

**CRYSTALLINE BACTERIAL BIOFILM
FORMATION ON URINARY CATHETERS BY
UREASE PRODUCING URINARY TRACT
PATHOGENS**



Thesis presented for the Degree of Philosophiae Doctor

by

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November 2007

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Acknowledgements

I would like to thank Dr David Stickler for his excellent supervision, help, advice and encouragement during the study which has helped to make it such an enjoyable experience.

To my family for their support and understanding throughout the study and my university life.

Thanks to my friends and colleagues in the research group at Cardiff University who made it such a great place to work and also provided invaluable help and advice when needed: Nora, Sheridan, Gareth, Sazzle, Gwenz, Nicola, Mike, John, Steve.

I would also like to thank Mike Turner, Rob Young and Dr A. C. Hann for all their advice and help with electron microscopy.

Special thanks must go to Mrs Joan Hubbard for all of her assistance and understanding with autoclaving.

Last but by no means least, I would like to thank my girlfriend Rebecca for her patience and support during the study.

Publications and presentations

Broomfield, R. J. and Stickler, D. J. (2005). P496. Crystalline bacterial biofilm formation on urinary catheters by urease producing urinary tract pathogens. *Clin Microbiol Infect* **11, Suppl 2:** 130. Presented at the 16th ECCMID (European Congress of Clinical Microbiology and Infectious Diseases), Copenhagen: 2 – 5 April 2005.

Broomfield, R. J. and Stickler, D. J. (2006). Crystalline bacterial biofilm formation on urinary catheters by urease producing urinary tract pathogens. Presented at the fourth all-Wales Microbiology Meeting, Gregynog Hall: 20 – 22 March 2006

Summary

Long-term bladder catheterisation inevitably results in bacteriuria and the formation of biofilms on the catheters. Crystalline biofilms form when urease-producing bacteria infect the catheterised urinary tract. These organisms generate alkaline urine causing the crystallisation of calcium and magnesium phosphates and inducing the complication of catheter blockage. The principal organism associated with the formation of crystalline biofilms on urinary catheters is *Proteus mirabilis*. While several other urinary tract pathogens are capable of producing urease, there is little epidemiological or experimental evidence to indicate whether or not they are involved in the encrustation and blockage of indwelling catheters.

The aim of this study was to compare the abilities of various urease-positive species to encrust and block catheters with crystalline biofilm. Experiments were performed in laboratory models of the catheterised bladder infected with a range of urease-producing species. The results of these experiments allowed the classification of the bacteria into three groups: rapid encrusters, slow encrusters and non-encrusters. Rapid encrusters (*Pr. mirabilis*, *Proteus vulgaris* and *Providencia rettgeri*) were able to raise the urinary pH to 8 – 9 and cause catheter blockage within 37 h. Slow encrusters (*Morganella morganii*, *Staphylococcus aureus* and *Staphylococcus saprophyticus*) were able to raise the urinary pH moderately (from 6.1 to 6.89 – 7.39 over 96 h) and cause the formation of some encrustation on the catheters. Non-encrusters (*Providencia stuartii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter freundii* and *Citrobacter koseri*) were not able to raise the mean urinary pH above mean levels of 6.45 and did not form crystalline biofilm.

Pr. mirabilis, *Pr. vulgaris* and *Pv. rettgeri* were also capable of rapidly encrusting silver-hydrogel coated latex catheters (Bard I.C. catheter) and nitrofurazone impregnated silicone catheters (Rochester NF catheter). There were no significant differences between the times these organisms took to block these catheters compared to all-silicone control catheters. The antimicrobial catheters also had no effect on the urinary pH generated by these organisms. The insensitivity of the three encrusting species to nitrofurazone (MICs 32 – 128 µg/ml) is clearly a major factor in the failure of these catheters to prevent encrustation.

The results of experiments in which the balloons of all-silicone catheters were inflated with solutions of triclosan (3 mg/ml in 0.1 M sodium carbonate) confirmed previous observations that catheter encrustation by *Pr. mirabilis* was prevented by this strategy. It also proved effective against *Pr. vulgaris*. In both cases, in contrast to the controls, the numbers of viable cells recovered from the residual urine fell steeply within 24 h, the pH of the urine dropped below its nucleation pH (pH 6.5) and the catheters drained freely for the seven day experimental period. The effect of triclosan on encrustation by *Pv. rettgeri* was minimal however, with no significant difference between blockage times or urinary pHs in the test and control models. While the *Proteus* sp. had MICs of triclosan of ≤ 0.2 µg/ml, the value for *Pv. rettgeri* was 64 µg/ml. Inflating catheter balloons with a solution which generated nitric oxide proved ineffective as a means of controlling catheter encrustation.

Previous studies have shown that a simple cellulose acetate / bromothymol blue sensor is capable of signalling infection by *Pr. mirabilis* and the early stages of catheter encrustation. Placed in the drainage bag it can give early warnings to patients, carers and nurses that catheters need to be replaced. While the use of the sensor in this way could avoid the clinical crises induced by catheter blockage, it would be of more value if an effective strategy to inhibit encrustation could be deployed when the problem is signalled. In the present study it was demonstrated that strips of the sensor polymer placed in the drainage bags changed from yellow to blue signalling the rise in urinary pH induced by infection with *Pr. mirabilis*, *Pr. vulgaris* or *Pv. rettgeri*. Electron microscopy confirmed that encrustation had started on the catheters at the times the sensors turned blue. Triclosan (3 mg/ml) introduced into the catheter balloons when the signal was observed was found to halt the development of the *Proteus* crystalline biofilms. It was concluded that an integrated sensor / modulator strategy was feasible for the control of encrustation by these species. The sensor was also effective in providing early warning of blockage by the slow encrusting species *M. morgani* and *Staph. saprophyticus*. Experiments with the non-encrusting species *E. coli*, *Ent. faecalis* and *K. pneumoniae* in the model system showed that the sensors remained yellow in colour throughout the tests. Some false positive signals were observed however in the cases of *Staph. aureus*, *Ps. aeruginosa* and *Pv. stuartii*. Better specificity might be achieved by placing the sensor at the catheter / drainage tube junction since this would allow monitoring of the voided urine rather than the pH of urine stored in the bag.

Pr. mirabilis is generally present as part of mixed communities of organisms in the urine of patients undergoing long-term catheterisation. The availability of collections of organisms from the urine of three such patients whose catheters block very slowly or not at all made possible a study on the effect of the other members of the bacterial community on the rate at which *Pr. mirabilis* produced crystalline biofilm. In one of the cases it was found that a community of *E. coli*, *Ps. aeruginosa* and *K. oxytoca* had a profound effect on the rate of encrustation by *Pr. mirabilis*. While the *Pr. mirabilis* was able to block catheters in a mean time of 40 h, the community delayed blockage until 147 h in one replicate and in two others the catheters in models inoculated with all four organisms drained freely for the full 168 h test period.

The results of this study show that while a large number of urease positive species colonize the catheterised urinary tract, only a few species are capable of causing rapid encrustation. Of these *Pr. mirabilis* is by far the most common and it should be the focus of strategies to control catheter encrustation. The sensor / modulator concept using the cellulose acetate / bromothymol blue polymer and triclosan is clearly a feasible strategy for the management of catheter encrustation. Further work on the identification of relatively benign species that are antagonistic to *Pr. mirabilis* would be of interest. It could lead to the development of a biological interference study to control the problem that complicates the care of many elderly and disabled people.

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List of Abbreviations

AAS	-	Atomic absorption spectrometry
AHL	-	Acyl-homoserine lactone
ANOVA	-	Analysis of variance
BTB	-	Bromothymol blue
CAUTI	-	Catheter-associated urinary tract infection
Ch	-	Charriere (outer circumference of catheter (mm))
cfu	-	Colony forming units
CI	-	Colistin inositol (agar)
CLED	-	Cysteine-lactose electrolyte deficient (agar)
DLC	-	Diamond-like carbon
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraacetic acid
EMRSA	-	Epidemic methicillin-resistant <i>Staph. aureus</i>
EPS	-	Extracellular polysaccharide
ESEM	-	Environmental scanning electron microscopy
HMDS	-	Hexamethyldisilazane
HV-SEM	-	High vacuum scanning electron microscopy
IgA	-	Immunoglobulin A
ISA	-	Iso-sensitest agar
ISB	-	Iso-sensitest broth
IUC	-	Indwelling urinary catheter
LV-SEM	-	Low vacuum scanning electron microscopy
MIC	-	Minimum inhibitory concentration

List of Abbreviations

MRSA	-	Methicillin-resistant <i>Staph. aureus</i>
MSIC	-	Minimum swarming inhibitory concentration
NO	-	Nitric oxide
pH _n	-	Urinary nucleation pH
pH _v	-	Voided urine pH
RNS	-	Reactive nitrogen species
SE	-	Standard error
TSA	-	Tryptone soya agar
TSB	-	Tryptone soya broth
UTI	-	Urinary tract infection

Section 1

Introduction

1. Introduction

Urinary catheters are the most commonly used prosthetic device, with over 30 million being deployed every year in the USA alone (Darouiche, 2001). They are widely used for the long-term management of urinary incontinence or retention in the elderly and patients disabled by strokes, spinal injuries, and neuropathies such as multiple sclerosis. In addition urinary catheters have a number of short-term uses in hospitalisation, such as allowing the measurement of urine output in severely ill patients or facilitating repair of the urethra following surgery (Kunin, 1988). Although catheters provide a convenient way to drain urine from the bladder, unfortunately they also undermine important defence mechanisms of the bladder and induce vulnerability to infection. As a result they are a major cause of the infections that are acquired by patients in healthcare facilities (Meers *et al.*, 1981; Jepsen *et al.*, 1982; Warren *et al.*, 1989). It is difficult to understand why at a time when spectacular advances have been made in other areas of medicine, that it is still not possible to perform the relatively simple task of draining urine from the bladder without putting patients at risk. There is a clear clinical need to understand the processes that induce the infection-associated complications in catheter management and devise novel strategies to control the problems that pose severe threats to the health of many elderly and disabled people.

1.1 The history of the urinary catheter.

Self-retaining Foley catheters were initially designed to control bleeding following transurethral resectioning of the prostate (Foley, 1929). It was soon realised however

that indwelling urinary catheters would be useful in the management of incontinence, allowing drainage of the bladder (Foley, 1937). The original Foley catheter was made of latex and consisted of a hollow tube down which the urine flows with a second smaller tube embedded in its wall to allow inflation of the retention balloon once the catheter was in place. The design of the indwelling catheter has remained essentially unchanged from Foley's original.

There are two types of indwelling catheterisation used currently, urethral and suprapubic (Figure 1.1). Urethral catheterisation involves the insertion of the catheter into the bladder via the urethra. This is the most commonly used form of catheterisation and can be performed by health care professionals without the need for extensive training. In suprapubic catheterisation the catheter is inserted directly into the bladder through the anterior abdominal wall. This is a surgical procedure, and can be subject to complications resulting in bladder damage (Hamid *et al.*, 2002; Witham and Martindale, 2002).

1.2 The prevalence of urinary catheterisation.

The use of indwelling urinary catheters (IUCs) is widespread throughout the world. Large numbers of hospital patients undergo indwelling bladder catheterisation. A prevalence study in hospitals in 8 European countries found that between 5 and 25 % of patients were catheterised (Jepsen *et al.*, 1982). Another large scale study by Zimakoff *et al.* (1993) found that 13.2 % of hospital patients in Denmark were catheterised. Other smaller scale studies conducted in Europe and the USA have also found similar levels of catheterisation in hospital patients (Gastmeier *et al.*, 2000;

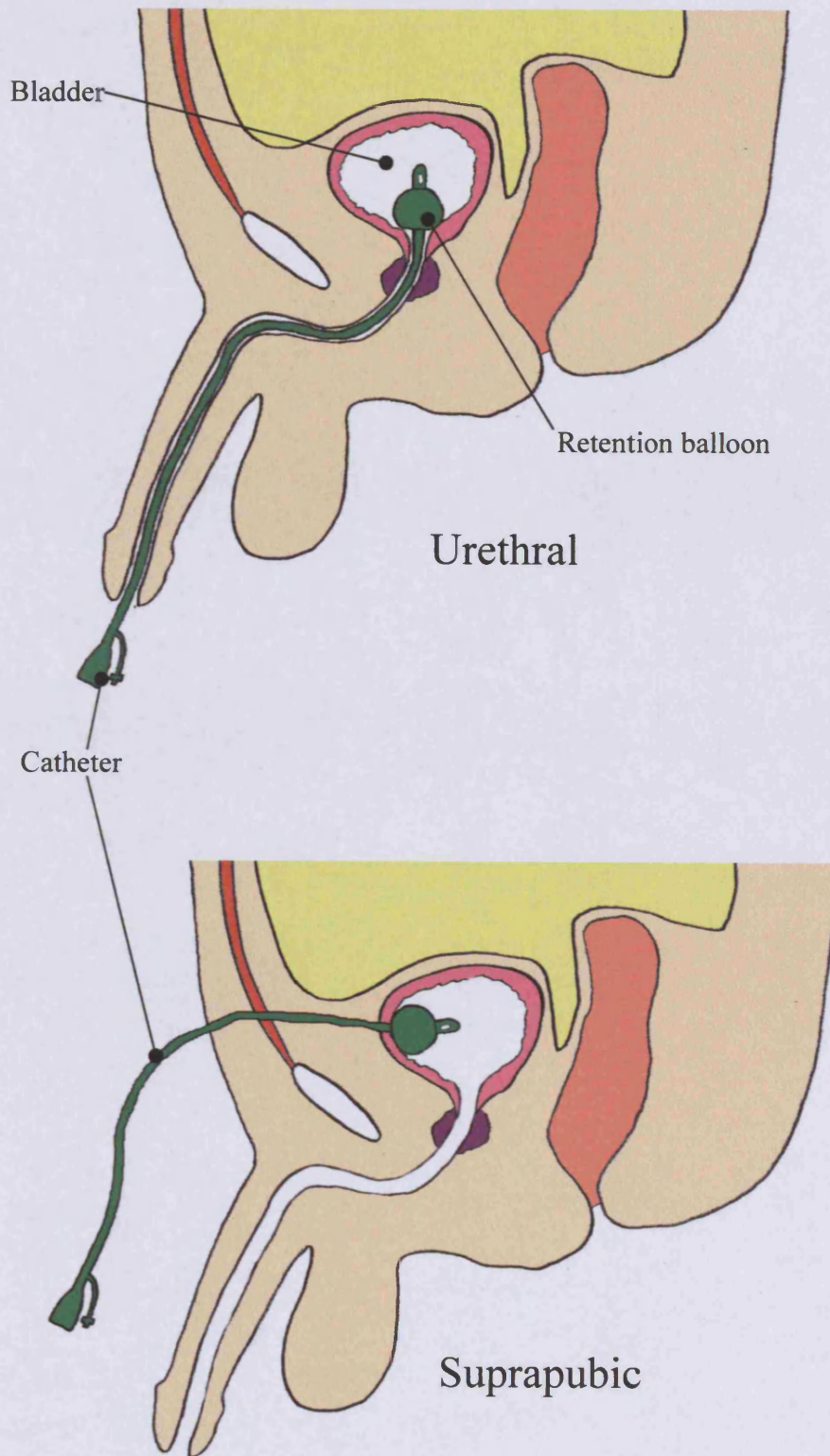


Figure 1.1: The placement of urethral and suprapubic catheters.

Saint *et al.*, 2000). Catheterisation in hospital patients is often short term (≤ 28 days). Merle *et al.* (2002) for example, found that the mean duration of catheterisation among 10,000 urologic patients over 6 years in a French hospital was 3 days, with a maximum of 70 days.

Catheterisation is also common in community care, particularly among patients with spinal cord injuries or neuropathies and in the elderly. Studies by Warren *et al.* (1989), Zimakoff *et al.* (1993) and Smith *et al.* (2000) reported the prevalence of urinary catheterisation to be between 4.9 % and 7.5 % for the residents of long-term care facilities. Kunin *et al.* (1992) found that 10.5 % of patients at nursing homes were catheterised on entry, and that these patients tended to remain catheterised during the study period (1 year). In a survey of the use of catheters in nursing homes in England McNulty *et al.* (2003) found that overall 9 % of residents were catheterised, although there was wide variation between nursing homes with some having as many as 40 % of residents catheterised. Getliffe and Mulhall (1991) and Zimakoff *et al.* (1993) also found that 4 % and 3.9 % respectively of patients who were receiving home care were catheterised. Long-term catheterisation (> 28 days) is common in community care patients (Getliffe and Newton, 2006) and is most often used in the management of bladder dysfunction (urinary retention or chronic urinary incontinence (Warren, 1992)).

1.3 Catheter associated urinary tract infections (CAUTIs).

Underlying many of the complications associated with catheter management is the common occurrence of catheter-associated urinary tract infection (CAUTI). Patients

often develop bacteriuria (the presence of bacteria in the urine) within a few days of catheterisation. Garibaldi *et al.* (1974) estimated risk of developing bacteriuria at between 3 and 10 % per day. Warren *et al.* (1987) found that bacteriuria was universal in a study on long-term catheterised women. Long-term catheterised patients (> 28 days) will inevitably develop bacteriuria (Stickler and Zimakoff, 1994). An analysis of pooled data by Saint (2000) revealed that patients catheterised for 2 – 10 days can be expected to develop bacteriuria in 26 % of cases.

While bacteriuria is normally asymptomatic, other more serious conditions such as bacteraemia (the presence of bacteria in the blood) (Warren *et al.*, 1987; Jewes *et al.*, 1988) and pyelonephritis (acute bacterial infection of the kidney) (Warren *et al.*, 1988) can develop from it. In addition bacteriuria involving urease positive organisms is associated with catheter obstruction (Mobley and Warren, 1987) and the formation of infection stones in the bladder (Bichler *et al.*, 2002). In the very long-term (years) bladder cancer is associated with chronic catheterisation (West *et al.*, 1999) (Section 1.3.3).

Nickel (1991) stated that “inserting and leaving a Foley catheter *in situ* is like building a bridge between the outside world and the sterile bladder, along which bacteria can travel”. Given the prevalence of bacteriuria in catheterised individuals, this statement would seem to be accurate. Despite this catheters remain a popular method for long-term bladder management, largely due to a lack of suitable alternatives.

With the increased occurrence of UTIs associated with catheterisation, unnecessary catheterisation should be avoided. Jain *et al.* (1995) found in a prospective study that

in 21 % of cases the use of an IUC was 'unjustified', and that in 47 % of cases continued catheterisation was 'unjustified'. Saint *et al.* (2000) and Hazelett *et al.* (2006) also found that catheter use was 'inappropriate' in 31 % and 49 % of cases respectively. The unnecessary use of catheters in these instances is both dangerous and costly, not only in terms of patient health but also financially (due to the cost of treating additional problems).

Suprapubic catheters are sometimes used as an alternative to urethral catheterisation. They are inserted directly into the bladder through the anterior abdominal wall. The onset of bacteriuria in suprapubic catheters is slower than that in urethral catheters, with 50% of patients infected on day 40 as opposed to 50% infection after one week with urethral catheters (Ringert and Gross, 1996). In the long-term however bacteriuria is still inevitable. Newman and Price (1977) reported a 95% incidence of bacteriuria in spinal cord patients with long-term suprapubic catheterization. Sheriff *et al.* (1998) reported similar problems to those found in long-term urethral catheters (bacteriuria, catheter blockage, urine leakage) although they also reported that the general level of satisfaction with the catheters was high. Complications related to the insertion of suprapubic catheters in spinal cord patients, resulting in bladder damage, have also been reported (Hamid *et al.*, 2002; Witham and Martindale, 2002). Suprapubic catheterisation has also been associated with high rates of bladder stone formation (Nomura *et al.*, 2000). Suprapubic catheterisation provides an alternative to the urethral catheter but it is subject to additional complications.

1.3.1 The routes of infection in IUCs.

There are three main routes of infection associated with IUCs (Figure 1.2). These are: (a) infection due to carriage of bacteria into the bladder as the catheter is inserted, (b) the periurethral route (bacteria migrating along the outside of the catheter and urethral mucosa sheath) and (c) the intraluminal route (through the lumen of the catheter).

The bacterial flora of the urethral meatus can be introduced into the bladder on catheterisation and produce bacteriuria (Barnes *et al.*, 1992; Schlager *et al.*, 1999). A system bypassing the first 1.5cm of the distal urethra was found to decrease the incidence of CAUTIs in men with spinal cord injury (Bennett *et al.*, 1997), indicating the importance of this route in CAUTIs.

Infection along the outside of the catheter and urethral mucosa sheath has also been shown to be an important route for catheter associated bacteriuria. Kass and Schneiderman (1957) found that after the application of a small amount of *Serratia marcescens* to the periurethral epithelium of 3 patients with IUCs the bacteria were cultured from the patients' urine between 1 and 3 days later. Although highly unethical by today's standards this study does indicate the speed with which bacteria are able to migrate along the outside of IUCs to the bladder. Daifuku and Stamm (1984) found that urethral colonization preceded bladder colonization in 12 of 18 women and 5 of 17 men with bacteriuria.

Infection through the lumen of the catheter itself is also an important route. The introduction of closed collection systems in the 1960's was responsible for delaying the onset of bacteriuria (Garibaldi *et al.*, 1974). Since then contamination of the



Figure 1.2: Structure of a urinary catheter and potential routes of infection. The catheter is inserted into the urethra of the patient and the retention balloon inflated with 10 ml of sterile water via a syringe attached to the balloon inflation port. Once in place urine flows into the catheter through the eyehole and down the lumen to an attached collection bag.

collection bag or a failure of the closed drainage system have been responsible for most intra-luminal infections. A study of the relative importance of infection routes by Tambyah *et al.* (1999) found that where the route of entry could be determined, 34 % of contaminants used the intra-luminal route, with extra-luminal contamination (along the urethra or contamination during insertion) accounting for the remaining 66 %.

1.3.2 Pathogens associated with CAUTI.

A wide range of microorganisms are involved in CAUTIs. In a large scale study carried out in 25 countries (141 hospitals) on nosocomial UTIs the major microorganisms isolated from urine samples were (on average), *Escherichia coli* (25.1 %), *Enterococcus* sp. (13.2 %), *Pseudomonas aeruginosa* (10.5 %), *Klebsiella* sp. (10 %), *Proteus* sp. (7.3 %), *Enterobacter* sp. (5 %) and *Staphylococcus aureus* (3.7 %) (Bouza *et al.*, 2001). A significant component of the isolates were yeast of the genus *Candida* (16.4 %). The vast majority (90.8 %) of these catheterisations were short term (< 30 days). The organisms isolated from long-term catheterisations are similar to those found in short term catheterisations, with the most common being *Providencia stuartii* (which has been particularly associated with infections in nursing homes (Muder *et al.*, 1992)), *Pr. mirabilis*, *Morganella morganii*, *Enterococcus* sp., *Ps. aeruginosa*, *E. coli*, *Klebsiella* sp. and *Citrobacter* sp. (Clayton *et al.*, 1982; Warren *et al.*, 1987; Kunin, 1989). *E. coli* has been found to be the most commonly isolated organism from long-term catheterised women, with *Pr. mirabilis* being more common in men (Nicolle *et al.*, 1996). Infections occurring in short term catheterisations are generally caused by a single species (Bouza *et al.*, 2001) while

long-term catheterisations are often polymicrobial, consisting of two or more species (Clayton *et al.*, 1982; Warren *et al.*, 1982; Jewes *et al.*, 1988). A study on patients who had acquired bacteriuria after short term catheterisation (Harding *et al.*, 1991) found that younger women (≤ 65) were significantly more likely to have resolved the infection 14 days after catheter removal than older women (> 65). Other risk factors involved in UTIs are: female gender, duration of catheterisation, lack of systemic antibiotics, and violations of catheter care (Stamm, 1991).

1.3.3 The impact of CAUTIs on morbidity and mortality.

Long-term IUCs have a major effect both on patient morbidity and mortality. Kunin *et al.* (1992) found the IUC to be an independent risk factor for death in long-term nursing home facilities. In a prospective study they found that patients catheterised for more than 76 % of their days resident in the nursing homes were three times more likely to die within one year than non-catheterised residents. As mentioned previously patients with long-term IUCs will inevitably develop CAUTI (Stickler and Zimakoff, 1994). Although asymptomatic bacteriuria in hospitalised patients was found to be unrelated to bacteraemia by Tambyah and Maki (2000) a study by Mylotte *et al.* (2002) on nursing home acquired bacteraemia found that for 51 % of clinical episodes the source of the infecting bacteria was the urinary tract (this was particularly true for *Pr. mirabilis* infections), although only 8 % of total mortality during the period of hospitalisation occurred in patients with bacteraemia from this source, compared with 58 % of total mortality from just 11 % of clinical episodes for respiratory sources. Bacteraemia has been found to progress into septicaemia (sepsis of the blood) in 10 – 16 % of urological patients (Grabe, 1987).

Acute and chronic pyelonephritis has been associated with long-term catheterisation (Warren *et al.*, 1988; Warren *et al.*, 1994). Autopsies carried out on elderly long-term catheterised nursing home patients found that acute renal inflammation was present in 38 % of patients who died with a urinary catheter in place vs. 5 % of non-catheterised patients (Warren *et al.*, 1988). A later study found that 10 % of patients catheterised for > 90 days during their last year of life had chronic pyelonephritis while none of the patients catheterised for \leq 90 days had chronic pyelonephritis (Warren *et al.*, 1994).

Chronic indwelling catheters have also been identified as an independent risk factor for bladder cancer in patients with spinal cord injuries (West *et al.*, 1999; Groah *et al.*, 2002; Hess *et al.*, 2003). Chronic infection and chronic inflammation of the bladder mucosa due to the presence of the catheter are thought to be important in the development of bladder cancer in long-term catheterised patients (Delnay *et al.*, 1999). The presence of nitrate reducing organisms such as *E. coli* or *Pr. mirabilis* has been shown to generate carcinogenic nitrosamines in the urine of paraplegic patients (Tricker *et al.*, 1991). Nitrosamines have been linked to the occurrence of a wide range of cancers (O'Brien *et al.*, 1996) and so their production in the catheterised urinary tract may well be responsible for bladder cancer.

1.4 Bacterial biofilms.

All patients undergoing long-term catheterisation will experience bacteriuria. While the catheter remains in place the bacteria are extremely difficult to eliminate from the urine with antibiotic therapy (Clayton *et al.*, 1982; Warren *et al.*, 1982). In most cases, for most of the time the patients do not experience symptomatic infection and it

is common practice not to attempt treatment with antibiotic therapy (Kunin *et al.*, 1987a). As a result urine containing large bacterial populations flows through the catheter for periods of up to 12 weeks at a time (the normal duration of placement for a long-term catheter). Bacteria have a propensity to colonize surfaces and live as sessile biofilm communities rather than as planktonic cells in suspension (Costerton *et al.*, 1987). In aquatic ecosystems, from rocks in streams to prosthetic devices implanted in the body, the vast majority of bacteria are found in biofilms on surfaces (Costerton *et al.*, 1995).

Catheters provide attractive, unprotected sites for bacterial colonization, particularly the rims of the eyeholes and the luminal surfaces (Stickler *et al.*, 2003b). Bathed in a constant gentle flow of a nutrient-sufficient medium, the sessile bacterial populations thrive. As a result extensive biofilm communities develop within days (Ganderton *et al.*, 1992). There are several major advantages for bacteria living in biofilms, including protection from antibacterial chemicals, bacteriophages and predators such as protozoa and phagocytes (Donlan and Costerton, 2002). Donlan and Costerton (2002) defined the biofilm as “a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription”.

1.4.1 Biofilm structure and formation.

Biofilm formation has been studied extensively in recent years, particularly in *Ps. aeruginosa*. The initial stage of biofilm formation is the attachment of cells to the

surface, via a conditioning layer, and the formation of dispersed monolayers. These monolayers then become confluent and microcolonies several cells thick then begin to form (Caldwell and Lawrence, 1986; Lawrence *et al.*, 1989). The form of mature biofilms is subject to some discussion. Lawrence *et al.* (1991) used scanning confocal laser microscopy to examine fully hydrated mature biofilms. They found that the mature biofilms had a distinct 'architecture'. The bacteria were embedded in an extracellular polysaccharide matrix (EPS) and channels and pores permeated the structure. Costerton *et al.* (1995) proposed a universal water-channel model of the mature biofilm based on the observation of *Ps. aeruginosa* biofilm formation (Figure 1.3). In this model bacterial microcolonies produce large quantities of EPS and begin to form cone shaped structures which extend away from the colonized surface. These then develop into pillars and mushroom shaped structures which may fuse together, with the mature biofilm consisting of these structures and a network of water channels between them. Costerton *et al.* (1995) suggested that these channels comprised a primitive circulatory system allowing exchange of nutrients and metabolites with the bulk fluid phase. It is important to note that much of the work on which this model was based was conducted in relatively low nutrient conditions. Some biofilms, such as those on teeth and urinary catheters, have been observed to form more densely packed structures with confluent layers of cells and little evidence of the water channels present in the Costerton model (Nyvad and Fejersdorf, 1989; Stickler *et al.*, 1993a). Wimpenny and Colasanti (1997) proposed a model of biofilm structure which attempted to explain these differences. Using a cellular automaton model they showed that the differences in biofilm structure could be explained by the availability of nutrients. In low nutrient environments the model predicted a structure similar to that of the Costerton model (see Figure 1.3) while in higher nutrient environments

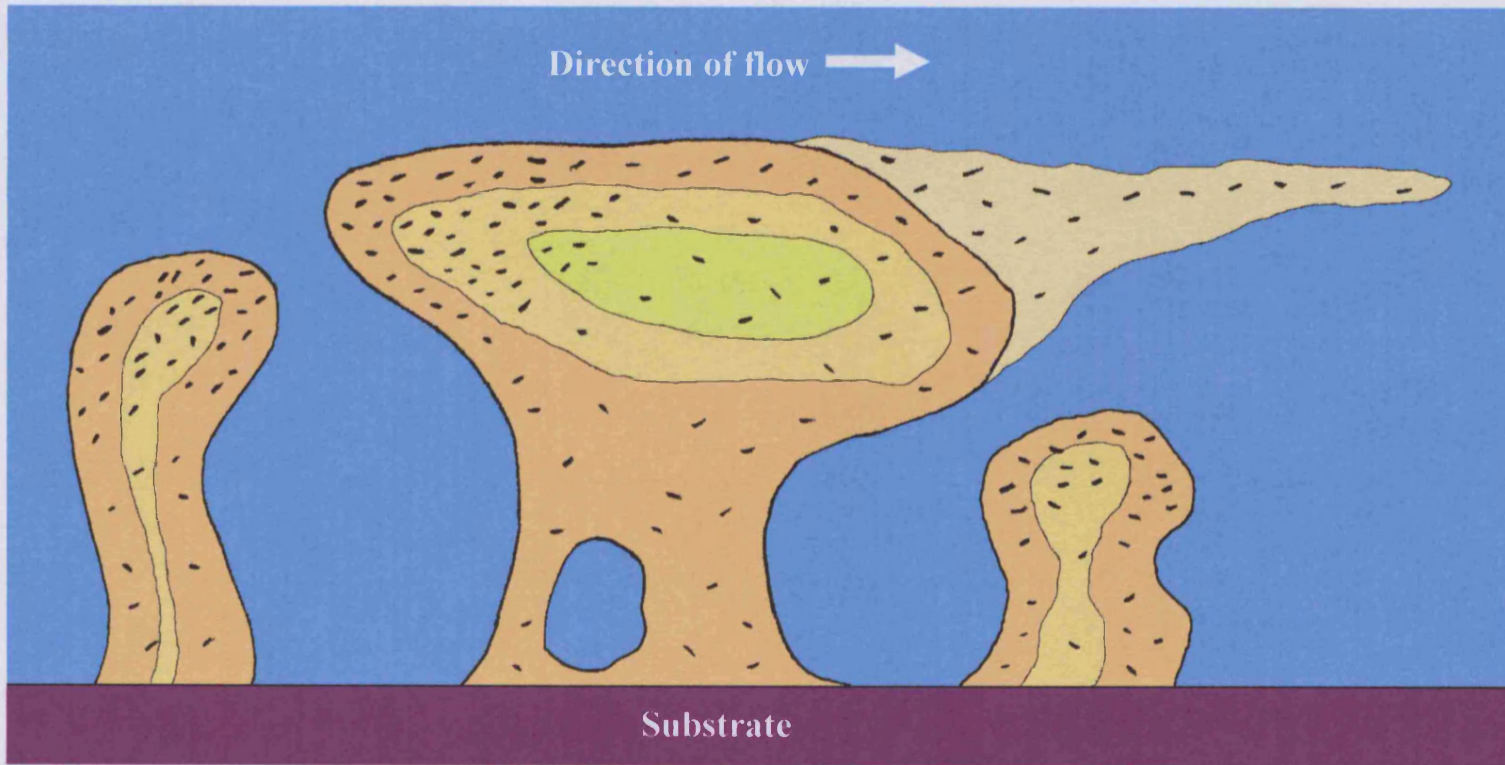


Figure 1.3: The structure of a mature biofilm as proposed by Costerton *et al.* (1995). The mature biofilm consists of matrix enclosed 'towers' and 'mushrooms' separated by water channels. Adapted from Donlan and Costerton (2002).

(such as the mouth or the urinary tract) the model predicted a dense biofilm with confluent layers of cells.

Bacteria in a biofilm show different phenotypes from those in planktonic suspension. Sauer *et al.* (2002) characterised 5 distinct stages in *Ps. aeruginosa* biofilm formation, with multiple phenotypes displayed which were distinct from the planktonic form. In the initial stages of biofilm formation bacteria often express specific surface contact factors, for example type I pili in *E. coli* (Pratt and Kolter, 1998). Costerton *et al.* (1995) stated that surface adhesion is 'remarkably unaffected by the physical or chemical nature of the surface concerned'. Studies by Downer *et al.* (2003) and Stickler *et al.* (2006b) however, have indicated that the properties of the surface (such as charge) have a significant effect on the adhesion of *Pr. mirabilis*. Price *et al.* (2005) also found that silane-treated silicone rubber was effective in reducing the adherence of *Candida* sp. in comparison to untreated controls. In implanted devices such as urinary catheters the surface of the device is often coated with a glycoproteinaceous film termed a 'conditioning film' which is formed from components of the surrounding body fluids (Gristina, 1987). This film has been shown to be important in the initial stages of biofilm formation, providing receptor sites for bacterial adhesion (Gristina, 1987; Lappin-Scott and Bass, 2001). While type I pili are important in adhesion, twitching motility, involving type IV pili rather than flagella (Wall and Kaiser, 1999), also appears to be associated with successful biofilm formation in *Pseudomonas* sp. (O'Toole and Kolter, 1998; Whitchurch *et al.*, 2002b) allowing colonization to spread over the substrate, particularly in the initial stage of biofilm formation.

The mature biofilm is composed of a hydrated EPS matrix in which the bacteria reside (Sutherland, 2001). Lawrence *et al.* (1991) determined that the biofilms they were studying were composed of 80 – 95 % non-cellular material (the matrix). This matrix often contains other compounds such as signalling molecules and DNA (Stickler *et al.*, 1998b; Whitchurch *et al.*, 2002a). Signalling molecules are used in bacterial communication known as quorum sensing. Quorum sensing is a cell density-dependent system which allows the coordination of behaviour (i.e. phenotype) in a bacterial population, for example the production of virulence factors (Parsek and Greenberg, 2000). Once a threshold concentration of the quorum sensing molecules has been reached a change in global cell regulation is triggered (Spoering and Gilmore 2006). While Gram-positive and Gram-negative bacteria use different molecules for quorum sensing the use of signalling molecules appears to be important for biofilm formation in both. The production of a competence signalling peptide in *Streptococcus* sp. has been shown to contribute to biofilm formation (Li *et al.*, 2002a; Petersen *et al.*, 2004) while acyl-homoserine lactones (AHLs) have been found in the biofilms formed by several Gram-negative species (Davies *et al.*, 1998; Stickler *et al.*, 1998b; Lehner *et al.*, 2005; Rice *et al.*, 2005). The disruption of biofilm formation using non-native acyl-homoserine lactone derivatives has been observed in *Ps. aeruginosa* biofilms (Geske *et al.*, 2005). Mature biofilms also show heterogeneity in physiological activity. Xu *et al.* (1998) showed that physiologically active cells were restricted to the top 30 μm of a 130 μm deep *Ps. aeruginosa* biofilm. This was determined to be due to the limited oxygen availability in the lower layers of the biofilm.

Bacteria emigrate from an established biofilm as individual cells or as aggregates of cells which slough off (Donlan and Costerton, 2002). Studies by Davies *et al.* (1998)

and Rice *et al.* (2005) showed that this may be mediated by AHLs. Detachment of large pieces of biofilm may aid in the development of infections in a medical setting (Donlan and Costerton, 2002).

1.4.2 Multi-species biofilms.

Bacterial biofilms such as in the bovine rumen, oral biofilms, and those occurring on urinary catheters are usually composed of several species (Stickler, 1999; Stoodley *et al.*, 2002; Li *et al.*, 2004). Co-aggregation, in which genetically distinct bacteria become attached to one another through specific molecular interactions, is thought to be important in the development of multi-species oral biofilms (Rickard *et al.*, 2003). This occurs either by single planktonic cells adhering to genetically distinct cells in a biofilm or by groups of already co-aggregated cells adhering to the biofilm (known as co-adhesion (Bos *et al.*, 1994)). Bacteria in mature oral biofilms are often seen in associations known as ‘corn-cobs’ such as those observed by Cobb *et al.* (2003). The extent to which this occurs in multispecies urinary catheter biofilms is unknown.

1.4.3 The resistance of biofilms to anti-microbial agents.

One of the most important features associated with biofilms is their increased resistance to antimicrobials relative to genetically identical bacteria in a planktonic state. Nickel *et al.* (1985b) and Ladd *et al.* (1987) demonstrated that *Ps. aeruginosa* biofilms growing on sections of catheter material were capable of surviving concentrations of tobramycin as high as 1000 mg/l for 12 h or more while planktonic cells were rapidly killed with just 50 – 100 mg/l. Pascual *et al.* (1993) also found

increased resistance in *Ps. aeruginosa* catheter associated biofilms. Biofilms have also been shown to increase resistance in a number of other organisms such as *Burkholderia cepacia*, *Staph. aureus* and *E. coli* (Desai *et al.*, 1998; Harrison *et al.*, 2004). The increased resistance of biofilms to antibiotics can be dramatic (Donlan and Costerton, 2002). Ceri *et al.* (1999) found that antibiotic resistance could be as much as 1000 times greater for biofilms when compared to planktonic cells.

There are a number of theories which attempt to explain the increased resistance of cells in biofilms to antimicrobial agents. The biofilm matrix has been proposed to constitute a diffusion barrier, slowing significantly the penetration of antimicrobial agents into the biofilm. Suci *et al.* (1994) found that the penetration of ciprofloxacin into a surface was slowed when a *Ps. aeruginosa* biofilm was present. A suspension of alginate (EPS) from *Ps. aeruginosa* was also able to inhibit the diffusion of gentamicin and tobramycin (Hatch and Schiller, 1998). The inhibition of antimicrobial diffusion has been linked with their binding to biofilm matrix constituents (Suci *et al.*, 1994; Mah and O'Toole, 2001). Increased production of EPS has been observed in *Ps. aeruginosa* biofilms in response to imipenem (Bagge *et al.*, 2004). Some biofilms however are not so effective in preventing the diffusion of antimicrobials (Dunne *et al.*, 1993) so limited diffusion into the biofilm cannot fully explain the increased resistance observed.

The growth rate of cells in a biofilm is often slowed compared to that in the planktonic state (Wentland *et al.*, 1996). It has been proposed that since the cells in a biofilm are growing more slowly the uptake of antibiotics will also be reduced, leading to increased resistance (Donlan and Costerton, 2002). Anwar *et al.* (1992)

found that older *Ps. aeruginosa* biofilms (10 day old) were significantly less susceptible to antibiotics than younger biofilms (2 day old). The slowest growing biofilm cells have also been found to be the most resistant in *E. coli* (Evans *et al.*, 1991). However they also found that slow growing planktonic and biofilm *Ps. aeruginosa* cells were equally resistant to ciprofloxacin while at increased growth rates the planktonic cells were more susceptible than the biofilm cells. This suggests that a property of the biofilm other than simply growth rate was responsible for the increased resistance, as pointed out by Mah and O'Toole (2001). Walters *et al.* (2003) however found that oxygen limitation and low metabolic activity but not poor antibiotic penetration were associated with increased *Ps. aeruginosa* biofilm resistance to ciprofloxacin (and tobramycin).

Brown and Barker (1999) suggested that the slower growth rate seen in some biofilm cells might be related to a stress response rather than simply to nutrient limitation. The changes in gene expression result in physiological changes which may serve to protect the cell from antimicrobials. For example *E. coli* cultures at high cell density have been shown to produce increased levels of catalase, which would help to protect against hydrogen peroxide (Liu *et al.*, 2000). Elkins *et al.* (1999) observed that *Ps. aeruginosa* biofilms exposed to hydrogen peroxide produced catalase in response, which may indicate that the response to antimicrobials is adapted to the type of agent used.

A review by Lewis (2005) put forward the theory that biofilm resistance can be attributed to 'persister' cells. In some of his earlier work he noted some *Ps. aeruginosa* biofilm cells were essentially 'invulnerable' when exposed to high levels

of ofloxacin while the bulk of the biofilm was quite vulnerable (Brooun *et al.*, 2000). These cells were thought to be phenotypic variants of the wild type rather than mutations since they allow the regeneration of the original population after treatment (Keren *et al.*, 2004). He also argued that the presence of ‘persisters’ could explain the relapsing nature of biofilms in infection after antibiotic treatment, with ‘persisters’ being protected by the biofilm matrix from host defences and then repopulating the biofilm (Lewis, 2005).

The mechanism of biofilm resistance thus continues to be the subject of much discussion and speculation and it may well be due to a combination of the factors mentioned rather than to a single process.

1.4.4 Catheter associated biofilms.

The importance of biofilm formation in infections resulting from long-term catheterisation was first realised in the 1980’s (Nickel *et al.*, 1985a). Nickel *et al.* (1989) found that the biofilms on the catheters they examined were composed of a wide range of species, some of which had not been detected using routine culture methods. Ganderton *et al.* (1992) reported that catheters removed from patients had biofilms present on their surfaces and that biofilms were occasionally present when bacteriuria had not been detected. They also reported that the biofilms observed were often polymicrobial and that there was a large variation in the depth of the biofilm which did not correspond to the time that the catheter had been indwelling. Analysis of catheter biofilms has shown little evidence of some of the more structured elements such as water channels which have been reported in biofilms found in other locations

(Stickler *et al.*, 1993a). Ohkawa *et al.* (1990) found biofilms on catheters that were indwelling for one week. They hypothesised that these biofilms acted as reservoirs of infection due to the protection given to bacteria within them from chemotherapeutic agents used in the treatment of urinary tract infections, as demonstrated by Pascual *et al.* (1993).

Biofilms are also able to rapidly colonize other areas of urinary tract management devices such as catheter drainage bags. Rogers *et al.* (1996) found *in vitro* biofilm formation occurred rapidly when bacteria were inoculated into drainage bags.

In addition to biofilms composed of bacteria in an EPS matrix, such as those observed by Ganderton *et al.* (1992), biofilms containing large amounts of crystalline material commonly occur in urinary catheters (Cox *et al.*, 1989)

1.5 Urinary catheter encrustation.

Many of the biofilms that form on long-term catheters are crystalline in nature (Stickler *et al.*, 1993b). They encrust the catheter lumen and can block the flow of urine. This results either in leakage of urine around the outside of the catheter, patients becoming incontinent, or retention of urine and painful distention of the bladder (Getliffe and Mulhall, 1991; Stickler and Zimakoff, 1994). Retention of urine due to catheter blockage is potentially dangerous, as it can cause a reflux of infected urine to the upper urinary tract leading to cases of pyelonephritis and septicaemia (Wagenlehner and Naber, 2000). Encrustations can also occur on the retention balloon and eye-hole of the catheter (Stickler and Zimakoff, 1994; Morris and

Stickler, 1998a). Due to their crystalline nature, encrustations can cause trauma to the bladder mucosa and to the urethra (Stickler and Zimakoff, 1994).

Analysis of encrusted catheters removed from patients has shown that the crystalline material is composed of a combination of magnesium ammonium orthophosphate hexahydrate (struvite) and hydroxyapatite (calcium phosphate) (Cox *et al.*, 1987). The struvite appears as large coffin-shaped crystals. The hydroxyapatite occurs as aggregates of microcrystals (Cox *et al.*, 1989).

Kunin *et al.* (1987b) characterised patients who were subject to persistent catheter blockage as 'blockers'. No significant difference was found in the makeup of urine from 'blockers' and 'non-blockers'. This was confirmed by the studies of Choong *et al.* (2001). In both instances urinary pH was found to be significantly higher for 'blockers' than 'non-blockers'. Studies by Mobley and Warren (1987) and Kunin (1989) found that the urine of patients who experienced catheter blockage was often colonized by urease positive bacteria, particularly *Pr. mirabilis*.

1.6 Urease producing urinary tract pathogens.

A wide range of bacterial species colonize the catheterised urinary tract and many of these species are able to produce the enzyme urease (Mobley and Warren, 1987). Urease (urea amidohydrolase; E.C.3.5.1.5) is a cytoplasmic nickel containing metalloenzyme which catalyses the hydrolysis of urea. The net result of this reaction is the formation of two molecules of ammonia and a molecule of carbonic acid as shown in Figure 1.4. The equilibration of ammonia and carbonic acid with their

respectively protonated and deprotonated forms results in an overall increase in pH (Mobley *et al.*, 1995). In urine this increased pH reduces the solubility of calcium and magnesium phosphates (hydroxyapatite and struvite) and as a result makes crystal formation more likely. This makes urease the most important factor in the formation of encrustation on urinary catheters

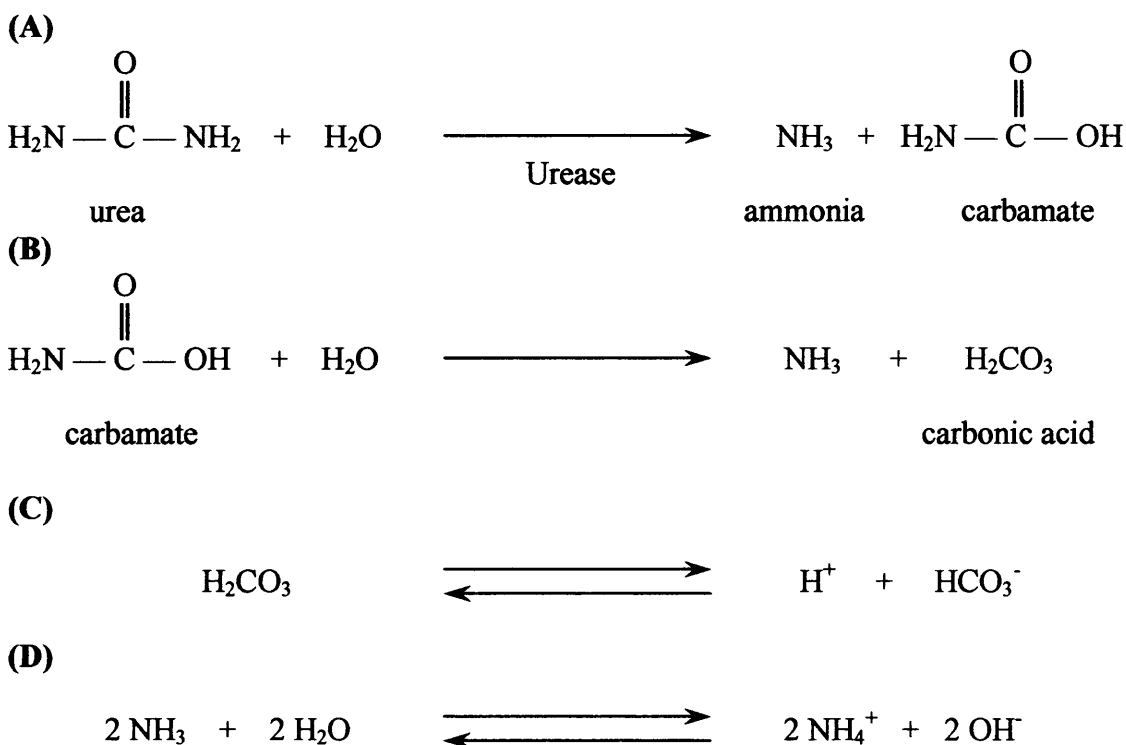


Figure 1.4: The hydrolysis of urea. Urease hydrolyses urea to produce ammonia and carbamate (A). Carbamate spontaneously decomposes to form ammonia and carbonic acid (B). The carbonic acid and ammonia molecules equilibrate with their deprotonated (C) and protonated (D) forms. Adapted from Burne and Chen (2000).

1.6.1 Urease producing *Pr. mirabilis* in catheter encrustation and blockage.

Pr. mirabilis is the species of bacteria present most often in the urine of patients who block their catheters (Mobley and Warren, 1987; Kunin, 1989). It is the organism most commonly found on encrusted catheters (Stickler *et al.*, 1993b). Experimental

work in bladder models has also shown that it is the urinary pathogen most capable of raising the pH of urine and generating crystalline catheter biofilms (Stickler *et al.*, 1998a). The urease produced by *Pr. mirabilis* has a relatively low substrate affinity (Jones and Mobley, 1987) but this is not an important factor in urine, where urea is present at high concentrations (up to 500mM (Gendlina *et al.*, 2002)). The urease of *Pr. mirabilis* is particularly potent, being able to hydrolyse urea several times faster than ureases produced by other species (Jones and Mobley, 1987). Urease production in *Pr. mirabilis* is induced by the presence of urea (Thomas and Collins, 1999) and only relatively low concentrations (4 mM) are required to initiate its gene transcription (D'Oriazo *et al.*, 1996). Expression of the urease gene cluster is regulated by the transcriptional activator UreR (D'Oriazo and Collins, 1993b). Dattelbaum *et al.* (2003) found that a mutant *Pr. mirabilis* strain deficient in UreR was unable to produce urease and was also less able to cause kidney infections in mouse models of urinary tract infection. This was also found to be the case with urease negative *Pr. mirabilis* in a study by Jones *et al.* (1990), indicating that urease production has an important role in the virulence of *Pr. mirabilis*. Mobley *et al.* (1991) suggested that the increased pH has a deleterious effect on the renal epithelium.

Pr. mirabilis swarmer cells (elongated polyploid hyperflagellated cells which can reach over 80 μm in length (Belas, 1996)) have been found to express far larger (30 fold) quantities of urease than normal vegetative cells (Allison *et al.*, 1992). Sabbuba *et al.* (2002) suggested that swarmer cells of *Proteus*, with their substantially increased levels of urease (the driving force of encrustation) and their ability to migrate rapidly over catheters, are well adapted to initiate ascending infection of the

catheterised urinary tract and produce catheter blocking crystalline biofilm. Swarmer cells also demonstrate increased expression of other virulence factors such as ZapA, an IgA degrading protease, which may have a role in infection (Walker *et al.*, 1999).

While it is quite clear that the urease enzyme of *Pr. mirabilis* is the main force driving crystalline biofilm formation, there is some evidence that the bacterial capsule has a role to play in the process. Experiments *in vitro* have demonstrated that the capsule produced by *Pr. mirabilis* forms a gel which encourages crystal growth (Clapham *et al.*, 1990). The purified exopolysaccharide from the capsule has also been shown to be uniquely capable of binding magnesium and accelerating struvite formation (Dumanski *et al.*, 1994).

As well as causing catheter encrustation urease producing *Pr. mirabilis* is also responsible for the formation of some types of bladder stones (Naas *et al.*, 2001). The stone both protects the bacteria and may serve as a reservoir of infection (Li *et al.*, 2002a). Sabbuba *et al.* (2004) found that the DNA profiles of pairs of *Pr. mirabilis* isolates taken from bladder stones and encrusted catheters from 6 patients were identical. This may mean that any new catheter inserted in these cases will soon become colonized and encrusted with bacteria from the bladder stones.

1.6.2 Urease production in urinary tract pathogens other than *Pr. mirabilis*.

There is strong epidemiological and experimental evidence that *Pr. mirabilis* is the main cause of catheter encrustation and blockage. While other urease producing

species can be found quite commonly infecting the catheterised urinary tract the extent of their involvement in generating crystalline biofilms is uncertain.

The production of urease in these species is controlled in a variety of ways. In a number of species closely related to *Pr. mirabilis*, such as *Pr. vulgaris* and *Pv. rettgeri* (formerly *Pr. rettgeri*), urease production is induced by the presence of urea (Mobley *et al.*, 1987; Mobley and Hausinger, 1989). Both of these species have been shown to be able to form crystalline biofilms similar to those of *Pr. mirabilis* on urinary catheters (Stickler *et al.*, 1998a). These two species are much less prevalent however in the catheterised urinary tract than *Pr. mirabilis* (Mobley and Warren, 1987). A study by Senior (1979) found that *Pr. mirabilis* accounted for 96.5 % of urinary tract infections by the *Proteeae* tribe (*Proteus*, *Providencia* and *Morganella* sp.) members while *Pr. vulgaris* and *Pv. rettgeri* accounted for less than 2 %. Senior and Leslie (1986) speculated that the low number of infections with *Pr. vulgaris* was related to its infrequent occurrence as a part of the gut microflora, which is often the source of bacteria infecting the urinary tract (Daifuku and Stamm, 1984). Urease producing isolates of *Pv. stuartii*, another member of the *Proteeae* tribe, have also been found to possess a plasmid-encoded urease similar to that of *Pr. mirabilis* (D'Oriazo and Collins, 1993a). Urease activity in these isolates was found to be induced by the presence of urea (D'Oriazo and Collins, 1993b). This plasmid-encoded urease was also found in a number of *E. coli* isolates.

