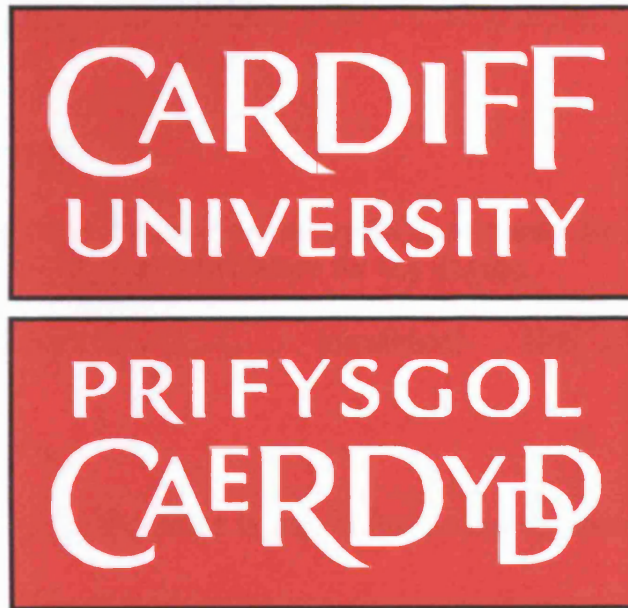# A STUDY OF THE DEVELOPMENT OF CRYSTALLINE *PROTEUS MIRABILIS* BIOFILMS ON URINARY CATHETERS

Thesis presented for the Degree of Philosophiae Doctor

By

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Summary

Infection by *Proteus mirabilis* can seriously complicate the care of patients undergoing long-term indwelling bladder catheterisation. The urease-producing bacteria colonize the catheter surfaces forming extensive biofilm communities and are capable of generating ammonia from urea and elevating the pH of the urine and biofilm. Under these conditions crystals of calcium and magnesium phosphates form in the urine and within the bacterial biofilm on the indwelling device leading to its encrustation and blockage. Urine can leak around the outside of the blocked catheter and patients become incontinent. Alternatively, urine is retained within the bladder, causing painful distension of the bladder. Reflux of infected urine to the kidneys can lead to serious symptomatic episodes such as pyelonephritis, septicaemia and endotoxic shock. All available types of indwelling catheter are vulnerable to this problem and currently there are no effective procedures available for its control.

While the basic mechanism has been established for catheter encrustation we still need to know more about some of the fundamental aspects of the process. Little is known about the early events and the precise mechanisms which *P. mirabilis* uses to colonize catheter surfaces. The factors that control the rate at which crystalline biofilm forms on the catheters are also unknown. The aims of this study were to establish the sequence of events in the early stages of crystalline *P. mirabilis* biofilm formation on the range of currently available catheters for use with patients; to determine the role of Mannose-Resistant *Proteus*-like fimbriae (MR/P fimbriae) in *P. mirabilis* crystalline biofilm formation on catheters; to investigate how the pH at which calcium and magnesium phosphates precipitate from urine, the nucleation pH (pH_n) can be manipulated and to determine the effect of this parameter on the rate of catheter encrustation.

Using a laboratory model of the catheterised bladder, scanning electron microscopy and X-ray microanalysis, the initial stages of *P. mirabilis* crystalline biofilm development was observed on catheter surfaces. All-silicone, silicone-coated latex, hydrogel-coated latex and hydrogel/silver-coated latex catheters rapidly acquired a microcrystalline 'foundation layer' comprised predominantly of calcium phosphate, upon which, *P. mirabilis* crystalline biofilm subsequently developed. A similar 'foundation layer' was observed on the encrusted surfaces of hydrogel/silver-coated catheters removed from long-term catheterised patients. The catheters impregnated with nitrofurazone briefly delayed the onset of crystalline biofilm formation, while all-silicone and hydrogel-coated latex catheters inflated with triclosan (3 mg/ml in Na_2CO_3) were able to maintain acidic urine pH and prevent crystalline biofilm development for the 7 day experimental period.

There is evidence that MR/P fimbriae are involved in initiating infection in non-catheterised urinary tracts. The role of these adhesins in crystalline biofilm formation on indwelling catheters however, has not been investigated. Using bladder models infected with a wild type *P. mirabilis* strain able to express MR/P fimbriae and its derived MR/P-negative mutant, time to catheter blockage experiments and scanning electron microscopy revealed that MR/P fimbriae were not essential for *P. mirabilis* colonization of catheter surfaces or the development of crystalline *P. mirabilis* biofilm. Although the wild type and mutant strain initiated biofilm formation in different ways both rapidly blocked all-silicone catheters with crystalline material. The overriding factor in catheter blockage was the generation of alkaline urine, raising the pH above that at which crystalline formations develop.

Previously it has been demonstrated that the pH_n of human urine can be elevated by dilution and by increasing its citrate content. In the present study the effect of dilution and adding citrate on the pH_n of artificial was assessed. Furthermore, the effect on the rate of encrustation on all-silicone catheters was examined in laboratory models supplied with these urines and infected with urease-positive *P. mirabilis*, *Providencia rettgeri* and *Proteus vulgaris*. The pH_n of urine could be elevated from pH 6.7 in neat urine to pH 8.4 in urine diluted to 1:6. When neat, 1:1, 1:2 and 1:3 diluted urines were supplied to bladder models significant increases in catheter lifespan were recorded at each ascending dilution. Increasing the citrate content of the 1:1 diluted urine from 0 to 3.0 g/L citrate elevated the pH_n from pH 7 to pH 9.1. Scanning electron microscopy of catheter sections revealed crystalline material in the biofilms could be virtually eliminated for at least 7 days in models supplied with urine with pH_ns of >pH 8.5. Time to catheter blockage experiments showed the rate of catheter encrustation became significantly reduced as the pH_n of urine increased. Catheters in models supplied with urine containing citrate concentrations of 1.5 mg/ml (pH_n >8.4) or more drained freely for the whole 7-day experimental period. Bladder models supplied with urine containing 1.5 mg/ml citrate and infected with *Proteus vulgaris* and a potent urease-producing isolate of *Providencia rettgeri* also drained freely for significantly longer than respective control experiments.

The urinary pH_n of long-term catheterised patients infected with *P. mirabilis* varies considerably. If the urinary pH_n in these patients could be elevated consistently above that produced in *P. mirabilis* infected urine, it would prevent or significantly reduce episodes of catheter blockage. The effect of supplementing the daily diet of a healthy volunteer with citrate–containing drinks on urinary pH_n and the rate of catheter encrustation was determined using the bladder model. A significant elevation in the pH_n to 8.1 was recorded in the urine when the volunteer consumed 2 L of a citrate-containing drink as part of their daily fluid intake. Furthermore, the mean lifespan of catheters from *P. mirabilis* infected bladder models supplied with this urine was nearly five times longer (P <0.05) than those supplied with urine collected on a standardised fluid intake of 1.3 L/24 h. This was encouraging as the pH of the bladder model urine was consistently more alkaline than is 'normal' for long-term catheterised patients infected with *P. mirabilis*. The citrate drinks strategy will not of course eliminate the causative organisms of encrustation from the catheterised urinary tract but would be a long-term suppressive therapy. Despite this, it has intrinsic advantages in that it doesn't involve the use of antimicrobials and it is effective whatever urease producer is inducing the encrustation. The selection of resistant organisms will thus not be a problem. Will the strategy be effective in the long-term? Will it create other problems? Only experience will answer these questions. At this stage however, it seems to offer the exciting prospect of control of a complication that undermines the quality of life for so many elderly and disabled people.

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1. Introduction

1.1. Urinary incontinence, the catheter and bladder management

The International Continence Society (ICS) describes urinary incontinence (UI) as 'a condition where involuntary loss of urine is a social or hygienic problem and is objectively demonstrable' (Andersen *et al.*, 1992). Individuals suffering from severe, chronic incontinence have a considerably impaired quality of life. The complications that can arise in the management of urinary incontinence also pose serious threats to the health of many elderly and disabled people.

1.1.1. The prevalence of urinary incontinence

A working party of the Royal College of Physicians estimated that around three million people in the UK suffer from urinary incontinence. It is particularly common in the elderly with as many as 25% of the patients in residential homes and 40% of those in nursing homes being affected (Royal College of Physicians, 1995). Demographic analysis suggests that by 2030 the proportion of over 60 year olds in Britain will increase to about one third of the population (Greengross *et al.*, 1997). Given the ageing population, urinary incontinence is likely to become more of a problem in the coming years.

1.1.2. The indwelling urinary catheter

Indwelling urinary catheters (IUC's) are used to manage chronic urinary incontinence and urinary retention in the elderly and for patients disabled by neurological disease, strokes and spinal cord injury. They also have important short-term roles in hospitals for the accurate urinary measurement of unconscious patients and as an aid to urethral repair after prostatic or gynaecological surgery (Stickler, 1996). Catheters have a long

history in urological care and their origins can be traced back over 2000 years to the

Hippocratic era of ancient Greece (Moog *et al.*, 2004). Initially they were fashioned

out of various materials (including tanned animal skins), although malleable metals

appeared to be the preferred option (Abdel-Halim, 1990). In the 19th century rubber

catheters were produced by the vulcanization of latex, and by the early 20th century

Dr. Frederick Foley designed and developed the self-retaining catheter (Foley, 1929;

Foley, 1937). The catheters used today are essentially the same as those developed by

Foley seventy years ago.

The basic design consists of a hollow drainage tube approximately 40 cm in length.

Catheters are available in various sizes having a range of external diameters, which

are measured in Charrière (Ch) units. One Ch unit is equivalent to 0.33 mm so the

commonly used size 12 Ch catheter has an external diameter of 4 mm. The tube is

usually inserted into the bladder via the urethra (Figure 1) and is held in position by

means of the retention balloon, which is generally inflated with 10 ml of water after

insertion. Urine drains through the catheter lumen from the bladder and collects in an

external drainage bag. Suprapubic catheterisation also involves the use of the Foley-

type catheter. In these cases the catheter is inserted through a narrow channel created

surgically in the abdominal wall, just above the pubic bone. The balloon is

subsequently inflated to hold the device in place and the catheter will remain

indwelling. Some patients prefer this method of catheterisation but IUC's are

generally used more widely (Kohler-Ockmore and Feneley, 1996).

Figure 1. Male urinary tract catheterised with a Foley-type catheter

1.1.3.   Prevalence of indwelling bladder catheters

Prevalence studies have revealed the extent to which Foley catheters are used for bladder management in healthcare facilities worldwide. Jepsen *et al.* (1982) for example performed such a study in hospitals in eight European countries and reported that 11% of patients were undergoing indwelling catheterisation. Zimakoff *et al.* (1993) found that 13.2% of hospital patients in Denmark were catheterised. A survey of residents in nursing homes in Maryland (USA) found that 7.5% had bladder catheters (Warren *et al.*, 1989). Figures from similar institutions in Denmark and the UK were 4.9% and 9% respectively (Zimakoff *et al.*, 1993; McNulty *et al.*, 2003). It is also clear that many patients in community care in their own homes have Foley catheters. Getliffe and Mulhall (1991) found for example that 4% of this group in the UK were catheterised.

The extensive use of indwelling bladder catheters has occurred in spite of concerns over the risks they pose to patients. In his 1958 editorial, Paul Beeson warned of the dangers of indwelling catheterisation and its relation to serious infections of the upper urinary tract. "At times the catheter is indispensable for therapy, and there are many good indications for its use. Nevertheless the decision to use this instrument should be made with the knowledge that it involves risk of producing a serious disease which is often difficult to treat" (Beeson, 1958). In spite of grave concerns surrounding the use of indwelling catheters, studies have indicated that many patients are catheterised unnecessarily, or remain catheterised for longer than is necessary. In a prospective study of 202 patients attending a US hospital, Jain *et al.* (1995) concluded that the decision to catheterise was unjustified in 21% of cases. In addition they concluded that 47% of the 912 patient days where catheters were in place were unjustified.

Recent guidelines on incontinence management published by The Department of Health reiterated the need for proper use of the indwelling catheter. They stressed that only after all other avenues are exhausted should catheterisation be considered, and that the patients need for continuing catheterisation should be regularly assessed and the catheter removed as soon as possible (Panknin and Althaus, 2001). However, Huang *et al.* (2004) demonstrated that many catheters are still left indwelling for longer than is necessary. They also demonstrated the benefits of proper catheter management with the timely removal of the devices, significantly reduces the rates of infection, the use of antimicrobial therapy and costs.

1.2. Complications of long-term catheterisation

Long-term catheterisation of the urinary tract causes increased morbidity and mortality in patients. It can lead to trauma of the lower urinary tract, bladder carcinoma, infective bladder and kidney stones, urinary tract infection, bacteriuria, pyelonephritis, bacteraemia and septicaemia. In addition they impose a heavy workload on healthcare workers and place a significant financial burden on healthcare services (Stickler and Zimakoff, 1994).

1.2.1. Urethral necrosis

The physical presence of a urinary catheter causes pressure necrosis, basically meaning erosion of the bladder and urethral mucosa (Larsen *et al.*, 1989; Larsen *et al.*, 1997; Stoffel and McGuire, 2006). Pressure necrosis of the lower urinary tract in spinal cord injured (SCI) patients can also be caused by extrusion of the inflated balloon from the bladder due to recurring detrusor contractions. Replacing the device with a larger bore catheter does not resolve the situation and can even exacerbate the

situation (Andrews and Shah, 1998). Although the problem can sometimes be resolved through suprapubic catheterisation, an irrevocably damaged urethra may require radical surgery to redirect urine flow and return continence (Andrews and Shah, 1998; Stoffel and McGuire, 2006). Cases of urethral necrosis have been reported being more prevalent in women patients as there is a lack of alternative external collection devices for this group (Andrews and Shah, 1998). Trop and Benett (1992) reported that 92% of women with long-term catheters eventually suffered with urethral erosion.

1.2.2. Bladder carcinoma

A number of studies have reported a high incidence of bladder cancer among individuals with spinal cord injuries (SCI). El-Masri and Fellows (1981) for example examined the medical records of 6000 patients who had attended the spinal injury unit at Stoke Mandeville Hospital and found 25 cases of bladder carcinoma. This rate was 20-fold greater than would be expected from consideration of the rate of disease in the general population. Similar results have been reported by spinal cord units in the USA (West *et al.*, 1999; Groah *et al.*, 2002). It is probable that several factors are responsible for the increased rate of bladder cancer in this group. It should also be recognized that many paraplegic are injured in their youth and some endure catheterisation for up to 30-40 years. Akaza *et al.* (1984) proposed that the mere implantation of the catheter, which could lead to chronic inflammation of the bladder mucosa for many years, was the stimulus for carcinogenesis. Tricker *et al.* (1991) found nitrosamines in the infected urine of catheterised spinal-injured patients. These powerful carcinogens are generated in the urine by the metabolic activity of the bacterial flora. They may well also have a role in the development of these cancers.

1.2.3. Urinary tract infection, bacteriuria and bacteraemia

Catheter-associated urinary tract infections (CAUTI) account for approximately 40% of hospital infections (Haley *et al.*, 1985; Harbarth *et al.*, 2003). While catheters are a convenient way to drain urine from a disabled bladder, they also form a bridge for bacteria from a contaminated external environment to infect a vulnerable body cavity. The incidence of bacteriuria in catheterised patients increases with duration of catheterisation. Garibaldi *et al.* (1974) examined daily urine specimens from 405 newly catheterised patients in a US community hospital. They reported that the prevalence of bacteriuria increased each day after catheterisation, and after a period of only 10 days 50% of these patients were bacteriuric. Once organisms have infected the residual urine in the bladder, they multiply rapidly and reach $> 10^5$ cfu/ml within a few days (Stark and Maki, 1984). Saint (2000) combined the data from several studies that examined the rates of bacteriuria in individuals catheterised from 2 to 10 days. The pooled cumulative incidence of bacteriuria in the duration of these short-term catheterised patients was 26%.

Other reports have demonstrated that after continuous catheterisation for 1 month virtually 100% of individuals will have bacteriuria, and in many cases mixed communities of species will be present in the urine (Warren *et al.*, 1982). Jewes *et al.* (1988) monitored 115 community dwelling patients in the UK and found that after 8 weeks with an indwelling catheter all except 2 patients (who were both on antibiotic therapy) were positive for bacteriuria. This study also reported bacteriuria increased with the duration of catheterisation. It is important to state however, that the majority of bacteriuric episodes do not develop into symptomatic urinary tract infection. Tambyah *et al.* (2000) studied 1497 patients and reported that 14.9% developed

CAUTI, described as $\geq 10^5$ organisms per ml urine. Only 7.7% of these however, were symptomatic infections. Kunin *et al.* (1987a) also found a small proportion of those with bacteiuria developed symptomatic UTI. They reported that although 74% of elderly catheterised nursing home patients were positive for CAUTI less than 2% presented with febrile episodes.

Several other studies have shown that CAUTI can lead to bloodstream infection (bacteraemia). A multi-centre prevalence survey on hospitalized patients carried out by Jepsen *et al.* (1982) reported that the development of bacteraemia was five-times higher in patients with UTI than those without, and this could be directly related to the use of an indwelling urinary catheter. Jewes *et al.* (1988) found that transient, asymptomatic bacteraemia occurred in 10.2% of catheterised patients after routine catheter changes. Coello *et al.* (2003) studied device related bacteraemia in 73 UK hospitals. Although they reported that intensive care units and special baby care units had high incidence of bacteraemia, they also found that in general medicine, general surgery, geriatric medicine and urology in non-teaching hospitals bacteraemia from CAUTI caused 20.9% of all episodes. The development of sepsis in 126 catheterised patients was studied retrospectively by Rosser *et al.* (1999). They reported that in 15.8 % of these patients, urosepsis (inflammation of the upper urinary tract causing bacterial seeding of the blood) was responsible, with patient age and duration of catheterisation among the significant risk factors. Those without sepsis had a catheter in place for a mean of 16.6 days while those that developed urosepsis had a catheter in place for a mean of 29.3 days.

1.2.4. Bladder and kidney stones

The formation of mineralized aggregates in the urinary tract of individuals undergoing long-term bladder catheterisation is relatively common. Their formation can be due to factors including nutrition or metabolic imbalance (idiopathic), or can be the result of chronic infection with urease-producing bacteria (Grifith *et al.*, 1976; Bichler *et al.*, 2002; Siener and Hesse, 2002; Siener *et al.*, 2005). In SCI patients elevated calcium levels in urine (hypercalcuria) caused by immobilization is common in the first three months after injury and the risk of forming metabolic bladder and kidney stones is highest in this period (DeVivo *et al.*, 1984; Chen *et al.*, 2001). The presence of an indwelling catheter however, significantly increases the risk of developing infected bladder and kidney stones. In a retrospective study of 1336 SCI patients over 25 years, Chen *et al.* (2001) reported that individuals with indwelling catheters were nine times more likely to form bladder stones in the first year than those who were catheter-free and had bladder control. In a 15-year study of 1359 SCI patients in Australia, Donellan and Bolton (1999) reported that 3.5% of patients were treated for upper urinary tract calculi. Those that developed kidney stones had a significantly higher incidence of indwelling catheter use (49%), and also bladder stones (52%). Ku *et al.* (2006) followed 140 male SCI patients over a 17-year period from 1987 to 2003 and recorded the incidence of renal and bladder stones. They reported that 28% and 15% of those studied developed renal and bladder stones respectively. The multivariate analysis of their data showed that age at injury, type of injury and the presence of an indwelling catheter were all significant risk factors in stone development.

Infective stones are caused by chronic infection of the urinary tract with urease-producing bacteria, particularly *Proteus mirabilis*. They consist of mineralised or amorphous aggregates of struvite (magnesium ammonium phosphate) and apatite (calcium phosphate) (McLean *et al.*, 1985; Bichler *et al.*, 2002). The formation of infective stones occurs when urease-producing bacteria hydrolyse urea and turn urine alkaline causing precipitation of the calcium and magnesium salts out of solution (see Section 1.6.). Dumanski *et al.* (1994) using purified *P. mirabilis* capsular polysaccharide (CPS), showed that the anionic nature of the bacterial CPS attracts magnesium ions onto the cell surface. This can act as a nidus for crystallization with the development of the crystalline microcolony, which continues as the bacteria proliferate (Stickler and Zimakoff, 1994). Sabbuba *et al.* (2004) used pulse field gel electrophoresis of restriction enzyme digests of bacterial DNA to show that the strains of *P. mirabilis* in infected bladder stones in catheterised patients had identical genotypes to strains colonizing their indwelling catheters. Bacteria dwelling in these infection stones are difficult to treat with antimicrobials and cause particular problems if situated in the kidney. Here they can cause episodes of pyelonephritis, renal abscesses and septicaemia (Stickler and Zimakoff, 1994).

1.2.5. Increased mortality

In a prospective study of catheterised patients at a US hospital, Platt *et al.* (1982) reported that mortality rates in patients with CAUTI were almost three times that in those without CAUTI. Kunin *et al.* (1992), in a prospective study of a large population of nursing-home residents in Ohio reported similar findings. Patients with an indwelling catheter for 76% or more of their time were found to be three times more likely to be dead within a year than matched controls. They also reported that

incidence of hospitalization, the length of hospitalization and requirement of antimicrobial therapy were all three times greater in the catheterised patients.

1.2.6. Pyelonephritis

Ascending infection from the bladder to the kidneys causing pyelonephritis is a serious complication of catheterisation. Persistent and recurring episodes of pyelonephritis are dangerous as they can lead to permanent renal damage and renal failure. Warren et al. (1988) in a post-mortem study of 75 elderly patients who had died in nursing homes, reported that 38% of those with an indwelling catheter in place at time of death had acute pyelonephritis compared to only 5% of those without an indwelling catheter. In a further autopsy study Warren et al. (1994) reported that patients catheterised for >90 days were significantly more at risk from chronic renal inflammation than those catheterised for <90 days. Larsen et al. (1997) reviewed the medical records of 142 chronically catheterised and non-catheterised SCI patients. The overall conclusions were that those with indwelling catheters had significantly more episodes of pyelonephritis and evidence of renal damage.

1.2.7. Catheter blockage

Blockage in the flow of urine through the catheter is a serious complication in the care of catheterised patients. It can lead to incontinence with urine leaking around the outside of the catheter. Alternatively urine is retained resulting in painful distension of the bladder. Reflux of infected urine to the upper tract can then induce episodes of pyelonephritis and bloodstream infections (Stickler and Zimakoff, 1994). Good management will ensure that patients are fitted with catheters of the appropriate size

and length, as catheters that are too long may develop 'kinks' or twists, which may prevent urine flow (Godfrey, 2000). Drainage bags should not be placed too far below the bladder as to create negative pressure and suck the walls of the bladder into the eye-let (Lowthian, 1998). The walls of the bladder can also occlude the eyehole when bladder spasms cause contraction of the bladder around the catheter (Lowthian, 1998). The most common cause of catheter blockage however, results from the build-up of crystalline bacterial biofilms that develop on the catheter surfaces (Section 1.6). As with infection-induced stone formation the problems arise from urease-producing bacteria that turn the urine alkaline. The elevated pH causes precipitation of magnesium and calcium phosphates from the urine which accumulate on the internal and external catheter surfaces (Hedelin *et al.*, 1984; Cox *et al.*, 1987; Morris and Stickler, 1997). Up to 50% of long-term catheterised patients block their catheters as a result of the build up of crystalline biofilm material on their devices (Cools and Van der Meer, 1986; Kunin *et al.*, 1987b; Kohler-Ockmore and Feneley, 1996).

Kunin *et al.* (1987b) used the terms 'blockers' and 'non-blockers' to describe the catheter encrusting habits of long-term catheterised patients. Blockers consistently and recurrently formed significant encrustation on their devices within days or weeks of the catheter being introduced. In contrast, the catheters of 'non-blockers' did not become occluded with encrustations. In a further study Kunin (1989) followed 32 long-term catheterised elderly women in a US nursing home half of whom were 'non-blockers' and the other half made up of 'blockers' and 'intermediates'. Catheters were removed at two-week intervals after which, the flow-rate and level of encrustation on the catheters were determined. They reported that 'blockers', 'non-blockers' and 'intermediates' retained their particular status during the study period. 'Blockers'

required more frequent catheter changes, and the removed devices had significant encrustation with poor flow rates. The micobiological evaluation of their urine showed that *Proteus* group organisms including (*P. mirabilis*, *Providencia stuartii*, and *Morganella morganii*) were isolated from 100% of 'blockers' urine, 80% of 'intermediates' and 60% of 'non-blockers', and the mean pH of urine from 'blockers' was significantly more alkaline than that of 'non-blockers'. Getliffe (1994a) studied the catheter encrustation in 42 community dwelling patients by visually examining catheters removed after 6-week intervals. Patients were classed as 'blockers' if they blocked two or more of their catheters during the study period or 'non-blockers' if they did not. They found that 42% of patients could be regarded as 'blockers', with a significantly higher proportion of women blocking their catheters than men. They also reported that there was no relationship between catheter size, type (hydrogel-coated and silicone-elastomer) and 'blocker' status. 'Blockers' often needed re-catheterisation < 6 weeks. Other studies have also reported similar levels of catheter blockage (Cools and Van der Meer, 1986). Kohler-Ockmore and Feneley (1996) studied the prevalence of morbidity in long–term catheterised patients over two successive years in three healthcare districts in the UK. This study found 48% of patients experienced catheter blockage.

1.2.8. Increased workload and financial burden

The complications of long-term catheterisation can significantly increase the workloads of healthcare workers and impose a heavy financial cost on healthcare services. In their 2-year study estimating the prevalence of morbidity in long-term catheterised patients Kohler-Ockmore and Feneley (1996) reported that one district nurse had 223 daytime emergency referrals in a 6-month study period. The total

number of emergency referrals for catheter complications in a population of 467

patients in the 6-month period was 506. The majority of these cases were due to

catheter blockage. The cost of nosocomial catheter-associated UTI in terms of

hospitalization was assessed by Tambyah *et al.* (2002). In their report which observed

1,497 newly catheterised patients it was estimated that each episode of CAUTI cost

$589. However, most of these infections were asymptomatic and not caused by potent

urease-producing species that significantly exacerbate CAUTI and cause the more

severe catheter related problems. The cost of treating CAUTI in these patients would

be expected to be much higher.

1.3. Infection and biofilms

The continent bladder helps to maintain a healthy urinary tract as the intermittent

voiding of urine flushes out invading microbes and keeps it free from infection most

of the time. The introduction of the indwelling catheter disrupts the normal voiding

action of the bladder and compromises the hosts natural defence mechanisms, leaving

the urinary tract vulnerable to infection.

1.3.1. Infection

The microflora that invades the catheterised urinary tract is diverse in nature. Initial

colonization is usually with single species such as *Eschericia coli*, *Enterococcus*

faecalis or *Staphylococcus epidermidis* (Tabmbyah *et al.*, 1999; Matsukawa *et al.*,

2005). With time the urinary flora becomes more complex and eventually mixed

communities develop which commonly include Gram-negative nosocomial organisms

such as *Proteus*, *Pseudomonas*, *Providencia* and *Klebsiella* (Clayton *et al.*, 1982;

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Warren *et al.*, 1982). Microbial infection of the urinary tract in the catheterised patient occurs in two ways. Infections via the intra-lumenal route occur when bacteria enter the bladder through the catheter from an infected drainage system. The extra-lumenal route of infection occurs when bacteria migrate through the periurethral space between the catheter and the urethral epithelium (Kass and Schneiderman, 1957; Nickel *et al.*, 1985; Stamm, 1991). The introduction of the closed drainage system to prevent contamination of the urinary tract reduced UTI via the intra-lumenal route (Kunin, 1966).

Keeping the urine collection system sterile however, especially in long-term catheterisation is virtually impossible as taps need to be opened to drain the urine and junctions between catheter and collection bags have to be disconnected when bags are changed. Nickel *et al.* (1985a) studied the importance of the intra-lumenal and extra-lumenal routes of infection by *Ps. aeruginosa* in catheterised rabbits. They reported that bacteriuria ($>10^5$ organisms per ml urine) occurred between 32-48 hours in the rabbits when the drainage system was infected, compared with 72-168 hours via the extra-lumenal route. They concluded that in patients catheterised for very short time periods, maintaining a sterile drainage system was fundamental in preventing bacteriuria. In long-term catheterisation however, the most important route of infection would be via the extra-lumenal route. It was interesting that electron microscopy of catheters removed from these rabbits showed that the spread of biofilm-associated organisms was instrumental in ascending the catheter and causing infection in both intra-lumenal and extra-lumenal routes. Other studies have shown that the main routes of infection are different between men and women. Daifuku and Stamm (1984) studied 35 catheterised men and women in an intensive care unit and

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reported that the majority of women studied became infected via the extra-lumenal route. These infections were caused by the spread of bacterial from the rectal area to the periurethral area. In contrast, men were more likely to become contaminated through cross infection via the intra-lumenal route. The infecting strains originated from the periurethral area but not by organisms migrating from their rectal skin. In a later study however, Tambyah *et al.* (1999) found no difference between men and women in this respect. Their results suggested that in both sexes the main route of infection was along the outside of the catheter, with two-thirds of patients acquiring infection in this way.

The complex urinary microflora of long-term catheterised patients is difficult to eliminate with antibiotics (Clayton *et al.*, 1982). In addition as most of these infections are asymptomatic for most of the time antibiotic therapy is often withheld. It is common practice to use antibiotics only when the evidence suggests that infection has spread to the kidneys or bloodstream (Kunin, 1987a). As a result, infected urine flows through the catheters for up to 3-month periods at a time. The catheters provide attractive, unprotected sites for bacterial adherence. Once attached and washed in a gentle flow of nutrient urine, the sessile organisms thrive and produce extensive biofilm communities (Stickler, 1996).

1.3.2. Biofilms

In aquatic habitats bacteria show a strong basic instinct to colonize available surfaces as biofilms. They can be found in environmental, industrial and medical situations. Costerton (1999) described the basic formation of the biofilm and its clinical relevance – "Bacteria that attach to surfaces aggregate in a hydrated polymeric matrix

of their own synthesis to form biofilms. Formation of these sessile communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections". In the biofilm mode of growth they are able to colonize favourable environments rather than be washed away, while the cells embedded in the biofilm are also protected from a range of environmental stresses. These include nutrient limitation, fluid shear forces, toxic chemicals, antimicrobial agents, desiccation, UV irradiation, predation by protozoa or phagocytes and changes in pH and temperature (De Beer, 1994; Costerton *et al.*, 1995; Wimpenny and Colasanti, 1997; Danese, 2002; Donlan, 2002).

The architecture, exopolysaccharide (EPS), diffusion gradients, bulk flow rates, spacial arrangement of bacterial cells and the inhabitant species of a biofilm promote a variety of microenvironments throughout the biofilm giving the structure a heterogeneous nature (Costerton *et al.*, 1995; Jenkinson and Lappin-Scott, 2001; Stoodley *et al.*, 2002; Donlan and Costerton, 2002; Stoodley and Stoodley, 2005). The distribution of microenvironments within biofilms has been demonstrated in a number of studies. Using microelectrodes Costerton *et al.* (1994) for example demonstrated the variability in dissolved oxygen levels throughout *Ps. aeruginosa* biofilms. They found significantly contrasting concentrations of dissolved oxygen at the biofilm/liquid interface (almost 100% of that recorded in the bulk liquid), while the biofilm/solid interface deep within the biofilm was virtually anaerobic. Such microenvironments within biofilms are known to allow diverse species to form mutually beneficial relationships where together they are capable of degrading complex compounds and nutrients that would not be utilized by individual species alone (Nielsen *et al.*, 2000).

The early concept of the biofilm structure came from research carried out on freshwater and laboratory biofilms (Costerton *et al.*, 1978; Lawrence *et al.*, 1991; Costerton *et al.*, 1995). The water mosaic model they described showed the biofilm as microcolonies of cells arranged in three-dimensional mushroom-like structures infiltrated with a network of water channels. The water channels were thought to be important as they allowed for the transport of nutrients, gasses and waste products into and out of the biofilm. Subsequent studies have shown that biofilm architecture is diverse, depending on a range of factors including the inhabitant species, cell-signalling, EPS production, and environmental factors including the availability of nutrients, and liquid shear forces (Stoodley *et al.*, 2002; Wimpenny and Colasanti, 1997).

1.3.2.1. Biofilm development

Many studies have attempted to elucidate the mechanisms by which biofilm formation is initiated. The cells from the surrounding bulk fluid come into contact with the surface usually as a result of hydrodynamic forces, Brownian motion or cell motility. Non-specific factors are then thought to facilitate adherence to the substrata including electrostatic charges and Lifshitz-Van der Waals forces, before specific interactions between the bacterial cell and the surface preserve its attachment (Busscher and van der Mei, 1997; Dunne, 2002). Initially cells form a reversible attachment to the surface allowing a possible return to the planktonic state. The cells that initiate biofilm formation become 'irreversibly' attached when EPS production is up-regulated anchoring them to the surface. These cells multiply and begin colonization of the surface developing into microcolonies that form the early architecture of the

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sessile community (Costerton, 1999; Sauer *et al.*, 2002; Stoodley *et al.*, 2002; Shachter, 2003).

Specific interactions between cell and surface are mediated through cell surface associated appendages such as pili, curli, fimbriae, various adhesins and flagella (Dunne, 2002). Pratt and Kolter (1998) demonstrated that flagella and type 1 pili in *E. coli* were essential in biofilm formation on PVC surfaces. Their evidence suggested that flagella were required to provide the initial contact and possibly overcome any repulsive forces encountered at the surface, while type 1 pili were necessary for adherence and biofilm formation. O'Toole and Kolter *et al.* (1998) demonstrated the importance of flagella and type IV pili in the early stages of *Ps. aeruginosa* biofilm development on PVC surfaces. Type IV pili are associated with 'twitching motility' which bacteria use to move over surfaces and is driven by extension and retraction of the surface attached pili (Burrows, 2005). O'Toole and Kolter *et al.* (1998) monitored biofilm development over the initial 8 hours and demonstrated that wild type strains were able to colonize the surface and form aggregates and microcolonies (the basic structure of biofilms) as cells congregated on the surface. A mutant strain unable to produce flagella was a poor surface colonizer. A pilus-minus mutant strain on the other hand, was able to colonize the surface in a similar fashion to the wild type initially, but did not show twitching motility and was unable to aggregate into microcolonies.

1.3.2.2. Biofilm phenotype

Cells in biofilms are known to be phenotypically different from planktonic cells and show different gene expression patterns than free-swimming cells. The early work of

Davies *et al.* (1993) demonstrated that attachment to a surface altered gene expression in *Ps. aeruginosa*. The expression of the genes involved in the synthesis of the EPS alginate was shown to be up-regulated in cells newly attached to a Teflon surface. Sauer and Camper (2001) examined physiological changes in *Ps. putida* in the switch from planktonic to sessile growth in first 6 hours of attachment to silicone surfaces. They found 15 proteins were up-regulated and 30 proteins were down regulated while overall 40 genes were differentially expressed following initial attachment. Further studies by Sauer *et al.* (2002) demonstrated that multiple phenotypes are expressed by *Ps. aeruginosa* during biofilm formation. They suggested there were five stages of biofilm development: (i) reversible attachment, (ii) irreversible attachment (iii) maturation-1 (iv) maturation-2, and (v) dispersion. On average a 35% difference in protein expression between the stages was observed. They also found that cells lost their motility at stage (ii) before regaining motility at stage (v). When protein profiles of cells at each biofilm stage were compared with planktonic cultures those in the maturation-2 stage were the most distinct from planktonic cells expressing over 800 proteins at a six-fold increase. The protein profiles of cells from the dispersion stage were more similar to those in planktonic cultures than those in the maturation-2 stage.

Schembri *et al.* (2003) used micro-array methods to monitor the global gene expression patterns (with particular emphasis on genes associated with cell structure and biofilm formation), of *E. coli* in sessile growth on glass surfaces compared to planktonic growth. They found that 79 genes were significantly altered in sessile growth compared to planktonic growth. The Ag43 surface protein known to promote auto-aggregation and biofilm formation was expressed at significantly higher levels in the sessile community than in planktonic growth. They also reported increased

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expression of the genes under the control of the *rpo*S-encoded sigma factor. These genes are involved in cellular responses to a range of stresses including chemical agents and nutrient depletion and are related to slow growth.

1.3.2.3. Quorum sensing

The expression of a number of bacterial genes is known to be under the control of cell-signalling chemicals known as quorum sensing (QS) molecules. These molecules diffuse freely across cell membranes and between cells. In dense bacterial populations, when the concentration of these QS molecules exceeds a threshold, a regulatory effect over genes under their control takes place (Kjelleberg and Molin, 2002; Abraham, 2006; Spoering and Gilmore, 2006). A number of cell signalling molecules in both Gram-negative and Gram-positive bacteria have been identified. In Gram-negative species, QS is under the control of acylated homoserine lactone (AHL) molecules, while in Gram-positive species QS is known to be controlled by the auto-inducer peptide (AI-2) and the competence signalling peptide (CSP). CSP is known to promote complementation (uptake of naked DNA from the environment), which may be a selective advantage to cells in stressful environments. It has also been demonstrated in having a role in biofilm formation (Petersen *et al.*, 2004). Although AHLs are absent in Gram-positive species, AI-2 is also found in Gram-negative species suggesting a means of interspecies communication (Abraham, 2006). The presence of signalling molecules or peptides in bacterial biofilms is well documented, and they are thought to have a multi-factorial role including, virulence, biofilm formation, disruption of other competing bacteria, cell lysis, and the dissemination of biofilm cells (Kjelleberg and Molin, 2002; Suntharalingam, 2005; Abraham, 2006; Spoering and Gilmore, 2006). McLean *et al.* (1997) demonstrated the presence of

AHLs in naturally occurring biofilms. Using *Agrobacterium tumefaciens* A136 as an AHL-responsive reporter strain in 'cross-feeding' assays, they showed that these molecules diffused through agar from biofilms forming on limestone rocks found in a Texas river. These biofilms were composed of *Pseudomonas* species including *Ps. putida* and *Ps. fluorescens*. Using similar 'cross-feeding' assays, Stickler *et al.* (1998a) demonstrated that AHLs could be detected in bacterial communities colonizing indwelling urinary catheters removed from patients.

Davies *et al.* (1998) studied the role of cell-signalling systems in *Ps. aeruginosa* biofilm formation. They compared the biofilm forming ability of a wild type strain to a *lasI* mutant that was unable to produce N-(3-oxododecanoyl)-L-homoserine lactone. Biofilms formed by the wild type were composed of mushroom shaped microcolonies with cells spatially separated within. The *lasI* mutant however, could only form flat shallow biofilms of tightly packed cells (approximately 20% of the thickness of the wild type biofilm) under the same growth conditions. When these biofilms were treated with the detergent sodium dodecyl sulphate (0.2 %) the wild type biofilm was unaffected. In contrast the biofilm formed by the mutant detached from the surface within 5 minutes of the detergent introduction. When synthetic AHL was introduced to the mutant culture it produced a biofilm identical to the wild type with spatially separated cells. The biofilm also became recalcitrant to treatment with the detergent, supporting the theory that biofilm structure is an important factor in resistance to antibacterial agents. It has also been suggested that quorum sensing is involved in the dispersal of cells from the biofilm (Donlan, 2001).

1.3.2.4. Biofilms on medical devices

Infections associated with implanted medical devices are characteristically persistent and refractile to antibiotic therapy. The nature of these infections results from the properties of the biofilms that develop on the surfaces of these devices. Biofilms that form on implanted medical devices are extremely difficult to eradicate as they evade host immune responses such as phagocytosis and also the effect of antimicrobial chemical agents (Costerton *et al.*, 1995; Stickler and McLean, 1995; Donlan, 2001). Biofilms forming on implanted medical and prosthetic devices are becoming ever more clinically relevant as an increasing number of individuals have such devices in place. These devices which include replacement joints, heart valves, artificial hearts, vascular shunts, contact lenses, stents and lines, venous and urinary catheters and breast implants are manufactured from a range polymers and metals. Darouiche (2001) postulated that 5 major principles are important in the formation of device related biofilms: (1) different bacteria may adhere differently to the same device material; (2) the same bacteria may adhere differently to different device material; (3) the same bacteria may adhere differently to the same device material placed under different circumstances, including the medium in which the device is placed (*e.g.* hydrophobic vs. hydrophilic medium), type of flow, and temperature; (4) *in vitro* inhibition of bacterial colonization of the device does not ensure anti-infective efficacy *in vivo*; and (5) the clinical benefit of a particular surface-modifying approach may vary from one application to another.

Essentially implants act as 'foreign bodies' once they are incorporated into host tissue. It has been demonstrated that a range of proteins present in body fluids

including collagen, albumin, fibrinogen, fibronectin, vitronectin, laminin and α-1-microglobulin readily adsorb onto the surfaces of implants forming 'conditioning layers' (Barbucci and Magnani, 1994; Stickler and McLean, 1995; Santin *et al.*, 1997; Reid, 1999). These adsorbed proteins can trigger inflammation when the immune system response attracts neutrophils and macrophages to the device (Stickler and McLean, 1995). Tang and Eaton (1993) used a mouse model (implanted with polyethelene terephthalate film) to demonstrate that the acute immune response was initiated by the fibrinogen content of the conditioning layer that adsorbed to the surface. In a further study Tang *et al.* (1996) identified that a specific region (D γ190-202) of fibrinogen was responsible for phagocyte interaction. The adsorbed proteins also offer 'binding sites' for bacteria to adhere when epitopes present on their surfaces are recognized by specific bacterial adhesins. This has been demonstrated in a number of studies. Host proteins including fibrinogen, fibronectin and collagen are known to interact with 'microbial surface components recognising adhesive matrix molecules' (MSCRAMMs) of *Staphylococcus aureus* allowing them to adhere to host cells and implanted devices that are coated with these proteins (Vaudaux *et al.*, 1993; Foster and Hook, 1998; Arrecubieta *et al.*, 2006).

1.3.2.5. Resistance to antimicrobial agents

Studies have shown that bacterial cells dwelling within the biofilm have an increased resistance to antibacterial agents, which is thought to be dependent on a number of factors. Poor diffusion into the biofilm (determined by the architecture and nature of the EPS matrix), the phenotypic nature of biofilm cells - a proportion of which will be dormant or very slow growing, and the composition of the inhabitant bacterial consortia have all been suggested as resistance mechanisms (Mah and O'Toole, 2001;

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Donlan and Costerton, 2002; Dunne, 2002). Nickel *et al.* (1985b) used a laboratory

model to grow *Ps. aeruginosa* (previously isolated from a CAUTI) biofilms on latex

discs using artificial urine as the growth media. They found that viable cells were

recoverable from the biofilm even after a 12-hour period of exposure to the antibiotic

tobramycin at a concentration of 1,000 µg/ml. This concentration was far in excess of

the 50 µg/ml tobramycin concentration required to kill the planktonic *Ps. aeruginosa*

cells removed from the same experimental system. Gristina *et al.* (1987) reported that

viable cells from *Staphylococcus epidermidis* biofilms grown on steel discs could be

recovered after 8 hours exposure to tobramycin at a concentration of 1,000 µg/ml.

When these cells were recovered from the biofilm and re-suspended in a planktonic

state the tobramycin minimum inhibitory concentration (MIC) and minimum

bactericidal concentration (MBC) were recorded at <0.5 µg/ml, and were identical to

the cells in the original broth cultures.

Diffusion limitation in biofilms can result from a reduction in the fluid flow within the

biofilm matrix and by the increased diffusion distances resulting from the biofilm

architecture (Stewart, 2003). Hoyle *et al.* (1992) showed that the EPS of *Ps.*

aeruginosa biofilms provided a physical barrier to the diffusion of the β-lactam

antibiotic piperacillin. Studies have also shown that the biofilm matrix can act as a

diffusion barrier by inactivating antibacterial compounds. Using microelectrodes to

monitor chlorine penetration into *Ps. aeruginosa* and *K. pneumoniae* biofilms, de

Beer *et al.* (1994) found that diffusion of chlorine into the biofilm decreased with

depth into the matrix. The chlorine concentration at the deepest parts of these biofilms

was as low as 20% of the concentration of the chlorine in the bulk liquid. They found

that alginate (a major exopolysaccharide associated with *Ps. aeruginosa* biofilms) was

able to bind chlorine and effectively neutralize its effect, reducing its penetration

through the biofilm. Even when antimicrobial agents penetrate the biofilm matrix readily and in significant concentrations it does not mean they will be effective on the sessile biofilm community. Dunne *et al.* (1993) demonstrated that *Staphylococcus epidermidis* biofilms were completely penetrable with vancomycin and rifampin at concentrations in excess of their respective MBC's. However, viable cells were retained within the biofilm matrix even after 72 h exposure to rifampin in excess of the MBC concentration. When the MIC's and MBC's of these biofilm cells were tested they were significantly higher than for planktonic cells. In these circumstances it is thought the 'phenotype' of the biofilm cells increases their resistance.

The MICs and MBCs of contemporary antibiotics are based on exponentially growing planktonic cell cultures (Fux *et al.*, 2005). Studies have shown however, that compared to cells at the peripheral areas of the community, bacterial cells deep within the biofilm matrix grow much slower or adopt a dormant non-growing phenotype (Wentland *et al.*, 1996; Sternberg *et al.*, 1999; Sauer and Camper, 2001). This slow growth is thought to be due to the reduced availability of nutrients (Donlan and Costerton, 2002; Mah and O' Toole, 2001). The growth rate of cells has been shown to greatly determine the effect the antimicrobial agents, and may therefore be a major factor why bacterial biofilms are extremely difficult to eradicate (Eng *et al.*, 1991). In addition to this, studies have shown that persister cells (dormant cells with spore-like attributes) are present in bacterial populations. Persister cells are highly tolerant to a range of environmental and physical challenges and are known to be present in biofilm communities (Keren *et al.*, 2004). This would further explain the inherent recalcitrant nature of bacterial biofilms.

Bacterial cells that are susceptible to antibacterials may also be 'protected' from these agents by other species within a mixed community biofilm. Anderl *et al.* (2000) examined the penetration of the β-lactam antibiotic ampicilin, and also ciprofloxacin into filter disks through biofilms of a β-lactamase producing wild type *K. pneumoniae* able to degrade ampicilin, and a mutant strain unable to produce β-lactamase. They reported that ciprofloxacin penetrated both biofilms while ampicillin only penetrated the mutant biofilm suggesting that β-lactamase activity was the important factor in preventing penetration. Other reports have also shown that synergistic interactions in multispecies biofilms also confer increased resistance to antimicrobial agents. Burmølle *et al.* (2006) demonstrated that multi-species biofilms composed of marine isolates of *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis* and *Acinetobacter iwoffii* produced biofilms of considerably more biomass when grown together than when grown as single species. When hydrogen peroxide or tetracycline was added to the biofilms grown in microtitre wells the relative activity of the multi-species biofilms was markedly higher compared with the single species biofilms. In conclusion, the overall evidence surrounding the increased resistance of biofilm associated cells to antimicrobials suggests that the basis for antibacterial resistance in bacterial biofilms is a multifactorial phenomena, and it is likely to be distinct depending on the antibacterial being considered.

1.4. Attempts to prevent catheter-associated urinary tract infection

A variety of approaches have been used to try and prevent the infection of the catheterised urinary tract. The most effective of these strategies has been the introduction of the closed drainage system. Attempts to block the routes of infection with antibacterial agents have met with limited if any success (Kunin, 1997).

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1.4.1.   Closed drainage system and drainage bag disinfection

In the early days of catheterisation, it was common practice for catheters to drain into open bottles or buckets under the patient's bed. Under these conditions infection was inevitable after 3-4 days. Kunin and McCormack (1966) showed that a novel disposable closed drainage system in which urine flowed from the catheter, through a tube attached to a collection bag, reduced infection rates to 20 - 50 % in patients being catheterised for short periods (up to 13 days). These systems were not closed for the duration of catheterisation of course as it was necessary, every eight hours or so, to open the tap on the bag to empty the urine. Attempts were made to prevent contamination of the urine in the bag by adding disinfectant to the bag each time it was emptied. Gillespie *et al.* (1983) used chlorhexidine to maintain the sterility of the drainage bag in catheterised patients. Although the drainage bags in these patients remained sterile, there was no significant difference in the rates of CAUTI in patients with chlorhexidine treated collection bags compared to patients whose collection bags were not treated with the disinfectant. In a similar study Thompson *et al.* (1984), evaluated the potential of hydrogen peroxide to disinfect urine collection bags of catheterised patients as a means to prevent CAUTI developing. This study also reported a significant reduction in bag contamination but there was no significant reduction in CAUTI between patients where collection bags were treated with hydrogen peroxide and controls. In general although the closed drainage system may prevent infection in the very short-term, those catheterised for extended periods will inevitably become bacteriuric via the extra-lumenal route.

Colonization of the peri-urethral skin by urinary tract pathogens is an important risk factor for CAUTI (Garibaldi *et al.*, 1980; Daifuku and Stamm, 1984). It would seem

to be sensible to perform regular cleaning of the urethral meatus with antibacterial agents. Well – controlled prospective trials of this approach however, have failed to demonstrate any benefit (Burke *et al.*, 1981; Burke *et al.*, 1983). In their earlier study Burke *et al.* (1981) demonstrated that twice-daily cleansing of the urethral meatus with povidone-iodine solution and application of povidone-iodine ointment was not effective in reducing the incidence of bacteriuria. In total, 16.0 % of patients undergoing this treatment acquired bacteriuria compared to 12.4 % in patients in the control group who received no meatal care, daily infection rates were 5.9 % and 4.5 % per day in test and control groups respectively. Cleansing once daily with soap and water resulted in 12.2 % of patients acquiring bacteriuria versus 8.1 % in the control group receiving no cleansing of the urethral meatus. In a later study Burke *et al.* (1983) used a poly-antibiotic ointment in meatal care, and also concluded that if there was any benefit at all in using this ointment it was small.

1.4.2. Bladder washouts

A number of antibacterial agents have also been evaluated as a means to control CAUTI. The technique of bladder washing generally involves the instillation of an antibacterial solution (50-100ml) through the catheter into the bladder. The catheter is then clamped and after 20 – 30 minutes the clamp released to allow the solution to drain out. There is little evidence that this procedure is effective in preventing or controlling CAUTI. For example Clayton *et al.*, (1982) reported that when a combination of kanamycin and colistin was used as a bladder washout on a patient infected with *K. pneumoniae*, the infecting organism was replaced by a kanomycin resistant strain of *P. mirabilis*. Davies *et al.* (1987) reported that repeated bladder washouts with chlorhexidine solution over a 3-week period in catheterised patients

had no effect on bacterial numbers determined from urine samples. Moreover, episodes of infection with *Proteus spp.* (important pathogens in the catheterised urinary tract), increased in patients using the chlorhexidine washout. Stickler *et al.* (1987) demonstrated in experiments in a laboratory model of the bladder that clinical isolates of *Providencia stuartii*, *Ps. aeruginosa*, *P. mirabilis*, *K. pneumoniae* and *Streptococcus faecalis* all resisted washouts with chlorhexidine solution. They also investigated the effects of chlorhexidine on the urinary flora of catheterised patients. It was demonstrated that any antibacterial activity in the urinary flora was minimal and temporary.

The infection control team at a Southampton Hospital devised a policy designed to comprehensively block all possible routes of infection of the catheterised bladder. The policy was based on the antiseptic chlorhexidine. Daily washes with chlorhexidine were used to disinfect the peri-urethral skin. A gel containing chlorhexidine was applied daily to the catheter insertion site and the antiseptic was instilled into the drainage bag each time it was emptied. After an apparent initial drop in the incidence of CAUTI an outbreak of infections involving over 90 patients occurred with a chlorhexidine resistant, multi-drug resistant strain of *P. mirabilis* (Walker and Lowes, 1985; Dance *et al.*, 1987). The outbreak was only controlled when the policy was abandoned. Warren *et al.* (1978) investigated the use of a double-lumen catheter to deliver a continuous irrigation of the bladder with neomycin and polymixin. They reported no differences in the rate of CAUTI in test and control patients. The incidence of infection for both groups was approximately 5% per day. More importantly there was a marked difference in the bacterial flora colonizing the

different groups, with levels of organisms resistant to the antibiotics significantly higher in the group using the irrigation.

1.4.3. Administration of prophylactic antibiotics

The evidence suggests that antibiotics are not effective in preventing bacteriuria in patients undergoing long-term catheterisation (Clayton *et al.*, 1982). Garibaldi *et al.* (1974) reported that the use of systemic antibiotics in catheterised patients had a limited effect, and only prevented bacteriuria in patients in the first 4 days of catheterisation. The rate of bacteriuria after this time was not significantly different between individuals receiving, and those not receiving antibiotic therapy. They also reported that those treated with antibiotics were more likely to harbour antibiotic resistant strains. In a follow-up study Britt *et al.* (1977), reported that patients receiving prophylactic antibiotics during short-term catheterisation after gynecological surgery had reduced episodes of bacteriuria compared to a placebo group (3 out of 96 patients versus 9 out of 100 patients respectively). However, at time of discharge from hospital there was no significant difference between bacteriuria in the groups (8 out of 82 in treated group versus 8 out of 75 in untreated group). The majority of CAUTI are asymptomatic, and the general consensus on the use of antibiotics is that they should only be administered when symptoms suggest that invasive infection of the kidneys or bloodstream has occurred (Warren, 1991; Tambyah and Maki, 2000; Trautner *et al.*, 2005).

1.5.   *Proteus mirabilis* – a particularly dangerous organism in the catheterised urinary tract

Proteus mirabilis is an opportunist pathogen and will cause infections in wounds, burns, and of the urinary tract, when its normal defence mechanisms have been impaired (Penner, 1984). It causes particularly severe problems in individuals undergoing long-term catheterisation. In these individuals it is responsible for blockage of the catheter, a complication which results in increased morbidity and mortality (Mobley and Warren, 1987; Kunin, 1989; Hedelin *et al.*, 1991; Kohler-Ockmore and Feneley, 1996; Stickler, 1996). The bacterium can be found in aquatic and soil environments, sewage, and as part of the natural gut flora of animals and humans (Penner, 1984). *P. mirabilis* possesses a battery of virulence factors including agglutinins, flagella, proteases, haemolysins, siderophores and the enzyme urease (Rozalski *et al.*, 1997). It is also capable of producing swarmer-cells which can migrate over surfaces and have been implicated in pathogenesis (Mobley and Belas, 1995).

Swarming behaviour in *P. mirabilis* occurs when vegetative swimmer-cells differentiate into elongated, highly flagellated and highly motile swarmer-cells. These differentiated cells can be 10-80 times the length of swimmer cells, possess 10^3-10^4 flagella (compared to 4-10 peritrichious flagella in swimmer-cell), and become multinucleate (Mobley and Belas, 1995). Various environmental cues and induced gene expression are required for the differentiation process including contact with a solid surface, a high population density, inhibition of flagella rotation and cell-cell signalling (Mobley and Belas, 1995; Rozalski *et al.*, 1997; Rather, 2005). The increased motility observed in the swarming phenomenon is the result of multi-

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cellular coordination. On solid surfaces adjacent swarmer-cells interweave their flagella to form helical structures and produce large amounts of extracellular slime that forms a matrix through which the integrated raft of cells can migrate rapidly via coordinated flagella movement (Stahl *et al.*, 1983; Jones *et al.*, 2004). After a period of swarming, migration stops and cells at the perimeter of the swarming edge divide into swimming-cells in a process called consolidation. When population density and slime production are sufficient cells will differentiate once again and resume swarming behaviour. Repetition of these events produces the characteristic rings of growth as the organism swarms over agar plates. The role of swarming in colonization of the catheterised urinary tract is not fully understood at present. A number of studies however, have shown that *P. mirabilis* can swarm over the surfaces of a wide variety of catheter materials. It is possible that swarming may be a factor in the migration of the bacteria from the urethral meatus to the bladder. Stickler and Hughes (1999) used 'catheter bridges' to demonstrate that *P. mirabilis* was able to swarm over a range of catheter surfaces including all-silicone, silicone-coated latex, hydrogel-coated latex and hydrogel/silver-coated latex. They found that *P. mirabilis* could migrate over 10 cm of catheter surface within a 24 hour period. In these experiments the speed of migration was highest over the hydrophilic surfaces of hydrogel catheters. In a similar study Sabbuba *et al.* (2002) used catheter bridges to demonstrate that *P. mirabilis* and *P. vulgaris* were capable of migrating over the surfaces of hydrogel-coated latex, hydrogel/silver-coated latex, silicone-coated latex and all-silicone catheter materials, faster than nine other urinary tract pathogens. Jones *et al.* (2004) reported that non-swarming transposon-generated mutants of *P. mirabilis* had impaired ability to migrate over catheters compared to the parental wild type strain. The role of

swarming in pathogenesis has also been considered in urinary tracts that have been compromised by the presence of an indwelling urinary catheter.

Allison *et al.* (1992) examined the role of swarming in the invasion of human urothelial cell cultures. Swarmer cells were found to be more invasive than vegetative cells. Additionally they showed mutants defective in motility and swarming were less invasive than wild type strains. They also examined the role of other virulence factors (flagellin and haemolysin) that are up-regulated in swarmers and found that they were not relevant to the entry into the epithelial cells. Jansen *et al.* (2003) used green fluorescent protein expression and confocal microscopy to observe *P. mirabilis* cell morphotypes invading the urinary tract of transurethrally infected mice. Their observations of kidney, ureter and bladder tissues revealed a very low percentage of swarmer cells (0.14%) were present (none at all observed in ureters), and concluded that the swimmer-cell was the predominant morphotype in ascending UTI. In contrast, Allison *et al.* (1994) intravenously infected mice with wild type *P. mirabilis*, two mutant strains with different abnormal swarming behaviour, a non-swarming but motile mutant and a swarming but non-haemolytic mutant. Histological examination of kidney sections of the mice revealed that wild type *P. mirabilis* and the non-haemolytic mutant swarmer-cells were the predominant morphotype found in the kidney tissue. The non-swarming mutant strains were less virulent compared to the swarming strains. In the same study they also observed the rates of ascending UTI by directly infecting the bladders of mice with the test strains. They demonstrated that wild type and non-haemolytic mutant were able to colonize the kidneys of infected mice while none of the non-swarming mutants were able to colonize the kidney.

Other virulence factors which are thought to be important in *P. mirabilis* pathogenesis are the surface associated appendages. Two major fimbrial types expressed by *P. mirabilis* are Mannose-Resistant *Proteus*-like fimbriae (MR/P fimbriae) and Mannose-Resistant *Klebsiella*-like fimbriae (MR/K fimbriae). The expression of MR/P fimbriae is phase-variable and has been suggested to play a role in acute pyelonephritis (Bahrani *et al.*, 1994; Li *et al.*, 2002). MR/P fimbriae belong to the type IV pili family, a group of retractable adhesive appendages, synthesized by a range of pathogenic and environmental Gram-negative bacteria (Burrows, 2005). Their presence can be detected by their ability to agglutinate a range of red blood cell types (including human, bovine, and chicken) in the presence and absence of mannose (Bahrani *et al.*, 1994). The process of extension and retraction through the cell envelope is driven by ATP-dependent assembly and dis-assembly of the pili structural subunits, and they are involved in specific and non-specific binding to tissue, abiotic surfaces such as glass, plastic, and metal (Burrows, 2005). Type IV pili allow twitching motility across surfaces, DNA uptake, and biofilm formation. It is thought they are important in cytotoxicity as they allow invading cells to be drawn adjacent to host cells where contact-dependent secretion systems can take place (O'Toole and Kolter, 1998; Burrows, 2005). It has been suggested that both MR/P and MR/K fimbriae may be important in CAUTI. Mobley and Chippendale (1990) used haemagglutination to examine the presence of MR/P and MR/K fimbriae expressed in *P. mirabilis* isolates from patients with acute pyelonephritis, catheter-associated UTI and from faecal samples. They demonstrated that MR/P fimbriae were expressed in all isolates while MR/K fimbriae were not always expressed.

Sareneva *et al.* (1990) demonstrated that an MR/P fimbriae expressing strain bound strongly to tubular epithelial cells of human kidney tissue and to exfoliated epithelial cells. In contrast strains expressing MR/K fimbriae bound to the Bowman's capsule, glomeruli and interstitial tissue of the kidney sections but only weakly to exfoliated epithelial cells. In the same study however, they showed the MR/P strain did not agglutinate human or bovine red blood cells in the presence of human urine (MR/K agglutination was not inhibited). They concluded that urine contains low molecular weight molecules that inhibit MR/P binding *in vivo*, which may be a factor in the low rates of *P. mirabilis* infection in normal individuals. As MR/K agglutination was not affected in the presence of urine they suggest these fimbriae were responsible for mediating initial adherence *in vivo*, especially in the catheterised urinary tract *i.e.* directly to the catheter surface or where localized damage to the urinary tract may occur. This hypothesis supports previous work carried out by Mobley *et al.* (1998) who monitored weekly urine samples from 51 long-term catheterised patients. They found that *Pv. stuartii* was the most prevalent isolate in urine samples and that strains expressing MR/K fimbriae were more likely to cause bacteriuric episodes of long duration (> 12 weeks) than episodes of short duration (1 week or less). Strains that did not express MR/K were more likely to be present in urine for short periods. They also demonstrated using *in vitro* adherence assays that MR/K strains adhered better to silicone-coated latex catheters than strains not expressing MR/K fimbriae.

Bahrani *et al.* (1991) infected mice with *P. mirabilis* to determine the role of MR/P fimbriae and flagella in ascending UTI. They used an enzyme-linked immunosorbent assay (ELISA) of the serum of the previously challenged mice to reveal an immune response against purified MR/P and flagella, thus demonstrating that both were

expressed during the ascending UTI. Bahrani *et al.* (1994) also transurethrally inoculated mice with a wild type MR/P$^+$ and an MR/P$^-$ mutant and assessed colonization and virulence in the urinary tract after a 1 week challenge. The results showed that wild type *P. mirabilis* colonized urine, bladder and kidneys in significantly higher numbers than the mutant strain. Histopathological findings suggested that the mutant strain also caused less damage to uroepithelium cells and did not cause pyelonephritis.

Zunino *et al.* (2001) transurethrally co-challenged sixteen mice with equal titres of a wild type clinical *P. mirabilis* isolate and its isogenic mutant unable to express MR/P fimbriae. They isolated bacteria colonizing the kidneys and bladder after 7 days and found the wild type out-competed the mutant in both parts of the urinary tract. In the same study they intravenously inoculated mice with the test organisms and found that after seven days 16 of 17 mice infected with the wild type had infected kidneys (presence of abscesses or recovery of bacteria from tissue), compared to only 12 of 24 mice inoculated with the mutant strain. They also demonstrated that the wild type strain adhered to uroepithelial cells in significantly higher numbers after a 1 hour incubation period.

Jansen *et al.* (2004) presented evidence that MR/P fimbriae expression is important during ascending UTI and biofilm formation. The authors used a wild type parent strain (*P. mirabilis* HI4320) and phase-locked (MR/P on or MR/P off) isogenic mutants of the parent strain. Results showed that wild type *P. mirabilis* expressed MR/P fimbriae when introduced into the urinary tract of mice, and the majority of bacteria colonizing bladder, ureter and kidney tissue (removed from the mice at 2 and

4 days), expressed MR/P fimbriae. They also found that all three strains were able to colonize the bladders of mice, but adhered to distinct areas demonstrating that other virulence factors are important in urinary tract colonization. In the same study they observed early-stage biofilm formation on glass cover slips. Phase locked on MR/P strains formed cell aggregates and typical 3-dimensional biofilms at 18 hours while wild type and MR/P phase-locked off strains only adhered to the glass surface as single cells. When they examined 48 hour biofilm formation in PVC microtitre wells the biofilms of the parent strain and the phase-locked off MR/P strain were similar, while the MR/P phase-locked on strain had formed a better biofilm. When the same strains were grown on cover slips for seven days however, the wild type formed much thicker biofilms (~ 63μm) compared to biofilms formed by phase-locked on and phase-locked off MR/P strains (~10μm). They concluded that MR/P fimbriae expression is important in auto-aggregation of cells and enhancement of biofilm formation in the early stages, but needs to be controlled for mature biofilm formation to take place. It seems therefore that while several studies have implicated MR/P fimbriae in the pathogenesis of UTI in the uncompromised urinary tract, there is no direct evidence supporting their role in CAUTI.

The most significant *P. mirabilis* virulence factor associated with complications in CAUTI is the enzyme urease, which is induced by urea (Nicholson *et al.*, 1993; Dattelbaum *et al.*, 2003). Urease action is the driving force for the formation of the crystalline biofilm that block catheters (Section 1.6). The enzyme is also thought to play a significant part in UTI. Jones *et al.* (1990) demonstrated the contribution of urease in infection using a mouse model infected with wild type *P. mirabilis* HI4320 strain and its urease-negative derivative. Quantitative results showed that not only was

the wild type strain more obstinate in the urinary tract of infected mice compared to the mutant strain, but was isolated in significantly higher numbers than the mutant from the urine, bladder and kidneys. In a similar study Johnson *et al.* (1993) also reported that the *P. mirabilis* strain was significantly more persistent in the bladder and kidneys of infected mice compared to the urease-negative strain. The wild type also caused significantly more episodes of renal pathology including pyelonephritis, renal abscesses and epithelial necrosis than the urease-negative strain. Urease is also known to be important for the function of other *P. mirabilis* virulence factors e.g. *P. mirabilis* protease enzymes that degrade host immunoglobulins IgA and IgG. These proteins are present in the urine and protect host membranes and tissues from bacteria and bacterial products (Rozalski *et al.*, 1997). *P. mirabilis* proteases work optimally at pH 8.0 and it is thought that elevation of urinary pH by urease action allows for maximum proteolytic enzyme activity on the IgA and IgG (Rozalski *et al.*, 1997).

1.6.   *Proteus mirabilis* and other urease producers in the encrustation of catheters

The care of patients undergoing long-term catheterisation is seriously compromised by infection with urease-producing bacteria, particularly *P. mirabilis*. Urease action on urea generates two molecules of ammonia to each molecule of carbon dioxide. These moieties form ammonium and bicarbonate ions and the net result is an increase in the concentration of OH^- ions and the generation of alkaline urine as shown below.

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$2NH_3 + 2H_2O \rightleftharpoons 2NH_4^+ + 2OH^-$$

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$
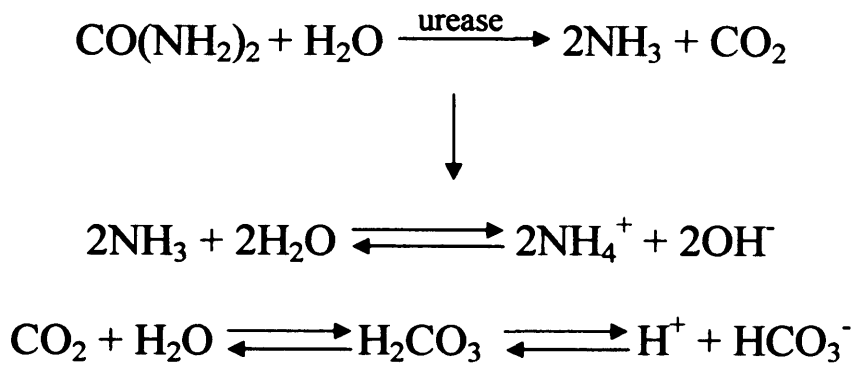
Under these conditions dissolved solutes including ionic calcium, magnesium and phosphate precipitate out of the urine in the form of ammonium magnesium phosphate or struvite, $[NH_4MgPO_46H_2O]$, and calcium phosphate species such as carbonate apatite $[Ca_{10}(PO_4)_6CO_3]$, (Hedelin *et al.*, 1984; Mobley and Warren, 1987; Kunin, 1989; McLean *et al.*, 1996). These phosphates are deposited on the internal and external surfaces of the catheter and become incorporated into the biofilm matrix forming crystalline biofilms (Cox *et al.*, 1987; Hedelin *et al.*, 1991; Morris *et al.*, 1997; Morris and Stickler, 2001). The crystalline biofilm can eventually prevent the device from draining. In the majority of these cases the only option is replacement of the catheter with a new device. Removing an encrusted catheter however, can be extremely painful for the patient, causing trauma to the urinary tract (Morris and Stickler, 1998b). Fragments of the crystalline biofilm can remain in the bladder, acting as a reservoir for the colonization and encrustation of the replacement device. Catheter replacement in patients infected with these urease-producing species is generally only effective for short periods until the replacement catheter itself succumbs to the ongoing encrustation process.

A number of urease-producing bacterial species including *P. mirabilis*, *P. vulgaris*, *Pv. rettgeri*, *Morganella morganii*, *K. pneumoniae*, and *Ps. aeruginosa* can be responsible for CAUTI. The most common organism isolated from the urine of long-term catheterised patients suffering from recurrent catheter blockage is *P. mirabilis* (Mobley and Warren, 1987; Kunin, 1989). It is also the species most commonly found on patients' encrusted catheters (Stickler *et al.*, 1993). Stickler *et al.* (1998) tested the encrusting ability of a number other urease-producing species using *in vitro* bladder models. They reported that *P. mirabilis*, *P. vulgaris* and *Pr. rettgeri* were all capable of elevating urinary pH and causing extensive catheter encrustation while *M. morganii*, *K. pneumoniae* and *Ps. aeruginosa* failed to produce alkaline urine and any appreciable crystalline biofilm. Hedelin *et al.* (1991) determined the composition of catheter encrustation, urinary pH and also the bacteriology of 11 long-term catheterised patients. They reported that patients with mean urinary pH values above a critical value of 6.8 were particularly subject to catheter encrustation and blockage. The elevated urinary pH was linked to the presence of persistent urease-producing *P. mirabilis*. They also found that calcium phosphate made up the bulk of deposits on catheters in patients with mean urinary pH <6.7, while magnesium phosphate made up the bulk of deposits > pH 6.8. In a subsequent study Burr and Nuseibeh (1993), determined a range of urinary parameters including calcium, magnesium, phosphate and pH in the urine samples collected from 40 long-term catheterised spinal cord injured patients, half of whom blocked their catheters regularly. From the data collected they calculated that the main risk factors connected to catheter blockage were a urinary pH > 6.9, and urinary concentrations of ammonium > 20 mM/L and calcium > 2.45 mM/L. Their general conclusions were that urease-producing bacteria

and urinary calcium concentration were the most important factors in catheter

blockage.

The pH value at which calcium and magnesium phosphates come out of solution in a

sample of urine has been termed the nucleation pH (pH_n) (Choong *et al.*, 1999). These

authors compared the voided pH (pH_v) and pH_n of urine samples from long-term

catheterised patients that either blocked or did not block their catheters. The results

were interesting in that the mean pH_ns of non-blockers and blockers were similar

(7.46 and 7.38 respectively). However, the pH_vs between these groups varied

significantly with non-blockers having more acidic (pH_v 5.97) urine than blockers

(pH_v 7.63). They concluded that the 'safety margin' (pH_n - pH_v) observed in blockers

urine was much reduced compared to that of the non-blockers, and this was important

in the development of encrustation.

A prospective study recently carried out by Mathur *et al.* (2006a), examined the

lifespan of catheters from 21 long-term catheterised patients with urease-producing

Proteus species in their urinary flora. They also determined the voided pH (pH_v) and

nucleation pH (pH_n) of weekly urine recorded in samples obtained from the patients.

They found that the times catheters took to block in the patients varied from 2-98

days. Patients were designated as slow encrusters (mean catheter life-span of >28

days) or rapid encrusters (mean catheter life-span of <28 days). The mean pH_v, pH_n

and safety margin (pH_n - pH_v) values were calculated for the two groups. The analysis

revealed that while rapid encrusters appeared to have a higher mean pH_v (7.20) than

slow encrusters (6.91) this difference was not statistically significant. The mean pH_n

of rapid encrusters (7.32) however, was significantly lower then the mean pH_n (8.08)

of those where catheters blocked less frequently ($P = 0.002$). Further analysis of this

data (Mathur *et al.*, 2006b) showed that there was a significant correlation between

catheter lifespan and urinary pH_n, the higher the pH_n the longer the catheter took to

block. Another important point from these studies was that there was considerable

variability in the pH_n values of the weekly urine samples obtained from these patients.

This led the authors to suggest that the 'critical pH' described by Hedelin *et al.* (1991)

or the risk factor of a pH >6.9 described by Burr and Nuseibeh (1993) were not set in

stone and could be manipulated. Strategies to elevate the pH_n and reduce the rates of

catheter encrustation in long-term catheterised patients thus seemed possible.

1.7. Urine composition, diet and their role in encrustation

Studies investigating ways of reducing the metabolic stone formation in the urinary

tract have shown that significant risk factors include high calcium and high oxalate

excretion in the urine, low citrate excretion and low urine output (Seltzer *et al.*, 1996;

Borghi, 2002; Siener and Hesse, 2002; Honow *et al.*, 2003; Meschi *et al.*, 2004;

Siener and Hesse, 2005). These studies have also demonstrated that urinary

composition can be manipulated successfully by dietary intervention, significantly

reducing the stone-forming potential in patients predisposed to metabolic urolithiasis.

Increasing urinary citrate excretion in stone-forming patients as a means of preventing

urolithiasis is already a recognised therapy. Citrate is a key intermediate in the citric

acid cycle essential in aerobic respiration, but is also excreted freely in urine. It is a

natural 'chelating agent', forming soluble complexes with various metal ions

including calcium and magnesium, and is an inhibitor of calcium oxalate stone

formation (Pak, 1987; Siener and Hesse, 2005).

Preventing or reducing encrustation in long-term catheterised patients infected with urease-producing bacteria by elevating citrate excretion is an attractive proposition. Wang *et al.* (1993) previously demonstrated that urease-induced crystallization in urine *in vitro* can be markedly delayed in urine supplemented with citrate. In synthetic and human urine containing no citrate the initiation of crystal formation by jack bean urease occurred at pH 6.54 and 6.68 respectively. When these urines were supplemented with 4 mM of citrate the initiation of crystal nucleation did not occur until pH 8.52 and 8.64 respectively. When they compared urine from two healthy volunteers with natural varying citrate concentrations, they also found that crystal nucleation occurred at a higher pH in the urine with the higher citrate concentration. In a further study Wang *et al.* (1994) also demonstrated that potassium citrate ingested orally significantly increased the concentration of citrate in urine in healthy volunteers.

