symptomatic bacteraemia may only be a very small proportion of the total bacteraemia associated with catheter use, and that even asymptomatic bacteraemia could lead to bacterial endocarditis in susceptible patients.

1.3.2. Pyelonephritis

Pyelonephritis is acute or chronic infection of the kidneys, and in catheterised patients it is usually caused by the ascent and invasion of bacteria from the bacteriuric bladder into the kidneys. In his editorial entitled “the case against the catheter” Beeson (1958) noted how the use of catheters increased the risk of acquiring pyelonephritis, and that the decision to catheterise a patient should be made in the knowledge that it can result in this serious disease. Two out of three febrile episodes in aged, long-term catheterised patients may arise from the urinary tract, and pyelonephritis is the source of many of these episodes (Warren, 2001). Warren *et al.* (1988) conducted a prospective clinicopathological study, examining the urinary tracts of 75 consecutively autopsied patients from a single Baltimore nursing home. The study revealed that 21 out of 55 catheterised patients had acute renal inflammation compared to one out of 20 non-catheterised patients. This study also highlighted that acute cystitis was associated with renal inflammation, 14 out of 18 kidneys in patients with acute cystitis also had renal inflammation. The duration of catheterisation is significantly associated with increasing the prevalence of chronic pyelonephritis and chronic renal inflammation (Warren *et al.*, 1994). A retrospective analysis of urologic complaints in male spinal injured patients revealed that those managed with an IUC

were significantly more likely to develop pyelonephritis than those who were not (Larsen *et al.*, 1997).

1.3.3. Cancer of the bladder

Studies of spinal cord injured (SCI) patients suggest that people who are catheterised for extended periods of time are at a higher risk of developing carcinoma of the bladder. One of the first to make this association was Melzak (1966), who showed that 11 out of 3800 patients at the national spinal injuries centre in Stoke-Mandeville Hospital (0.28%) developed bladder cancer. He noted that the only common factors amongst all patients that developed bladder cancer was a long-standing, chronic bladder infection and the presence of a long-term suprapubic or urethral indwelling catheter. More recent work has confirmed these findings. A retrospective population-based analysis of invasive treatments for carcinoma of the bladder revealed that spinal injured patients with IUCs were more than twice as likely to develop bladder cancer than patients using any other form of bladder management (West *et al.*, 1999). In a study of 3670 patients with SCI, indwelling catheters were shown to be a significant independent risk factor for increasing the risk of mortality by bladder cancer (Groah *et al.*, 2002). Bladder cancer occurred after 12 or more years of catheterisation and in patients as young as 29, the risk of developing bladder cancer in patients catheterised for more than 20 years was 398.1 per 100,000 person-years.

The precise mechanisms that contribute to the increased incidence of bladder cancers among SCI patients are unknown. One theory is that malignant transformation occurs following infection and chronic stimulation of the urothelium, which is brought about by the presence of the catheter (Akaza *et al.*, 1984; West *et al.*, 1999). A second

possible explanation is based on the theory that nitrate-reducing bacteria contaminating urine can produce nitrosamines, which are notorious carcinogens. Studies on patients in a paraplegic ward (Tricker *et al.*, 1991; Stickler *et al.*, 1992) confirmed that nitrate-reducing bacteria commonly infect the urine of SCI patients and that nitrosamines can be detected in patients' urine. The urine from a control group from the same hospital was found to be sterile and contained no nitrosamines, supporting the hypothesis that endogenously formed nitrosamines may be a risk factor for bladder cancer.

1.3.4. Infection stones

The formation of stones in the bladder and kidneys is a common problem amongst catheterised patients. The incidence of bladder stones in SCI patients has decreased over the past 30 years, however, an estimated 15% of all individuals who suffered a spinal cord injury between 1985 and 1996 still developed a bladder stone (Chen *et al.*, 2001). This study highlighted that the risk ratios for initial stone formation was more than three times higher in patients with IUCs than for individuals managed by intermittent catheterisation, catheter free care and condom drainage. A similar study by Larsen *et al.* (1997) revealed that bladder and renal stones were three times more prevalent in catheterised than non-catheterised patients. DeVivo *et al.* (1984) established that SCI patients with a bladder stone at hospitalisation were 8.6 times more likely to develop renal stones before discharge than patients who were free of bladder stones.

These stones can vary from being solid highly mineralised bodies to dispersed aggregates of crystals in an organic matrix (Stickler and Zimakoff, 1994), and are

formed due to chronic infections by urease producing bacteria (Griffith *et al.*, 1976). Microcolonies of urease producers are formed when the bacteria produce extracellular polymeric substances (EPS) that glue aggregates of bacteria together. These encapsulated microcolonies of urease producing bacteria, particularly of *Pr. mirabilis*, play a central part in the development of these stones. Positively charged calcium and magnesium ions are attracted to the anionic polysaccharide capsule. The production of the urease enzyme by the bacteria splits urea to ammonia and carbon dioxide, elevating the pH. The localised alkaline environment causes precipitation of calcium phosphate (apatite) and magnesium ammonium phosphate (struvite) crystals in the urine and in the EPS encapsulating the microcolonies. Continued bacterial growth in the aggregate colonies, along with the production of urease and EPS provides an ideal environment for the formation and attraction of crystals that together produce an infection stone (Clapham *et al.*, 1990).

Stone formations in the bladder are not usually life threatening, whereas renal stones can lead to dangerous complications such as chronic pyelonephritis and renal dysfunction. The bacteria in the stones are protected from adverse agents such as host factors and antibacterials by crystals and EPS. Bacteria from the stone can therefore easily re-infect the urine following antibacterial treatment regimes, and due to their high risk of recurrence, infection stones are often described as 'stone cancer' (Hedelin, 2002).

1.3.5. Catheter blockage

Catheter blockage due to encrustation is one of the biggest problems associated with the use of IUCs. As in the production of infection stones, encrustation is formed by

the precipitation of calcium and magnesium phosphates. The encrustation leads to persistence of infection and causes irritation to the bladder mucosa. The mineralised deposits can block the catheter lumen or eye-hole resulting in either urinary incontinence or retention. Urinary incontinence due to urine by-passing the catheter complicates the nursing care of the patient, and can lead to breakdown of the skin, bedsores and all the consequent problems. Urinary retention is painful and distressing and if it goes unnoticed, urinary reflux leads to ascending infection and the development of pyelonephritis, which can lead to episodes of fever, sepsis and shock (Kunin, 1987c). When blockage occurs, removal of the mineralised catheter is the only option, and often causes trauma to the urethra and bladder mucosa. A recent clinical study revealed that 62% of patients suffering from recurrent catheter blockage had bladder stones (Sabbuba *et al.*, 2004).

This problem is particularly distressing for community based patients and carers, where instant professional help is not available. Recurrent catheter blockage is also costly to health services, in terms of both time and resources, necessitating unscheduled visits and time-consuming procedures (Getliffe, 1990). The primary concern regarding catheter blockage, however, should always be the misery it causes the patient.

1.4. The control of catheter associated urinary tract infections

The maintenance of a closed drainage system is important in preventing UTI in the first week of catheterisation. Over the years several other methods trying to prevent the development of CA-UTI have been attempted.

1.4.1. Disinfection of the drainage bag

Extensive research efforts in the 1980's focused on disinfecting drainage bags with agents such as hydrogen peroxide, iodophors, chlorhexidine and trichloroisocyanuric acid (Stamm, 1991). The belief that eliminating bacteria in the drainage bag would lower the incidence of CA-UTI was, however, short-lived. Although these agents reduced contamination in the drainage bags, their effect on the overall rate of infection was minimal due to their infrequency as the source of infection. A study by Thompson *et al.* (1984) highlighted that only 7% of organisms contaminating the bag were also responsible for the bacteriuria. Gillespie *et al.* (1983) and Thompson *et al.* (1984) showed that disinfecting patients' drainage bags with either chlorhexidine or hydrogen peroxide had no significant effect on the rate of bacteriuria compared to patients' bags that were not disinfected.

1.4.2. Bladder washouts

Attempts to delay infection by irrigating the bladder with antimicrobial solutions appeared promising when they were first tested. Studies on the effect of intermittent irrigation showed that chlorhexidine and silver nitrate were effective in reducing the urinary bacterial counts of patients with infected catheterised urinary tracts, but that saline and acetic acid solutions were not (Bruun and Digranes, 1978). Chlorhexidine and silver nitrate lowered the numbers of all organisms isolated prior to bladder irrigation. It was concluded that although the antibacterial efficacy of chlorhexidine was not as good as the silver nitrate, its use would be favoured due to the pain associated with irrigating the bladder with silver nitrate. It is worth noting that this was a very small clinical study, with less than 10 patients receiving treatment with each type of washout solution.

Stickler *et al.* (1987) used a physical model of the catheterised bladder to assess the activity of povidone-iodine, phenoxyethanol, chlorhexidine, chlorhexidine supplemented with EDTA and tris, noxythiolin and neomycin as bladder instillation treatments for UTI in catheterised patients. Single applications of these antibacterials for 30 min were not capable of sterilising urine in the bladder infected singly with a range of six uropathogens. Phenoxyethanol was, however, effective at dealing with *Pv. stuartii* and *Ps. aeruginosa*. It was noted that daily washouts with these solutions would at best only produce a temporary decrease in the numbers of bacteria, which would have recovered back to their original numbers by the time for the next washout the following day. Similar work by King and Stickler (1991) established that only mandelic acid could eliminate a range of bacterial species commonly responsible for CA-UTI under conditions simulating heavy infections.

A clinical trial demonstrated that instilling chlorhexidine or saline into the bladder was not effective in reducing bacteriuria in patients catheterised for 10 days or more (Davies *et al.*, 1987). Kennedy *et al.* (1992) showed that bladder washouts with Suby G (a 3.23% citric acid solution) and Solution R (a 6% citric acid solution) did not eliminate bacteria or have any effect on urease producing bacteria. A study in which 106 hospital and district nurses were interviewed about their experiences with bladder washouts revealed that more than half were recommending chlorhexidine washouts for long term catheterised patients (Roe, 1989). This is despite the fact that no antiseptic, especially chlorhexidine can eradicate infections in long-term catheterised patients (Stickler and Thomas, 1980; Slade and Gillespie, 1985). Nearly half of the nurses had a non-rational reason to support the prescribing of these solutions, and some relied exclusively on manufacturers' recommendations, which concerning the

use of chlorhexidine as a washout solution is not supported by clinical and scientific evidence (Roe, 1989). There is still confusion about when bladder washouts should be performed and their effectiveness (Evans and Godfrey, 2000). Although bladder washouts may benefit patients whose catheters block regularly, for patients whose catheters drain freely their use is unnecessary and it is best to maintain the closed drainage system (Roe, 1989).

1.4.3. Cleansing

The importance of infections stemming from the colonization of the peri-urethral pathway in CA-UTI has been confirmed, and is especially significant in female patients (Garibaldi *et al.*, 1980). It was therefore logical to assume that cleansing or using antibacterial ointments on this area would prevent bacterial meatal entry. However, Burke *et al.* (1981) showed that daily meatal cleansing with soap and water or with a povidone-iodine solution followed by the application of povidone-iodine ointment was ineffective at lowering rates of bacteriuria, and actually increased rates in a subset of high-risk female patients. The use of a poly-antibiotic ointment twice daily was shown to decrease bacteriuria by 1% compared to the control group, indicating that meatal care with such ointments confer little benefit (Burke *et al.*, 1983).

1.4.4. Systemic and oral administration of antibiotics

Antibiotics are not generally prescribed to control CA-UTI, and there is a consensus that patients with asymptomatic bacteriuria should not be treated with antibiotics. Their administration should be to those patients showing clinical symptoms of infection (Warren, 1991). Britt *et al.* (1977) evaluated the effectiveness of short-term

systemic antibiotic prophylaxis in controlling catheter-associated bacteriuria in women undergoing gynecological operations. They discovered that nine out of 100 women in the placebo group developed bacteriuria compared to three out of 96 receiving the antibiotics. However, the benefit of receiving the antibiotics was short lived since at the time of discharge from the hospital, both these groups had similar rates of positive cultures. It is now accepted that administration of antibiotics only produces short-term benefits, and that after only a few days the sensitive uropathogens are replaced by resistant ones (Garibaldi *et al.*, 1974; Clayton *et al.*, 1982; Kunin, 1987c; Warren, 1996). It must also be noted that if bacteria growing on catheters have formed biofilms, although antibiotics eliminate microorganisms in the urine, bacteria in the biofilm remain viable and can cause re-infection after the antibiotic course had concluded (Nickel, 1991). The routine use of antibiotics is therefore limited by their lack of effectiveness and the possible emergence of resistance (Garibaldi *et al.* 1974; Stamm, 1991; Warren 1996). The use of prophylactic antibiotics should only be considered as a preventative method in high-risk patients for short-term periods of catheterisation (Britt *et al.*, 1977; Stamm, 1991).

1.4.5. The incorporation of antimicrobials into the catheter

The search for an IUC that would prevent infection led to antimicrobial agents being used to coat or impregnate catheters. An ideal antimicrobial agent should therefore; have a broad spectrum of antimicrobial activity, persist for the lifetime of the catheter, not be altered by contact with body fluids and not select for drug resistant organisms (Stickler, 2000).

The wide antimicrobial spectrum of silver against nosocomial pathogens, along with its ability to satisfy many of the above criteria led to several manufacturers coating urethral catheters with silver oxide or metallic silver coated surfaces. Gabriel *et al.* (1996) showed that primary adherence of *E. coli* was reduced 25% - 50% using hydrogel silver-coated compared to hydrogel-coated latex catheters. It must be noted, however, that this study only monitored the adherence of bacteria to the catheters over a very short time period (2 h).

Clinical studies on silver-modified catheters have provided inconsistent results. A large randomised clinical trial failed to demonstrate the efficacy of silver impregnated IUCs in preventing CA-UTI (Riley *et al.*, 1995). This study showed that not only did the silver catheters increase the incidence of bacteriuria in men, they also significantly increased the rate of staphylococcal bacteriuria. Bologna *et al.* (1999) noted a trend towards a reduction in UTI with the use of hydrogel-silver coated catheters, although the results were not significantly different from those not coated with silver. Liedberg and Lundeberg (1990) showed that there was a significant decrease in UTI when silver alloy coated catheters were used in short-term catheterised patients (six days or less).

A meta-analysis of eight clinical trials performed by Saint *et al.* (1998) compared whether silver-coated IUC were likely to lead to lower rates of UTI than standard catheters. Their results revealed that silver alloy catheters were significantly more effective in preventing bacteriuria, whereas silver oxide catheters were not. Saint *et al.* (1998), along with others (Schierholz *et al.* (1999) and Niel-Weise *et al.* (2002)) note that the findings of this study should be interpreted with caution. The clinical trials

were all subject to different interventions and were performed on different study populations, the results using the silver alloy catheters were all carried out by the same institution and the analysis only compared bacteriuria, and not the clinical outcome. A review by Niel-Weise *et al.* (2002) highlighted a lack of high standard trials related to the effectiveness of silver coatings on IUCs, and concluded that because of this, there is insufficient evidence to recommend the use of these devices.

The conflicting results regarding the use of silver-coated catheters indicate that they may give minimal protection against CA-UTI. There is no evidence that they are effective in preventing infection in patients undergoing long-term catheterisation. At best they may delay bacteriuria in short-term catheterised patients. This said, there are different methods of introducing silver into the catheterised urinary tract that may prove more effective. A silver iontophoretic catheter with two silver wires connected to an electric power source has been shown to have a broad spectrum of activity against Gram-negative and positive bacteria (Raad *et al.*, 1996). A lecithin, silver citrate and liquid silicone mixture has also been shown to be more effective at preventing intraluminal ascent of bacteria than other silver coatings (Kumon *et al.* 2001).

Early work impregnating catheters with antibiotics showed no efficacy in preventing CA-UTI (Butler and Kunin, 1968). The possible outcome of impregnating catheters with antibiotics could be particularly hazardous. The release of sub-inhibitory concentrations of antibiotics from the catheter, together with the presence of high numbers of different bacterial species in the catheterised urinary tract could lead to the selection of bacteria with decreased antibiotic sensitivities, aggravating the problems

of CA-UTI and drug resistance. Notwithstanding these concerns, antibiotic impregnated catheters continue to be developed.

The antimicrobial activity and durability of minocycline and rifampin impregnated catheters were tested in spinal injured patients for a maximum of two weeks. On removal of the catheters, sections were cut from the devices and tested for antibacterial activity against lawns of bacteria. Zones of inhibition were observed against 10 tested uropathogenic species including *Ps. aeruginosa* and *Pr. mirabilis* (Darouiche *et al.* 1997). A clinical trial evaluating the short-term efficacy of these catheters revealed that patients who received the antimicrobial impregnated catheters had significantly lower rates of Gram-positive bacteriuria compared to the control group, but similar rates of Gram-negative bacteriuria and candiduria (Darouiche *et al.*, 1999b). Testing organisms isolated before and after the insertion of minocycline and rifampin impregnated central venous catheters (CVCs) revealed similar antibiotic sensitivities (Darouiche *et al.*, 1999a). Although this indicates that bacterial strains with decreased susceptibilities had not developed, the use of such catheters should be coupled with a continued surveillance programme that monitors resistance.

Darouiche *et al.* (1997) and Darouiche *et al.* (1999b) both comment that using combinations of antimicrobials with different modes of action may prevent the development of bacterial resistance. Tambe *et al.* (2001), however, discovered a 10- to 16-fold increase in minimum inhibitory concentration (MIC) after 10 - 20 subcultures through sub-inhibitory concentrations of the combination of minocycline and rifampicin, but noted that resistant bacteria had not yet emerged in the clinical setting.

An *in vitro* study evaluated the activity of nitrofurazone-coated urinary catheters against six species of susceptible and multidrug resistant (MDR) bacteria (Johnson *et al.*, 1999). Using an agar diffusion assay it was established that the nitrofurazone-coated catheters showed comparable inhibition zones with susceptible and MDR strains for each species tested except for vancomycin resistant *Enterococcus faecium*. A limited number of bacterial species were tested in this study, and some very important urinary tract pathogens including *Ps. aeruginosa* and *Pr. mirabilis*, species intrinsically resistant to nitrofurazone, were not tested. The complications associated with *Pr. mirabilis* indicate that the impact of these catheters on long-term catheter care will be negligible.

Reid *et al.* (1994) using an *in vitro* assay demonstrated that pre-treatment of silicone-coated latex catheters with ciprofloxacin significantly reduced adhesion and survival of a clinical isolate of *Ps. aeruginosa*. Stickler *et al.* (1994) confirmed that pre-treatment of sections of silicone-coated latex catheters with ciprofloxacin inhibited bacterial colonization of suspensions of cells in buffer, although cells in urine colonized both control and ciprofloxacin treated catheter sections equally well. Subsequent experiments in physical models of the bladder demonstrated that within 48 h *Ps. aeruginosa*, *Pv. stuartii*, *Pr. mirabilis* and *E. coli* were all capable of colonizing control and treated catheter sections.

Using an agar inhibition method, the antimicrobial efficacy of Foley catheters (latex and silicone) impregnated with 1) chlorhexidine and silver sulfadiazine (CXS) and 2) chlorhexidine, silver sulfadiazine and triclosan (CXST) were compared to the silver hydrogel-coated latex catheter and nitrofurazone-coated silicone catheters (Gaonkar *et*

al., 2003). The CXS and CXST catheters both had an increased spectrum of antimicrobial activity compared to the silver- and nitrofurazone-coated catheters. This group also devised a novel *in vitro* urinary tract model to study the ability of these catheters to prevent extraluminal migration of bacteria into the bladder. Overall, the CXST catheters exhibited a broader antimicrobial spectrum and significantly increased the duration of antimicrobial activity compared to the silver- and nitrofurazone-coated catheters (Gaonkar *et al.*, 2003). The group concluded that the greater efficacy of the CXST catheters compared to the CXS catheters might be due to the synergistic effects of all the different agents. It is surprising, however, that they did not test the migration of *Pr. mirabilis* along the various antibacterial catheters or suggest that triclosan alone could be responsible for the increased efficacy. The extrapolation that zone of inhibition size could predict the clinical efficacy of a catheter in some of the above experiments is dangerous (Stickler, 2000), and should generally only be used as a starting point on which to base further investigations.

Reid (1999) made a suggestion that oral administration of antibiotics could be a way of coating the surfaces of medical devices. Reid *et al.*, (2001) showed that oral administration of ciprofloxacin and ofloxacin in 40 patients led to the antibiotics being adsorbed onto their ureteral stents. This method should be approached with caution as such indiscriminate and overuse of antibiotics would surely provide the perfect environment for the selection of bacteria with decreased antibiotic sensitivities, exacerbating and not controlling the problem of CA-UTI.

1.4.6. Other measures for controlling CA-UTI

The selection of resistant organisms after the introduction of antiseptic agents should be considered carefully. Stickler and Thomas (1980) tested the sensitivity of over 800 isolates of Gram-negative bacteria to different antiseptic agents, and revealed that approximately 10% of isolates exhibited a degree of resistance to cationic agents. These resistant organisms were *Proteus*, *Providencia* or *Pseudomonas* species, and were also generally resistant to five to seven antibiotics. The resistant strains were mostly isolated from patients where chlorhexidine washes were used to clean the urethral meatus prior to catheter insertion, chlorhexidine creams were used to facilitate catheter insertion and in some cases chlorhexidine was added to the drainage bags. From these results it was concluded that antiseptic policies that rely on the intensive use of cationic antiseptics such as chlorhexidine could well result in the selection of bacterial flora that are notoriously drug resistant. Clinical evidence supporting this warning soon followed.

Instilling chlorhexidine or saline into the bladder was not effective in reducing bacteriuria in patients catheterised for 10 days or more, and there was an increase in patients with *Proteus* bacteriuria (Davies *et al.*, 1987). Out of 24 patients, six had *Proteus* bacteriuria pre-washout but this increased to 11 patients after completing a course of chlorhexidine instillations. Dance *et al.* (1987) report on an outbreak of UTI between 1980 and 1985 involving strains of *Pr. mirabilis* resistant to gentamicin and several other antibiotics that affected 90 patients in Southampton. This outbreak occurred shortly after the introduction of extensive chlorhexidine use into the catheter care policies. It is possible that overuse of chlorhexidine impregnated catheters could result in a similar scenario. Gaonkar *et al.* (2003) note that “choosing antimicrobials

with a lower risk of inducing microbial resistance for incorporation into medical devices is critical”.

There is little doubt that many patients are catheterised unnecessarily (Saint *et al.*, 2000; Gokula *et al.* 2004). A study by Saint *et al.* (2000) revealed that physicians and medical students were often unaware that their in-patients had IUCs. It was also noted that when catheters were used inappropriately, they were ‘forgotten’ about more frequently than the appropriately used ones, and if physicians are unaware that a catheter is in place they are unlikely to order its removal. Currently there are no effective measures for preventing CA-UTI, the best way to decrease the incidence of infection associated with these devices is to avoid their use, or reduce the length of time they remain *in situ*.

1.5. Bacterial biofilms

Costerton *et al.* (1995) defined biofilms as “matrix-enclosed populations adherent to each other and/or to surfaces or interfaces”. In aquatic environments bacteria prefer to grow on surfaces rather than in suspension. For this reason larger numbers of bacteria are found as sessile biofilm communities than as single planktonic cells. Biofilms can be found in all aquatic environments from streams to sewers, and food fermenters to ship hulls. Advances in modern medicine have led to the increased use of medical devices and prostheses over the past decades, which continue to grow 7% per annum (Ried *et al.*, 1995). Persistent microbial infection, however, is one of the leading causes of failure of these devices, and biofilms are typically central to the microbial pathogenesis of these troublesome infections (Donlan and Costerton, 2002).

Microorganisms undergo profound changes as they transform from planktonic organisms to surface attached communities. These changes produce new phenotypic characteristics for cells growing in biofilms and occur in response to environmental factors (O'Toole *et al.*, 2000). The catheterised urinary tract provides an ideal environment for biofilm formation, with infected urine flowing over the catheter surface for prolonged periods of time. Clinical and laboratory studies have revealed that biofilms form readily on catheter surfaces. Nickel *et al.* (1989) used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to show that the biofilm mode of growth is a predominant factor in the pathogenesis and persistence of CA-UTI. Ohkawa *et al.* (1990) using SEM revealed the presence of biofilms on the catheters of 13 out of 14 patients catheterised for 14 days or more. Ganderton *et al.* (1992) found biofilms on 44 out of 50 catheters that had been indwelling for three to 83 days. Extensive biofilm formation has also been observed on catheters removed from physical models of the catheterised urinary tract within 24 h of heavy inoculation with *Pr. mirabilis* (Stickler *et al.*, 2003).

The first step in biofilm formation is bacterial attachment to a surface. The surfaces of implanted devices are coated with glycoproteinaceous conditioning films from the surrounding body fluids containing fibronectin, fibrinogen and other proteins almost immediately after they are inserted. The components of the conditioning films provide receptor sites for bacterial adhesion (Gristina, 1987). The conditioning film plays an important role in influencing subsequent adsorption and attraction of microbes to the medical devices (Lappin-Scott and Bass, 2001). Ohkawa *et al.* (1990) collected IUCs from patients that were catheterised for short periods of time. The studies revealed fibrin on the surface of catheters removed from the patients within three days. They

concluded that this material originated from host cells injured during catheter insertions, and that this film was important in gluing microorganisms and crystals from the urine onto the catheter. The protein pattern of conditioning films on encrusted and non-encrusted ureteral stents have different electrophoresis profiles (Santin *et al.*, 1999). Western blots revealed that adsorption of Tamm-Horsfall protein and α 1-microglobulin were limited to non-encrusted devices.

Microbial cells in the vicinity of a medical device by direct contamination, transcutaneous spread or by seeding from infected bodily fluids that flow over the surface of the device, are drawn to the surface by physical forces such as hydrophobic interactions and electrostatic attractions (Stickler and McLean, 1995). When a bacterium is in very close proximity to the surface, short-range chemical interactions (ionic, hydrogen and covalent bonding) may occur (Gristina, 1987). Specific adhesions mediated by fimbriae are not strong. Firm adhesion requires the non-specific interaction of the elastic polymers of the extracellular polymeric substances (EPS) layer, even when the fimbriae dictate the site of eventual adhesion with specific receptors (Costerton, 1999).

The attached bacterial cells must synthesise new EPS in order to 'cement' their adhesion and progress from reversible attachment to the irreversible adhesion phase of biofilm formation (Costerton, 1999). Cell division then produces sister cells within the EPS, initiating the development of adherent microcolonies (Costerton *et al.*, 1987). Depending on the bacterial species and the level of nutrients in the surrounding media, the microcolonies will contain 10 - 25% cells and 75 - 90% EPS (Costerton, 1999). Glycocalyx, slime, capsule and sheath are all terms referring to EPS associated with

individual or cell aggregates, and biofilms (Characklis and Cooksey, 1983). The glycocalyx is defined as “tangled fibres of polysaccharides or branching sugars” (Costerton *et al.*, 1978). However, in microbial adhesion and biofilm formation in general, other macromolecules besides polysaccharides and sugars are found within the organic matrix, including glycoproteins, proteins and nucleic acid (Characklis and Cooksey, 1983). The organic matrix will therefore be referred to as EPS. Cell division within these microcolonies and recruitment of bacteria from the planktonic phase eventually results in extensive surface colonization. Nickel *et al.* (1992) showed that bacteria could migrate along a catheter surface against gravity and the flow of urine. Their study showed that after an initial “log phase” characterised by biofilm build up at the site of contamination, there is a “creep phase” characterised by biofilm movement.

Biofilms are not continuous layers of bacteria and EPS as once thought. Direct observations of living biofilms, particularly by confocal laser microscopy and other non-invasive physical methods have revealed that they are elaborate and complex structures. Caldwell *et al.* (1992a) and Caldwell *et al.* (1992b) showed how confocal laser microscopy used in conjunction with 3-D image analysis could create computer reconstructions of 3-D biofilms, allowing their development to be visualised directly. Biofilms may be comprised of a single or multiple bacterial species. Costerton *et al.* (1995) described how many microcolonies develop into mushroom like structures, and that most cells are at the top of the mushroom, whilst there are very few down the ‘stalk’. These microcolonies are arranged both horizontally in thin biofilms, and vertically in thick biofilms. Water-filled channels were also observed throughout the biofilms that could take nutrients into, and take waste out of the biofilm. It was noted,

however, that it is yet to be established whether such elaborate microcolonies and fluid channels are found in all biofilms.

Recent evidence has suggested that biofilm development might be regulated at the level of population-dependent gene expression controlled by cell-to-cell signalling molecules (O'Toole *et al.*, 2000). Acylated homoserine lactones (AHLs) are signal molecules that mediate population density-dependent (quorum sensing) gene expression in many Gram-negative bacteria (Fuqua *et al.*, 1996). Davies *et al.* (1998) showed that a mutation blocking the generation of the quorum sensing signalling molecules in *Ps. aeruginosa* prevented the formation of a normally differentiated biofilm, producing thin biofilms of densely packed cells. Stickler *et al.* (1998a) were the first to demonstrate that AHLs were produced by biofilms in the clinical setting. It was shown that *Ps. aeruginosa* biofilms that had developed in urinary catheters *in vivo* produced AHLs. Once the biofilm has reached a critical mass, a dynamic equilibrium is achieved when the outermost layer of the biofilm begins to generate planktonic organisms (Dunne, 2002). Using digital time-lapse microscopy imaging (DTLM) Lappin-Scott and Bass (2001) were able to show that loss of bacteria and small clusters of bacteria via erosion was a continuous process, as opposed to sloughing of large microcolonies. The organisms that are released from the biofilm are then free to colonize new surfaces (Dunne, 2002).

The biofilm mode of growth protects bacteria against antimicrobial agents and host defence mechanisms making them difficult to control and eradicate. Clinical observations have revealed that within the protection of the biofilm, bacteria that are sensitive to an antimicrobial agent in standard tests survive urinary concentrations of

antibiotics generated by conventional treatments. Nickel *et al.* (1985a) demonstrated that whilst a *Ps. aeruginosa* planktonic culture had tobramycin MIC of less than 1 µg/ml and a MBC of 50 µg/ml, the same strain growing as a thick biofilm on the surface of a latex catheter could survive exposure up to 1000 µg/ml. This concentration is at least five times higher than can be clinically achieved in the patients' urine. The failure of antibacterial washout solutions to clear established bladder infections can also be attributed to the increased resistance of bacteria growing in biofilms (Stickler *et al.*, 1987). Biofilm formation on surfaces is regarded as a universal bacterial strategy for both survival and optimal positioning with regard to available nutrients (Costerton *et al.*, 1987).

Although it is now accepted that bacteria in biofilms are generally more resistant to antibacterials than their planktonic counterparts, susceptibility tests of microorganisms using planktonic growth conditions are still used to predict their efficacy against biofilm bacteria. This is particularly true for medical devices, when antimicrobial therapy is based on antibiotic sensitivity data obtained using planktonic organisms in nutritionally rich media (Dunne, 2002). In reality these growth conditions could not be further from the conditions found in biofilms on medical devices. Anwar *et al.* (1990) suggested that a new more realistic *in vitro* test should be developed to study the dose and frequency of antibacterial agents required to treat infections involving medical devices. Costerton *et al.* (1987) considered that tests using planktonic cultures for biofilm-related problems yield useful information, such as their efficacy against planktonic cells that detach from the biofilm, or that negative results rules out their usefulness for treating biofilms. If the objective of an antimicrobial treatment is to treat biofilms however, the agent must be tested against bacteria in biofilms.

1.6. The resistance of bacterial biofilms

Bacteria in all sorts of biofilms share one common characteristic,- their ability to resist antimicrobial activity. When biofilms are formed on medical devices this characteristic extends to their ability to evade host defence mechanisms. Although current evidence does not exclude the possibility that conventional resistance mechanisms such as efflux pumps are expressed in biofilms, it is necessary to look beyond them to understand biofilm resistance (Stewart and Costerton, 2001). The protection conferred to bacteria in biofilms is due to a multi-cellular mode of growth. Several hypotheses have been proposed to try and explain the mechanisms responsible for the increased resistance shown by bacteria in biofilms (Donlan and Costerton, 2002).

A biofilm's most distinguishing feature is its ability to produce an exopolysaccharide matrix. For years the main hypothesis regarding the resistance of biofilms centred on the ability of the matrix to protect cells embedded in the biofilm by slowing down or preventing penetration of antimicrobials into the biofilm. Some research has shown that the biofilm can affect the diffusion of antimicrobial agents, for example Hoyle *et al.* (1992) showed that *Ps. aeruginosa* biofilms retarded the diffusion of piperacillin. Using a microelectrode, de Beer *et al.* (1994) demonstrated that only one fifth of the chlorine concentration in the bulk of the liquid penetrated the biofilms of *Ps. aeruginosa* and *K. pneumoniae*. The chlorine penetration profile of the biofilm also suggested that the chlorine was being consumed within the matrix. Other work however, has shown that the biofilm poses no barrier to the diffusion of other antimicrobial agents. Vancomycin and rifampicin are both able to diffuse through *Staphylococcus epidermidis* biofilms (Dunne *et al.*, 1993). However, although

rifampicin can penetrate *S. epidermidis* biofilms, it fails to effectively kill the bacteria in the biofilm (Zheng and Stewart, 2002). These authors suggest that the slow growth of the bacteria in the biofilm may be responsible for this protection. Hydrogen peroxide can penetrate thin but not thick biofilms of *Ps. aeruginosa* (Cochran *et al.*, 2000). The increased resistance of thin biofilms to hydrogen peroxide compared to planktonic cells must therefore be attributed to another resistance mechanism.

Inactivation of antimicrobial compounds could have a significant effect on their ability to kill bacteria in biofilms. Catalases have been shown to protect *Ps. aeruginosa* biofilms by preventing full penetration of hydrogen peroxide (Stewart *et al.*, 2000). Anderl *et al.* (2000) tested the penetration of ciprofloxacin and ampicillin into *K. pneumoniae* biofilms. Ciprofloxacin was shown to penetrate the biofilms quickly, whilst ampicillin failed to do so. Ampicillin penetrated the biofilms created by a β -lactamase deficient *K. pneumoniae* mutant, but failed to kill the bacteria within the biofilm. Bagge *et al.* (2004) monitored β -lactamase expression in *Ps. aeruginosa* biofilms exposed to antibiotics. β -lactamase expression was induced in the whole biofilm when exposed to high concentrations of imipenem, but only at the peripheries of the microcolonies at sub-MIC concentrations. Only the bacteria at the peripheries of the microcolonies were induced to form β -lactamases when exposed to ceftazidime, even at high concentrations. These experiments confirm that β -lactamase production in biofilms can increase in response to antibiotic exposure and show the heterogeneity of β -lactamase induction in growing biofilms.

These conflicting results indicate that the ability and extent to which antimicrobial agents penetrate a biofilm depends on the type of antimicrobial agent and the target

organism in the biofilm. It appears that slowing down or inhibiting antimicrobial penetration alone cannot explain the inherent resistance of bacteria in biofilms to antimicrobials.

Bacteria growing as biofilms are expected to experience an altered chemical environment depending on their position within the biofilm. Studies monitoring the oxygen content of biofilms have revealed that the outer layer of the biofilm can completely consume the available oxygen, leading to an anaerobic environment within the deep layers of the biofilm (de Beer *et al.*, 1994). Some form of micro-scale nutrient gradient is also expected within the biofilm, and the concentrations of substrates present are mirrored by that of metabolic products (Stewart and Costerton, 2001). It has been suggested that physiological changes (slower growth rates or activation of a starved state) in response to altered chemical environment, nutrient limitation or accumulation of inhibitory waste products may be responsible for the increased resistance of bacterial biofilms. Most antibacterial agents are more active against rapidly growing cells. Penicillin and ampicillin will not kill non-growing cells, whilst other β -lactams, cephalosporins, aminoglycosides and fluoroquinolones can kill non-growing cells, but not as effectively as rapidly growing ones (Lewis, 2001). Noting that planktonic stationary phase *K. pneumoniae* cultures were not killed by ampicillin or ciprofloxacin, especially in experiments with medium lacking carbon and nitrogen sources, Anderl *et al.* (2004) suggested that tolerance of biofilm bacteria may be explained by at least some cells in the biofilm being in stationary phase due to nutrient limitation. Their suggestion was supported by their experiments showing that glucose could not penetrate the entire biofilm and that oxygen only penetrated the upper biofilm level. This study showed that whilst the specific growth rate for

planktonic bacteria was 0.59 h^{-1} , it was only 0.032 h^{-1} for biofilm bacteria. These results suggested that local nutrient limitation within the biofilm that causes bacteria to enter stationary phase results in bacteria less susceptible to antibiotics.

Several studies have examined the effect of growth rates on the sensitivity of biofilm cells to antibacterial agents. Eng *et al.* (1991) controlled bacterial growth rates by limiting nutrient availability and showed that only the fluoroquinolones (ciprofloxacin and ofloxacin) were bactericidal against stationary phase Gram-negative organisms. The other antibiotics tested included gentamicin, nafcillin, cefotaxime, piperacillin, imipenem, meropenem and rifampicin. However, an increase in nutrients, and subsequent rise in growth rates resulted in the increased activity of multiple classes of antimicrobial agents. The susceptibility of *Ps. aeruginosa* and *E. coli* in chemostat grown cultures (planktonic cells) to ciprofloxacin has been shown to be directly related to the growth rates, with increased growth rates leading to increased bacterial sensitivities (Evans *et al.*, 1991). Increasing the growth rates in biofilms of non-mucoid strains of *Ps. aeruginosa* increased their susceptibility to ciprofloxacin, but had a minimal effect on the susceptibilities of mucoid strains of *Ps. aeruginosa*. Further studies revealed the increased susceptibility of *S. epidermidis* biofilms and planktonic cultures to ciprofloxacin and tobramycin at higher growth rates (Duguid *et al.*, 1992a; Duguid *et al.*, 1992b).

Desai *et al.* (1998) compared the resistance of planktonic and biofilm cultures of *Bulkholderia cepacia* to ciprofloxacin and cefazidime at different stages during exponential growth. Bacteria growing as biofilms were about 15-times more resistant to the antibiotics than the equivalent planktonic bacterial cultures, and resistance to

the antibiotics increased progressively during exponential growth phase. The dramatic change in resistance before a measurable reduction in growth indicates that the changes in resistance are attributed to factors other than changes in growth rates. These authors suggested that the increased resistance might be attributed to a mechanism that detects a change in environmental conditions, and then initiates the increased resistance.

A more recent hypothesis regarding the increased resistance of biofilms involves the concept that a sub-population of biofilm cells differentiate into a spore-like state, which are highly protected. This is an attractive concept as it could explain why biofilms are resistant to such a wide range of antimicrobials (Stewart, 2001). This hypothesis unlike the previous ones is not activated by diffusion limitation (antimicrobials or nutrients) and is supported by experiments performed by Cochran *et al.* (2000). These authors remarked that since thin biofilms of *Ps. aeruginosa* were still resistant to hydrogen peroxide and minocycline, that the resistance could not be attributed to transport problems through the biofilms, and they suggested that the resistance of these biofilms was due to an inherent phenotypic change that occurs on cell attachment to a surface. Further work by Brooun *et al.* (2000), showed a “plateau” on a biofilm killing plot of quinolones against *Ps. aeruginosa* biofilms. They suggested that although most cells were killed by the quinolones, a resistant sub-population persisted despite continued exposure to the antibiotic. Since this mechanism is genetically encoded, it could reveal new targets for novel anti-biofilm approaches (Cochran *et al.*, 2000).

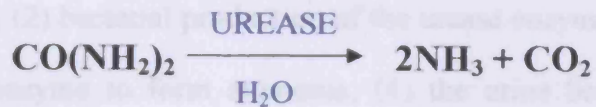
Reviewing the different mechanisms of biofilm resistance, Mah and O'Toole (2001) concluded that no one answer will do in response to the question of why cells growing in a biofilm mode of growth have an increase resistance to antibacterial agents. It is most likely that several mechanisms are responsible, and that they are dependent on the species of bacteria in the biofilm and the type of antimicrobial agent.

1.7. Catheter blockage and crystalline bacterial biofilm formation

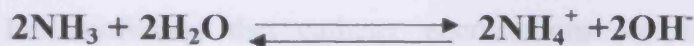
One of the most serious complications involving the long-term use of IUCs is the formation of encrustation on the catheter balloon and luminal surfaces. Kohler-Ockmore and Feneley (1996) carried out a two-year prospective study investigating the complications associated with the long-term use of IUCs. For the 457 patients that were being monitored, 506 emergency referrals were recorded in the first six months of the study period. Of these complications, 149 were referred for emergency hospital treatment and 60% of these complications were due to catheter blockage. A more detailed 12-week study of 54 patients in the second year revealed that 48% experienced catheter blockage. Getliffe (1990) and Getliffe (1994) reported that 54% and 43% of long-term catheterised patients in the community suffered from recurrent encrustation and blockage. A retrospective study of 69 long-term catheterised nursing home patients revealed that only 18% of catheters were replaced according to the once-a-month protocol, and in 54% of cases the catheters needed to be changed before this time because of catheter obstruction (Cools and Van der Meer, 1986). Most of the obstructed catheters had to be replaced in the second or third week of catheterisation. Mobley and Warren (1987) over a one-year period studied 32 catheterised women who had silicone-coated latex catheters in place for 100 days or more, 23 patients experienced at least one catheter obstruction (a total of 67 blockages).

Kunin *et al.* (1987a) used the terms “blocker” and “non-blocker” to broadly distinguish these two groups of patients. The “blockers” were patients that formed sufficient catheter encrustation to alter the flow of urine through the catheter within the first two weeks of insertion, whilst the urine flow through the catheter of “non-blockers” was not affected by encrustation. Kunin *et al.* (1987b) found this clinical distinction useful in deciding what catheter material to use in both patient groups. The “non-blockers”, who constituted half the patient population, did well regardless of the catheter material, whilst there was significantly less encrustation with silicone catheters than silicone- and teflon-coated latex catheters for the “blockers” after a 14-day trial period. To gain further insight into this phenomenon, Kunin (1989) studied patients that were grouped as “blockers”, “intermediates” and “non-blockers”. There were no significant differences among the groups for age, activities of daily living, mental status, use of antibiotics, clinical manifestations of urinary infection or incidence of fever. “Blockers” excreted urine that was significantly more alkaline, and that contained less magnesium, urea and phosphate. *Pr. mirabilis* and *Pv. stuartii* colonized the urine of “blockers” significantly more often than “non-blockers”. Patients tended to remain as “blockers” or “non-blockers” consistently over time. Mobley and Warren (1987) also found that *Pr. mirabilis*, but not the other urease-positive species was significantly associated with catheter obstruction. Similarly, bacteriological testing of catheters removed from patients undergoing long-term bladder management by Stickler *et al.* (1993) revealed that the most extensive encrustation was present on catheters colonized by pure cultures of *Pr. mirabilis*, whilst significant crystalline material was absent in non-*Proteus* biofilms.

Production of the urease enzyme is central to the development of catheter encrustation (Mobley *et al.*, 1995). Infections of the urinary tract with urease producers, particularly *Pr. mirabilis*, hydrolyse the urea in the urine increasing urinary ammonia, bicarbonate, carbonate and alkalinity (Griffith *et al.*, 1978).



Further hydrolysis yields:



The resulting alkalinity produces an environment in which magnesium and calcium phosphates are insoluble. Crystals of struvite (magnesium ammonium phosphate) and hydroxyapatite (a form of calcium phosphate) appear in the urine. Using jack bean urease, Hedelin *et al.* (1985) were able to show that crystallisation of calcium phosphates precedes the crystallisation of struvite. Studying encrustation on catheters taken from patients at a chronic care facility, Bruce *et al.* (1974) cultured *Proteus* from seven of the 14 patients, and in six of these seven patients high levels of calcium, phosphorus and magnesium were found in the catheter encrustation.

A SEM study of 32 encrusted urinary catheters showed that the encrustation consisted of blocky struvite crystals surrounded by very small amorphous crystallites of hydroxyapatite (Cox *et al.*, 1989a). The study showed that layers of tightly packed bacteria were visible underneath the minerals, and that crystals were often engulfed in

a bacterial layer. This process not only binds the crystals of both minerals together, it helps bind the continually forming crystalline deposits to the catheter surface. Cox *et al.* (1989a) therefore proposed that the sequence of events leading to the encrustation of catheters comprises of five steps: (1) colonization of the catheter surface by a urease producer, (2) bacterial production of the urease enzyme, (3) hydrolysis of urea by the urease enzyme to form ammonia, (4) the urine becomes alkaline and (5) struvite and hydroxyapatite crystals are deposited. A better understanding of biofilm formation by urease producing bacteria and the use of chemical analysis and electron microscopy has confirmed that catheter encrustations are mineralised bacterial biofilms.

Flow cell studies with *Pr. mirabilis* have shown that cells attach to the glass slides either singly or as small clumps, where they grow slowly and multiply (McLean *et al.*, 1991). Deposition of organic matter onto the glass slides was shown to immediately precede struvite crystallisation. The struvite crystals were shown to form within the *Pr. mirabilis* biofilm, and the biofilm afforded a significant degree of protection to these crystals against dissolution under conditions of increased flow rates. Studies by Ohkawa *et al.* (1990) noted that microorganisms and crystals were attached onto the catheter surface of IUCs of patients that had been catheterised for a week or more, the study also highlighted an intimate association between the bacteria and struvite crystals. *In-vitro* experiments have also shown the importance of the EPS to stabilise crystal growth (Clapham *et al.*, 1990). These experiments showed that crystals formed in the EPS have growth habits that reflect a higher supersaturation than those formed in the surrounding urine, i.e. that the bacterial presence in the biofilm provides an environment which facilitates struvite crystal growth.

Winters *et al.* (1995) using physical models of the catheterised bladder showed that heavy infection with *Pr. mirabilis* could encrust and block urinary catheters within 48 h. This work also showed that bacteria and crystals that form in the urine can adhere to the catheter, and that continued colonization and bacterial growth on the catheter and crystal surfaces produced an alkaline mineralised biofilm. SEM of freeze-fractured cross sections of the catheters showed that the encrustation completely occluded the lumen. X-ray microanalysis revealed that the material in and around the cell-like structures was calcium, phosphates and oxygen together with a small amount of magnesium. Further experiments using the models showed that urease positive *M. morganii*, *K. pneumoniae* and *Pv. stuartii* failed to raise the urinary pH and form crystalline bacterial biofilms (Stickler *et al.*, 1998b). In contrast *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* increased the urinary pH from 6.1 to 8.3 - 8.6, and extensive catheter encrustation was observed within 24 h. It was concluded that while many bacterial species can form biofilms on catheters, only organisms that generate alkaline urinary conditions could produce crystalline biofilms capable of causing catheter blockage. The urease enzyme produced by *M. morganii* and *K. pneumoniae* hydrolyse urea around ten times slower than the enzyme produced by *Pr. mirabilis* (Jones and Mobley, 1987). In addition, the urease enzyme of *K. pneumoniae* is induced by nitrogen starvation, whilst the urease enzyme of *Pr. mirabilis* is induced by urea to levels of up to 25-fold over those of non-induced cultures (Mobley *et al.*, 1995).

Studies of urinary samples from catheterised patients in a long-term rehabilitation hospital revealed that all samples yielded bacterial growth, and that *Pr. mirabilis* was the most commonly isolated organism (in 51% of all samples) (Hedelin *et al.*, 1984). Analysis of the encrustation from the patients' catheters showed that in addition to the

calcium and magnesium phosphates, smaller amounts of oxalate, urate and protein were present. Studies following 11 patients with long-term IUCs for a prolonged period of time revealed that urinary pH was related to the persistent presence of *Pr. mirabilis*, and that precipitation of phosphates on catheters was linked to the urinary pH (Hedelin *et al.*, 1991). The critical pH in this study was around 6.8, patients with a mean urinary pH below this had minute amounts of catheter encrustation, whilst above this pH, considerable catheter encrustation was observed but with marked individual variation. *Proteus* species once present in the catheterised urinary tract tends to persist to initiate a vicious circle of crystalline biofilm formation and local irritation (Hedelin, 2002).

Morris *et al.* (1997) tested 18 different types of catheters, including those coated with hydrogel or silver. Using a laboratory model of the catheterised urinary tract it was shown that none of the catheters were capable of resisting encrustation by *Pr. mirabilis* biofilms. Electron micrographs established that the catheter blockage was due to crystalline bacterial biofilm formation, and that the blockage generally occurred in the first few centimetres below the eye-hole. This was confirmed by calcium and magnesium analysis that revealed the encrustation was mainly on the 10 cm of catheter below the eye-hole.

1.8. *Proteus mirabilis*

Pr. mirabilis is a motile Gram-negative bacterium commonly found in the intestines of humans and animals, in soil and in polluted water (Coker *et al.*, 2000). It is not a common cause of UTI in normal healthy hosts, however, in patients who have urinary tracts compromised by functional or anatomical abnormalities or chronic

instrumentation it becomes an important cause of morbidity (Mobley *et al.*, 1995). Clinical studies have revealed the high incidence of complications associated with infections by this bacterium in long-term catheterised patients (Mobley and Warren, 1987; Kunin, 1989; Stickler *et al.*, 1993). Developing a catheter care regime which eliminated *Pr. mirabilis* would significantly reduce the complications associated with long-term catheter use.

Pr. mirabilis exists as short (2 - 4 μm) motile rods in liquid medium. However, it undergoes dramatic changes in response to growth on a solid surface, exhibiting a form of multicellular behaviour termed swarming. A short period after *Proteus* cells are inoculated onto suitable agar plates, the short vegetative rods at the colony's edge differentiate into long (20 - 80 μm) swarmer cells. The swarmer cells rapidly move away from the colony margin in a co-ordinate manner until they stop and revert back into the shorter vegetative cells by cell division, in a process termed consolidation. These vegetative cells grow normally for a certain period of time before the cells at the edge of the colony swarm again (Allison *et al.*, 1992). It is these periodic cycles of swarming and consolidation that produce concentric ring patterns on certain agar media.

Clinical studies have suggested that bacteria contaminating the urethral meatus can migrate along the external surface of the catheter into the bladder (Garibaldi *et al.*, 1980; Stamm, 1991). *In-vitro* testing has revealed that clinical strains of *Pr. mirabilis* can migrate over urethral catheters at a rate of up to 10 cm per day (Stickler and Hughes, 1999). Migration was significantly more rapid over hydrogel-coated latex catheters than over all-silicone or silicone-coated latex catheters. Similar experiments

by Sabbuba *et al.* (2002) later revealed that *Pr. vulgaris* could also successfully swarm over all four types of catheters tested. Testing the mobility of *Pr. mirabilis* in the presence of other species revealed that it was capable of transporting non-motile species such as *K. pneumoniae* and *S. aureus* over the catheter surface.

Stickler and Hughes (1999) using scanning electron microscopy revealed that *Pr. mirabilis* moves along the catheter surface as discrete rafts of typically elongated swarmer cells. By developing a new vapour fixation technique for preparing samples to observe under SEM, Jones *et al.* (2004) were able to show that adjacent swarmer cells formed helical connections by interweaving flagellar filaments. It was suggested that this highly ordered structural formation might contribute to the stability of the multicellular rafts, facilitate communication between swarmer cells, and may play an important role in organising the migration of the rafts. The importance of these connections were emphasised by the failure of mutants lacking the helical connections to swarm successfully.

As well as the production of urease and the ability to swarm, other *Pr. mirabilis* virulence factors include adherence to host mucosal surfaces, damage and invasion of host tissues, evasion of the host immune system and iron acquisition (Coker *et al.*, 2000). Allison *et al.* (1992) reported that *Pr. mirabilis* co-ordinately synthesised proteins required for swarming (flagellin) and virulence (haemolysin, urease and proteases) during cycles of cellular differentiation and migration. The virulence factors are linked to the predilection of *Pr. mirabilis* for infections of the upper urinary tract.

1.9. The control of catheter encrustation

A range of methods have been devised to try and control the problem of catheter blockage. Simply replacing a blocked catheter commonly results in rapid encrustation of the new catheter, gaining the patients a reputation as a “blocker” (Kunin, 1987c). However, nursing staff responsible for catheter management are often uncertain about the best way to manage encrusted catheters due to a lack of data about which method if any is best, and clinical experience with the poor performance of current treatments (Capewell and Morris, 1993).

1.9.1. Fluid intake and diet

The rationale of increasing a catheterised patients fluid intake is to reduce the concentration of urinary components such as calcium, magnesium and phosphates. A prospective study of 47 community patients with long-term catheters revealed that “blockers” were characterised by high urinary pH and ammonium concentration, although the study did not detect any significant differences in fluid intake or urinary osmolarity between patients categorised as “blockers” and “non-blockers” (Getliffe, 1994). Getliffe (2003) noted this was probably due to the fact that even very dilute urine still contains sufficient quantities of urinary components to produce a crystalline biofilm under alkaline conditions. Morris and Stickler (2001) carried out experiments in models of the catheterised urinary tract infected with *Pr. mirabilis*. By manipulating the concentration of artificial urine that was being supplied to the models, they showed that under conditions simulating increased fluid intake the deposition of crystalline biofilm on the catheters was significantly reduced.

Burr and Nuseibeh (1993) concluded from a study of 44 spine-injured catheterised patients that the main factors contributing to catheter blockage are bacterial urease activity and urinary calcium concentrations. They also noted that elevation of urinary pH following ingestion of effervescent preparations (such as those containing sodium carbonate), drug or diet induced increases in urinary calcium or magnesium excretion and inadequate fluid intake were contributing factors. Findings by Getliffe (1994) also showed that patients termed “blockers” consumed more dietary-fibre than “non-blockers”.

Although there is evidence from surveys and laboratory experiments that increasing fluid intake will result in more urinary calcium and magnesium remaining in solution, it is extremely difficult to persuade many catheterised patients (especially the elderly) to drink more. Perhaps it would be more persuasive if evidence was available from a controlled clinical trial which showed that increasing fluid intake reduced catheter encrustation.

1.9.2. Dietary acidification of the patient's urine

Attempts have been made to acidify patient's urine through the ingestion of acidifying agents such as ascorbic acid. McDonald and Murphy (1959) demonstrated that ascorbic acid could significantly lower the urinary pH in patients receiving a daily total of 2.5g ascorbic acid administered in doses every 4 h. Further studies by Travis *et al.* (1965) showed that orally administered ascorbic acid even in higher doses than in the previous experiment failed to consistently lower the urinary pH below 5.5 when given four times daily. From this they concluded that if ascorbic acid was to be used,

large amounts should be given to patients, and that it should be administered at 4 h intervals day and night.

Murphy *et al.* (1965) analysed the results from urine cultures made at their hospital over a three-year period. The authors noted that giving ascorbic acid orally every 4 h in patients with *Proteus* infected urine failed to have a significant acidifying effect. They noted that this contrast to results in uninfected urine must be attributed to the alkalinising effect of ureases produced by infecting organisms.

In-vitro experiments were performed to determine whether acidifying the urine could prevent a rise in pH when the urease enzyme was present in the urine (Bibby and Hukins, 1993). It was found that under these conditions, the assumptions of Le Chatelier's principle fitted, whereby a decrease in the pH resulting from acidification was countered by further urea being converted into ammonia. It was determined that to prevent the rise in pH resulting from complete conversion of urea in a day supply of urine would require 2.7 L of mono-basic acid at a reasonable concentration. Bibby and Hukins (1993) concluded that prevention of catheter encrustation by acidifying the patient's urine was not feasible without the elimination of the urease-producing bacteria.

The rationale behind encouraging patients to drink cranberry juice is based on its possible bacterial anti-adherence effects. *In-vitro* studies (Sobota, 1984) showed that urine obtained from healthy mice and humans, who had been drinking cranberry juice, inhibited the adherence of *E. coli* onto uroepithelial cells. In a later paper (Schmidt and Sobota, 1988) this anti adherence activity was also demonstrated against clinical

isolates of *Proteus*, *Klebsiella*, *Enterobacter* and *Pseudomonas*. Morris and Stickler (2001) used their laboratory model of the catheterised bladder to examine whether drinking cranberry juice produces urine that inhibits the development of crystalline *Pr. mirabilis* biofilm on catheters. They reported that there was significantly less encrustation ($P = 0.007$) on catheters from models receiving urine from volunteers who had drunk 2 x 500 ml of water, than on catheters incubated with models supplied with urine pooled from individuals who had drunk 2 x 500 ml of cranberry juice. The amount of encrustation on the two groups of catheters however, were substantially less ($P = 0.001$) than on catheters incubated in models supplied with urine from volunteers who had not supplemented their normal fluid intake. They concluded that the important factor in preventing catheter encrustation is a high fluid intake. To date, there is no clinical evidence supporting the use of cranberry juice as a preventative measure for controlling catheter encrustation.

1.9.3. Bladder washouts

Bladder washout solutions are frequently used in attempt to prevent catheter encrustation. Neutral solutions such as chlorhexidine or saline are not effective at preventing catheter encrustation (Getliffe, 2003). Getliffe *et al.* (2000) using *in-vitro* models of the catheterised bladder showed that Suby G was effective at dissolving catheter encrustation. It must be noted however, that Jack bean urease and not urease producing bacteria were used in this study. A randomised cross over study was conducted to study three bladder-washout solutions –saline, Suby G and Solution R (Kennedy *et al.*, 1992). The study revealed that neither of the washout solutions administered twice weekly for three weeks had a demonstrable effect in preventing crystal formation or catheter ensrustation, nor was there a significant difference in

catheter encrustation between acidic washout solutions and the neutral saline washout solution. Treatment with the acidic washout solutions resulted in a high frequency of red cells in the urine, suggesting that these solutions adversely affect the bladder endothelium.

1.9.4. Catheter coatings

In-vitro experiments comparing encrustation on latex catheters coated with modified hydrogel to all-silicone and silicone elastomer-coated catheters revealed that there was no significant difference in the quantities of encrusting deposits formed on the three materials (Cox *et al.*, 1989b). *In-vitro* tests with 18 different catheters including those coated with hydrogel or silver revealed that they were all susceptible to catheter blockage by encrustation (Morris *et al.*, 1997). Currently there is no experimental or clinical evidence to suggest that any of the catheters on the market can successfully resist colonization by crystalline *Pr. mirabilis* biofilms.

1.9.5. Urease inhibitors

Bibby and Hukins (1993) noted how difficult preventing the rise in urinary pH was in the presence of the urease enzyme. Several studies have focused on inhibiting this enzyme as a way of controlling catheter encrustation. The urease inhibitor acetohydroxamic acid (AHA) when administered orally, is absorbed into the gastrointestinal tract and excreted in the urine. When AHA was administered to 23 patients with renal calculi and urinary infection by urease producing organisms, it successfully reduced urinary ammonia and alkalinity in each patient (Griffith *et al.*, 1978). Williams *et al.* (1984) studied the effects of AHA on the growth of struvite stones in the urinary tract. Their findings revealed that AHA effectively inhibits stone

growth in patients infected with urea-splitting bacteria. Burns and Gauthier (1984) administered AHA to five patients that required frequent catheter changes because of severe encrustation and catheter blockage. It was shown that AHA significantly decreased the amount of encrustation on the catheters, and that all patients required less-frequent catheter changes during therapy. The reported complications associated with the administration of AHA vary from study to study; Burns and Gauthier (1984) reported only minimal adverse effects, Griffith *et al.* (1978) state that a dose of 1 g of AHA daily was well tolerated whilst Williams *et al.* (1984) reported that patients receiving AHA had increased adverse effects such as tremulousness and deep vein phlebothrombosis. Investigating the ability of urease inhibitors to control catheter encrustation by *Pr. mirabilis* in a physical model of the catheterised urinary tract, Morris and Stickler (1998) observed that AHA (1 mg/ml) and fluorofamide (1 µg/ml) restricted the increase in urinary pH from 9.1 to 7.6. These urease inhibitors also significantly reduced the deposition of calcium and magnesium salts onto the catheter. As fluorofamide is a more potent inhibitor of the *Pr. mirabilis* urease enzyme, much lower doses are required to prevent catheter encrustation. If the safety of fluorofamide could be confirmed it may have clinical applications in controlling catheter encrustation. Efforts to find more potent urease inhibitors have focused on synthesising a range of different dipeptidyl hydroxamic acids, and testing their activity against *Pr. mirabilis* ureases (Odake *et al.*, 1992). The use of non-antibacterial agents such as urease inhibitors to control catheter encrustation is an attractive prospect as it rules out the possibility of generating problematic resistant mutations.

1.9.6. What else can be done ?

In their study of community patients with long-term IUCs, Getliffe (1994) noticed a prevailing tendency towards only replacing a catheter when it blocks, resulting in “crisis care” of patients classed as “blockers”. Evans and Godfrey (2000) suggest that every effort should be made to try avoiding this type of “crisis management”, and that nurses should attempt to predict when individual patients’ catheters are going to block. Keeping a record of an individual’s blockage pattern over three to five catheter episodes identifies a characteristic pattern of catheter life (Norberg *et al.*, 1983). This information could help develop individual planned re-catheterisation programmes, changing the catheters before they were likely to block. This type of monitoring programme would reduce the distress catheter blockage causes to patients and ease the pressure on the health services with the need for fewer urgent unscheduled catheter changes. Evans and Godfrey (2000) also suggest that testing the patients’ urinary pH may assist in predicting the catheter life for individual patients.

1.10. A novel method for controlling catheter encrustation

Bibby *et al.* (1995) were the first to suggest that the catheter retention balloon could be filled with an antimicrobial solution instead of water as a novel way of controlling catheter encrustation. They suggested that the delivery of the antimicrobial agent could be achieved by diffusion through the balloon. Using a simple *in-vitro* model with an all-silicone Foley catheter inflated with a mandelic acid solution, they showed that low concentrations of this agent could pass through the catheter balloon into the surrounding liquid for up to four weeks. The concentration of the antimicrobial agent in the catheter balloon could be monitored. If it fell below a level at which it was effective, it could easily be replaced or replenished without disrupting the closed

drainage system. Another benefit of this approach was that it did not require any modifications to catheters currently used. Oral antibiotic intake destabilises the gut flora and can result in overgrowth of certain organisms or colonization by resistant organisms (Levy, 2000). Treatment of CA-UTI by delivering antibacterial agents directly into the bladder could avoid altering the gut flora by oral administration of drugs.

Mandelic acid has previously been used to perform bladder washouts, but unfortunately it is not very active against *Pr. mirabilis*. A search of the literature revealed that *Pr. mirabilis* was very sensitive to the biocide triclosan. Studies investigating the concentration of triclosan required to inhibit growth and swarming of 42 animal isolates of *Pr. mirabilis* and *Pr. vulgaris* were performed by Firehammer (1987). These revealed that growth of all but one isolate was inhibited by less than 1 µg/ml in broth and on agar, and that swarming was inhibited at concentrations two- to four-fold less than the MIC. Reported MICs of triclosan against national collection of type cultures (NCTC) of *Pr. mirabilis* are 0.3 µg/ml (Bhargava and Leonard, 1996). Catheter isolates of *Pr. mirabilis* have also been shown to be very sensitive (MIC, 0.5 µg/ml) to triclosan (Stickler, 2002).

The phenylether or chlorinated bisphenol, triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether), is a synthetic antimicrobial agent. It has a broad spectrum of activity encompassing many, but not all types of Gram-positive and Gram-negative non-sporulating bacteria, and some fungi and viruses (Bhargava and Leonard, 1996; Jones *et al.*, 2000; Schwiezer, 2001). Triclosan is added to toothpastes and mouthwashes to prevent or reduce plaque (Fine *et al.*, 1998), and is used in personal

care products such as deodorants, soaps and handwashes. It has been incorporated into fabrics and plastics such as surgical drapes, children's toys and cutting boards, and searches of patent databases reveal further unique applications for triclosan-impregnated material, ranging from antimicrobial concrete to triclosan impregnated bowling ball inserts (Schweizer, 2001). Triclosan's safety has been established through extensive acute and long-term toxicity, carcinogenicity, reproduction and teratology studies (Bahrgava and Leonard, 1996).

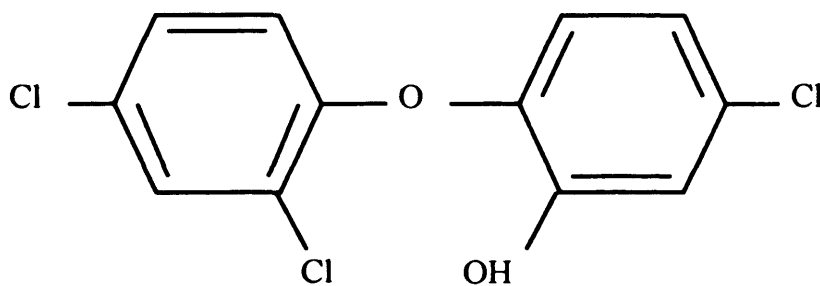


Figure 2: Chemical structure of triclosan.

In-vitro studies evaluating triclosan-impregnated sutures revealed that they were effective at preventing microbial adherence of selected nosocomial pathogens, which may reduce the risk of superficial incisional site infections in at-risk surgical populations (Edmiston *et al.*, 2004). Continuous ambulatory peritoneal dialysis catheters impregnated with triclosan, chlorhexidine and silver sulfadiazine tested in rats were shown to be highly effective at lowering bacterial colonization at the catheter exit point compared to control catheters (Kim *et al.*, 2002). Using *in-vitro* experiments, Gaonkar *et al.*, (2003) established that urinary catheter-tubing impregnated with triclosan, chlorhexidine and silver sulfadiazine exhibited a broad-spectrum of antimicrobial resistance.

1.11. Aims of study

In view of its overriding importance in the process of encrustation and blockage of urinary catheters, it was decided to develop a strategy for the control of the problem which specifically targeted *Pr. mirabilis*. The strategy was based on the biocide triclosan, which has particular activity against *Pr. mirabilis*. It also involved a novel approach to introducing the active agent into the catheterised bladder which did not involve disturbing the integrity of the closed drainage system.

AIMS:

- To establish whether *Pr. mirabilis* strains isolated from the catheterised urinary tract are fully sensitive to triclosan.
- To determine the minimal bactericidal concentrations for triclosan of clinical *Pr. mirabilis* isolates growing in urine.
- To investigate what concentrations of triclosan can be delivered through the retention balloon of catheters.
- To determine if filling the catheter retention balloon with triclosan will delay or prevent catheter blockage by crystalline *Pr. mirabilis* biofilms.
- To ascertain what concentrations of triclosan are required in the urine to eliminate established *Pr. mirabilis* biofilms from catheters.

- To investigate whether resistant cells emerge when populations of *Pr. mirabilis* are exposed to triclosan, and if so, whether the triclosan resistant cells exhibit cross-resistance to antibiotics.
- To examine how the triclosan strategy influences the development of catheter biofilm communities by a variety of species that cause CA-UTI.

2. MATERIALS AND METHODS

2.1. Chemicals

Most chemicals used in this study, unless otherwise stated, were purchased from Fisher Scientific Ltd. (Loughborough, UK). The triclosan and triclosan formulations were obtained from CIBA speciality chemicals (Basel, Switzerland).