Urease production in the commonly occurring urinary tract pathogen *M. morganii* (formerly *Pr. morganii*) appears to be constitutive (Rosenstein *et al.*, 1981). The urease produced by *M. morganii* is distinct from that of other uropathogens (Hu *et al.*,

1990) and has a higher substrate affinity than that of the ureases from other *Proteeae* tribe members and also a larger molecular weight (Jones and Mobley, 1987). Unlike the ureases of most other Gram-negative bacteria the urease of *M. morganii* has a low optimum pH of 5.5 which appears to provide protection from acidic conditions (Young *et al.*, 1996). *M. morganii* urease has also been reported to hydrolyse urea more slowly than that of *Pr. mirabilis* (Jones and Mobley, 1987). Stickler *et al.* (1998a) found that *M. morganii* was unable to raise the pH of artificial urine significantly over 24 h incubation periods.

There is also some evidence that *Staph. saprophyticus* may produce urease constitutively (Sissons *et al.*, 1990). This species is not commonly found in the catheterised urinary tract but is regularly found to be the cause of uncomplicated UTI in young women (Ferry *et al.*, 1988).

A number of urease positive urinary tract pathogens control the production of their urease as a part of the nitrogen regulatory system (Mobley *et al.*, 1995). Urease production in *Klebsiella* sp. has been a particular focus of research. The production of urease in *K. pneumoniae* has been shown to be blocked by mutations of the global nitrogen regulation system (ntr) cascade (*ntrA* and *ntrC*) (Collins *et al.*, 1993). In addition urease expression has been found to be low in *K. aerogenes* when grown in nitrogen rich media (Mulrooney *et al.*, 1989). The expression of urease by *Ps. aeruginosa* has also been found to be regulated by available nitrogen (Totten *et al.*, 1990). Due to the high levels of available nitrogen present in the urinary tract, bacteria in which urease synthesis is controlled by the nitrogen regulatory system are unlikely to produce high levels of urease in this environment. However *K. oxytoca*

has been reported to cause hyperammonaemia, the presence of high concentrations of ammonia in the blood which is usually associated with *Proteus* infections, in urinary stasis (Cheang *et al.*, 1998).

Staph. aureus is another pathogen commonly isolated from the catheterised urinary tract. Isolates of *Staph. aureus* are occasionally found to be capable of producing urease, although the control mechanisms have not been investigated fully. There is some evidence of pH dependent expression of urease similar to that seen in *Streptococcus salivarius*, with apparent inhibition of urease synthesis at high pHs (Sissons *et al.*, 1990). Resch *et al.* (2005) observed that the production of urease was one of a number of enzymes upregulated in *Staph. aureus* biofilms when compared to planktonic cells. Urease production is also characteristic of one of the two most important strains of epidemic methicillin-resistant *Staph. aureus* (EMRSA-16) affecting UK hospitals. EMRSA-16 accounted for 24 % of all MRSA isolates in England and Wales in 2000 (Murchan *et al.*, 2004).

1.7 Factors that modulate the rate of catheter encrustation.

Although urease is the most important factor in the formation of encrustation the chemical composition of the urine is also significant. In particular the concentration of calcium ions in the urine, in conjunction with elevated urine pH, has been identified as a major factor in the formation of encrustation. Burr and Nuseibeh (1993) found that elevated calcium levels in urine combined with the presence of a urease producing microorganism were the most important factors in catheter blockage. Studies on human urine *in vitro* have confirmed the importance of calcium

and also magnesium concentrations (Hugosson *et al.*, 1990). This is unsurprising given that the encrustation is formed from calcium and magnesium phosphates which become insoluble at elevated urine pHs. However it does not explain the wide variations in the rates at which catheter encrustation occurs.

It is obvious to nursing staff responsible for the care of catheterised patients that even among patients infected with *Pr. mirabilis* there is considerable variation in the times catheters take to block (i.e. the catheter lifespan). A recent prospective study by Mathur *et al.* (2006a) characterised the variability in the lifespan of catheters in patients having *Pr. mirabilis* in their urine. The time the catheter took to block with encrustation varied from 2 – 98 days. The rate of crystalline biofilm formation on catheters is clearly extremely variable. The factors that modulate the rate at which *Pr. mirabilis* encrusts catheters are poorly understood.

As mentioned previously the urine of long-term catheterised patients is often colonized with mixed communities of organisms. *Pr. mirabilis* is commonly found with two or three other organisms in patients' urine (Clayton *et al.*, 1982; Warren *et al.*, 1987). The effect of these other species on the rate at which *Pr. mirabilis* is able to cause encrustation has not been investigated.

It has often been noted that high urinary pH is associated with catheter blockage (Hedelin *et al.*, 1991; Kohler-Ockmore, 1991; Burr and Nuseibeh, 1997). As catheter blockage is brought about by the elevation of urinary pH, it would seem likely that the higher the urinary pH generated by the bacterial flora, the shorter will be the catheter lifespan. Some attempts have been made to identify a critical pH value above which

encrustation and blockage will occur. Hedelin *et al.* (1991) suggested that crystal formation in urine occurs predominantly above a critical value of pH 6.8. This conclusion was based on their observations that very little encrustation was found on catheters of patients with a mean urinary pH below this value. Choong *et al.* (2001) reported that patients who blocked their catheters rapidly (within 6 weeks) had a mean urinary pH of 7.63. The results of the studies by Mathur *et al.* (2006a & b) however suggested a more subtle relationship between the urinary pH and the encrustation process. The data showed that in patients infected with *Pr. mirabilis* the nucleation pH of urine (pH_n) is the important factor in predicting the rate of catheter encrustation. The nucleation pH of a sample of urine is defined as the pH above which calcium and magnesium phosphates come out of solution (Choong *et al.*, 1999). Statistical analysis of data from the Mathur *et al.* (2006a) study showed that there was a highly significant ($P=0.004$) positive correlation between pH_n and catheter lifespan. The higher the pH_n the slower the rate of encrustation and the greater the catheter lifespan.

1.8 Strategies to control catheter encrustation and blockage.

Community nurses and continence advisors in the UK have recorded their uncertainties over the management of patients who suffer from recurrent catheter blockage (Capewell and Morris, 1993). This is a reflection of the situation that although several strategies have been advocated for the control of the problem, there is little hard evidence from controlled clinical trials that any of them are effective.

1.8.1 Bladder washouts.

Bladder washouts with various solutions have been used for many years to try and deal with the problems of CAUTI and catheter encrustation. The technique involves the introduction of 50 – 100 ml of the solution into the bladder by injection through the catheter. The catheter is then clamped so that the solution is retained in the bladder. After 20 – 30 minutes the clamp is removed to allow the solution to drain into the urine collection bag.

Acidic bladder washouts have been used for a number of years in attempts to control the formation of encrustation on urinary catheters (Getliffe, 1996). Getliffe (1994) compared the effects of using washout solutions of Suby G (3.2 % citric acid), mandelic acid and physiological saline on the formation of encrustation by *Pr. mirabilis* in an *in vitro* model. Both Suby G and mandelic acid were able to reduce the encrustation present on the catheters after instillation while saline had no effect. In a second study Getliffe *et al.* (2000) found similar results, with Suby G and Solution R (6 % citric acid) being able to reduce the encrustation present on catheters in urine containing jack bean urease. Clinical studies on the effectiveness of bladder washouts however, have not conclusively shown that these strategies have any effect *in vivo* (Kennedy *et al.*, 1992). A review of the available data (Mayes *et al.*, 2003) for citric acid washout solutions (Suby G and Solution R) found that the evidence for the use of these solutions was weak and suggested that they should not be used routinely but instead evaluated on a patient by patient basis. It has also been observed that the acidification of urine by citric acid solutions is quickly countered by urease producing bacteria once the solution is removed, and that these solutions are not effective in

removing urease producing bacteria from the urinary tract (Bibby and Hukins, 1993). Bladder washouts have also been linked to irritation of the bladder epithelium (Elliott *et al.*, 1989). Despite this acidic bladder washouts remain popular in the management of catheter encrustation (Rew, 1999; Evans and Godfrey, 2000).

Chlorhexidine is a biocide which is often used as an infection control measure in catheterised patients (Stickler, 2002). Chlorhexidine containing bladder washout solutions have been in use since the 1970's (Guttmann, 1973). The intention of its use in combating encrustation is to eliminate the infecting urease producing bacteria. While chlorhexidine can be effective against Gram-positive bacteria, it is often less so against Gram-negative bacteria (this is most likely due to an intrinsic property of the Gram-negative cell wall (Stickler, 2002)), importantly including *Pr. mirabilis* (Stickler *et al.*, 1971). Despite their widespread use (Stickler and Chawla, 1987) there is little clinical or *in vitro* evidence that chlorhexidine washouts are effective in either sterilizing urine or against the bacterial biofilms which act as a reservoir of infection (Davies *et al.*, 1987; Stickler *et al.*, 1987 a & b; King and Stickler, 1991; Stickler *et al.*, 1991). The over use of chlorhexidine can also lead to increased resistance amongst Gram-negative bacteria, as was shown by the experiences of the Southampton infection control team. They devised a comprehensive strategy to reduce the occurrence of CAUTI, with chlorhexidine used for disinfection of the urethral meatus, included in gel lubricants used to allow easier passage of the catheter into the bladder, and added to the urine drainage bags (Southampton Infection Control Team, 1982). Despite initial success in reducing the occurrence of CAUTI this strategy lead to the selection of drug resistant Gram-negative bacteria (Walker and

Lowes, 1985) culminating in an outbreak of chlorhexidine and antibiotic resistant *Pr. mirabilis* infections (Dance *et al.*, 1987).

1.8.2 Dietary acidification of urine.

The formation of catheter encrustations is inextricably linked to increased urine pH (Hedelin *et al.*, 1991). The dietary acidification of patient's urine has as a result been viewed as a potentially useful strategy in combating the formation of catheter encrustation

McDonald and Murphy (1959) demonstrated that the administration of a daily total of 2.5 g of ascorbic acid at 4 hour intervals could significantly lower patient's urine pH. A later study by Travis *et al.* (1965) showed that this therapy was not able to consistently reduce urine pH below 5.5 even when higher doses were used. An analysis of the results of the use of orally administered ascorbic acid on urine pH showed that in patients with *Proteus* infected urine the therapy failed to have any acidifying effect (Murphy *et al.*, 1965). This was attributed to the action of the ureases produced by *Proteus* sp.

Bibby and Hukins (1993) attempted to determine whether it was possible to prevent an increase in urinary pH by urinary acidification when urease was present. They found that in order to counter the effect of the urease, large quantities of strong acid would be required. This is due to the effect of Le Chatelier's principle, since any acidification of urine under these conditions results in the conversion of more urea to ammonia to oppose the change. In fact it was determined that to prevent a rise in pH

resulting from the complete conversion of the urea in a days supply of urine would require 2.7 L of strong acid. As a result it was concluded that without the removal of urease producing bacteria the acidification of a patient's urine was not feasible.

Cranberry juice has often been advocated by health care practitioners to control catheter encrustation (Morris, 1999). While there is some evidence that cranberry juice is effective in combating uncomplicated UTI in young women it is unclear whether there is any effect in other patient groups (Jepson *et al.*, 2004). Urine from volunteers who had drunk cranberry juice a few hours earlier has been shown to inhibit the adherence of bacteria to the uroepithelium *in vitro* (Schmidt and Sobota, 1988). However urine collected in a similar way showed no effect on the formation of crystalline biofilms by *Pr. mirabilis* in an *in vitro* model when compared to the urine of volunteers who had drunk the same amount of water. Both sets of urine extended the blockage time when compared to the urine of volunteers who had not increased their fluid intake, emphasising the importance of maintaining dilute urine in combating catheter encrustation (Morris and Stickler, 2001).

1.8.3 Increased fluid intake.

Increased fluid intake has been associated with a reduction in the occurrence of catheter blockage (Burr and Nuseibeh, 1995). Burr and Nuseibeh (1997) found that consistent fluid intake rather than excessive consumption of fluid was important in maintaining dilute urine over 24 h periods. Suller *et al.* (2005) showed that increased fluid intake can raise the pH_n of urine significantly as well as reducing the concentration of calcium and magnesium ions available to form encrustations.

The influence of citrate in limiting the formation of catheter encrustations has been investigated recently. Wang *et al.* (1994) showed the potential of increased citrate consumption to reduce the formation of crystals in urine *in vitro*. Some doubts were expressed about its clinical usefulness since the consumption of excess citrate raises urinary pH (Burr and Nuseibeh, 1997). Suller *et al.* (2005) however, demonstrated that increased citrate consumption caused the pH_n of urine to be raised considerably, more than compensating for the increased urine pH. Stickler and Morgan (2006) showed that diluting urine and increasing its citrate concentration, elevated its pH_n and prevented *Pr. mirabilis* from forming crystalline biofilms in an *in vitro* model. It would be interesting to see if this effect is reproducible *in vivo*.

1.8.4 Urease inhibitors.

Given the association of urease producing pathogens with the formation of catheter encrustations, inhibitors of urease activity would be useful in combating encrustation. Acetohydroxamic acid has been used successfully to combat bladder stone formation and urinary catheter encrustation (Burns and Gauthier, 1984; Williams *et al.*, 1984). The mechanism of action of acetohydroxamic acid is shown in Table 1.1. Unfortunately acetohydroxamic acid has some rather unpleasant toxic side effects which, such as severe tiredness and unusual hair loss, have limited its clinical use (Williams *et al.*, 1984). Fluorofamide is another urease inhibitor which has the potential to reduce catheter encrustation. Studies in a rat model of infection and in an *in vitro* model have both shown its possible usefulness in combating encrustation (Millner *et al.*, 1982; Morris and Stickler, 1998b). Fluorofamide has not as yet been

Name	Class	Mode of Action
Chlorhexidine	Antibacterial	Disruption of cytoplasmic membrane function
Nitrofurazone	Antibacterial	Targets enzymes involved in carbohydrate metabolism and DNA synthesis
Nitric Oxide	Antibacterial	Unknown
Silver	Antibacterial	Toxic metal
Triclosan	Antibacterial	Inhibition of fatty acid synthesis
Acetohydroxamic Acid	Urease inhibitor	Binds to Nickel ion in urease inhibiting activity
Fluorofamide	Urease inhibitor	Unknown

Table 1.1: Antibacterial and urease inhibiting substances in use or proposed for use in CAUTI.

fully tested and so its toxicity is unknown and as a result is not currently used in clinical practice.

1.8.5 Antimicrobial catheters.

Attempts to produce catheters which resist infection initially focused on producing catheters with surfaces designed to inhibit the initial attachment of bacteria to the catheter surface. Latex catheters with coatings of teflon or hydrophilic hydrogel however, have not been shown to be effective in preventing either biofilm formation or blockage (Kunin *et al.*, 1987b; Bull *et al.*, 1991; Morris *et al.*, 1997).

A number of silver-alloy coated catheters began to become available during the early 1990's. These catheters are designed to inhibit the formation of biofilm by the gradual release of silver ions into the urine. While a number of studies have suggested that silver coated catheters may be able to reduce to occurrence of bacteriuria in short term catheterisation (Karchmer *et al.*, 2000; Rupp *et al.*, 2004) other studies have shown little or no difference between standard and silver coated catheters (Bologna *et al.*, 1999; Lai and Fontecchio, 2002; Srinivasan *et al.*, 2006). In addition silver coated catheters were not effective in preventing catheter encrustation and blockage in an *in vitro* model of the catheterised urinary tract (Morris *et al.*, 1997). There is no clinical evidence that silver coated catheters would be more effective than standard catheters in the prevention of CAUTI or blockage in long-term catheterised patients.

Nitrofurazone is another antimicrobial agent which has been incorporated into urinary catheters. The mode of action of nitrofurazone is not well defined but it is thought to target bacterial enzymes including those involved in carbohydrate metabolism and DNA synthesis (Shah and Wade, 1989; McOsker and Fitzpatrick, 1994). It is active against a wide range of Gram-positive and Gram-negative bacteria. Johnson *et al.* (1993) found that a nitrofurazone impregnated catheter was effective *in vitro* against many bacteria which commonly infect the catheterised urinary tract with the notable exception of *Pseudomonas* sp. and *Proteus* sp. Clinical trials of nitrofurazone impregnated catheters have shown some indications that these catheters may be effective in delaying the occurrence of hospital acquired CAUTI (Al-Habdan *et al.*, 2003; Lee *et al.*, 2004).

A systematic review by Johnson *et al.* (2006) of studies into the use of silver coated and nitrofurazone impregnated catheters was not able to make any definitive recommendations about their clinical use. They did conclude however that there was some evidence for the use of these catheters in short-term catheterisation. As Trautner *et al.* (2005a) pointed out even if definitive data become available for the effectiveness of these catheters in short-term catheterisation this does not necessarily mean that it will have any effect in chronic long-term catheterised patients.

Catheters incorporating chlorhexidine and combinations of chlorhexidine and silver and chlorhexidine, silver and triclosan are currently being developed for clinical use (Whalen *et al.*, 1997; Gaonkar *et al.*, 2003; Richards *et al.*, 2003). These catheters have shown some promise in *in vitro* studies but the widespread use of biocide

containing catheters in long-term catheterisation may well lead to the emergence of resistant organisms as has already been observed with chlorhexidine (Section 1.8.1).

Chakravarti *et al.* (2005) reported on the *in vitro* effect of a catheter fitted with silver electrodes. Electrification of the catheter using a 9 V battery caused the steady release of silver ions into the urine. This method was found to extend the lifespan of catheters when challenged with *Pr. mirabilis* when compared to controls.

1.8.6 Use of the catheter retention balloon to deliver antimicrobial agents to the bladder.

Bibby *et al.* (1995) first suggested that the catheter retention balloon could be filled with an antimicrobial solution as a method of controlling encrustation. The balloon membrane might then act as an effective diffusion barrier, allowing small amounts of the antimicrobial to pass into the urine in the bladder over an extended period from the large reservoir of antimicrobial solution in the balloon. Stickler *et al.* (2003a) found that the instillation of a solution containing 10 mg/ml triclosan into the balloon of a standard all-silicone catheter was able to keep catheters draining freely for 7 days when challenged with *Pr. mirabilis* in an *in vitro* model. The control catheters quickly became encrusted and blockage occurred within 24 h. A later study found that lower concentrations of triclosan were also effective in preventing blockage by *Pr. mirabilis* (Jones *et al.*, 2005). Jones *et al.* (2006) observed that while this strategy was effective against several other commonly occurring urinary tract pathogens a number of other species were able to form extensive biofilms on the surface of triclosan impregnated catheters. The resistant species however were not able to form

crystalline biofilms. Research is needed into the ability of triclosan to prevent the formation of crystalline biofilms by species other than *Pr. mirabilis* which are able to raise urinary pH significantly.

Urinary pH testing with indicator strips is commonly used to predict catheter encrustation in the management of urinary catheters (McCarthy and Hunter, 2001; Getliffe, 2002). An inherent problem of this technique is that since the urinary pH is subject to some variation during the day pH testing may miss points at which the urine is particularly alkaline, during which encrustation is more likely to occur (Burr and Nuseibeh, 1997). Measurements of urinary pH also need to be taken quickly since samples exposed to air may show an increase in pH (Choong *et al.*, 1999). Stickler *et al.* (2006a) developed a sensor designed to be placed in the urine collection bag and so provide constant monitoring of voided urinary pH. This sensor was shown to predict catheter blockage an average of 43 h before it occurred for *in vitro* models inoculated with *Pr. mirabilis*. A clinical assessment of the performance of this sensor showed it was able to signal both infection with *Pr. mirabilis* and the early stages of catheter encrustation, allowing time to take preventative action before the development of clinical crises (Stickler *et al.*, 2006c). The use of this pH sensor shows promise in the management of catheter encrustation and blockage. It would be useful to assess its effectiveness in predicting catheter blockage for a range of urease producing bacteria both *in vitro* and *in vivo* in order to provide constructive data for recommendations on its clinical use. The use of the pH sensor in determining when intervention with triclosan containing solutions is appropriate could also be useful, since the blanket use of the triclosan solution in long-term catheterisation could lead

to resistance problems similar to those encountered with chlorhexidine (Section 1.8.1). This combined strategy should be examined *in vitro*.

Carlsson *et al.* (2005) also used the approach of filling the catheter balloon with an antimicrobial solution. They evaluated the use of a solution which releases nitric oxide into the surrounding urine in a static model of the catheterised urinary tract. This approach was effective in reducing the numbers of *E. coli* in the model. It would be interesting to see what effect this approach has on species capable of causing encrustation in a continuous flow model of the catheterised urinary tract.

1.8.7 Bacterial interference with pathogenesis in CAUTI.

Several studies have investigated the possibility of interfering with CAUTI using non-pathogenic bacteria. This approach involves deliberate infection of the catheterised urinary tract with a non-pathogenic bacterial strain in order to prevent or reduce infection with pathogenic microorganisms and as a result prevent symptomatic infections (Darouiche and Hull, 2000). Work in this area has been carried out both *in vitro* and *in vivo* using a non-pathogenic *E. coli* strain (Darouiche *et al.*, 2001; Trautner *et al.*, 2003; Darouiche *et al.*, 2005). Colonization by pathogenic strains was shown to be reduced *in vitro* for pathogenic *E. coli*, *Ent. faecalis* and *Pv. stuartii* when a pre-existing non-pathogenic *E. coli* strain was present on catheters (Trautner *et al.*, 2003). This approach has also shown some success *in vivo* where patients with bladders colonized with non-pathogenic *E. coli* were half as likely as controls to develop symptomatic UTI during the subsequent year (Darouiche *et al.*, 2005). Some *in vitro* work has also been carried out using a non-pathogenic *E. coli* strain able to

produce bacteriocins (bacterially produced antibacterial agents, known as colicins in *E. coli*). This strain was able to completely prevent catheter colonization *in vitro* by colicin-susceptible *E. coli* but not by colicin-resistant *E. coli* (Trautner *et al.*, 2005b).

A similar strategy has been tried using non-pathogenic *Lactobacillus* sp. in an attempt to control *Pr. mirabilis* ascending UTI in a mouse model of infection (Fraga *et al.*, 2005). Although this strategy was effective in reducing the numbers of *Pr. mirabilis* present in the bladders of the mice it did not have any significant effect on the occurrence of kidney colonization.

Given the interactions observed between bacteria in these studies it would be interesting to investigate what effects, if any, the composition of the bacterial community present has in modulating *Pr. mirabilis* associated catheter blockage.

1.9 Aims of the study.

The enzyme urease is the force driving the encrustation and blockage of Foley catheters. While *Pr. mirabilis* produces this enzyme in abundance and is clearly a major cause of this complication in catheter care, there are many other urinary tract pathogens that register as urease positive in conventional bacterial identification tests. The extent to which any of these species are involved in blocking catheters is unknown. In view of this uncertainty, the aims of the work reported in this thesis were:

AIMS:

- To compare the relative abilities of a wide range of urease positive species to cause the formation of encrustations and blockage on urinary catheters.
- To test the ability of antimicrobial catheters to delay catheter blockage by *Pr. mirabilis* and other species found to be able to produce alkaline urine and crystalline bacterial biofilms.
- To examine the efficacy of delivering triclosan or nitric oxide into the bladder via the catheter retention balloon in preventing catheter blockage by species able to generate crystalline biofilm.
- To examine the feasibility of an integrated sensor/modulator strategy to detect the early stages of catheter encrustation and then inhibit further deposition of crystalline material thus preventing blockage.
- To examine the response of the sensor of catheter encrustation to bacterial species that are not capable of causing catheter blockage.
- To observe the effect of the other urinary tract pathogens present in the mixed bacterial communities that commonly infect catheterised patients on the encrustation and blockage of urinary catheters by *Pr. mirabilis*.

Section 2

Materials and Methods

2.1 Chemicals.

Most chemicals used in this study, unless otherwise stated, were purchased from Fisher Scientific Ltd. (Loughborough, UK).

2.2 Media.

All of the agar media and broths used in this study were purchased from Oxoid Ltd. (Basingstoke, UK). Chromogenic UTI agar was used in the isolation of bacteria from clinical catheter samples and enumeration of individual species in bladder model experiments performed with more than one species (Figure 2.1). Chromogenic UTI agar allows differentiation between bacterial species based on colony colour and / or morphology. This is due to chromogenic substrates incorporated into the agar, which allow the detection of enzymes specific to a particular genus or species. Cysteine-Lactose Electrolyte Deficient (CLED) agar was used for the culturing and enumeration of the various bacterial species used in this study (Figure 2.1). CLED agar inhibits or greatly reduces the swarming behaviour of several of the species used in this study (for example *Pr. mirabilis*, *Pr. vulgaris*), thereby allowing individual colonies to be counted. CLED agar supplemented with 2 ml/L Tween 80 (Sigma-Aldrich chemical company, Poole, UK) was used for enumeration in experiments involving triclosan. Tween 80 neutralizes triclosan, thereby allowing the enumeration of triclosan sensitive species from experiments involving triclosan. Tryptone Soya Agar (TSA) was used to detect swarming organisms from clinical catheter samples. TSA was also used for Dienes typing of *Pr. mirabilis* isolates (Figure 2.2). Colistin Inositol (CI) agar (Clayton, 1984) was used to enumerate *Pr. mirabilis* in urine from

bladder models that were inoculated with mixed bacterial communities. CI agar consists of 28 g/L Nutrient Agar, 10 g/L myo-inositol (Sigma), 0.02 g/L bromothymol blue (BTB) (Sigma) and 0.01 g/L colistin methanesulfonate (Sigma). Urea Agar, containing 2 % v/w urea, was used for the detection of the enzyme urease. Hydrolysis of the urea by urease causes an increase in pH, resulting in the agar changing from straw-yellow colour to pink due to a pH indicator in the agar (Figure 2.3). Iso-Sensitest Agar (ISA) was used as the agar base in the determination of minimum inhibitory concentrations (MIC) of triclosan (CIBA speciality chemicals, Basel, Switzerland) and nitrofurazone (Sigma). Iso-Sensitest Broth (ISB) was used to grow cultures for MIC testing. Tryptone Soya Broth (TSB) was used to prepare inocula for use in experiments and was also used as an ingredient in the artificial urine.

2.3 Artificial urine.

Artificial urine was prepared using a method based on that described by Griffith *et al.* (1976). The constituents used are listed in Table 2.1. The pH of the artificial urine was adjusted to 6.10 using 3 M sodium hydroxide prior to the addition of the gelatine. Once the gelatine had dissolved the artificial urine was sterilised using a capsule filter with a 0.2 µm pore size (Sartorius, Goettingen, Germany). TSB (1 g/L of urine) was prepared separately, sterilized by autoclaving at 121 °C for 15 minutes and added aseptically to the sterile artificial urine.

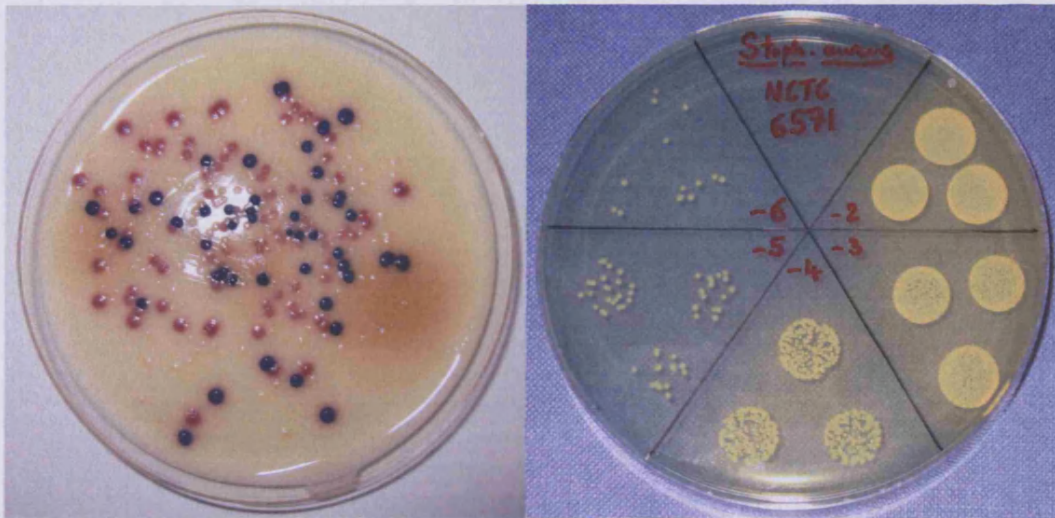


Figure 2.1: Enumeration methods. A spread plate of diluted urine with several species present is shown on the left and a drop count plate from urine containing one species is shown on the right.

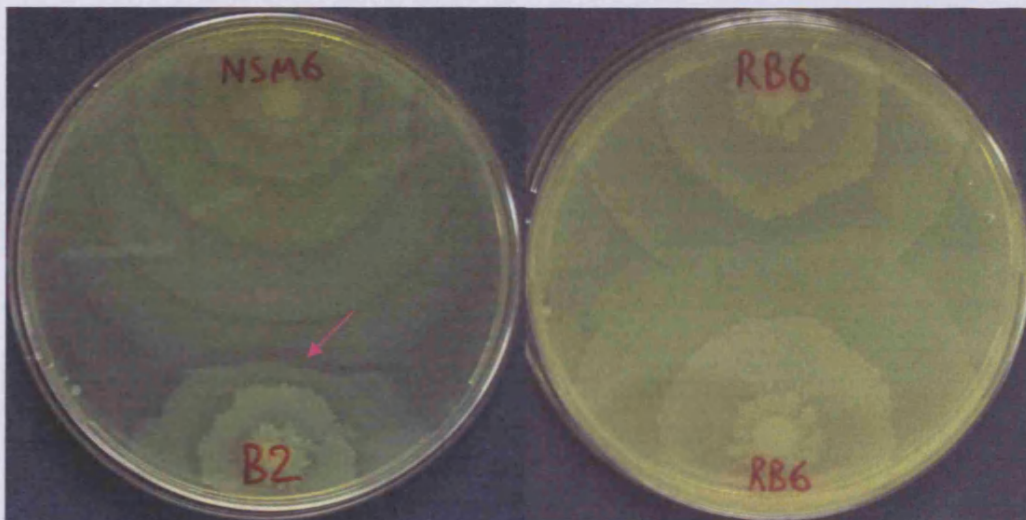


Figure 2.2: Dienes typing of *Pr. mirabilis* isolates on TSA. Different strains (left) will not swarm into each other leaving a gap between the swarming fronts (indicated ←), while isolates from the same strain will swarm into each other (right).

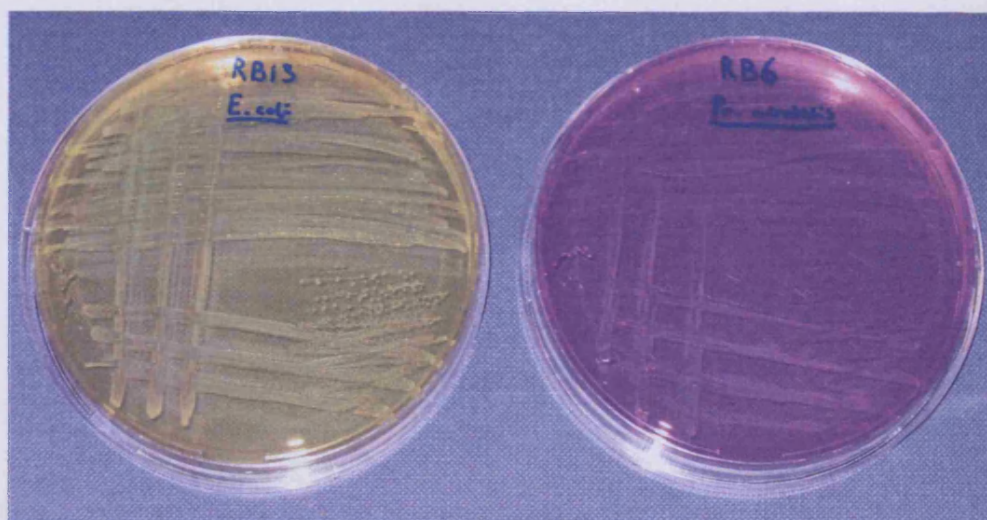


Figure 2.3: *E. coli* (urease -ve), left, and *Pr. mirabilis* (urease +ve), right, streaked onto urea agar

Chemical name	Formula	grams/litre
Sodium sulphate	Na_2SO_4	2.30
Calcium chloride dihydrate	$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	0.65
Magnesium chloride	$\text{MgCl} \cdot 6 \text{H}_2\text{O}$	0.651
Sodium chloride	NaCl	4.60
tri-Sodium citrate	$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2 \text{H}_2\text{O}$	0.65
Sodium oxalate	$(\text{COONa})_2$	0.02
Potassium dihydrogen orthophosphate	KH_2PO_4	2.80
Potassium chloride	KCl	1.60
Ammonium chloride	NH_4Cl	1.00
Urea	$\text{CO}(\text{NH}_2)_2$	25.00
pH to 6.10	-	-
Gelatine		5.00

Table 2.1: Chemicals used in the preparation of artificial urine. After preparation 1 g/L TSB is added prior to use.

2.4 Bacterial isolates.

Bacterial isolates were collected from the catheters of long term catheterised patients. Sections (approximately 1 cm long) cut from the catheters were placed in 10 ml of ¼ strength Ringer's solution (Oxoid). These were then sonicated gently at 35 kHz continuously for 5 minutes (Camlab Transsonic T310, Camlab Ltd., Cambridge, UK) and vortex mixed for 2 minutes in order to dislodge and break-up the biofilm. Serial dilutions of the resulting cell suspensions were then made and 100 µl aliquots were spread plated onto Chromogenic UTI agar. A loop of the original solution was also streaked onto TSA in order to detect any swarming species. Colonies with different colours / morphologies were then Gram stained and identities confirmed using the appropriate BBL Crystal™ identification test kits (Becton Dickinson, Oxford, UK). Additional isolates were also kindly supplied by Dr N. Morris, Dr N. Sabbuba, Dr S. Jones, Dr M. Suller, Mr S. Morgan and Miss S. Macloed. All of these were recent isolates from the catheters or urine of long term catheterised patients. A single strain (ZEC17, *Staphylococcus saprophyticus*), kindly supplied by Dr Zeliha Caliskan, was isolated from the urine of a patient with a urostomy. The identities of these organisms were confirmed using Gram staining and the appropriate BBL Crystal™ kits. The bacterial isolates were stored in pure culture at -80 °C in a 5 % glycerol solution.

2.5 Bacterial enumeration methods.

Viable cell numbers in cell suspensions were determined using either a drop count method, as described by Miles and Misra (1938), or by spread plating (Figure 2.1). Both methods required the preparation of a dilution series of the bacterial suspension

in ¼ strength Ringer's solution. In the drop count method triplicate 10 µl aliquots of each dilution were dropped onto pre-dried CLED agar plates. Each dilution was done in triplicate and dropped onto separate plates. In the spread plate method 10 µl aliquots of bacterial suspension were spread over the surface of pre-dried Chromogenic UTI agar plates. This was done for each appropriate dilution and in triplicate. Plates were incubated aerobically at 37 °C for 24 – 48 h.

2.6 Catheters.

All of the catheters used in experiments were size Ch 14. All-silicone and hydrogel-coated latex (Biocath) catheters were purchased from Bard Ltd. (Crawley, UK). Releen Nitrofurazone catheters were kindly provided by Coloplast (Humlebæk, Denmark). All of the catheters were connected to 2 L urine drainage bags (Bard) during experiments.

2.7 Determination of MIC's.

The MIC of triclosan to *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* isolates was determined on ISA plates. The method used was adapted from the method described by Andrews (2001). Stock solutions of triclosan were made in dimethyl sulfoxide (DMSO). The appropriate amounts were then added to molten ISA and mixed before pouring, resulting in plates containing the desired concentrations of triclosan. The plates were then dried in a laminar flow cabinet for 20 minutes. Overnight cultures of the strains to be tested were grown in ISB. The cultures were then adjusted with ISB to contain approximately 10⁸ cfu/ml. They were then diluted 1:10 with sterile

deionised H₂O. The prepared ISA plates were then inoculated with 1 µl aliquots of the diluted cultures (approx. 10⁴ cfu) using a Denley multipoint inoculator (Denley instruments Ltd., Billingham, UK). This was done in triplicate for each triclosan dilution. Control plates of ISA alone and ISA containing the maximum volume of DMSO used (without triclosan) in the screen were also prepared and inoculated as above. The inoculated plates were then incubated for 18 h at 37 °C. After 18 h the plates were examined and the MIC of each isolate determined as the lowest triclosan concentration at which no growth occurred (method as shown in Figure 2.4). For those isolates able to swarm the minimum swarming inhibitory concentration (MSIC) was also determined. This was determined as the lowest triclosan concentration at which no swarming occurred.

The MIC of nitrofurazone to *Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri*, *M. morganii*, *Staph. aureus* and *Staph. saprophyticus* isolates was determined using the same method. Nitrofurazone stock solutions were also prepared using DMSO.

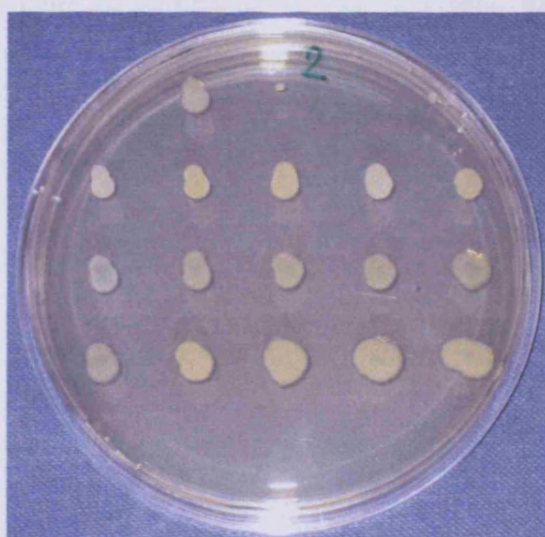


Figure 2.4: ISA plate containing triclosan after 18h incubation at 37°C

2.8 Atomic absorption spectrometry (AAS).

AAS was used to determine calcium and magnesium concentration in samples. AAS was carried out in an aa/ae spectrophotometer 457 (Thermo Electron Corporation, Waltham, Massachusetts, USA).

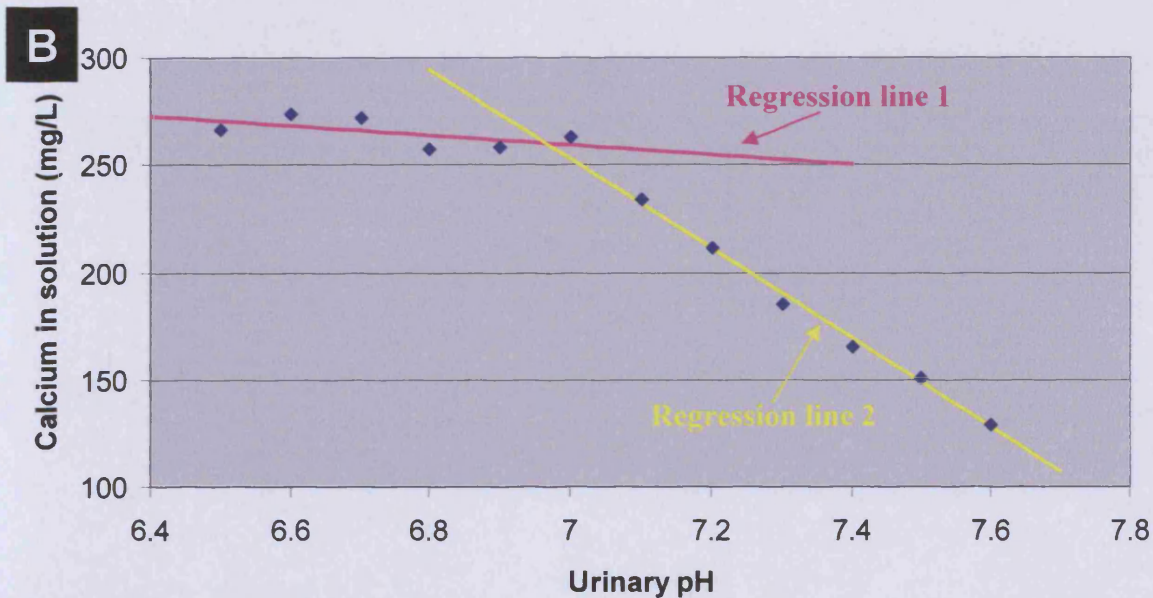
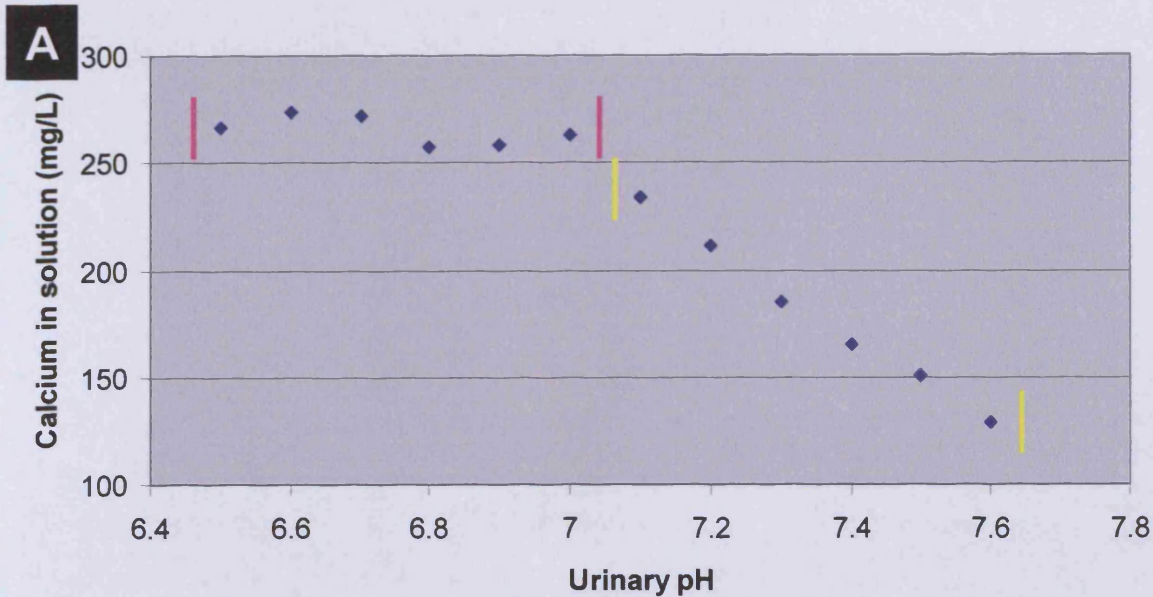
2.8.1 Determination of encrustation rates using AAS.

Calcium and magnesium encrustation rates ($\mu\text{g}/\text{min}$) for bacterial isolates on all-silicone catheters were determined using AAS. The rates calculated were the mean accumulation over the period the experiment was conducted (96 h or blockage) and do not indicate when the encrustation occurred during this period. Catheters were removed from the models at the completion of experiments and the external surface wiped clean of encrustation. The catheter balloons were removed and discarded. The catheters were then cut into small sections (approximately 1 cm long) which were then all placed in 100 ml of a 4 % nitric acid solution (made with polished water). The solutions containing catheter sections were then sonicated at 35 kHz for 5 minutes and vortex mixed for 2 minutes in order to dislodge and break-up the biofilm. Since AAS requires calcium and magnesium levels to be within a specific range, a series of dilutions in polished water was made in order to have a sample that fell within this range. The readable ranges on the spectrophotometer were between 0.5 and 2.0 ppm for calcium (at 422.7 nm) and between 0.05 and 0.4 ppm for magnesium (at 285.2 nm). Prior to analysis the spectrophotometer was calibrated between these ranges with standards purchased from Spectosol (BDH Chemicals Ltd., Poole, UK). These 1000 ppm standards were diluted with 1 % w/v nitric acid. The nitric acid and

polished water were also assayed for calcium and magnesium. Knowing the length of time each catheter had been *in situ* in the infected models, the encrustation rates were calculated as μg of calcium or magnesium / catheter / hour.

2.8.2 Determination of the nucleation pHs (pH_n) of calcium and magnesium in artificial urine at 37 °C.

The nucleation pH (pH_n) is defined as the pH at which calcium or magnesium crystallises from the urine (Choong *et al.*, 1999). Samples of artificial urine (1L) were heated to 37 °C in a water bath. The pH of the urine was increased in increments of 0.1 pH by the addition of 10 M sodium hydroxide up to a pH of 8.5. At each pH increment sub-samples (10 ml) were removed and centrifuged at 4000 g for 5 min to sediment any crystalline material. The concentrations of calcium and magnesium in the resulting supernatant were assayed using AAS. The pH_n was defined by an abrupt change in the slope of the graph showing a decrease in dissolved calcium or magnesium caused by precipitation of crystals, as shown in the example in Figure 2.5. As described by Choong *et al.* (2001) plotting pH of urine against the values of calcium or magnesium in solution at each pH produces two straight lines which intersect at the pH_n . Regression lines were calculated by least square analysis for the two parts of the graph and used to determine the pH at their intersection.

**C**

Regression line 1 : calcium (mg/L) = $420 - 23 \text{ pH}$

Regression line 2 : calcium (mg/L) = $1709 - 208 \text{ pH}$

To calculate pH at intersection (pH_n):

$$420 - 23 \text{ pH} = 1709 - 208 \text{ pH}$$

$$\text{pH}_n = 1289/185 = \underline{6.97}$$

Figure 2.5: An example of the method used to calculate nucleation pH (pH_n). (A) The AAS data is plotted and the investigator puts the data into 2 groups – data prior to the beginning of a significant decline in calcium solubility (I) and data post the beginning of a significant decline in calcium solubility (II). (B) Linear regression analysis is conducted on both sets of data producing two regression equations. (C) The pH value at the point of intersection between the two regression lines is calculated (pH 6.97 in this case). This is the nucleation pH (of calcium) in this solution N.B. in order to use this method there must be a distinct point at which the metal ion solubility begins to drop

2.9 The *in vitro* model of the catheterised urinary tract.

An *in vitro* model of the catheterised urinary tract was used to study biofilm formation on urinary catheters. The model was described by Stickler *et al.* (1999), and consists of a glass chamber surrounded by a water jacket maintained at 37 °C, representing the bladder (Figure 2.6). After each model was sterilised (autoclaved at 121 °C for 15 mins) a Foley catheter was inserted aseptically through an inlet at the base of the bladder chamber, via a section of silicone tubing (representing the urethra). Once in place the retention balloon was inflated with 10 ml of sterile solution. The solution used to inflate the balloon was generally sterile water, but in some experiments solutions containing triclosan or sodium nitrite and ascorbic acid were used (see Sections 2.9.1 & 2.9.2). Once secured in place by the balloon the catheter was connected to a drainage tube and 2 L urine collection bag. The models were then connected to glass aspirators containing sterile artificial urine (representing the kidneys) via sterile silicone tubing (representing the ureters). Figure 2.7 shows a schematic diagram of the bladder model setup.

The models were inoculated with a 4 h logarithmic phase culture, incubated at 37 °C, of the isolate being tested in artificial urine containing approximately 10^8 cfu/ml. The size of the inoculum depended on the type of infection being modelled. For models of the heavily infected bladder an inoculum of 10 ml of culture was used, models of a developing infection used 100 µl of inoculum, and models of bacterial communities used 1 ml of inoculum for each isolate. Once inoculated, artificial urine was quickly supplied to the model using a peristaltic pump (503U, Watson and Marlow, Falmouth, UK) until the urine level in the model was just below the catheter eye-hole. The

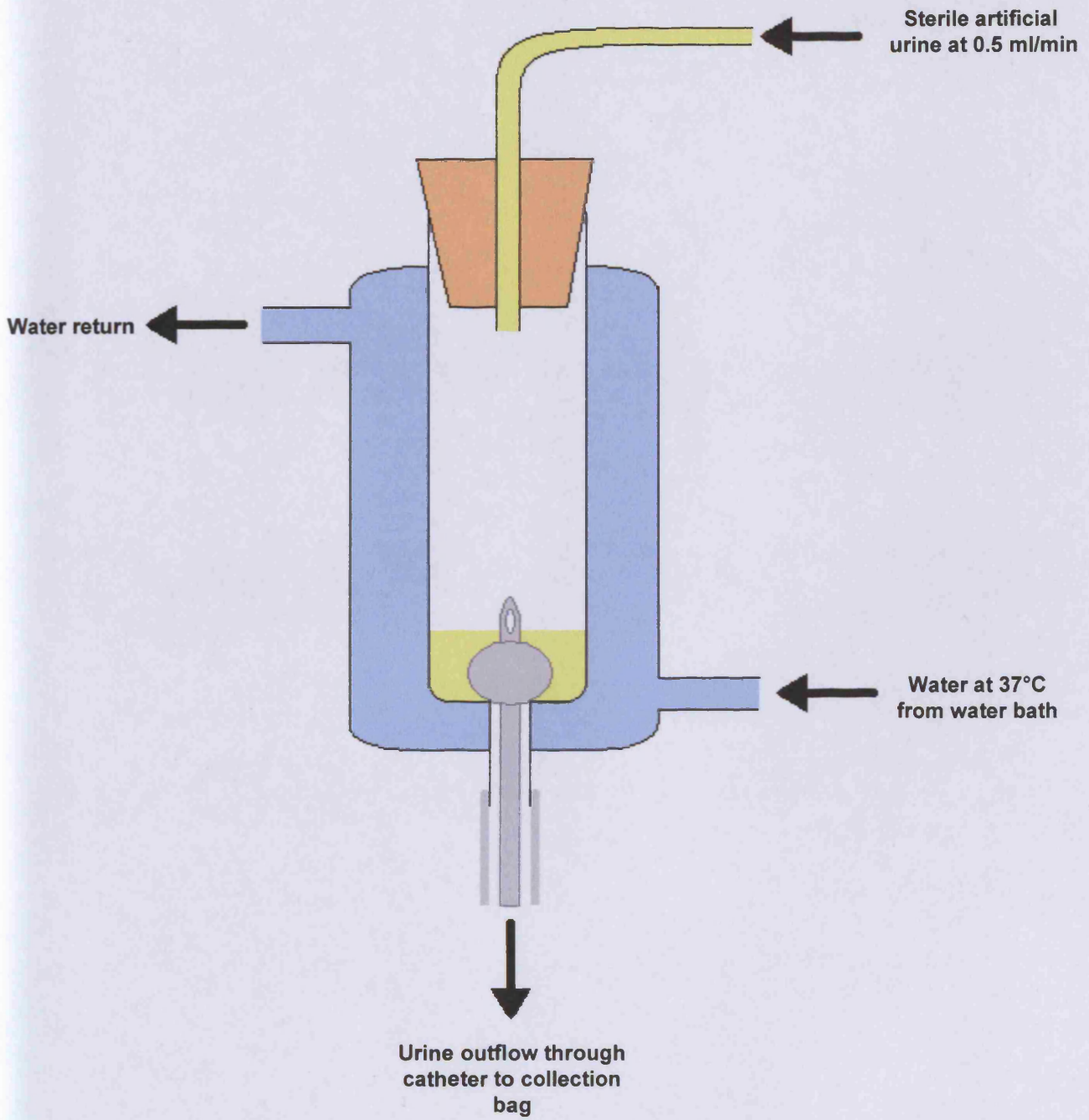


Figure 2.6: The bladder model

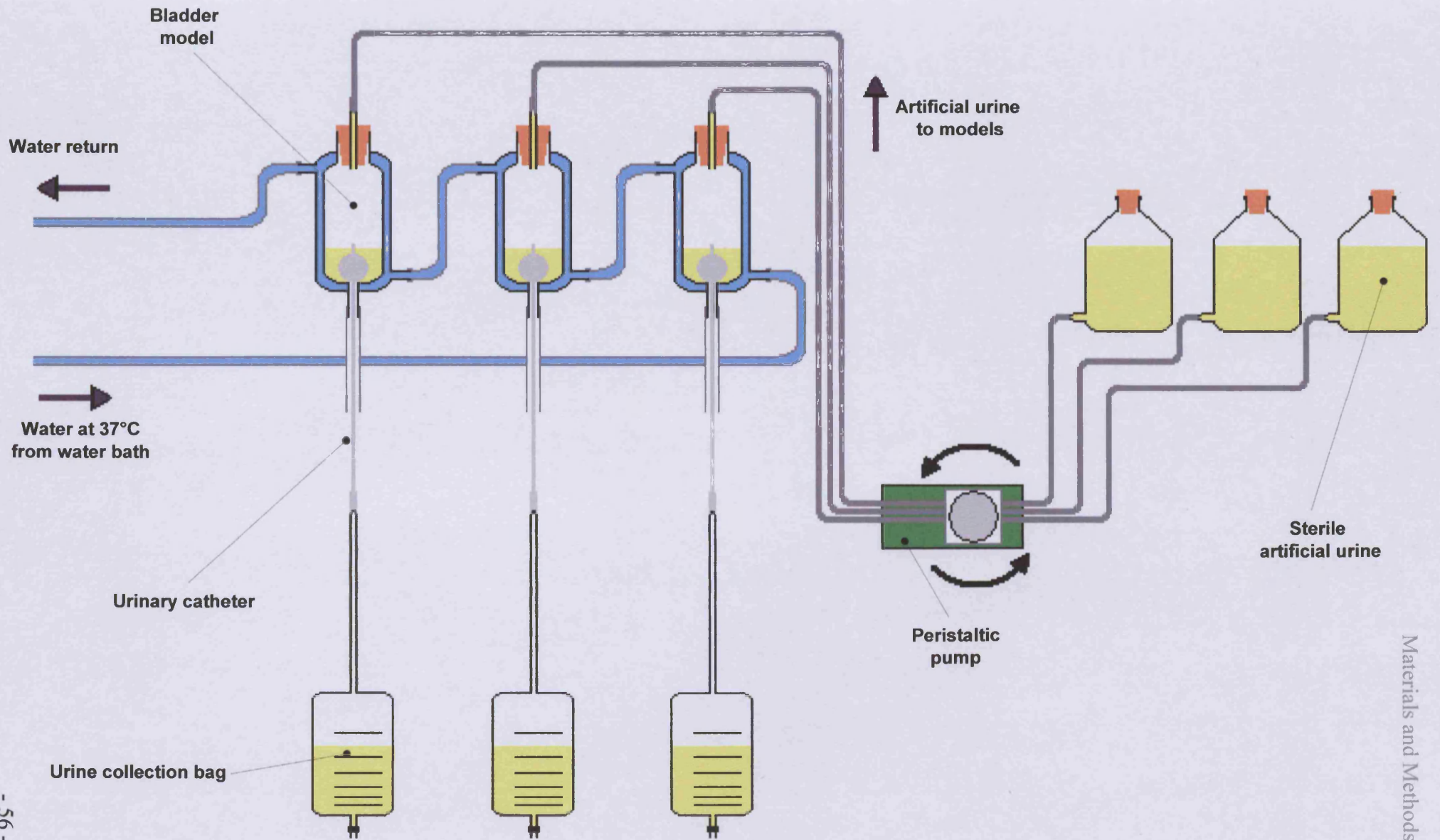


Figure 2.7: The bladder model setup

viable cell count and pH of the inoculum was determined. The inoculated model was allowed to stand for an hour in order to allow the culture to become established in the model. Urine was then supplied, via the peristaltic pump, to the model at a rate of 0.5 ml/min. The viable cell count and pH level of the residual urine in the model was determined at the point when the pump was started and at 24 h intervals for the duration of the experiment. Viable cell counts and pHs were also determined from urine in the model at the times any catheters blocked during the experiment.