Suller *et al.* (2005) also demonstrated the relationship between the concentration of important urinary solutes and pH_n using *in vitro* experiments. By manipulating the calcium, magnesium and phosphate concentrations in urine they showed that a degree of control over urinary pH_n could be achieved relatively easily. The concentration of the solutes had an inverse relationship on urinary pH_n. In addition, when citrate was added to the urine, its ability to form soluble complexes with the metal ions resulted in significant increases in pH_n. What was important in this study was that the pH_n of volunteers' urine could be elevated simply by increasing their consumption of water. In addition, when they increased the dietary citrate intake of the healthy volunteer, by introducing 500 ml of orange juice into the diet each evening, they found that citrate excreted in the urine gradually increased during the test period from 0.35 mg/ml at the

beginning to 1.21 mg/ml at the end (1 week). Importantly, the pH_n of the urine voided increased from pH 7.24 to over pH 8.2 during this period.

1.8. Attempts to control catheter encrustation

A variety of different approaches have been implemented to try and control catheter encrustation. The fact that urease-induced catheter blockage is still prominent in the catheterised community however, suggests that effective measures have yet to be identified.

1.8.1. Acidification of urine

Solutions aimed at dissolving catheter encrustation have been used with varying degrees of success. There are however, a number of citric acid based solutions currently available including Suby G (approximately 3.2% citric acid, ~ pH 4) and solution R (approximately 6% citric acid). Saline solutions are also widely used in bladder irrigation to remove obstruction and prevent clot retention after surgery but are thought to have little effect on encrustation (Evans and Godfrey, 2000; Shaw *et al.*, 2004; Getliffe, 1994 and Getliffe *et al.*, 2000). Evidence demonstrating the efficacy of the citric acid solutions is hard to find. As with the antiseptic bladder washouts the technique usually involves the instillation of 50-100 ml of the solution into the bladder through the catheter. The solution is retained in the bladder for about 15 minutes by clamping the catheter. The idea is that in this time the encrustation will dissolve in the citric acid (Getliffe *et al.*, 2000). Kennedy *et al.* (1992) performed a clinical study to assess the ability of three bladder maintenance solutions (Suby G, Solution R and saline) to reduce catheter encrustation. They concluded that none of

the solutions tested were effective at preventing catheter encrustation. They also failed to eliminate bacteria from the urinary tract and had no effect on urease-producing species. Irrigation of the urinary tract with these acidifying washouts may also have adverse effects on the patient and are thought to include damage to the bladder mucosa due to the acidic nature of the washout and physical damage caused by the actual method of installation (Kennedy *et al.*, 1992). Despite these findings bladder maintenance solutions are still widely used. McNulty *et al.* (2006) reported that 55% of nursing staff and 45% of other staff in registered care homes in three health districts in England regularly used bladder washouts or catheter maintenance solutions.

The possibility of lowering urinary pH through consumption or intravenous addition with acidifying compounds to prevent alkalization of the urine has also had little success. McDonald and Murphy (1959) administered daily doses of ascorbic acid (2.5 g) to patients and found it reduced urinary pH from about 6.0 to 5.6. A later study by Murphy *et al.* (1965) however, established that if the patients were infected with urease-producing bacteria, the oral ascorbic acid failed to reduce the urinary pH. This effect was confirmed by Bibby and Hukins (1993). These workers showed that the addition of acid (HCl) to urine containing the enzyme urease was rapidly neutralized by the hydrolysis of more urea to ammonia. They calculated that to neutralize the amount of ammonia produced from the urea excreted per day by a normal adult, something like 2-3 L of 0.1M HCl would be required. Dietary acidification of urine is clearly not a feasible strategy to control catheter encrustation.

Many nurses advise their patients to drink cranberry juice as a possible remedy for catheter encrustation. Morris and Stickler (2001) examined the efficacy of this strategy. They collected urine from volunteers during control periods when they were drinking their normal volume of fluids and when they had supplemented their daily fluids with either 500 ml of cranberry juice or water. The amounts of calcium and magnesium deposited on catheters were determined when the urines from these volunteers were supplied to bladder models infected with *P. mirabilis*. The results demonstrated that drinking 500 ml of cranberry juice on top of the normal fluid intake reduced encrustation on catheters compared to that observed under the normal fluid intake. However, they found that drinking an extra 500 ml of water was just as effective. These experiments also showed that the mean urinary pH of volunteers was not significantly acidified after drinking cranberry juice. It was concluded that increased fluid intake was the most important factor in reducing catheter encrustation.

1.8.2. Novel biomaterials

The development of a novel urinary catheter biomaterial with properties that can inhibit bacterial colonization has so far proved unsuccessful. In recent years, catheters with hydrophilic hydrogel coatings have become commonplace in the catheterised community with some studies suggesting they reduce bacterial attachment (Park *et al.*, 2002; Tunney and Gorman, 2002). However, comprehensive studies have shown that to date no catheter material has been produced that can avoid colonization by crystalline *P. mirabilis* biofilms (Morris *et al.*, 1997; Morris and Stickler, 1998a; Stickler *et al.*, 2002). A clinical study by Bull *et al.*, (1991) found that while patients preferred hydrogel-coated latex to silicone-coated latex catheters, encrustation and blockage was more prevalent in the hydrogel-coated devices.

The reason why novel coatings are ineffective could partly be due to the promotion of bacterial adherence by the host protein conditioning layer that covers the surface of many devices soon after their insertion (Barbucci and Magnani, 1994; Stickler and McLean, 1995; Santin *et al.*, 1997). In relation to this, Tenke *et al.* (2004) examined the encrustation resisting abilities of catheters coated with heparin in bladder models infected with *P. mirabilis*. The basis of the heparin coating relies on its anticoagulant properties that inhibit host protein adhesion and its strong electronegativity which repels bacterial cells (Tenke *et al.*, 2004). Although this coating showed significant reductions in encrustation on ureteral stents from clinical trials, heparin-coated catheters in the bladder models infected with *P. mirabilis* blocked at similar times to untreated silicone catheters.

1.8.3. Antibacterial catheters

Over the years several attempts have been made to produce catheters impregnated or coated with antimicrobial agents, the primary aim being to prevent CAUTI. Only two such catheters however, a silicone catheter impregnated with nitrofurazone and a catheter coated in metallic silver, have been used in clinical practice. Although their use is widespread particularly in the USA, there is considerable controversy over their efficacy. Previous clinical trials have suggested significant benefits in using antibacterial catheters to prevent CAUTI. However, these studies have consistently failed to stand up to professional scrutiny. In a comprehensive Cochrane database review of clinical trials involving silver-alloy catheters Brosnahan *et al.* (2004) concluded that the evidence in reducing infection was weak and the trials were of poor quality. Trautner *et al.* (2005) suggested that clinical trials using bacteriuria as the measure for infection are fundamentally flawed, as >90 % of bacteriuric episodes

in catheterised patients are asymptomatic. They point out that trials showing that antimicrobial catheters significantly reduced episodes of symptomatic bacteraemia or pyelonephritis would be far more valuable. Many trials are also carried out in short-term catheterisation events (<14 days) and wrongly presume that the same effect will occur in long-term (>30 days) catheterised patients (Trautner *et al.*, 2005). Johnson *et al.* (2006) reviewed the efficacy of nitrofurazone and silver-containing catheters. They concluded that on the evidence presented the currently marketed antimicrobial catheters may prevent bacteriuria in hospitalized patients during short-term catheterisation. They concluded that the majority of studies failed to provide data regarding their effect on morbidity, mortality, symptomatic UTI and bacteraemia. They also stated that the effectiveness of these catheters is variable and depends on the type of antimicrobial coating, type of patient control group, and type of control catheters compared against. There is no evidence to date that nitrofurazone or silver-alloy catheters prevent *P. mirabilis* infection. Conversely, there is evidence to suggest that *P. mirabilis* is able to block both these catheter types with crystalline biofilm (Morris *et al.*, 1997; Morris and Stickler, 1998a).

Attempts to control catheter encrustation with antimicrobial catheters must ensure that the antimicrobial agent is active for at least 6-12 weeks, the scheduled placement period of catheters. Bibby *et al.* (1995) suggested that the catheter balloon could be used as a reservoir for large amounts of an antibacterial agent and that the membrane of the balloon could act to control the slow release of the agent into the urine over prolonged periods. They were also able to show that mandelic acid could diffuse through the balloon in this way. Unfortunately mandelic acid has poor activity on *P. mirabilis* (King and Stickler, 1992). In contrast the biocide triclosan is extremely

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active against this species with minimum inhibitory concentrations of 0.5 µg/ml having been reported against isolates of *P. mirabilis* from encrusted catheters (Stickler, 2002). Stickler *et al.* (2003a) demonstrated that triclosan prevented *P. mirabilis* crystalline biofilm formation on all-silicone catheters inserted into *in vitro* bladder models. In this study catheter balloons were inflated with 10 mg/ml triclosan solution for the duration of the experiments or inflated with a similar concentration of the triclosan solution for 24 hours before it was replaced with sterile water. The catheters in both sets of experiments drained freely for a 7-day experimental period and scanning electron microscopy observations of catheter sections revealed little evidence of bacterial colonization or encrustation. Jones *et al.* (2006) demonstrated that inflating the retention balloons of catheters with triclosan prevented biofilm formation of a number of other urinary tract pathogens including *E. coli*, *K. pneumoniae* and *S. aureus* on catheter surfaces using similar laboratory models. They also showed that concentrations of triclosan recovered in the voided urine in the models varied between 0.02 and 0.16 mg/L. However, a number of bacterial species including *Ps. aeruginosa* and the potent urease producer *Pv. rettgeri*, were highly resistant to triclosan. In light of this, the future use of triclosan may be limited as selection for these organisms could occur. Another problem with prolonged exposure of bacterial species to antibacterials (especially in sub-inhibitory doses), is the development of resistance in susceptible species. If triclosan is utilised in the control of encrustation in long-term catheterised patients it would therefore be essential to monitor the urinary flora of resident organisms.

1.8.4. Urease inhibitors

Several chemical compounds that can be consumed orally that inhibit urease action have been evaluated as potential agents in preventing catheter encrustation. Morris and Stickler (1998b) studied the effect of varying concentrations of acetohydroxamic acid (AHA) and fluorofamide on P. mirabilis induced catheter encrustation using laboratory bladder models. They demonstrated that urine supplemented with either of these urease inhibitors was able to reduce urine alkalinity and encrustation. In their study concentrations of AHA (0.1 mg/ml) and fluorofamide (1.0 µg/ml) significantly reduced the amount of calcium and magnesium salts deposited on catheters compared to that in controls in 24-hour experiments. Previous studies have revealed however, that several hydroxamates are mutagenic and possible carcinogens, which has prevented their clinical use (Rosenstein and Hamilton-Miller, 1984). Although fluorofamide is not reported to be mutagenic, it is slightly toxic (Millner et al., 1982). As long-term catheterised patients infected with urease-producing species would require ongoing treatment to prevent their catheters from encrusting, the possible toxicity of these urease inhibitors means they are unlikely to be considered as an option. Wang et al. (2006) reported that resveratrol (a naturally occurring plant compound found in several food stuffs including grapes) was active against several virulence factors of P. mirabilis including urease activity. The effect of resveratrol on catheter encrustation however, has not yet been studied. As a naturally occurring compound resveratrol appears an attractive alternative in preventing catheter encrustation and further studies are therefore warranted on its efficacy.

1.9. What are the current problems and areas of uncertainty?

Despite many attempts to prevent and control catheter encrustation it remains a major complication in the care of many elderly and disabled people. While the basic mechanism has been established we still need to know more about some of the fundamental aspects of the process. Little is known about the early events and the precise mechanisms which *P. mirabilis* uses to colonize catheter surfaces. The factors that control the rate at which the crystalline biofilm forms on the catheters are also unknown. The expansion of our knowledge and understanding of these processes would be advantageous in devising future strategies aimed at reducing catheter encrustation.

1.10. Aims of the study

- To establish the sequence of events in the early stages of crystalline *P. mirabilis* biofilm formation on the range of catheters currently available for use with patients.

- To determine the role of Mannose-Resistant *Proteus*-Like (MR/P) fimbriae in the colonization of catheters.

- To investigate how the nucleation pH of urine can be manipulated and determine the effect of variation in this parameter on the rate of catheter encrustation.

2. Materials & Methods

2.1. Chemicals

The chemicals used in this study, unless otherwise stated were purchased from Fisher Chemicals (Loughborough, UK).

2.2. Catheters

All catheters used in this study were purchased from Bard Ltd (Crawley U.K), except for nitrofurazone impregnated catheters which were obtained from Coloplast (Humlebaek, Denmark). All catheters were of size 14 Ch.

2.3. Growth media

A number of different culture media were used for isolation, culture and enumeration of bacterial species described in this thesis. Cysteine Lactose Electrolyte Deficient (CLED) agar (Oxoid Ltd, Basingstoke UK), inhibits swarming behaviour and was used in obtaining single colonies in enumeration and identification purposes. Chromogenic UTI (CUTI) agar (Oxoid) has been developed especially for the identification of urinary tract pathogens, and was used in conjunction with CLED agar for identification and enumeration of catheter biofilm associated bacteria from clinical catheters. CUTI agar is CLED based allowing for growth of single colonies, but also contains two chromogenic substrates X-Glucose, and Red-Galactose. These chromatogens are cleaved by bacterial enzymes β-glucosidase and β-galactosidase respectively, giving colonies a characteristic morphological colour depending on which enzyme(s) they produce. Tryptone Soya Agar (TSA, Oxoid) was used to determine the presence of swarming behaviour from bacteria isolated from clinical

catheter specimens, and also to determine *P. mirabilis* growth and swarming behaviour in triclosan minimum inhibitory concentration (MIC) tests.

Tryptone Soya Broth (TSB, Oxoid) was used as a nutritional supplement in artificial urine. Luria-Bertani broth (LB broth, Sigma-Aldrich) was used as growth media for all haemagglutination assays and for *P. mirabilis* strains tested in autoaggregation assays.

Artificial urine (supplemented with 1 g/L TSB) was the preferred growth media used in the preparation of *P. mirabilis* cultures to be inoculated into bladder models. TSA supplemented with 5% defibrinated horse blood (Fisher Scientific) and 0.3% yeast extract (Oxoid) and Brain Heart Infusion Agar (BHI) supplemented with 0.3% yeast extract were the two types of media used to culture the control strains of *Streptococcus sanguis* GW2 and *Actinomyces naeslundii* 12104 (kindly donated by Dr. Pauline Handley, University of Manchester, UK) used in coaggregation/autoaggregation assays.

2.4.   Bacterial strains

A number of bacterial strains were used during this study. *P. mirabilis* NSM6 was a clinical isolate from an encrusted catheter donated by Dr. Nicola Morris. *P. mirabilis* HI4320 (wild type) and its fimbrial mutant *mrp⁻* HI4320 and urease-negative mutant *ure-* HI4320, were kindly supplied by Professor Harry L. T. Mobley, Division of Infectious Diseases, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland. The wild type strain was isolated from the urine of an infected catheterised patient. Other *P. mirabilis* isolates (used in haemagglutination assays) originating from catheter biofilm, urine and stool samples of catheterised

patients and also from environmental sources were supplied by Dr. Nora Sabbuba and Dr. Marc Suller. *P. mirabilis* SJ2 was donated by Dr. Steve Jones. *Providencia rettgeri* (SDM1) and *Proteus vulgaris* (SDM2) were both isolated from catheters of patients attending urology outpatients clinic at Southmead Hospital, Bristol, UK. The stocks of bacterial isolates were stored at - 80°C in 5% glycerol. Confirmation of isolates were identified by Gram stain and the appropriate BBL CRYSTAL biochemical analysis kit (Becton Dickenson, Maryland, USA).

2.5. Growth conditions of isolates and strains used in bladder models

Prior to use in experiments all strains were grown initially overnight on CLED agar at 37°C. In the majority of cases cultures were prepared for the bladder model by subsequently transferring 4 or 5 of the overnight colonies into a culture flask containing sterile artificial urine (100 ml), and incubated on a rotary shaker at 150 rpm for 4 hours giving a cell density of approximately 10^8 colony forming units per ml (cfu/ml).

The inocula of HI4320 and *mrp⁻* HI4320 strains for experiment in the bladder model were prepared by static incubation for 12 hours in culture flasks containing sterile artificial urine (100 ml) at 25°C or in sterile artificial urine supplemented with 4 µg/ml of the urease inhibitor fluorofamide (Tocris Cookson Ltd., Bristol, UK).

2.6. Viable cell counts

Cell numbers from cultures and bacterial suspensions were estimated using the methods described by Miles and Misra (1938). Samples to be enumerated were serially diluted in quarter strength ringers solution (Oxoid) to a maximum of 10^{-7} of

the original dilution with triplicate 3 X 10 µl aliquots of each dilution deposited onto dry CLED agar plates. The enumeration of bacteria isolated from biofilms residing on clinically derived catheters was carried out by spread plate method. Here, serial dilutions were carried out on samples as described above and 100 µl aliquots were deposited onto separate dry CLED and CUTI plates and spread aseptically with a glass spreader to ensure adequate dispersal of the inocula.

2.7. Isolation and enumeration of bacteria from patient's catheters

Catheters removed from patients attending clinics at Southmead Hospital were stored at 4°C overnight until transportation to the laboratory for characterisation and enumeration of their bacterial flora. Catheters were sectioned at two sites along the catheter length (unless otherwise stated) with 1 cm specimens removed from the eyehole area and from a site 15 – 16 cm down from the eyehole. Sites were chosen at opposite ends of the catheters to lower the risk of overlooking spatially dispersed isolates. Sections were sonicated for five minutes (Transsonic T310, Camlab Ltd., Cambridge, UK) in sterile universal containers with 10 ml of quarter strength ringers solution (Oxoid LTD, Basingstoke, UK), to break up the crystalline deposits. Sonicated samples were thoroughly vortex-mixed for two minutes and serially diluted from neat to a maximum of 10^{-7} dilutions. A series of plates containing CLED agar and Chromogenic UTI agar were spread with 100 µl of each dilution. In addition, a TSA plate was inoculated with 10 µl of the neat sample to detect any swarming bacteria. Plates were incubated for 24 hours and 48 hours. Identification of bacterial isolates was determined using Gram staining and BBL CRYSTAL test kits. The numbers of viable cells per millimetre of catheter length (cfu/mm) were calculated. The isolates from the biofilm communities were stored at -80°C in 5% glycerol. The

determination of biofilm communities on hydrogel silver-coated catheters described in results section 3.1.4. were carried out by Dr. Nora Sabbuba, Cardiff University.

2.8.   Minimum inhibitory concentration (MIC) of triclosan

The MIC of triclosan against *P. mirabilis* NSM6 and two other clinical isolates (*P. mirabilis* HI4320 and *P. mirabilis* SJ2) was tested in TSA agar. Initially 0.5 g of triclosan (CIBA speciality chemicals, Basel, Switzerland), was dissolved in 10 ml of 100% dimethyl sulfoxide (DMSO), giving a concentration of 50 mg/ml. Aliquots of this solution were then mixed aseptically into molten TSA agar, and poured into Petri dishes to give a range of plates containing 0.1, 0.2, 0.3 and 0.4 μg/ml final triclosan concentrations. Plates were left to stand overnight. *P. mirabilis* strains were grown on CLED agar overnight at 37°C with 4-5 colonies subsequently transferred to culture flasks containing TSB (100 ml) and incubated for 4 hours at 37°C under gentle aeration. At 4 hours, aliquots of the cultures were diluted in TSB 100-fold and then 10 μl aliquots containing approximately 10^4 cells spotted in triplicate onto the relevant agar plates. Control plates used in these tests included DMSO in TSA (at the same DMSO concentration as in the 0.4 μg/ml triclosan agar), and TSA only. All were incubated overnight at 37°C prior to inspection.

2.9.   Artificial urine

A standard artificial urine based on that described by (Griffith *et al. 1976*) was prepared in deionised water using the following major constituents g/L: 0.49 calcium chloride; 0.65 magnesium chloride hexahydrate; 4.6 sodium chloride; 2.3 di-sodium sulphate; 0.65 tri-sodium citrate dihydrate; 0.02 di-sodium oxalate; 2.8 potassium di-

hydrogen phosphate; 1.6 potassium chloride; 1.0 ammonium chloride; and 25.0 urea. The urine pH was adjusted to pH 6.1 using NaOH before addition of 5.0 g/L gelatine and supplemented with 1g/L TSB. In experiments where fluorofamide was used to inhibit the effect of urease activity, the urea analogue was dissolved in deionised water and sterilised using a 0.2 μm pore size capsule filter (Sartorious, Goëttingen, Germany) prior to its addition to the standard urine at 4 μg/ml final concentration. When dilutions of the standard urine were required in experiments, the constituents were fractioned accordingly upon addition. Urine that contained increased citrate concentrations was achieved by manipulation of the tri-sodium citrate content. In experiments where citrate content of the artificial urine was manipulated the pH was adjusted to pH 6.3.

2.10. The bladder model set-up and experimental procedure

The physical models of the catheterised bladders have been previously been described by Stickler *et al.* 1999. They were assembled in the same way for all experiments. Essentially, they consist of a glass chamber (bladder) contained within a water-jacket maintained at 37°C. The entrance of the chamber is sealed with a rubber stopper. Silicone tubing (ureter) connects the chamber to a urine reservoir (kidneys) via a 503U peristaltic pump (Watson & Marlow, Falmouth, UK). At the base of the chamber is an outlet port fitted with a 30 cm length of silicone tubing acting as a urethra. Prior to experiments the models were sterilised by autoclaving at 121°C for 15 minutes. Catheters were inserted aseptically via the urethra. Catheter balloons were inflated normally with 10 ml sterile deionised water and attached to drainage tube and collection bag. The models were typically assembled in sets and held in position with clamps and stands. The collection bag tubes were secured (taped) to the workbench

under enough tension as to allow the retention balloon of the catheter to form a watertight seal with the internal surface of the chamber. The bladder chambers were then primed with urine from the aspirator via the peristaltic pump up to the catheter eyehole level. At this stage the pump was switched off and a 10 ml aliquot of urine removed from the model and replaced with a 10 ml inoculum of the relevant bacterial culture. Models were allowed to incubate for 1 hour to allow the bacterial population to establish itself in the chamber before the pump was restarted (time 0, experiment start). The rate that urine was supplied to the bladder models at the start was pre-determined and varied according to each experiment. The experimental set-up of bladder models and a schematic diagram of the bladder model are shown in Figure 2 and 3 respectively.
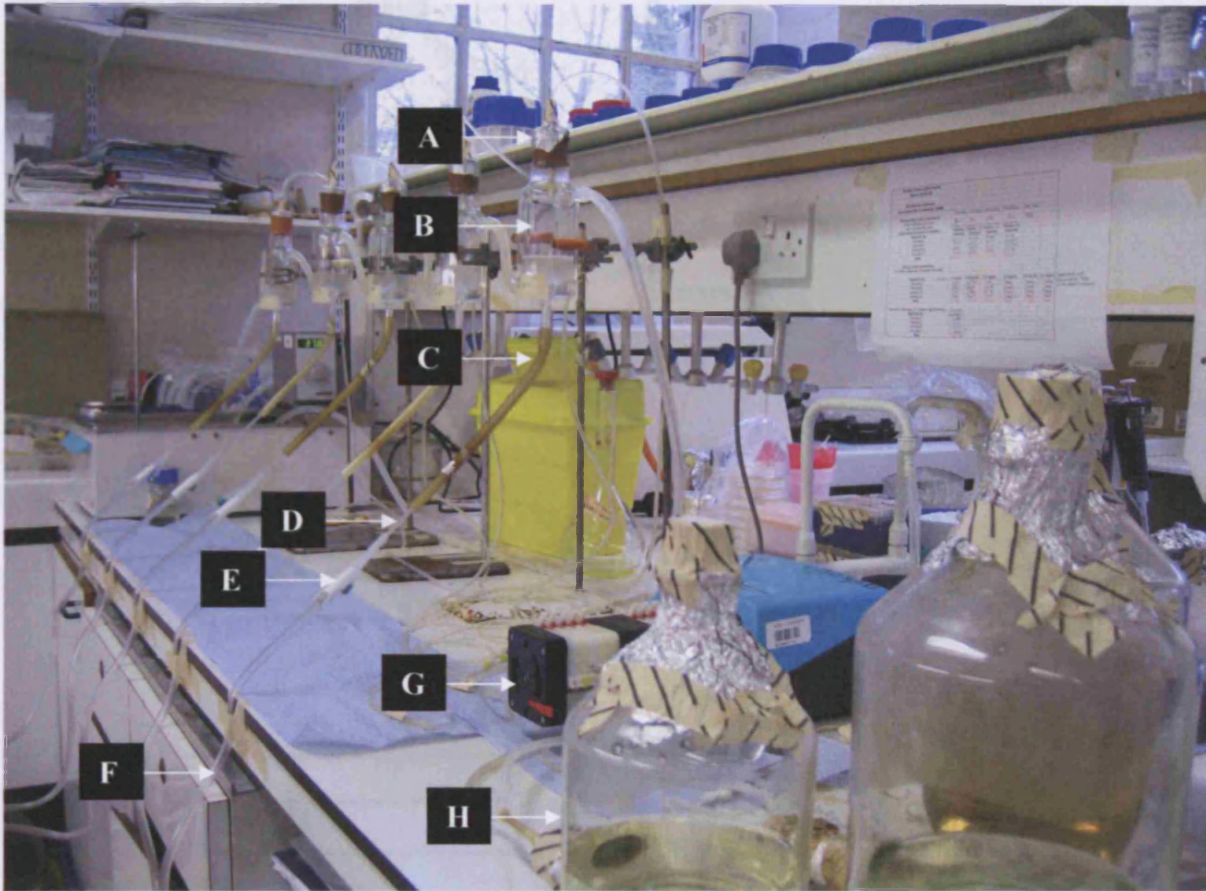
Figure 2.   Experimental bladder models assembled in series

The photograph shows: (A) The inlet supplying urine and bladder model seal, (B) bladder chamber, (C) urethra, (D) catheter leaving urethra, (E) catheter/drainage tube junction and inflation port, (F) drainage tube leading to drainage bag (bag not shown), (G) peristaltic pump, (H) aspirator containing sterile urine.

Ureter

Bladder

Retention balloon

Urethra

Water in 37°C

Pump

Aspirator

Urine

Catheter

Balloon inflation port

Drainage bag

Drainage tap

Figure 3. Bladder model set-up

Bladder models were typically arranged in series and inter-connected using silicone tubing attached to the water jacket inlets/outlets. Models were held in position via clamp/stand. Aspirators supplied sterile urine to the models via peristaltic pump through silicone tubing acting as the ureter. Catheters were secured to the workbench using tape under enough tension to ensure a watertight seal between the retention balloon and the bladder model inner surface.

2.11. Production of catheter sections for analysis

Catheters were sectioned for a number of purposes including high and low vacuum electron microscopy, the enumeration of adhering bacteria on the catheter surface and characterisation of the bacterial flora from biofilms on catheters of clinical origin. Sections 1cm in length, were cut from four regions of the catheter: A-the eyehole region; B-immediately below the eyehole; C-immediately below the retention balloon; D-16 cm down from the eyehole. Figure 4 shows a Foley type catheter with the location of the sample sites indicated. Before removing catheters from bladder models the urine was removed from the bladder chamber using a pipette. Catheters were carefully disconnected from the drainage bag and the contents of the retention balloon removed using a 10 ml syringe. The end of the catheter (housing the balloon inflation port) was separated with a scalpel, allowing the catheter body to be carefully removed through the inlet end of the bladder model chamber with forceps. Catheter sections used in low vacuum SEM analysis or for enumeration of attached bacteria were sectioned transversely only. Sections destined for high vacuum SEM analysis were additionally sectioned longitudinally (B-D only) in order to expose the internal lumen.

2.12. Enumeration of bacterial cells on catheters from experiments in the bladder model

Catheter sections (described in 2.11.), were removed from the models at the end of each experiment and placed into sterile universal containers pre-filled with 10ml Ringer's solution (Oxoid UK, Ltd). The samples were subsequently exposed to 5 minutes of gentle sonication in a sonic waterbath (Camlab, Cambridge UK) followed

by 2 minutes of vortex mixing and serially diluted with 10 µl aliquots dropped onto

CLED agar plates incubated overnight at 37°C for enumeration. The calculated viable

cfu/ml was proportional to numbers of viable cells colonizing the catheter surface per

millimetre of catheter length.

2.13. High vacuum scanning electron microscopy (HVSEM)

Catheter sections were fixed in 2.5% glutaraldehyde (Agar Scientific, Essex, UK) in

0.1M Sörrensen buffer pH 8 for 4 hours at room temperature or overnight at 4°C.

Sörrensen buffer was prepared by initially making 1 L stock solutions in deionised

water of: (A) 14.196 g di-sodium hydrogen orthophosphate and (B) 13.609 g

potassium di-hydrogen orthophosphate. Stock solutions were mixed at a ratio of

95%:5% (A:B) to give the required pH. Subsequently, sections were washed in 0.1M

Sörrensen buffer for 15 minutes and exposed to a second fixing/background staining

process using a 2% osmium tetroxide in 0.1M sörrensen buffer (1:1) for 1 hour. A

further 15 minute wash in deionised water was carried out prior to dehydration of the

samples using an ascending ethanol series (70%, 90% and 2 x 100%), and finally

sublimation dehydration using hexamethyldisilazane (HMDS), i.e. 100%

ethanol:HMDS (1:1), and finally 100 % HMDS x 2 (15 minutes each step). The

samples were left to air dry overnight before being gold sputter coated using an

Edwards S150B sputter coater (BOC Edwards, Crawley, UK), and visualised using a

Philips XL-20 scanning electron microscope (FEI UK Ltd., Cambridge, UK)

accelerating voltage 15kv-25kv. Sörrensen buffer pH 6 (15%:85%, A:B) was used in

the fixation of samples from urease-inhibited experiments where urine remained

acidic.
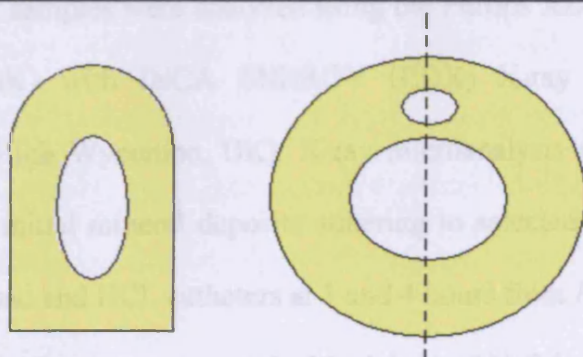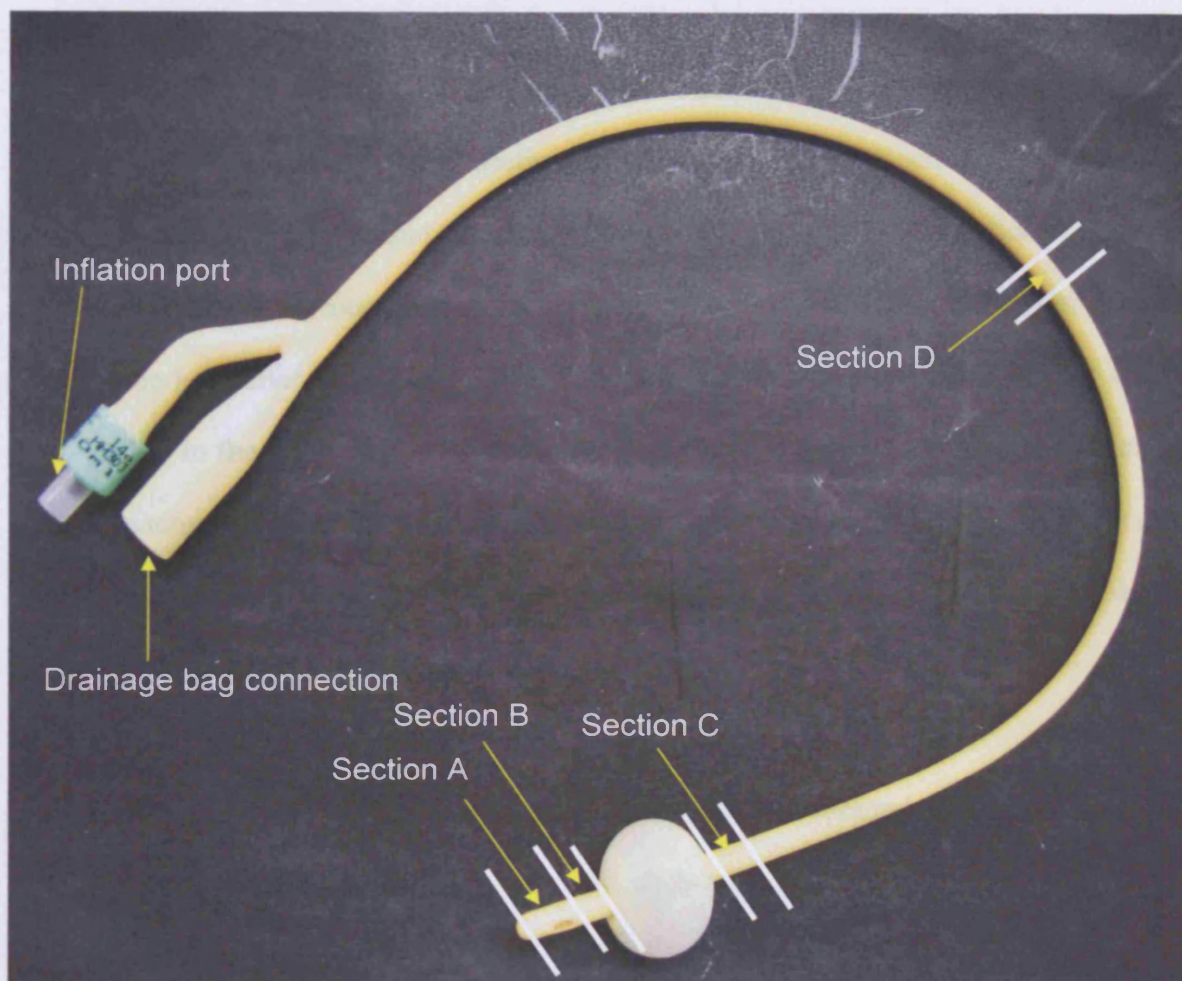
Figure 4. **Catheter sectioning sites (image shows hydrogel-coated latex catheter)**

Section A (eyehole region), section B (immediately below eyehole), section C (immediately below retention balloon), and section D (16 cm below eyehole). Below main image are examples of catheter sections which include the eyehole and lumenal section (with dotted line indicating sectioning for HVSEM).

2.14. Low vacuum scanning electron microscopy (LVSEM)

Sections of catheter analysed under low vacuum SEM were not subjected to any further processing after sectioning. Samples were placed in eppendorf tubes containing moist tissue paper to alleviate the effects of dehydration on specimens while awaiting examination. In the majority of cases, samples were examined within the hour after removal from the bladder model, but on occasion overnight storage at 4°C in the moistened eppendorf tube was required before microscopy. The extent of encrustation/biofilm formation on the catheter sections was determined using a JEOL JSM 5200 scanning electron microscope (JEOL (UK) Ltd., Welwyn Garden City, UK), accelerating voltage 20-25kv.

2.15. Environmental scanning electron microscopy (ESEM) and X-ray microanalysis

A selection of samples were analysed using the Philips XL-30 ESEM (FEI UK LTD., Cambridge UK) with INCA ENERGY (EDX) X-ray analysis system (Oxford Instruments, High Wycombe, UK). X-ray microanalysis was used to determine the nature of the initial mineral deposits adhering to selected catheter sections removed from all-silicone and HCL catheters at 1 and 4 hours from *P. mirabilis* NSM6 infected bladder models. Sections were washed in deionised H_2O before dehydration for 24 hrs at 37°C and then carbon sputter-coated using a K450 sputter-coater (Emitech, Kent, UK) prior to analysis.

2.16. Determination of the calcium and magnesium concentration in solution at various pH's

Urine (250 ml, pH 6.1) was heated in a water-bath at $37^{\circ}C$ for 1 hour and the pH increased stepwise in 0.5 increments to pH 9 using 1M NaOH solution. At each 0.5 interval 10 ml of the urine was removed into Falcon tubes and centrifuged for 5 minutes at 3600 rpm using a PK 120 centrifuge (ALC International, Milan, Italy) to pellet precipitated material. Aliquots of the supernatant were then serially diluted in universal containers with polished deionised H_2O to a range including; neat artificial urine, 1:10, 1:50, 1:100, 1:200 and 1:500 concentrations to give 4 ml final volumes which were stored at $4^{\circ}C$. Using an aa/ae spectrophotometer 457 (Thermo Electron Corporation, Waltham, Massachusetts, US) the calcium and magnesium concentrations in the urine at each pH increment was determined. Dilutions were necessary as calcium and magnesium concentrations were required to be within a specific range (between 0.5 and 2 ppm for calcium at 422.7 nm, and between 0.05 and 0.4 ppm for magnesium at 285.2 nm). The atomic absorbance spectrometer was initially calibrated to these ranges with standards purchased from Spectosol (BDH Chemicals Ltd., Poole, UK). The polished deionised H_2O was also tested for its calcium and magnesium content with results adjusted accordingly.

2.16.1. Determination of calcium, magnesium and citrate content in human urine

Calcium and magnesium concentrations were determined from 5 ml aliquots of collected human urine that were centrifuged at 3600 rpm for 10 min using a PK 120 centrifuge (ALC International), and sterilised using a Minisart millipore filter $0.2\mu m$ pore size (Sartorious) and stored at 4 $^{\circ}C$ prior to spectroscopic analysis. Samples were

serially diluted using polished deionised H_2O to give final concentrations of neat urine, 1:10, 1:50, 1:100, 1:200, 1:400 and 1:800 and their calcium and magnesium content determined by atomic absorbance spectroscopy. Citrate was determined using a citrate assay kit (Boehringer Mannheim, Darmstadt, Germany). Samples (1 ml) were filtered through a Minisart millipore filter 0.2µm pore size (Sartorious) as soon as possible or after storage at 4°C. Samples stored at 4°C were returned to approximately room temperature before being assayed.

2.17. Haemagglutination assay

The presence of MR/P fimbriae was detected by their ability to agglutinate chicken red blood cells (CRBC) in the presence and absence of mannose. The mannose resistant haemagglutination (MRHA) assays here are based on methods described by Bahrani *et al.* (1994). *P. mirabilis* isolates were grown statically in flasks containing 100 ml sterile Luria Bertani Broth, and passaged three times for 48 hours each at 37°C to achieve optimum conditions for MR/P fimbrial expression. Cells were harvested at 12000 x g for 1 minute using a Genofuge 16M centrifuge (TECHNE, Cambridge, UK), and re-suspended to approximately 10^9 cfu/ml in phosphate buffered saline (PBS, pH 7.2 0.85%), and PBS + 50 mM mannose.

Doubling dilutions from neat to 1:16 of the *P. mirabilis* suspensions were then set up in a 96 well u-bottomed microtitre plate, (Bibby Sterlin Ltd., Staffordshire, UK) with an equal volume of 3% CRBC suspension (Research Diagnostics Inc., Concord, MA, USA). The complete microtitre plate was assembled so that bacterial strains were incubated with CRBC in PBS only, CRBC in PBS + 50 mM mannose, tannic acid-treated CRBC in PBS, and tannic acid-treated CRBC in PBS + 50 mM mannose. The

loaded microtitre plates were incubated gently on a platform shaker for 1 hour at room temperature. Haemagglutination was determined by blood cells forming a diffuse matt in the microtitre well while the formation of a tight button of blood cells in the bottom of wells indicated that no haemagglutination had occurred. Wells containing corresponding buffers and CRBC were set up as controls.

2.18. Coaggregation/autoaggregation assay

To observe if the phenomenon of autoaggregation occurred in *P. mirabilis* isolates expressing MR/P fimbriae, visual autoaggregation assays were carried out on wild type *P. mirabilis* HI4320, and the *mrpA* mutant *P. mirabilis* HI4320 in comparison with two known coaggregating strains of *Streptococcus sanguis* GW2 and *Actinomyces naeslundii* ATCC 12104 (Handley *et al.*, 1985). Methods were based on those described Cisar *et al.* (1979). *P. mirabilis* strains were grown statically and passaged in LB Broth for 6 days (under conditions described in Section 2.17), to allow for the optimum expression of MR/P fimbriae. Overnight plate cultures of *Streptococcus sanguis* GW2 and 48 hour plate cultures of *Actinomyces naeslundii* ATCC 12104 were used to inoculate separate flasks of BHI broth (100 ml). The flasks were incubated statically at 37°C overnight in an aerobic Gaspak ™ Jar (Becton Dickenson) containing a BBL ™ dry anaerobic indicator strip (Becton Dickenson), a BBL ™ Gaspak ™ anaerobic hydrogen and carbon dioxide generator envelope (Becton Dickenson), and 0.5% palladium catalyst. The growth of urinary strains and oral strains were orchestrated so that they would be ready for the assay at the same time.

Cells were harvested at 5000 x g at 4°C for 10 minutes using an Avanti® J-E centrifuge (Beckman Coulter UK Ltd., High Wycombe, UK) and washed three times in coaggregation buffer consisting of: 1 mM Trizma® Base, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.15 M NaCl. Urinary strains to be assessed in artificial urine (*P. mirabilis ure* HI4320 only) were washed in artificial urine. Bacterial suspensions were adjusted to optical densitiy readings of 1.5 using a Helios γ UV-Visible Spectrophotometer (Unicam, Cambridge, UK) set at 650 nm. Assays were performed in sterile test-tubes with the coaggregation control consisting of 2 ml of each oral strain (4 ml total), while autoaggregation assays consisted of 4 ml of the urinary *P. mirabilis* strains. Test tubes were vortex mixed for 10 s followed by gentle rolling between the palms of the hands for 20 s and the level of coaggregation in oral strains and autoaggregation in urinary strains visually assessed using the following criteria:

Score	Level of aggregation
0	No aggregates visible in suspension
+1	Small, uniform aggregates in turbid suspension
+2	Easily visible aggregates in turbid suspension
+3	Clearly visible aggregates that settle leaving an almost clear supernatant
+4	Large aggregates that settle almost instantaneously to leave clear supernatant

Scores were completed at one hour intervals from the first time of mixing (t=0) up to 6 hours and then finally at 24 hours. The pH of cell suspensions were taken at t=0 and at 24 hours.

2.19. Determination of nucleation pH (pH$_n$)

Evaluation of the nucleation pH (pH$_n$) of artificial and human urines was based on the method described by Choong *et al.* (1999). In experiments examining the effect of increased fluid output and increased citrate concentration, artificial urine was maintained at 37°C with samples subsequently alkalinized in increments of 0.20 pH units with 1 M sodium hydroxide solution up to a maximum pH of 10. At each increment the optical density was measured at 600 nm against a urine blank with a Unicam Helios γ spectrophotometer (Cambridge, UK).

In subsequent experiments examining the pH$_n$ of human volunteer urine the pH of samples was reduced to pH 5.0 (urine blank) using concentrated hydrochloric acid before being alkalinised in 0.25 increments using 10 M sodium hydroxide. Samples were read against the blank at 540 nm. The pH$_n$ of a urine sample was determined from the resulting plot of pH versus optical density and was defined by an abrupt change in the slope of the graph showing an increase in turbidity. Plotting pH versus optical density produces two straight line segments which intersect at the pH$_n$. Regression lines were calculated by least squares analysis for these two portions of the graph and used to determine the pH at their intersection. If the pH$_n$ of urines calculated by regression analysis failed to correlate with the abrupt rise in absorbance recorded from spectrophotometer readings, the pH$_n$ was determined as the mean value between the initial reading when an abrupt and sustained increase in absorbance occurred and the preceding reading.

2.20. Statistical analysis of data

Minitab® Release 14 (Minitab Inc., PA, USA) was the statistical software package of choice used on data in this thesis. Unless otherwise stated experiments were

performed in triplicate and where appropriate the standard error (SE) of the mean was indicated. Analysis of variance (ANOVA) executed at the 95% confidence interval level was the statistical test of choice for all experiments. Least squares regression was the statistical test used to determine the nucleation pH of all relevant urines. Pearson correlation of rank was used to determine relationship between nucleation pH and catheter lifespan in relevant experiments. To allow ANOVA, least squares regression and Pearson correlation of rank to be performed the data was analysed with the Minitab programme to confirm normal distribution of the residuals and the equal variances of the data. If these assumptions were violated and the data could not be transformed alternative statistical tests were applied as indicated.

3. Results

3.1. The development of crystalline *P. mirabilis* biofilms on indwelling urinary catheters

Previous studies in the bladder model have concentrated on comparing the rates at which different catheters block with crystalline *P. mirabilis* biofilm (Morris *et al.* 1997; Morris and Stickler, 1998a). The aim of the work reported in this section was to investigate the sequence in which cells and crystals are laid down on catheter surfaces and initiate crystalline biofilm formation. The experiments were performed under conditions which simulate the common situation in which patients' catheters are recurrently blocking and have to be replaced frequently. In these cases new catheters are introduced into bladders in which urine is heavily infected with *P. mirabilis* and the urinary pH is already elevated (Mathur *et al.*, 2006a).

3.1.1. Crystalline biofilm development in bladder model experiments

Bladder models were fitted with a range of Foley type catheters including: all-silicone (AS); hydrogel-coated latex (HCL); silicone-elastomer coated latex (SCL); hydrogel/silver-coated latex (HSCL) and a nitrofurazone impregnated catheter (NF). Additionally, a series of all-silicone and hydrogel-coated latex catheters (AST and HCLT respectively), whose retention balloons were inflated with a 10 ml solution of 3 mg/ml triclosan in $0.1M$ Na_2CO_3 were also included. Models were inoculated with 10 ml of a *P. mirabilis* NSM6 4 hour culture (approximately 10^8 cfu/ml), and supplied with artificial urine at a rate of 0.5 ml/minute. Triplicate experiments were performed in which catheters were removed from models for examination after pre-determined intervals of 1, 2, 4, 12, 18 h. In a further series of experiments catheters were removed from models that were operated until they blocked or after 7 days if blockage did not

occur. Sections (1 cm long) were cut from catheters as described in Figure 4 and processed for either low or high vacuum SEM analysis. In the cases of AS, SCL and HCL catheters sections from the third replicate experiment were processed for enumeration of adherent bacteria. Throughout the experiments, the pH and viable cell populations of the residual urine in the model chamber were determined. For all catheters studied control models were supplied with sterile urine but not inoculated with *P. mirabilis* NSM6. The results presented in Figure 5 summarize the data on the mean times each type of catheter took to block. It can be seen that the five commercially available Foley-type catheters all blocked within 40 h. In contrast AST and HCLT catheters that were inflated with 3 mg/ml triclosan drained freely for the full 7 day experimental period.

Mean viable cell counts and pH's of the residual urine sampled throughout the experiments are presented in Tables 1 and 2 respectively. Initially the mean viable cell numbers in urine of all the models were approximately 10^8 cfu/ml and mean pH values in excess of pH 8. In the urine from models fitted with AS, SCL, HCL and HSCL catheters the mean viable counts fluctuated initially but remained between 10^7-10^8 cfu/ml. Similarly the mean pH of urine from these models remained above 7.0 and in all cases was > 8.0 at time of catheter blockage. In models fitted with NF catheters mean viable cell counts and pH of urine at 4 hours fell to approximately 10^6 cfu/ml and < pH 7 respectively before recovering to high 10^7 cfu/ml and > pH 8 at 18 hours. When the AS and HCL catheters were inflated with triclosan rather than water, within 4 hours the number of viable cells in the residual urine fell markedly and the urine had become acidic.

The sensitivity of various *P. mirabilis* strains to triclosan including NSM6 had been previously reported by Jones *et al.* (2005). The sensitivity of *P. mirabilis* NSM6 to triclosan was also confirmed here prior to experiments and was determined at being between 0.1 and 0.2 µg/ml. Figure 6 shows a selection of agar plates containing a range of triclosan concentrations and illustrates the effect of triclosan on the growth and swarming behaviour on *P. mirabilis* NSM6 and *P. mirabilis* SJ2.
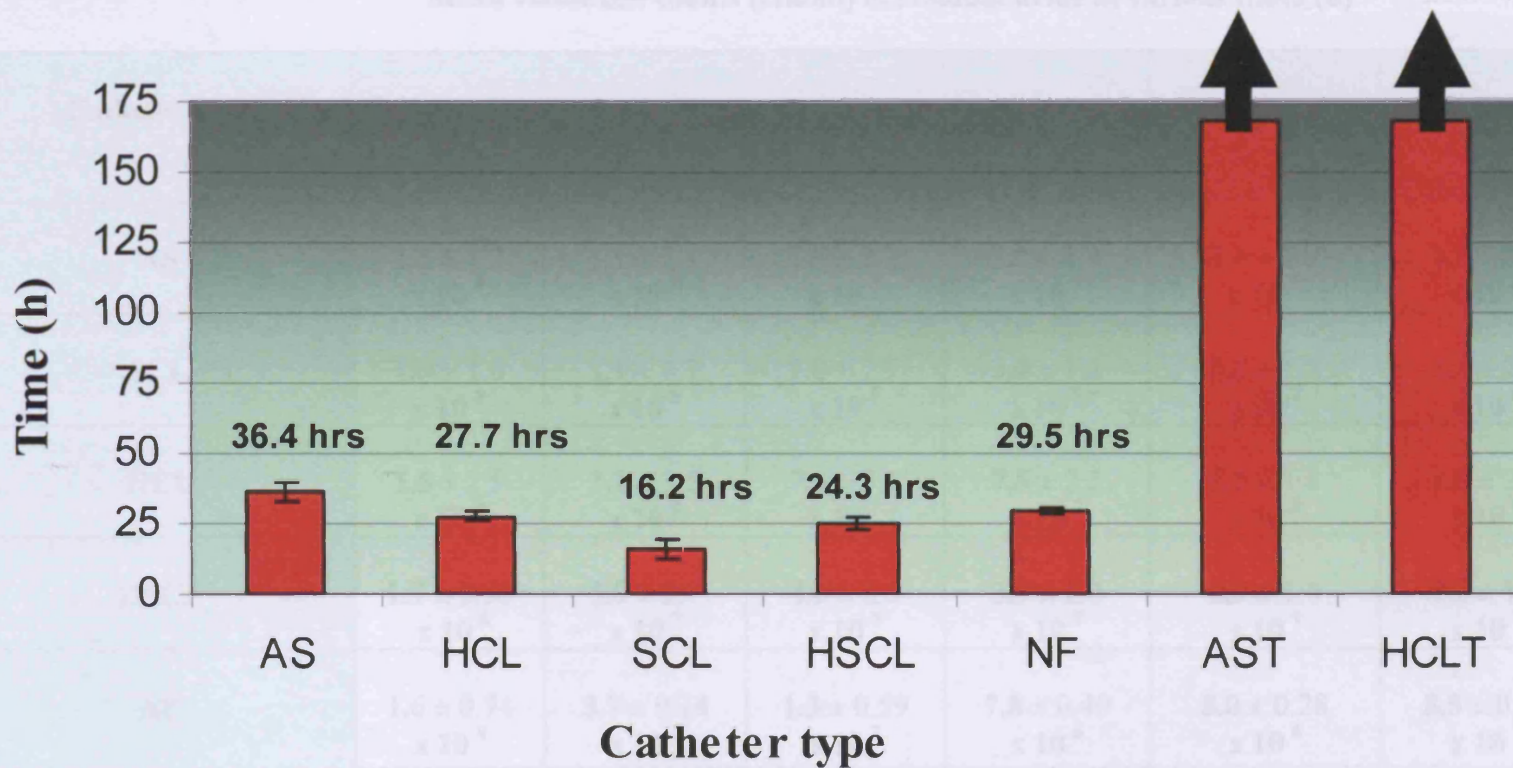
Figure 5.   **Mean times to catheter blockage in models inoculated with *P. mirabilis* NSM6**

All models were inoculated with 10 ml of artificial urine containing approximately 10^8 cfu/ml. Mean values ± standard error of mean were calculated for results of three replicated experiments. AS indicate all-silicone catheters, HCL, hydrogel-coated latex, SCL, silicone elastomer-coated latex, HSCL, hydrogel-silver coated latex, NF, nitrofurazone impregnated catheters, AST and HCLT indicate that the retention balloons of the catheters were inflated with 3 mg/ml triclosan rather than water. The arrows indicate that in these cases the catheters drained freely for the 7 day period.

Catheter type	0	1	2	4	12	18	Blockage *
AS	1.5 ± 1.2 $\times 10^8$	1.1 ± 2.1 $\times 10^8$	7.8 ± 2.2 $\times 10^7$	5.8 ± 1.4 $\times 10^7$	7.4 ± 1.9 $\times 10^7$	1.3 ± 3.5 $\times 10^8$	1.1 ± 2.2 $\times 10^8$
SCL	1.6 ± 1.0 $\times 10^8$	1.1 ± 2.7 $\times 10^8$	7.0 ± 2.7 $\times 10^7$	3.0 ± 1.1 $\times 10^7$	5.6 ± 1.2 $\times 10^7$	1.3 ± 2.5 $\times 10^8$	7.2 ± 2.9 $\times 10^7$
HCL	1.5 ± 2.5 $\times 10^8$	1.1 ± 3.5 $\times 10^8$	7.6 ± 3.1 $\times 10^7$	7.8 ± 2.5 $\times 10^7$	1.1 ± 1.8 $\times 10^8$	1.0 ± 2.3 $\times 10^8$	6.5 ± 0.3 $\times 10^7$
HSCL	1.7 ± 0.58 $\times 10^8$	5.9 ± 2.7 $\times 10^7$	4.6 ± 2.5 $\times 10^7$	3.7 ± 2.0 $\times 10^7$	6.9 ± 1.4 $\times 10^7$	7.1 ± 1.2 $\times 10^7$	9.7 ± 1.7 $\times 10^7$
NF	1.6 ± 0.74 $\times 10^8$	3.7 ± 0.24 $\times 10^7$	1.3 ± 0.59 $\times 10^7$	7.8 ± 0.40 $\times 10^6$	8.0 ± 0.28 $\times 10^6$	8.5 ± 0.23 $\times 10^7$	6.5 ± 0.81 $\times 10^7$
AST (triclosan)	2.2 ± 2.2 $\times 10^8$	2.0×10^5 †	9.0×10^4 †	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
HCLT (triclosan)	1.0 ± 3.4 $\times 10^8$	1.5 ± 1.7 $\times 10^7$	1.8 ± 0.23 $\times 10^6$	9.0 ± 0.15 $\times 10^5$	9.0 ± 0.6 $\times 10^3$	1.0 ± 0.5 $\times 10^4$	5.0 ± 0.6 $\times 10^3$

* Samples were taken as soon as possible after blockage of catheters had occurred. † No viable organisms were detected in two of the three replicates

Table 1.   Mean viable cell counts of *P. mirabilis* NSM6 from residual urine in the bladder models
Start time samples were obtained by collecting the initial 5ml of voided urine from the drainage bag. All further samples from bladder model chamber were taken when experiments were concluded. The mean values were calculated ± standard error of the mean from three replicated experiments.

Mean pH of residual urine at various times (h)

Catheter type	Start pH	1	2	4	12	18	Blockage *
AS	8.48 ± 0.03	7.85 ± 0.23	7.26 ± 0.29	7.14 ± 0.21	7.49 ± 0.14	7.7 ± 0.35	8.19 ± 0.08
SCL	8.63 ± 0.03	8.1 ± 0.12	7.5 ± 0.35	7.19 ± 0.17	7.2 ± 0.19	7.92 ± 0.10	8.03 ± 0.13
HCL	8.46 ± 0.04	7.6 ± 0.22	7.6 ± 0.25	7.4 ± 0.25	7.6 ± 0.03	8.1 ± 0.18	8.2 ± 0.17
HSCL	8.51 ± 0.10	7.52 ± 0.34	7.32 ± 0.17	7.06 ± 0.23	7.46 ± 0.16	7.96 ± 0.17	8.25 ± 0.16
NF	8.56 ± 0.02	7.06 ± 0.02	6.5 ± 0.08	6.4 ± 0.08	7.38 ± 0.23	8.29 ± 0.18	8.66 ± 0.15
AST	8.52 ± 0.01	7.02 ± 0.17	6.6 ± 0.17	6.32 ± 0.06	6.2 ± 0.03	6.19 ± 0.04	† 6.13 ± 0.03
HCLT	8.48 ± 0.08	7.3 ± 0.19	6.75 ± 0.27	6.48 ± 0.2	6.19 ± 0.03	6.25 ± 0.03	† 6.11 ± 0.01

* Samples were taken as soon as possible after blockage of catheters had occurred. † pH at 7 days

Table 2. Mean urine pH values of residual bladder model urine
Start time samples were obtained by collecting the initial 5ml of voided urine from the drainage bag. All further samples from bladder model chamber were taken when experiments were concluded. The mean values were calculated ± standard error of the mean from three replicated experiments.
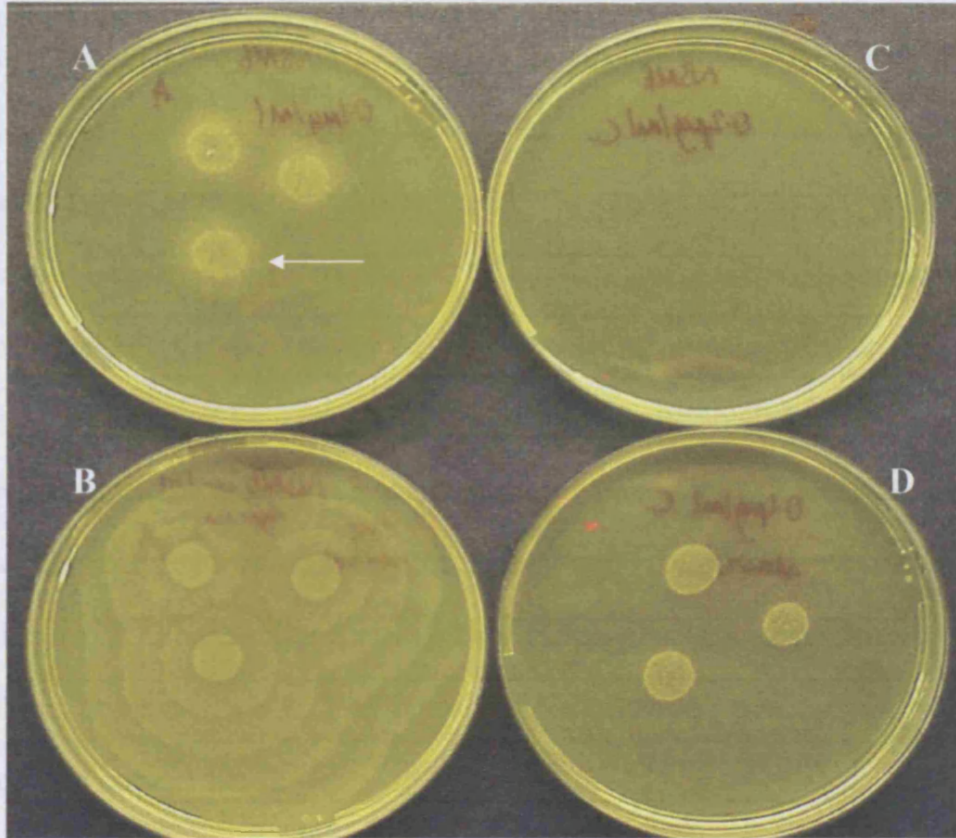
Figure 6. **Effect of triclosan on swarming and growth of *P. mirabilis* strains**

(A) *P. mirabilis* NSM6 limited swarming in the presence of 0.1 µg/ml triclosan (arrow indicates extent of swarming edge). (B) *P. mirabilis* NSM6 positive control without triclosan (concentric rings of swarming phenomenon are evident). (C) No growth of *P. mirabilis* NSM6 in the presence of 0.2 µg/ml triclosan. (D) Growth but no swarming of *P. mirabilis* SJ2 in the presence of 0.1 µg/ml triclosan. All strains incubated for 24 hours at 37°C on TSA.

3.1.1.1. Low vacuum SEM evaluation of crystalline biofilm development

The low magnification electron micrographs presented in Figures 7 and 8 illustrate the development of crystalline material around the catheter eye-lets and on the lumenal surfaces just below the eyeholes (section B). The images indicate that in contrast to the control catheters (Figure 9), the AS, SCL, HCL, and HSCL catheters all became encrusted within 4 h exposure in the infected bladder models. Similar images (not shown) were obtained for section C. The encrustation continued to accumulate as time progressed until the catheters eventually occluded. Encrustation at site D developed at a much slower rate. Nitrofurazone catheters appeared relatively free from encrustation over the initial 12 h period. It is clear however, that after 12 h encrustation proceeded rapidly as at mean times of 29.5 h extensive encrustation was seen occluding the eyehole and catheter lumen.

The micrographs presented in Figure 10 demonstrate that when the AS and HCL catheters were inflated with triclosan they remained free of encrustation even after seven days residence in *P. mirabilis* infected bladder models.
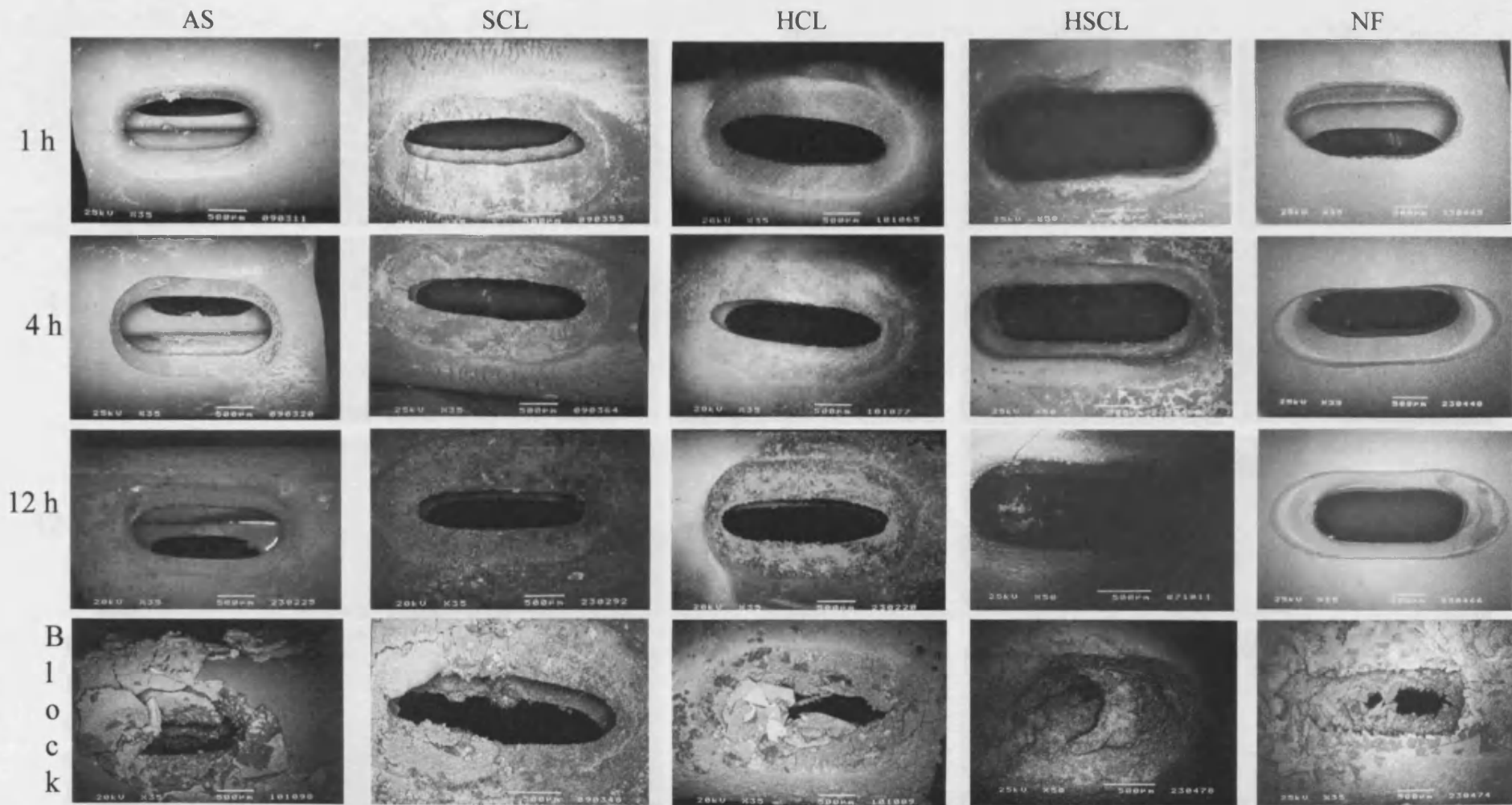
Figure 7. LVSEM images of *P. mirabilis* NSM6 induced encrustation at the eyehole of urinary catheters

The images show the extent of encrustation developing around the eyelets of: AS (All-silicone); SCL (silicone elastomer-coated latex); HCL (hydrogel-coated latex); HSCL (hydrogel/silver-coated latex); NF (nitrofurazone-impregnated) catheters after various residence times *P. mirabilis* infected models.
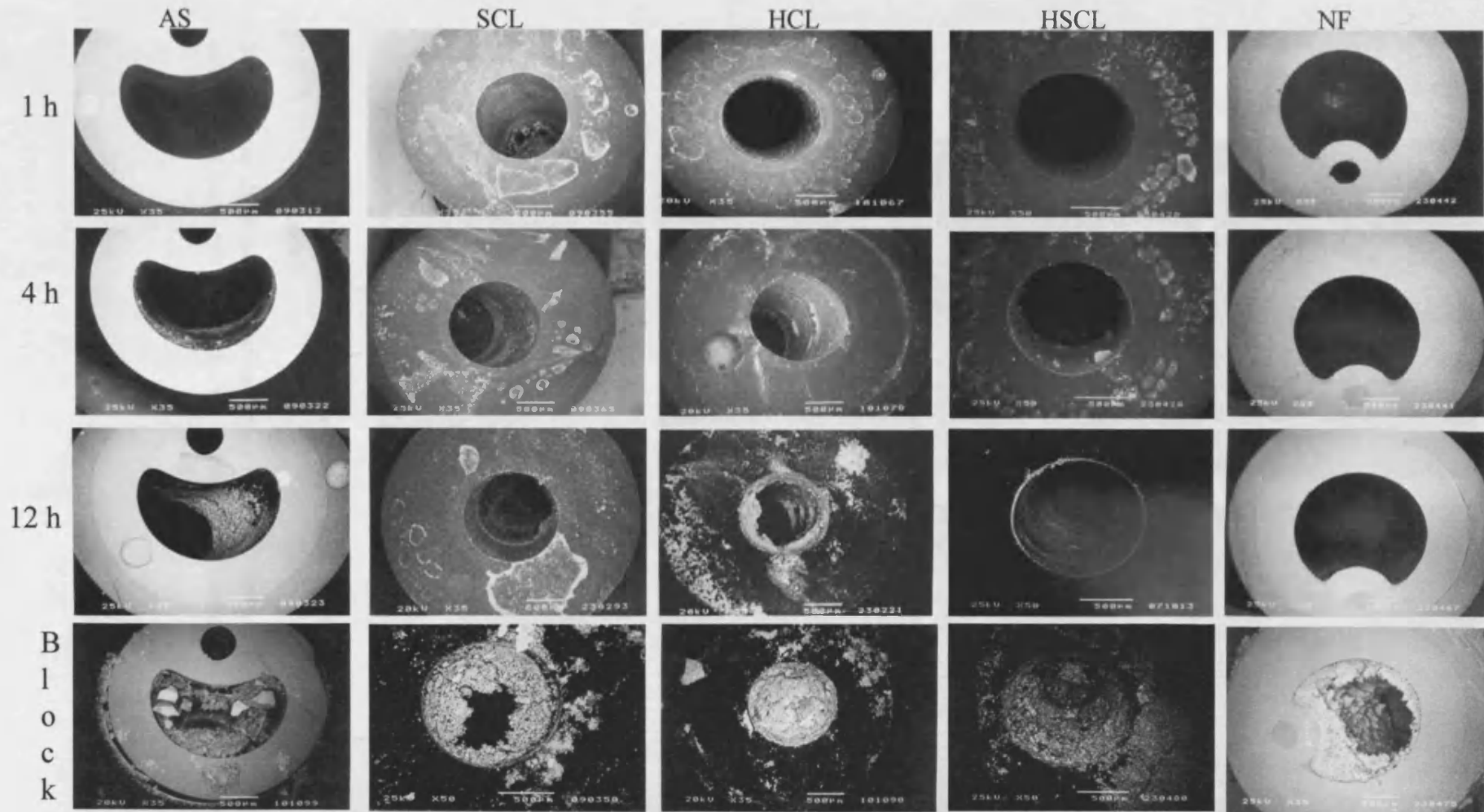
Figure 8. LVSEM images of *P. mirabilis* NSM6 encrustation at the lumenal site (B) on a range of urinary catheters

The images show the extent of encrustation in the lumen just below the eyehole of: AS (All-silicone); SCL (silicone elastomer-coated latex); HCL (hydrogel-coated latex); HSCL (hydrogel/silver-coated latex); NF (nitrofurazone-impregnated) catheters after various residence times *P. mirabilis* infected models.
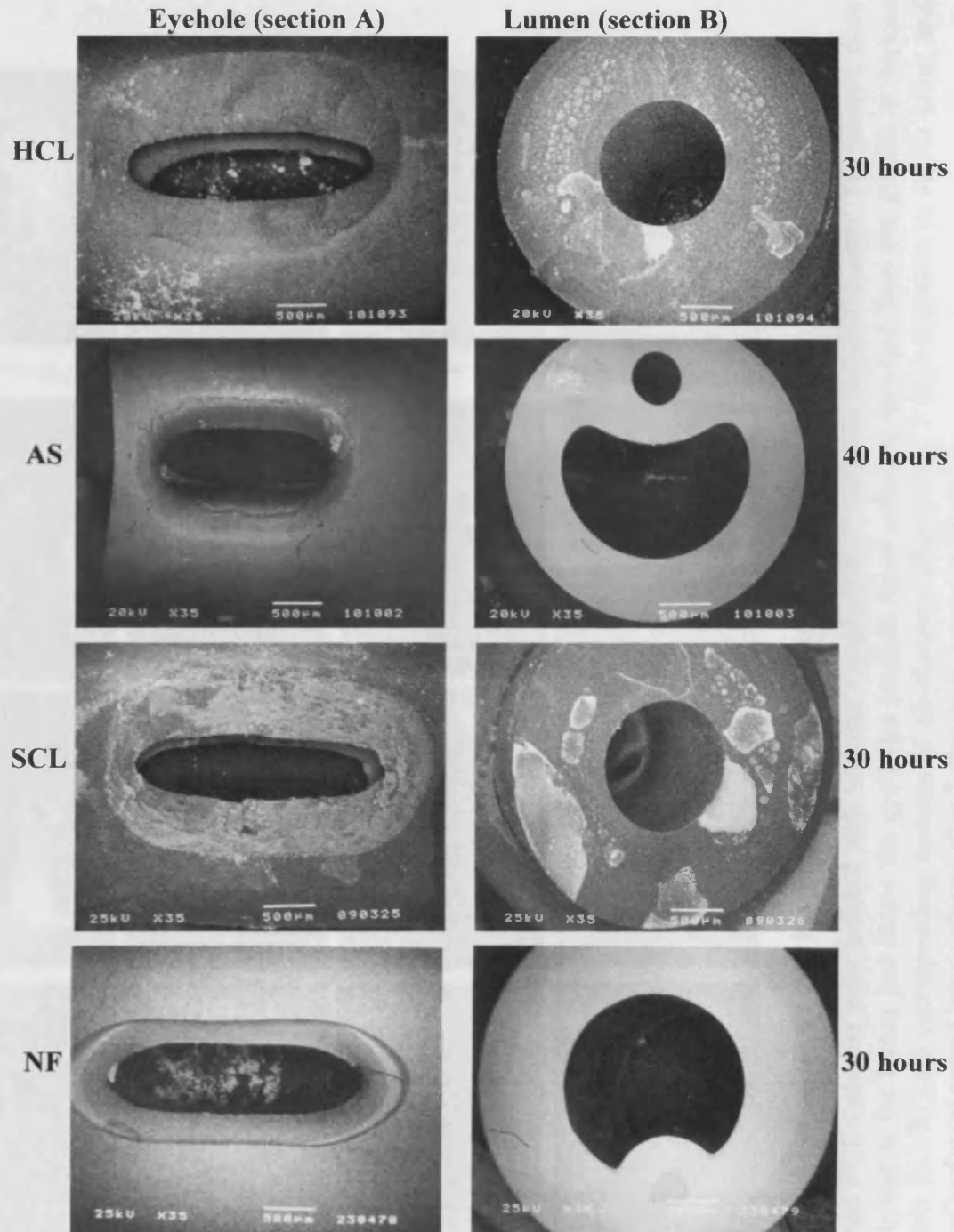
Figure 9. **LVSEM of catheters that had been incubated in control models**

The images show sections of catheters removed from after 30 – 40 hours from models that had been supplied with urine but not inoculated with *P. mirabilis*.
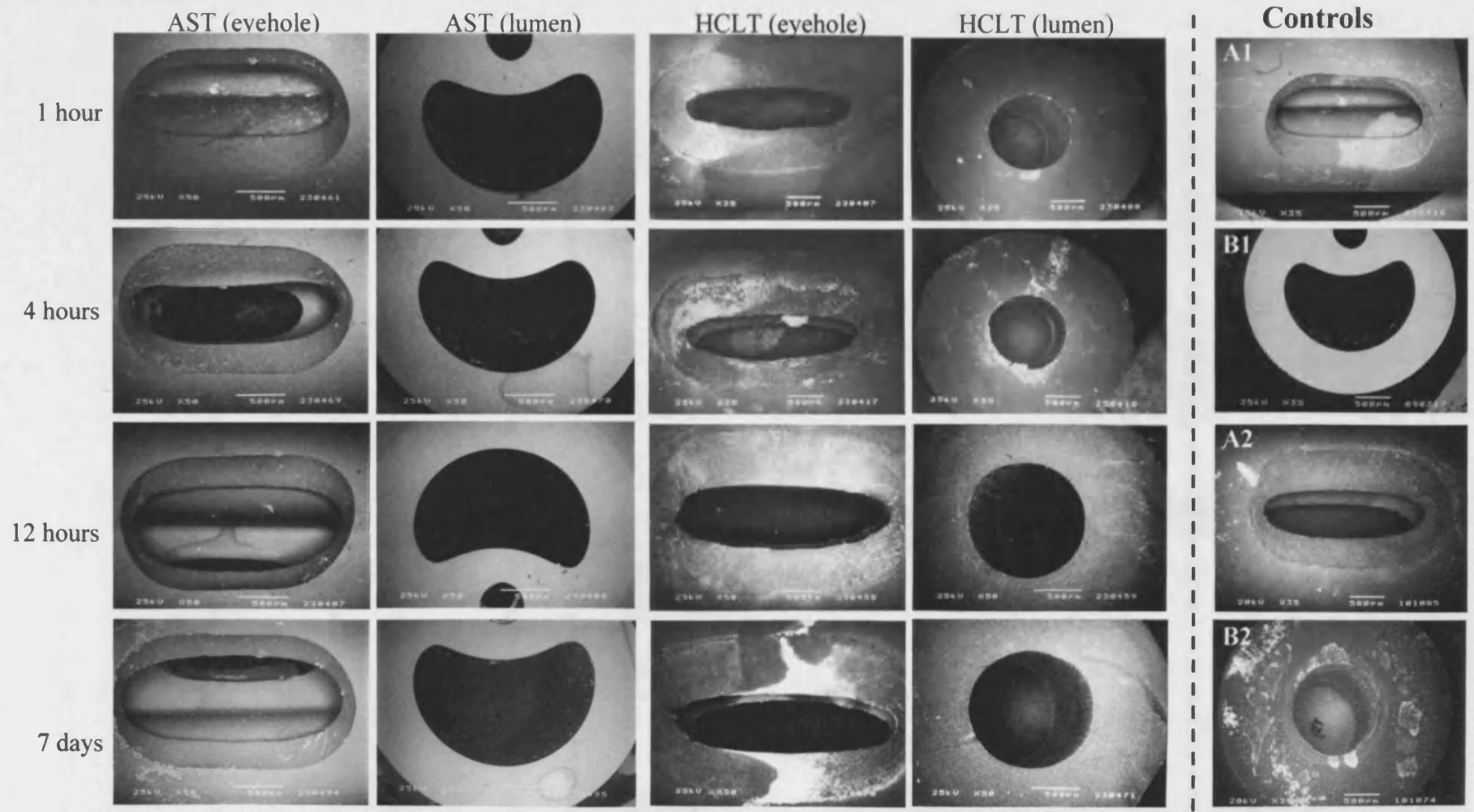
Figure 10.   LVSEM micrographs of catheters that had been inflated with triclosan

The retention balloons of AS and HCL catheters were inflated with triclosan (3mg/ml) in *P. mirabilis* infected models. The images show the surfaces around the eye-lets and in the lumen just below the eye-holes (section B) of catheters removed from the models at various times up to seven days. Controls (A1/B1) are corresponding sections of all-silicone catheter after 7 days exposure to sterile urine, and A2/B2 corresponding sections of hydrogel coated latex catheter after 7 days exposure to sterile urine.