2.2. Culture media

The agar media used in this study were purchased from Oxoid (Basingstoke, UK). Cysteine-Lactose Electrolyte Deficient (CLED) Agar was used to culture a range of urinary pathogens. CLED Agar inhibits the swarming of most *Pr. mirabilis* strains (Figure 3a). Colistin Inositol (CI) Agar (Clayton, 1984) was used for the enumeration of strains of *Pr. mirabilis* that swarmed on CLED Agar. CI Agar is made up using 28 g/L Nutrient Agar, 10 g/L myo-inositol (Sigma chemical company, Poole, UK), 0.02 g/L bromothymol blue (Sigma) and 0.01 g/L colistin methanesulfonate (Sigma). Nutrient Agar was used for viable cell counting of *Klebsiella pneumoniae* cultures. Tryptone Soya Agar (TSA) was used to demonstrate the swarming of *Pr. mirabilis* (Figure 3b) and in the method for determining the minimum inhibitory concentrations (MIC) of triclosan to test organisms. Iso-sensitest Agar was used for the antibiotic sensitivity testing. Urea Agar, a nutrient agar containing 2% (v/w) urea was used to check for the production of the urease enzyme. When inoculated with urease-producing organisms the medium changes from straw-colour to bright pink (Figure 3c). Non-urease producing organisms produce no colour change (Figure 3d).

Tryptone Soya Broth (TSB) was used to grow cultures for experimental purposes and to supplement the artificial urine.

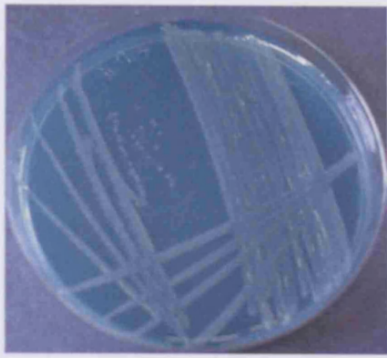


Figure 3a: *Pr. mirabilis* culture on a CLED Agar plate.

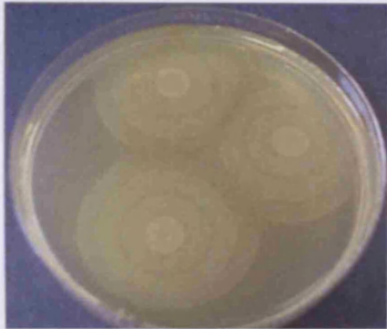


Figure 3b: A *Pr. mirabilis* 4 h culture spot inoculated onto a TSA plate.

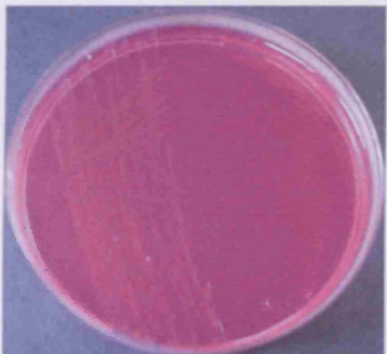


Figure 3c: *Pr. mirabilis* streaked onto a urea Agar plate

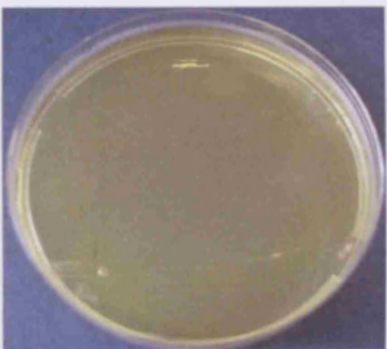


Figure 3d: *E. coli* streaked onto a urea Agar plate.

Figure 3. The appearance of urinary tract pathogens on different agar.

2.3. Preparation of artificial and human urine

The artificial urine used in this study was made up as described by Griffith *et al.* (1976). It was made up using the chemical quantities given in Table 1. Before the gelatine was added, the pH was adjusted to 6.10 with sodium hydroxide (3 M). Once the gelatine had dissolved, the artificial urine was filter sterilized using a 0.2 µm Sartorius (Goettingen, Germany) capsule filter. The TSB was prepared separately and aseptically added to the sterile artificial urine. The pH was measured with an Accumet basic AB15 pH meter.

To prepare sterile human urine, urine was collected from healthy human volunteers and pooled. It was filtered through cotton wool to remove general debris and then sterilized by filtration through a 0.8 µm capsule filter (Sartorius), and then through a 0.2 µm capsule filter (Sartorius).

2.4. Bacteria

The bacteria used in this study were clinical isolates from the indwelling catheters of patients undergoing long-term catheterisation. They were kindly supplied by Dr. N. Morris, Dr. N. Sabbuba, Dr. S. Jones, Dr M. Suller and Miss S. Macleod of Cardiff School of Biosciences. In each case, identities of isolates were confirmed by Gram-staining and then using the appropriate BBL™ crystal identification kits (Becton Dickinson, Oxford, UK). The *E. coli* reference strain (NCTC 10418) was used as a control organism in the antibiotic sensitivity testing. Isolates were stored at -80°C in cryogenic vials containing 5% glycerol.

Chemical	Chemical formula	Grams per litre
Sodium sulphate	Na ₂ SO ₄	2.3
Calcium chloride dihydrate	CaCl ₂ .2H ₂ O	0.65
Magnesium chloride	MgCl ₂ .6H ₂ O	0.651
Sodium chloride	NaCl	4.6
Trisodium citrate	Na ₃ C ₆ H ₅ O ₇ .2H ₂ O	0.65
Sodium oxalate	(COONa) ₂	0.02
Potassium dihydrogen orthophosphate	KH ₂ PO ₄	2.8
Potassium chloride	KCl	1.6
Ammonium chloride	NH ₄ Cl	1
Urea		25
pH to 6.10 using sodium hydroxide (NaOH)		
Gelatine		5
Filter sterilize		
Tryptone soya broth (TSB)		1

Table 1: The chemical quantities required for preparing artificial urine.

placed into the bacterial lawn and incubated overnight at 37°C. The following day the plates were checked for any zones of inhibition around the catheter sections.

2.5. Catheters

During the experimental work, size 14 Ch. all-silicone, silicone-coated latex or hydrogel-coated latex (Becton) catheters were used. All catheters were purchased

2.4.1. Viable bacterial cell counts

The method described by Miles and Misra (1938) was used to determine the number of viable bacterial cells present in cultures and cell suspensions. Serial dilutions of the bacterial suspensions were made in quarter strength Ringer's solution (Oxoid). Triplicate aliquots (10 µl) were dropped onto pre-dried CLED Agar plates that had been divided into different sections for each dilution. Each set of dilutions was prepared in triplicate and inoculated onto different plates.

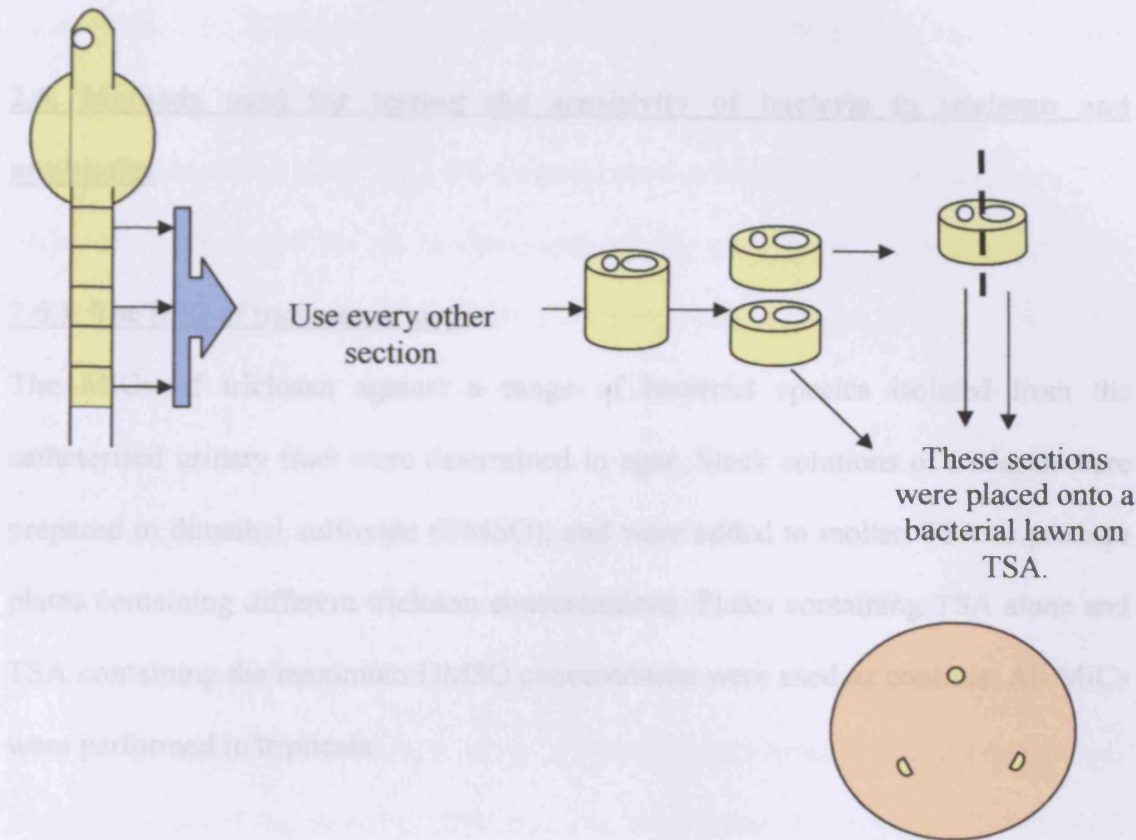
2.4.2. Preparation of bacterial lawns for testing antibacterial activity of catheter sections

Young log phase cultures of *Pr. mirabilis* B2 grown in TSB for 4 h at 37°C were used as the inocula. Aliquots (100 µl) were dropped onto plates of TSA. Bacterial lawns were prepared by spreading the culture using a glass rod and allowing them to dry in a laminar flow cabinet. Catheters were cut into sections of 1 cm, every other 1 cm section of the catheter was used and the other discarded. The test sections were cut further into 0.5 cm sections, one of these 0.5 cm section was left whole whilst the other was cut in half lengthwise, leaving the catheter inflation line in one half but not the other (Figure 4). Since it was not possible to cut the latex based catheters lengthwise, two whole 0.5 cm sections were used. The catheter sections were then placed onto the bacterial lawns and incubated overnight at 37°C. The following day the plates were checked for any zones of inhibition around the catheter sections.

2.5. Catheters

During the experimental work size 14 Ch all-silicone, silicone-coated latex or hydrogel-coated latex (Biocath) catheters were used. All catheters were purchased

Donor (Bent Ltd, Crawley, UK). In the bladder model experiments the catheters were connected to 2000 ml urine drainage bags (Bard).



In the case of the clinical isolates of *P. mirabilis*, tests were performed using cultures

The zone of inhibition was measured as the diameter of the zone around the catheter section that had been left intact, measuring across the lumen through the inflation line.

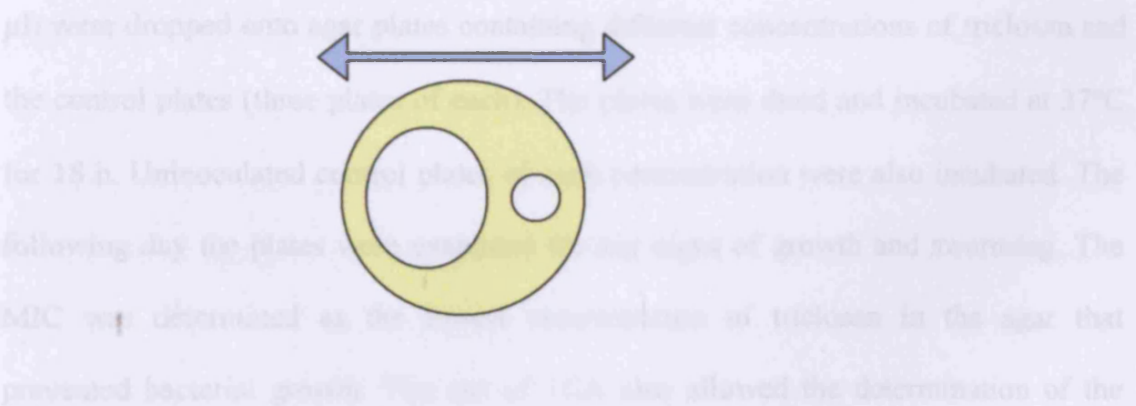


Figure 4: Testing the antibacterial activity of catheter sections.

Minimum inhibitory concentration (MIC) of infection against *P. mirabilis*.

from Bard Ltd. (Crawley, UK). In the bladder model experiments the catheters were connected to 2000 ml urine drainage bags (Bard).

2.6. Methods used for testing the sensitivity of bacteria to triclosan and antibiotics

2.6.1. The MIC of triclosan in agar

The MICs of triclosan against a range of bacterial species isolated from the catheterised urinary tract were determined in agar. Stock solutions of triclosan were prepared in dimethyl sulfoxide (DMSO), and were added to molten TSA to produce plates containing different triclosan concentrations. Plates containing TSA alone and TSA containing the maximum DMSO concentration were used as controls. All MICs were performed in triplicate.

In the case of the clinical isolates of *Pr. mirabilis*, tests were performed using cultures of the test organisms in 100 ml TSB that had been incubated at 37°C for 4 h with gentle aeration. These cultures were diluted 1:100 with TSB and triplicate aliquots (10 µl) were dropped onto agar plates containing different concentrations of triclosan and the control plates (three plates of each). The plates were dried and incubated at 37°C for 18 h. Uninoculated control plates of each concentration were also incubated. The following day the plates were examined for any signs of growth and swarming. The MIC was determined as the lowest concentration of triclosan in the agar that prevented bacterial growth. The use of TSA also allowed the determination of the minimum inhibitory swarming concentration (MISC) of triclosan against *Pr. mirabilis*.

Alternatively aliquots (1 μ l) of overnight cultures of test organisms in TSB (10 ml) were applied to agar plates using the Denley multipoint inoculator (Denley instruments Ltd., Billingham, UK). This allowed several strains to be inoculated onto one agar plate, allowing a high throughput of strains and species. Each strain was inoculated onto three plates for each concentration of triclosan and control plates. The plates were incubated for 18 h and examined for growth the following day. This method was used to test the MIC of the triclosan resistant mutants of *Pr. mirabilis* and for the clinical isolates of eight other species of bacteria.

2.6.2. The MIC of triclosan in artificial urine

Triclosan stock solutions were prepared in DMSO at 1000 times the final concentrations required in the artificial urine. Aliquots (10 μ l) of these stock solutions were added to artificial urine (10 ml) in sterile glass universal containers and mixed. Inocula (10 μ l) of the test organisms that had been grown overnight in TSB at 37°C were added to each concentration of triclosan, mixed and incubated with gentle shaking at 37°C in an orbital shaker for 6 h. Control cultures were set up where the *Pr. mirabilis* cultures were used to inoculate 10 ml artificial urine and 10 ml artificial urine containing 10 μ l DMSO. For each batch of tests, uninoculated tubes containing 10 ml artificial urine and 10 ml artificial urine and DMSO were also placed in the incubator as controls. Following the 6 h incubation period, the cultures were observed for turbidity as a sign of bacterial growth. The pHs of the cultures were also recorded. The MIC of triclosan was recorded as the lowest concentration of triclosan that inhibited growth.

2.6.3. Determination of the bactericidal activity of triclosan

Tests were performed in order to determine the bactericidal efficacy of triclosan. To standardise the bacterial inoculum in this procedure, calibration curves of optical density against viable cell counts were prepared. This was achieved by harvesting bacterial growth from an overnight culture grown on a CLED Agar plate and re-suspending the cells in 10 ml sterile de-ionized water. This suspension was diluted stepwise 1:1 with sterile de-ionized water down to 1:16. The optical density of each of these dilutions, including the neat sample was measured at 500 nm, and viable cell counts on CLED Agar were also performed on each suspension. This was done in triplicate for each strain examined in the bactericidal tests (Appendix 1). The resulting calibration curves were used to prepare standardised inocula of 1×10^9 cfu/ml for use in the bactericidal tests.

The method used for the bactericidal tests was based on the British Standard EN 1276. The standard suspensions (1 ml) of the test organisms were used to inoculate solutions (9 ml) in glass vessels containing various concentrations of triclosan in de-ionized water, and all resulting in a final DMSO concentration of 1%. The suspensions were mixed and incubated at ambient temperature. At various time intervals, samples (1 ml) were removed and added to the neutraliser (9ml). The neutraliser used for triclosan contained 1.5% (w/v) lecithin and 5% (v/v) Tween 80 (polysorbate 80) (Suller and Russell, 1999). Control suspensions where the standard inoculum (1 ml) was added to de-ionized water (9 ml) were run in parallel with each test. Samples were taken from the control suspensions at the same time intervals as from the test suspensions.

2.6.4. Neutralisation of triclosan

The efficacy of the neutraliser was confirmed using a method based on that described by Langsrud and Sundheim (1998). Incubation mixtures were prepared containing the neutraliser (8 ml), triclosan (100 µl of a 10 mg/ml solution in DMSO) and sterile de-ionized water (900 µl). Inocula of *Pr. mirabilis* B2 cells (1 ml of overnight TSB cultures grown at 37°C) were added to the triclosan and neutraliser mixtures. After 5 min contact time at ambient temperature, samples were removed to enumerate the numbers of viable cells. Two controls were run parallel to this experiment, a positive control where sterile de-ionized water (100 µl) was added to the mixture instead of the triclosan solution, and a negative control where sterile de-ionized water was added instead of the neutraliser.

To examine the possible toxicity of the neutraliser and of the 1% DMSO used in these tests and in the bactericidal testing procedure, tests based on the method by Messenger *et al.* (2001) were performed. Overnight TSB cultures (1 ml) grown at 37°C were added to the neutraliser (9 ml) and to mixtures containing a final concentration of 1% DMSO. Following a 15 min contact time, viable counts were performed. Controls using 9 ml sterile de-ionized water instead of the neutraliser or the DMSO solution enabled the bacterial reduction to be calculated. These tests were carried out on all strains that came into contact with the neutraliser or with 1% DMSO.

2.6.5 The MICs of antibiotics to test strains

The MICs of ampicillin, ciprofloxacin, gentamicin, nalidixic acid, nitrofurantoin, trimethoprim, cefalexin and norfloxacin to a range of test organisms were determined using E-test strips (AB Biodisk, Sweden). The methods used were based on those used

by Brown and Brown (1991). Agar plates with a depth of 4 ± 0.5 mm were prepared by pouring 15 ml of molten Iso-sensitest Agar into petri dishes. The inoculum was prepared by suspending bacterial colonies from an overnight agar plate into sterile de-ionized water. To achieve the correct turbidity, the suspension was visually compared to a 0.5 McFarland standard. A swab was dipped into the inoculum suspension and the excess was removed. This swab was then used to streak the entire surface of the Iso-sensitest Agar plates three times (rotating the plate 90 degrees each time to distribute the inoculum evenly). The plates were allowed to dry completely before applying the E-test strips with forceps. One strip was placed on each agar plate and the plates were incubated for 18 h at 37°C. The MIC value was determined by observing where the edge of the inhibition ellipse intersected the side of the strip. Any swarming by *Proteus* in the inhibition ellipse was ignored. The MIC of the reference strain *E. coli* NCTC 10418 to the test antibiotics was also determined using this method.

2.7. The bladder model

2.7.1. The bladder model set up

The *in vitro* bladder model set-up was used to study the ability of a range of urinary tract pathogens to form biofilms on urinary catheters, and to observe what effect introducing triclosan into the catheterised urinary tract had on their development. The model of the catheterised bladder was described by Stickler *et al.* (1999), and consists of a glass chamber (the bladder) that is maintained at 37°C by a water jacket. Water at 37°C was supplied to the outer jacket from a circulating water bath. The models were sterilised by autoclaving (121°C for 15 min), and a Foley catheter was inserted aseptically into each model through a section of tubing (the urethra) attached

to the glass outlet at the base of the model. To reduce the chance of contamination the catheters were inserted into the models in a laminar flow cabinet. The catheters are secured into position by inflating the catheter balloon with 10 ml of sterile water. The catheters were then connected to drainage bags that collect the urine draining through the catheters from the model (Figure 5).

Sterile artificial urine stored in sterile aspirators was supplied to the bladder models via sterile silicone tubing by a peristaltic pump (503U, Watson and Marlow, Falmouth, UK) to maintain a constant flow of urine into the bladder. A residual volume of 20 ml was pumped into the bladder models (just below the level of the catheter eyelet) and the pump supplying the urine was switched off. The models were then inoculated with a 4 h log-phase culture in artificial urine (10 ml), and the culture left for 1 h to establish itself in the system before resuming the supply of urine at a rate of 0.5 ml per minute. The viable cell count and pH of the inoculating culture was determined. The viable cell count and pH was also taken from the residual urine in the bladder when the catheter blocked, or at the end of the experiment if the catheter did not block. Unless otherwise stated models were fitted with size 14 Ch catheters and experiments were replicated three times.

2.7.2 Electron microscopy

2.7.2.1. Low vacuum scanning electron microscopy

Low vacuum scanning electron microscopy (LV-SEM) was used to visualise the extent of encrustation on the catheter eye-hole and in its inner-lumen. Six 1 cm sections (Figure 6) were cut from catheters that had been carefully taken out of the bladder models. These samples needed no further preparation and were placed onto

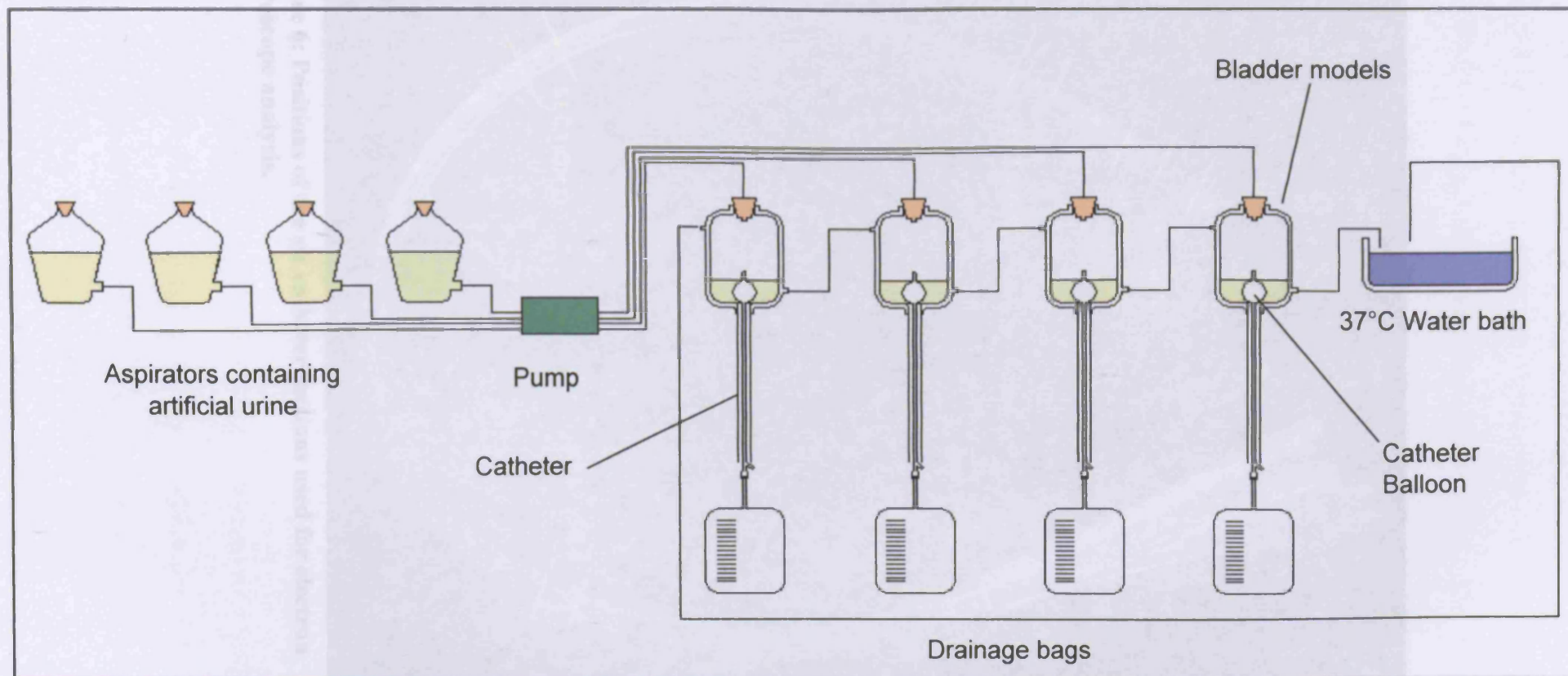


Figure 5: The bladder model set-up. The models consist of a glass chamber maintained at 37°C by a water jacket. Urine stored in the aspirators is pumped into the model and then drains through a catheter inserted into the base of the chamber. The urine draining through the catheter collects in drainage bags linked to the catheters. Figure adapted from Stickler *et al.* (1999).

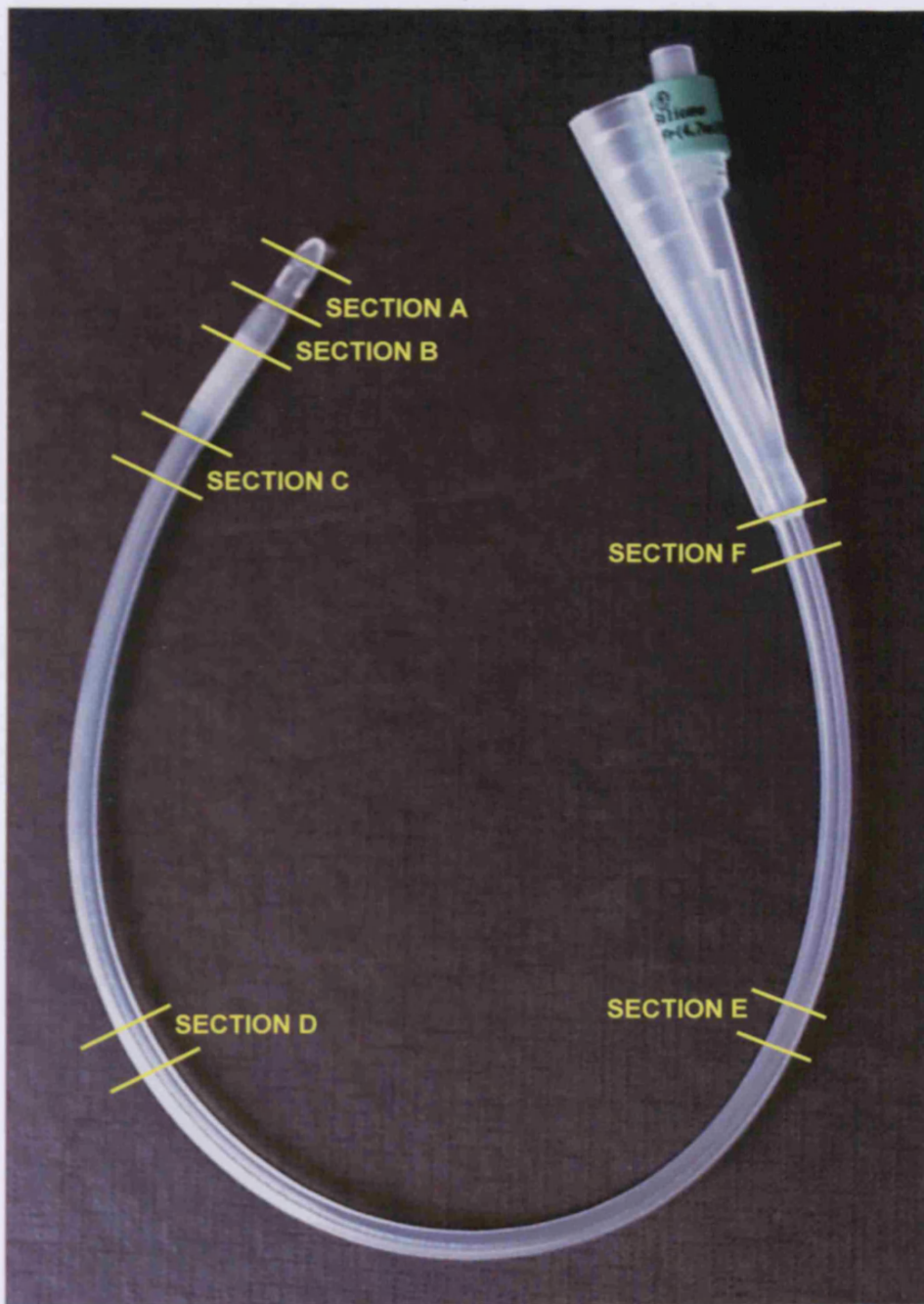


Figure 6: Positions of the six catheter sections used for electron microscope analysis.

black carbon disks stuck onto a metal stub. The sections were then viewed under the JEOL JSM 5200lv SEM on low vacuum mode (3%) at 20 kv. The first section was examined looking down through the eye-hole and the remaining sections were observed looking down through the lumen.

2.7.2.2. Critical point drying and high vacuum scanning electron microscopy

The same catheter sections as in Figure 6 were used, but the 1 cm sections were also cut in half longitudinally. The catheter sections were fixed either with or without ruthenium hexaammine trichloride (RHT). All the electron microscopy reagents were purchased from Agar scientific (Stansted, UK), except for the RHT, which was obtained from Johnson Matthey Materials Technology Ltd., Hertfordshire, UK.

Fixing without RHT

The 0.1 M Sørensen phosphate buffer was made by adding 95 ml of 0.1 M disodium hydrogen orthophosphate to 5 ml of 0.1 M potassium dihydrogen orthophosphate. The sections were fixed in 2.5% glutaraldehyde in 0.1 M Sørensen phosphate buffer (pH 8.5) at room temperature for 1 h. Samples were then washed in the 0.1 M Sørensen phosphate buffer and stored in fresh buffer overnight at 4°C. The samples were then post-fixed by immersion for 30 min at room temperature in 1% osmium tetroxide in 0.1 M Sørensen phosphate buffer. Special care was taken when handling osmium as it can fix corneas and skin. It was used in the fume hood and gloves were worn at all times. The osmium was pipetted off and the specimens were washed twice in de-ionized water for a total of 15 min before being dehydrated in a graded series of ethanol solutions (70 - 100%). The samples were placed firstly in 70% ethanol then 90% ethanol and twice in 100% ethanol each for 10 - 15 min. Once the dehydration

process was complete the samples were critically point dried using liquid carbon dioxide (Balzers CPD 030 critical point dryer). The specimens were finally mounted with carbon cement onto aluminium stubs and left to dry overnight in a dessicator. The samples were gold-coated (Edwards sputter coater S150B) and examined using the JEOL XL20 scanning electron microscope.

Fixing with RHT

The primary fixation step for this experiment was carried out in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer and RHT (0.14 g of RHT was added into 20 ml of the fixative 1 h before it was needed, to allow the pH to stabilise) and left overnight at room temperature. The fixative was then washed off with 0.05 M sodium cacodylate buffer. The samples were then processed in the same way as fixing without RHT from the post fixing with osmium tetroxide step.

2.7.3. Calcium and magnesium analysis of the catheter biofilm by atomic absorption spectroscopy

This method was used to determine the extent of encrustation on catheters removed from the bladder models. After removing the balloon the catheter was cut into 2 cm sections and placed into 100 ml of 4% (v/v) nitric acid in double de-ionized water. This sample was then sonicated at 35 khz for 5 min to facilitate the dissolution of the crystals embedded in the biofilm, and samples were kept refrigerated at 4°C until the samples could be analysed. To carry out the analysis, the nitric acid from the bottles containing the catheter sections was diluted 1:10, 1:50, 1:100, 1:200 and 1:400 using 4% nitric acid to bring the concentration into the readable range of the spectrophotometer. These samples were then analysed using the atomic absorption

spectrophotometer (aa/ae Spectrophotometer, Thermoelectron, UK). The readable ranges were between 0.5 ppm and 2 ppm for calcium (at 422.7 nm) and between 0.05 ppm and 0.4 ppm for magnesium (at 285.2 nm). Prior to sample analysis, the spectrophotometer was calibrated within these ranges with standards purchased from Spectrosol (BDH Chemicals Ltd., Poole, UK). These 1000 ppm standards were diluted with 1% (v/v) nitric acid. This enabled the assay of the calcium and magnesium accumulating on each catheter to be determined.

2.8. Triclosan extraction from artificial urine and chemical analysis

Dr. Carsten Muller of Cardiff School of Biosciences designed the experimental procedure for this method to determine the concentration of triclosan in artificial urine samples. The chemicals used for this test were AnalaR grade chemicals purchased from BDH. 50 ml of each artificial urine sample was acidified with eight drops of concentrated hydrochloric acid. The sample was then poured into a 100 ml separating funnel and extracted twice with dichloromethane (10 ml). This method involved shaking the separating flask containing the artificial urine sample and the dichloromethane fifty times, and then leaving the flask for 20 min to allow the organic and inorganic layers to separate out. The triclosan preferentially dissolves in the dichloromethane, hence the bottom layer containing the triclosan in dichloromethane was then taken off and kept. The sample remaining in the funnel was extracted again in the same way with dichloromethane. The combined bottom layers of dichloromethane containing triclosan were dried with anhydrous sodium sulphate, - the salt was added until the anhydrous salt appeared in the extract. The sample was decanted into a 50 cm³ round-bottomed flask and dried down on a rotary evaporator. The sample remaining in the round-bottomed flask was re-suspended twice in 0.5 ml

of high-pressure liquid chromatography (HPLC) solvent (70% acetonitrile (HPLC quality)). This 1 ml sample was then run on HPLC. The control was 10 µg/ml triclosan in absolute ethanol. The HPLC machine (Dionex AGP) was used with a Spectra-physics auto-sampler (AS3500) and a Spectra-physics 100 UV detector at 279 nm. A Phenomenex lichrosphere (5uRP-8e, 250 x 4mm) column was used and was fitted with a Phenomenex ODS security cartridge, which prevented the column getting damaged. The data was collected and interpreted using HPLC technologies prime chromatography software.

As chemicals are detected by HPLC, the chromatograph displays a peak. Each peak on a chromatograph indicates the presence of a chemical in the sample and is labelled with a retention time (the retention time for triclosan in this experiment was around 5.75). The retention time is the time it takes for a chemical to come out of the HPLC column. The triclosan control samples were clearly identified on the HPLC chromatograph by a clean peak. By standardising the HPLC parameters, the retention time for the peak of a given chemical compound is kept the same. Therefore, by comparing the area underneath the peak of the control triclosan samples to the test samples at the same retention time, it was possible to quantify the concentration of triclosan in the unknown samples. To calculate the percentage efficiency of the extraction, two artificial urine samples were spiked to contain 1 µg/ml of triclosan and were put through the above procedure. By working out the percentage efficiency of the extraction, the results for the amount of triclosan in each sample could be corrected.

2.9. Physical properties of catheter balloons

The physical properties of catheter balloons that had been inflated with water and triclosan solutions were tested. Catheters were inflated with water, a triclosan solution and the solvent used in the triclosan solution. Catheter balloons were submerged in water and kept in a dark cupboard. The catheters were observed daily for any signs of change in the balloon morphology and the water in which the balloons were soaking was changed weekly. After eight weeks, the catheter balloons were deflated and a section of each catheter balloon was stretched using the Lloyds testing instrument (LRK10) (Figure 7). This allowed a comparison between the load (N) that each section could take, its tear strength (N/mm) and its percentage elongation. Even small samples of balloon material are very hard to break, hence a nick (half way across the balloon test section) was placed half way down the sample to facilitate tearing.

2.10. Isolation of mutants with increased resistance to triclosan

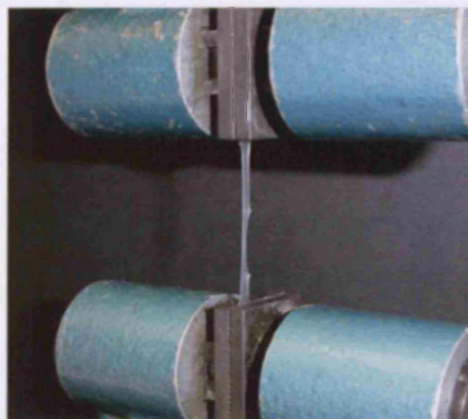
Two methods were employed to attempt to isolate *Pr. mirabilis* mutants with decreased susceptibilities to triclosan.

2.10.1. The disk diffusion method

Sterile paper disks (Whatman, Kent, UK) were soaked in super-MIC concentrations of 0.5, 1, 2, 5 and 10 µg/ml triclosan in 1% DMSO for 5 min. The disk soaking solutions were prepared by diluting triclosan stock solutions in DMSO that had been prepared at 100 times the desired experimental concentration 1:100 with sterile de-ionized water. The disks were placed onto TSA plates swabbed with a 4 h culture of *Pr. mirabilis*. Following a pre-diffusion period of 1 h, the plates were sealed with nescofilm and



The catheter balloon before stretching.



The catheter balloon just before it snapped.

Figure 7: The LRK10 Lloyd's testing instrument. This instrument was used to test the physical properties of the catheter balloons.

incubated at 37°C for up to five days. Plates were examined daily for resistant colonies growing within the inhibition zone around the disks.

2.10.2. The heavy inoculum method

100 µl of a 4 h TSB culture was swabbed onto TSA plates containing super-MIC concentrations of 0.5, 1, 2, 5 and 10 µg/ml triclosan (all contained less than 1% DMSO). Plates were left for one hour before they were sealed and incubated at 37°C for up to five days. Plates were examined daily for any resistant colonies.

Any apparently resistant organisms produced using these methods were sub-cultured onto TSA plates. Frozen stocks of each mutant were also prepared (in cryogenic vials containing 5% glycerol). To prevent the loss of resistance in these mutants, these frozen cultures and plates contained 0.1 µg/ml of triclosan until the stability of the resistance was confirmed.

2.10.3. Determining the stability of the resistant mutants

To determine whether the bacterial resistance was stable, mutant strains were cultured in the absence of triclosan. If the resistance is stable, the MIC will not decrease when sub-cultured in the absence of the selective pressure. Following the initial plate to broth (10 ml TSB) sub-culture, 14 overnight broth-to-broth sub-cultures were performed, testing the MIC after the 1st, 3rd, 7th, 11th and 15th sub-culture (Figure 8). After every sub-culture, each TSB culture was plated out onto TSA and CLED Agar to check the culture's purity. At the end of the experiment, the identity of a selection of mutants was confirmed using the BBL™ crystal identification kits.

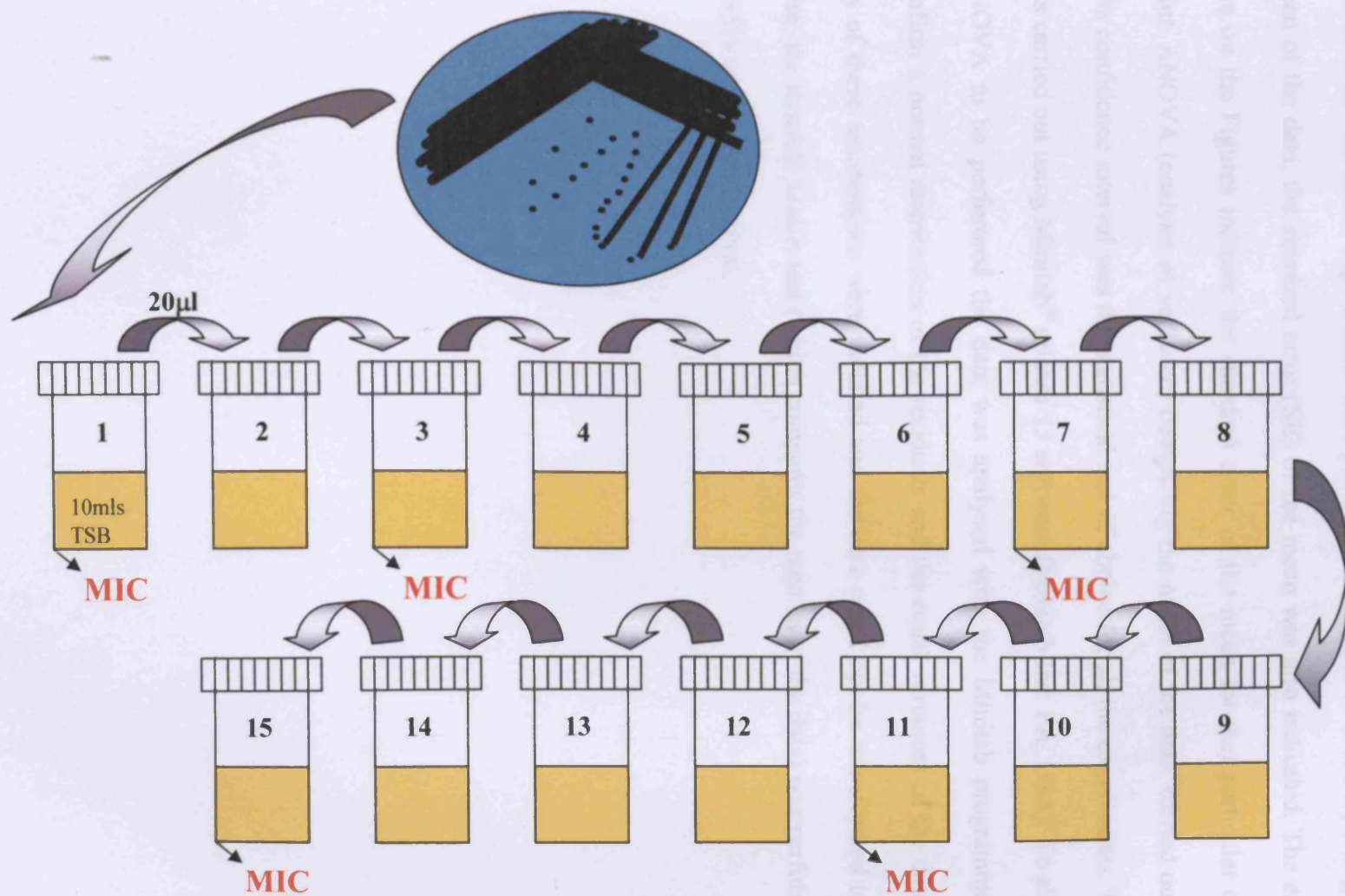


Figure 8: Method for determining the stability of the mutants. Following the initial plate to broth sub-culture, 14 overnight broth-to-broth sub-cultures were performed, testing the MIC after the 1st, 3rd, 7th, 11th and 15th sub-culture.

2.11. Statistical analysis

Unless otherwise stated experiments were performed in triplicate. When reporting the mean of the data, the standard error (SE) of the mean was also indicated. The error bars on the Figures indicate the standard error of the mean for that particular data point. ANOVA (analysis of variance) comparing the mean of the data, carried out at 95% confidence interval was the statistical test of choice for all the experiments. This was carried out using Minitab[®] release 13 software (Minitab Inc. PA, USA). To allow ANOVA to be performed the data was analysed with the Minitab programme to confirm a normal distribution of the residuals and the equal variances of the data. If any of these assumptions were violated and the data could not be transformed to fit them, the Kruskal Wallis test (which compares the median of the data) was performed at 95% confidence interval.

3. RESULTS

3.1. The sensitivity of *Pr. mirabilis* to triclosan

Clinical and laboratory studies have clearly identified *Pr. mirabilis* as the organism mainly responsible for the encrustation and blockage of catheters (Mobley and Warren, 1987; Kunin, 1989; Stickler *et al.*, 1993). If this organism is to be a primary target for novel strategies to control the complication of catheter encrustation, then it is important to investigate the sensitivity of strains that are currently forming crystalline biofilms on patients' catheters to triclosan.

Firehammer (1987) reported that low concentrations of triclosan ($< 1 \mu\text{g/ml}$) inhibited the growth of animal isolates of *Pr. mirabilis* and that swarming was prevented at sub-MIC concentrations. More recently Stickler (2002) confirmed that several *Pr. mirabilis* isolates from encrusted catheters were also sensitive to triclosan (MIC, $0.5 \mu\text{g/ml}$). The aim of this section was to examine the activity of triclosan on catheter isolates of *Pr. mirabilis* in more detail.

The MIC and MISC of a collection of *Pr. mirabilis* strains that had been isolated from encrusted urinary catheters were determined and the results are given in Table 2. These data confirm the sensitivity of *Pr. mirabilis* catheter isolates to triclosan, the MISC of the isolates being between 0.1 and $0.2 \mu\text{g/ml}$ and the MIC between 0.1 and $0.3 \mu\text{g/ml}$.

The tube dilution technique was used to examine the bacteriostatic activity of triclosan against *Pr. mirabilis* in artificial urine. A typical set of results is illustrated in Figure 9. The MICs in artificial urine of *Pr. mirabilis* B2, NP6, NP14, NP37, NP43 and NP55 ranged from 0.05 - $0.2 \mu\text{g/ml}$, confirming the sensitivity of *Pr. mirabilis* to triclosan in

<i>Pr. mirabilis</i> strain	Minimum inhibitory swarming concentration ($\mu\text{g/ml}$)	Minimum inhibitory concentration ($\mu\text{g/ml}$)
NP 1	0.1	0.2
NP 6	0.1	0.1
NP 14	0.2	0.2
NP 15	0.1	0.2-0.3
NP 16	0.1	0.2
NP 17	0.1	0.2
NP 30	0.1	0.2
NP 33	0.1	0.2
NP 35	0.1	0.2
NP 36	0.1	0.2
NP 37	0.1	0.1
NP 38	0.2	0.2
NP 41	0.1	0.2
NP 43	0.1	0.2
NP 44	0.2	0.2
NP 45	0.1	0.2
NP 47	0.1	0.2
NP 50	0.1	0.1-0.2
NP 52	0.1	0.2
NP 55	0.1	0.2
B 2	0.1	0.2
NSM 6	0.1	0.2
NSM 42	0.1	0.2
<i>E. coli</i> (NCTC 10148)	N/A	0.2

Table 2: The MIC and MISC of triclosan in agar of 23 *Pr. mirabilis* isolates from patient's catheters. The MIC value of triclosan against the *E. coli* reference strain (NCTC 10418) is also included. The results are based on experiments replicated three times.

Figure 9: A *Pr. mirabilis* B2 artificial from urine swab, containing different concentrations of triclosan.

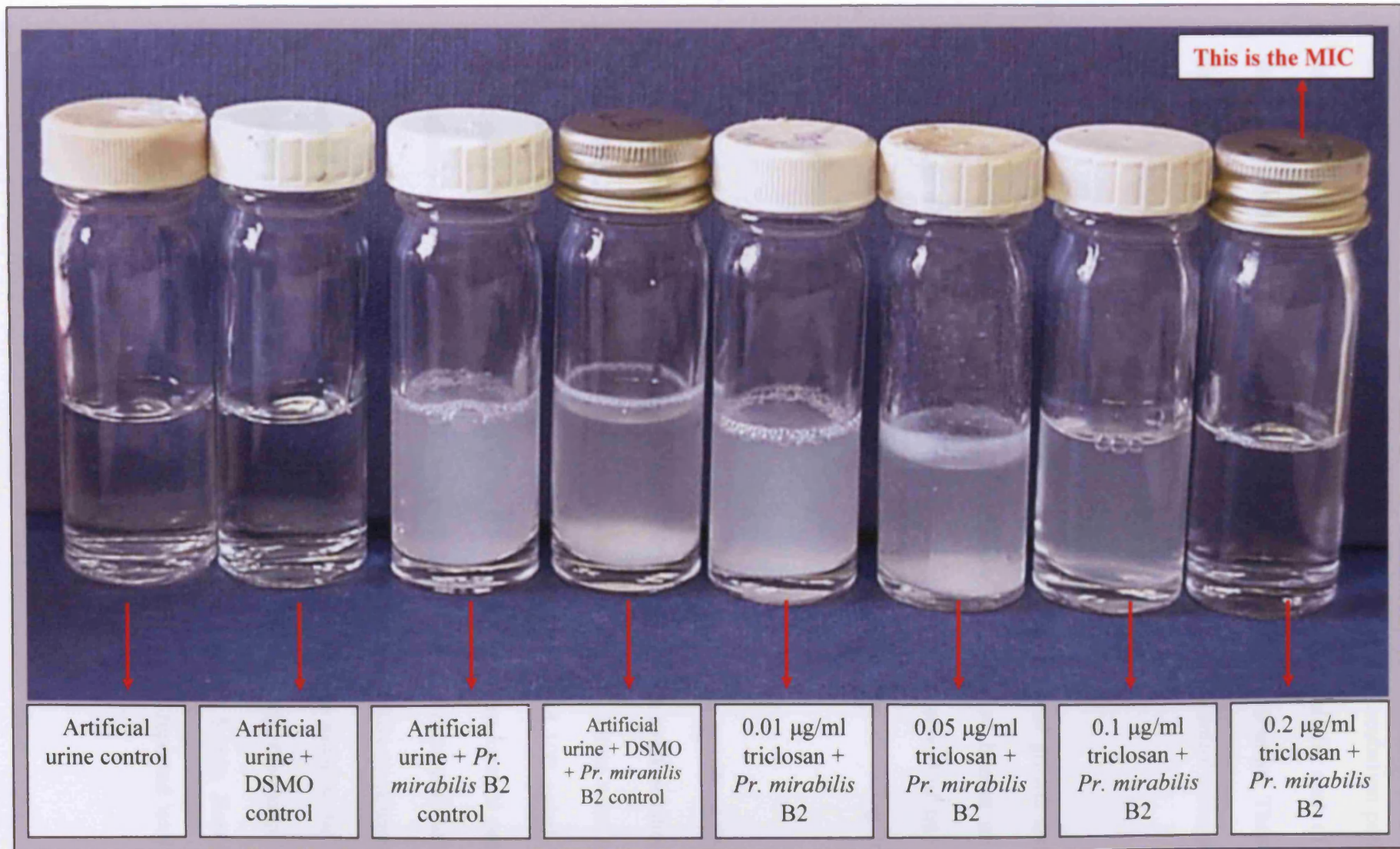


Figure 9: A *Pr. mirabilis* B2 artificial urine culture series containing different concentrations of triclosan.

artificial urine. The pH of each culture was measured after the incubation period, the results are presented in Figure 10. It can be seen that the mean pH for each of the six strains exposed to 0.2 µg/ml triclosan was still acidic after incubation. These mean values were all significantly ($P < 0.05$) lower than the pH of the control cultures for all six *Pr. mirabilis* strains.

The next step was to determine the effect of triclosan on bacterial growth and pH in pooled human urine. The growth of *Pr. mirabilis* B2 in pooled human urine was followed, monitoring the viable cell count and the pH over a period of time in the presence and absence of triclosan. Experiments were performed adding triclosan either at stationary phase or at mid-log phase.

Aliquots (2 ml) of an overnight culture of *Pr. mirabilis* B2 grown in 100 ml TSB were used to inoculate sterile pooled human urine (200 ml). Triclosan stock solutions were prepared at 200 times the final required concentration in DMSO. These were added to give final triclosan concentrations in the urine of 0.1, 1, 10 and 100 µg/ml, and all resulting in a final DMSO concentration of 0.5%. A human urine control containing no triclosan and a 0.5% DMSO control were also tested. Prior to incubation at 37°C in an orbital shaker, the flasks were shaken and a sample was taken to determine the viable cell count and the pH. Samples were taken every hour to monitor the pH and the viable cell counts. Before performing the viable cell counts, the samples were neutralised by placing 1 ml into 9 ml of the neutraliser for 5 min. Experiments determining the efficacy and non-toxicity of the neutraliser are reported later in this section.

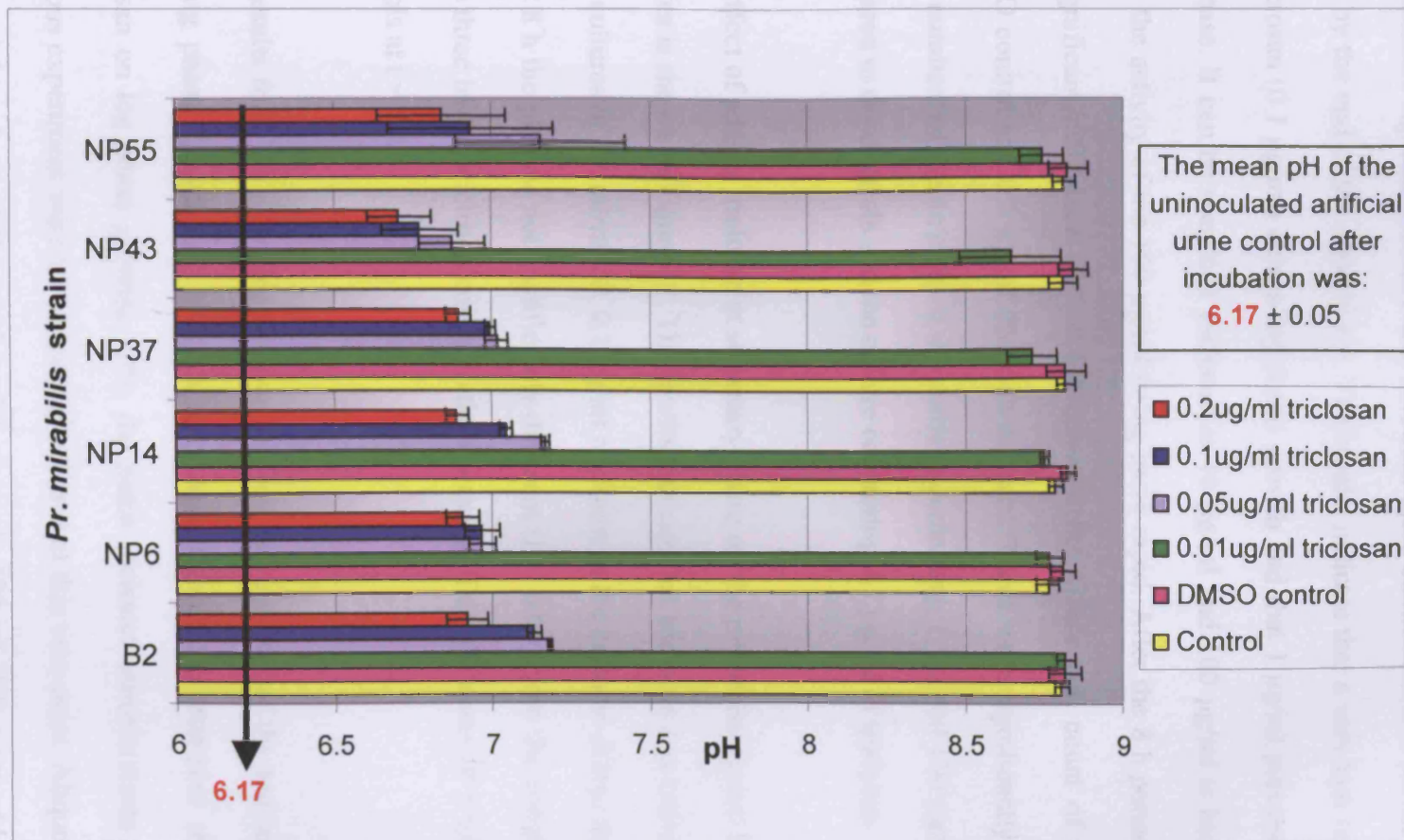


Figure 10: The effect of different triclosan concentrations on the pH values of artificial urine culture series' inoculated with *Pr. mirabilis*. Artificial urine culture series' were set up containing different concentrations of triclosan, and inoculated with one of six different *Pr. mirabilis* strains. These results show the pH values of the cultures after a 6 h incubation period. The results are a mean of three experiments. The black arrow indicates the mean pH of the uninoculated artificial urine controls after incubation.

Figure 11 shows the data from the experiment adding triclosan at $t = 0$ h (stationary phase). The results show that the control and DMSO control cultures produce characteristic sigmoidal curves, and that both these control cultures reached stationary phase by the end of the experiment. The results indicate that a very low concentration of triclosan ($0.1 \mu\text{g/ml}$) delays log phase growth and that $1 \mu\text{g/ml}$ prevents entry into log phase. It can be seen that triclosan at $10 \mu\text{g/ml}$ and $100 \mu\text{g/ml}$ is bactericidal in urine, the activity of the $100 \mu\text{g/ml}$ being more rapid. After the 8 h period, there was no significant difference ($P > 0.05$) between the viable cell count of the control, DMSO control and $0.1 \mu\text{g/ml}$ triclosan cultures. There were significantly lower ($P < 0.05$) numbers of viable cells in the cultures containing 1, 10 and $100 \mu\text{g/ml}$ triclosan compared to the controls and the culture containing $0.1 \mu\text{g/ml}$ of triclosan.

The effect of adding triclosan at stationary phase on the pH of the pooled human urine cultures is shown in Figure 12. The results indicate that triclosan can control the pH of urine cultures of *Pr. mirabilis*. $0.1 \mu\text{g/ml}$ triclosan in the cultures delays the rise in pH but at 8 h the pH was not significantly different ($P > 0.05$) from the controls. The pH of the three higher triclosan concentrations was significantly lower ($P < 0.05$) than the controls at $t = 8$ h.

The results from the experiment above showed that cultures of the test strain were at mid-log phase growth at $t = 2.5$ h after inoculation. To determine the effect of triclosan on log phase growing cells, the same triclosan concentrations used in the previous experiment were tested against cultures at this time point. Aliquots (2 ml) of an overnight culture of *Pr. mirabilis* B2 grown in 100 ml TSB were added to sterile pooled human urine (200 ml). These cultures were shaken and a sample was taken to

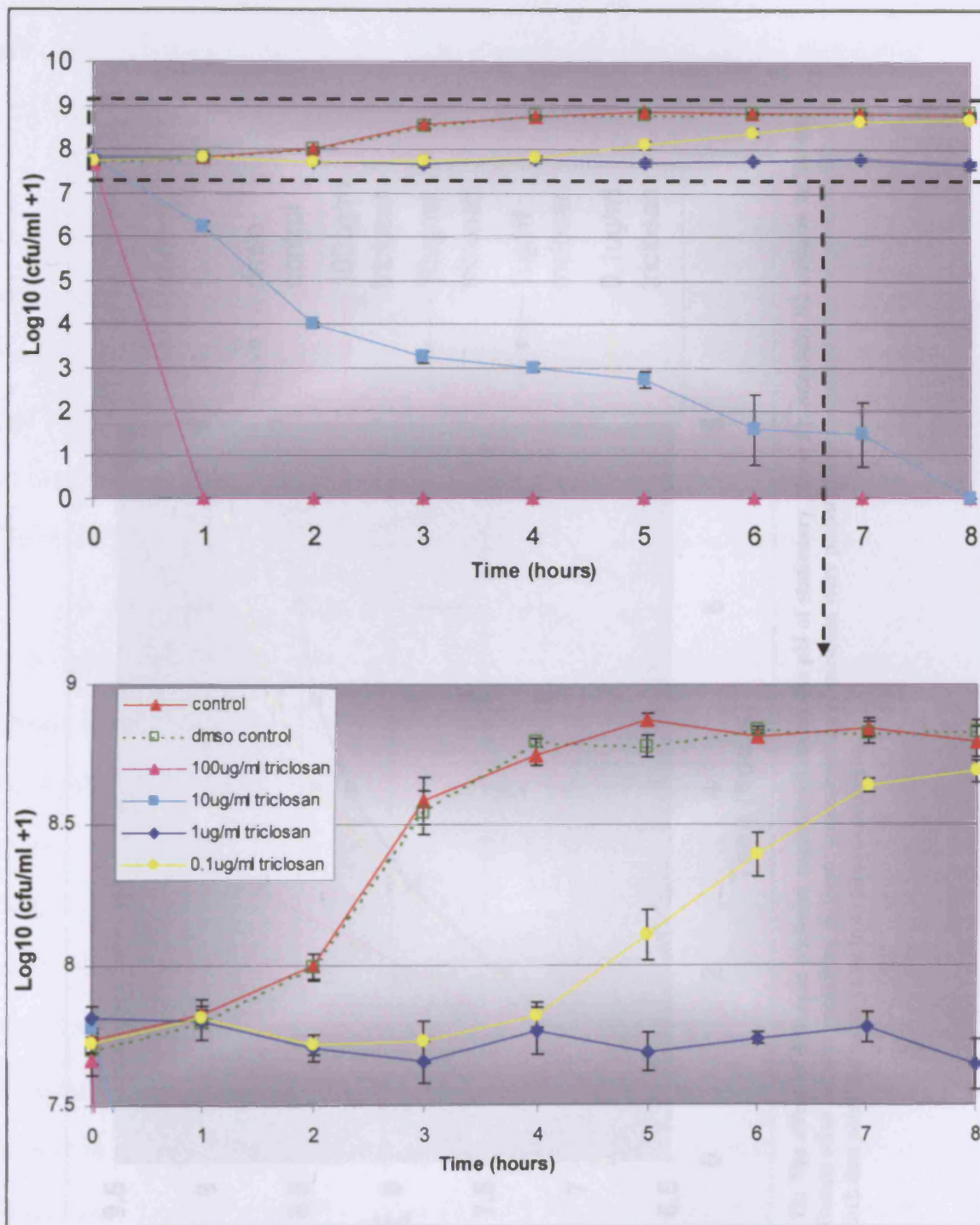


Figure 11: The effect of different triclosan concentrations on stationary phase *Pr. mirabilis* B2 cultures in pooled human urine. Human urine cultures containing different concentrations of triclosan were prepared and inoculated with *Pr. mirabilis* B2 at t = 0 h. Each data point is a mean value from three experiments.

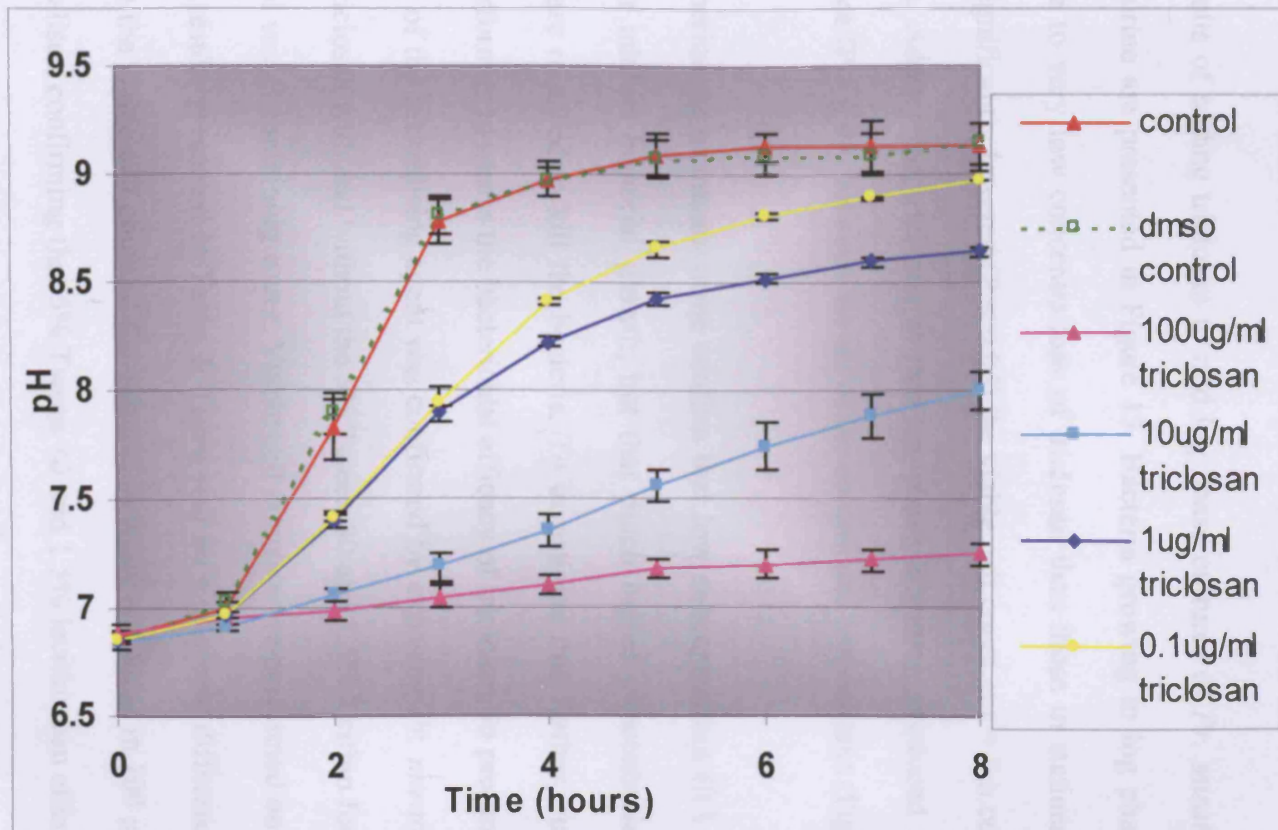


Figure 12: The effect of different triclosan concentrations on the pH of stationary phase *Pr. mirabilis* B2 cultures in human urine. Human urine cultures containing different concentrations of triclosan were prepared and inoculated with *Pr. mirabilis* B2 at t = 0 h. Each data point is a mean value from three experiments.

determine the viable cell count and the pH (at $t = 0$ h) before the cultures were placed in the 37°C orbital shaker. Samples were taken and analysed hourly throughout the experiment. In addition a sample was taken immediately after adding the triclosan or DMSO at $t = 2.5$ h.

The results of adding triclosan to mid-log phase cultures of *Pr. mirabilis* in pooled human urine are presented in Figure 13. Bacteria growing in log phase were more sensitive to very low concentrations of triclosan than those in stationary phase. 0.1 µg/ml significantly lowered ($P < 0.05$) the viable cell count at $t = 8$ h compared to the controls. Adding the triclosan at mid-log phase however, produced no significant difference ($P > 0.05$) between the pH of the control and test cultures (Figure 14).

The experiments in human urine confirm that low concentrations (0.1 - 1 µg/ml) of triclosan inhibits bacterial growth, but that much higher concentrations (10 - 100 µg/ml) are required to kill the bacteria. To investigate this further, suspension tests were performed to assess the bactericidal efficacy of triclosan. In preliminary tests the efficacy of the neutralising agent was confirmed by exposing *Pr. mirabilis* B2 to 100 µg/ml triclosan with and without the 5% Tween 80 and 1.5% lecithin for 5 min, and to a control vessel containing water. Viable cell counts were performed on these cultures and the results presented in Table 3. There was no significant difference ($P = 0.919$) between the viable cell count of the cultures in water and those in 100 µg/ml triclosan in neutraliser, confirming that 5% Tween 80 and 1.5% lecithin can effectively quench bactericidal activity of triclosan at the concentration tested.