2.9.1 Preparation of Triclosan solutions used to inflate catheter balloons.

Stock solutions containing triclosan were prepared for use as an alternative to water for inflating catheter balloons. Stock solutions were of 3 mg/ml (or 6 mg/ml) triclosan in 0.1 M (or 0.2 M) Na_2CO_3 . Hy-Shield SkinSure plus (Hygieia Healthcare Ltd., Bideford, UK) cream diluted with sterile water was also used to prepare solutions containing 3 or 6 mg/ml triclosan. For the Na_2CO_3 solutions 100 ml of 0.1 M (or 0.2 M) Na_2CO_3 was autoclaved at 121 °C for 15 min. Once cooled 0.3 g (or 0.6 g) of triclosan was added and the suspension stirred using a magnetic stirrer until the biocide had dissolved. The 6 mg/ml triclosan in 0.2 M Na_2CO_3 required some heating in order for the triclosan to dissolve. For experiments using 3 mg/ml triclosan in 0.1 M Na_2CO_3 control experiments using catheters were conducted, using 0.1 M Na_2CO_3 alone and sterile water for balloon inflation. For the Hy-Shield SkinSure plus cream solutions sterile water was added to the cream in plastic universal containers. For the 3 mg/ml solution 14 ml of water was added to 6 ml of cream, and for the 6 mg/ml solutions 8 ml of water was added to 12 ml of cream. The cream and water were then mixed by vigorous shaking.

2.9.2 Preparation of nitric oxide producing solutions used to inflate catheter balloons.

Stock solutions capable of producing nitric oxide when combined were prepared for use as an alternative to water for inflating catheter balloons. Stock solutions of ascorbic acid (20 mM) and sodium nitrite (10 mM) were prepared in sterile saline (0.9 % w/v) and the pHs adjusted to 2.5 with conc. HCl. Immediately prior to inoculation of the model equal volumes of these stock solutions and 10 ml of the resulting solution (10 mM ascorbic acid, 5 mM sodium nitrite) were used to inflate catheter balloons.

2.9.3 The pH sensor.

A number of bladder models were run with sections of pH sensor placed in the drainage bag. The sensors were made using the method described in Stickler *et al.* (2006a). The sensors were kindly provided by Dr G. Adusei (Cardiff School of Dentistry). Sensors were prepared by dissolving cellulose acetate (Acordis Ltd., Coventry, United Kingdom) in acetone and adding a mixture of Bromothymol blue (BTB) and sulphuric acid. Under these conditions, the BTB becomes covalently bound to the cellulose acetate. Polyethylene glycol was added as a plasticizer and to control the rate of movement of ions through the matrix, thus controlling the response rate of the material to changes in pH. The mixture was spread over a glass plate to allow the polymer mixture to set. Strips (5 cm long by 2 cm wide by 1 mm thick) of the calcium acetate-BTB polymer were then prepared, washed in water to remove residual acid, and stored at 4 °C. Strips of sensor approximately 1 cm long by 2 mm wide by 1 mm thick were cut for insertion into drainage bags. The sensors were

inserted aseptically into the drainage bags through the outlet tap. Digital photographs of the sensor were taken at the point when urine began to be pumped at 0.5 ml/min and at 24 h intervals afterwards. The pH of the urine in the drainage bag was also determined at these points. For some experiments additional photographs and pH readings were taken at 2, 4 and 12 h after the start of the experiments.

2.9.4 Production of pH sensor colour chart.

A chart showing the colour of the pH sensor at urinary pHs of between 6.1 and 9.4 was produced to allow comparison with pH sensors placed in catheter drainage bags. This was done by adjusting the pH of 500 ml of artificial urine at room temperature (approx. 25 °C) from pH 6.1 to pH 6.2 and then to pH 9 in 0.2 pH increments using 3 M NaOH. Samples (10 ml) of artificial urine were taken at each point and a section of pH sensor added. After 24 h at room temperature the sensor strips were removed from the urine, blotted to remove excess liquid, and then digital photographs taken. The pictures of the sensor were then combined to produce a colour chart (see Figure 3.41).

2.10 Electron microscopy and digital photography.

Electron microscopy and digital photography were used to allow visual comparison of biofilm formation and encrustation levels. Sections of catheter were cut as indicated in Figure 2.8. The sections (1 cm long) were taken from the catheter eyehole (section 1), immediately below the catheter eyehole (section 2), immediately below the catheter balloon (section 3), and 15 cm below the base of the balloon (section 4). In

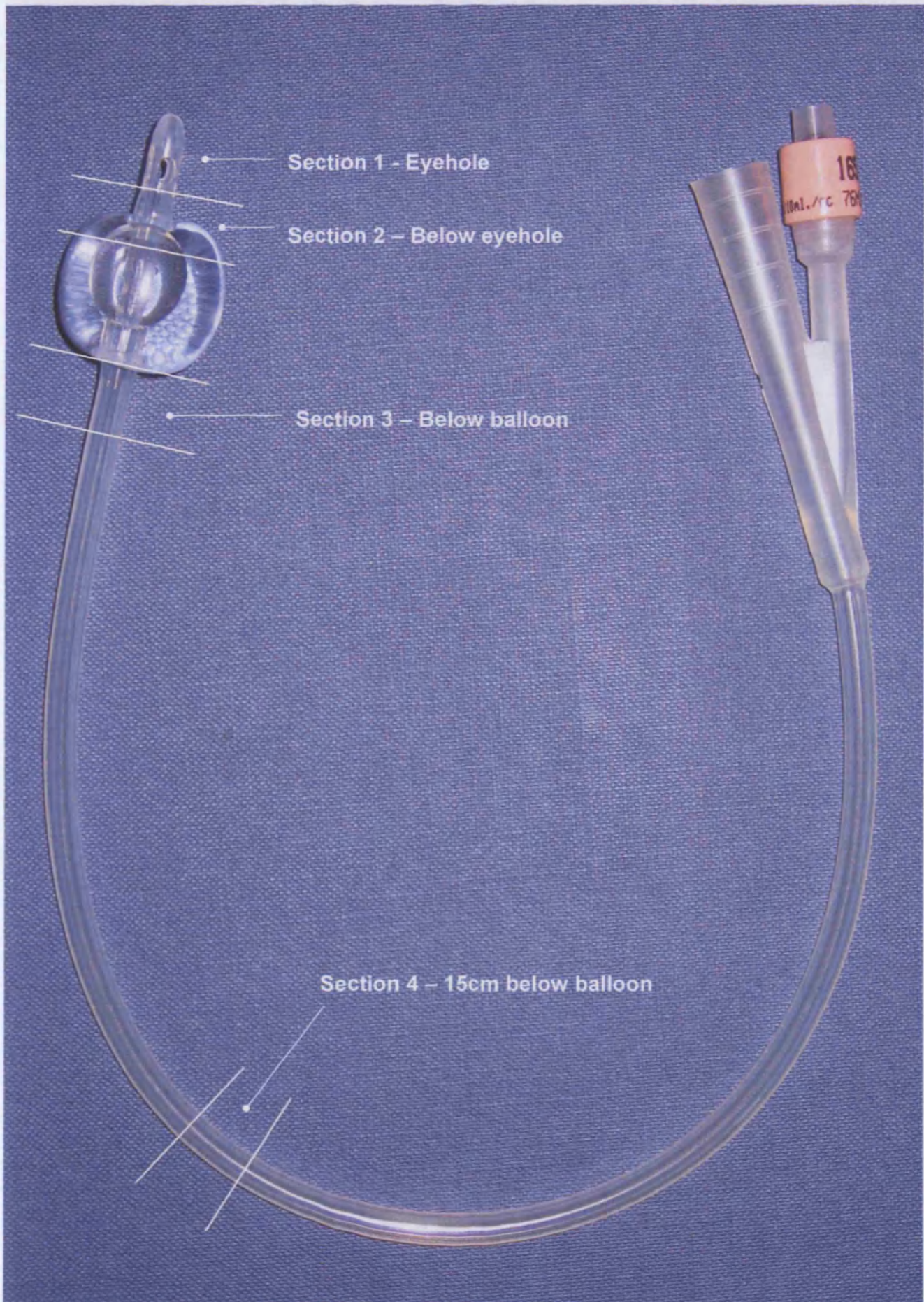


Figure 2.8: Catheter sections used for SEM and digital photos

some experiments electron microscopy and digital photography were also used to examine pH sensor strips removed from the catheter drainage bag.

2.10.1 Digital photography and Low vacuum scanning electron microscopy (LV-SEM).

Catheters for photography and LV-SEM were carefully removed from the bladder model and sections cut. If required the pH sensor was also removed from the drainage bag. Excess fluid was carefully drained by blotting before either photography or LV-SEM was carried out. A Canon IXUS 500 digital camera (Canon UK Ltd, Reigate, UK) was used for digital photography. LV-SEM was carried out using a JEOL JSM 5200 lv SEM (JEOL (UK) Ltd., Welwyn Garden City, UK) on low vacuum mode (3 %) at 20 kv. Images of section 1 were taken looking onto the eyehole, while for the other catheter sections images were taken looking down the catheter lumen.

2.10.2 Environmental scanning electron microscopy (ESEM).

ESEM combined with X-ray analysis was used to determine the composition of crystals forming on the surface of catheter eyeholes. The ESEM was carried out using a Veeco FEI (Philips) XL30 ESEM Field Emission Gun (FEI UK Ltd., Cambridge, UK) with an INCA ENERGY (EDX) X-ray analysis system (Oxford Instruments, High Wycombe, UK), and was operated by Mr P. Fisher (School of Earth Sciences, Cardiff University). The eyehole section was dried overnight in a 37 °C incubator before the analysis was carried out. Samples were carbon coated and analysed in high

vacuum SEM mode. An example of the use of ESEM combined with X-ray analysis in the identification of apatite microcrystals is shown in Figure 2.9. The morphology of struvite crystals in urine, identified by fourier transform infrared microscopy by Daudon *et al.* (1991), is shown in Figure 2.10.

2.10.3 High vacuum scanning electron microscopy (HV-SEM).

Catheters for HV-SEM were carefully removed from the bladder model and sections cut. Sections 2, 3, and 4 were cut in half longitudinally. The sections were then placed in a 2.5 % glutaraldehyde solution in 0.1 M Sørensen phosphate buffer (adjusted to the pH of the urine in the bladder model from which the catheter was removed by altering the proportions of monobasic (NaH_2PO_4) and dibasic (Na_2HPO_4) sodium phosphate used to make the buffer) and left to fix at 4 °C overnight. The samples were then washed in 0.1 M pH adjusted Sørensen phosphate buffer for 15 minutes. The samples were then post-fixed by immersion in 1 % osmium tetroxide in 0.1 M Sørensen phosphate buffer for 1 h. The osmium was then removed and the samples washed by immersion in deionised water for 15 minutes. The samples were then dehydrated using a series of ethanol solutions. The samples were first immersed in a 70 % ethanol solution for 15 minutes. This was then replaced with a 90 % ethanol solution for 15 minutes, followed by 2 washes in 100 % ethanol for 15 minutes each. Silicone based catheters were then dried using a critical point dryer (Blazers CPD 030 critical point dryer, Bal-Tec AG, Blazers, Liechtenstein). Latex based catheters were dried using Hexamethyldisilazane (HMDS) (Agar Scientific), in a fume cupboard. After the final 100 % ethanol wash the sections were immersed in a

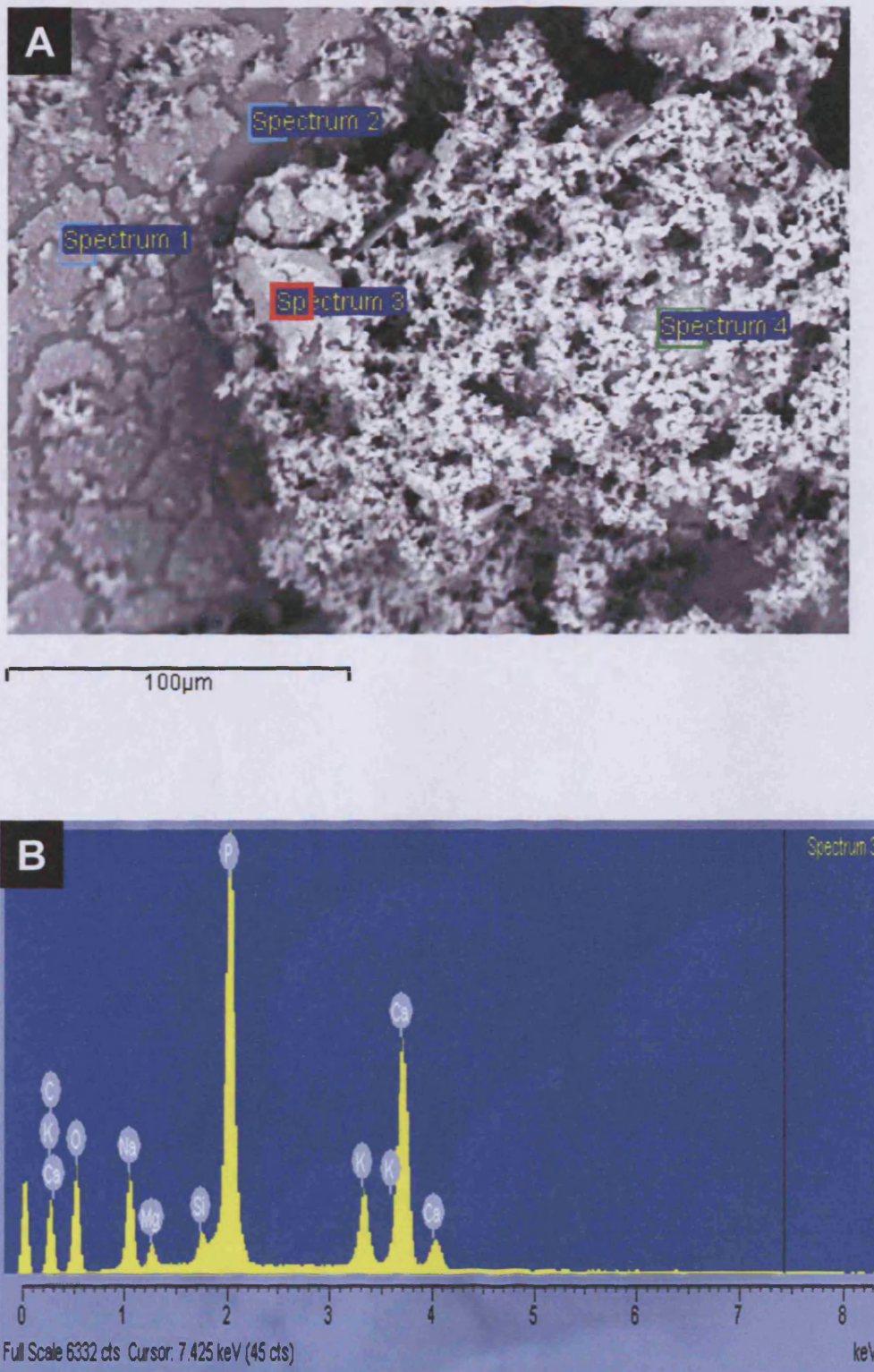


Figure 2.9: An example of the use of X-ray microanalysis in identifying calcium phosphate microcrystals using ESEM. Scanning microscopy is used to identify an area of interest (A). X-ray microanalysis of the sample area results in the emission of x-rays with energies characteristic to the elements being ionised. The spectrum obtained (B) can be used to construct a table of element abundance from the area analysed, as shown in Figure 3.8. The spectrum shown in (B) is from the area surrounded by red lines in (A). The electron micrograph and spectrum were kindly provided by Mr S. Morgan (Cardiff School of Biosciences).

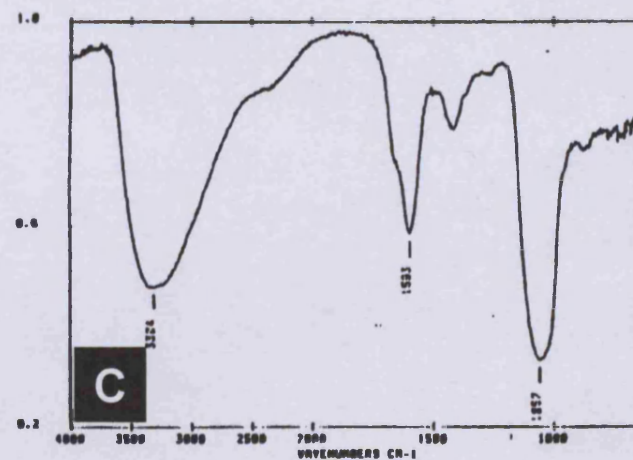
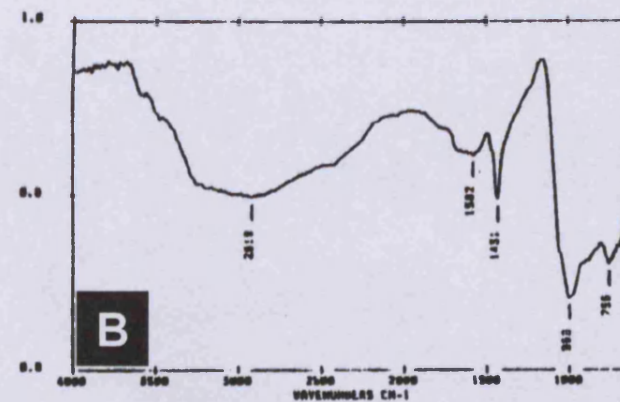
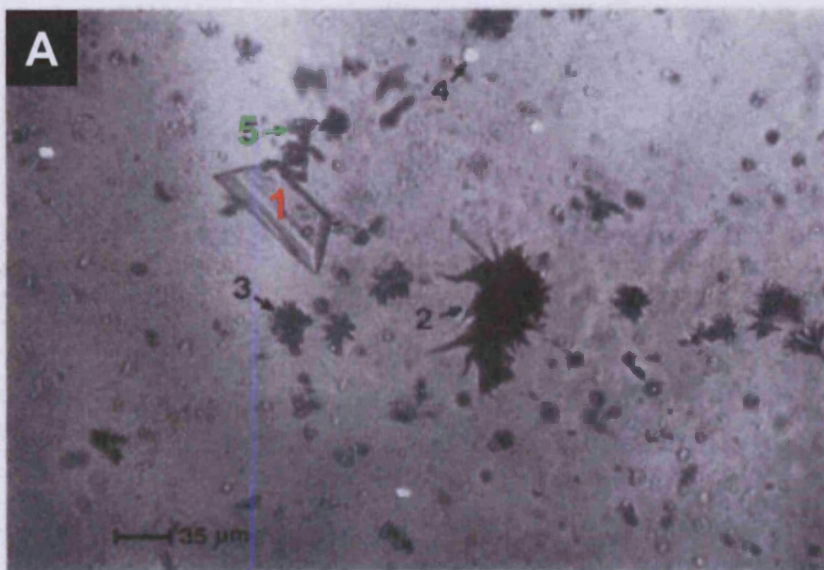


Figure 2.10: Examples of struvite and calcium phosphate microcrystals in infected human urine. Fourier transform infrared microscopy was used to obtain both image (A) and the spectra (B & C). The spectrum obtained from the crystal labeled 1 in (A) is shown in (B). Comparison with reference spectra indicated that this crystal is composed of struvite. Similarly the spectrum obtained from the crystal labeled 5 in (A) is shown in (C) and indicates that it is composed of calcium phosphate microcrystals. These examples are adapted from Daudon *et al.* (1991).

1:1 solution of ethanol : HMDS for 15 minutes. This was followed by 2 washes in HMDS alone for 15 minutes each. The final wash was not removed, but was left partially covered in the fume cupboard to evaporate slowly over 1 – 2 days. Once dried, using critical point drying or HMDS, the catheter sections were attached to aluminium stubs using adhesive black carbon disks. Eyehole sections were attached so that micrographs could be taken looking onto the eyehole and the other sections attached so that the length of the lumen section was visible. The catheter sections attached to stubs were then sputter coated with gold (Edwards sputter coater S150B). HV-SEM images were taken using a Philips XL-20 HV-SEM (FEI UK Ltd., Cambridge, UK).

2.11 Assay of urease production.

Test strains were streaked out for single colonies (from freezer stocks) to CLED agar and incubated overnight at 37 °C. Artificial urine (50 ml) was then inoculated with a loop full of the bacterial culture and incubated with aeration for 4 h at 37 °C. The cultures were centrifuged at 4000 rpm for 30 seconds to remove crystals from the suspension, then the supernatant was poured into another container and centrifuged at 4000 rpm for 5 minutes and the cells resuspended in 25 ml of ice cold 0.1 M sodium phosphate buffer (pH 7.3) 10 mM EDTA (0.01 M). A urease assay based on the modified Bertholt reaction (Creno *et al.*, 1970) was then used to determine the enzyme activity of the whole cell suspension. 200 µl of culture extract was then added to a reaction mixture containing 50 mM urea and 100 mM sodium phosphate buffer (pH 7.3) and incubated in test tubes at 37 °C for 10 minutes. The reaction was terminated by addition of phenol (0.5 %) sodium nitroprusside (0.0025 %) solution (2

ml). 2 ml of 0.25 % sodium hydroxide 0.21 % sodium hypochlorite solution was then added. Colour development was initiated by incubation for 6 minutes at 56 °C (Figure 2.11). The solution was then placed in a spectrophotometer and read at 600 nm against a reagent blank. The amount of ammonia present in the solution (and therefore the amount of urea hydrolysed) was then calculated by comparison to a standard curve created using ammonium chloride solutions substituted for culture extract (example shown in Figure 2.12). Protein levels in the cell resuspensions in 0.1 M sodium phosphate buffer (pH 7.3) 10 mM EDTA were assayed using a commercial protein determination test kit (Sigma). The amount of urea hydrolysed per mg protein was then calculated ($\mu\text{moles of urea hydrolysed} / \text{min} / \text{mg protein}$).

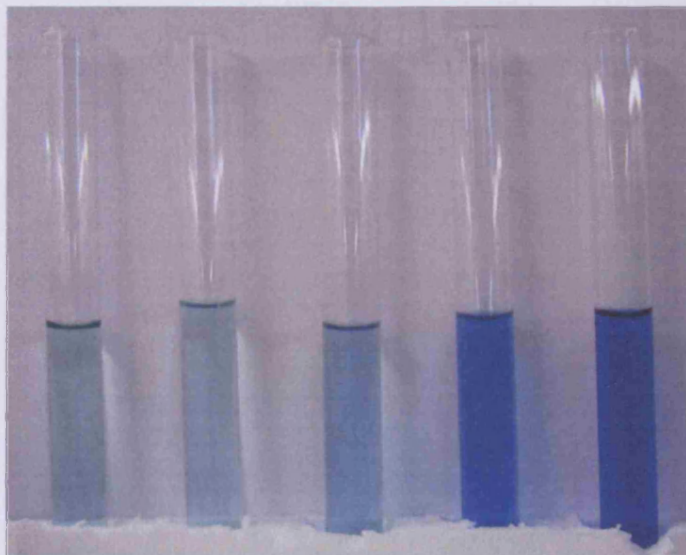


Figure 2.11: Solutions containing increasing amounts of ammonia (left to right) after colour development.

2.12 Statistical analysis.

Unless otherwise stated experiments were performed three times independently and where appropriate the standard error (SE) of the mean was indicated. ANOVA (analysis of variance) carried out at 95 % confidence interval was the statistical test of

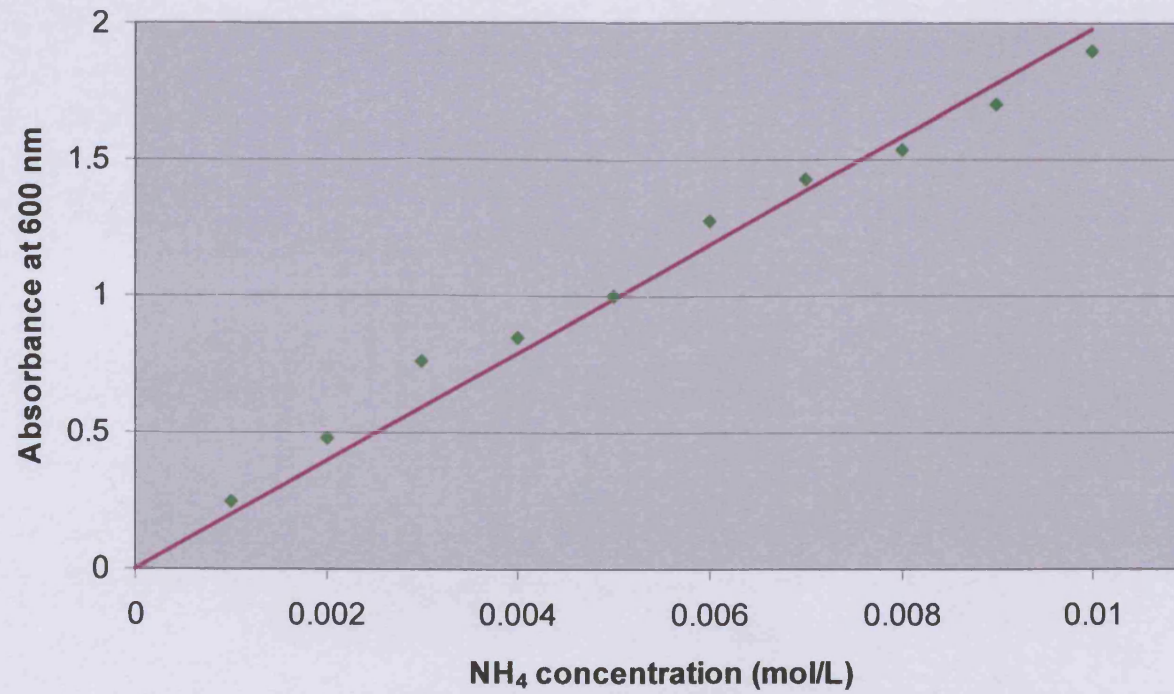


Figure 2.12: Example of a calibration curve used to determine the amount of urea hydrolysed in samples from the urease assay. Known concentrations of NH₄ were used in place of bacterial culture extract in the urease assay to obtain this data.

choice for all the experiments. This was carried out using Minitab[®] release 14 software (Minitab Inc., State College, Pennsylvania, USA). In order to use ANOVA the data analysed had to conform to the normal distribution and have homogeneity of variance. This was checked using Minitab, and if these assumption were violated and after transformation of the data they still did not fit, then the non-parametric Kruskal-Wallis test was used instead, again at a 95 % confidence interval. When only two sets of data were to be compared a two sample t-test (parametric) or Mann-Whitney test (non-parametric) were used.

Section 3

Results

3.1 Comparison of the ability of urease producing species to encrust and block all-silicone catheters.

There is substantial epidemiological and experimental evidence that the major cause of catheter encrustation is *Proteus mirabilis* (Mobley and Warren, 1987; Kunin, 1989; Stickler *et al.* 1993b; Morris and Stickler, 1998a; Stickler *et al.*, 1998a). The extent to which other urinary tract pathogens capable of producing urease are involved in the encrustation and blockage of patients' catheters is not clear. In view of this uncertainty, a wide range of bacterial species that had been isolated from catheter-associated urinary tract infections was examined for their ability to produce the enzyme urease in standard laboratory bacteriological identification tests. The ability of a collection of urease positive isolates to produce crystalline biofilms on catheters was then examined in the bladder models.

3.1.1 The ability of urease producing urinary tract bacteria to cause catheter encrustation and blockage.

Bladder models were inoculated with 10 ml of a 4 h mid-logarithmic culture (inoculated with a loopful of test organism grown from freezer culture overnight on agar) of test organism in artificial urine (approximately 10^8 cfu/ml). At 24 h intervals, up to a maximum of 96 h, or at blockage if it occurred, urine samples were taken from the bladder chamber. The pHs of the urine samples were measured and the viable bacterial cell populations determined by the drop count method. The extent of encrustation present on catheter sections was visualised using digital photography and HV-SEM. The amount of calcium and magnesium present on the catheters was determined by AAS and the rate of encrustation calculated. The urease activity of the

4 h logarithmic cultures in artificial urine was also assayed. Statistical analysis (ANOVA) was used to determine if there was any significant difference between the mean blockage times, urinary pHs, encrustation rates, and urease activity for the different test species.

Figure 3.1 shows the urinary pHs recorded for the experiments for nine urease producing species in comparison to a urease negative control (*E. coli*). The blockage time and final urinary pH (96 h or blockage) is shown in Table 3.1. Three urease producing species (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*) caused catheter blockage within 96 h. In models inoculated with the six remaining urease producing species (*Pv. stuartii*, *M. morgani*, *Staph. aureus*, *Staph. saprophyticus*, *Ps. aeruginosa* and *K. pneumoniae*) the catheters drained freely for the full 96 h test period. There was no significant difference ($P=0.059$) between the mean blockage times for *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*. The mean urinary pH at 96 h was compared to that of the *E. coli* control for those species which did not block. In the case of *M. morgani*, *Staph. aureus* and *Staph. saprophyticus* the final mean urinary pHs were significantly different ($P<0.05$) from that of the urine in the *E. coli* control. The final mean urinary pHs produced by these three species were not however significantly different ($P>0.05$) from each other. The final mean urinary pHs recorded at 96 h in models inoculated with *Pv. stuartii*, *Ps. aeruginosa* and *K. pneumoniae* were not significantly different from each other or from that of the *E. coli* control ($P>0.05$). The final mean urinary pH for those species which blocked was significantly different from the control ($P<0.05$). All three of the species which caused catheter blockage produced final mean urinary pHs which were significantly different from those of *M. morgani*, *Staph. aureus* and *Staph. saprophyticus*

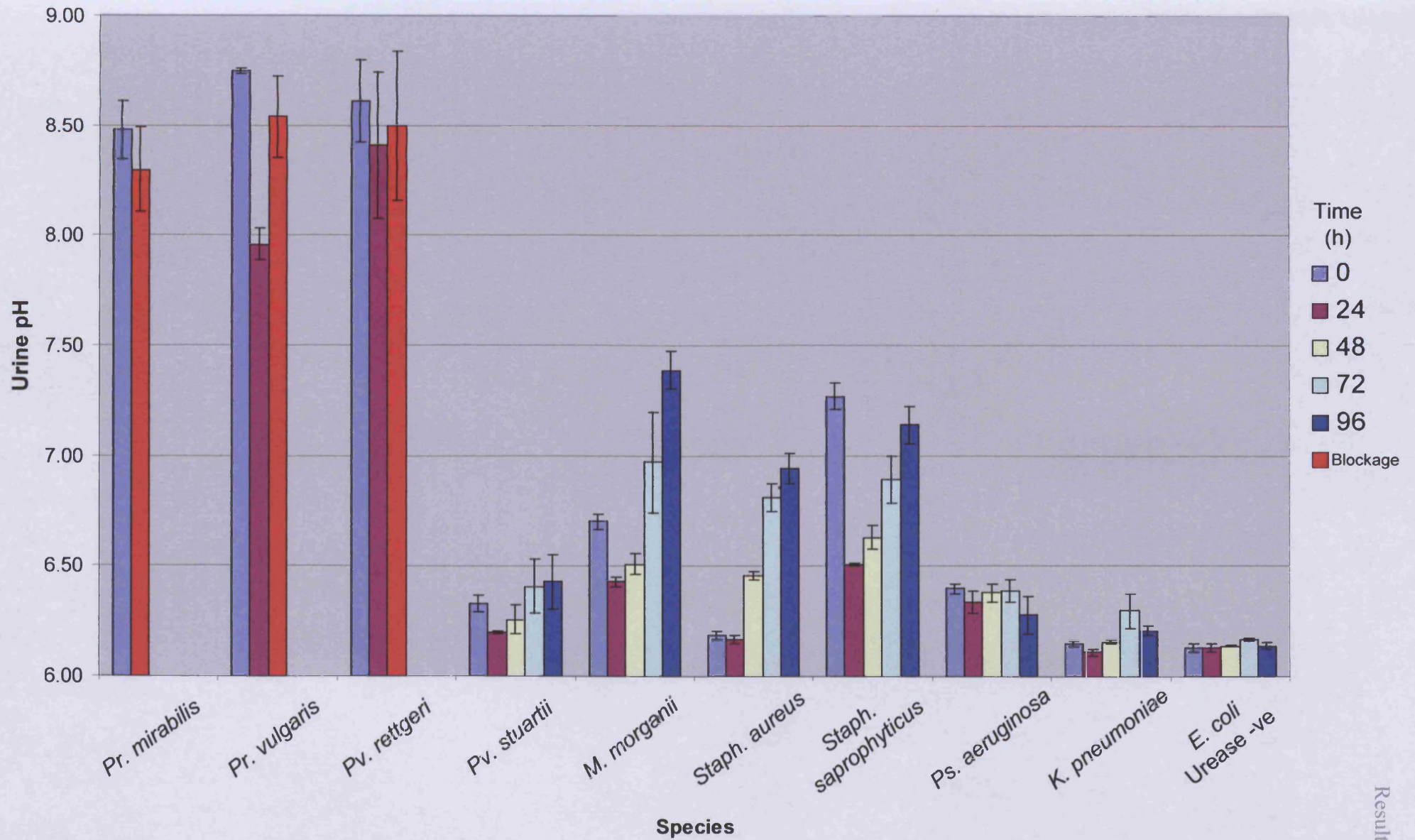


Figure 3.1: The mean* pHs recorded for urine from the bladder chamber of models infected with a range of urease producing species. Experiments were stopped at 96 h if blockage had not occurred by this point. A urease negative *E. coli* isolate was used as a control. * The mean values \pm SE were calculated for the results of triplicate experiments.

Species (isolate)	Mean blockage time, if applicable (h) (\pm SE)	Final urinary pH (at blockage or 4 days) (\pm SE)
<i>Proteus mirabilis</i> (RB6)	19.8 (\pm 2.91)	8.34 (\pm 0.19) ▲
<i>Proteus vulgaris</i> (SDM2)	36.4 (\pm 3.85)	8.42 (\pm 0.21) ▲
<i>Providencia rettgeri</i> (SDM1)	32.2 (\pm 6.19)	8.36 (\pm 0.19) ▲
<i>Providencia stuartii</i> (RB14)	-	6.44 (\pm 0.06) ●
<i>Morganella morganii</i> (RB15)	-	7.39 (\pm 0.08) ■
<i>Staphylococcus aureus</i> (P10 6/9) (urease +ve)	-	6.89 (\pm 0.06) ■
<i>Staphylococcus saprophyticus</i> (ZEC17)	-	7.14 (\pm 0.09) ■
<i>Pseudomonas aeruginosa</i> (RB16)	-	6.32 (\pm 0.05) ●
<i>Klebsiella pneumoniae</i> (SDM17)	-	6.31 (\pm 0.05) ●
<i>Escherichia coli</i> (RB13) (urease -ve control)	-	6.15 (\pm 0.02) ●
<i>Staphylococcus aureus</i> (W112) (urease -ve control)	-	6.09 (\pm 0.01)
Uninoculated control	-	6.11 (\pm 0.01)

Table 3.1: The mean* times catheters took to block and urinary pHs in models infected with a range of urinary pathogens. * mean values \pm SE were calculated from the results of three replicate experiments. - indicates catheters drained freely for the 96 h experimental period.

- Indicates that the mean final urinary pHs for this group of species were not significantly different from the urease negative control *E. coli* or from each other ($P > 0.05$).
- Indicates that the mean final urinary pHs for this group of species were significantly greater than the urease negative control *E. coli* ($P < 0.05$) but were not significantly different from each other ($P > 0.05$).
- ▲ Indicates that the mean final urinary pHs for this group of species were significantly greater than the ■ group ($P < 0.05$) but were not significantly different from each other ($P > 0.05$).

($P < 0.05$). There was no significant difference between the final mean urinary pHs produced by the species that blocked catheters ($P > 0.05$).

Figure 3.2 shows the encrustation rates for calcium and magnesium. Catheters were removed from the model at blockage or 96 h and the amounts of calcium and magnesium present were determined using AAS. The data were then related to the time the catheter had been in the model to determine the encrustation rate. There was no significant difference ($P > 0.05$) between the calcium encrustation rates for *Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri* and *M. morgani* while they were all significantly different ($P < 0.05$) from the *E. coli* control. The calcium encrustation rates of *Staph. aureus*, *Pv. stuartii*, *Ps. aeruginosa* and *K. pneumoniae* were not significantly different ($P > 0.05$) from each other or the control *E. coli*. There was also no significant difference ($P > 0.05$) between the encrustation rates of *M. morgani*, *Ps. aeruginosa* and *Pv. stuartii*. The magnesium encrustation rates of *M. morgani*, *Staph. aureus*, *Pv. stuartii*, *Ps. aeruginosa* and *K. pneumoniae* were not significantly different ($P > 0.05$) from each other or the control *E. coli*. Magnesium encrustation rates for *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* were not significantly different ($P > 0.05$) from each other but were significantly different ($P < 0.05$) from the other species and the control.

3.1.2 The effect of pH on the solubility of calcium and magnesium in artificial urine.

The pH at which calcium and magnesium phosphate crystals form in urine (the nucleation pH, pH_n) is characteristic of a particular urine. The more dilute the urine, the higher the pH_n (Suller *et al.*, 2005). The pH_n of the artificial urine used in these

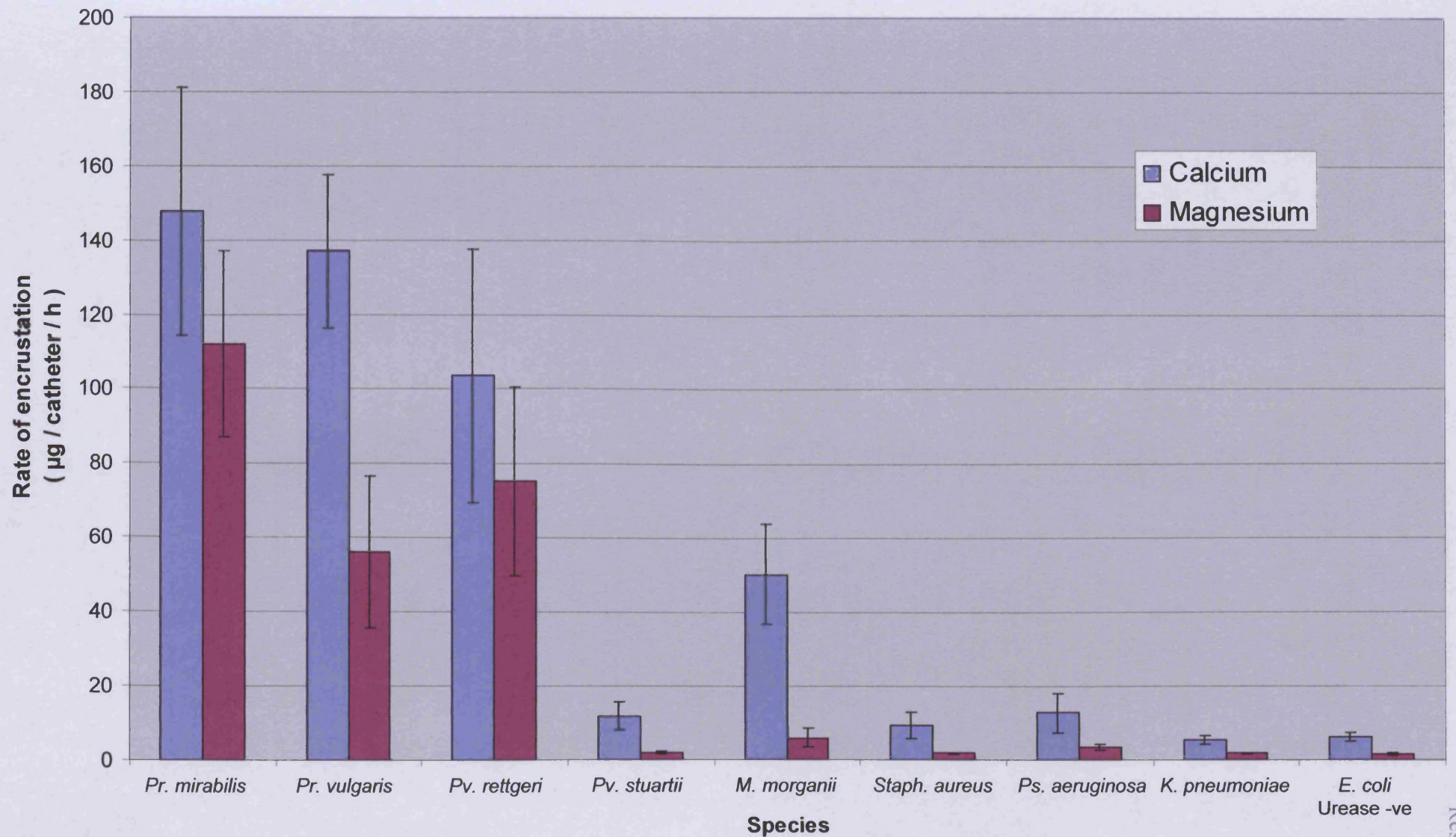


Figure 3.2: Mean* \pm SE rates of calcium and magnesium accumulation on catheters in bladder models infected with various bacterial species. Experiments were stopped at 96 h if blockage had not occurred by this point. A urease negative *E. coli* isolate was used as a control. * Mean values calculated from the results of triplicate experiments. The rates calculated were the mean accumulation over the period the experiment was conducted (96 h or blockage) and do not indicate when the encrustation occurred during this period.

experiments was determined as described in Section 2.8.2. The concentration of calcium and magnesium remaining in solution as the urinary pH was increased are presented in Figure 3.3. Using the method described by Choong *et al.* (1999) (Figure 2.5) the mean $\text{pH}_n \pm \text{SE}$ for calcium was calculated to be 6.54 ± 0.01 . The pH_n for magnesium was clearly higher than this figure but could not be calculated since there was no distinct point where the concentration began to drop steeply.

3.1.3 The urease activity of the test species.

The urease activity produced by the test species when growing in the artificial urine was determined using 4 h logarithmic phase cultures. The results presented in Figure 3.4 show that many of the species that produce detectable urease in bacteriological identification tests, produce relatively low amounts of the enzyme when grown in urine. In fact the rate of hydrolysis of urea was not significantly different from the urease negative controls for *Pv. stuartii*, *K. pneumoniae*, *K. oxytoca*, *Serratia marcescens*, *Citrobacter freundii*, *C. koseri*, *Enterobacter cloacae* and *Ps. aeruginosa* ($P=0.270$) or for *Staph. aureus* from the *Staph. aureus* urease negative control ($P>0.05$). The activities of *Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri*, *M. morganii* were all significantly greater ($P<0.05$) than *Staph. aureus* and the *Staph. aureus* urease negative control. There was no significant difference ($P>0.05$) between *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*, while the rate for *M. morganii* was significantly different ($P<0.05$) to both *Proteus* species but not significantly different ($P>0.05$) from *Pv. rettgeri*.

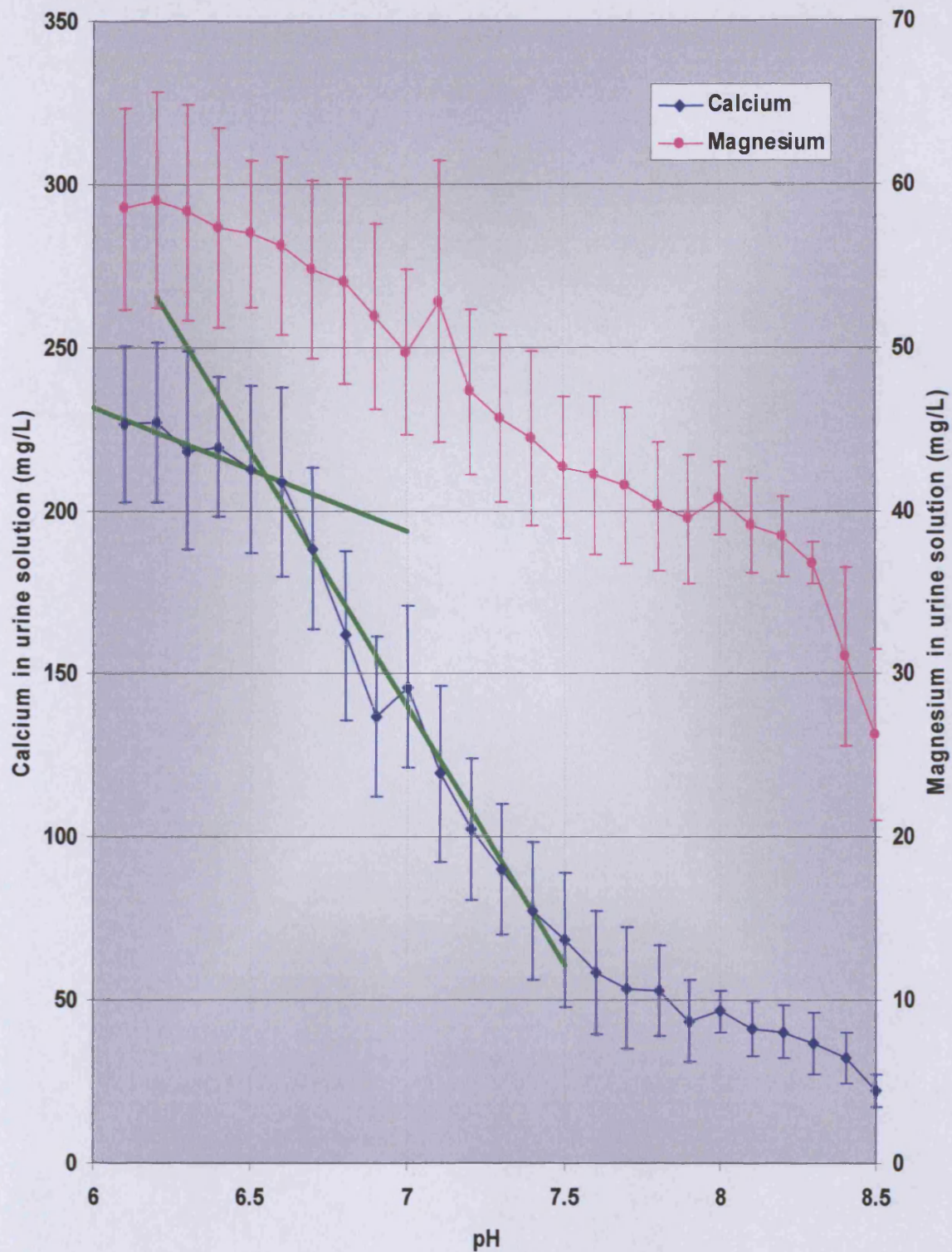


Figure 3.3: Calcium and magnesium concentrations in artificial urine at each pH after removal of crystals by centrifugation. Each data point is the mean of 3 replicates (SE indicated). The green lines show the regression lines used to calculate the nucleation pH of calcium in artificial urine (the urine pH at their point of intersection, pH 6.54). The data for magnesium was not suited to this analysis due to a lack of a distinct point where its solubility began to drop rapidly (such as can be seen for calcium at pH 6.6).

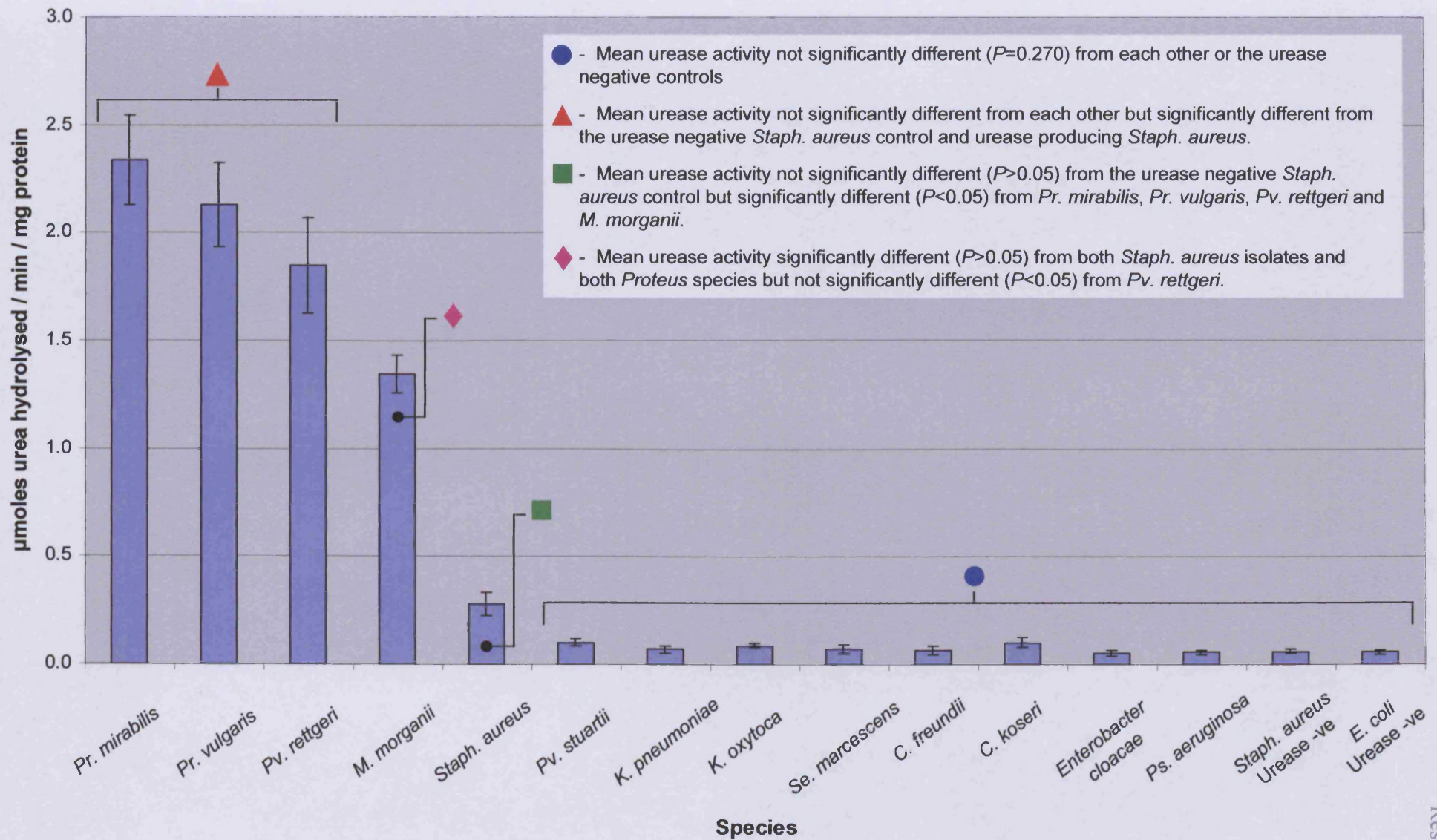


Figure 3.4: Rates of urea hydrolysis for urease producing urinary tract bacteria grown in artificial urine (4 h shake flask cultures). Urease negative *Staph. aureus* and *E. coli* isolates were used as controls.

3.1.4 Visualization of the catheter biofilms.

Digital photographs were taken of catheters removed from the bladder model at 96 h or blockage for nine urease producing species and a urease negative *E. coli* control (Figures 3.5 & 3.6). The 3 species which caused blockage (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*) all show large amounts of encrustation, particularly on the eyehole and lumen sections nearest the eyehole (Figure 3.5). The images of *M. morgani* show that some encrustation is present after 96 h (Figure 3.5) while *Staph. aureus* and *Staph. saprophyticus* also show signs of encrustation (Figure 3.6). *Ps. aeruginosa* and *K. pneumoniae* produced little biofilm at the eyehole or immediately below it. Extensive biofilms were found however, lower down the catheter lumen (Figure 3.6). *Pv. stuartii* failed to generate biofilm, the catheter appeared similar to those removed from *E. coli* infected models (Figure 3.5).