3.1.1.2. High vacuum SEM of crystalline biofilm development

A knowledge of the nature of the catheter surfaces that are exposed to bacterial colonization is basic to an understanding of how they come to be colonized by biofilm. The images presented in Figures 11 and 12 reveal the surface topography of eyehole and lumenal sites of AS, SCL, HCL and HSCL catheters. While the all-silicone catheters have relatively smooth surfaces, irregularities were found. All of the latex-based catheters had irregular topographies, especially where the eye-lets had been cut into the latex. In addition, the common occurrence of diatomaceous skeletons embedded in the lumenal surfaces of the latex catheters exacerbated the surface roughness of the latex catheters.

All-silicone (AS), silicone-coated latex (SCL), hydrogel-coated latex (HCL) and hydrogel/silver-coated latex catheters (HSCL)

Figures 13-17 show HVSEM micrographs of the development of *P. mirabilis* NSM6 crystalline biofilm on AS, SCL, HCL and HSCL catheters respectively. Crystalline material formed a comprehensive 'foundation layer' on the exposed eyehole surface and lumenal surfaces of sections B and C. Sections D on all catheter types acquired only isolated patches of encrusted deposits at this time. Although occasional *P. mirabilis* cells were found colonizing catheter surfaces at 2 h they were rare. By 4 h *P. mirabilis* microcolonies were observed on all catheter types. Bacterial colonization occurred predominantly on the foundation layer present on the catheter surfaces. It was clear however, that cells also bound to areas of the catheter surfaces that were free of encrustation.

By 12 h all catheter types from infected models were extensively colonized with crystalline material and large numbers of bacterial cells. Between 12 and 18 h encrustation developed rapidly, and catheters removed at 18 h in infected models were heavily colonized by mature crystalline biofilms.

HVSEM micrographs of control catheters (including NF catheters) in models that were not infected with *P. mirabilis* are shown in Figure 19. These catheters did not develop the extensive encrustation observed in catheters from infected models although small isolated amorphous deposits were occasionally visible. In one instance crystalline formations were observed around the eye-let of the AS control catheter after 7 days exposure to sterile urine. The largest of these deposits approximately 200 x 100 μm was composed of needle-shaped crystals aggregated into 'rosette-like' formations typical of crystalline calcium phosphate.

Nitrofurazone impregnated catheters

The overall development of encrustation on NF catheter surfaces is shown in Figure 18. As was observed using LVSEM, HVSEM analysis showed that the initial encrustation process on NF catheters was delayed in comparison to that observed on AS, SCL, HCL and HSCL catheters. Surfaces of NF devices removed from infected bladder models at 1, 2 and 4 h appeared free of encrustation (except for extremely rare patches at the eye-hole section), and of any bacterial colonization. Encrustation and bacterial colonization was first observed at 12 h when accumulations of microcrystals formed patches of foundation layer around the eye-let of the catheter. *P. mirabilis* microcolonies were abundant around the eye-let area adhering to the mineralised layer and directly to the catheter surface. Encrustation was relatively rare at the lumenal

sections B and C, but *P. mirabilis* microcolonies were abundant. Colonization was less abundant at section D. From 12 h onward, as seen in AS, SCL, HCL and HSCL catheters, the formation of crystalline biofilm on NF devices also developed rapidly. Surfaces of NF catheters after 18 h in infected bladder models had acquired a mature crystalline biofilm that seemed comparable to those formed on AS, SCL, HCL and HSCL catheters.
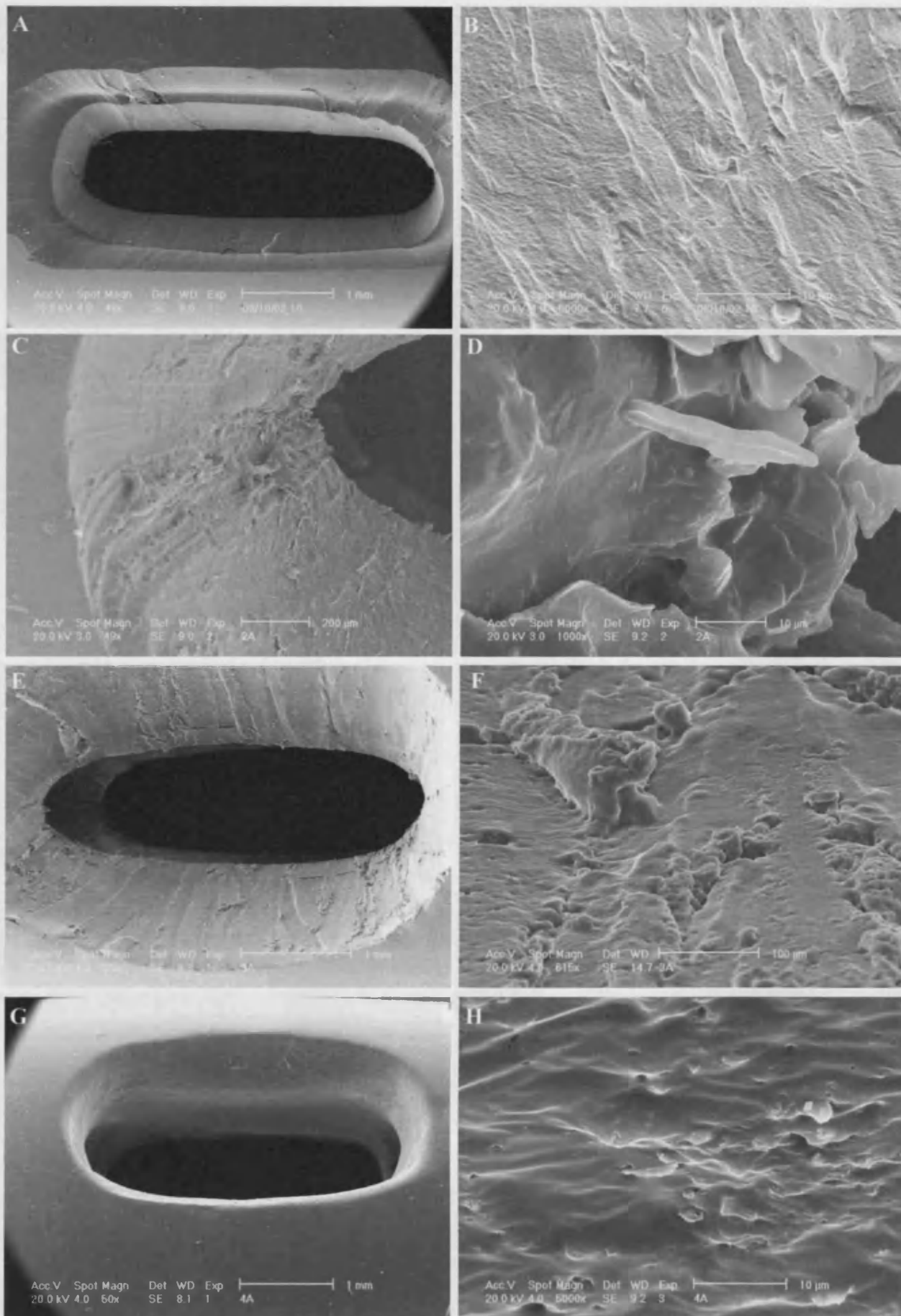
Figure 11.   Surface topography (eyehole region) of unused catheters fresh from packaging

The HVSEM micrographs show the surface features of : (A + B) all-silicone catheter; (C + D) silicone-coated latex catheter; (E + F) hydrogel-coated latex catheter; and (G + H) hydrogel/silver-coated catheter.
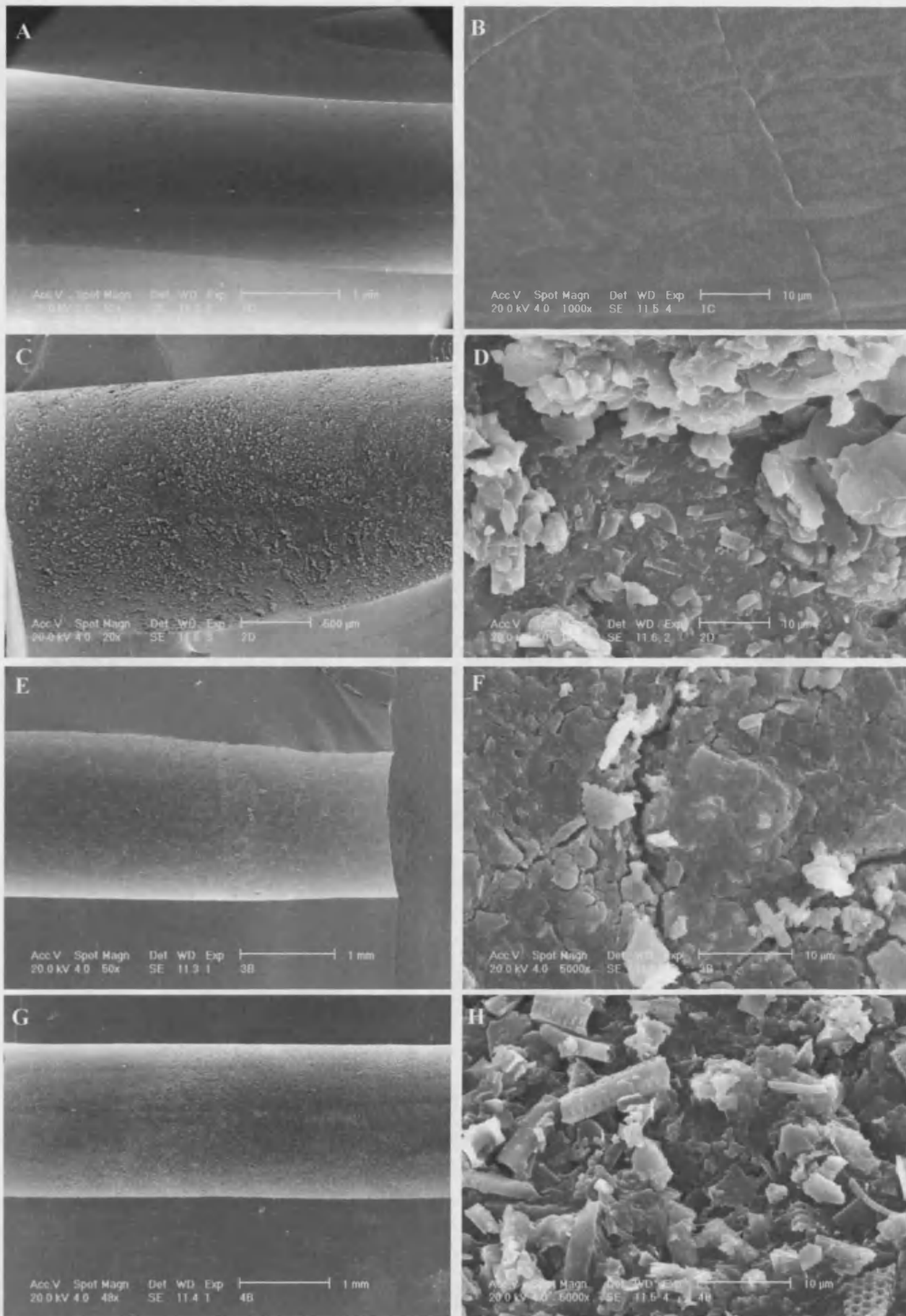
Figure 12.   **Surface topography (lumenal region) of unused catheters fresh from packaging**

The HVSEM micrographs show the surface features of: (A +B) all-silicone catheter; (C + D) silicone-coated latex catheter; (E + F) hydrogel-coated latex catheter; and (H + I) hydrogel/silver-coated catheter.
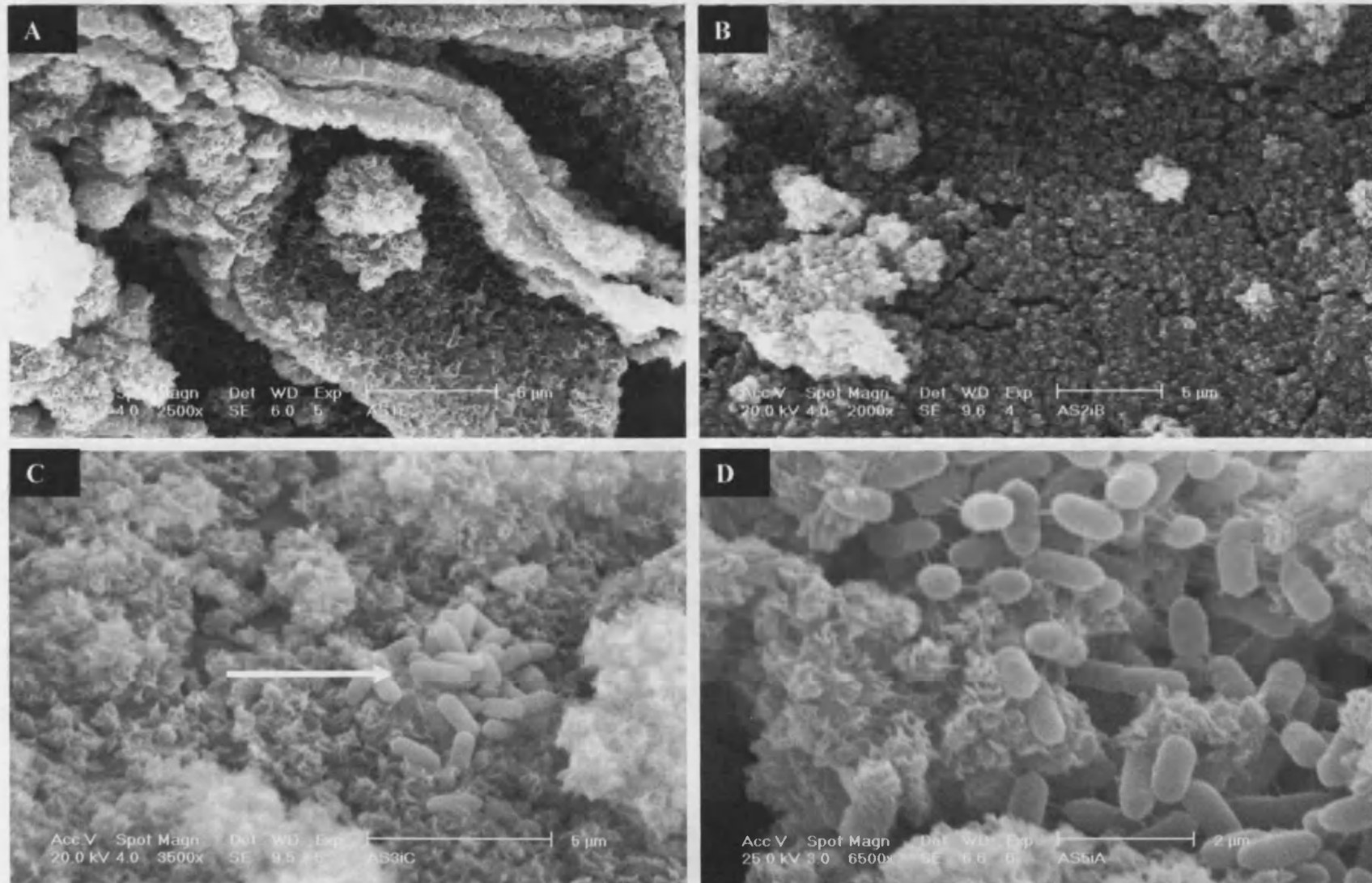
Figure 13.   *P. mirabilis* **NSM6 crystalline biofilm development on all-silicone catheters**

The micrograph series shows: (A) the microcrystalline foundation layer on the surface of the eyehole area after 1 h residence in bladder model; (B) lumenal area of surface just below the eyehole after 2 h residence in the bladder model; (C) microcolonies of *P. mirabilis* NSM6 microcolonies (arrow) on the foundation layer at a lumenal site just below the retention balloon at 4 h; (D) a mature crystalline biofilm at eyehole section at 18 h.
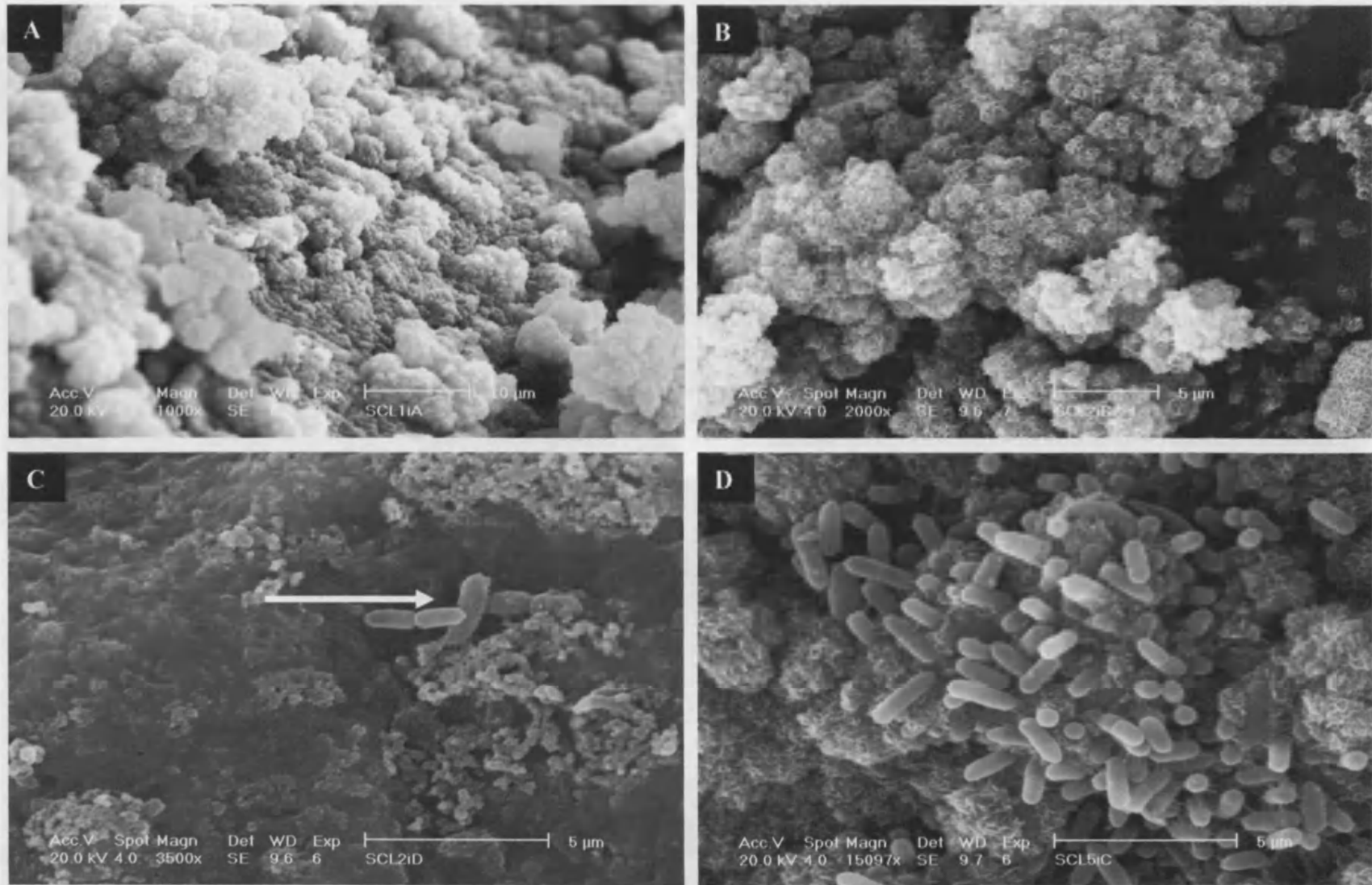
Figure 14. Crystalline biofilm development on SCL catheters in *P. mirabilis* NSM6 infected bladder models

The HVSEM micrographs series shows: (A) the microcrystalline foundation layer on eyehole section of catheter after 1 h in the bladder model; (B) crystalline deposits accumulating on a lumenal surface just below the retention balloon at 2 h; (C) *P. mirabilis* cells (arrow) on encrusted lumenal surface 16 cm below the retention balloon after 2 h; (D) mature crystalline biofilm on lumenal section just below the retention balloon after 18 h.
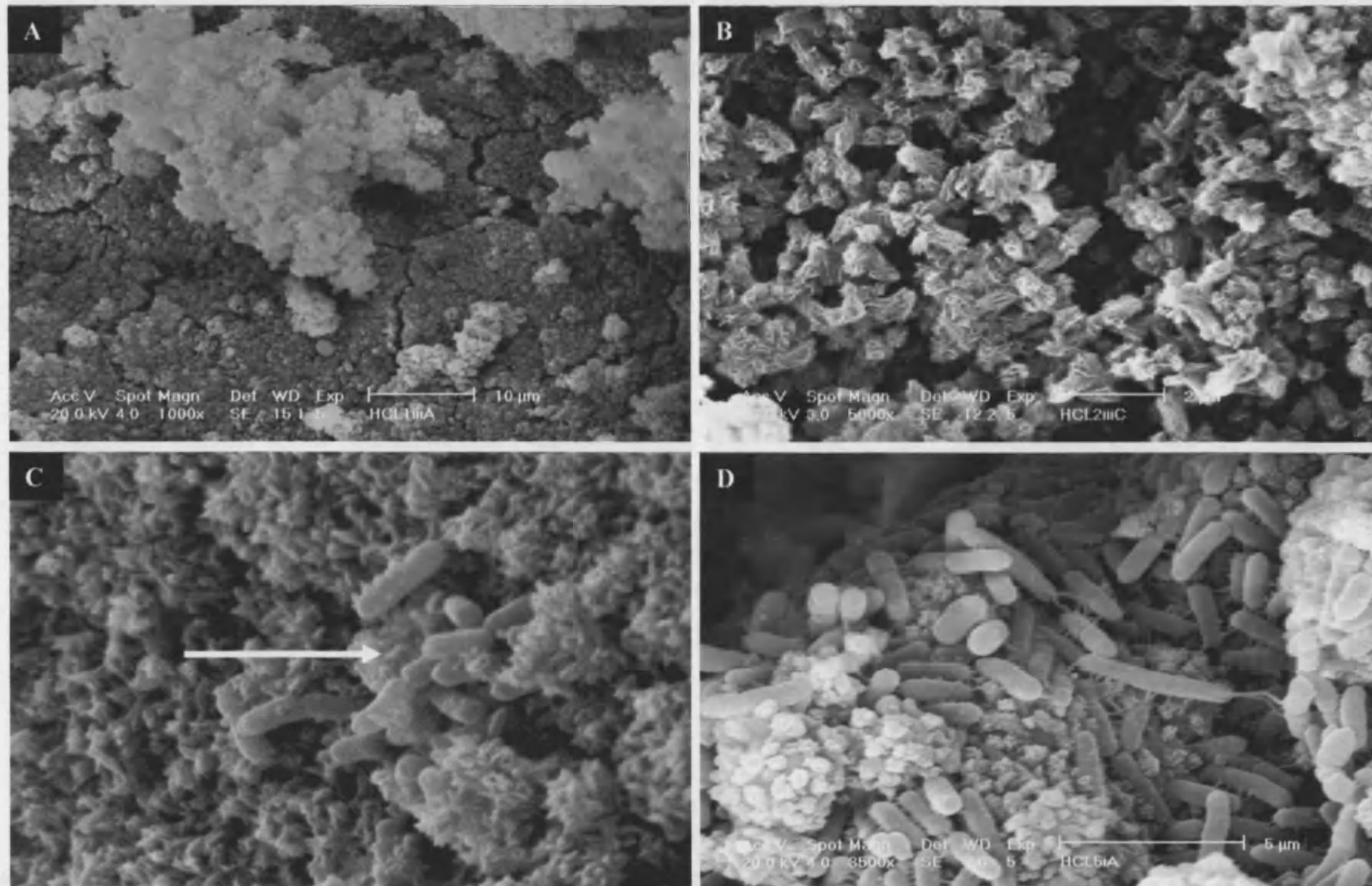
Figure 15.   **Crystalline biofilm development on HCL catheters in *P. mirabilis* NSM6 infected bladder models**
The HVSEM micrographs series shows: (A) the microcrystallinefoundation layer on eyehole section of catheter after 1 h in bladder model; (B) the microcrystalline structure of the deposits on lumenal surface just below the retention balloon at 2 h; (C) a microcolony on the foundation layer (arrow) at eyehole after 4 h; (D) mature crystalline biofilm on eyehole section after 18 h.
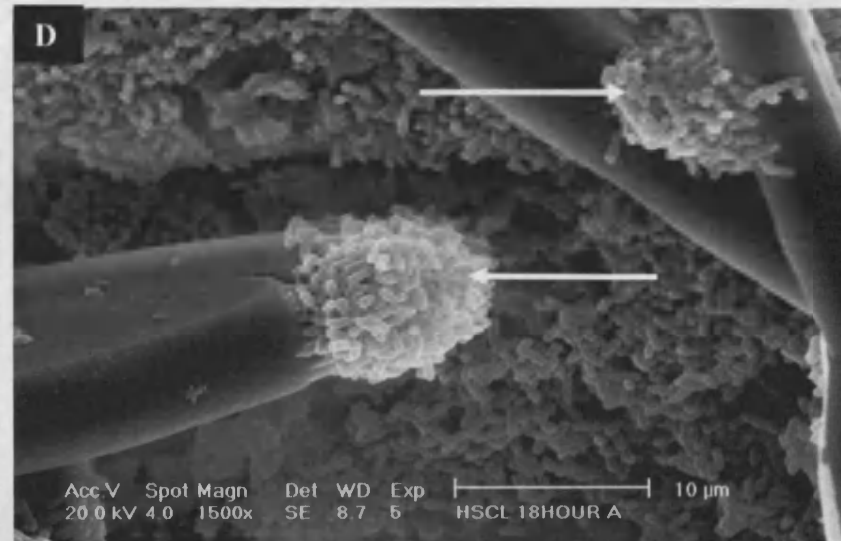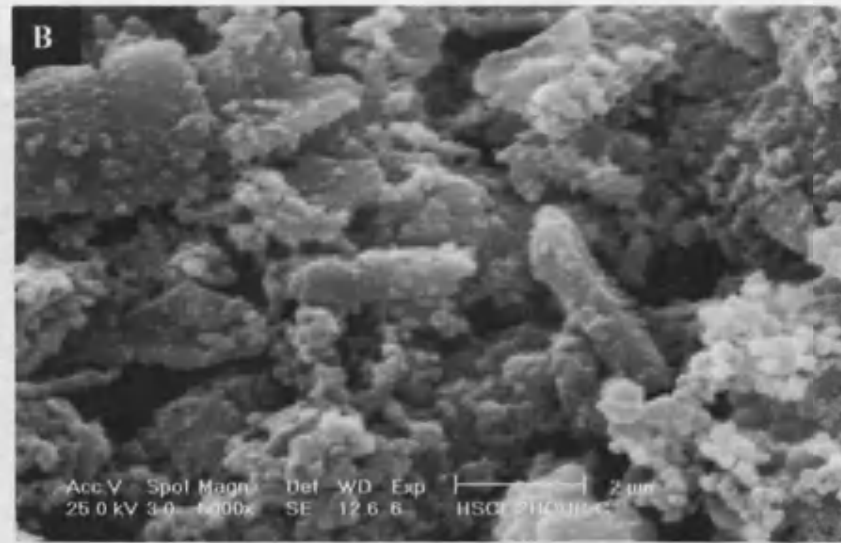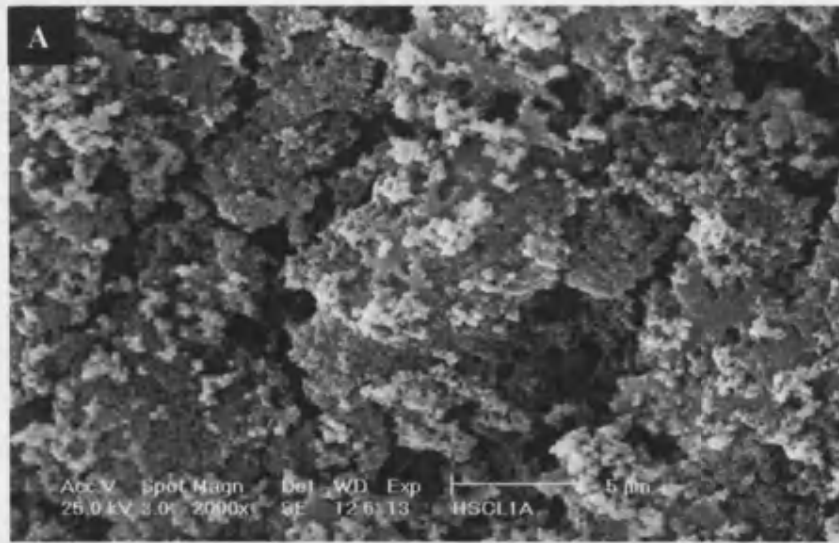
Figure 16. Crystalline biofilm development on HSCL catheters in *P. mirabilis* NSM6 infected bladder models

The HVSEM micrographs series shows: (A) the foundation layer on eyehole section of catheter after 1 h in an infected bladder model; (B) the microcrystalline deposits accumulating on lumenal surface just below the retention balloon at 2 h; (C) a microcolony on the foundation layer at eyehole after 4 h in bladder model. Cells also appear to have amorphous deposits on their surfaces; (D) a mature crystalline biofilm on eyehole section after 18 h. *Proteus* microcolonies (arrows) can be seen colonising needle-like crystals (typical of crystalline calcium phosphate).
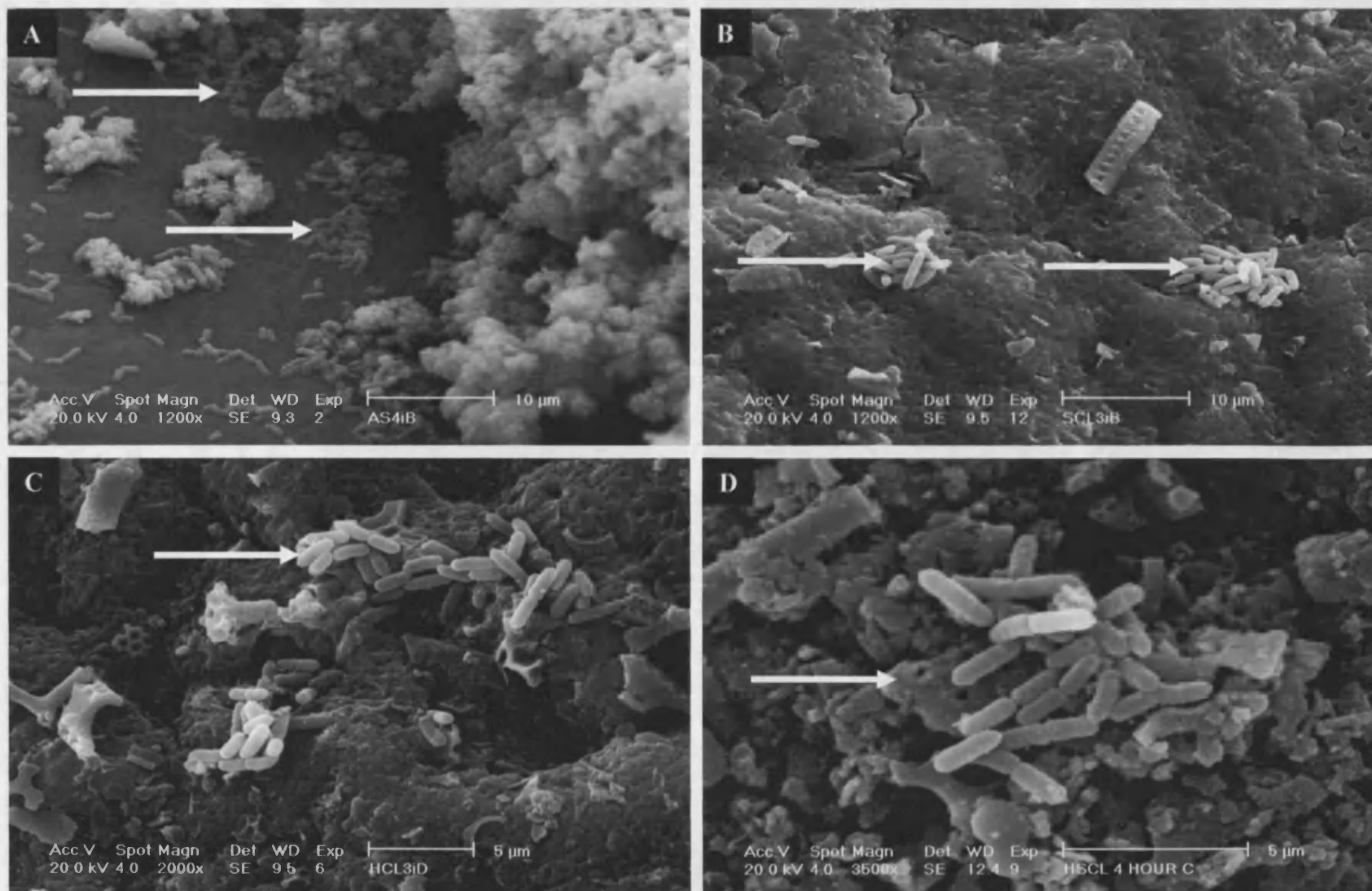
Figure 17. *P. mirabilis* **NSM6 adherence to areas of catheter surfaces not affected by encrustation**

The HVSEM micrographs shows: (A) *P. mirabilis* cells (arrows) adhering directly to AS lumenal surface below the eyehole at 12 h in model; (B) *P. mirabilis* cells (arrows) adhering directly to SCL catheter surface below the eyehole at 4 h; (C) *P. mirabilis* cells (arrow) adhering directly to HCL catheter surface 16 cm from the eyehole at 4 h. (D) *P. mirabilis* cells adhering diatomaceous debris (arrow) on HSCL catheter surface below the retention balloon after 4 h.
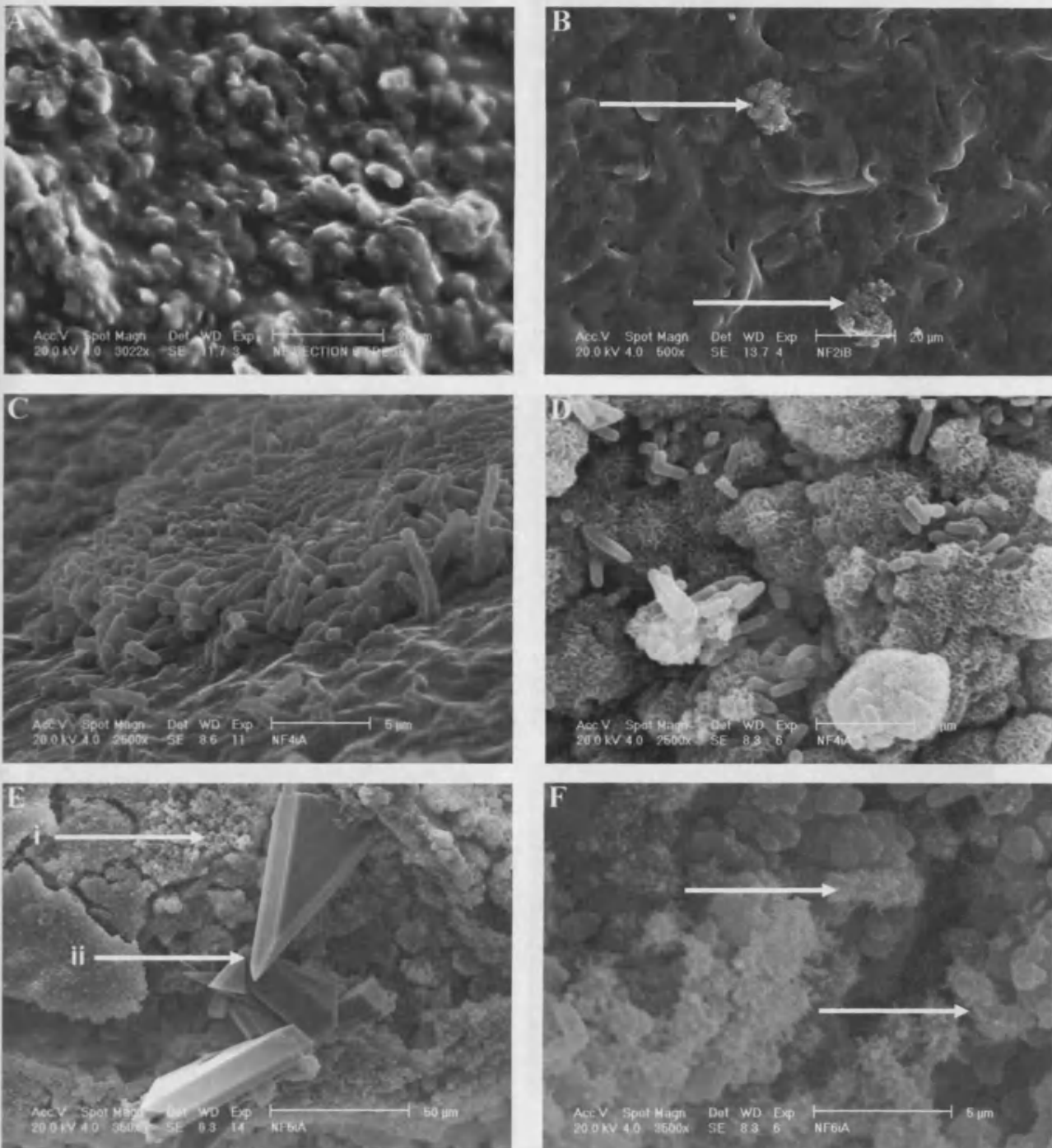
Figure 18. HVSEM images of *P. mirabilis* NSM6 crystalline biofilm formation on nitrofurazone-impregnated catheters

The micrograph series shows: (A) the topography of unused lumenal catheter surfaces; (B) a lumenal section (just below the eyehole) after 2 h infected bladder model showing isolated encrusted deposits (arrows). Similar images (not shown) were obtained at 1 and 4 h; (C + D) are eyehole sections at 12 h, *Proteus* cells can be seen directly colonizing the catheter surface and the mineralised foundation layer; (E) is a mature biofilm after 18 h in the model with typical microcrystalline (arrow i) and large crystal formations (arrow ii); (F) mature crystalline biofilm at 27.5 hrs (blockage) with *Proteus* cells (arrows) covered in what appear to be mineral deposits.

97
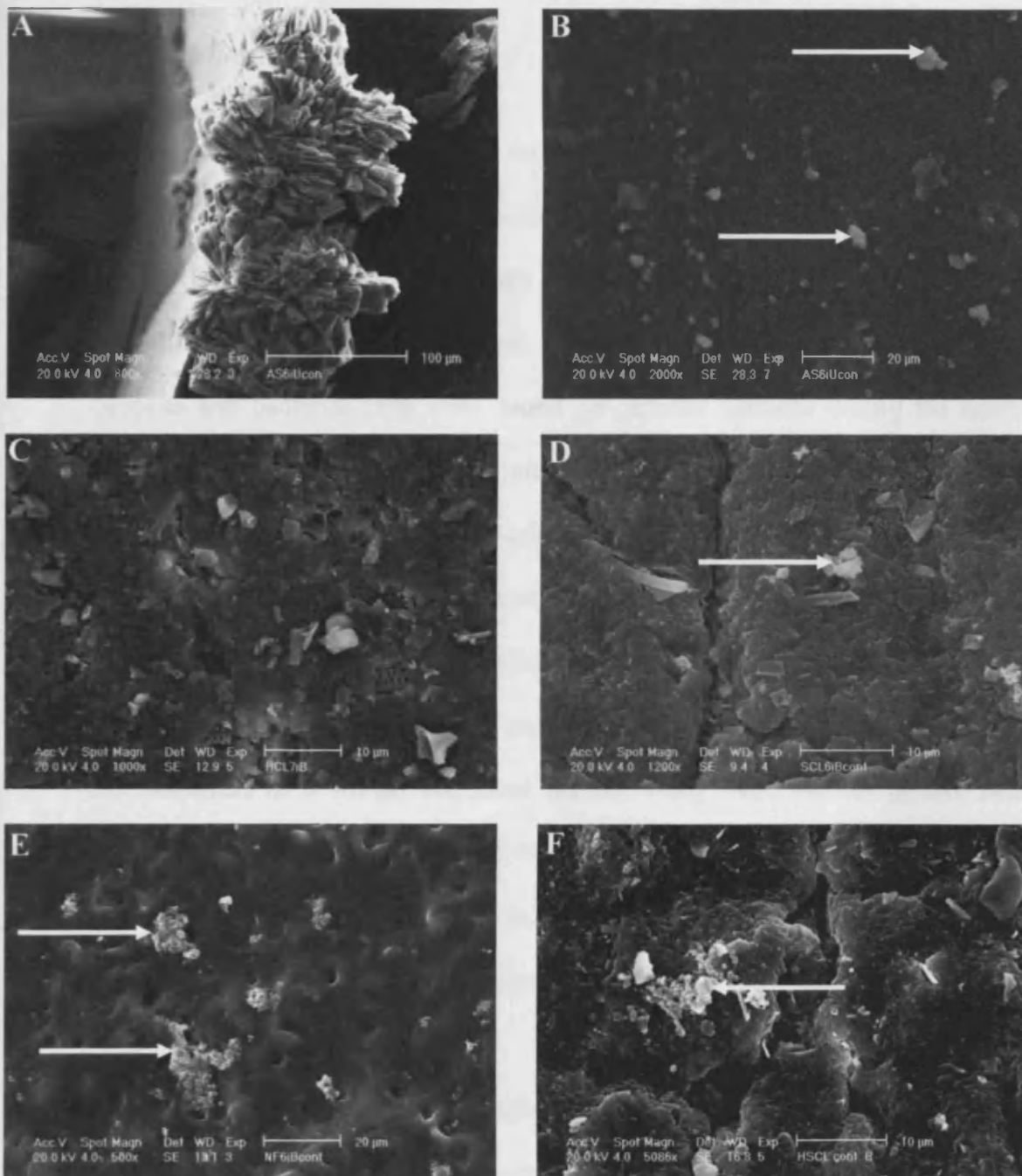
Figure 19. **The condition of control catheter surfaces from bladder models exposed to sterile artificial urine**

The HVSEM micrograph series show: (A) the crystalline formation (typical of calcium phosphate) found at eyehole of all-silicone catheter after 7 days exposure to sterile urine; (B) the lumenal surface of the same catheter shown in micrograph A. Arrows indicate amorphous material; (C) a hydrogel-coated latex catheter after 7 days exposure to the urine; (D) the silicone-coated latex catheter surface at 30 h; (E) a nitrofurazone impregnated catheter at 30 h; (F) the surface of HSCL catheter at 30 h. All arrows indicate amorphous deposits that resemble calcium phosphate.

Triclosan loaded catheters

Crystalline biofilm did not develop on AS or HCL catheters loaded with triclosan (3 mg/ml). The HVSEM micrographs illustrating the effect of triclosan on crystalline biofilm formation on these catheters are shown in Figures 20 and 21. Although crystalline biofilm formation did not occur in triclosan-loaded catheters, encrusted deposits and bacterial cells were found on catheter surfaces during the course of experiments. Encrusted deposits forming a foundation layer were present in AST and HCLT catheters around respective eye-lets after 1 h and 2 h residence in infected bladder models. This level of encrustation was similar to levels observed around eye-lets in corresponding catheters not inflated with triclosan. The encrustation at the eye-let area on triclosan loaded devices was reduced at 4 h and 12 h. It became 'patchy' and remained in a similar condition for the 7-day experimental period. Mineral deposits at lumenal sections did not form an extensive foundation layer on any sites (B-D) at any of the time periods examined. Here encrustation appeared as small random patches over all three lumenal sites.

P. mirabilis colonization of the triclosan loaded devices also failed to materialise to the extent observed in non-triclosan treated models. No evidence of bacterial colonization was observed on catheter sections from either AST or HCLT models up to 4 h. However, on sections removed after 12, 18 h and 7 days in infected models bacterial cells were observed. On all-silicone catheters inflated with triclosan *P. mirabilis* appeared as scattered single cells or in small groups < 5 cells. Many of these cells appeared 'unhealthy' in nature with distorted cell morphologies. *P. mirabilis* appeared to colonize hydrogel-coated latex catheters inflated with triclosan more efficiently than was seen in AST catheters, and was able to form patches of biofilm at

a number of sites on catheters removed from 12 h, 18 h and 7 days. These biofilms were not widespread over the catheter or crystalline in nature, and were restricted to specific areas of the eye-lets forming biofilms approximately 200 x 150 μm in size. The lumenal areas of the HCLT catheter appeared less affected by colonization. In catheters examined after 12 h, 18 h and 7 days in infected models only relatively low levels of cells were found adhering to the surfaces. As with the AST catheters, cells on HCLT lumenal sites often appeared 'unhealthy'.
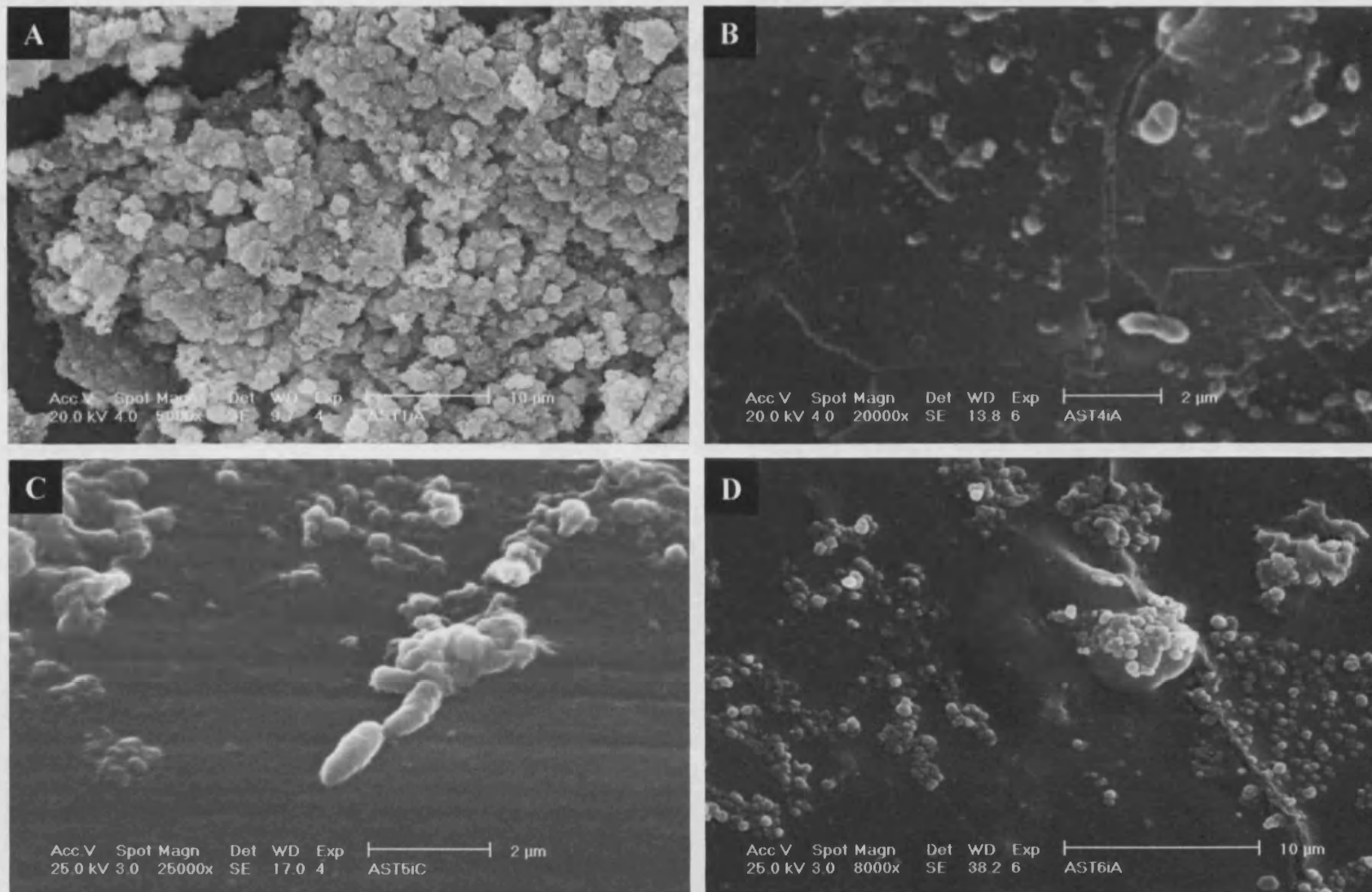
Figure 20. **The effect of triclosan on *P. mirabilis* NSM6 crystalline biofilm development on AS catheters**

The retention balloons were inflated with triclosan (3 mg/ml) instaed of water. The HVSEM micrographs show: (A) an encrusted surface of eyehole section after 1 h residence in bladder model; (B) the eyehole surface after 12 h in the bladder model; (C) an area of the lumen (just below retention balloon) after 18 h in the bladder model; (D) the sparse mineral deposition on catheter eyehole surface after 7 days in bladder model.
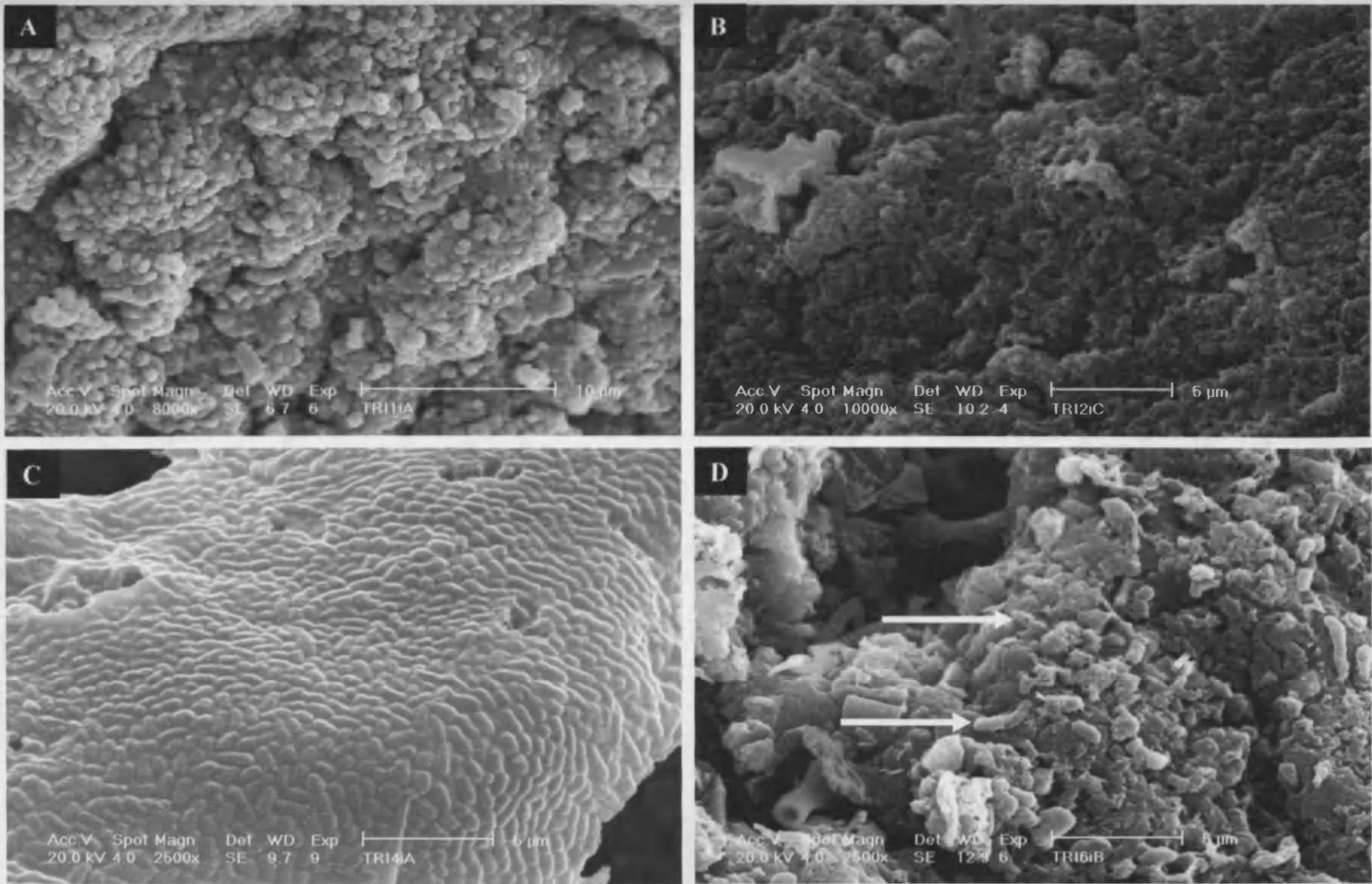
Figure 21.   **The effect of triclosan on *P. mirabilis* NSM6 crystalline biofilm development on HCL catheters**

Retention balloons of catheters were inflated with triclosan (3 mg/ml) instead of water. The HVSEM micrographs show: (A) the foundation layer on eyehole surface after 1 h in bladder model; (B) the lumenal section at 2 h in bladder model; (C) an isolated area of eyehole colonized with non-crystalline biofilm after 12 h in bladder model; (D) the lumenal surface (just below eyehole) at 7 days with *Proteus* cells (arrows) scattered over the surface.

3.1.1.3. Adherence of *P. mirabilis* to catheter surfaces

In one of the replicate experiments in which the AS, SCL, and HCL catheters were examined for biofilm development, the numbers of viable cells recovered from the four catheter sections were determined. The results are presented in Figure 22. The overall pattern of colonization on these catheters confirmed what was found using high vacuum SEM, in that sections at the eyehole and first few centimetres down the lumen appeared to be colonized first. Overall, numbers of viable cells recovered from sections of all catheters remained relatively stable over the initial 4 hours (approximately 10^4 cfu/mm), after which a steady increase in the number of cells adhering to sections was observed until time of blockage. Cell numbers peaked at approximately 10^7 cfu/mm at sites (A) and (B), and (C) and were approximately 10^6 at sections (D).

3.1.2. X-ray microanalysis

The chemical nature of the crystalline material that formed on the catheter surfaces before bacterial colonization occurred was examined using X-ray microanalysis. Sections of AS and HCL catheters (1 cm) were removed from *P. mirabilis* NSM6 infected models after 1, 2 and 4 h and examined in the microscope. Figures 23 and 24 show the results of X-ray microanalysis of hydrogel-coated latex catheters after 1 h and an all-silicone catheter after 4 h in *P. mirabilis* NSM6 infected bladder models respectively. Results indicated that the accumulations found on the surfaces were relatively high in calcium and phosphorous suggesting the presence of calcium phosphate. The presence of other urinary constituents including potassium, magnesium and sodium were also found but in lower quantities.

Catheter section A

Catheter section B

Catheter section C

Catheter section D
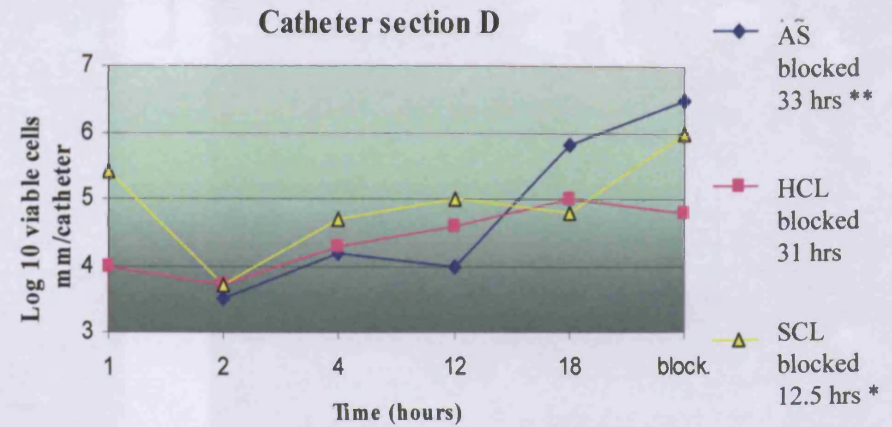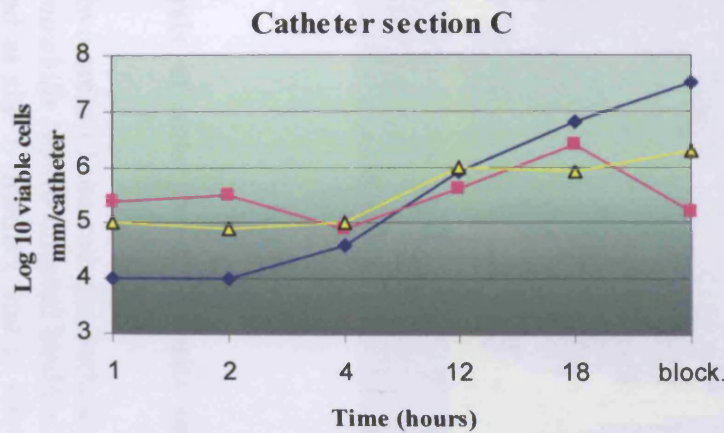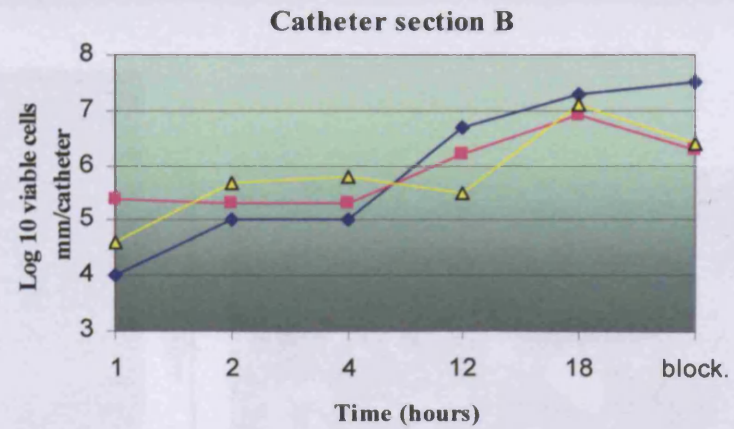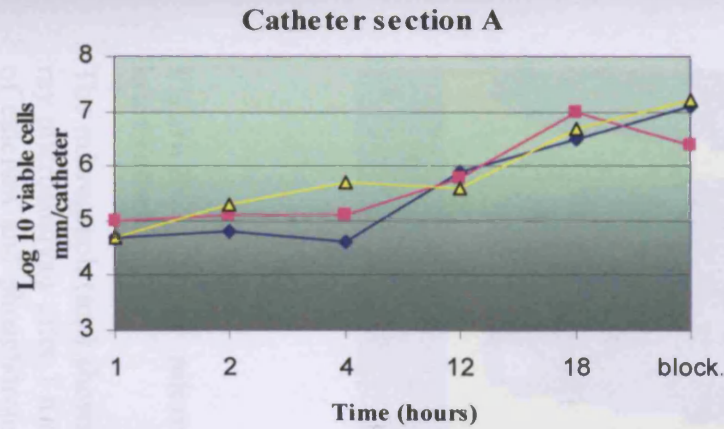
Legend:
- AS blocked 33 hrs **
- HCL blocked 31 hrs
- SCL blocked 12.5 hrs *

Figure 22. *P. mirabilis* **NSM6 colonisation of all-silicone, silicone-coated latex and hydrogel-coated latex catheter surfaces**

Experiments were concluded at 1, 2, 4, 12, 18 hours and at catheter blockage. Catheter sections (1 cm) were removed from: (A) eyehole, (B) just below the eyehole, (C) just below the balloon region and (D) 16 cm below the eyehole. Sections were processed with viable cells mm/ catheter length determined. *SCL blockage model occluded at 12.5 hours . ** Viable cells <10^3 cfu/mm catheter length at 1 hour period at site D. Data from 1 replicate only for each catheter at each time interval. All models inoculated with 10 ml of a 4 hour NSM6 culture containing 10^8 cfu/ml.
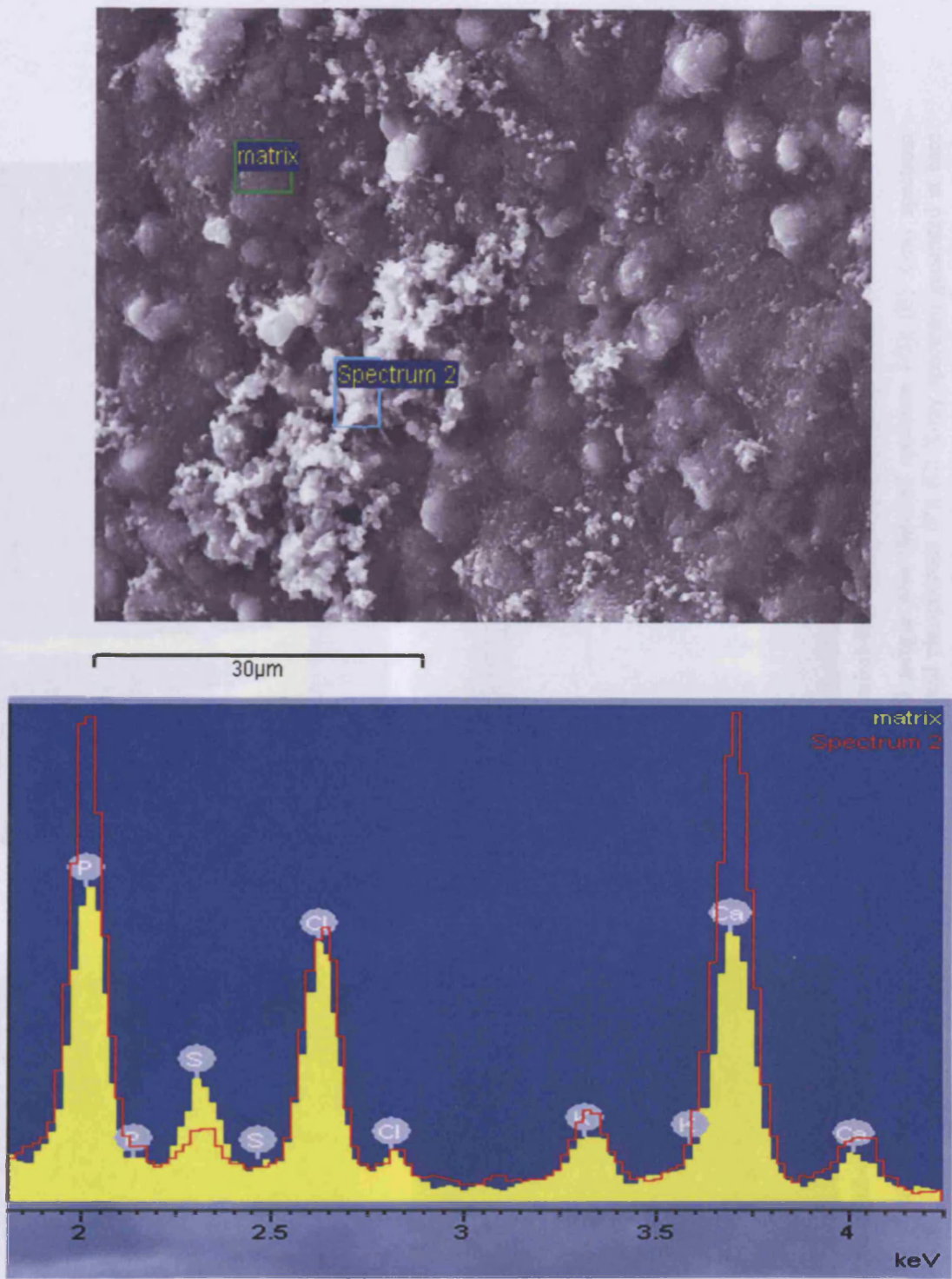
Figure 23. **X-ray microanalysis of amorphous deposits on hydrogel-coated latex catheter**

The micrograph (top) shows the two areas of the catheter surface examined using x-ray microanalysis after 1 h in *P. mirabilis* NSM6 infected bladder models. The levels of calcium and phosphorous found at site 1 (matrix), and at site 2 (spectrum2) are shown as peaks in the generated graph.
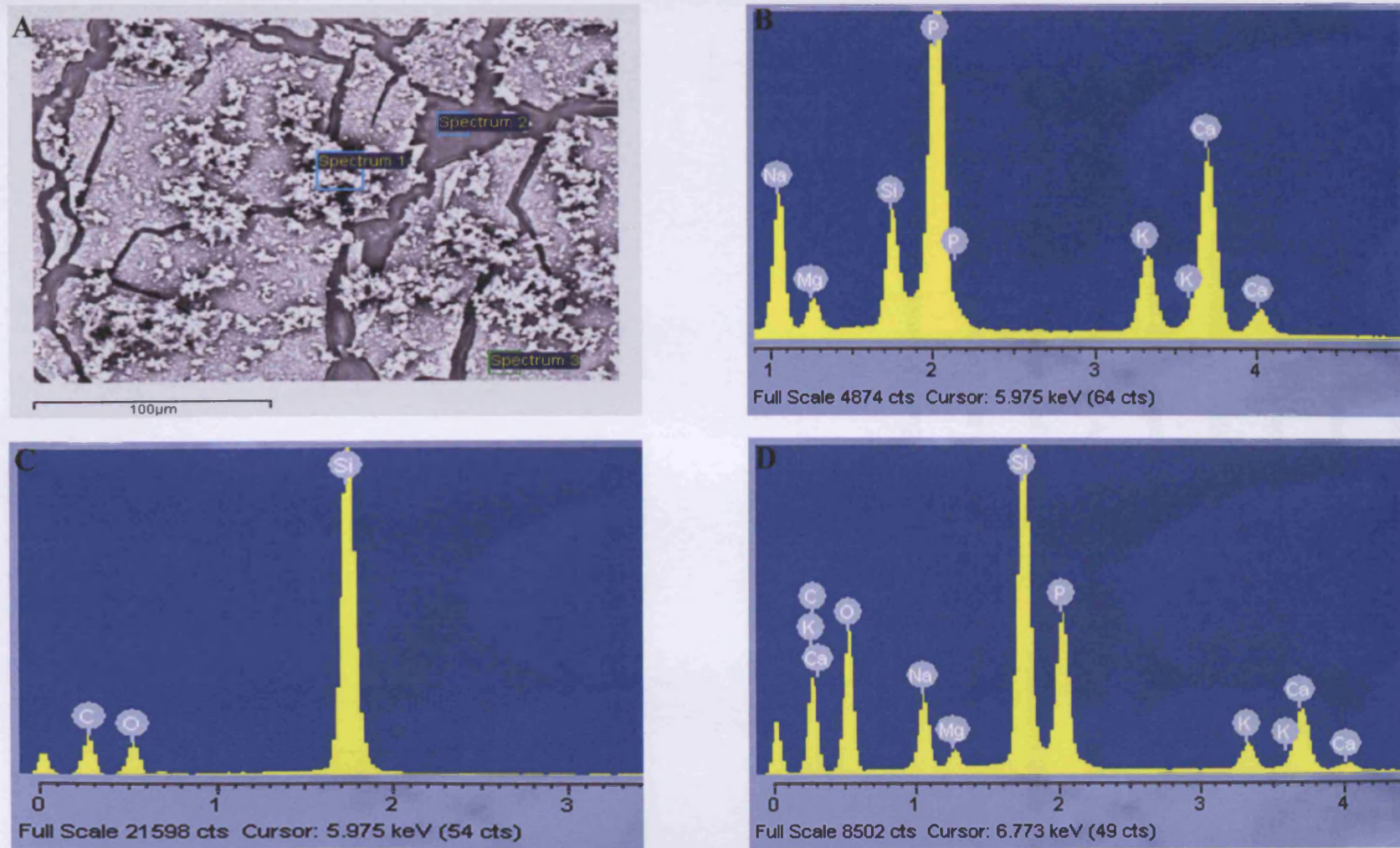
Figure 24. X-ray microanalysis of all-silicone catheter surface after 4 h exposure to *P. mirabilis* NSM6 infected urine

The figure shows: (A) ESEM image of encrustation on lumenal section of catheter showing 3 sample sites (labelled spectrum 1-3); (B) X-ray spectrum generated from foundation layer at site 1. Peaks indicated the layer was rich in calcium (Ca) and phosphorous (P); (C) X-ray spectrum generated at bare catheter surface at site 2, indicating presence of silicone (Si); (D) X-ray spectrum of site 3 high in calcium and phosphorous content. Site 3 also indicated high silicone content probably due to the x-ray beam penetrating the foundation layer.

3.1.3. Nucleation pH (pH$_n$) of artificial urine

Calcium and magnesium phosphates form the bulk of urease-induced crystalline biofilms that encrust urinary catheters (Cox *et al.*, 1989; Burr and Nuseibeh, 1993 & 1997). To determine when these urinary solutes precipitated from urine used in bladder model experiments, levels of Ca and Mg in the urine over an ascending pH range were determined using atomic absorbance spectroscopy. The pH$_n$ of urine was also determined. The results of AAS and regression analysis on urine are shown in Figure 25 A and 25 B. The pH$_n$ of artificial urine was determined at approximately pH 6.7. AAS analysis confirmed that Ca and Mg precipitation from urine began to occur at approximately the same pH range.