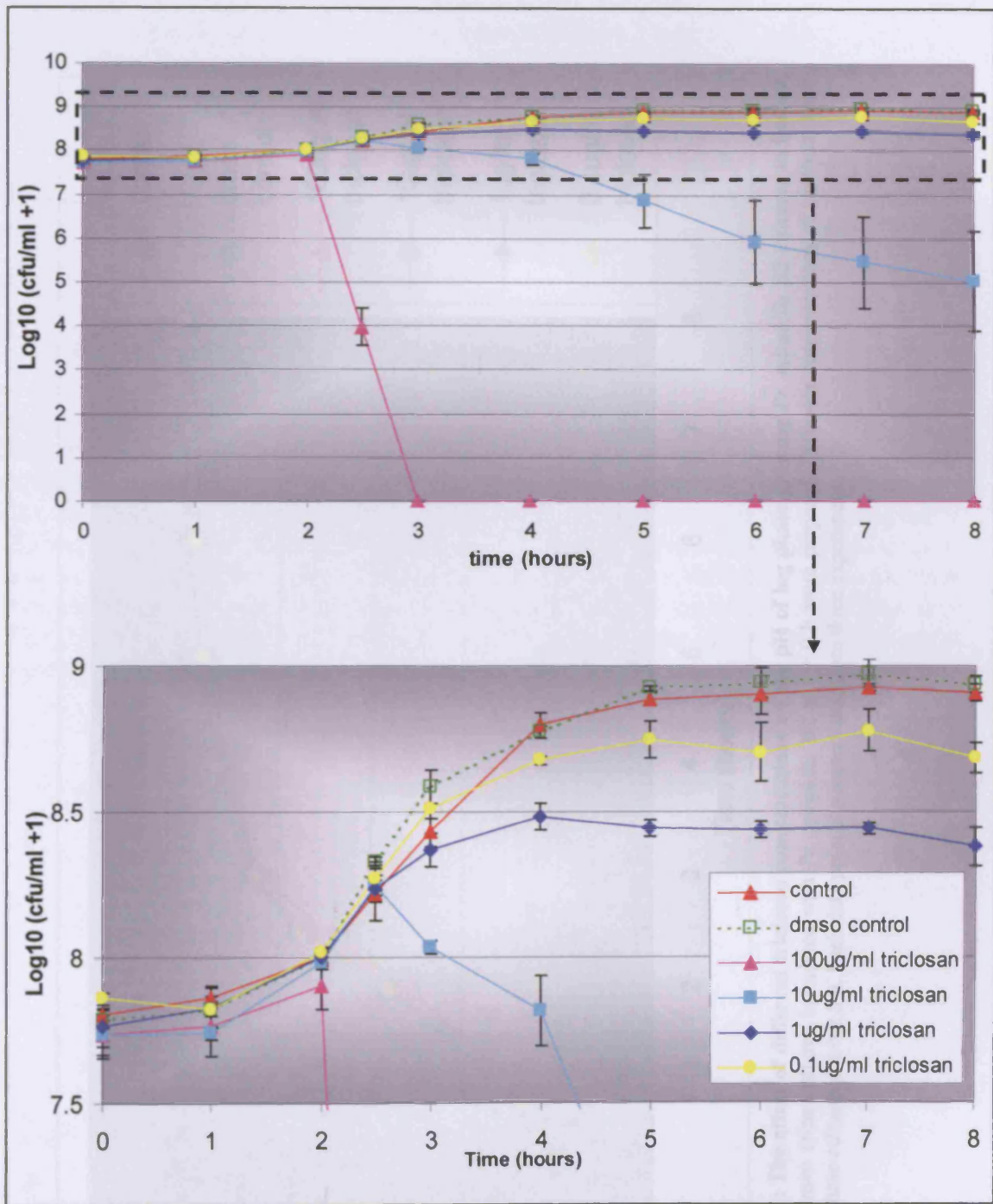


Figure 13: The effect of different triclosan concentrations on log phase growing *Pr. mirabilis* B2 in pooled human urine. Human urine cultures inoculated with *Pr. mirabilis* B2 at t = 0 h were prepared. Different concentrations of triclosan were added to these cultures at t = 2.5 h. Each data point is a mean value from three experiments.

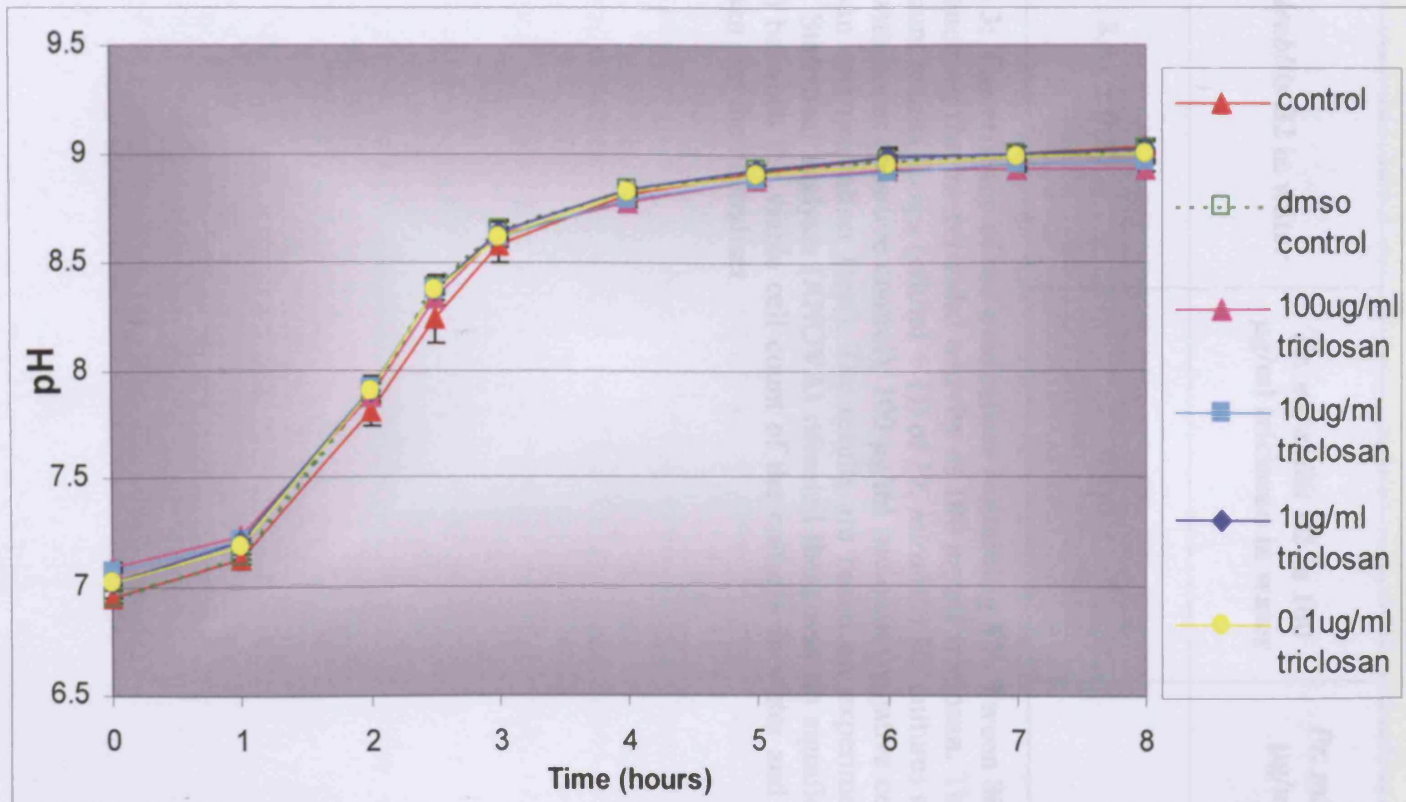


Figure 14: The effect of different triclosan concentrations on the pH of log phase growing *Pr. mirabilis* B2 cultures in human urine. Human urine cultures inoculated with *Pr. mirabilis* B2 at t = 0 h were prepared. Different concentrations of triclosan were added to these cultures at t = 2.5 h. Each data point is a mean value from three experiments.

The effect of the neutraliser and the 1% DMSO used in the suspension tests was

Viable cell count of test suspensions ($\text{Log}_{10} (\text{cfu/ml} + 1)$)		
<i>Pr. mirabilis</i> B2 in water	<i>Pr. mirabilis</i> B2 in 100 $\mu\text{g/ml}$ triclosan in water	<i>Pr. mirabilis</i> B2 in 100 $\mu\text{g/ml}$ triclosan and neutraliser
8.81 ± 0.10	0 ± 0	8.78 ± 0.12

Table 3: The efficacy of the neutraliser containing 5% Tween 80 and 1.5% lecithin at quenching the bactericidal activity of 100 $\mu\text{g/ml}$ triclosan. The data shows viable cell count results ($\text{Log}_{10} (\text{cfu/ml} + 1)$) of *Pr. mirabilis* B2 cultures after a 5 min contact time with water (positive control), 100 $\mu\text{g/ml}$ triclosan (negative control) or 100 $\mu\text{g/ml}$ triclosan and neutraliser (test). The results are based on experiments replicated three times. Statistical analysis (ANOVA) revealed there was no significant difference ($P = 0.919$) between the viable cell count of the cultures in water and those in 100 $\mu\text{g/ml}$ triclosan and the neutraliser.

strains tested produced a five-log reduction in viable cell counts in 5 min with this concentration, contact times of 30 and 60 min were also included in the experiment.

The suspension test results with five different *Pr. mirabilis* strains are given in Figure 15. The mean \log_{10} reduction in viable cell count increases with time for all five strains, but there is considerable variability in the \log_{10} reduction between strains.

The effect of the neutraliser and the 1% DMSO used in the suspension tests was determined for all strains that were to be analysed using this method. Analysis of the data presented in Table 4 shows there was no significant difference ($P > 0.05$) between the control cultures and the neutraliser and DMSO treated cultures. These results confirm that the 1% DMSO and the neutraliser were non-toxic to all the strains tested.

In suspension tests the contact time between the antiseptic solution and the bacterial inoculum is normally 5 min. A general accepted requirement for an effective antiseptic is that in suspension tests a \log_{10} reduction in bacterial concentration equal to or greater than five is produced i.e. at least 99.999% of bacterial cells are killed (BS EN 1276 1997; Reybrouck, 1998). The maximum concentration used in the suspension tests in this study was 100 $\mu\text{g/ml}$ triclosan. Since none of the *Pr. mirabilis* strains tested produced a five-log reduction in viable cell counts in 5 min with this concentration, contact times of 30 and 60 min were also included in the experiments. The suspension test results with five different *Pr. mirabilis* strains are given in Figure 15. The mean \log_{10} reduction in viable cell count increases with time for all five strains, but there is considerable variability in the \log_{10} reduction between strains.

Species	Viable cell count of test suspension (Log ₁₀ cfu/ml ± standard error)		
	Control	Neutraliser	DMSO
<i>Pr. mirabilis</i> B2	8.30 ± 0.06	8.25 ± 0.07	8.29 ± 0.07
<i>Pr. mirabilis</i> B2 mutant strain. M44	8.37 ± 0.06	8.42 ± 0.05	8.40 ± 0.05
<i>Pr. mirabilis</i> B2 mutant strain. M48	8.68 ± 0.04	8.65 ± 0.02	8.67 ± 0.02
<i>Pr. mirabilis</i> B2 mutant strain. M55	8.15 ± 0.03	8.15 ± 0.02	8.13 ± 0.03
<i>E.coli</i> NSM 16	8.61 ± 0.10	8.60 ± 0.04	8.54 ± 0.08
<i>K. pneumoniae</i> CC 36	8.10 ± 0.03	8.09 ± 0.02	8.13 ± 0.05
<i>Pv. stuartii</i> NSM 14	8.48 ± 0.21	8.46 ± 0.23	8.47 ± 0.20
<i>Ent. faecalis</i> CC 39	7.99 ± 0.03	7.98 ± 0.07	7.97 ± 0.01
<i>Ser. marcescens</i> CC 13	8.35 ± 0.04	8.34 ± 0.03	8.36 ± 0.05
<i>M. morgani</i> MS 2	8.68 ± 0.13	8.67 ± 0.12	8.69 ± 0.14
<i>S. aureus</i> NSM 5	8.25 ± 0.03	8.20 ± 0.06	8.24 ± 0.06
<i>Ps. aeruginosa</i> NSM 57	8.20 ± 0.03	8.22 ± 0.02	8.21 ± 0.04
<i>Pr. mirabilis</i> NSM 6	8.19 ± 0.06	8.20 ± 0.02	8.16 ± 0.08
<i>Pr. mirabilis</i> NSM 42	8.41 ± 0.15	8.39 ± 0.13	8.37 ± 0.17
<i>Pr. mirabilis</i> NP 55	8.72 ± 0.13	8.70 ± 0.11	8.71 ± 0.11
<i>Pr. mirabilis</i> NP 37	8.49 ± 0.09	8.44 ± 0.08	8.47 ± 0.06

Table 4: The viable cell count (Log₁₀ cfu/ml) of test suspensions of different organisms after 15-minutes contact time with water (control), neutraliser and 1% DMSO. Analysis of the data presented in this table showed there was no significant difference ($P > 0.05$) between the control suspensions and the neutraliser or DMSO treated suspensions. This data confirms the non-toxicity of the neutraliser and of 1% DMSO. The results are a mean of three experiments.

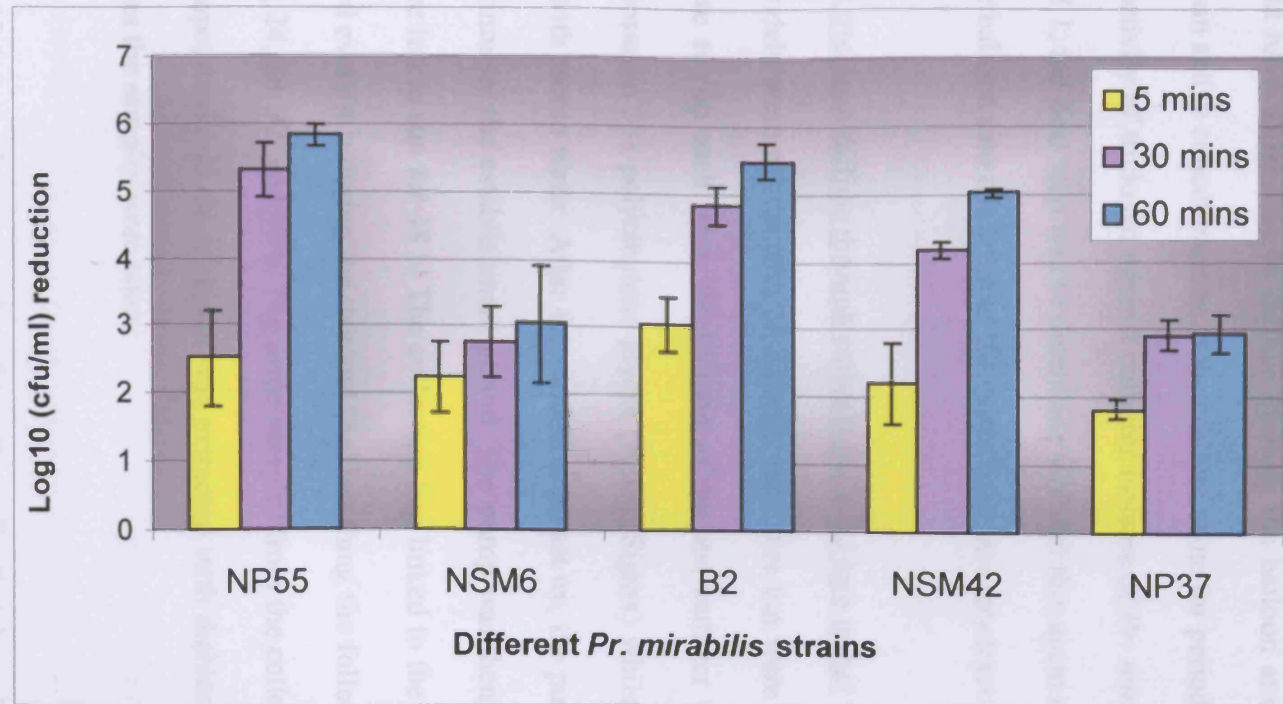


Figure 15: Log_{10} (cfu/ml) reduction for five strains of *Pr. mirabilis* after three contact times with 100 $\mu\text{g/ml}$ triclosan. Suspension tests were performed to determine the bactericidal efficacy of triclosan against five stains of *Pr. mirabilis*. The *Pr. mirabilis* strains were exposed to 100 $\mu\text{g/ml}$ triclosan for 5, 30 and 60 min. Results are a mean of values obtained from three experiments.

3.2. Experiments to determine the feasibility of using the catheter retention balloon as a novel way of introducing triclosan into the catheterised urinary tract

Potentially the catheter balloon provides a large reservoir for agents that might act as inhibitors of the encrustation process. If it is to be exploited in this way however, the agent would have to be able to diffuse through the balloon at a controlled rate that would sustain active concentrations in the bladder urine for periods of up to 12 weeks. Since the activity of triclosan against catheter isolates of *Pr. mirabilis* was confirmed in section 3.1, the next step was to determine whether this agent could diffuse through the catheter balloon into the urine of the catheterised urinary tract.

3.2.1. Does triclosan diffuse through catheter balloons into urine?

Bladder models were set up with all-silicone catheters but were not inoculated. Two models were set up each time; the balloon of the test catheter was inflated with 10 mg/ml triclosan in 5% polyethylene-glycol (PEG) (Sigma) whilst the control balloon was filled with sterile water. After the models were set up, the pump was switched off for 1 h to simulate the establishment period. The pump was then switched on and the models were left to run for 48 h. The collection bag linked to the triclosan test models was emptied every 4 h during the day and the first thing the following morning, i.e. at time = 4, 8, 24, 28, 32, and 48 h. The urine samples from the collection bags were kept at room temperature and the triclosan was extracted with dichloromethane as soon as possible after the samples were taken.

At time = 48 h, samples were taken from the collection bags linked to the control catheters. These samples along with two fresh artificial urine samples were put through the same extraction procedure as those samples suspected of containing triclosan.

These controls ensured that there was nothing in the artificial urine or in the catheter polymer that would give a background reading from the high pressure liquid chromatography (HPLC) close to the triclosan peak. After extraction of triclosan from each of the artificial urine samples, HPLC was used to determine the amount of triclosan in each of the samples (Section 2.8). This experiment was replicated twice.

The concentration of triclosan in the artificial urine samples taken from collection bags of catheters inflated with 10 mg/ml triclosan in 5% PEG over a 48 h period are shown in Figure 16. The results from the spiked artificial urine samples determined that the percentage efficacy of the extraction process was 81.3%, the results in Figure 16 have been corrected to take this into account. No triclosan was detected in the control artificial urine or in the samples taken from the control models fitted with catheters inflated with sterile water.

3.2.2. Does triclosan impregnate the catheters?

After establishing that triclosan could pass through the balloons of all-silicone catheters, further bladder models were set up to determine if catheters would become impregnated with triclosan. Bladder models were run in the same way as in section 3.2.1, using all-silicone catheters and both types of latex based catheters. Models were not inoculated and were left to run for 48 h. At the end of the experiment the catheters were taken out of the models and the test catheters that had been inflated with triclosan had their balloons flushed out. This was done by inflating and deflating the catheter balloon with fresh sterile water 10 times. To determine if the triclosan had impregnated the catheter material, test and control catheter sections were placed onto *Pr. mirabilis* B2 bacterial lawns (section 2.4.2).

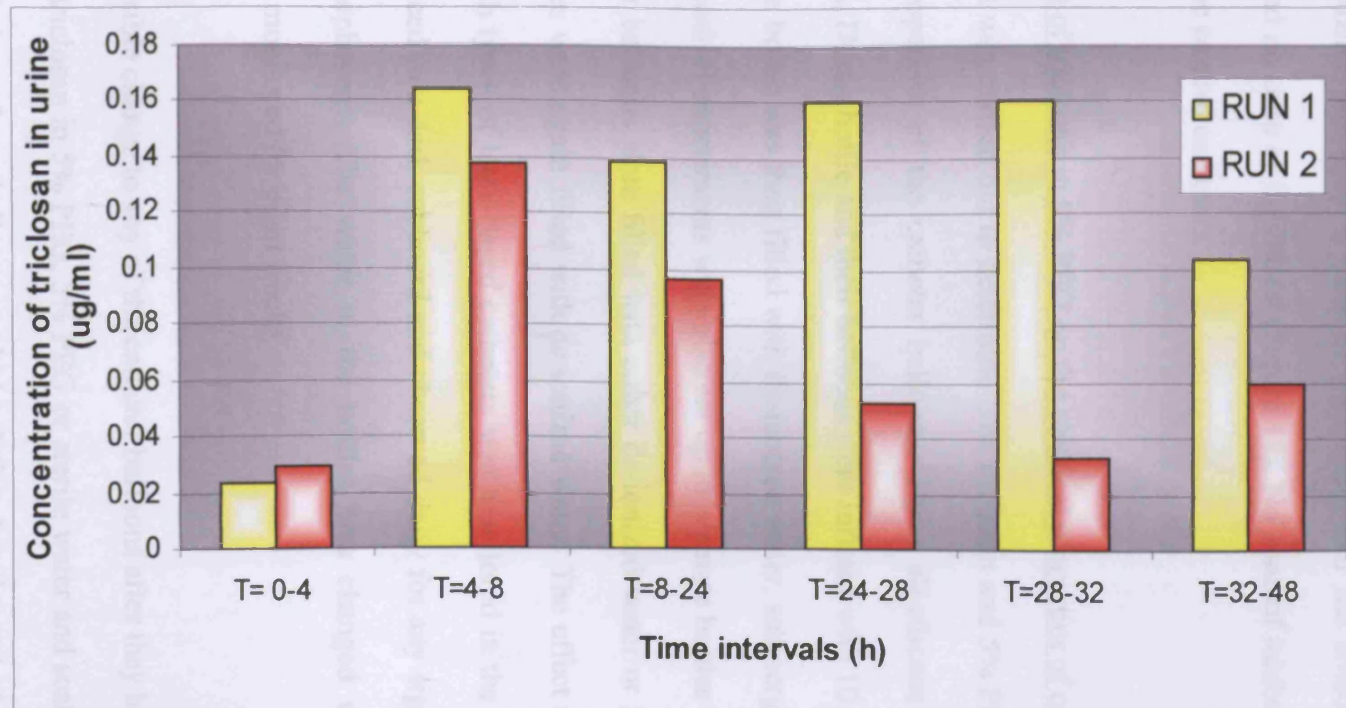


Figure 16: The concentration of triclosan ($\mu\text{g/ml}$) in the artificial urine samples taken from bladder models fitted with all-silicone catheters inflated with 10 mg/ml triclosan in 5%PEG over a 48 h period. These values have been corrected to take into account the efficacy of the extraction process.

Each impregnated all-silicone catheter section produced a zone of inhibition, indicating that triclosan impregnated the length of the all-silicone catheters. In contrast, only the top section from directly beneath the balloon of the triclosan impregnated hydrogel-coated latex catheter produced a zone of inhibition, and the silicone-coated latex catheter produced no zone of inhibition (Figure 17). No zone of inhibition was visible around any of the control catheters.

3.2.3. The effect of triclosan in 5% PEG on the physical properties of catheter balloons

This experiment was carried out to determine how triclosan and 5% PEG might affect the physical properties of the catheter balloons. Three all-silicone catheters were placed into a 1 L Duran bottle and their balloons were inflated with 10 mg/ml triclosan in 5% PEG. The bottle was then filled with de-ionized water, submerging the catheter balloons. Two control experiments were also set up in separate bottles where three all-silicone catheter balloons were filled with either de-ionized water or 5% PEG before the Duran bottles were again filled with de-ionized water. The effect of triclosan and 5% PEG on both types of latex-based catheters was monitored in the same way. The bottles were placed in a dark cupboard and observed daily for any signs of change in the balloon morphology. The water in the bottles was changed weekly and the experiment was monitored for eight weeks.

There was no visible change to any of the catheter balloons after they had been inflated with 10 mg/ml triclosan in 5% PEG, 5% PEG or sterile water and soaked in water for eight weeks. All the catheter balloons could be deflated at the end of the experiment. The physical properties of the balloons were analysed using the Lloyds testing instrument. This instrument works by placing samples of materials into tight grips.

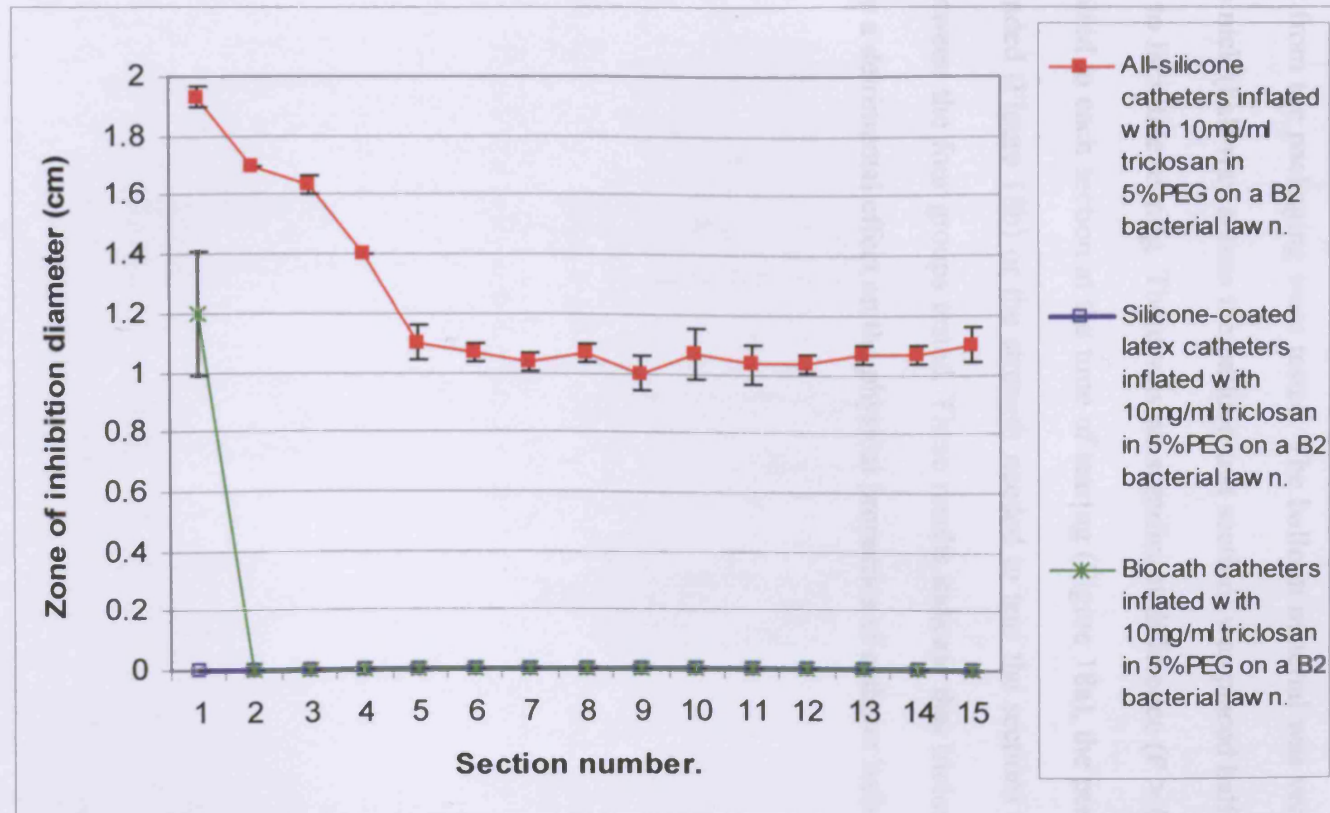


Figure 17: The mean zone of inhibition size (cm) produced by 0.5 cm sections taken down the length of three different catheter types that had been inflated with triclosan in the bladder models for 48 h. Catheter sections were placed onto *Pr. mirabilis* B2 bacterial lawns. Section 1 was directly below the catheter balloon, sections 2 – 15 were taken every other 1 cm down the length of the catheter. The results are a mean of values obtained from three experiments.

These grips that are linked to a computer system pull the sample until it tears, giving information about the physical properties of the material. The balloons of the latex based catheters were too strong to be tested using this instrument, but the balloons of the three sets of test all-silicone catheters along with a control catheter balloon taken straight from the packaging were tested. The balloon material was very hard to break, hence a nick (half way across the balloon test section) was placed half way down each sample to facilitate tearing. There was no significant difference ($P > 0.05$) in the load (N) applied to each section at the time of tearing (Figure 18a), the percentage it could be extended (Figure 18b) or the strength needed to tear the sections (N/mm) (Figure 18c) between the four groups tested. These results indicate that triclosan and PEG do not have a detrimental effect on the physical properties of catheter balloons.

3.3. The effect of incorporating triclosan into the polyurethane urinary tract on the

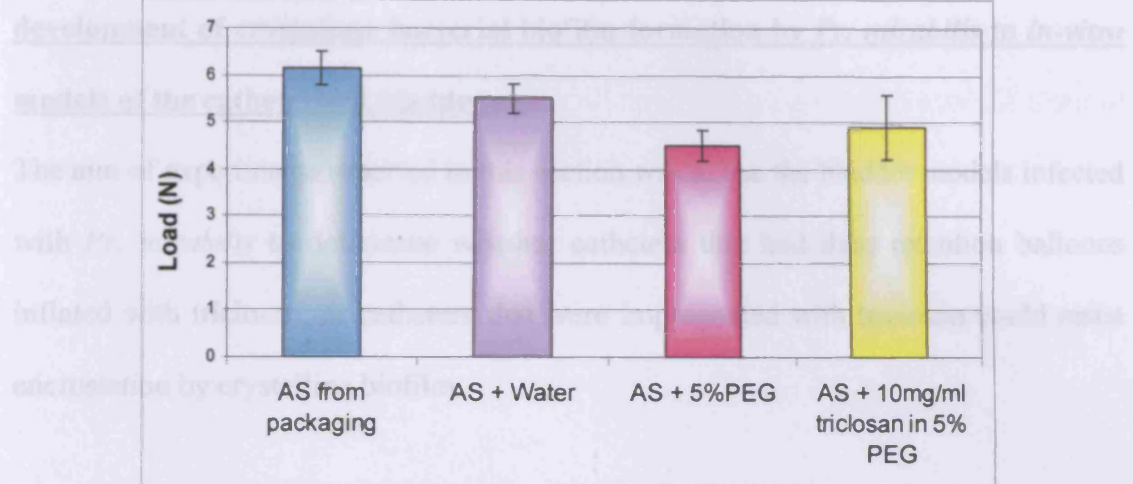


Figure 18a: Load (Newtons) applied to catheter sections at breakage.

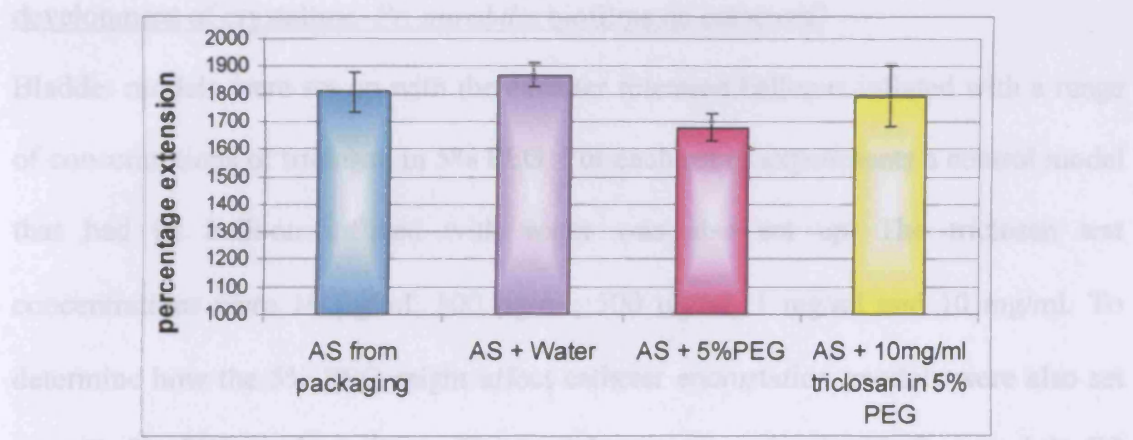


Figure 18b: Percentage extension results.

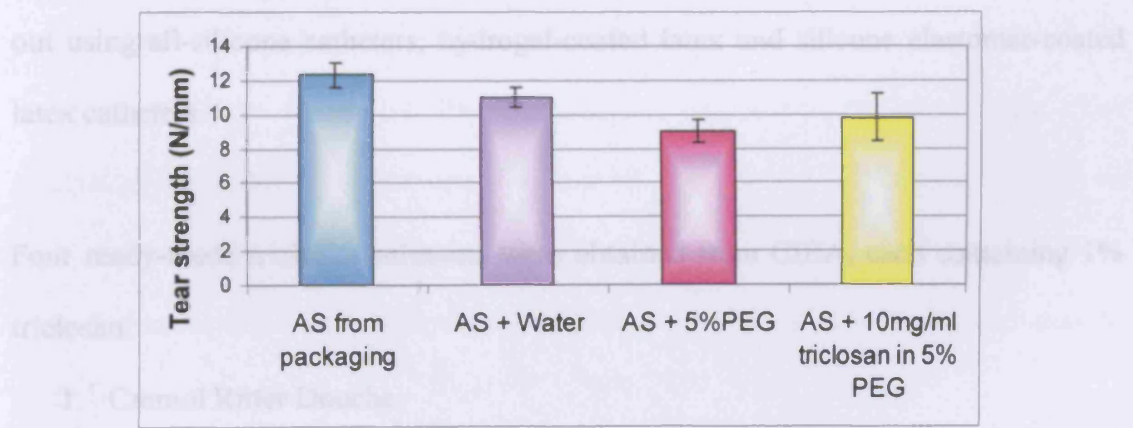


Figure 18c: Tear strength (N/mm) results.

Figure 18: The effect of triclosan on the physical properties of all-silicone (AS) catheter balloons. Load (N), percentage extension and tear strength (N/mm) results from all-silicone catheter balloons taken straight from the packaging, and from balloons that were submerged in water for eight weeks filled with either water, 5% PEG or 10 mg/ml triclosan in 5% PEG. The results are a mean of values obtained from three experiments.

3.3. The effect of introducing triclosan into the catheterised urinary tract on the development of crystalline bacterial biofilm formation by *Pr. mirabilis* in in-vitro models of the catheterised bladder

The aim of experiments reported in this section was to use the bladder models infected with *Pr. mirabilis* to determine whether catheters that had their retention balloons inflated with triclosan, or catheters that were impregnated with triclosan could resist encrustation by crystalline biofilm.

3.3.1. Does filling the retention balloon with triclosan solutions inhibit the development of crystalline *Pr. mirabilis* biofilms on catheters?

Bladder models were set up with the catheter retention balloons inflated with a range of concentrations of triclosan in 5% PEG. For each set of experiments a control model that had its balloon inflated with water was also set up. The triclosan test concentrations were 10 µg/ml, 100 µg/ml, 500 µg/ml, 1 mg/ml and 10 mg/ml. To determine how the 5% PEG might affect catheter encrustation, models were also set up with 5% PEG in the balloon. The models were inoculated with *Pr. mirabilis* B2 and run until blockage or for a maximum of seven days. This experiment was carried out using all-silicone catheters; hydrogel-coated latex and silicone elastomer-coated latex catheters.

Four ready-made triclosan solutions were obtained from CIBA, each containing 1% triclosan.

1. Cremol Ritter Douche.
2. Procutol Wash lotion.
3. Henkel Ecolab skinsan scrub.

4. Dial complete foaming hand wash.

The ability of these commercially available triclosan formulations to prevent encrustation in all-silicone catheters was also tested using the bladder models. Control catheters were inflated with water, and were run alongside test catheters whose balloons were inflated with one of the ready-made triclosan formulations. Models were run until blockage or for a maximum of seven days, and experiments were performed in triplicate.

Figures 19a, 20a and 21a show the time to blockage results for the three different catheter types inflated with different concentrations of triclosan in 5% PEG and inoculated with *Pr. mirabilis* B2. Only two (Henkel Ecolab skinsan scrub and Dial complete foaming hand wash) out of the four pre-made CIBA formulations were tested as the others were too viscous to inflate the retention balloon. The 5% PEG in the balloon produced no significant difference ($P > 0.05$) in the time to blockage for the three catheter types compared to the controls. 10 and 100 $\mu\text{g/ml}$ triclosan in 5% PEG in the catheter balloon significantly increased ($P < 0.05$) the time to blockage of the all-silicone catheters and latex-based catheters respectively compared to the control models. Some of the higher concentrations of triclosan tested enabled the catheters to drain freely for the duration of the seven day experiment. The concentrations required to prevent blockage of the latex-based catheters for seven days were higher than with the all-silicone catheters. Using the two CIBA formulations to inflate the catheter balloon also allowed the all-silicone catheters to drain freely for seven days.

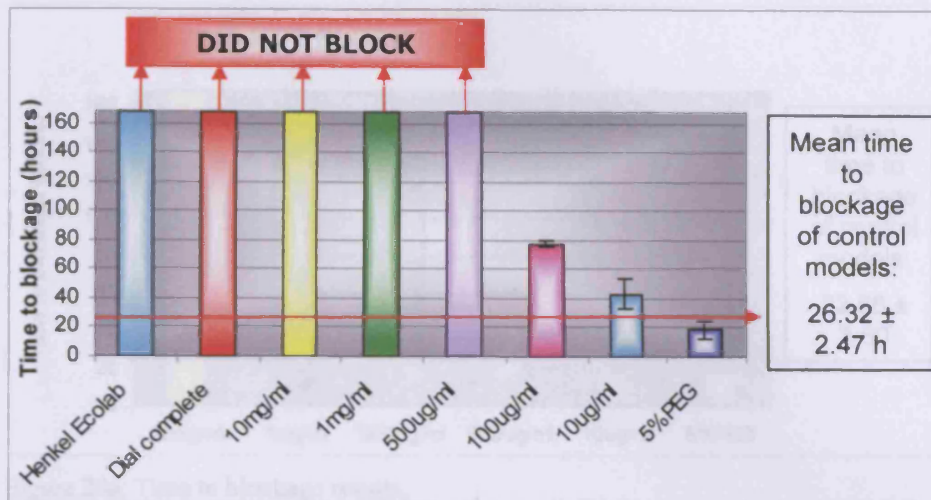


Figure 19a: Time to blockage results.

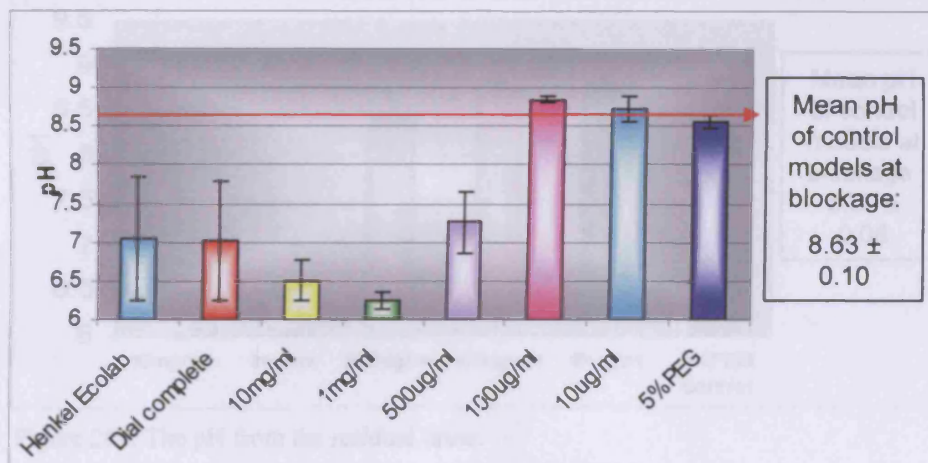


Figure 19b: The pH from the residual urine.

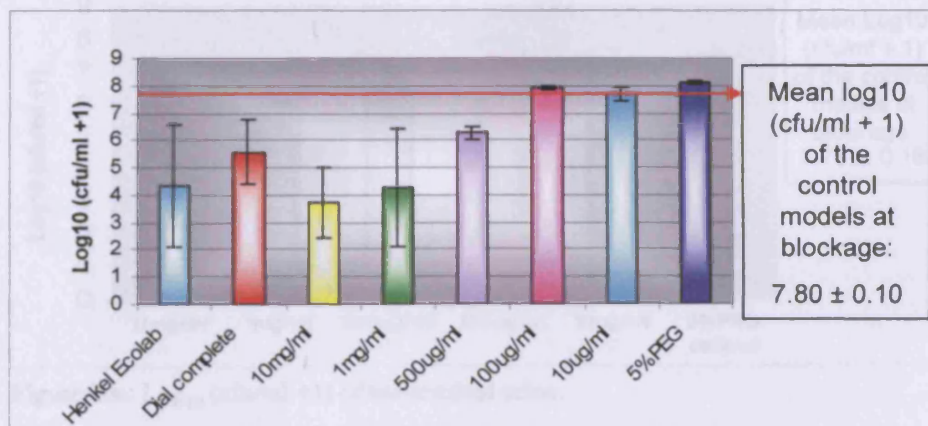


Figure 19c: Log₁₀ (cfu/ml + 1) in the residual urine.

Figure 19: Time to blockage, pH and viable cell count results from models fitted with all-silicone catheters inflated with the CIBA formulations or different concentrations of triclosan in 5% PEG after blockage or after seven days. Models were inoculated with *Pr. mirabilis* B2. The red arrows indicate the results of the control models. Each value is a mean from triplicate experiments.

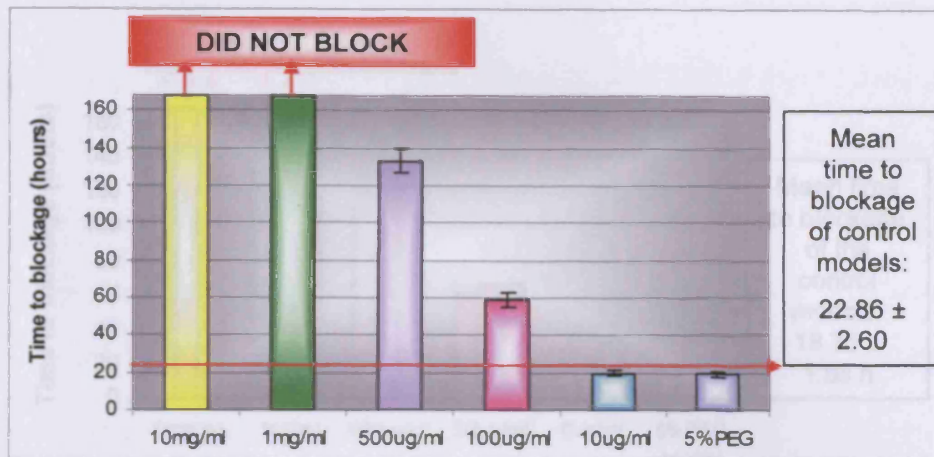


Figure 20a: Time to blockage results.

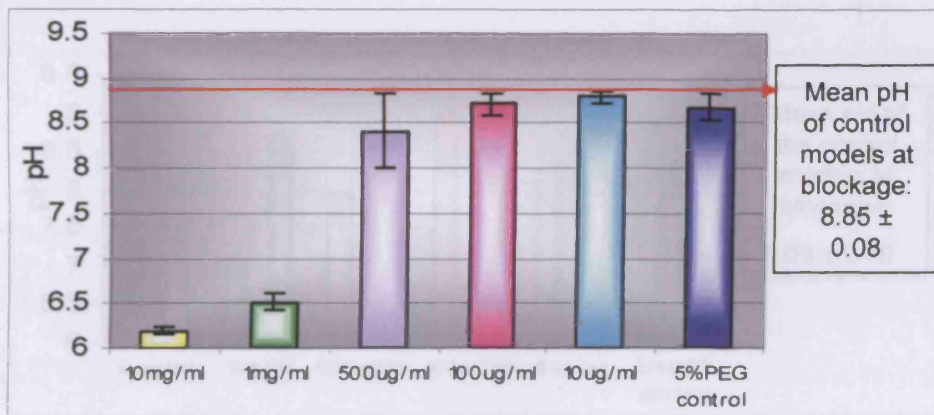


Figure 20b: The pH from the residual urine.

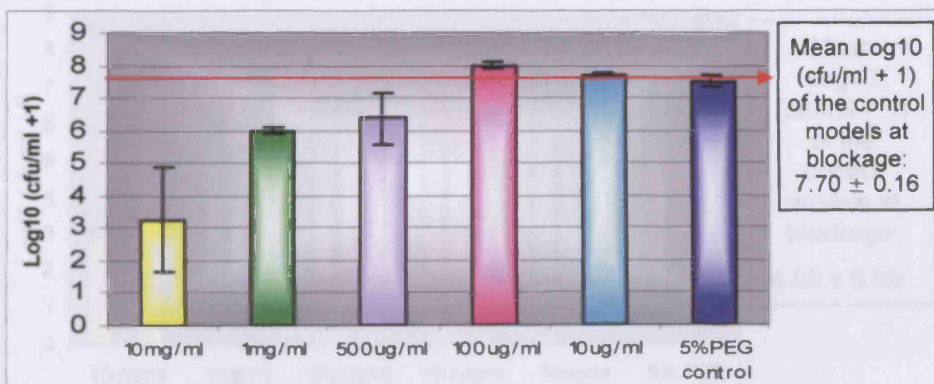


Figure 20c: Log₁₀ (cfu/ml + 1) of the residual urine.

Figure 20: Time to blockage, pH and viable cell count results from models fitted with hydrogel-coated latex catheters inflated with different concentrations of triclosan in 5% PEG after blockage or after seven days. Models were inoculated with *Pr. mirabilis* B2. The red arrows indicate the results of the control models. Each value is a mean from triplicate experiments.

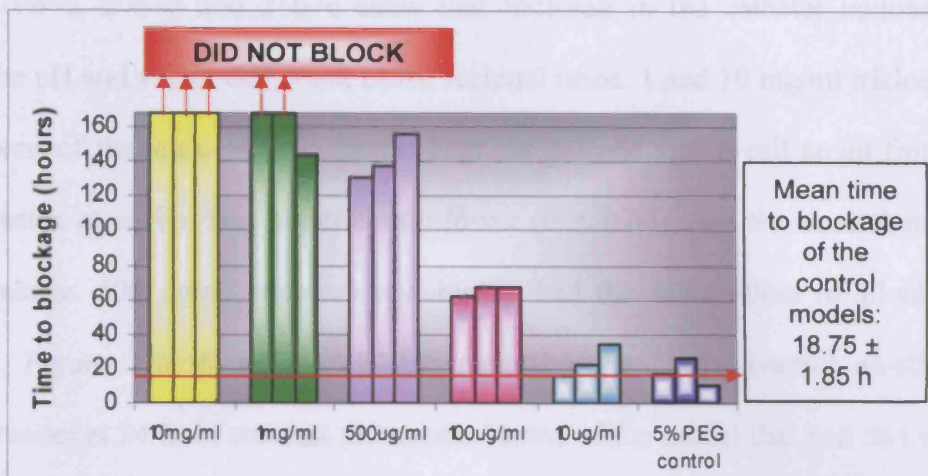


Figure 21a: Time to blockage results.

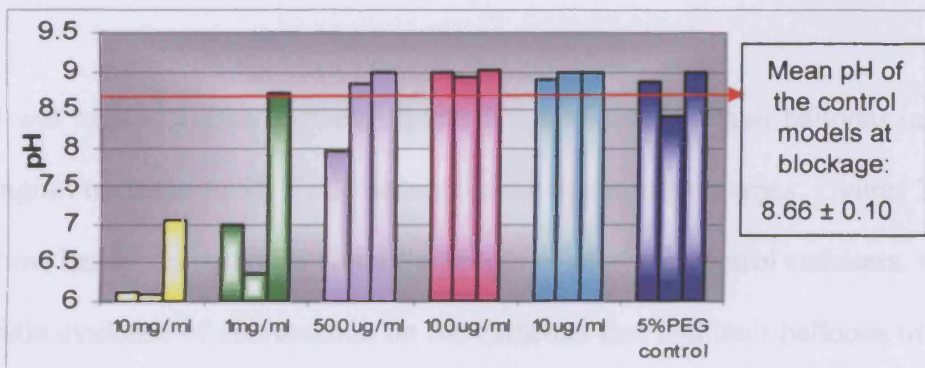


Figure 21b: The pH from the residual urine.

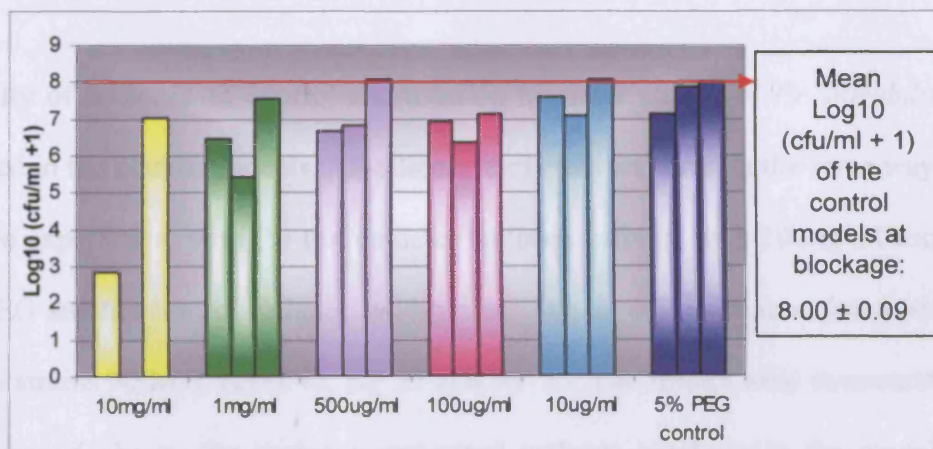


Figure 21c: Log₁₀ (cfu/ml + 1) in the residual urine.

Figure 21: Time to blockage, pH and viable cell count results from models fitted with silicone-coated latex catheters inflated with different concentrations of triclosan in 5% PEG after blockage or after seven days. Models were inoculated with *Pr. mirabilis* B2. The red arrows indicate the results of the control models.

Figures 19b+c, 20b+c and 21b+c show that triclosan in the catheter balloon can control the pH and viable cell count of the residual urine. 1 and 10 mg/ml triclosan in the balloons of the latex-based catheters kept the pH and viable cell count from the residual urine at seven days significantly lower ($P < 0.05$) than the control models after blockage. 500 $\mu\text{g/ml}$ triclosan and higher had the same effect in all-silicone catheters. Figure 22a shows the cloudy residual urine in the control all-silicone catheter model at 24 h, in contrast the residual urine of the model that had its balloon inflated with 10 mg/ml triclosan in 5% PEG was clear at 24 h, and remained like this until the end of the seven day experiment.

LV-SEM was carried out on control and test catheters that had their balloons inflated with 10 mg/ml triclosan in 5% PEG for each of the three catheter types. Figures 23, 24 and 25 show heavy encrustation down the length of all three control catheters, whilst there is little evidence of encrustation on the catheters that had their balloons inflated with 10 mg/ml triclosan in 5% PEG (Figures 26, 27 and 28).

The ability of triclosan to control encrustation by other strains of *Pr. mirabilis* was also tested in the bladder models. All-silicone catheters were run in the same way as in the above experiment, with the test catheter balloons inflated with 10 mg/ml triclosan in 5% PEG and the control catheters with water. The models were inoculated with *Pr. mirabilis* strains NSM 6, NSM 42, NP 37 and NP 55. The results were consistent with those obtained above, the triclosan prevented catheter blockage in the models for seven days (Figure 29a), and significantly ($P < 0.05$) lowered the pH and viable cell count of the test models compared to the controls (Figures 29b+c).

Control



Triclosan

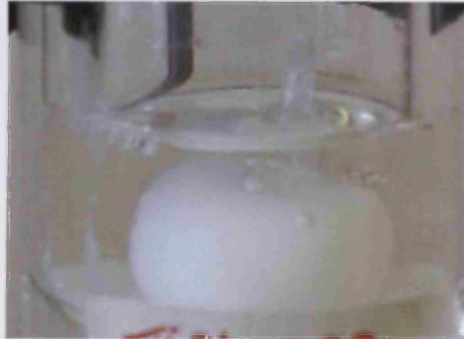
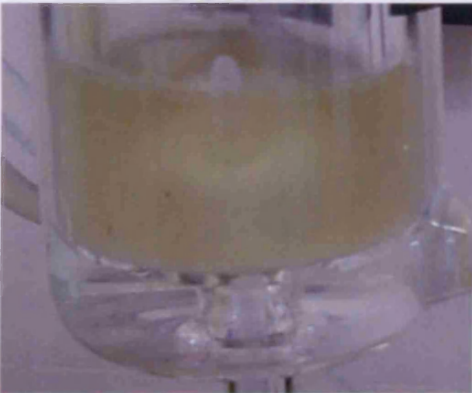


Figure 22a: The residual urine of models supplied with artificial urine and fitted with catheters inflated with 10 mg/ml triclosan in 5% PEG or water 24 h after inoculation with *Pr. mirabilis*.

Control



Triclosan



Figure 22b: The residual urine of models supplied with pooled human urine and fitted with catheters inflated with 10 mg/ml triclosan in 5% PEG or water 24 h after inoculation with *Pr. mirabilis*.

Figure 22: The effect of triclosan on the turbidity of the residual urine in *Pr. mirabilis* infected bladder models supplied with artificial and human urine.

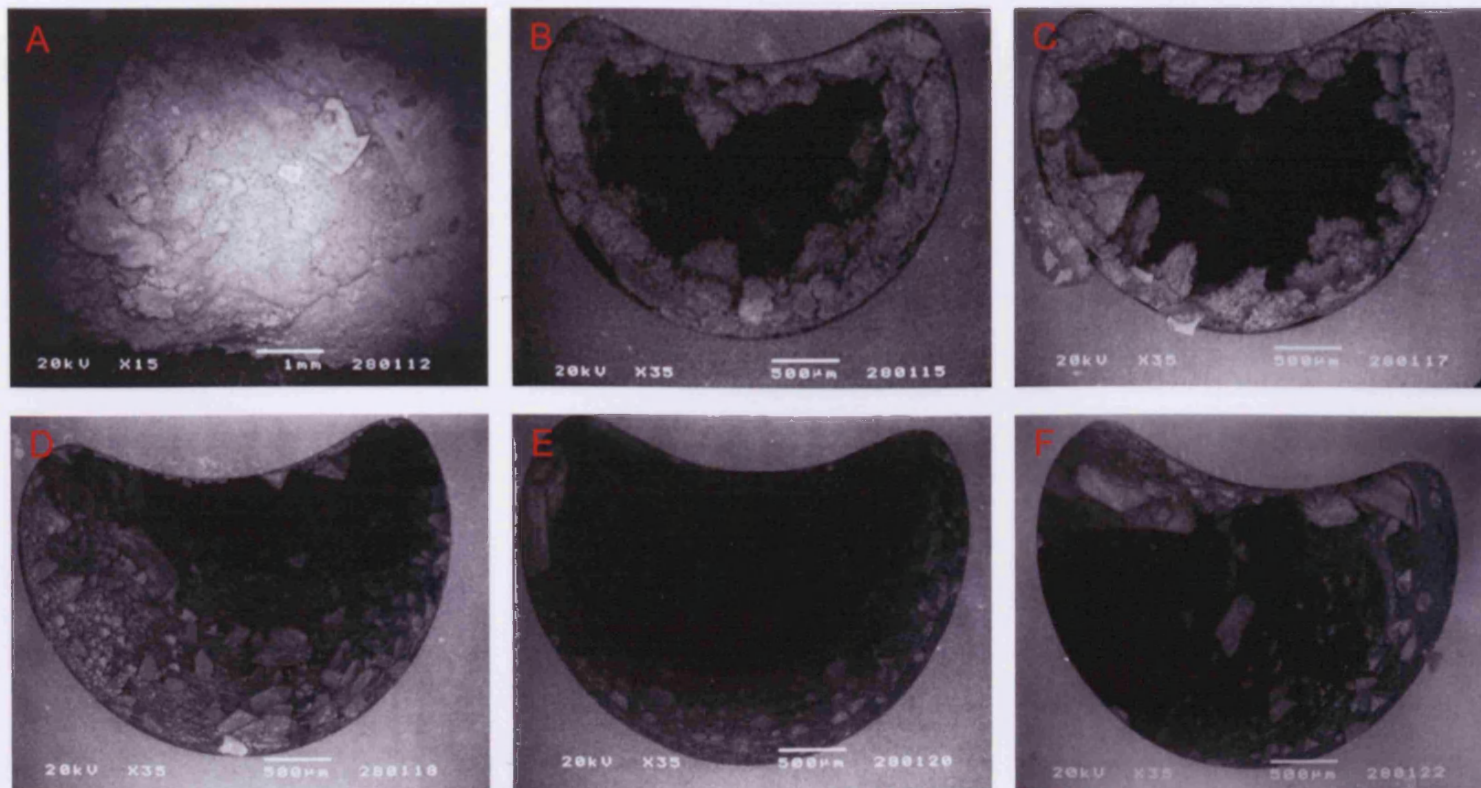


Figure 23: Electron micrographs of a blocked control all-silicone catheter taken from the bladder model. 6 pictures were taken along the length of the catheter. The positions of sections A-F are indicated in Figure 6.

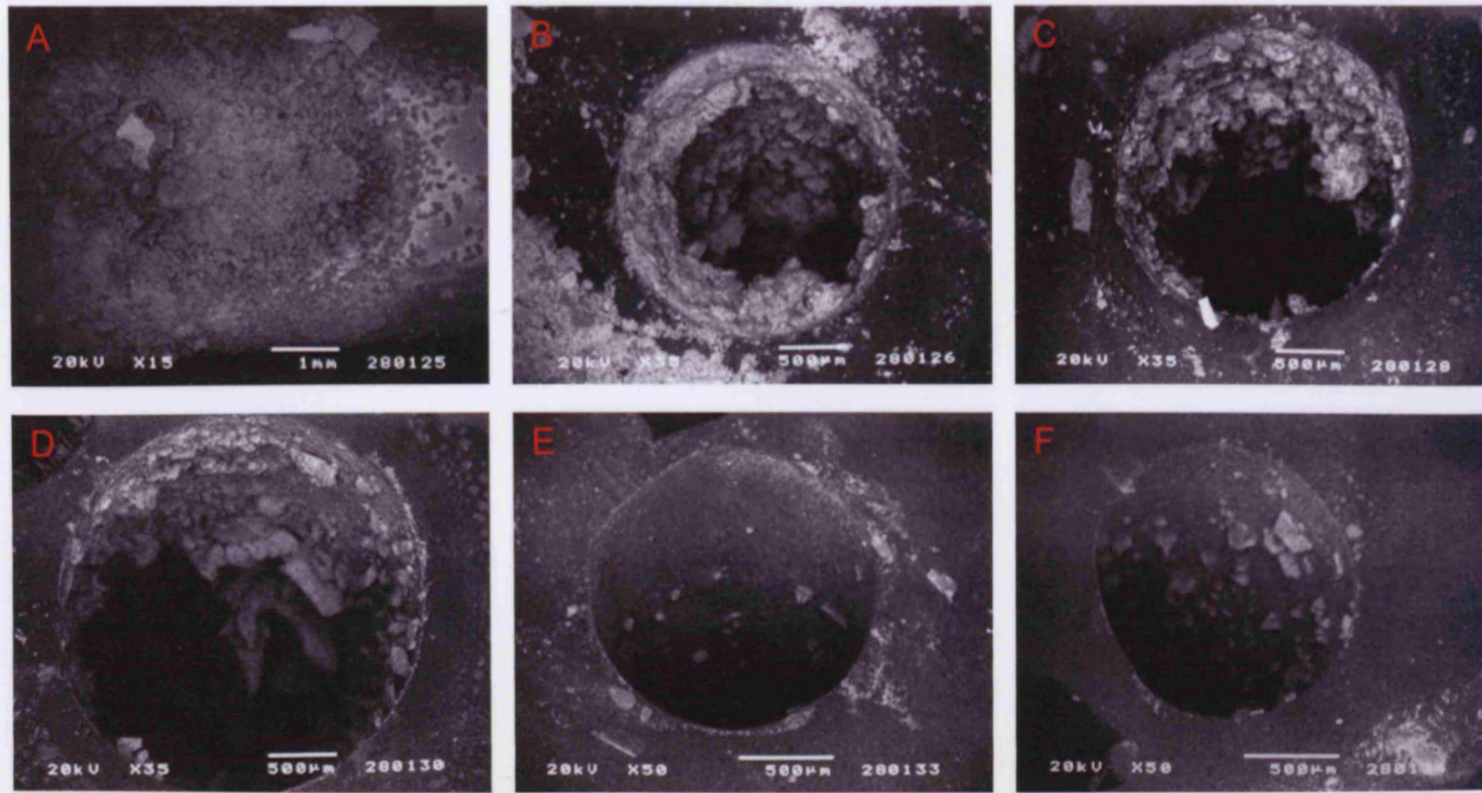


Figure 24: Electron micrographs of a blocked control hydrogel-coated latex catheter taken from the bladder model. 6 pictures were taken along the length of the catheter. The positions of sections A-F are indicated in Figure 6.

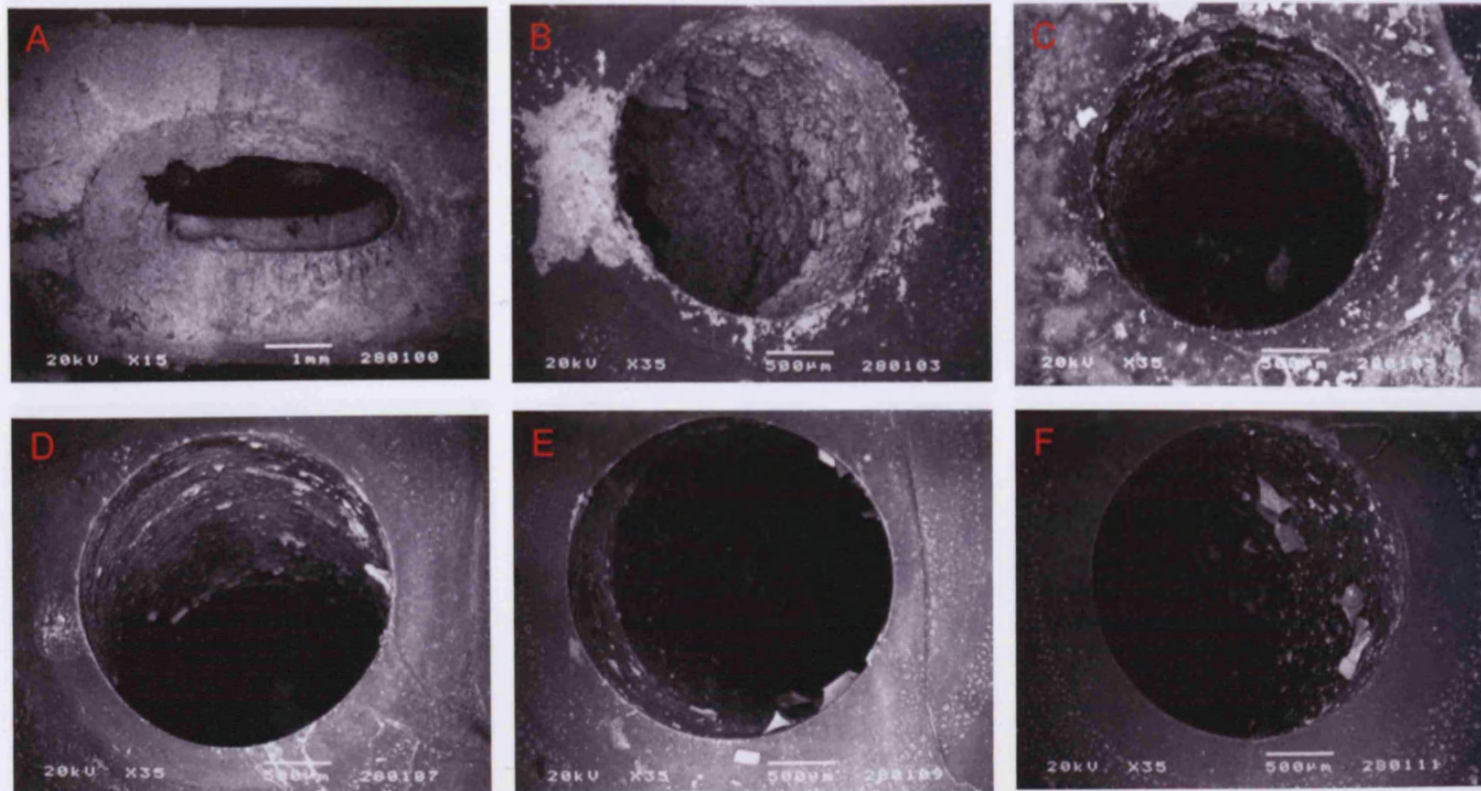


Figure 25: Electron micrographs of a blocked control silicone-coated latex catheter taken from the bladder model. 6 pictures were along the length of the catheter. The positions of sections A-F are indicated in Figure 6.