HV-SEM carried out on catheter sections confirmed the crystalline nature of some of the biofilms. Figure 3.7 shows HV-SEM of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* biofilms at blockage. All three catheters show heavy encrustation consisting of microcrystals typical of apatite. The biofilms produced by *M. morgani*, *Staph. aureus* and *Staph. saprophyticus* also contained large crystalline forms (Figure 3.8). X-ray micro-analysis of the large crystalline arrays produced by *Staph. aureus* indicated that these structures were composed of calcium phosphate (Figure 3.9). No crystalline material was observed in the biofilms formed by *Pv. stuartii*, *K. pneumoniae* or *Ps. aeruginosa* (Figure 3.10). The biofilms formed by the urease negative controls *E. coli* and *Staph. aureus* are clearly non-crystalline (Figure 3.11a &

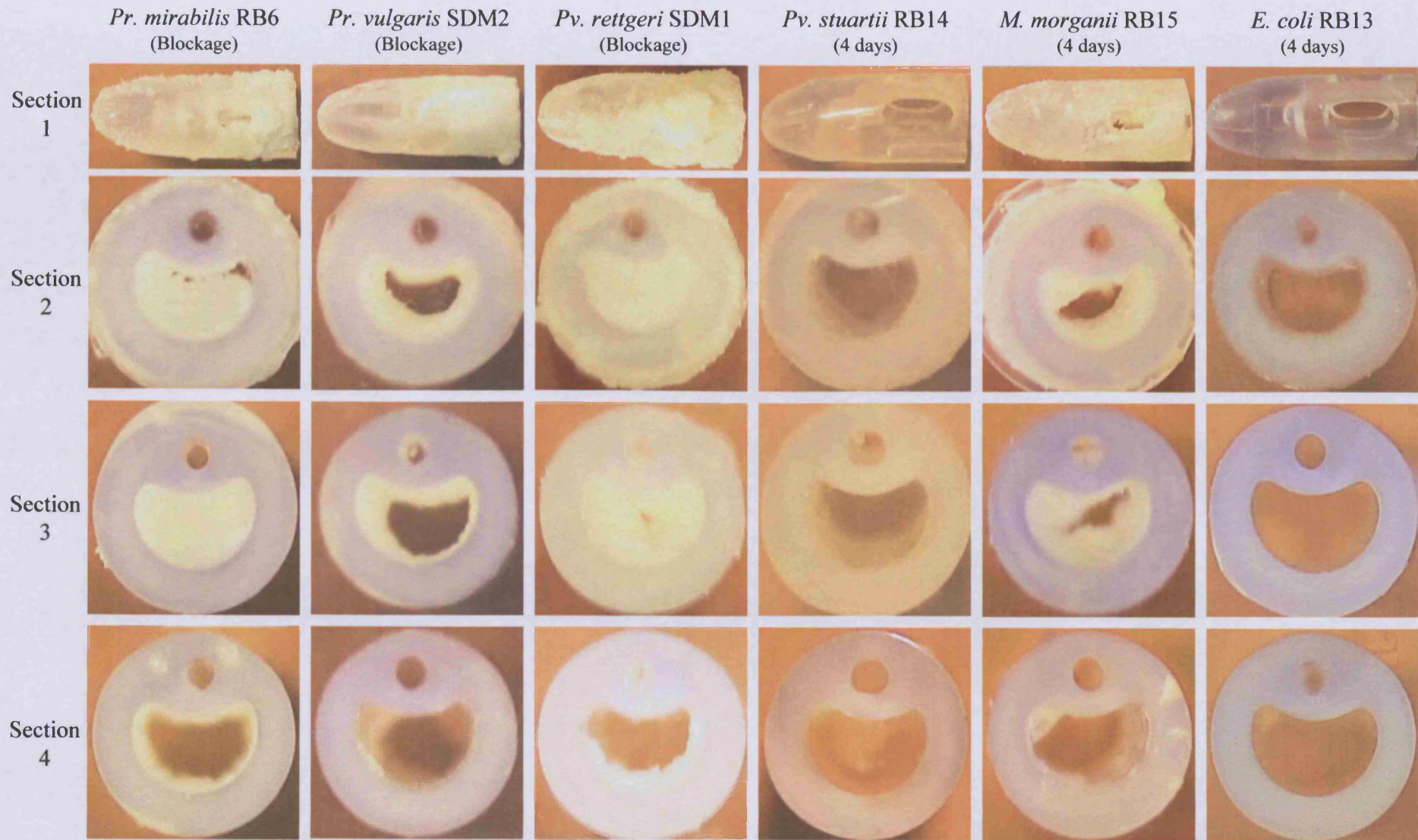


Figure 3.5: Comparison of encrustation at 4 days or blockage against *Pr. mirabilis* (+ve control) and *E. coli* (-ve control)

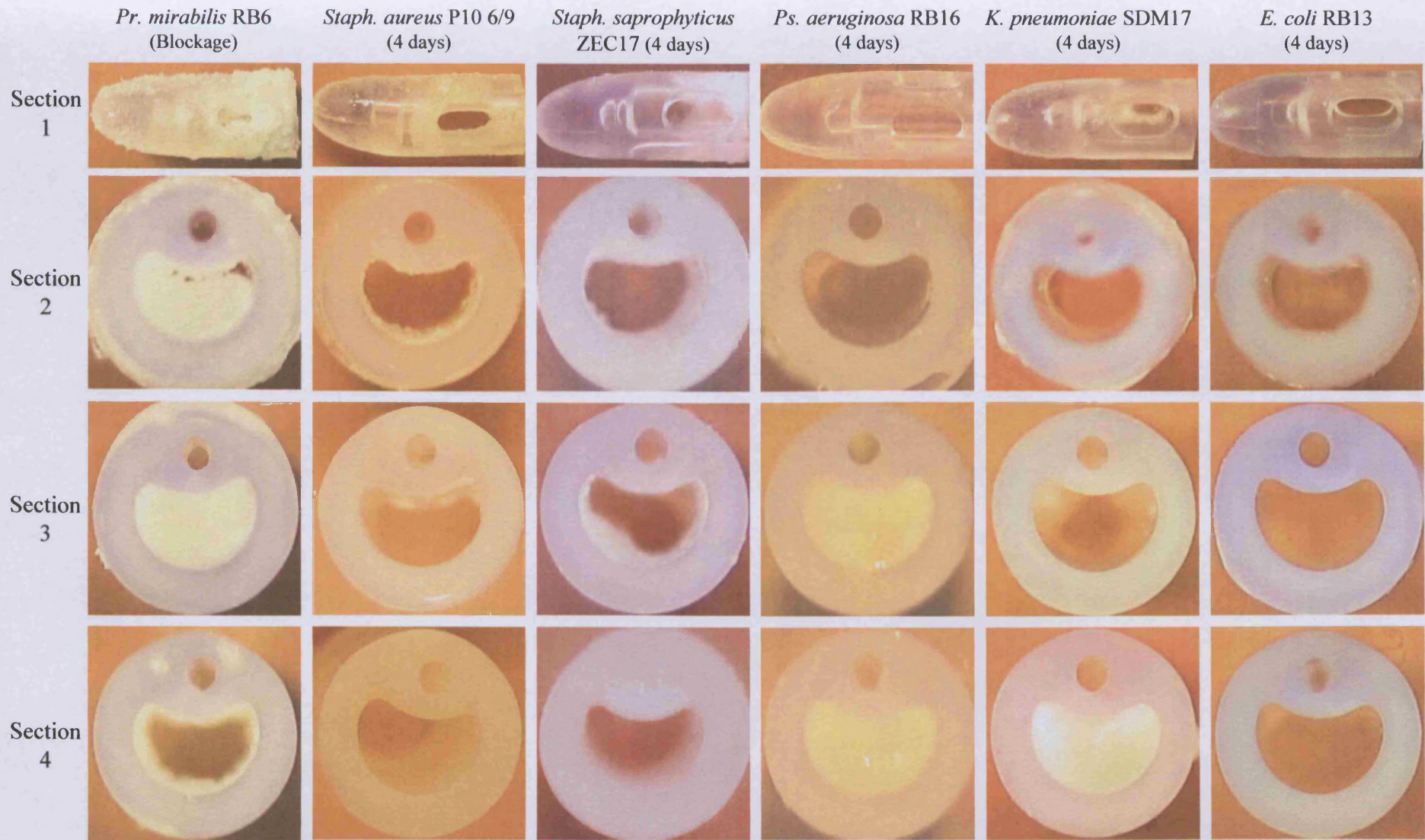
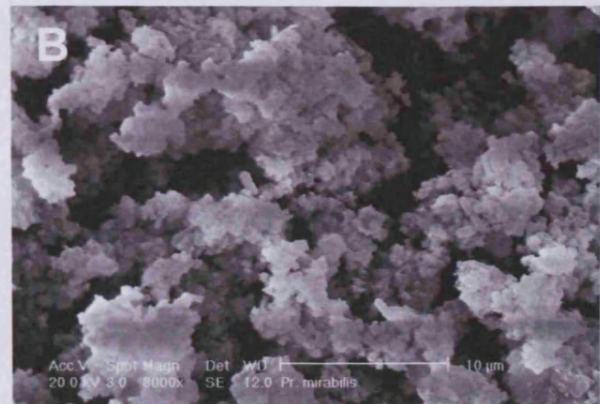
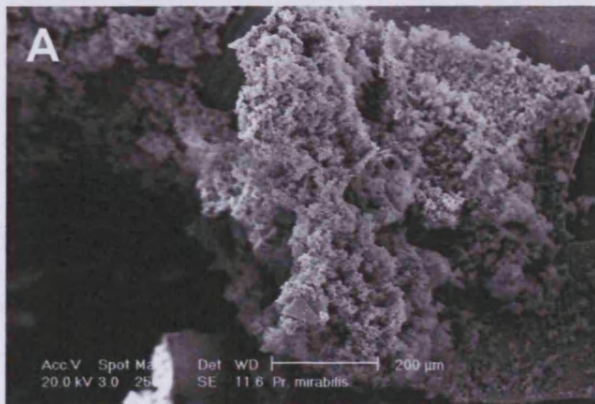
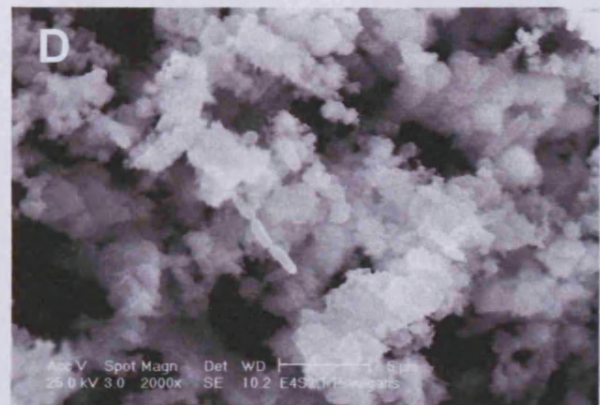
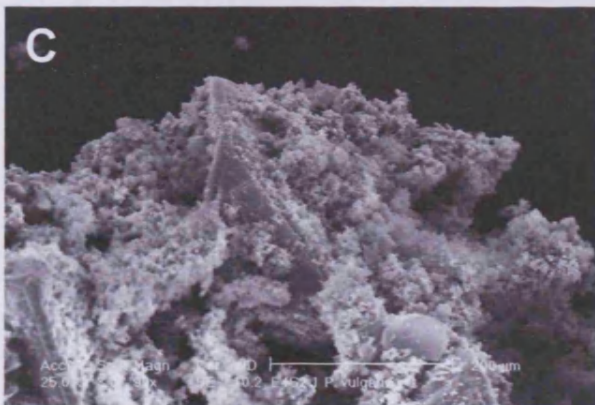


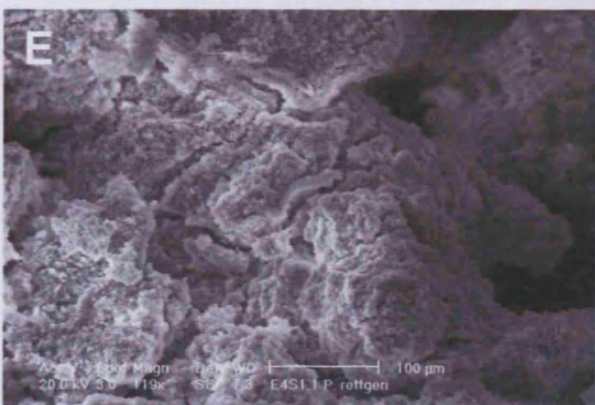
Figure 3.6: Comparison of encrustation at 4 days or blockage against *Pr. mirabilis* (+ve control) and *E. coli* (-ve control)



A & B: Encrusted *Pr. mirabilis* biofilm on an all-silicone catheter (removed from the bladder model at blockage). The biofilm can be seen to be heavily encrusted (A). Microcrystals typical of apatite can be seen at high magnification (B).

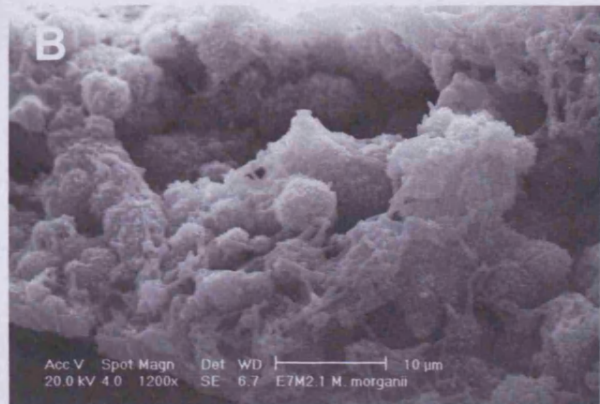


C & D: Encrusted *Pr. vulgaris* biofilm on an all-silicone catheter (removed from the bladder model at blockage). Crystalline material typical of struvite and apatite can be seen (C). Bacterial cells and apatite are visible at high magnification (D).

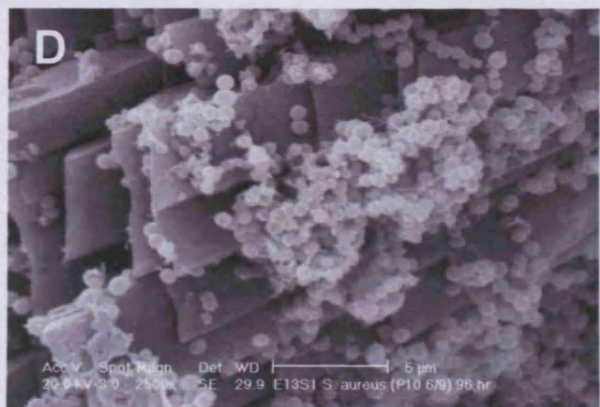
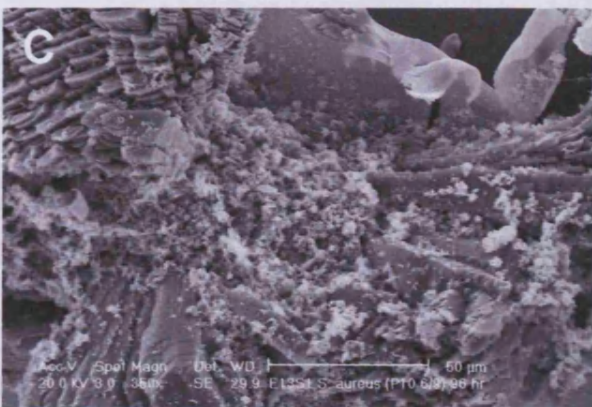


E & F: Encrusted *Pv. rettgeri* biofilm on an all-silicone catheter (removed from the bladder model at blockage). The extensive crystalline biofilm (E) when visualised at high magnification is composed of bacterial cells and microcrystals (F).

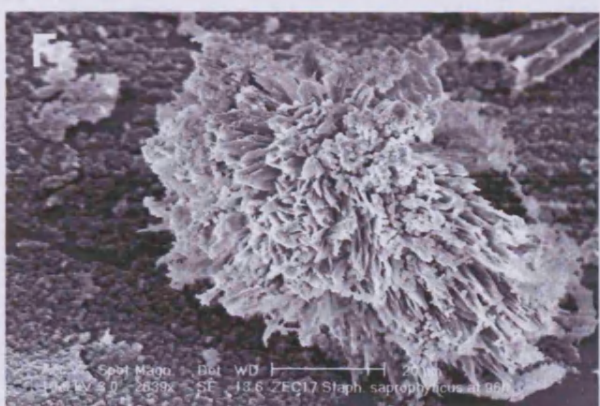
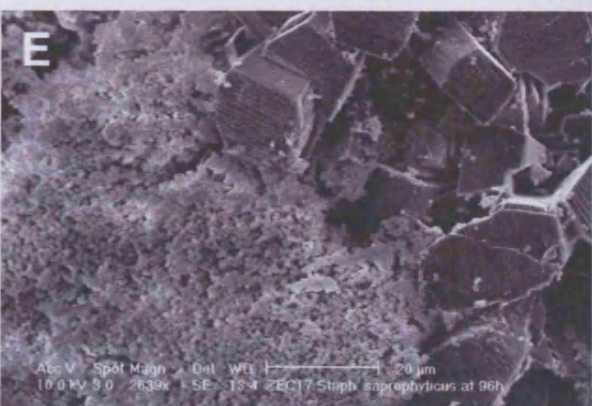
Figure 3.7: HV-SEM images of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* biofilms on all-silicone catheters removed from bladder models at blockage.



A & B: The crystalline nature of an *M. morganii* biofilm on an all-silicone catheter (removed from the bladder model after 4 days).

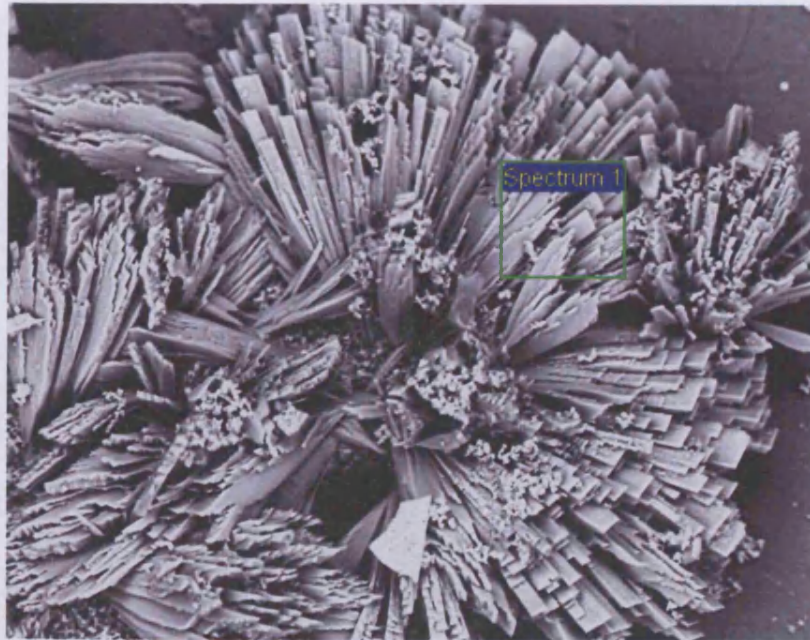


C & D: *Staph. aureus* biofilm on an all-silicone catheter (removed from the bladder model after 4 days). A variety of crystalline forms can be seen together with large numbers of cocci.



E & F: *Staph. saprophyticus* biofilm on an all-silicone catheter (removed from the bladder model after 4 days). Crystalline structures are clearly present in the bacterial biofilm

Figure 3.8: HV-SEM images of *M. morganii*, *Staph. aureus* and *Staph. saprophyticus* biofilms on all-silicone catheters removed from bladder models after 4 days.



Element	Atomic %	Weight %
Carbon	7.46	4.38
Oxygen	68.51	53.6
Sodium	0.14	0.16
Magnesium	0.06	0.08
Silicone	0.13	0.18
Phosphorous	10.86	16.45
Calcium	12.83	25.15



Element	Atomic %	Weight %
Carbon	6.88	3.77
Oxygen	63.11	46.1
Phosphorous	11.49	16.25
Calcium	18.52	33.88

Figure 3.9: X-ray microanalysis data for large crystal formations on a urease positive *Staph. aureus* biofilm. The element abundance data presented in the tables is taken from the areas labeled in green on the electron micrographs.

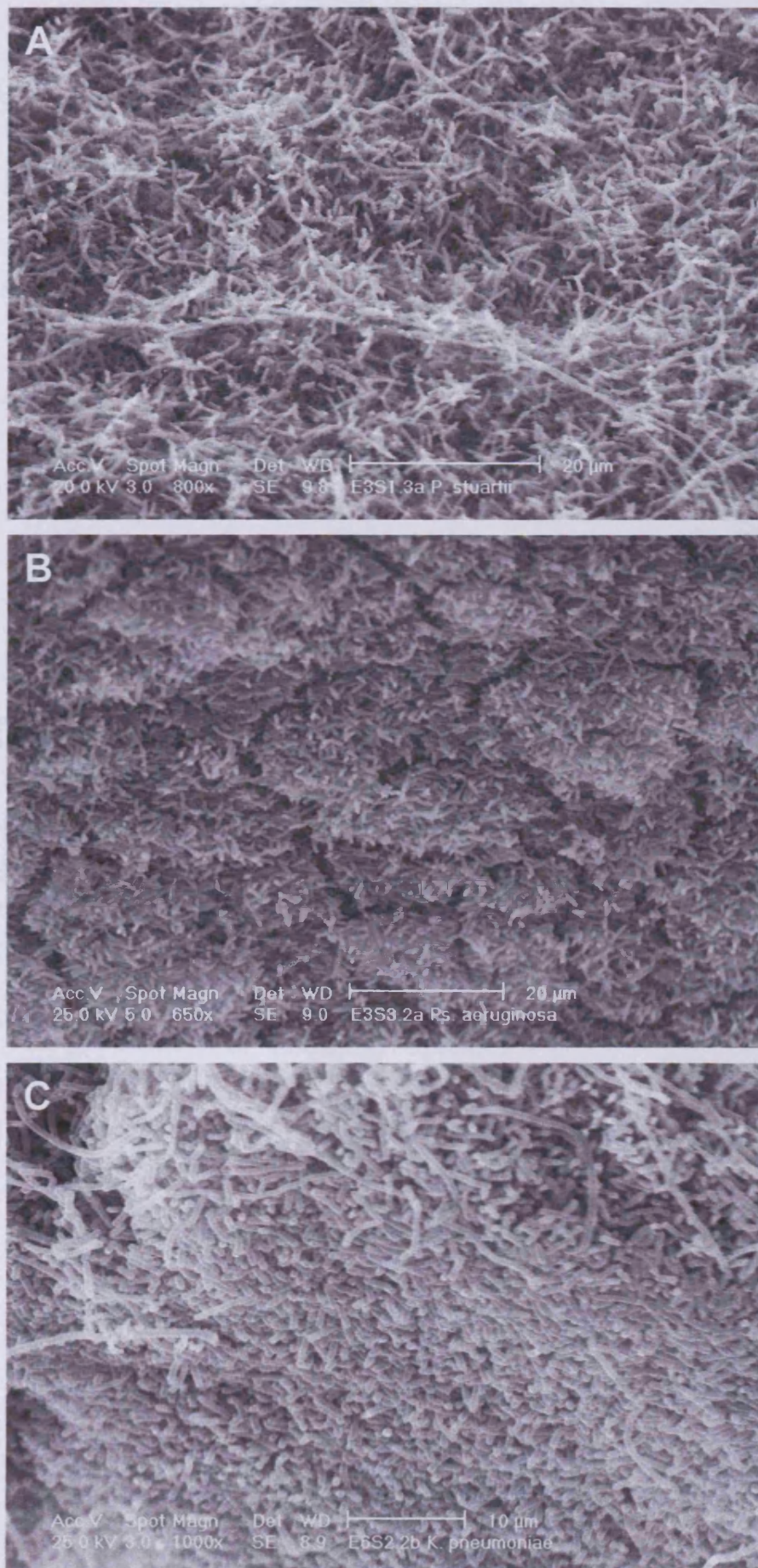


Figure 3.10: HV-SEM images of *Pv. stuartii* (A), *Ps. aeruginosa* (B) and *K. pneumoniae* (C) biofilms on all-silicone catheters removed from bladder models after 4 days.

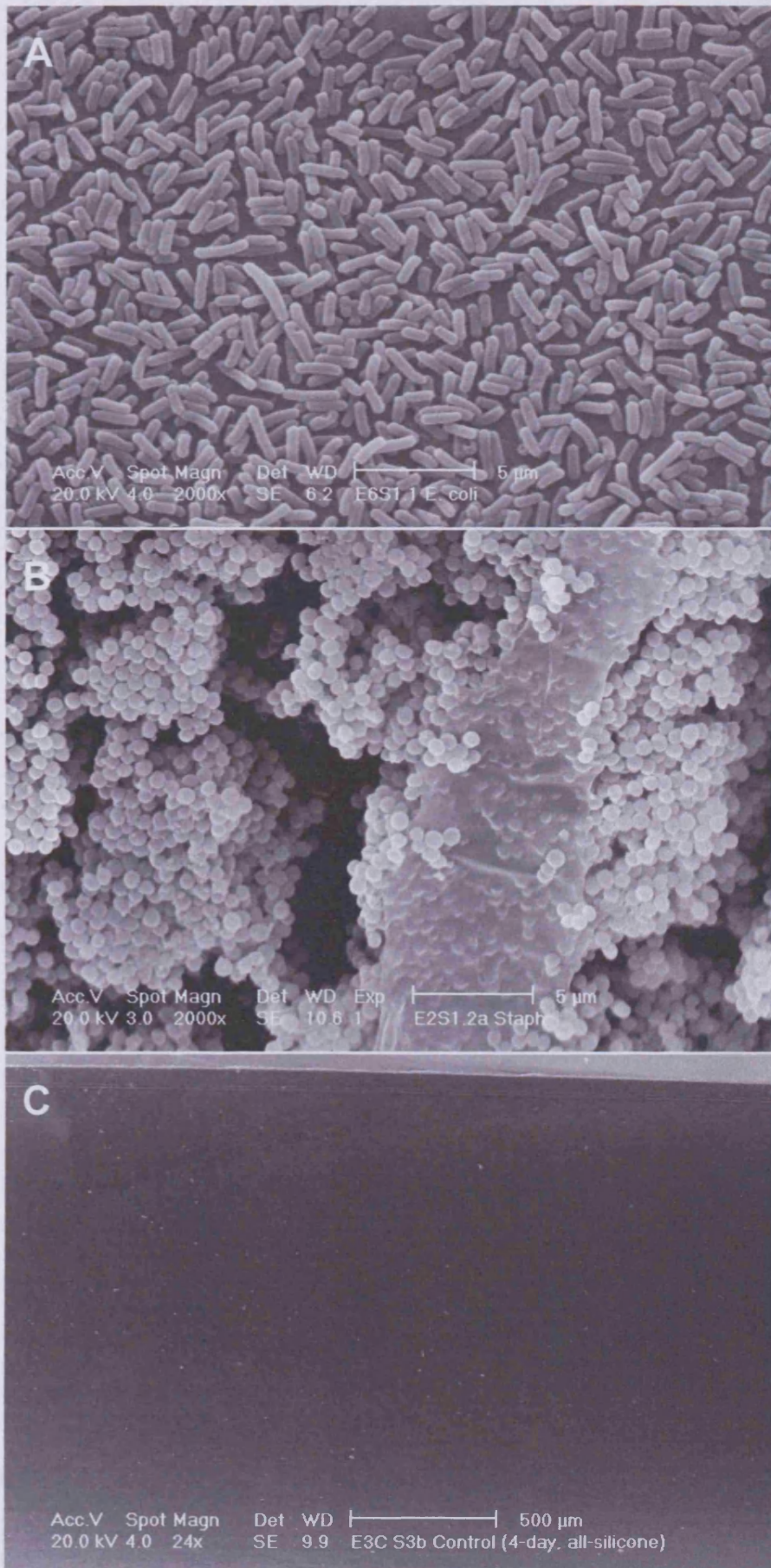


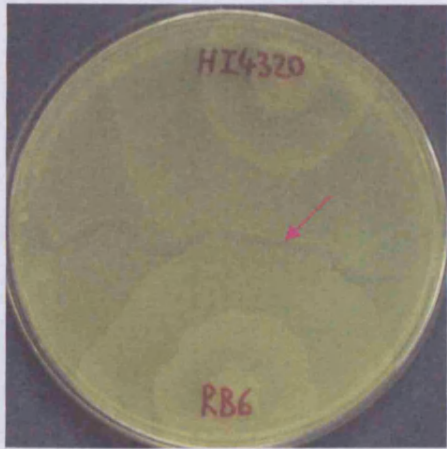
Figure 3.11: HV-SEM images of control catheters removed from the bladder model after 4 days. Controls were performed using urease negative *E. coli* (A) and *Staph. aureus* (B) isolates, and also an uninoculated model (C). The surface of the uninoculated catheter is quite bare, although some small pieces of debris are visible.

b). Examination of the uninoculated control catheter shows the nature of the catheter surface (Figure 3.11c).

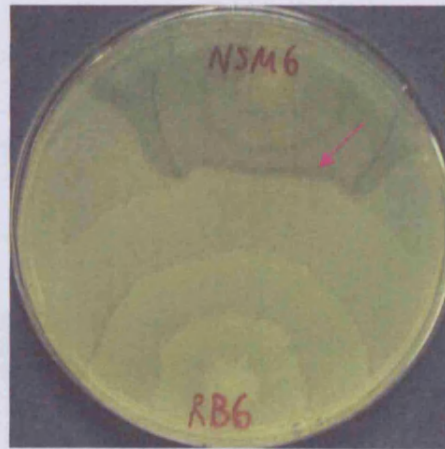
3.1.5 An assessment of the abilities of three isolates of each urease producing species to raise urinary pH and cause catheter encrustation.

To examine whether the results obtained from the independently replicated experiments with just one isolate of each test species were typical for that species, the abilities of a further three isolates of each species to produce crystalline biofilms was tested in the bladder models. The isolates of any given species were all obtained from different patients. In the case of *Pr. mirabilis*, Dienes typing confirmed that the three isolates chosen were distinct strains (Figure 3.12). The experiments were performed once for each isolate and samples were collected as described in Section 3.1.1. Statistical analysis (Two sample t-test) was used to determine if there was any significant difference between the blockage times and final bladder model pHs for the isolates tested in Section 3.1.1 and the pooled data from the three isolates of the same species.

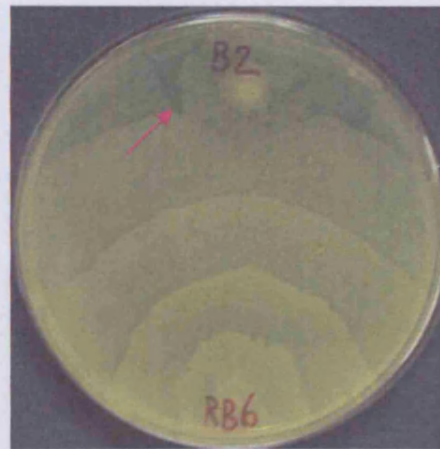
Table 3.2 shows a summary of the data obtained. For those species which blocked within 96 h (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*) there was no significant difference ($P>0.05$) between the mean blockage times of the original and pooled alternative isolates. With the exception of *Staph. aureus* none of the species tested showed a significant difference between the original and pooled alternative isolates for the mean final pH values.



HI4320 (top) vs. RB6 (bottom)



NSM6 (top) vs. RB6 (bottom)



B2 (top) vs. RB6 (bottom)



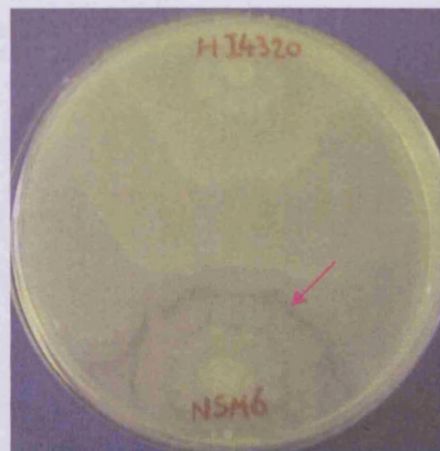
RB6 (top) vs. RB6 (bottom)



HI4320 (top) vs. B2 (bottom)



NSM6 (top) vs. B2 (bottom)



HI4320 (top) vs. NSM6 (bottom)

Figure 3.12: Dienes typing of swarming *Pr. mirabilis* isolates on TSA. Combinations of the four *Pr. mirabilis* isolates tested all show the characteristic gap between the swarming fronts (indicated ←), and are therefore considered to be different strains. No gap is evident at the point the swarming fronts meet for the control of a single isolate swarming towards itself.

Species	Data from triplicate experiments on original test isolates of urease producing species			Pooled data from single experiments with three alternative isolates of each urease producing species			Result of statistical analysis* (Two sample t-test)	
	Isolate code	Mean blockage time, if applicable (h) (\pm SE)	Final urinary pH (at blockage or 4 days) (\pm SE)	Isolate codes	Mean blockage time, if applicable (h) (\pm SE)	Final urinary pH (at blockage or 4 days) (\pm SE)	Blockage time (if applicable)	Final urinary pH
<i>Proteus mirabilis</i>	RB6	19.8 (\pm 2.91)	8.34 (\pm 0.19)	B2, NSM6, HI4320	22.0 (\pm 2.00)	7.95 (\pm 0.06)	NSD ($P=0.614$)	NSD ($P=0.185$)
<i>Proteus vulgaris</i>	SDM2	36.4 (\pm 3.85)	8.42 (\pm 0.21)	NSM19, H2V, W	28.7 (\pm 2.85)	8.44 (\pm 0.27)	NSD ($P=0.213$)	NSD ($P=0.972$)
<i>Providencia rettgeri</i>	SDM1	32.2 (\pm 6.19)	8.36 (\pm 0.19)	NSM26, RE12, AAAAG	26.0 (\pm 8.19)	8.35 (\pm 0.28)	NSD ($P=0.565$)	NSD ($P=0.963$)
<i>Providencia stuartii</i>	RB14	-	6.44 (\pm 0.06)	NSM71, SM53, SM72	-	6.27 (\pm 0.04)	-	NSD ($P=0.108$)
<i>Morganella morganii</i>	RB15	-	7.14 (\pm 0.12)	NSM63, SM18, SM40	-	7.28 (\pm 0.05)	-	NSD ($P=0.575$)
<i>Staphylococcus aureus</i>	P10 6/9	-	6.89 (\pm 0.06)	NSM5, NSM7, N49	-	6.27 (\pm 0.06)	-	HSD ($P<0.001$)
<i>Pseudomonas aeruginosa</i>	RB16	-	6.32 (\pm 0.05)	RB4, SM10, SDM5	-	6.29 (\pm 0.03)	-	NSD ($P=0.723$)
<i>Klebsiella pneumoniae</i>	SDM17	-	6.31 (\pm 0.05)	NSM22, SM28, RB8	-	6.27 (\pm 0.02)	-	NSD ($P=0.568$)

Table 3.2: Comparison between blockage time (if appropriate) and final urinary pH (at blockage or 96 h) recorded in triplicate experiments on original test isolates and single experiments on three alternative isolates of each species.

* - indicates catheters drained freely for the 96 h experimental period; NSD: No significant difference; HSD: Highly significant difference

Digital photographs taken of catheters removed from the bladder model at blockage or after 96 h are presented in Figures 3.13 – 3.20. The isolates of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* tested all show large amounts of encrustation to be present on the catheter sections, in particular the eyehole sections (Figures 3.13 – 3.15). The *M. morgani* isolates showed some variation (Figure 3.16), with some alternative isolates (NSM63 & SM18) apparently less encrusted than the original isolate (RB15) after 96 h. There was also a difference between the original isolate and the alternative isolates for *Staph. aureus* (Figure 3.17). While the main isolate (P10 6/9) showed some encrustation little sign of crystalline material is apparent on the alternative isolates (N49, NSM5 & NSM7) after 96 h. Figure 3.21 shows that, while the mean urinary pH at 96 h of the original *Staph. aureus* isolate is significantly greater ($P < 0.001$) than that of the alternative isolates, the mean cfu/ml of the original isolate is significantly less ($P < 0.001$) than that of the alternative isolates. For *Ps. aeruginosa* there was no apparent difference between any of the isolates after 96 h, with little biofilm present on the top two sections and very thick mucoid biofilm present on the third section (Figure 3.18). The images of *Pv. stuartii* (Figures 3.19) show little evidence of biofilm produced by any of the isolates. In the case of *K. pneumoniae* two of the four isolates appeared to form biofilm on section 3 of the catheters (Figure 3.20).

3.1.6 An assessment of the ability of less commonly occurring urease producing bacteria to raise urinary pH.

The collection of bacteria that has been built up in the Cardiff laboratory over the years contains several other urease producing species that although not common, have

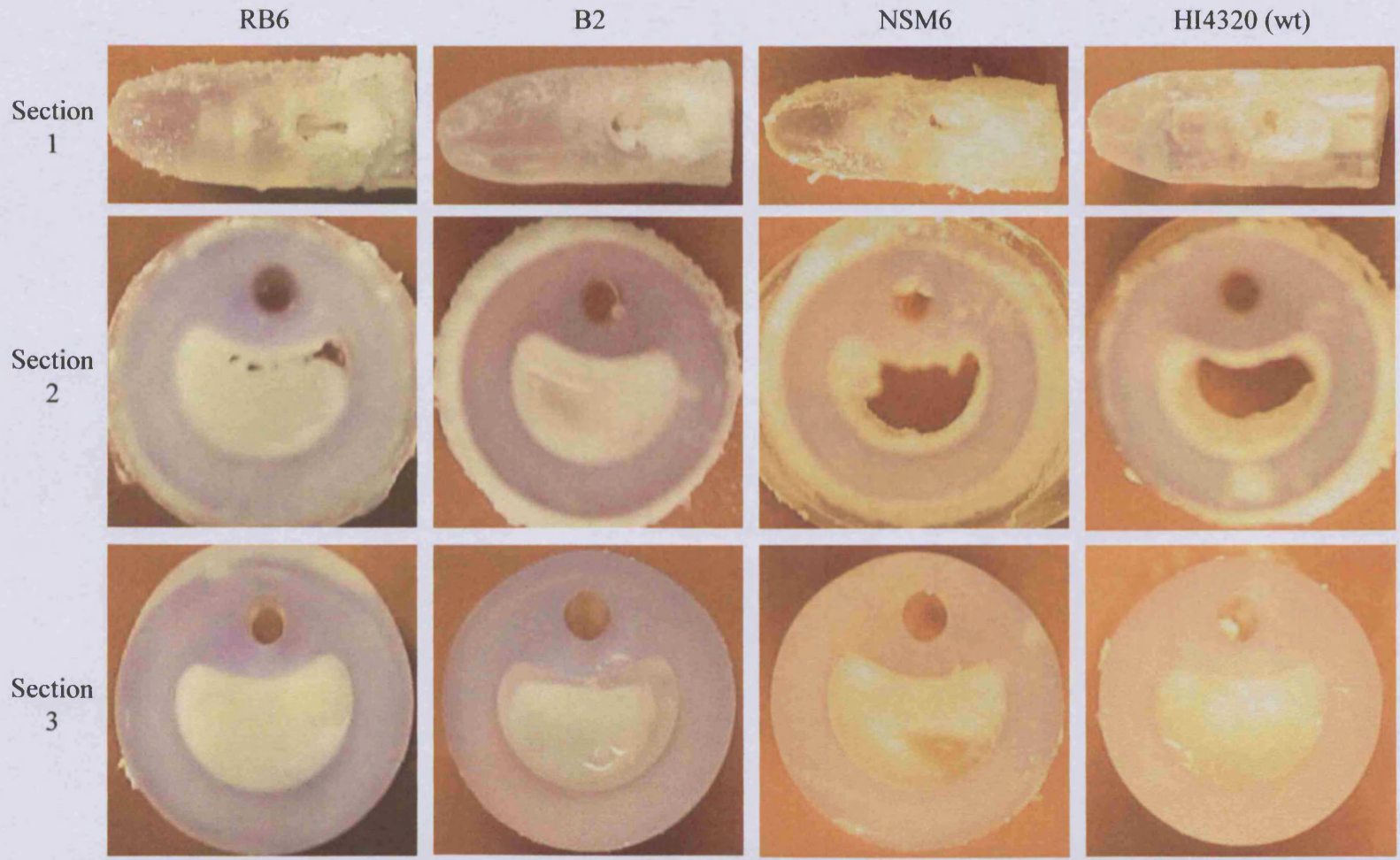


Figure 3.13: Comparison of encrustation at catheter blockage for four *Pr. mirabilis* isolates

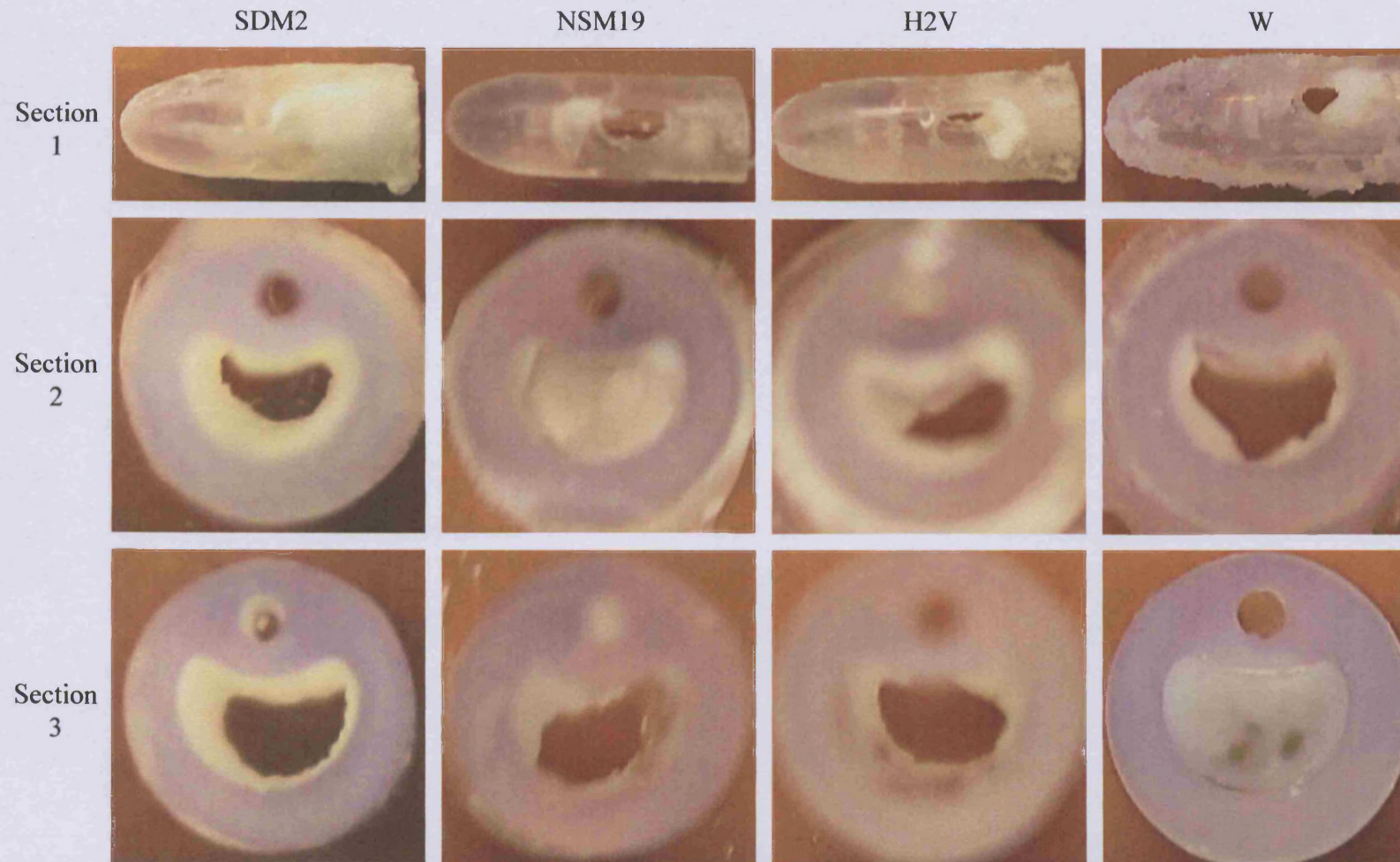


Figure 3.14: Comparison of encrustation at catheter blockage for four *Pr. vulgaris* isolates

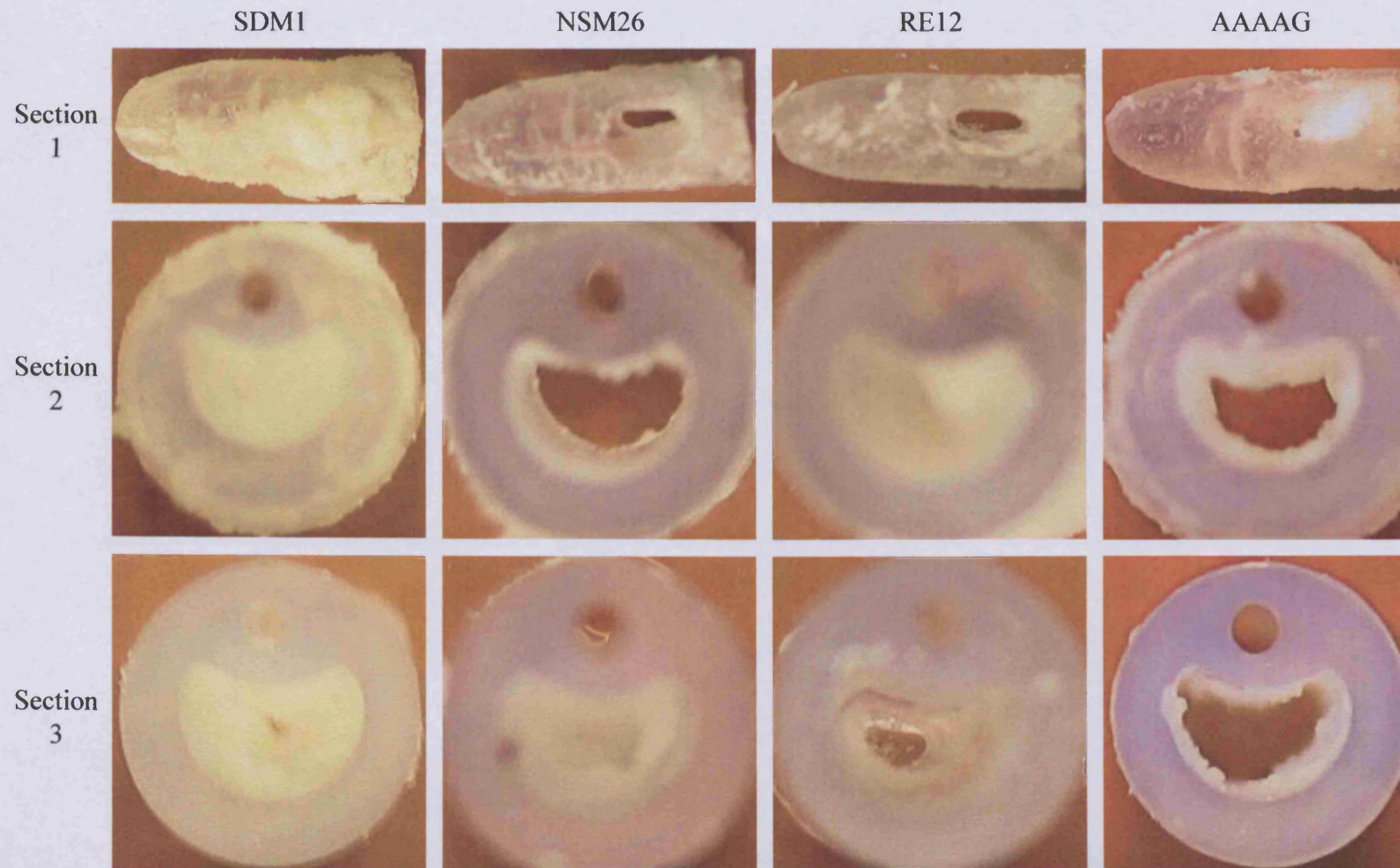


Figure 3.15: Comparison of encrustation at catheter blockage for four *Pv. rettgeri* isolates

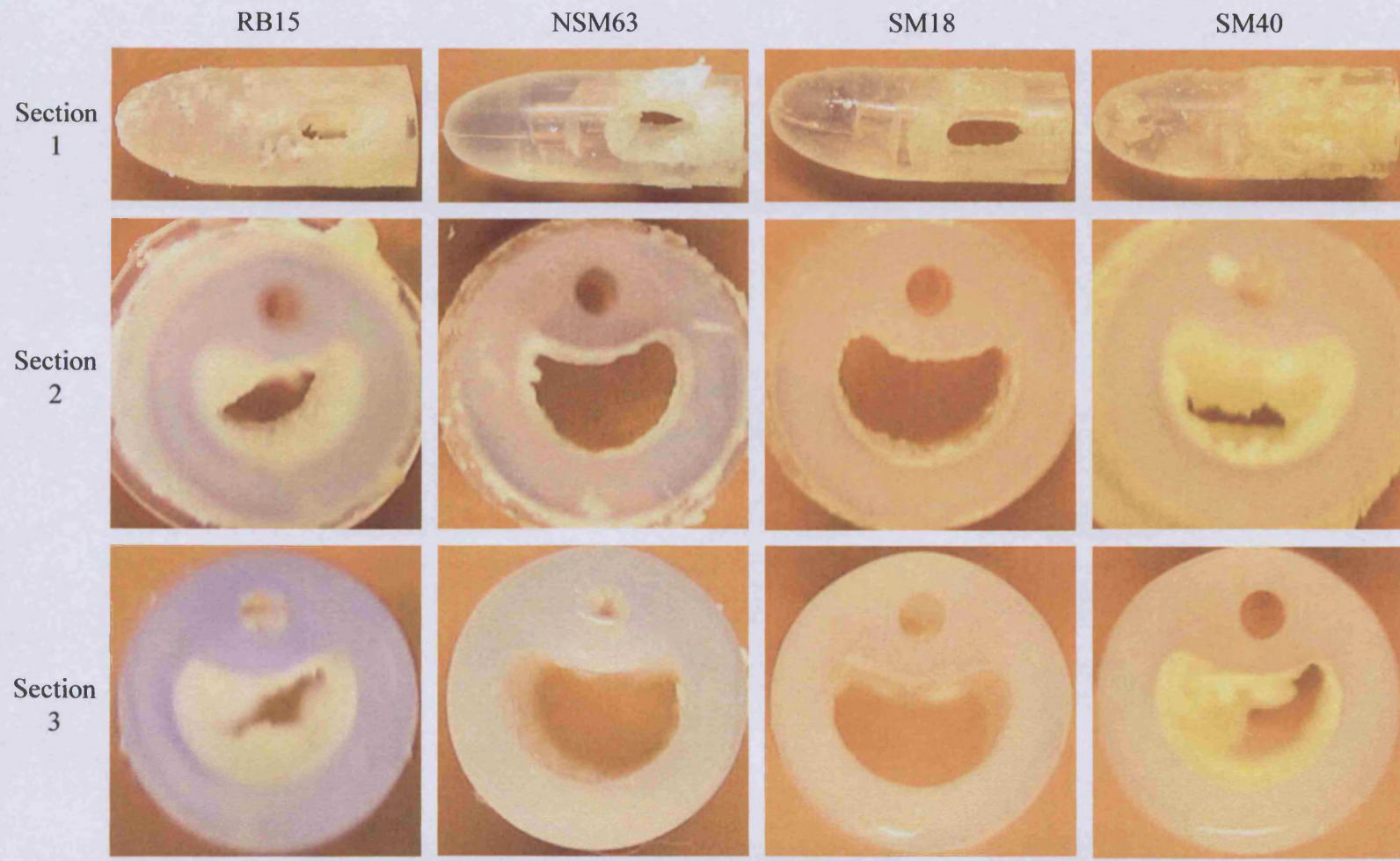


Figure 3.16: Comparison of encrustation after four days for four *M. morganii* isolates