The effect of pH on calcium and magnesium content in urine

A
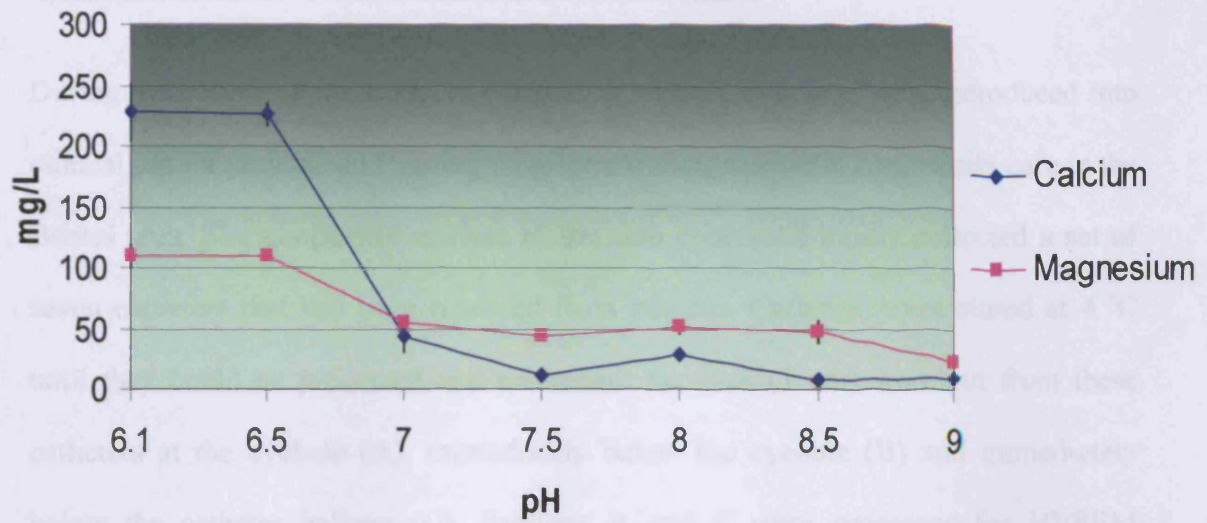
B

Nucleation pH of standard artificial urine
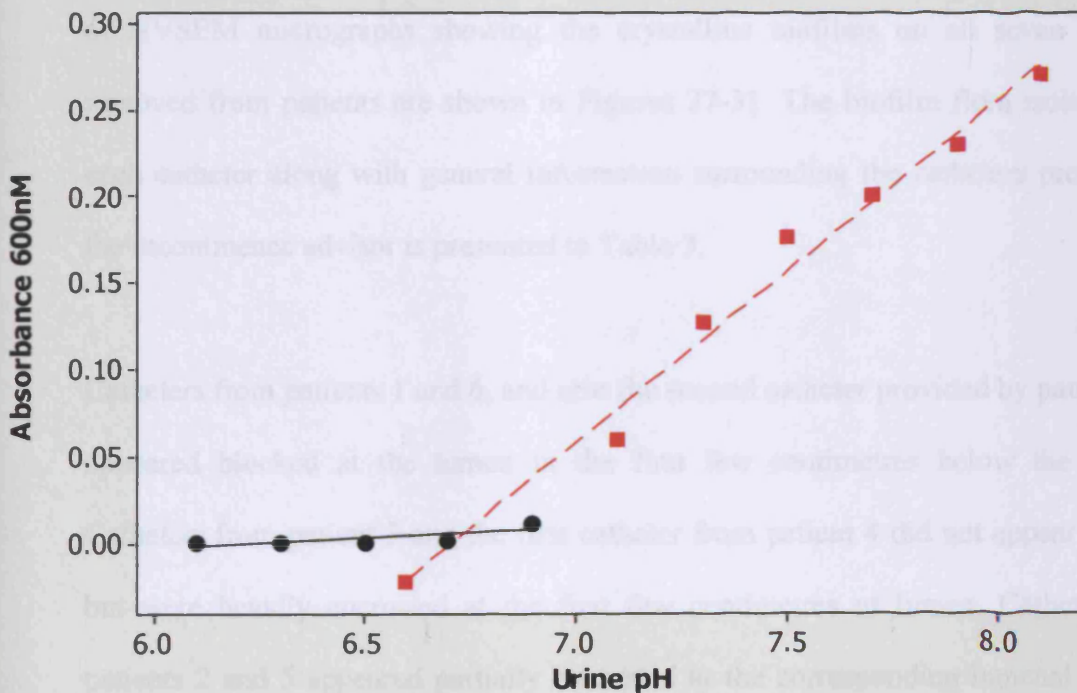
Figure 25.   (A) Calcium and magnesium precipitation from artificial urine at elevated pH. (B) Determination of pH_n

Figure 25A shows the calcium and magnesium concentrations remaining in solution as urine pH becomes elevated. Initial Ca and Mg precipitation occurred between pH 6.5 and pH 7. Figure 25B shows the pH_n of artificial urine determined using regression analysis with values based on pH v absorbance. The pH_n is where the two regression lines meet and was determined at pH 6.7.

3.1.4. Investigation of HSCL catheters of clinical origin

During the course of the study, the BARD IC. HSCL catheters were introduced into

clinical use for patients undergoing long-term catheterisation in community care in the

Bristol area. The continence advisor in the area concerned kindly collected a set of

seven catheters that had been removed from patients. Catheters were stored at 4 °C

until they could be processed and examined. Sections (1 cm) were cut from these

catheters at the eyehole (A), immediately below the eyehole (B) and immediately

below the catheter balloon (C). Sections A and C were processed for HVSEM

analysis while section (B) was processed to determine the biofilm flora. Digital

photographs of sections A and C were taken and are shown in Figure 26. A selection

of HVSEM micrographs showing the crystalline biofilms on all seven catheters

removed from patients are shown in Figures 27-31. The biofilm flora isolated from

each catheter along with general information surrounding the catheters provided by

the incontinence advisor is presented in Table 3.

Catheters from patients 1 and 6, and also the second catheter provided by patient 4, all

appeared blocked at the lumen in the first few centimetres below the eyehole.

Catheters from patient 3 and the first catheter from patient 4 did not appear blocked,

but were heavily encrusted at the first few centimetres of lumen. Catheters from

patients 2 and 5 appeared partially encrusted at the corresponding lumenal areas. In

conjunction to what was observed visually and through digital photography, HVSEM

analysis revealed the bulk of encrusted biofilm was found on the lumen sections of the

catheters. The mineralised deposits appeared typical of the calcium phosphate and

magnesium phosphate that are normally associated with urease-induced encrustation

and was always associated with substantial bacterial colonization. Large areas of the

catheter where encrusted biofilm was present appeared to possess a mineralised foundation layer upon which the bacterial biofilm had developed. This type of crystalline biofilm formation appeared similar to that observed using HVSEM in encrusted catheters removed from previous bladder model experiments. As in the *in vitro* models, areas where biofilm appeared to be directly colonizing the catheter surface were also observed in the HSCL catheters removed from patients. *P. mirabilis* and *Providencia rettgeri* (species known to be associated with catheter encrustation and blockage), were isolated from catheters 3 and 5. Although no species associated directly with catheter encrustation were isolated from the remaining catheters, HVSEM revealed species other than those isolated were present in the biofilms on these catheters.
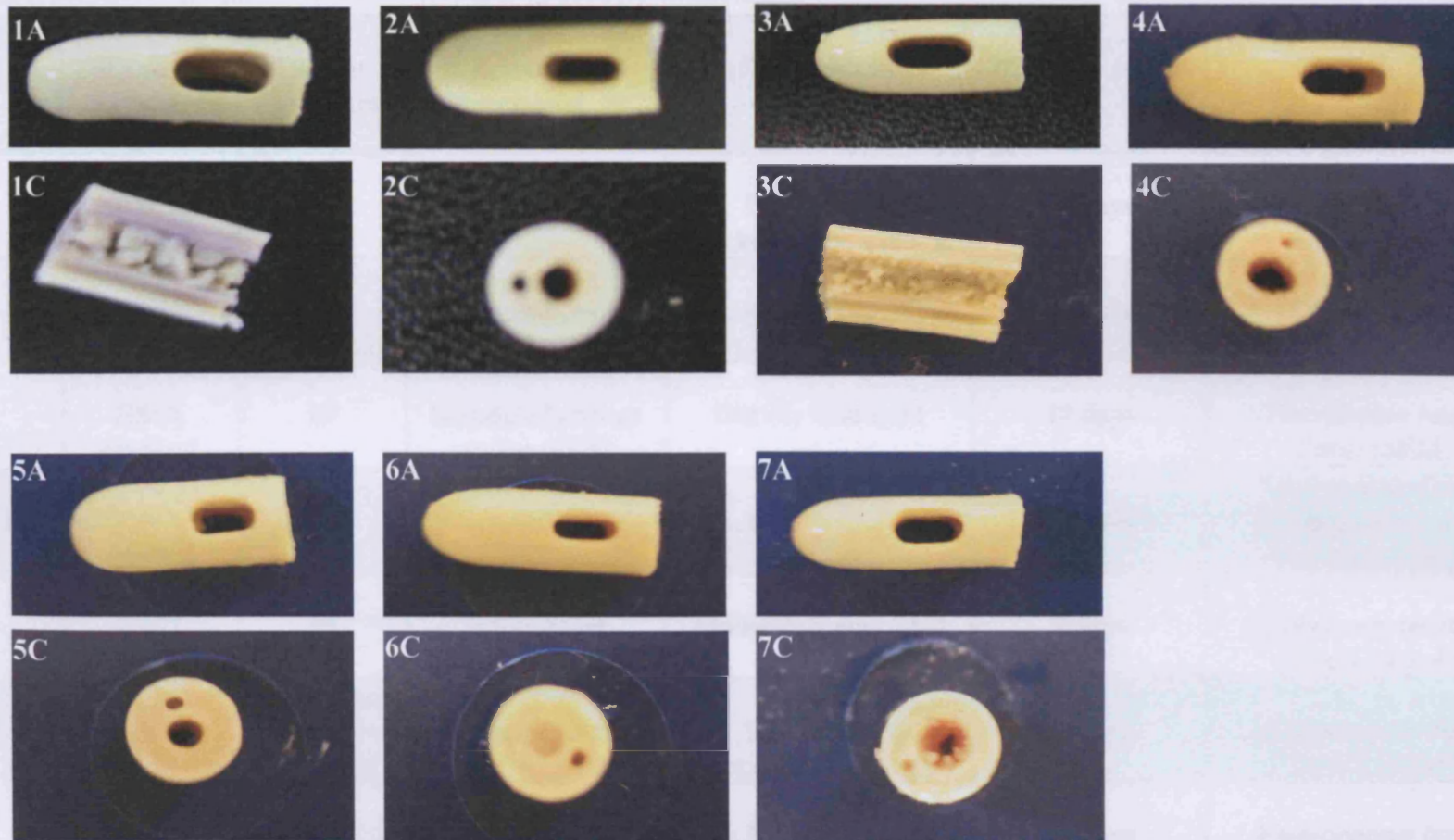
Figure 26. **Digital images of hydrogel/silver-coated latex catheters removed from catheterised patients**

Sections 1A-7A show eyehole regions and sections 1C-7C show regions directly below the retention balloon. Sections 1C and 3C have been transversely sectioned and show encrusted deposits lining the catheter lumen. Catheters 4 and 7 were two separate catheters removed from the same patient over a 1 month period.

Information table regarding Bard IC. Hydrogel/silver-coated latex catheters

Patient No.	Type of cathteter	Patient age (years)	Reason for removal	Catheter condition	Duration *in situ*	Biofilm flora
1	HSCL urethral	53	Failure to drain	Encrustation blocked below eyehole	10 days	*Enterococcus faecalis* *Micrococcus luteus* Bacilli (SEM)†
2	HSCL urethral	Information not provided	Scheduled change (urine pH 8)	Lightly encrusted	14 days	*Enterococcus faecalis* *Staphylococcus hominis* Bacilli (SEM) †
3	HSCL urethral	89	Scheduled change (urine pH 9)	Heavily encrusted	14 days	*Providencia rettgeri* Cocci (SEM) †
4	HSCL urethral	81	Scheduled change	Heavily encrusted	14 days	*Enterococcus faecalis* *Staphylococcus aureus* Also bacilli (SEM) †
5	HSCL urethral	94	Information not provided	Moderately encrusted	5 days	*Staphylococcus vitulans* *Proteus mirabilis*
6	HSCL urethral	Information not provided	Failure to drain	Encrustation blocked below eyehole	14 days	*Enterococcus durans* Bacilli (SEM) †
4*	HSCL urethral	81	Failure to drain	Encrustation blocked below eyehole	11 days	*Enterococcus faecalis* *Staphylococcus aureus*

4* 2nd catheter from patient 4. † Species not isolated from catheter but observed as part of the biofilm flora using HVSEM analysis

Table 3.   **Profiles of hydrogel/silver-coated latex catheters from catheterised patients**

The condition of devices that were not blocked was assessed visually. Urine pH is indicated whenever information was available.
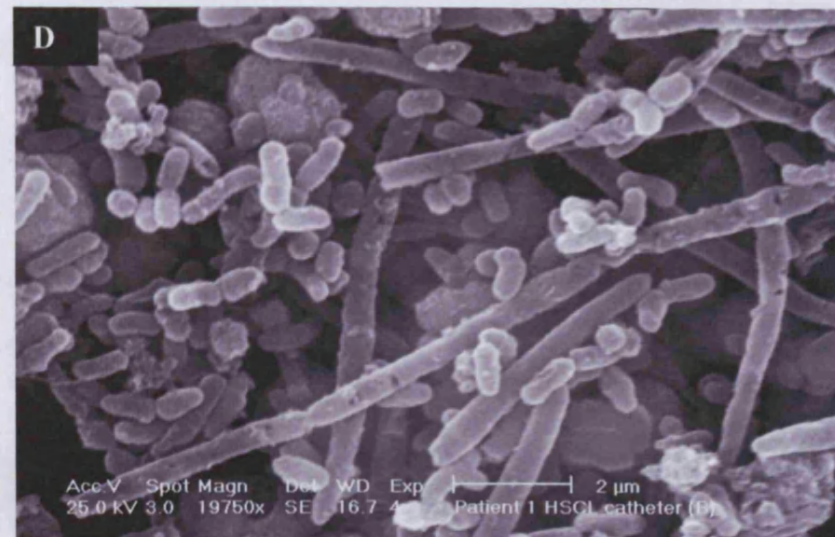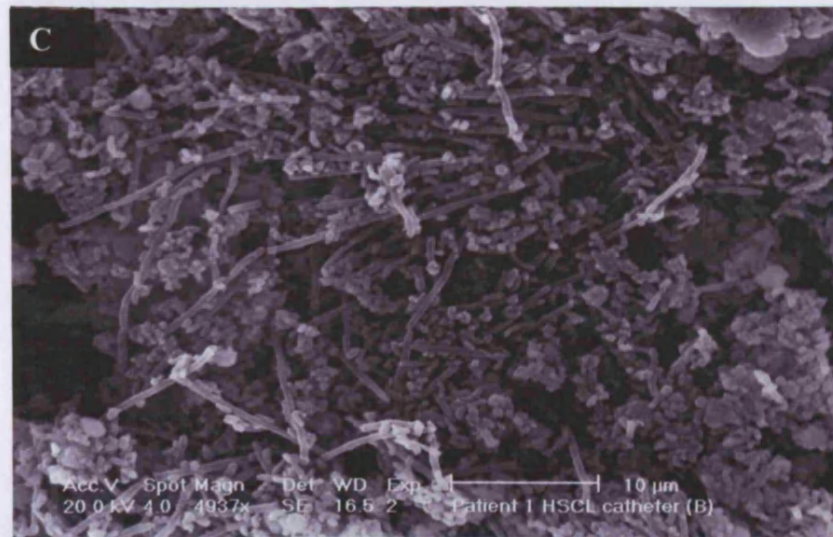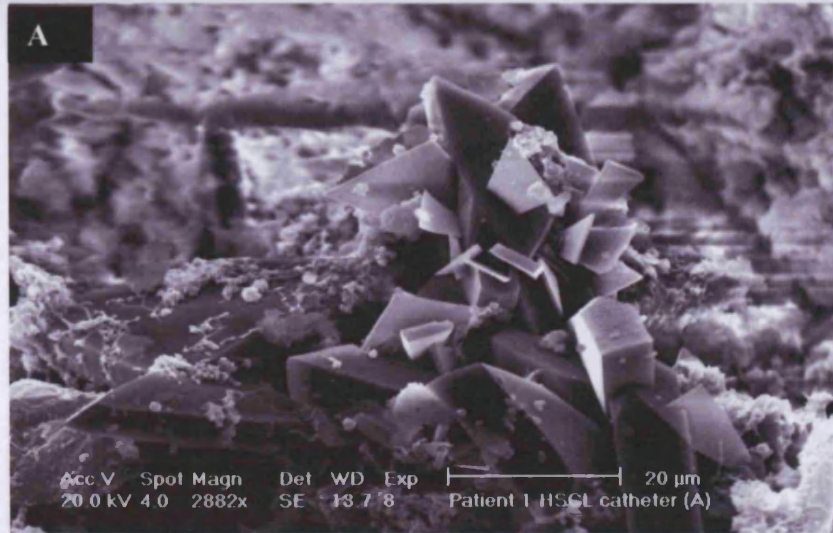
Figure 27.   Crystalline biofilms on hydrogel/silver-coated latex catheters from catheterised patients

The micrographs show (A + B) crystalline material around eye-let of patient 1 catheter. (C + D) illustrate a mixed community biofilm on a lumenal section just below the retention balloon of a catheter also from patient 1.
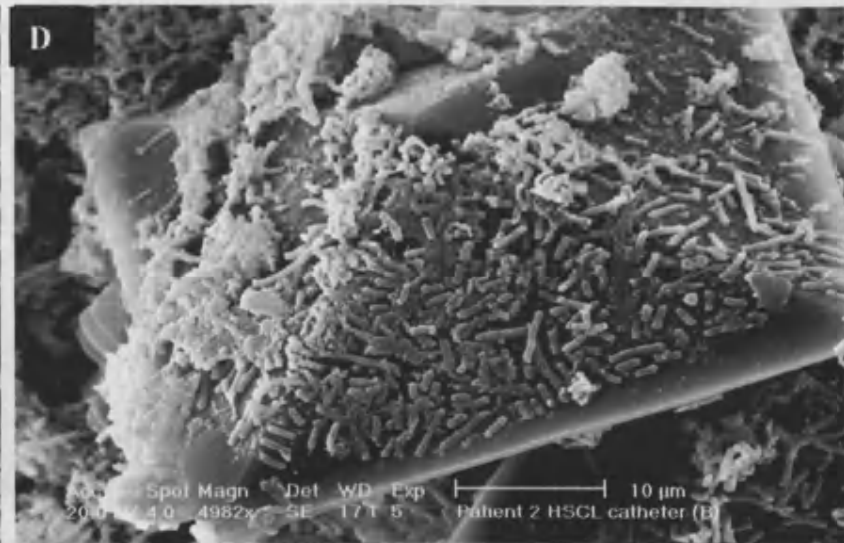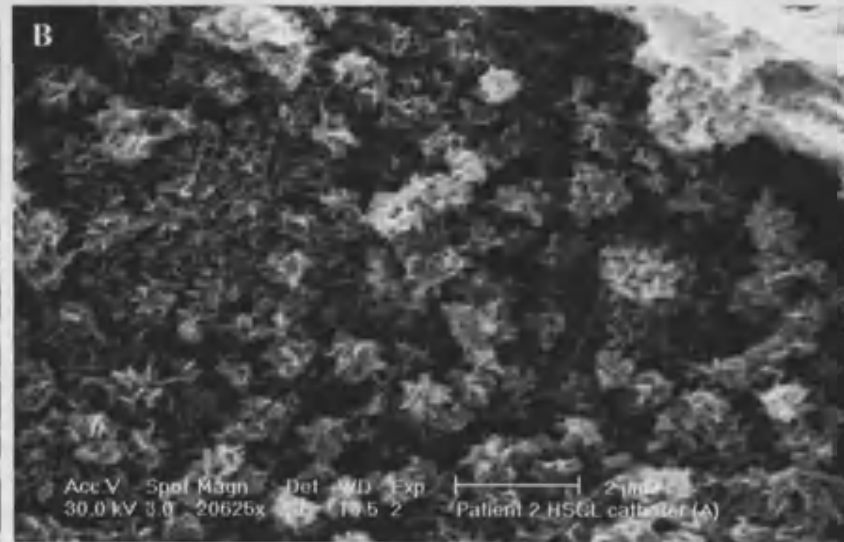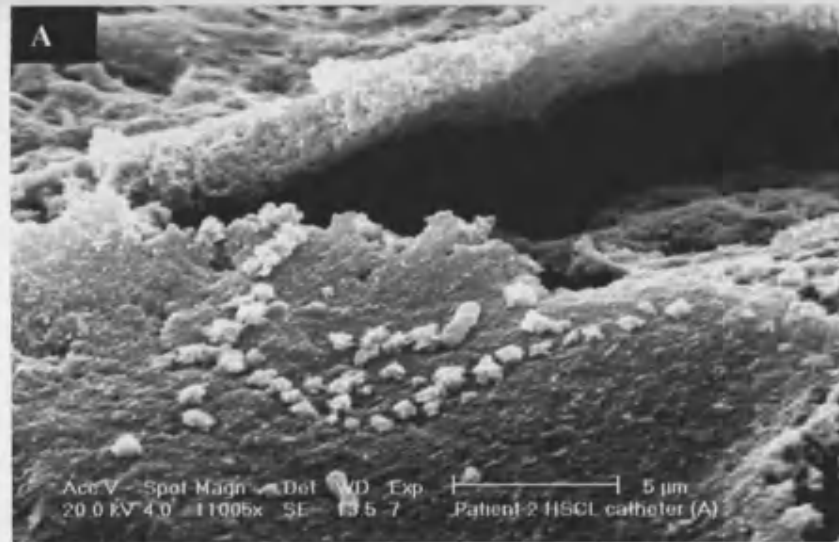
Figure 28. Crystalline biofilms on hydrogel/silver coated latex catheters from catheterised patients

The micrographs show: (A) a foundation-like layer on surface of the eye-hole bevel of catheter from patient 2: (B) a microcrystal layer at also at the eyehole of catheter from patient 2; (C + D) are lumenal sections of catheter from patient 2 just below the retention balloon showing mature biofilm crystalline deposits.
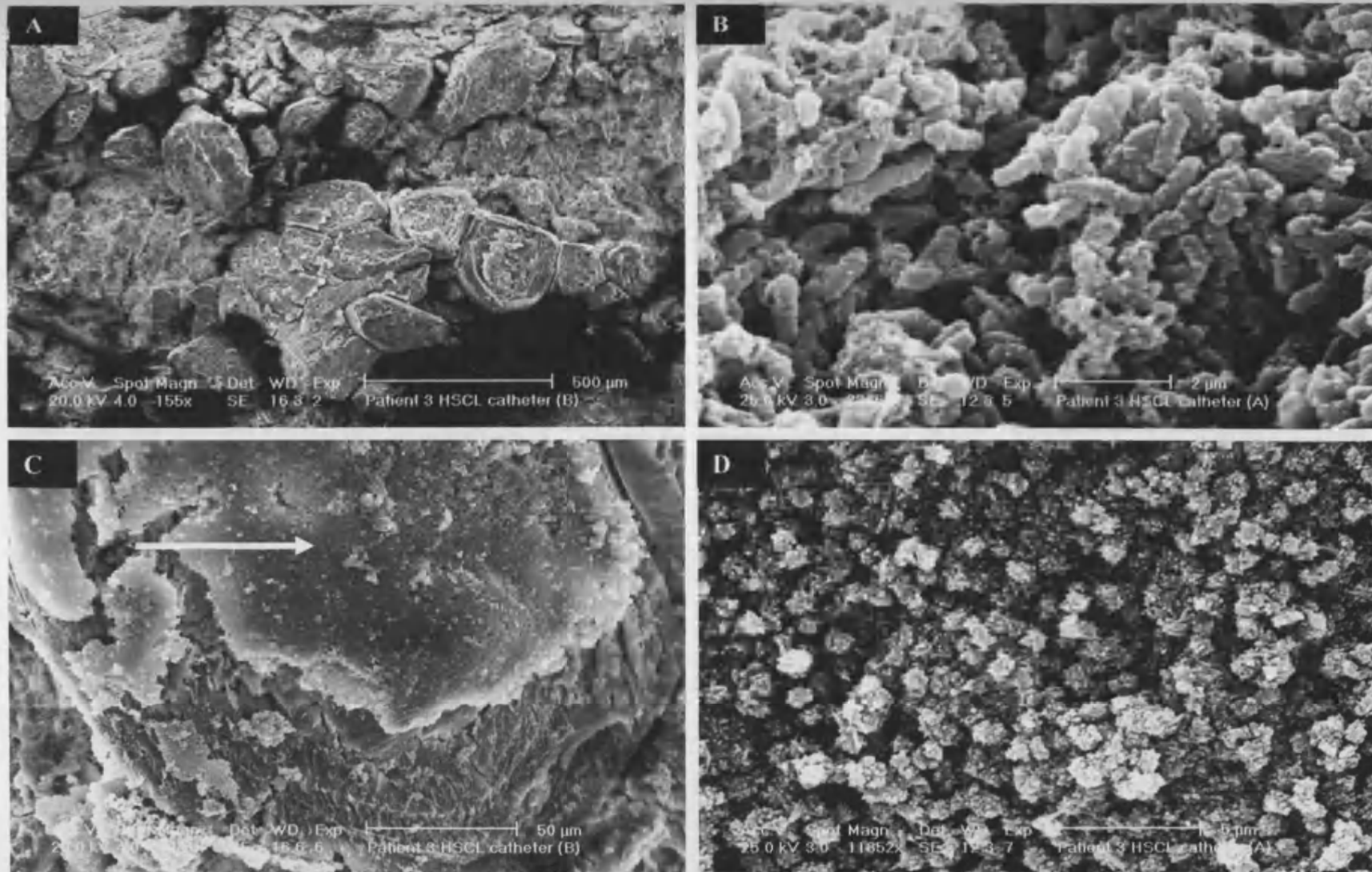
Figure 29. Crystalline biofilms on hydrogel/silver coated latex catheters from catheterised patients

The micrographs of the catheter from patient 3 show: (A) the crystalline deposits in lumenal section just below the retention balloon; (B) an encrusted biofilm at eyehole section of catheter; (C) a crystalline structure covered in bacterial biofilm (arrow) several layers thick; (D) a microcrystal-like layer found at t eyehole.
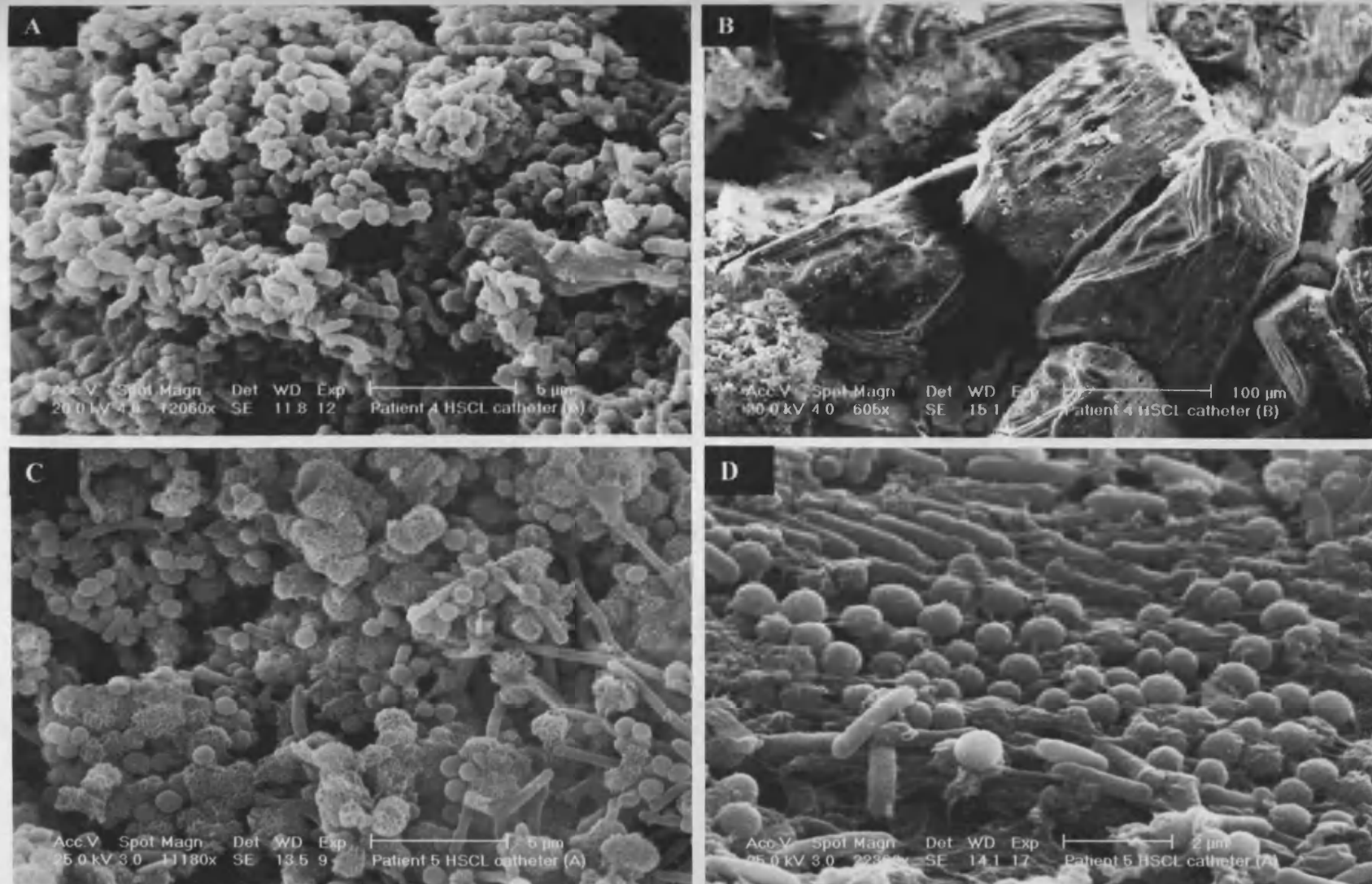
Figure 30.   Crystalline biofilms on hydrogel/silver coated latex catheters from catheterised patients

The micrographs show: (A) a mixed species biofilm around eye-let of section of catheter from patient 4; (B) large crystalline deposits in lumenal section just below the retention balloon of catheter from patient 4; (C) an encrusted mixed community biofilm at eyehole section of catheter from patient 5. This crystalline material is typical of calcium phosphate can be seen amongst the biofilm cells; (D) shows bacterial cells (bacilli and cocci) adhering to eyehole surface of catheter from patient 5.

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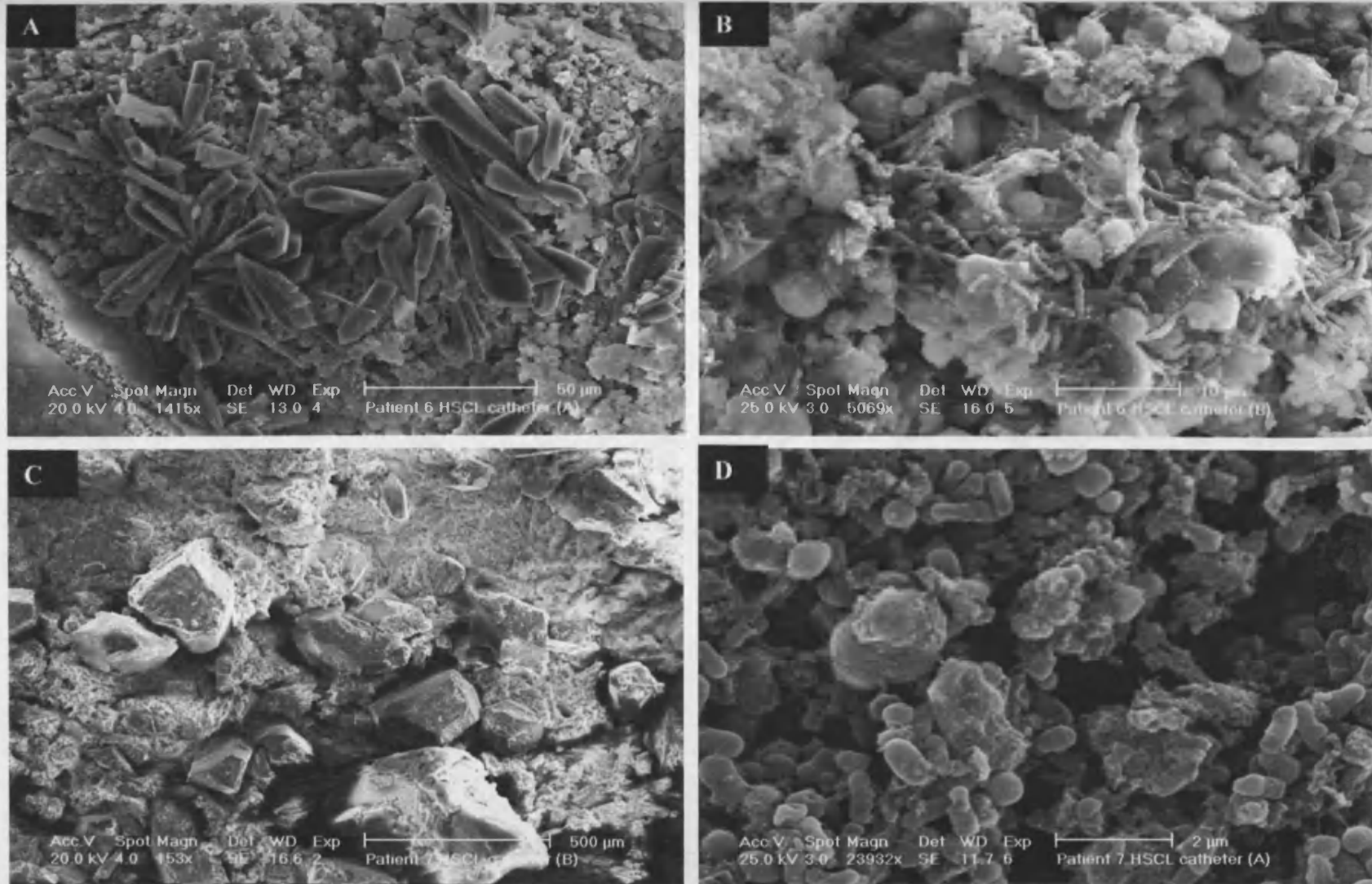
Figure 31. Crystalline biofilms on hydrogel/silver coated latex catheters from catheterised patients

The micrographs show: (A) large crystalline structures similar to calcium phosphate crystals at the eyehole area of catheter from patient 6; (B) typical biofilm structure found in lumen of catheter from patient 6; (C) large crystalline deposits found in lumen of the 2nd catheter from patient 4; (D) high magnification of a mixed-species crystalline biofilm from eyehole section of the 2nd catheter from patient 4.

3.2. Role of Mannose-Resistant *Proteus*-like fimbriae (MR/P fimbriae) in *Proteus mirabilis* colonization of all-silicone catheter surfaces

Results reported in Section 3.1. suggest that when a catheter is introduced into a bladder containing urine in which a population of *P. mirabilis* cells has produced alkaline conditions, crystalline biofilm formation on the catheter is initiated by the deposition of a microcrystalline foundation layer of calcium phosphate. Like many other bacteria *P. mirabilis* can produce appendages that are believed to be involved in attaching cells to surfaces. The most studied of these are the MR/P fimbriae (Mannose-Resistant *Proteus*-like fimbriae). There is evidence that these structures are involved in cell-aggregation, attachment to surfaces, colonization and invasion of the urinary tract (Mobley & Chippendale, 1990; Bahrani *et al.*, 1994; Jansen *et al.*, 2004). The extent to which they are involved in the encrustation of catheters however, is uncertain. They might be responsible for initiating attachment of cells directly to the catheters or to the microcrystalline foundation layer. On the other hand they might have no role in the process of crystalline biofilm formation.

3.2.1. MR/P expression in *P. mirabilis* isolates from environmental and clinical sources

A collection of twenty seven *P. mirabilis* isolates from a range of clinical and environmental sources were assayed for their ability to express MR/P fimbriae. Results of duplicate haemagglutination assays for all *P. mirabilis* isolates tested are presented in Table 4. All the test organisms were incubated under optimum conditions for MR/P expression and assayed for their ability to agglutinate chicken red blood cells (CRBC) in the presence and absence of 50 mM mannose. Figure 32 shows the

haemagglutination profiles of the wild type *P. mirabilis* HI4320 and its *mrpA* mutant strain.

3.2.2. Effect of fluorofamide on pH of urine

To prevent *P. mirabilis* elevating the urinary pH in the bladder model experiments, a urease inhibitor fluorofamide (Morris & Stickler, 1998b) was used as a urine supplement. To determine the concentration of fluorofamide required in urine a series of WT *P. mirabilis* HI4320 urine cultures containing fluorofamide at 2, 3 and 4 μg/ml were incubated at 37°C with gentle aeration. After 4 h incubation the pH and viable cell counts were determined for each culture. Results showed mean viable cell counts (not shown) from cultures were not significantly different at the end of the 4 h incubation period ($P = 0.9$). The mean pH values in cultures containing 2 and 3 μg/ml fluorofamide were close to the nucleation pH of urine (pH 6.7), reaching pH 6.72 and 6.68 respectively. The mean pH of cultures supplemented with 4 μg/ml fluorofamide was pH 6.46 after 4 h incubation. It was decided therefore that fluorofamide at 4 μg/ml urine would be used to prevent significant pH increases in urine infected with *P. mirabilis*.

3.2.3. Preparation of the inocula of WT and *mrpA P. mirabilis* HI4320

To minimise disruption to fimbriae and also to synchronise cultures with experiment start times, *P. mirabilis* cultures used for bladder model inocula were grown statically over a 12 h period at 25°C. Growth curves were plotted for both strains in the presence and absence of 4 μg/ml fluorofamide with pH values of cultures also recorded. The results (Figure 33) show that viable cell densities reached 10^8 cfu/ml in

all cultures at the end of the incubation period with no significant difference between means (P = 0.81). The mean pH values for WT and *mrpA* cultures (8.71 and 8.69 respectively), incubated in urine only were not significantly different (P = 0.77). The mean urine pH's for WT and *mrpA* cultures incubated in urine containing 4 μg/ml fluorofamide slightly exceeded the pH_n reaching 6.75 and 6.78 respectively, but were not significantly different (P = 0.51).

Bacterial strain	Agglutination ± mannose	Origin
WT HI4320	+	CAUTI
mrpA HI4320	-	CAUTI
SM13	+	CAUTI
SM27	+	CAUTI
AO	+	CAUTI
AAAS (non-swarmer)	+	CAUTI
AAAX	+	CAUTI
NP5	+	CAUTI
SDM18 (non-swarmer)	+	Catheter biofilm
RB6	+	Catheter biofilm
RB20	+	Catheter biofilm
AK	+	Catheter biofilm
SDM15	+	Catheter biofilm
SDM16	+	Catheter biofilm
NSM6	+	Catheter biofilm
NP41	+	Catheter biofilm
NP11	+	Penarth seawater
NP10	+	Penarth seawater
NP31	+	Swansea seawater
NP34	+	Swansea seawater
NP23	+	Seine river water
NP24	+	Seine river water
NP21	+	Seine river water
NP39	+	Cardiff seawater
NP27	+	Sewage
NP29	+	Sewage
NP54	+	Sewage
NP40	+	Sewage
NP22	+	Sewage

(+) Agglutination in presence and absence of 50 mM mannose, (-) no agglutination in presence and absence of mannose. CAUTI = isolated from catheter-associated urinary tract infection

Table 4.   **MR/P expression in *P. mirabilis* isolates from a range of environmental and clinical sources**
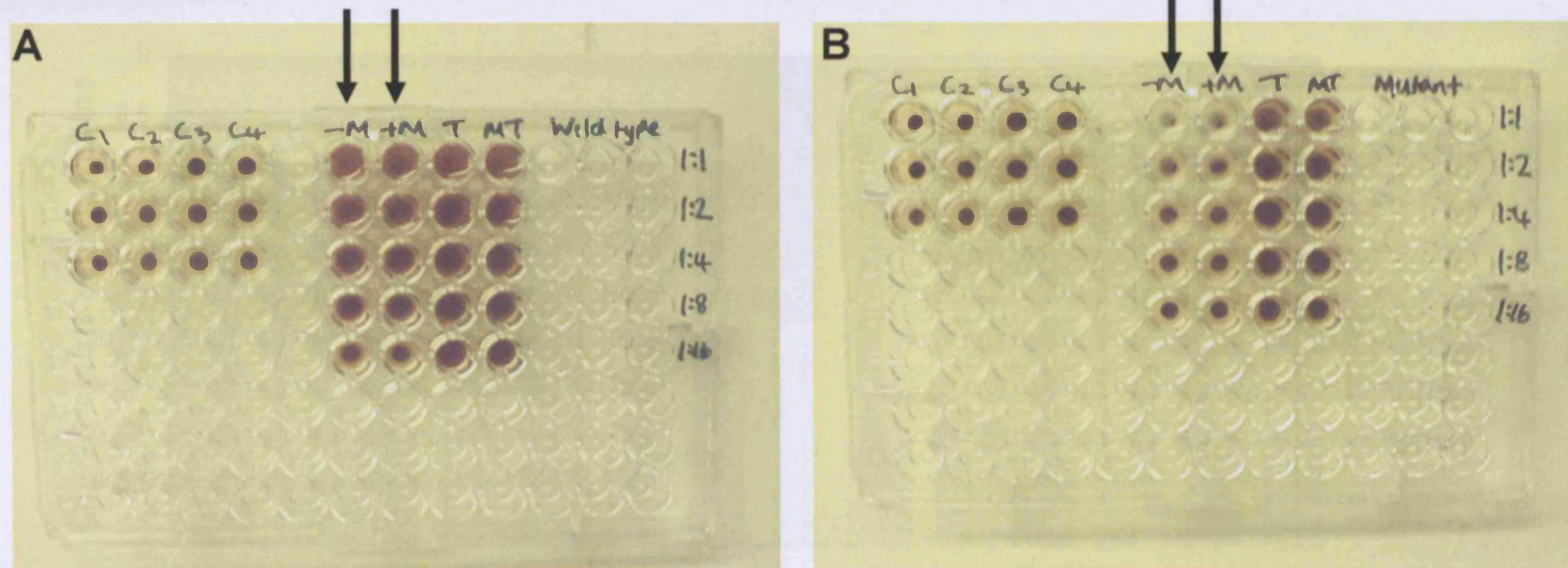
Figure 32. **Haemagglutination profiles of wild type and *mrpA* mutant *Proteus mirabilis* HI4320**

The images show: (A) the wild type *P. mirabilis* HI4320 agglutination of CRBC in the absence and presence of 50 mM mannose (-M and +M respectively) seen as a diffuse matt of cells within the microtitre well (arrows); (B) no agglutination of CRBC in absence or presence of 50 mM mannose by *mrpA* mutant indicated by the tight button of cells formed in the microtitre wells (arrows). Tannic acid sensitised cells (T and MT) are agglutinated in the absence and presence of 50 mM mannose in both strains indicating the presence of agglutinins other than MR/P fimbriae. Bacterial suspensions 1:1 to 1:16 were used in the tests. Control wells (C1-C4) contained CRBC, mannose treated CRBC, tanned CRBC and mannose treated tanned CRBC and were not inoculated with *P. mirabilis*.
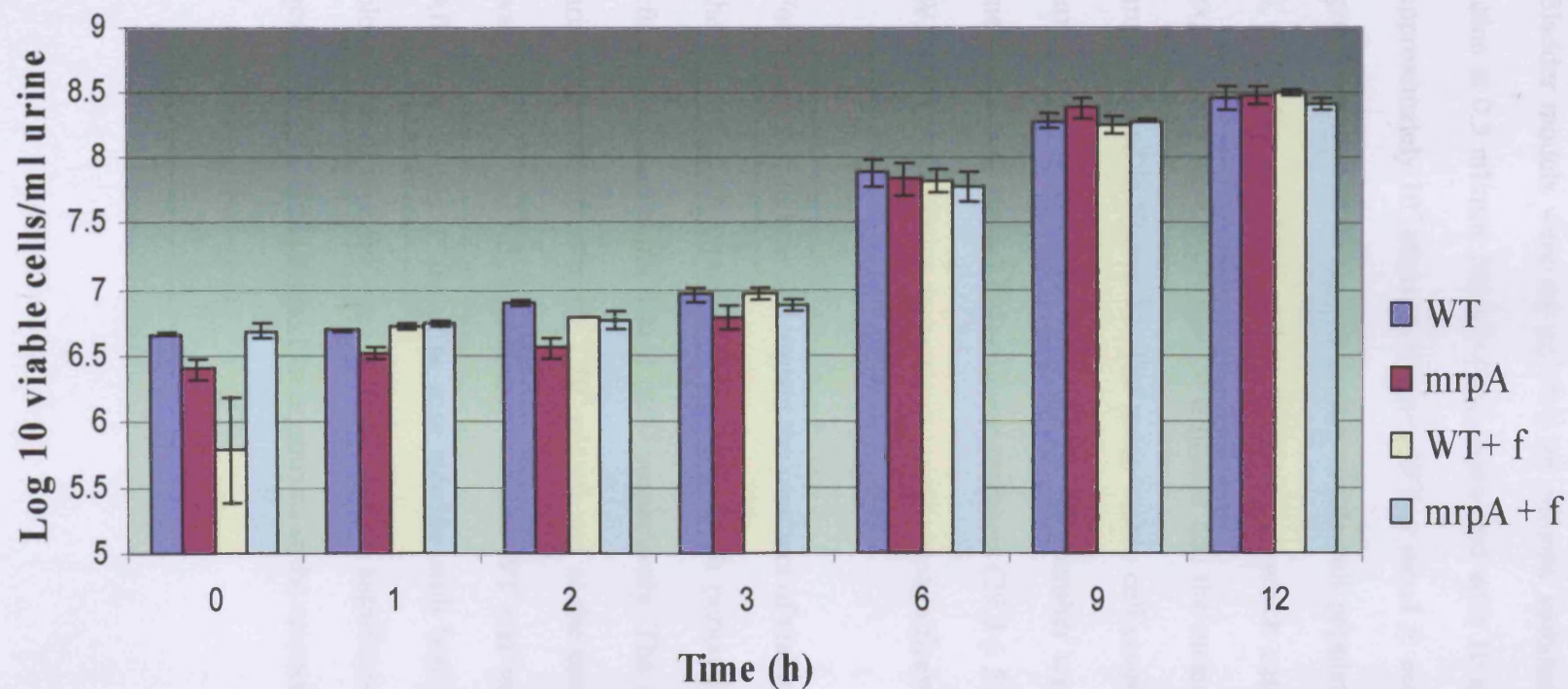
Figure 33. **Mean viable counts of WT and *mrpA P. mirabilis* in static cultures at 25 °C**

P. mirabilis wt and *mrpA* HI4320 were incubated statically for 12 h in flasks containing artificial urine with and without 4 µg/ml fluorofamide. Viable cell numbers were recorded at various intervals up to 12 h. There were no significant differences ($P = 0.81$) in cell numbers between cultures after 12 h incubation. The mean values ± SE were calculated for the results of three replicated experiments.

3.2.4. Ability of WT and *mrpA P. mirabilis* HI4320 to encrust all-silicone catheters

Bladder models were set up with all silicone catheters and supplied with artificial urine at 0.5 ml/min. Models were inoculated with 10 ml of a 4 h culture (containing approximately 10^8 cfu/ml), of either WT or *mrpA P. mirabilis* HI4320 incubated with gentle aeration at 37°C. The urinary viable cell populations and pH were recorded at 0, 4, 8, 12, and 24 h and at the times at which catheter blockage occurred. The experiments were performed in triplicate and the mean times catheters took to block are presented in Figure 34. The mean viable cell counts and mean pH values of the urine samples recovered from the model chamber are presented in Figure 35. The mean blockage times of all-silicone catheters (29.9 ± 5.61 h and 41.9 ± 1.43 h for the WT and mutant strains respectively) were not significantly different ($P = 0.1$).

For both the wild type and mutant the numbers of viable cells in the residual urine and the urinary pH declined over the initial 4 h period from 10^8 cfu/ml to around 10^7 cfu/ml and from > pH 8 to < pH 7 respectively. The numbers of viable cells in the urine then stabilized at 10^7 - 10^8 cfu/ml and at the times of catheter blockage, there was no significant difference between the WT and mutant populations ($P = 0.28$). After 4 h the pH of the urine rose steadily with both organisms and at the time of blockage had reached > pH 8. There was no significant difference between the pH's generated ($P = 0.98$) by the two organisms at the respective blockage times.
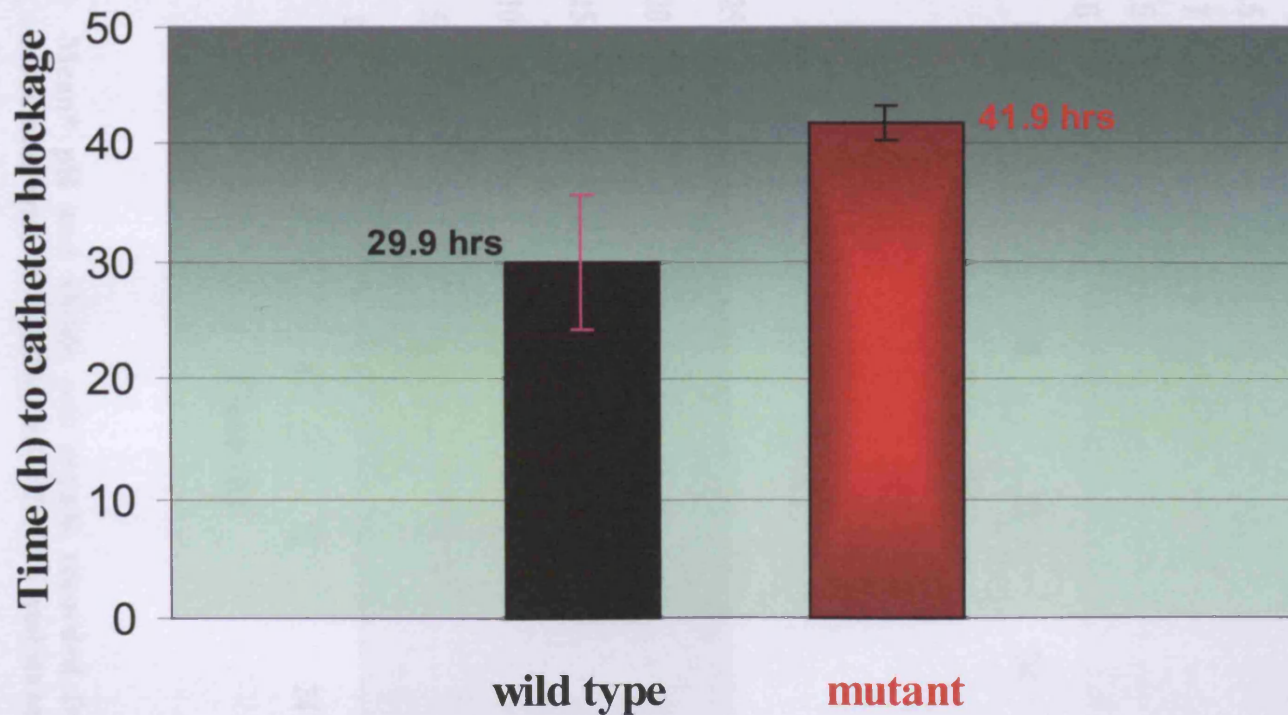
Figure 34. **Mean* blockage times of all-silicone catheters in bladder models inoculated with wild type or *mrpA Proteus mirabilis* HI4320**

Bladder models fitted with all-silicone catheters were inoculated with 10 ml (10^8 cfu/ml) of the test strains. Urine was supplied at 0.5 ml/min and models were run until catheters blocked. There were no significant differences between mean blockage times ($P = 0.1$) between WT or *mrpA* strains. Standard error of the mean of three replicates is shown. * The mean values ± SE were calculated from the results of three replicate experiments.
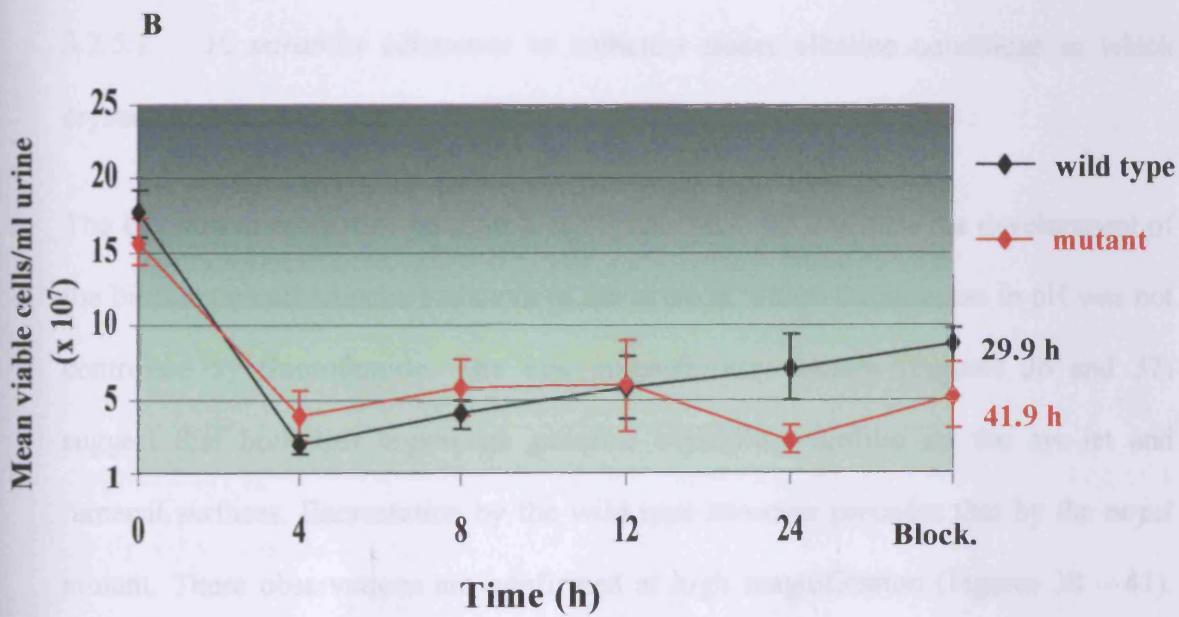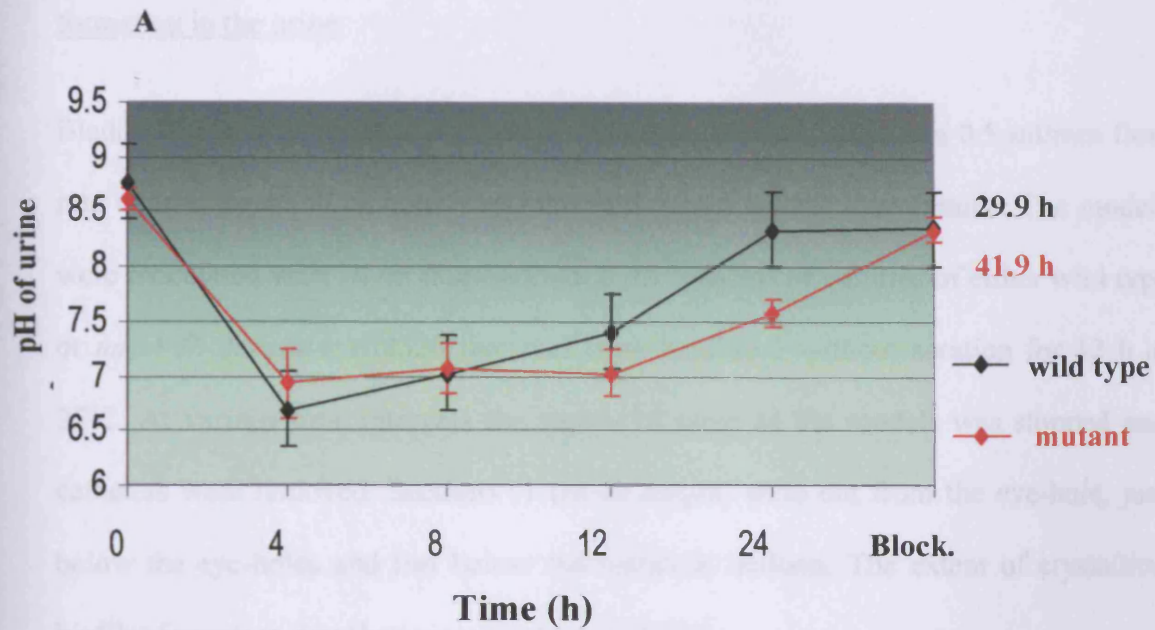
Figure 35.   Mean* pH and viable cell counts recorded from residual urine in models inoculated with *P. mirabilis* HI4320 WT and its *mrpA* mutant

(A) shows the mean pH of residual urine in models; (B) the mean numbers of viable cells in the urine. *The mean values ± SE were calculated from the results of three replicate experiments.

3.2.5. *P. mirabilis* adherence to catheters in the presence and absence of crystal formation in the urine

Bladder models fitted with all-silicone catheters were supplied at a 0.5 ml/min flow rate with either urine or urine supplemented with 4 µg/ml fluorofamide. The models were inoculated with 10 ml (approximately 10^8 cfu/ml) of cultures of either wild type or *mrpA P. mirabilis* HI4320 that had been incubated without aeration for 12 h at 25°C. At various time intervals the supply of urine to the models was stopped and catheters were removed. Sections (1 cm in length) were cut from the eye-hole, just below the eye-holes and just below the retention balloon. The extent of crystalline biofilm formation was then visualized by HVSEM.

3.2.5.1.   *P. mirabilis* adherence to catheters under alkaline conditions in which crystals form in the urine

The electron micrographs presented in Figures 36 – 41 illustrate the development of the biofilms on all-silicone catheters in the urine in which the increase in pH was not controlled by fluorofamide. The low magnification images (Figures 36 and 37) suggest that both test organisms generate crystalline biofilm on the eye-let and lumenal surfaces. Encrustation by the wild type however precedes that by the *mrpA* mutant. These observations are confirmed at high magnification (Figures 38 – 41). Crystalline biofilm of the WT strain is clearly visible on both the eye-let and lumenal sections at 8 h, but doesn't appear until 12 h in the case of the mutant. It is also apparent that at 12 – 18 h the WT biofilms have large populations of cells whereas the mutant biofilms are mainly crystalline in nature and contain relatively few cells. At 30

h however, while the mutant biofilm remained mainly crystalline in nature there were patches that were densely populated by cells (Figure 42).

3.2.5.2. *P. mirabilis* adherence to catheters in acidic urine

The electron micrographs presented in Figures 43 – 48 show the development of biofilm on all-silicone catheters in models infected with the test *P. mirabilis* strains in which acidic pH of the urine was maintained by the urease inhibitor fluorofamide. The low magnification micrographs of the eye-let and lumenal surfaces (Figures 43 and 44) suggest that extensive colonization by the wild type is not apparent until 24 h and that even at 48 h the *mrpA* mutant has produced only disperse patches of biofilm. At high magnification (Figures 45 - 48) there is little sign of crystalline material in the biofilms. In the case of the wild type the bacterial colonization at 4 and 12 h is in the form of micro-colonies that were associated with surface irregularities, especially in the eye-hole region. More extensive coverage is visible at 24 h and 48 h over both the eye-let and lumenal surfaces. Very little colonization of catheters by the *mrpA* strain was observed in the first 12 h. At 24 h micro-colonies had developed on parts of the eye-let and by 48 h these had formed a discontinuous, patchy, crystal-free biofilm several cells thick. On the lumenal surface, colonization at 48 h was limited to a dispersed population of single cells.

The numbers of viable cells recovered from the residual urine and the urinary pH of the models used in HVSEM are presented in Figure 49. It can be seen that the fluorofamide maintained acidic conditions in the urine, thus assuring that crystal formation would not have taken place in the urine throughout the experiment.
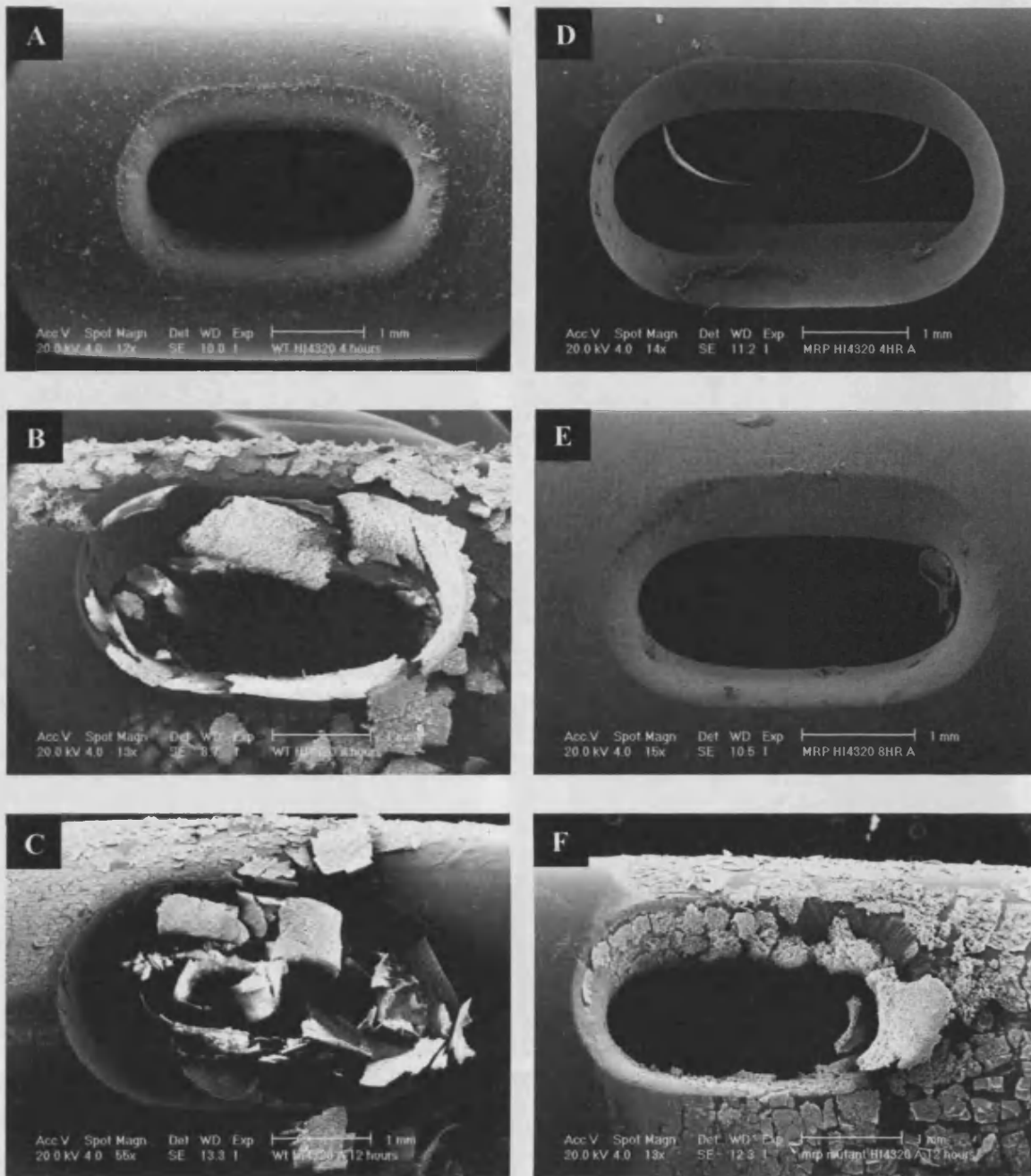
Figure 36. Crystalline biofilm formation of all-silicone catheters by WT and *mrpA* HI4320 *P. mirabilis*

Models were supplied with urine and inoculated with 10^8 cfu/ml of the test strains. The micrographs (A–C) illustrate encrustation at 4, 8 and 12 h in WT infected bladder models. (D-F) illustrate encrustation at 4, 8 and 12 h in *mrpA* infected bladder models.
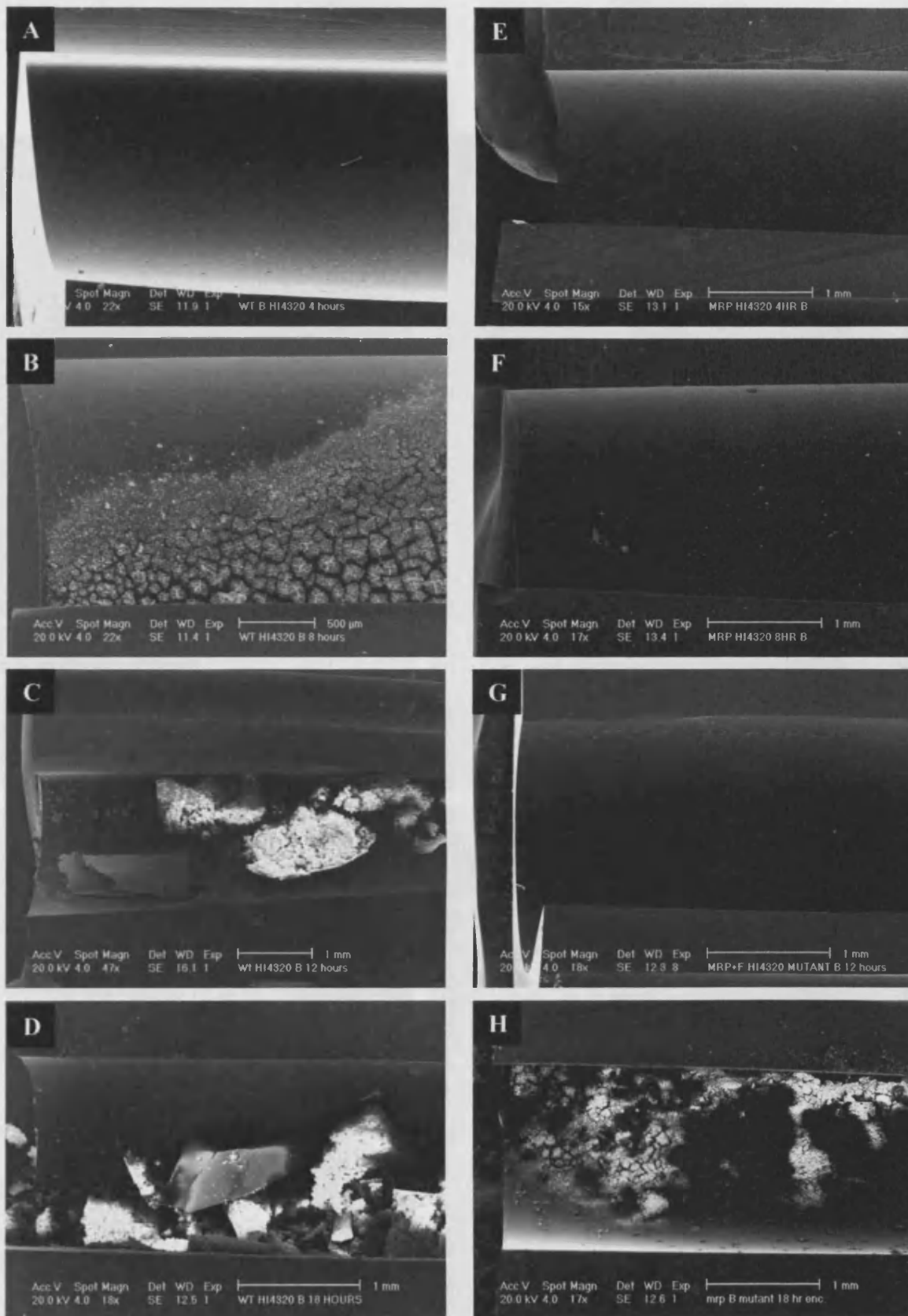
Figure 37. Crystalline biofilm formation of the lumenal surfaces of all-silicone catheters by WT and *mrpA* HI4320 *P. mirabilis* under alkaline conditions

Models were supplied with urine and inoculated with 10^8 cfu/ml of the relevant test organisms. The micrographs show encrustation developing at lumenal sections just below the eyehole regions of catheters. (A-D) illustrate encrustation at 4, 8, 12 and 18 h in WT infected bladder models. (E-H) illustrate encrustation at 4, 8, 12 and 18 h in *mrpA* infected bladder models.