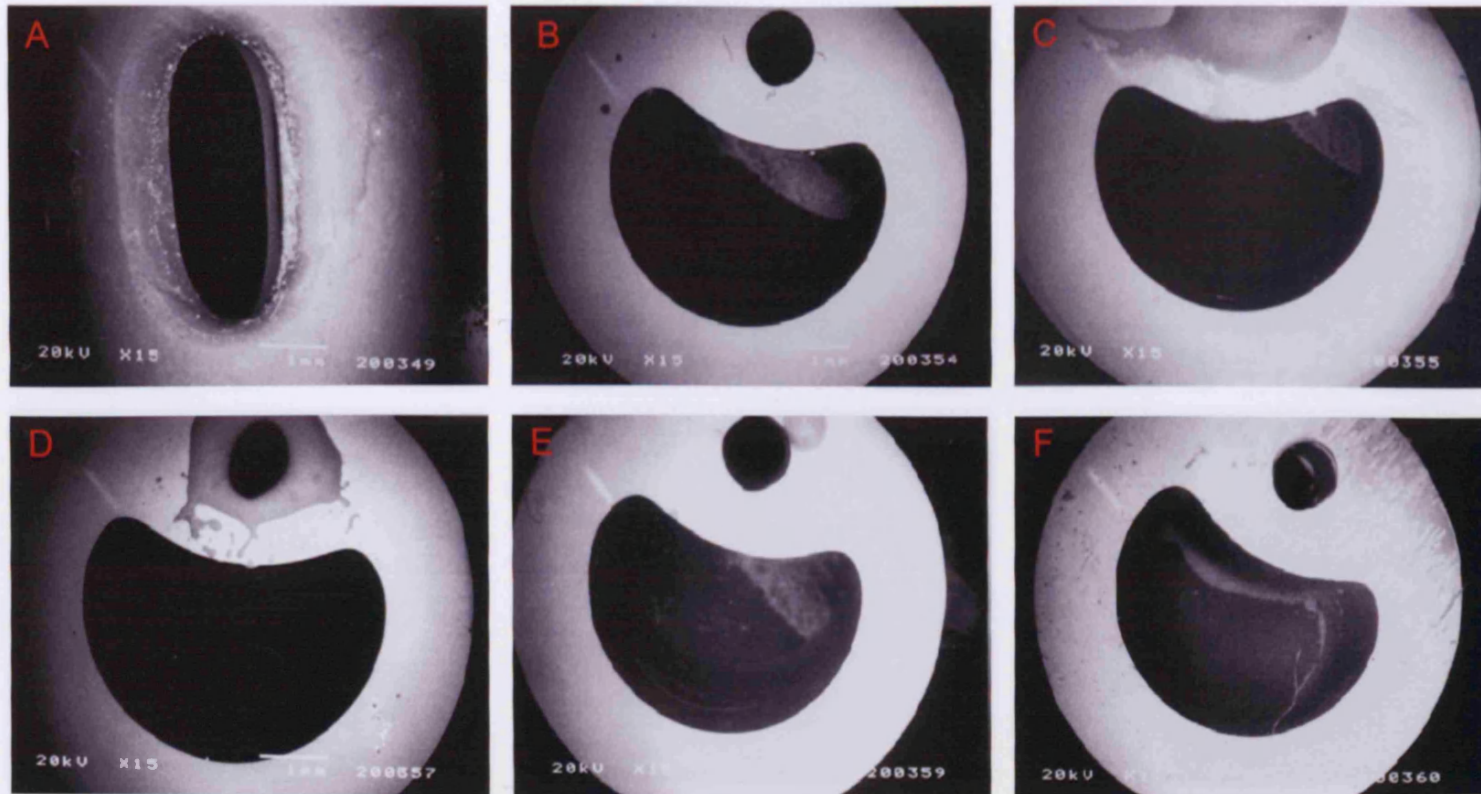


Figure 26: Electron micrographs of an all-silicone catheter that had its balloon inflated with 10 mg/ml triclosan in 5% PEG after 7 days in the bladder model. 6 pictures were taken along the length of the catheter. The positions of sections A-F are indicated in Figure 6.

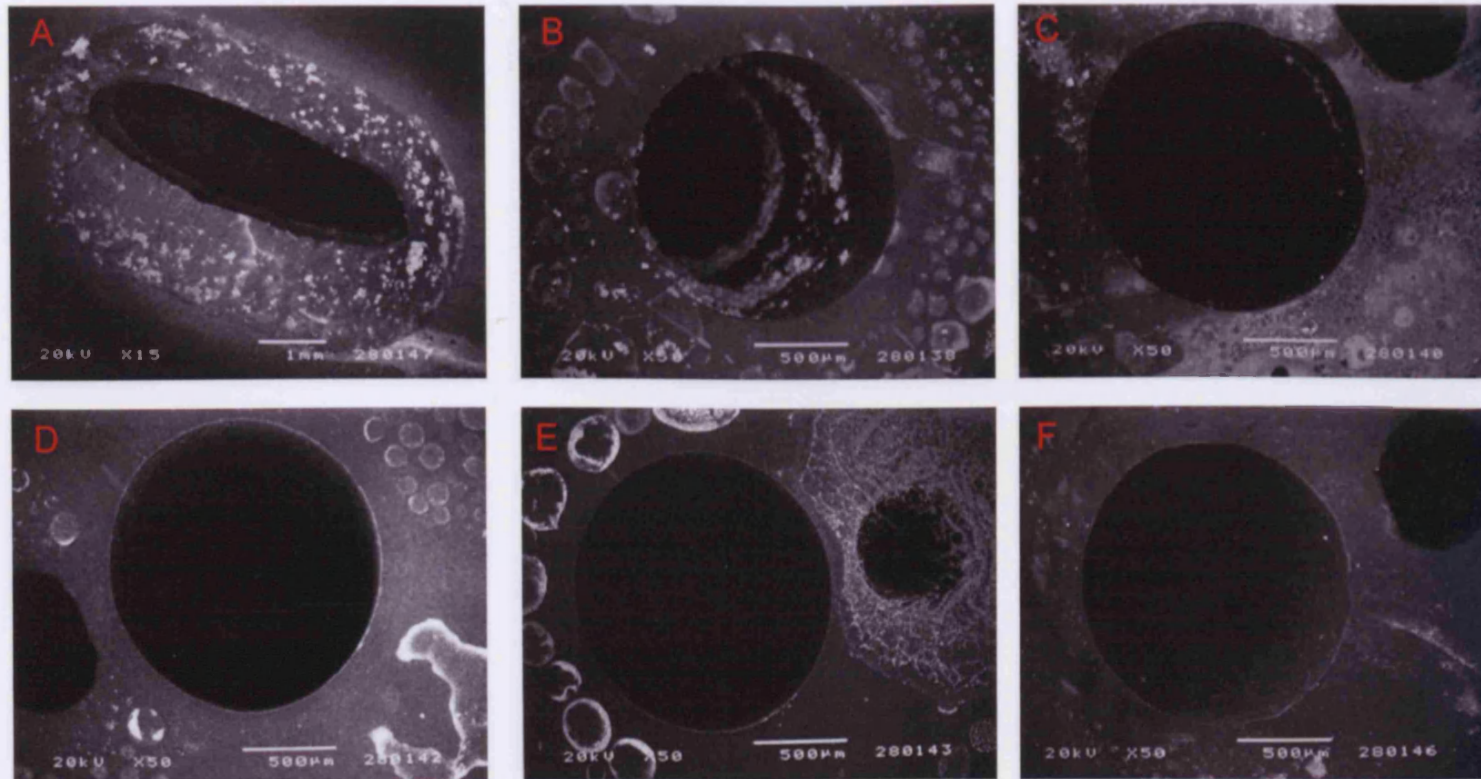


Figure 27: Electron micrographs of a hydrogel-coated latex catheter that had its balloon inflated with 10 mg/ml triclosan in 5% PEG after 7 days in the bladder model. 6 pictures were taken along the length of the catheter. The positions of sections A-F are indicated in Figure 6.

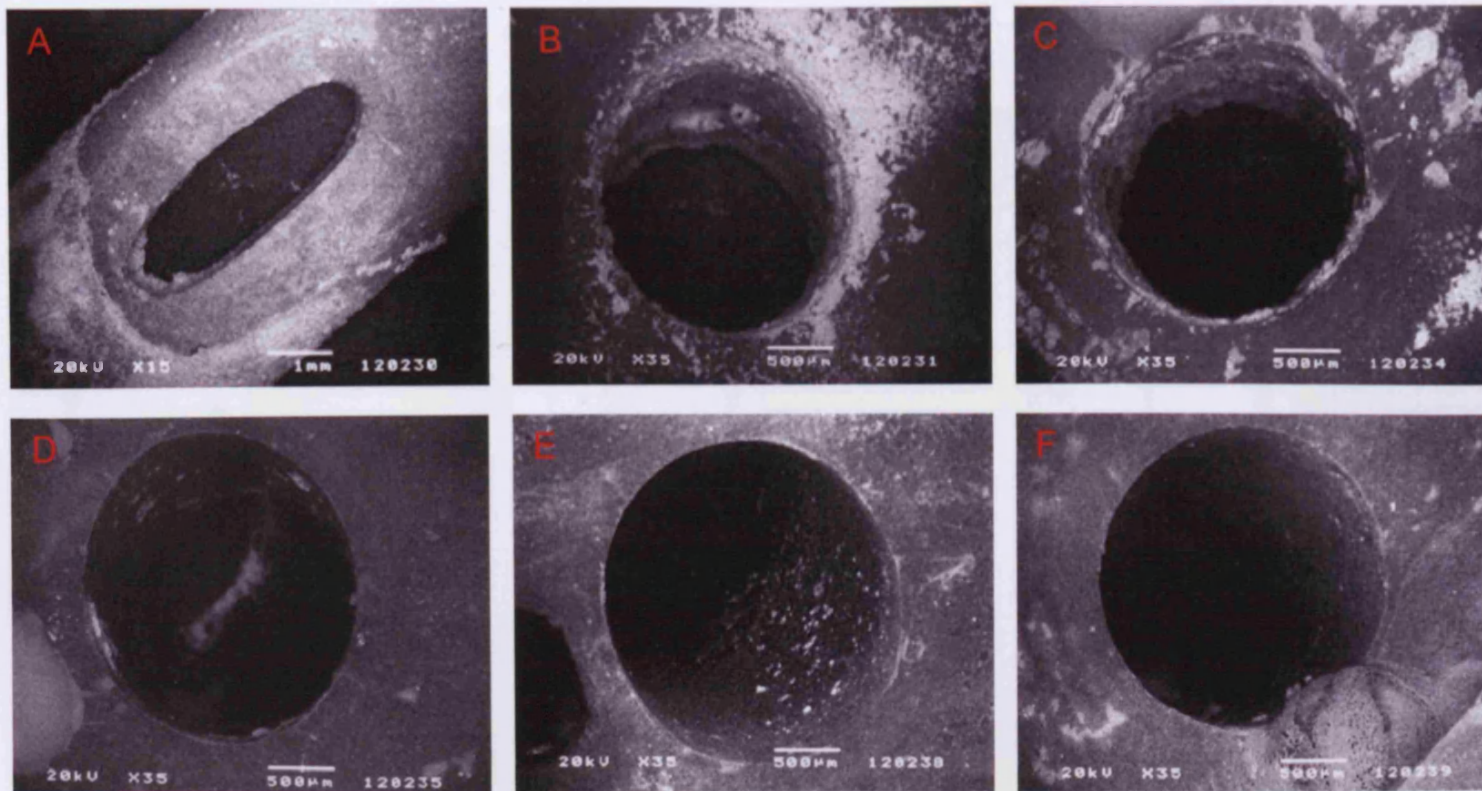


Figure 28: Electron micrographs of a silicone-coated latex catheter that had its balloon inflated with 10 mg/ml triclosan in 5% PEG after 7 days in the bladder model. 6 pictures were taken along the length of the catheter. The positions of sections A-F are indicated in Figure 6.

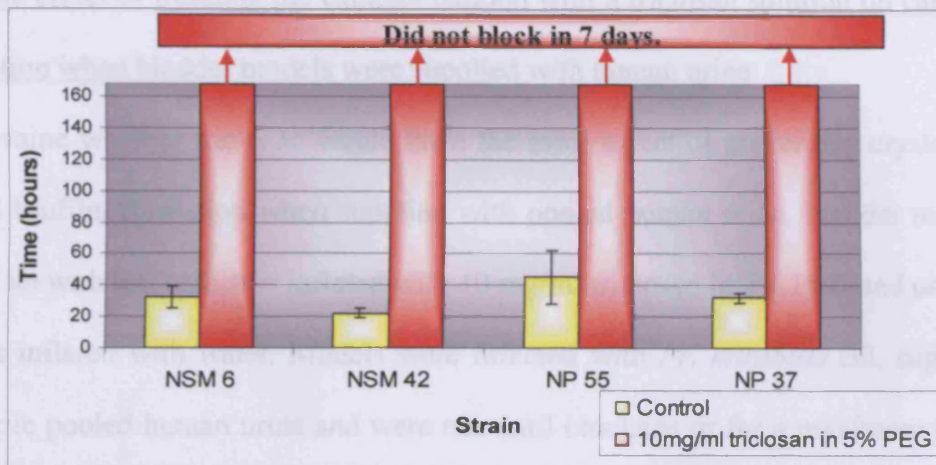


Figure 29a: Time to blockage results.

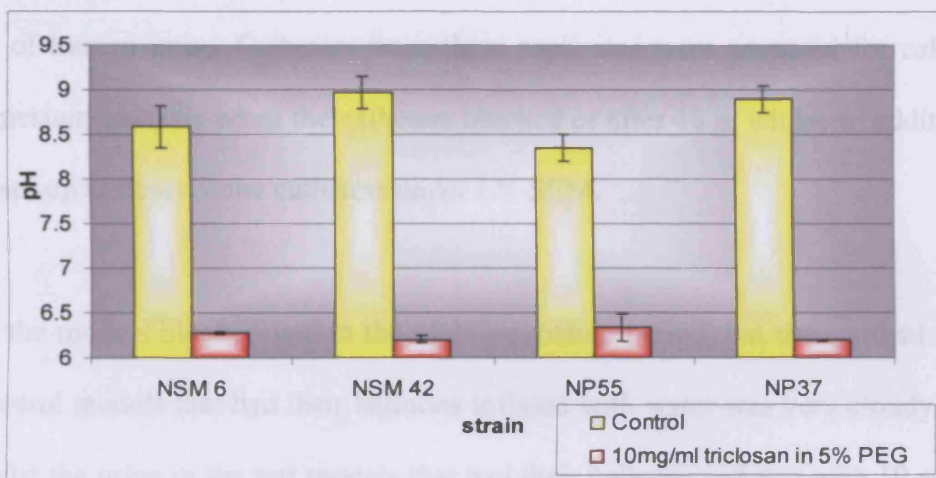


Figure 29b: The pH from the residual urine.

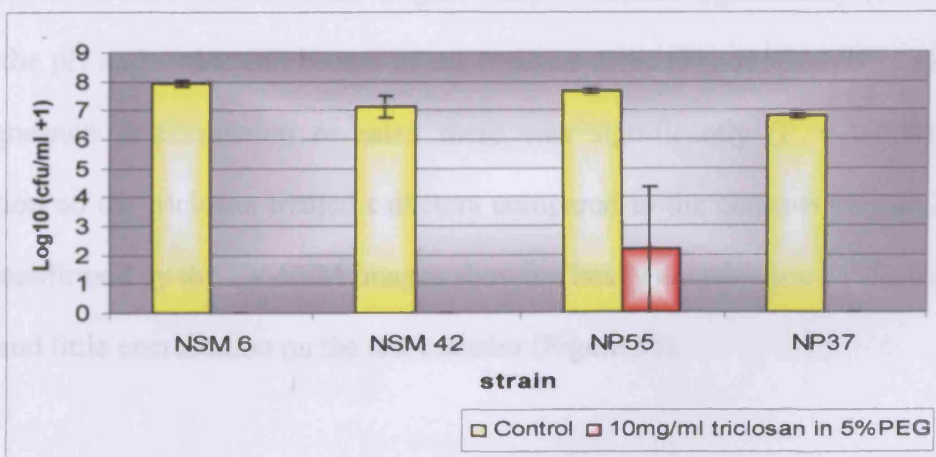


Figure 29c: Log₁₀ (cfu/ml +1) of the residual urine.

Figure 29: Time to blockage, pH and viable cell count results from test and control models fitted with all-silicone catheters and inoculated with four different *Pr. mirabilis* strains. Catheters in control models were inflated with water, catheters in test models were inflated with 10 mg/ml triclosan in 5% PEG. The results are a mean of triplicate experiments.

3.3.2. The effect of inflating the catheter balloon with a triclosan solution on catheter encrustation when bladder models were supplied with human urine

To determine whether triclosan would have the same effect of preventing crystalline bacterial biofilm formation when supplied with pooled human urine, bladder models were set up with test catheters inflated with 10 mg/ml triclosan in 5% PEG and control catheters inflated with water. Models were infected with *Pr. mirabilis* B2, supplied with sterile pooled human urine and were run until blockage or for a maximum of 48 h. This limited time period is due to the difficulty in collecting and sterilizing large volumes of human urine. Catheters from three replicates were prepared for calcium and magnesium analysis when the catheters blocked or after 48 h, whilst an additional run was set-up to observe the catheters under LV-SEM.

None of the models blocked within the 48 h incubation period, but the residual urine of the control models that had their balloons inflated with water was very cloudy after 48 h, whilst the urine in the test models that had their balloons inflated with 10 mg/ml triclosan in 5% PEG remained clear (Figure 22b). Triclosan significantly ($P < 0.05$) lowered the pH and viable cell counts of the residual urine (Figures 30a+b). Calcium and magnesium determination revealed there was significantly ($P = 0.000$) less encrustation on the triclosan treated catheters compared to the controls (Figure 30c), this was confirmed by the LV-SEM images showing heavy encrustation on the control catheter and little encrustation on the test catheter (Figure 31).

3.3.3. The ability of triclosan impregnated catheters to prevent blockage

From previous experiments (section 3.2.2) it became apparent that inflating the catheter balloon with 10 mg/ml triclosan in 5% PEG in an uninoculated model can

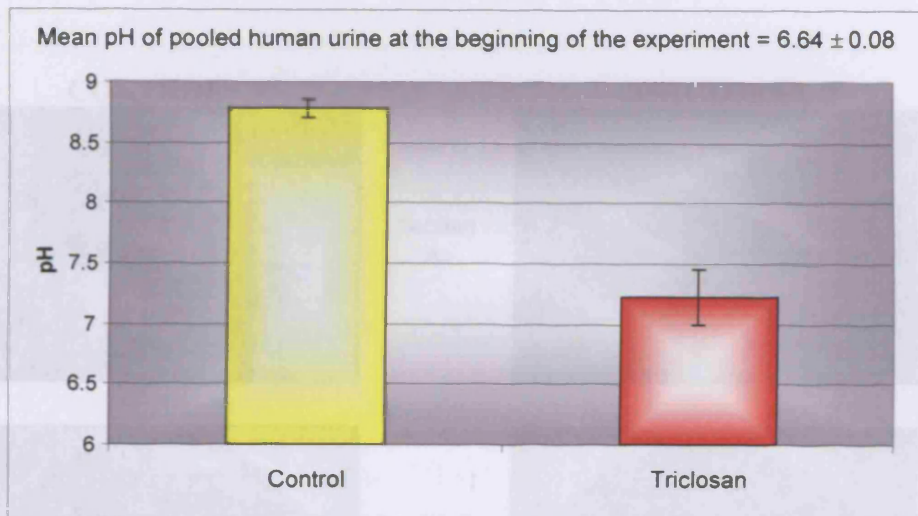


Figure 30a: The pH of the residual urine in the models at 48 h.

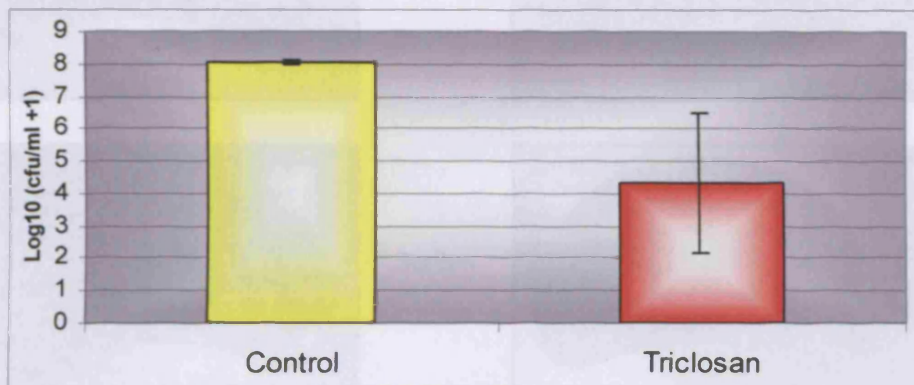


Figure 30b: Log₁₀ (cfu/ml + 1) of the residual urine at 48 h.

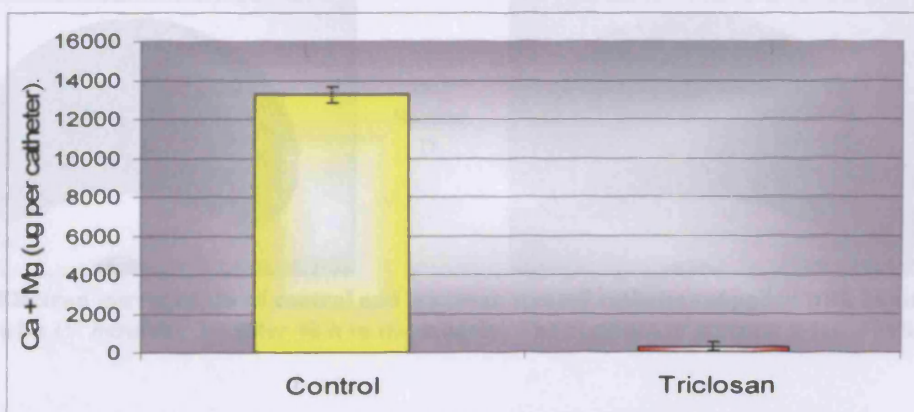


Figure 30c: Amount of calcium and magnesium (μg per catheter) recovered from the catheters at 48 h.

Figure 30: pH, viable cell count and calcium and magnesium results from test and control catheters taken from models supplied with human urine. Control catheters were inflated with water, test catheters were inflated with 10 mg/ml triclosan in 5% PEG. Experiments were run for a maximum of 48 h. Results are a mean of experiments replicated four times.

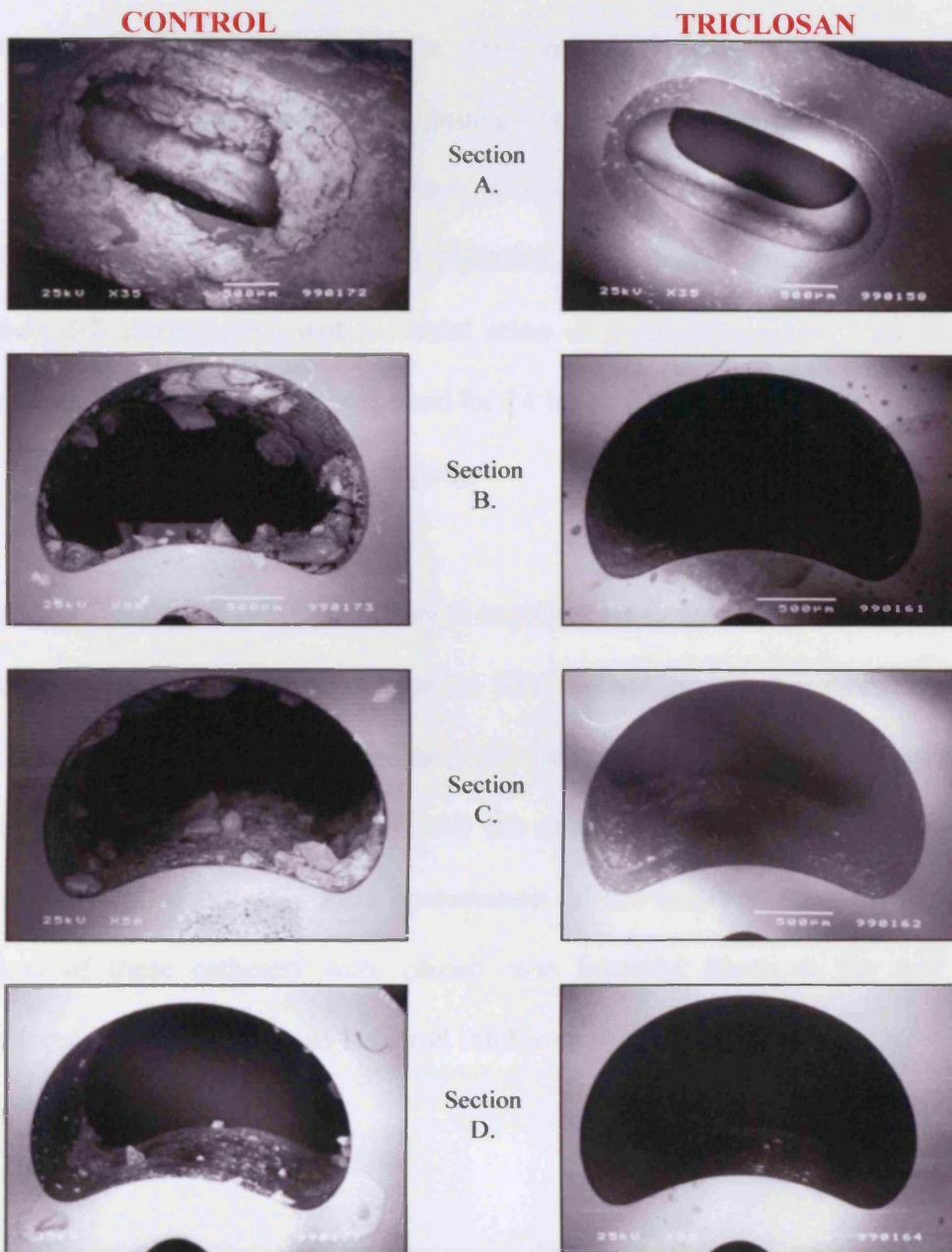


Figure 31: Electron micrographs of control and triclosan treated catheters supplied with human urine and inoculated with *Pr. mirabilis* B2 after 48 h in the models. The positions of sections A-D are indicated in Figure 6.

impregnate the length of all-silicone catheters. This method along with filling the catheters in their packaging with 10 mg/ml triclosan in 5% PEG was used to impregnate all-silicone catheters. After deflation, the catheter balloons were flushed out 10 times with sterile water. The impregnated catheters were then taken from the models or packaging, and placed in fresh models. A control model that had its balloon inflated with sterile water was also included. Each model was infected with *Pr. mirabilis* B2 and supplied with artificial urine until blockage or for a maximum of seven days. Catheters were impregnated for 24 h using the bladder model method, and for 1 min, 1 h and 24 h in their packaging.

None of the replicates from the catheters impregnated in the models for 24 h blocked within the seven day experimental period. The residual urine in the models remained clear throughout the experiment and no viable cells were cultured from the urine at the end of the experiment (Figure 32b). All the urine samples remained acidic (Figure 32c) and LV-SEM revealed little encrustation on the catheter sections (Figure 33). Sections of these catheters were placed onto bacterial lawns at the end of the experiment, revealing zones of bacterial inhibition around sections down the length of the catheter (Figure 34).

The results of impregnating the catheters in the packaging were more variable than those impregnating the catheters in the models. One or two replicates for each impregnating time period blocked whilst the others drained freely for seven days (Figure 32a). The pH and viable cell count of the residual urine from the catheter impregnated in the model was significantly lower ($P < 0.05$) than from models fitted with catheters impregnated for 1 min in the packaging, but not significantly different

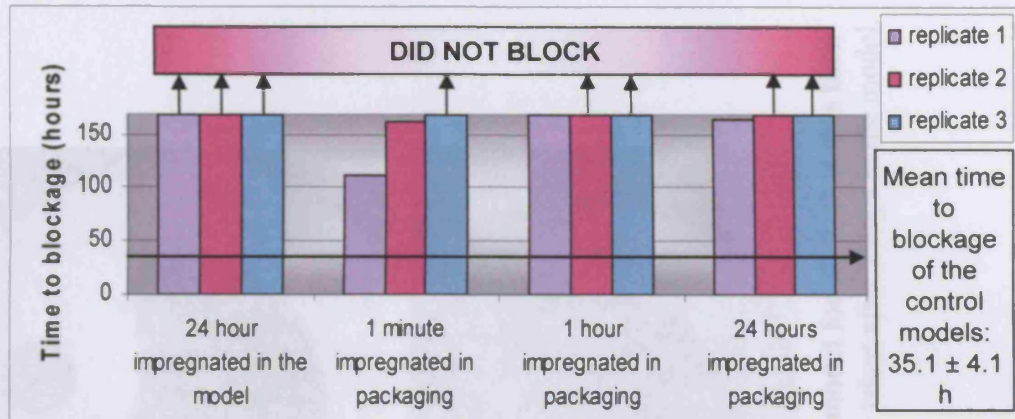


Figure 32a: Time to blockage results.

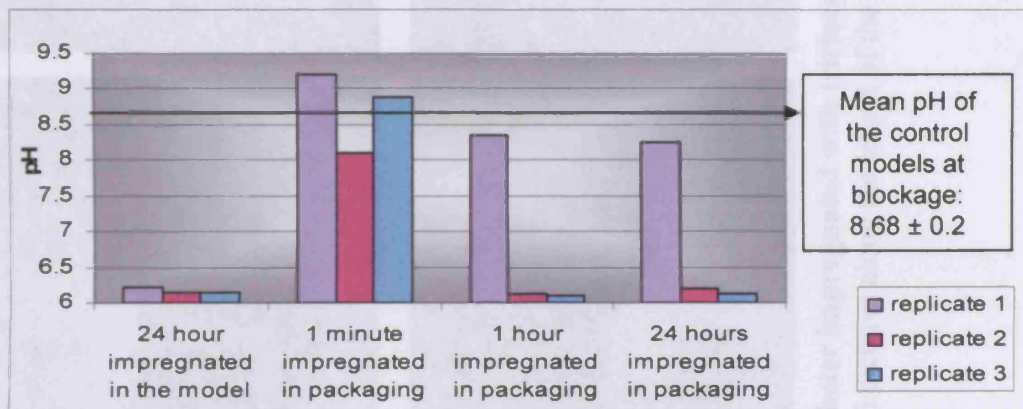


Figure 32b: The pH from the residual urine.

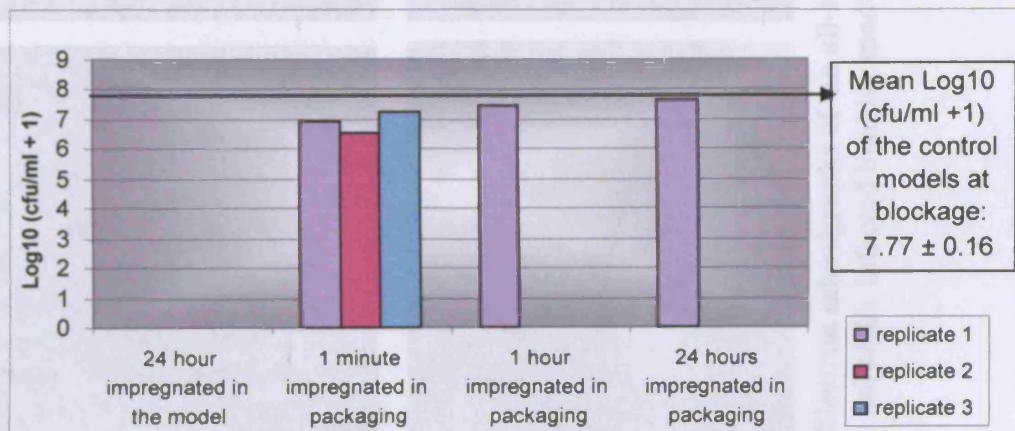


Figure 32c: Log₁₀ (cfu/ml + 1) in the residual urine.

Figure 32: Time to blockage, pH and viable cell count results from models fitted with all-silicone catheters impregnated for different time periods with triclosan in the models and in their packaging. Models were inoculated with *Pr. mirabilis* B2. The black arrows indicate the mean results of the control models.

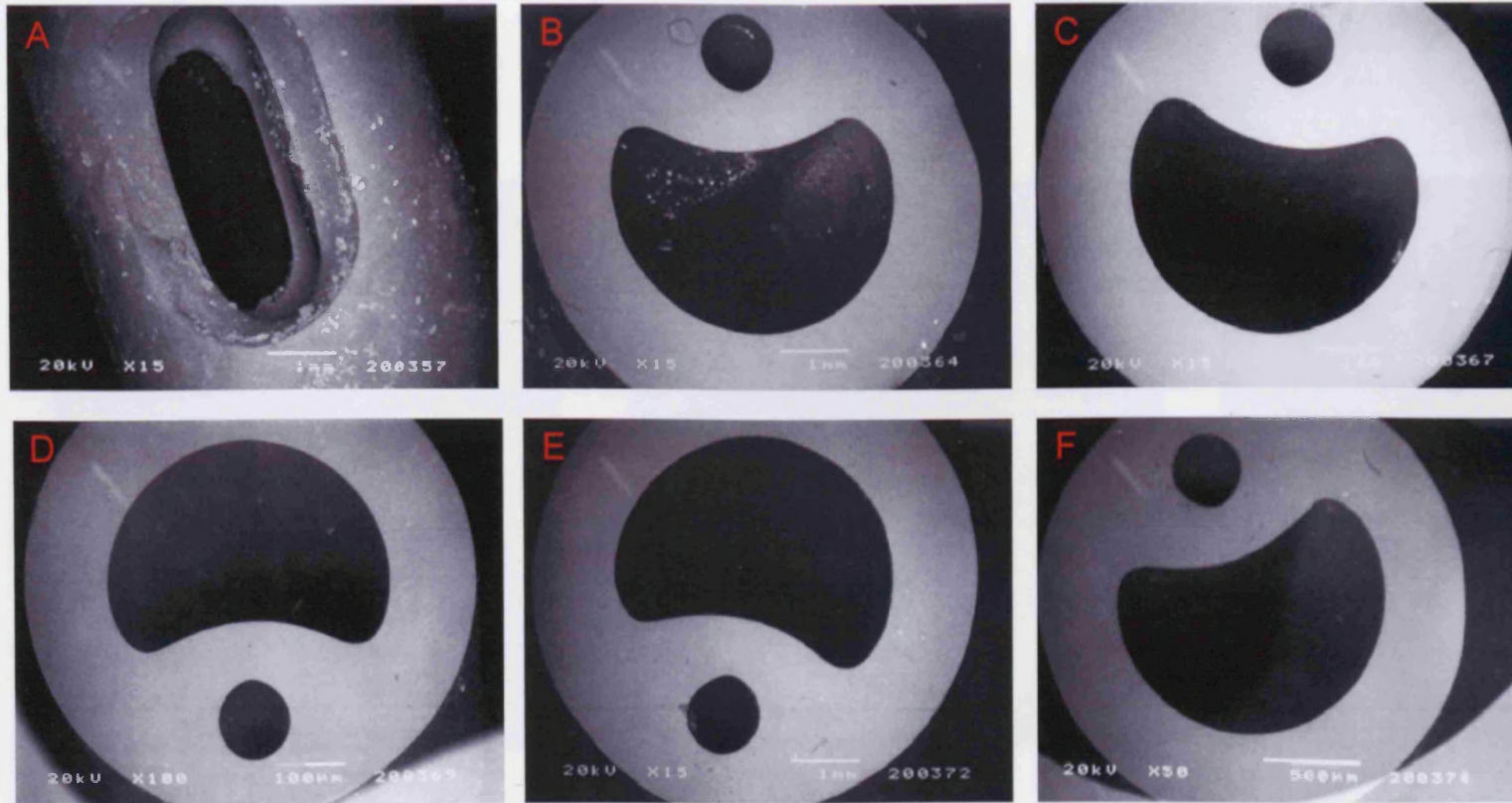


Figure 33: Electron micrographs of an all-silicone catheter impregnated with triclosan inside the model for 24 h, that was then tested in *Pr. mirabilis* infected bladder models. These 6 pictures along the length of the catheter were taken after 7 days in the model.

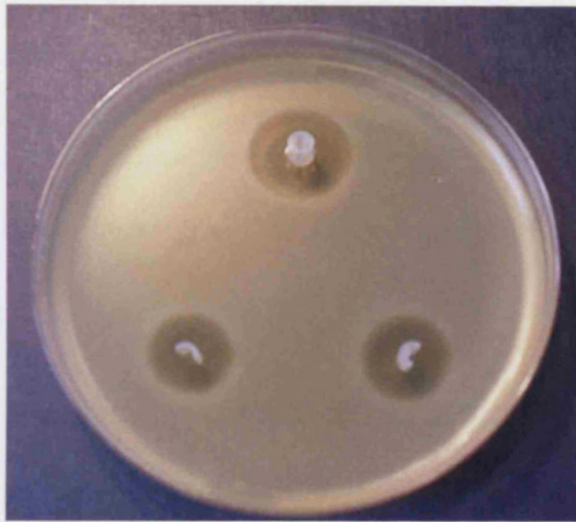


Figure 34a.

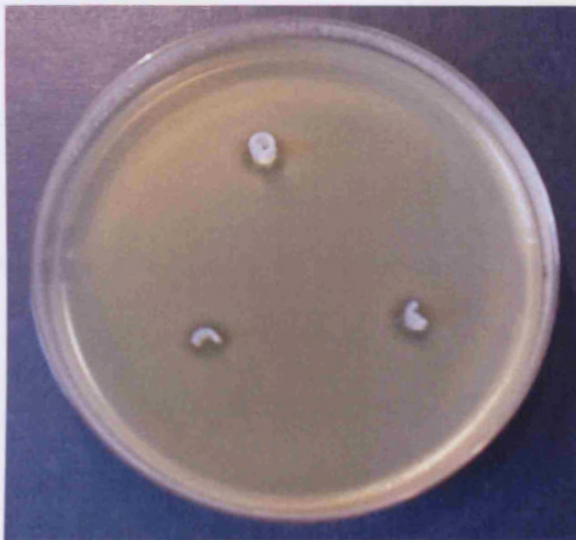


Figure 34b.

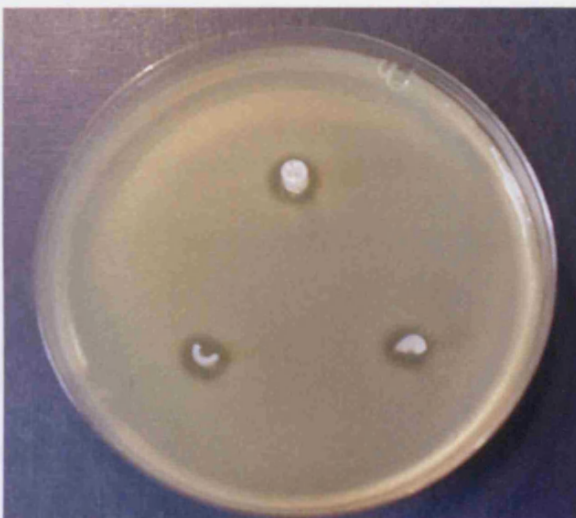


Figure 34c.

Figure 34: Images showing zones of inhibition from an all-silicone catheter that was impregnated with triclosan in the bladder model for 24 h, and was then tested in a *Pr. mirabilis* infected bladder model. The images show zones of inhibition created on *Pr. mirabilis* lawns by sections taken from a catheter after seven days in the infected bladder model. The top image (a) shows the section from under the balloon, the central image (b) is of a section from the middle of the catheter, and the final image (c) is the section above where the catheter attaches to the drainage bag.

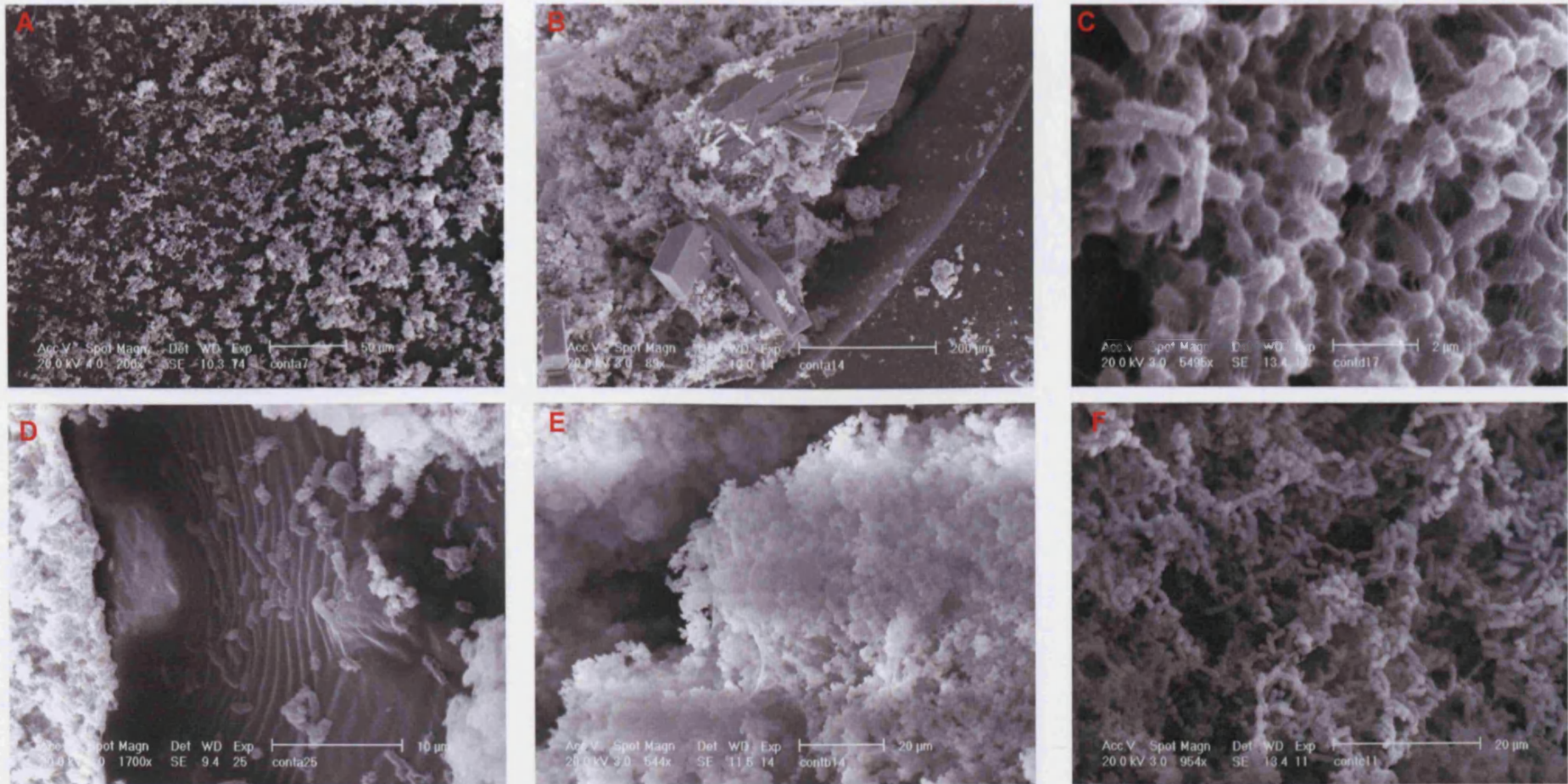


Figure 35: Scanning electron micrographs of a control all-silicone catheter at blockage fixed without RHT. Images A, B and D were taken from the catheter eye-hole. Images C and E were taken from the section directly beneath the catheter balloon and catheter eye-hole respectively. Image F was taken from a section half way down the length of the catheter. Large numbers of bacilli can be seen in images A, C and F, the ones in image C appear to be covered by a polysaccharide matrix. Image D shows how bacteria become trapped within rough surfaces. Large struvite crystals and small crystallites of hydroxy-apatite are visible in images B and E.

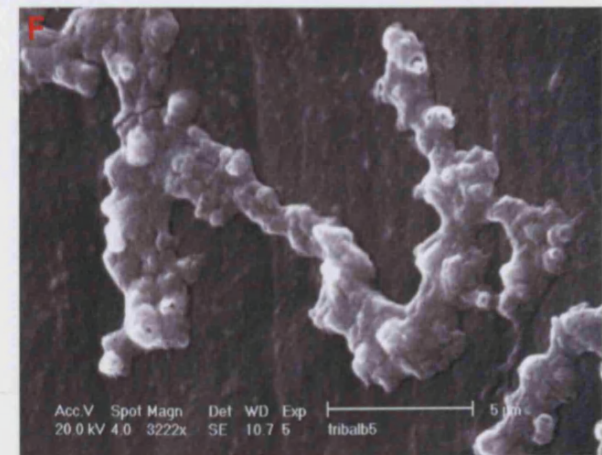
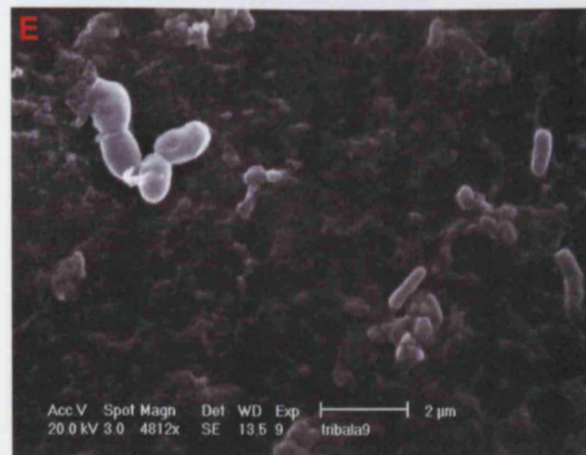
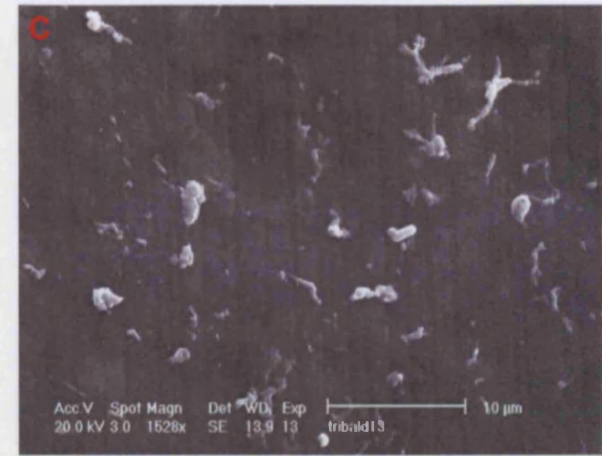
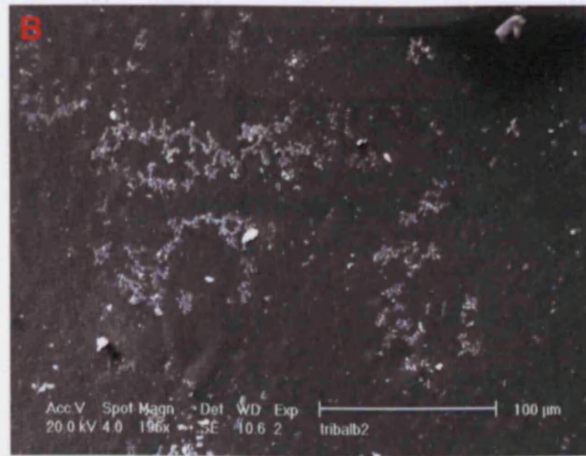
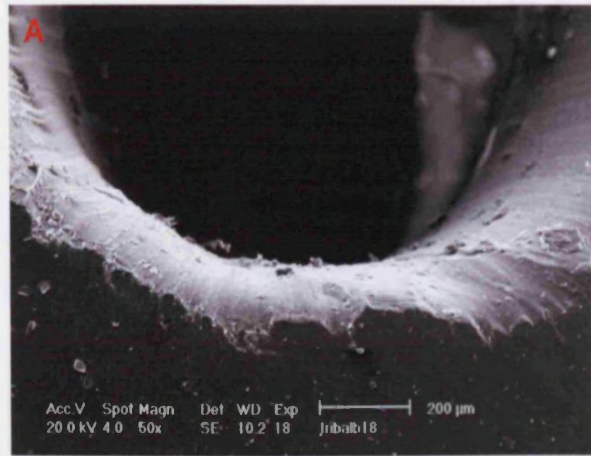


Figure 36: Scanning electron micrographs of an all-silicone catheter inflated with 10 mg/ml triclosan in 5% PEG that drained freely in the model for 7 days and was fixed without RHT. These images are taken of the catheter eye-hole and at intervals down the length of the catheter. Image A reveals little sign of catheter encrustation, but highlights the surface irregularities resulting from engineering techniques used to create the eye-hole. Only very small groups of bacteria were visible, many of which appeared smaller than on the control catheter, and often appeared to be deformed.

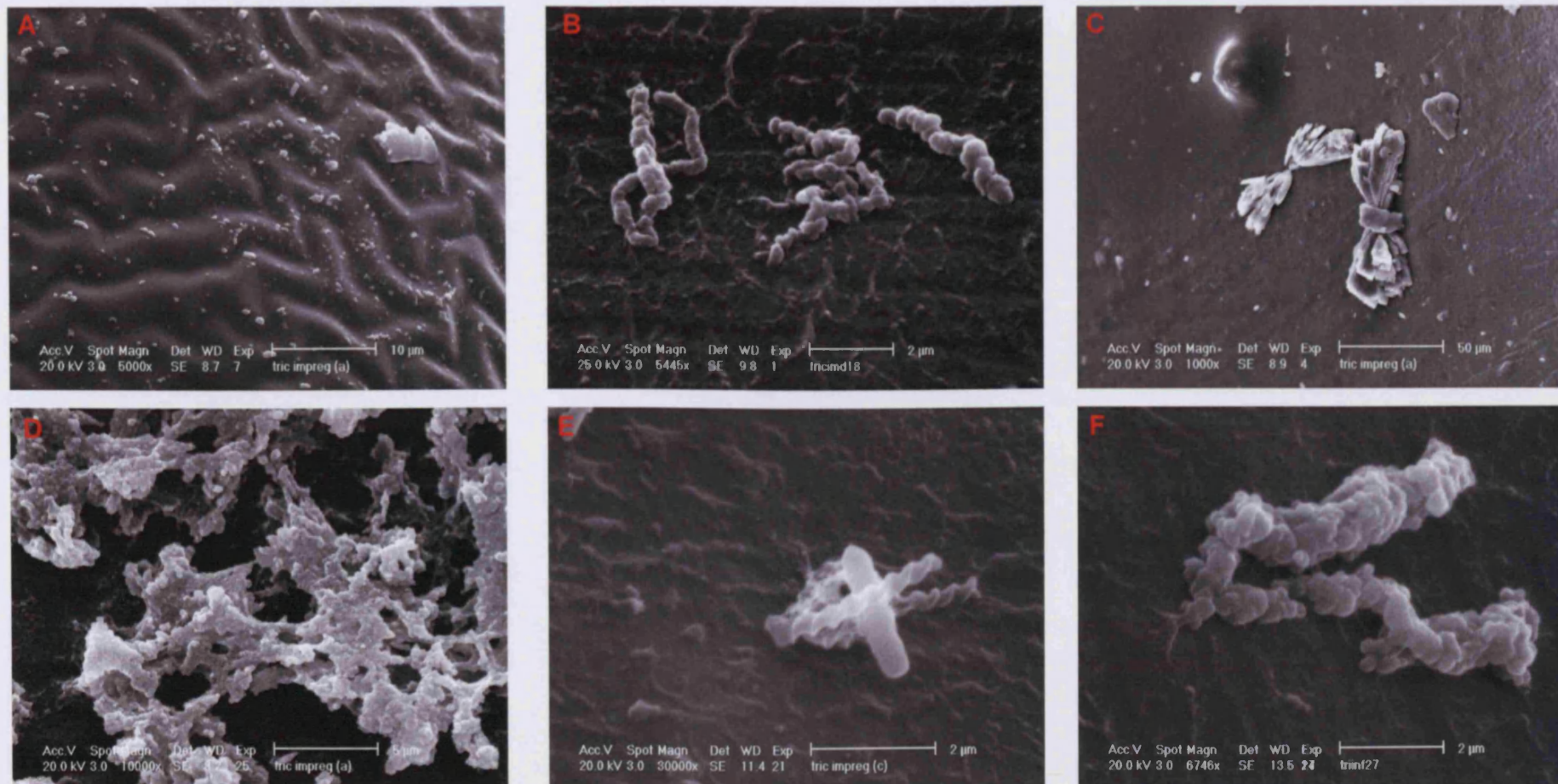


Figure 37: Scanning electron micrographs of an all-silicone catheter impregnated in the model for 24 h that then prevented catheter blockage in a *Pr. mirabilis* infected model for 7 days. The catheter was fixed without RHT. These images are taken of the catheter eye-hole and at intervals down the length of the catheter. Only very small groups of bacteria were visible. Many of the bacteria appeared smaller than in the control catheter, and often appeared to be deformed.

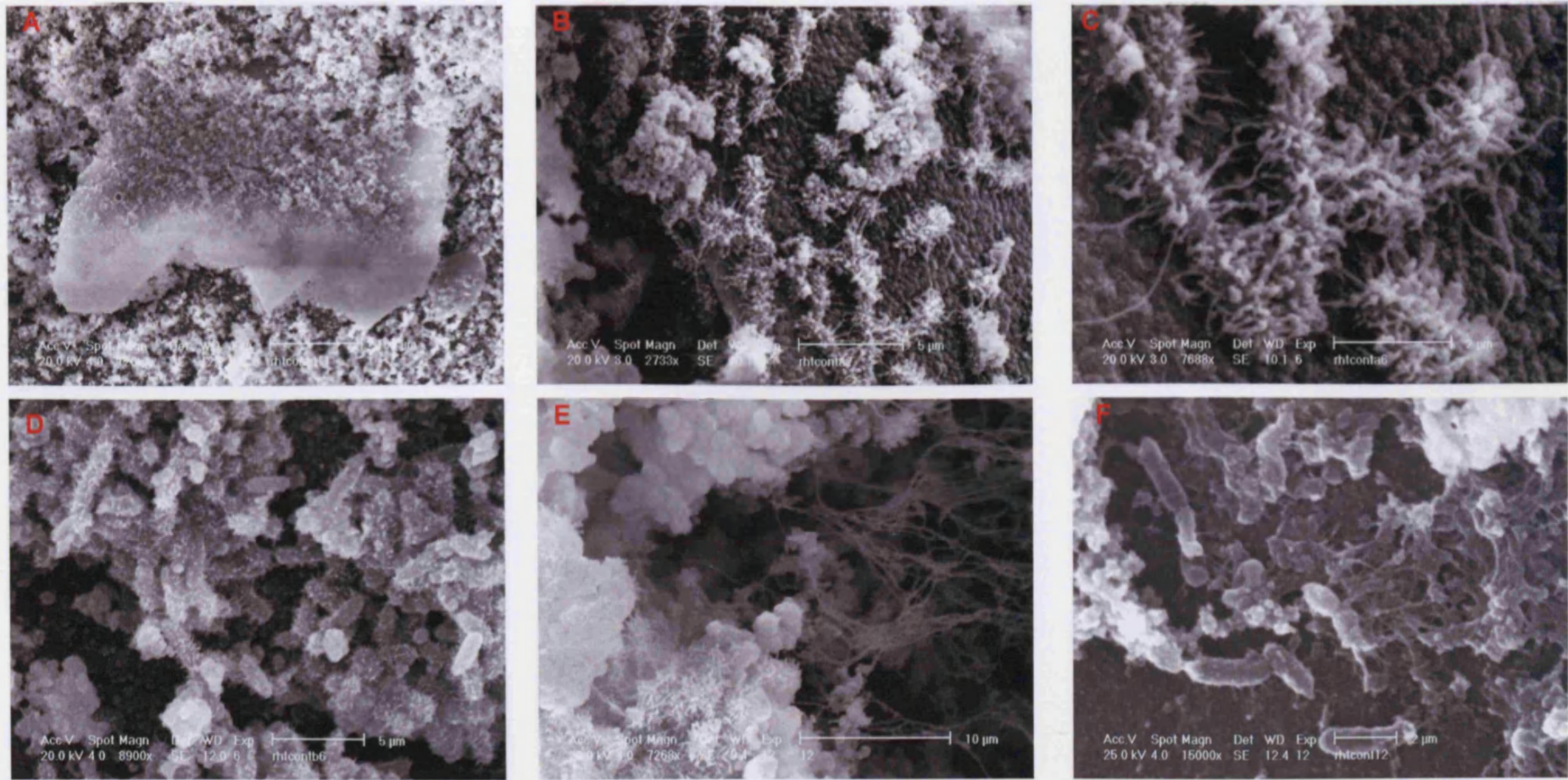


Figure 38: Scanning electron micrographs of a control all-silicone catheter at blockage fixed with RHT. Large numbers of *Pr. mirabilis* could be seen colonizing the catheter eye-hole and the length of the catheter. Bacteria can be seen surrounding and colonizing a large crystal in image A. Images B, C and D show high magnification images of the *Pr. mirabilis* cells. Image E shows a web like polysaccharide matrix binding groups of bacterial cells. A gluey polysaccharide matrix can be seen covering the bacteria in image F.

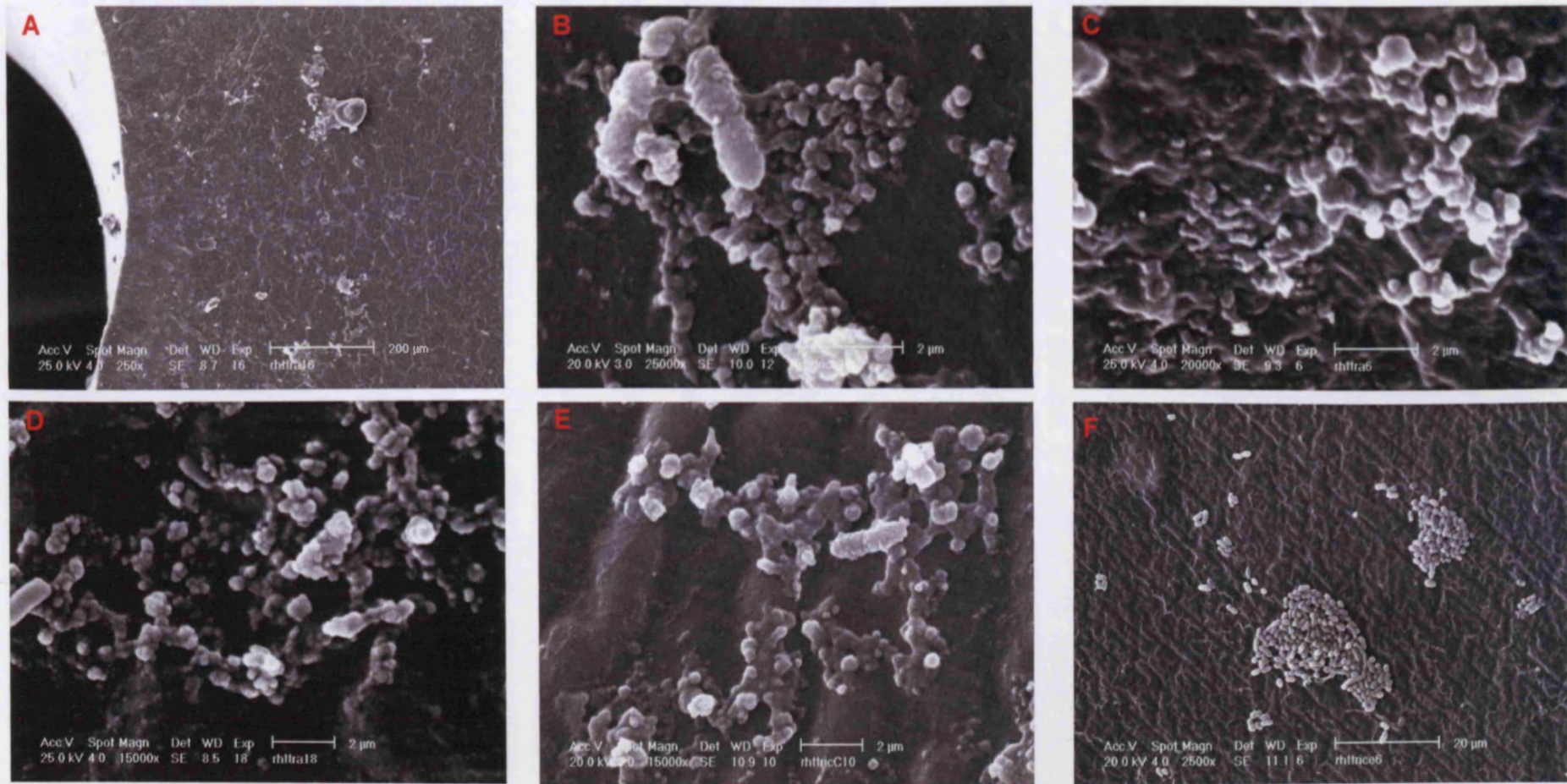


Figure 39: Scanning electron micrographs of an all-silicone catheter inflated with 500 μg/ml triclosan in 5% PEG that drained freely in the model for 7 days and was fixed with RHT. These images are taken of the catheter eye-hole and at intervals down the length of the catheter. Image A reveals little sign of encrustation on the catheter eye-hole. Very little crystal formation was observed down the length of the catheter. Groups of bacterial cells were however visible but they appeared deformed and smaller than normal cells.

triclosan treated catheters (Figures 36, 37, 39), many of which appeared deformed. Hardly any crystalline material was observed on the triclosan test catheters.

3.3.5. The effect of introducing triclosan into the balloon on established *Pr. mirabilis* catheter biofilms

Bladder models were set up with all-silicone catheters, the balloons of both control and test models were filled with sterile water. 18 h after the models had been inoculated with *Pr. mirabilis* B2, the water inflating the catheter balloon of the test model was replaced with 10 mg/ml triclosan in 5% PEG. All models were then run until blockage or for a maximum of seven days.

The data obtained from three replicate experiments are presented in Figure 40. It is clear that exposing an 18 h biofilm to triclosan did not prevent subsequent blockage of the catheter. The control catheters blocked at a mean time of 30 h compared to the 90 h of the treated catheters. So once the biofilm has started to develop on the catheter, the triclosan strategy is less effective. The pH of the urine in the test models was alkaline (mean pH = 8.2) and not significantly different ($P = 0.513$) from the pH of the urine in the control models. There was also little effect on the cells in urine suspension in the bladder models, there being no significant difference ($P = 0.496$) between the mean log viable cell count from urine of control and test models.

3.3.6. The concentration of triclosan required in the urine to inhibit an established biofilm from further development and subsequent catheter blockage

The aim of this experiment was to use the bladder models to allow a crystalline biofilm to form on all-silicone catheters, then to change the urine supply to one that

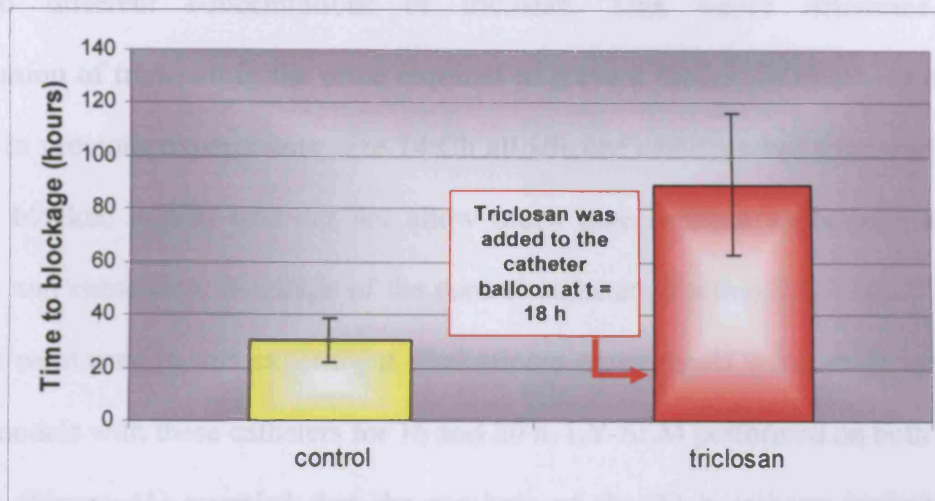


Figure 40a: Time to blockage results.

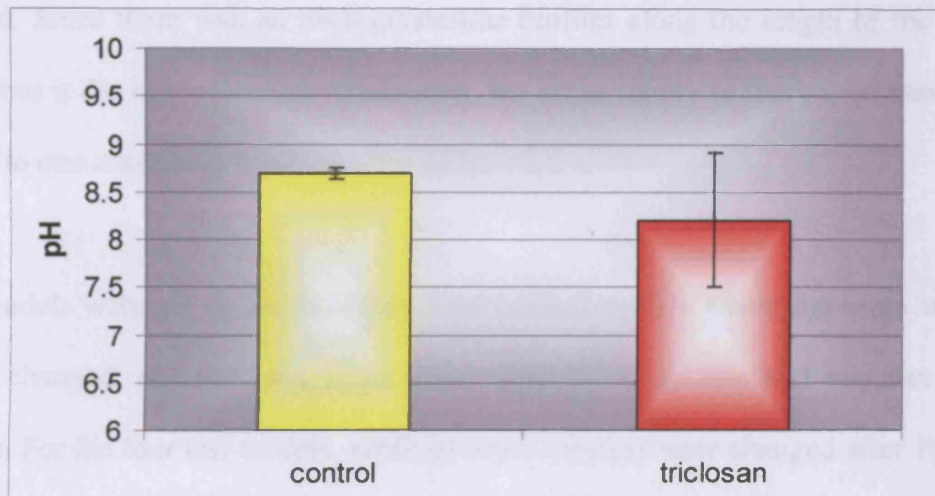


Figure 40b: The pH from the residual urine.

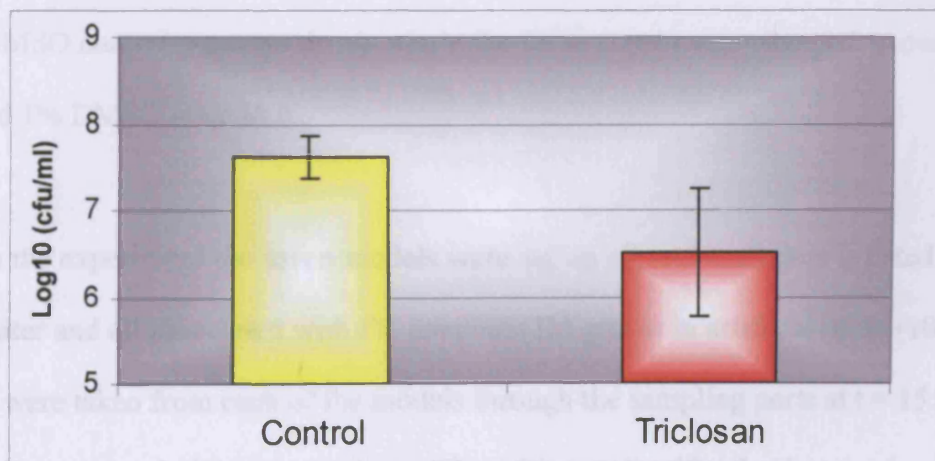


Figure 40c: The Log₁₀ (cfu/ml) of the residual urine.

Figure 40: The effect of introducing triclosan into catheter balloons where encrustation by *Pr. mirabilis* had already begun. Control catheters were inflated with water. 18 h after inoculation with *Pr. mirabilis* the water inflating the test catheter balloons was replaced with 10 mg/ml triclosan in 5% PEG. The subsequent time to blockage, pH and viable cell count in the residual urine are shown. The results are means of triplicate experiments.

contained different concentrations of triclosan. This would determine the concentration of triclosan in the urine required to prevent further development of the biofilm. In previous experiments, size 14 Ch all-silicone catheters had been used, but as these blocked rapidly and did not allow much time between application of the triclosan and subsequent blockage of the control catheters (section 3.3.5), size 16 Ch catheters were used in this experiment. Preliminary experiments were set up running control models with these catheters for 16 and 20 h. LV-SEM performed on both these catheters (Figure 41) revealed that the eye-hole of the 20 h catheter looked very encrusted. Since there was an even crystalline biofilm along the length of the 16 h catheter but it did not look close to blocking, the urine supply in this experiment was changed to one containing triclosan after 16 h.