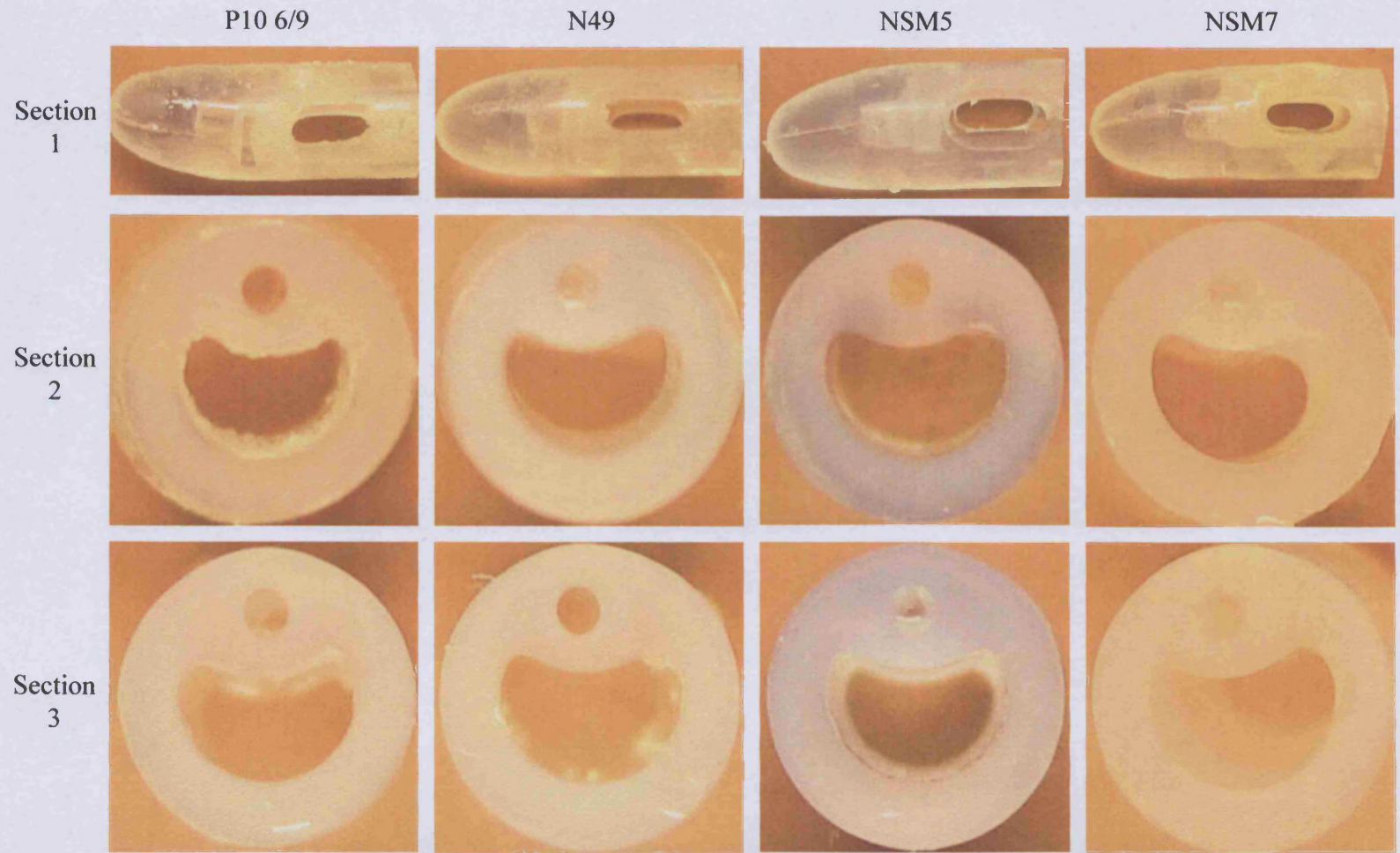


Figure 3.17: Comparison of encrustation after four days for four *Staph. aureus* isolates

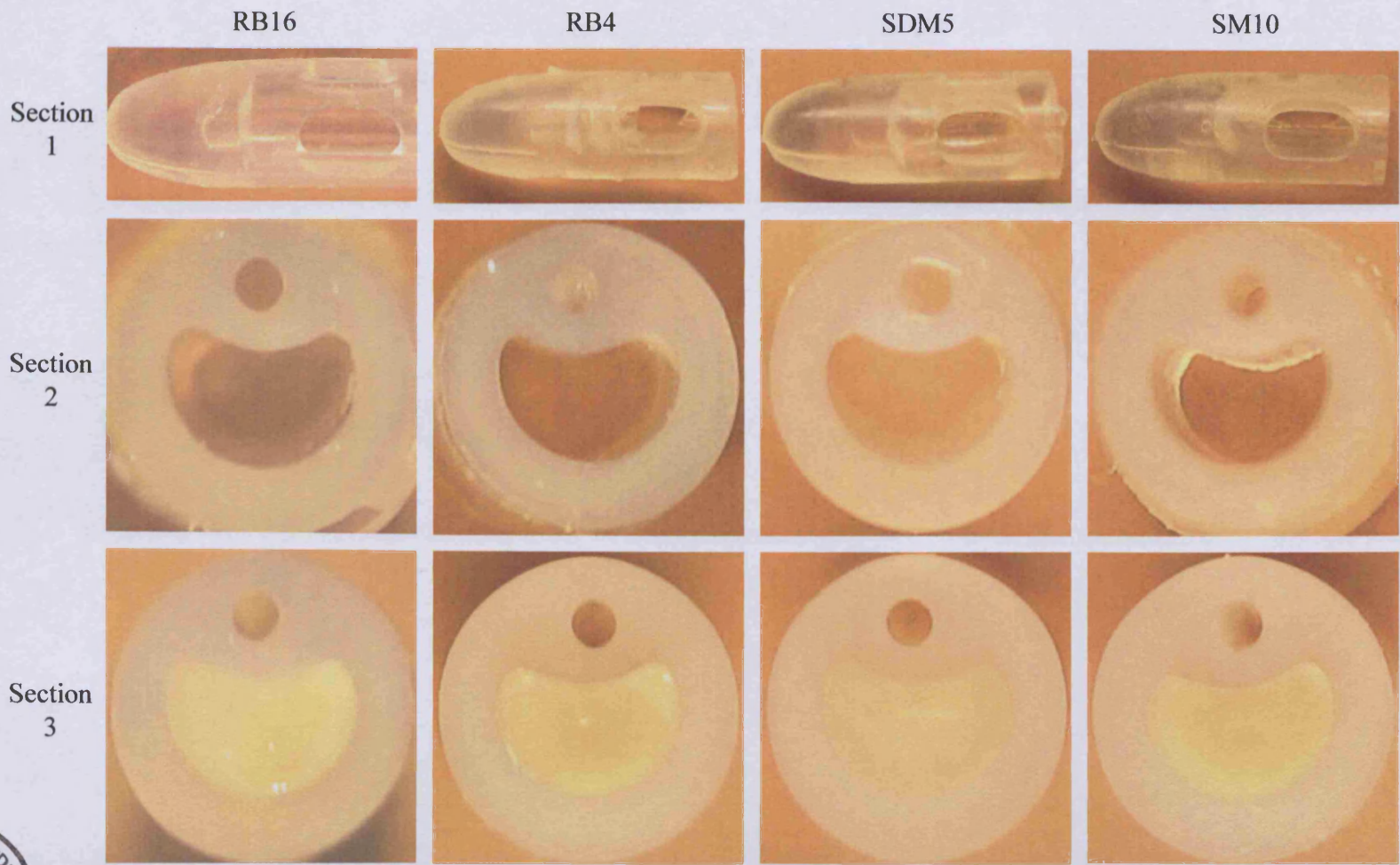


Figure 3.18: Comparison of biofilm formation after four days for four *Ps. aeruginosa* isolates



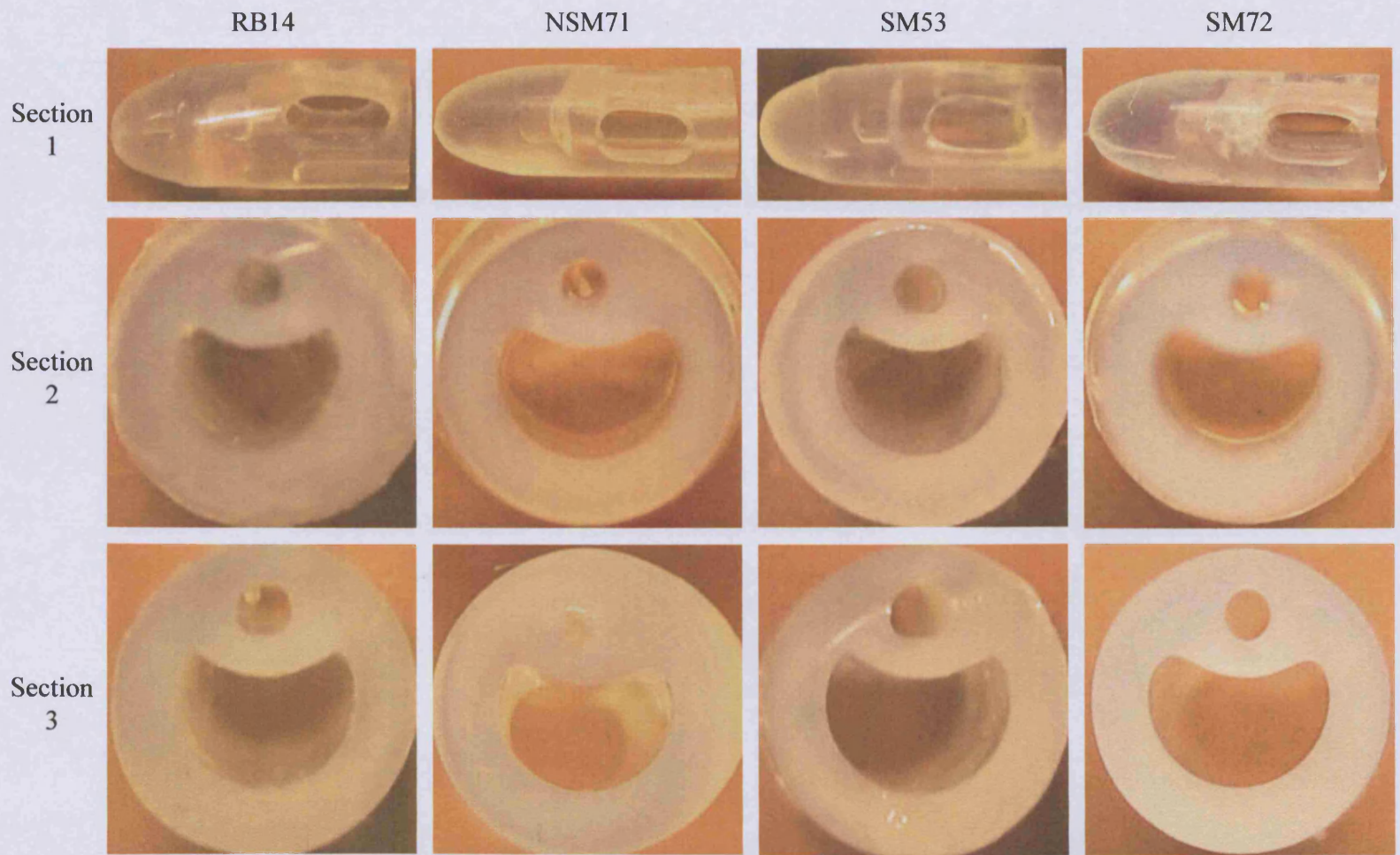


Figure 3.19: Comparison of biofilm formation after four days for four *Pv. stuartii* isolates

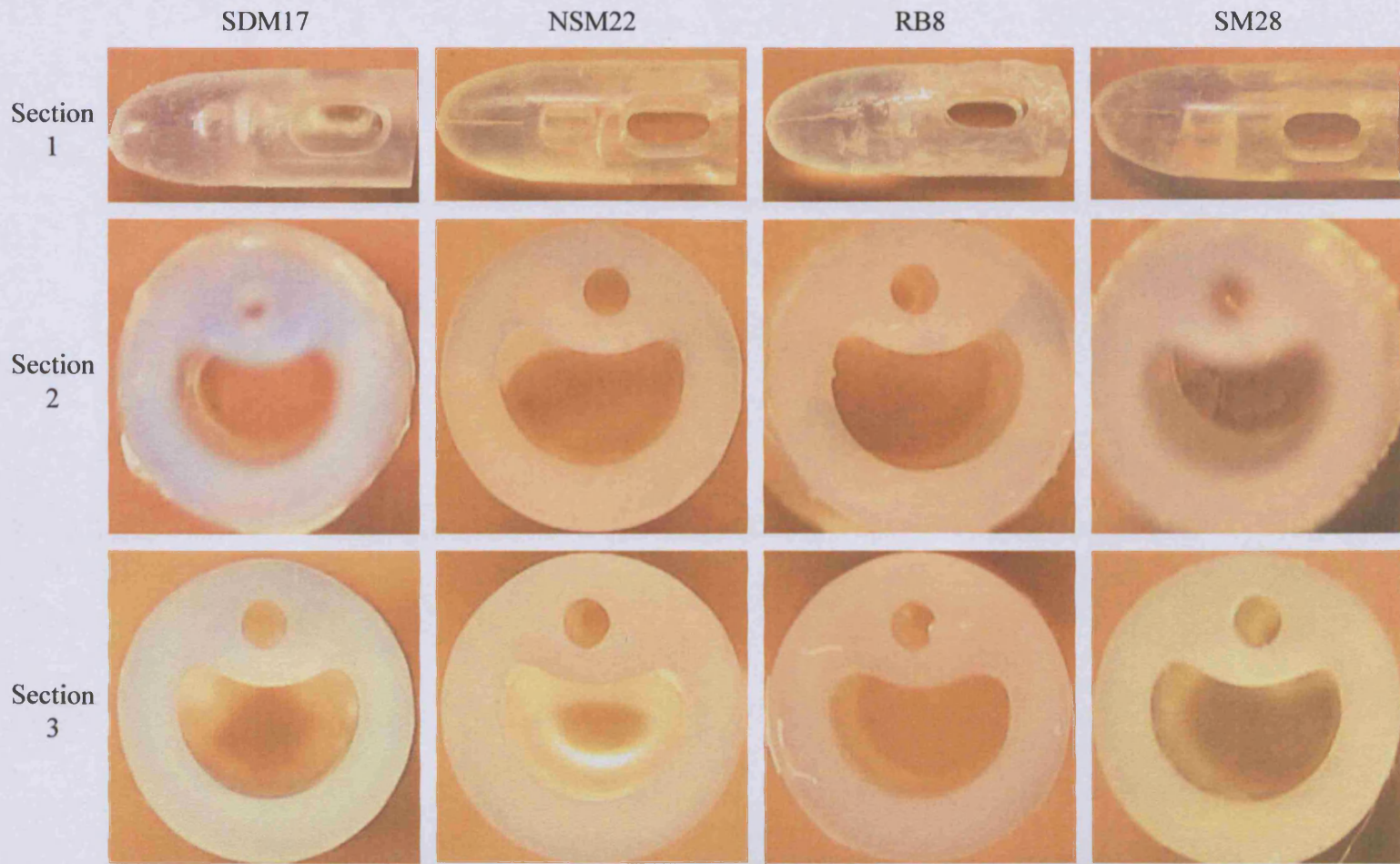


Figure 3.20: Comparison of biofilm formation after four days for four *K. pneumoniae* isolates

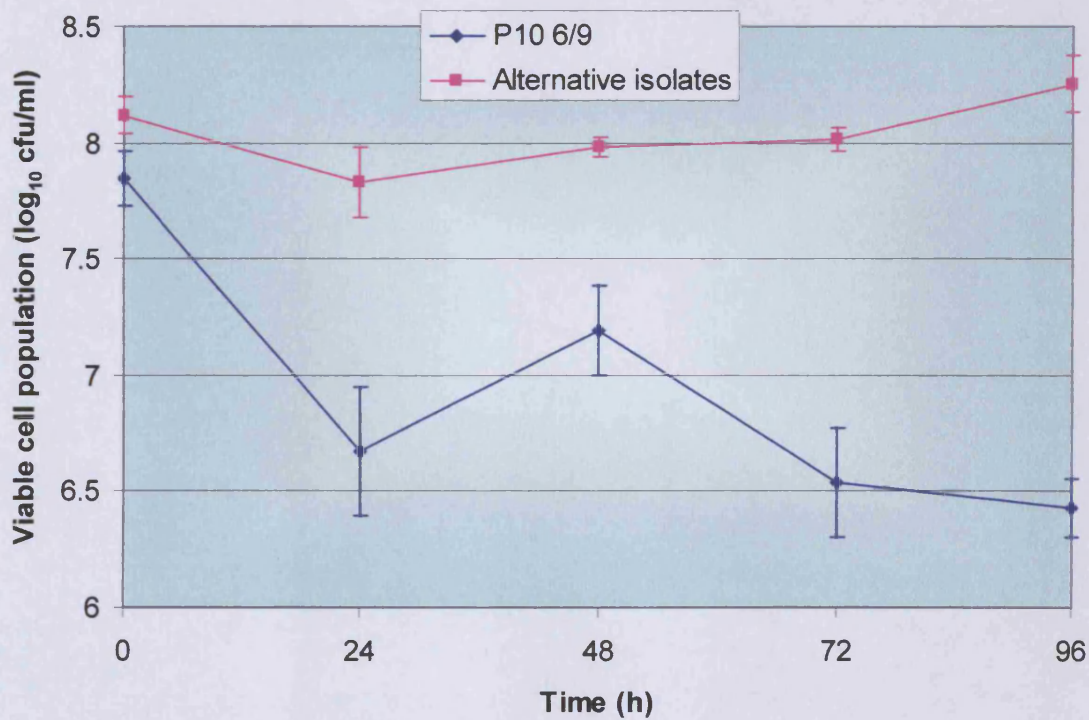
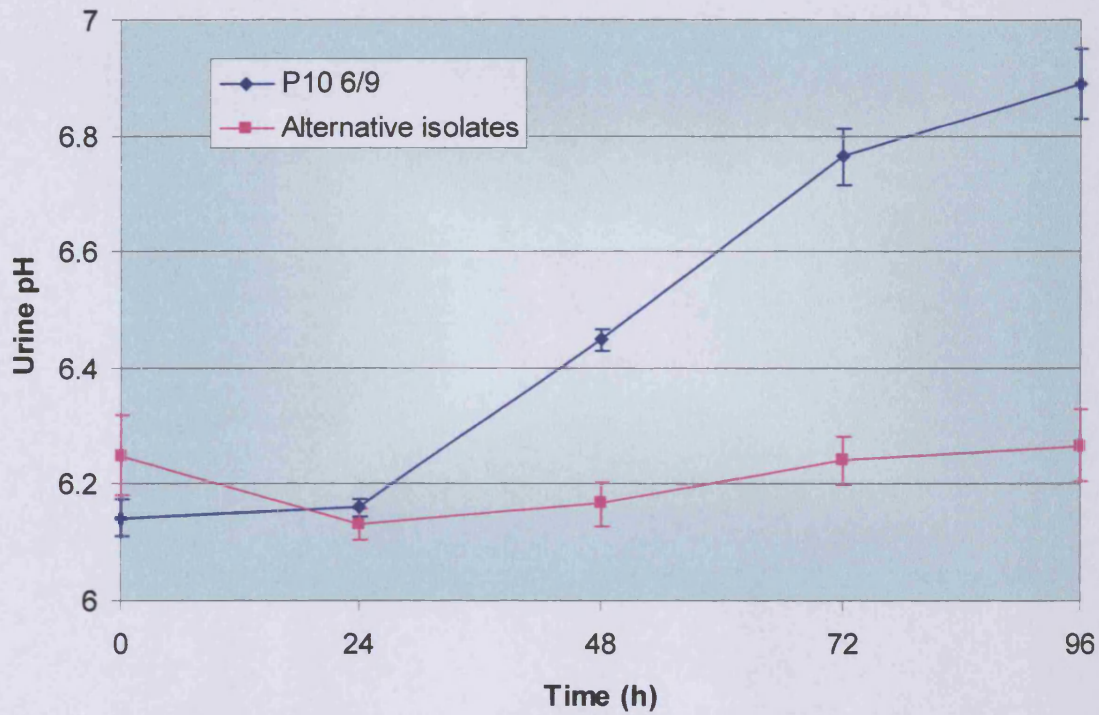


Figure 3.21: Variation in urine pH and viable cell numbers vs. time for *Staph. aureus* (P10 6/9) and three alternative urease producing *Staph. aureus* isolates (NSM5, NSM7, N49) in the bladder model over four days. Experiments using the alternative isolates were performed once for each isolate and the data combined. Statistical analysis indicates that the means of viable cell numbers and urinary pH for *Staph. aureus* P10 6/9 at 96 h are significantly different ($P < 0.001$) than those of the alternative isolates.

been isolated from time to time from catheter associated UTIs. Nine isolates of five such species (*Ser. marcescens*, *K. oxytoca*, *Enterobacter cloacae*, *C. freundii* and *C. koseri*) were also tested in the bladder model for their ability to produce crystalline biofilms.

The results obtained with three isolates of *Ser. marcescens* are presented in Figure 3.22. There was little evidence of biofilm on the catheter sections (Figure 3.22a). The urinary pH remained stable at approximately 6.2 over 96 h, while the numbers of bacteria also remained stable at just above 10^8 cfu/ml (Figure 3.22b). This was also true for the other isolates tested (*K. oxytoca*, *Enterobacter cloacae*, *C. freundii* and *C. koseri*), with the urinary pH remaining stable at approximately 6.2 and the bacterial numbers remaining stable at just above 10^8 cfu/ml (Figures 3.23 – 3.25).

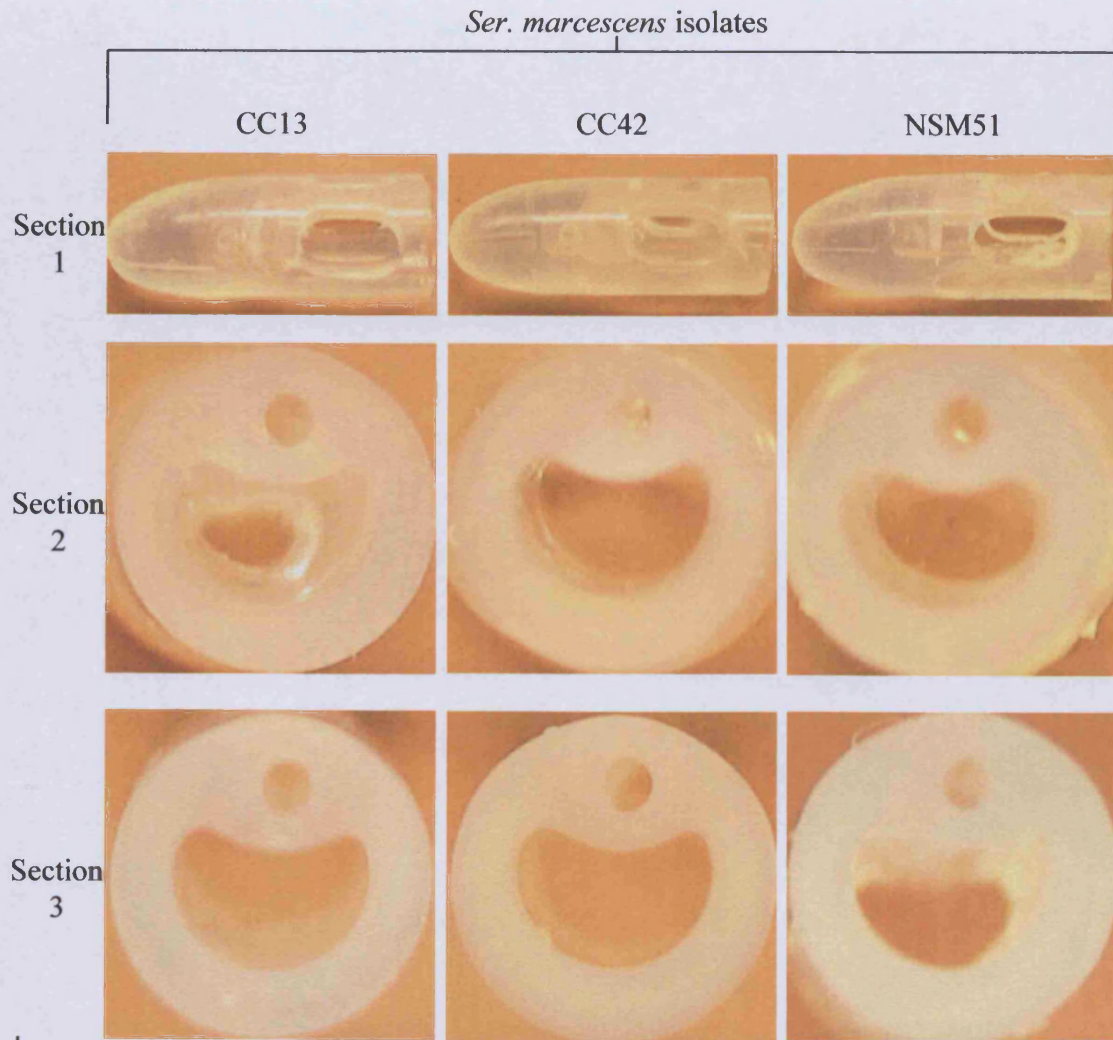


Figure 3.22a: Sections of catheter removed from *Ser. marcescens* infected bladder models after four days.

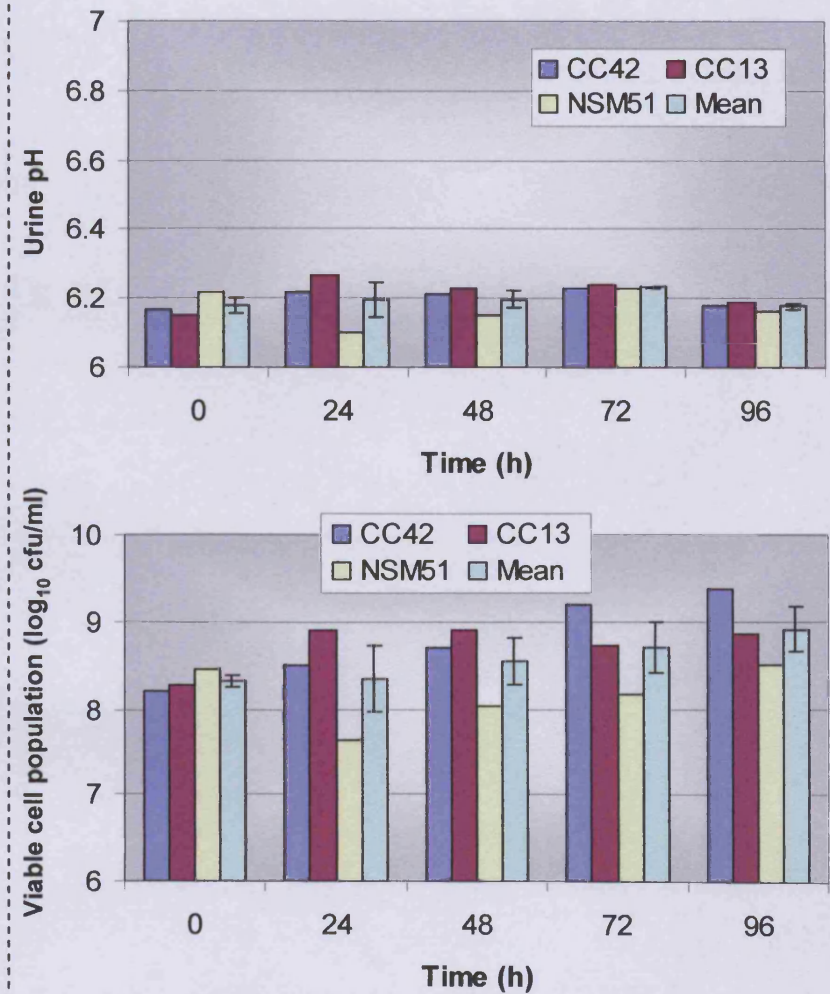


Figure 3.22b: The pHs and viable cell populations of the residual urine over a 96 h test period in bladder models inoculated with *Ser. marcescens* isolates. Experiments were performed once for each isolate. The mean values from the three isolates combined (\pm SE) is also shown.

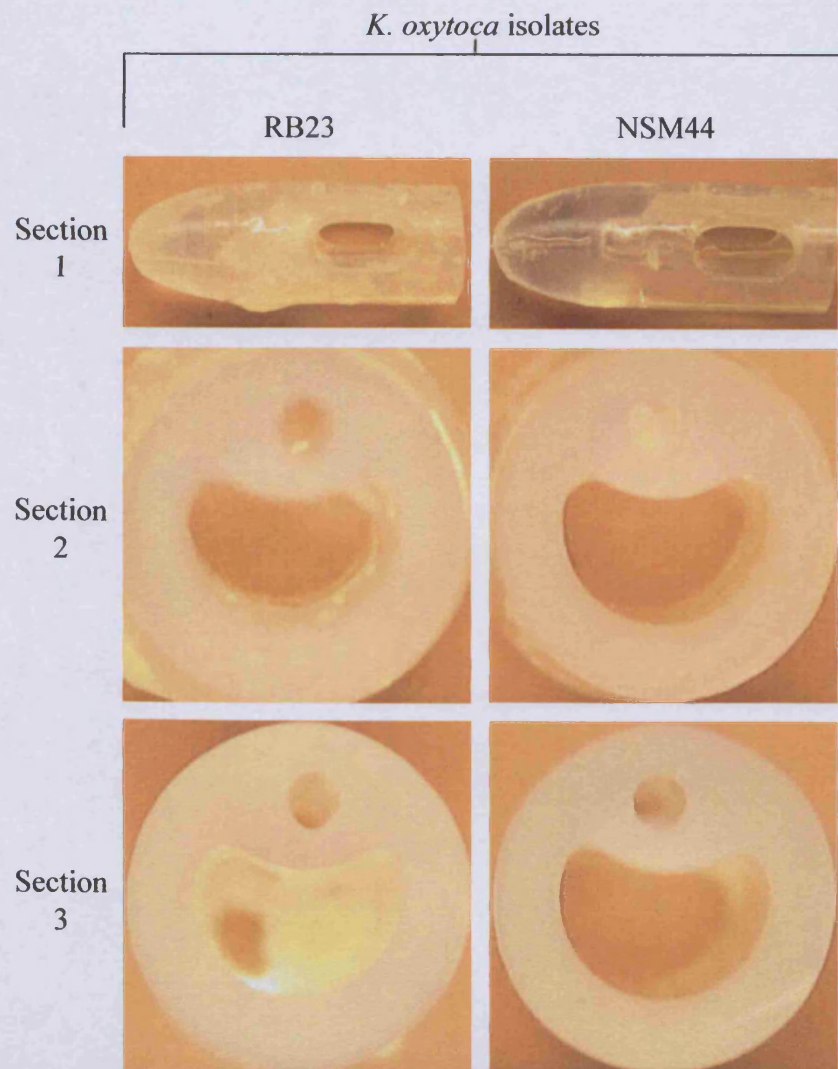


Figure 3.23a: Sections of catheter removed from *K. oxytoca* infected bladder models after four days.

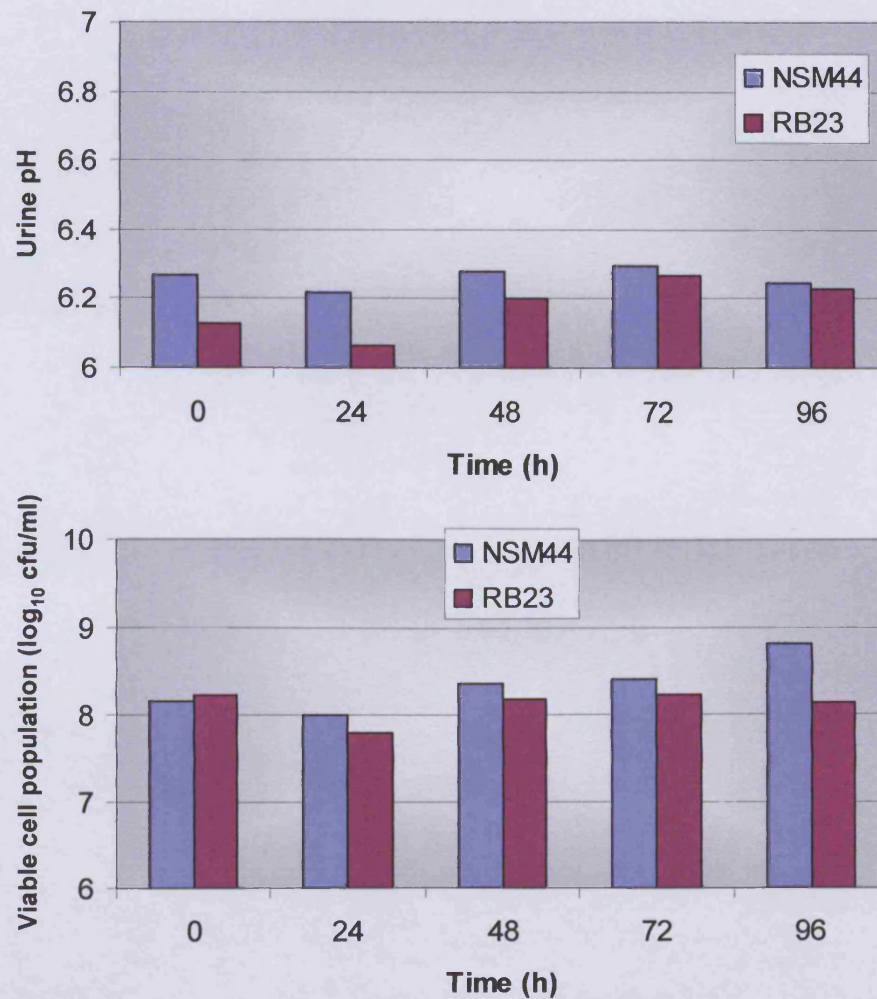


Figure 3.23b: The pHs and viable cell populations of the residual urine over a 96 h test period in bladder models inoculated with *K. oxytoca* isolates. Experiments were performed once for each isolate.

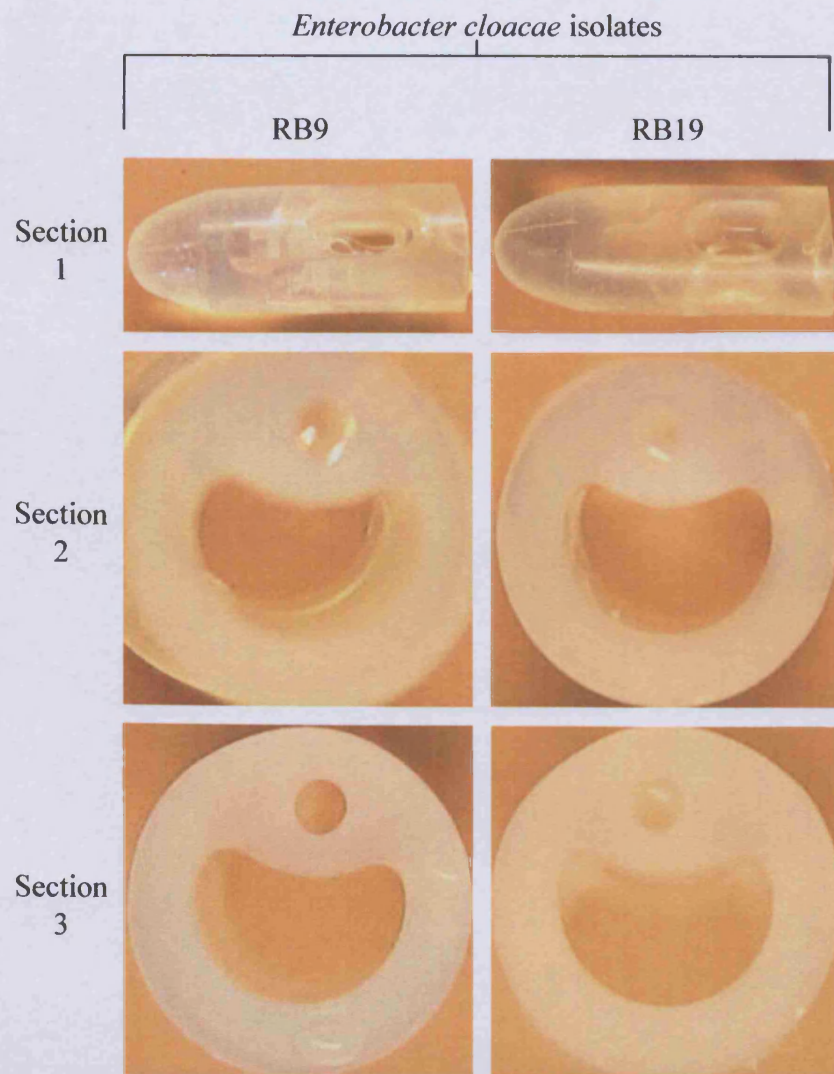


Figure 3.24a: Sections of catheter removed from *Enterobacter cloacae* infected bladder models after four days.

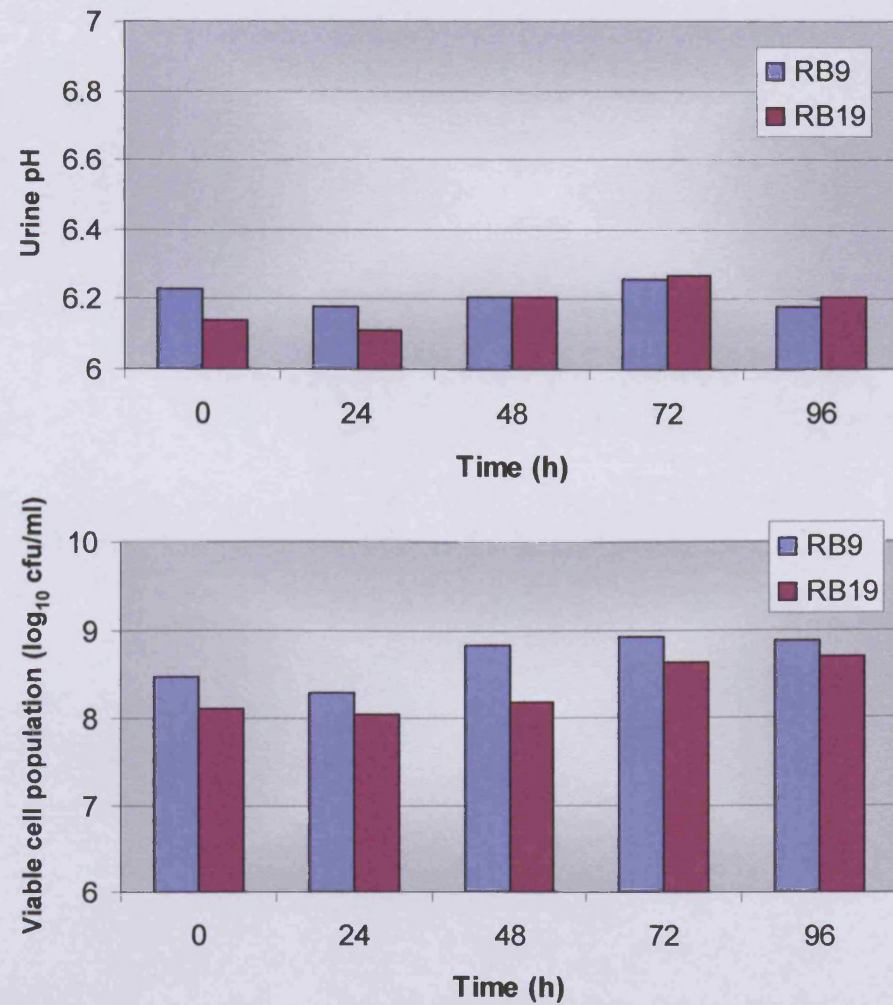


Figure 3.24b: The pHs and viable cell populations of the residual urine over a 96 h test period in bladder models inoculated with *Enterobacter cloacae* isolates. Experiments were performed once for each isolate.

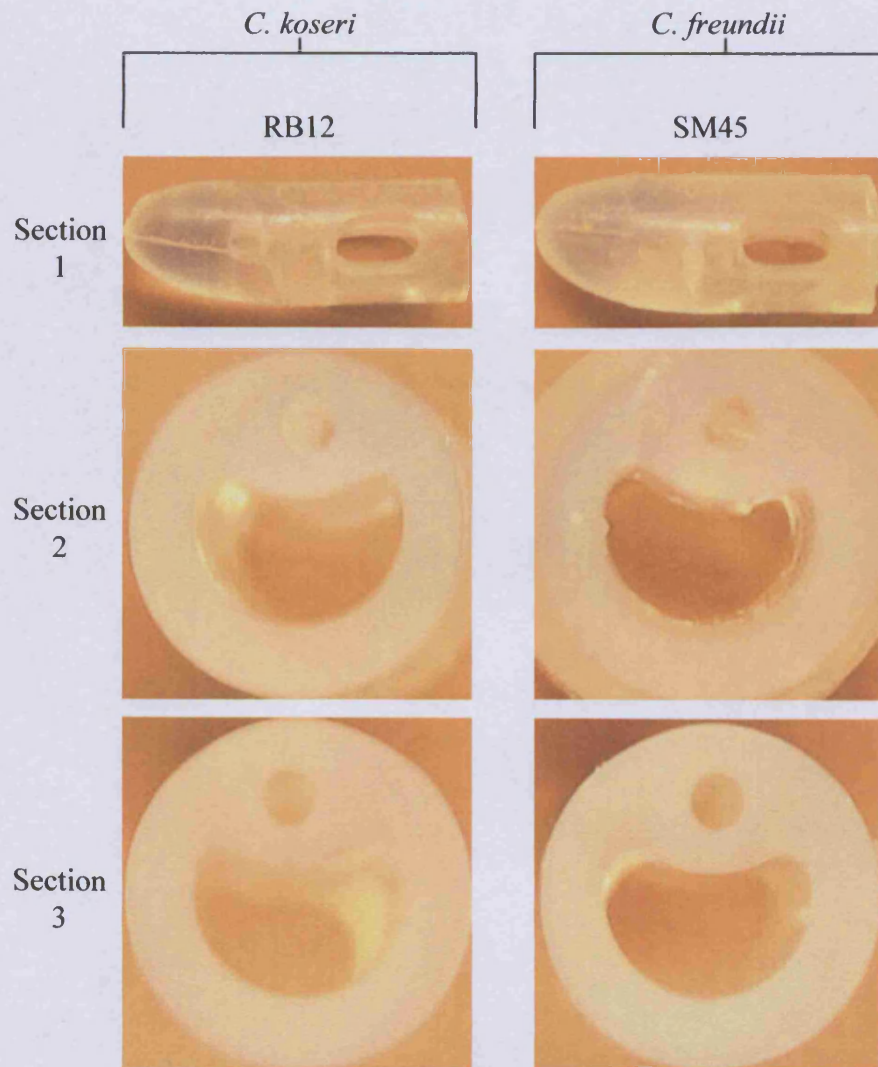


Figure 3.25a: Sections of catheter removed from *C. freundii* (SM45) and *C. koseri* (RB12) infected bladder models after four days.

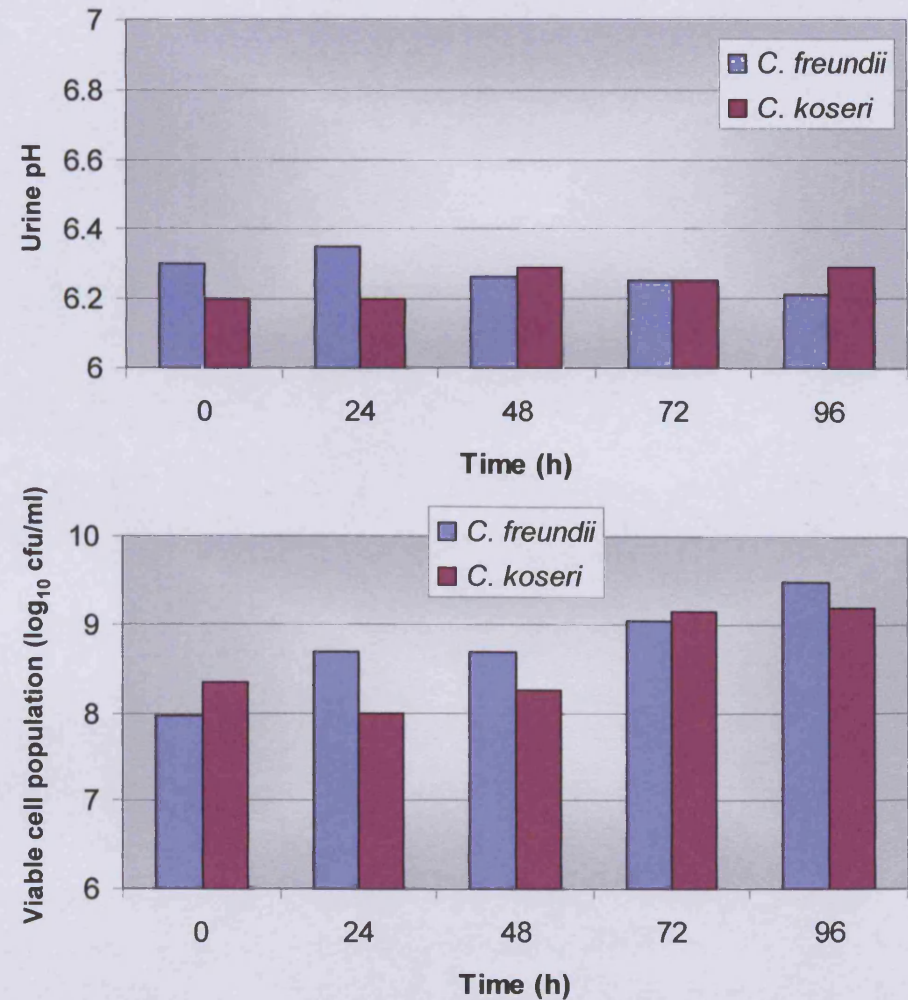


Figure 3.25b: The pHs and viable cell populations of the residual urine over a 96 h test period in bladder models inoculated with *C. freundii* (SM45) and *C. koseri* (RB12). Experiments were performed once for each isolate.

3.2 The development of crystalline biofilm on silver-hydrogel and nitrofurazone catheters.

Antimicrobial catheters (silver-hydrogel coated latex (Bard IC catheter) and the nitrofurazone impregnated all-silicone catheter (Rochester Medical NF catheter)) have recently been introduced into clinical practice. In view of the controversy surrounding their efficacy in preventing catheter-associated UTI (Trautner *et al.*, 2005a; Johnson *et al.*, 2006) it was decided to examine their ability to resist encrustation by crystalline biofilm. Experiments were performed in the bladder models with five of the urease producing species that had been shown to be capable of forming crystalline biofilm in urine (Section 3.1).

A summary of the results indicating the mean times catheters took to block, calculated from three replicate experiments, is presented in Table 3.3. The experiments were conducted as stated in Section 3.1.1, until 168 h or blockage, with an all-silicone catheter inoculated as a control. The results show that the three most active urease producers *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* blocked the silicone control, the silver and the nitrofurazone catheters within mean times of 30 h. There was no significant difference in mean times to blockage for these species for the silicone and nitrofurazone catheters ($P > 0.05$). *Pr. mirabilis* and *Pv. rettgeri* however blocked the silver catheters significantly more rapidly than *Pr. vulgaris* ($P < 0.05$). *M. morgani* took significantly longer to block the silicone and nitrofurazone catheters, 149 and 131 h respectively, ($P < 0.05$) and failed to block the silver catheters within the 168 h experimental period. All three types of catheter drained freely for 168 h in models infected with *Staph. aureus*.

Catheter type	Mean blockage time (h) (\pm SE)					Statistical analysis of blockage times (ANOVA)
	<i>Pr. mirabilis</i> (RB6)	<i>Pr. vulgaris</i> (SDM2)	<i>Pv. rettgeri</i> (SDM1)	<i>M. morgani</i> (RB15)	<i>Staph. aureus</i> (P10 6/9)	
All-silicone	20.0 (\pm 2.6)	25.0 (\pm 2.0)	26.7 (\pm 7.9)	149 (\pm 13.0)	Did not block after 168 h	HSD ($P < 0.001$)
Silver-hydrogel coated latex	15.7 (\pm 2.0)	23.7 (\pm 1.3)	15.3 (\pm 0.9)	Did not block after 168 h	Did not block after 168 h	SD ($P = 0.012$)
Nitrofurazone	26.7 (\pm 3.2)	27.0 (\pm 2.5)	29.7 (\pm 6.1)	131.3 (\pm 12.2)	Did not block after 168 h	HSD ($P < 0.001$)
Statistical analysis of blockage times (ANOVA)	NSD ($P = 0.068$)	NSD ($P = 0.444$)	NSD ($P = 0.256$)	NSD ($P = 0.379$) (between all-silicone and nitrofurazone)	-	

Table 3.3: Blockage times (\pm SE) for five urease producing species on all-silicone, silver-hydrogel and nitrofurazone catheters recorded in triplicate experiments. Statistical analysis was carried out on the blockage times between the different species on each catheter type and for each species on the three catheter types

*NSD: No significant difference; SD: Significant difference; HSD: Highly significant difference; - indicates no statistical analysis attempted

Figure 3.26 shows the variation in urinary pH for the five test species during the experimental period for each of the catheters tested. For all-silicone catheters (Figure 3.26a) there was no significant difference ($P>0.05$) between the mean final urinary pHs of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*, while all three species were significantly different ($P<0.05$) from the mean final urinary pHs of *M. morgani* and *Staph. aureus*. There was no significant difference ($P>0.05$) between the mean final urinary pHs of *M. morgani* and *Staph. aureus*. The silver-hydrogel catheters (Figure 3.26b) showed a similar pattern, with no significant difference ($P>0.05$) between the mean final urinary pHs of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*, while all three species were significantly different ($P<0.05$) from the mean final urinary pH of *Staph. aureus*. The mean final urinary pH of *M. morgani* was not significantly different ($P>0.05$) from that of *Pr. vulgaris* and *Pv. rettgeri* but was significantly different ($P<0.05$) from those of *Pr. mirabilis* and *Staph. aureus*. For nitrofurazone catheters (Figure 3.26c) there was no significant difference ($P>0.05$) between the mean final urinary pHs of *Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri* and *M. morgani* while all four species were significantly different ($P<0.05$) from the mean final urinary pH of *Staph. aureus* (Figure 3.26c).

The mean urinary viable cell counts are presented in Figure 3.27. Each of the species shows a consistent pattern irrespective of the catheter type being tested. Both *Proteus* species show a similar pattern, with an initial cfu/ml of just above 10^8 , falling slightly at blockage. For models inoculated with *Pv. rettgeri* the cell numbers present remained stable at approximately 10^7 cfu/ml for the duration of the experiment. *M. morgani* numbers fell in the first 24 h from an initial cfu/ml of above 10^8 to below 10^7 , before recovering steadily back to around 10^8 cfu/ml. The numbers of *Staph.*

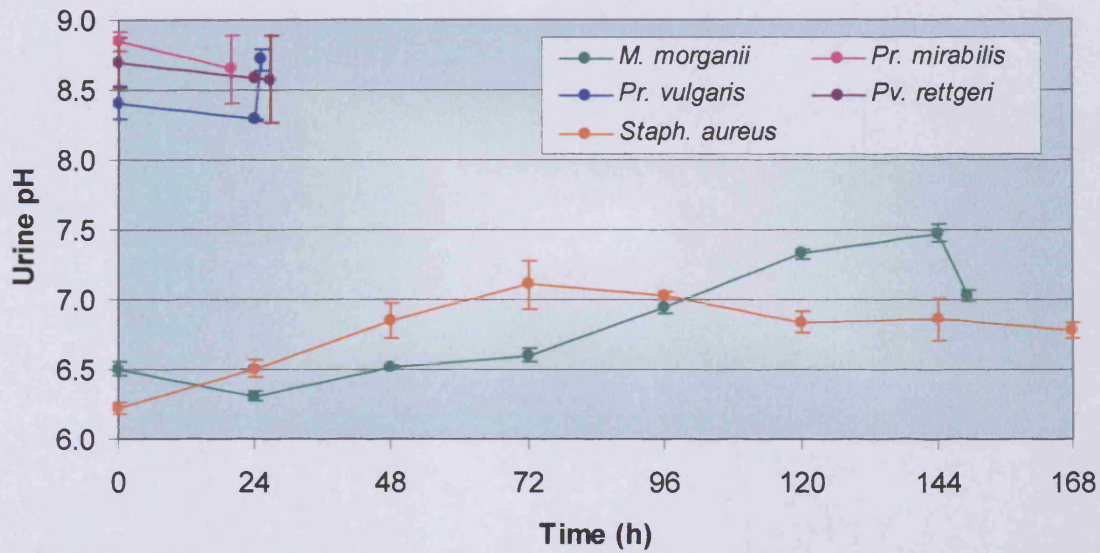


Figure 3.26a: Variation in urinary pH vs. time for five urease producing species until blockage or 168 h on all-silicone catheters.

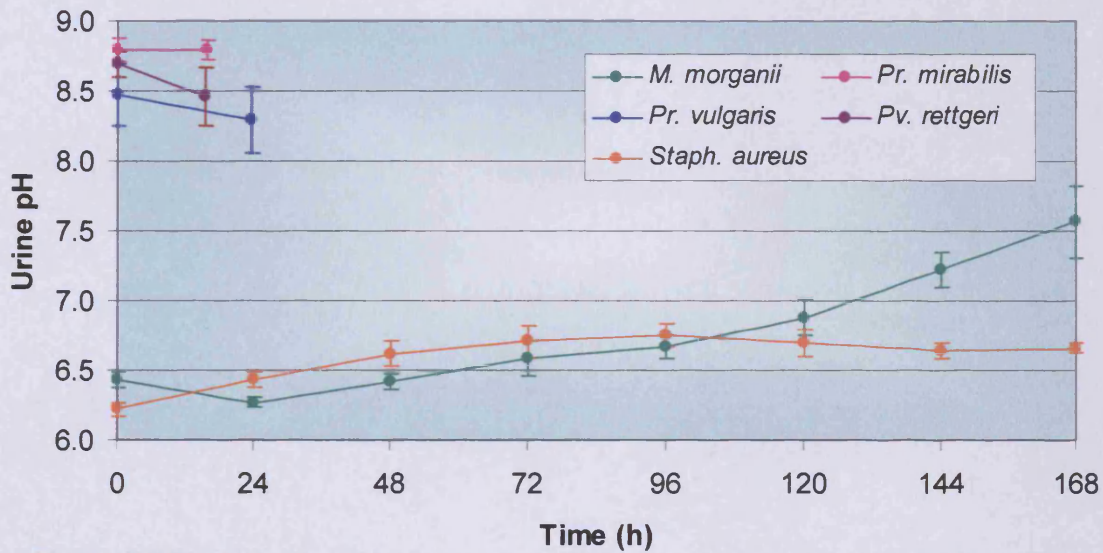


Figure 3.26b: Variation in urinary pH vs. time for five urease producing species until blockage or 168 h on silver-hydrogel coated latex catheters.