130
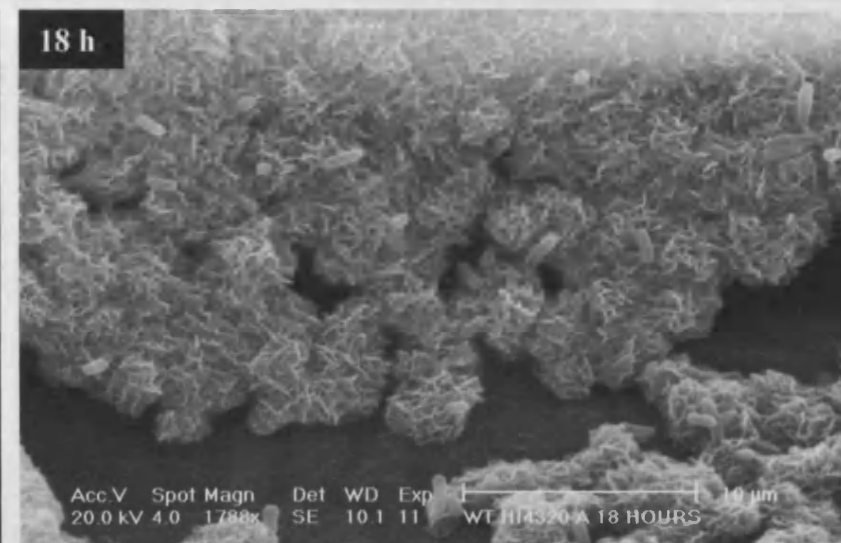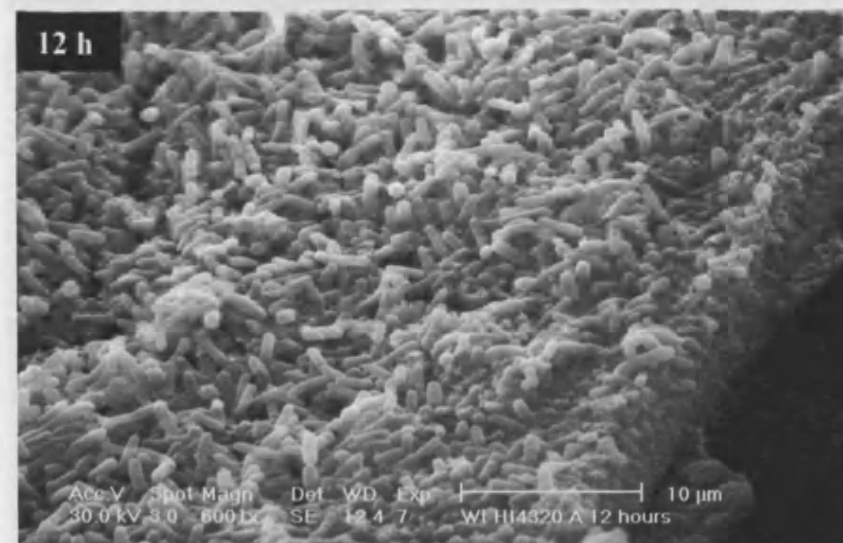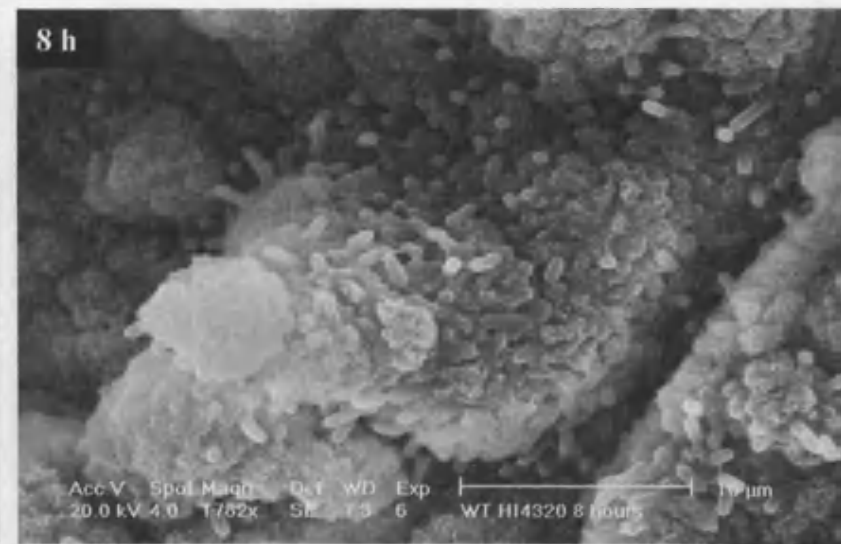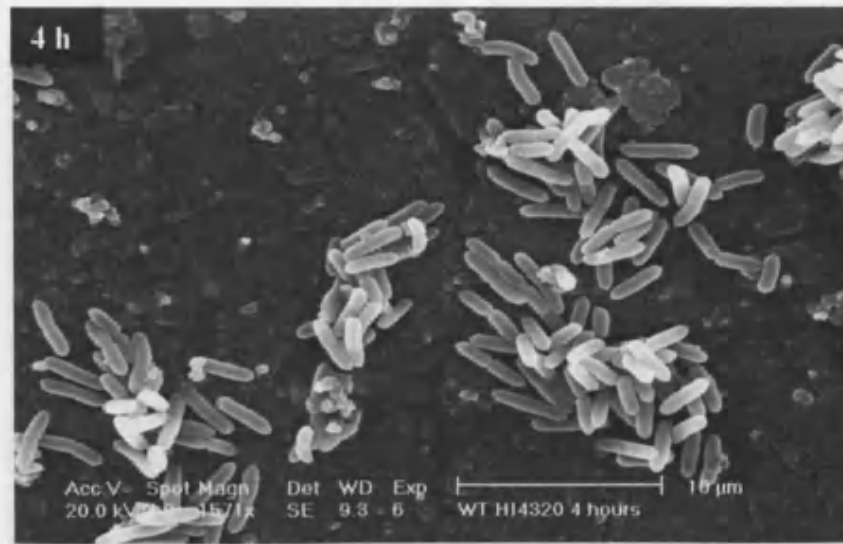
Fig 38. **Wild type HI4320 *P. mirabilis* colonization of the eye-hole of all-silicone catheters in alkaline urine**

The micrographs show the accumulation of *Proteus* cells and microcrystals over an 18 h period.
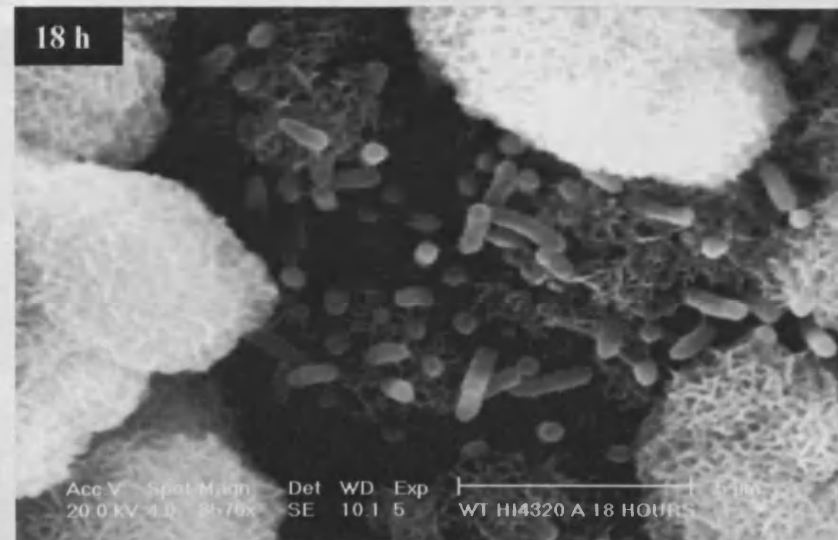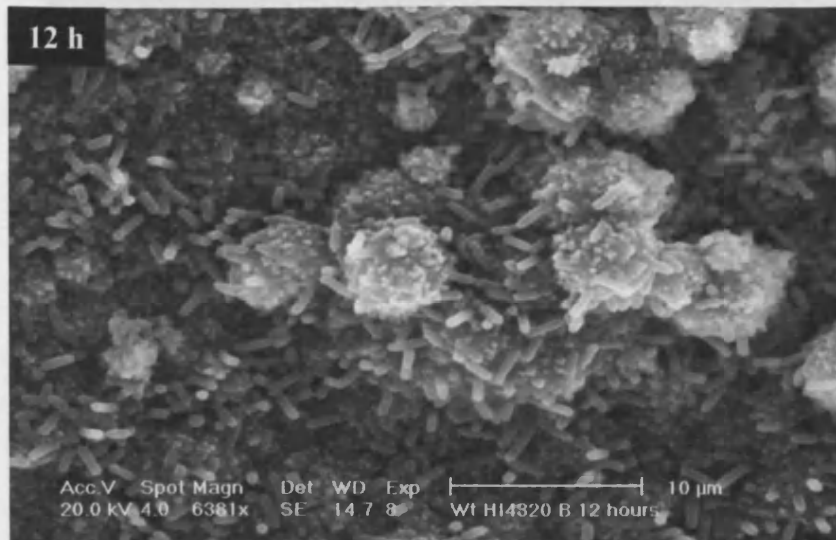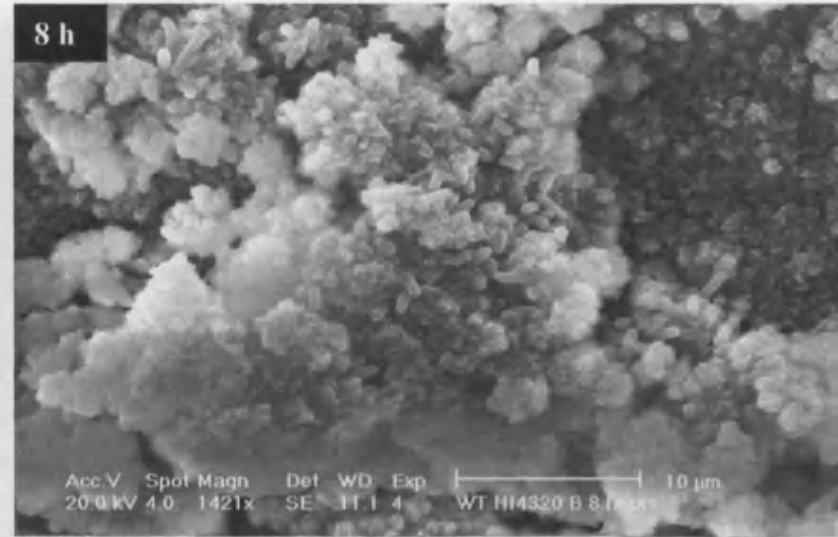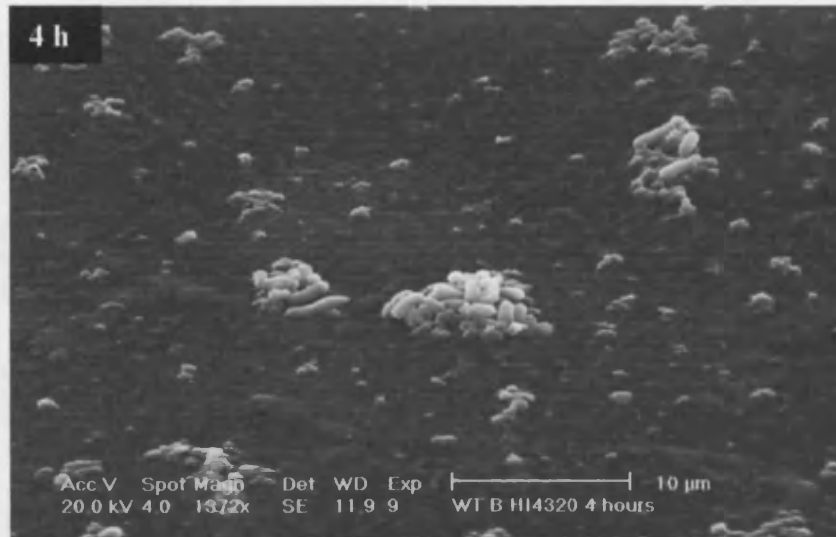
Fig 39.   **Wild type HI4320 *P. mirabilis* colonization of the lumenal surfaces of all-silicone catheters in alkaline urine**

The micrographs show the accumulation of *Proteus* cells and microcrystals over an 18 h period.
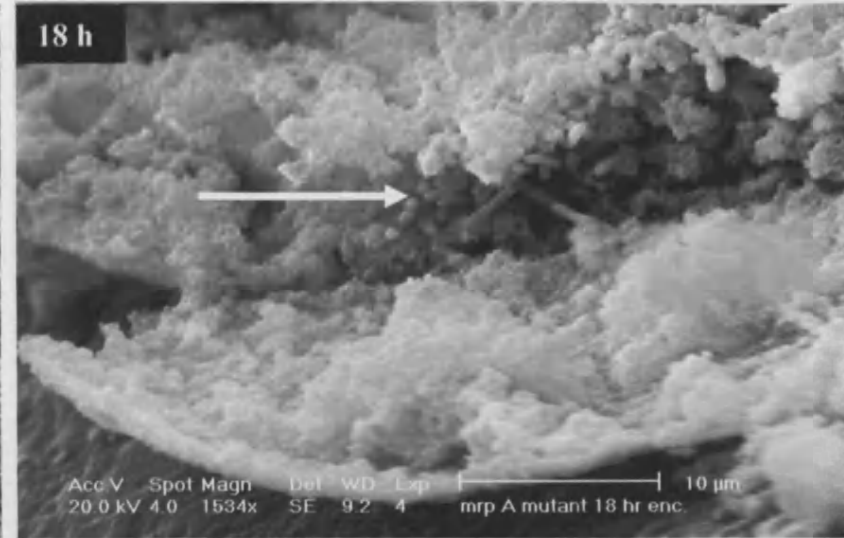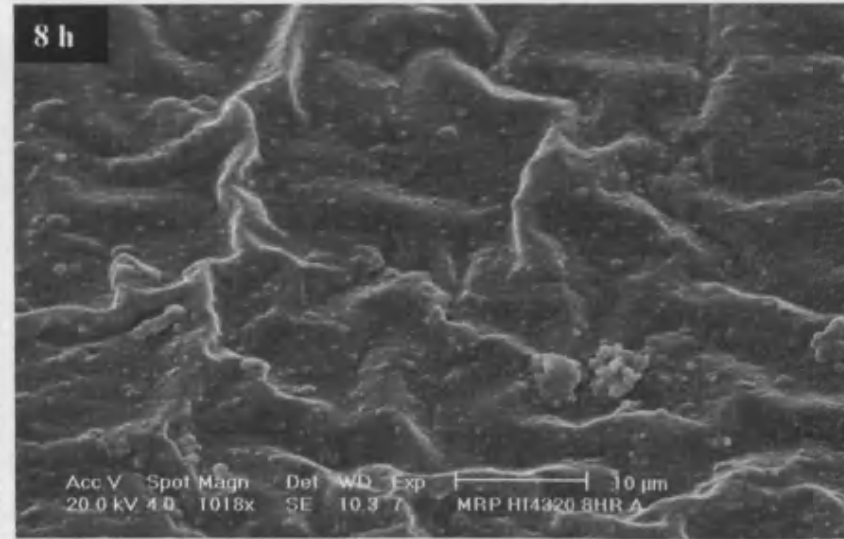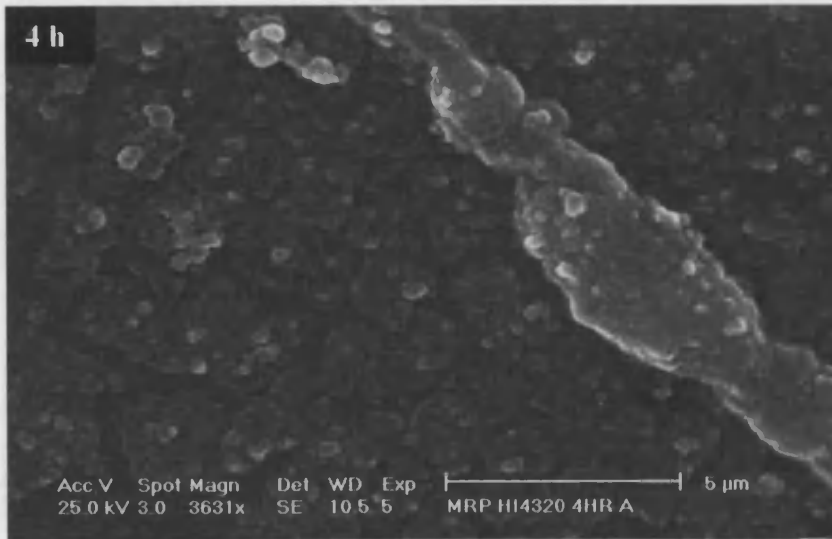
Fig 40. **Mutant HI4320 *P. mirabilis* colonization of eye-lets of all-silicone catheters in alkaline urine**

The micrographs show the accumulation of *Proteus* cells (arrows) and microcrystals over an 18 h period.
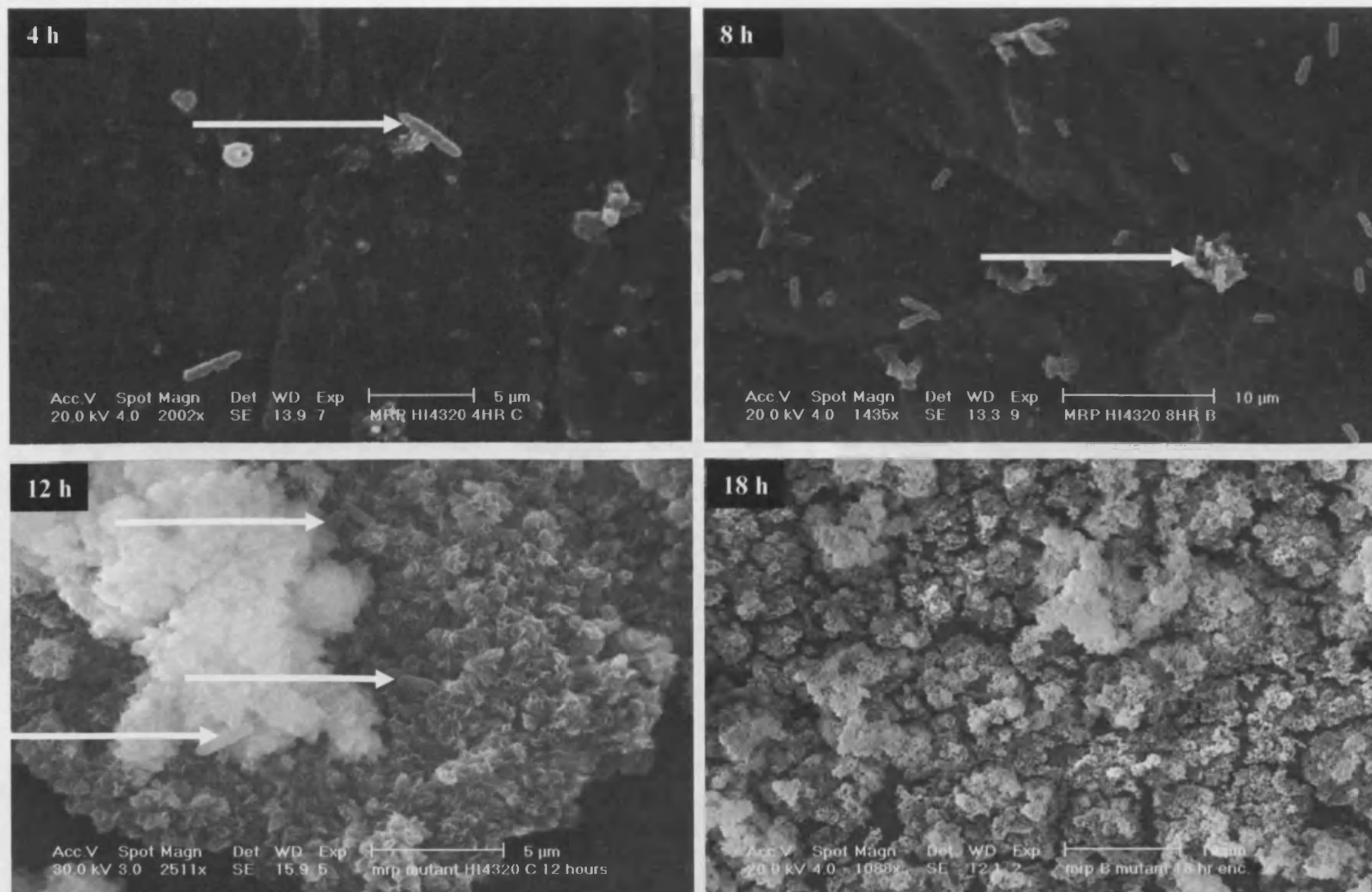
Fig 41.   **Mutant HI4320 *P. mirabilis* colonization of the lumenal surfaces of all-silicone catheters in alkaline urine**

The micrographs show the accumulation of *Proteus* cells (arrows) and microcrystals over an 18 h period.
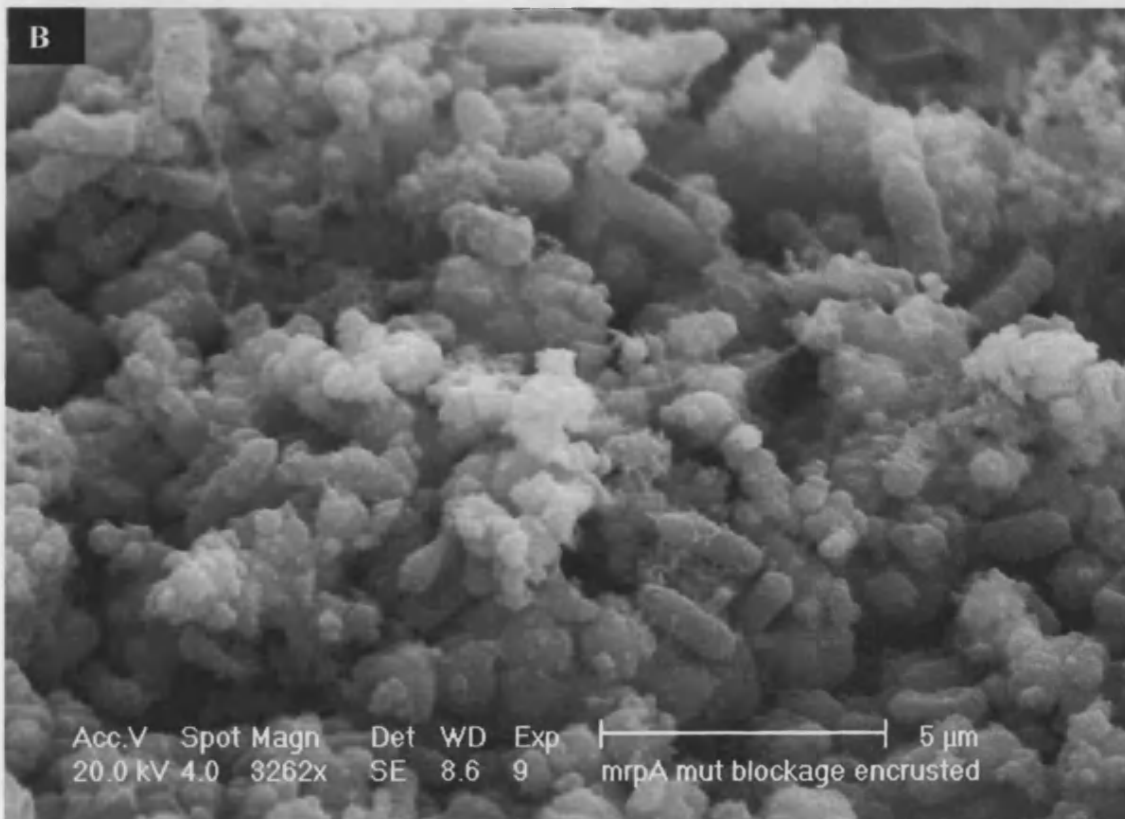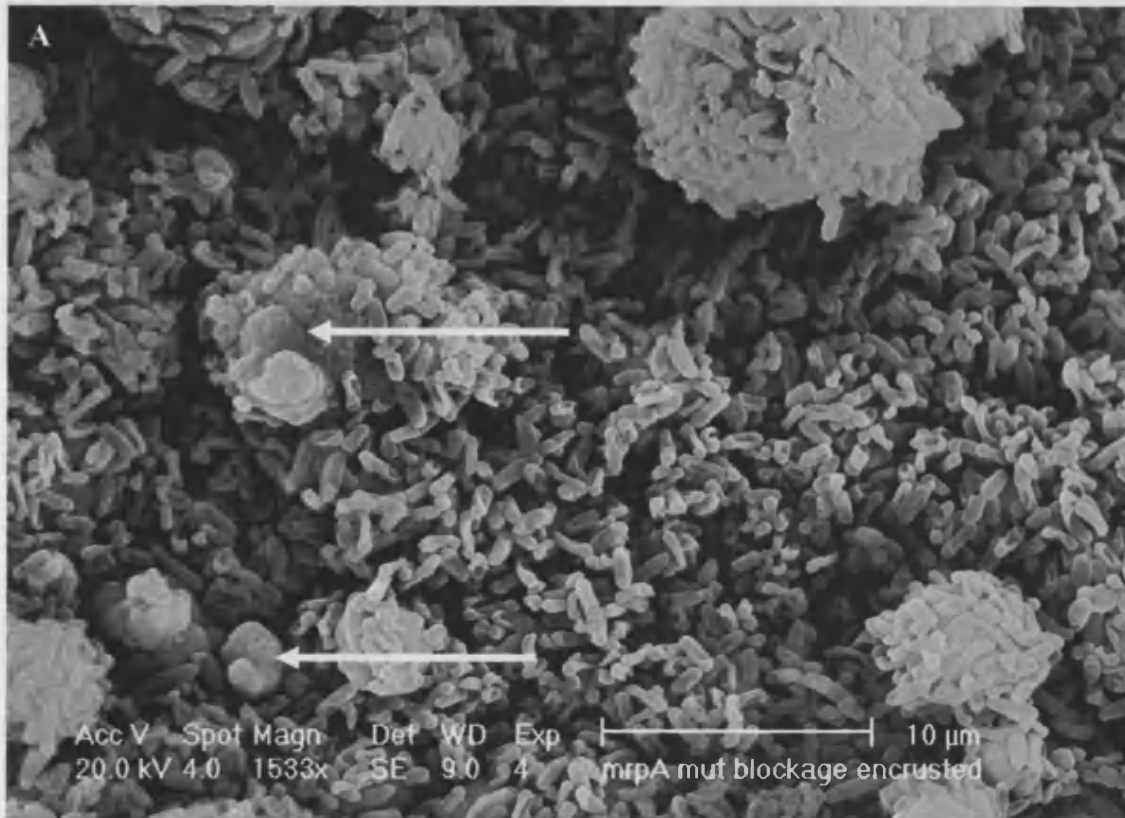
Figure 42. **Mutant HI4320 colonization of the eye-let of all-silicone catheters in alkaline urine at 30 h**

Bladder models inoculated with *mrpA* HI4320 were run until catheters blocked (30 h). The micrographs show: (A) a patch of biofilm several layers thick heavily populated with bacteria. Arrows indicate encrusted deposits within the biofilm; (B) shows a high magnification image of *P. mirabilis* cells and microcrystals forming the crystalline biofilm at the eye-let of the same catheter.

135
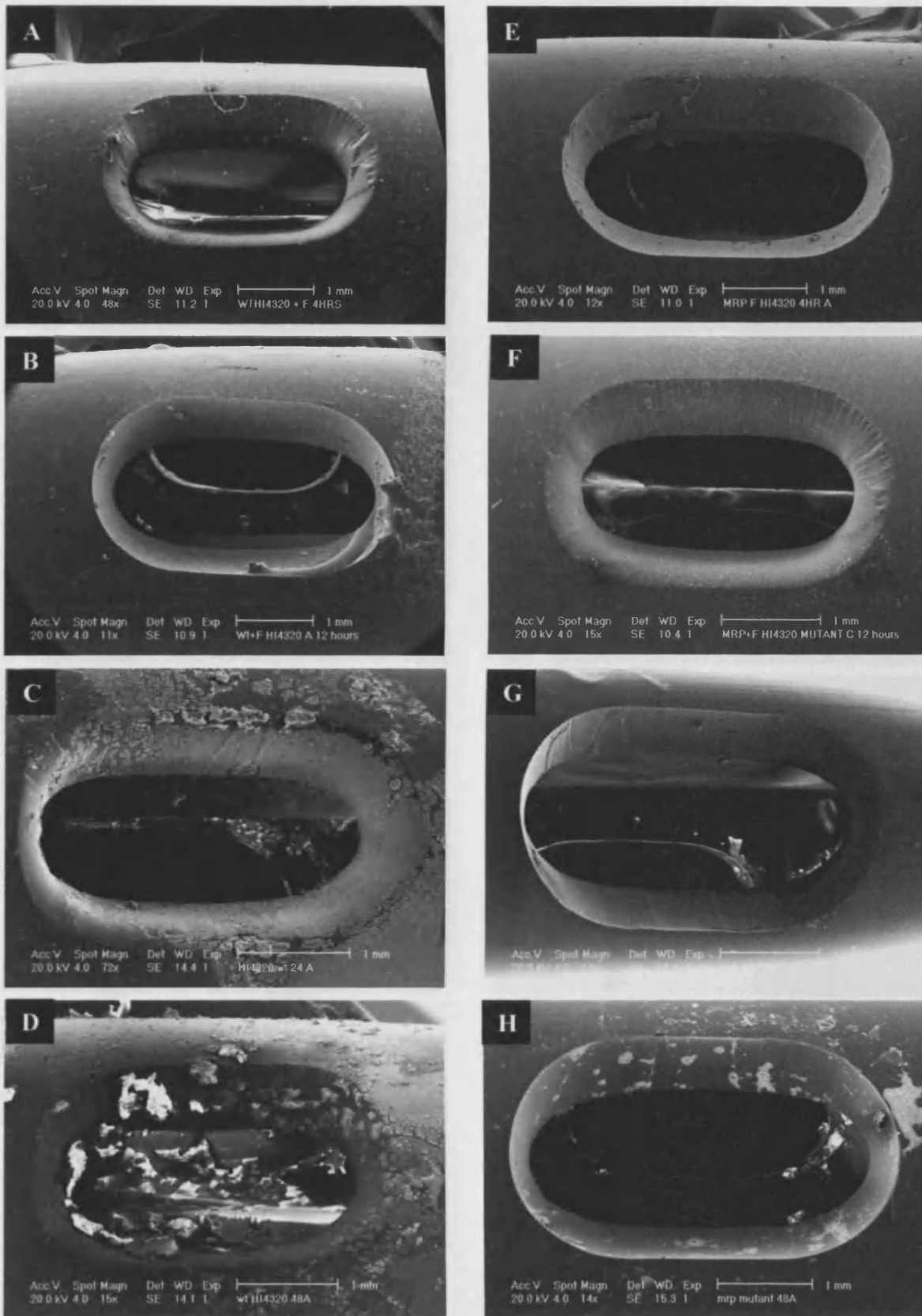
Figure 43. Colonization of the eye-let of all-silicone catheters by WT and *mrpA P. mirabilis* HI4320 in acidic urine

Models inoculated with test strains were supplied with artificial urine supplemented with fluorofamide (4 µg/ml). The micrographs illustrate: (A-D) colonization around the eye-let area of catheter sections at 4, 12, 24 and 48 h in WT HI4320 infected bladder models; (E-H) colonization at 4, 12, 24 and 48 h incubation respectively with *mrpA* HI4320 infected bladder models.

136
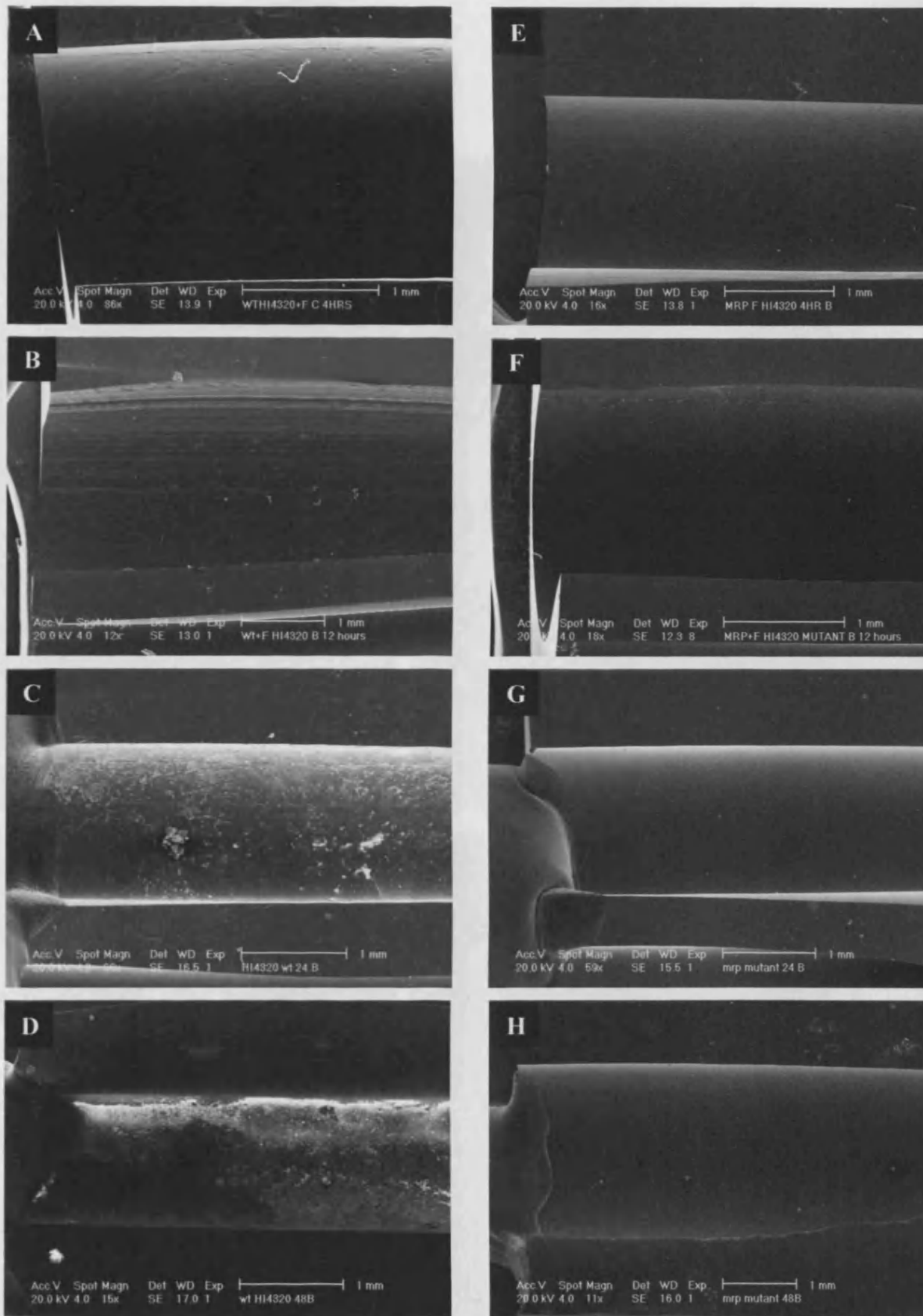
Figure 44. **Colonization of the lumenal surfaces of all-silicone catheters by WT and *mrpA P. mirabilis* HI4320 in acidic urine**

Models were inoculated with test strains and supplied with artificial urine supplemented with fluorofamide (4 µg/ml). The micrographs illustrate: (A-D) colonization of the lumenal surface of the catheters at 4, 12, 24 and 48 h in WT HI4320 infected bladder models; (E-H) colonization of the lumenal surface of the catheters at 4, 12, 24 and 48 h in *mrpA* HI4320 infected bladder models.

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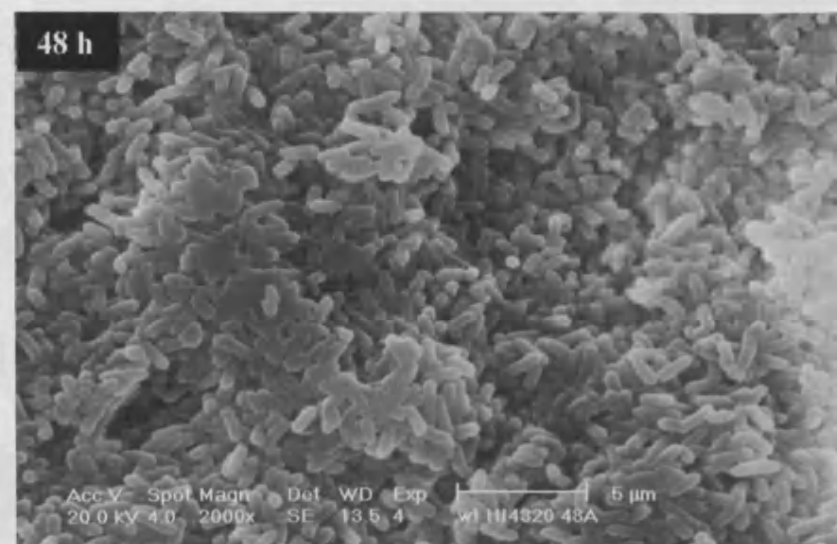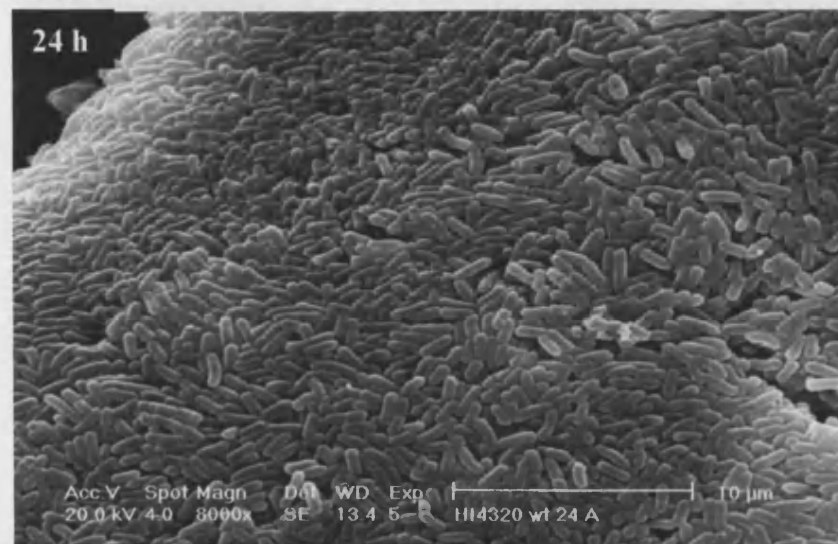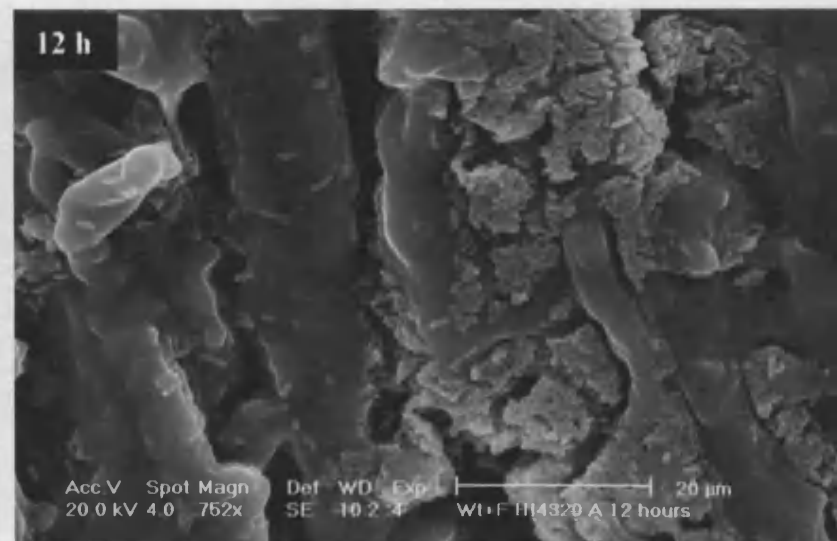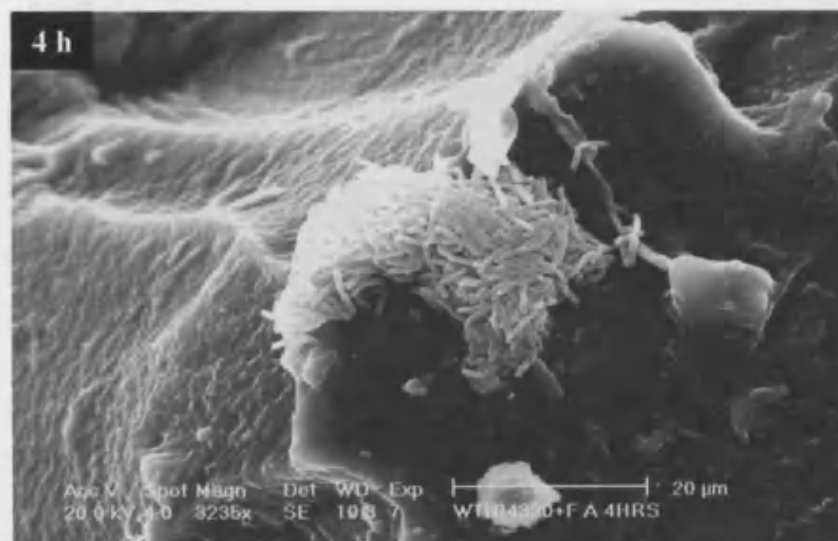
Figure 45. **Colonization of the eye-let of all-silicone catheter surfaces in WT HI4320 *P. mirabilis* infected bladder models in acidic urine**

Models were supplied with urine supplemented with fluorofamide (4 µg/ml).
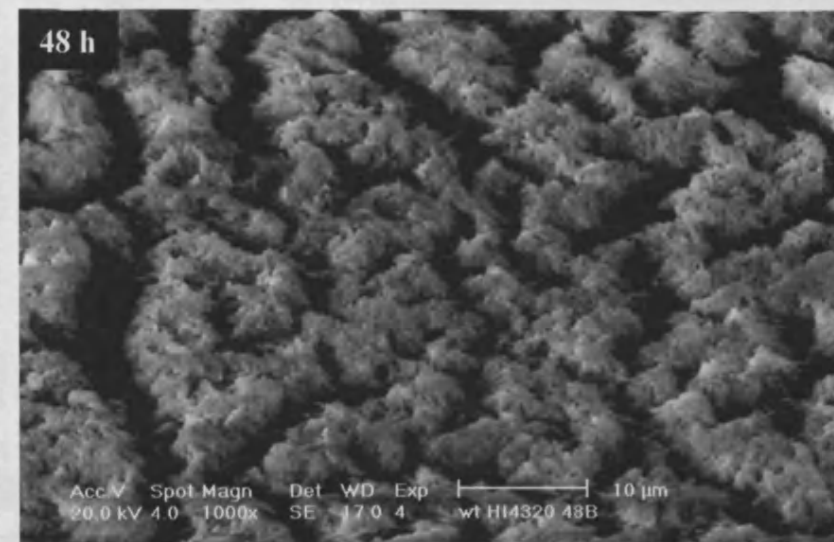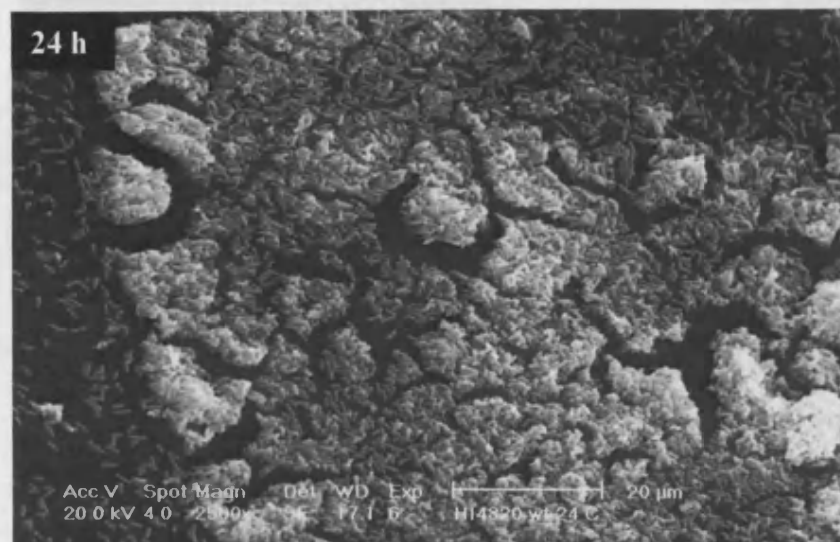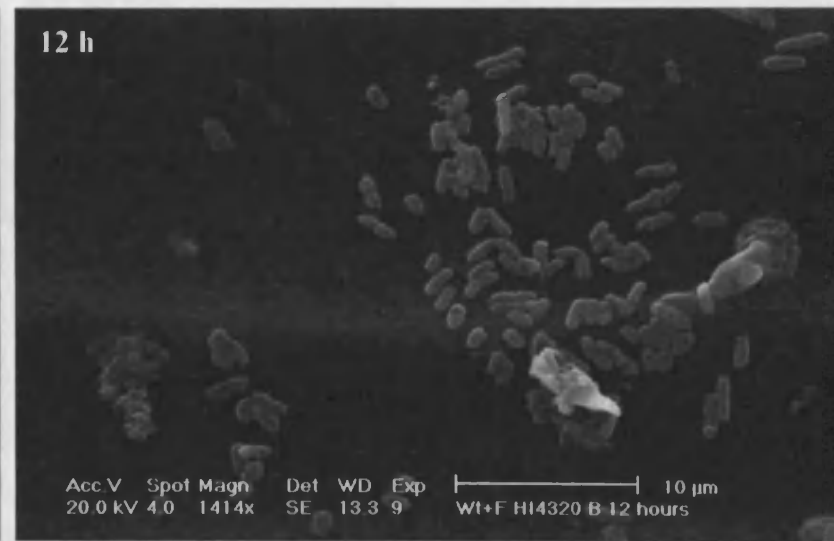
Figure 46. Colonization of the lumenal surface of all-silicone catheters in WT HI4320 *P. mirabilis* infected bladder models in acidic urine

Models were supplied with urine supplemented with fluorofamide (4 μg/ml).
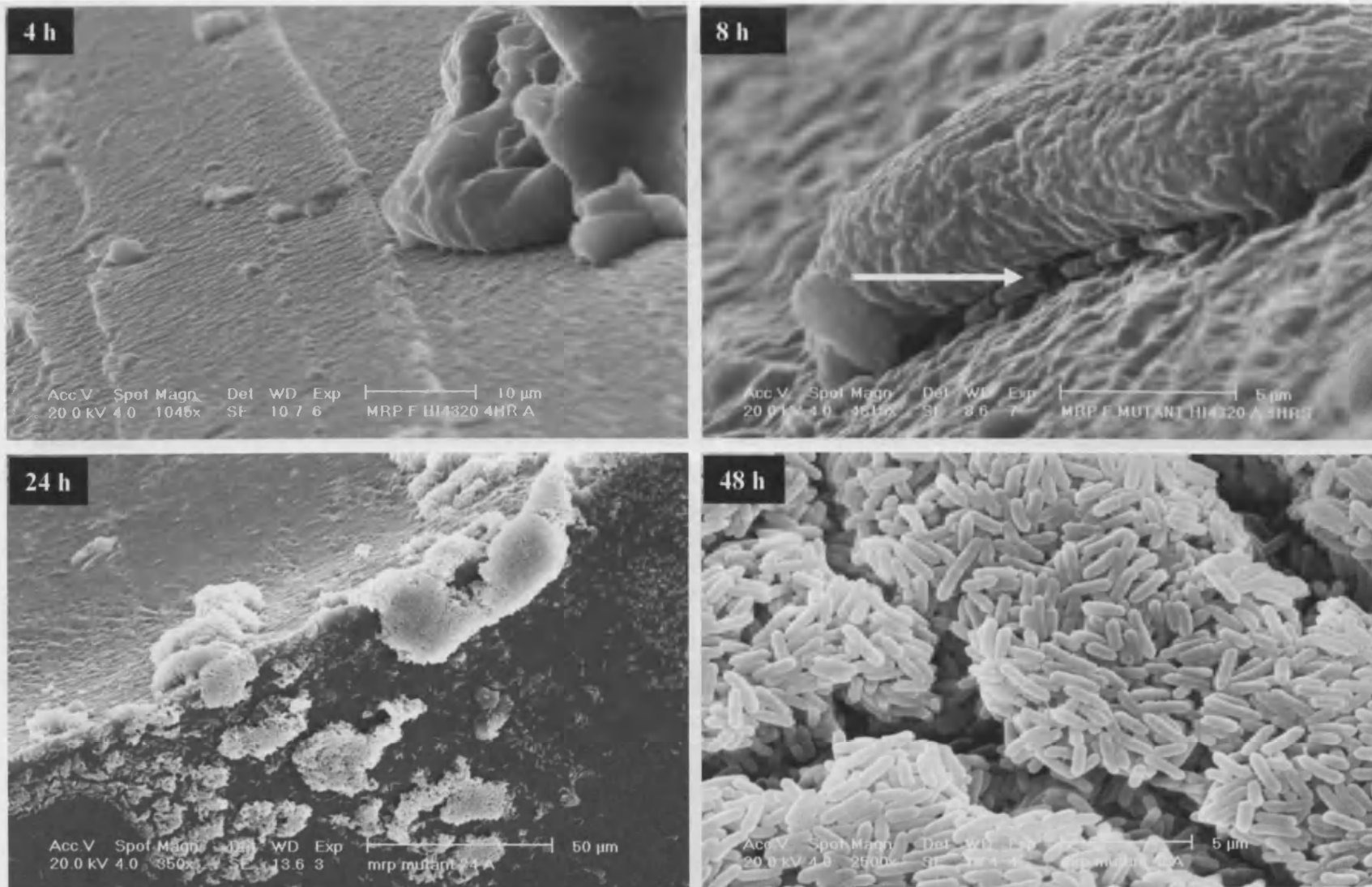
Figure 47. Colonization of the eye-hole of all-silicone catheter surfaces in *mrpA* HI4320 *P. mirabilis* infected bladder models in acidic urine

Models were supplied with urine supplemented with fluorofamide (4 µg/ml). The HVSEM images show typical areas around the eye-let of the catheter. The image taken at 8 h shows a rough area of the eye-let surface where *P. mirabilis* cells (arrow) have been trapped.
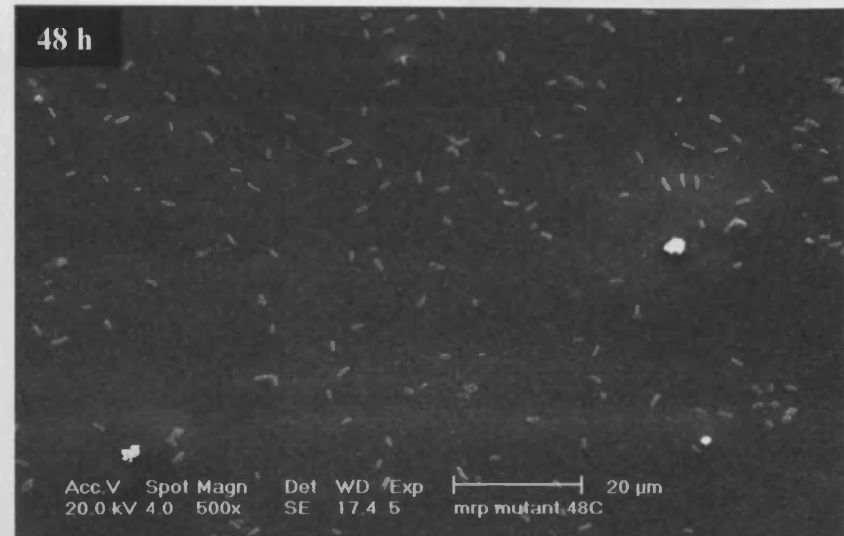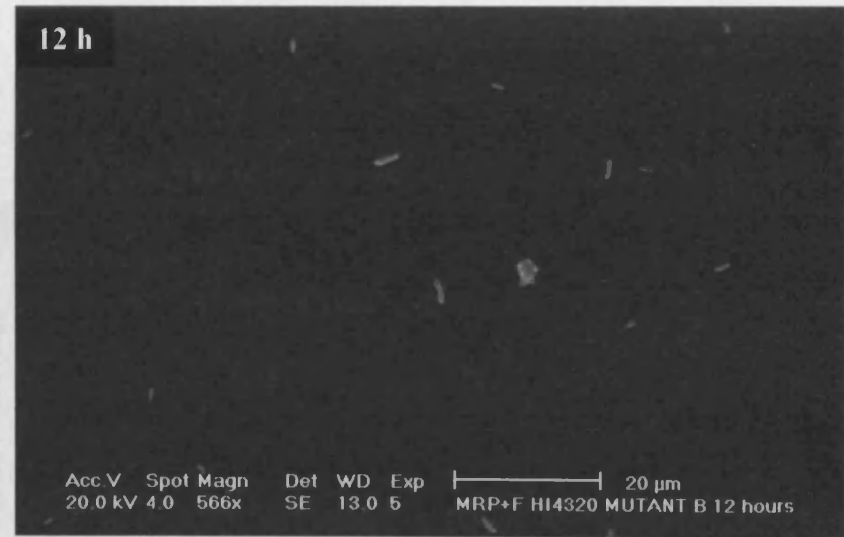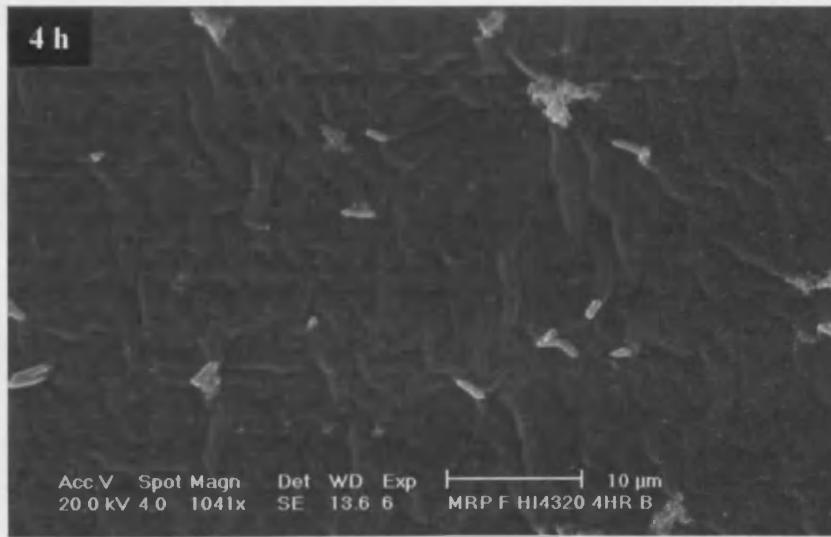
Figure 48. Colonization of the lumenal surfaces of all-silicone catheters in *mrpA* HI4320 *P. mirabilis* infected bladder models in acidic urine

Models were supplied with urine supplemented with fluorofamide (4 μg/ml). The image at 24 h shows one of the rare patches of biofilm just below the retention balloon.

A

B
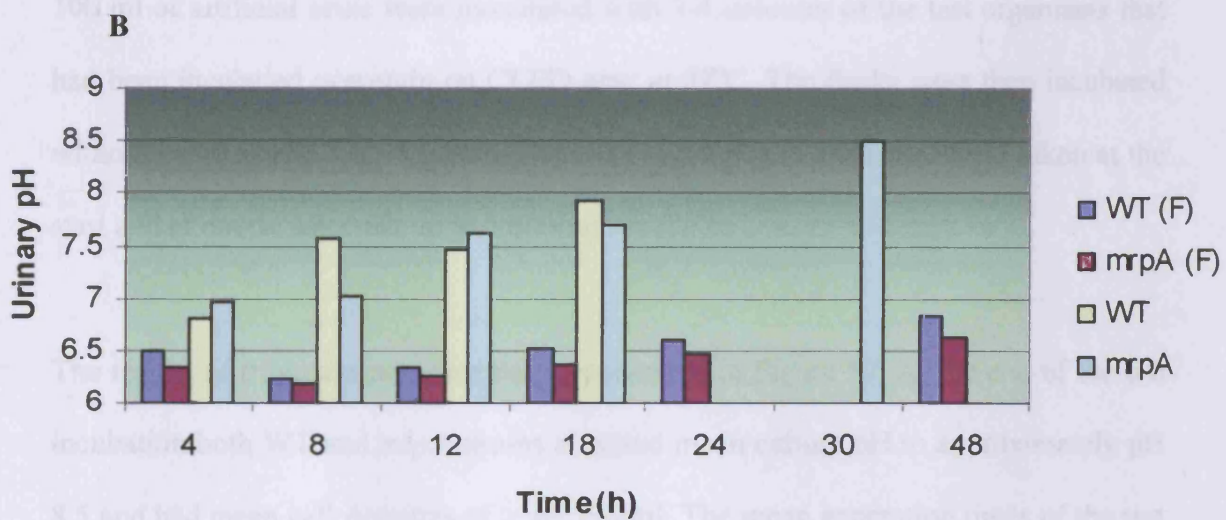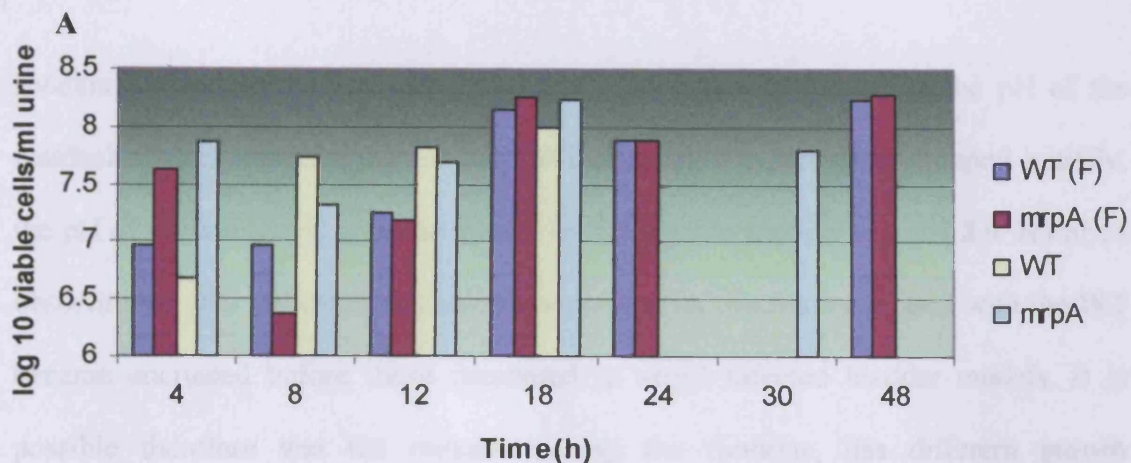
Figure 49. **The viable cell counts (A) and pH (B) of residual urine in models infected with the wild type and *mrpA* mutant of *P. mirabilis* HI4320**

The presence of fluorofamide in the urine supplied to the models is indicated by (F) after strain designation. Experiments were run for 4, 8, 12, 18, 24 and 48 h with fluorofamide supplemented urine and 4, 8, 12, 18 and 30 h with control urine. All models inoculated with 10 ml of the test organisms ($\geq 10^8$ cfu/ml). The data shown is from one experiment in each case.

3.2.6.   Growth of WT and *mrpA* HI4320 in urine batch culture at 37°C

Evidence presented in Sections 3.2.4. and 3.2.5. showed that while the pH of the residual bladder model urine for both WT and *mrpA* experiments dropped initially, the pH of urine infected with the mutant took longer to recover to > pH 8.0. HVSEM observations also indicated that catheters resident in models inoculated with the WT became encrusted before those incubated in *mrpA* infected bladder models. It is possible therefore that the mutant lacking the fimbriae, has different growth characteristics and or urease activity than its parent wild type. To examine these possibilities the test strains were grown in urine in batch culture. Flasks containing 100 ml of artificial urine were inoculated with 3-4 colonies of the test organisms that had been incubated overnight on CLED agar at 37°C. The flasks were then incubated without aeration at 37°C. Viable cell counts and the pH of cultures were taken at the start and at hourly intervals up to a maximum of 6 h.

The results of triplicate experiments are presented in Figure 50. At the end of the 6 h incubation both WT and *mrpA* strains elevated mean culture pH to approximately pH 8.5 and had mean cell densities of > 10^8 cfu/ml. The mean generation times of the test organisms incubated under the these conditions were not significantly different ($P = 0.43$) and were calculated as 1.68 ± 0.08 and 1.55 ± 0.13 h^{-1} for WT HI4320 and *mrpA* HI4320 strains respectively.
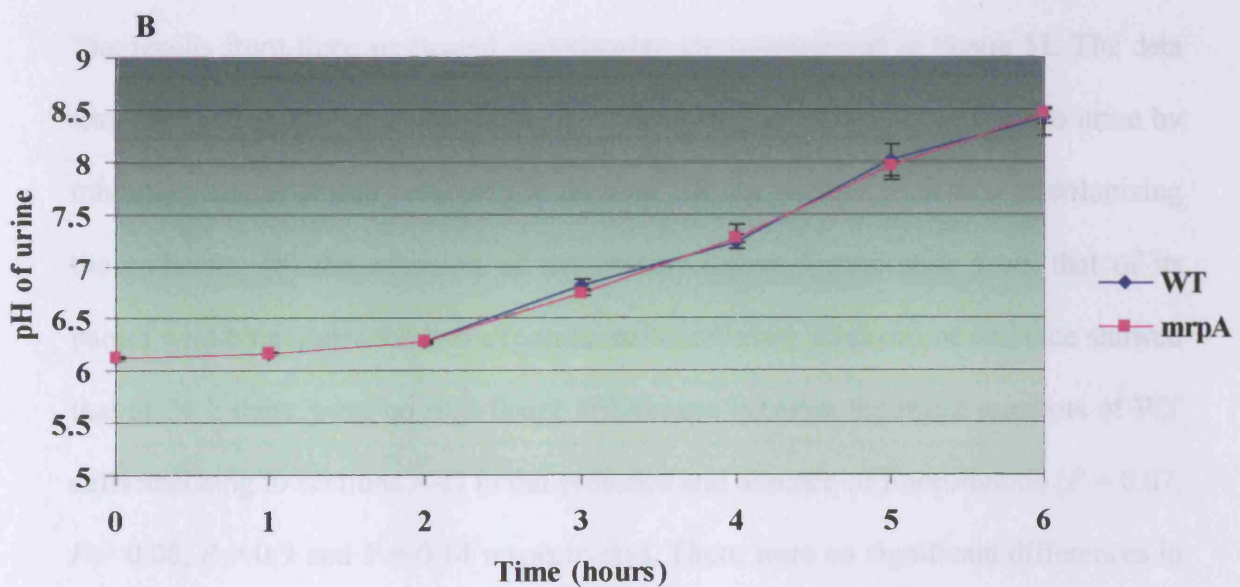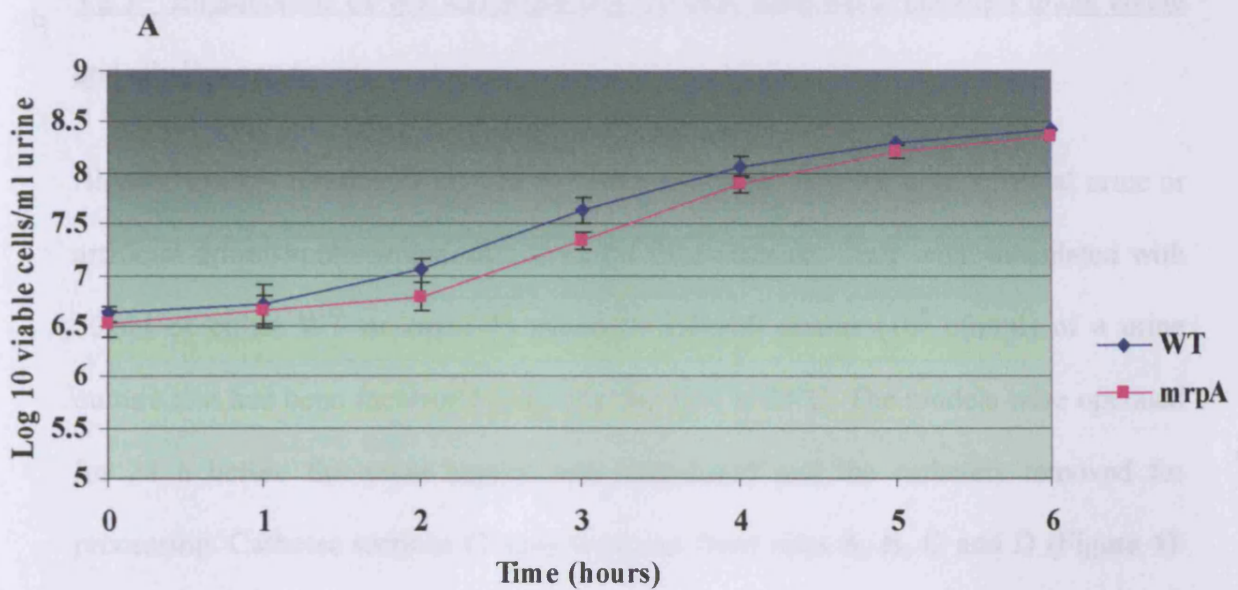
Figure 50. **Growth curves and urease activity of WT and *mrpA* HI4320 in urine batch culture at 37°C**

The mean values ± SE of the viable cell counts (A) and pH (B) were calculated from the results of three replicated experiments.

3.2.7.   Enumeration of WT and *mrpA* HI4320 cells adhering to catheters under acidic and alkaline conditions

Bladder models fitted with all-silicone catheters were supplied with artificial urine or artificial urine supplemented with 4 µg/ml fluorofamide. They were inoculated with 10 ml of either WT or *mrpA P. mirabilis* HI4320 strains (10^8 cfu/ml) of a urine culture that had been incubated statically for 12 h at 25°C. The models were operated for 24 h before the urine supply was terminated and the catheters removed for processing. Catheter sections (1 cm) were cut from sites A, B, C and D (Figure 4). The numbers of viable bacterial cells adhering to these sections were then determined.

The results from three replicated experiments are summarized in Figure 51. The data was analysed to determine whether (a) maintaining acidic conditions in the urine by inhibiting urease activity affects the numbers of the mutant or wild type colonizing the catheters; (b) the adhesion of the mutant differs significantly from that of its parent wild type under the two experimental conditions. Analysis of variance showed that at 24 h there were no significant differences between the mean numbers of WT cells attaching to sections A-D in the presence and absence of fluorofamide ($P = 0.07$, $P = 0.08$, $P = 0.9$ and $P = 0.14$ respectively). There were no significant differences in the mean numbers of *mrpA* HI4320 viable cells attaching to catheter sections A, C and D under the two experimental conditions ($P = 0.08$, $P = 0.4$ and $P = 0.4$ respectively). In the case of section B however, significantly more mutant cells attached in alkaline than acidic urine ($P = 0.007$). No significant differences were recorded in the mean numbers of WT v *mrpA* HI4320 adhering to sections A-D in alkaline ($P = 0.4$, $P = 0.9$, $P = 0.5$ and $P = 0.2$ respectively), or acidic conditions ($P = 0.2$, $P = 0.6$, $P = 0.9$ and $P = 0.2$ respectively). When experiments were stopped mean cell densities in the

residual urines were all $10^7 - 10^8$ cfu/ml and were not significantly different for: WT

(alkaline urine) v WT (acidic urine, $P = 0.3$); *mrpA* (alkaline urine) v *mrpA* + (acidic

urine, $P = 0.07$); WT (alkaline urine) v *mrpA* (alkaline urine, $P = 0.09$); WT (acidic

urine) v *mrpA* (acidic urine, $P = 0.9$).

3.2.8. Expression of *P. mirabilis* MR/P fimbriae by cells growing in the bladder models

Although WT *P. mirabilis* HI4320 expresses MR/P fimbriae when grown in LB broth

under optimum conditions required for the fimbrial expression (Section 2.17), it was

unclear if MR/P were expressed by WT HI4320 under bladder model conditions. To

determine this, bladder models fitted with all-silicone catheters were inoculated with

either 10 ml of cultures of WT or mutant HI4320 (approximately 10^8 cfu/ml) that had

been incubated for 12 h without aeration at 25°C. Urine supplemented with 4 µg/ml

fluorofamide was supplied to models at 0.5 ml/min. At 24 and 48 h the residual urine

was removed from the models. The cells were harvested by centrifugation and re-

suspended in phosphate buffered saline (PBS, pH 7.2), or PBS containing 50 mM

mannose to approximately 10^9 cfu/ml and the presence of MR/P fimbriae determined

by haemagglutination testing.

Figure 52 shows the haemagglutination profiles of WT HI4320 and *mrpA* HI4320 at

24 and 48 h in infected bladder models. The results indicate that MR/P fimbriae were

not expressed in WT or *mrpA* cells at 24 h. At 48 h however, WT HI4320 cells

agglutinated CRBC in the presence and absence of mannose indicating that MR/P

fimbriae were being expressed.

3.2.9.   Auto-aggregating ability of WT and *mrpA P. mirabilis* HI4320 strains

Evidence presented in Section 3.2.6. suggested that the growth characteristics and urease production were similar between the WT and *mrpA* test organisms. This indicated that the slow recovery of pH levels in *mrpA* infected bladder models, and also the delayed encrustation of catheters in bladder models infected with the mutant (reported in 3.2.4 and 3.2.5.), may have been caused by other factors. As MR/P fimbriae have been associated with cell aggregation (Section 3.2.), it was hypothesized that cell aggregates forming in the bladder models may be responsible for the quicker recovery of urine pH in bladder model urine and earlier encrustation of catheters in the WT bladder models. To determine whether WT *P. mirabilis* expressing MR/P fimbriae was better at forming cell aggregates than the mutant HI4320 strain, auto-aggregation assays were carried out on the two test organisms. Strains were incubated in Luria Bertani broth at $37^{\circ}C$ for 6 days and passaged at 48 h intervals for optimum conditions for MR/P expression. Subsequently, isolates were assessed visually (Section 2.18) for formation of cell aggregates in test tubes under assay conditions over a 24 h period. Known co-aggregating strains of *Actinomyces naeslundii* ATCC 12104 and *Streptococcus sanguis* GW2 were used as a positive control to indicate the aggregation phenomenon.

The results of duplicate auto-aggregation assays are presented in Table 5. It was clear that the WT *P. mirabilis* HI4320 was able to form visible cell aggregates considerably better than the mutant HI4320 strain under the test conditions. Cell aggregations were clearly visible in the test-tubes containing WT HI4320 within 5 minutes of the experiment procedure. Although some evidence of auto-aggregation occurred in the test-tubes containing the *mrpA* HI4320 strain at the identical time period, it was at a

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much reduced level. Any auto-aggregation that occurred in assays did so within the initial 5 minute period after the experimental procedure took place and did not increase as time elapsed. Figure 53 shows a selection of photographs of test-tubes used in the auto-aggregation experiments carried out in aggregation buffer.
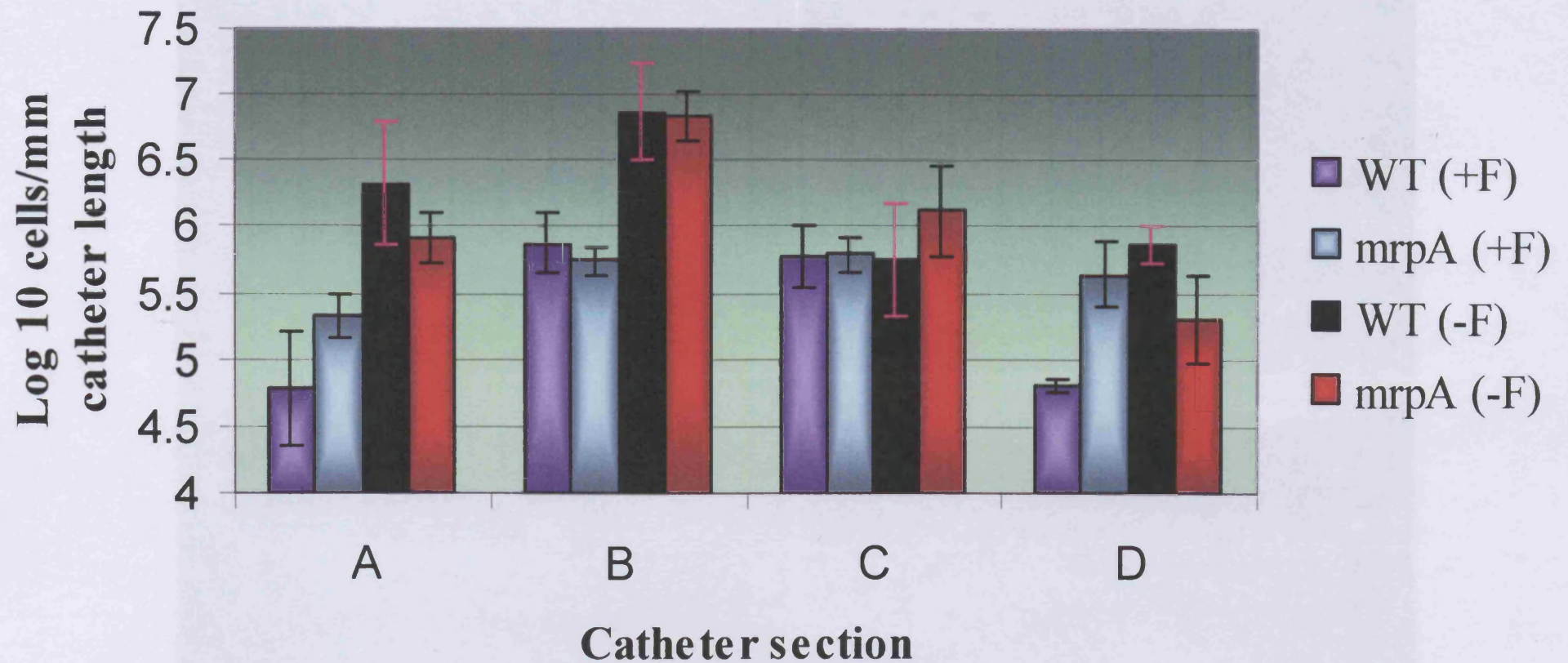
Figure 51. **Viable cell counts from all-silicone catheter sections after 24 h in infected bladder models**

Bladder models were inoculated with 10^8 cfu/ml of wt or *mrpA* HI4320 strains and supplied with urine only or urine containing 4 µg/ml fluorofamide (indicated as –F and +F respectively). The mean values ± SE were calculated from the results of three replicated experiments.
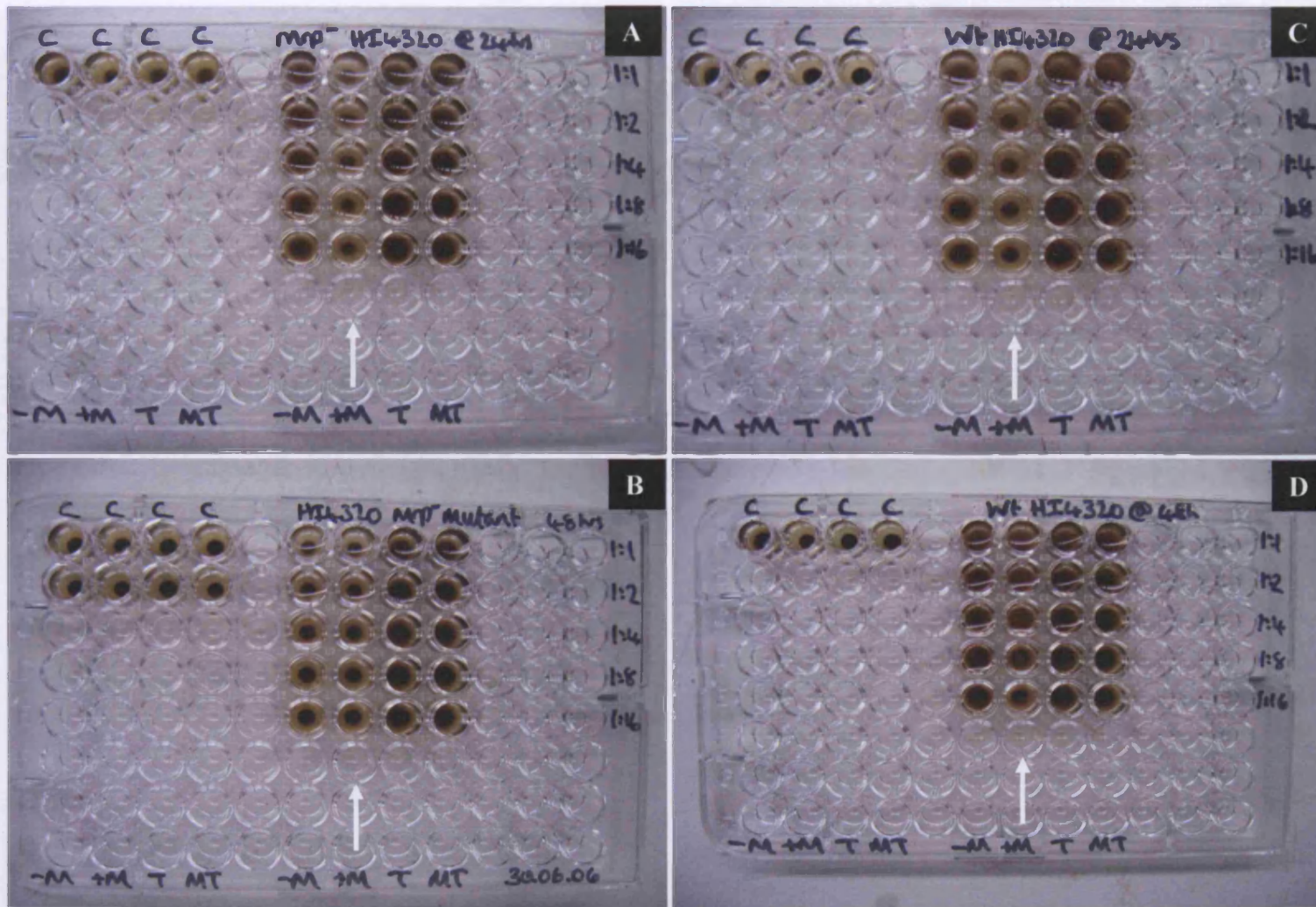
Figure 52. **Haemagglutination patterns of wt and *mrpA P. mirabilis* HI4320 from residual bladder model urine**

Bladder models inoculated with wt or *mrpA* HI4320 were supplied with urine containing 4 µg/ml fluorofamide. Cells were harvested at 24 and 48 h and tested for MR/P expression. The microtitre plates show: (A + B) tight button (no agglutination) of CRBC incubated in the presence of 50 mM mannose (arrows) with *mrpA* HI4320 from 24 and 48 h bladder models respectively; (C) shows no CRBC haemagglutination in wt HI4320 cells from the 24 h bladder model (arrow) incubated with 50 mM mannose; (D) shows diffuse matt (agglutination) of CRBC in all wells incubated with wt HI4320 from 48 h residual bladder model urine. The agglutination of CRBC in the absence of mannose or of CRBC sensitized with tannic acid indicate the presence of other agglutinins.