Seven models were set up for each test. Two control models where the urine supply was not changed, one that was taken down after 16 h and one that was run until blockage. For the four test models, artificial urine supplies were changed after 16 h to contain 0.1, 1, 10 and 100 $\mu\text{g/ml}$ triclosan, all with a final DMSO concentration of 1%. A DMSO control was also set up where the urine supply was changed to one that contained 1% DMSO after 16 h.

To begin the experiment the seven models were set up all with catheters inflated with sterile water and all inoculated with *Pr. mirabilis* B2 grown in artificial urine (10 ml). Samples were taken from each of the models through the sampling ports at $t = 15$ h for viable cell counts and pH determinations. The tubing and artificial urine supply of the DMSO control and triclosan test models were changed at $t = 16$ h. One of the control models was also taken down at this time point, and the catheter was prepared for

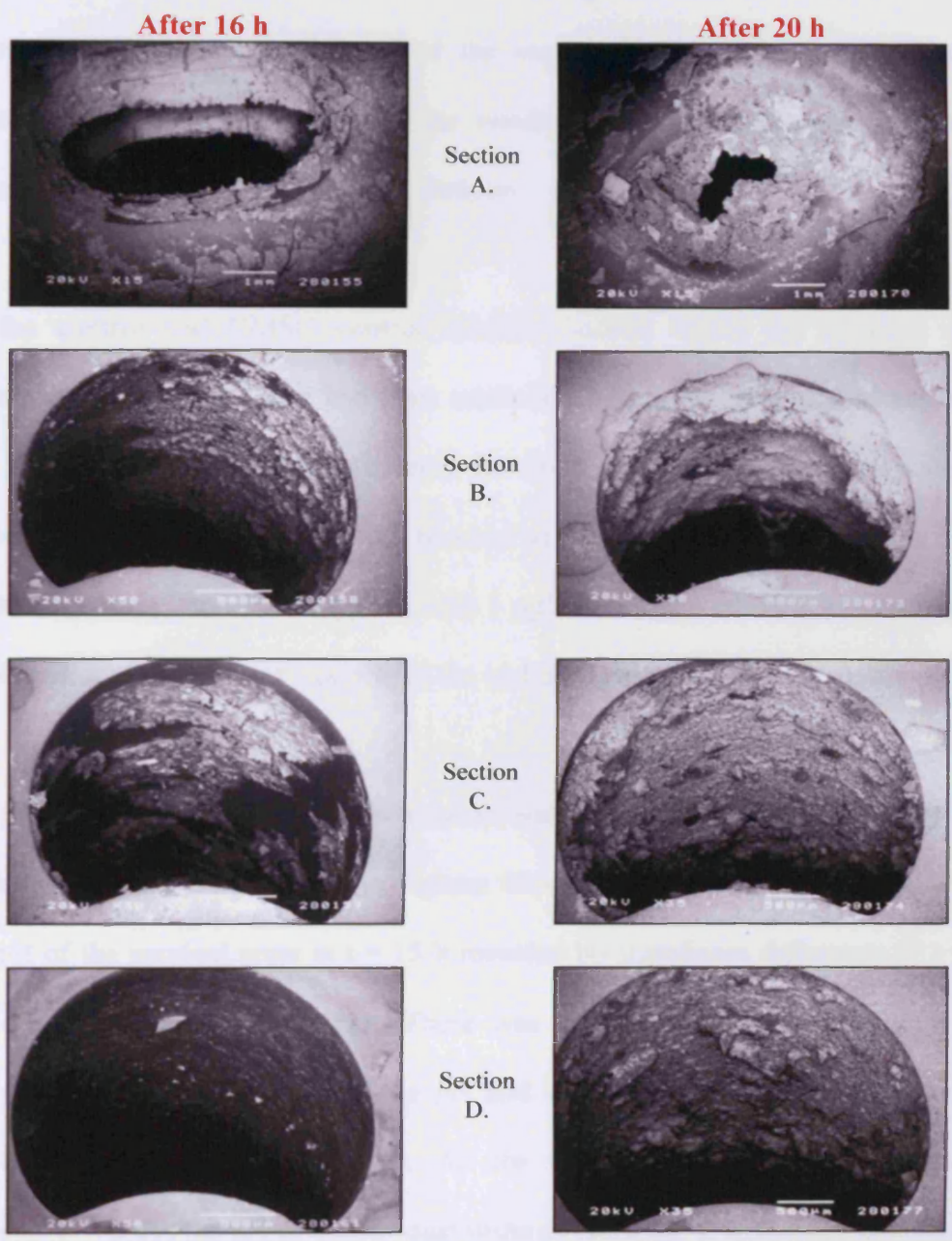


Figure 41: Electron micrographs down the length of size 16 Ch all-silicone catheters inflated with water that had been in *Pr. mirabilis* B2 infected bladder models for 16 h and 20 h. The positions of sections A-D are indicated in Figure 6.

calcium and magnesium analysis. Samples were taken through the sampling ports at $t = 42$ h and the models were left to run until blockage or for a maximum of 66 h. When the models blocked or at the end of the experiment, viable cell counts and pH determinations were performed on the residual urine. These catheters were also prepared for calcium and magnesium analysis.

All the control and DMSO control models blocked within the duration of the experiment. The models that had their artificial urine supply changed to contain 10 and 100 $\mu\text{g/ml}$ triclosan drained freely for 66 h. With 0.1 $\mu\text{g/ml}$ triclosan in the artificial urine, two of the replicates blocked at 41 and 55 h whilst the other did not block. One of the replicates supplied with 1 $\mu\text{g/ml}$ triclosan after 16 h blocked (at 50 h) whilst the others drained freely until the end of the experiment.

The pH and viable cell count of each model was monitored at 15 h, 42 h, 66 h and at blockage, the data are presented in Figures 42 - 45. Analysis of the viable cell count and pH of the residual urine at $t = 15$ h revealed no significant difference ($P > 0.05$) between any of the seven groups. There was no significant difference ($P > 0.05$) between the time to blockage or the pH and viable cell counts of the control and DMSO control models at blockage. All the triclosan concentrations significantly lowered ($P < 0.05$) the pH of the residual urine at blockage or at 66 h compared to the control models at blockage, whilst 10 and 100 $\mu\text{g/ml}$ triclosan were required to significantly lower ($P < 0.05$) the viable cell count of the urine compared to the controls.

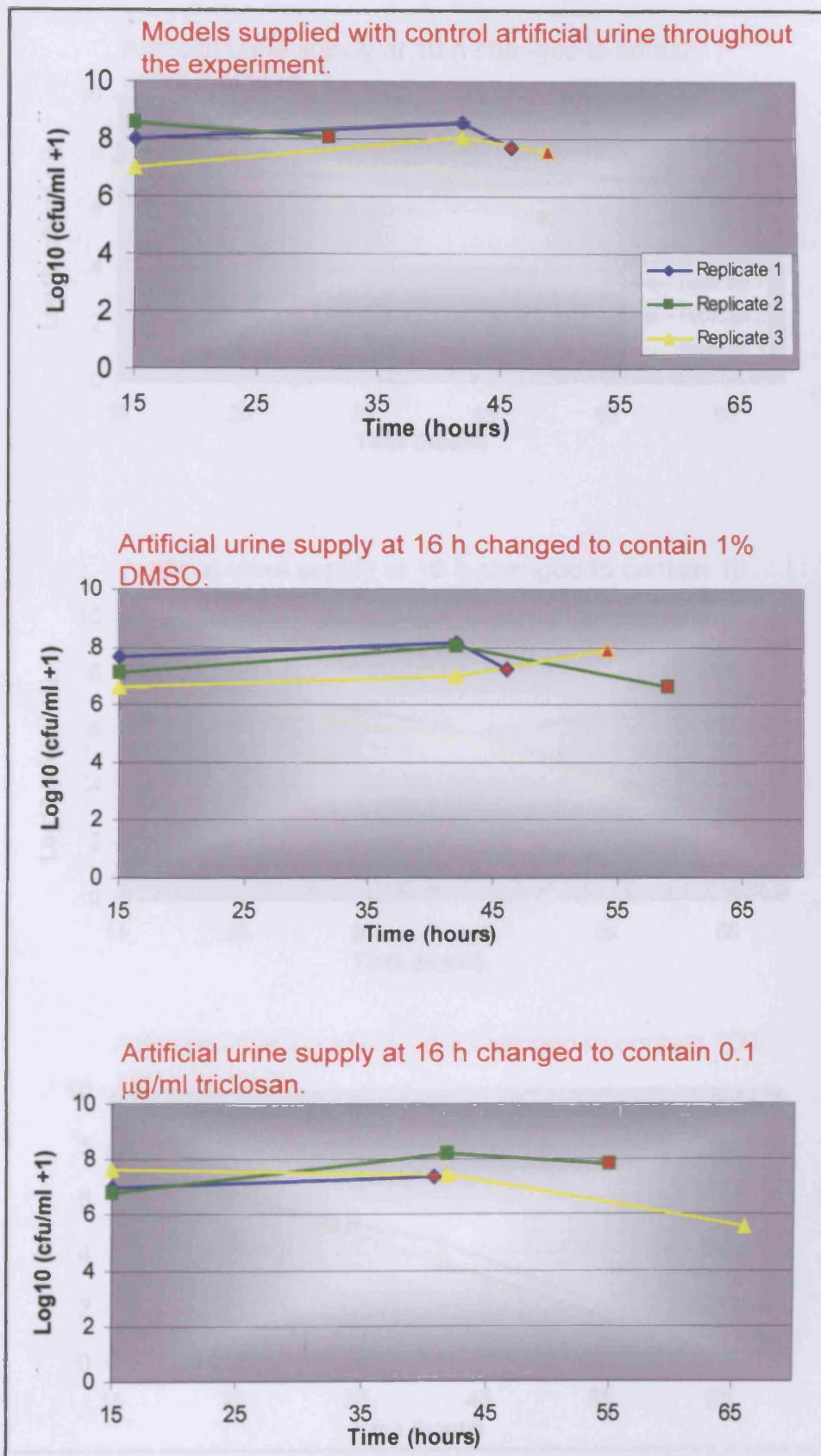


Figure 42: The viable cell count from the residual urine of models in which the urine supply was changed to contain different triclosan concentrations 16 h after the bladder models were inoculated with *Pr. mirabilis* B2. Red points indicate the time of catheter blockage. Models were run for a maximum of 66 h.

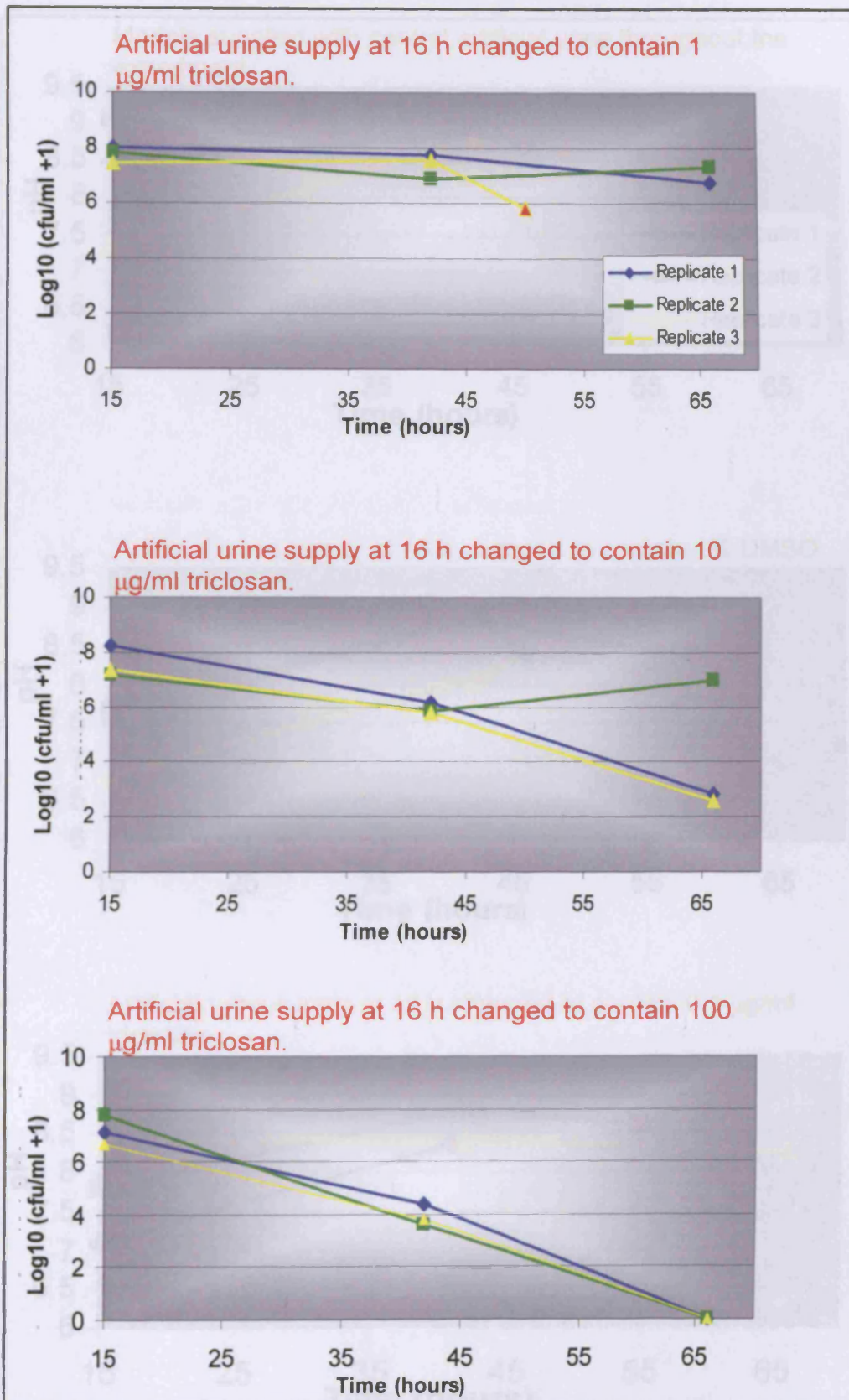


Figure 43: The viable cell count from the residual urine of models in which the urine supply was changed to contain different triclosan concentrations 16 h after the bladder models were inoculated with *Pr. mirabilis* B2. Red points indicate the time of catheter blockage. Models were run for a maximum of 66 h.

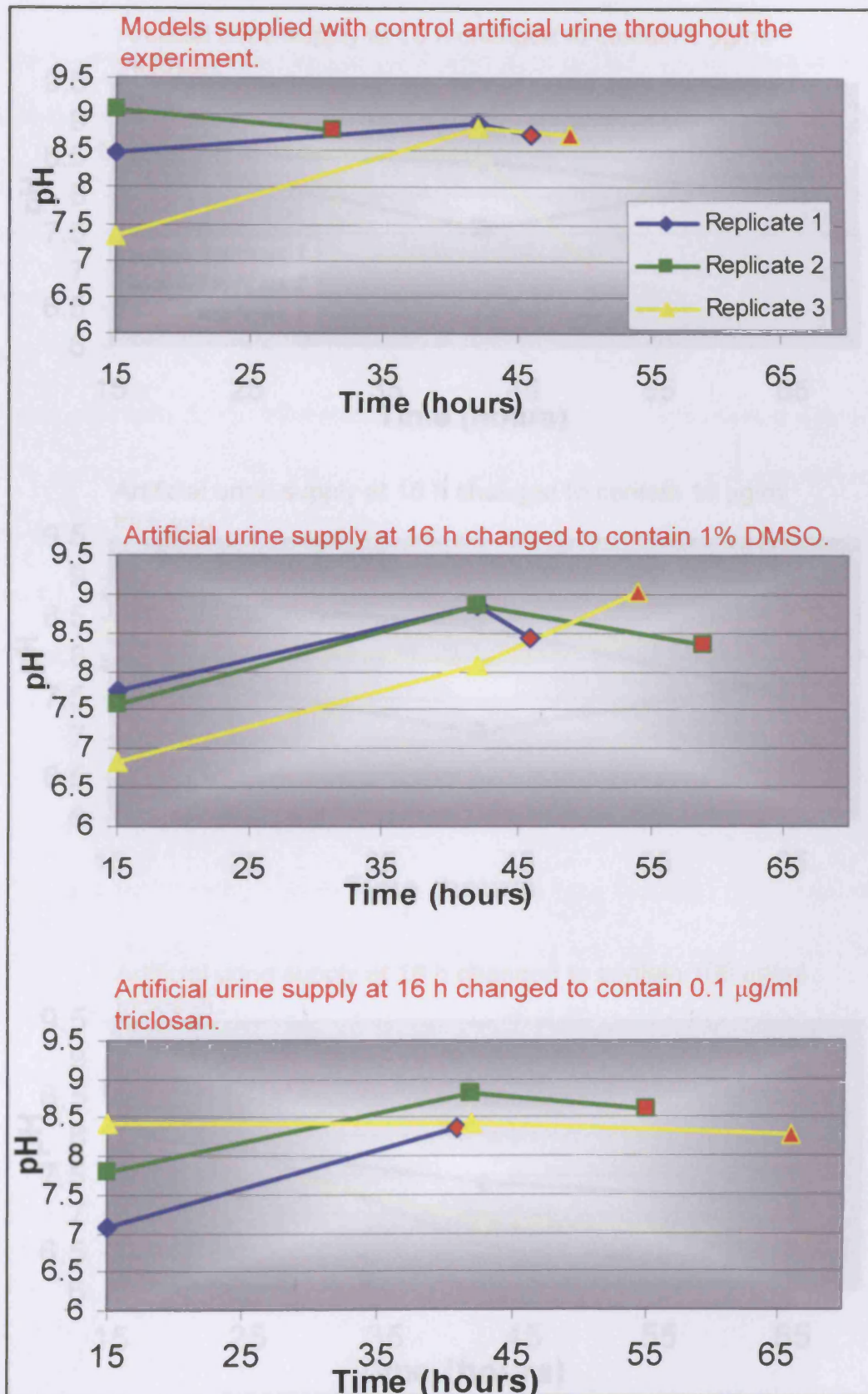


Figure 44: The pH from the residual urine of models in which the urine supply was changed to contain different triclosan concentrations 16 h after the bladder models were inoculated with *Pr. mirabilis* B2. Red points indicate the time of catheter blockage. Models were run for a maximum of 66 h.

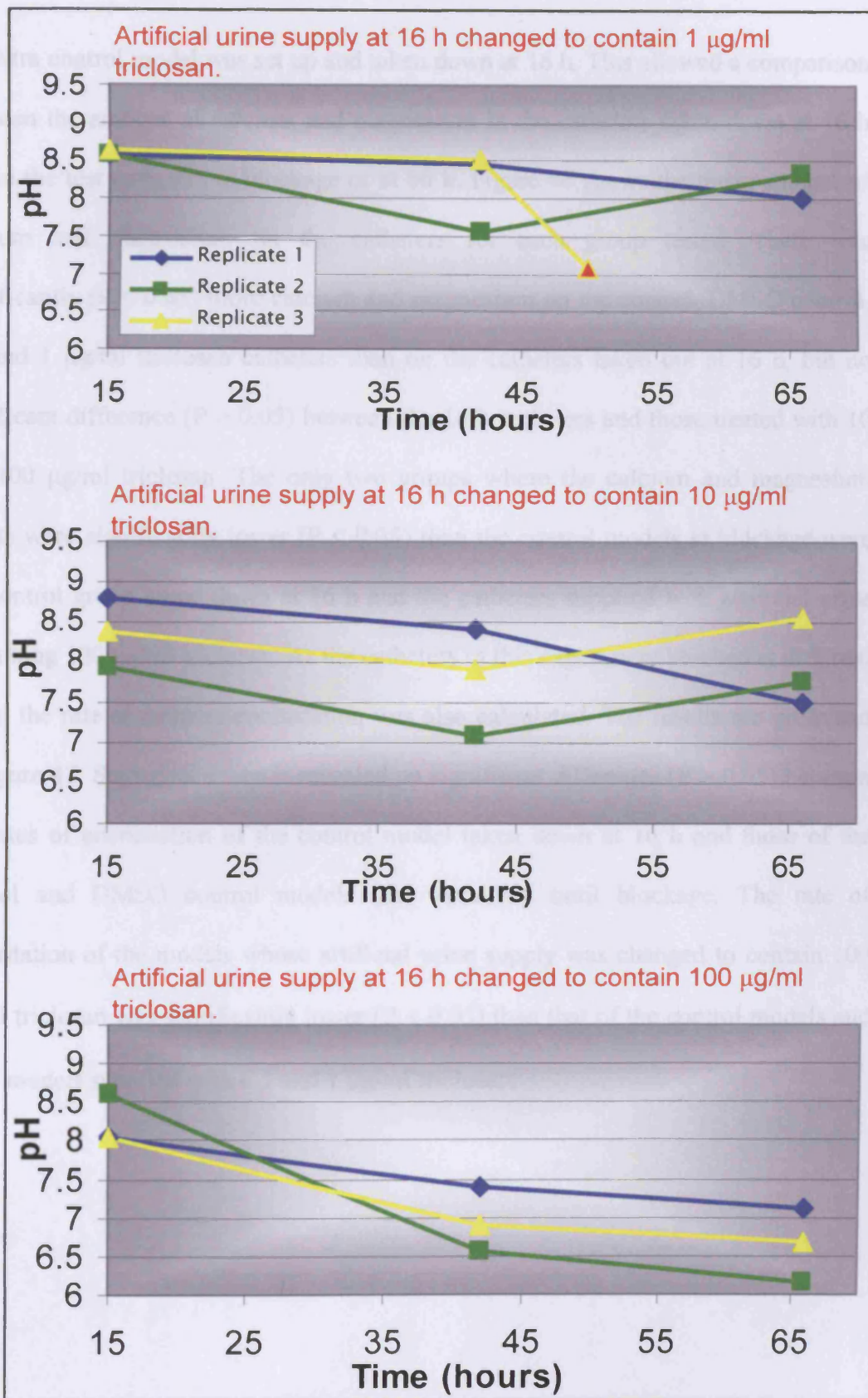


Figure 45: The pH from the residual urine of models in which the urine supply was changed to contain different triclosan concentrations 16 h after the bladder models were inoculated with *Pr. mirabilis* B2. Red points indicate the time of catheter blockage. Models were run for a maximum of 66 h.

An extra control model was set up and taken down at 16 h. This allowed a comparison between the amount of calcium and magnesium in the catheters taken down at 16 h and in the test catheters at blockage or at 66 h. Figure 46 shows the mean amount of calcium and magnesium on the catheters for each group tested. There was significantly ($P < 0.05$) more calcium and magnesium on the control, DMSO control, 0.1 and 1 $\mu\text{g/ml}$ triclosan catheters than on the catheters taken out at 16 h, but no significant difference ($P > 0.05$) between the 16 h catheters and those treated with 10 and 100 $\mu\text{g/ml}$ triclosan. The only two groups where the calcium and magnesium results were significantly lower ($P < 0.05$) than the control models at blockage were the control group taken down at 16 h and the catheters supplied with artificial urine containing 100 $\mu\text{g/ml}$ triclosan. As the catheters in this experiment blocked at different times, the rate of catheter encrustation was also calculated. The results are presented in Figure 47. Statistical analysis revealed no significant difference ($P > 0.05$) between the rates of encrustation of the control model taken down at 16 h and those of the control and DMSO control models that were run until blockage. The rate of encrustation of the models whose artificial urine supply was changed to contain 100 $\mu\text{g/ml}$ triclosan was significantly lower ($P < 0.05$) than that of the control models and those models supplied with 0.1 and 1 $\mu\text{g/ml}$ triclosan.

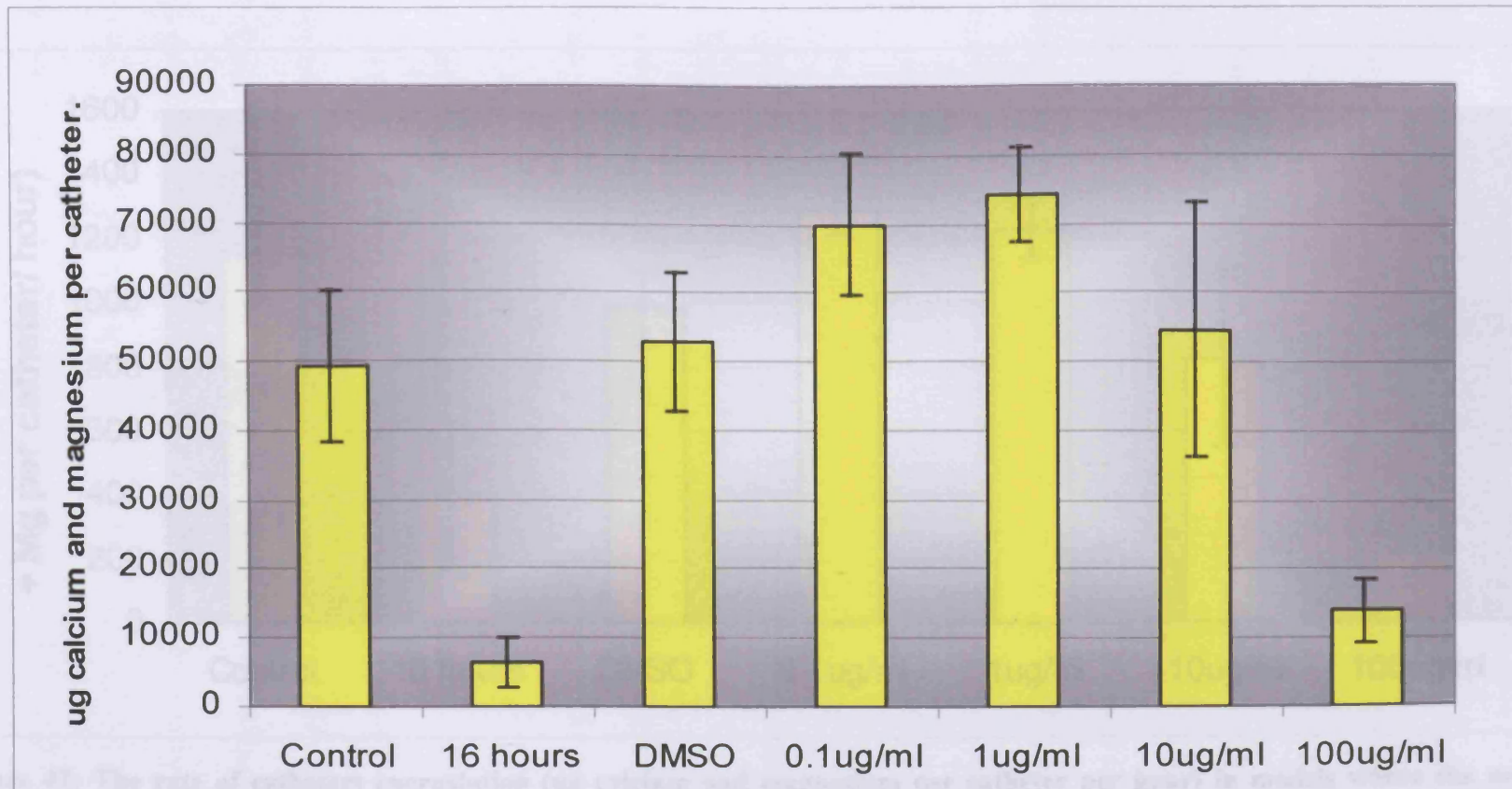


Figure 46: The amount of calcium and magnesium (μg per catheter) from catheters in which the urine supply was changed to contain different triclosan concentrations 16 h after the bladder models were inoculated with *Pr. mirabilis* B2. Three control models were also set up, one was taken down at 16 h, one was run until blockage and the other was supplied with artificial urine containing 1% DMSO. The results are a mean of triplicate experiments.

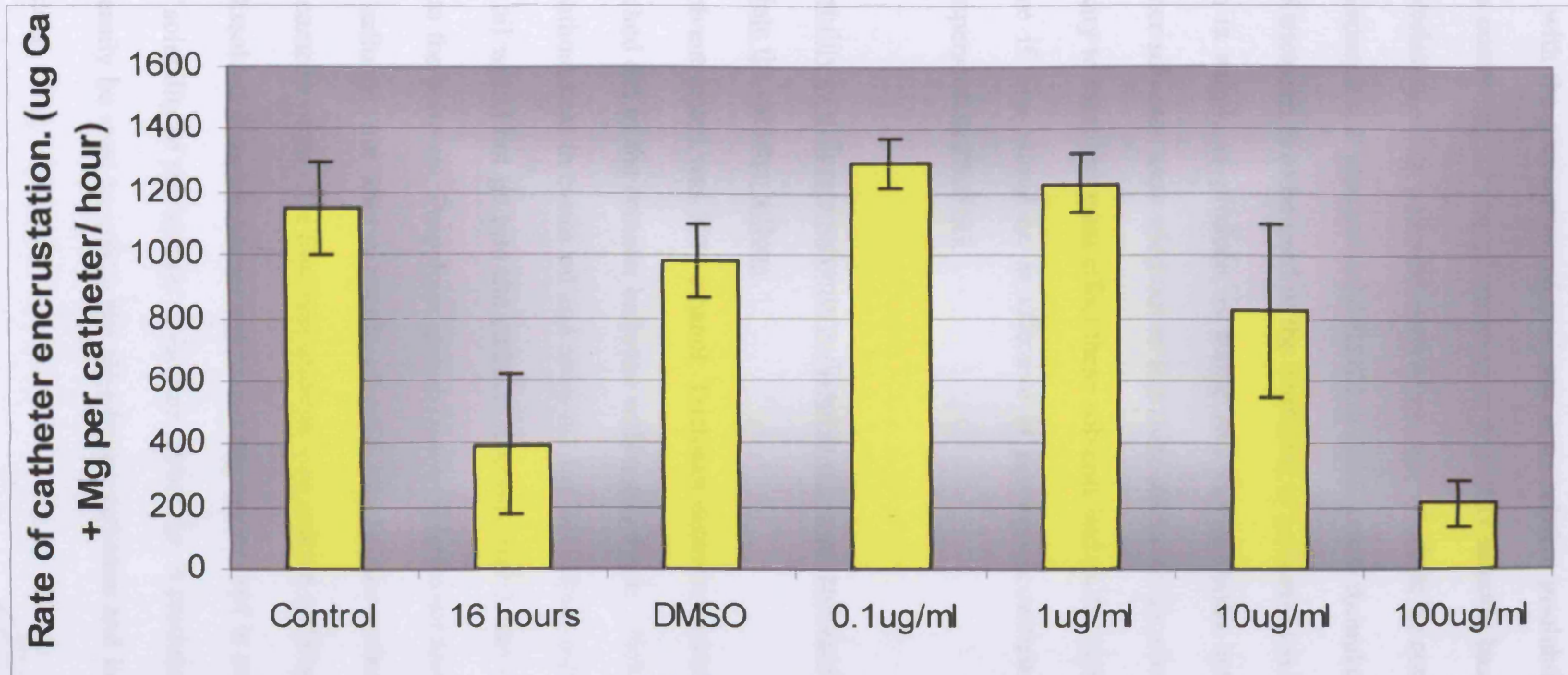


Figure 47: The rate of catheters encrustation (μg calcium and magnesium per catheter per hour) in models where the urine supply was changed to contain different concentrations of triclosan 16 h after the bladder models were inoculated with *Pr. mirabilis* B2. Three control models were also set up, one was taken down at 16 h, one was run until blockage and the other was supplied with artificial urine containing 1% DMSO. The results are a mean of triplicate experiments.

3.4. Formulation of the triclosan solution

A problem with the preparation of triclosan is its relative insolubility in water. The formulation containing 10 mg/ml triclosan in 5% PEG solution has to be heated and stirred to produce a white colloidal suspension, and needs to be continually stirred to keep it in suspension. It also proved difficult to sterilize this formulation by membrane filtration. If triclosan is to be used in the treatment of patients, it is important that the formulation is simple to produce, is stable and can be easily sterilized. For these reasons, other solvents were tried out to test their ability to dissolve triclosan. It was then necessary to establish what effect these solvents had on the catheter balloons and to determine if they would be as effective at preventing catheter blockage as the triclosan suspension in 5% PEG.

3.4.1. The ability of different solvents to dissolve triclosan and the feasibility of using them to inflate the catheter balloon

The first solvent tested was 70% ethanol. Triclosan dissolved quickly in this but the alcohol leached out of the catheter balloons within one week. Triclosan could also be dissolved without heat in castor oil and olive oil, but both these oils are very viscous. The castor oil would not go into the catheter balloons and it was difficult to get the olive oil into the balloons. Propylene glycol dissolved triclosan and could be used to inflate the balloons, but after a couple of days soaking the catheters in water, the balloons became swollen. The final test solution was sodium carbonate; 0.1 M sodium carbonate dissolved 3 mg/ml triclosan with stirring but no heat in under 1 h. This was the limit of solubility of the biocide in sodium carbonate. It produced a clear solution that could easily be used to inflate the all-silicone catheters and both types of latex based catheters.



3.4.2. The effect of 3 mg/ml triclosan in 0.1 M sodium carbonate on the catheter balloons

Using the method described in section 3.2.3, the effect of triclosan in 0.1 M sodium carbonate on the catheter balloon was determined. There were no visible changes to any of the catheter balloons inflated with 3 mg/ml triclosan in 0.1 M sodium carbonate, 0.1 M sodium carbonate or water after eight weeks soaking in water. All catheter balloons could still be deflated at the end of the experiment. The volume of liquid left in the catheter balloons after two months soaking in water was recorded and the data are presented in Figure 48. There was no significant difference ($P > 0.05$) between the volumes of liquid left in any of the three groups for both types of latex catheters. In the case of the all-silicone catheters there was significantly ($P < 0.05$) more fluid in the 0.1 M sodium carbonate balloons (with and without triclosan) than in the catheters that had their balloons inflated with water.

3.4.3. The ability of triclosan in 0.1 M sodium carbonate used to inflate the catheter balloon at preventing encrustation by *Pr. mirabilis*

Bladder models were set up fitted with all-silicone catheters. Control catheters were inflated with water whilst the test catheters were inflated with 10 µg/ml, 100 µg/ml, 500 µg/ml, 1 mg/ml and 3 mg/ml triclosan all in 0.1 M sodium carbonate. A control catheter inflated with 0.1 M sodium carbonate was also tested. The models were inoculated with *Pr. mirabilis* B2 and run until blockage or for a maximum of seven days.

There was no significant difference ($P > 0.05$) between the time to blockage (Figure 49a) or the pH and viable cell count (Figures 49b+c) from the residual urine of the

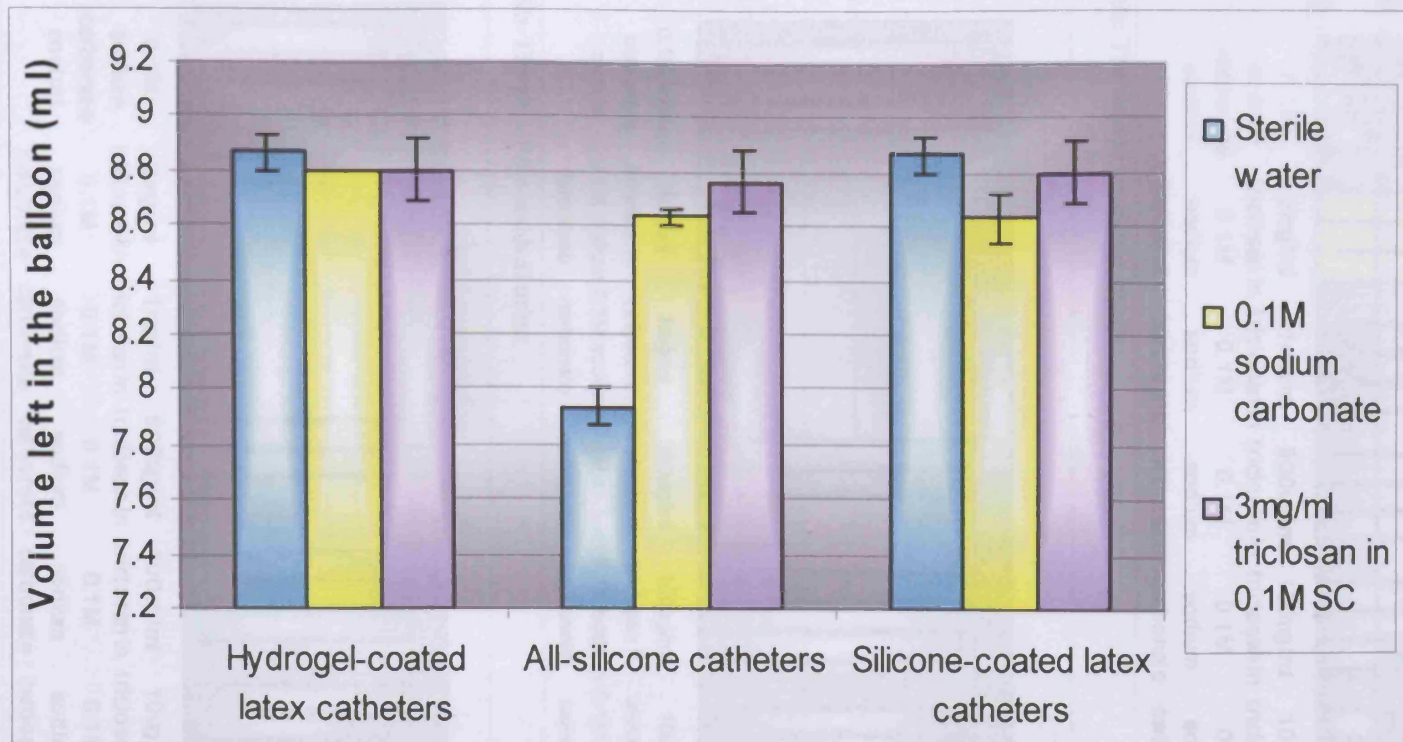


Figure 48: Volume of liquid left in the balloons of three different types of catheters that were inflated with 10 ml sterile water, 0.1 M sodium carbonate or 3 mg/ml triclosan in 0.1M sodium carbonate for two months. The mean values are from triplicate experiments.

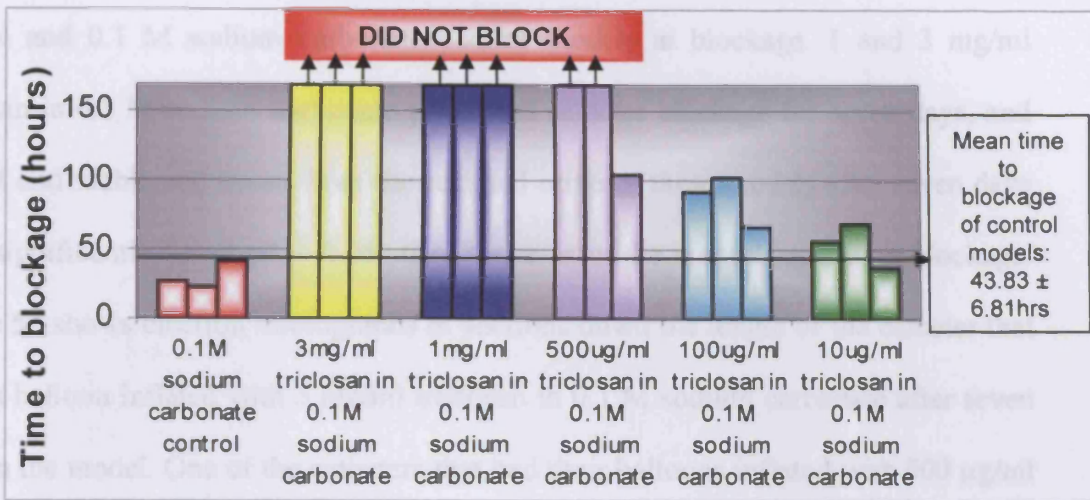


Figure 49a: The time to blockage results.

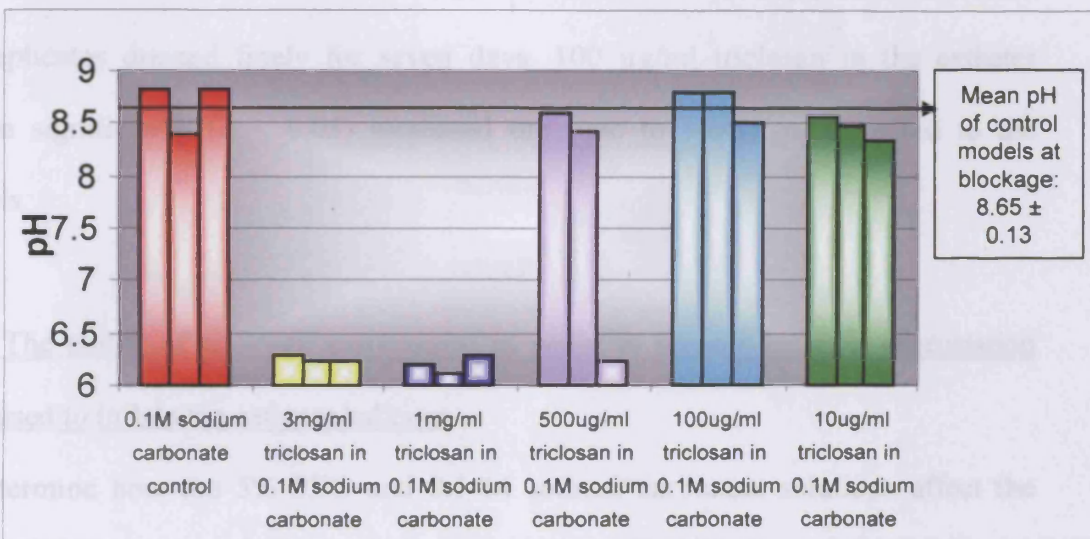


Figure 49b: The pH of the residual urine.

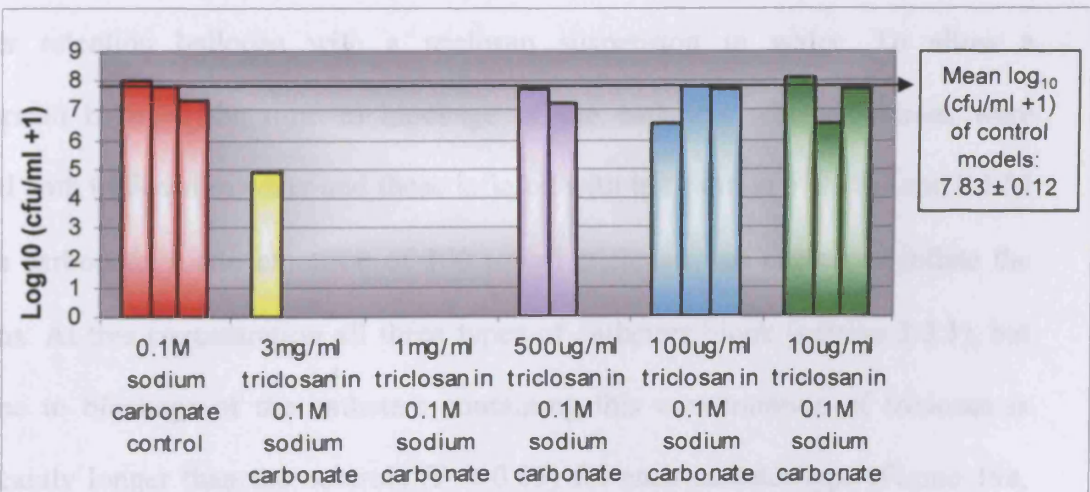


Figure 49c: Log₁₀ (cfu/ml +1) of the residual urine.

Figure 49: Time to blockage, pH and viable cell count results from models fitted with all-silicone catheters inflated with triclosan in sodium carbonate and inoculated with *Pr. mirabilis* B2. The black arrows indicate the mean results of the control models.

control and 0.1 M sodium carbonate control models at blockage. 1 and 3 mg/ml triclosan in 0.1 M sodium carbonate prevented catheter blockage for seven days, and the pH and viable cell count from the residual urine of these models after seven days were significantly lower ($P < 0.05$) than those from the control models at blockage. Figure 50 shows electron micrographs of sections down the length of the catheter that had its balloon inflated with 3 mg/ml triclosan in 0.1 M sodium carbonate after seven days in the model. One of the catheters that had their balloons inflated with 500 μ g/ml triclosan in 0.1 M sodium carbonate blocked within the seven days, whilst the other two replicates drained freely for seven days. 100 μ g/ml triclosan in the catheter balloon significantly ($P < 0.05$) increased the time to blockage compared to the controls.

3.4.4. The ability of triclosan suspensions in water to prevent catheter encrustation when used to inflate the catheter balloons

To determine how the 5% PEG and 0.1 M sodium carbonate solutions affect the ability of triclosan to prevent catheter encrustation, models were set up filling the catheter retention balloons with a triclosan suspension in water. To allow a comparison between the time to blockage of the catheters whose balloons were inflated with triclosan in water and those inflated with triclosan in 5% PEG and 0.1 M sodium carbonate, a concentration of 100 μ g/ml triclosan was chosen to inflate the balloons. At this concentration all three types of catheters block (section 3.3.1), but the time to blockage of the catheters containing this concentration of triclosan is significantly longer than the controls ($P < 0.05$) for each catheter type (Figure 19a, 20a, 21a). All three different types of catheters were tested with the 100 μ g/ml suspension of triclosan in water, models were run until blockage. All-silicone

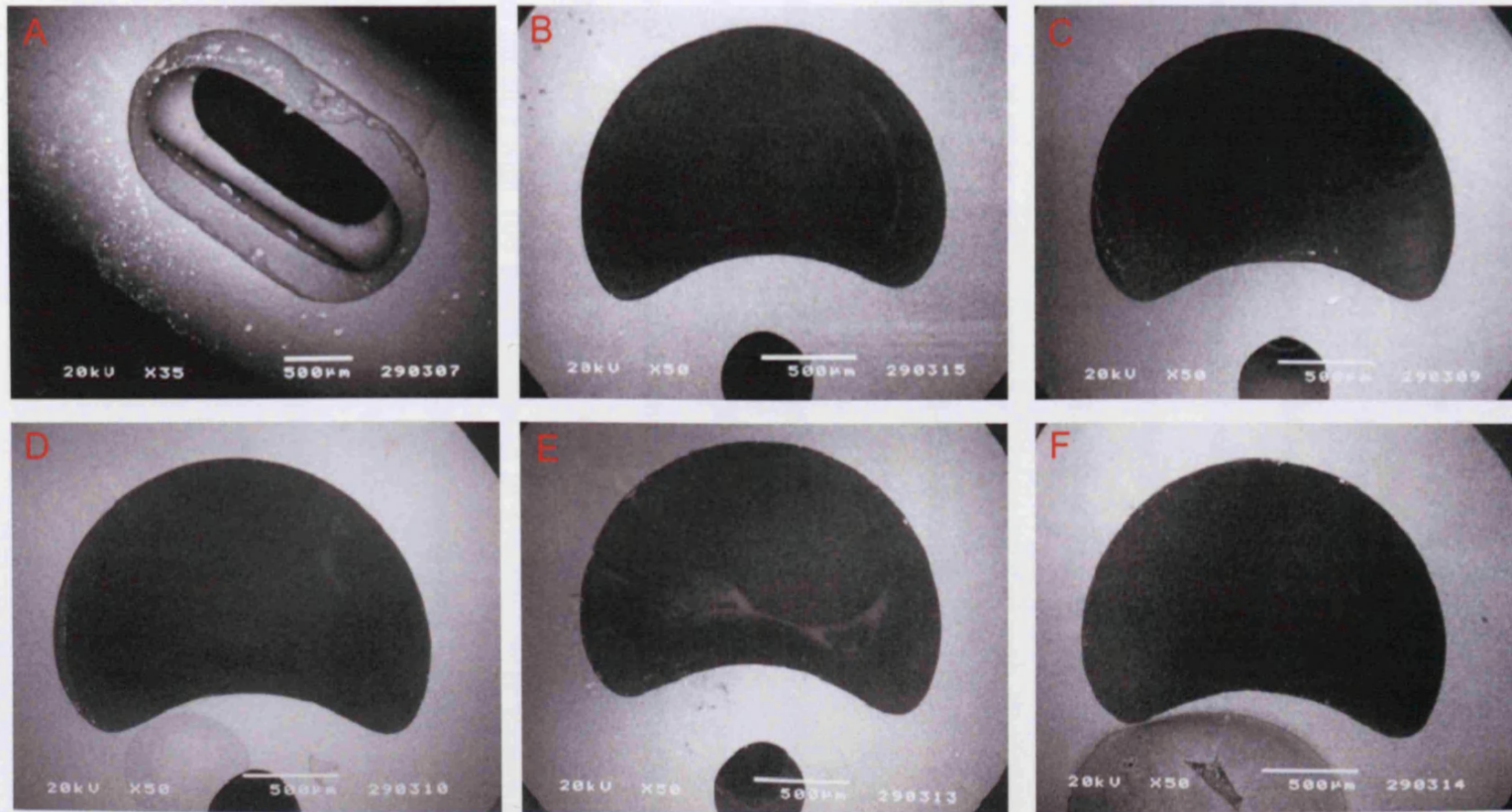


Figure 50: Electron micrographs of an all-silicone catheter that had its balloon inflated with 3 mg/ml triclosan in 0.1 M sodium carbonate after 7 days in a *Pr. mirabilis* infected bladder model. 6 pictures were taken along the length of the catheter. The positions of sections A-F are

catheters were also set up inflating the catheter with a 10 mg/ml triclosan suspension in water. Models were run until blockage or for a maximum of seven days.

There was no significant difference ($P > 0.05$) between the time to blockage, pH or viable cell count in the residual urine (Figures 51) of all-silicone catheters using 100 $\mu\text{g/ml}$ triclosan in suspension compared to the same concentration in 5% PEG or 0.1 M sodium carbonate. The 10 mg/ml triclosan in aqueous suspension in all-silicone catheters allowed the urine to drain freely for seven days. There was no significant difference ($P > 0.05$) between the viable cell counts and pH from the residual urine of these models at seven days compared to those inflated with 10 mg/ml triclosan in 5% PEG or 3 mg/ml triclosan in 0.1 M sodium carbonate.

Using the hydrogel-coated latex catheters, there was no significant difference ($P > 0.05$) in the time to blockage, pH or viable cell count between catheters inflated with 100 $\mu\text{g/ml}$ triclosan in aqueous suspension and the same concentration in 5% PEG. The pH and viable cell count from the residual urine of models fitted with the catheters inflated with 100 $\mu\text{g/ml}$ triclosan in 5% PEG were significantly lower ($P < 0.05$) than those inflated with triclosan in suspension for the silicone-coated latex catheters. The time to blockage for the silicone-coated latex catheters were also significantly ($P < 0.05$) increased using triclosan in 5% PEG.

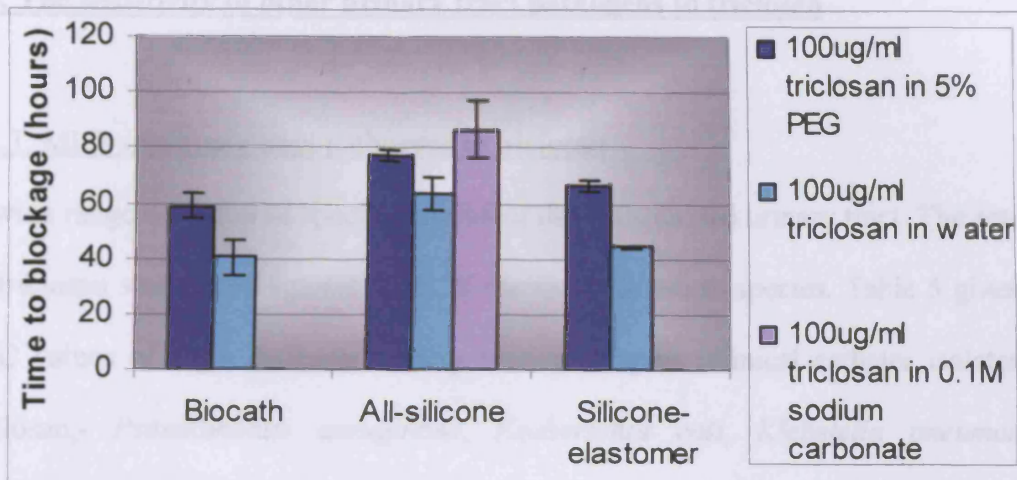


Figure 51a: The time to blockage results.

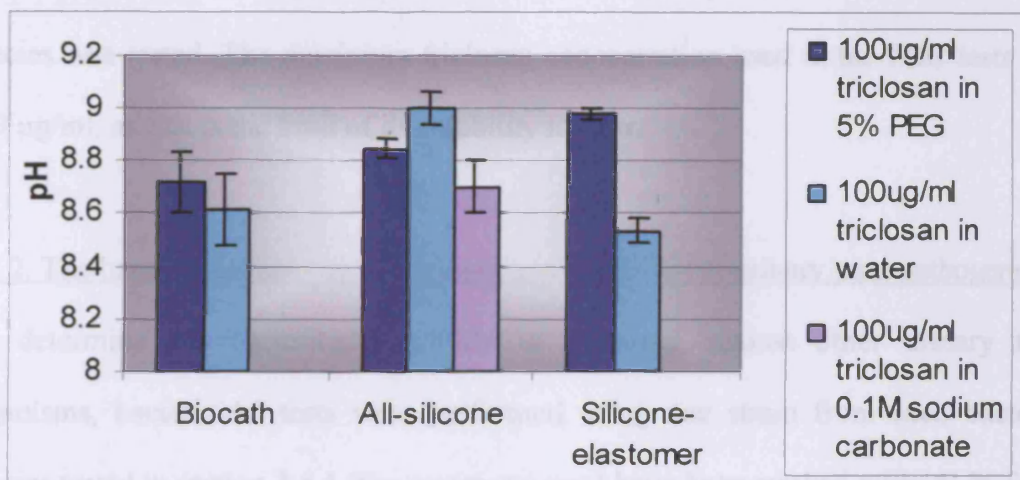


Figure 51b: The pH from the residual urine.

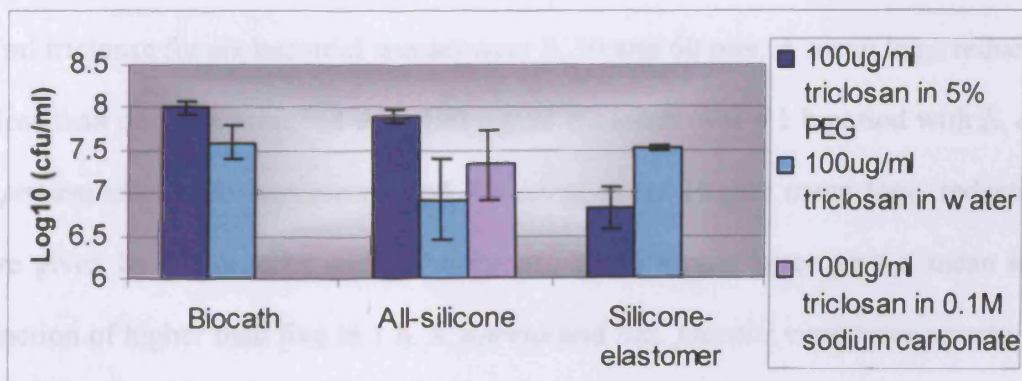


Figure 51c: Log₁₀ (cfu/ml) in the residual urine.

Figure 51: Time to blockage, pH and viable cell count results from models fitted with three different catheter types and inflated with 100 μ g/ml triclosan in water or 5% PEG, the all-silicone catheter results with 100 μ g/ml triclosan in 0.1M sodium carbonate are also included. All-silicone, Biocath (hydrogel-coated latex catheters) and silicone-elastomer (silicone-coated latex catheters) catheters were tested. Models were inoculated with *Pr. mirabilis* B2. Results are means of triplicate experiments.

3.5. The sensitivity of other urinary tract pathogens to triclosan

3.5.1. MICs of urinary tract pathogens to triclosan

A wide range of bacterial species can infect the catheterised urinary tract. The activity of triclosan was tested against eight of the more common species. Table 5 gives the MIC values of eight different urinary tract pathogens (clinical catheter isolates) to triclosan,- *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Providencia stuartii*, *Serratia marcescens*, *Staphylococcus aureus* and *Enterococcus faecalis* were all tested. Where possible five isolates of each species was tested. The maximum triclosan concentration used in the MIC tests was 100 µg/ml, as this is the limit of its solubility in agar.

3.5.2. The bactericidal activity of triclosan against different urinary tract pathogens

To determine the bactericidal activity of triclosan against other urinary tract organisms, bactericidal tests were performed using one strain from each bacterial species tested in section 3.5.1 (the organisms used have been marked with ‘*’ in Table 5). Figure 52a shows the mean log₁₀ reductions in the numbers of viable cells with 100 µg/ml triclosan for six bacterial species over 5, 30 and 60 min. A mean log₁₀ reduction of less than one was achieved with 100 µg/ml triclosan over a 1 h period with *E. coli*, *K. pneumoniae*, *Ser. marcescens* and *Ps. aeruginosa*. Higher mean log₁₀ reductions were given by *Pv. stuartii* and *M. morganii*, but only the latter gave a mean log₁₀ reduction of higher than five in 1 h. *S. aureus* and *Ent. faecalis* were more sensitive to the lethal effects of triclosan, hence lower concentrations were tested all over a 5 min time period (Figure 52b). Mean log₁₀ viable bacterial cell count reductions of over five were achieved with 20 and 30 µg/ml triclosan for *Ent. faecalis* and *S. aureus*

Species	Strain no.	Triclosan MIC ($\mu\text{g/ml}$)	Urease producer?
<i>E. coli</i>	NSM 16*	0.2	X
	N 123	0.2	X
	N 55	0.3	X
	N 39	0.2	X
	SM 1	0.2	X
<i>Pv. stuartii</i>	NSM 14*	0.3-0.4	X
	NSM 40	0.2	X
	NSM 58	0.2	X
	NSM 71	0.2	X
	NSM 24	0.2-0.3	X
<i>K. pneumoniae</i>	SM 28	0.7	✓
	NSM 48	0.3-0.4	✓
	NSM 37	0.4-0.6	✓
	NSM 41	0.5-0.6	✓
	CC36*	0.5-0.8	✓
<i>Ent. faecalis</i>	N 21	7	X
	N70	7	X
	CC39*	7	X
	CC40	7	X
	CC43	7	X
<i>S. aureus</i>	N 112	0.1	X
	N 114	0.1	X
	NSM 5*	0.4-0.6	✓
	SM 9	0.2	X
	NSM 8	0.1-0.2	X
<i>Ser. marcescens</i>	NSM 63	>100	✓
	N 53	>100	✓
	CC 13*	>100	✓
	CC 42	>100	✓
<i>M. morgani</i>	NSM 63	>100	✓
	NSM 21	>100	✓
	NSM 18	>100	✓
	NSM 13	>100	✓
	MS 2*	>100	✓
<i>Ps. aeruginosa</i>	NSM 10	>100	✓
	NSM 20	>100	✓
	NSM 46	>100	✓
	NSM 57*	>100	✓
	NSM 62	>100	✓

Table 5: The MIC ($\mu\text{g/ml}$) of triclosan for eight different urinary tract pathogens. The values were obtained from three replicate tests. Single strains of each species marked with '' were tested in further experiments. The ability of each strain to produce the urease enzyme is also shown.**

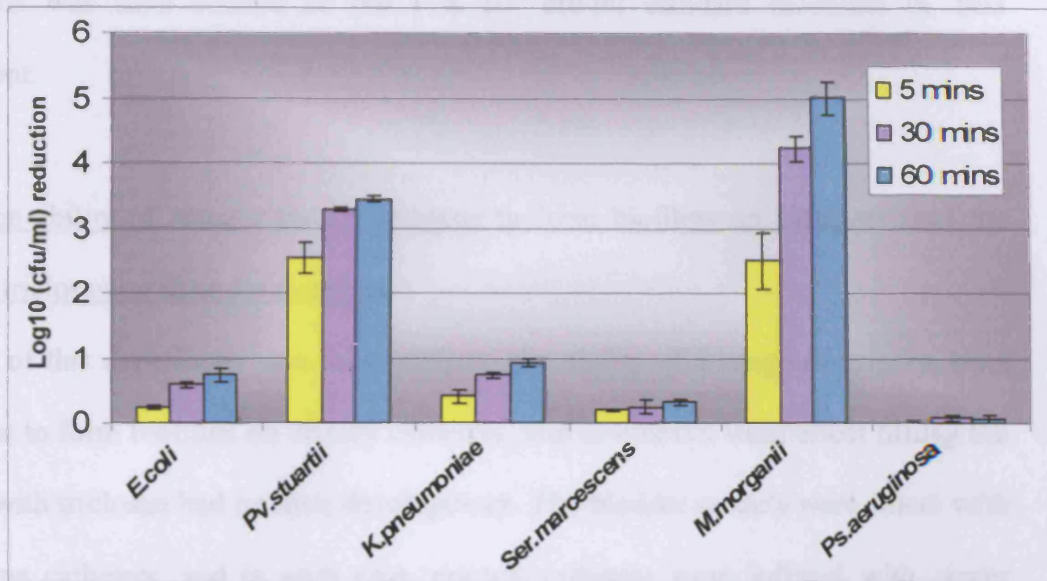


Figure 52a: Mean \log_{10} (cfu/ml) reduction of six species after different contact times with 100 $\mu\text{g/ml}$ triclosan

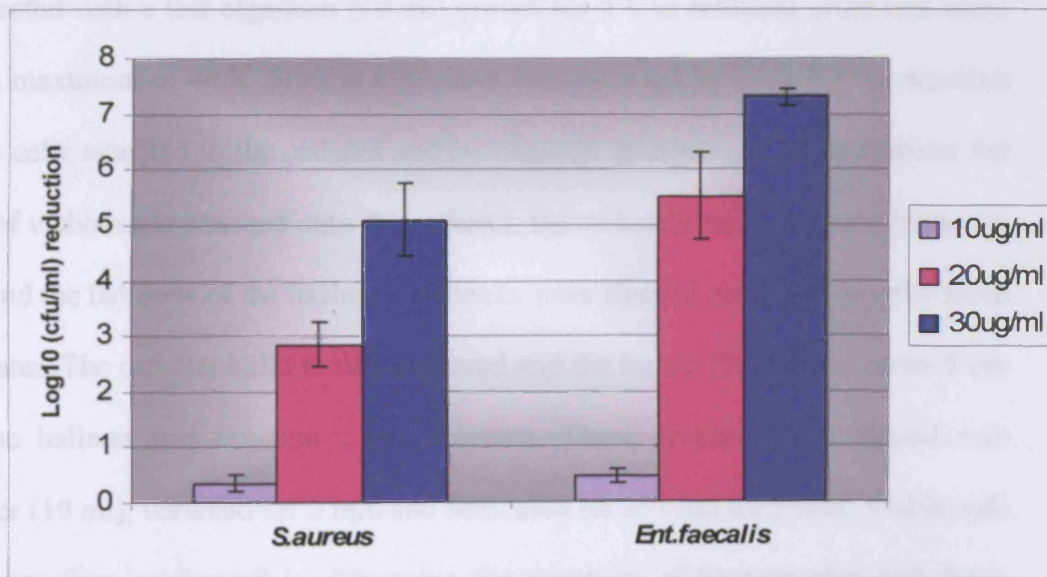


Figure 52b: Mean \log_{10} (cfu/ml) reduction after a 5 min contact time with 10, 20 and 30 $\mu\text{g/ml}$ triclosan.

Figure 52: The bactericidal effect of triclosan on urinary tract pathogens.

respectively. Neat suspensions of *Ent. faecalis* only contained about 5×10^7 cfu/ml; hence this was used instead of the 1×10^9 cfu/ml standard inoculum in this experiment.

3.5.3. The ability of urinary tract pathogens to form biofilms on catheters and the effect of triclosan on their development

The aim of this experiment was to investigate the ability of a range of urinary tract pathogens to form biofilms on urinary catheters, and to observe what effect filling the balloon with triclosan had on their development. The bladder models were fitted with all-silicone catheters, and in each case, control catheters were inflated with water while the test catheters were inflated with 10 mg/ml triclosan in 5% PEG. Models were infected with a test organism (10 ml) grown for 4 h in artificial urine and were run for a maximum of 48 h. Biofilm formation was assessed by counting the number of viable cells attached to the catheter and by electron microscopy. To determine the number of viable cells attached onto the catheter, the catheters were removed from the models and the balloons of the triclosan catheters were flushed out 10 times with fresh sterile water. The catheter balloon was removed and the top of the catheter up to 5 cm below the balloon was cut into 1 cm sections. These sections were placed into neutraliser (10 ml), vortexed for 2 min and sonicated (at 35 khz) for 5 min. Viable cell counts were then performed to determine the numbers of bacteria that had been shaken off the catheter sections. This experiment was replicated four times, so that the catheters from one run could be observed under LV-SEM.

The same eight bacterial strains that were tested in section 3.5.2 were tested in the bladder models. *Pr. mirabilis* B2 was also run in the models for a maximum of 48 h

with and without triclosan. None of the eight different bacterial species blocked the catheter within 48 h. All the *Pr. mirabilis* control models blocked within this time (mean 29 ± 9.24 h), but the triclosan models drained freely for 48 h.

The pH of the inoculating culture, and the pH of the residual urine from the control and test bladder models from each organism at 24 and 48 h are shown in Figure 53. The pHs of the inoculating cultures with *Pr. mirabilis* were all above 8.6 and the pH of the control models remained above 8 throughout the experiment until blockage. Triclosan significantly reduced the pH in the residual urine of the *Pr. mirabilis* infected models at 48 h compared to the pH of the controls at blockage ($P = 0.046$). The pH values for all the other bacterial species remained acidic throughout the experiment, even with the urease-producing organisms. The pH of the residual urine for the triclosan treated *Pv. stuartii* was significantly lower than the control at 48 h ($P = 0.017$). There was no significant difference ($P > 0.05$) between the pH values of the test and control models at 24 or 48 h for any of the other bacterial species tested.

Figure 54 shows the $\log_{10} (\text{cfu/ml} + 1)$ data of the inoculating culture and of the residual urine from the control and test bladder models from each organism at 24 and 48 h. For the *Pr. mirabilis*, *Pv. stuartii*, *E. coli*, *S. aureus*, *Ent. faecalis* and *K. pneumoniae* there were significantly lower numbers ($P < 0.05$) of viable cells in the residual urine of the triclosan treated models compared to the controls at 24 and 48 h. There was no significant difference ($P > 0.05$) between the viable cell count from the residual urine of control and triclosan treated models inoculated with *M. morgani*, *Ser. marcescens* or *Ps. aeruginosa* at 24 or 48 h.