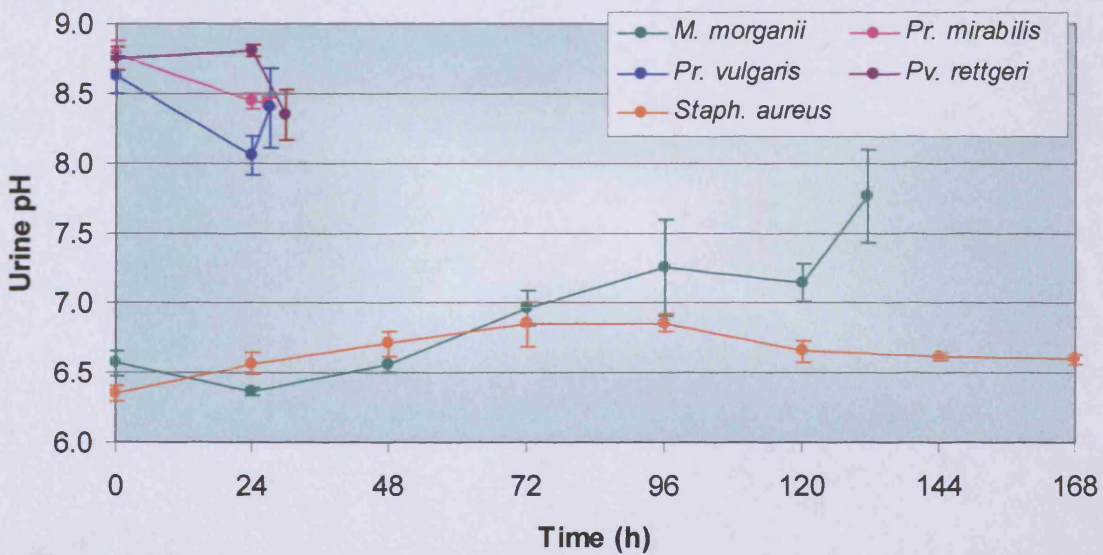


Figure 3.26c: Variation in urinary pH vs. time for five urease producing species until blockage or 168 h on nitrofurazone impregnated all-silicone catheters.

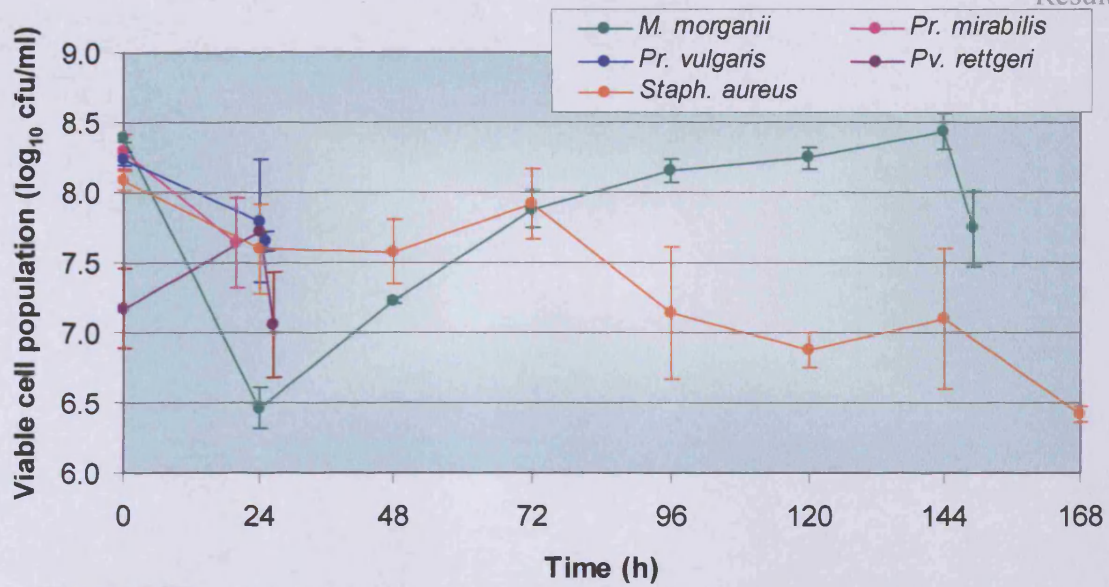


Figure 3.27a: Variation in viable cell numbers vs. time for five urease producing species until blockage or 168 h on all-silicone catheters.

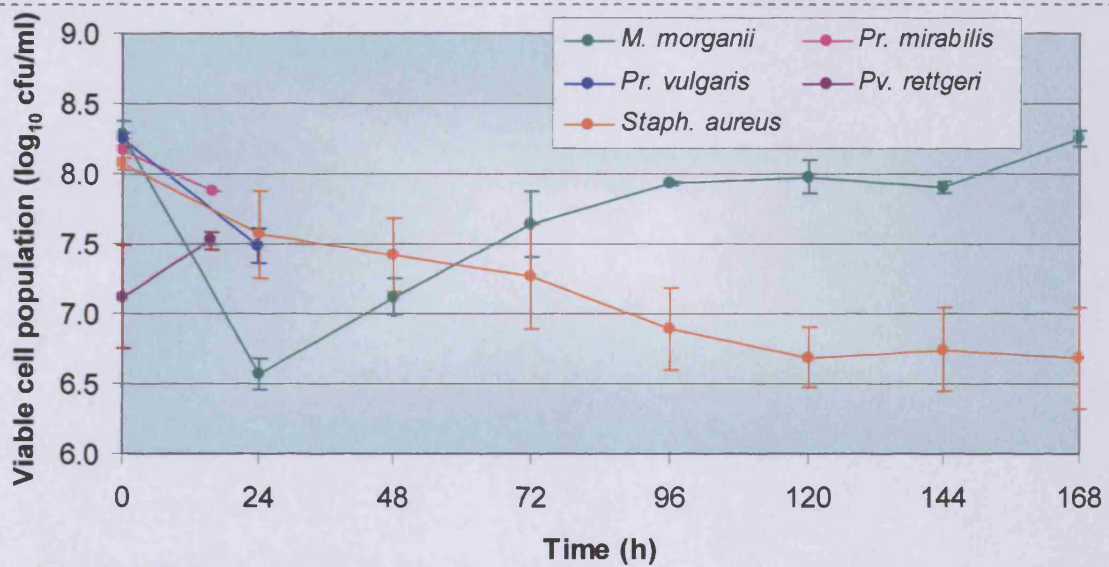


Figure 3.27b: Variation in viable cell numbers vs. time for five urease producing species until blockage or 168 h on silver-hydrogel coated latex catheters.

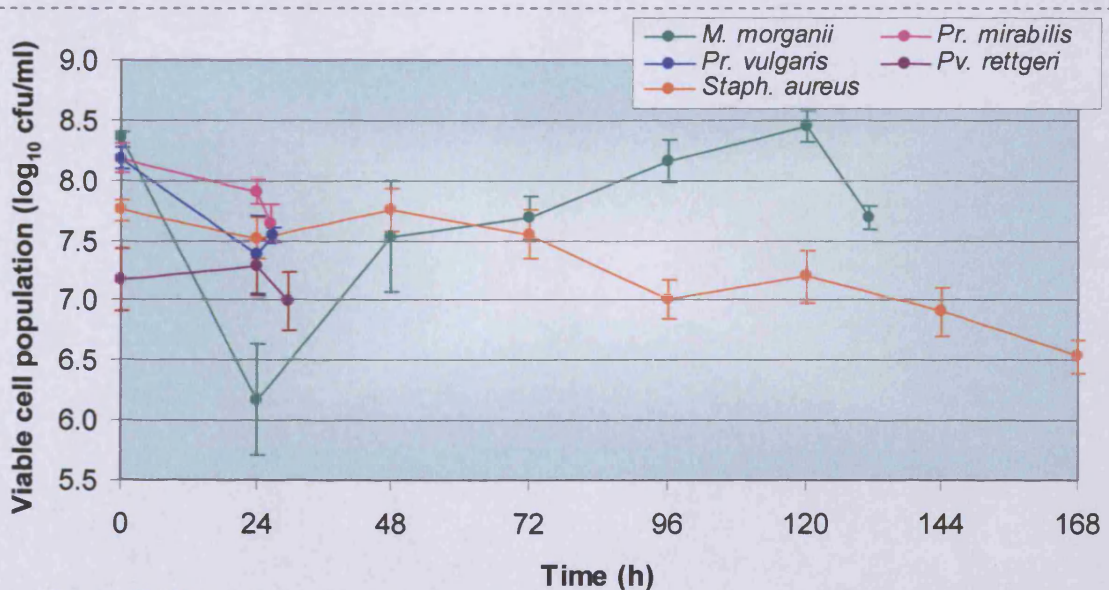


Figure 3.27c: Variation in viable cell numbers vs. time for five urease producing species until blockage or 168 h on nitrofurazone impregnated all-silicone catheters.

aureus cells present show a steady fall from around 10^8 cfu/ml initially to below 10^7 cfu/ml after 168 h.

Digital photography, LV-SEM and HV-SEM were used to visualise the biofilms present on catheters at blockage or after 168 h. The images of all-silicone catheters were similar to those present in Section 3.1.

3.2.1 Visualisation of the encrustation present on silver-hydrogel catheters.

Digital photographs and LV-SEM images of silver-hydrogel catheters removed from the model at blockage or after 168 h are presented in Figures 3.28 & 3.29. The catheters infected with *Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri* and *M. morgani* are heavily encrusted, in particular around and below the catheter eyehole, while very little encrustation is present on the un-blocked *Staph. aureus* catheter. HV-SEM images of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* biofilms at blockage (Figure 3.30) show that the encrustation present on the catheters was mostly composed of apatite microcrystals, with a few struvite crystals also observed in the *Pr. vulgaris* biofilm on the eyehole section. Images of the *M. morgani* biofilm (Figure 3.31) showed that while the encrustation was mostly composed of apatite microcrystals some large calcium phosphate crystals were also present. HV-SEM of the *Staph. aureus* biofilm after 168 h showed that the encrustation present on the eyehole section was mostly composed of large calcium phosphate crystals (Figure 3.31). In the catheter lumen the encrustation was mainly composed of apatite microcrystals with some large calcium phosphate crystals also present.

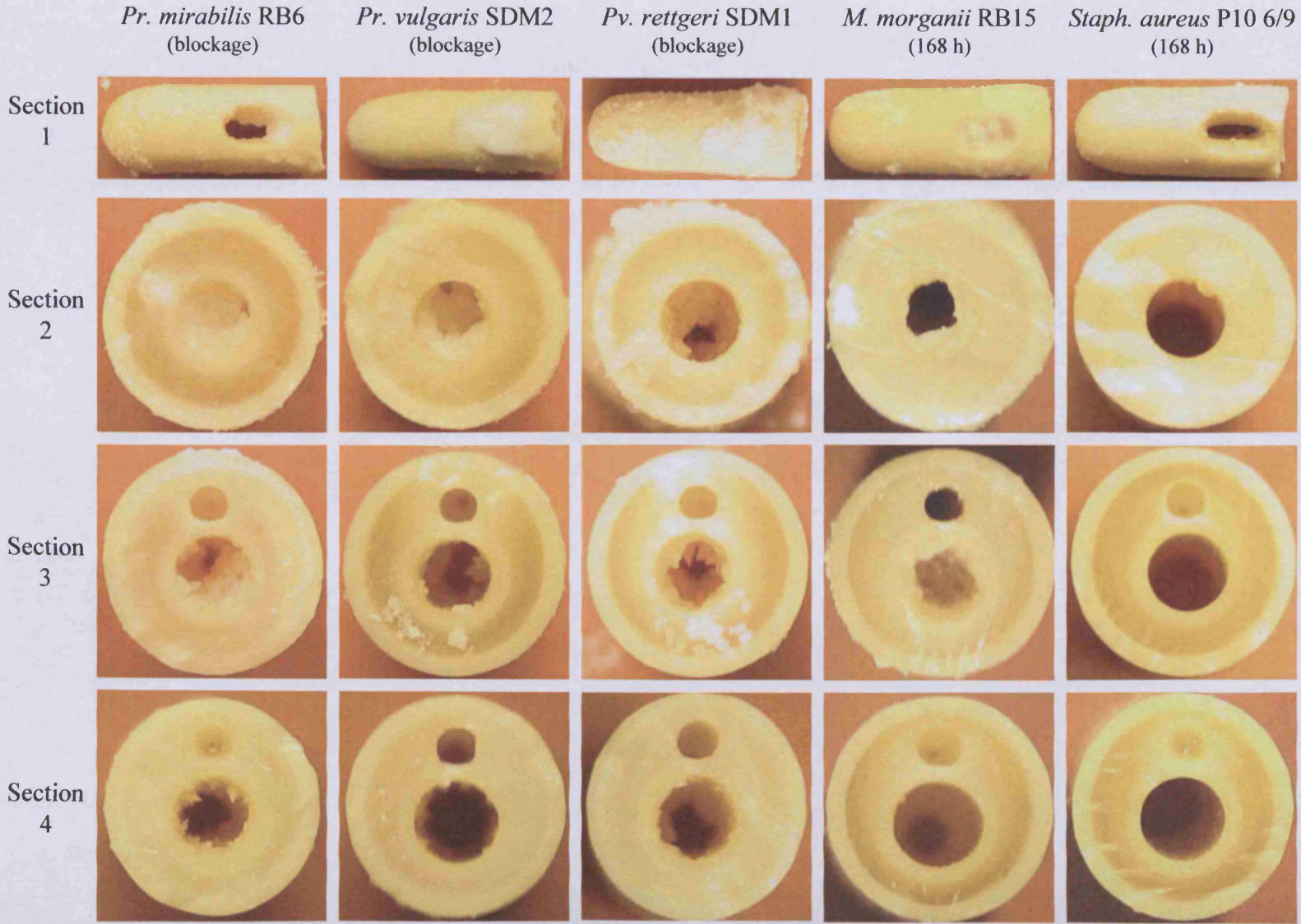


Figure 3.28: Digital camera images of silver hydrogel catheters removed from the model at blockage or after 168 h.

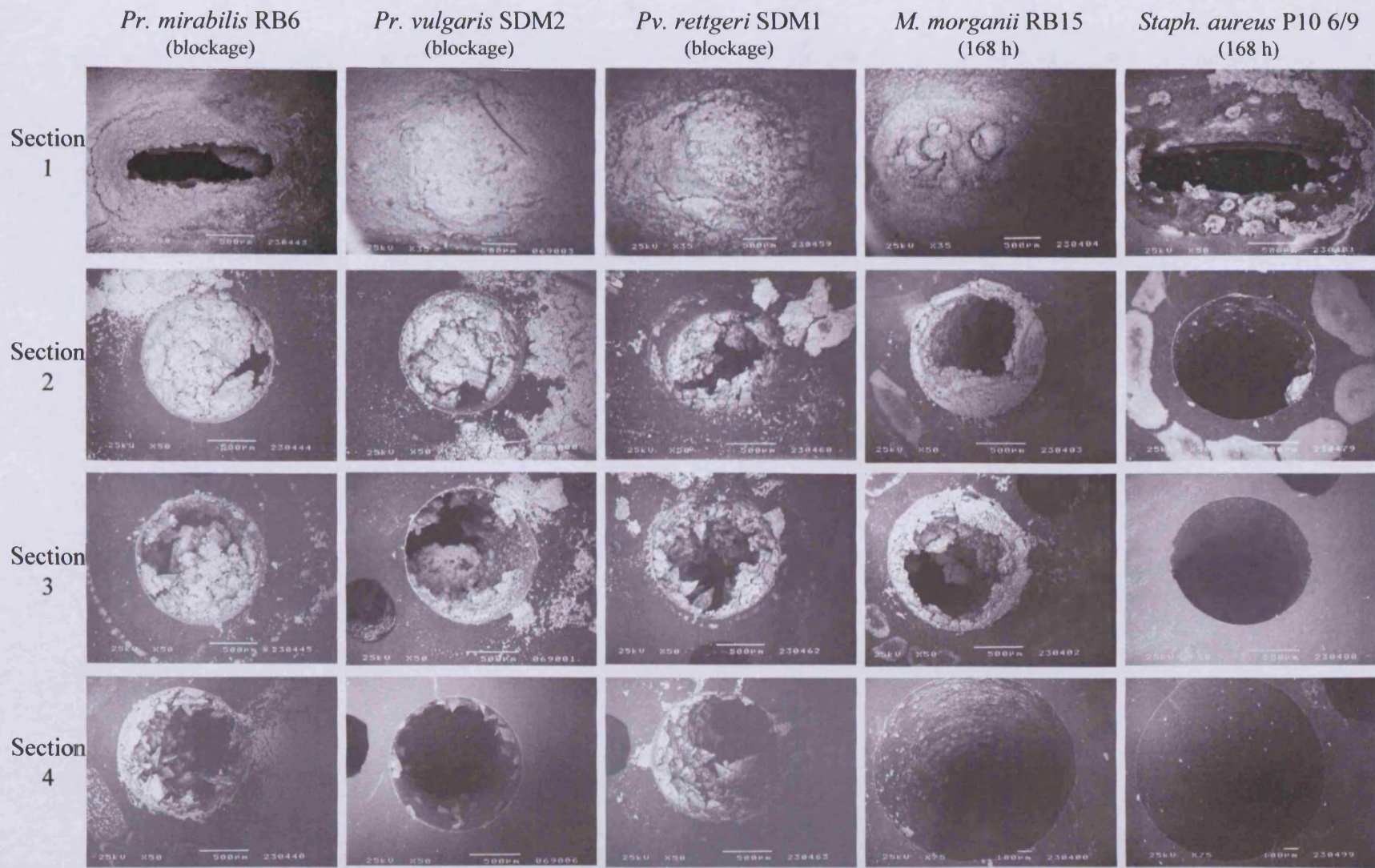
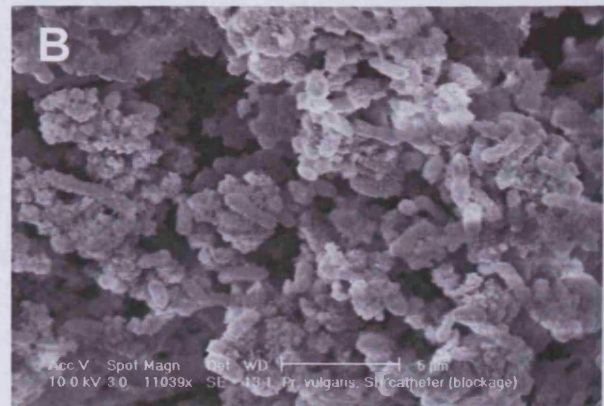
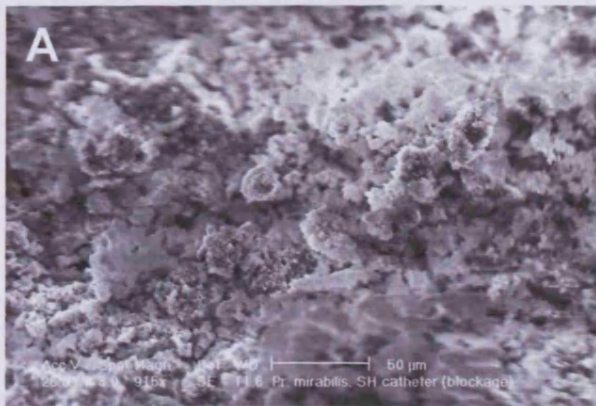
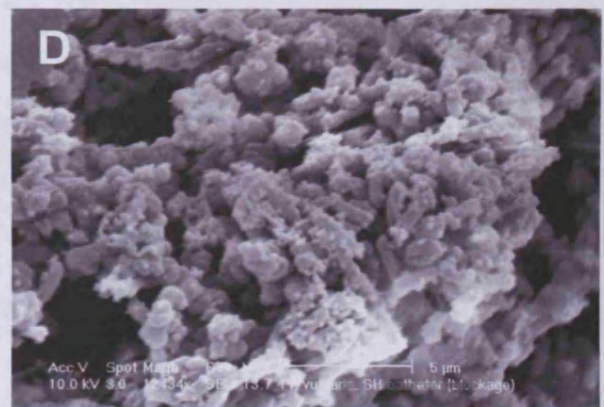


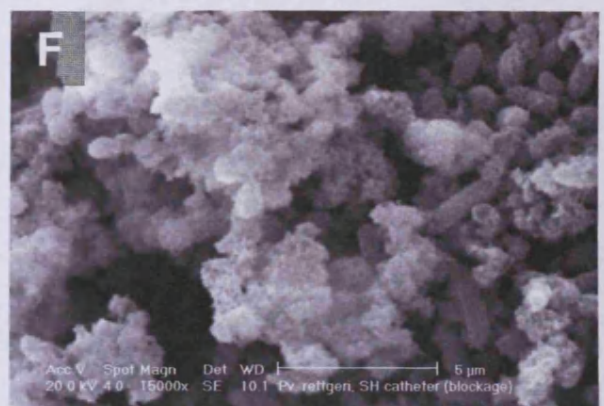
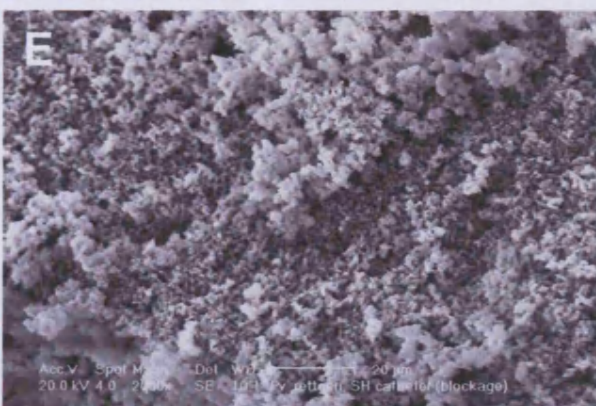
Figure 3.29: LV-SEM images of silver-hydrogel catheters removed from the model at blockage or after 168 h.



A & B: Encrusted *Pr. mirabilis* biofilm on a silver-hydrogel catheter. Biofilm heavily encrusted with apatite microcrystals is shown in A. No struvite crystals were observed in the biofilm. A close-up image of the biofilm is shown in B, showing *Pr. mirabilis* rods in association with apatite microcrystals.

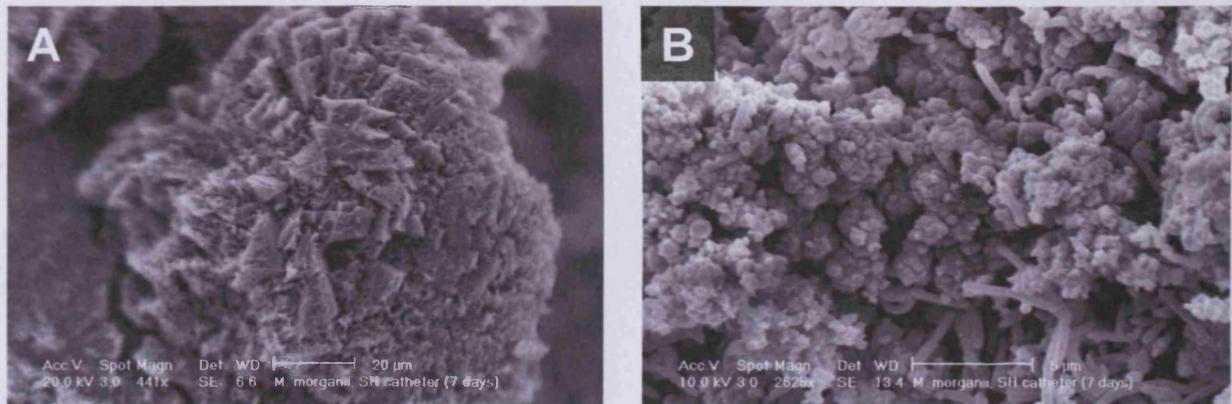


C & D: Encrusted *Pr. vulgaris* biofilm on a silver-hydrogel catheter. A large struvite crystal is present (indicated by ←, C), surrounded by apatite microcrystals. Few struvite crystals were observed in the biofilm. *Pr. vulgaris* cells were seen in association with apatite microcrystals at higher magnification (D).

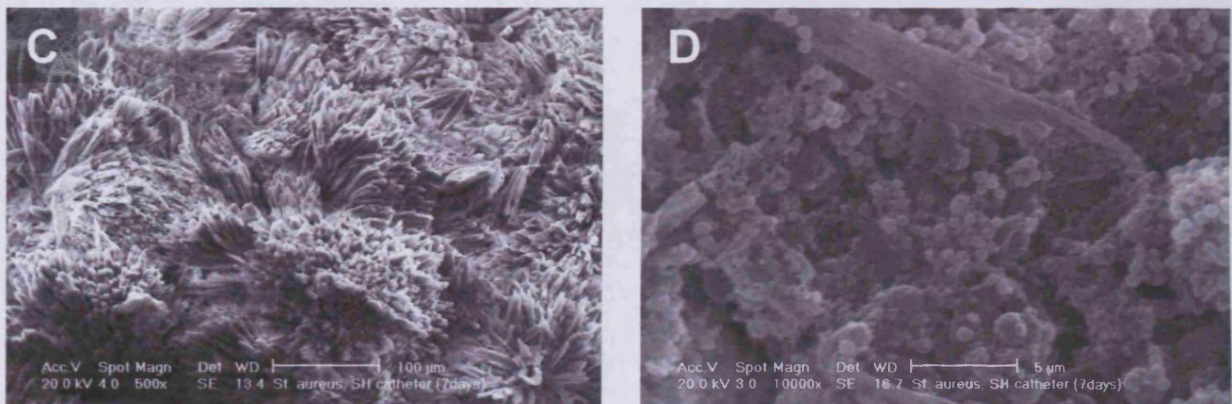


E & F: Encrusted *Pv. rettgeri* biofilm on a silver-hydrogel catheter. Biofilm heavily encrusted with apatite microcrystals is shown in E. No struvite crystals were observed in the biofilm. A high magnification image of the biofilm is shown in F, showing *Pv. rettgeri* rods in association with apatite microcrystals.

Figure 3.30: HV-SEM images of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* biofilms on silver-hydrogel coated latex catheters removed from bladder models at blockage.



A & B: *M. morgani* biofilm on a silver-hydrogel catheter. Some arrays of large calcium phosphate crystals are present around the eyehole (A). The biofilm was also encrusted with apatite microcrystals crystals (B).



C & D: *Staph. aureus* biofilm on a silver-hydrogel catheter. Many arrays of large calcium phosphate crystals are present, in particular around the eyehole (C). Further down the catheter the biofilm was encrusted with apatite microcrystals in addition to some large calcium phosphate crystals (D).

Figure 3.31: HV-SEM images of *M. morgani* and *Staph. aureus* biofilms on silver-hydrogel coated latex catheters removed from bladder models after 168 h.

3.2.2 Visualisation of the encrustation present on nitrofurazone catheters.

Digital photographs and LV-SEM images of nitrofurazone catheters removed from the model at blockage or after 168 h are presented in Figures 3.32 & 3.33. The catheters infected with *Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri* and *M. morgani* are heavily encrusted, in particular around and below the catheter eyehole, while very little encrustation is present on the un-blocked *Staph. aureus* catheter. HV-SEM images of *Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri* and *M. morgani* biofilms at blockage (Figures 3.34 & 3.35) show that the encrustation present on the catheters was mostly composed of apatite microcrystals, with a few struvite crystals also observed in the *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* biofilms on the eyehole sections. HV-SEM of the *Staph. aureus* biofilm after 168 h showed that the encrustation present on the eyehole section was mostly composed of apatite microcrystals, with some large calcium phosphate crystals also present (Figure 3.35). In the catheter lumen the encrustation was composed of apatite microcrystals.

3.2.3 The MIC of nitrofurazone for encrusting species used in experiments with nitrofurazone catheters.

The MIC of nitrofurazone for four of isolates of each of the species tested on the nitrofurazone catheters was determined using the method described in Section 2.7. The highest concentration tested was 128 µg/ml, since this is approaching the limit of solubility for nitrofurazone in agar. The results are displayed in Table 3.4. The MIC was greatest for *Pv. rettgeri* isolates (64 – 128 µg/ml), with *Pr. mirabilis* (64 µg/ml), *M. morgani* (32 – 64 µg/ml) and *Pr. vulgaris* (32 – 64 µg/ml) isolates exhibiting high

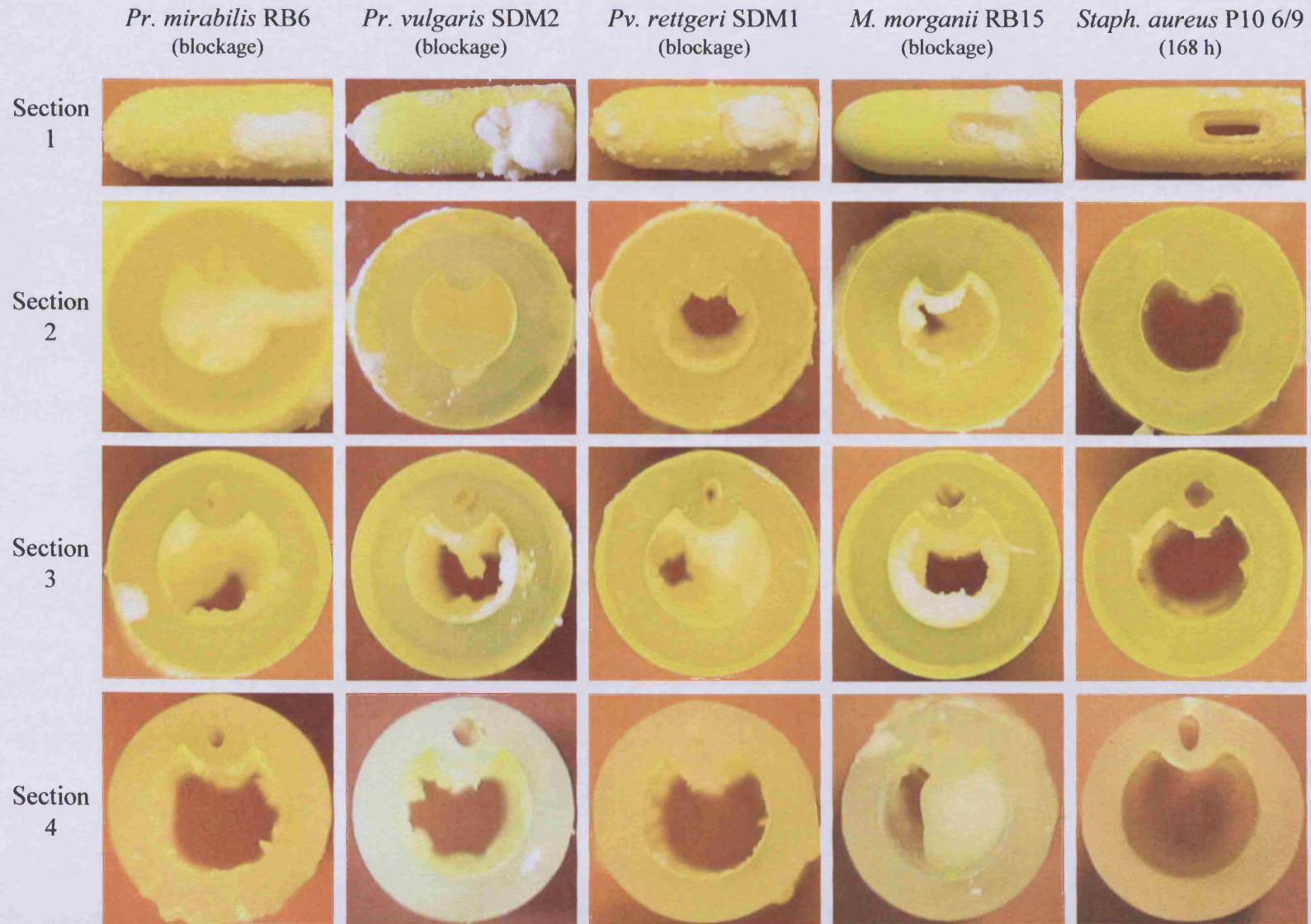


Figure 3.32: Digital camera images of nitrofurazone catheters removed from the model at blockage or after 168 h.

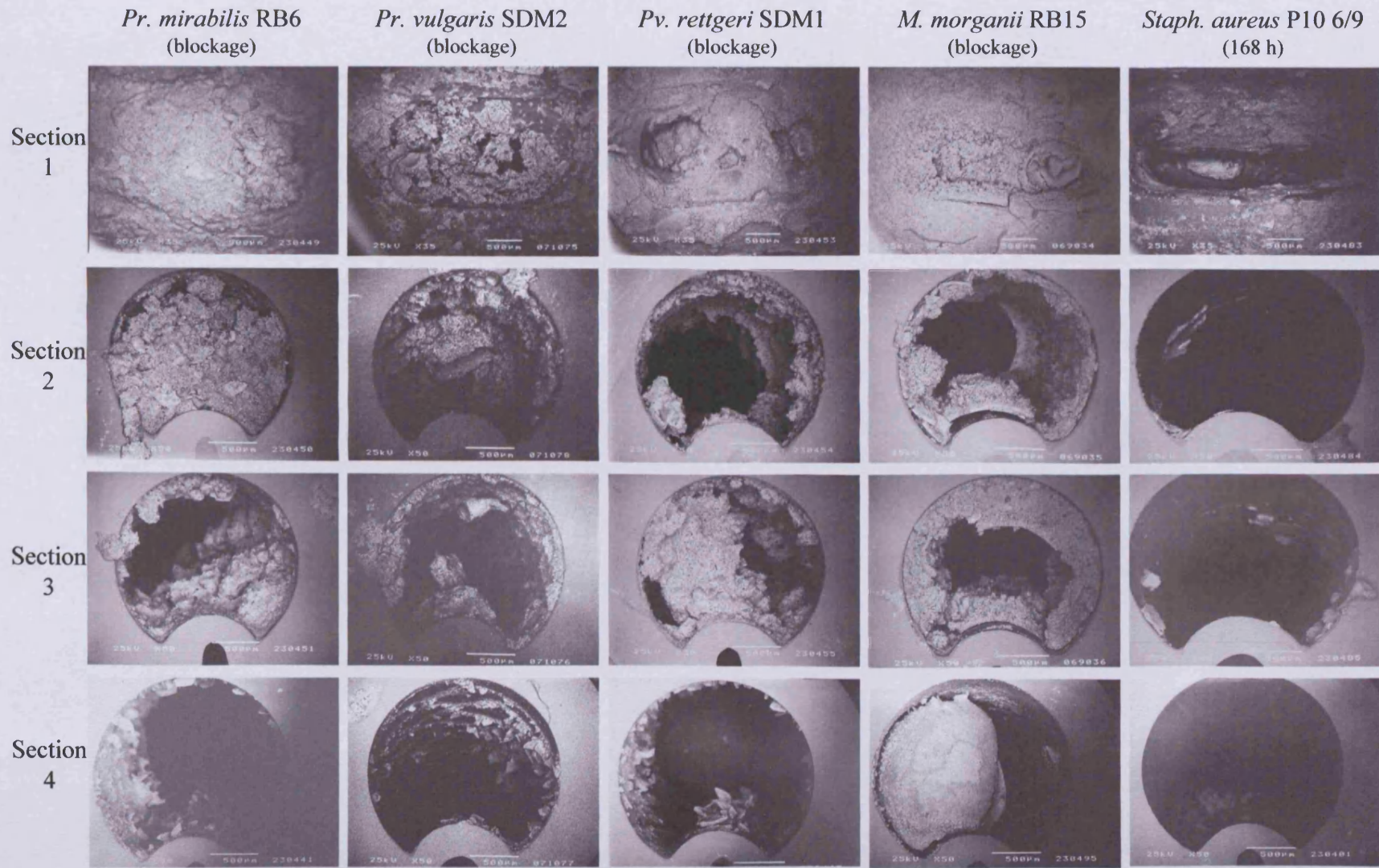
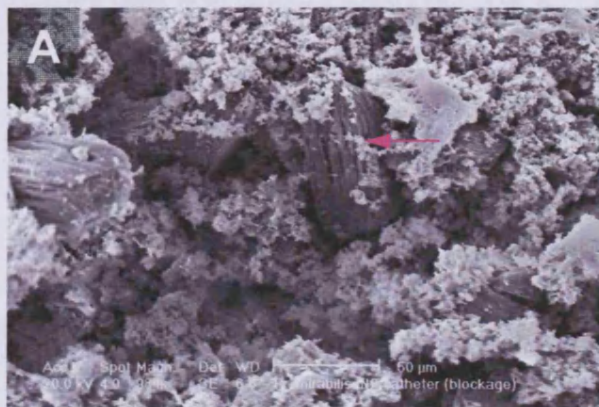


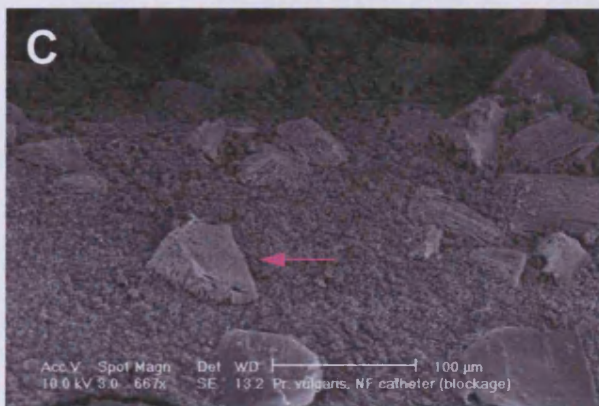



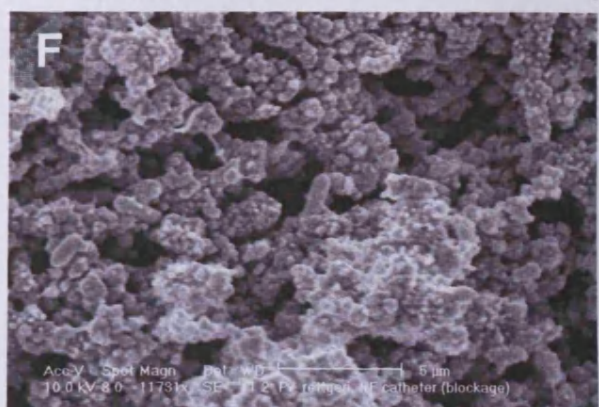
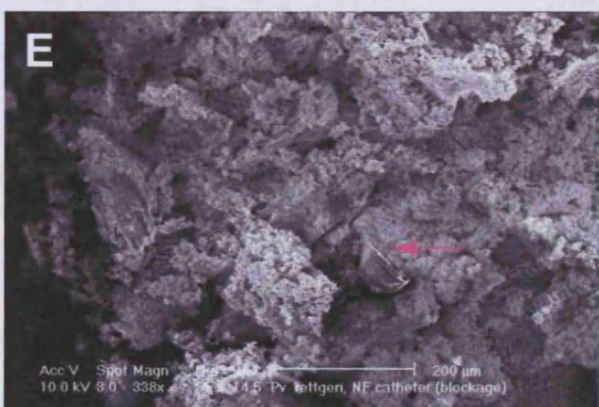
Figure 3.33: LV-SEM images of nitrofurazone catheters removed from the model at blockage or after 168 h.



A & B: Encrusted *Pr. mirabilis* biofilm on a nitrofurazone catheter. Several large struvite crystals are present (indicated, , A), surrounded by apatite microcrystals. A relatively large number of struvite crystals were observed in the biofilm. Biofilm heavily encrusted with apatite microcrystals is shown in B, with some bacterial cells present (indicated, ).



C & D: Encrusted *Pr. vulgaris* biofilm on a nitrofurazone catheter. A relatively large number of struvite crystals were present (indicated, , C), surrounded by apatite microcrystals. Few struvite crystals were observed in the biofilm. Biofilm heavily encrusted with apatite microcrystals is shown in D.




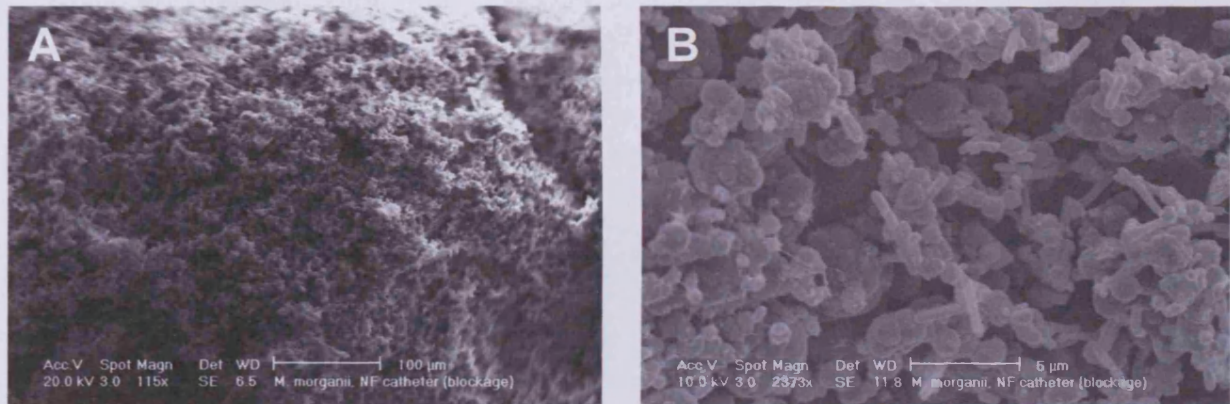
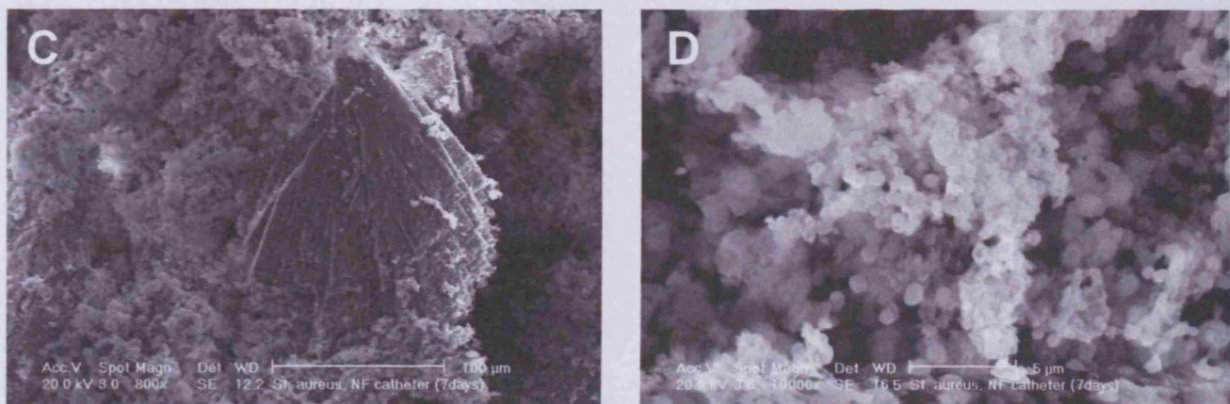
E & F: Encrusted *Pv. rettgeri* biofilm on a nitrofurazone catheter. A number of large struvite crystals are present (indicated, , E), surrounded by apatite microcrystals. Few struvite crystals were observed in the biofilm. Biofilm heavily encrusted with apatite microcrystals is shown in F.

Figure 3.34: HV-SEM images of *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri* biofilms on nitrofurazone catheters removed from bladder models at blockage.



A & B: *M. morganii* biofilm on a nitrofurazone catheter. The biofilm is heavily encrusted with apatite microcrystals (A). A close-up image of the biofilm is shown in B, showing *M. morganii* rods in association with apatite microcrystals.



C & D: *Staph. aureus* biofilm on a nitrofurazone catheter. Some arrays of large calcium phosphate crystals are present around the eyehole together with apatite microcrystals (C). Further down the catheter the biofilm was encrusted with apatite microcrystals (D).

Figure 3.35: HV-SEM images of *M. morganii* and *Staph. aureus* biofilms on nitrofurazone catheters removed from bladder models at blockage (*M. morganii*) or after 168 h (*Staph. aureus*).

Species	Isolate	Nitrofurazone MIC ($\mu\text{g/ml}$)	Nitrofurazone MSIC ($\mu\text{g/ml}$) (if appropriate)
<i>E. coli</i> (control)	NCTC 10418	4	-
<i>Staph. aureus</i> (control)	NCTC 6571	8	-
<i>Pr. mirabilis</i>	RB6	64	16
<i>Pr. mirabilis</i>	NSM6	64	16
<i>Pr. mirabilis</i>	B2	64	4
<i>Pr. mirabilis</i>	HI4320 wild type	64	16
<i>Pr. vulgaris</i>	SDM2	32	-
<i>Pr. vulgaris</i>	H2V	32	-
<i>Pr. vulgaris</i>	NSM19	64	-
<i>Pr. vulgaris</i>	W	32	-
<i>Pv. rettgeri</i>	SDM1	64	-
<i>Pv. rettgeri</i>	RE12	128	-
<i>Pv. rettgeri</i>	NSM26	128	-
<i>Pv. rettgeri</i>	AAAAG	128	-
<i>M. morgani</i>	RB15	64	-
<i>M. morgani</i>	SM18	64	-
<i>M. morgani</i>	SM40	32	-
<i>M. morgani</i>	NSM63	64	-
<i>Staph. aureus</i>	P10 6/9	16	-
<i>Staph. aureus</i>	NSM5	16	-
<i>Staph. aureus</i>	NSM7	16	-
<i>Staph. aureus</i>	N49	8	-
<i>Staph. saprophyticus</i>	ZEC17	4	-

Table 3.4: MIC of nitrofurazone (and if appropriate MSIC) for selected isolates. Control plates containing the largest levels of solvent used (DMSO) and agar only plates all showed growth for all isolates.

MICs in comparison to the *E. coli* NCTC 10418 (4 µg/ml) and *Staph. aureus* NCTC 6571 (8 µg/ml) controls. *Staph. aureus* isolates exhibited MICs similar to that of the control *Staph. aureus* (8 – 16 µg/ml). A single isolate of *Staph. saprophyticus* was also included in the screen, as this isolate was also identified as being capable of causing encrustation. Since this species is rarely isolated from long-term catheterised patients only one isolate was available for testing. The isolate was found to be quite sensitive to nitrofurazone (MIC 4 µg/ml).

It is clear that the four main urease producers have (*Pr. mirabilis*, *Pr. vulgaris*, *Pv. rettgeri* and *M. morgani*) have relatively high MICs for nitrofurazone. This lack of sensitivity could well explain why there was no significant difference between the mean times to blockage recorded for the all-silicone and nitrofurazone impregnated silicone catheters ($P>0.05$), as shown in Table 3.3.

3.3 The control of catheter encrustation by the delivery of antimicrobial agents through the retention balloon.

A strategy to control catheter encrustation by inflating the retention balloons with solutions of antimicrobial agents rather than with water was first suggested by Bibby *et al.* (1995). The idea was that the balloon would constitute a large reservoir from which antimicrobials could be delivered to the urine and thus inhibit the encrustation process. It was also suggested that the membrane of the balloon might provide a diffusion barrier that could control the rate of release of agents into the residual urine over protracted periods. Subsequent experimental work in laboratory models of the catheterised bladder showed that the biocide triclosan, which is extremely active against *Pr. mirabilis*, can diffuse through the balloons of silicone and latex catheters and inhibit encrustation (Stickler *et al.*, 2003a; Jones *et al.*, 2005). Unfortunately the formulation of triclosan used in this *in vitro* work (10 mg/ml in 5 % aqueous polyethylene glycol) is not suitable for producing the sterile solutions that would be necessary for use with patients. Because of the possibility of the degradation of triclosan by heating, sterilization by autoclaving is not a possibility. The polyethylene glycol formulation produces an emulsion rather than a solution and on membrane filtration, the phases separate and the filtrate is an aqueous solution containing very little triclosan. Williams (2006) investigated various ways of producing sterile stable solutions of triclosan and found that solutions containing up to 3 mg/ml can be prepared in 0.1 M sodium carbonate. It was decided to examine the ability of this formulation to inhibit catheter encrustation by the three species previously shown to be capable of rapidly blocking catheters.

3.3.1 The ability of triclosan 3 mg/ml in 0.1 M sodium carbonate to inhibit crystalline biofilm formation by species capable of causing rapid catheter blockage.

Bladder models were inoculated with 10 ml of a 4 h logarithmic culture of *Pr. mirabilis* (RB6), *Pr. vulgaris* (SDM2) or *Pv. rettgeri* (SDM1) in artificial urine. The catheter retention balloons of the test catheters were inflated using 10 ml of a 3 mg/ml triclosan solution in 0.1 M Na₂CO₃ (pH 11) prepared as described in Section 2.9.1. Catheters with retention balloons inflated with 0.1 M Na₂CO₃ or sterile water were used as controls. At 24 h intervals, up to a maximum of 168 h, or at blockage if it occurred, urine samples were taken from the bladder chamber. The pH values of the urine samples were measured and the viable bacterial cell populations were determined. The experiments were all carried out as three independent replicates and a summary of the data on the time the catheters took to block is presented in Table 3.5.

Figure 3.36 shows the urinary pH and viable bacterial cell population data collected for models inoculated with *Pr. mirabilis* RB6. The catheters with retention balloons inflated with control solutions (0.1 M Na₂CO₃ or water) all blocked within 40 h, with no significant difference between the mean blockage times ($P=0.467$). In contrast urine was still flowing freely through the catheters with balloons inflated with triclosan in 0.1 M Na₂CO₃ after 168 h. Statistical analysis revealed that there was no significant difference between the means of the initial urinary pHs ($P=0.783$) and initial cell populations ($P=0.288$) of the test and control models. Inflation of the balloons with the triclosan solution clearly had a profound effect, significantly reducing the pH ($P=0.04$) and viable cell population ($P<0.05$) of the residual urine.

Species (isolate)	Mean blockage time (h) (\pm SE)		
	Water control	0.1 M Na ₂ CO ₃ control	Triclosan (3 mg/ml)
<i>Pr. mirabilis</i> (RB6)	23.7 (\pm 6.9)	18.0 (\pm 1.5)	Did not block after 168 h
<i>Pr. vulgaris</i> (SDM2)	27.0 (\pm 2.0)	38.7 (\pm 7.7)	Did not block after 168 h
<i>Pv. rettgeri</i> (SDM1)	30.7 (\pm 0.3)	32.3 (\pm 6.0)	41.7 (\pm 8.5)

Table 3.5: The effect of inflating catheter retention balloons with triclosan (3 mg/ml in 0.1 M Na₂CO₃) on catheter blockage times in models infected with *Pr. mirabilis*, *Pr. vulgaris* or *Pv. rettgeri*.

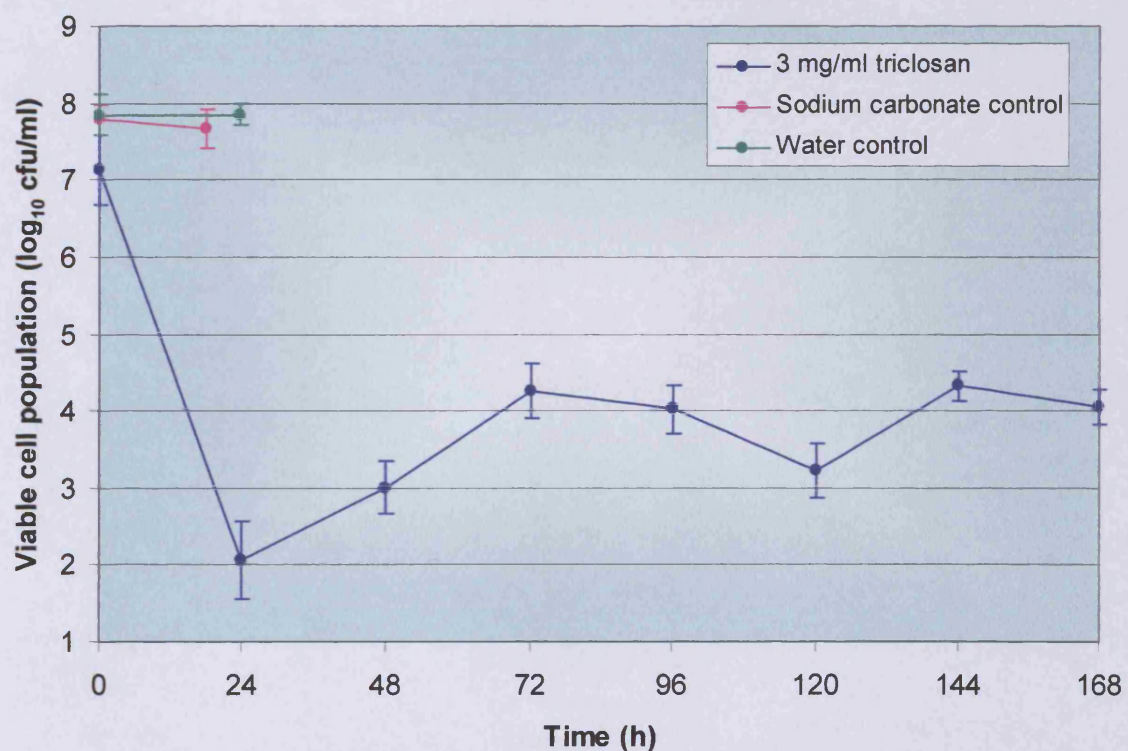
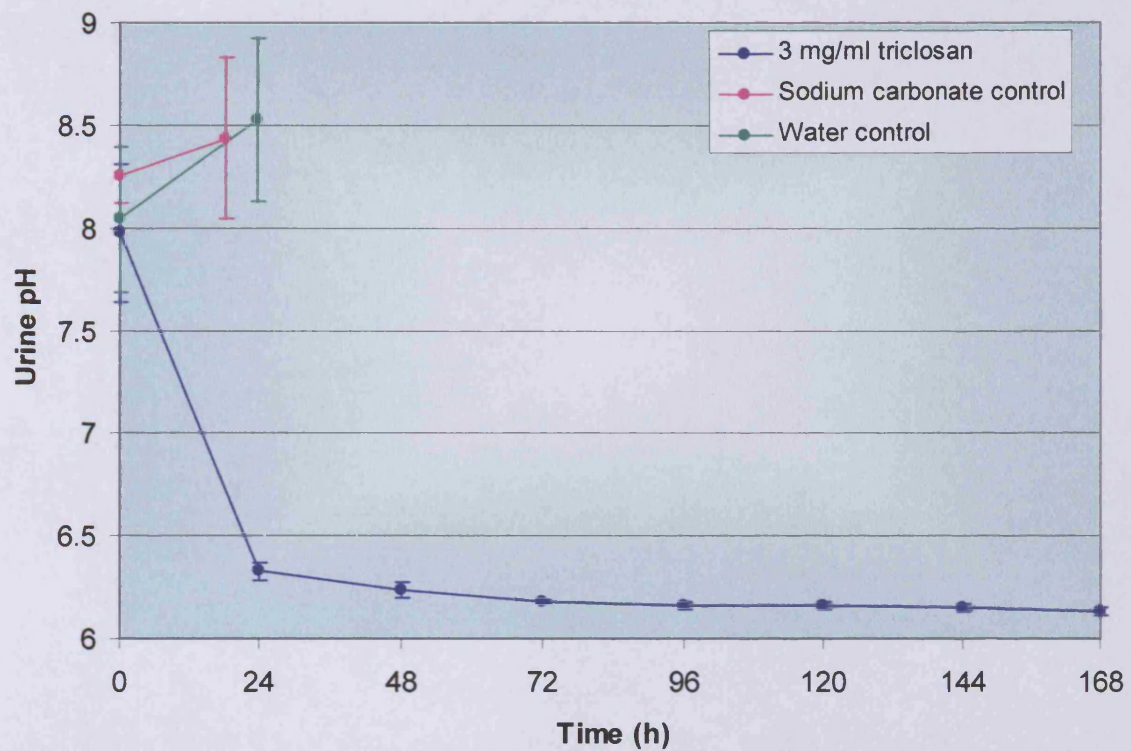


Figure 3.36: Variation in urinary pH and cell population vs. time for models inoculated with *Pr. mirabilis* RB6 until catheter blockage or 168 h. The catheter retention balloons were inflated with either 10ml of 3 mg/ml triclosan in 0.1 M Na₂CO₃ or a control solution (0.1 M Na₂CO₃ or sterile water).