Auto-aggregation of WT and *mrpA P. mirabilis* HI4320

Bacterial Strain	5 minutes	24 h
A. naeslundii + *S. sanguis* (control)	+ 4	+ 4
	+ 4	+ 4
P. mirabilis WT HI4320 (1)	+2/+3	+2/+3
P. mirabilis WT HI4320 (2)	+ 3	+ 3
P. mirabilis mrpA HI4320 (1)	+ 1	+ 1
P. mirabilis mrpA HI4320 (2)	+ 1	+ 1

+1 small uniform aggregates in turbid suspension, +2 easily visible aggregates in turbid suspension, +3 clearly visible aggregates that settle leaving an almost clear supernatant,+4 large aggregates that settle almost instantaneously to leave clear supernatant

Table 5. **Aggregation profiles of WT and *mrpA P. mirabilis* HI4320 in co-aggregation buffer**

P. mirabilis strains were grown for 6 days in LB broth under conditions for optimum MR/P fimbriae expression. The results of the two aggregation assays for each strain along with co-aggregating controls are indicated. Visual assessment of auto-aggregation occurring in the test-tubes from 1-6 h (not shown) was not different from observations taken at 5 minutes or 24 h.
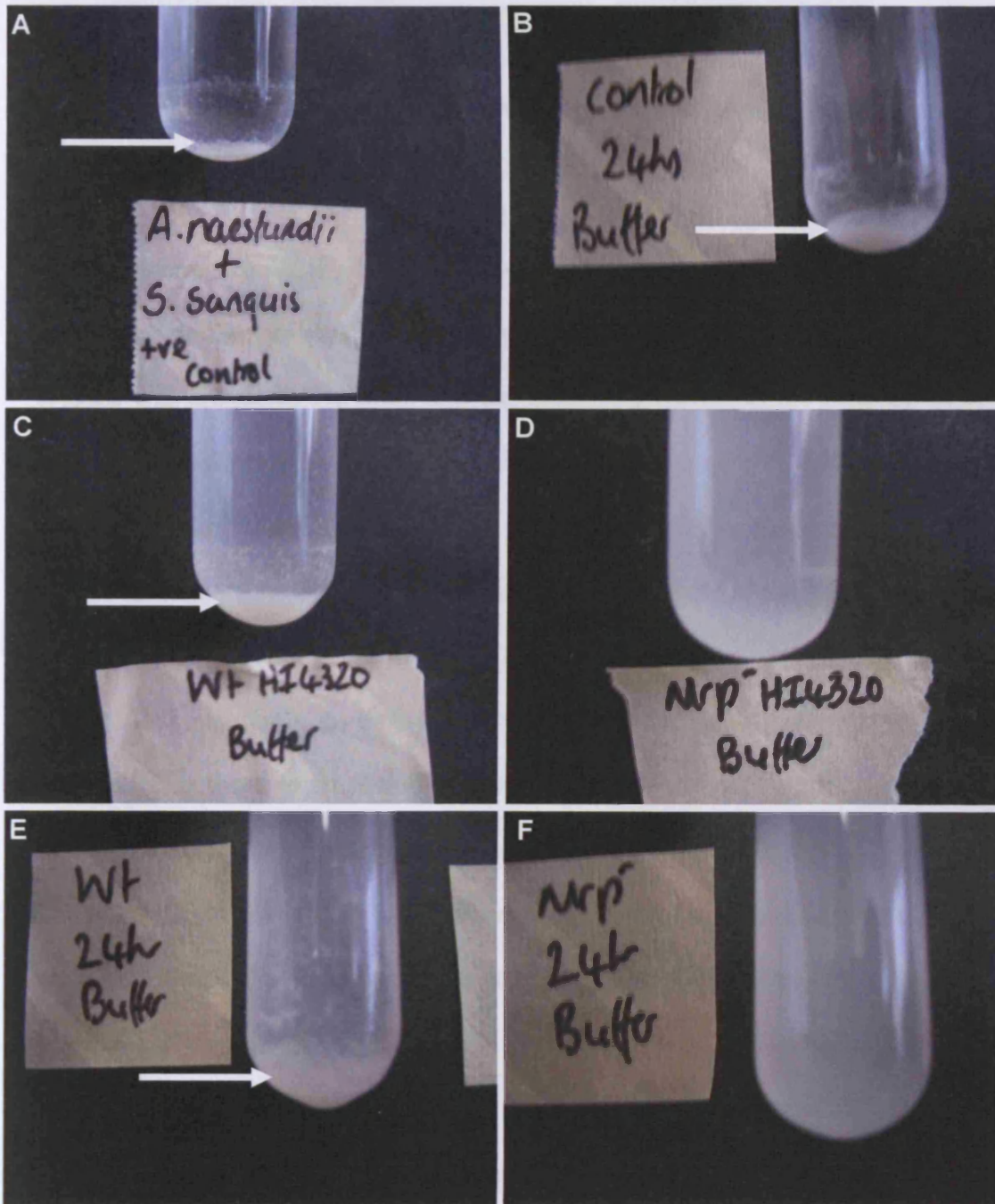
Figure 53. **Auto-aggregation of WT and *mrpA P. mirabilis* HI4320**

The digital images of test-tubes show: (A + B) the auto-aggregation phenomenon occurring in control strains (arrows indicate cell aggregates); (C) WT HI4320 after 5 minutes (arrow indicate prominent cell aggregates); (D) *mrpA* HI4320 after 5 minutes; (E) WT HI4320 at 24 h; (F) *mrpA* HI4320 at 24 h.

3.3. Modulation of crystalline *Proteus mirabilis* biofilm development on urinary catheters

The relationship between pH_v and pH_n is a major factor in determining the solubility of calcium and magnesium phosphate in urine. When the pH_v rises above the pH_n, crystals will appear in the urine and the greater pH_v exceeds the pH_n, the more of these salts will crystallize out of solution (Choong *et al.*, 2001). The recent prospective clinical study by Mathur *et al.* (2006a) showed that the variation in the rate of catheter encrustation in individuals infected with *Proteus* is also a function of the difference between pH_v and pH_n. They reported that patients whose urines had pH_v values consistently lower than their pH_n's, did not block their catheters. On the other hand, catheter encrustation and blockage was rapid in those patients having urine with no such safety margin between the mean pH_v and pH_n values. Mathur *et al.* (2006a) also found that the pH_n of individuals urine varied widely from week to week, suggesting that it should be possible to devise a strategy to increase the safety margin between pH_v and pH_n by elevating pH_n. In a study of urine from healthy volunteers, Suller *et al.* (2005) demonstrated that it was possible to manipulate pH_n. Simply diluting urine by increasing the fluid intake and by raising its citrate content, the pH_n of urine could be elevated to values that are rarely achieved even in *P. mirabilis* infected urine. The aim of experiments reported in this section was to establish whether the manipulation of the pH_n of urine in the ways suggested by Suller *et al.* (2005) modulates the rate at which crystalline biofilm form on urinary catheters in laboratory models infected with *P. mirabilis*.

3.3.1.   The effect of diluting urine on its pH_n and the rate of catheter encrustation

The composition of the artificial urine used in these studies presented in Sections 3.1 and 3.2. was devised to represent that of the concentrated urine produced by elderly long-term catheterised patients who commonly drink very little. To simulate the effect of increasing the fluid intake, a series of dilutions of the basic urine (from 1:1 to 1:6) were made with de-ionized water. The pH_n values of these diluted urines were determined and the results are presented in Table 6. It can be seen that the dilution of the urine has a profound effect on pH_n, the values increasing from 6.7 (neat urine) to 8.4 (1:6 dilution).

Experiments were then performed in bladder models fitted with all-silicone catheters and inoculated with *P. mirabilis* NSM6 to investigate the effect of increasing fluid intake on the times catheters took to block. Sets of four models were supplied with neat, 1:1, 1:2 and 1:3 dilutions of the artificial urine at corresponding flow rates of 720, 1440, 2160 and 2880 ml/24 h. Samples of urine from the voided urine were taken at 24 h intervals for pH measurement and viable cell counting. The models were run until catheters blocked with crystalline biofilm. The results from four replicated experiments are summarized in Figures 54 and Tables 7 and 8.

It is clear that diluting urine and increasing its flow rate to the bladder models extends the times catheters took to block. Statistical analysis confirmed that the mean times to blockage in the four sets of models were all significantly different ($P < 0.05$). Figure 54 also shows the mean time to blockage (calculated for four replicates) of catheters in models supplied with urine at 1:1 dilution but at the original flow rate of 720 ml/24 h. The mean value of 50.5 ± 4.6 h was not significantly different ($P > 0.9$) from the

value of 49.5 ± 6.0 h recorded in models supplied with 1:1 dilution of urine at 1440

ml/24 h. This result suggests that the dilution of the urine rather than the flow rate is

the major factor in controlling the rate of catheter encrustation. Data presented in

Table 7 show that pH levels remained > pH 8 in all models throughout experiments.

The mean viable cell counts from voided urine (Table 8) remained > 10^7 cfu/ml in all

models throughout experiments.

Dilutions of artificial urine	Nucleation pH (pH$_n$)
Neat	6.7
1:1	6.9
1:2	7.3
1:3	7.5
1:4	7.9
1:5	8.1
1:6	8.4

Table 6.   **The effect of dilution of urine on pH$_n$**

The pH$_n$ was determined using least squares regression analysis.
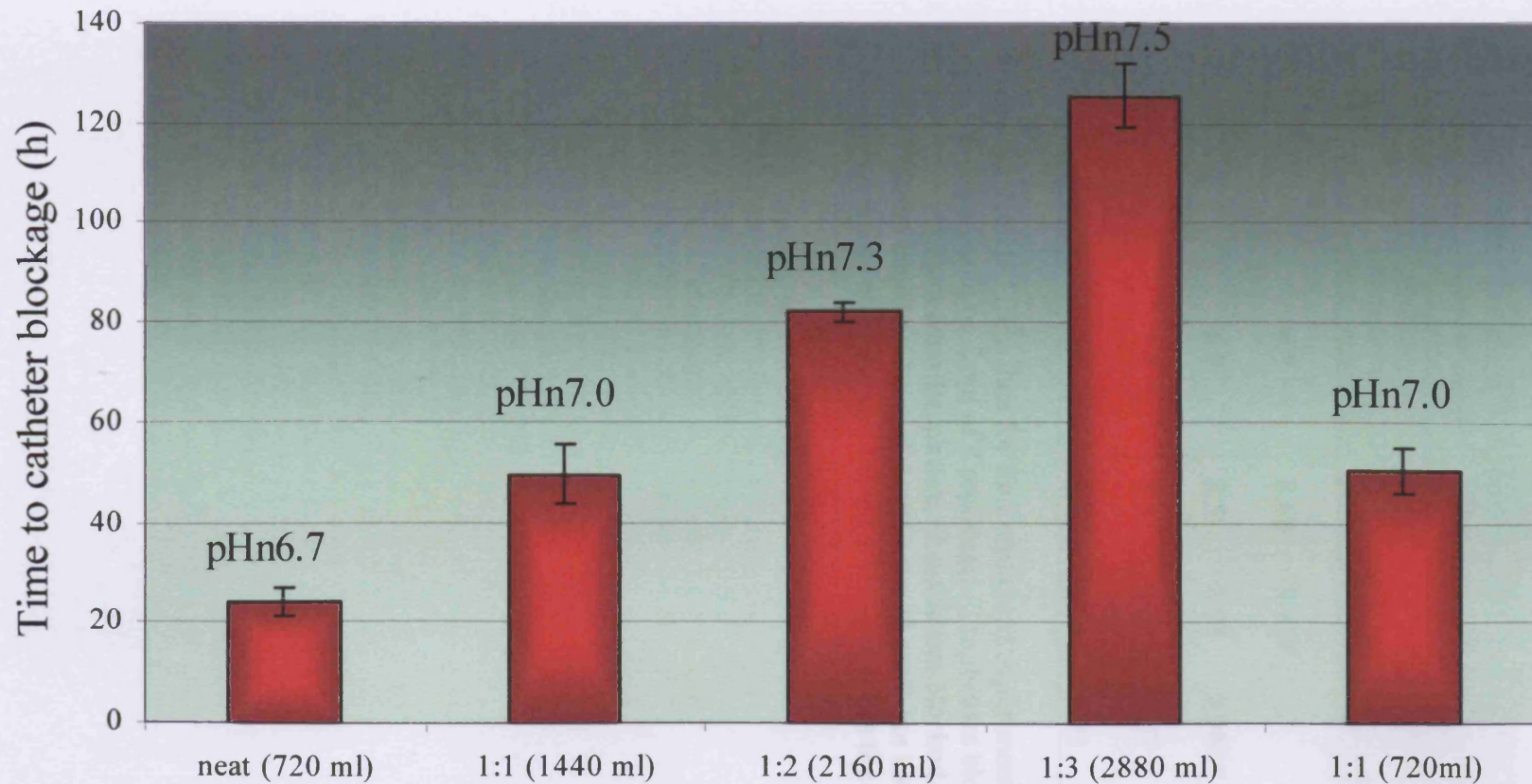
Figure 54. The effect of diluting urine and increasing its flow-rate to bladder models colonized by *P. mirabilis* NSM6 on the rate of catheter blockage

The values indicated are means and standard error of the means calculated from four replicated experiments. The pH_n values of urines supplied to the models are also indicated.

Table 7. The nucleation pHs of urine supplied to the models and the [†]mean pH (pHv) of urine voided from the models infected with *P. mirabilis* NSM6

Urine output (ml/24h)	Nucleation pH (pH$_n$) of urine	pH of urine voided from model at various times during life-span of catheters					
		1h	24h	48h	72h	96h	120h
720	6.7	8.62	8.42*	-	-	-	-
1440	6.9	8.51	8.75	9.08**	-	-	-
2160	7.3	8.82	8.72	8.97	9.07	-	-
2880	7.5	8.82	8.76	8.93	8.95	9.04	9.1***

† Means calculated from data for four replicated experiments unless otherwise stated
*Mean based on 2 out of 4 replicates (2 replicates blocked before 24 h)
** Data from 1 replicate, (3 replicates blocked before 48 h)
***Mean based on 3 out of 4 replicates (1 replicate blocked before 120 h)
- Indicates that no urine was voided at these times as the catheters had blocked

Table 8.   The [†]mean number of viable cells/ml of urine voided from models infected with *P. mirabilis* NSM6 and supplied with urine at different flow-rates

Urine output (ml/24h)	Viable cells (cfu x 10^7/ml) in urine voided from model at various times during the life-span of catheters					
	1h	24h	48h	72h	96h	120h
720	22.0	5.3 *	-	-	-	-
1440	15.0	5.3	8.0**	-	-	-
2160	14.0	2.7	4.0	2.3	-	-
2880	14.0	2.7	6.2	11.0	7.1	1.3 ***

† Means calculated from data for four replicated experiments unless otherwise stated
*Mean based on 2 out of 4 replicates (2 replicates blocked before 24 h)
** Data from 1 replicate, (3 replicates blocked before 48 h)
***Mean based on 3 out of 4 replicates (1 replicate blocked before 120 h)
- Indicates that no urine was voided at these times as the catheters had blocked

3.3.2. Effect of citrate on urine pH_n and the rate of catheter encrustation

Suller et al. (2005) showed that the pH_n of urine could be raised by increasing its citrate concentration. They also demonstrated in experiments with healthy human volunteers that the citrate content and pH_n of urine could be elevated simply by consuming 500 ml of orange juice on a daily basis. The aims of the following experiments were to determine the effect increased citrate concentrations in artificial urine would have on its pH_n, and on the rate of catheter encrustation when the urine was supplied to laboratory models infected with P. mirabilis NSM6.

The artificial urine diluted to 1:1 was produced containing citrate concentrations ranging from 0, to 3.0 g/L. The pH_n of each specific urine was determined using the optical density method and least squares regression analysis. Urine containing the different citrate concentrations was then supplied to bladder models at a rate of 1440 ml daily urine output (flow-rate of 1.0 ml/min). The models were inoculated with 10 ml of a 4 h culture of P. mirabilis NSM6 (approximately 10^8 cfu/ml). The bladder models were run until the all-silicone catheters blocked or up to a maximum of 7 days when experiments were stopped manually. Four replicated experiments were performed for each citrate concentration. Viable cell counts and pHs of the voided urine were determined at the start of the experiments and at 24 h intervals thereafter.

The data presented in Table 9 show the effect of citrate content on pH_n. It can be seen that the pH_n values increased from 6.7 (no citrate) to 9.1 (3.0 g/L citrate). The mean blockage times of the all-silicone catheters in the bladder models supplied with urine containing the various citrate concentrations are shown in Figure 55. It was apparent that the citrate content in the urine had a profound effect on the time it took catheters

to block with crystalline biofilm. Catheters in models supplied with urine containing

no citrate, 0.41 and 1.0 g/L citrate had mean blockage times that ranged from 32.3 ±

3.5 h, to 107.3 ± 5.5 h. Statistical analysis (ANOVA) revealed that in these bladder

models the times to catheter blockage became significantly ($P < 0.05$) longer with

each ascending citrate concentration in the urine. What was more encouraging was

that in bladder models supplied with the urine containing 1.5, 2.0 and 3.0 g/L citrate,

the catheters drained freely for the full 7-day experimental period.

Increasing the citrate content of urine also clearly affected the extent to which

phosphates were precipitated in the residual urine of the infected models. Figure 56

illustrates the relative turbidity of the urine in models supplied with urine containing

0.41 to 3.0 g/L citrate. The data presented in Table 10 and Table 11 show that the pH

of urine remained >pH8 and viable cell populations > 10^7 cfu/ml in all cases

throughout the experimental period.

Citrate concentration in 1:1 diluted artificial urine (g/L)	Nucleation pH (pH$_n$)
0	7.0
0.41	7.4
1.0	8.2
1.5	8.3
2.0	8.5
3.0	9.1

Table 9.   **Nucleation pH values of urine containing various citrate concentrations**

Citrate concentrations were adjusted by increasing the levels of tri-sodium citrate when preparing the urine.
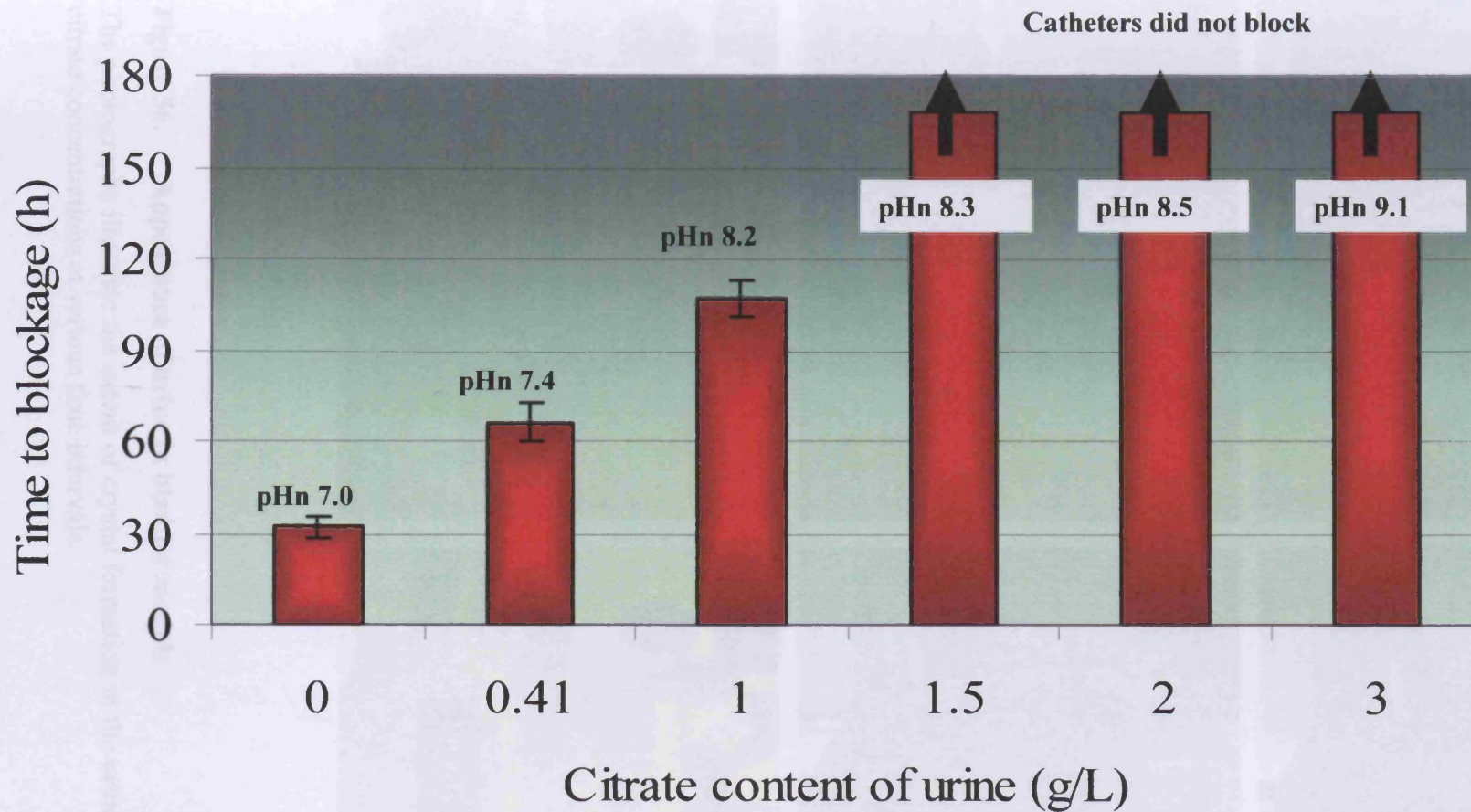
Figure 55. **Blockage times of all-silicone catheters in models inoculated with *P. mirabilis* NSM6 and supplied with urine containing various citrate concentrations**

Bladder models were inoculated with 10 ml of a 4 h culture of *P. mirabilis* NSM6 and supplied with a 1:1 dilution of the standard urine supplemented with various citrate concentrations. The values indicated are the means and standard error of the means calculated from four replicated experiments. The pH_n of urines supplied to the models are also indicated.
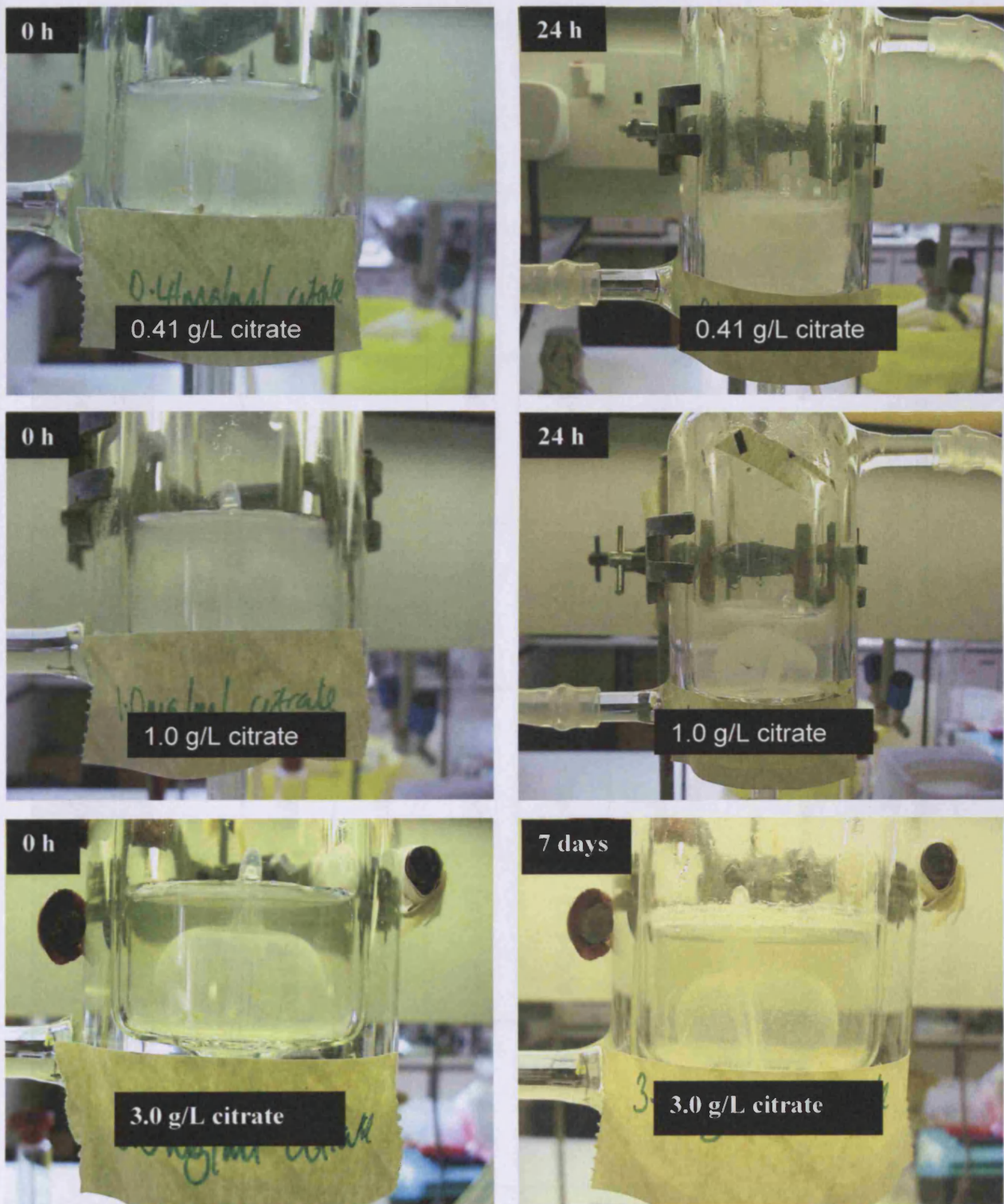
Figure 56.   Appearance of urine in bladder models

The photographs illustrate the extent of crystal formation in the urine containing a range of citrate concentrations at various time intervals.

[Citrate] in 1:1 diluted urine (g/L)	pH of residual urine from models at various times (h) during the life-span of catheters							
	0	24	48	72	96	120	144	168
0	8.49 ±	8.72 ± 0.12	-	-	-	-	-	-
0.41	8.53 ± 0.05	8.85 ± 0.05	8.87 ± 0.02 †	8.95 ± 0.07 ††	-	-	-	-
1.0	8.56 ± 0.05	8.65 ± 0.12	8.91 ± 0.04	9.02 ± 0.02	9.05 ± 0.01 †	-	-	-
1.5	8.52 ± 0.03	8.47 ± 0.04	8.8 ± 0.04	8.88 ± 0.01	8.89 ± 0.02	8.96 ± 0.02	8.95 ± 0.04	8.7 ± 0.02
2.0	8.56 ± 0.05	8.48 ± 0.07	8.74 ± 0.01	8.91 ± 0.03	8.96 ± 0.04	8.98 ± 0.04	9.0 ± 0.03	8.82 ± 0.07
3.0	8.49 ± 0.03	8.3 ± 0.09	8.72 ± 0.01	8.81 ± 0.02	8.95 ± 0.03	8.97 ± 0.02	8.98 ± 0.01	8.75 ± 0.1

Table 10.   The *mean pH of residual bladder model urine in bladder models supplied with urine containing a range of citrate concentrations

Models were fitted with all-silicone catheters and inoculated with 10 ml of 4 h *P. mirabilis* NSM6 culture. * The means ± SE of the means were calculated (unless otherwise stated) from the results of four replicated experiments. (†) mean based on three replicates (††) mean based on two replicates (-) all catheters in series had blocked by this time

[Citrate] in 1:1 diluted urine (g/L)	Mean viable cell populations (cfu x 10^7 cfu/ml) of residual bladder model urine at known time intervals (h)							
	0	24	48	72	96	120	144	168
0	14.3 ± 1.7	8.6 ± 1.2	-	-	-	-	-	-
0.41	14.3 ± 2.0	15.5 ± 5.7	14.3 ± 6.1 †	7.5 ± 3.6 ††	-	-	-	-
1.0	18.0 ± 1.1	4.7 ± 2.2	11.0 ± 5.9	14.0 ± 4.0	10.0 ± 2.5 †	-	-	-
1.5	11.3 ± 0.9	8.65 ± 2.1	16.1 ± 4.1	22.5 ± 1.9	12.1 ± 3.8	28.2 ± 8.6	22.0 ± 3.0	54.5 ± 3.7
2.0	11.0 ± 1.4	8.3 ± 2.2	11.5 ± 2.1	5.6 ± 0.59	12.1 ± 6.0	23.8 ± 4.4	16.3 ± 4.6	45.0 ± 12.0
3.0	16.3 ± 1.0	8.68 ± 1.8	12.9 ± 2.5	11.1 ± 6.2	14.2 ± 2.8	12.7 ± 2.2	22.8 ± 5.9	6.23 ± 0.65

Table 11.   **The * mean viable cell populations in residual bladder model urine in bladder models supplemented with various citrate concentrations**

Models were fitted with all-silicone catheters and inoculated with 10 ml of 4 h *P. mirabilis* NSM6 culture. The * means ± SE of the means were calculated (unless otherwise stated) from the results of four replicated experiments. (†) mean based on three replicates (††) mean based on two replicates (-) all catheters in series had blocked by this time

3.3.2.1. Electron microscopy of catheter biofilms formed in urine containing a range of citrate concentrations

A set of bladder models fitted with the all-silicone catheters were infected with *P. mirabilis* NSM6 and supplied with urine containing 0.41, 1.0 and 1.5 g/L citrate. When the catheter in the model supplied with the urine containing 0.41 g/L citrate blocked, catheters were removed from all three models. Additional catheters in bladder models supplied with urine containing 1.5, 2.0 and 3.0 g/L citrate were removed for examination after 7 days. Sections (1 cm in length) from the eyehole region and from immediately above the retention balloon were cut from each catheter and the extent of encrustation visualized directly by LVSEM.

The micrographs (Figures 57 and 58) show that crystalline biofilm formation was noticeably less developed on catheters supplied with urine containing the higher citrate concentrations. The crystalline biofilm that developed on the catheter supplied with 0.41 g/L citrate had completely blocked the eye-let of the catheter at 64 h and the lumenal section was also severely encrusted. Although the crystalline biofilm that had developed on the surfaces of the catheter supplied with 1.0 g/L citrate at a similar time (72 h) was significant at the eye-let area, it was however, noticeably reduced at the lumenal site. Relatively little encrustation was visible at 72 h on the surfaces of the catheter supplied with urine containing 1.5 g/L citrate. The micrographs presented in Figure 58 show that although the catheter from the model supplied with urine containing 1.5 g/L citrate was still draining freely at 7 days, significant encrustation had formed on its surfaces. Catheters removed from bladder models supplied with urine containing 2.0 and 3.0 g/L citrate at the end of the 7 day experimental period

however, showed no signs of crystalline biofilm development. Viable cell populations

in residual bladder urine in all experiments at time of stoppage were $> 10^7$ cfu/ml and

the pH's of residual urine $>$pH 8.
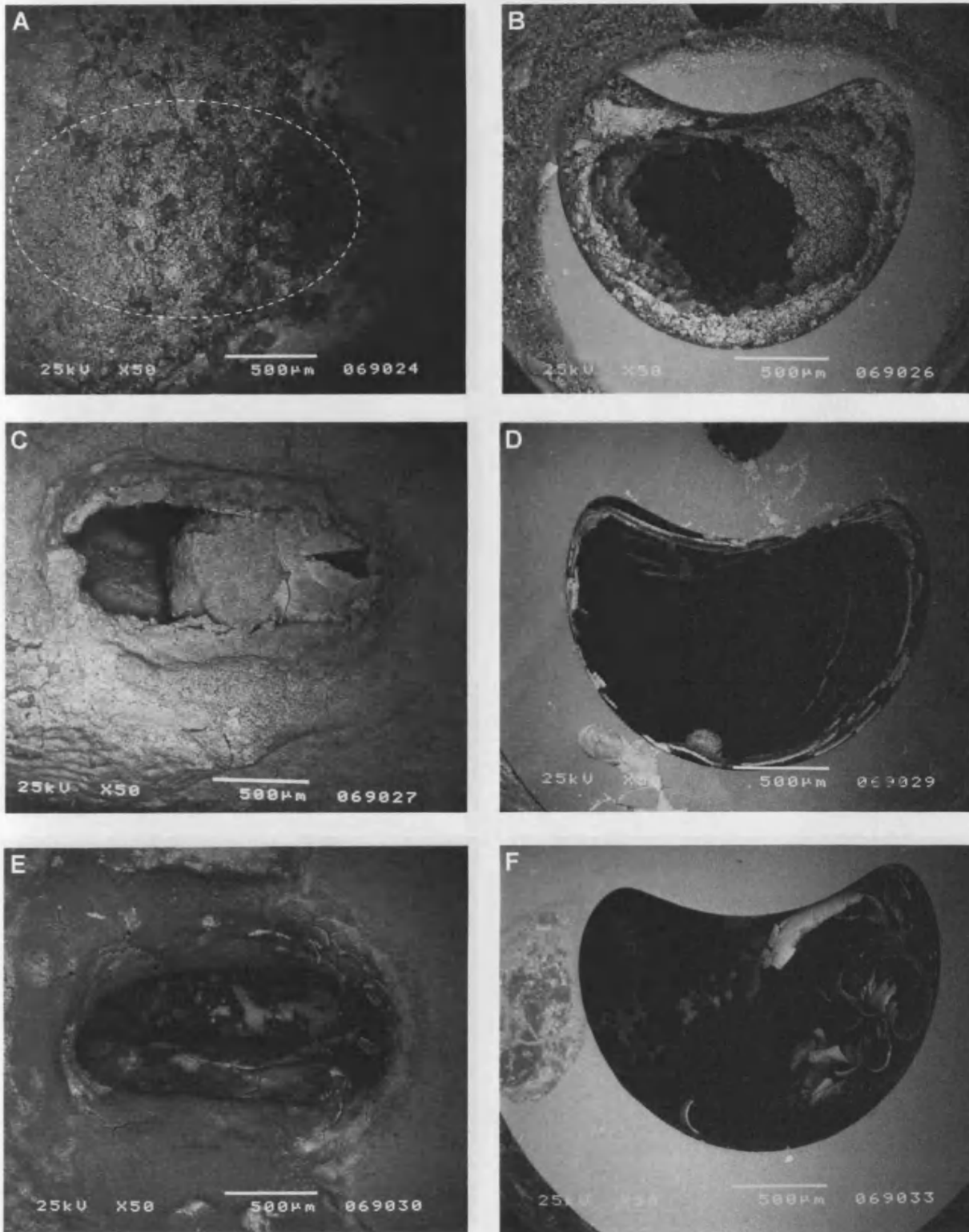
Figure 57. Scanning electron micrographs showing encrustation on catheters from models supplied with urine containing 0.41, 1.0 and 1.5 g/L citrate

The micrographs show the encrustation at the eye-holes and on the lumenal surface of cross-sections taken just above the retention balloons. A and B are of a catheter which blocked after 64 h in a model supplied with urine containing 0.41 g/L citrate (the outline of the eye-hole is shown). C + D are of a catheter removed after 72 h from a model supplied with urine containing 1.0 g/L citrate; E and F are of a catheter removed after 72 h from a model supplied with urine containing 1.5 g/L citrate.

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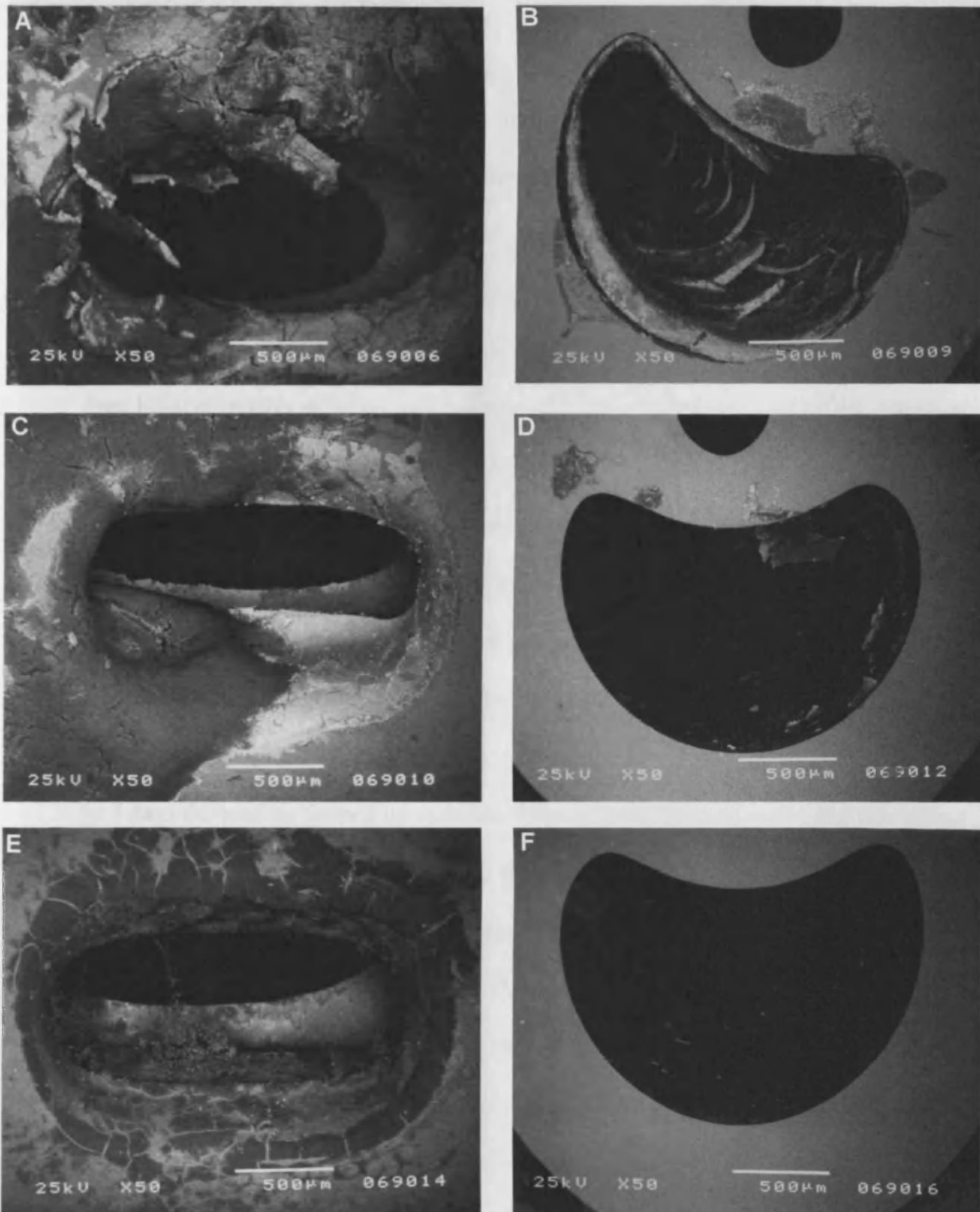
Figure 58. Scanning electron micrographs showing encrustation on catheters from models supplied with urine containing 1.5, 2.0 and 3.0 g/L citrate

The micrographs reveal the extent of encrustation at the eye-holes and on the lumenal surfaces of cross-sections taken just below the retention balloons after 7 days incubation in the models. A and B are of a catheter from a model supplied with urine containing 1.5 g/L citrate. C and D are from a model supplied with urine containing 2.0 g/L citrate. E and F are from a model supplied with urine containing 3.0 g/L citrate.

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A selection of HVSEM micrographs of catheter sections removed from models supplied with urine supplemented with the various citrate concentrations are shown in Figure 59 - 66. Catheters from bladder models supplied with urine containing 0.41 g/L citrate for 24 h were colonized with a typical *P. mirabilis* crystalline biofilm (Figure 59 A and B). In contrast, the *P. mirabilis* biofilms on the catheter surfaces from bladder models supplied with urine containing 1.0, 1.5, 2.0 and 3.0 g/L citrate at the 24 h period showed no evidence of crystalline deposits (Figures 59 C and D, 60 and 61). By 48 h crystalline material had started to form in the biofilms on catheters from models supplied with urine containing 1.0 and 1.5 g/L citrate (Figure 62). There was no sign of crystalline biofilm on catheters examined from bladder models supplied with urine containing 2.0 and 3.0 g/L citrate at 48 h (Figure 63).

At 7 days the biofilm formed on catheters from models supplied with urine containing 1.5 g/L citrate was crystalline in nature (Figure 64). The micrographs reveal distinct differentiated regions within its structure. The biofilm was made up of a foundation layer upon which a typical crystalline *P. mirabilis* biofilm had formed. Above this was a crystal-free mass of elongated *P. mirabilis* swarmer-like cells. In contrast, the 7-day biofilms colonizing catheter surfaces from models supplied with urine containing 2.0 g/L citrate (Figure 65) were almost completely free of microcrystalline structures with only rare instances of relatively small amounts of what appeared to be calcium phosphate present. Swarmer-like cells could not be found. The biofilm on the surfaces of catheters removed from models supplied with urine containing 3.0 g/L citrate at 7 days (Figure 66) was completely free of any microcrystalline deposits. Populations of viable cells in the residual bladder urine in all the experiments at time of stoppage were $> 10^7$ cfu/ml while the pH of residual urine was >pH 8.
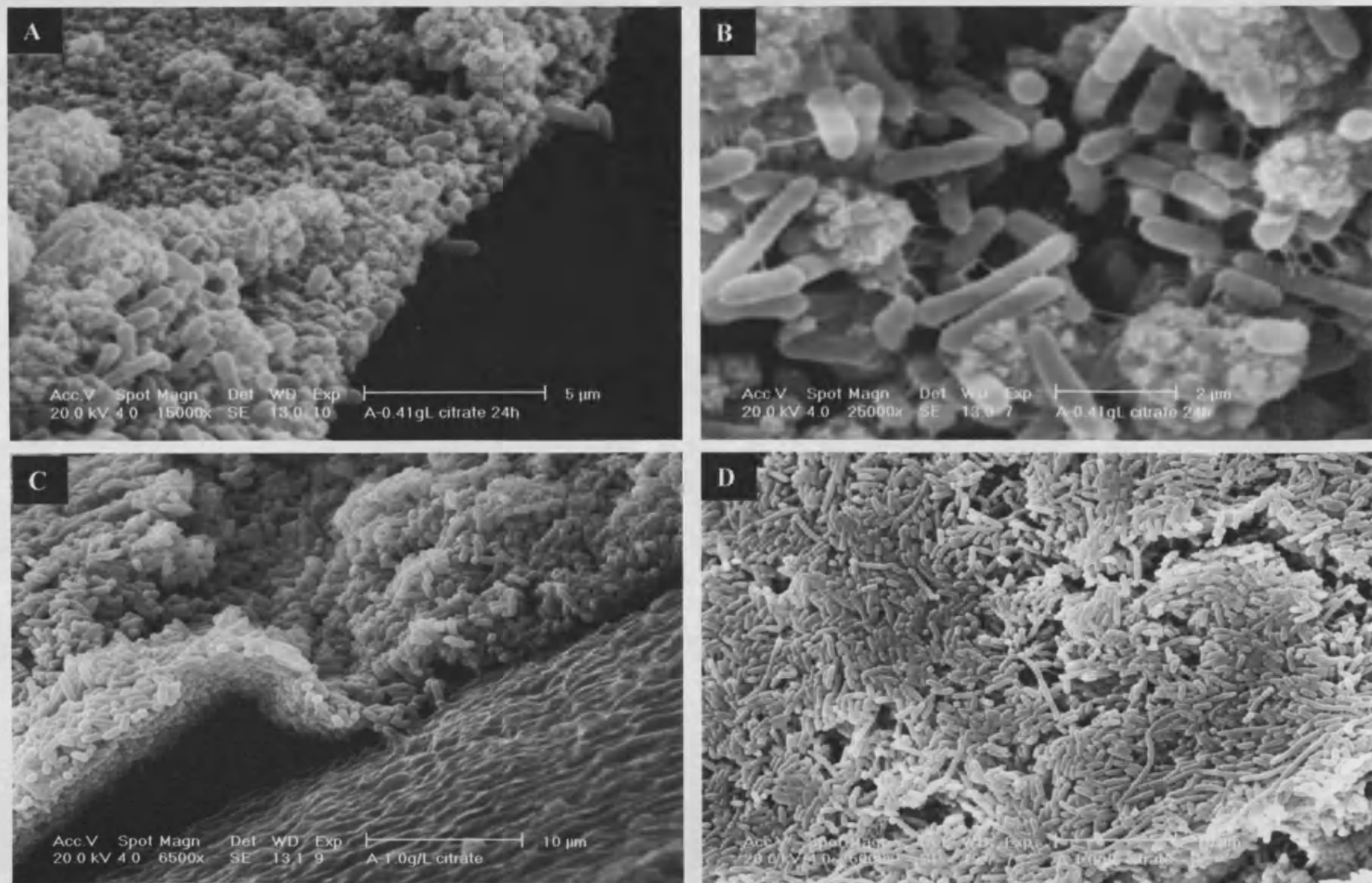
Figure 59. *P. mirabilis* **NSM6 biofilm on all-silicone catheters in urine with various citrate concentrations**

The micrographs show: (A+ B) the crystalline biofilm at the eye-let of a catheter at 24 h in an infected model supplied with 0.41 g/L citrate; (C+D) the non-crystalline biofilm at 24 h on the eye-let of catheter from model supplied with 1.0 g/L citrate.
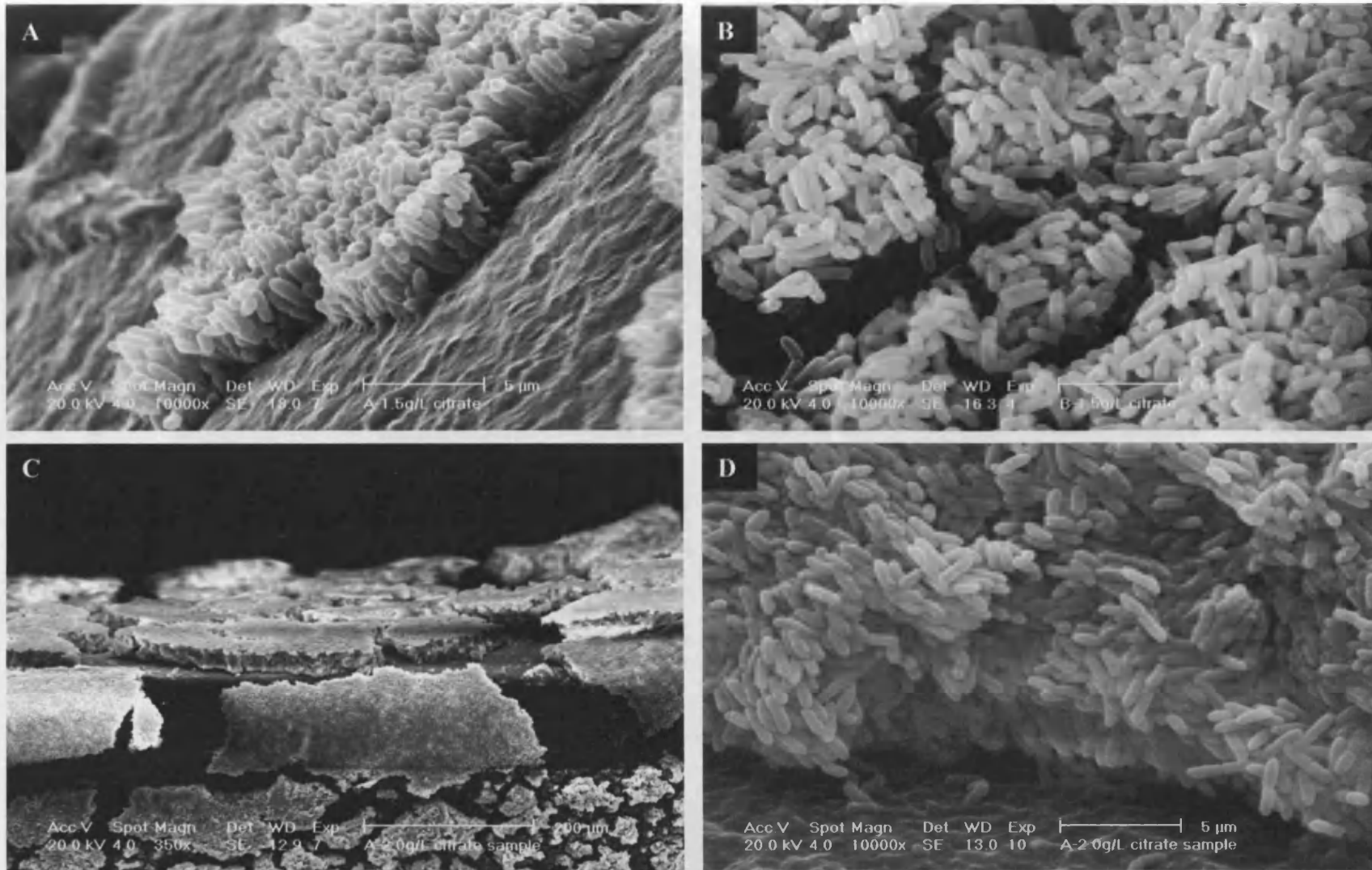
Figure 60. *P. mirabilis* **NSM6 biofilm on all-silicone catheters in urine with various citrate concentrations**

The micrographs show: (A+ B) the non-crystalline biofilm at the eye-let of a catheter at 24 h in an infected model supplied with 1.5 g/L citrate; (C+D) the non-crystalline biofilm at 24 h on the eye-let of catheter from model supplied with 2.0 g/L citrate.
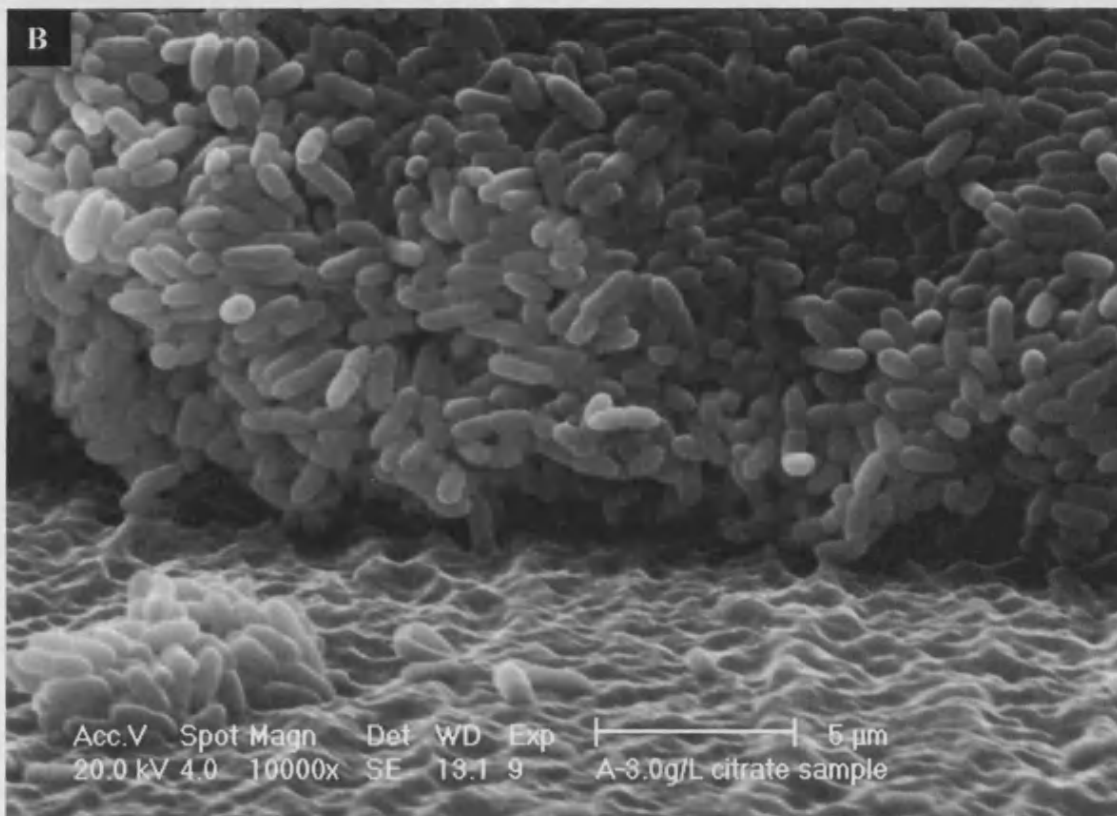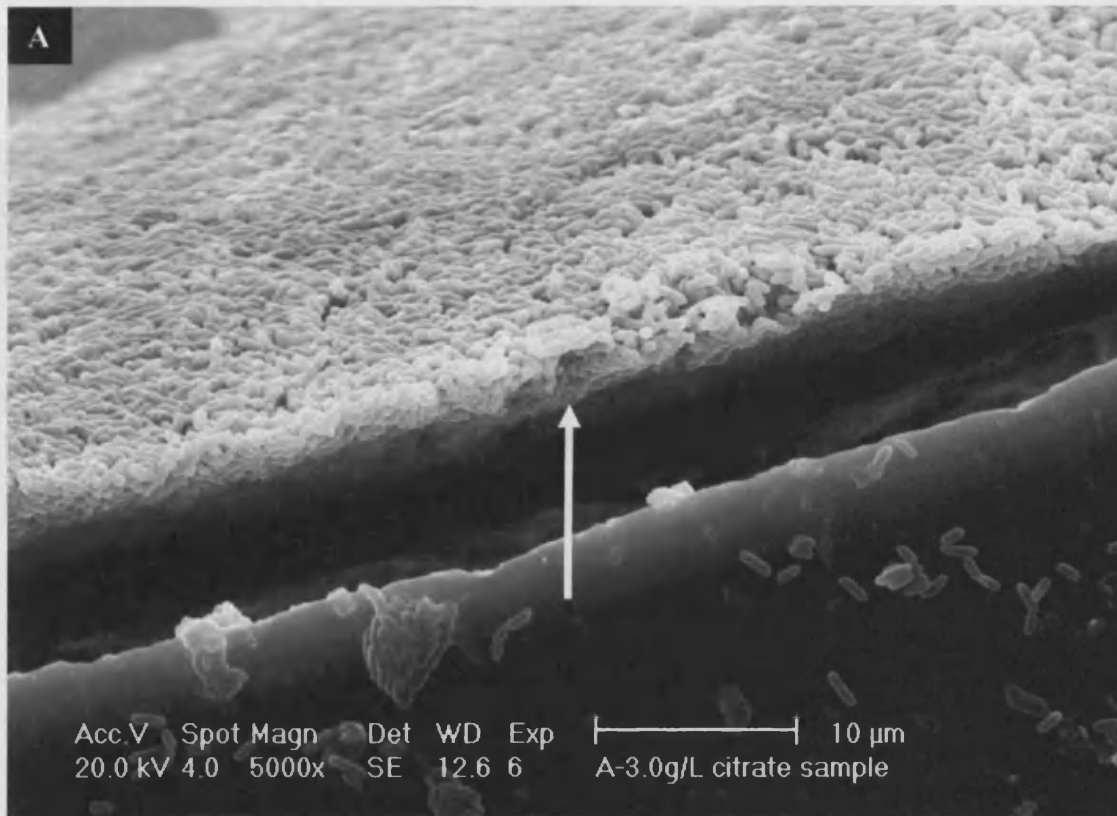
Figure 61. *P. mirabilis* **NSM6 biofilm on all-silicone catheters in urine with various citrate concentrations**

The micrographs show: (A) the non-crystalline biofilm (arrow) at the eye-let bevel of a catheter at 24 h in an infected model supplied with 3.0 g/L citrate; (B) a high magnification image of the non-crystalline biofilm at 24 h on the eye-let of the same catheter.
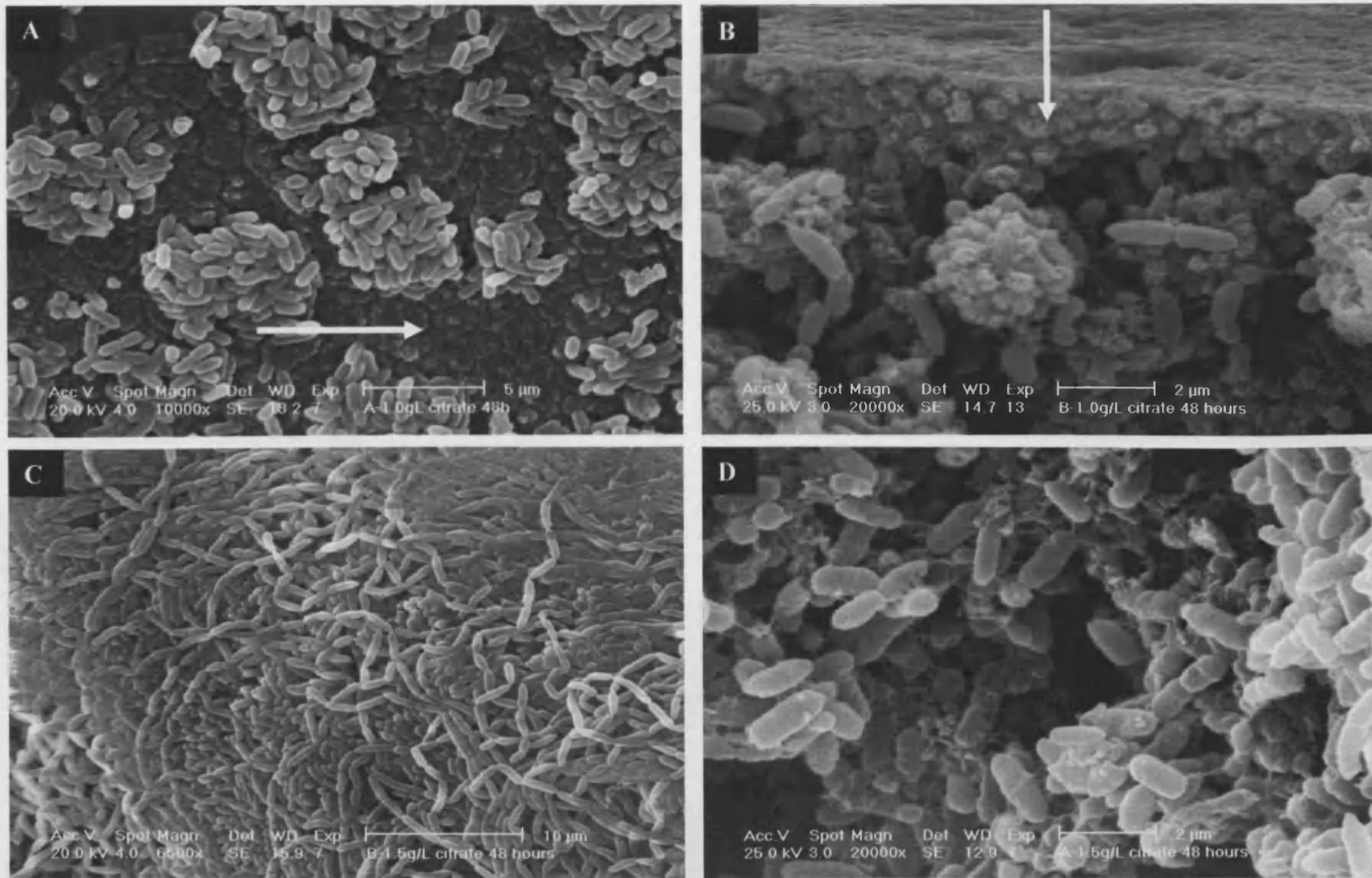
Figure 62. *P. mirabilis* **NSM6 biofilm on all-silicone catheters in urine with various citrate concentrations**

The micrographs show: (A) the micro-crystalline deposits (arrow) present at the eye-let of a catheter at 48 h in infected bladder model; (B) the crystalline biofilm (arrow) on same catheter on the lumenal section just below the eyehole (models were supplied with urine containing 1.0 g/L citrate); (C+D) the non-crystalline and crystalline areas of biofilm respectively at 48 h on the eye-let of a catheter from a bladder model supplied with urine containing 1.5 g/L citrate.
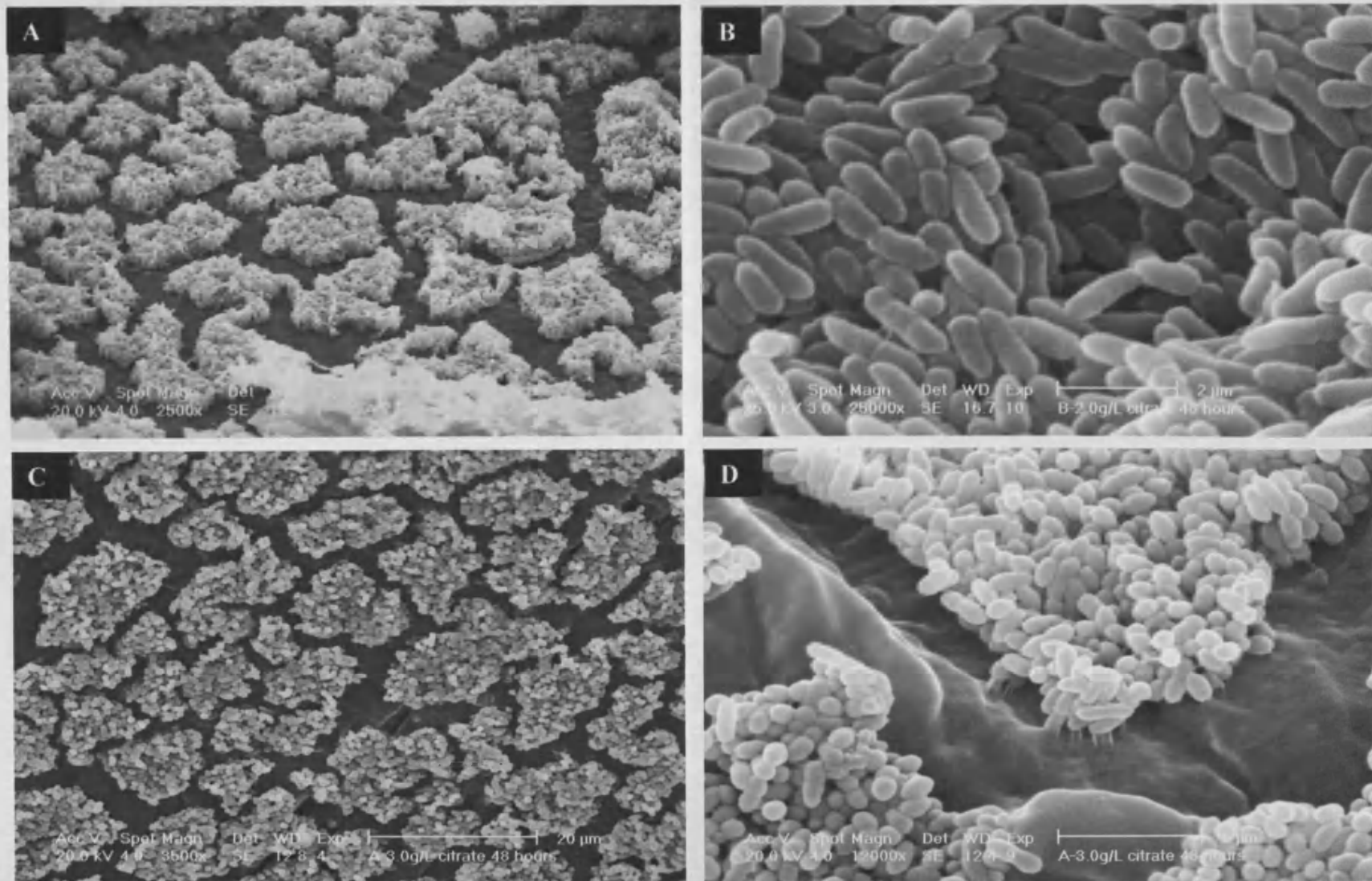
Figure 63. *P. mirabilis* **NSM6 biofilm on all-silicone catheters in urine with various citrate concentrations**

The micrographs show: (A+ B) the non-crystalline biofilm at the eye-let and lumenal section respectively of a catheter at 48 h in an infected model supplied with urine containing 2.0 g/L citrate; (C+D) the non-crystalline biofilm on the eye-let at 48 h on a catheter from a bladder model supplied with urine containing 3.0 g/L citrate.
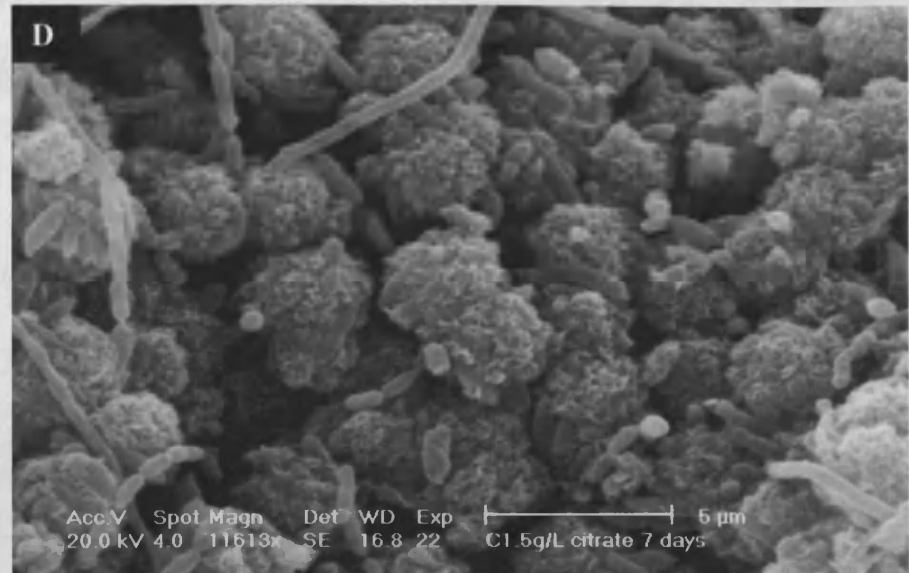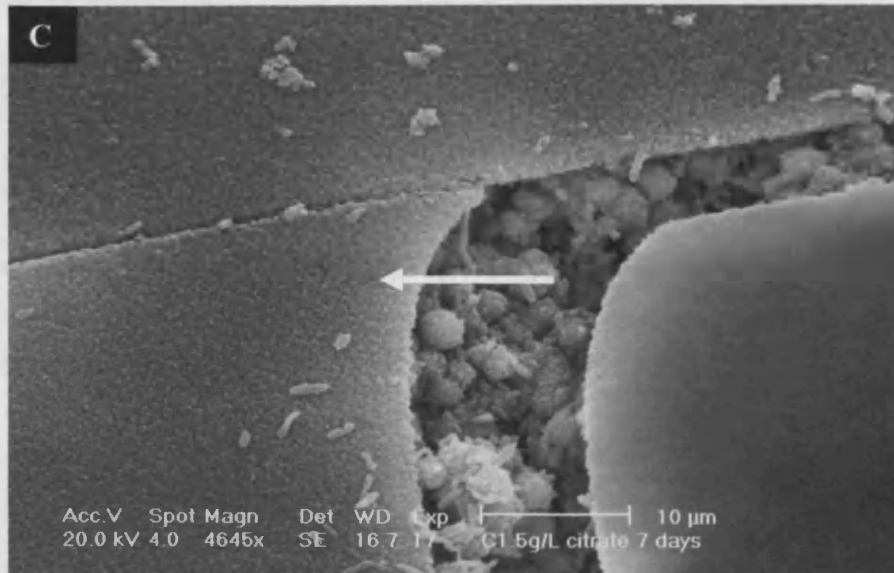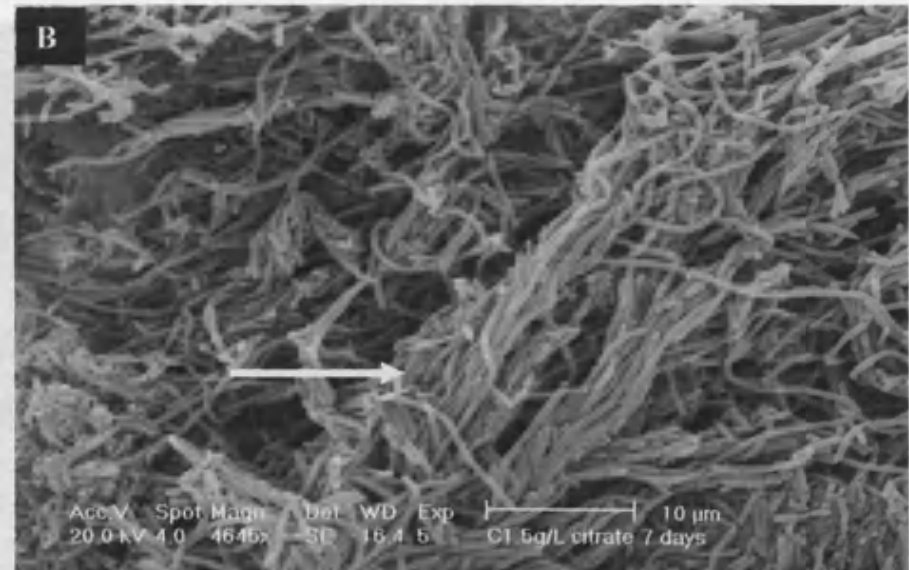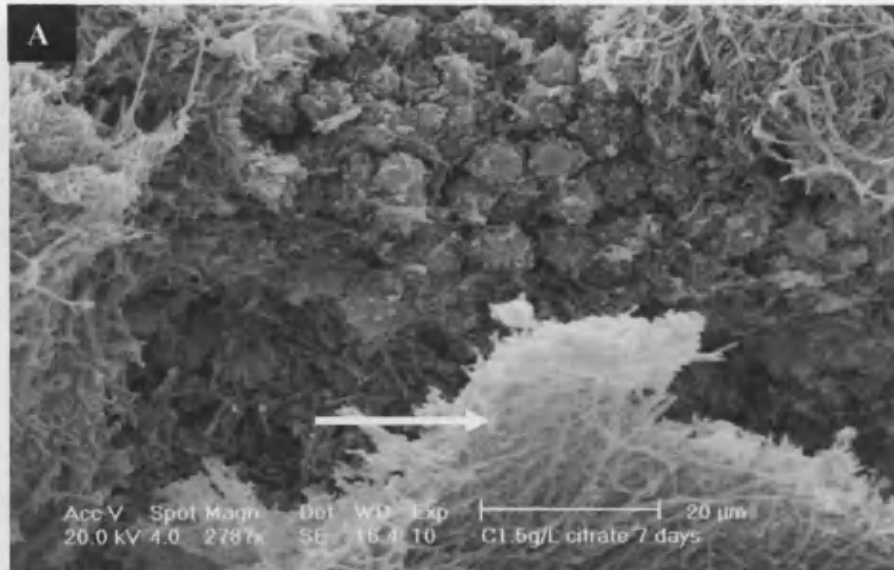
Figure 64. *P. mirabilis* **NSM6 biofilm on all-silicone catheter surfaces in urine containing 1.5 g/L citrate at 7 days**

The micrographs show the differentiated nature of the biofilm observed on catheters at 7 days. (A+B) show the rafts of swarmer-like cells (arrows) at the lumenal section just below the retention balloon. (C) shows the underneath of the 'foundation layer' (arrow) with crystalline biofilm visible through the fractured layer. (D) shows an area of typical *P. mirabilis* crystalline biofilm on the catheter lumen.
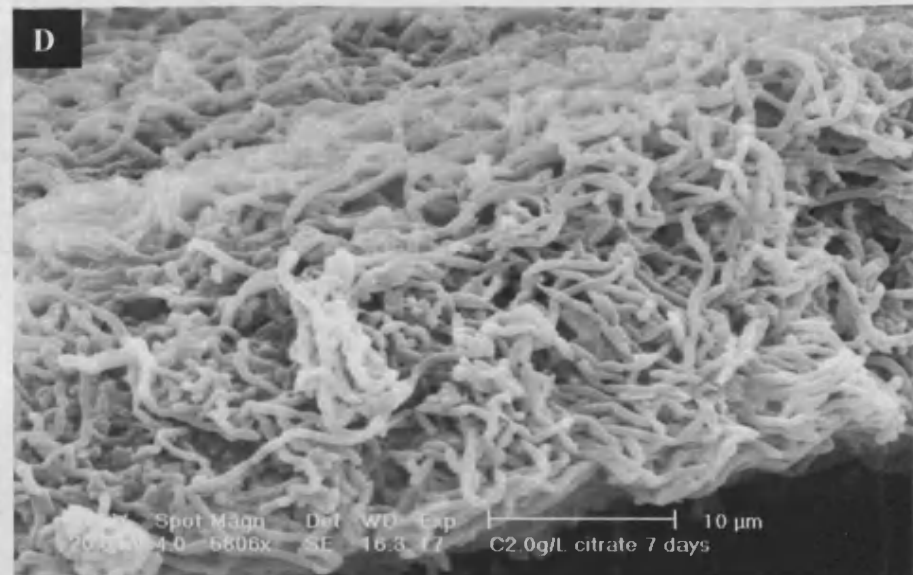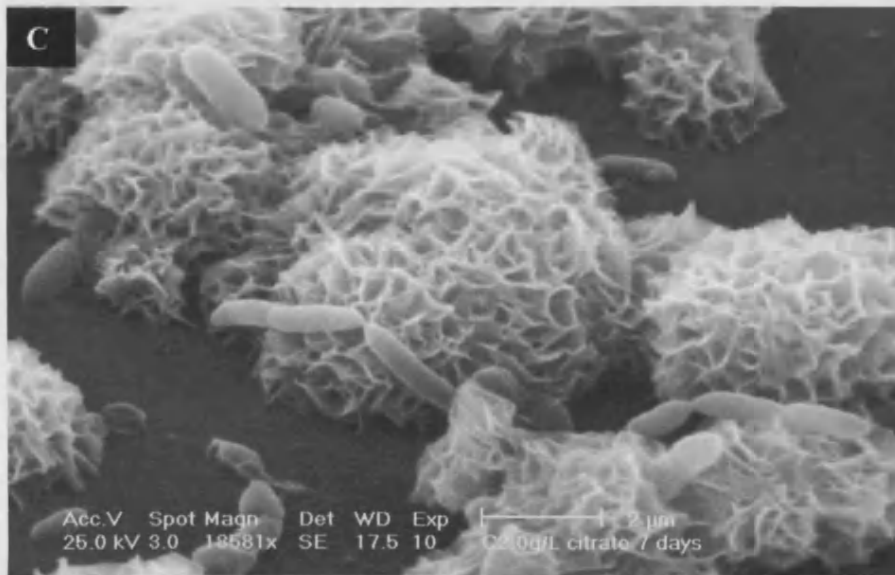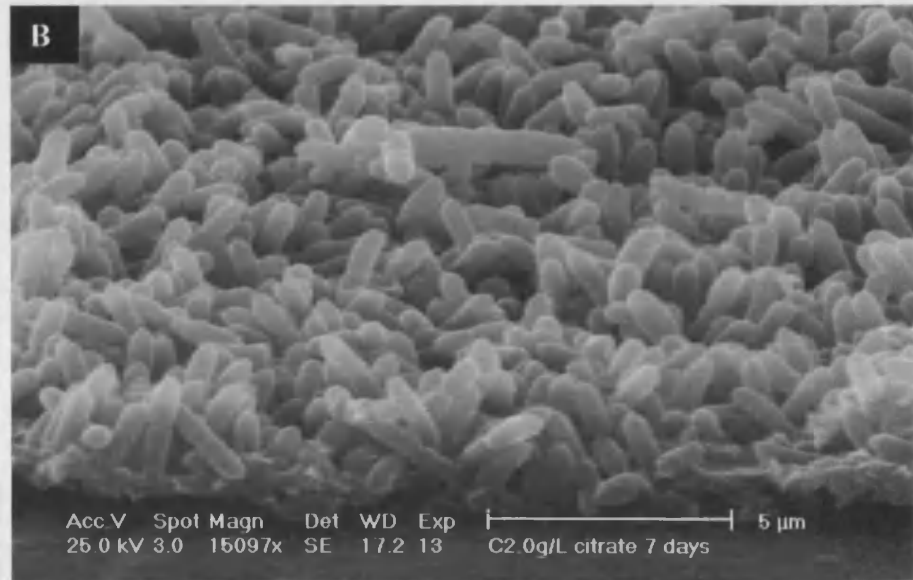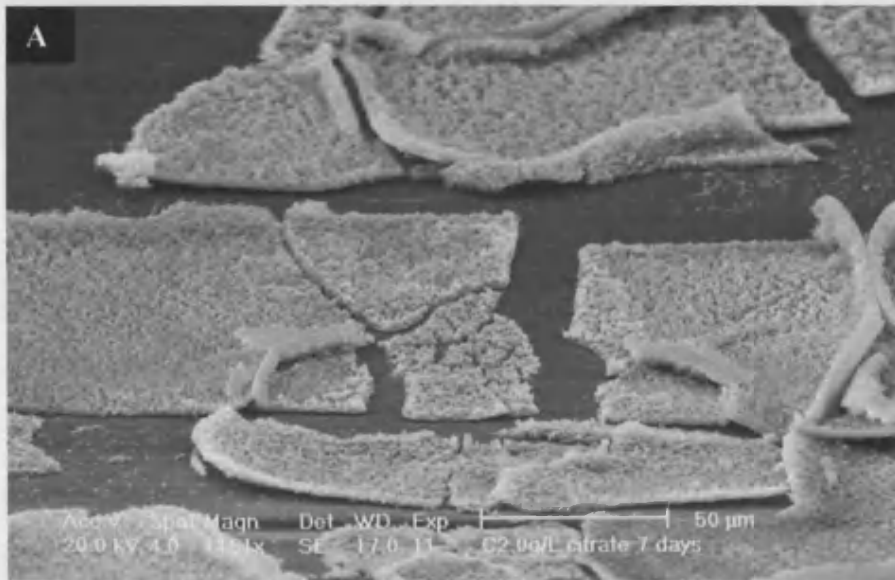
Figure 65.   *P. mirabilis* **NSM6 biofilm on all-silicone catheter surfaces in urine containing 2.0 g/L citrate at 7 days**

The micrographs show: (A+B) high and low magnification of an area of biofilm on the lumenal section just below the retention balloon; (C) the rare microcrystals observed on the lumen; (D) elongated cells at another site on the catheter lumen.
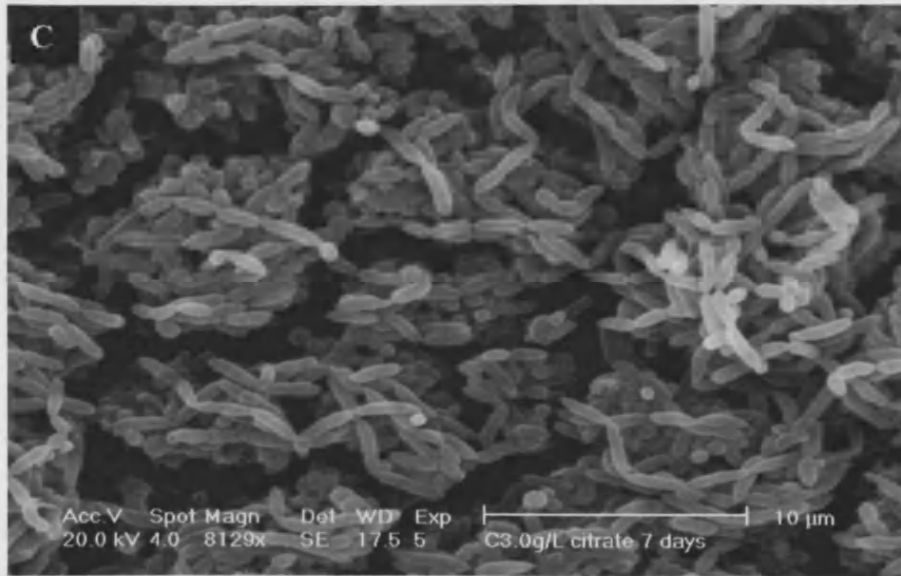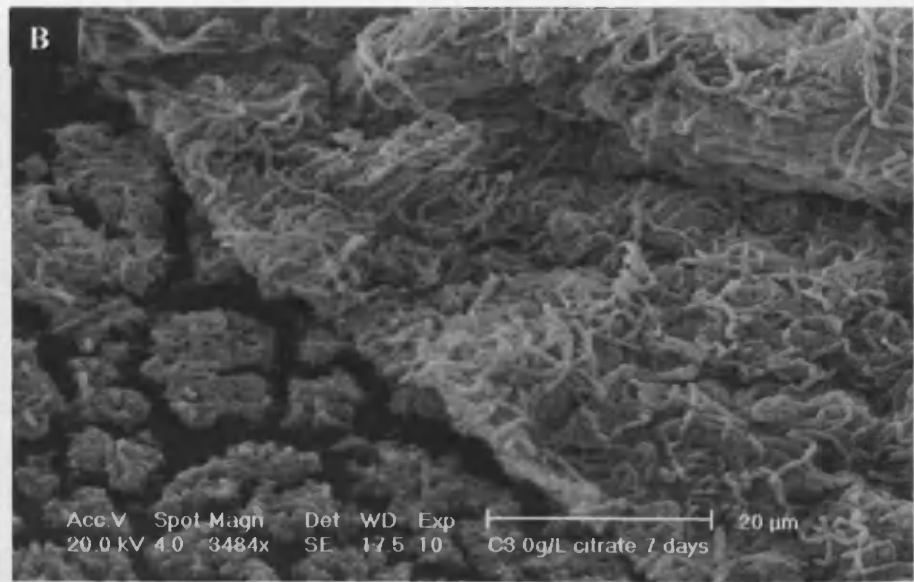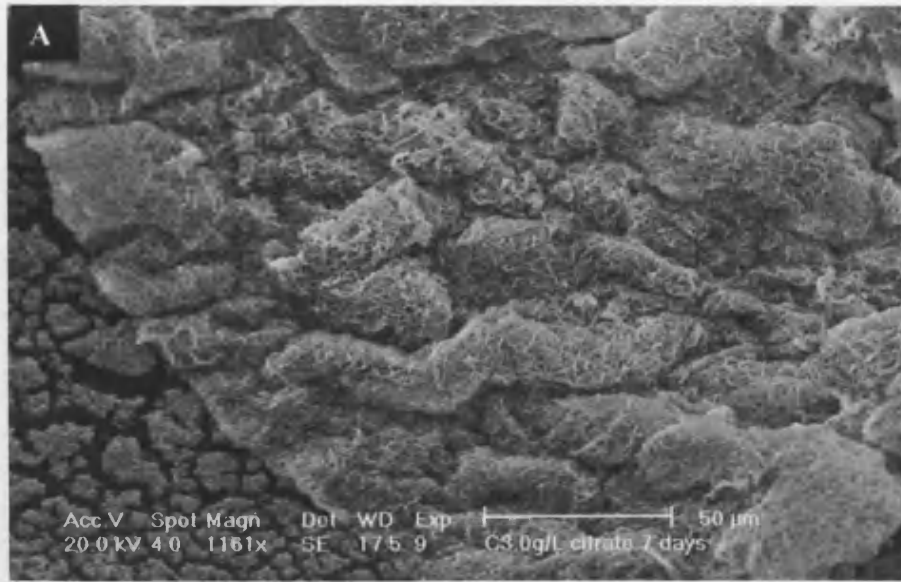
Figure 66.   *P. mirabilis* **NSM6 biofilm on all-silicone catheter surfaces in urine containing 3.0 g/L citrate at 7 days**

The micrographs show: (A-D) low and high magnification of the typical crystalline-free biofilm at various sites on the catheter lumen.