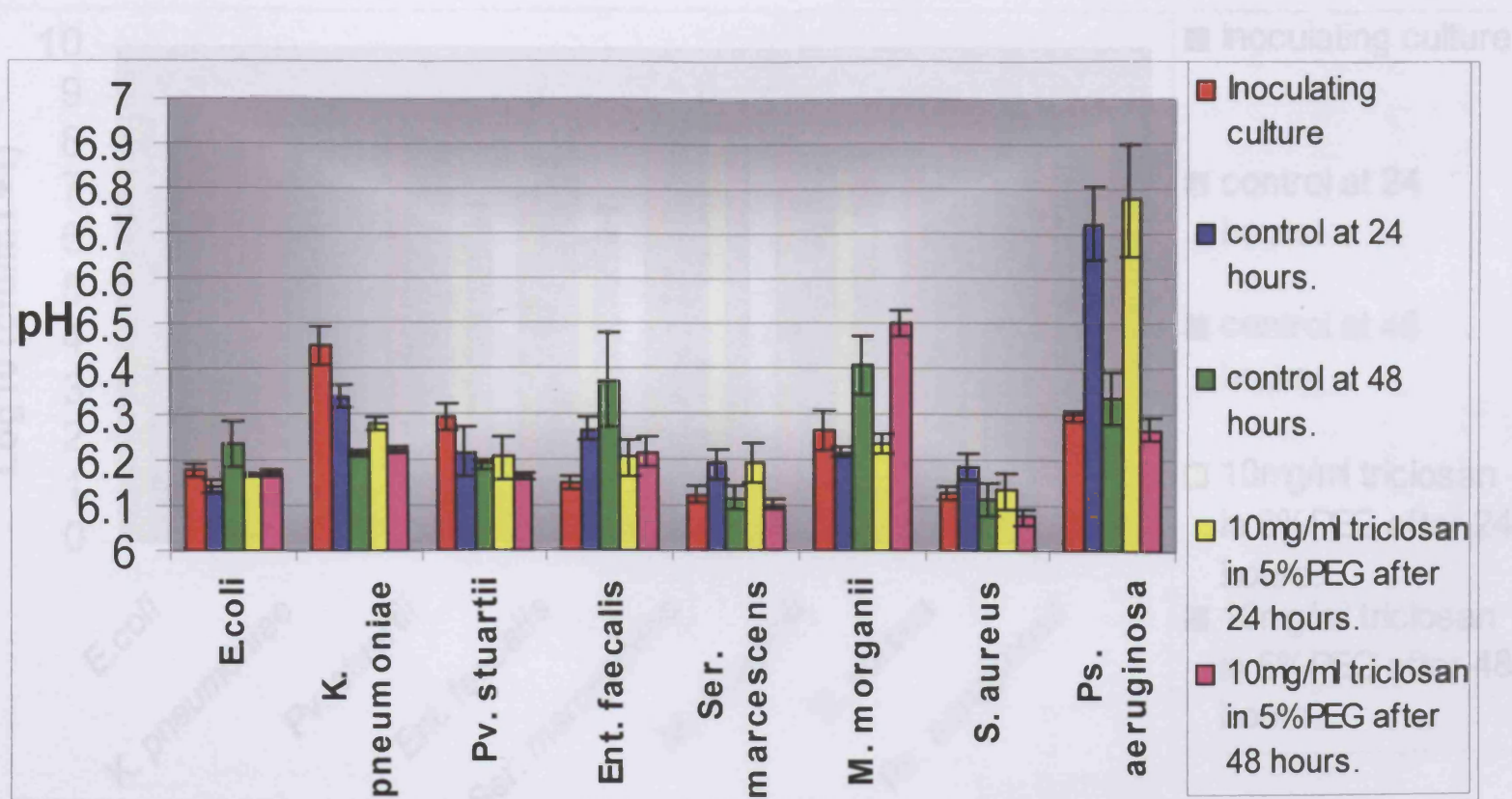


Figure 53: The mean pH of the inoculating culture and the residual urine of the control and triclosan test models inoculated with 8 different urinary tract pathogens after 24 and 48 h. The balloons of control catheters were inflated with water, the balloons of the test catheters were inflated with 10 mg/ml triclosan in 5% PEG. The values are means from four replicate experiments.

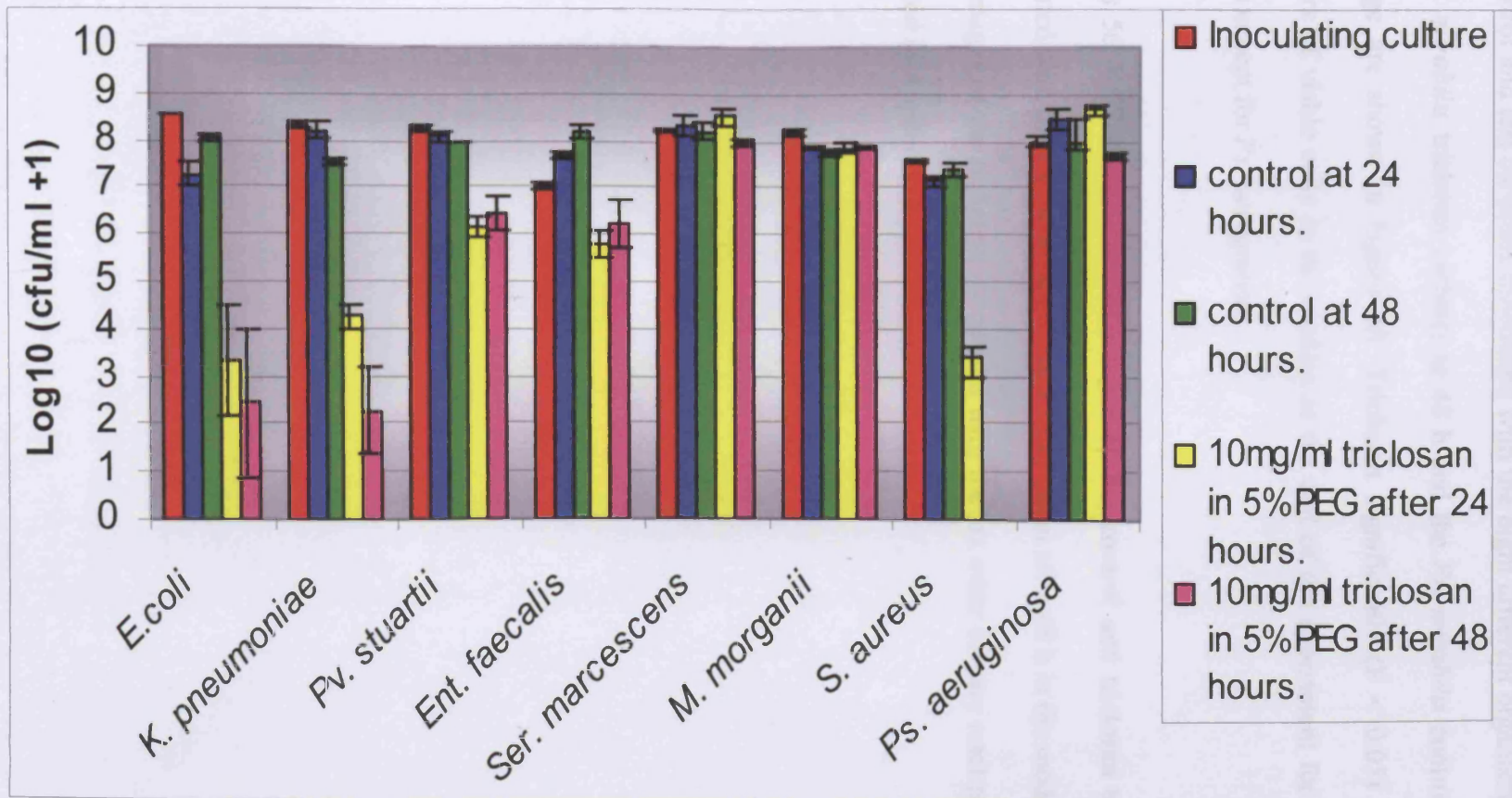


Figure 54: Mean \log_{10} (cfu/ml +1) bacterial count of the inoculating culture and the residual urine of the control and triclosan test models inoculated with eight different urinary tract pathogens after 24 and 48 h. The balloons of control catheters were inflated with water, the balloons of the test catheters were inflated with 10 mg/ml triclosan in 5% PEG. The values are means from four replicate experiments.

The $\log_{10} (\text{cfu/ml} + 1)$ for the numbers of bacteria in the biofilm on the top sections of the control and test catheters inoculated with the eight different organisms after 48 h, the *Pr. mirabilis* triclosan catheter at 48 h and the *Pr. mirabilis* control catheter at blockage are shown in Figure 55. Triclosan significantly ($P < 0.05$) reduced the numbers of viable cells in the biofilm at the end of the experiment for all bacterial species except for *Ps. aeruginosa*.

Figures 56 + 57 show LV-SEM images of the control and triclosan test catheters inoculated with the *Ps. aeruginosa* and *E. coli* taken after 48 h in the models. The LV-SEM images of the catheters inoculated with the six other urinary tract pathogens are presented in Appendix 2.

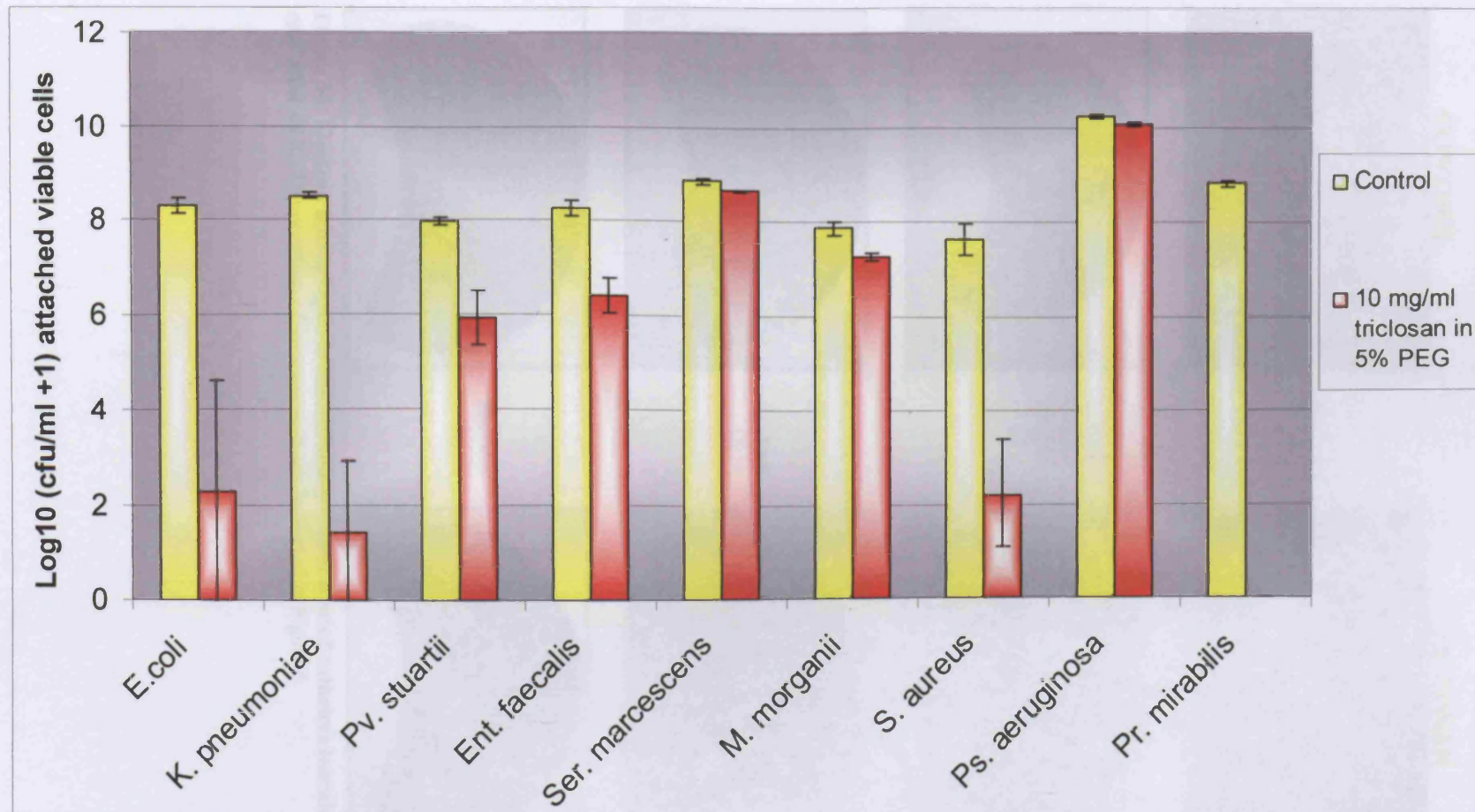


Figure 55: Mean \log_{10} (cfu/ml + 1) of the numbers of bacteria recovered from the top section of the catheter (up to 5 cm below the balloon) in the control and test models inoculated with different bacterial species after 48 h, or after blockage for the *Pr. mirabilis* controls. Control models were inflated with water, test catheters were inflated with 10 mg/ml triclosan in 5% PEG. The results show the number of viable cells attached from 5 cm below the catheter balloon and upwards. The values are means from three replicate experiments. Error bars represent the standard errors.

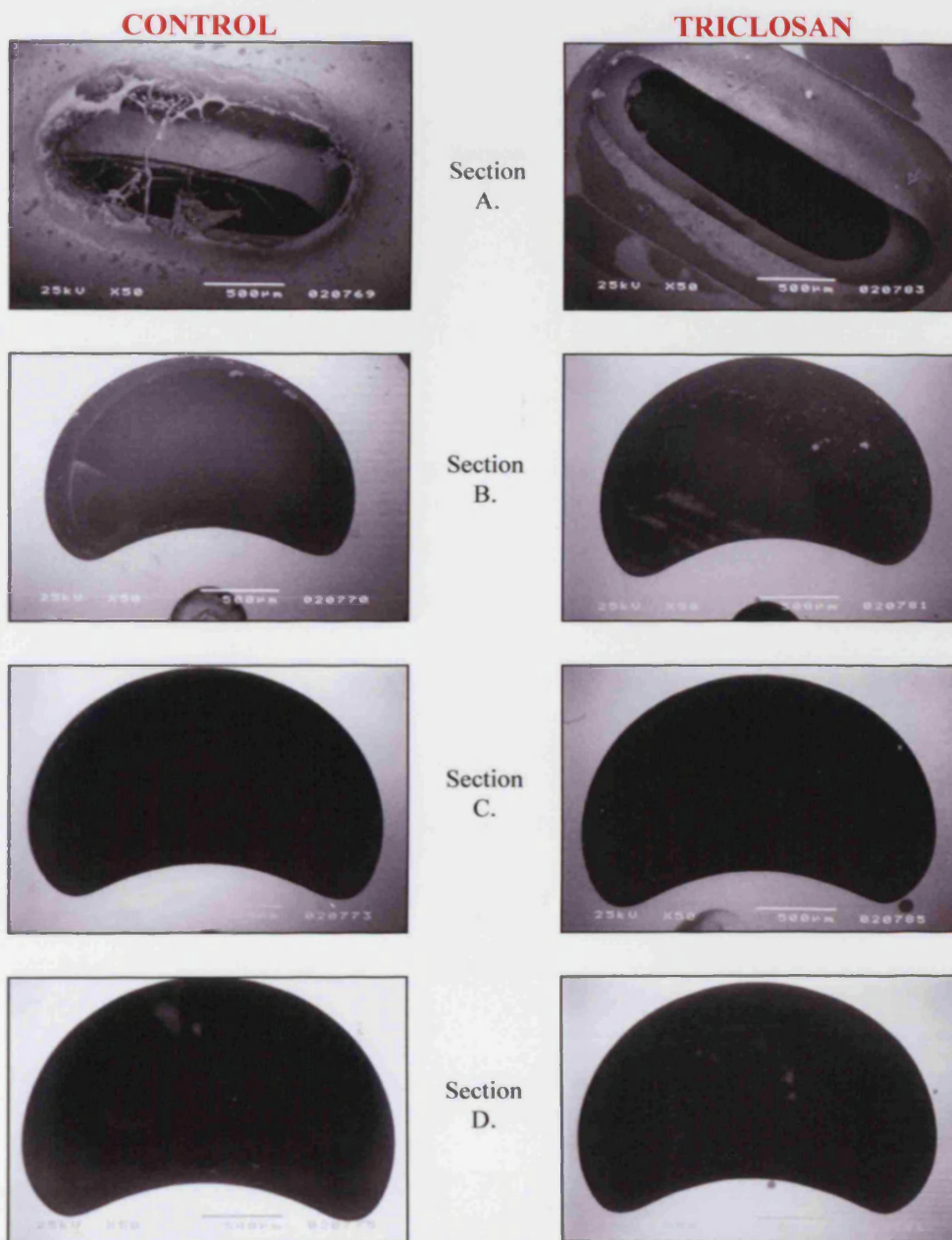


Figure 56: Electron micrographs of control and triclosan treated catheters inoculated with *E. coli* after 48 h. The positions of sections A-D are indicated in Figure 6.

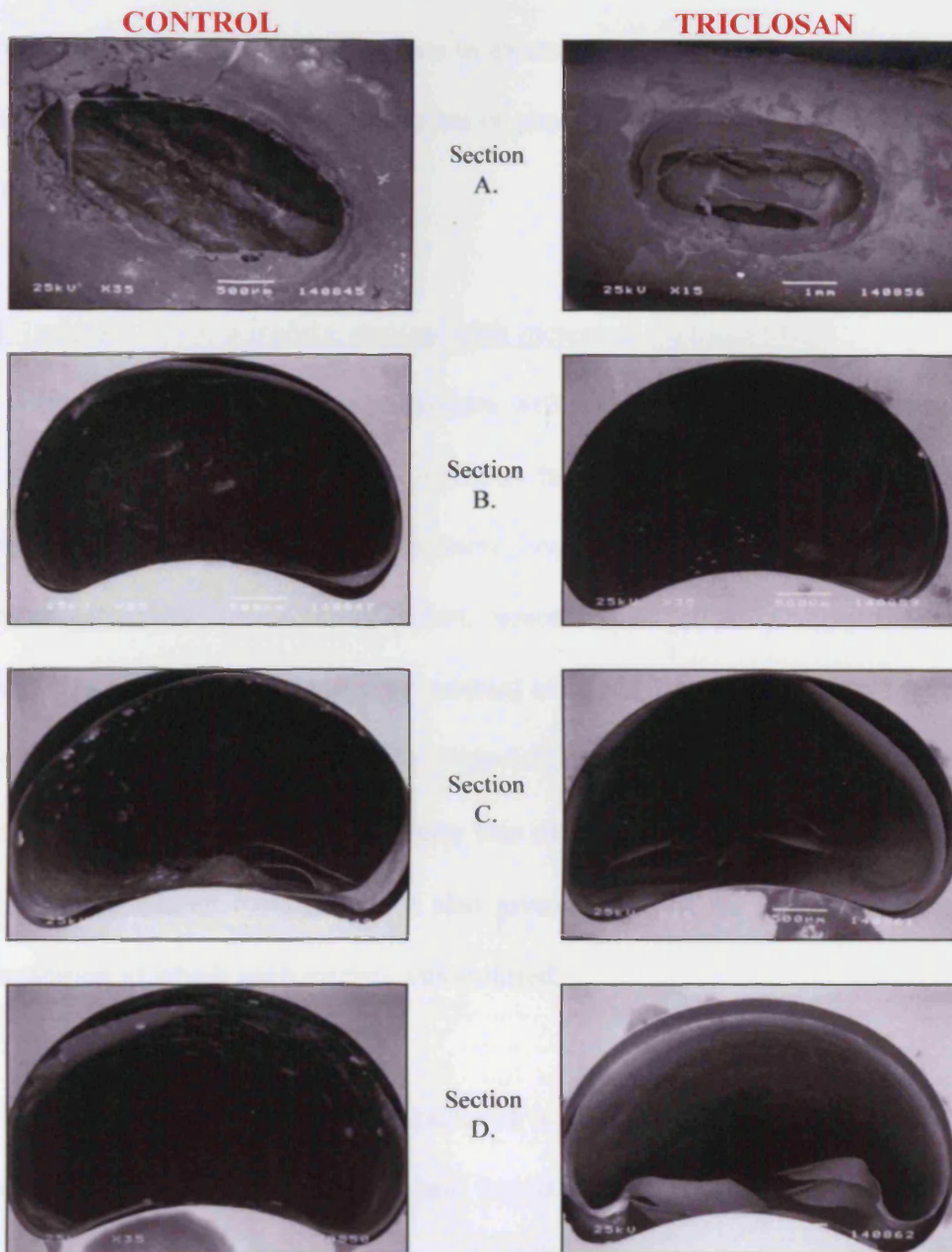


Figure 57: Electron micrographs of control and triclosan treated catheters inoculated with *Ps. aeruginosa* after 48 h. The positions of sections A-D are indicated in Figure 6.

3.6. An investigation of resistance to triclosan in *Pr. mirabilis*

Due to the increased concern regarding resistance in bacteria to biocides and antibiotics, the aim of this section was to examine whether exposure of *Pr. mirabilis* to triclosan could result in the emergence of strains with an increased resistance to the biocide.

3.6.1. Isolation of *Pr. mirabilis* mutants with increased triclosan MICs

Two different methods of isolating mutants with elevated triclosan MICs were tested. The first method relied on isolating mutants from a zone of inhibition created by a triclosan soaked disk placed onto a bacterial lawn. All five *Pr. mirabilis* strains tested swarmed into these zones of inhibition, hence the isolation of mutants using this method was not possible. The second method involved spreading a heavy inoculum of the test strains onto plates containing super-MIC concentrations of triclosan (0.5 – 10 µg/ml). 55 mutants were isolated using this method. Table 6 shows the MISC and MIC for each mutant isolated and it also gives details of the parent strains and the concentration at which each mutant was isolated.

A selection of 14 mutants were sub-cultured in the absence of triclosan for 15 days. Table 7 shows the MIC for each mutant tested after 1, 3, 7, 11 and 15 sub-cultures. There were no substantial decreases in the MIC of any mutants tested. Each of the mutant sub-cultures were streaked out onto TSA and CLED Agar and they revealed no signs of contamination. The purity of the cultures whose MICs rose considerably (M12, 17, 19, 35, 40, 44, 55) were confirmed using the BBL crystal™ identification kits. This set of experiments confirmed the stability of the mutants.

Mutant number	Parental strain	Concentration of triclosan at which the mutant was isolated	MIC $\mu\text{g/ml}$	MSC $\mu\text{g/ml}$
M1	NP43	0.5 $\mu\text{g/ml}$	5-40	0.8-10
M2	NP43	0.5 $\mu\text{g/ml}$	5	0.8-1
M3	NP43	0.5 $\mu\text{g/ml}$	60-80	10-20
M4	NP43	0.5 $\mu\text{g/ml}$	5	0.3-0.4
M5	NP43	0.5 $\mu\text{g/ml}$	0.5-5	0.1-0.3
M6	NP43	0.5 $\mu\text{g/ml}$	5	0.3-0.5
M7	NP43	0.5 $\mu\text{g/ml}$	5	0.3-0.5
M8	NP43	0.5 $\mu\text{g/ml}$	0.3-0.6	0.3
M9	NP43	1 $\mu\text{g/ml}$	40-60	20-40
M10	NP43	1 $\mu\text{g/ml}$	5	0.3-0.4
M11	NP43	1 $\mu\text{g/ml}$	20-40	5-10
M12	NP43	1 $\mu\text{g/ml}$	40-60	20-40
M13	NP43	2 $\mu\text{g/ml}$	40-60	40
M14	NP43	2 $\mu\text{g/ml}$	2-5	0.6-1
M15	NP43	5 $\mu\text{g/ml}$	40	0.6-2
M16	NP43	5 $\mu\text{g/ml}$	40	10
M17	NP43	10 $\mu\text{g/ml}$	40-60	0.8-5
M18	NP14	0.5 $\mu\text{g/ml}$	0.3-0.4	0.2
M19	NP14	0.5 $\mu\text{g/ml}$	40-60	20-40
M20	NP14	0.5 $\mu\text{g/ml}$	0.4-0.5	0.3-0.4
M21	NP14	1 $\mu\text{g/ml}$	5	2-5
M22	NP14	1 $\mu\text{g/ml}$	0.3-0.4	0.2-0.3
M23	NP14	2 $\mu\text{g/ml}$	5-10	5
M24	NP14	2 $\mu\text{g/ml}$	5	2-5
M25	NP37	0.5 $\mu\text{g/ml}$	5-10	2
M26	NP37	0.5 $\mu\text{g/ml}$	20	10
M27	NP37	1 $\mu\text{g/ml}$	5-10	2-5
M28	NP37	1 $\mu\text{g/ml}$	5-10	2-5
M29	NP37	1 $\mu\text{g/ml}$	10	5
M30	NP37	2 $\mu\text{g/ml}$	5-10	2-5
M31	NP37	2 $\mu\text{g/ml}$	5	2-5
M32	NP55	0.5 $\mu\text{g/ml}$	5-10	5
M33	NP55	0.5 $\mu\text{g/ml}$	5-10	5
M34	NP55	0.5 $\mu\text{g/ml}$	5-10	5
M35	NP55	0.5 $\mu\text{g/ml}$	40	40
M37	NP55	0.5 $\mu\text{g/ml}$	5-10	2
M38	NP55	1 $\mu\text{g/ml}$	5-10	5
M39	NP55	1 $\mu\text{g/ml}$	20-60	20-40
M40	NP55	1 $\mu\text{g/ml}$	40-60	10-40
M41	NP55	2 $\mu\text{g/ml}$	0.2	0.1
M42	NP55	2 $\mu\text{g/ml}$	0.8-5	0.5-1
M43	NP55	2 $\mu\text{g/ml}$	5-40	1-20
M44	B2	0.5 $\mu\text{g/ml}$	40	10-20
M45	B2	0.5 $\mu\text{g/ml}$	2-5	0.4-0.6
M46	B2	0.5 $\mu\text{g/ml}$	2-5	0.4-0.6
M47	B2	0.5 $\mu\text{g/ml}$	2-5	0.2-0.4
M48	B2	0.5 $\mu\text{g/ml}$	2-5	0.8-2
M49	B2	0.5 $\mu\text{g/ml}$	0.5-5	0.2-0.5
M50	B2	0.5 $\mu\text{g/ml}$	5	0.8
M51	B2	0.5 $\mu\text{g/ml}$	0.3-0.8	0.2-0.4
M52	B2	2 $\mu\text{g/ml}$	20-60	20-40
M53	B2	2 $\mu\text{g/ml}$	40	10-40
M54	B2	2 $\mu\text{g/ml}$	0.2-0.3	0.1
M55	B2	5 $\mu\text{g/ml}$	40	20

Table 6: Table showing the parental strain of each mutant, the concentration at which the mutants were isolated, their MIC and MISC. The results are from experiments performed in triplicate in agar.

Mutant no.	MIC ($\mu\text{g/ml}$) after 1, 3, 7, 11 and 15 subcultures in the absence of triclosan				
	MIC 1	MIC 3	MIC 7	MIC 11	MIC15
M 12	60	60	60	>100	>100
M 17	60	60	60	>100	100
M 19	40	40	40	100	80
M 21	5	5	5	10	5
M 23	5	10	10	20	10
M 26	20	20	20	20	10
M 29	5	5	5	5	5
M 31	5	5	2	2	2
M 35	40	40	40	>100	>100
M 40	100	>100	>100	>100	>100
M 42	5	5	10	10	5
M 44	40	40	60	100	>100
M 48	2	2	2	2	2
M 55	40	40	60	>100	>100

Table 7: The MIC of 14 *Pr. mirabilis* mutants after 1, 3, 7, 11 and 15 subcultures in the absence of triclosan. Since there were no considerable decreases in the MIC when the mutants were sub-cultured in the absence of a selective pressure, they were considered to be stable.

3.6.2. Bactericidal activity of triclosan against the mutants

Three of the mutants M44, M48 and M55 derived from *Pr. mirabilis* B2 were tested in further experiments. The mean \log_{10} viable cell count reductions for these mutants and their wild-type strains given by 100 $\mu\text{g/ml}$ triclosan over three time periods are shown in Figure 58. Tables 8, 9 and 10 show the P-values from statistical analysis individually comparing each strain tested in the suspension tests throughout this project at 5, 30 and 60 min.

The MICs for M44, M48 and M55 in artificial urine were 5 - 8, 3 and 4 - 8 $\mu\text{g/ml}$ respectively. These MICs are significantly higher ($P < 0.05$) than that of *Pr. mirabilis* B2 which was 0.2 $\mu\text{g/ml}$. Figure 59 shows the pH values from the cultures over a range of triclosan concentrations. Much higher triclosan concentrations were required to maintain acidic conditions in the artificial urine inoculated with the mutants compared to the parental strain. 0.2 $\mu\text{g/ml}$ of triclosan kept the artificial urine culture inoculated with B2 acidic, whilst to keep the cultures inoculated with the mutants acidic, concentrations of 6, 3 and 8 $\mu\text{g/ml}$ triclosan were required for M44, M48 and M55 respectively.

3.6.3. The ability of triclosan in the balloon to control catheter encrustation when the bladder models were inoculated with *Pr. mirabilis* triclosan resistant mutants

Three mutants M44, M48 and M55 and their parental strain *Pr. mirabilis* B2 were run in the bladder models fitted with all-silicone catheters. Control models with sterile water in the balloon and test models with 10 mg/ml triclosan in 5% PEG in the balloon were set up for each mutant and the wild-type strain. Models were run until blockage or for a maximum of 48 h. Calcium and magnesium analysis was performed

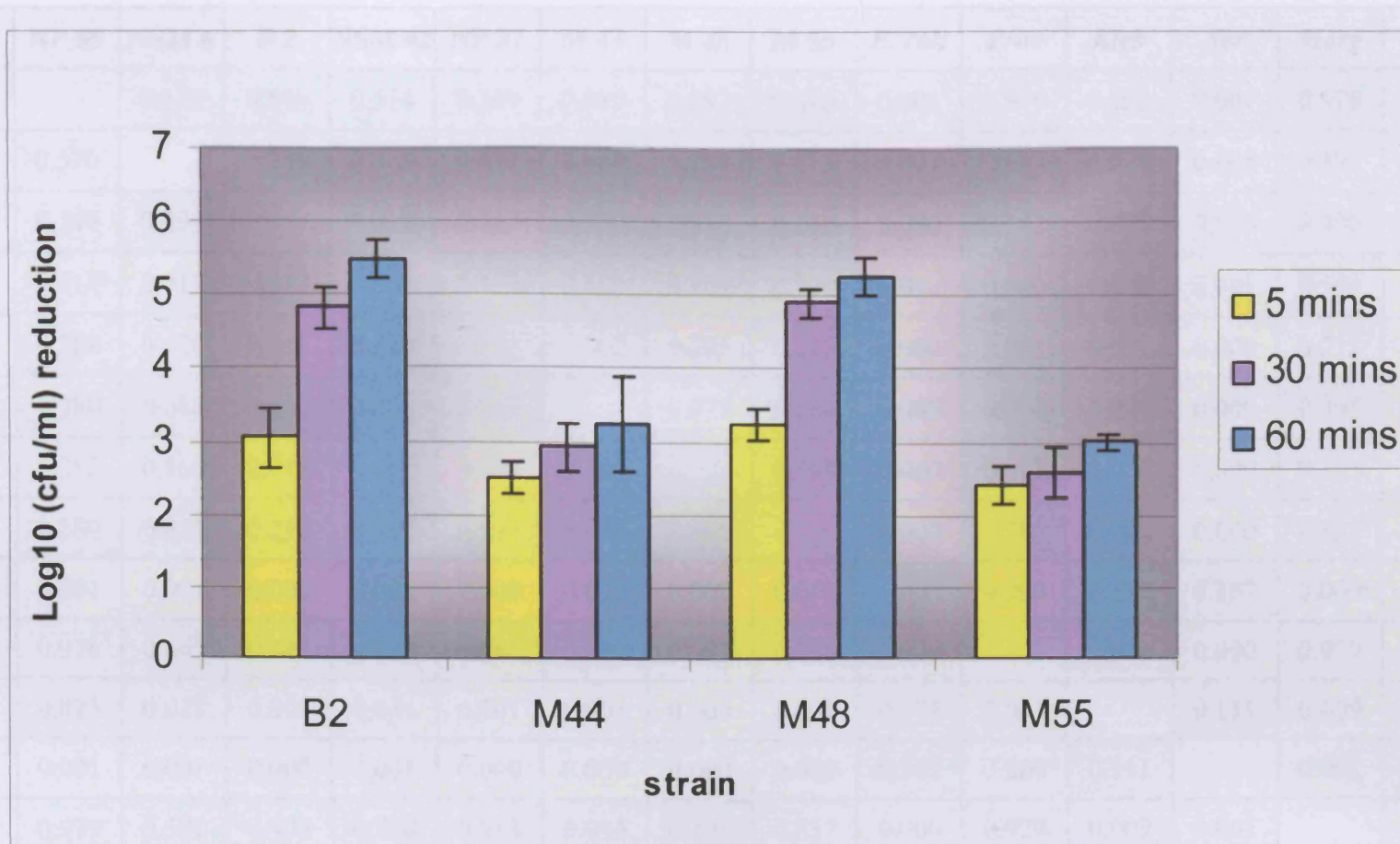


Figure 58: The bactericidal activity of triclosan against *Pr. mirabilis* B2 and three of its mutant strains after different contact times with 100 µg/ml triclosan. The mean values are from experiments performed in triplicate.

Table 8. The T-values from statistical analysis individually comparing each strain tested with 100 µg/ml triclosan at the time examining the bactericidal effects of triclosan after a 5 minute contact time. The table includes the *Pr. mirabilis* strains B2, M44, M48, M55, NSM 0 and NSM 42. Three mutant strains of *Pr. mirabilis* (M4, M55, and M57). The *Pr. aeruginosa*, *E. coli*, *K. pneumoniae*, *M. luteus*, *Pr. stuartii* and *St. pneumoniae* strains tested have also been included.

	NP 55	NSM 6	B 2	NSM 42	NP 37	M 44	M 48	M 55	<i>E. coli</i>	<i>Prov</i>	<i>Kleb</i>	<i>Ser</i>	<i>Morg</i>	<i>Ps</i>
NP 55		0.570	0.546	0.514	0.389	0.980	0.382	0.880	0.001	0.976	0.023	0.001	0.979	0.023
NSM 6	0.570		0.299	0.719	0.490	0.688	0.160	0.813	0.001	0.643	0.028	0.001	0.591	0.002
B 2	0.546	0.299		0.317	0.048	0.300	0.716	0.250	0.000	0.342	0.003	0.000	0.400	0.000
NSM 42	0.514	0.719	0.317		0.579	0.680	0.189	0.790	0.002	0.640	0.044	0.001	0.508	0.004
NP 37	0.389	0.490	0.048	0.579		0.062	0.005	0.126	0.000	0.061	0.001	0.000	0.213	0.000
M 44	0.980	0.688	0.300	0.680	0.062		0.071	0.784	0.000	0.900	0.001	0.000	0.995	0.000
M 48	0.382	0.160	0.716	0.189	0.005	0.071		0.065	0.000	0.092	0.000	0.000	0.195	0.000
M 55	0.880	0.813	0.250	0.790	0.126	0.784	0.065		0.000	0.705	0.002	0.000	0.857	0.000
<i>E. coli</i>	0.001	0.001	0.000	0.002	0.000	0.000	0.000	0.000		0.000	0.174	0.267	0.000	0.005
<i>Prov</i>	0.976	0.643	0.342	0.640	0.061	0.900	0.092	0.705	0.000		0.001	0.000	0.929	0.000
<i>Kleb</i>	0.023	0.028	0.003	0.044	0.001	0.001	0.000	0.002	0.174	0.001		0.111	0.009	0.018
<i>Ser</i>	0.001	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.267	0.000	0.111		0.001	0.006
<i>Morg</i>	0.979	0.591	0.400	0.508	0.213	0.995	0.195	0.857	0.000	0.929	0.009	0.001		0.000
<i>Ps</i>	0.023	0.002	0.000	0.004	0.000	0.000	0.000	0.000	0.005	0.000	0.018	0.006	0.000	

Table 8: The P-values from statistical analysis individually comparing each strain tested with 100 µg/ml triclosan in the tests examining the bactericidal effects of triclosan after a 5 minute contact time. The table includes five *Pr. mirabilis* strains B2, NP37, NP55, NSM 6 and NSM 42. Three mutant strains of *Pr. mirabilis* B2: M44, M48 and M55. The *Ps. aeruginosa*, *E. coli*, *K. pneumoniae*, *M. morgani*, *Pv. stuartii* and *Ser. marcescens* strains tested have also been included.

	NP 55	NSM 6	B 2	NSM 42	NP 37	M 44	M 48	M 55	<i>E. coli</i>	<i>Prov</i>	<i>Kleb</i>	<i>Ser</i>	<i>Morg</i>	<i>Ps</i>
NP 55		0.018	0.389	0.057	0.007	0.011	0.427	0.007	0.000	0.001	0.000	0.000	0.078	0.000
NSM 6	0.018		0.025	0.057	0.809	0.807	0.018	0.797	0.001	0.513	0.002	0.009	0.058	0.000
B 2	0.389	0.025		0.101	0.010	0.012	0.829	0.007	0.000	0.001	0.000	0.000	0.156	0.000
NSM 42	0.057	0.057	0.101		0.008	0.022	0.033	0.011	0.000	0.001	0.000	0.000	0.815	0.000
NP 37	0.007	0.809	0.010	0.008		0.975	0.003	0.483	0.001	0.147	0.001	0.000	0.011	0.000
M 44	0.011	0.807	0.012	0.022	0.975		0.007	0.521	0.000	0.513	0.000	0.001	0.027	0.000
M 48	0.427	0.018	0.829	0.033	0.003	0.007		0.004	0.000	0.000	0.000	0.000	0.074	0.000
M 55	0.007	0.797	0.007	0.011	0.483	0.521	0.004		0.001	0.043	0.001	0.003	0.014	0.000
<i>E. coli</i>	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.001		0.000	0.089	0.037	0.000	0.001
<i>Prov</i>	0.001	0.513	0.001	0.001	0.147	0.513	0.000	0.043	0.000		0.000	0.000	0.005	0.000
<i>Kleb</i>	0.000	0.002	0.000	0.000	0.001	0.000	0.000	0.001	0.089	0.000		0.012	0.000	0.000
<i>Ser</i>	0.000	0.009	0.000	0.000	0.000	0.001	0.000	0.003	0.037	0.000	0.012		0.000	0.179
<i>Morg</i>	0.078	0.058	0.156	0.815	0.011	0.027	0.074	0.014	0.000	0.005	0.000	0.000		0.000
<i>Ps</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.179	0.000	

Table 9: The P-values from statistical analysis individually comparing each strain tested with 100 µg/ml triclosan in the tests examining the bactericidal effects of triclosan after a 30 minute contact time. The table includes five *Pr. mirabilis* strains B2, NP37, NP55, NSM 6 and NSM 42. Three mutant strains of *Pr. mirabilis* B2: M44, M48 and M55. The *Ps. aeruginosa*, *E. coli*, *K. pneumoniae*, *M. morgani*, *Pv. stuartii* and *Ser. marcescens* strains tested have also been included.

	NP 55	NSM 6	B 2	NSM 42	NP 37	M 44	M 48	M 55	<i>E. coli</i>	<i>Prov</i>	<i>Kleb</i>	<i>Ser</i>	<i>Morg</i>	<i>Ps</i>
NP 55		0.032	0.330	0.008	0.001	0.018	0.143	0.000	0.000	0.000	0.000	0.000	0.052	0.000
NSM 6	0.032		0.053	0.034	0.911	0.854	0.068	0.658	0.008	0.513	0.020	0.003	0.092	0.004
B 2	0.330	0.053		0.164	0.003	0.033	0.600	0.001	0.000	0.002	0.000	0.000	0.273	0.000
NSM 42	0.008	0.034	0.164		0.002	0.032	0.402	0.000	0.000	0.000	0.000	0.000	1.000	0.000
NP 37	0.001	0.911	0.003	0.002		0.672	0.003	0.823	0.002	0.132	0.002	0.001	0.005	0.000
M 44	0.018	0.854	0.033	0.032	0.672		0.045	0.821	0.018	0.513	0.003	0.001	0.064	0.001
M 48	0.143	0.068	0.600	0.402	0.003	0.045		0.001	0.000	0.003	0.000	0.000	0.526	0.000
M 55	0.000	0.658	0.001	0.000	0.823	0.821	0.001		0.000	0.014	0.000	0.000	0.002	0.000
<i>E. coli</i>	0.000	0.008	0.000	0.000	0.002	0.018	0.000	0.000		0.000	0.299	0.023	0.000	0.005
<i>Prov</i>	0.000	0.513	0.002	0.000	0.132	0.513	0.003	0.014	0.000		0.000	0.000	0.004	0.000
<i>Kleb</i>	0.000	0.020	0.000	0.000	0.002	0.003	0.000	0.000	0.229	0.000		0.002	0.000	0.001
<i>Ser</i>	0.000	0.003	0.000	0.000	0.001	0.001	0.000	0.000	0.023	0.000	0.002		0.000	0.032
<i>Morg</i>	0.052	0.092	0.273	1.000	0.005	0.064	0.526	0.002	0.000	0.004	0.000	0.000		0.000
<i>Ps</i>	0.000	0.004	0.000	0.000	0.000	0.001	0.000	0.000	0.005	0.000	0.001	0.032	0.000	

Table 10: The P-values from statistical analysis individually comparing each strain tested with 100 µg/ml triclosan in the tests examining the bactericidal effects of triclosan after a 60 minute contact time. The table includes five *Pr. mirabilis* strains B2, NP37, NP55, NSM 6 and NSM 42. Three mutant strains of *Pr. mirabilis* B2: M44, M48 and M55. The *Ps. aeruginosa*, *E. coli*, *K. pneumoniae*, *M. morgani*, *Pv. stuartii* and *Ser. marcescens* strains tested have also been included.

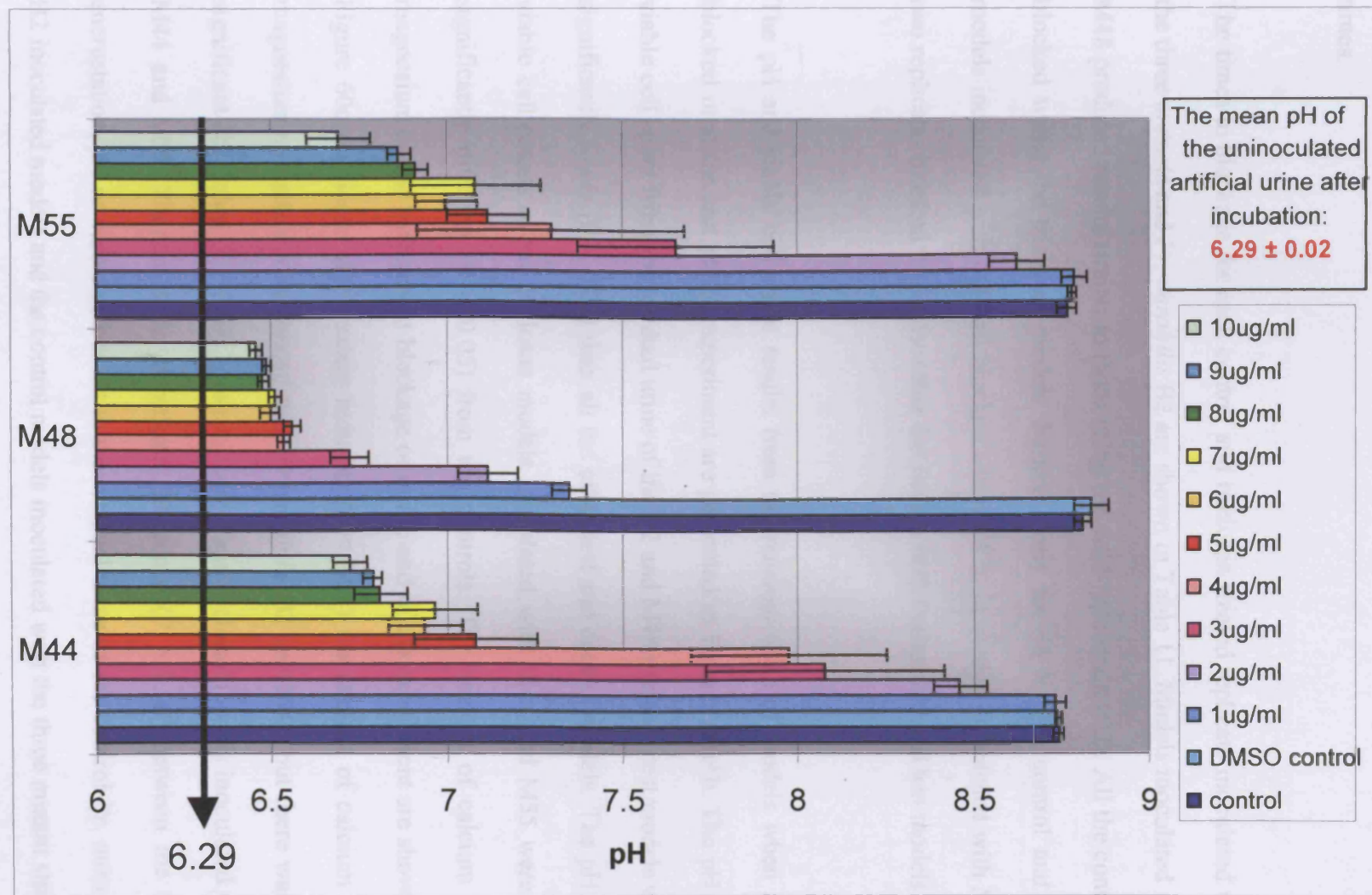


Figure 59: The pH values of artificial urine cultures containing different triclosan concentrations and inoculated with *Pr. mirabilis* mutant strains. Artificial urine culture series were set up containing different concentrations of triclosan, and inoculated with the mutant strains. These results show the pH values of the cultures after the 6 h incubation period. The results are a mean of three experiments. The black arrow indicates the mean pH of the uninoculated artificial urine controls after incubation

on the catheters when they blocked or after 48 h. The experiment was replicated three times.

The times to blockage for each control and triclosan treated replicate inoculated with the three mutants and *Pr. mirabilis* B2 are shown in Table 11. Models inoculated with M48 produced results similar to those using the wild-type strain (B2). All the controls blocked whilst the triclosan models drained freely for 48 h. The control and test models inoculated with M55 all blocked within 48 h. In models inoculated with M44 two replicates blocked whilst the other did not for both the control and test models.

The pH and viable cell count results from the residual urine of models when they blocked or at the end of the experiment are presented in Figures 60a+b. The pH and viable cell count from the residual urine of the B2 and M48 triclosan test models were significantly lower ($P < 0.05$) than all the other test and control models. The pH and viable cell counts of the triclosan models inoculated with M44 and M55 were not significantly different ($P > 0.05$) from the controls. The amount of calcium and magnesium on the catheters at blockage or at the end of the experiment are shown in Figure 60c. Triclosan significantly reduced ($P < 0.05$) the amount of calcium and magnesium on catheters inoculated with *Pr.mirabilis* B2 and M48, but there was no significant difference ($P > 0.05$) between control and triclosan models inoculated with M44 and M55. There was no significant difference ($P > 0.05$) between the total encrustation, pH or viable cell count in the residual urine of the control *Pr. mirabilis* B2 inoculated models and the control models inoculated with the three mutant strains. The pH, viable cell count and total encrustation was however significantly higher ($P < 0.05$) in the triclosan models inoculated with mutants M44 and M55 than in triclosan

Replicate no.	Strain	Control / test model	Time to blockage (h)
Replicate 1	B2	control	25.5
Replicate 2	B2	control	35
Replicate 3	B2	control	35
Replicate 1	B2	10mg/ml triclosan in 5% PEG	Did not block in 48 h
Replicate 2	B2	10mg/ml triclosan in 5% PEG	Did not block in 48 h
Replicate 3	B2	10mg/ml triclosan in 5% PEG	Did not block in 48 h
Replicate 1	M44	control	Did not block in 48 h
Replicate 2	M44	control	29
Replicate 3	M44	control	30
Replicate 1	M44	10mg/ml triclosan in 5% PEG	40
Replicate 2	M44	10mg/ml triclosan in 5% PEG	33
Replicate 3	M44	10mg/ml triclosan in 5% PEG	Did not block in 48 h
Replicate 1	M48	control	38
Replicate 2	M48	control	34
Replicate 3	M48	control	28
Replicate 1	M48	10mg/ml triclosan in 5% PEG	Did not block in 48 h
Replicate 2	M48	10mg/ml triclosan in 5% PEG	Did not block in 48 h
Replicate 3	M48	10mg/ml triclosan in 5% PEG	Did not block in 48 h
Replicate 1	M55	control	31
Replicate 2	M55	control	42
Replicate 3	M55	control	36
Replicate 1	M55	10mg/ml triclosan in 5% PEG	46
Replicate 2	M55	10mg/ml triclosan in 5% PEG	41
Replicate 3	M55	10mg/ml triclosan in 5% PEG	44

Table 11: Time to catheter blockage data from the control and triclosan test bladder models inoculated with mutants M44, M48 and M55 and their wild-type strain *Pr. mirabilis* B2. The catheters of test models were inflated with 10 mg/ml triclosan in 5% PEG, and those of the control models with water. The models were run for a maximum of 48 h and the experiment was performed in triplicate.

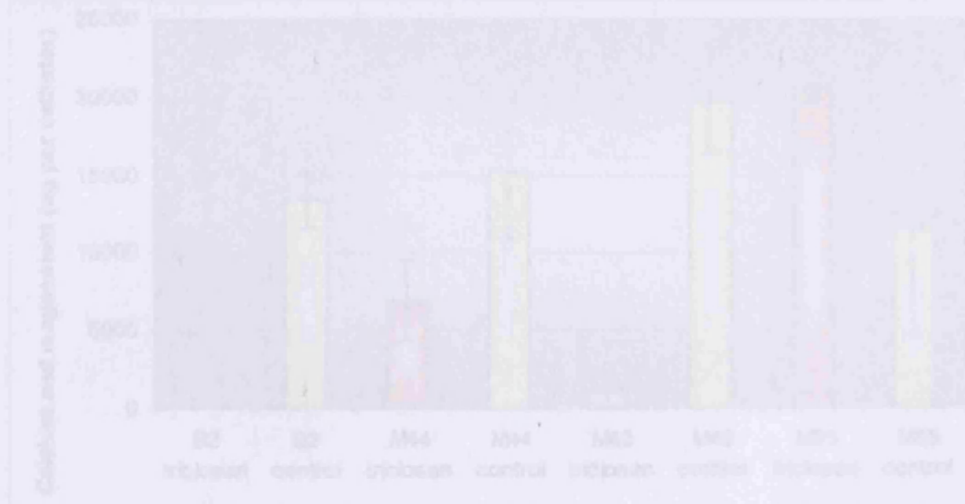


Figure 10: Mean amount of culture and negative results (µg per catheter)

Figure 10: pH, viable cell count and resident and negative results from models inoculated with *Pr. mirabilis* B2 and three of its mutant strains. The bellows of control catheters were inflated with water, the bellows of the test catheters were inflated with 10 mg/ml triclosan in 5% PEG. The models were run for a maximum of 48 h. The results are a mean from three replicate experiments.

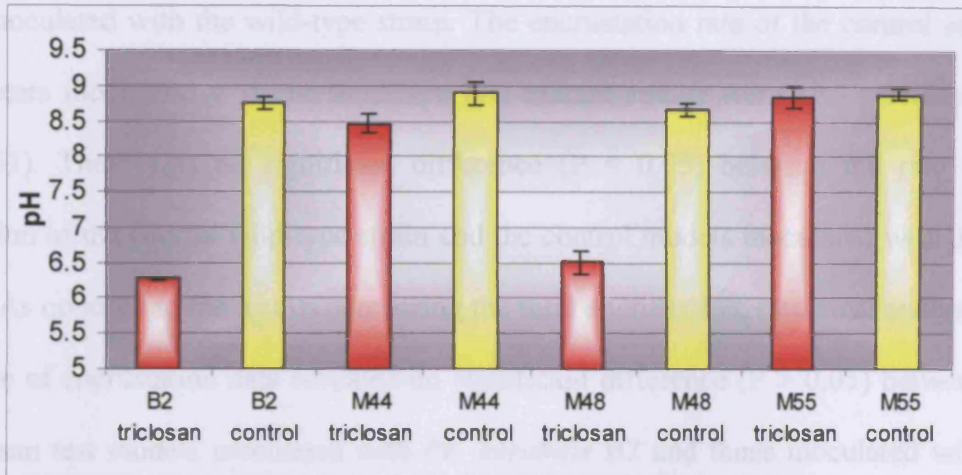


Figure 60a: The mean pH of the residual urine.

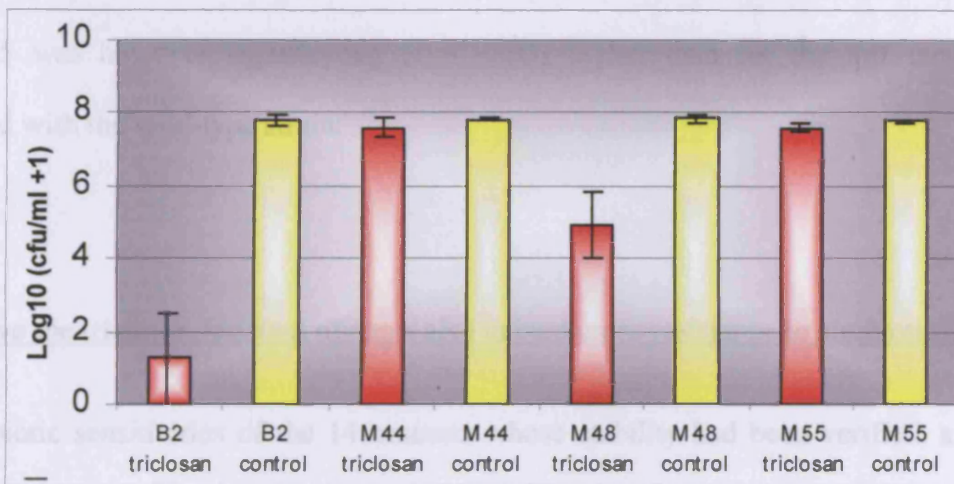


Figure 60b: Mean log₁₀ (cfu/ml + 1) bacteria in the residual urine.

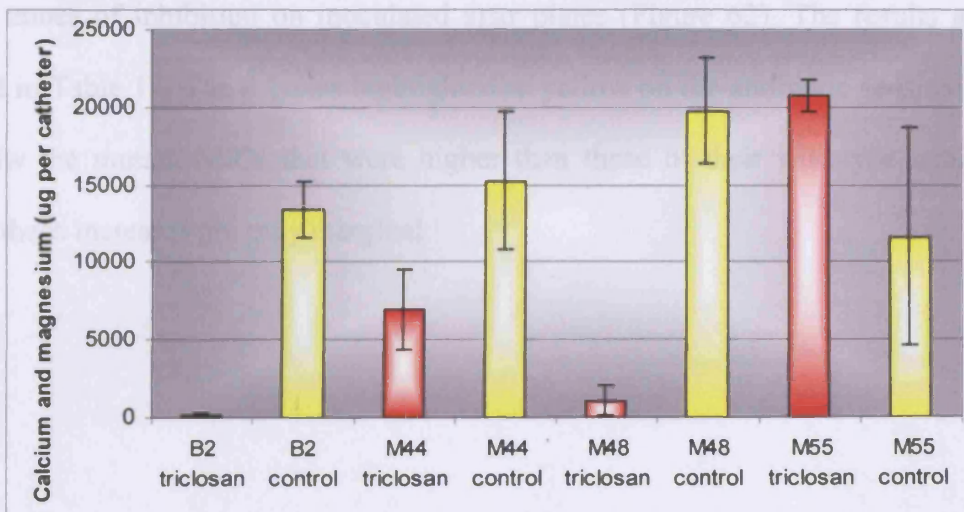


Figure 60c: Mean amount of calcium and magnesium (μg per catheter).

Figure 60: pH, viable cell count and calcium and magnesium results from models inoculated with *Pr. mirabilis* B2 and three of its mutant strains. The balloons of control catheters were inflated with water, the balloons of the test catheters were inflated with 10 mg/ml triclosan in 5% PEG. The models were run for a maximum of 48 h. The results are a mean from three replicate experiments.

models inoculated with the wild-type strain. The encrustation rate of the control and test catheters inoculated with the wild-type and mutant strains were also calculated (Figure 61). There was no significant difference ($P < 0.05$) between the rate of encrustation of the control wild-type strain and the control models inoculated with the mutants. As opposed to the results comparing the total encrustation, statistical analysis of the rate of encrustation data revealed no significant difference ($P > 0.05$) between the triclosan test models inoculated with *Pr. mirabilis* B2 and those inoculated with mutants M44 and M48. The rate of encrustation for the triclosan models inoculated with M55 was however significantly ($P < 0.05$) higher than for the test model inoculated with the wild-type strain.

3.6.4. Have the triclosan resistant mutants also gained cross-resistance to antibiotics?

The antibiotic sensitivities of the 14 mutants whose stability had been verified, and their 5 wild-type strains were tested using antibiotic E-test strips. The E-tests produce elliptical zones of inhibition on inoculated agar plates (Figure 62). The results are presented in Table 12. The 8 boxes highlighted in yellow on the antibiotic sensitivity table show the mutant MICs that were higher than those of their wild-type strain, however these increases are only marginal.

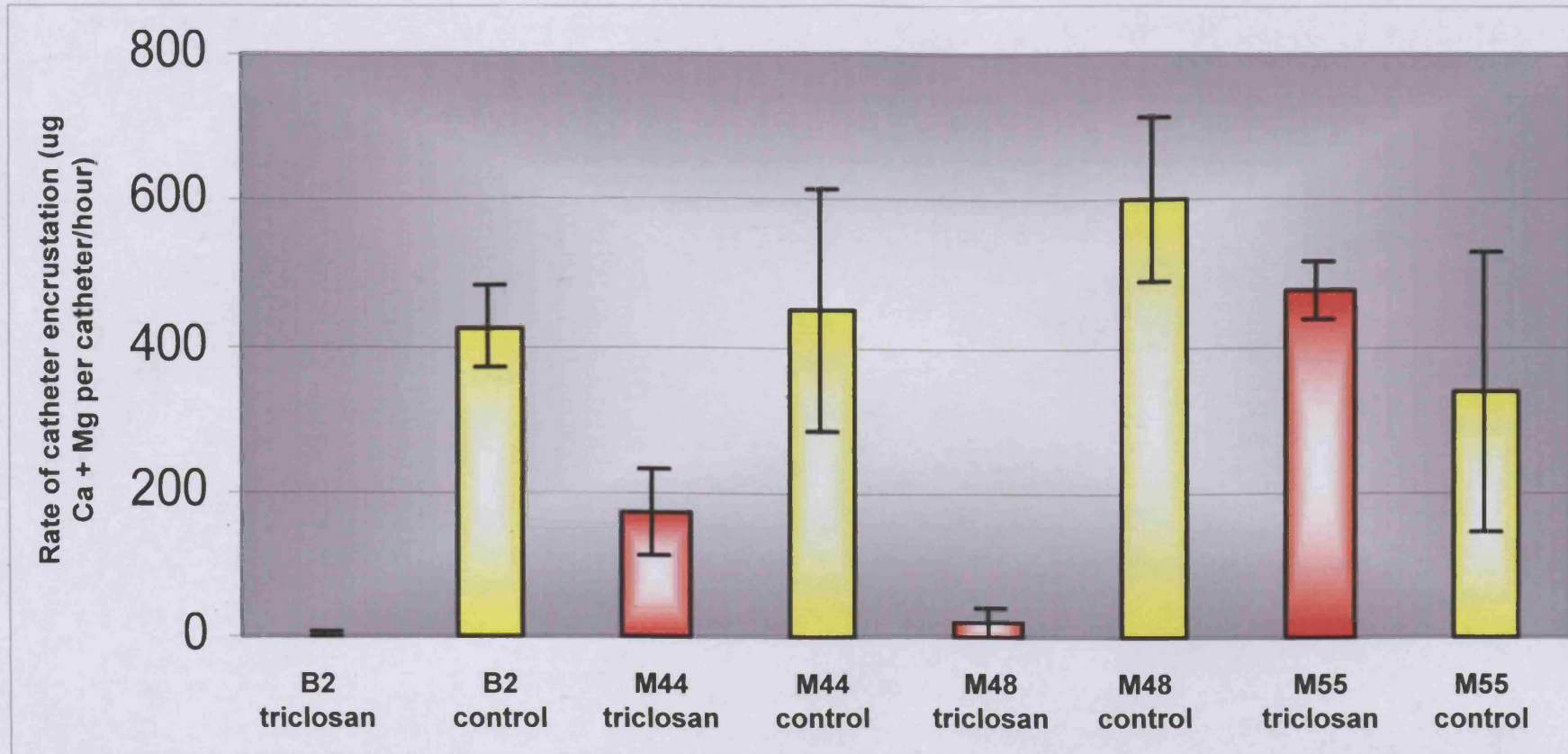


Figure 61: The rate of catheter encrustation (μg calcium and magnesium per catheter per hour) in control and test models inoculated with three mutant strains and the wild type strain *Pr. mirabilis* B2. Control models had catheter balloons inflated with water, test models had catheters inflated with 10 mg/ml triclosan in 5% PEG. The results are a mean from three replicate experiments.

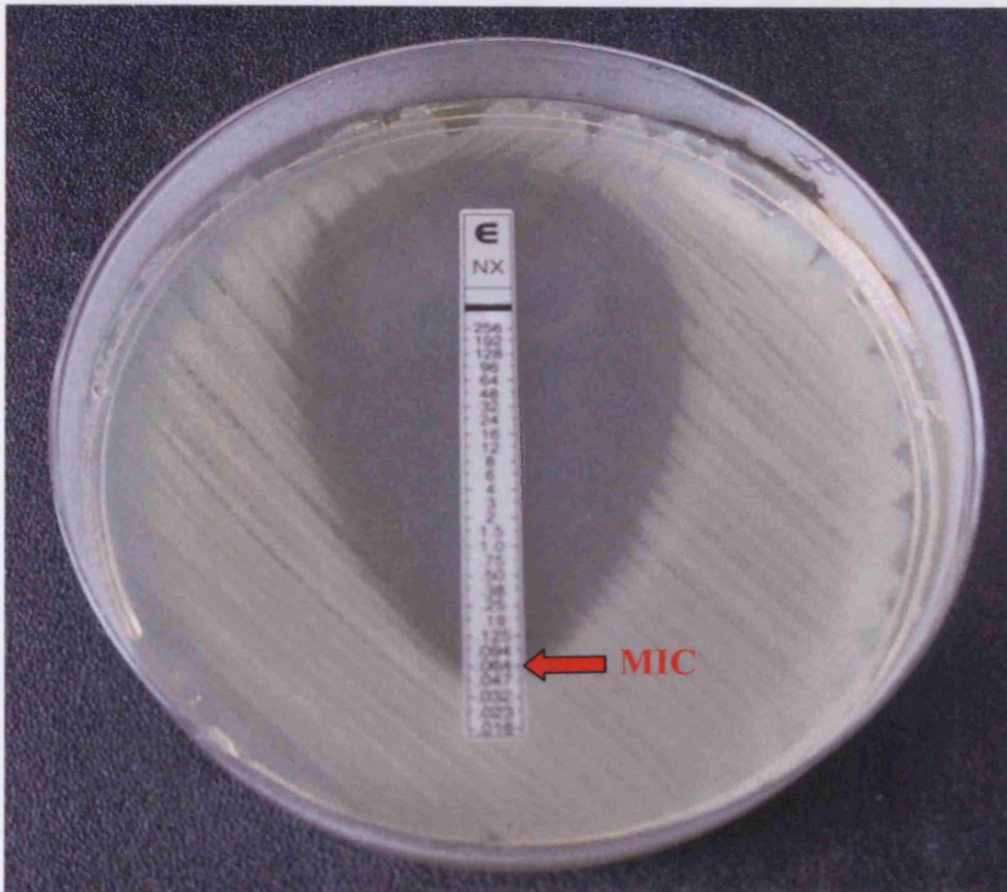


Figure 62: A picture illustrating the typical elliptical zone of inhibition created by the E-test strips. The MIC value was determined by observing where the edge of the inhibition ellipse intersected the E-test strip.

ANTIBIOTICS								
Test strain	trimethoprim	ampicillin	ciprofloxacin	nitrofurantoin	norfloxacin	cefalexin	nalidixic acid	gentamicin
<i>E.coli</i> 10418	0.032 - 0.047	0.5 - 0.75	0.003 - 0.004	2 - 3	0.023	2 - 3	0.38 - 0.58	0.094
<i>Pr. mirabilis</i> B2	R	R	0.023 - 0.032	64 - 96	0.047	6	2 - 3	0.38
M44	R	R	0.032	32 - 48	0.047	4 - 6	1.5 - 2	0.38
M48	R	R	0.016 - 0.023	24 - 48	0.047	3 - 4	1.5 - 2	0.25 - 0.38
M55	R	R	0.023 - 0.032	48	0.047	4	1.5	0.38 - 0.5
<i>Pr. mirabilis</i> NP14	R	16	0.064	32 - 48	0.25	3	3	0.125 - 0.25
M19	R	12 - 16	0.047 - 0.064	32 - 48	0.19 - 0.25	4	2 - 3	0.125 - 0.19
M21	R	16	0.047 - 0.064	48	0.19 - 0.25	3 - 4	3	0.125 - 0.19
M23	R	12 - 16	0.047 - 0.064	32 - 64	0.19 - 0.25	3	2 - 4	0.094 - 0.125
<i>Pr. mirabilis</i> NP37	R	R	0.016 - 0.023	24	0.047 - 0.064	4 - 24	4 - 12	0.38 - 4
M26	R	R	0.012	24 - 32	0.032	3	1.5 - 2	0.125 - 0.19
M29	R	R	0.008 - 0.012	16 - 24	0.032 - 0.047	2 - 4	2 - 3	0.19 - 1
M31	R	R	0.008 - 0.012	24	0.023 - 0.032	3 - 12	1.5 - 2	0.125 - 0.25
<i>Pr. mirabilis</i> NP43	0.38	0.38 - 0.5	0.012	48	0.047	4	2	0.094 - 0.125
M12	0.19 - 0.25	0.125 - 0.19	0.006 - 0.008	12 - 32	0.023	1.5 - 2	1.5 - 2	0.064 - 0.094
M17	0.25 - 0.38	0.19 - 0.25	0.006 - 0.012	12 - 32	0.023	1 - 2	2	0.064 - 0.094
<i>Pr. mirabilis</i> NP55	R	R	0.016	64 - 96	0.047 - 0.064	4 - 6	3	0.125 - 0.25
M35	R	R	0.016 - 0.023	32 - 96	0.032 - 0.047	3	2 - 3	0.25
M40	R	R	0.012 - 0.023	64	0.032 - 0.047	3 - 6	1.5 - 2	0.125 - 0.19
M42	R	R	0.016	48 - 64	0.032	4	2 - 3	0.25

Table 12: The MIC of eight antibiotics against *Pr. mirabilis* wild-type and mutant strains. The results highlighted in blue are those of the wild-type strains. The results highlighted in yellow are the mutant MICs that were above those of the wild-type strains. All results were determined using antibiotic E-test strips, and experiments were performed in triplicate. All MIC measurements are in µg/ml. R indicates that the bacterial isolates were resistant to the test antibiotics.