A very similar set of data was produced from the experiments with *Pr. vulgaris* SDM2 (Figure 3.37). None of the test catheters blocked within the 168 h test period. The control catheters all blocked within 60 h and showed no significant difference ($P=0.216$) between mean blockage times. There was no significant difference ($P=0.255$) between the mean initial urinary pHs of the test and control models. The median final urinary pH of the Na_2CO_3 control was significantly greater ($P=0.04$) than that of the test model. The means of the initial and final cell populations of the control models were not significantly different ($P>0.05$) from each other while the mean initial and final cell populations of the test model were significantly different ($P<0.05$) to both of the controls.

While the triclosan formulation controlled the generation of alkaline urine and catheter encrustation by both *Pr. mirabilis* and *Pr. vulgaris* it had little effect on the process in models infected with *Pv. rettgeri* SDM1 (Figure 3.38). Catheter blockage occurred in all of the test and control models. There was no significant difference between the median blockage times ($P=0.575$), mean initial urinary pHs ($P=0.887$), mean final pHs ($P=0.844$) and mean final cell populations ($P=0.102$) of the test and control models. The mean initial cell population of the control models was significantly different ($P<0.05$) from that of the test models while the control models were not significantly different ($P>0.05$) from each other.

The MIC of triclosan for four of isolates of each of the three species was determined using the method described in Section 2.7. The lowest concentration tested was 0.05 $\mu\text{g/ml}$ and the highest concentration tested was 128 $\mu\text{g/ml}$, since this is approaching the limit of solubility for triclosan in agar. The results (Table 3.6) confirm the

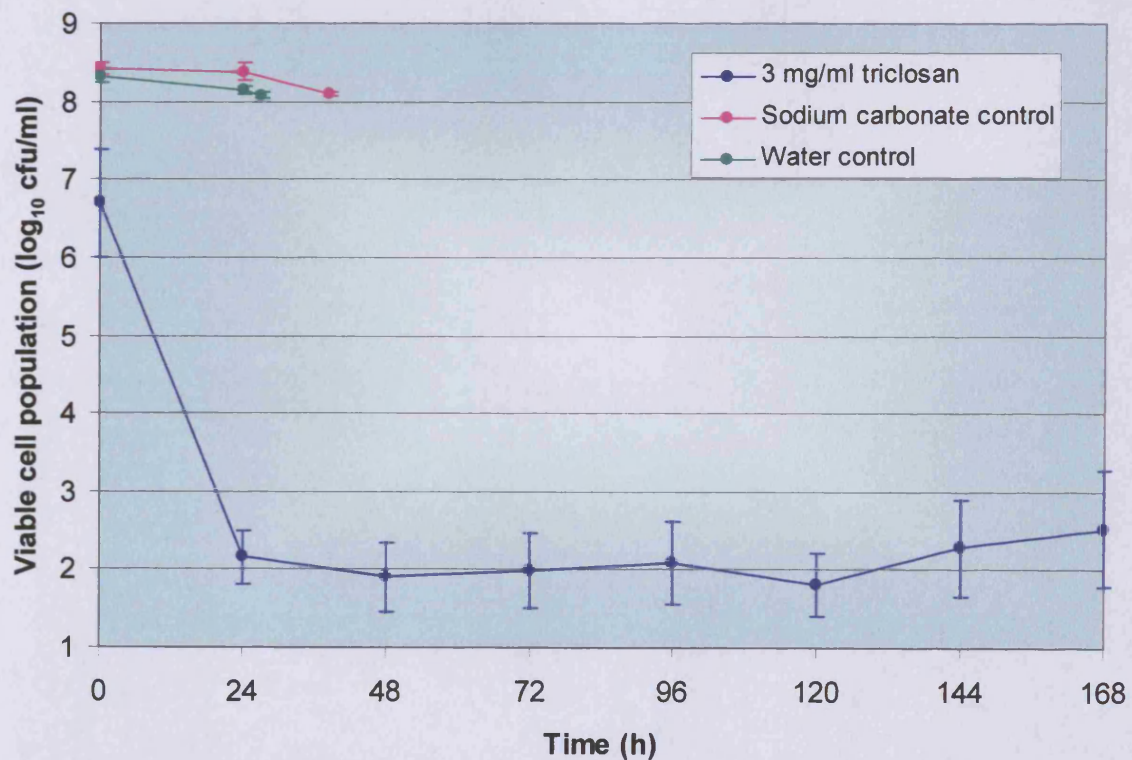
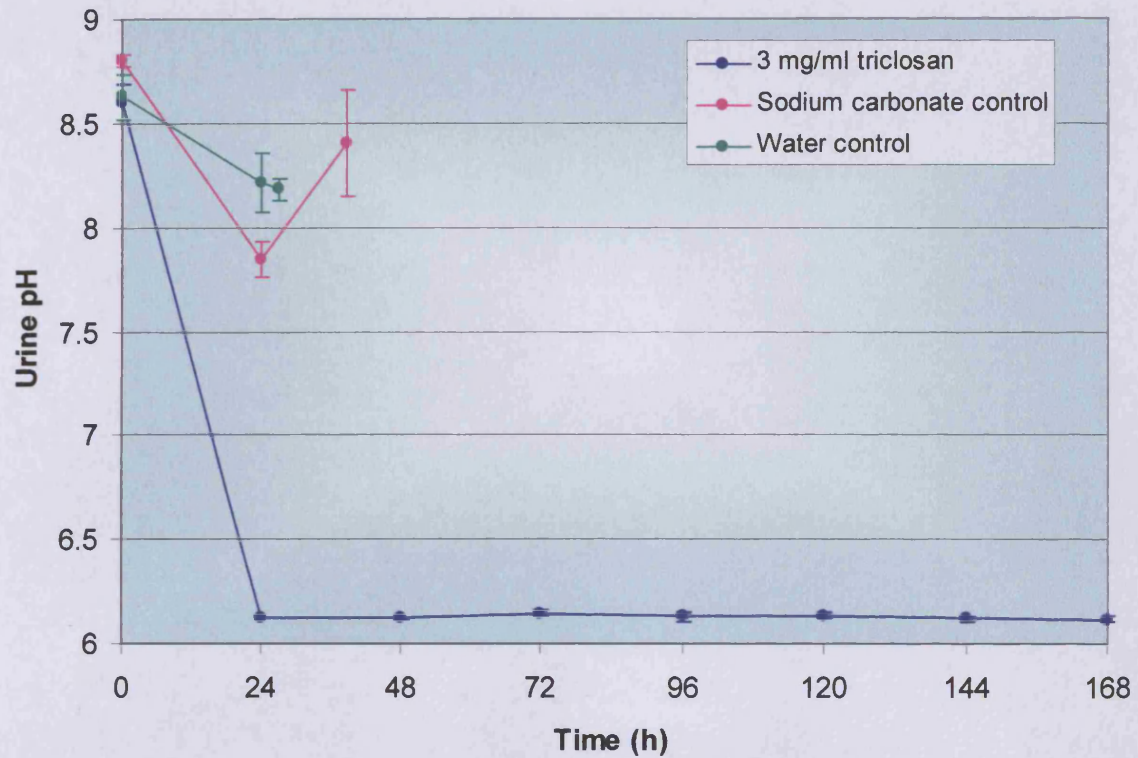


Figure 3.37: Variation in urinary pH and cell population vs. time for models inoculated with *Pr. vulgaris* SDM2 until catheter blockage or 168 h. The catheter retention balloons were inflated with either 10ml of 3 mg/ml triclosan in 0.1 M Na_2CO_3 or a control solution (0.1 M Na_2CO_3 or sterile water).

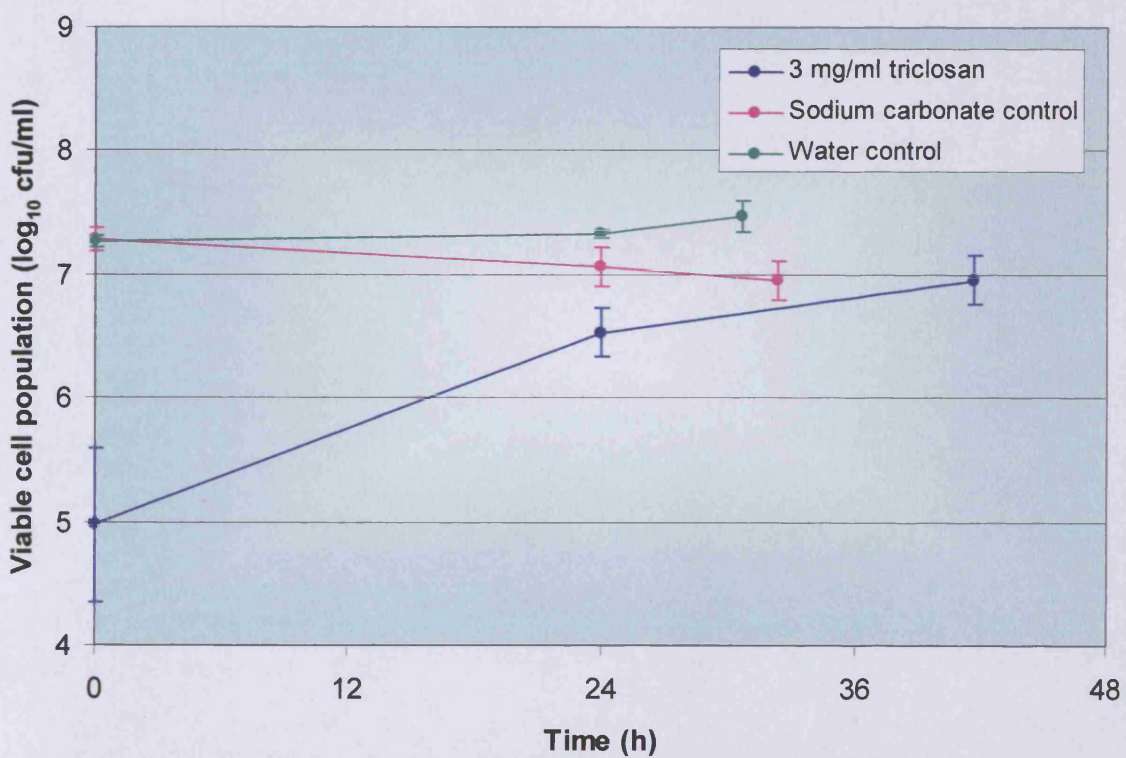
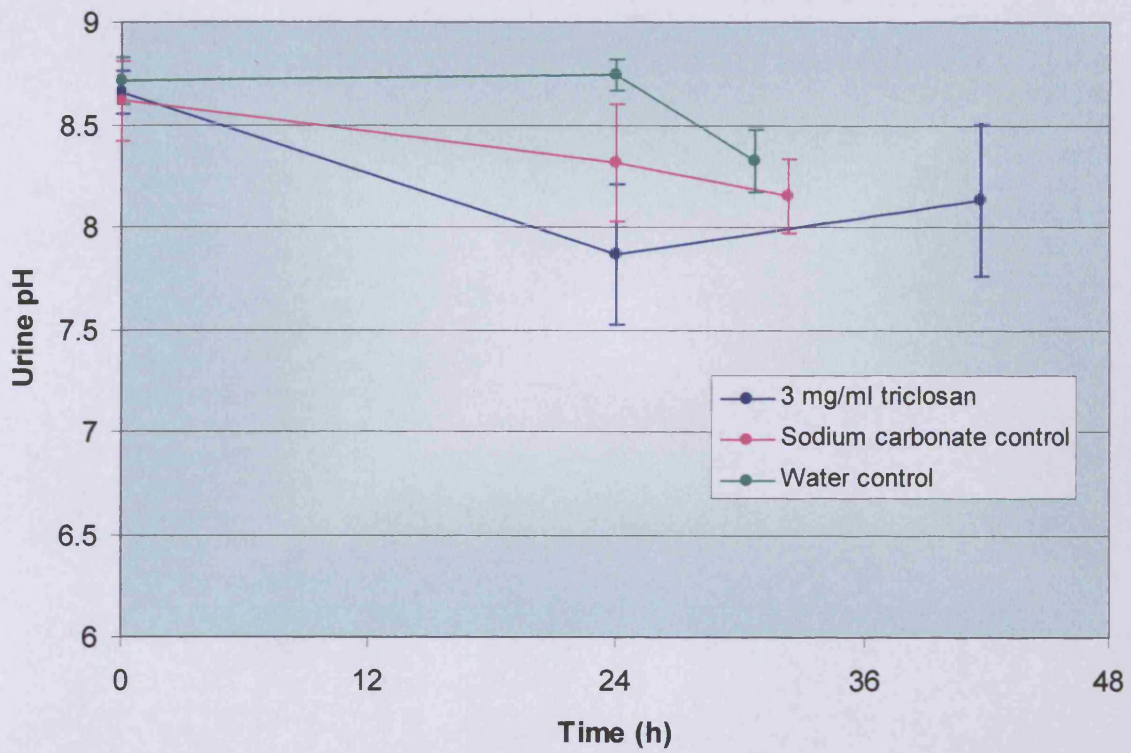


Figure 3.38: Variation in urinary pH and cell population vs. time for models inoculated with *Pv. rettgeri* SDM1 until catheter blockage. The catheter retention balloons were inflated with either 10ml of 3 mg/ml triclosan in 0.1 M Na₂CO₃ or a control solution (0.1 M Na₂CO₃ or sterile water).

Species	Isolate	Triclosan MIC ($\mu\text{g/ml}$)	Triclosan MSIC ($\mu\text{g/ml}$) (if appropriate)
<i>E. coli</i> (control)	NCTC 10418	0.1	-
<i>Staph. aureus</i> (control)	NCTC 6571	0.05	-
<i>Pr. mirabilis</i>	RB6	0.1	0.1
<i>Pr. mirabilis</i>	NSM6	0.2	0.1
<i>Pr. mirabilis</i>	B2	0.1	0.1
<i>Pr. mirabilis</i>	HI4320 wild type	0.1	0.1
<i>Pr. vulgaris</i>	SDM2	0.1	-
<i>Pr. vulgaris</i>	H2V	0.05	-
<i>Pr. vulgaris</i>	NSM19	0.1	-
<i>Pr. vulgaris</i>	W	0.1	-
<i>Pv. rettgeri</i>	SDM1	64	-
<i>Pv. rettgeri</i>	RE12	64	-
<i>Pv. rettgeri</i>	NSM26	64	-
<i>Pv. rettgeri</i>	AAAAG	64	-

Table 3.6: MIC and, if appropriate, MSIC of triclosan for selected isolates. Control plates containing the largest levels of solvent used (DMSO) and agar only plates all showed growth for all isolates.

extreme sensitivity of *Pr. mirabilis* and *Pr. vulgaris* to this biocide. *Pv. rettgeri* is much more resistant to triclosan (MIC 64 µg/ml) and this clearly explains the failure of the triclosan formulation to prevent catheter encrustation by this organism.

3.3.2 The effect of a nitric oxide generating solution on catheter encrustation by *Pr. mirabilis*.

A recent study by Carlsson *et al.* (2005) showed that filling catheter retention balloons with a solution that generated nitric oxide significantly reduced the number of *E. coli* present in the residual urine, in a static model of the catheterised urinary tract. As nitric oxide has a broad spectrum of antimicrobial activity and readily diffuses through silicone it was decided to test this strategy against *Pr. mirabilis* using our continuous flow model.

The nitric oxide generating solution used was 10 mM ascorbic acid, 5 mM sodium nitrite in physiological saline at pH 2.5, as described by Carlsson *et al.* (2005). Immediately prior to inoculation of the bladder model equal volumes of stock solutions of pH 2.5 20 mM ascorbic acid in physiological saline and pH 2.5 10 mM sodium nitrite in physiological saline were mixed and the resulting solution used to inflate the retention balloon. Catheters with balloons inflated with pH 2.5 10 mM ascorbic acid in physiological saline, pH 2.5 5 mM sodium nitrite in physiological saline, pH 2.5 physiological saline only, and sterile water were used as controls. Bladder models were inoculated with 100 µl of a 4 h logarithmic culture of *Pr. mirabilis* RB6 in artificial urine. At 24 h intervals until blockage urine samples were taken from the bladder chamber. The pHs of the urine samples were measured and

the viable bacterial cell populations determined. The extent of encrustation present on catheter sections was visualised using digital photography.

Figure 3.39 shows the blockage times and variation in bladder chamber urine pH for test and control models inoculated with *Pr. mirabilis* RB6. There was no significant difference ($P=0.109$) between the mean blockage times or the mean final urine pHs ($P=0.182$) of the test and control catheters. Statistical analysis also showed no significant difference in the mean initial urinary cell populations ($P=0.266$) and mean final urinary cell populations ($P=0.378$) of the test and control models (data not shown).

Digital photographs of test and control catheters removed from the model at blockage are shown in Figure 3.40. All of the catheters show large amounts of encrustation, particularly on the eyehole and lumen sections nearest the eyehole. It is clear that the nitric oxide generating solution had little effect on the formation of crystalline biofilm by *Pr. mirabilis*.

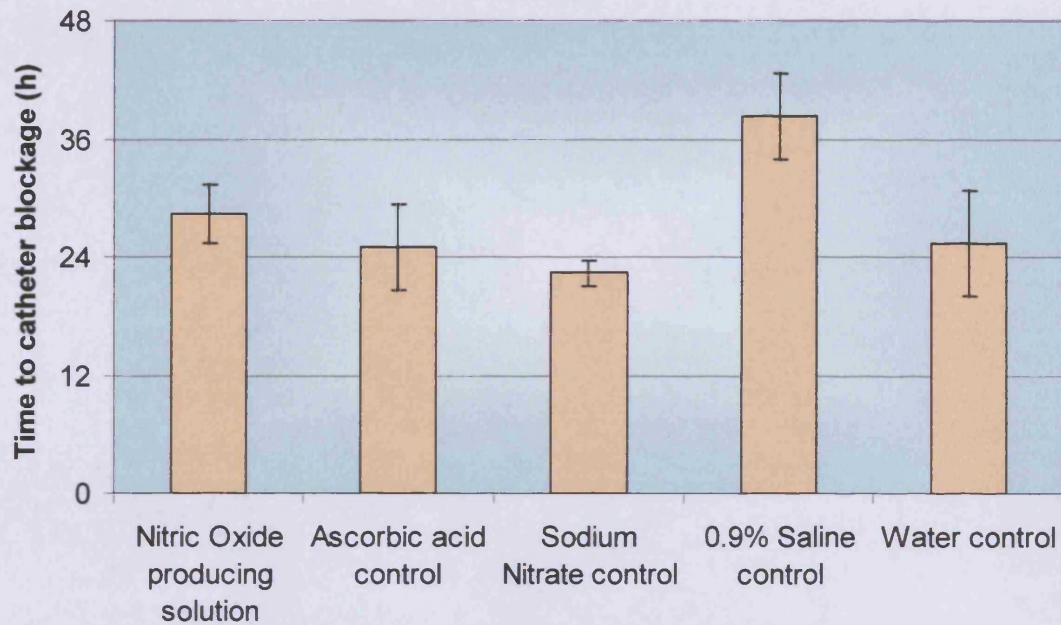


Figure 3.39a: Mean blockage times for catheters with retention balloons filled with a nitric oxide generating solution or one of four controls. All bladder models were inoculated with 100 μ l of a 4 h logarithmic phase culture of *Pr. mirabilis* RB6 in artificial urine. The mean values \pm SE were calculated from the results of three replicate experiments.

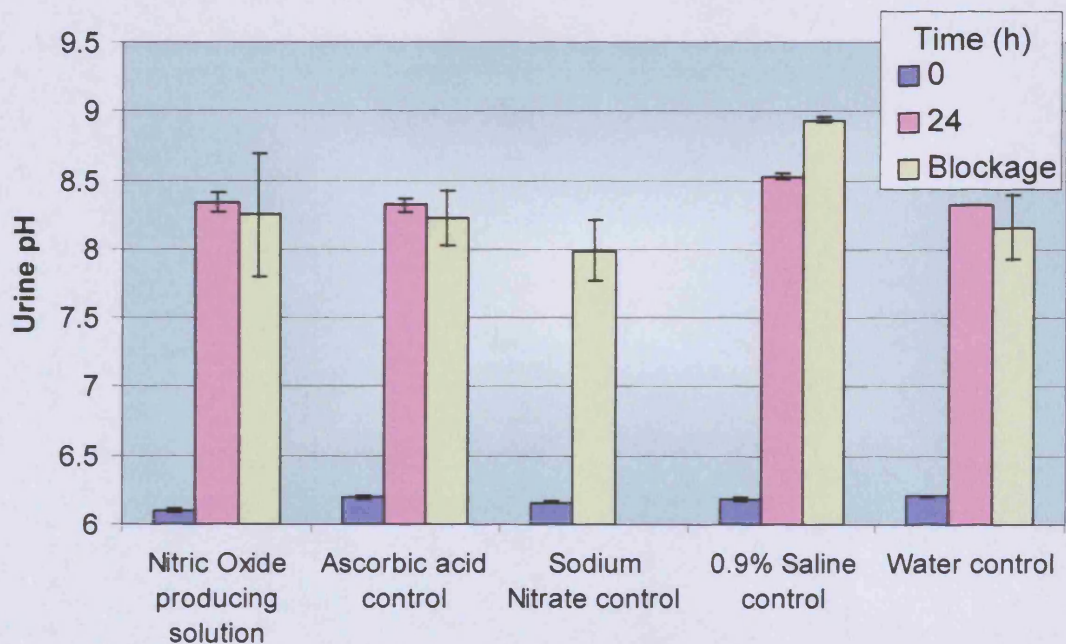


Figure 3.39b: Variation in urine pH in the bladder model until blockage for catheters with retention balloons filled with a nitric oxide generating solution or one of four control solutions. All bladder models were inoculated with 100 μ l of a 4 h logarithmic phase culture of *Pr. mirabilis* RB6 in artificial urine.

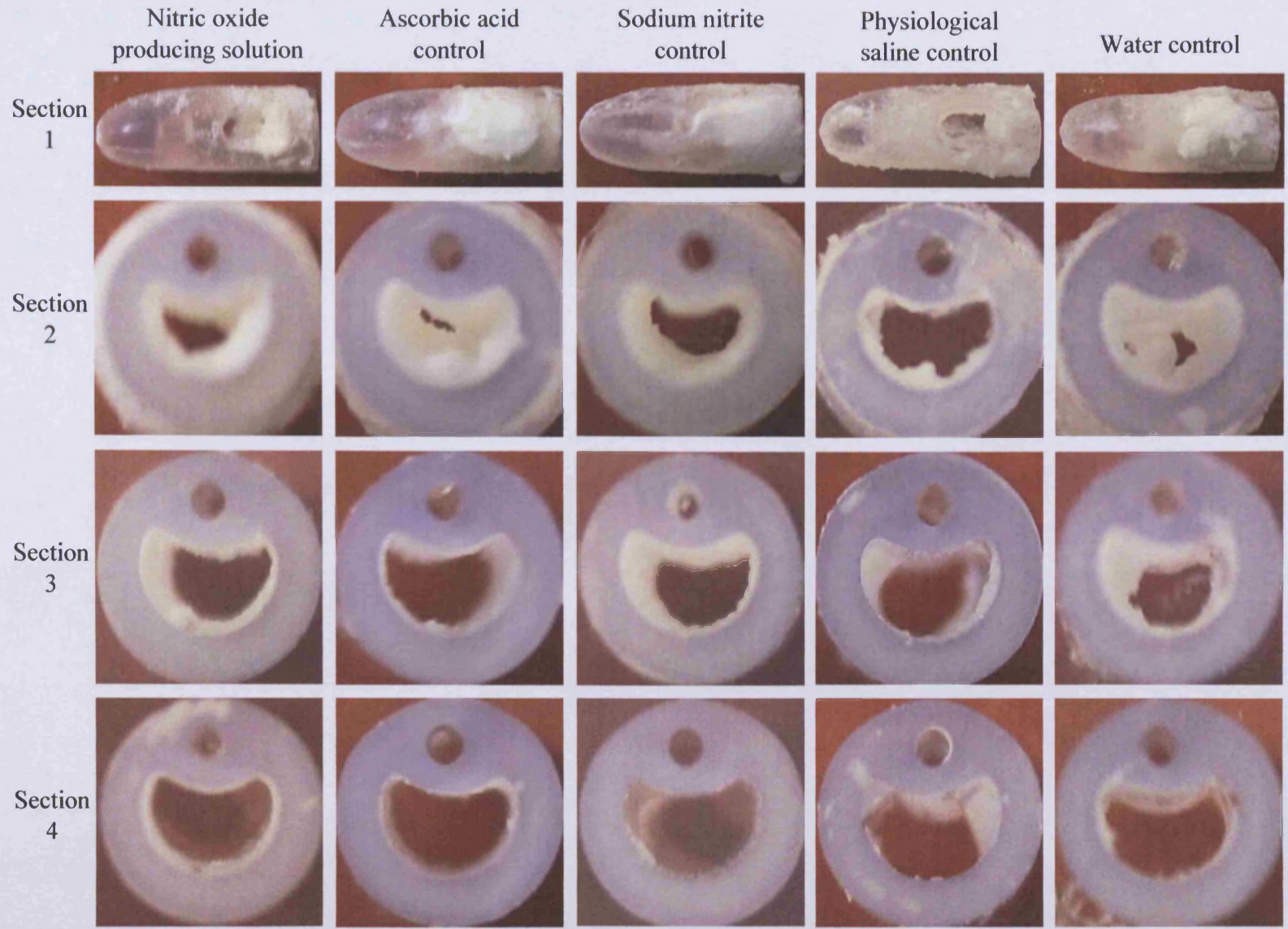


Figure 3.40: Digital camera images comparing encrustation at blockage for all-silicone catheters with retention balloons filled with either a nitric oxide producing solution or a control solution. All bladder models were inoculated with 100 µl of a 4 h logarithmic phase *Pr. mirabilis* (RB6) culture.

3.4 An integrated sensor/modulator strategy for the control of catheter encrustation.

The complication of catheter blockage and encrustation is unpredictable and commonly results in emergency referrals of patients with urinary retention or incontinence owing to obstruction of the flow of urine (Kohler-Ockmore and Feneley, 1996). Stickler *et al.* (2006a) developed a simple sensor to detect the early stages in the formation of crystalline biofilm on catheters and thus give early warning of an impending clinical crisis. The sensor is composed of cellulose acetate to which the pH indicator bromothymol blue is covalently bound. It is a simple strip of yellow plastic that can be located in the urine collection bag. In laboratory and clinical studies (Stickler *et al.*, 2006a; Stickler *et al.*, 2006c) it has been shown to change from yellow to blue in response to infection by urease producing organisms such as *Pr. mirabilis*. It has also been demonstrated that when the sensor changes colour, crystalline biofilm has started to form on the catheter. The idea is that when the sensor in the bag turns blue, the triclosan strategy (Section 3.3) will be implemented.

An important practical question is; when the sensor gives the signal, will the emptying of the water from the balloon of the current catheter and refilling with the triclosan be effective in preventing catheter blockage? In other words will the triclosan strategy be effective in stopping additional encrustation forming on the catheter? On the other hand will it be necessary to replace the existing catheter with one inflated with triclosan in order to prevent blockage? A programme of work was performed in the bladder models in order to resolve these issues. The experiments involved the three species that had been shown to encrust catheters rapidly. The triclosan was prepared

from a proprietary handwash solution (Hy-Shield SkinSure plus, Hygieia Healthcare Ltd.) diluted to 3 mg/ml or a solution at 3 mg/ml in 0.1 M sodium carbonate.

Bladder models were inoculated with 100 µl of a 4 h logarithmic phase culture of *Pr. mirabilis* RB6, *Pr. vulgaris* SDM2, or *Pv. rettgeri* SDM1 in artificial urine. The catheter retention balloons of the test and control catheters were inflated with 10 ml of sterile water. The pH sensor was placed aseptically into the urine collection bag and its appearance monitored until it corresponded to pH 8 on a sensor colour chart (Figure 3.41). At this point 5 ml of water was removed from the balloon of the test catheter and replaced with 5 ml of either Hy-Shield Skinsure plus cream diluted with sterile water to contain 6 mg/ml triclosan or 6 mg/ml triclosan in 0.2 M Na₂CO₃. The water remaining in the balloon was mixed with the triclosan solution by removing 5 ml of solution and then re-inflating the balloon several times. Urine samples were taken from the bladder chamber and urine collection bag at the start of the experiment, at the point the sensor became positive, 8 h after triclosan intervention and at 24 h intervals post triclosan intervention until 96 h or blockage. Additional urine samples were taken from the urine collection bag at 2, 4 and 12 h (and 18 h if the sensor had not become positive by that point) after the start of the experiment. The pHs of all the urine samples were measured and the viable bacterial cell populations of urine samples from the bladder chamber determined. Digital photographs of the pH sensors were taken at each point the urine collection bag pH was measured. Digital photography and HV-SEM were used to assess the extent of encrustation on catheters and sensors at the point the sensor became positive and at blockage or 96 h post triclosan intervention for the control and test catheters. Statistical analysis was used where appropriate to determine if there was any significant difference between the

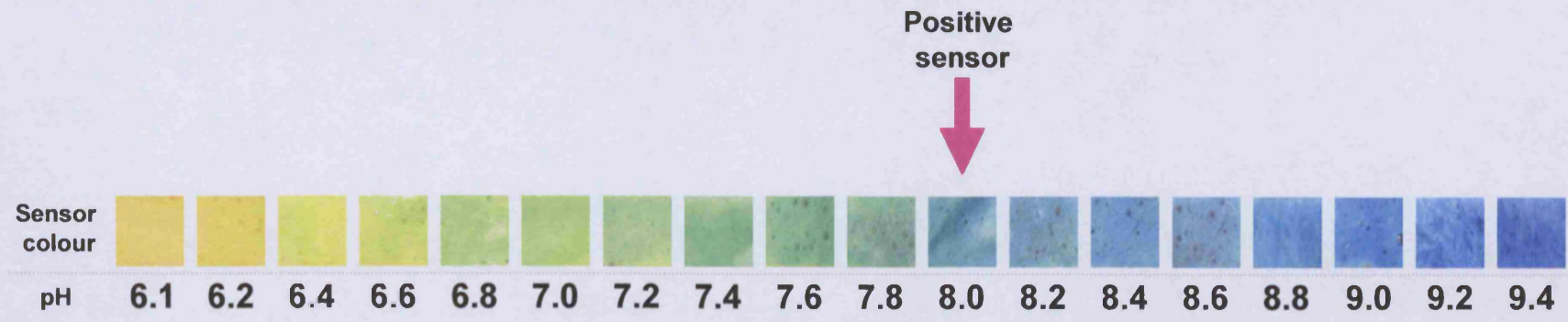



Figure 3.41: The appearance of the pH sensor over the pH range 6.1 – 9.4 in artificial urine. The colour chart was used for comparison to pH sensors inserted into urine collection bags. The pH sensor was considered to be ‘positive’ when the colour corresponded to pH 8 on the pH sensor colour chart (indicated ).

mean (Two Sample t-test) or median (Mann-Whitney) blockage times and urinary pHs of the test and control catheters.

Figure 3.42 shows the variation in urinary pH in the bladder chamber and urine collection bag for models inoculated with *Pr. mirabilis* with triclosan intervention (Hy-Shield) applied at the time the sensor became positive. Blockage occurred in the test catheters of two of the replicates while one model drained freely for the duration of the experiment. In all three test catheters the urine pH in the collection bags remained above 8.5 after triclosan intervention while the urine pH in the bladder model quickly fell to below 7.5. The pH of the residual urine in the test model which drained freely for the duration of the experiment remained around pH 7 after intervention, although the final reading showed a sharp rise in urinary pH (up to 8.5).

The data from the experiments with *Pr. vulgaris* are shown in Figure 3.43. Blockage occurred in the test catheter of one of the replicates while two models drained freely for the duration of the experiment. For the test catheter in which blockage occurred the time of blockage was three times that of the control catheter. In all three test catheters the urine pH in the collection bags remained above 8 after triclosan intervention while the urine pH in the bladder model quickly fell to below 7. The pH of the residual urine in all three test models remained below pH 7 after intervention.

In experiments with *Pv. rettgeri* blockage of all three test catheters occurred within 24 h of the sensor becoming positive (Figure 3.44). There was no significant difference between the mean blockage times of the test and control catheters ($P=0.509$). Statistical analysis also revealed no significant difference between the median final

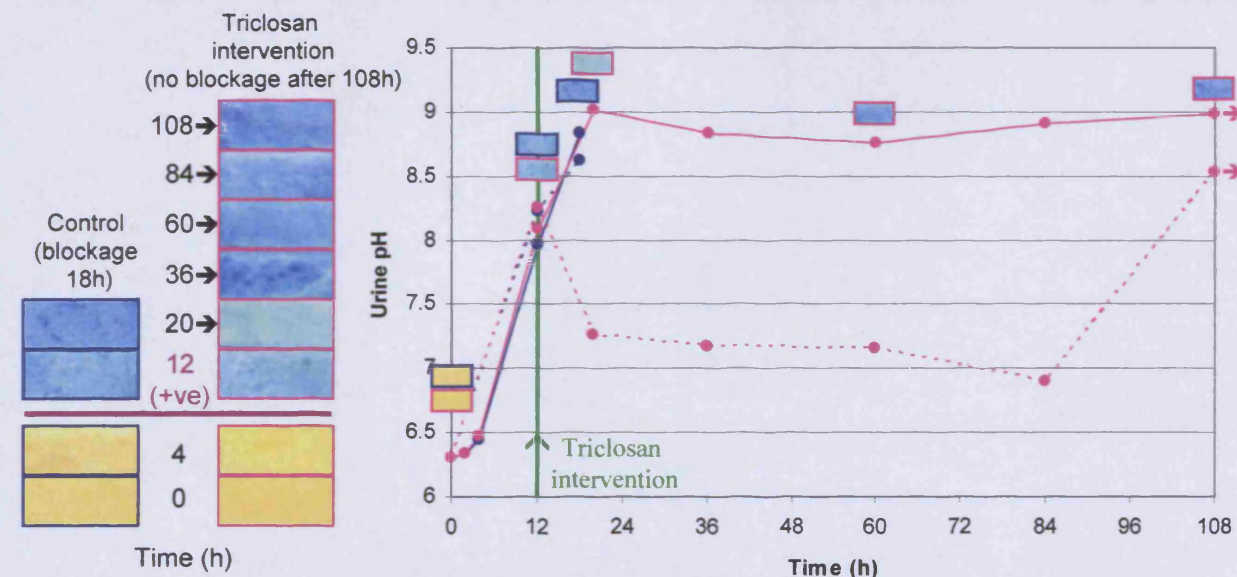
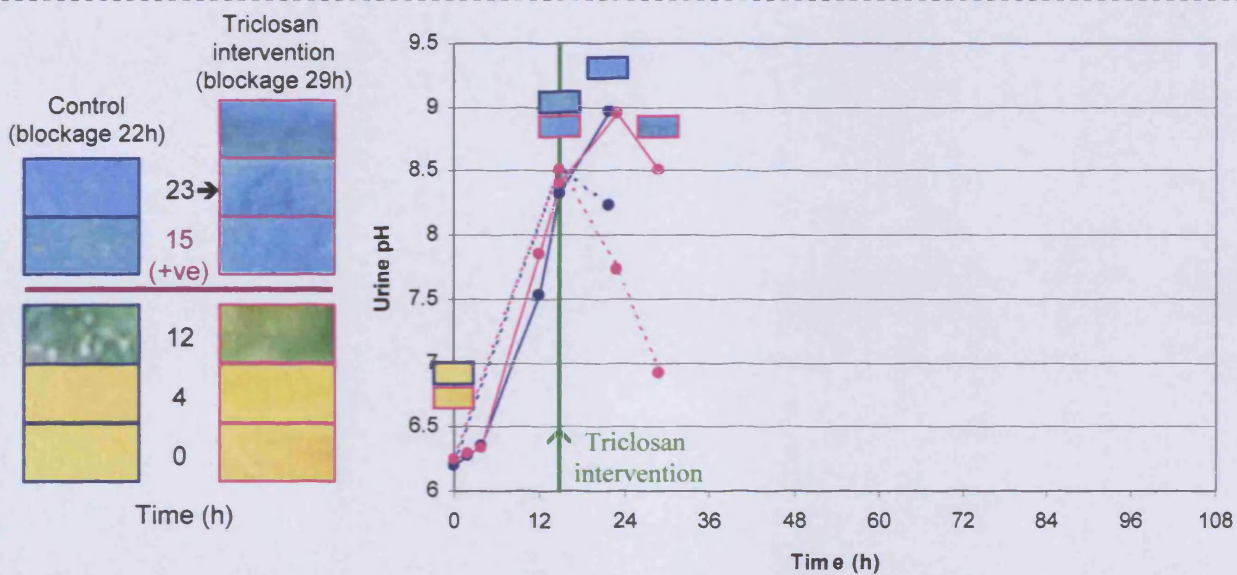
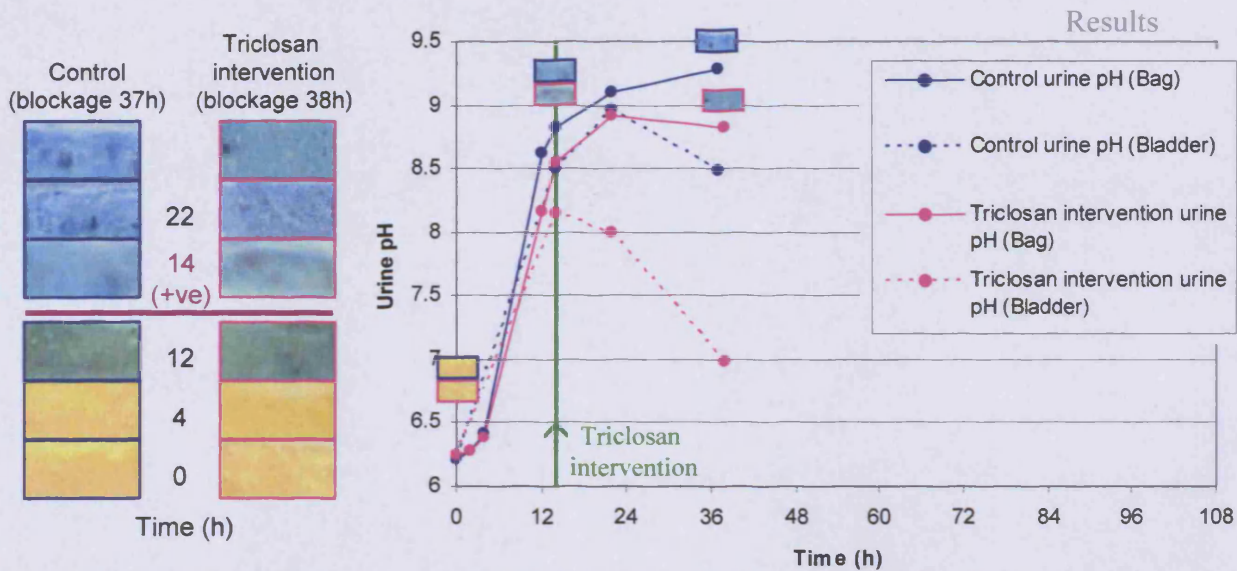


Figure 3.42: Variation in urinary pH vs. time for models inoculated with *Pr. mirabilis* RB6 until blockage or 96 h post triclosan intervention. 3 individual replicates are shown. When the sensor gave the positive signal the water in the test catheter balloons was replaced with triclosan at 3 mg/ml (diluted Hy-Shield). The appearance of the pH sensors placed in the catheter bag is also shown. Selected reduced size images of the sensors are shown at the appropriate time points on the graphs (border colour indicates which line they correspond to). *→ indicates that the catheter was draining freely at the end of the experiment.

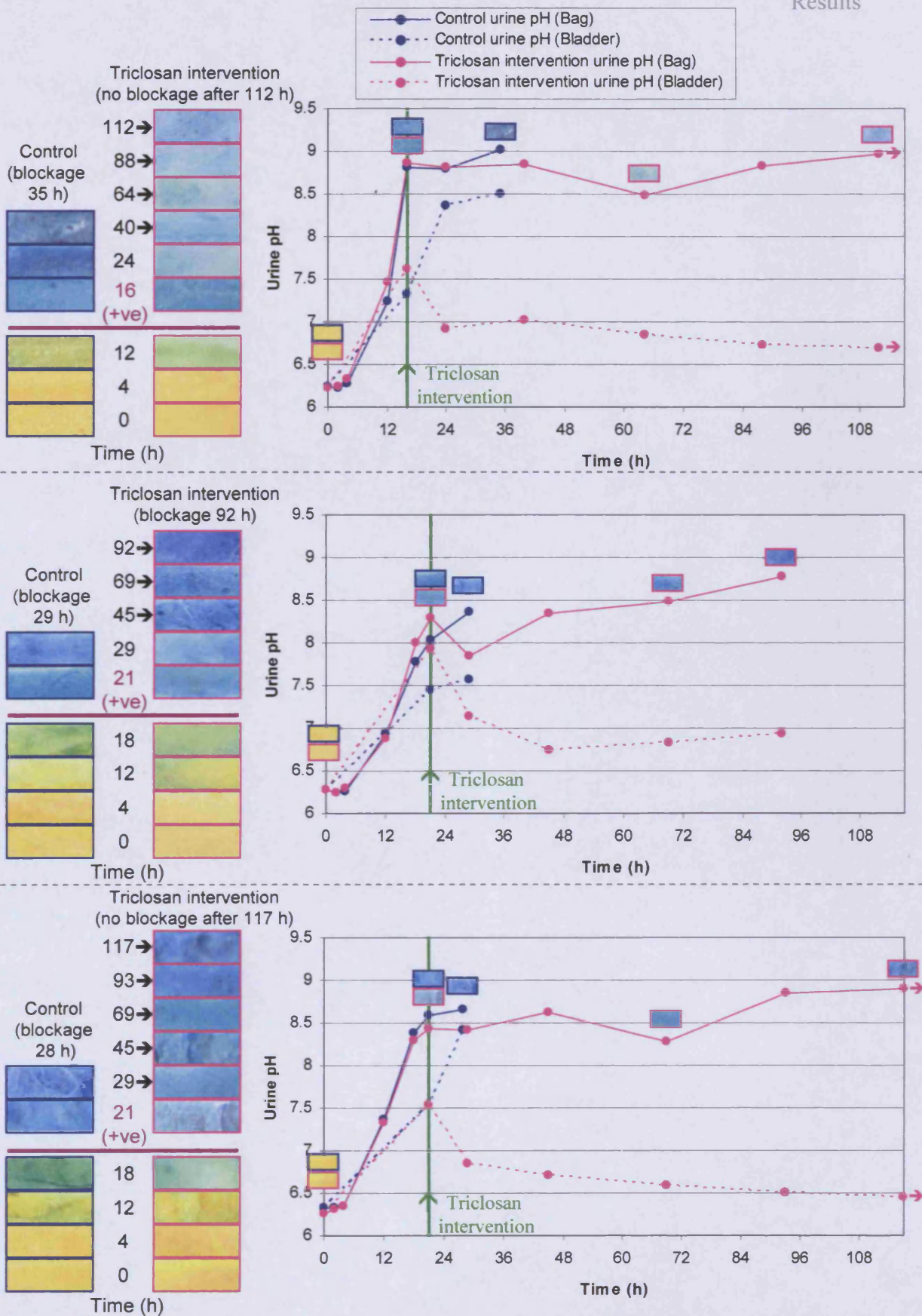


Figure 3.43: Variation in urinary pH vs. time for models inoculated with *Pr. vulgaris* SDM2 until blockage or 96 h post triclosan intervention. 3 individual replicates are shown. When the sensor gave the positive signal the water in the test catheter balloons was replaced with triclosan (3 mg/ml). The colour of pH sensors placed in the catheter bag is also shown. Selected reduced size images of the sensors are shown at the appropriate time points on the graphs (border colour indicates which line they correspond to).

*→ indicates that the catheter was draining freely at the end of the experiment.

Results

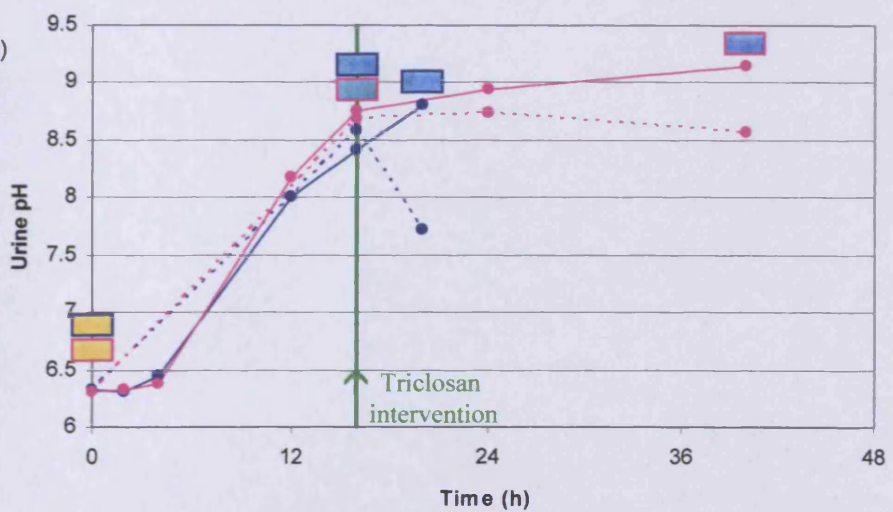
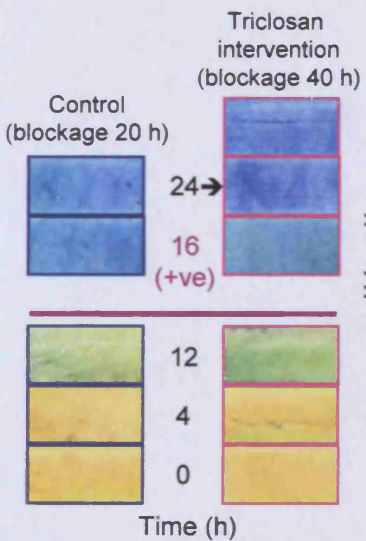
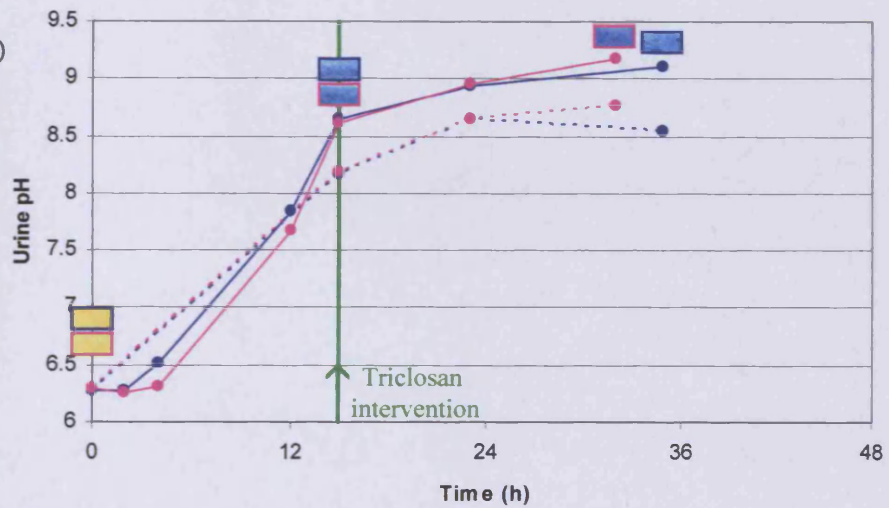
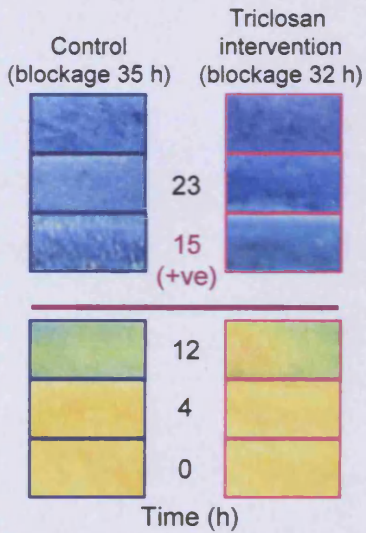
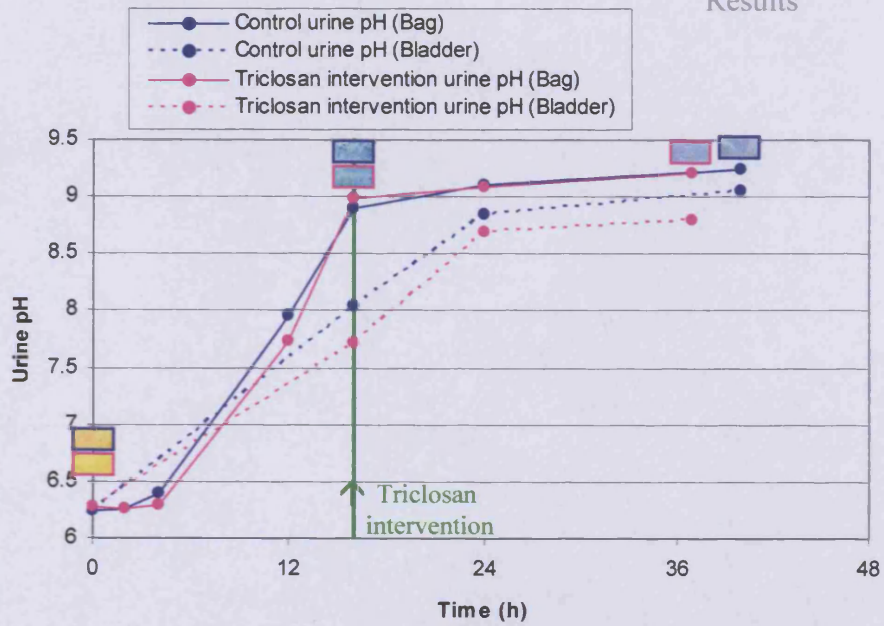
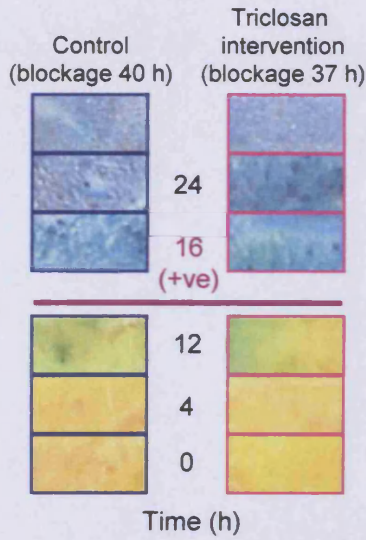


Figure 3.44: Variation in urinary pH vs. time for models inoculated with *Pv. rettgeri* SDM1 until blockage. 3 individual replicates are shown. When the sensor gave the positive signal the water in the test catheter balloons was replaced with triclosan (3 mg/ml). The appearance of the pH sensors placed in the catheter bag is also shown. Selected reduced size images of the sensors are shown at the appropriate time points on the graphs (border colour indicates which line they correspond to).

urinary pH in the collection bags ($P=0.663$) and mean final urinary pH in the bladder chamber ($P=0.543$) of the test and control models.