3.3.3. The effect of elevated urinary citrate levels on the rate of catheter encrustation caused by other urease-producing bacteria

The results produced in Sections 3.1. and 3.2. demonstrated that by diluting urine and increasing its citrate content a profound effect on the rate of encrustation in *P. mirabilis* infected bladder models could be achieved. Although *P. mirabilis* is the major cause of encrustation and blockage of urinary catheters other urease producing organisms can be found in the urine of catheterised patients. Some have been found to be capable of generating crystalline biofilm (Kunin, 1989; Stickler *et al.*, 1998b). Recently (Broomfield, personal communication) showed that *Providencia rettgeri* SDM1 and *Proteus vulgaris* SDM2 were capable of encrusting and blocking catheters rapidly. With this in mind, the following experiments were performed to assess the effect of the manipulation of the pH_n of urine has on the rate of catheter encrustation in laboratory bladder models infected with these organisms.

Bladder models fitted with all-silicone catheters were inoculated with a 10 ml inoculum of a 4 h culture of either *Providencia rettgeri* SDM1 or *Proteus vulgaris* SDM2 (approximately 10^8 cfu/ml). The test strains had previously been isolated from the same encrusted catheter. Models were supplied at a flow rate of 1.0 ml/min with a 1:1 dilution of artificial urine supplemented with 0.205 or 1.5 g/L citrate. Experiments were run until catheter blockage or until a maximum of 7 days. The viable cell populations and pH of residual bladder model urine were determined at the start of the experiment and every 24 h thereafter. The catheter blockage times were recorded.

The mean times the catheters took to block calculated from the results of three replicated experiments are presented in Figure 67. In models supplied with urine

containing 0.205 g/L citrate the median times to catheter blockage for *Pv. Rettgeri*

SDM1 and *P. vulgaris* SDM2 were 41.28 ±1.07 h and 59.07 ±15.46 h respectively.

Statistical analysis (Kruskal – Wallis test) indicated that the median values were not

significantly different (P >0.05). In the bladder models supplied with urine containing

1.5 g/L citrate two of the three catheters in models infected with *Pr. rettgeri* SDM1

drained freely for the full 7 days. The third replicate blocked at 108 h. All three

catheters in models infected with *P. vulgaris* SDM2 remained patent for the whole 7

days.

The mean viable cell populations and pHs of the residual bladder model urines (Table

12) showed that at times *Pv. rettgeri* SDM1 was able to reach higher cell densities and

generate more alkaline conditions in the models than *P. vulgaris* SDM2. Mean viable

cell populations in residual urine from *Pv. rettgeri* SDM1 infected models varied

between 10^8 and 10^9 cfu/ml, while mean viable cell populations in residual urine from

P. vulgaris SDM2 infected models varied between 10^7 and 10^8 cfu/ml. Mean pH

values of residual urine in *Pv. rettgeri* SDM1 bladder models frequently exceeded pH

9 while mean pH's of residual urine in *P. vulgaris* SDM2 infected models reached

values which exceeded pH 8.
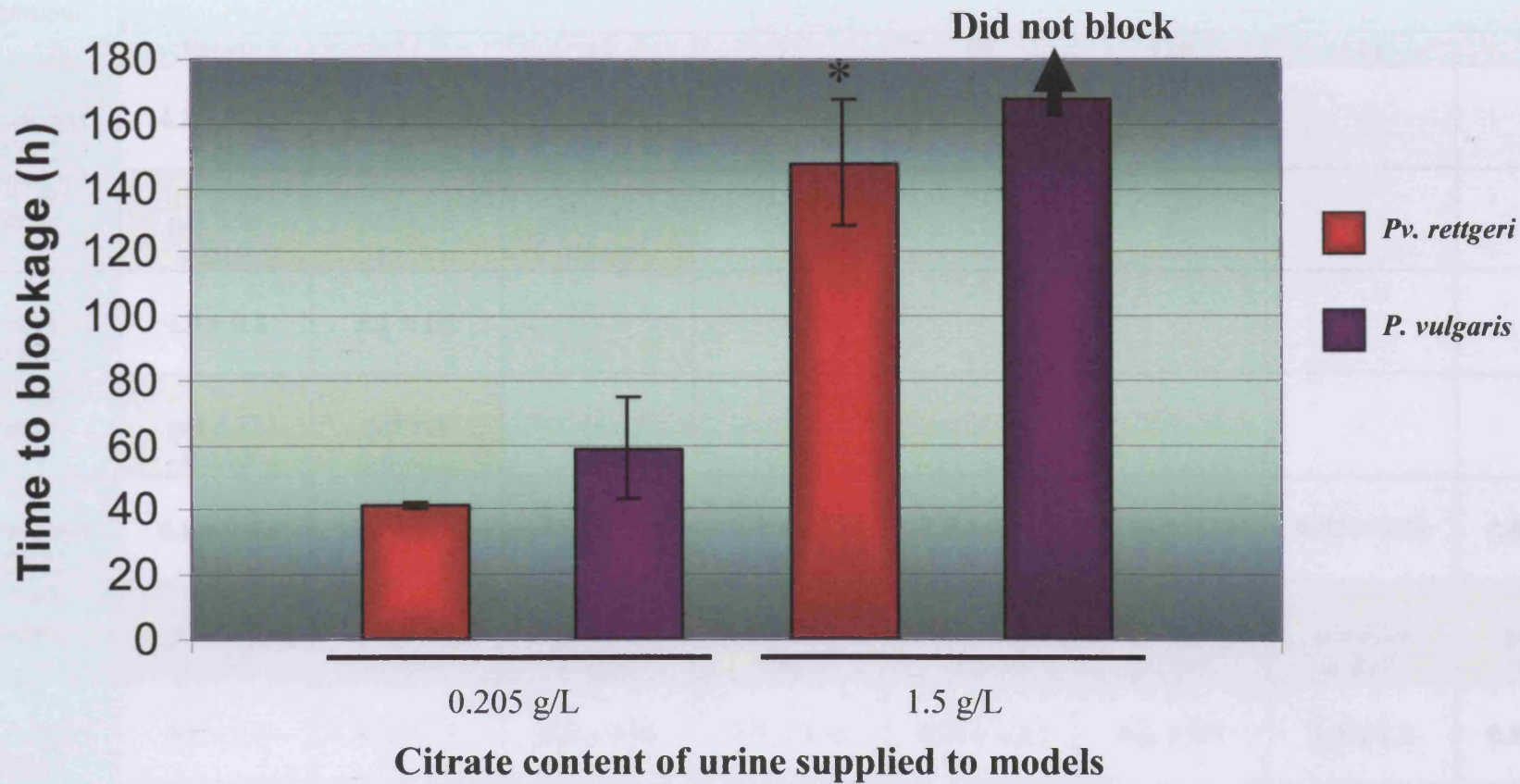
Figure 67. **The effect of urinary citrate content on the rate of catheter blockage by *P. vulgaris* SDM2 and *Pv. rettgeri* SDM1**

Bladder models fitted with all-silicone catheters were inoculated with 10 ml of a 4 h culture of the test strains and supplied at 1.0 ml/min with urine containing 0.205 and 1.5 g/L citrate. The standard error of the mean blockage times of three replicated experiments is shown.

* Two of the three catheters drained freely for the 7 day experimental period.

Test organism	Mean viable cell population (x 10^8 cfu/ml) and pH of residual urine throughout experiments (h)							
	Start	24	48	72	96	120	144	168
P. vulgaris SDM2 0.205 g/L citrate	4.2 ± 0.35	0.76 ± 0.04	2.1 ± 0.85 †	-	-	-	-	-
	pH 8.59 ± 0.09	pH 8.13 ± 0.15	pH 8.82 † ± 0.05	-	-	-	-	-
Pv. rettgeri SDM1 0.205 g/L citrate	4.0 ± 0.8	3.1 ± 1.2	-	-	-	-	-	-
	pH 8.23 ± 0.03	pH 9.1 ± 0.1	-	-	-	-	-	-
P. vulgaris SDM2 1.5 g/L citrate	4.1 ± 0.47	2.9 ± 1.06	3.0 ± 0.92	1.9 ± 1.15	1.5 ± 0.32	0.74 ± 0.11	0.82 ± 0.29	0.80 ± 0.29
	pH 8.43 ± 0.09	pH 7.67 ± 0.12	pH 8.6 ± 0.06	pH 8.51 ± 0.13	pH 8.65 ± 0.03	pH 8.7 ± 0.02	pH 8.74 ± 0.07	pH 8.67 ± 0.14
Pv. rettgeri SDM1 1.5 g/L citrate	4.7 ± 1.1	6.6 ± 3.3	5.1 ± 0.86	3.7 ± 0.95	13.0 ± 0.59	6.2 ± 0.9	2.5 ± 1.6	0.94 ± 0.05
	pH 8.27 ± 0.21	pH 8.75 ± 0.04	pH 8.89 ± 0.04	pH 9.04 ± 0.03	pH 9.09 ± 0.04	pH 9.14 ± 0.01 †	pH 9.09 ± 0.12 †	pH 9.03 ± 0.13 †

(†) mean based on two replicates, (–) indicates that by this time all models had blocked

Table 12. **Mean viable cell population and pH of residual urine in *P. vulgaris* SDM2 and *Pv. rettgeri* SDM1 infected bladder models**

Models were fitted with all-silicone catheters and inoculated with 10 ml of a 4 h culture of *P. vulgaris* SDM2 or *Pv. rettgeri* SDM1 (approximately 10^8 cfu/ml). Experiments were supplied at 1.0 ml/min with 1:1 diluted artificial urine containing 0.205 or 1.5 g/L citrate. The mean ± SE of the mean calculated from three replicated experiments are shown.

3.4. The manipulation of urinary pH_n through increased dietary fluid and citrate intake and its effect on the rate of catheter encrustation

The results reported in Section 3.3. showed that the manipulation of the pH_n of artificial urine by its dilution and increase in its citrate content, had a significant effect on the rate of catheter encrustation in laboratory bladder models. Seltzer *et al.* (1996) previously reported that individuals prone to calcium nephrolithiasis who were 'hypo-citraturic' became 'normo-citraturic' after consuming regular lemonade drinks containing a high citrate content. More recently Siener *et al.* (2005) showed that strict dietary management significantly altered the urinary composition, reducing the stone formation in recurrent calcium oxalate stone-formers. Suller *et al.* (2005) showed that the pH_n and citrate content of urine in healthy volunteers could be manipulated by increasing their fluid intake and by supplementing their diet with regular citrate containing drinks. As dietary manipulation of urinary composition can be beneficial to urolithiasis it may also be effective in controlling the problem of calcified formations on urinary catheters. The aim in this section was to investigate the effect that the dietary manipulation (based on the methods described by Seltzer *et al.*, 1996 and Suller *et al.*, 2005), would have on the pH_n of urine and the rate of catheter encrustation when this urine was supplied to catheterised laboratory bladder models.

3.4.1. Urine profiles of healthy volunteers

Initially the urine profiles of five healthy volunteers were determined. The data was used to examine the variability in urine composition among individuals and also to determine any 'abnormalities' in the urinary composition of the volunteer intended to be utilized in further experiments. There were no dietary or lifestyle restrictions imposed. Urine samples from each individual were collected between 0600 – 1700 hours on three separate days. Urinary parameters including the voided pH (pH_v), the nucleation pH (pH_n), the pH safety margin (pH_n - pH_v), calcium, magnesium and citrate content were established for each urine void. The volume of urine voided on each occasion was also recorded.

The urinary profiles determined for all volunteers (Table 13) illustrate the variability of urinary parameters observed among those examined. The mean pHs of voided urines ranged from pH 5.75 – 6.72, mean pH_ns ranged from pH 6.77 – 8.02 and the mean pH safety margins varied between 0.29 to 1.74 pH units. The mean urine output among volunteers ranged from 706 to 1881 ml over the 11-hour sampling period. Mean magnesium concentrations recorded in urine were similar amongst volunteers ranging from 0.04 – 0.07 g/L, while mean calcium concentrations in urine showed more variability ranging from 0.05 to 0.22 g/L. The mean citrate concentrations among urines ranged from 0.25 – 0.37 g/L. The mean values for the amount of Ca, Mg and citrate excreted by individuals during the sampling period ranged from 0.08 – 0.22 g, 0.035 – 0.09 g and 0.18 – 0.56 g respectively, an approximate 3-fold variation between the lowest and highest excretions in all three parameters. Volunteer 1 was used in all subsequent fluid and citrate intake experiments.

Urinary profiles of healthy volunteers

Volunteer	pH$_v$	pH$_n$	pH margin (pH$_n$-pH$_v$)	Urine output (ml)	[Ca] g/L	[Mg] g/L	[Citrate] g/L	Ca excreted (g/11h)	Mg excreted (g/11h)	Citrate excreted (g/11h)
1	5.75 ± 0.19	7.25 ± 0.07	1.5 ± 0.22	706 ± 71.6	0.17 ± 0.015	0.07 ± 0.008	0.25 ± 0.023	0.12 ± 0.02	0.049 ± 0.007	0.18 ± 0.07
2	6.03 ± 0.16	7.76 ± 0.16	1.74 ± 0.19	1335 ± 222.05	0.08 ± 0.017	0.04 ± 0.007	0.31 ± 0.05	0.08 ± 0.04	0.035 ± 0.008	0.34 ± 0.07
3	6.72 ± 0.22	8.02 ± 0.12	1.3 ± 0.22	1881 ± 162.5	0.05 ± 0.008	0.049 ± 0.008	0.33 ± 0.05	0.1 ± 0.06	0.09 ± 0.027	0.56 ± 0.09
4	6.31 0.18	6.77 ± 0.12	0.47 ± 0.15	795 ± 54.1	0.22 ± 0.016	0.051 ± 0.008	0.37 ± 0.07	0.18 ± 0.01	0.039 ± 0.011	0.22 ± 0.04
5	6.5 ± 0.11	6.79 ± 0.1	0.29 ± 0.07	1183 ± 224.5	0.19 ± 0.017	0.05 ± 0.006	0.28 ± 0.04	0.22 ± 0.04	0.058 ± 0.008	0.31 ± 0.06

Table 13.   **The * mean values of urinary constituents and parameters of 5 healthy volunteers**

Urine was collected between 0600 – 1700 hours on three separate occasions for each volunteer. *The means ± SE of the means were calculated from the results of three replicated sampling periods.

3.4.2. The effect of increased citrate intake on urinary citrate and pH$_n$

The next step was to identify a commercially available soft drink high in citrate content to use as a dietary supplement in the experiments to manipulate pH$_n$. The citrate content of a selection of commercially available soft drinks was assessed using the citrate assay kit described in Section 2.16.1. Samples (1 ml) were filtered through a 0.2 μ/m Minisart micropore filter (Sartorious) to remove any large particles from the sample before testing. The results of the citrate assays on the test fruit juices are presented in Table 14. It is clear that the juices from lemons and limes contains substantially more citrate than the other fruits.

A selection of juices with high citrate content were then considered for inclusion in the experimental citrate drink. After a series of informal tasting sessions it was decided that the citrate drink would consist of a mixture of lemon and orange juice at a ratio of 1:4 respectively. A total of 750 ml of the lemon/orange drink was consumed by a healthy volunteer in 250 ml aliquots at three evenly spaced intervals throughout the day (morning, afternoon and evening), supplementing the daily diet with approximately 11g of citrate. The routine was implemented for four days with all urine voided between (0600 – 1700 hrs) on days two, three and four collected. No other restrictions on diet or lifestyle were enforced. The urinary profile of the volunteer was determined after the citrate drink regimen and compared with the urinary profile obtained previously from the same volunteer under normal dietary conditions (Section 3.4.1.). The mean citrate concentration in urine and also the total (mean) daily citrate excreted under the normal and increased citrate diets within the sampling period are shown in Figure 68. Comparison of mean pH$_v$, pH$_n$, pH safety margin and urine output before and after the citrate diet are shown in Figure 69.

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Although citrate concentrations (g/L urine) were consistently higher in urine samples obtained on the increased citrate diet compared to those on the normal diet, difference in the mean values were not significant ($P = 0.09$). The mean values of total citrate excreted in urine over the 11-hour sampling period were higher in urine voided on the citrate supplemented diet but again not significantly different ($P = 0.11$). There were no significant differences in pH_v ($P > 0.05$ Kruskal-Wallis analysis), and no significant differences ($P > 0.05$) between mean pH_n values (pH 7.26 ± 0.07 before citrate diet, 7.46 ± 0.09 after citrate diet). When the mean pH safety margins of urine before and after the citrate diet (1.50 ± 0.22 and 1.44 ± 0.2 respectively) were compared the values were not significantly different ($P > 0.05$). Comparisons of both Ca and Mg concentrations in urines before and after the citrate diet (not shown) also indicated there were no significant differences ($P > 0.05$). The mean volumes of urine voided were 706 ± 71.6 ml and 810 ± 86.9 ml before and after consumption of the citrate drink respectively, and were not significantly different ($P = 0.41$).

Type of juice	Mean citrate content g/L
Coleman's Lime Jif	58.7 ± 4.35
Tesco Lemon Juice	44.9 ± 4.15
Del Monte Pure Grapefruit Juice	12.7 ± 0
Tesco Raspberry & Pear Juice	1.6 †
Tesco 'Pure Florida' Orange. & Grapefruit	7.2 †
Tesco Pure Pink Grapefruit Juice	12.9 ± 0.2
Tesco 'Pure Florida' Orange Juice	7.0 ± 0.2

Table 14. **The *mean citrate content of a range of commercially available fruit juices**

* The means ± SE of the means (unless otherwise stated) were calculated from two replicated assays. † result from single assay.
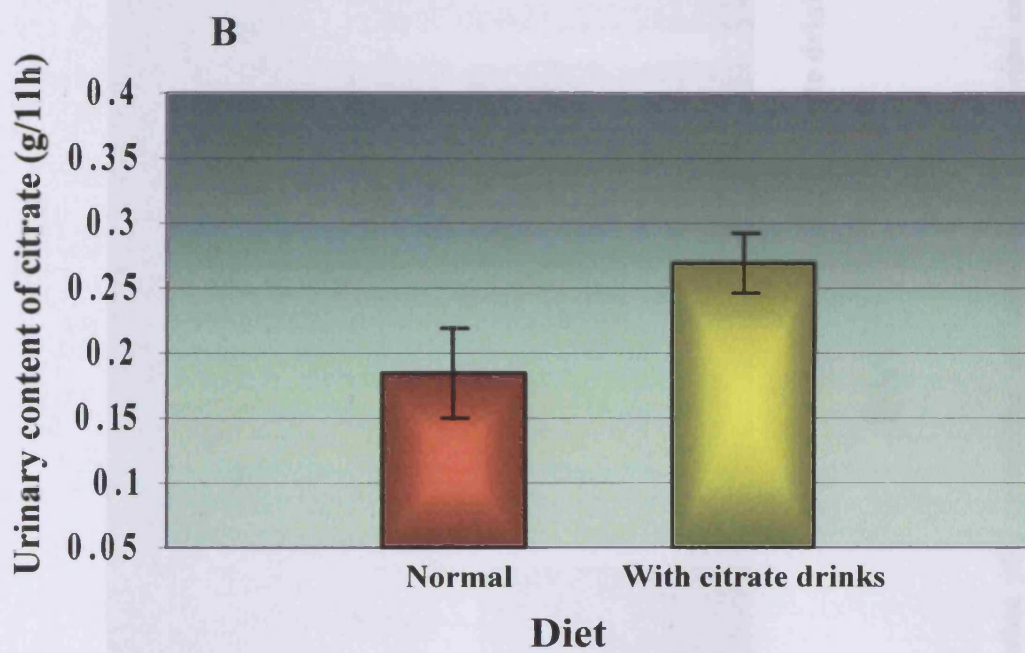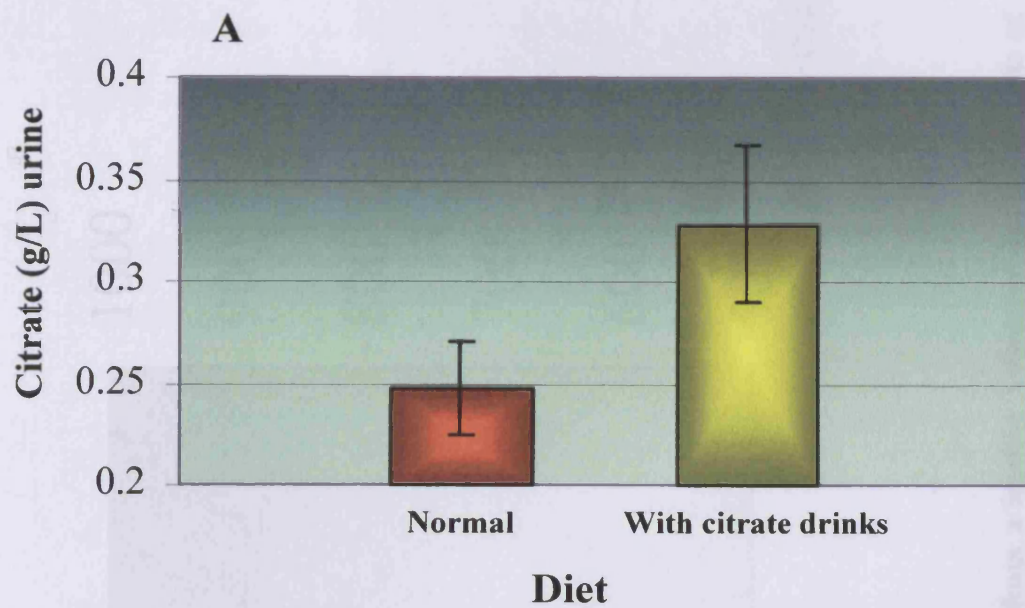
Figure 68. The *mean citrate concentration and mean (total) citrate excreted in urine

Urine was collected between 0600 – 1700 h on three separate occasions. (A) shows the mean citrate concentration in voided urine under the normal and increased citrate regimen. (B) shows the mean of the total citrate excreted on the normal and increased citrate diets. * The means ± SE of the means calculated from the results of three replicated sampling periods are illustrated.
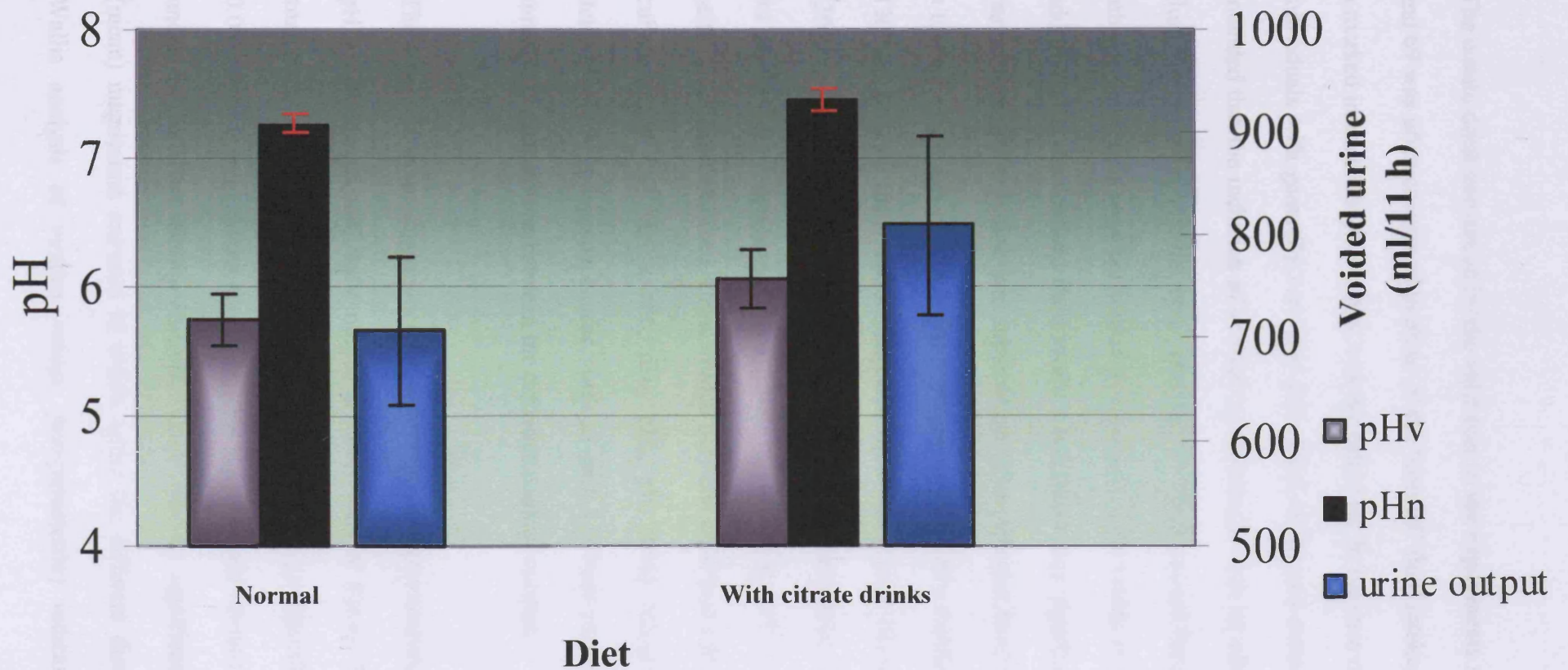
Figure 69. The *mean voided urine pH, nucleation pH and volume of urine collected from a healthy volunteer during periods of normal and elevated intakes of citrate

All voided urines were collected between 0600 – 1700 on 3 separate occasions before and after consuming 3 daily doses of the citrate drink. *The means ± SE of the means calculated from the results of three replicated sampling periods are indicated.

The citrate drink consumed by the volunteer in the experiments reported in Figure 68 and 69 was often consumed in place of the 'normal' fluid intake, the result being that a marked increase in fluid output was not achieved. In the case of elderly catheterised individuals with generally very low daily fluid intake and concentrated urine, it was intended that the inclusion of a citrate drink should form an addition to their normal fluid consumption, and not be a substitute for their normal fluid intake. With this in mind, experiments were performed to determine if the citrate-containing drink used in addition to a standardised fluid intake would have any significant effect on urinary parameters. The volunteer was subjected to three different fluid intake routines lasting a total of 4 days per regimen. The routines consisted of a standardised fluid intake of 1300 ml per 24 h (SFI), the standardised fluid intake with 750 ml of additional water (2050 ml per 24 h, SFI + W), and the standardised fluid intake with an additional 750 ml of the citrate containing drink (2050 ml per 24 h, SFI + C). No other dietary restrictions were enforced. Urine voided between 0600 and 1700 h on days 2 - 4 was collected and Ca, Mg, citrate, pH_v, pH_n, pH safety margin and urine volume determined for independent urine voids in each 11- hour period. A break of at least three days was allowed between the different dietary routines.

The results of the mean urinary Ca, Mg and citrate concentrations and mean pH_v, pH_n, pH safety margin and urine output are presented in Figure 70 and 71. Statistical analysis (ANOVA) of the data revealed there were no significant differences ($P > 0.05$) in the mean calcium concentrations (g/L) or total (mean) calcium excreted (g) under the different dietary regimes. There was no significant difference in total (mean) magnesium excreted in urines under the different diets, although Kruskal-Wallis analysis of median values (non-parametric) indicated magnesium was

significantly more concentrated ($P < 0.05$ at the 97.5% CI level) in urine from the SFI diet. The mean citrate concentration in urine from the SFI + W regimen (0.17 ± 0.02 g/L) was found to be significantly higher ($P < 0.05$) than that recorded in urine from the SFI + C regimen (0.12 ± 0.01 g/L). This may have been due to the increased volume of urine voided under the SFI + C diet however (1578 ± 67.23 ml), compared to the SFI + W diet (1266 ± 129.91 ml), because when their respective mean values of total citrate excreted were compared the difference was not significant ($P > 0.05$). The mean citrate excreted under SFI + W however, was significantly higher ($P < 0.05$) than citrate excreted under SFI regimen.

The mean pHs of voided urine ranged from pH 5.5 – pH 6 and was highest in urine collected under the SFI + C diet. When the nucleation pHs were determined for the different urines the mean pH_ns were 7.25 ± 0.12, 7.59 ± 0.17 and 8.05 ± 0.12 from SFI, SFI + W and SFI + C routines respectively and was significantly higher ($P < 0.05$) under the SFI + C regimen than under the SFI diet. The mean pH safety margins were 1.74 ± 0.12, 2.19 ± 0.14 and 2.29 ± 0.14 pH increments for urine collected under the SFI, SFI + W and SFI + C routines respectively. The mean value for the urine collected under the SFI + C regimen being significantly higher ($P < 0.05$) than that collected on the SFI diet. Statistical analysis also showed that the mean volume of urine voided under the SFI regimen was significantly lower ($P < 0.05$) than that voided on SFI + W and SFI + C diets.
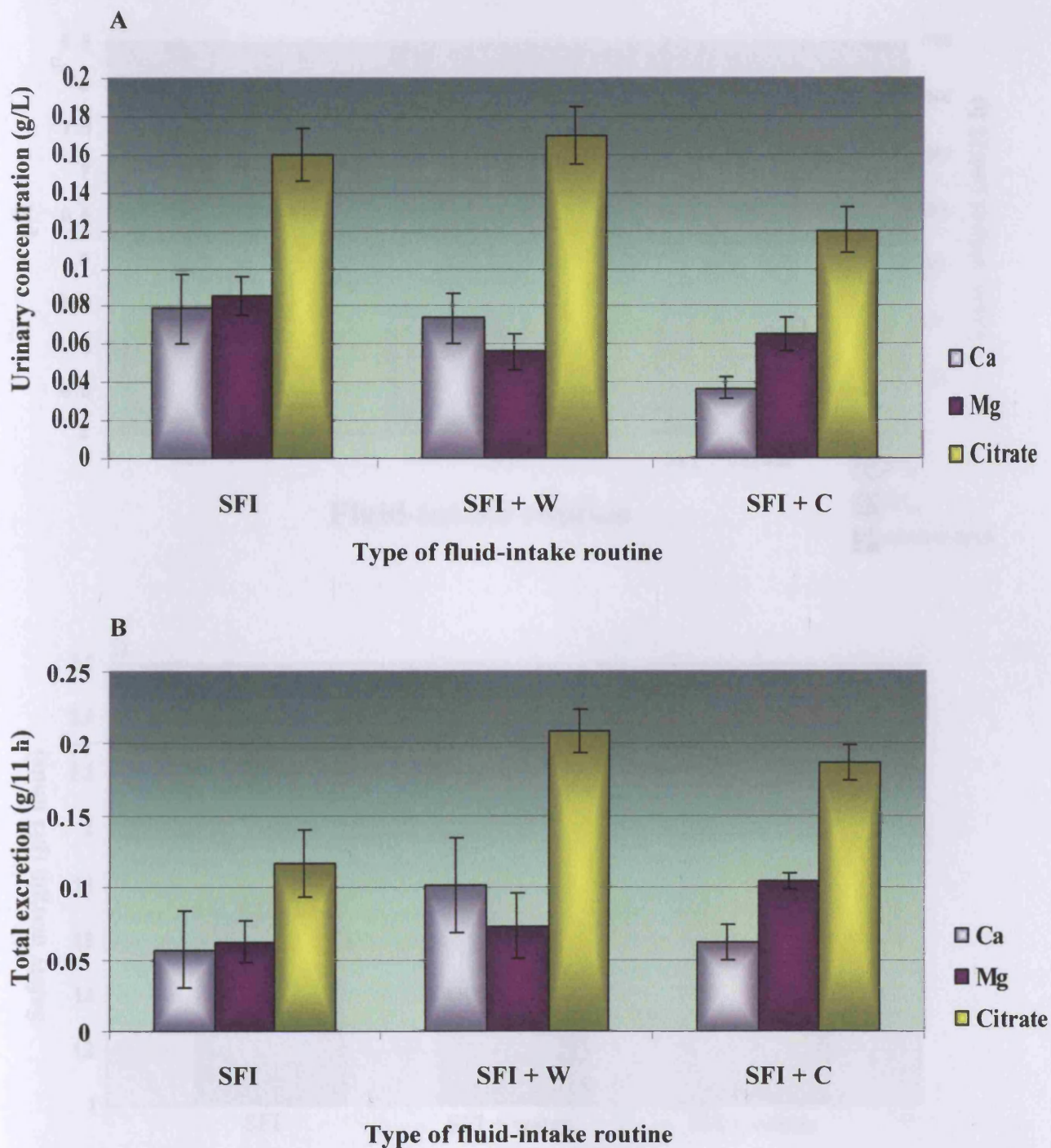
Figure 70. The *mean Ca, Mg and citrate concentration in urine and *mean total Ca, Mg and citrate excreted under different fluid intake regimens

Urines were collected between 0600 – 1700 h on three separate occasions under the different fluid intake regimens. (A) shows the mean Ca, Mg and citrate concentrations in the different urines. (B) shows the total Ca, Mg and citrate excreted during the same period. *The means ± SE of the means calculated from three replicated sampling periods are indicated.
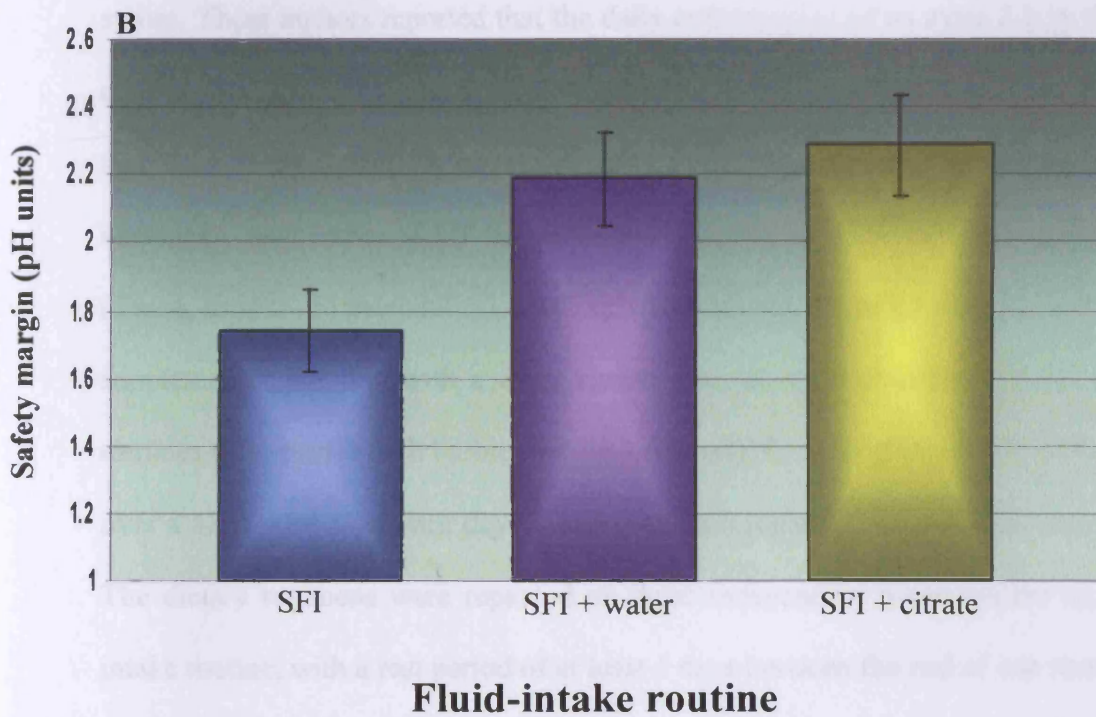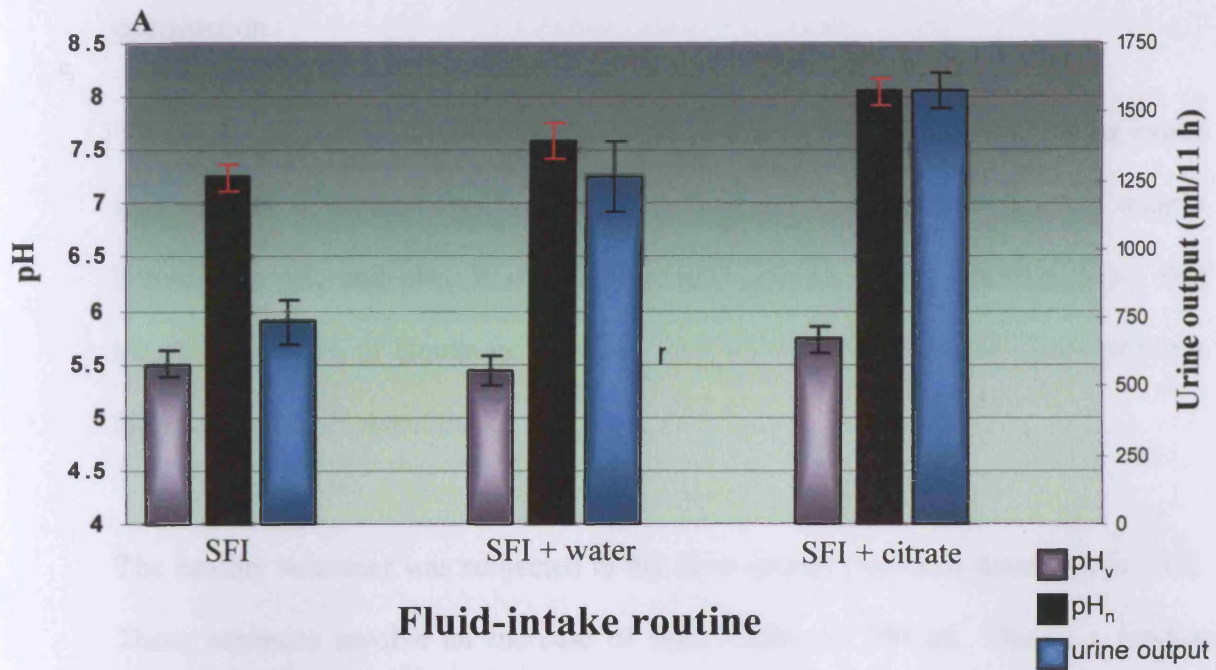
Figure 71. The *mean pH_v, pH_n, urine output and pH safety margin of urines voided under different fluid intake regimens

All voided urine was collected between 0600 – 1700 hours on 3 separate occasions for each dietary regimen. (A) shows the mean pH_v, pH_n, and urine output. (B) shows the mean safety margin ($pH_n – pH_v$) in pH increments. *The means ± SE of the means calculated from three replicated sampling periods are indicated.

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3.4.3. The effect of increased fluid and citrate intake on the rate of catheter encrustation

The results presented in Figure 71 show that by increasing the fluid and citrate intake it is possible to elevate significantly the pH_n of the resulting urine and the margin between its pH_v and pH_n. It is therefore now possible to test the hypothesis that increasing the pH_n of human urine in this way will reduce the rate at which catheters are encrusted by *P. mirabilis*.

The healthy volunteer was subjected to the three dietary regimens described in 3.4.2. These regimens involve an increase of fluid intake of 750 ml. This is a modest increase compared to that suggested by Seltzer *et al.* (1996) as a treatment for kidney stones. These authors reported that the daily consumption of an extra 2 L in the form of lemonade, effectively reduced stone-forming potential. A fourth regimen based on that devised by Seltzer *et al.* (1996) was therefore given to the volunteer. This involved a fluid intake of 140 ml lemon juice (Tesco) and 250 ml Summer fruit drink (Tesco, used to aid palatability), made up to a final volume of 2 L with tap water. This supplemented the diet with a daily citrate dose of approximately 6.0 g/L. Dietary routines were started 24 h before the first urine sample was collected. All voided urine over a 48 h period between days 2 and 3 of each regimen was collected and pooled. The dietary regimens were repeated on three independent occasions for each fluid intake routine, with a rest period of at least 4 days between the end of one routine and the start of another. The volunteer was encouraged not to change any other aspects of their dietary or lifestyle habits.

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The pooled urines were filter sterilized, firstly through cotton wool to remove large debris, and then through a 0.8 µm and finally 0.2 µm micro-pore filters (Sartorious). Aliquots (40 ml) of the filtered urine were taken for determination of pH_n. A sample (5 ml) was removed for AAS determination of calcium and magnesium content, and a further 5 ml sample removed for citrate and pH_v determination. Total urine output over the 48 h period was also recorded. The sterilized urines were supplied at a standard flow rate of 720 ml/24 hours (0.5 ml/minute) to bladder models fitted with all-silicone catheters and inoculated with 10 ml of 4 h culture of *P. mirabilis* NSM6 (approximately 10^8 cfu/ml). Experiments were based on three replicates for each urine type with mean times to catheter blockage recorded. Viable cell populations and pHs of voided bladder model urine were taken at the start of the experiment and at 24 h intervals thereafter.

Table 15 shows the profiles and characteristics of the urine collected during the four different dietary regimens. The mean values of pH_v ranged from pH 5.91 to pH 6.77 but were not significantly different ($P > 0.05$) from each other. The mean pH safety margins were also not significantly different ($P > 0.05$), ranging from pH units of 1.1. in urine collected under the SFI + C regimen to 1.81 in the urine collected under the lemonade diet. The mean nucleation pH of the urine however, increased significantly ($P < 0.05$) between urine voided under the SFI diet (pH 7.27 ± 0.15) compared to urine voided under the lemonade diet (8.10 ± 0.17). The mean urine output per 48 h under the different dietary conditions ranged from 3633 ± 90 ml under the SFI diet to 7433 ± 230 ml under the lemonade diet. Statistical analysis (ANOVA) revealed that the mean volume of urine voided under the lemonade diet was significantly higher ($P < 0.05$) than that of the other dietary regimens. The mean urine output under the SFI + C regimen was also significantly higher than urine output under the SFI routine ($P <$

0.05). Statistical analysis revealed the mean Ca concentration in urine (ANOVA) and median values of total Ca excreted under each regimen over the 48 h period (Kruskal –Wallis) were not significantly different ($P > 0.05$). The mean Mg concentration was significantly higher ($P < 0.05$) in SFI urine compared to SFI + C urine and urine voided under the lemonade diet, but the mean total amounts of Mg excreted over the three 48 h periods were not significantly different ($P > 0.05$) among urines. Although there was considerable disparity in the amount of citrate consumed among the dietary regimens, statistical analysis revealed there were also no significant differences ($P >$ 0.05) in mean citrate concentrations or in mean values of total citrate excreted over the 48 h periods.

The mean times all-silicone catheters took to block in bladder models supplied with the pooled urine from the different dietary regimens are presented in Figure 72A. The mean catheter blockage times were: 25.22 ± 5.11 h (SFI); 35.56 ± 2.27 h (SFI + W); 44 ± 8.14 h (SFI + C) and 118.08 ± 20.06 h (lemonade diet). Although variability was observed in the mean lifespan of catheters, differences were not significant ($P > 0.05$) among mean catheter blockage times in bladder models supplied with urine collected under the SFI, SFI + W and SFI + C regimens. The mean lifespan of catheters in bladder models supplied with urine from the lemonade diet however, was significantly ($P < 0.05$) longer compared to that from models supplied with urine from the three other regimes.

The individual blockage times of each specific catheter were plotted against the pH_n of the urine supplied to its respective bladder model (Figure 72B). Pearson's correlation performed on the log of the urine pH_n versus the log of the catheter

blockage time indicated that the correlation between urinary pH_n and the rate of catheter encrustation was highly significant ($P < 0.001$, correlation coefficient value 0.844). The mean pH and mean viable cell populations from residual bladder model urine during the course of experiments (Table 16) show that the mean cell densities in residual urine remained between 10^7-10^8 in all bladder models, while the mean pH's of the bladder model urine in the experiments remained above pH 8 and were consistently > pH 8.5 at time of catheter blockage.

Constituents and parameters of voided urine

Urine type	pH$_v$	pH$_n$	pH safety margin	Urine output (ml/48 h)	[Ca] g/L	[Mg] g/L	[Citrate] g/L	Excreted Ca (g/48 h)	Excreted Mg (g/48 h)	Excreted citrate (g/48 h)
SFI	5.91 ± 0.26	7.27 ± 0.15	1.36 ± 0.09	3633 ± 90	0.076 ± 0.03	0.059 ± 0.01	0.16 ± 0.02	0.28 ± 0.101	0.21 ± 0.03	0.58 ± 0.06
SFI + W	6.1 ± 0.21	7.7 ± 0.09	1.61 ± 0.22	4116 ± 160	0.046 ± 0.003	0.048 ± 0.008	0.19 ± 0.03	0.19 ± 0.02	0.2 ± 0.04	0.78 ± 0.15
SFI + C	6.77 ± 0.05	7.86 ± 0.19	1.1 ± 0.17	4833 ± 320	0.093 ± 0.023	0.032 ± 0.004	0.23 ± 0.05	0.48 ± 0.06	0.16 ± 0.01	1.1 ± 0.18
Lemonade diet	6.29 ± 0.35	8.10 ± 0.17	1.81 ± 0.23	7433 ± 230	0.04 †	0.023 ± 0.003	0.12 ± 0.02	0.17 ± 0.019	0.3 ± 0.01	0.88 ± 0.16

SFI = standardised fluid intake (1300ml/24 hours), SFI + W = standardised fluid intake + 750 ml water, SFI + C = standardised fluid intake + 750 ml citrate drink, Lemonade diet = 2L of lemonade drink + any other fluid consumed † all 3 values 0.04 g/L

Table 15.   The effect of four different fluid and citrate intake routines on the characteristics of urine

Urine was collected over 48 h and pooled. This was performed on three separate occasions for each fluid and citrate intake regimen. The means ± SE of the means were calculated from the results of three replicated sampling periods.
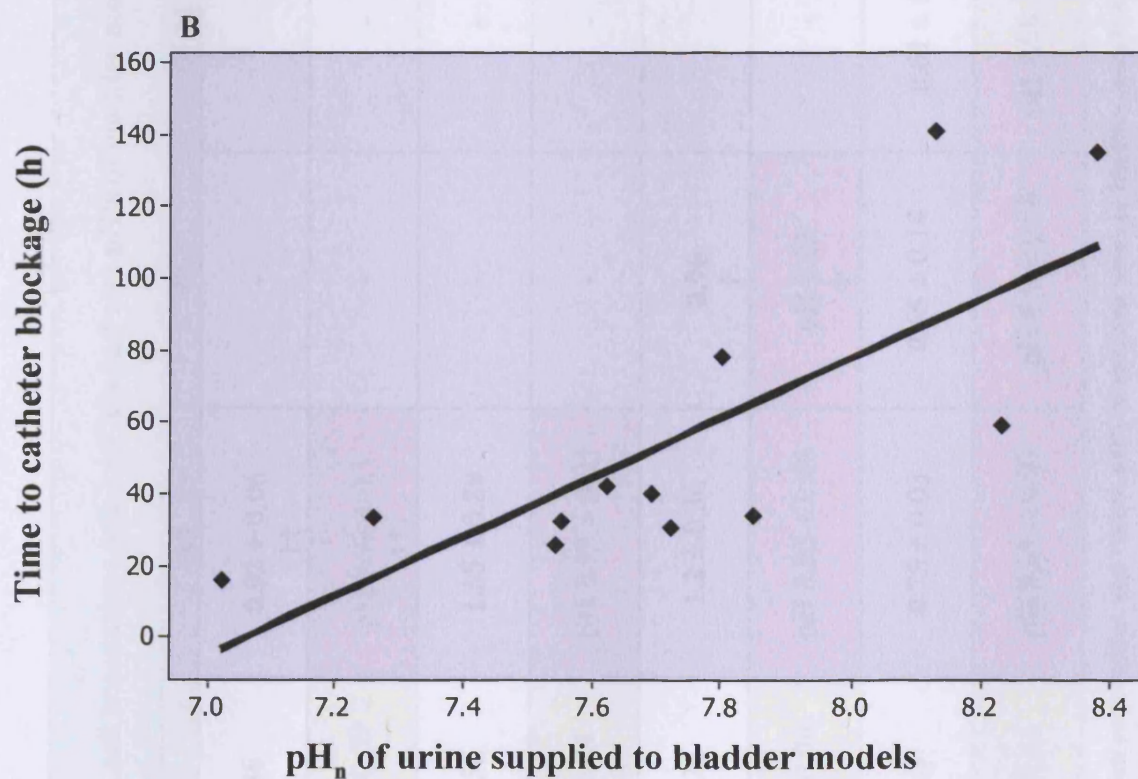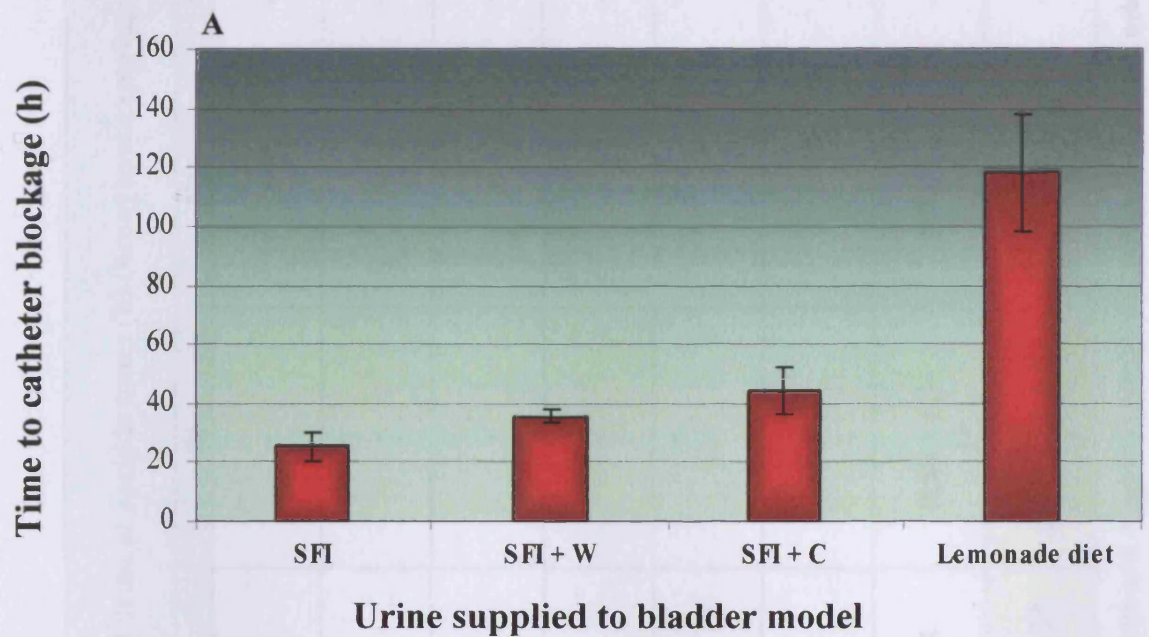
Figure 72. (A) The *mean times to catheter blockage in bladder models supplied with urine voided from various dietary conditions and infected with *P. mirabilis* NSM6 (B) correlation of the urine pH_n with catheter blockage times

Bladder models fitted with all-silicone catheters and inoculated with *P. mirabilis* NSM6 were supplied with 48 h pooled urine from different dietary routines. (A) * the means ± SE of the the means were calculated from three replicated experiments. (B) shows the relationship between urinary pH_n and catheter blockage time.

Fluid intake	Mean viable cell populations (cfu x 10^8 cfu/ml) and pH's of bladder model urine at various times (h) throughout experiments						
	0	24	48	72	96	120	144
SFI	2.3 ± 0.46	0.92 ± 0.06 ††	-	-	-	-	-
	pH 8.53 ± 0.09	pH 8.92 ± 0.1 ††	-	-	-	-	-
SFI +W	1.67 ± 0.56	1.55 ± 0.89	-	-	-	-	-
	pH 8.4 ± 0.14	pH 8.93 ± 0.05	-	-	-	-	-
SFI + C	1.36 ± 0.24	1.2 ± 0.36	0.96 †	-	-	-	-
	pH 8.62 ± 0.04	pH 8.95 ± 0.08	pH 9.02 †	-	-	-	-
2 L lemonade	1.96 ± 0.3	0.75 ± 0.03	0.65 ± 0.14	0.61 ± 0.14	0.99 ± 0.31 ††	3.0 ± 1.5 ††	-
	pH 8.84 ± 0.12	pH 8.91 ± 0.09	pH 8.9 ± 0.08	pH 8.98 ± 0.08	pH 9.02 ± 0.02 ††	pH 9.02 ± 0.09 ††	-

Table 16.   The *mean viable cell populations and *mean pH's of residual urine in bladder models infected with *P. mirabilis* NSM6 and supplied with pooled urine voided under various fluid intake regimens

Bladder models were inoculated 10 ml of a 4 h culture of *P. mirabilis* NSM6. * The means ± SE of the means (unless otherwise stated) were calculated from the results of three replicated experiments. (†) one replicate only (††) means based on values from two replicates (-) all models had blocked.

4. Discussion

4. Discussion

The early stages in the process of biofilm formation on indwelling urological devices are not well understood. In the cases of prosthetic devices implanted into the vascular system or as joint replacements, there is strong evidence that the initial step is their rapid coating with the host proteins such as albumin, fibrinogen, fibronectin and laminin from body fluids (Donlan and Costerton, 2002). There is some evidence to suggest that similar events occur when devices are exposed to urine. Ohkawa *et al.* (1990) noticed that fibrillar deposits were present on the surfaces of almost all indwelling urethral catheters removed from patients after ≤3 days. They used immuno-fluorescent staining and microscopy to demonstrate these deposits consisted of fibrin. In another study Santin *et al.* (1997) showed proteins including albumin and α-1-microglobulin were present on the surfaces of ureteral stents removed from patients.

A study of the early stages of catheter encrustation by crystalline biofilm in patients would require the insertion and removal of catheters at intervals over the first few hours of their placement. This would be a clinically unnecessary invasive intervention and ethical considerations would make such a study difficult to perform. One advantage of the bladder model is that catheters can be inserted and removed for examination at will. The experiments can also be performed under standardized, reproducible conditions that are impossible to achieve in a clinical investigation.

In patients suffering from recurrent catheter encrustation and blockage, catheters are exposed immediately on placement to alkaline urine cultures of *P. mirabilis*. Under these circumstances the urine will contain crystalline material in addition to bacterial

cells. The conditions chosen for the experiments in the bladder model were intended

to simulate these circumstances. The process was observed on a range of catheters

manufactured from latex and silicone and on devices impregnated with antimicrobial

agents. The catheterised bladder model was used in conjunction with a variety of

scanning electron microscopy techniques and X-ray microanalysis. These methods

provided substantial information about the early stages of *P. mirabilis* crystalline

biofilm development under conditions intended to reflect the circumstances applying

in patients who are enduring the complication of recurrent catheter encrustation.

4.1. The development of crystalline *Proteus mirabilis* biofilms on indwelling urinary catheters

The results of the initial set of experiments reported in Figure 5 and Tables 1 and 2

show that when all the currently available catheters were inserted into bladder models

containing residual urine at pH 8.5 and *P. mirabilis* at 10^8 cfu/ml they block within 40

hours. These findings confirm those reported by Morris *et al.* (1997) and Morris and

Stickler (1998a), that *P. mirabilis* rapidly blocked AS, SCL, HCL and HSCL catheters

with crystalline biofilm. This present study demonstrated that catheters impregnated

with nitrofurazone were also vulnerable to blockage with *P. mirabilis* crystalline

biofilm. In contrast, AS and HCL catheters inflated with triclosan were not affected

by crystalline biofilm and drained freely for the 7-day experimental period. It was also

significant that the pH of the urine in models fitted with the triclosan impregnated

catheters dropped rapidly from 8.5 to between 6.0 – 6.5 within 4 h and remained acid

for the 7-day experimental period. In the case of the HCLT catheter the number of

viable cells in the residual urine dropped from 10^8 cfu/ml to 10^3-10^6 cfu/ml. The AST

catheter had an even greater bactericidal effect. While the mean number of viable

cells present at the start of the experiment was 2×10^8 cfu/ml, at 4 h and thereafter for

7 days no viable cells were recovered from the urine. Catheters inflated with triclosan

are clearly able to resist *P. mirabilis* crystalline biofilm development, these results

confirming the findings of Stickler *et al.* (2003a).

4.1.1. Observations on the development of catheter encrustation using LVSEM

The examination by LVSEM of catheters removed from the models at 1 h, 4 h, 12 h,

and at blockage or 7 days revealed the development of the encrustation around the

eye-lets and on the lumenal surfaces. The striking feature of the micrographs is that

although there are signs of encrustation after 1 h on most of the catheters, little further

accumulation of crystalline material seems to take place in the next 11 h. It is after 12

h that the bulk of crystalline biofilm develops. The results presented in Table 2 show

that in all cases when the supply of urine is turned on, the pH of the residual urine in

the bladder chamber drops. It is only after 12 h that the pH rises to around 8.0 and

above. The higher the urinary pH is above its nucleation pH (pH_n) the more calcium

and magnesium will be deposited from the urine (Suller *et al.*, 2005), thus explaining

the accelerated encrustation after 12 h.

The experiments with the NF catheters are interesting as they suggest little if any

encrustation takes place in the first 12 h, by 29 h however, the eye-holes and central

channels are blocked by extensive crystalline deposits. The results presented in Table

2 show that from 2 - 4 h the urinary pH fell below its pH_n of 6.7 (Figure 25), by 12 h

it has recovered to 7.4 and by 18 h to 8.3. The viable cell count also drops below 10^7

cfu/ml in these cultures until 18 h (Table 1). These results suggest that the

nitrofurazone eluting from these catheters has an initial effect, perhaps temporarily

slowing the growth of *P. mirabilis* in the continuous culture system. It is clear that by 18 h the culture has recovered and encrustation is proceeding rapidly. It is possible that the majority of the nitrofurazone contained in these catheters elutes over the initial 12 h. This seemed to be the case as it was clear from observations that the voided urine in these experiments displayed a strong yellow pigmentation (nitrofurazone is yellow in colour) over this period. It is likely that most of the nitrofurazone eluted from the catheter in this time, after which *P. mirabilis* was able to proliferate rapidly, returning the residual urine to > pH 8.0. The micrographs of the triclosan-treated catheters confirm that at 7 days there was no sign of encrustation on the eye-lets or in the lumen. It is likely therefore that these catheters would drain freely for considerably longer than the 7-day experimental period.

4.1.2. Observations on the development of crystalline catheter biofilms using HVSEM

The surfaces of unused catheters were examined using HVSEM (Figures 11 and 12) and showed that a variety of surface-associated irregularities were present among the catheter types. The rough nature of catheter surfaces was previously described by Stickler *et al.* (2003b) who reported that rough areas of catheter surfaces encouraged bacterial colonization. They used HVSEM images to show that the *P. mirabilis* colonization of latex-based catheters begins in the crevices and depressions of the surfaces around the eye-lets of the catheter. The poor quality of the surface at the eye-lets of catheters is caused through the mechanical insertion of the eyehole and was evident on all catheters in this present study. Apart from physically 'trapping' bacterial cells, it is possible that the 'excess' catheter material that protrudes out from the catheter body at the eye-let may act as a nidus for crystal nucleation. The eye-let

of the AS, NF and HSCL catheters appeared to be much smoother than the corresponding areas of SCL and HCL catheters, although they did display some surface irregularities. The lumenal sections of the AS catheter also appeared to be generally smoother than other catheters. The central channel of the NF impregnated catheter (Figure 18A) however, was rough and had a granular appearance which may have been a result of the impregnated antimicrobial. The main body of latex-based catheters were also extremely rough and often littered with diatomaceous silica-skeletons. The source of these silica-skeletons is the diatomaceous earth used in the manufacturing process of latex-based catheters to prevent the catheter material sticking to specific machine parts during catheter production. If bacterial colonization and encrustation of catheters are to be tackled seriously it is fundamental that these devices are made to high standards. Stickler *et al.* (2003b) pointed out that the catheters currently available, with their roughly engineered eye-holes and narrow central channels are extremely vulnerable to blockage by crystalline biofilm. The images presented in Figures 11 and 12 confirm that there is plenty of scope to improve catheter design and manufacturing standards.

The HVSEM images presented in Figures 13 – 16 suggest a common sequence of events in the development of the crystalline biofilm on the AS, SCL, HCL and HSCL catheters. After only one hour in the models, the catheter surface at the eye-lets and the lumen just above the retention balloon is covered with a layer of material that seems to be comprised of aggregates of micro-crystals. The material forming this "foundation layer" is similar in structure to crystalline forms of calcium phosphate found by He *et al.* (2003). Calcium phosphate (hydroxyapatite) is a fundamental composite in the formation of bones and teeth and these workers observed the protein-

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mediated formation of this compound *in vitro*. The structure of calcium phosphate formed here was apparently identical to that of microcrystals forming the "foundation layer" shown in Figures 13 - 16. Subsequently X-ray microanalysis confirmed that this layer was comprised predominantly of calcium and phosphate (Figures 23 and 24). There was little sign of struvite crystals in the early stage which was not surprising as the urine used in these experiments (like real urine) contained higher concentrations of calcium than magnesium. Another factor is that calcium phosphate precipitates from urine at a slightly lower pH than the magnesium salt (Suller *et al.*, 2005). Crystal formations on catheter surfaces however, were not exclusive to catheters removed from infected bladder models. The HVSEM micrographs of control catheters (Figure 19) incubated in sterile urine for seven days showed that crystal formations also developed on these catheters. Material resembling crystalline calcium phosphate was predominantly observed at the eye-let area (Figure 19 A) which is often the area associated with a rough topography. This adds further weight to the argument that rough surfaces are likely to act as a foci for crystal nucleation.

The *P. mirabilis* colonization of these catheters (Figures 13 - 16) also displayed a common theme, and microcolonies were observed on the calcified layer on all these four catheter types at 4 h. The "foundation layer" however, was not essential for *P. mirabilis* adherence. Isolated patches of the catheter surfaces free of microcrystalline deposits were also colonized by *P. mirabilis* (Figure 17). As was observed with the crystalline deposits, the eye-let and lumenal areas just above the retention balloon were the first areas to become colonized by bacterial cells. At 18 h these areas of the catheters were comprehensively covered with a densely populated *P. mirabilis* crystalline biofilm matrix. The numbers of viable cells adhering to AS, SCL, HCL

catheter sections (Figure 22) correlated with the HVSEM observations. These experiments showed that colonization was heaviest at the eye-let area and first few centimetres down the lumen, reaching around 10^7 cfu/mm catheter length. The numbers of viable cells adhering to the lumen 16 cm down from the eye-hole generally remained an order of magnitude lower. The results also indicated that cells were recovered from the catheter surfaces between 1 – 4 h although HVSEM observations at these times showed only low levels of colonization. The increase in cell numbers visualized in the HVSEM observations from 12 h onwards generally correlated well with the numbers of cells recovered from the catheter sections. It was noticed in some instances that the surfaces of *P. mirabilis* cells initially colonizing the HSCL catheter (Figure 16) appeared extremely irregular. Winters *et al.* (1995) demonstrated that *P. mirabilis* cells can act as a nidus for the nucleation of calcium phosphate which envelope the cells as the crystalline biofilm develops. It is possible therefore that the uneven nature of the surface was due to calcium phosphate deposits. The nature of the deposits on the catheter surface surrounding these cells seemed to indicate this was the case. The irregular appearance of these cells however, may have been a result of the antibacterial effect of silver eluting from the HSCL catheter. Silver is active on a number of cellular targets and is known to react with the amino acid residues of proteins and interfere with DNA replication. They have also been associated with disruption to membranes, the cell envelope and the cell wall, which can manifest in surface abnormalities (Russell *et al.*, 1994; Percival *et al.*, 2005).

The HVSEM observations showing *P. mirabilis* crystalline biofilm development on NF catheters (Figure 18) suggested that encrustation and colonization on this catheter was initially delayed. There was no evidence of *P. mirabilis* colonization on these

catheters up until 12 h, and any encrustation was negligible. The eye-let and lumenal section just above the retention balloon at 12 h was colonized by *P. mirabilis* microcolonies adhering directly to the catheter surface and to isolated patches of crystalline material that had accumulated at these sites. However, at 18 h the crystalline biofilm was well established and was indistinguishable from the crystalline biofilms observed on the other commercially available catheter types at 18 h. As the NF catheters went on to block at a mean time of 29.5 it appears that any effect of nitrofurazone on *P. mirabilis* is short-lived and is unable to prevent rapid crystalline biofilm formation on these catheter surfaces.