4. DISCUSSION

4. Discussion

Indwelling bladder catheters are the most commonly deployed prosthetic medical devices (Darouiche, 2001). The incidence of infection, morbidity and mortality associated with their use is unacceptable (Kunin, 1988). Complications resulting from CA-UTI are not only distressing to the patients but are also a considerable drain on health service resources. Shih *et al.* (2003) carried out a prospective study examining the costs of labour, supplies and services involved in caring for long-term care residents with and without urinary incontinence. The cost of caring for patients with urinary incontinence was shown to be an additional \$4,957 per patient per annum. Tambyah *et al.* (2002) investigated the additional direct costs of hospitalisation due to CA-UTIs in 1,497 newly catheterised patients. They reported that the extra cost of hospitalisation incurred in the management of CA-UTI in 123 infected patients was \$20,662 in extra diagnostic tests and \$35,872 in extra medication costs. The mean cost for dealing with each CA-UTI was computed to be \$589.

Results from a pilot study investigating the costs associated with long-term catheterisation in community based patients demonstrated a wide variation in direct costs associated with different patients over a three month period (Evans *et al.*, 2000). In the seven patients studied, the costs varied from between £118.80 to £2588.26, the most significant part of this being staffing costs. The cost burden associated with dealing with these problems is likely to increase in future years with demographic changes to the human population. Whereas people over the age of 60 currently constitute a fifth of the British population, by 2030 this will rise to a third (Greengross

et al., 1997). A novel strategy for controlling the complications of CA-UTI would thus be beneficial to patients and the health service.

4.1. Novel methods for controlling biofilm formation on urinary catheters

Conceptually, the simplest approach to prevent biofilm formation would be to impregnate medical devices with a broad-spectrum antimicrobial agent that eluted into the surrounding environment. In this way planktonic bacteria in the vicinity of the device could be attacked before they colonize surfaces and adopt the antibacterial resistant biofilm phenotype (Danese, 2002). Although a simple idea, there are a number of technical obstacles that have limited the effectiveness of antimicrobial impregnated or coated IUCs. Firstly, impregnating or coating a catheter should not alter the physical properties of the device or change its compatibility with the host tissue. Secondly, the catheters must contain sufficient amounts of the chosen antimicrobial to allow sustained release of active concentrations of the agent for the lifetime of the device (Danese, 2002). Difficulties in delivering bacteriostatic or bactericidal concentrations of antimicrobial agents from catheters for long periods of time have limited the usefulness of antimicrobial catheters in patients undergoing long-term bladder management.

Cho *et al.* (2001) and Cho *et al.* (2003) developed catheters that were coated with the antibiotic gentamicin sulphate. The catheters were each loaded with 1.0 mg of the antibiotic. *In vitro* tests showed that on day one 200 µg of the antibiotic was released from the catheter and that by day seven the amount being eluted had reduced to 50 µg/day. Further *in vivo* studies with catheterised rabbits established that the gentamicin catheters inhibited CA-UTI for at least five days, suggesting they may be

useful in controlling infection in patients undergoing short-term catheterisation, but giving no indication regarding their usefulness for patients undergoing long-term catheterisation. Similarly although there is some evidence that silver-coated catheters are effective in preventing UTIs in short term catheterised patients, there is no evidence suggesting their usefulness for patients undergoing long-term bladder management (Saint *et al.*, 1998).

Darouiche *et al.* (1997) examined the antimicrobial durability of IUCs impregnated with rifampin and minocycline by testing the antimicrobial activity of catheters that had been inserted into spinal injured patients. At different time intervals the catheters were removed and sections of the catheters were placed onto bacterial lawns of different urinary tract pathogens. The catheters produced detectable zones of inhibition against 10 uropathogens for up to 14 days, however after 21 days insertion, their activity was negligible. Johnson *et al.* (1999) used an *in vitro* agar diffusion assay to determine the durability of the activity of nitrofurazone and silver-coated urinary catheters. The activity was assessed by daily transference of catheter sections onto fresh agar plates. The nitrofurazone catheters were active against the test species (*E. coli*, *K. pneumoniae*, *Citrobacter. freundii*, *S. aureus*, *Ent. faecium* and coagulase-negative staphylococci) for a maximum of just five days. The silver catheters showed no activity after day one.

Richards *et al.* (2003) showed that it was possible to incorporate chlorhexidine digluconate (CHG) into a silicone elastomer. This material was then compression moulded to form a urinary catheter. *In vitro* tests with these catheters revealed that the concentration of CHG released on day one was equivalent to $2670 \mu\text{g}/\text{cm}^2$, but by day

three this had dropped to less than $20 \mu\text{g}/\text{cm}^2/\text{day}$. From day 12 CHG was not detectable in the buffer used to suspend the catheter material. Schierholz *et al.* (1997) incorporated antistaphylococcal agents (flucloxacillin, ciprofloxacin, gentamicin and fosfomycin) into polyurethanes and monitored the drug release rates. In all polymers tested, more than half the total antibiotic concentrations were released after five days.

DiTizio *et al.* (1998) used liposomal technology to try and control the rate of release of antibacterial agents from catheters. They developed a liposomal hydrogel system that consisted of a poly (ethylene glycol)-gelatin hydrogel in which liposomes containing ciprofloxacin were sequestered. This was cross-linked to the surface of silicone catheters and *in vitro* assays were performed to monitor the release of ciprofloxacin. Whereas catheters coated in a simple hydrogel containing ciprofloxacin lost all their antibiotics within 4 h, the drug was released steadily from the liposomal coating for up to seven days. The longevity of the protective effect was severely limited however, by the amount of ciprofloxacin that could be loaded into the coating. It was only possible to incorporate $185 \mu\text{g}/\text{cm}$ of ciprofloxacin into the catheter coating and achieve a release rate of $1.9 \mu\text{g}/\text{cm}/\text{hr}$.

Another novel approach for controlling the rate of release of active concentrations of antibacterial agents is to sandwich a layer of an active antimicrobial agent within a material that controls its rate of diffusion into surrounding fluids. The aim of the research by Tcholakian and Raad (2001) was to develop a central venous catheter that could resist infection for longer time periods. They tested catheters that had a layer of minocycline and rifampin sandwiched within a silicone sheath which formed the external and luminal catheter surfaces. These catheters demonstrated significant

antimicrobial activity after more than 325 days incubation in human serum. As a control for the above experiment, polyurethane catheters coated with chlorhexidine were tested. Maki *et al.* (1997) had reported that these coated polyurethane catheters could reduce infection rates by at least fourfold compared to uncoated catheters. However, as opposed to the catheters with a sandwiched antimicrobial layer, these catheters lost all their antimicrobial activity within 14 days.

In view of the problems of exploiting conventional antibacterial agents in the protection of long-term prosthetic devices, some workers have turned to interesting alternative approaches. Singh *et al.* (2002) for example showed that lactoferrin blocks *Ps. aeruginosa* biofilm development. They discovered that lactoferrin which is an iron chelator, stimulated bacterial twitching motility and that this prevented bacteria from attaching to and colonizing surfaces. The incorporation of iron chelators into biomaterials could therefore inhibit bacterial attachment and subsequent biofilm formation.

Several studies have suggested that using benign bacteria to prevent symptomatic infections by interfering with the growth of pathogenic bacteria may reduce CA-UTI. Darouiche *et al.* (2001) used the non-pathogenic *E. coli* 83972 strain to test the safety and efficacy of bacterial interference in preventing symptomatic CA-UTI in spinal injured patients. This non-pathogenic strain was isolated from a young girl who had asymptomatic bacteriuria for three years without altering renal function (Andersson *et al.*, 1991). The *E. coli* suspensions were instilled into patients' bladders, and the rate of symptomatic UTI in successfully colonized patients was compared to their rates of symptomatic infection prior to colonization, and to the rate of symptomatic infection

in patients that were unsuccessfully colonized by the non-pathogenic *E. coli* strain. The results revealed that the rate of symptomatic UTI was 33-fold lower in patients that were colonized by the non-pathogenic strain versus those that were not successfully colonized. This suggested that bacterial interference could be a safe and effective way of controlling CA-UTI. The same non-pathogenic *E. coli* strain was also tested *in vitro*. These studies revealed that a coating of this strain of *E. coli* on IUCs significantly impeded bacterial colonization by *Ent. faecalis*, *Pv. stuartii*, uropathogenic *E. coli* and *Candida albicans* (Trautner *et al.*, 2002; Trautner *et al.*, 2003). It was a pity that the ability of the non-pathogenic *E. coli* to inhibit catheter colonization by *Pr. mirabilis* was not investigated. Trautner and Darouiche (2004) commented that since it has become apparent that bacteria can eventually overcome most methods that we come up with for treating them, it may be time for us to stop trying to outsmart them, and to try and get them to outsmart each other.

Davies *et al.* (1998) demonstrated that a mutation blocking the generation of quorum sensing signalling molecules in *Ps. aeruginosa* prevented the formation of a normally differentiated biofilm, resulting in the production of thin biofilms with densely packed bacterial cells. More recently furanones have been shown to control *E. coli* biofilm formation by inhibiting quorum sensing (Ren *et al.*, 2001). McLean (2004) described a simple screening method that could be used to search for potential quorum sensing inhibitory activity by plants and bacteria. The samples were covered with a soft agar overlay containing indicator bacteria. These bacteria regulate pigment production by N-hexanoyl-HSL quorum sensor and are inhibited by AHL inhibitors, therefore a lack of pigment production by the indicator culture indicates quorum-sensing inhibition.

Stewart (2003) suggested that an alternative approach of controlling biofilm formation would be to unglue the whole biofilm structure, either by dissolving the matrix polymer or by blocking its synthesis. Previously, Yasuda *et al.* (1993) had reported that treatment of *Ps. aeruginosa* biofilms with clarithromycin, a macrolide antibiotic with no antibacterial activity against *Ps. aeruginosa*, resulted in eradication of the membranous structures that covered the colonies in the biofilm. Using an *in vivo* rat infection model they showed that the therapeutic effect of ofloxacin against biofilms of *Ps. aeruginosa* was enhanced with oral co-administration of clarithromycin. They suggested that the clarithromycin induced loss of the glycocalyx was responsible for the increased therapeutic efficacies of the ofloxacin against biofilms of *Ps. aeruginosa*. Yasuda *et al.* (1994) showed that low concentrations of clarithromycin also eradicated the glycocalyx matrix of *S. epidermidis* biofilms and resulted in the increased penetration of antibiotics through biofilms. Goto *et al.* (1999) investigated the activity of several classes of antibiotics alone or combined with a macrolide antibiotic on *Ps. aeruginosa* biofilm development in artificial urine on Teflon catheters. *In vitro* studies revealed that macrolide antibiotics only potentiated some classes of antibiotics. A clinical trial revealed that patients with IUCs treated with oral ciprofloxacin combined with clarithromycin achieved a higher clinical efficacy than ciprofloxacin alone, although the complete bacterial elimination rate did not differ between the two groups (Tsukamoto *et al.*, 1999). The authors noted that whilst this trial suggested that clarithromycin may increase the anti-biofilm efficacy of ciprofloxacin in the clinical setting, further work should be carried out investigating the optimal duration and dosage of clarithromycin that should be used.

There is certainly a need to develop novel strategies for inhibiting biofilm formation and to test them against crystalline *Pr. mirabilis* biofilms on catheters. It is important to bear in mind however that long-term devices, such as Foley catheters, need protection against encrustation for weeks and months rather than just for days. To date it has not been possible to load the devices with sufficient active agents or to control the release of the agent so that effective concentrations are sustained in the surrounding body fluids for the lifetime of the device. Exploitation of the catheter balloon as the reservoir for an active agent could solve both these problems. The balloon can be inflated with 10 ml of solutions containing high concentrations of an agent. The wall of the balloon provides a diffusion barrier which could control the release of active agents into the bladder urine. It was decided to investigate this approach using the agent triclosan that had been previously reported to be very active on *Pr. mirabilis* strains isolated from animal sources (Firehammer, 1987).

4.2. The sensitivity of *Pr. mirabilis* to triclosan

The results presented in Table 2 confirmed that strains of *Pr. mirabilis* isolated from CA-UTI are extremely sensitive to triclosan. Swarming was inhibited by 0.1 - 0.2 µg/ml triclosan and the MICs ranged from 0.1 – 0.3 µg/ml. These results correlate well with those reported by Firehammer (1987), Bhargava and Leonard (1996) and Stickler (2002). MICs are typically used as a measurement of antibiotic sensitivity. It is important to bear in mind that biocides are usually used in concentrations well in excess of their MIC. Therefore, while MIC values provide useful starting points in studies on biocides, it is also necessary to examine their bactericidal effects (Russell, 2003).

There is ample evidence that the problem of catheter encrustation is brought about when urease activity of *Pr. mirabilis* raises the pH of the patients urine above the pH at which calcium and magnesium normally remain in solution (Kunin, 1989; Hedelin *et al.*, 1991; Choong *et al.* 2001). Strategies to control catheter encrustation must be able to control the rise in urinary pH in the catheterised urinary tract infected with *Pr. mirabilis*. The MICs in artificial urine were carried out to establish whether triclosan would be as effective at inhibiting *Pr. mirabilis* growth in artificial urine, and to determine if this in turn would prevent an increase in the pH of the cultures. For six strains of *Pr. mirabilis*, the MICs of triclosan in artificial urine were similar to the concentrations inhibiting their growth in agar. It was also clear that at 0.2 µg/ml, triclosan was able to maintain the pH of the *Pr. mirabilis* urine cultures below 7.0 (Figure 10). These experiments were performed in glass vessels and it is interesting to note that when they were performed in plastic universal containers the resulting MICs in artificial urine were 5 - 10 µg/ml.

Several studies have established that triclosan is bacteristatic at low concentrations, but that higher concentrations are required for a bactericidal effect against *S. aureus* and *E. coli* (McMurry *et al.*, 1998a; Suller and Russell, 1999; Suller and Russell, 2000). The 'suspension tests' monitoring the lethal effects of triclosan against *Pr. mirabilis* reported in Figure 15 were all carried out exposing 1×10^8 cfu/ml bacterial cells to 100 µg/ml triclosan. No published data could be found regarding the lethal effects of triclosan against *Pr. mirabilis*. None of the five strains tested gave a 5-log₁₀ (cfu/ml) reduction with 100 µg/ml triclosan in 5 min, therefore, contact times of 30- and 60 min were also included. These results demonstrate that although concentrations of 0.2 µg/ml inhibit the growth of *Pr. mirabilis*, concentrations around 100 µg/ml are

required to produce bactericidal effects, and that the lethal activity of triclosan against *Pr. mirabilis* is relatively slow acting. Suller and Russell (2000) for example demonstrated that 10 µg/ml triclosan produced a 6-log₁₀ reduction in viability of a standard strain of *S. aureus* after just a one-minute contact time.

In the experiments to establish what effect triclosan had on the growth of *Pr. mirabilis* cultures in pooled human urine, adding very low concentrations of triclosan (0.1 µg/ml) at stationary phase inhibited log phase growth for 2 h (Figure 11). Adding 1.0 µg/ml triclosan kept the cultures in stationary phase, but as expected from the bactericidal tests, higher triclosan concentration (10 or 100 µg/ml) were required to produce a lethal effect. Within 1 h of adding 100 µg/ml triclosan to the urine cultures, viable *Pr. mirabilis* cells could not be recovered. The higher temperatures used in these experiments (37°C) may explain the enhanced lethal effects in the urine cultures compared to the bactericidal tests. The results presented in Figure 12 show that the higher triclosan concentrations (1, 10 and 100 µg/ml) kept the pH values of the cultures significantly ($P < 0.05$) lower than the control culture.

Adding 100 µg/ml triclosan to mid-log phase growing *Pr. mirabilis* urine cultures resulted in a rapid 4-log reduction in the viable cell count (Figure 13). By $t = 3$ h, no viable cells could be detected in the culture. The results with the other triclosan concentrations were similar to those adding triclosan at stationary phase. The stationary and log phase cultures appear to be equally susceptible to triclosan. Suller and Russell (2000) also observed that strains of *S. aureus* in stationary and exponential growth phase were equally susceptible to triclosan. Similarly Gomez-

Escalada *et al.* (2002) showed that triclosan had a rapid lethal effect against *E. coli* in all stages of growth.

Adding triclosan at mid-log phase had no effect on the terminal pH of the urine cultures (Figure 14). The pH curves for the test and control cultures were identical. These data suggest that in the experiment where triclosan was added at stationary phase, it prevented a rise in pH by preventing bacterial growth and the production of the urease enzyme. The bacterial cultures growing at log phase would have already produced a large amount of urease by the time the triclosan was added. The results show that even after adding 100 µg/ml triclosan there was enough urease in the culture to keep the pH rising at the same rate as the control. Microbial urease enzymes appear to be cytoplasmic proteins (Mobley *et al.*, 1995). The increase in culture pH following bacterial death is likely to be due to the liberation of urease enzymes from lysed bacterial cells.

All samples taken from the control and test human urine cultures were neutralised prior to commencing the viable cell counts, as were the suspensions in the experiments determining the lethal effects of triclosan. Neutralisation is often overlooked when testing the activity of antimicrobial agents. The neutralisation of an antimicrobial agent is essential to stop its activity at a given exposure time, its continued activity due to no or incomplete neutralisation leads to misleading results (McDonnell *et al.*, 1998). The neutraliser used in this study effectively quenched the bactericidal activity of triclosan at concentrations up to 100 µg/ml (Table 3). No viable cells were detectable in the suspensions where *Pr. mirabilis* B2 was added to triclosan and water (final concentration 100 µg/ml), and there was no significant

difference ($P = 0.919$) between the number of viable cells in the control suspension and in the mixture containing $100 \mu\text{g/ml}$ triclosan in the neutraliser. The non-toxicity of the neutraliser (and the 1% DMSO used in the suspension tests) was confirmed for every strain that was tested (Table 4).

4.3. The feasibility of using the catheter retention balloon as a novel way of introducing triclosan into the catheterised urinary tract

Having confirmed the sensitivity of *Pr. mirabilis* catheter isolates to triclosan and shown that concentrations as low as $0.2 \mu\text{g/ml}$ could maintain urine cultures below pH 7, the next phase of the research involved investigating the feasibility of delivering triclosan to the infected bladder through the catheter balloon. If triclosan was to be used in this way, it had to be able to diffuse through the catheter balloon without having any detrimental effect on the balloon's function. Triclosan is only sparingly soluble in water, and DMSO is too harmful to be used in the catheter balloon, therefore, preliminary experiments were carried out using 10 mg/ml triclosan in 5% PEG. PEG was chosen as it is known to be a good solvent for triclosan and has also been shown to modify the diffusion characteristics of silicone (Ciba-Geigy corporation, 1988; Kim *et al.*, 2000). The triclosan in PEG had to be heated and stirred to produce a white colloidal suspension, and needed to be continually stirred to keep it in suspension.

In the experiments to determine the concentration of triclosan passing through the catheter balloon of all-silicone catheters, the concentration of triclosan in each sample was determined by HPLC. Figure 16 shows that low concentrations of triclosan ($0.02 - 0.16 \mu\text{g/ml}$) were detected in each of the artificial urine samples taken over the 48 h

period. The low concentration of triclosan in the first sample taken from both replicates suggests that the triclosan may take a couple of hours to diffuse through the catheter balloon. The difference between the amount of triclosan released by the two different replicates may be attributed to differences in balloon thickness. None of the control urine samples produced a peak at the retention time close to the triclosan peak. This experiment established the effectiveness of this method for extracting and quantifying the concentration of triclosan in artificial urine samples, and confirmed that triclosan could diffuse from the retention balloon of all-silicone catheters. The maximum concentration of triclosan being delivered through the balloon over the 48 h test period was 0.16 $\mu\text{g/ml}$, with an average daily flow rate of 0.5 ml per minute, 115 μg triclosan is released from the catheter daily. Given that the balloon is inflated with 10 ml of a 10 mg/ml triclosan solution, if the same release rate was maintained, it would take over 850 days for all the triclosan to be released from the catheter balloon.

The extraction of triclosan from the artificial urine was time consuming, therefore, only two replicates were performed. Further replicates may have indicated a more definite trend in the way triclosan was released from catheter balloons. Other experiments based on this method that would provide useful information would be those investigating the concentration of triclosan that can pass through the balloons of latex-based catheters. Although setting up models for longer periods of time may provide useful information, running the bladder models for extended periods of time can be difficult due to contamination problems. Future experiments could determine whether using triclosan in different solvents or formulations would increase the concentration of triclosan passing through the balloon into the urine. For example it may be possible to use different pore-formers to increase the concentration of triclosan

being released from the balloon. Kim *et al.* (2000) tested the feasibility of controlling the release of antibiotics from polymers using pore-formers. These are compounds that facilitate the release of drugs by forming channels in the polymer matrix. Using these agents can help release active agents out of polymers regardless of their solubility. Kim *et al.* (2000) showed that it was possible to control the release of antibiotics from a polymer matrix by changing the particle size and loading dose of the pore former.

Preliminary experiments to determine the MIC of triclosan against *Pr. mirabilis* in artificial urine revealed that triclosan could bind to the plastic universal containers. Experiments were therefore carried out to determine if inflating the catheter balloon with a triclosan solution would result in triclosan becoming impregnated in the catheter material. The balloons of the catheters that were impregnated in the models were thoroughly rinsed out 10 times with sterile water. This was to ensure that any zone of inhibition created by the catheter sections resulted from the triclosan being impregnated in the catheter sections and not from any triclosan residues left in the inflation line. Only the top section from directly beneath the catheter balloons produced a zone of inhibition with the hydrogel-coated latex catheters, whilst none of the silicone elastomer-coated latex catheter sections produced a zone of inhibition (Figure 17). With the all-silicone catheters, one of the 0.5 cm sections was cut in half lengthways, so that one half included the catheter inflation line, whilst the other did not. All these catheter sections produced zones of inhibition, indicating that triclosan completely impregnated the full length of the all-silicone catheters.

If a new solution is to be used instead of water it must not compromise the function of the retention balloon. It is also important that the catheter balloon can still be deflated after 8 - 12 weeks. In the experiment to determine how triclosan and 5% PEG might affect the catheter balloons, no change was observed in the balloon morphology of the three catheter types inflated with the three solutions (10 mg/ml triclosan in 5% PEG, 5% PEG and deionized water). All the catheter balloons could still be deflated after the eight-week period. The physical properties of the catheter balloons inflated with the different solutions were tested using the LRK10 Lloyds testing instrument. This allowed a comparison of the load (N) that each section could take, its tear strength (N/mm) and its percentage elongation. Because of the strength of even small samples of balloon material, a standard incision was placed half way down the sample to facilitate tearing. It was impossible to tear the catheter balloons of the latex-based catheters, therefore only results on the effect of triclosan and 5% PEG on the physical properties of all-silicone catheter balloons were obtained. As well as testing the all-silicone catheters that had been inflated with the different solutions for eight weeks, a catheter balloon section taken straight from its packaging was also tested. There was no significant difference ($P > 0.05$) between the tear strength (N/mm), load (N) or percentage extension between the four groups (Figure 18). These results indicated that triclosan and 5% PEG do not have a detrimental effect on the physical properties of the all-silicone catheter balloons.

4.4. The effect of introducing triclosan into the catheterised urinary tract on the development of crystalline bacterial biofilm formation by *Pr. mirabilis* in *in-vitro* models of the catheterised bladder

Experiments were performed to determine how inflating catheter balloons with triclosan in 5% PEG would affect crystalline biofilm formation by *Pr. mirabilis* B2 on all-silicone catheters (Figure 19). The control catheters inflated with water blocked with crystalline biofilm after a mean time of 26 h. At blockage the pH of the residual urine in the models had increased from 6.1 to a mean of 8.63, and the mean viable cell count was 7.80×10^7 cfu/ml. The all-silicone catheters inflated with even the lowest test concentration of triclosan (10 µg/ml) took a significantly longer ($P < 0.05$) time to block compared to the control models. Catheter balloons inflated with triclosan concentrations of 500 µg/ml or higher in 5% PEG enabled the catheters to drain freely for the duration of the experiment. When catheters were inflated with 1 and 10 mg/ml triclosan in 5% PEG the mean pH value of the residual urine stayed acidic for seven days, and the residual urine in the models remained clear throughout the experiment (Figure 22). The mean number of viable cells in the urine of these models was reduced to around 10^4 cfu/ml at seven days.

LV-SEM revealed significant crystalline biofilm formation on the control catheters at blockage (Figure 23), whilst Figure 26 reveals little evidence of biofilm colonization or encrustation on all-silicone catheters inflated with 10 mg/ml triclosan in 5% PEG after seven days. Electron micrographs of the critically point dried control catheter sections fixed with and without the RHT revealed a crystalline bacterial biofilm where *Pr. mirabilis* bacilli were associated with crystalline material (Figures 35 and 38). Large blocky crystals as seen in Figure 35b were clearly recognisable as struvite

crystals, whilst crystallites powdery in appearance, typical of hydroxyapatite can be seen in Figure 35e. Large crystals of struvite could often be seen embedded in the hydroxyapatite (Figure 35b). These observations are similar to those made by Cox *et al.* (1989a) and Stickler *et al.* (1993). Although it is reasonable to assume that these are crystals of calcium and magnesium phosphates, x-ray microanalysis of the catheter sections, similar to that performed by Winters *et al.* (1995) could have confirmed their identities.

Cox (1990) investigated the surface morphologies of different types of Foley catheters by SEM. Their study revealed the rough irregular nature of catheter surfaces. From their observations on images of used and unused catheter surfaces, Axelsson *et al.* (1977) had previously concluded that the ideal catheter surface should be free from surface irregularities, and noted that if this were the case, it would lower the incidence of complications associated with the long-term use of catheters. The manufacturing process for catheters commonly involves mechanically punching out the catheter eye-holes. Electron micrographs of unused catheters have revealed that this results in catheters with narrow eye-holes that have rough irregular surfaces (Stickler *et al.*, 2003). Electron micrographs from the study by Stickler *et al.* (2003) also illustrated how *Pr. mirabilis* biofilm formation begins from microcolonies formed within the depressions of the irregular surface before spreading across the eye-hole rims. The electron micrographs in this study have also shown that the catheter eye-hole can be rough and irregular (Figure 36a), and that bacteria can become trapped within these surface imperfections (Figure 35d). Observations on electron micrographs in Figure 41 also shows that the biofilm appears to spread down the catheter. Stickler *et al.* (2003) concluded that developing catheters with larger internal lumens and smoother

surfaces would substantially reduce the problems associated with these devices. Developing manufacturing techniques capable of achieving these goals should be possible, if only the medical device industry was prepared to take up the challenge.

Stickler *et al.* (2003) used RHT to stabilise polyanionic components of the bacterial polysaccharide matrix prior to fixation in osmium. To determine if there was any benefit to using RHT, two critically point dried control catheters were observed by SEM in this study, fixed either with or without RHT. Although Figure 35c shows a web-type matrix covering the *Pr. mirabilis* bacilli fixed without RHT, it was not possible to get a detailed view of the cells appearance at higher magnifications. In contrast, much more detail could be seen on the *Pr. mirabilis* cells on the catheter sections fixed with RHT. The images show that *Pr. mirabilis* has elaborate surface structures, with hair like extensions coming off the cell wall (Figure 38b,c). Figure 38e shows an extensive polysaccharide matrix, which appeared to be attached to hydroxyapatite crystals and *Pr. mirabilis* cells. From these images it was concluded that fixation with RHT produces better and more detailed images, and should be included in the fixation of all catheters prepared for critical point drying in future experiments.

The images shown in Figure 36 and 37 are of catheters that had drained freely in the bladder models for seven days. The catheter in Figure 36 had its balloon inflated with 10 mg/ml triclosan in 5% PEG throughout the seven day test period. The images shown in Figure 37 are of the catheter that was impregnated with triclosan in the bladder models for 24 h, and then the triclosan was replaced with water and the model was inoculated with *Pr. mirabilis* B2. Both catheters were fixed without RHT. In

contrast to the control catheters, very little crystalline material was visible on the catheter sections. Large clusters of cells could be seen on the control catheter sections, whilst cells on the triclosan treated catheters were attached as individuals or in small groups. The morphology of the bacilli also differed on the triclosan treated catheters compared to the control catheters, many of the images (Figures 36e, f, 37b, d, f) show structures which appear to be clusters of cells fused together. Figure 36e, shows what appear to be normal looking bacilli next to very small looking cells. The images of catheter sections taken from the impregnated catheter and the catheter that had its balloon inflated with 10mg/ml triclosan are very similar. This suggests that over the 24 h period sufficient triclosan must have been absorbed by the silicone of the impregnated catheter to protect it from bacterial colonization and encrustation.

The lowest concentration of triclosan in the catheter balloon which inhibited the blockage of all-silicone catheters was 500 µg/ml in 5% PEG. The images presented in Figure 39 show electron-micrographs of a catheter which drained freely for seven days that had its balloon inflated with 500 µg/ml triclosan and that was fixed with RHT. These images also show disfigured and very small bacterial cells (Figure 39b, c, d, e). Unlike the control RHT treated catheter sections, no hair-like structures could be seen on the *Pr. mirabilis* cells colonizing the triclosan treated catheter. Triclosan thus appears to have an effect on the structure of the *Pr. mirabilis* bacilli. It would be interesting to compare the development of *Pr. mirabilis* biofilms in control catheters inflated with water and in test catheters inflated with triclosan solutions. Studies taking the control and test catheters inflated with the maximum concentrations of triclosan out of the bladder models at regular intervals after inoculation would give more information about how triclosan affects the process of biofilm formation. We

know that initially only a low concentration of triclosan passes through the catheter balloon. Therefore, it would be interesting to investigate the early stages of colonization. This would determine if the initial concentration of triclosan passing through the catheter is high enough to prevent cells from attaching to the surface, or whether cells attach but fail to develop a biofilm because triclosan is released. The use of transmission electron microscopy (TEM) would also give more details on the effect triclosan has on the morphology of *Pr. mirabilis* cells.

The bladder model experiments are designed to simulate placing a new catheter into a patient already with significant *Pr. mirabilis* bacteriuria and a high urinary pH. To allow a comparison between different experiments, chemically defined artificial urine was used in most bladder model experiments, a standard inoculum was added and all experiments were run under standard conditions. Catheter blockage is easily detected in the bladder models because the urine level of the reservoir rises above the eyehole. It is difficult to correlate the times of catheter blockage in the models to the time it will take the catheters to block in patients. However, comparing the times to blockage of test models to a control model gives an indication whether a new catheter care regime is likely to prevent or delay catheter blockage. All-silicone catheter balloons inflated with the higher concentrations of triclosan prevented catheter blockage for the duration of the seven day experiment even after what was considered to be a severe challenge with a dense population (10^8 cfu/ml) of *Pr. mirabilis*. The results presented in Figure 19 thus suggest that this approach could have practical applications in controlling catheter encrustation.

Experiments using the two ready-made formulations obtained from CIBA containing 1% (w/v) triclosan to inflate the catheter balloons of all-silicone catheters gave similar results to those using the highest concentrations of triclosan in 5% PEG (Figure 19). Placebo formulations not containing triclosan were not available to test, therefore the inhibitory effect of inflating the balloons with these formulations cannot conclusively be attributed to the effects of triclosan alone. In all other bladder model experiments, the activity of the triclosan solvent was tested in the balloon to determine if they had any inhibitory effects. Catheters inflated with 5% PEG did not perform significantly differently ($P > 0.05$) from those inflated with water.

Having established that all-silicone catheters inflated with triclosan could control catheter encrustation, further experiments were performed using hydrogel- and silicone-coated latex catheters (Figures 20 and 21). The mean time to blockage for control models of both types of latex catheters was under 24 h. Concentrations of 1 mg/ml triclosan were required in the latex catheter balloons to prevent blockage for the full seven days. This may be attributed to the fact that unlike the all-silicone catheters, the latex-based catheters do not become impregnated with the antibacterial agent (Figure 17). The electron micrographs presented in Figures 24, 25, 27 and 28 illustrate the encrustation occurring on the latex-based catheters, confirming the lack of colonization of the eye-holes and luminal surfaces when triclosan was used to inflate the balloons.

The mean pH value from the residual urine of the control models for both types of latex catheters was above pH 8.5 at blockage (Figures 20b and 21b), and their viable cell counts were around 1×10^8 cfu/ml (Figures 20c and 21c). The higher triclosan test

concentrations (1 and 10 mg/ml triclosan in 5% PEG) significantly reduced ($P < 0.05$) the pH and viable cell count in the residual urine at the end of the experiment compared to the control models at blockage. The number of viable cells in the residual urine of the triclosan models varied from one run to the other. This could be because of differences in the thickness of the catheter balloons from one catheter to another, resulting in different concentrations of triclosan being delivered from the balloon to the urine.

It was considered important to investigate what effect using triclosan in the catheter balloon would have on the development of crystalline biofilms by other strains of *Pr. mirabilis*. The test models fitted with all-silicone catheters inflated with 10 mg/ml triclosan in 5% PEG and inoculated with each of the four different *Pr. mirabilis* strains drained freely for the duration of the seven day experiment. The control models all blocked in a mean of 22 – 46 h (Figure 29a). The pH and viable cell count in the residual urine of the test models at seven days was also significantly lower ($P < 0.05$) than in the control models at blockage (Figures 29b and 29c).

The time to blockage of the control models from the experiment with different triclosan concentrations in the balloon were $18.75 \text{ h} \pm 1.85 \text{ h}$ for the silicone-coated latex catheters, $22.86 \text{ h} \pm 2.60 \text{ h}$ for the hydrogel-coated latex catheters and $26.32 \text{ h} \pm 2.47 \text{ h}$ for the all-silicone catheters. Statistical analysis of these data however revealed that there was no significant difference ($P = 0.082$) between the times to blockage of the control models fitted with the three different catheter types. An earlier study with *Pr. mirabilis* infected bladder models by Morris *et al.* (1997) reported that while latex catheters blocked at mean times of around 20 – 30 h, the Bard size 14 ch all-silicone

catheter took 50 h to block. These findings were consistent with the clinical experience reported by Kunin *et al.* (1987 b) that encrustation and blockage was a greater problem with latex than silicone catheters. A possible explanation for the poorer performances of the recent all-silicone catheters in the bladder models could be due to changes in the manufacturing process for these catheters.

There are constituents present in human urine which are not present in artificial urine. Therefore, it was important to test this novel strategy in models supplied with human urine. Because of the difficulties of collecting and sterilizing the large volumes of urine required to run the bladder models for seven days, in these experiments the effect of triclosan was assessed by examining the amounts of calcium and magnesium forming on test and control catheters over a 48 h period. None of the control or test models supplied with human urine blocked within the 48 h experimental time period. However, as with the artificial urine experiments, the control models were very cloudy at the end of the experiment, whilst the test models remained clear (Figure 22). The test models fitted with catheter balloons inflated with 10 mg/ml triclosan in 5% PEG also significantly reduced ($P < 0.05$) the pH and viable cell count in the residual urine compared to the control models (Figure 30a and 30b). Calcium and magnesium analysis of the catheters established that there was significantly less ($P = 0.000$) encrustation on the test catheters compared to the controls (Figure 30c), these observations were confirmed by LV-SEM images (Figure 31) showing little encrustation on the test catheter and heavy encrustation on the control. This established that although models supplied with pooled human urine take longer to block than those supplied with artificial urine, there was significant encrustation on

the control catheters, which was inhibited by the use of triclosan in the catheter balloon.

In the experiments reported in Figure 32, the catheters used were inflated with triclosan for various time periods, the balloons were emptied, washed and re-inflated with water. The idea was to test whether simply impregnating the silicone of the catheter in this way could inhibit encrustation. Test catheters impregnated with triclosan in the models for 24 h drained freely for the duration of the seven day experiment, and the residual urine in the models remained acidic (Figure 32a and 32b). No viable cells were cultured from the residual urine at the end of the experiment (Figure 32c). The viable cell counts were lower after seven days than with catheters inflated with 10 mg/ml triclosan in 5% PEG. This can be explained by the experiments monitoring the concentration of triclosan that passed through the catheter balloon, which showed that there was an initial delay in the release of triclosan. Triclosan is absorbed throughout the length of the catheter impregnated in the model at the time of inoculation, which might reduce the initial delay in the release of triclosan from the catheter material. The sections of the triclosan-impregnated catheter were placed onto *Pr. mirabilis* B2 bacterial lawns at the end of the seven day experiment. Large zones of inhibition were observed around all the sections (Figure 34), indicating that triclosan remained in the catheter material after seven days *in situ* in the model. Electron micrographs of the triclosan impregnated catheter (Figure 33) at the end of the experiment revealed no sign of encrustation.

The results with catheters that had been impregnated in their packaging for different time periods were more variable than those with the catheters impregnated in the

models. One or two replicates for each time period blocked whilst the others drained freely for seven days (Figure 32a). The enhanced performance of the catheters impregnated in the models for 24 h compared to the same time period impregnated in the packaging could be attributed to the triclosan in the artificial urine which had been flowing through the catheter adsorbing to the drainage lumen. Another possibility is that a concentration gradient between the triclosan solution in the balloon and the residual urine in the model may have led to higher concentrations of triclosan impregnating these catheters. The results also show that to protect catheters from encrustation by pre-loading them with triclosan prior to their placement in the bladder, it would be necessary to retain the triclosan solution in the balloon for about an hour. This might well restrict the practicality of using this particular strategy on patients.

Experiments using the bladder models thus far focused on preventing the development of crystalline bacterial biofilms by *Pr. mirabilis*. Since these results were so successful, the possibility of using triclosan to remove established biofilms or to inhibit their further development was considered. The effect of adding triclosan into the balloons of catheters on which crystalline *Pr. mirabilis* biofilm had formed for 18 h was investigated. Replacing water in the balloons of encrusted catheters with 10 mg/ml triclosan in 5% PEG after the 18 h pre-incubation period failed to have a significant effect ($P > 0.05$) on the total time to blockage or pH and viable cell count in the residual urine (Figure 40). From the time the triclosan was added to the test model at $t = 18$ h to the time it took for the catheters to block, there was no significant difference ($P = 0.103$) between the test and control catheters. These results indicate this novel strategy would be less successful at preventing catheter blockage once encrustation had begun. The results indicate that if the strategy is to be used in patients

it will be necessary to replace the encrusted catheter and inflate the new catheter with the triclosan solution.

It is now accepted that if the objective of an antimicrobial agent is to treat biofilms, then the agent must be tested against bacteria growing as communities on surfaces (Donlan and Costerton, 2002). It is therefore important that more realistic *in vitro* tests be devised to study the ability of antimicrobial agents to treat infections involving medical devices (Anwar *et al.*, 1990). The aim of the experiments reported in Figures 42 – 47 was to determine the concentration of triclosan required in the artificial urine supplying the bladder models to eradicate or prevent further development of an established biofilm. Preliminary experiments revealed that with size 16 Ch catheters there was an even crystalline biofilm along the length of the catheter 16 h after inoculation (Figure 41), therefore the urine supply was changed to contain triclosan at this time. The control and DMSO control models blocked within the duration of the experiment, whilst 10 and 100 µg/ml triclosan in the urine prevented catheter blockage for the 66 h experimental period. For the models supplied with 0.1 µg/ml triclosan, two of the three test models blocked and one of the models supplied with 1 µg/ml triclosan blocked. By running a second control model, and taking it down at the time the urine supplies were changed to contain triclosan, it was possible to compare the amount of encrustation that had developed on the catheters from that time. The amounts of calcium and magnesium on the control, DMSO control, 0.1 µg/ml and 1 µg/ml triclosan catheters was significantly higher ($P < 0.05$) than on the catheters taken out at 16 h (Figure 46). There was no significant difference ($P > 0.05$) between the amounts of calcium and magnesium on the catheters taken down at 16 h compared to those treated with 10 and 100 µg/ml triclosan. It therefore appears that 10 µg/ml is

the minimum triclosan concentration required in the urine to inhibit and prevent further development of *Pr. mirabilis* crystalline biofilms. In this experiment some catheters drained for the full 66 h whilst others blocked at different times, in view of this the rates of catheter encrustation for the test and control models were also determined (Figure 47). The rate of encrustation was significantly lower ($P < 0.05$) than the controls in only those models supplied with 100 $\mu\text{g/ml}$ triclosan. Concentrations of 10 and 100 $\mu\text{g/ml}$ triclosan in the artificial urine also significantly reduced ($P < 0.05$) the viable cell count and pH of the urine compared to the controls.

As well as determining the biofilm inhibitory concentration of triclosan against *Pr. mirabilis*, these experiments also showed that the *in vitro* models could easily be adapted to determine the concentration of antimicrobial agents required in the urine of patients to inhibit further biofilm development. This method could easily be modified to determine the concentration of antimicrobials required in urine to prevent crystalline bacterial biofilm formation or it could also be used to determine the concentration of antimicrobial agents required for treating CA-UTI.

4.5. Formulation of triclosan

If using triclosan formulations to inflate catheter balloons is to form a new strategy for controlling catheter encrustation, the solution must be as easy to use in the retention balloon as water. For this reason the triclosan solution should fit the following criteria: it should need no preparation prior to use, it should be sterile, be stable in solution, and should easily inflate the catheter balloon. Unfortunately the formulation of triclosan used in this work (10 mg/ml in 5% aqueous PEG) does not meet all these criteria. The triclosan needs to be stirred and heated (60°C) to get it to dissolve and

even then it only forms an emulsion that settles out on standing, rather than form a solution. Because of the possibility of degradation at high temperatures, sterilization by heating was not an option. Membrane filtration led to separation of the phases, the resulting filtrate was found to be an aqueous solution containing very little triclosan. Since the intended use for this solution is in busy hospitals and by nurses looking after community based patients, the use of this formulation was not feasible. Alternative solutions for dissolving triclosan which could be used instead of 5% PEG were investigated.

Whilst searching for new solvents, only those that would be safe to use in the bladder were considered. The search for a new solvent involved several stages. Firstly, determining the ability of different solvents to dissolve triclosan. Secondly, catheters were inflated with the new solutions to make sure they could inflate the balloon, and the catheters were soaked in water for eight weeks to establish what effect the solvent had on the catheter balloons. Finally any solution that passed the first stage were tested to determine if they would be as effective at preventing catheter blockage as the triclosan suspension in 5% PEG.

The first solution tested in this experiment was triclosan in 70% ethanol. Although triclosan dissolved quickly, the alcohol leaked out of the catheter balloons within 1 week. The use of castor oil or olive oil to dissolve triclosan was an attractive prospect because of their non-toxicity. Both these oils were capable of dissolving triclosan without heating, but their viscosity made it hard or impossible to inflate the catheter balloon with them, therefore their use was ruled out. Although propylene glycol could easily dissolve triclosan, and could be used to inflate the catheter balloon, after a

couple of days soaking the catheters in water, the catheter balloons became swollen. 0.1 M sodium carbonate could dissolve 3 mg/ml triclosan with stirring but no heat in under 1 h. The resulting clear solution could inflate the all-silicone catheters and both types of latex-based catheters. There were no visible changes to the balloons of all-silicone or latex-based catheters inflated with 0.1 M sodium carbonate with and without triclosan after being soaked in water for eight weeks. All the catheters could be deflated at the end of the eight-week period and the use of the sodium carbonate with and without triclosan resulted in significantly more ($P < 0.05$) fluid being retained in the all-silicone catheters than in those inflated with water (Figure 48). The loss of water from silicone catheter balloons can be a problem; therefore this secondary consequence of using sodium carbonate would be an added beneficial effect.

After identifying triclosan in sodium carbonate as a possible formulation that could be used to inflate the catheter balloons, its ability to inhibit crystalline biofilm formation was tested in the bladder models. The limit of solubility of triclosan in 0.1 M sodium carbonate was found to be 3 mg/ml. The range of concentrations tested in the bladder models was therefore from 10 μ g/ml to 3 mg/ml. The data from these experiments (Figure 49) revealed no significant difference ($P > 0.05$) between the sodium carbonate controls and the models that had water in the balloon in terms of time to blockage and viable cell count and pH in the residual urine at blockage. The use of 1 and 3 mg/ml triclosan in 0.1 M sodium carbonate successfully prevented catheter blockage for the duration of the seven-day experiment. The pH and viable cell count from the residual urine in these models after seven days were significantly lower ($P < 0.05$) than those from the control models at blockage. One replicate that had its

balloon inflated with 500 µg/ml triclosan in 0.1 M sodium carbonate blocked and the others drained freely for the duration of the experiment, whilst none of the all silicone catheters inflated with 500 µg/ml triclosan in 5% PEG blocked. However, there was no significant difference ($P > 0.05$) between the time to blockage, pH or viable cell count at blockage using the 100 µg/ml triclosan in 5% PEG or in 0.1 M sodium carbonate. Statistical analysis of the results from the experiments with catheter balloons inflated with 10 mg/ml triclosan in 5% PEG and 3 mg/ml triclosan in 0.1 M sodium carbonate revealed there was no significant difference ($P > 0.05$) between the pH and viable cell count after seven days. This set of experiments confirmed that triclosan in both solvents was effective in controlling urinary pH, *Pr. mirabilis* growth and catheter blockage. Figure 50 shows little encrustation on the all-silicone catheter that had its balloon inflated with 3 mg/ml triclosan in 0.1M sodium carbonate after seven days in the bladder model.

To determine if the type of solvent used affected the ability of triclosan to prevent catheter blockage, bladder model experiments were carried out with all-silicone, silicone-coated latex and hydrogel-coated latex catheters having their balloons inflated with simple suspensions of triclosan in water. The results of the time to blockage, pH and viable cell count in the residual urine (Figures 51) of all-silicone catheters using 100 µg/ml triclosan in aqueous suspension were not significant different ($P > 0.05$) from the same concentration in 5% PEG or 0.1 M sodium carbonate. Test models were also set up with all-silicone catheter balloons inflated with 10 mg/ml triclosan in aqueous suspension and these drained freely for the duration of the seven day experiment. There was no significant difference ($P > 0.05$) between the pH and numbers of viable cells cultured from the residual urine of these models (data not

shown) compared to those inflated with 10 mg/ml triclosan in 5% PEG in previous experiments. There was no significant ($P > 0.05$) difference in the results obtained using hydrogel-coated latex catheters that had their balloons inflated with 100 $\mu\text{g/ml}$ triclosan in 5% PEG or in aqueous suspension, however, the use of 5% PEG improved the ability of triclosan to prevent catheter blockage with silicone-coated latex catheters. The time to blockage increased significantly ($P < 0.05$) using 100 $\mu\text{g/ml}$ triclosan in 5% PEG compared to the same concentration in aqueous suspension, and the pH and viable cell count in the residual urine were also significantly ($P < 0.05$) lower using triclosan in 5% PEG.

Triclosan is only poorly soluble in water and at 10 mg/ml it forms a particulate suspension. In experiments with this suspension, it was observed that leakage occurred from some catheters at the inflation port. This was probably due to the undissolved triclosan particles preventing the valves at the inflation port sealing properly. For this reason it is unlikely that simple aqueous suspensions of triclosan could be used to inflate patients' catheters.

4.6. The sensitivity of other urinary tract pathogens to triclosan

The aim of this part of the study was to determine the sensitivity to triclosan of other urinary tract pathogens that commonly infect catheterised patients. The ability of these organisms to form biofilms on urinary catheters was examined and the effect of triclosan on the development of these biofilms tested. The MIC of triclosan against the eight different uropathogenic species tested is shown in Table 5. *E. coli*, *Pv. stuartii*, *K. pneumoniae* and *S. aureus* were all very sensitive to triclosan. The MIC of triclosan for *S. aureus* was between 0.1 - 0.6 $\mu\text{g/ml}$. Bhargava and Leonard (1996) reported that

the MIC of triclosan against culture collection strains of *S. aureus* was 0.01 - 0.1 µg/ml, Suller and Russell (2000) however reported that the MIC of triclosan for clinical isolates of *S. aureus* was between 0.025 and 1 µg/ml. The MIC of triclosan for *E. coli* was 0.2 - 0.3 µg/ml which correlates well with those values reported by Bhargava and Leonard (1996) of 0.1 - 0.3 µg/ml. Bhargava and Leonard (1996) reported MICs of triclosan against *K. pneumoniae* of 0.3 µg/ml, however higher concentrations were required to inhibit the growth of catheter isolates of *K. pneumoniae* (0.3 - 0.8 µg/ml). All strains of *Ent. faecalis* tested had MICs of 7 µg/ml, this value is slightly lower than the MIC of 10 µg/ml triclosan against *Ent. faecalis* reported by Heath and Rock (2000). The MIC of triclosan against *Ser. marcescens*, *M. morgani* and *Ps. aeruginosa* was over 100 µg/ml. These findings correlate well with the values reported by Bhargava and Leonard (1996), they noted that the MIC for *Ps. aeruginosa* was over 1000 µg/ml, and over 100 µg/ml for *Ser. marcescens*.

The bactericidal effect of triclosan against one strain of each species was also tested (Figure 52). As expected *Ps. aeruginosa* was the most resistant to the lethal effects of triclosan. Statistical analysis of the data (Table 10) showed that the mean log₁₀ (cfu/ml) reduction with *Ps. aeruginosa* was significantly lower than all the other strains tested after 60 min contact time. For *Ser. marcescens* the mean log₁₀ (cfu/ml) reduction given by 100 µg/ml triclosan was less than one for each contact time tested. Although the MIC for *M. morgani* was over 100 µg/ml, the strain tested in the suspension test gave a log₁₀ (cfu/ml) reduction of over five after a 60 min contact time with 100 µg/ml triclosan. On the other hand, although the MICs for *E. coli* and *K. pneumoniae* were very low, the mean log₁₀ (cfu/ml) reduction for each contact time with 100 µg/ml triclosan was less than one. Although the *Ent. faecalis* was moderately

susceptible to the inhibitory effects of triclosan, it was very susceptible to its lethal effects, 20 µg/ml gave a log₁₀ (cfu/ml) reduction of over five after 5 min contact time. Since only one strain of each species was tested however, the results may not be representative of the entire species, therefore future studies may consider performing suspension tests with more strains of each species to conclude whether there are any trends between inhibitory and lethal effects. Messenger *et al.* (2001) tested the bactericidal activity of triclosan against several micro-organisms. Although it is not possible to compare data from this study directly with their data since different triclosan concentrations and different contact times were tested, similar trends were observed. After a one minute contact time with 500 µg/ml triclosan, the *S. aureus* and *Ent. faecalis* were very sensitive to the lethal effects of triclosan resulting in a log₁₀ reduction in viable bacteria (cfu/ml) of between 1 and 2. The *E. coli* and *Ps. aeruginosa* however appeared more resistant to the lethal effects, the log₁₀ (cfu/ml) reduction in viable bacterial cells was less than one.

In the experiments with these species in the bladder models, it was clear that none of them were capable of producing alkaline conditions in urine. The pH of the inoculating culture and the pH of the residual urine from the control and test bladder models inoculated with each organism after 24 and 48 h are shown in Figure 53. As in previous experiments, the pH of the residual urine in the control models infected with *Pr. mirabilis* increased to over 8 within 24 h and the catheters blocked with crystalline biofilm within 48 h (data not shown). All the triclosan test models inoculated with *Pr. mirabilis* drained freely for 48 h, as did the control and triclosan models inoculated with the other eight bacterial species. The residual urine in the *Pr. mirabilis* test model

and the test and control models infected with the eight different uropathogens remained acidic throughout the experiment.

Figures 56 and 57 show electron micrographs of test and control catheters taken out of *E. coli* and *Ps. aeruginosa* inoculated bladder models after 48 h. The images were taken at four different positions down the length of the catheters. Images of control and test catheters inoculated with the six other bacterial species are shown in Appendix 2. A biofilm can be seen on the eye-hole of the control *E. coli* catheter, but there is no sign of biofilm formation on the triclosan test catheter. A substantial biofilm can be seen on the eye-hole of the control and test *Ps. aeruginosa* catheters, and a thin biofilm can be seen along the length of the catheters. In contrast to the *Pr. mirabilis* infected models, none of the other bacterial species tested produced a crystalline biofilm. Therefore, despite being capable of producing urease, *Ser. marcescens*, *S. aureus*, *K. pneumoniae*, *Ps. aeruginosa* and *M. morgani* failed to produce a crystalline bacterial biofilm or turn the residual urine alkaline. It would have been interesting to fix, critically point dry and gold-coat the control and triclosan catheters inoculated with the different organisms to observe the sections under HV-SEM. This may have revealed biofilms that were not visible by LV-SEM. It would also be interesting to observe any differences in the morphology of the bacteria on the triclosan treated compared to the control catheters.

The finding that *Pr. mirabilis* rapidly elevates the urinary pH forming crystalline biofilms, while other urease producers fail to do so correlates well with clinical observations. For example Mobley and Warren (1987) found a significant association between catheter obstruction and the presence of *Pr. mirabilis* in urine, but not

between any other urease-positive species. Whilst bacteriological analysis of catheters removed from patients undergoing long-term bladder management revealed that catheters colonized with *Pr. mirabilis* formed crystalline biofilms, significant crystalline material was absent in non-*Proteus* biofilms (Stickler *et al.*, 1993). The results of these experiments also confirms and extends those reported by Stickler *et al.* (1998b) showing that *Pr. mirabilis* produced more encrustation on catheters in the bladder model than other urease positive organisms.

Inflating the catheter balloons with triclosan significantly ($P < 0.05$) reduced numbers of viable cells in the biofilm for all strains tested except *Ps. aeruginosa* (Figure 55). Although there were slightly fewer viable cells on triclosan treated *Ps. aeruginosa* catheters compared to the control catheters, the difference was not significant ($P = 0.063$). Figure 54 shows the $\log_{10}(\text{cfu/ml} + 1)$ data of the inoculating culture, and of the residual urine from the control and test bladder models from each organism at 24 and 48 h. For the *Pr. mirabilis*, *Pv. stuartii*, *E. coli*, *S. aureus*, *Ent. faecalis* and *K. pneumoniae* there were significantly lower numbers ($P < 0.05$) of viable cells in the residual urine of the triclosan treated models compared to the controls at 24 and 48 h. This suggests that this novel approach of controlling catheter encrustation could also help control a range of other urinary tract pathogens.

Most of the information regarding the use of triclosan to control biofilms has centred on its ability to control dental plaque. For example, investigating the effect of triclosan on the microbial composition of dental plaque, Walker *et al.* (1994) reported that in subjects assigned to use a triclosan containing dentrifice, there was a more pronounced decrease in culturable flora after three and six months compared to the

control subjects. The antibacterial activity of an amine fluoride, sodium fluoride and triclosan containing mouthwash was investigated by Arweiler *et al.* (2002). Compared to the control, the test mouthwash displayed significantly better antibacterial and anti-plaque activity. Few studies have considered using triclosan to prevent or treat other forms of bacterial biofilms. Jones *et al.* (1994) showed that liposomes carrying triclosan could inhibit the growth of biofilms of *S. epidermidis*. It was reported that even after exposing the biofilms to the triclosan liposomes for short periods of time, triclosan concentrated preferentially in the biofilm rather than in the liposome lipid carriers. A study comparing the effects of incorporating triclosan into plastics (at the same concentrations it is used in many consumer plastic products) revealed no significant difference between attached bacterial population onto the triclosan incorporated plastics compared to control plastic sections (Junker and Hay, 2004). It was suggested that the ability of biofilms to form on these plastic surfaces was due to the limited desorption of triclosan from the material.

The results presented in Figures 52, 54 and 55 illustrate the intrinsic resistance of *Ps. aeruginosa* to triclosan. Chuanchuen *et al.* (2001) demonstrated that triclosan was the substrate for multiple *Ps. aeruginosa* efflux pumps, and showed that under laboratory conditions, *Ps. aeruginosa* exposure to triclosan could select for multi drug resistant mutants. As yet, however, there is no evidence to suggest that this has happened in a non-laboratory setting. Subsequent studies using defined mutant strains showed that the ability of *Ps. aeruginosa* to survive in concentrations of triclosan exceeding 1000 µg/ml was attributed solely to the expression of efflux pumps (Chuanchen *et al.*, 2003). The authors comment that since triclosan is an excellent substrate for efflux pumps that a link between triclosan and antibiotics must exist, and note that at the

very least, in triclosan treated environments, *Ps aeruginosa* “should be well equipped to tip the ecological balance in their favour”. Schweizer (2001) also comments that the widespread use of triclosan could lead to the selection of triclosan resistant bacteria such as *Ps. aeruginosa*. This should be an important consideration when thinking about introducing triclosan into any setting. *Ps. aeruginosa* is a clinically significant pathogen, and infections associated with this bacterium are often difficult to treat due to its many acquired and intrinsic antibiotic resistances. If clinical trials introducing triclosan into the catheter care regime go ahead, its effect on the microbial ecology of the urinary tract would have to be monitored closely for any untoward shifts in microbial flora. If the overgrowth of *Ps. aeruginosa* becomes a problem, formulating a solution combining triclosan with an anti-pseudomonal agent may become an option. It is, however, impossible to guess how introducing triclosan into catheter care will affect the microbial balance, the only way of finding out is to carry out closely monitored clinical trials.

Stickler *et al.* (1998b) showed that whilst urease positive *M. morgani*, *K. pneumoniae* and *Ps. aeruginosa* failed to raise the urinary pH and form crystalline biofilms, strains of *Pr. mirabilis*, *Proteus vulgaris* and *Providencia rettgeri* generated alkaline urine and crystalline biofilms. *Pr. vulgaris* and *Pv. rettgeri* are however, not frequently isolated from the catheterised urinary tract (Kunin, 1989), and a clinical study of long-term catheterised patients revealed no trend between the presence of *Pr. vulgaris* and *Pv. rettgeri* in the catheterised urinary tract and catheter obstruction (Mobley and Warren, 1987). Experiments determining the sensitivity of both these species to triclosan should be carried out in future studies. The insensitivity of one of these bacterial species to triclosan could result in the overgrowth of bacteria capable of

forming encrustation in the triclosan treated catheterised urinary tract. Bladder models should also be run inoculated with these organisms to determine how inflating the catheter balloon with a triclosan solution would affect biofilm development.

After the first couple of weeks of catheterisation, most patients become colonized with several different bacterial species. Warren *et al.* (1982) showed that in patients with chronic IUCs, bacteriuria was polymicrobial with up to five different bacterial species in 77% of all weekly urine specimens. Therefore, one of the most important experiments to carry out in future studies should involve monitoring the effect of triclosan on the development of catheter biofilms in bladder models infected with mixed bacterial communities. This may allow us to gain an insight into any untoward changes to look out for in clinical trials with the triclosan inflated catheters.

4.7. Triclosan mode of action and concerns about resistance

Bacteria employ three general classes of resistance mechanisms; target alteration, reduction in target access or inactivation of the inhibitor (Chapman, 1998). Bacterial resistance to biocides can either be intrinsic or acquired (Russell, 1995). Acquired resistance to antimicrobial agents results from either bacterial acquisition of plasmids or transposons, or can arise from a mutation within a bacterial population exposed to an antimicrobial agent (Russell, 1995). The use of biocides as disinfectants is an important part of our armoury in the fight against infectious diseases (Heinzel, 1998). There is an increasing concern that exposure of bacterial communities to sub-lethal concentrations of biocides could result in the development of strains that have decreased sensitivities to these agents. The importance of preserving the efficacy of biocides is heightened by the decreasing rate at which new disinfectants are being

introduced (Chapman, 1998). Although a change in biocide sensitivity is unlikely to influence the lethal effects of biocides (due to the high concentrations of biocides usually in use), it could alter the susceptibility of bacterial communities to other antibacterial agents, particularly when the biocide and other antibacterial agents share the same target site or when induction of efflux pumps can confer resistance to several agents.

Some authors have raised concerns that the extensive use of triclosan will select for bacteria resistant to it and cross-resistant to other antibiotics or antimicrobial agents (Levy, 2001; White and McDermott, 2001). Similarly, research by other groups has led them to suggest that if stable low-level triclosan resistant mutants occur in the environment, they may encourage preferential survival of mutants resistant to other antimicrobial agents (Heath *et al.*, 1998; McMurry *et al.*, 1998a). Some authors have emphasised that like all antimicrobial agents, the use of triclosan should be limited to clinically useful situations but have cast doubts over the proposal that triclosan use will lead to antibiotic resistance (Gilbert and McBain, 2001; McBain *et al.*, 2003; Russell, 2003).

Studies by Villalain *et al.* (2001) confirmed that the membrane destabilising effects of triclosan leads to leakage of cytoplasmic constituents. It has also been shown that triclosan has a specific bacterial target site. McMurry *et al.* (1998a) reported that at low concentrations, the inhibition of growth of *E. coli* by triclosan resulted from a block in lipid synthesis. The agent specifically inhibited the NADH-dependent enoyl-acyl carrier protein (ACP) reductase, FabI. This enzyme plays a crucial role in completing rounds of fatty acid elongation (Heath and Rock, 1995). McMurry *et al.*

(1998a) and Levy *et al.* (1999) showed that mutations in/or over-expression of the gene that encodes enoyl-ACP reductase, prevented the blockage of lipid synthesis by triclosan. Analysis of the crystalline structure of wild-type and mutant *E. coli* FabI protein revealed that all mutations were at residues that lined the cleft of the enzyme to which the nicotinamide co-factor and substrate bind. This was the first evidence that triclosan was not a non-specific biocide, and that it acted upon a specific bacterial target.

Levy *et al.* (1999) and Roujeinikova *et al.* (1999a) carried out structural analysis and inhibition experiments on the enoyl-ACP reductase complex of *E. coli* with triclosan and NAD⁺. The electron density maps of the enoyl-ACP-reductase complexed with NAD⁺ and triclosan revealed that triclosan binds to the complex adjacent to the nicotinamide ring of the nucleotide cofactor in the enzyme's active site, taking the place of where the substrate usually binds. The similarity between the structure of triclosan and the enzyme's natural substrate showed that triclosan could bind tightly to this active site (Figure 63). This indicated that triclosan acted as a competitive inhibitor of the ACP-reductase, being able to form a stable complex with it and its cofactor NAD⁺. Heath *et al.* (1998) also reported that enoyl-ACP-reductase is the primary site for triclosan action, and concluded that the effect triclosan has on the structure and function of membranes is a consequence of its specific inhibitory action against fatty acid biosynthesis.

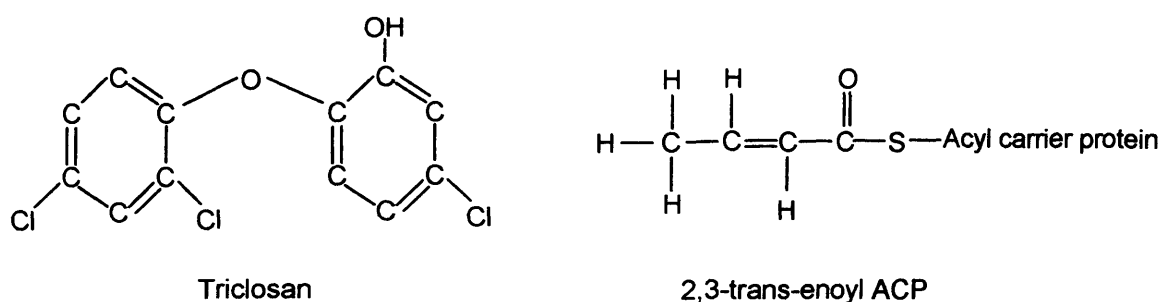


Figure 63: Chemical structure of triclosan and of the substrate for the enoyl-ACP reductase enzyme

It was not clear however, whether this single enzyme was responsible for both the bacteriostatic effect exhibited by triclosan at low concentrations and its bactericidal effects at higher concentrations. Antibiotics generally only have a single mode of action and therefore a single cellular target. Mutations which lead to increased antibiotic MICs are usually an indication of therapeutic failure. In contrast with biocides, an increase in MIC is generally not correlated with a decrease in bactericidal activity since these antibacterial agents have multiple target sites (McDonnell and Pretzer, 1998). In any case, when used as disinfectants, biocides such as triclosan are used in concentrations far in excess of the MIC. Suller and Russell (1999) investigated the susceptibilities of methicillin resistant *S. aureus* (MRSA) and methicillin sensitive *S. aureus* (MSSA) to different biocides including triclosan. They noted that although the triclosan MIC results for MRSA were higher than 1 µg/ml (compared with 0.025 µg/ml for MSSA), the MRSA and MSSA remained equally susceptible to its killing effects at 2, 5 and 10 µg/ml. A survey of bacterial tolerance to biocides in industrial environments where the agents were being manufactured recovered just two isolates of *Pv. stuartii* and *Acinetobacter johnsonii* that exhibited increased tolerance in terms of MICs to triclosan. Neither strain was more difficult to kill by “in-use” concentrations of triclosan than corresponding culture collection strains of the same species (Lear *et al.*, 2002). A strain of MRSA with an increased triclosan MIC was isolated from a hospital patient; however the rate of kill by triclosan for this isolate remained unchanged (Cookson *et al.*, 1991).

Suller and Russell (2000) noted that if the sole target of triclosan was this enoyl-reductase enzyme involved in lipid synthesis, then organisms growing exponentially should be more susceptible. They found however that exponential and stationary

phase cells of *S. aureus* were equally sensitive to triclosan. Similarly, the results reported in Figures 11 and 13 showed that stationary and log phase cultures of *Pr. mirabilis* in human urine were equally sensitive to the lethal effects of triclosan. McDonnell and Pretzer (1998) proposed that triclosan, as other biocides has multiple cellular targets, primarily the cytoplasmic membrane but also intracellular targets such as the enoyl-ACP reductase.

McMurry (1998b) established that over-expression of the multi-drug efflux pump locus *acrAB*, or of *marA* or *soxS*, both of which are positive regulators of *acrAB* could decrease triclosan susceptibility two fold. It must be noted that concentrations of triclosan used in clinical practice are much higher than the concentrations used in experiments by McMurry *et al.* (1998a) and McMurry *et al.* (1998b), although since triclosan persists in the environment, it is reasonable to assume that some areas will come into contact with lower triclosan concentration. Braoudaki and Hilton (2004) showed that exposure of *E. coli* 0157 to sub lethal triclosan concentrations under laboratory conditions resulted in the development of strains with increased triclosan resistance. These strains also demonstrated decreased susceptibilities to other antibiotics and biocides, prompting the authors to note that inappropriate use of triclosan could contribute to the problem of microbial resistance. The development of resistant bacteria in the laboratory is not conclusive evidence that resistant bacteria will develop in the clinical setting. In the 'real world', such mutants would also be faced with challenges not present in the laboratory setting including competition from other organisms as well as a host of environmental factors.

The discovery of a specific target site for triclosan led to concerns about it sharing its target sites with other therapeutic agents. The target site for isoniazid, currently the most widely used anti-tuberculosis drug is the enoyl reductase (InhA) of *Mycobacterium tuberculosis*. McMurry *et al.* (1999) demonstrated that InhA (the mycobacterial analogue of the FabI protein) is a target for triclosan and isoniazid in *M. smegmatis* and concluded that the overuse of triclosan could select for isoniazid resistant mycobacteria. It must be noted, however, that *M. tuberculosis* is intrinsically resistant to triclosan (Fraise, 2002). Further experiments have revealed that the enoyl reductase of *E. coli* is also a target for hexachlorophene and the experimental diazaborine family of antibacterial agents (Roujeinikova *et al.*, 1999b; Heath *et al.*, 2000).

There is an increasing view that triclosan should be retained for important applications. The growing public interest in preventing the transmission of infectious diseases, together with clever marketing techniques has led to an increase in products that contain “antibacterial agents” to “kill germs”. Hundreds of products have flooded the market claiming to combat germs. These products range from toilet seats to toys. Studies investigating the antibacterial activity of such products generally fail to demonstrate their efficacy. For example, Braid and Wale (2002) investigated the antibacterial activity of triclosan impregnated food storage boxes against *S. aureus*, *E. coli*, *Ps. aeruginosa*, *Bacillus cereus*, and *Shewanella putrefaciens* in conditions simulating domestic use. At 4°C they found the boxes exhibited no antibacterial effect against these species. Triclosan incorporated plastics have been moulded into products such as sinks showers and spas. Junker and Hay (2004) simulated the conditions in which these plastics are used, and observed no significant difference between attached

biofilm populations on triclosan-incorporated and control plastic sections after one to three weeks exposure to drinking water. It was also found that only trace amounts of triclosan desorped from the triclosan-incorporated plastics. The authors therefore questioned the long-term utility of triclosan-incorporated materials and suggest that the efficacy of such agents has yet to be proved.

Favero (2001) made the important point that incorporation of antimicrobial agents into plastics such as toys could not be expected to kill transient bacteria. Water must be present to transport the antibacterial agents across the bacterial cell wall. So these products have to be washed with soap and water to remove any microbial contamination. The inclusion of antimicrobial agents into products such as toys, telephones and mattresses offers no obvious public health benefit. Common sense approaches to hand washing and home hygiene usually provide a healthy environment in homes and other settings (Favero, 2001). There is now a general consensus that frivolous and unnecessary applications of triclosan should be eliminated. The aim for the future should therefore be to retain triclosan for important and valuable applications (Russell, 2004). These include dental hygiene products (Fine *et al.*, 1998) and hand and body washes to control MRSA (Brady *et al.*, 1990; Webster *et al.*, 1994; Zafar *et al.*, 1995; Irish *et al.*, 1998).

Recent surveys on the use of triclosan and other biocides have failed to establish a relationship between their use and antibiotic or antibacterial resistance. In a randomised study, Cole *et al.* (2003) collected environmental and clinical samples from the homes of antibacterial (triclosan and other biocides) product users and nonusers. They could find no relationship between the use of the antibacterial

products and antibiotic resistance. A prevalence survey of households also revealed no significant difference in antibiotic resistance or the numbers and kinds of bacteria found in the homes where antimicrobial agents were or were not being used (Marshall, 2003). A study of bacterial tolerance to biocides in industrial environments failed to demonstrate an association between the presence of residual biocides and the emergence of resistant bacteria (Lear *et al.*, 2002). Research by McBain *et al.* (2003) examined the effect triclosan may have on domestic-drain biofilm microcosms. The results showed that low-level exposure of environmental biofilms to triclosan did not affect antimicrobial susceptibility and they concluded that the emergence of antibiotic resistance through triclosan use in the kitchen is highly improbable.

The incorporation of triclosan into oral products also raised concerns about its use resulting in the development of intrinsically triclosan-resistant communities or of mutants with reduced susceptibility to triclosan and other antibacterials. Studies over extended time periods (28 weeks) revealed that the use of dentifrice containing 0.3% triclosan did not result in untoward shifts in micro-flora of supragingival plaque towards pathogenic bacterial species (Zambon *et al.*, 1990). Further studies confirmed these findings and demonstrated that the use of triclosan did not result in the emergence of resistant strains (Walker *et al.*, 1994; Zambon *et al.*, 1995; Fine *et al.*, 1998). The consensus view seems to be that there is no evidence that the use of triclosan in dental hygiene products results in the development of resistant, opportunistic or pathogenic microorganisms in the mouth (Expert Review Panel, 2000). A review by Sreenivasan and Gaffar (2002) of microbiological results obtained from clinical studies also concluded that the use of oral care products containing antimicrobial agents resulted in no adverse alterations in the bacteria found in dental

plaque, or in the emergence of resistant microflora. McBain *et al.* (2004) used the gradient plate technique to investigate the effect of triclosan exposure on the antimicrobial susceptibilities of important dental bacteria. Few major changes in triclosan susceptibility were observed, and triclosan exposure caused no significant decrease in the susceptibility of the bacteria towards chlorhexidine, metronidazole or tetracycline. The incorporation of triclosan into dental care products and its beneficial effects in reducing plaque is now considered as one of the justified uses of triclosan.

The work reported in section 3.6 of this thesis addressed the question of whether exposure of *Pr. mirabilis* to triclosan could result in the selection of resistant mutants, and whether any such mutants could gain cross-resistance to antibiotics. Two methods were used to select for mutants with decreased triclosan sensitivities, the 'disk diffusion' and the 'heavy inoculum' method. Interestingly *Pr. mirabilis* swarmed into the zones of inhibition created by the triclosan disks, ruling out the possibility of using this method to generate mutants. The heavy inoculum method was much more productive however and 55 mutants from five different strains of *Pr. mirabilis* were isolated. The MICs and MISCs of each of these mutants are given in Table 6. All mutants had elevated MICs compared to their corresponding wild-type strain. The MIC values ranged from twice to 200 times greater than their respective wild-type strain. A selection of 14 mutants isolated from each of the five wild-type strains with varying degrees of increased resistance to triclosan were chosen for further study. Some resistance phenotypes can be unstable and lost in the absence of a selective pressure (Chapman, 1998). The MIC of the 14 selected mutants was determined after 1, 3, 7, 11 and 15 subcultures in the absence of triclosan. The results (Table 7) reveal that the MICs were generally very stable.

Three of the mutants (M44, M48 and M55) derived from the wild-type strain *Pr. mirabilis* B2 were tested for their sensitivity to triclosan in bactericidal suspension tests. The mean \log_{10} (cfu/ml) reductions given by 100 $\mu\text{g/ml}$ triclosan for these mutants and the wild-type strain *Pr. mirabilis* B2 over three different contact times are presented in Figure 58. Tables 8-10 show the P-values from statistical analysis individually comparing each strain tested in the suspension tests throughout this project at 5, 30 and 60 min. After 5 min contact time, there was no significant difference ($P > 0.05$) in the reduction of viability between the mutants and the wild-type. The \log_{10} (cfu/ml) reductions of M44 and M55 were significantly lower ($P < 0.05$) than the wild-type strain after 30 and 60 min contact time. It must be noted however, that the bactericidal activity was not significantly different ($P > 0.05$) from other wild-type *Pr. mirabilis* strains (NSM 6 and NP 37) after these contact times.

The MICs of triclosan against the three selected *Pr. mirabilis* B2 mutants in artificial urine (3 - 8 $\mu\text{g/ml}$) were significantly higher ($P < 0.05$) than for the wild-type strain (0.2 $\mu\text{g/ml}$). In the case of the mutants, the concentration of triclosan required to keep the artificial urine acidic was 3 - 8 $\mu\text{g/ml}$ (Figure 59). In contrast, only 0.2 $\mu\text{g/ml}$ was required to prevent alkaline conditions developing by the wild-type *Pr. mirabilis* B2 (Figure 10). The ability of these selected mutants to form biofilms and the effect of inflating the balloon with triclosan on their development was determined using the bladder models. Models inoculated with M48 produced results similar to those using the wild-type strain B2 (Table 11). All the controls blocked whilst the triclosan models drained freely for 48 h. All control and test models inoculated with M55 blocked within the 48 h experimental time period. Two replicates blocked whilst the other did not for both the control and test models inoculated with M44. In models

inoculated with M44 and M55, the pH and viable cell counts of the triclosan and control models were not significantly different ($P > 0.05$) at the end of the experiment (Figure 60). The results presented in Figures 60 and 61 also show that triclosan significantly reduced ($P < 0.05$) the calcium and magnesium deposited on catheters in models inoculated with *Pr. mirabilis* B2 and M48, but had no significant effect ($P > 0.05$) in models inoculated with M44 and M55. It is interesting that the MICs of triclosan for M44 and M55 were both 40 $\mu\text{g/ml}$, compared to 2 – 5 $\mu\text{g/ml}$ for M48. It appears then that if the clinical use of triclosan generated mutants with MICs in the region of 40 $\mu\text{g/ml}$ or above, the success of the triclosan strategy to control catheter encrustation could be undermined.

The relative sensitivities of the five wild-type strains and their 14 triclosan resistant mutants to eight antibiotics that are used to treat UTIs caused by Gram-negative bacilli were examined using E-test strips. The antibiotic E-test strips were introduced in the late 1980's. They are plastic strips that release a continuous concentration gradient of a given antimicrobial agent when placed on an agar plate. Research comparing the discriminatory power of the E-tests with conventional susceptibility testing methods soon followed. Baquero *et al.* (1992) showed that the E-tests provided quantitative data similar to using the agar dilution method, and that they were far superior to the disk diffusion tests. A review of the numerous studies comparing the E-tests to other susceptibility testing methods concluded that the E-test compared favourably to other methods (Sanchez and Jones, 1992).

The antibiotic sensitivities of the mutants and the wild-type strains are shown in Table 12. There was some variation between the antibiotic sensitivities of the wild-type *Pr.*

mirabilis strains. For example, *Pr. mirabilis* NP14 and NP43 were sensitive to ampicillin whilst the other three strains were resistant, and *Pr. mirabilis* NP43 was the only strain sensitive to trimethoprim. However, the strains were all equally sensitive to triclosan in terms of MICs (0.1 - 0.2 µg/ml). The eight boxes highlighted in yellow (Table 12) show the mutants with MICs against particular antibiotics higher than that of their corresponding wild-type strain. These increases are only slightly above those of the wild-type strains however, and these types of variations can be expected using the E-test strips with *Proteus*. This data therefore suggests that although these mutants have an increased tolerance to triclosan, there does not appear to be any cross-resistance to the antibiotics tested. The E-test strips give detailed information about the MIC of different antibiotics to bacterial strains, and they are quick and simple to use. They are however expensive to use, therefore only 14 of the mutants were tested. If time and money had not been limited, the stability of all the mutants should have been verified and their antibiotic sensitivities determined.

Training experiments have been used to select for mutants of *Acinetobacter johnsonii* and *Pseudomonas stutzeri* with increased sensitivities to triclosan (Lear *et al.*, 2002). This involves exposing bacterial strains repeatedly to increasing concentrations of antimicrobial agents and checking for any elevated tolerances. In future experiments it would be interesting to see if the training method could be applied to the bladder models. Bladder models with catheters inflated with triclosan could be set up and inoculated, after running the models for a given time, organisms isolated from the urine and any catheter biofilms at the end of the experiment could be used to re-inoculate a series of other bladder-models.

The stability of the mutants' MICs indicates that it would be possible for any mutants that were generated to spread in the absence of triclosan. The results also show that should *Pr. mirabilis* strains with increased triclosan resistance develop, they could interfere with the effectiveness of this novel way of controlling catheter encrustation. However, it must be considered that the generation of mutants in the 'real world' would be in conditions very different from the conditions used in the laboratory setting. It is important that this study has highlighted the potential for the development of *Pr. mirabilis* strains with decreased sensitivities to triclosan, however, the only way to determine if resistance will occur outside the laboratory is to run clinical trials and monitor performance in clinical practice. These studies must screen for any alterations in susceptibilities of organisms to triclosan before and after introducing the strategy into the catheter care regime. Although the data is limited in this present study, it does suggest that should *Pr. mirabilis* mutants arise, their antibiotic susceptibilities will remain unchanged. Future studies may consider testing the sensitivities of the mutants and wild-type strains to other biocides to determine if there is any cross-resistance. Although this study failed to establish a link between triclosan resistance and increased antibiotic tolerance in *Pr. mirabilis*, its effect on other organisms present in the catheterised urinary tract was not tested. Future studies should consider investigating if there is any link between triclosan use and possible increased antimicrobial tolerance to other agents with other bacterial species. Any clinical study should monitor the antimicrobial sensitivities of all the organisms isolated from the catheterised urinary tract.

4.8. Concluding remarks and future work

From the results using the *in-vitro* bladder models inoculated with catheter isolates of *Pr. mirabilis*, the potential for using triclosan in the catheter balloon to prevent crystalline bacterial biofilms has been established. The catheters inflated with the higher concentrations of triclosan prevented catheter blockage for the duration of the seven days experiments. The ability of the catheters inflated with triclosan to resist encrustation by a severe challenge with a pure culture of *Pr. mirabilis* suggests that this approach could have practical applications in controlling catheter encrustation.

Unlike other ways of preventing biofilm formation on urinary catheters, this method provides a multi-pronged bacterial attack. Triclosan diffuses from the balloon and targets planktonic bacteria in the residual urine. In the case of all-silicone catheters, the cells in any biofilms attempting to form on the catheter will be attacked from above by triclosan in the urine and from below by triclosan diffusing from the silicone. Using the balloon also allows the catheter to be loaded with large amounts (up to 100 mgs) of the active agent. This is a much higher concentration than can be used to coat the surface of catheters. For example, Cho *et al.* (2001) were only able to load 1.05 mg gentamicin onto the surface of gentamicin-coated urethral catheters. Even with larger catheters, Darouiche *et al.* (1997) estimated that the total amounts of minocycline and rifampin bound to the length of a size 18 Ch silicone Foley catheter was only 22.3 and 16.4 mg respectively.

The failure of any method currently available to successfully control catheter encrustation fuels the need for the results of this study to be confirmed by a controlled clinical trial. The simplicity of this method that involves using catheters that are

currently in use should facilitate its clinical development. It is important that clinically useful agents such as triclosan are not exploited indiscriminately, therefore the use of triclosan in the catheter balloon should be limited to those patients classified as “blockers”. Before inflating the patients’ catheter balloons with the triclosan solution, the clinical study should firstly monitor patients with their catheter balloon inflated with water. This would provide information about how long the catheters take to block, the urinary pH and the organisms that colonize the catheterised urinary tract before and after triclosan was used to inflate the catheter balloon. The numbers of bacteria colonizing the catheters should also be enumerated before and after introducing triclosan into the catheter care regime. The monitors of the clinical trial would have to make sure that there were no changes to the patients’ daily routine during the study that would affect the outcome of the trial, and any antibiotic use should be noted. A simple sensor that signalled the early stages of encrustation on catheters could give a useful early warning of impending blockage. Such a device would provide a “sensor/ modulator” strategy so that triclosan is exploited only in those situations where it is needed.

Sections of this study have highlighted areas that should be monitored carefully when a clinical trial is held. Any change in patients’ bacterial flora should be carefully observed to check that there is not an increase in bacterial strains resistant to triclosan. By determining the patients’ flora before inflating the balloon with triclosan, any untoward changes in the microbial flora can be monitored. The triclosan sensitivity of bacterial strains isolated prior to and during the study should be continually monitored. No cross-resistance to antibiotics was seen in mutants with higher tolerances to triclosan in this study, however any strains that appear to have increased

triclosan sensitivities should be tested against other antimicrobials. Although it is possible to speculate about a shift in microbial flora, or the development of resistant mutants, the only way we will ever know if these problems are likely to occur are by setting up clinical trials and by monitoring any subsequent clinical practice. If the clinical trial successfully controls catheter encrustation, it would be interesting to determine what the cost savings would be to the health service by lowering the incidence of complications associated with these devices.

Laboratory investigations are needed to continue research in this area, in particular to investigate new agents that could be used in the catheter balloon with triclosan to increase its antimicrobial spectrum. The process of choosing antimicrobial agents to test would be much simpler if there was a rational way of selecting agents that could diffuse through the catheter balloon. Malcolm *et al.* (2003) assayed the release characteristics of eight low-molecular weight drugs from a silicone matrix-type intravaginal ring *in-vitro*. The major factors involved in influencing the permeation of the drugs through the silicone-elastomer were determined. An equation was produced that allowed the release rates of new substances from the silicone-elastomer to be predicted based on their molecular weights and melting points. It would be interesting to collaborate with polymer chemists and perform similar experiments assaying the ability of different antimicrobial agent to diffuse through the balloons of the all-silicone and latex-based catheters. It may be possible to develop an equation which would allow us to predict which type of agents would diffuse best through the latex balloons.

The use of agents other than antimicrobials in the catheter balloon should also be considered. For example, the toxicity of urease inhibitors has restricted their use (Williams *et al.* 1984), therefore this non-systemic way of introducing the agents into the catheterised urinary tract may prevent these side effects. If high enough concentrations of the urease inhibitors could be passed through the catheter balloon, it could be an effective way of controlling catheter encrustation. It may also be possible to use other agents that disrupt biofilm development such as quorum sensing inhibitors. Experiments should investigate the feasibility of delivering antibiotics to treat CA-UTI through the retention balloon. This would allow antibiotics to be delivered directly into the catheterised bladder without exposing the gut flora to their action. It might also be possible to exploit the catheter balloon to deliver pharmacologically active compounds into the bladder.

In future experiments the bladder models should be used to develop a training method that better simulates how resistance in biofilms could develop in the clinical setting. It would also be interesting to observe the control and test catheters inoculated with the different uropathogens using HV-SEM, and to observe in closer detail the effect triclosan has on biofilm formation by *Pr. mirabilis*. Apart from *Pr. mirabilis*, the other urease producing bacteria were only run in the bladder models for 48 h, future experiments could determine if they form crystalline biofilms on longer incubation in the bladder models. The bactericidal activity of triclosan was tested against one strain of each different uropathogenic species, to establish if there are any trends between the inhibitory and lethal effects, more strains of each species should be tested. The bladder model could also be used to determine the concentrations of other agents that are required in the urine to eradicate biofilms or inhibit their development.

There are many other questions that remain to be investigated. For example, can chemical agents be added to the triclosan solution in sodium carbonate to potentiate its delivery into the bladder? Are *Pv. stuartti* and *Pr. vulgaris* sensitive to triclosan? Does triclosan prevent biofilm development by *Pv. stuartti* and *Pr. vulgaris*? What concentration of triclosan passes through the balloon of latex based catheters? What concentration of triclosan impregnates the all-silicone catheter? Is triclosan in sodium carbonate as effective at preventing crystalline biofilm formation in latex catheters as triclosan in 5% PEG?

It is important that the triclosan solution in sodium carbonate can be easily sterilized. Since we have established that triclosan binds to plastic and silicone, it will be necessary to check that the triclosan does not bind to the membrane of the filter. Simple experiments could be carried out to determine the concentration of triclosan in the sample before and after filtration.

Currently there are no effective methods for controlling the problem of catheter blockage by encrustation. If the triclosan strategy can be transferred successfully from the laboratory to the clinic, it could bring major improvements in the bladder management of many elderly and disabled patients. It is important to bear in mind that no particular strategy is likely to provide a “once and for all” final solution to the problem. History warns us that bacteria, with their immense facility for genetic variation, manage to overcome most of the approaches designed for their elimination. It is crucial therefore, that research in this field continues to develop further schemes

to control a problem that profoundly affects the quality of life and well-being of so many people.

4.9. General conclusions

1. Isolates of *Pr. mirabilis* from encrusted catheters were all extremely sensitive to triclosan. Concentrations of 0.1 – 0.2 µg/ml of the agent in agar inhibited swarming and 0.1 – 0.3 µg/ml inhibited growth.
2. The artificial urine MIC tests showed that concentrations of 0.2 µg/ml triclosan prevented the generation of alkaline conditions by *Pr. mirabilis*.
3. In batch cultures in human urine, 0.1 – 1 µg/ml of triclosan had inhibitory effects on the growth of *Pr. mirabilis*, but concentrations of 10 – 100 µg/ml were required to produce a bactericidal effect.
4. Suspension tests in aqueous solutions of triclosan showed that 100 µg/ml were required to produce a bactericidal effect.
5. Experiments in models of the catheterised bladder demonstrated that inflation of all-silicone catheter balloons with triclosan (10 mg/ml in 5% PEG) resulted in diffusion of the biocide through the balloon into the urine. Using the information gained from this experiment about the release rates of triclosan from all-silicone catheters, it was calculated that if the same release rate was maintained, it would take over 850 days for all the triclosan to be released from the catheter balloon. The biocide also became impregnated into the silicone along the whole length of the catheter.
6. The physical properties of catheter balloons inflated with 5% PEG or 10 mg/ml triclosan in 5% PEG and soaked in water for eight weeks were not significantly (P

> 0.05) different from those of the control catheters inflated with water or from catheters taken straight from their packaging.

7. On incubation, the urinary pH in the control models infected with *Pr. mirabilis* rose from 6.1 to over 8, which precipitated encrustation. The control models fitted with all-silicone, silicone-coated latex and biocath catheters all blocked. LV-SEM revealed that the *Pr. mirabilis* control models was heavily encrusted at blockage.
8. By inflating catheter balloons with triclosan solutions it was possible to prevent catheter blockage for the duration of the seven day bladder model experiments. Electron microscopy on test catheters taken out of the models after seven days revealed little sign of encrustation. By introducing triclosan into the catheterised urinary tract it was also possible to maintain acidic urinary conditions and control the numbers of viable *Pr. mirabilis* cells. The ability of triclosan to prevent catheter blockage after a severe challenge with *Pr. mirabilis* suggests that it could have practical applications in controlling catheter encrustation.
9. Catheters impregnated with triclosan in the bladder models or in their packaging were shown to prevent catheter blockage or significantly ($P < 0.05$) delay catheter blockage, as well as significantly ($P < 0.05$) lowering the viable cell count and pH of the residual urine. The results showed that to protect catheters from encrustation by pre-loading them with triclosan prior to their placement in the bladder, it would be necessary to retain the triclosan in the catheter balloon for over an hour, restricting the clinical usefulness of this strategy.
10. Introducing triclosan into the balloon of catheters where encrustation had already begun, had no significant ($P > 0.05$) effect on delaying subsequent catheter blockage. This suggested that if the strategy is to be effective in patients who are

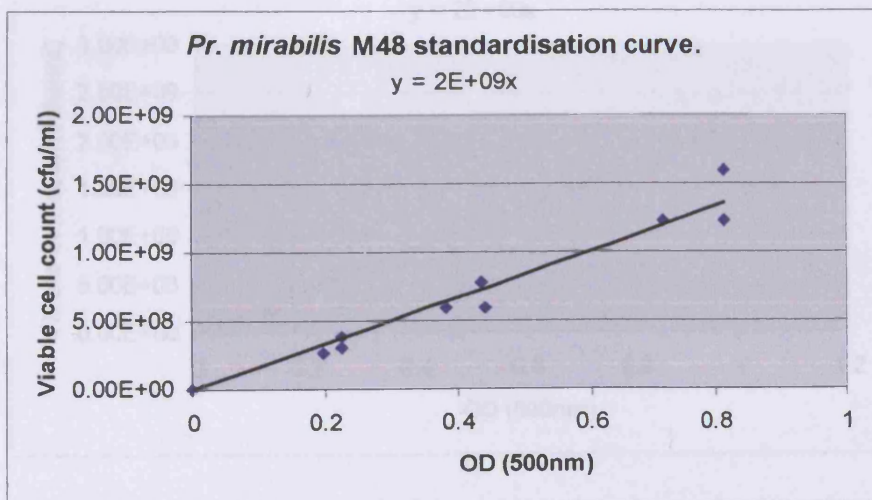
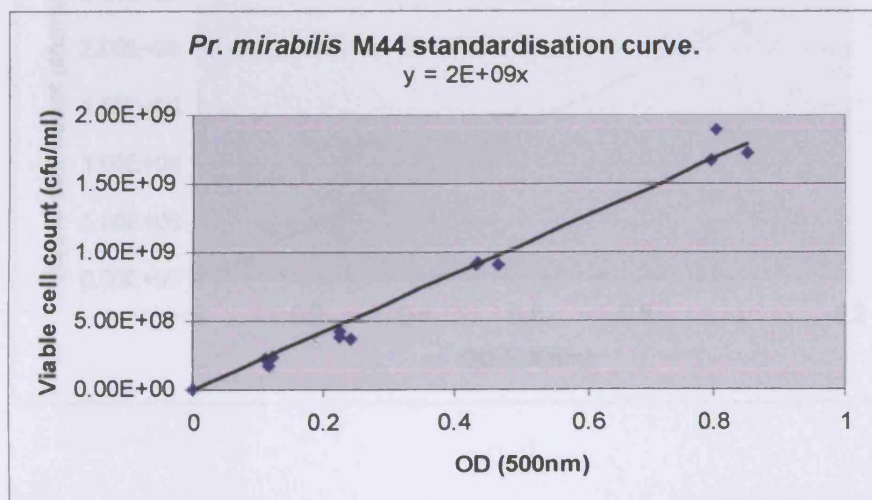
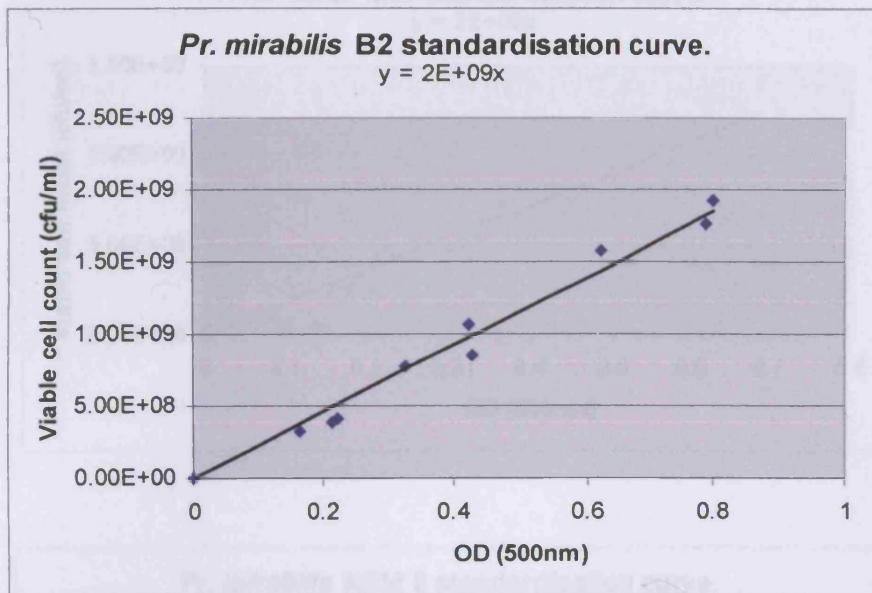
“blockers” it will be necessary to change catheters and then inflate the new catheter with the triclosan solution.

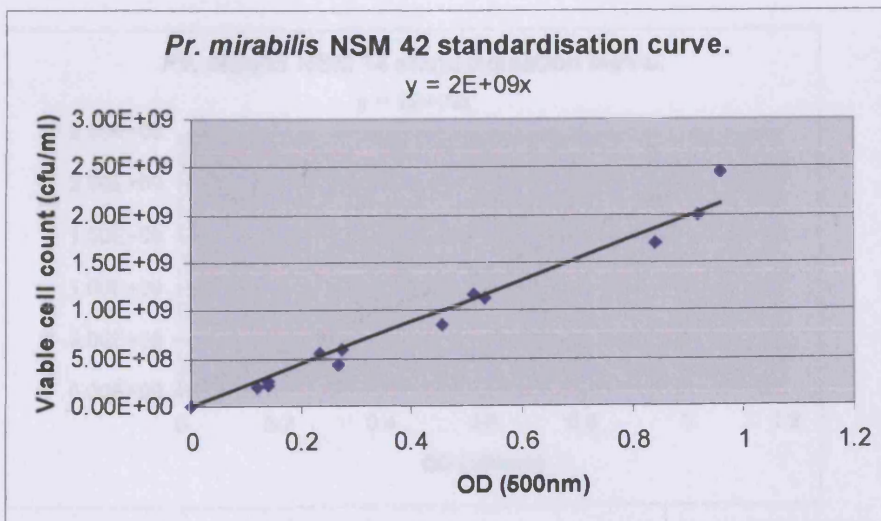
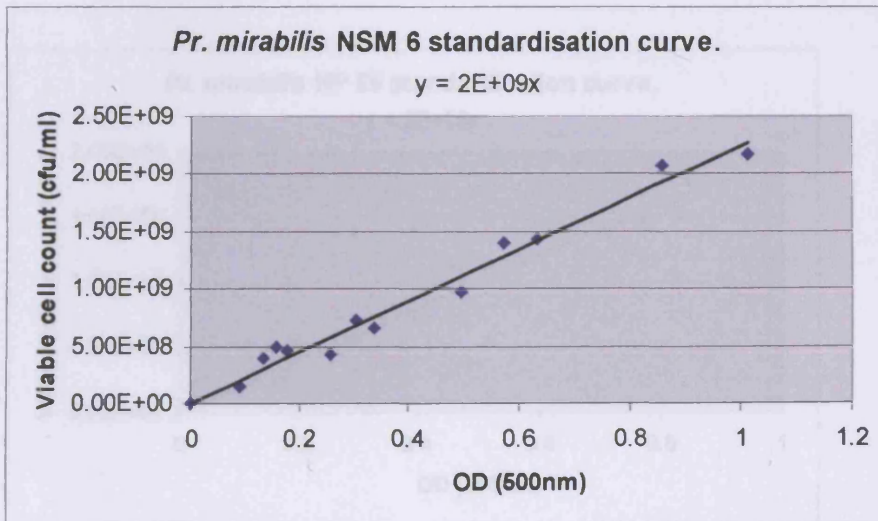
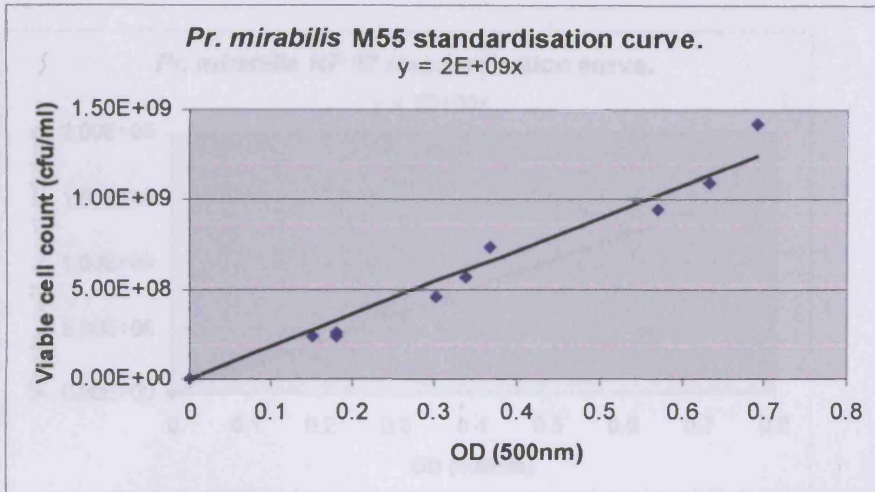
11. Triclosan in 0.1M sodium carbonate solution is much simpler to produce and sterilize than the emulsion in 5% w/v PEG. It is equally as effective in preventing crystalline biofilm formation and would be an appropriate formulation for use in clinical trials.
12. Of the nine different uropathogens tested in the bladder models, only *Pr. mirabilis* was able to produce alkaline urine and form crystalline bacterial biofilms. This is despite the ability of the strains of *Ser. marcescens*, *S. aureus*, *K. pneumoniae*, *Ps. aeruginosa* and *M. morgani* tested to produce the urease enzyme in conventional bacterial identification tests. With the exception of *Ps. aeruginosa*, the development of biofilms by all these species was inhibited by triclosan.
13. If clinical trials introducing triclosan into the catheter care regime go ahead, the microbial ecology of the urinary tract should be monitored closely for any untoward shifts in microbial flora.
14. In the laboratory it is possible to generate mutants of *Pr. mirabilis* that are resistant to triclosan. Tests in the bladder model indicate that these mutants could form crystalline biofilms on catheters that had been treated with triclosan. All the strains of *Pr. mirabilis* from encrusted catheters were shown to be extremely sensitive to triclosan, but if resistant strains arise in clinical practice, the efficacy of the strategy would be undermined.
15. It is important that this study has highlighted the potential for the development of *Pr. mirabilis* strains with decreased sensitivities to triclosan, however, the only way to determine if resistance will occur outside the laboratory is to run clinical trials and monitor performance in clinical practice.

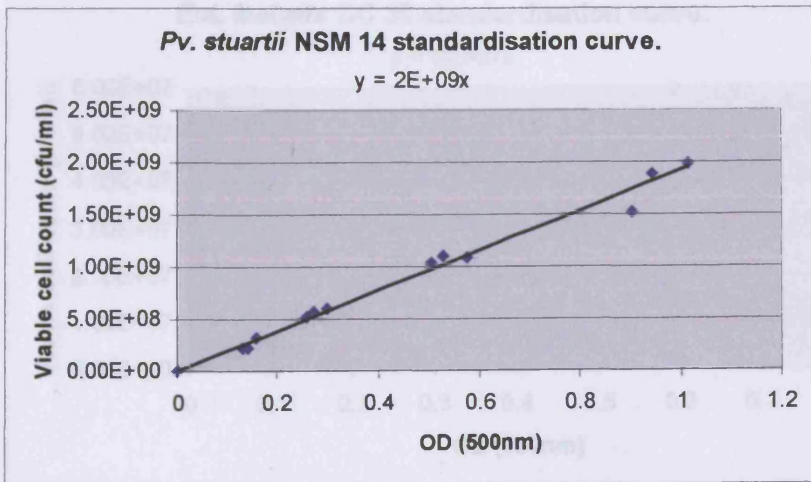
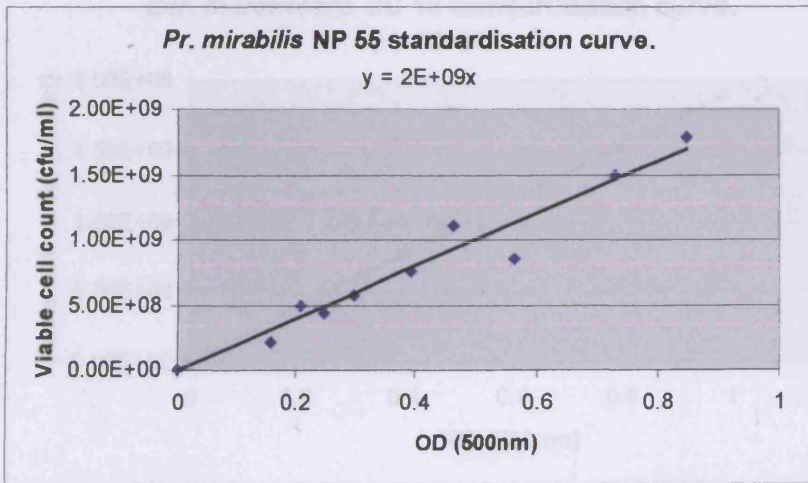
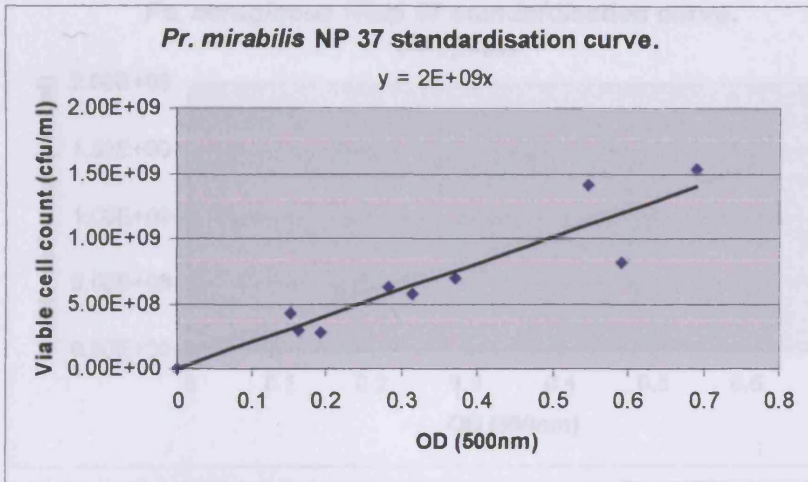
16. The triclosan resistant mutants generated in the laboratory were not cross-resistant to any of the eight antibiotics tested.
17. The results from this study suggests that if this novel strategy for controlling catheter encrustation could be transferred successfully from the laboratory to the clinic, it could improve the quality of life and well-being of many elderly and disabled people who are currently enduring the complications associated with long-term indwelling bladder catheterisation.

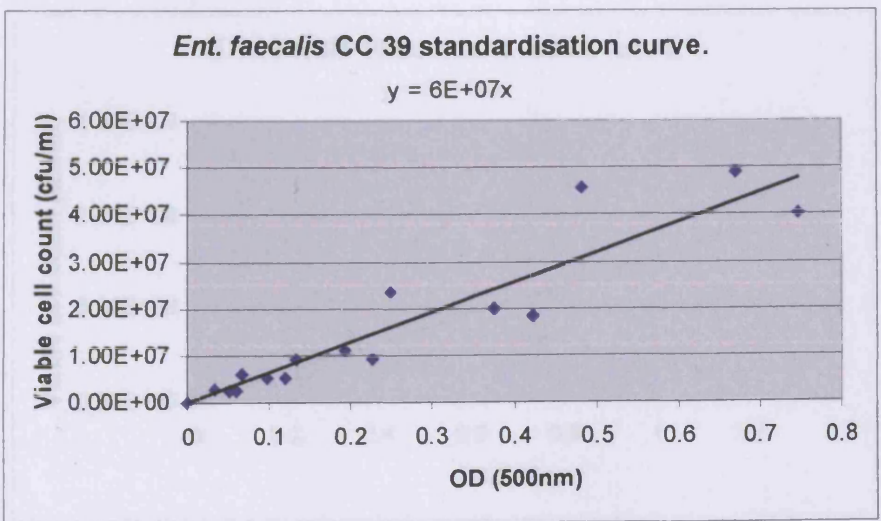
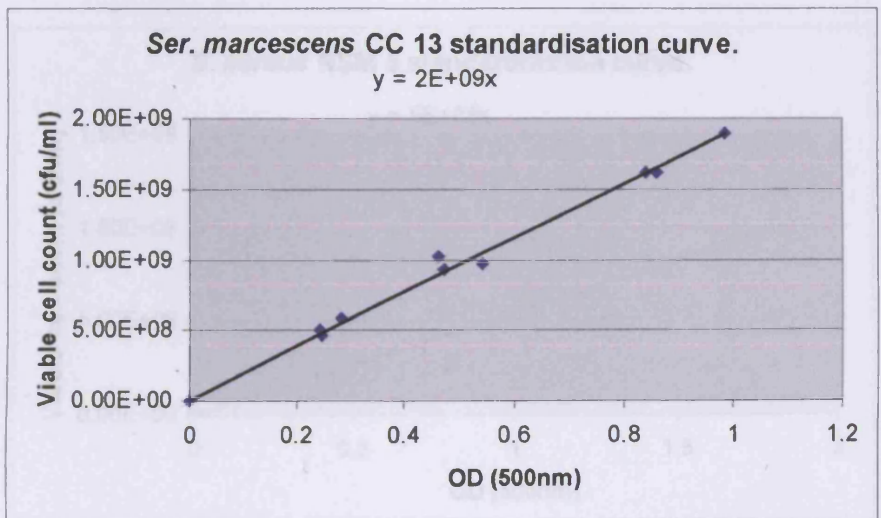
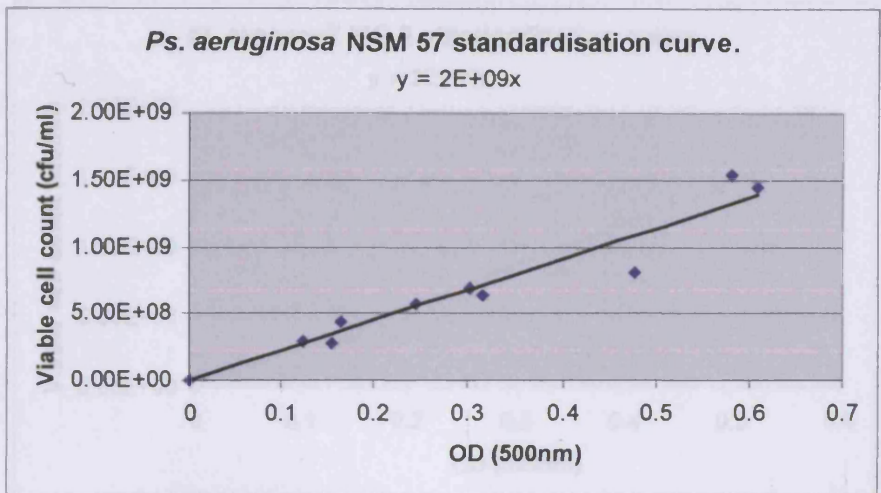
5. APPENDICES

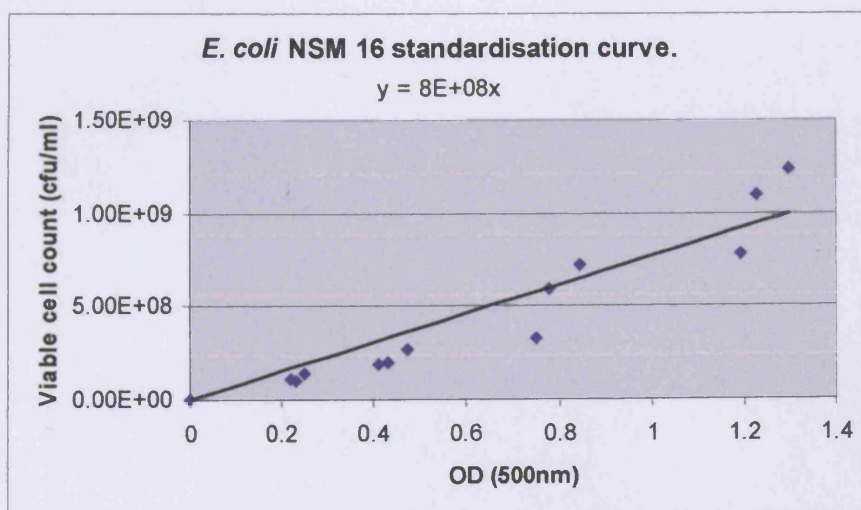
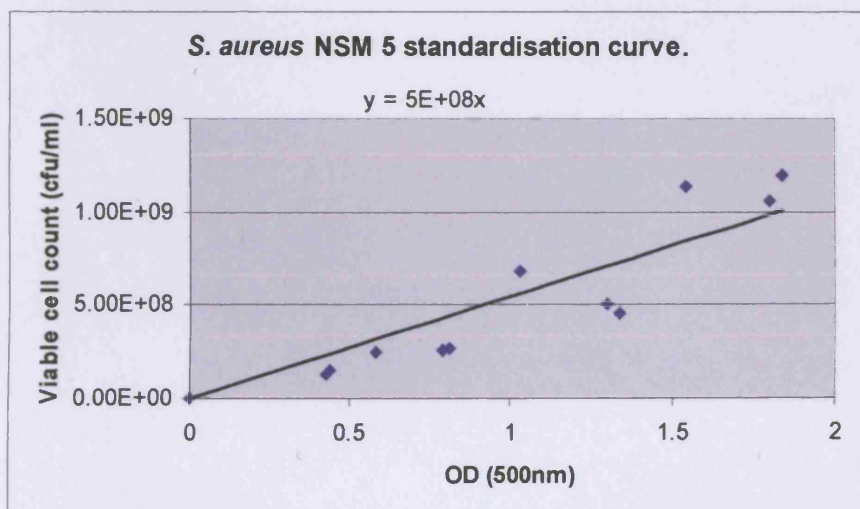
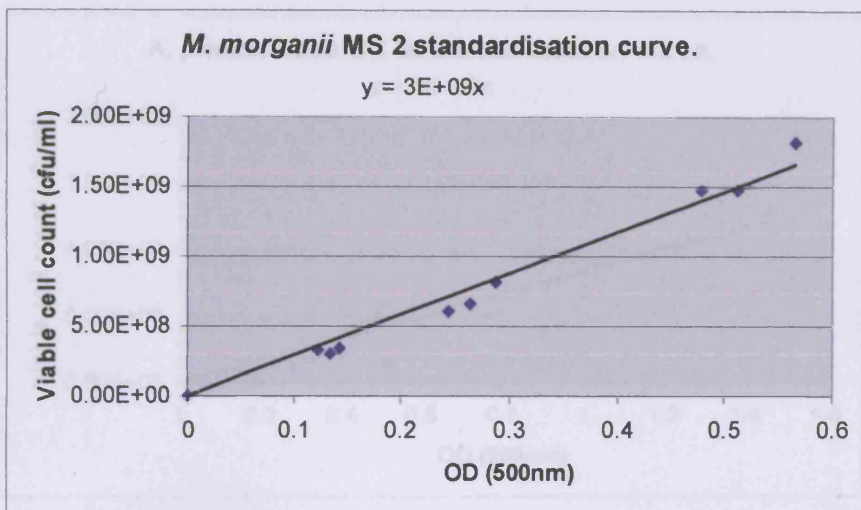
Appendix 1: Calibration curves used to prepare standardised inocula of 1×10^9 cfu/ml for use in the bactericidal tests. The standardisation curves were produced from three individual experiments. On each graph, the equation for the gradient of the curve is indicated.

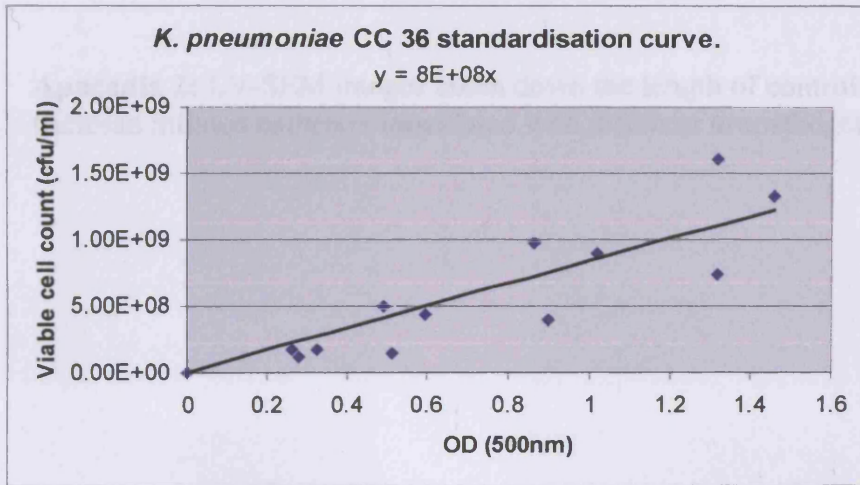




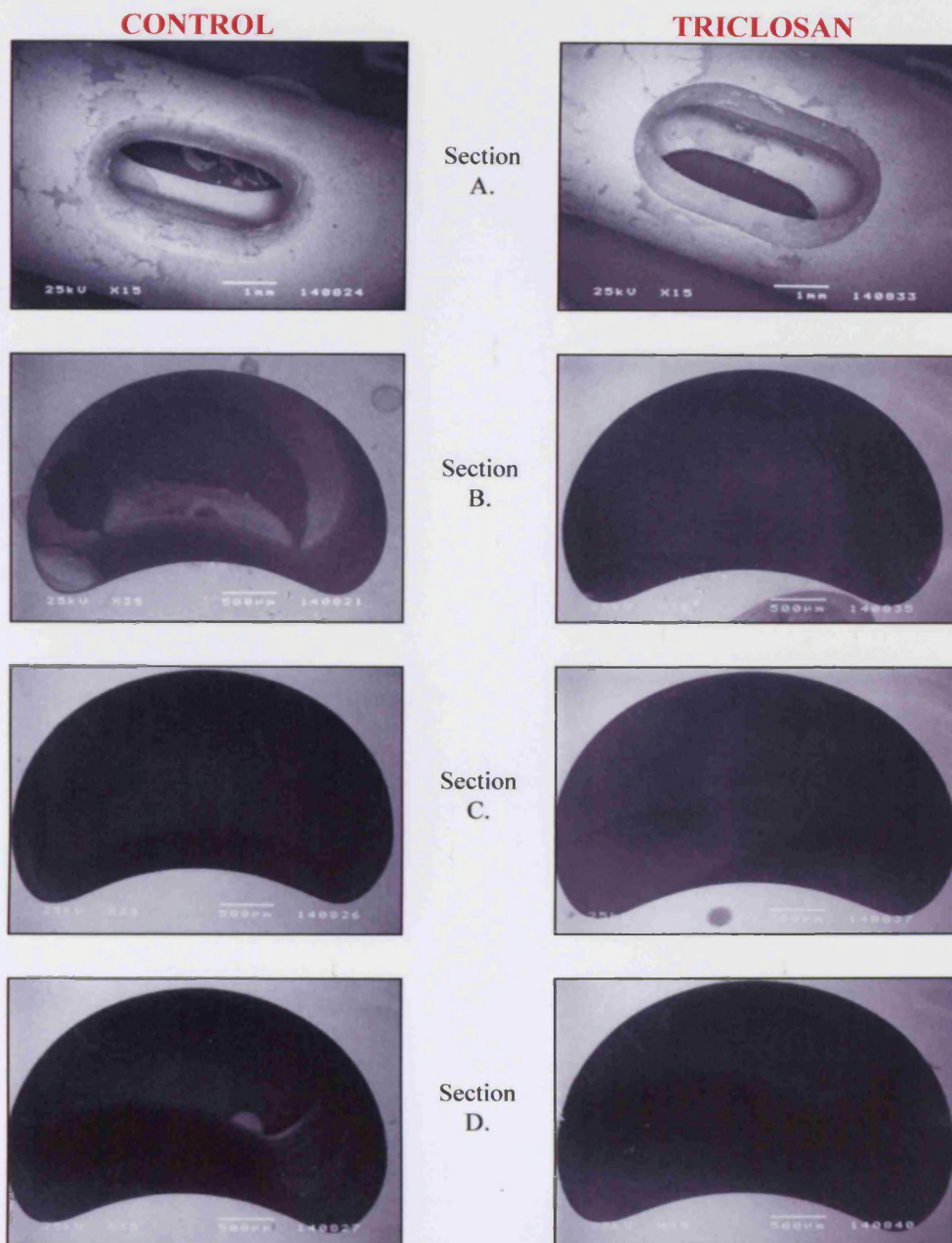




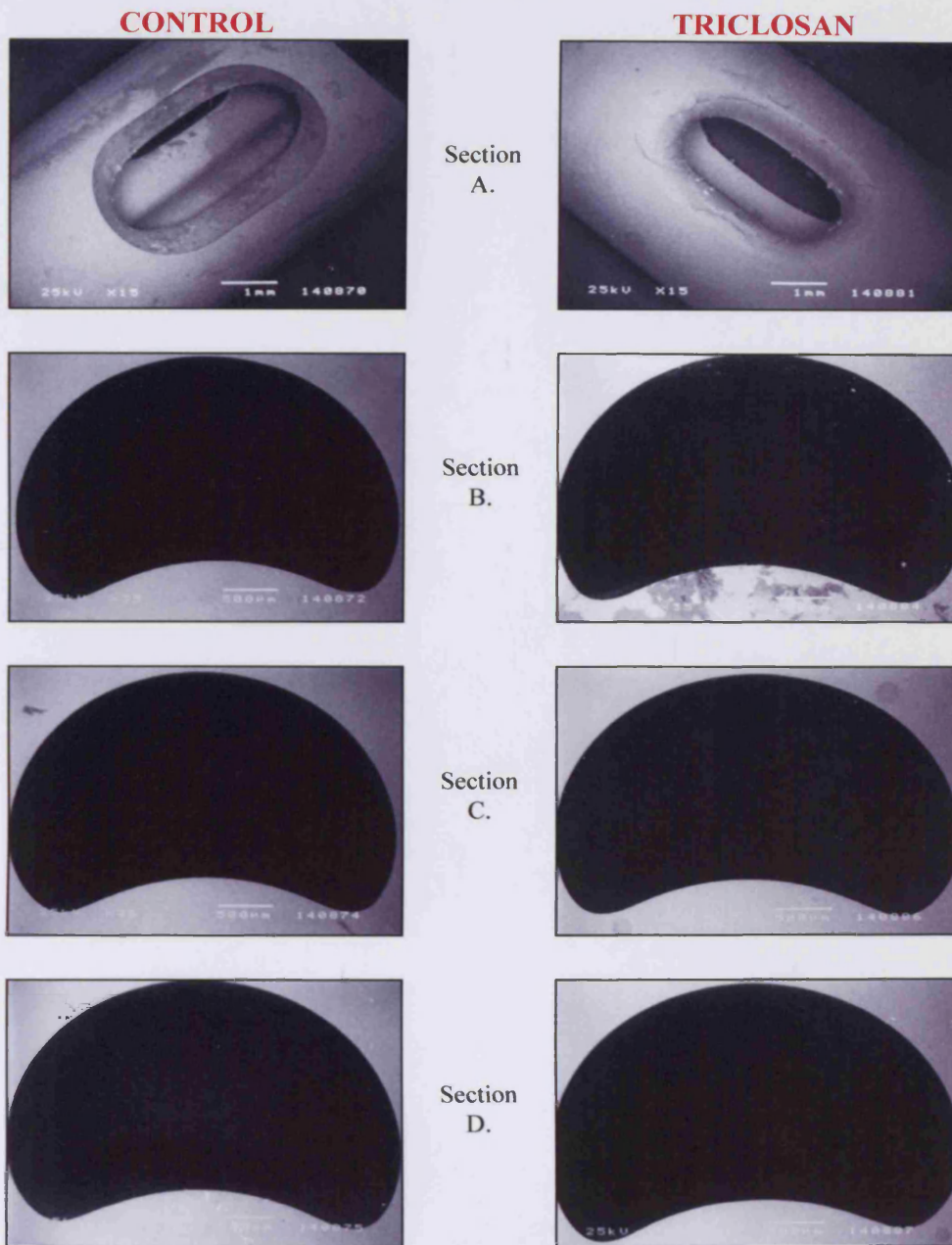




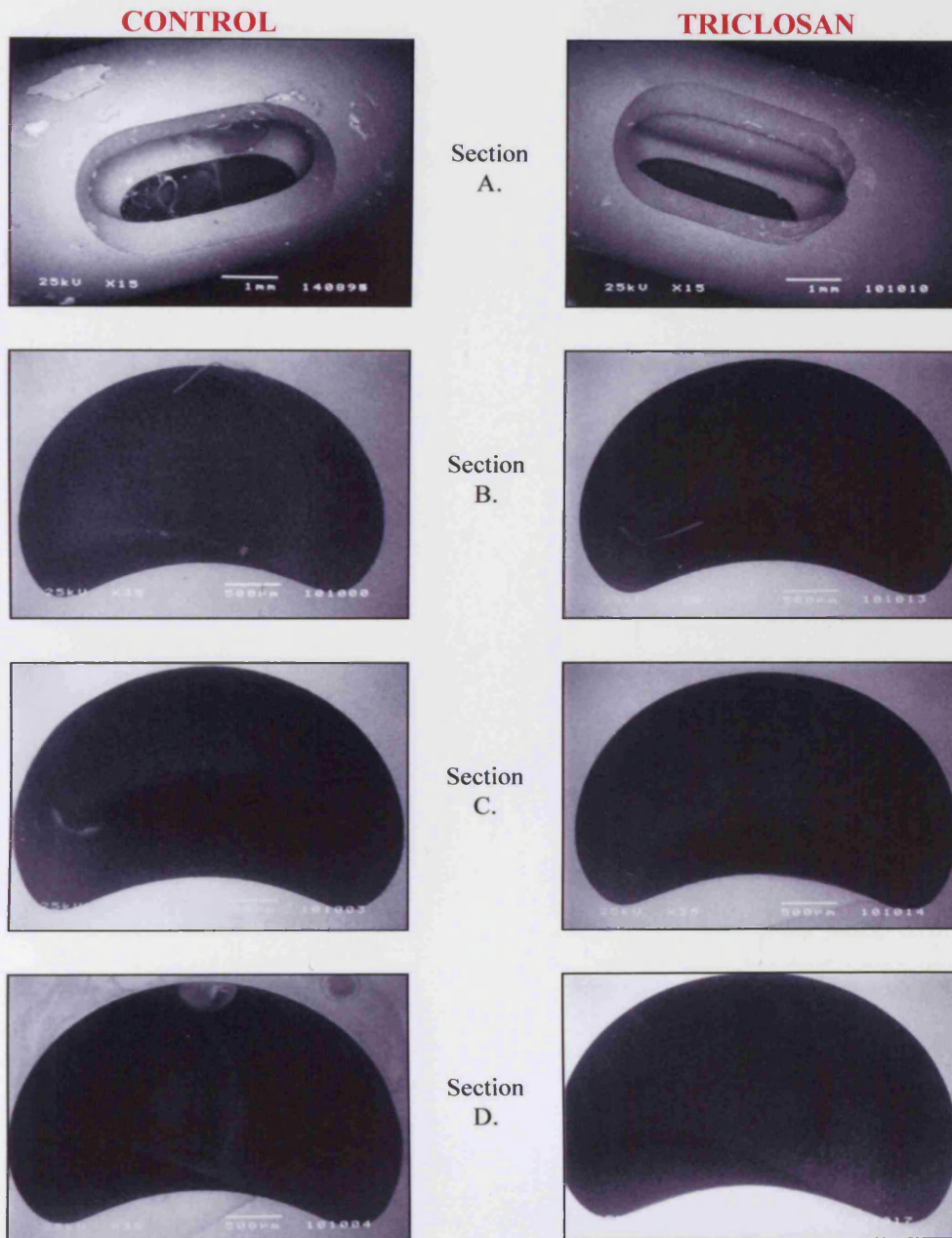
Appendix 2: LV-SEM images taken down the length of control and triclosan inflated catheters inoculated with different uropathogens.



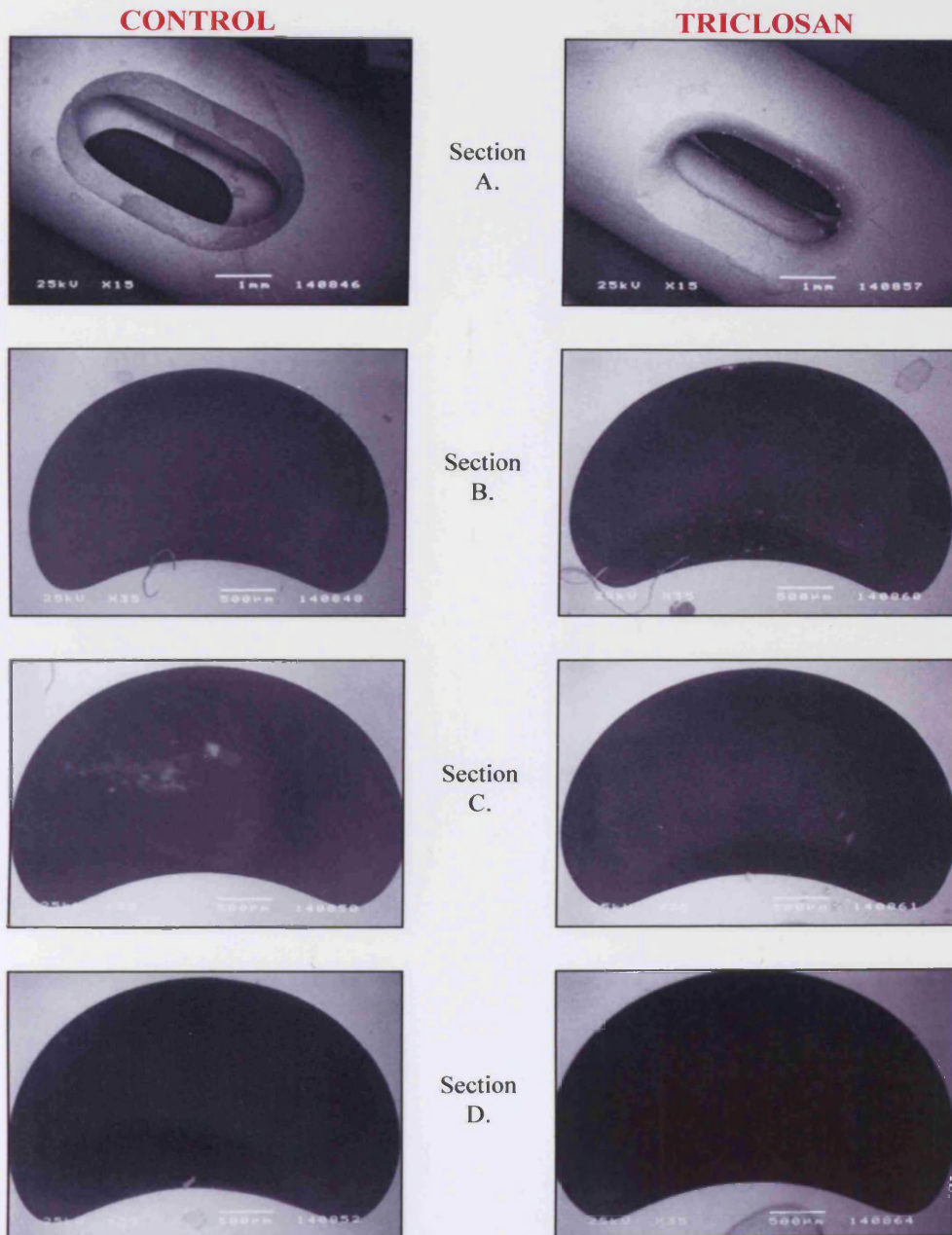
Appendix 2a: Electron micrographs of control and triclosan treated catheters inoculated with *Ser. marcescens* after 48 h. The positions of sections A-D are indicated in Figure 6.



Appendix 2b: Electron micrographs of control and triclosan treated catheters inoculated with *Ent. faecalis* after 48 h. The positions of sections A-D are indicated in Figure 6.

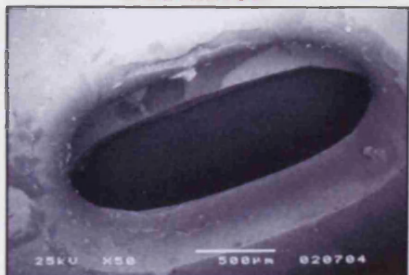


Appendix 2c: Electron micrographs of control and triclosan treated catheters inoculated with *M. morganii* after 48 h. The positions of sections A-D are indicated in Figure 6.



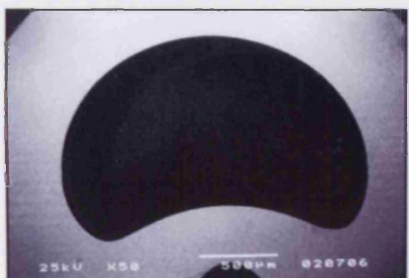
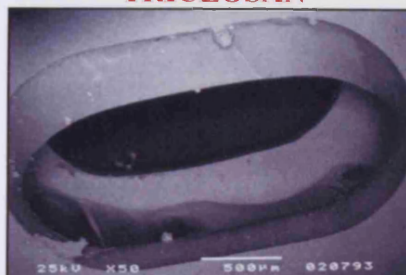
Appendix 2d: Electron micrographs of control and triclosan treated catheters inoculated with *Pv. stuartii* after 48 h. The positions of sections A-D are indicated in Figure 6.

CONTROL

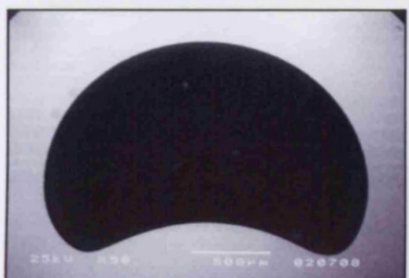


Section
A.

TRICLOSAN



Section
B.



Section
C.

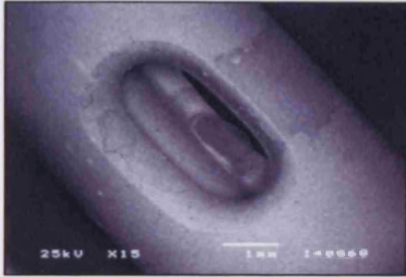


Section
D.



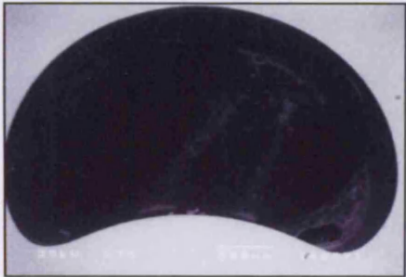
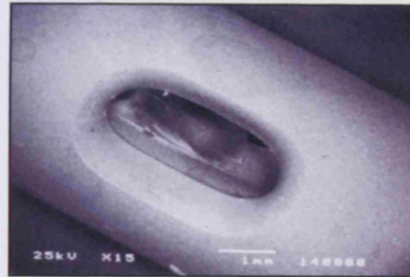
Appendix 2e: Electron micrographs of control and triclosan treated catheters inoculated with *K. pneumoniae* after 48 h. The positions of sections A-D are indicated in Figure 6.

CONTROL

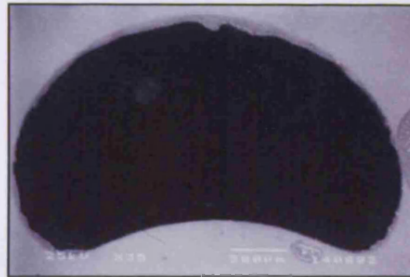


Section
A.

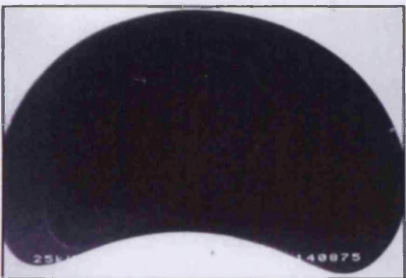
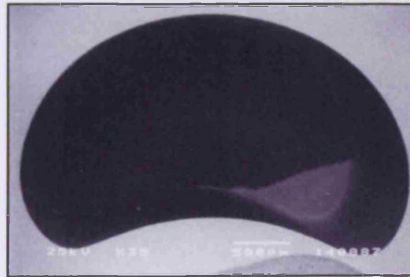
TRICLOSAN



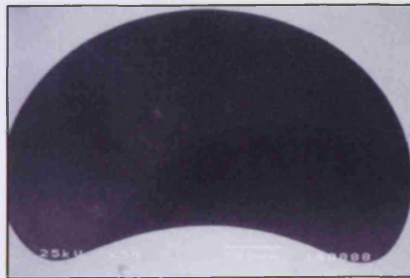
Section
B.



Section
C.



Section
D.



Appendix 2f: Electron micrographs of control and triclosan treated catheters inoculated with *S. aureus* after 48 h. The positions of sections A-D are indicated in Figure 6.

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