4.1.3. *P. mirabilis* crystalline biofilm development on triclosan treated catheters

The HVSEM micrographs of crystalline biofilm development on triclosan treated catheters (Figures 20 and 21) showed that the microcrystalline "foundation layer" did initially form on the surfaces of AST and HCLT catheters. The "foundation layer" however, appeared to be transient in its nature. It seemed to dissolve into the surrounding fluid as the pH of the residual urine in the bladder chamber became acidic. The result of this was a gradual depletion of the "foundation layer" with catheters removed from the infected bladder models after 7 days showing very little in the way of deposited crystalline material. It was interesting that corresponding sections of the NF catheter failed to acquire this early "foundation layer" even though the pH of the residual bladder model urine was similar to that observed in the triclosan-treated experiments over the initial 4 h. A possible reason for this could be due to the release of nitrofurazone from the catheter surface into the urine. If the catheter surface becomes unstable and degrades as the nitrofurazone is released,

crystalline deposits and bacterial cells would be prevented from adhering as they would have no permanent surface to colonize.

Significant bacterial colonization of the AST and HCLT catheters failed to develop. *P. mirabilis* colonization of the surfaces of AST catheters was rarely observed and consisted of single cells or groups of < 5 cells. No biofilm-associated cells were observed on AST catheters even on those removed from 7-day experiments. The physical appearance of many of the cells that were present appeared 'unhealthy' however, which might have been due to the effect of triclosan. A number of cells colonizing the lumenal sections of the HCLT catheters also had a similar unhealthy appearance. Triclosan has more than one mode of action, but is known to disrupt cell wall integrity via inhibition of fatty-acid biosynthesis (Russell, 2004). There were instances however, where patches of *P. mirabilis* biofilm were observed on HCLT catheters. These were not crystalline in nature and were found consistently on the eye-let region of catheters removed at 12 and 18 hours, and at 7 days. Cells within the biofilm appeared healthy, which covered small areas of around 200 x 150 μm. Previous work by Jones *et al.* (2004) demonstrated that although triclosan diffused through the balloons of latex-based catheters, unlike silicone catheters it did not impregnate the catheter material. It is possible that under these circumstances cells could colonize these catheters but their subsequent development into mature crystalline biofilms was prevented by the bacteriostatic effect of the continued diffusion of the triclosan from the balloon into the urine.

4.1.4. Lessons for catheter design

The rapid development of a calcified "foundation layer" on catheters in these *in vitro* experiments is an important finding. It has implications for the design of catheter biomaterials resistant to encrustation. Novel catheters with surfaces aimed at inhibiting the formation of the protein "conditioning layer", preventing bacterial adherence or with an antibacterial immobilised to the surface are likely to be ineffective. The "foundation layer" would immediately alter the surface property of a catheter and allow bacteria to colonize the device in the absence of the "conditioning layer". It would also form a protective barrier between the immobilised antibacterial and bacterial cells. It is unlikely that a single catheter material will be developed with comprehensive properties allowing it to resist the adherence of host proteins, bacterial cells and the foundation layer. The experiments using catheters impregnated with triclosan suggested that *P. mirabilis* crystalline biofilm formation is preventable on currently available catheter materials. The antibacterial was effective because it quickly reduced bacterial populations in the residual urine, which in turn caused the urinary pH to drop below its pH_n. Alternative antimicrobial catheters would have to have similar properties in order to produce effective results. A major concern surrounding the use of antimicrobial catheters however, is the persistent exposure of bacterial cells to sub-inhibitory doses of the antibacterial agent.

The levels of resistance to triclosan recorded for mutant strains of various species have not generally been a concern since when triclosan is used as a disinfectant it is usually applied at concentrations way above the MICs (Lear *et al.*, 2002). The concentrations that elute into the urine from the catheter balloon however, are close to the MICs of clinical isolates (Jones *et al.*, 2006). A strain of *P. mirabilis* with an MIC

of 40 µg/ml for triclosan was obtained by exposure to the biocide in the laboratory. This strain was able to produce crystalline biofilm and block catheters that had been injected with triclosan in the bladder model (Jones *et al.*, 2005). The selection of such resistant strains of *P. mirabilis* in clinical practise would thus undermine the strategy. Another potential problem is that one of the species capable of encrusting catheters *Pv. rettgeri*, and at present occasionally found in the catheterised urinary tract, is resistant to triclosan (MIC, 64 µg/ml, Broomfield, 2007). There is thus the potential that the extensive use of the triclosan strategy could select for resistant mutants of *P. mirabilis* and catheter-encrusting species that are intrinsically resistant. A less problematic strategy for encrustation control would be to prevent crystal formation in the urine by maintaining the pH of urine below that of its pH_n. Achieving this without the use of antimicrobials would simultaneously avoid the issue of antimicrobial resistance and the need for novel materials that inhibit crystalline biofilm development.

4.1.5. Observation of HSCL catheters removed from long-term catheterised patients

The Bardex I.C. hydrogel/silver-coated latex catheter contains metallic silver in a gold/palladium coating. The extended and slow release of silver ions from the matrix into the surrounding fluid provides antibacterial activity over both internal and external surfaces (Davenport and Keely, 2005). Recent studies claiming that the antimicrobial layer stops bacterial adhesion and reduces infection have been heavily criticised for their poor quality and inappropriate control measures (Brosnahan *et al.*, 2004). For example, Ahearn *et al.* (2000) reported that HSCL catheters were less prone to adherence by *Ps. aeruginosa* than control catheters. However, the control catheters were all-silicone and not a standard hydrogel-coated latex catheter. Catheter

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sections were incubated in minimal media instead of urine, and therefore failed to take

into account the effect of any conditioning layer developing on the catheter that may

have altered its surface properties. Furthermore, the effect of a 'vigorous washing'

stage on these two very different catheter materials was also ignored. Previous bladder

model studies carried out by Morris and Stickler (1998a) clearly demonstrated that *P.*

mirabilis rapidly blocks HSCL catheters with crystalline biofilm. The colonization of

the HSCL catheters by *P. mirabilis* in these present bladder model studies (Figure 16

and Figure 17 D) firmly supports these findings. The catheters were coated in a

calcium phosphate "foundation layer" which was rapidly colonized by *P. mirabilis*

cells. The micrographs presented in Figures 27-31 confirm that in patients these

catheters also become covered in foundation layers. Cells colonize the crystalline

layer and extensive crystalline biofilm causes rapid catheter blockage.

The information provided in Table 3 illustrates that all the HSCL catheters from

clinical origin were colonized with multi-species crystalline biofilm. The catheters

removed from patients 1 and 6, and also a 2nd catheter removed from patient 4, had

blocked rapidly with crystalline biofilm after insertion (10, 11 and 14 days

respectively, Figures 26 – 1 A + C, 6 A + C and 7 A + C). Another catheter had

multiple bacterial species and significant encrustation after only 5 days *in situ* (Figure

30 C + D). The bacteriological examination of the catheter sections removed from the

devices only confirmed the presence of two urease-producing species (catheters from

patient 3 and 5). However, all catheters had been stored at 4 °C for a number of

weeks, and it was possible that a proportion of the bacterial species present in the

biofilm were no longer viable and could not be isolated. This appeared to be the case

as HVSEM examination of catheter sections revealed the presence of bacilli and cocci

shaped bacteria in the biofilm communities of all five other catheters, even though the

bacteriological studies only resulted in the isolation of coccal species (Figures 27 - 31). Considering the level of encrustation observed on these catheters it was likely that all patients were colonized by urease-producing bacteria. The pH of urine at the time of catheter removal from patient 2 (8.0) suggested the presence of a urease-producer, while patient 3 who was infected by the urease-producing *Pv. rettgeri* had a urine pH of 9.0. The crystalline material associated with these biofilms were similar of that found within *in vitro* crystalline biofilms and comprised of "rosette-like" formations typical of calcium phosphate (Figure 27 A and B and 31 A), and the microcrystalline calcium phosphate (hydroxyapatite) accumulations similar to the "foundation layer" (Figures 28 A and B, 29 D and 30 C). Large crystals typical of those formed by struvite (Figures 28 D, 30 B) were also observed on a number of the catheters (Harrison, 1957).

While the sensitivity of organisms such as *E. coli* and *Ps. aeruginosa* to silver is known (MIC of $AgNO_3$, 8 - 80 mg/L) there is little information in the literature on the sensitivity of *P. mirabilis* (Chopra, 2007). It would be useful to know the MICs of silver ions for a range of urinary tract pathogens especially *P. mirabilis* and other potent urease producers. Chakravarti *et al.* (2005) reported that catheters fitted with silver electrodes connected to a 9 v battery released 0.25 µg/ml of silver ions into the residual urine of *P. mirabilis* infected bladder models, which had a significant effect on the rate of catheter encrustation. BARD has not yet provided information on the extent of silver released from their catheters. During the present study however, urine that had passed through the catheters in the bladder models was collected and assayed for its silver content by atomic absorbance spectroscopy. Values of only 0.01 – 0.02 µg/ml were recorded. These are unlikely to have a significant antibacterial effect.

4.2. The role of MR/P fimbriae in *P. mirabilis* crystalline biofilm formation

Little is known about the role of *P. mirabilis* MR/P fimbriae in the catheterised urinary tract especially in the development of *P. mirabilis* crystalline biofilm on catheter surfaces. Previous studies have shown that these type IV fimbriae are expressed in the majority of *P. mirabilis* isolates and are associated with cell-aggregation, attachment to surfaces and biofilm formation. MR/P fimbriae have also been shown to be an important virulence factor in the infection of the 'normal' urinary tract (Mobley and Chippendale, 1990; Bahrani *et al.*, 1994; Caliskan, 2004; Jansen *et al.*, 2004). It would improve our understanding of *P. mirabilis* catheter-associated urinary tract infection (CAUTI) if the role of these fimbriae in the colonization and crystalline biofilm development of urinary catheters was clear.

The availability of a wild type *P. mirabilis* isolate and its isogenic *mrpA* mutant enabled the investigation of the role of MR/P fimbriae in the early stages of crystalline and non-crystalline biofilm formation on all-silicone catheters. Initially the bladder model system was used to generate the alkaline conditions similar to the catheter replacement events discussed in Section 4.1. The crystalline biofilm on the catheters removed from the infected bladder models at various times were examined using scanning electron microscopy. In addition to this, the formation of non-crystalline biofilms by the two test strains was also observed when urine was supplemented with a urease inhibitor that kept the urine acidic.

4.2.1. Expression of MR/P fimbriae in *P. mirabilis* isolates

Initially twenty-seven *P. mirabilis* isolates from a range of environmental sources including river water, sewage, catheter-biofilm and CAUTI, were tested for the ability to produce MR/P fimbriae. The results of the haemagglutination assays presented in Table 4 show that all the wild type *P. mirabilis* isolates tested were able to express MR/P fimbriae. This confirmed the findings of Mobley and Chippendale (1990) and Caliskan (2004). The results of the haemagglutination assay presented in Figure 32, confirmed that the WT and the *mrpA* mutant *P. mirabilis* isolates used throughout this study were positive and negative for MR/P expression respectively.

4.2.2. Inhibition of urease with fluorofamide

Previously, Morris and Stickler (1998b) reported that fluorofamide is a potent inhibitor of *P. mirabilis* urease and is effective in preventing catheter encrustation without having bactericidal effects. It was decided that fluorofamide would be the urease inhibitor used in this present study to observe biofilm development by the test strains in the absence of crystal formation. The results of the 4 h batch culture experiments incubated at 37 °C (Section 3.2.2) indicated that 4 µg/ml of fluorofamide was able to inhibit *P. mirabilis* urease action. Cultures incubated under these conditions reached $>10^8$ cfu/ml (not shown) but remained acidic (<pH 6.5) preventing the urinary pH from exceeding the pH_n (~pH 6.7).

4.2.3. Preparation of WT and *mrpA* cultures for bladder model experiments

Cultures used in bladder model experiments that were designed to determine the role of MR/P fimbriae in crystalline biofilm formation were grown statically over a 12 h period at 25°C. The results presented in Figure 33 show that fluorofamide at 4 µg/ml concentration had no bactericidal affects on WT or *mrpA* strains incubated under these conditions. Cultures incubated in the presence and absence of fluorofamide were able to reach similar cell densities (10^8 cfu/ml, $P = 0.81$) during the incubation period. Although the mean pH of the WT and mutant batch cultures incubated with fluorofamide reached 6.75 and 6.78 respectively (similar to the pH_n of the urine), no evidence of crystalline material was apparent in the respective cultures. When 10 ml of these cultures were initially inoculated into the bladder chamber the residual urine remained below the pH_n of 6.7 (not shown) and any crystal formation in the urine was negligible.

4.2.4. The ability of WT and *mrpA P. mirabilis* to encrust all-silicone catheters

The results presented in Figure 34 demonstrated that both WT and *mrpA P. mirabilis* strains were able to block AS catheters. The mean catheter blockage times for the WT and *mrpA* strains (29.9 ± 5.61 h and 41.9 ± 1.43 h respectively were not significantly different ($P = 0.1$). It was interesting however, that the WT strain produced alkaline conditions in the residual urine earlier than the *mrpA* strain. The urinary pH and viable cell populations recorded during the course of the experiments (Figure 35) indicate that both strains were similarly affected by washout when the urine supply was turned on. The mean pH of the residual urine (Figure 35 A) infected with the WT strain was

~8.3 at 24 h compared to ~7.5 in the *mrpA* infected models. This suggests that extensive crystalline material would have formed earlier in the WT infected models.

4.2.5. *P. mirabilis* biofilm formation on catheters under conditions favouring crystal formation in urine

The electron micrographs presented in Figures 36 - 41 illustrated that while both strains form extensive crystalline biofilms on the catheters, the timing of the sequence of events is rather different with the mutant than the wild type. At 8 h the WT has produced considerable encrustation on the surfaces around the eye-holes and on the catheter lumen (Figures 36 and 37). At this stage there is little sign of crystalline material on the catheter from the models incubated with the mutant. The pH of the residual urine at 8 h was ~ pH 7.5 and ~ pH 7.0 for WT and *mrpA* respectively (Figure 48), which would account for the differences in crystalline material on the catheter surfaces. At 12 h the pH of the residual urine in the bladder model chamber exceeded 7.5 in both cases. Although the catheter eye-let surfaces showed similar levels of encrustation at this time, the lumenal sections were heavily encrusted by the WT while those incubated with the *mrpA* mutant strain appeared free of crystalline material. It was not until 18 h that encrustation was apparent on the central channels of catheters incubated with the *mrpA* strain.

The micrographs presented in Figures 38 and 39 indicated that the WT had produced the typical crystalline biofilm on the eye-let and lumenal section of the catheters by 8 h. Over this period however, few *mrpA* cells managed to colonize the catheters (Figures 40 and 41). By 12 h the *mrpA* mutant had generated the characteristic crystalline calcium phosphate "foundation layer" on catheters. Some *mrpA* cells were

visibly attached to the foundation layer at 12 and 18 h, but it was not until 30 h that extensive colonization by cells became visible (Figure 42). This was interesting as viable cell counts recorded in the residual bladder model urine at the time when the experiments were stopped for catheter removal (Figure 49) show that population densities were similar between WT and *mrpA* strains at these times. Figure 49 also shows that the pH of the urine in the model inoculated with the mutant had reached 8.5 by 30 h. As this is well above the pH_n of the urine supplied to the model, at this late stage of the process large amounts of crystalline material would be forming and the rate of encrustation accelerating. Thus although the ways in which the crystalline biofilm develops may differ, there was no significant difference in the mean times the wild type and its MR/P⁻ mutant took to block catheters.

The observations made in the experiments where the urine was allowed to become alkaline suggest that while MR/P fimbriae are not essential for crystalline biofilm formation on catheters, they may have a role in cell-crystal aggregation. Stickler *et al.* (2006) reported that cell-crystal aggregations forming in alkaline urine were responsible for *P. mirabilis* adherence to agarose and agarose-hydrogel surfaces. These surfaces were resistant to colonization by *P. mirabilis* cells when the crystals were absent in acidic urine. Calcium phosphate is a known aggregator of bacterial cells (Berry *et al.*, 1997), and it would be interesting to carry out adherence experiments similar to those described by Stickler *et al.* (2006) with the WT and *mrpA* strains on various surfaces in acidic and alkaline urine. The adhesive properties of bacterial cells to surfaces have been evaluated using atomic force microscopy (Dufrene *et al.*, 2001). It may be possible to determine differences in the affinity of

MR/P$^+$ and MR/P$^-$ *P. mirabilis* strains to calcium phosphate and catheter surfaces using similar methods.

4.2.6. Biofilm formation in acidic urine

The electron micrographs shown in Figures 43 – 48 record the colonization of AS catheters by WT and *mrpA* strains in the absence of crystal formation. Even under low magnification (Figures 43 and 44) the differences in the extent of colonization was clearly visible. The eye-let and lumenal surfaces of catheters exposed to WT infected urine for 24 and 48 h had acquired comprehensive biofilms while catheters exposed to the *mrpA* strain had only acquired patchy colonization at the eye-let at 48 h. Under high magnification (Figures 45 - 48), the colonization by the two strains was much clearer. The WT strain was able to form isolated microcolonies on the catheter surfaces within 4 h with the numbers of adhered cells slowly increasing during the initial 12 h (Figure 45 and 46). These cells were often associated with areas of the catheter surface that had a rough topography, suggesting that these areas are capable of initiating bacterial colonization. At 24 h the WT strain had formed a comprehensive non-crystalline biofilm several cells deep on the eye-let and the first few centimetres of the lumenal area. By 48 h extensive biofilm had formed on the lumenal section.

In contrast, the *mrpA* strain (Figures 47 and 48) showed little evidence of colonization over the initial 12-h period in the acidic urine, and was observed as isolated cells which in some instances appeared trapped in rough areas of the surface. At 24 h *mrpA* colonization was relatively rare and was limited to a small area at the rim of the eye-let approximately 200 μm x 200 μm where it formed a patchy biofilm several cells

thick. The lumenal sections also showed rare patches of biofilm. At 48 h more 'patches' of biofilm were observed at the eye-let but failed to form the continuous comprehensive biofilm structure of the WT strain. The lumenal section was still poorly colonized at this time and had not acquired a biofilm.

4.2.7. Enumeration of WT and *mrpA* cells adhering to catheter surfaces

The results presented in Figure 51 showed little difference in numbers of the wild type and mutant cells adhering to catheters under alkaline and acidic urine conditions at 24 h. The only significant difference was observed at section B when the mean numbers of *mrpA* cells attaching to this section in alkaline conditions was significantly different ($P = 0.007$) than in acidic urine. With hindsight it would have been interesting to have evaluated cell adhesion at various times up to 24 h. The results at 24 h however, do not support the conclusions from the electron microscopy that the WT strain adheres more effectively to catheters under both alkaline and acidic conditions. The main conclusions must be that cells that lack MR/P fimbriae are still capable of adhering to catheters under both sets of conditions.

4.2.8. MR/P expression in the bladder model

The results of the haemagglutination tests on cells growing in the urine in the bladder models (Figure 52) confirm that the *mrpA* mutant did not produce MR/P fimbriae during the 48 h incubation period. It was also apparent however, that the WT did not express the MR/P fimbriae until 48 h. The WT cells adhering to the catheter under acidic conditions at 24 h (Figures 45 and 46) were thus not expressing MR/P fimbriae, another indication that these adhesins are not necessary for catheter colonization. A

complicating issue in trying to resolve the factors involved in adherence of *P. mirabilis* to catheters is that both the WT and mutant (at 24 and 48 h) were able to agglutinate tannic acid-treated red blood cells in the presence and absence of mannose, suggesting they were expressing MR/K fimbriae (Bahrani *et al.* 1991). Mobley *et al.* (1988) reported that MR/K fimbriae were responsible for the adherence of *Pv. stuartii* to siliconized-latex catheters. Further experiments will be needed to establish whether they are involved in attaching *P. mirabilis* to catheters.

4.2.9. The ability of WT and *mrpA* to form cell-cell aggregations

The results of the auto-aggregation assays presented in Table 5 and Figure 53 demonstrated that WT *P. mirabilis* cells incubated under conditions for MR/P expression were able to form visible aggregates in test-tubes comparable to the co-aggregating control species. The *mrpA* strain incubated under identical conditions did not form similar cell-cell aggregations. Previous studies with *Ps. aeruginosa* showed that similar adhesins were responsible for biofilm formation as they initiate cell-cell aggregation and generate microcolonies – the basic structure of the bacterial biofilm (O'Toole and Kolter, 1998). The MR/P fimbriae may thus have a similar role in *P. mirabilis*.

Although all the strains of *P. mirabilis* found on urinary catheters seem to be capable of producing MR/P fimbriae, their importance in crystalline biofilm formation on patients' catheters is still uncertain. It would be interesting to know if they are expressed by cells growing in the residual urine in the bladders of catheterised patients. If this is the case the ability for MR/P expressing strains to quickly form cell-cell aggregations is a possible mechanism for persisting in environments under liquid

shear forces. In the catheterised bladder the cell aggregations may settle out of suspension and avoid these forces. In addition, their ability to initiate biofilm development on catheter surfaces would enable them to benefit from the protection of the biofilm matrix. It is possible that MR/P fimbriae also allow co-aggregation with other genetically distinct species (Rickard *et al.*, 2003). This would allow *P. mirabilis* cells expressing MR/P fimbriae entering an infected catheterised urinary tract to interact with biofilm communities already present on the indwelling device. It would be interesting to carry out co-aggregation assays with MR/P$^+$ *P. mirabilis* strains with other urinary tract isolates, and also test the ability of WT and *mrpA* mutant strains to infiltrate the biofilms of other urinary tract isolates. It is possible that the expression of MR/P fimbriae by *P. mirabilis* in clinical episodes of infection is advantageous in allowing them to produce the recalcitrant and persistent infections associated with *P. mirabilis* CAUTI. The fact that cells lacking or not expressing MR/P fimbriae can also block catheters suggests that *P. mirabilis* can generate crystalline biofilm in a number of different ways. The overriding factor in the development of these biofilms is the generation of alkaline urine, the elevation of pH_v above pH_n by the bacterial urease and precipitation of crystalline material.

4.3.   Modulation of crystalline *P. mirabilis* biofilm

Nursing staff responsible for catheter management are well aware that even for patients designated as recurrent "blockers", the times catheters take to block (*i.e.* catheter lifespan) varies considerably. In their prospective study with patients with *P. mirabilis* infections, Mathur *et al.* (2006a) reported that the times catheters took to block varied from 2 – 98 days. As catheter encrustation is brought about by the rise in urinary pH caused by the activity of urease, it would seem probable that the higher the pH generated by *P. mirabilis*, the more rapidly the catheters will block. Mathur *et al.* (2006a and 2006b) however, showed that the relationship between urinary pH and the rate of the encrustation process was rather more subtle. The data showed that in patients infected with *P. mirabilis* the nucleation pH (pH_n) is the important factor in controlling the rate of catheter blockage. Statistical analysis showed that there was a highly significant ($P = 0.004$) positive correlation between pH_n and catheter lifespan. Thus the higher the pH_n of a patient's urine, the slower the encrustation process and the longer the catheter lifespan. The study also showed that the pH_n of a patient's urine could vary considerably from week to week. These findings suggested that it might be possible to manipulate pH_n as a therapeutic approach to deal with catheter encrustation.

Suller *et al.* (2005) demonstrated that it was indeed possible to manipulate pH_n very simply. Studies using healthy volunteers showed that by increasing the fluid intake, thus diluting the urine, elevated its pH_n. Increasing the concentration of the chelating agent citrate in the urine by taking citrate-containing drinks further elevated the pH_n. These observations provided the rationale for the experiments in the bladder model exploring the effect of elevating urinary pH_n on the rate of catheter encrustation. The

pH_n of artificial urine was manipulated in a controlled manner by dilution and by increasing its citrate content.

4.3.1. The effect of fluid intake on the urinary pH_n and catheter encrustation

The results in Table 6 illustrate that simply diluting the urine has a profound effect on the pH_n. The pH at which crystals formed in the urine increased from 6.7 in the neat urine, to 8.4 in the urine diluted to 1:6. When the neat, 1:1, 1:2 and 1:3 diluted urines were supplied to models the rate of catheter encrustation (estimated by mean time to catheter blockage), was also greatly affected. The mean blockage times were significantly different ($P < 0.05$) between each of the four dilutions (Figure 54). The mean catheter lifespan being extended from 24.13 ± 2.9 h in models supplied with neat urine, to approximately five times longer (125.5 ± 6.4 h) in bladder models supplied with urine at a 1:3 dilution. The analysis of the urine from these experiments (Table 7) showed the mean pH of the voided urine was consistently alkaline remaining > pH 8.4 in all cases. Furthermore, in models supplied with 1:1, 1:2 and 1:3 diluted urines the mean pH of the urine was > pH 8.7 at 24 h and > pH 8.9 at 48 h. The mean viable cell populations (Table 8) recorded in the residual urine of experiments were also consistent and remained at $10^7 - 10^8$ cfu/ml confirming they were heavily infected throughout the experimental period.

Burr and Nusiebeh (1997) examined the relationship between catheter blockage and urine composition in patients with spinal cord injuries with long-term indwelling catheters. They reported that patients with catheters lasting for at least 6-weeks (non-blockers) had mean urine outputs of > 3 L/24 h. They recommended that a high uniform rate of fluid intake should be mandatory for those prone to catheter blockage.

The evidence reported in this present study supports this recommendation. The flow rates of urine supplied to the models were increased proportionately as urine became more dilute, in order to simulate higher fluid intakes and increased urine production. Flow rates ranged from 720 ml/24 h for the neat urine to 2880 ml/24 h for the 1:3 diluted urine. The mean blockage times of catheters in control experiments with a flow rate of 720 ml/24 h and supplied with 1:1 diluted urine (50.5 ± 4.6 h) was not significantly different ($P = 0.9$) from the mean blockage time of 49.5 ± 6.0 h of catheters from bladder models supplied with identical urine but at the higher flow rate of 1440 ml/24 h. These data (Figure 54) suggest that the urine concentration and its pH_n rather than flow rate are the important modulating factors in controlling the formation of the crystalline biofilms.

4.3.2. The effect of citrate on the urinary pH_n and catheter encrustation

The results presented in Table 9 demonstrate that elevating the citrate content of urine had an even more dramatic effect on pH_n than simply diluting the urine. The pH_n at which crystal formation occurred in urine increased from ~pH 7.4 at 0.41g/L citrate to ~pH 9.1 at 3.0 g/L citrate. Mean catheter blockage times (Figure 55) were 32.3 ± 3.5 h, 66.25 ± 6.7 h and 107.3 ± 5.5 h in models supplied with urine containing 0, 0.41, and 1.0 g/L citrate respectively. Furthermore, in models supplied with urine containing 1.5, 2.0 and 3.0 g/L citrate (pH_n values of 8.3, 8.5 and 9.1 respectively) catheters did not occlude with crystalline biofilm and drained for the full 7-day experimental period. The increased citrate concentrations in the urine did not affect the ability of P. mirabilis to elevate the pH of residual bladder model urine, or its ability to proliferate in the urine (Tables 10 and 11). The pH of urine was consistently > pH 8.0 while viable cell populations remained > 10^7 cfu/ml in all cases throughout

the experimental periods. The ability of citrate to inhibit crystal formation in the urine was apparent in the residual bladder model urine during the course of the experiments (Figure 56). It was clear that urine containing the higher citrate concentrations were able to delay or prevent the onset of visible crystal formation occurring in the residual bladder model urine.

Suller *et al.* (2005) previously demonstrated that urinary citrate concentrations could be elevated in healthy volunteers by supplementing their diet with 500 ml of orange juice daily. The citrate concentration of urine was raised from 0.35 to 1.2 g/L and the urinary pH_n increased from 7.25 to 8.2. In this present study supplementing the 1:1 dilution urine with 1.0 g/L citrate also elevated the urinary pH_n to ~pH 8.2. This is interesting as Mathur *et al.* (2006a) reported that the mean pH_n of patients who slowly encrusted their catheters was 8.08. Examination of their regression analysis of pH_n against catheter lifespan suggest that if the mean pH_n of a patients' urine is 8.2, then the mean catheter lifespan will be in the region of 5 weeks, even in the presence of *P. mirabilis*. This compares with the mean lifespan of catheters of 107 h in the models supplied with urine having a pH_n of 8.2. It must be appreciated however, that the experiments in the models simulated a heavy pure culture *P. mirabilis* infection consistently generating alkaline urine that was between pH 8.5 - 9.0. Another significant observation from the Mathur *et al.* 2006a, 2006b papers is that the mean pH_v of urine only exceeded 8.0 in 3 of the 21 patients studied. If the mean urinary pH_n of patients who rapidly encrust their catheters could be consistently elevated to around 8.2 by increasing their fluid and citrate intake the complication might be controlled in most sufferers. The observations in this study should encourage a clinical trial to

examine the effect of increasing a patient's fluid intake with citrate-containing drinks on the encrustation and blockage of catheters.

4.3.3. The development of P. mirabilis crystalline biofilm in urine containing various citrate concentrations

The low vacuum electron micrographs presented in Figure 57 and 58 illustrate the differences in the rate of catheter encrustation when urine containing the range of citrate concentrations was supplied to bladder models. Catheters in models supplied with urine containing 0.41 g/L citrate blocked at the eye-let and first few centimetres of the lumen with typical encrustation at 64 h. Appreciably less encrustation was observed on catheters that had been removed at 72 h from models supplied with urine containing 1.0 and 1.5 g/L citrate. Catheters in models supplied with urine containing 1.5, 2.0 and 3.0 g/L citrate drained freely for the whole 7 days. Examination of these catheters showed that while some encrustation was visible on the one from the model supplied with urine containing 1.5 g/L citrate those from models supplied with urine containing 2.0 and 3.0 g/L citrate appeared free of crystalline deposits. Analysis of the residual urine from models at the start and at conclusion (not shown), showed that all cases the residual urine was heavily infected by P. mirabilis, urinary pH and viable cell populations being > pH 8 and > 10^7 cfu/ml respectively.

The HVSEM micrographs shown in Figures 59 – 66 illustrate that the manner of P. mirabilis crystalline biofilm development on the catheters was determined by the citrate content and pH_n of the urine. The catheter removed from the model supplied with urine containing citrate at 0.41 g/L had acquired a crystalline "foundation layer" upon which, a 'typical' crystalline P. mirabilis biofilm was developing at 24 h (Figure

59 A + B). This could be expected as the pH of the residual bladder model urine at this time was 8.33, and considerably higher than the urine pH_n of pH 7.4, conditions under which crystals would have been forming readily in the urine. Catheters removed from models supplied with urine containing 1.0 – 3.0 g/L citrate at 24 h were colonized by extensive *P. mirabilis* biofilms, but showed no evidence of crystalline material (Figures 59 – 61). This was not surprising since urines containing 2.0 and 3.0 g/L citrate have pH_n values comparable or above those of the residual bladder urine (pH 8.52 v pH_n 8.5, and pH 8.2 v pH_n 9.1 respectively). In the bladder models supplied with urine containing 1.0 and 1.5 g/L citrate however, the pH of the residual urine in both these bladder models exceeded the urinary pH_n (pH 8.66 v pH_n 8.2, and pH 8.89 v pH_n 8.3 respectively). At 48 h biofilms on catheters that had developed in urine containing 1.0 g/L citrate contained extensive crystalline deposits, while those that had developed in urine containing 1.5 g/L citrate comprised of distinct non-crystalline and crystalline regions (Figure 62). In contrast to this, biofilms that developed on catheters in models supplied with urine containing citrate at 2.0 and 3.0 g/L remained free of crystalline deposits at the 48 h period (Figure 63).

Mature 7-day biofilms on catheters from models supplied with urine containing 1.5 g/L citrate comprised three distinct regions. These included what appeared to be a crystalline layer resembling the calcium phosphate 'foundation layer', a crystalline biofilm matrix containing integrated cells and calcium phosphate microcrystals, and a non-crystalline region made up of swathes of swarmer-like *P. mirabilis* cells. Little is known about the role of swarmer-cells in *P. mirabilis* biofilm formation, but this evidence suggests they may have a role in the development of *P. mirabilis* biofilms in the absence of crystalline deposits, or in elevated citrate concentrations. It was

interesting to observe the presence of a crystalline foundation layer on the catheter surfaces from experiments supplied with urine containing 1.5 g/L citrate, as it was clear from the HVSEM analysis that at 24 h (Figure 60) initial *P. mirabilis* biofilm formation occurred in the absence of crystalline deposits. It is possible that an increase in localised urease concentration within these biofilms as they mature allow crystals to nucleate and grow at the catheter surface eventually forming a calcified layer between surface and biofilm. It is also possible that microcrystals forming within the biofilm are attracted to the silicone surface itself. This may be possible as microcrystals adhered readily to the catheter surfaces in experiments described in Section 4.1. suggesting that the nature of the catheter surface itself may attract crystals that have formed in the urine. The 7-day biofilms that formed on the surfaces of catheters in models supplied with urine with 2.0 g/L citrate were generally free of crystalline material, with only rare instances of crystalline deposits (Figure 65 C) which resembled typical amorphous calcium phosphate. The biofilms developing on catheters removed from bladder models supplied with urine at 3.0 g/L citrate showed no evidence of crystalline material (Figure 66). The evidence provided by electron microscopy confirms that citrate is able to delay or prevent *P. mirabilis* crystalline biofilm formation on AS catheters by elevating the urinary pH_n. The possible use of this naturally occurring urinary constituent to prevent catheter encrustation and blockage should be investigated in properly controlled clinical trials.

4.3.4. The effect of citrate on the rate of catheter encrustation by *Pv. rettgeri* and *P. vulgaris*

Elevating the pH_n of urine by increasing its citrate content also had a significant effect on the rate of catheter encrustation caused by clinical isolates of *P. vulgaris* and *Pv.*

rettgeri. The results presented in Figure 67 showed that both test strains rapidly blocked AS catheters in models supplied with 1:1 diluted urine containing 0.205 g/L citrate. The mean lifespans of catheters were 59.06 ± 15.46 and 41.28 ± 1.06 in models infected *P. vulgaris* and *Pv. rettgeri* respectively and were not significantly different (Kruskal–Wallis, *P* >0.05). When the citrate content of the urine was increased to 1.5 g/L, in the case of *P. vulgaris* all three catheters drained freely for the 7-day experimental period. Two of the three catheters in models infected with *Pv. rettgeri* also drained freely for the whole 7 days, while the third bladder model blocked with crystalline biofilm after ~4.5 days. The results presented in Table 12 show that the *Pv. rettgeri* isolate was a potent urease producer and quickly adapted to the bladder model conditions generating mean urinary pH's consistently > pH 9.0 and producing mean viable cell populations of $10^8 - 10^9$ cfu/ml. In contrast, the mean pH of the residual urine in experiments infected with *P. vulgaris* reached a maximum of pH 8.74 at 6 days, while viable cells varied from $10^7 - 10^8$ c/ml over the 7-day period. The significant reduction in the rate of catheter encrustation is particularly important for *Pv. rettgeri*. This isolate was able to rapidly block catheters impregnated with triclosan solution in other bladder model experiments (Broomfield, 2007). This supports the theory that the antimicrobial resistance patterns of catheter encrusting species are unlikely to affect the efficacy of the elevated pH_n strategy in preventing encrustation.

Elevating the urinary pH_n by diluting urine and by increasing its citrate content significantly reduces the *in vitro* rate of encrustation and crystalline biofilm formation by a range of urease-producing organisms on all-silicone catheters. Although increased hydration reduces the rate of catheter encrustation, this method alone may

not elevate urinary pH_n to levels high enough to reduce or prevent catheter encrustation and blockage in the majority of long-term catheterised patients infected with *P. mirabilis*. Examination of the data from the clinical study by Mathur *et al.* (2006b) suggests that if the mean pH_n of urine could be elevated to around 8.1, then catheter encrustation could be prevented in around 80% of patients infected with *P. mirabilis*. To increase the pH_n of the urine to 8.1 fluid output would have to be approximately 4 L per 24 h (1:5 dilution). It would be very difficult to maintain this level of hydration in patients. However, the experiments showed that by generating a fluid output of ~1.5 L per 24 h whilst simultaneously increasing its citrate content to 1.0 g/L, a pH_n of ~8.1 could be achieved. Preventing crystalline biofilm formation by elevating the pH_n of urine is an attractive alternative to using antimicrobial agents. It avoids the difficult issue of resistance and is likely to be effective whatever organisms are responsible for catheter encrustation and for all types of catheter.

4.4. The effect of manipulating urinary pH_n through dietary fluid and citrate intake

Manipulating the constituents of urine through dietary change has proved beneficial in reducing the metabolic risk factors associated with calcified stone formations in the upper urinary tract (Seltzer *et al.*, 1996; Siener *et al.*, 2005). Increasing urinary citrate excretion, whilst reducing urinary calcium concentration are some of the fundamental aims in these diet-related therapies. Infection-associated stone formations are similar in nature to the calcified deposits that affect urinary catheters, and it is possible therefore that similar methods may be beneficial in the prevention of catheter encrustation. In support of this theory, Morris and Stickler (2001) demonstrated that increasing fluid intake in healthy volunteers had a significant effect on the rate of catheter encrustation in bladder model experiments. Suller *et al.* (2005) demonstrated that the pH_n of urine in healthy individuals could be favourably manipulated simply by increasing fluid intake and by elevating urinary citrate content. Furthermore, Mathur *et al.* (2006a) reported that the pH_n of long-term catheterised patients was extremely variable and therefore was open to manipulation. If both the daily fluid intake and urinary citrate concentration in catheterised patients could be increased simultaneously by regularly consuming citrate-containing drinks, it may be possible to generate urine with an elevated pH_n thus inhibiting crystal formation even in the presence of *P. mirabilis* infection. At present however, there is no evidence that this type of dietary intervention achieves a reduction in the rate of catheter encrustation. If evidence could be provided to indicate that supplementing the diet with citrate-containing drinks reduces the rate catheter encrustation in bladder model experiments, it would provide a platform to warrant proper clinical trials.

4.4.1. Urinary profiles of healthy volunteers

The results presented in Table 13 illustrate that the urinary profiles of the healthy volunteers were highly variable. The mean concentrations of calcium, magnesium and citrate excreted in the urine of the volunteers over the study period varied by approximately three to four-fold, ranging from $0.05 - 0.22$ g, $0.035 - 0.09$ g and $0.18 - 0.56$ g respectively. The mean pH_ns of the urines ranged from 6.77 to 8.02, while the mean pH safety margins ranged from 0.29 to 1.74 pH units. It was interesting that on a number of occasions the pH_v of urine from volunteers 4 and 5 were comparable to the pH_n, and in one instance the pH_v of the urine from volunteer 4 had a negative safety margin of -0.35 pH increments. In addition to this, six of the eleven urine samples provided by volunteer 4, and eleven of the thirteen urine samples provided by volunteer 5 had acidic pH_n values. This is important as in these cases relatively small increases in urinary pH would be sufficient to cause crystalline precipitation. Overall the data give an indication of why the rate of encrustation among catheterised patients can be so variable.

In *P. mirabilis* infected urine the pH_v will become elevated and the important safety margin then becomes that between the pH_n and the pH_v generated by the action of urease. In the study by Mathur *et al.* (2006a) the mean pH_v of the urine of patients infected with *P. mirabilis* that rapidly encrusted their catheters was 7.2 and mean safety margins were +0.13 pH increments. If the safety margins presented in Table 13 are re-calculated according to this mean pH_v of 7.2, values of -0.43, -0.41, +0.05, +0.56 and +0.82 are obtained for volunteers 4,5,1,2 and 3 respectively. Extrapolating these data suggest volunteers 1, 4 and 5 immediately fall into the category of those that would rapidly encrust their catheters. The importance of maintaining a high fluid

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output to dilute urine and elevate pH_n was evident in the urinary profiles of volunteers

2 and 3. These volunteers had the two highest mean urine outputs (1335 and 1881

ml/11 h sampling period respectively) and had the two lowest mean urinary calcium

and magnesium concentrations and the highest pH_ns. It was interesting that although

volunteers 2 and 3 had the most dilute urines, they also had the 3rd and 2nd highest

mean citrate concentrations respectively. This is interesting as maintaining or

increasing citrate concentrations in urine whilst reducing calcium and magnesium

concentrations would be advantageous in elevating urinary pH_n. As no dietary

restrictions were enforced on the participants of this study however, it was not certain

whether volunteers 2 and 3 consumed considerably more citrate as part of their diet,

or consumed considerably less calcium and magnesium than other participants. It does

suggest however, that increasing urinary citrate under conditions of increased

hydration is possible.

4.4.2. The effect of citrate-containing drinks on urinary citrate content and pH_n

The results of the citrate assays presented in Table 14 showed that a number of fruit

juices and concentrates were high in citrate and had potential for use as the citrate-

containing drink. The assays also ensured an approximate amount of consumed citrate

could be calculated when the citrate-containing drink was taken. The drink

subsequently used was made from a combination of lemon concentrate and orange

juice (1:4) and was based on its citrate content and palatability. The drink was taken

in 3 x 250 ml aliquots throughout the day supplementing the diet with ~11 g of citrate

per 24 h. The results presented in Figure 68 show that the mean citrate concentration

of urine increased 32 % (0.25 to 0.33 g/L respectively) after taking the citrate-

containing drink. The mean total amount of citrate increased by 50 % (0.18 to 0.27 g

respectively) during the sampling period. However, these differences were not significant ($P = 0.09$). Statistical analysis of the other urinary variables shown in Figure 69 revealed there were no significant differences in pH_v, the pH safety margin or pH_n for the two types of urine ($P > 0.05$). The results presented in Figure 68 and 69 however, were based on data obtained when the citrate drink was consumed in place of other fluids on the 'normal' diet and the volumes of urine voided on the two diets were not significantly different ($P > 0.05$). If the pH_n of patients' urine is to be elevated increased hydration as well as increased citrate will be important.

When fluid intakes were 'standardised' to 1300 ml/24 h and 750 ml of the citrate drink consumed in addition to this, significant differences in a number of urinary parameters were observed (Figures 70 and 71). Importantly, the mean pH_n of urine from the increased citrate diet (8.05) was significantly higher ($P < 0.05$) than the mean pH_n of the urine from the standardised diet (7.25). It was also higher (but not significantly so) than the mean pH_n (7.59) of the control urine (standardised fluid intake diet when supplemented with 750 ml of water). The reasons for the increase in urinary pH_n after consumption of the citrate-containing drink however, could not be explained by increases in excreted citrate. The mean citrate concentration in urine collected after consuming the citrate-containing drink was significantly lower ($P < 0.05$) than that in urine collected in the standardised diet supplemented with 750 ml of water. When the mean total amounts of citrate excreted were compared between these two diets however, they were not significantly different ($P > 0.05$). It was noticed however, that the mean volume of urine voided under the citrate diet was higher than that voided in the SFI + W diet (~1600 ml/11 h v 1270 ml/11 h period respectively). This may have diluted the urine slightly as the mean calcium concentration in the

urine from the increased citrate diet was basically half that observed in the standard and standard + water diets. It was disappointing that the levels of citrate in urine on the SFI + C regimen, did not reach those found by Suller *et al.* (2005) who reported a gradual increase in citrate concentrations of urine rising to ~ 1.2 g/L after a week of consuming 500 ml of orange juice daily. It is possible that the duration of the dietary regimen in this study (4 days) was not long enough to elevate citrate concentrations significantly in the urine. In hindsight urine could have been collected after one week of consuming the citrate drinks. The amount of citrate excreted in the urine of an individual however, will depend on a number of other factors. As an intermediate in the citric acid cycle, citrate is utilised according to energy requirements and will be affected by the day-to-day activities of an individual. Genetic factors are also involved in the concentration of citrate that an individual will excrete in their urine (Shah *et al.*, 2005). Excretion of citrate into the urine is highly regulated by the kidneys and a major influence is the intracellular acid-base balance in the kidney, which is affected by potassium concentration. Citrate excretion is reduced when potassium concentrations are depleted (Simpson, 1983). Various forms of potassium citrate are used to increase citrate excretion in patients prone to metabolic kidney stone formation. These dietary supplements may also be useful in the treatment of long-term catheterised patients infected with *P. mirabilis*.

4.4.3. Increasing fluid and citrate intake and its effect on pH_n and the rate of catheter encrustation

The profiles of the urine of the healthy volunteer under four dietary routines presented in Table 15 show that the concentrations of urinary constituents and the parameters of other urinary variables altered considerably under the different dietary routines. It was

clear that urine with low Ca and Mg concentrations, high pH safety margins and elevated pH_ns were generally associated with the increases in urine output. The urine from the SFI + C regimen however, had the lowest mean pH safety margin (1.1 pH increments), while the mean Ca concentration of ~0.093 g/L was the highest observed. The low pH safety margin was attributed to high mean urinary pH_v of 6.77, which may have been caused by the high citrate intake. Elevation of the pH_v by high citrate intake may be of concern in catheterised patients. If citrate therapy was introduced urinary pH would have to be monitored. The higher concentration of Ca may have been influenced by other dietary factors. It was disappointing to find that both the total excreted citrate over the 48 h period and the concentration of citrate in these urines were not significantly different ($P > 0.05$) among the different urines - even though there was clear disparity between the amounts of citrate consumed through dietary fluids. This may again have been due to the relatively short dietary periods. The effect of the different diets on urinary pH_n was substantial. Urine collected from the SFI + W and SFI + C and lemonade regimens all had higher mean pH_n values compared to that of the SFI regimen (+0.43, +0.59 and +0.83 pH units respectively), only the pH_n of the urine from the lemonade diet however, was significantly higher ($P < 0.05$). The important finding was that the mean pH_n of urine while the volunteer was on the lemonade diet was approximately 8.1. In the recent study by Mathur *et al.* (2006a) a mean pH_n of around 8.1 was high enough to prevent long-term catheterised patients infected with *P. mirabilis* from rapidly encrusting their catheters (mean catheter lifespan > 28 days). If a high fluid/citrate intake similar to the lemonade diet in this study was introduced to clinical patients infected with *P. mirabilis* that rapidly encrust their catheters, it is possible that the episodes of encrustation and catheter blockage would be reduced.

When the urine collected from the different dietary routines was supplied to *P. mirabilis* infected bladder models (Figure 72 A), the mean lifespan of the all-silicone catheters increased in relation to their respective urinary pH_ns. Although the mean time to catheter blockage for the SFI, SFI + W and SFI + C experiments were not significantly different ($P > 0.05$), the catheters supplied with urine from the SFI + W (pH_n 7.7) and SFI + C (pH_n 7.86) routines took an average of 1.41, 1.74 times longer to block respectively than those supplied with urine collected from the SFI diet. Catheters from models supplied with urine collected from the lemonade diet however, drained for a mean of >118 h, an average of 4.68 times longer than those from the SFI experiments ($P < 0.05$). The results presented in Table 16 show that *P. mirabilis* was able to generate consistently alkaline conditions in the residual urine of the models >pH 8.5. Throughout the experimental period therefore significant amounts of crystalline material would have been precipitating in the urine. Mean viable cell populations remained between $10^7 - 10^8$ cfu/ml and confirmed that the residual urine in bladder models remained heavily infected. As previously stated the pH of urine voided by long-term catheterised patients infected with *P. mirabilis* is quite variable and is rarely consistently >pH 8.5. In the study by Mathur *et al.* (2006b) only 3 of the 21 patients infected with *P. mirabilis* had mean pH_v values > 8.0. The ability to raise the pH_n of volunteers urine to 8.1 suggests that the lemonade diet could resolve the problem of catheter encrustation in 85% of patients and substantially slow down the process in the others.

When the urinary pH_n of each individual 48 h pooled urine sample was plotted against the individual catheter blockage times (Figure 72 B), there was a highly significant correlation ($P < 0.001$). The effect of the pH_n of artificial urine on catheter blockage

time was previously reported in Section 3.3. The correlation between the pH_n of real urine and catheter blockage time revealed in this present section is important as it demonstrates that this relationship is also true in urine that has been manipulated through dietary intervention. Previous studies have reported that maintaining a high and uniform fluid intake (> 3 L/24 h) is a critical factor in reducing the rate of catheter encrustation (Burr and Nuseibeh, 1997; Morris and Stickler, 2001). The mean volume of voided urine under the lemonade diet in this study was > 7 L/48 h. It would be interesting to carry out further studies on generating elevated citrate concentrations in urine through supplementing the diet with citrate while maintaining a daily fluid output of 2 - 3 L in a range of volunteers. It may simply be the case that elevated citrate excretion occurs after longer periods on increased citrate diets ($\geq$ 1 week). Supplementing the lemonade diet or high fluid diets with potassium citrate may be another option to increase citrate excretion.

4.5. General discussion and future work

The ability to stick to surfaces and adopt the biofilm mode of growth is a basic survival mechanism for bacteria in aquatic environments (Donlan and Costerton, 2002). The picture that is emerging from recent work on *P. mirabilis* is that this ingenious organism can initiate biofilm development on urinary catheters in a variety of ways. An important finding of the work presented in Section 3.1 of this thesis is that the initial process in *P. mirabilis* crystalline biofilm development on AS, HCL, SCL and HSCL catheters can begin with the formation of a coating of calcium phosphate on the catheter. This "foundation layer" however, was not essential for *P. mirabilis* to colonize the devices as cells were also observed adhering directly to the catheter. It was also apparent that cells become entrapped in the rough irregular areas on the catheter surface. It has previously been recognised that *P. mirabilis* colonization of polymer materials to which cells fail to attach, can occur when cell-crystal aggregates that form in alkaline urine are deposited on surfaces (Stickler *et al.*, 2006). Other studies have indicated the initial stages of biofilm development on indwelling devices is the formation of a "conditioning layer" derived of host proteins (Barbucci and Magnani, 1994; Stickler and McLean, 1995; Santin *et al.*, 1997; Reid, 1999). Furthermore, it has recently been shown that *P. mirabilis* can integrate itself into established biofilms of other urinary pathogens such as *E. coli*, *K. pneumoniae* and *M. morganii* (Macleod, 2007).

The initial sequences in *P. mirabilis* adherence to catheter surfaces and subsequent crystalline biofilm formation may thus occur in at least six different ways:

1. *P. mirabilis* cells may adhere directly onto the catheter surface possibly through specific adhesin-mediated attachment (Figure 73 A).

2. The inherently rough areas of a catheter surface may facilitate 'non-specific' *P. mirabilis* adherence by physically trapping bacterial cells (Figure 73 B).

3. Cell-crystal aggregations formed in alkaline urine promote colonization when they become deposited on the surface (Figure 73 C).

4. *P. mirabilis* cells infiltrate the existing biofilm previously formed by genetically distinct species to form a crystalline mixed-species biofilm (Figure 74 A).

5. Host proteins form a "conditioning layer" on the catheter surface and promote *P. mirabilis* adherence through specific adhesins and receptors (Figure 74 B).

6. Calcium phosphate microcrystals that form in the alkaline urine are deposited on the catheter surface and form a "foundation layer" which *P. mirabilis* cells colonize (Figure 74 C).

A. Direct attachment

generation of alkaline urine
and crystalline biofilm

crystals

P. mirabilis cells

microcolony embedded in matrix

Catheter surface

B. Indirect attachment

generation of alkaline urine
and crystalline biofilm

cells entrapped in surface irregularities   microcolony

Catheter surface

C. Attachment mediated
through cell-crystal
aggregations

crystalline biofilm development

alkaline urine

Cell-crystal
aggregate

crystalline microcolony

Catheter surface

Figure 73. *P. mirabilis* **adhesion to catheter surfaces via direct, indirect and cell-crystal aggregation methods**

A. Cells directly adhere to catheter by specific cell-catheter surface interactions. **B.** Cells become entrapped in the inherently rough areas of catheter surfaces. **C.** Cell-crystal aggregations forming in the alkaline urine become deposited on the surface.

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A. Infiltration of existing biofilm

generation of alkaline urine
and crystalline biofilm

existing biofilm of another species
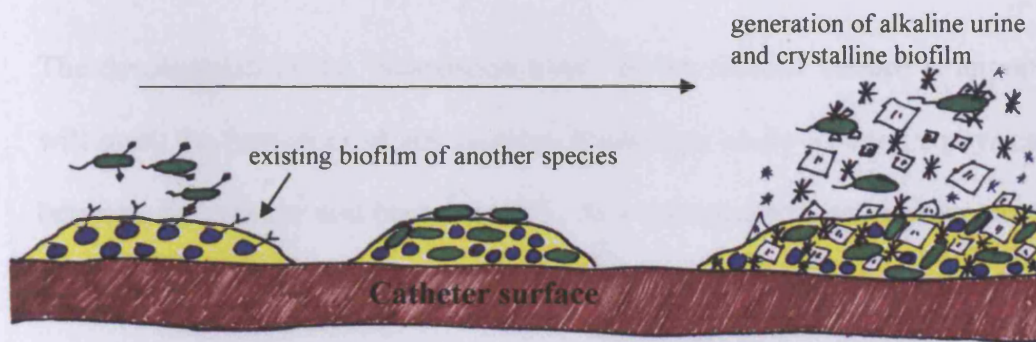
Catheter surface

B. Attachment to host protein
"conditioning layer"

generation of alkaline urine
and crystalline biofilm

host proteins

conditioning layer forms
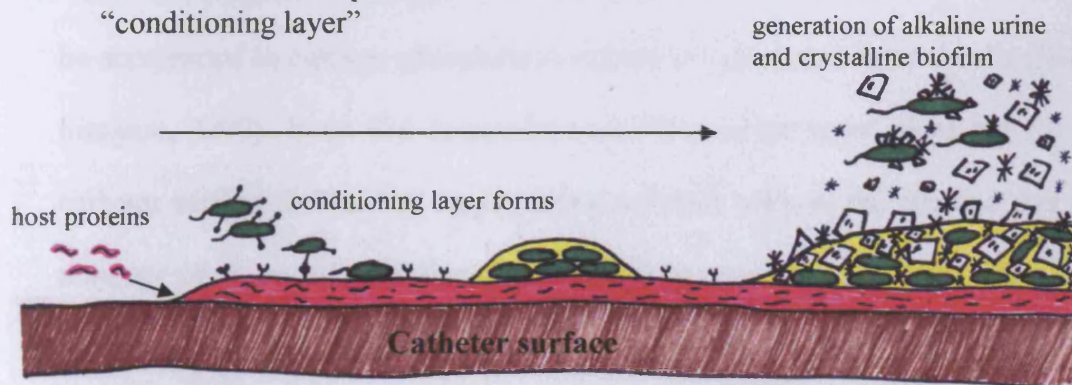
Catheter surface

C. Colonization of calcium phosphate
"foundation layer"

crystalline biofilm development

alkaline urine

foundation layer forms
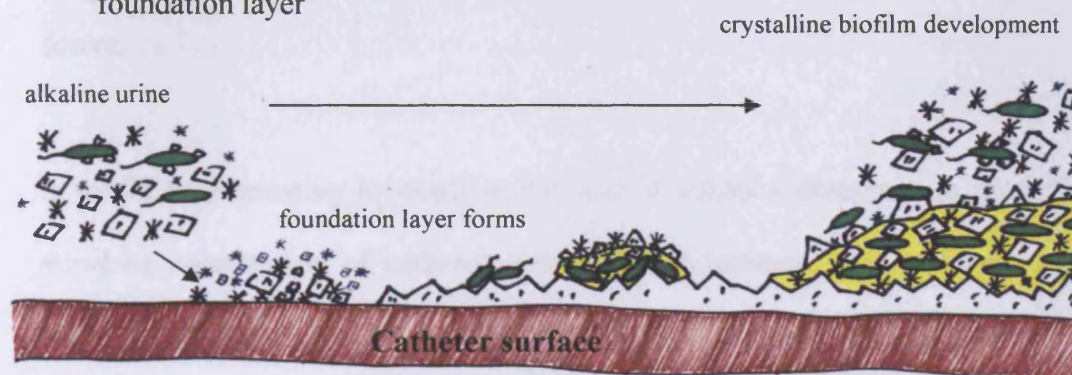
Catheter surface

Figure 74. ***P. mirabilis* adhesion to catheter surfaces via existing biofilm, "conditioning layer" and "foundation layer"**

4. *P. mirabilis* cells infiltrate an existing biofilm through specific cell-cell interactions. **5.** Cells colonize the protein "conditioning layer" through specific cell-protein interactions. **6.** Crystalline "foundation layer" forms on the surface and is subsequently colonized by *P. mirabilis*.

4.5.1. The importance of the "foundation layer" and calcium phosphate in *P. mirabilis* adhesion to catheter surfaces

The development of the "foundation layer" on the catheter surface is important as it will mask the properties of any material it develops on by forming a physical barrier between the catheter and bacterial cells. As a consequence, catheter materials aimed specifically at inhibiting bacterial adhesion or with an antibacterial immobilized on the surface, may be ineffective in patients with existing *P. mirabilis* infections. Once crystalline deposits are present on the catheter surface, bacterial colonization may also be accelerated as calcium phosphate is known to aggregate bacterial cells (Berry and Siragusa, 1997). Even if a comprehensive "foundation layer" does not form on a catheter surface, cell-crystal aggregations will still form in the alkaline urine in the majority of *P. mirabilis* infected patients. This crystalline material will shield *P. mirabilis* cells from the catheter surface in much the same way. Developing catheter materials that inhibit this calcium phosphate instigated adherence will be difficult as larger crystals or cell-crystal aggregations are likely to be deposited via gravitational forces.

It would be interesting to examine the role of calcium phosphate in promoting *P. mirabilis* colonization of catheter materials. For instance, how and why does the calcium phosphate layer form on the surface? What are the processes involved in cell attachment to the crystalline coating? Are the cells passively trapped in the crystalline material or are there physico-chemical attractive forces between the cells and the crystals? Are cellular adhesins involved in the adherence of cells to calcium phosphate? Experiments similar to those designed by Stickler *et al.* (2006) using parallel plate flow cells could be used to try and answer these questions. The affinity

of calcium phosphate for various catheter materials could also be assessed. This sort of information could help in developing catheters that do not encourage crystal adherence. Atomic force microscopy has been used in studies measuring the attractive forces between bacterial cells and various materials (Dufrene *et al.*, 2001) and it is possible that these methods could be exploited in these sort of experiments.

4.5.2.   Other factors promoting *P. mirabilis* adhesion to urinary catheters

The development of a protein "conditioning layer" would also offer protection to *P. mirabilis* in much the same way as the "foundation layer" by forming a physical barrier between cells and the catheter surface. Evidence suggests that adsorbed proteins on indwelling vascular devices specifically interact with the surface adhesins of *S. aureus* and facilitate initial adherence (Vaudaux *et al.*, 1993; Foster and Hook, 1998; Arrecubieta *et al.*, 2006). It is possible that similar interactions allow *P. mirabilis* to colonize urinary catheter surfaces, and it would be interesting to evaluate the rate of *P. mirabilis* colonization of various protein-coated surfaces using flow cell chambers and phase-contrast microscopy. Using urease-negative mutants or urease inhibitors in the experiments would be a way of avoiding the generation of alkaline urine and crystalline material.

The deliberate introduction of non-pathogenic *E. coli* into the bladder has been reported to reduce episodes of symptomatic UTI by virulent *E. coli* (Darouiche *et al.*, 2000). In the case of catheter encrustation it might be interesting to observe whether infection with a urease-negative mutant *P. mirabilis* strain prevented subsequent colonization by a wild-type urease-positive encrusting strain. The acquisition of virulence factors from pathogenic urinary tract isolates by deliberately introduced

non-pathogenic strains however, is something that will require stringent examination before this type of strategy could be implemented.

Work on the development of crystalline catheter biofilms has, up to now, largely concentrated on pure cultures of *P. mirabilis* (Morris *et al.*, 1997; Morris and Stickler, 1998a). Such single species biofilms can be found on catheters, but in long-term patients the catheter biofilms are commonly composed of polymicrobial communities containing other uropathogens (Ohkawa *et al.*, 1990; Ganderton *et al.*, 1992; Matsukawa *et al.*, 2005). The development of these mixed-species biofilms on urinary catheters has not been studied in detail and is an area that requires attention. Recent work by Macleod (2007) examined the effect other species have on the rate of catheter encrustation by *P. mirabilis*. Experiments in laboratory models showed that co-infection with *P. mirabilis* and *M. morganii* or *E. coli* for example, had no effect on the rate at which *P. mirabilis* blocked catheters. Co-infection of *P. mirabilis* with *Enterobacter cloacae* or *Ps. aeruginosa* however, significantly increased the times catheters took to block. Further experiments showed that the growth of *Ent. cloacae*, *M. morganii, K. pneumoniae* or *E. coli* biofilms for 72 hours on catheters prior to super-infection with *P. mirabilis* delayed catheter blockage significantly. In all these experiments however, *P. mirabilis* was able to generate alkaline urine and block the catheters. This work suggests that while there is antagonism between *P. mirabilis* and other urinary organisms, it is temporary and whatever the pre-existing urinary flora, infection with *P. mirabilis* is likely to lead to catheter encrustation and blockage.

Notwithstanding these results it would be of interest to reconstitute the mixed communities of organisms found in patients who are not infected with *P. mirabilis*

and do not encrust their catheters and examine their ability to inhibit the integration of

P. mirabilis into their biofilms. Techniques such as fluorescent labelling of cells and

scanning confocal laser microscopy has been used with great success to examine the

distribution of cells in mixed species biofilm and could prove useful in following the

development of these biofilm communities (Møller et al., 1998; Palmer et al., 2001).

If bacterial factors could be identified which prevent crystalline biofilm formation, a

biological interference strategy might be possible to control the problem. At present

however, there are few signs that such an approach is feasible.

4.5.3.   The development of catheters that resist encrustation

The realization of the many ways in which P. mirabilis can initiate crystalline biofilm

on catheters has profound implications for the development of devices that resist

encrustation, and indicates the enormous challenge facing manufacturers.

Fundamentally, catheter materials will have to prevent P. mirabilis and other urinary

pathogens from sticking. Finding a material with properties that could achieve this

may be difficult as different bacteria may adhere differently to the same material

(Darouiche, 2001). If only P. mirabilis adhesion is inhibited the bacterium would

encrust catheters by infiltrating the biofilms of other species (Figure 74 A). Catheter

materials with hydrophilic and strongly electron-donating properties that prevent P.

mirabilis adhesion would be ineffective once the bacterium enters the bladder and

generates alkaline urine causing cell-crystal aggregates to form and become deposited

on the surface as shown in Figure 73 C (Downer et al., 2003, Stickler et al., 2006).

Catheters need to be free of surface irregularities that otherwise allow P. mirabilis and

other uropathogens to become physically entrapped or provide a favourable surface

for cells to colonize (Figure 73 B). At present current manufacturing processes are not

capable of producing perfectly smooth catheters, and surface irregularities especially around the eye-let have been identified as sites of initial bacterial adherence (Stickler *et al.*, 2003b). If antimicrobial catheters are to be used it is important that the compounds diffuse directly into the urine, specifically target *P. mirabilis* and prevent a rise in urine pH and crystal development. It is most important that the generation of alkaline urine and crystal formation is prevented as crystalline biofilm will form even on smooth surfaces to which cells don't stick by the ways shown in Figure 73 C and 74 C). Furthermore, antimicrobial compounds will have to remain active for the life-time of the device (12 weeks) at concentrations consistently higher than the MIC.

4.5.4. Using the retention balloon to delivering compounds that inhibit *P. mirabilis* crystalline biofilm

Delivering agents such as triclosan into the bladder urine through the catheter retention balloon may be more effective than having antimicrobials immobilised onto the catheter surface in the treatment of patients infected with *P. mirabilis*. Compounds that diffuse through the balloon and into the bladder urine would be less affected by a "foundation" or "conditioning layer". The issues surrounding the use of triclosan strategies in preventing *P. mirabilis* crystalline biofilm development have been discussed in studies by Jones (2005) and Williams (2006). The main problems are finding materials that ensure triclosan will diffuse through the balloon at concentrations well above the MIC over prolonged periods (*i.e.* ~3 months), the effect of constant exposure of bladder epithelial cells to the antimicrobial and the selection of resistant strains. A recent study using bladder models infected with polymicrobial communities of urinary tract isolates showed that while triclosan eliminated susceptible isolates of *P. mirabilis*, *P. vulgaris*, *K. pneumoniae* and *E. coli*, it selected

for triclosan resistant isolates of *Pv. rettgeri* and *M. morganii* that were capable of generating alkaline urine (Williams, 2006). It is possible that future strategies may have to involve a combination of triclosan and other broad spectrum antimicrobials to reduce the emergence of resistant strains and to be effective against the range of urease-producing organisms. If this strategy was implemented, close monitoring of the susceptibility of the urinary tract flora to triclosan and other antimicrobials would be required.

The catheter balloon could also be used to deliver compounds that inhibit crystalline biofilm formation in other ways. Williams (2006) examined the ability of acetohydroxamic acid and fluorofamide to diffuse through silicone balloons. Unfortunately there was no sign that these urease inhibitors could diffuse into the urine and prevent the rise in urinary pH. Other urease inhibitors are available however, for example Wang *et al.* (2006) reported that resveratrol, a naturally occurring compound found in black grapes was a potent inhibitor of the production of urease by *P. mirabilis*. It would be interesting to test the diffusibility of these molecules through catheter balloons.

Analogues of quorum sensing (QS) molecules that are effective at disrupting biofilm formation offer another approach. The halogenated furanones derived from the macro algae *Delisea pulchra* have shown promise in this area, and are known to disrupt QS in a range of Gram-negative species. Furthermore, Gram *et al.* (1996) demonstrated that *P. mirabilis* swarming behaviour was inhibited on agar plates inoculated with these *Delisea pulchra* derived furanones. Unfortunately these compounds are highly toxic and may not be suitable for use in human subjects (Hentzer and Givskov, 2003).

Wu *et al.* (2004) however, reported that synthetic furanones were effective at disrupting *Ps. aeruginosa* biofilms in the lungs of infected mice, enhancing the bacterial clearance rates. It may be possible that in future non-toxic synthetic QS analogues may be developed that prevent biofilm formation on indwelling urinary catheters. It would be interesting to test the deliverability of such compounds via the retention balloon. A successful strategy for the prevention of *P. mirabilis* crystalline biofilms forming on the catheters of patients may require a combination of QS analogues and antimicrobials delivered through the catheter balloon. The QS analogue would keep bacterial cells in their planktonic state allowing the antimicrobial to kill them efficiently. The recent study by Williams and Stickler (2007) showed that polyurethane balloons were much more permeable than silicone or latex balloons to antibacterial agents. If catheter manufacturers could be persuaded to use polyurethane in the manufacture of catheters, it might be possible to deliver a variety of interesting active compounds directly into the bladder.

4.5.5. The role of fimbriae in *P. mirabilis* crystalline biofilm formation

The role of MR/P fimbriae in crystalline biofilm development or initial colonization of all-silicone catheters was not clearly determined in this study and warrants further investigation. Although the WT strain appeared to initially colonize catheter surfaces and integrate with crystalline material better than the *mrpA* mutant strain, evidence showing MR/P fimbriae were being expressed when this occurred however, was lacking. It would be useful to repeat the experiments with a range of different catheter materials in acidic and alkaline urine using phase-locked *P. mirabilis* mutants where MR/P fimbriae are either constitutively expressed or not expressed at all. It was clear from these experiments however, that the overriding factor in catheter blockage was

the generation of alkaline urine and not the extent of bacterial colonization. The take home message seems to be that preventing crystalline material from forming in the urine rather than preventing bacterial colonization is the most important factor in avoiding catheter blockage.

Cell surface adhesins are known to be important in coaggregation of oral bacterial species as they allow genetically diverse oganisms to co-adhere and form mutually beneficial biofilm communities. The autoaggregation assays in this present study showed that the WT *P. mirabilis* was able to form more obvious cell aggregations than the *mrpA* strain. It is possible that these adhesins may also have a role in the development of mixed-species catheter biofilms. The use of phase-locked mutants to determine the role of MR/P and other fimbriae in mixed-species biofilm formation may be one way of investigating the role of various adhesins in this phenomenon.

4.5.6. Preventing *P. mirabilis* crystalline biofilm development by manipulating the pH$_n$ of urine

Virtually all catheters that remain in place for >28 days become colonized with bacterial biofilm. In the majority of cases however, the individuals will avoid episodes of symptomatic UTI. In addition, results from experiments in this study have shown that catheters colonized by non-crystalline *P. mirabilis* biofilms drained freely until they were stopped manually at 7 days. These observations suggest that rather than trying to stop *P. mirabilis* colonization of the catheter, simply preventing the biofilm from becoming crystalline is a more realistic option for reducing episodes of catheter blockage. The results from this present study indicate that this can be achieved by elevating the pH$_n$ of urine. It is encouraging, that the preliminary results of a clinical

study that began recently at the Biomed Centre, Southmead Hospital Bristol (U.K.) has indicated that the urinary pH_n of long-term catheterised patients with *P. mirabilis* infection can be elevated using citrate drinks (Dr. A. Khan, personal communication). The mean urinary pH_n of patients' urine in three separate control periods were 7.68, 7.55 and 7.66 compared to 7.98, 8.07 and 8.12 when a group of 13 patients were given an extra 1 L/24 h of water, 1 L/24 h of a citrate-containing drink or 1 L/24 h of water plus potassium citrate respectively. The extension of this trial to include the effect on the rate of catheter encrustation in these patients is obviously the next step. It could be difficult to persuade some elderly patients to take such large volumes of extra fluid. Some might have physical problems in drinking such volumes. Suitable automatic drink dispensers could be developed to make it easier for these patients to accept and comply with these regimens.

Although all long-term catheterised patients infected with *P. mirabilis* may not respond significantly to this therapy, it may be possible to elevate the urinary pH_n in these patients by a combination of treatments. These may involve smaller increases in fluid intake supplemented with potassium citrate therapy whilst utilising the catheter balloon to deliver citrate directly into the bladder itself. The effect of citrate on bladder epithelial cells may have to be considered before citrate could be utilised in the catheter balloon. The ability of citrate to diffuse through various catheter materials however could be determined in bladder model experiments, while its effect on *P. mirabilis* crystalline biofilm development is determined. It is possible that in future, monitoring of patients' urinary pH_n will be part of their management. Dietary routines that elevate the pH_n of patients' urine could then be tailored to suit the individual needs of each person. The citrate drinks strategy will not of course eliminate the

causative organisms of encrustation from the catheterised urinary tract. It will need to be a long-term suppressive therapy. Despite this it has intrinsic advantages in that it doesn't involve the use of antimicrobials and it is effective whatever urease producer is inducing the encrustation. The selection of resistant organisms will thus not be a problem. Bacteria have developed many ingenious ways of overcoming the challenge of antimicrobial chemical agents but it is difficult to imagine that they can do anything about the laws of physical chemistry that govern the solubility of calcium and magnesium phosphates in urine.

Will the strategy be effective in the long-term? Will it create other problems? Only experience will answer these questions. In a retrospective study Kang *et al.* (2007) however, reported no adverse side-effects in patients who had been consuming 2 L of the lemonade drink to control kidney stones for up to 8 years (mean > 3 years). Careful watch would need to be kept of course, for adverse reactions in catheterised patients on the citrate drink regimen. At this stage however, it seems to offer the exciting prospect of control of a complication that undermines the quality of life for so many elderly and disabled people.

5. Conclusions

5. Conclusions

- When all-silicone, silicone-coated latex, hydrogel-coated latex and hydrogel/silver-coated latex catheters are inserted into the model bladders which contain alkaline urine cultures of *P. mirabilis*, a 'foundation-layer' comprised predominantly of calcium phosphate micro-crystals is first laid down on the catheter surfaces. The bacterial cells then colonize the material and the formation of the crystalline biofilm that encrust and block catheters begins.

- The 'foundation layer' was not essential for the development of these biofilms as cells were also observed adhering directly to the catheter or being trapped in irregular areas on the catheter surfaces.

- The 'foundation layer' will immediately alter the surface properties of an indwelling device by creating a calcified façade. This has important consequences for the efficacy of materials aimed at preventing bacterial adherence or those with antimicrobials immobilised onto their surfaces as *P. mirabilis* cells will be shielded from the catheter surface by the layer of microcrystals.

- Approaches to developing urological devices that are not vulnerable to encrustation need to take into account that *P. mirabilis* can initiate crystalline biofilm formation on catheters in a variety of ways depending on the prevailing conditions in the urinary tract.

- Currently available antimicrobial catheters impregnated with nitrofurazone or manufactured from hydrogel/silver-coated latex are extremely vulnerable to *P.*

mirabilis adherence, colonization and crystalline biofilm formation *in vitro*. Furthermore, hydrogel/silver-coated latex catheters removed from long-term catheterised patients were extremely prone to multi-species crystalline biofilm development and rapid catheter blockage.

- Inflating the catheter balloon with triclosan solution prevents *in vitro* crystalline *P. mirabilis* biofilm development by a combination of its antibacterial action and ability to maintain the residual urine at an acidic pH preventing crystal formation in the urine.

- Mannose resistant (MR/P) fimbriae are not essential for *P. mirabilis* colonization of catheter surfaces or the development of crystalline *P. mirabilis* biofilm. Although the wild-type MR/P producing *P. mirabilis* and MR/P-negative mutant strains initiated biofilm formation in different ways, the overriding factor in catheter blockage is not bacterial colonization of the device but the generation of alkaline urine above that of the urine pH_n.

- The pH at which crystals of calcium and magnesium phosphate form in the urine, the nucleation pH (pH_n) of that urine can be manipulated. *In vitro* it can be achieved simply by diluting the urine and by increasing its citrate concentration. This effect can also be achieved by encouraging volunteers to consume citrate-containing drinks in addition to their normal fluid intake.

- Experiments in bladder models infected with *P. mirabilis* showed that raising the pH_n of urine by dilution or adding citrate, significantly extended the times

catheters took to block. This strategy similarly reduced the rate of encrustation by

P. vulgaris and *Pv. rettgeri*.

- Data from experiments in bladder models supplied with urine from volunteers on different fluid intake regimens showed that the mean lifespan of catheters increased significantly with the rise in pH_n of the urine.

- The pH_n of the urine from volunteers who drank 2 L of a lemonade drink/day in addition to their normal fluid intake, rose to a mean value of 8.1. This is above the mean pH_v of 85% of catheterised patients infected with *P. mirabilis*. Such a strategy should thus resolve the complication of catheter encrustation in the majority of patients.

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