A repeat set of experiments was performed with *Pr. mirabilis* using triclosan at 3 mg/ml in 0.1 M Na_2CO_3 . The results (Figure 3.45) show that while the control catheters all blocked within 24 h of the positive sensor signal, none of the test catheters had blocked 96 h post triclosan intervention. The urinary pH in the bladder chamber fell to around pH 6.5 (24 h after triclosan intervention) in all of the test models and remained around pH 6.5 for the remainder of the experiment. The urinary pH in the collection bags initially rose to above pH 8.5 (8 h after triclosan intervention) before falling below pH 8 (24 h after triclosan intervention) but then rose back to above pH 8.5 for the remainder of the experiment.

The pH sensors (Figures 3.42 – 3.45) appear in general to have reflected the pH of the urine in the collection bag. It is important to note however that even in those cases where the triclosan reduced the pH of the residual urine in the bladder, the pH of the urine in the collection bag remained around 8 to 9 and the sensor stayed blue. These observations suggest that to monitor catheter encrustation effectively after the triclosan intervention it will be necessary to replace the urine collection bag with one containing a new sensor.

Digital photographs of catheters removed from the model at the times the sensors became positive, at blockage or at 96 h post triclosan intervention are shown in Figures 3.46 & 3.47. All of the catheters removed from the model at the times the sensor gave positive signals show some encrustation on both the catheter eyehole and

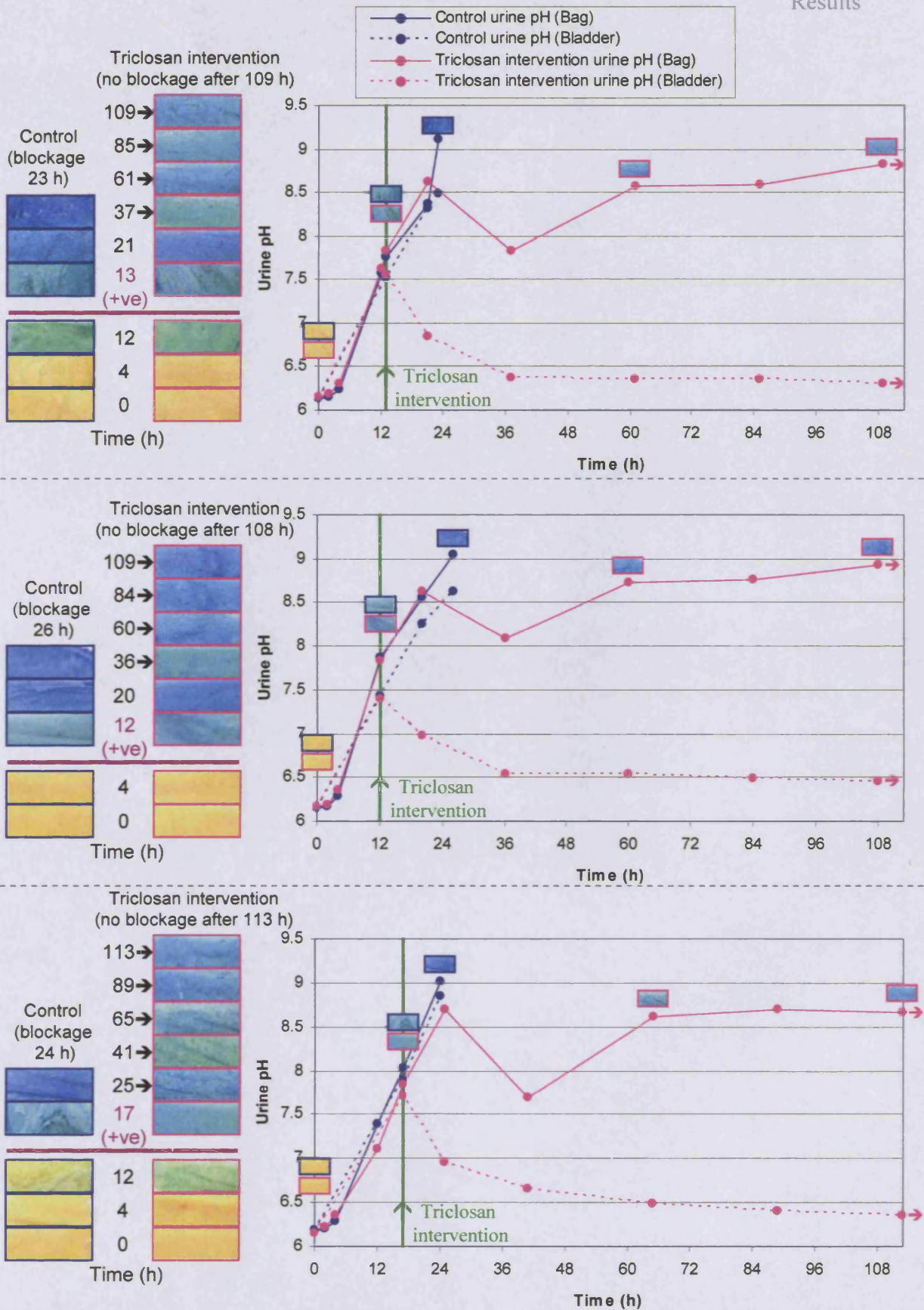


Figure 3.45: Variation in urinary pH vs. time for models inoculated with *Pr. mirabilis* RB6 until blockage or 96 h post triclosan intervention. 3 individual replicates are shown. When the sensor gave the positive signal the water in the test catheter balloons was replaced with triclosan (3 mg/ml in 0.1 M Na₂CO₃). The appearance of the pH sensors placed in the catheter bag is also shown. Selected reduced size images of the sensors are shown at the appropriate time points on the graphs (border colour indicates which line they correspond to).

*→ indicates that the catheter was draining freely at the end of the experiment.

Pr. mirabilis triclosan intervention (Hy-Shield)

Pr. mirabilis triclosan intervention (Na_2CO_3)

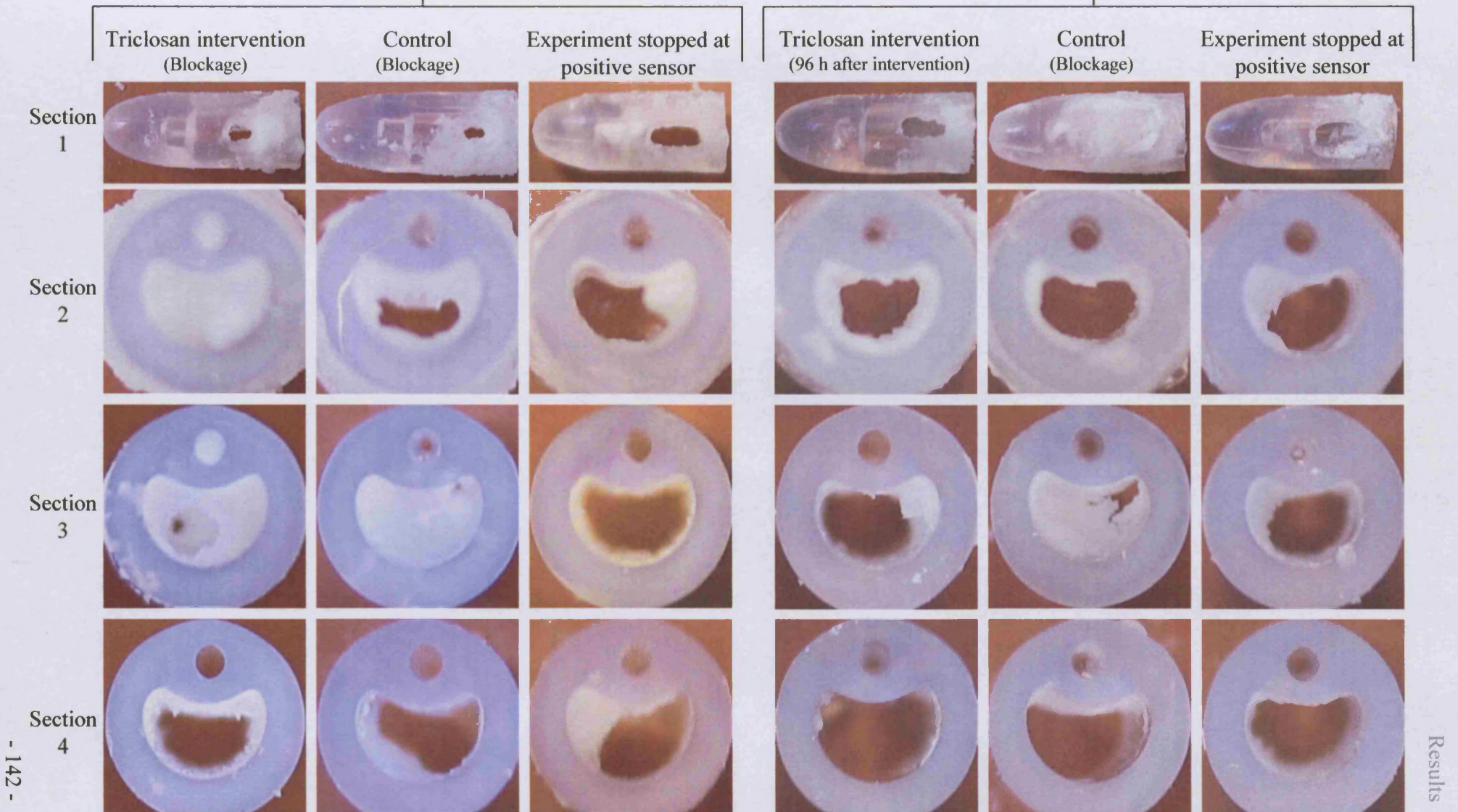


Figure 3.46: Comparison of encrustation on catheter removal at various times for test and control models inoculated with *Pr. mirabilis* RB6.

Pr. vulgaris triclosan intervention (Hy-Shield)

Pv. rettgeri triclosan intervention (Hy-Shield)

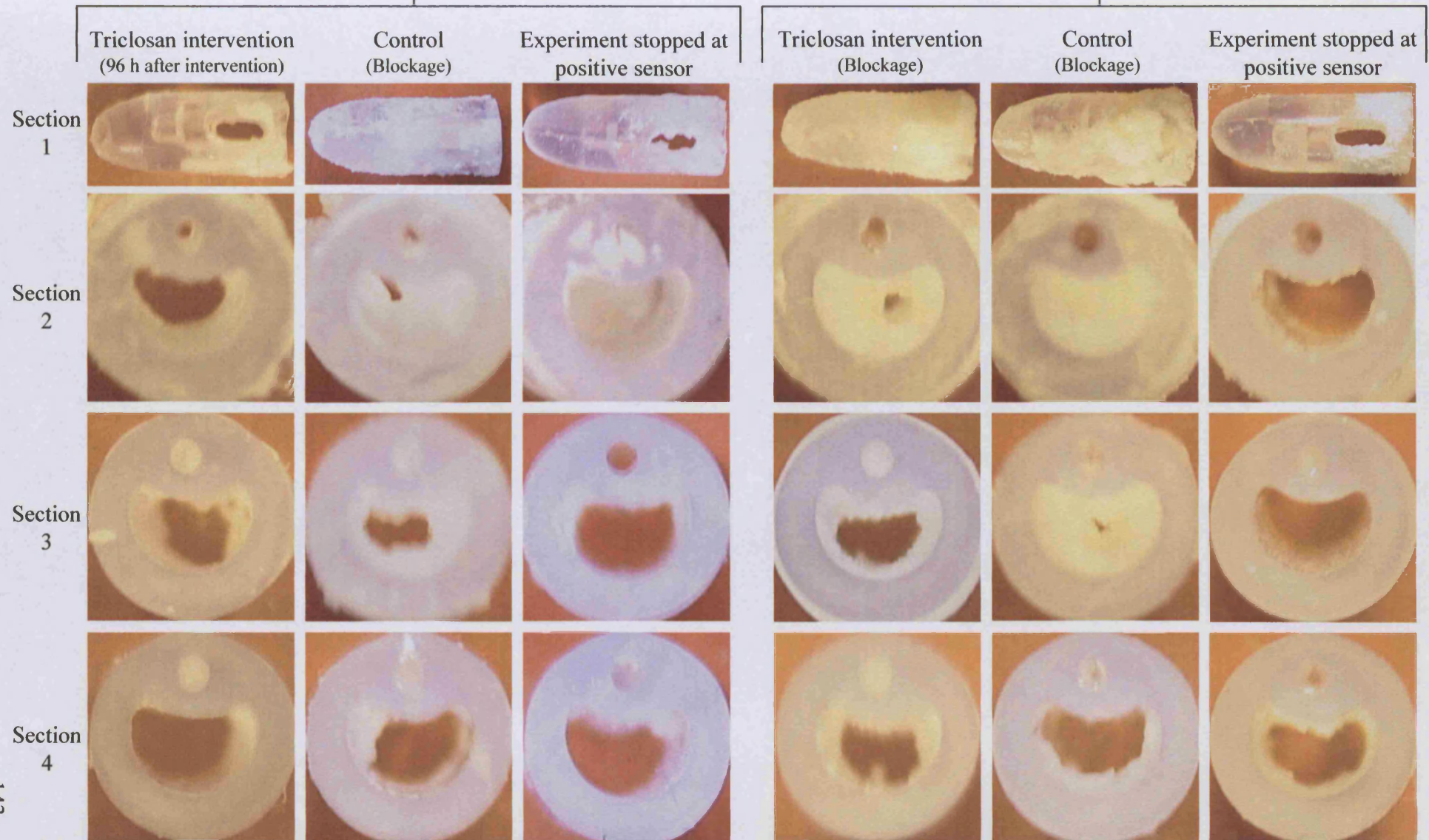
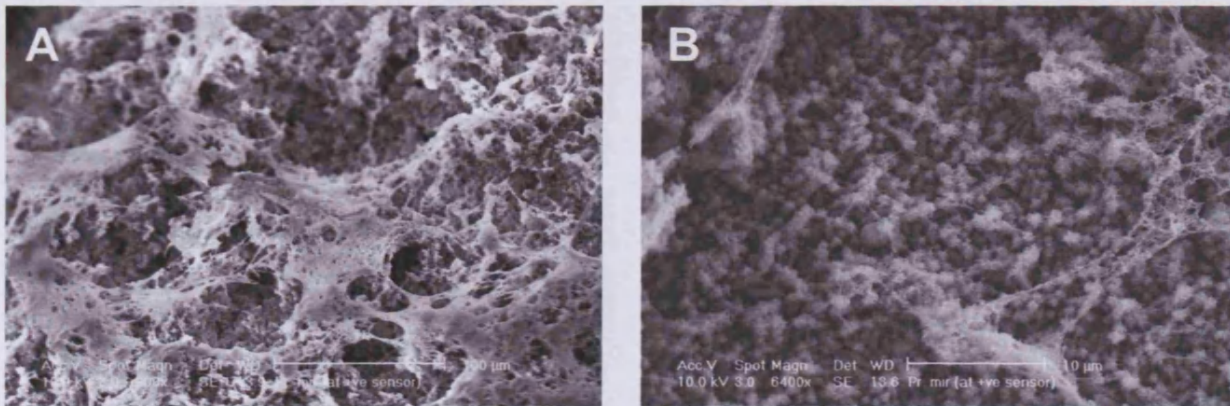


Figure 3.47: Comparison of encrustation on catheter removal at various times for test and control models inoculated with *Pr. vulgaris* SDM2 and *Pv. rettgeri* SDM1.

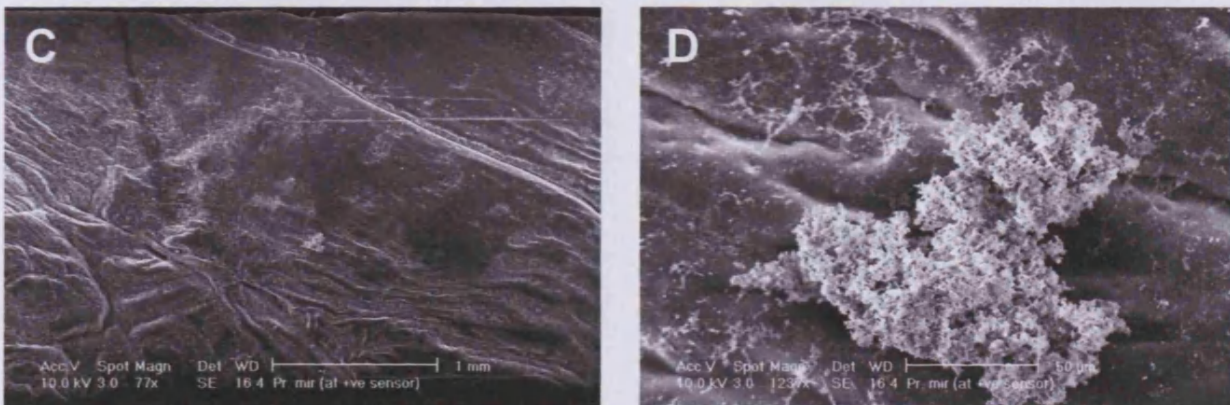
luminal sections. Those catheters which blocked show large amounts of encrustation, particularly on the eyehole sections, while those which drained freely for the duration of the experiment show little difference in encrustation to the sections of catheter removed from the model when the sensor turned blue.

HV-SEM was carried out on catheters and sensors removed from the model system at the point the sensor became positive and at blockage or 96 h post triclosan intervention in experiments using diluted Hy-Shield cream as the triclosan intervention solution. A film of what appears to be EPS was seen on the biofilms formed by all three test species on catheter sections removed from the model at positive sensor (Figures 3.48 – 3.50). Crystalline biofilms consisting of apatite microcrystals were observed on all of the catheter sections examined at the point the sensor became positive. Little evidence of biofilm was seen on the surface of sensors removed from the bag at the point the sensor became positive or on the sensor from the *Pv. rettgeri* triclosan intervention test model (Figures 3.48 – 3.50). The sensors removed from the collection bags of *Pr. mirabilis* and *Pr. vulgaris* triclosan intervention test models (neither of which blocked during the experiment) showed heavy encrustation on the sensor surface which was composed of apatite microcrystals (Figures 3.48 & 3.49).

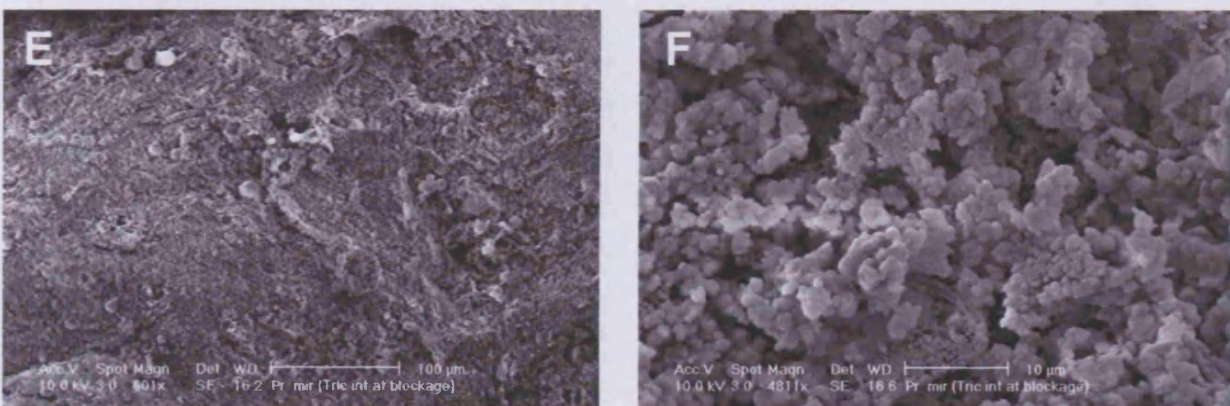
The results of the experiments show that the sensor is able to give early warning of encrustation for all of the three species tested. The Hy-Shield formulation however is not effective in stopping encrustation once it has begun. In contrast triclosan in Na_2CO_3 was effective in preventing *Pr. mirabilis* from forming further encrustation on the catheter during the remainder of the experiment. This suggests that



A & B: Encrusted *Pr. mirabilis* biofilm on an all-silicone catheter at the point the sensor became positive. Some EPS can be seen on the biofilm surface, overlaying the encrustation composed of apatite microcrystals (A). Closer examination of the biofilm shows bacterial cells in association with apatite microcrystals (B).

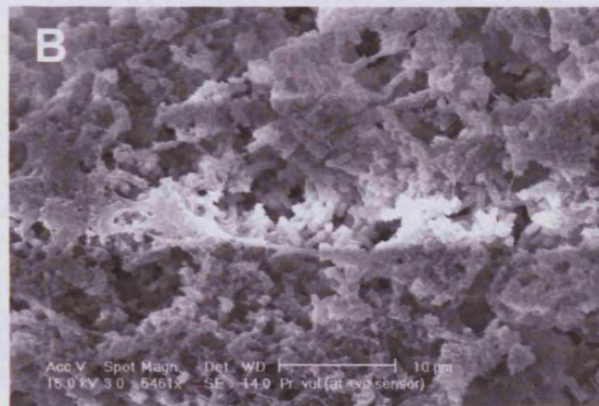
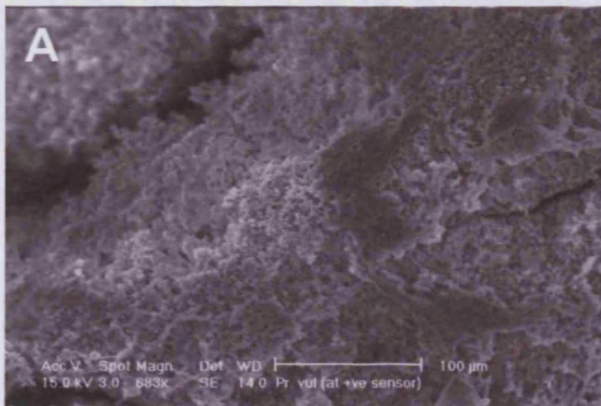


C & D: The surface of a pH sensor from the catheter bag of a bladder model inoculated with *Pr. mirabilis* at the point the sensor became positive. Little encrustation or biofilm can be seen on the sensor (C). Some small pieces of biofilm encrusted with apatite microcrystals were present (D).

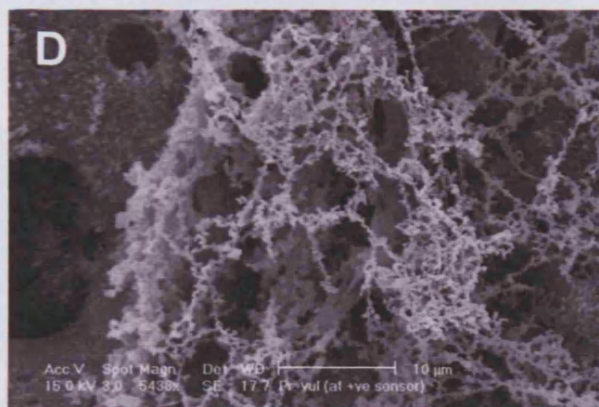
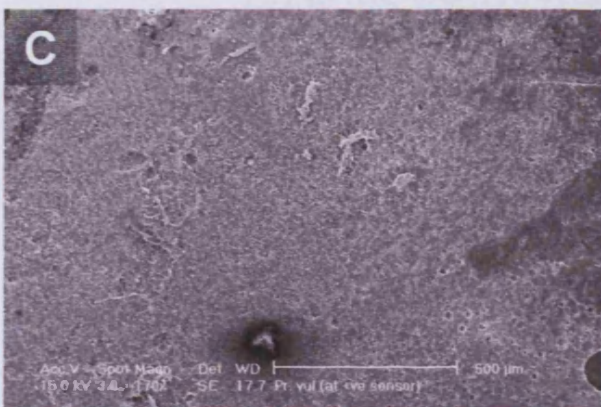


E & F: The surface of a pH sensor from the catheter bag of a bladder model inoculated with *Pr. mirabilis* 96 h after triclosan intervention. The surface of the sensor was heavily encrusted (E). Closer examination of the encrusted biofilm showed the encrustation was composed of apatite microcrystals (F).

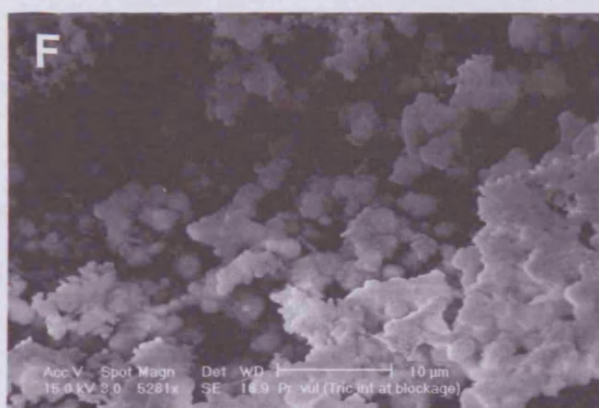
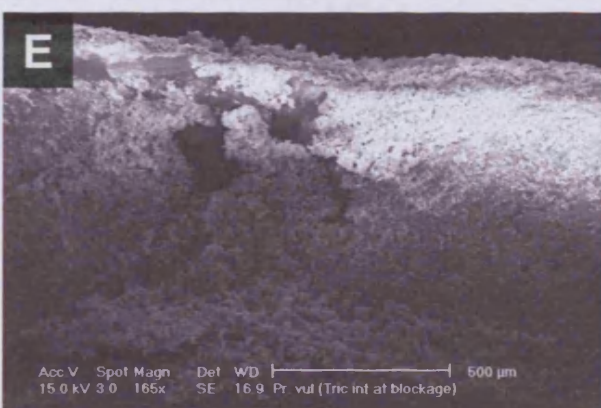
Figure 3.48: HV-SEM images of catheters and pH sensors, from the urine collection bags, from bladder models inoculated with *Pr. mirabilis*. Images A – D are of a catheter and its corresponding pH sensor from an experiment stopped at the point the sensor became positive. Images E & F are of a sensor from an experiment stopped 96 h after the introduction of triclosan into the catheter balloon.



A & B: Encrusted *Pr. vulgaris* biofilm on an all-silicone catheter at the point the sensor became positive. Some EPS can be seen on the biofilm surface, overlaying the encrustation composed of apatite microcrystals (A). Bacterial cells in association with apatite microcrystals are shown in B.

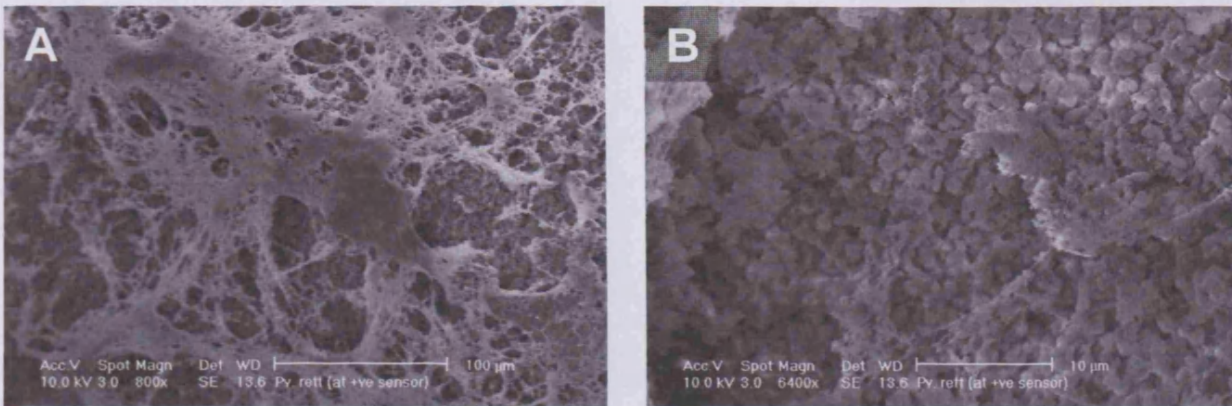


C & D: The surface of a pH sensor from the catheter bag of a bladder model inoculated with *Pr. vulgaris* at the point the sensor became positive. Some biofilm can be seen on the sensor (C). Small pieces of biofilm encrusted with apatite microcrystals were present (D).

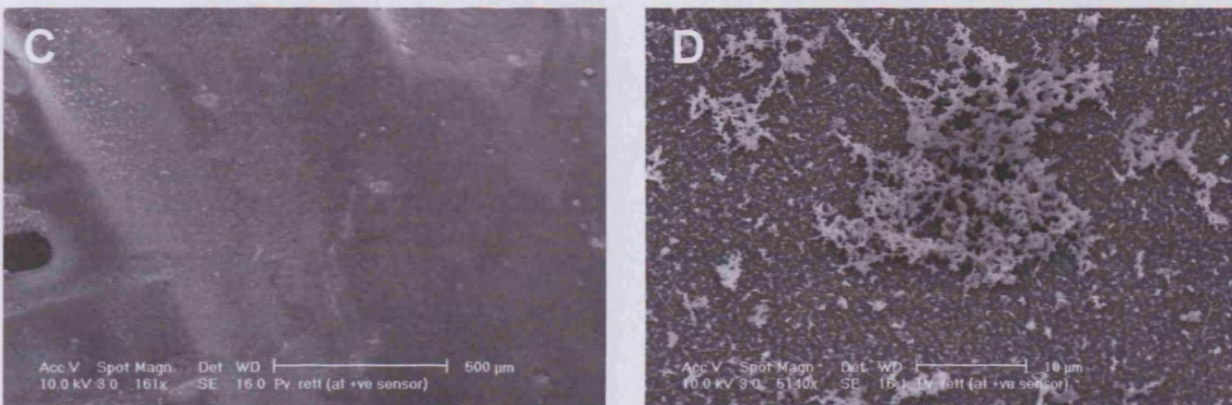


E & F: The surface of a pH sensor from the catheter bag of a bladder model inoculated with *Pr. vulgaris* 96 h after triclosan intervention. The surface of the sensor was heavily encrusted (E). Closer examination of the encrusted biofilm showed the encrustation was composed of apatite microcrystals (F).

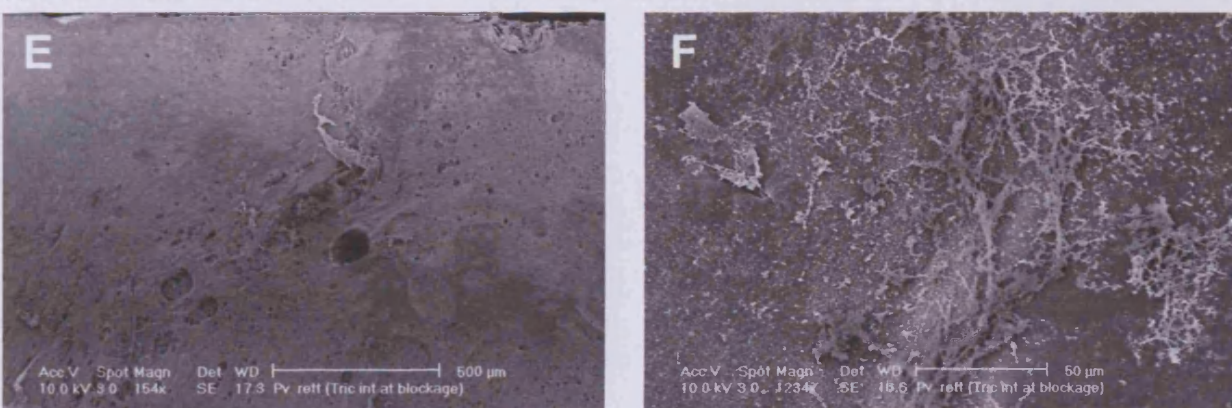
Figure 3.49: HV-SEM images of catheters and pH sensors, from the urine collection bags, from bladder models inoculated with *Pr. vulgaris*. Images A – D are of a catheter and its corresponding pH sensor from an experiment stopped at the point the sensor became positive. Images E & F are of a sensor from an experiment stopped 96 h after the introduction of triclosan into the catheter balloon.



A & B: Encrusted *Pv. rettgeri* biofilm on an all-silicone catheter at the point the sensor became positive. Some EPS can be seen on the biofilm surface, overlaying the encrustation composed of apatite microcrystals (A). Closer examination of the biofilm shows bacterial cells in association with apatite microcrystals (B).



C & D: The surface of a pH sensor from the catheter bag of a bladder model inoculated with *Pv. rettgeri* at the point the sensor became positive. Little encrustation or biofilm can be seen on the sensor (C). Closer examination of the sensor shows some biofilm encrusted with apatite microcrystals (D).



E & F: The surface of a pH sensor from the catheter bag of a bladder model inoculated with *Pv. rettgeri* at catheter blockage (triclosan intervention). Little encrustation or biofilm is present on the sensor (E). Thin biofilm with some apatite microcrystal encrustation is shown in F.

Figure 3.50: HV-SEM images of catheters and pH sensors, from the urine collection bags, from bladder models inoculated with *Pv. rettgeri*. Images A – D are of a catheter and its corresponding pH sensor from an experiment stopped at the point the sensor became positive. Images E & F are of a sensor removed the urine collection bag at catheter blockage from an experiment in which triclosan was introduced into the catheter balloon.

intervention with triclosan in Na_2CO_3 could allow the catheter to remain in place after a positive sensor signal when infection is due to triclosan sensitive species such as *Pr. mirabilis* or *Pr. vulgaris* (but not *Pv. rettgeri*).

3.5 The effectiveness of the pH sensor in predicting catheter blockage by species that encrust catheters slowly.

Experiments were performed to test the ability of the pH sensor to predict catheter blockage by *M. morgani*, *Staph. aureus* and *Staph. saprophyticus*. These species had been previously identified as able to encrust catheters but more slowly than those tested in Section 3.4. Bladder models were inoculated with 100 μ l of a 4 h logarithmic phase culture of each test organism in artificial urine. Urine samples were taken from the bladder chamber and urine collection bag at 24 h intervals until 168 h or blockage. The pHs of all the urine samples were measured and the viable bacterial cell populations of urine samples determined. Digital photographs of the pH sensors in the urine bags were taken each time point the urine pH was measured. Digital photography was also used to assess the extent of encrustation on catheters at blockage or 168 h.

Figure 3.51 shows the variation in urinary pH in the bladder chamber and collection bag of models infected with *M. morgani* RB15. It can be seen that the pH of the urine in the collection bag was more alkaline than the urine in the bladder chamber for the duration of the experiment. All three catheters blocked and the mean time to blockage was 149.3 h. The sensor became positive prior to blockage in all three replicates. The mean time (\pm SE) of the interval between the positive signal and catheter blockage was 37.3 h (\pm 2.85 h).

The urinary pH data recorded from models inoculated with *Staph. aureus* P10 6/9 is displayed in Figure 3.52. None of the three replicate catheters blocked during the 168

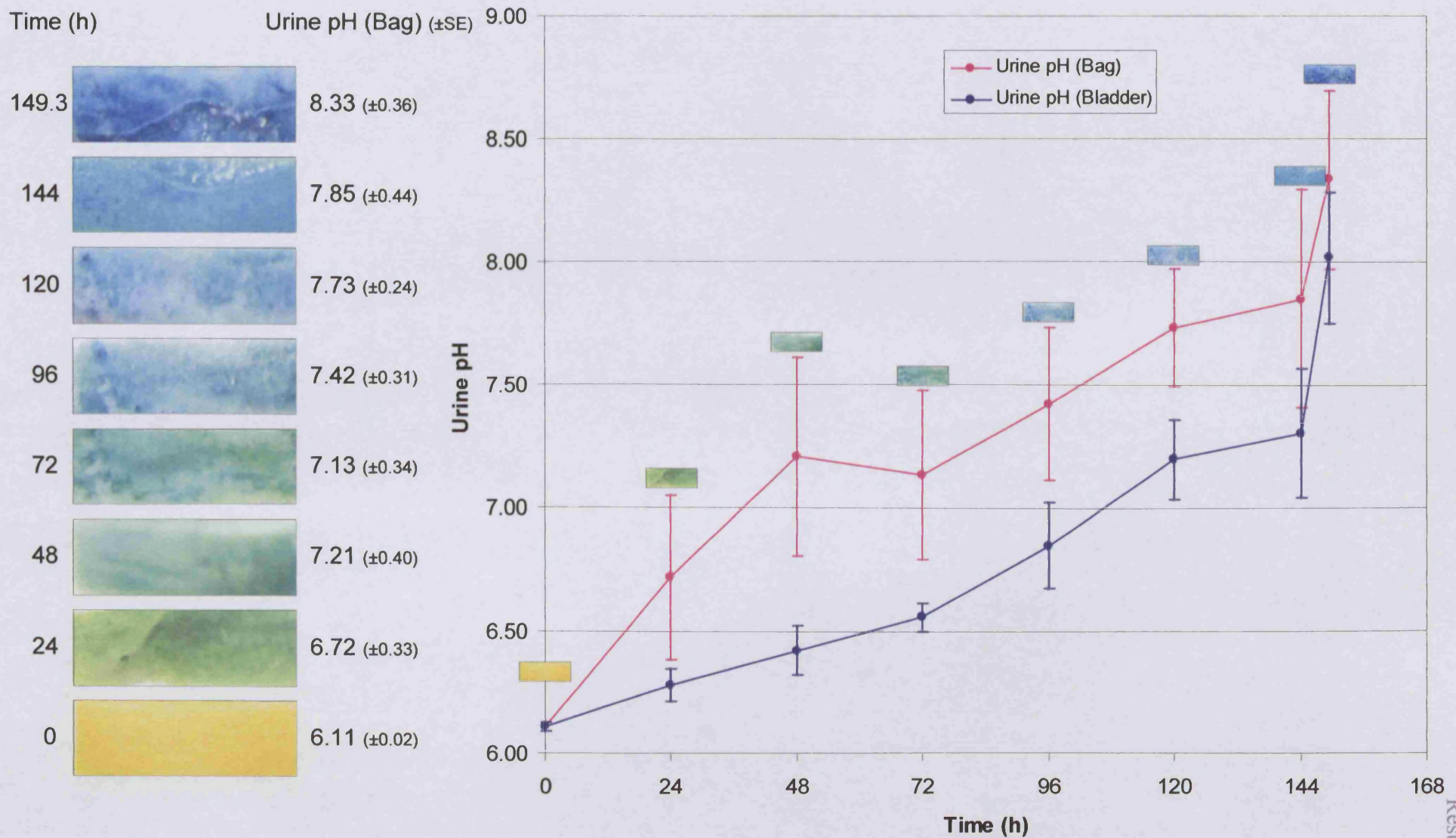


Figure 3.51: Urinary pH variation in the bladder and urine collection bag of models inoculated with *M. morganii* RB15 until blockage (149.3 h \pm 5.2). The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated. The sensor signal was considered to be positive at 96 h.

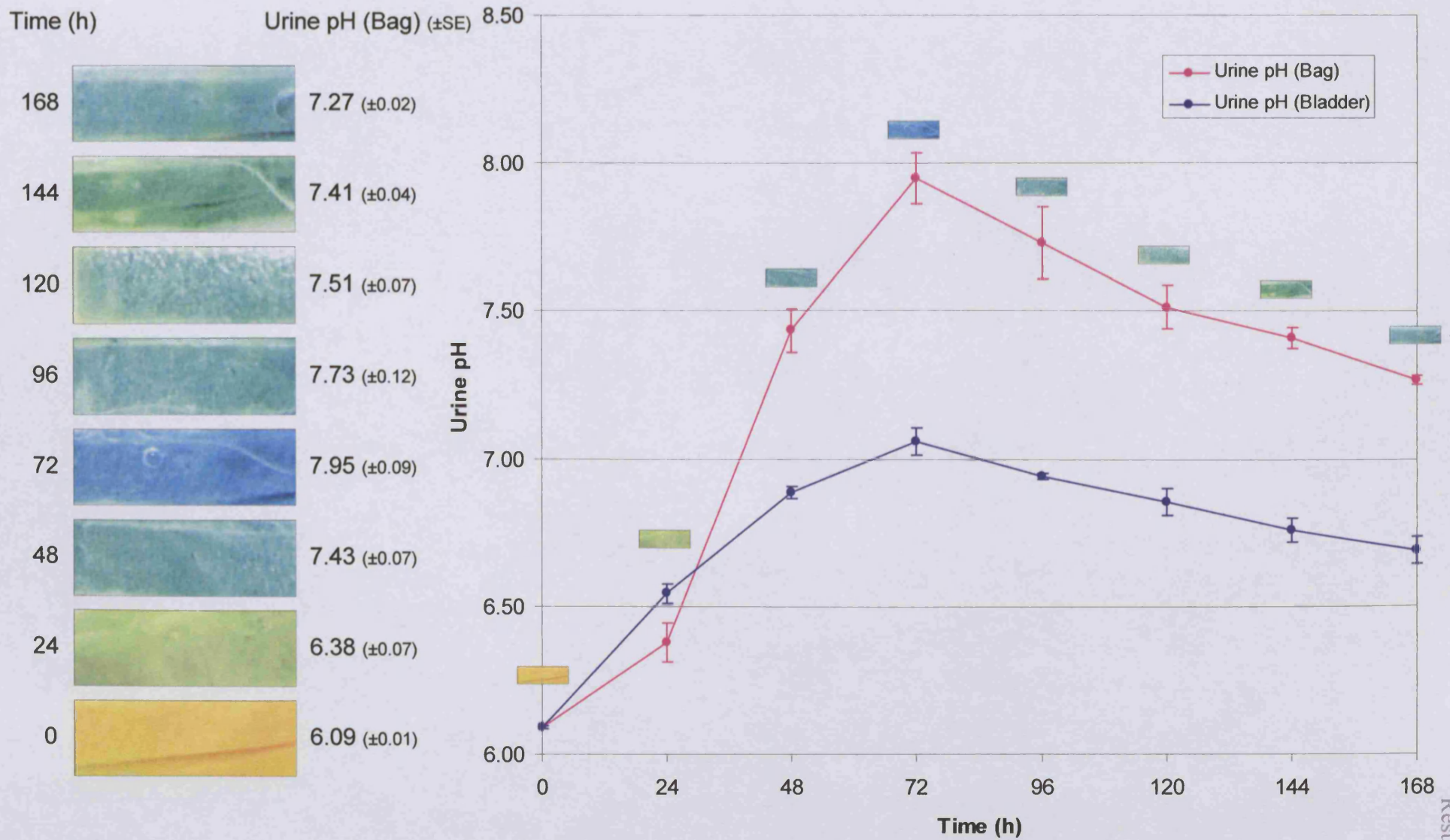


Figure 3.52: Urinary pH variation in the bladder and urine collection bag of models inoculated with *Staph. aureus* P10 6/9 over 168 h. The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated.

h experimental period. The sensors in two of the three replicates became positive during the experiment, although the signal intensity reduced as the urinary pH in the collection bag fell towards the end of the experiment. One sensor eventually became negative again.





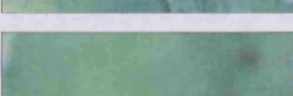


Figure 3.53 shows the urinary pH data recorded for models infected with *Staph. saprophyticus* ZEC17. All three catheters blocked with a mean time of 137 h. The sensor became positive prior to blockage in all three replicates, with the mean time (\pm SE) interval between the sensor judged to be positive and the catheter blocking being 41.0 h (\pm 15.0 h).

Digital photographs taken of catheters removed from the model at blockage or after 168 h are shown in Figure 3.54. Extensive encrustation is visible on the blocked *M. morgani* and *Staph. saprophyticus* catheters, particularly on the eyehole and lumen sections nearest the eyehole. The images of catheters from models inoculated with *Staph. aureus* show that although the catheters were not blocked at 168 h, crystalline biofilm was close to occluding the catheter lumen.

3.5.1 The reaction of the pH sensor to commonly occurring urinary tract pathogens that do not produce crystalline biofilms.

The colour of pH sensors placed in the urine collection bags of models infected with five commonly occurring urinary tract pathogens (*E. coli*, *Ent. faecalis*, *Ps. aeruginosa*, *Pv. stuartii* and *K. pneumoniae*) that had previously been shown not to generate catheter encrustation was monitored over 120 h. The experiments were

Time (h) Urine pH (Bag) (\pm SE)

137		7.85 (\pm 0.10)
120		7.76 (\pm 0.04)
96		7.86 (\pm 0.05)
72		7.64 (\pm 0.15)
48		7.45 (\pm 0.15)
24		6.30 (\pm 0.06)
0		6.14 (\pm 0.01)

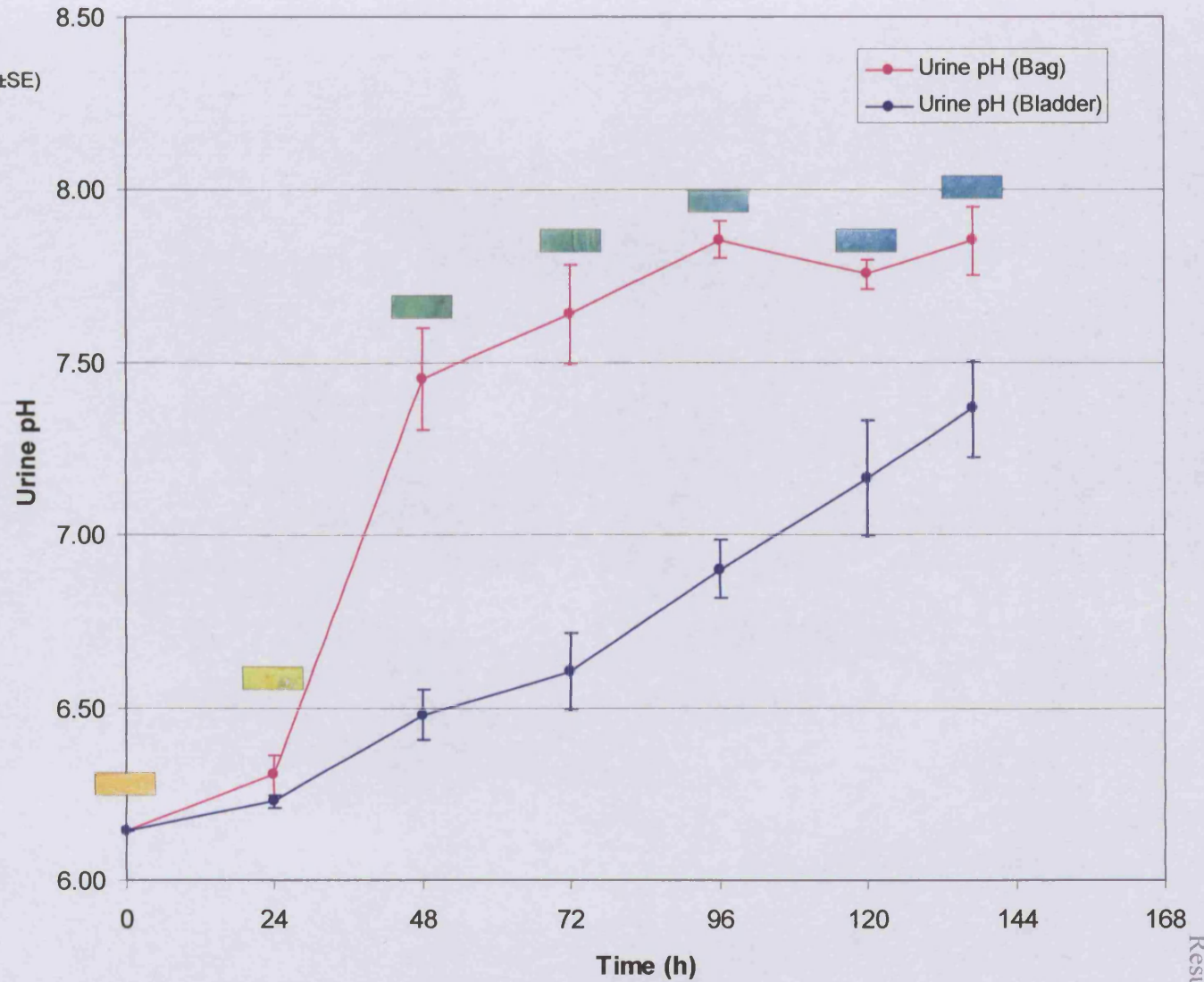


Figure 3.53: Urinary pH variation in the bladder and urine collection bag of models inoculated with *Staph. saprophyticus* ZEC17 until blockage (137.0 h \pm 15.0). The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated. The signal from the sensor was judged to be positive at 96 h.

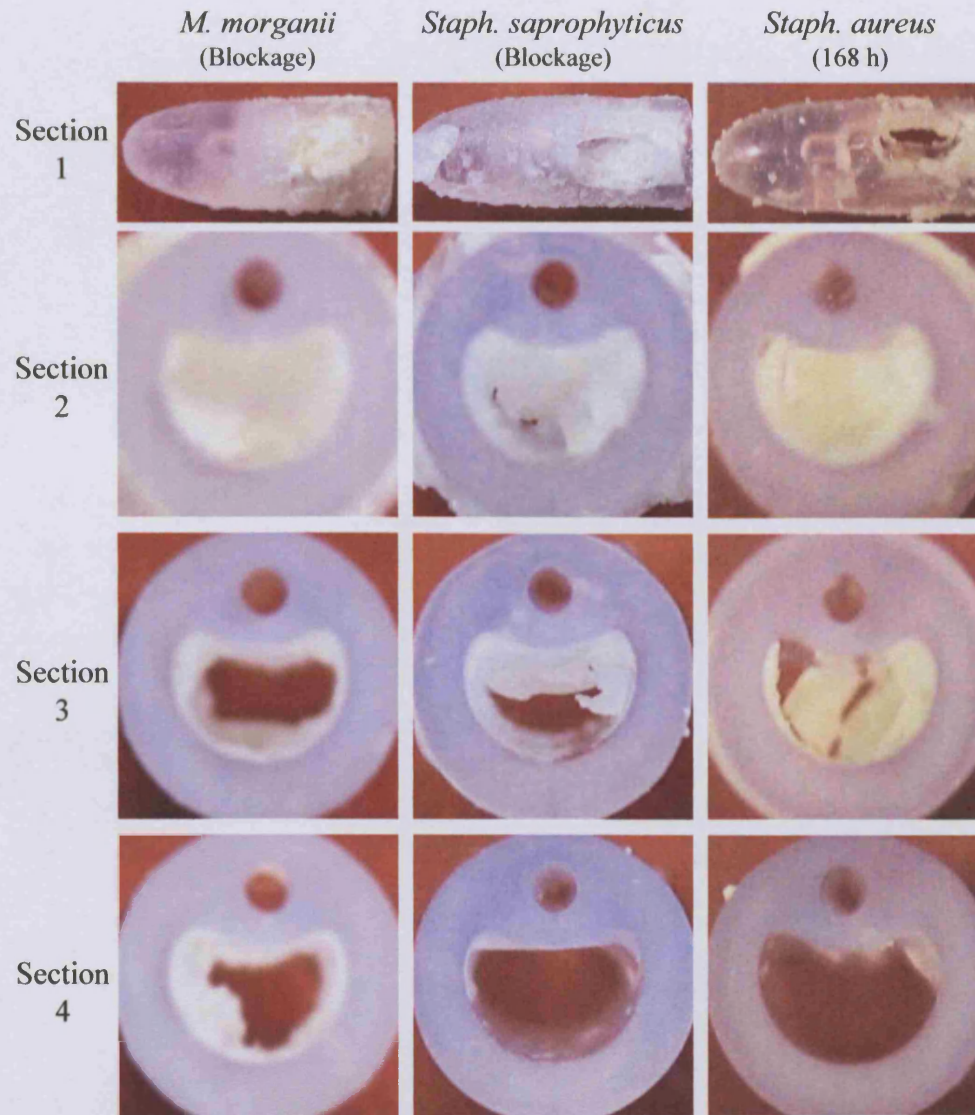


Figure 3.54: Digital photographs of catheters removed from the bladder model at blockage or after 168 h.

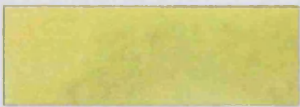
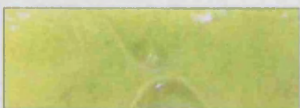
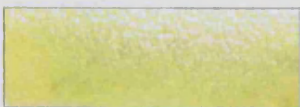

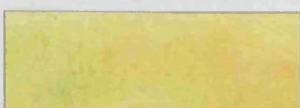

performed in triplicate for each species and samples were collected as described in Section 3.5. Three replicate models were operated for each species for 120 h.

The results of the experiments involving urease negative *E. coli* RB13 and *Ent. faecalis* RB5 are shown in Figures 3.55 & 3.56. For both species the pH of the urine remained below 6.2 in both the bladder and collection bag for the duration of the experiments. The sensor remained negative, with little difference in colour throughout the experiments.

In the experiments involving *Ps. aeruginosa* RB16, *Pv. stuartii* RB14 and *K. pneumoniae* SDM17 (Figures 3.57 – 3.59) the urine pH in the bladder chamber did not exceed 6.4 during the experiment. The pHs recorded for the urine in the collection bags were higher and this was reflected in the colour of the pH sensors. The sensors of one of the three replicates of both the *Ps. aeruginosa* and *Pv. stuartii* infected models became positive during the experiments. None of the sensors in the *K. pneumoniae* infected models became positive, although colour change from yellow to green was observed.

Digital photographs taken of catheters removed from the model after 120 h are shown in Figure 3.60. No encrustation was present on any of the catheters and little biofilm was observed on or immediately below the catheter eyehole. Extensive mucoid biofilm was observed on catheter sections from further down the lumen of catheters from *Ps. aeruginosa* and *K. pneumoniae* infected models.

Time (h) Urine pH (Bag) (\pm SE)

120		6.14 (\pm 0.01)
96		6.15 (\pm 0.01)
72		6.14 (\pm 0.03)
48		6.17 (\pm 0.02)
24		6.09 (\pm 0.01)
0		6.08 (\pm 0.01)

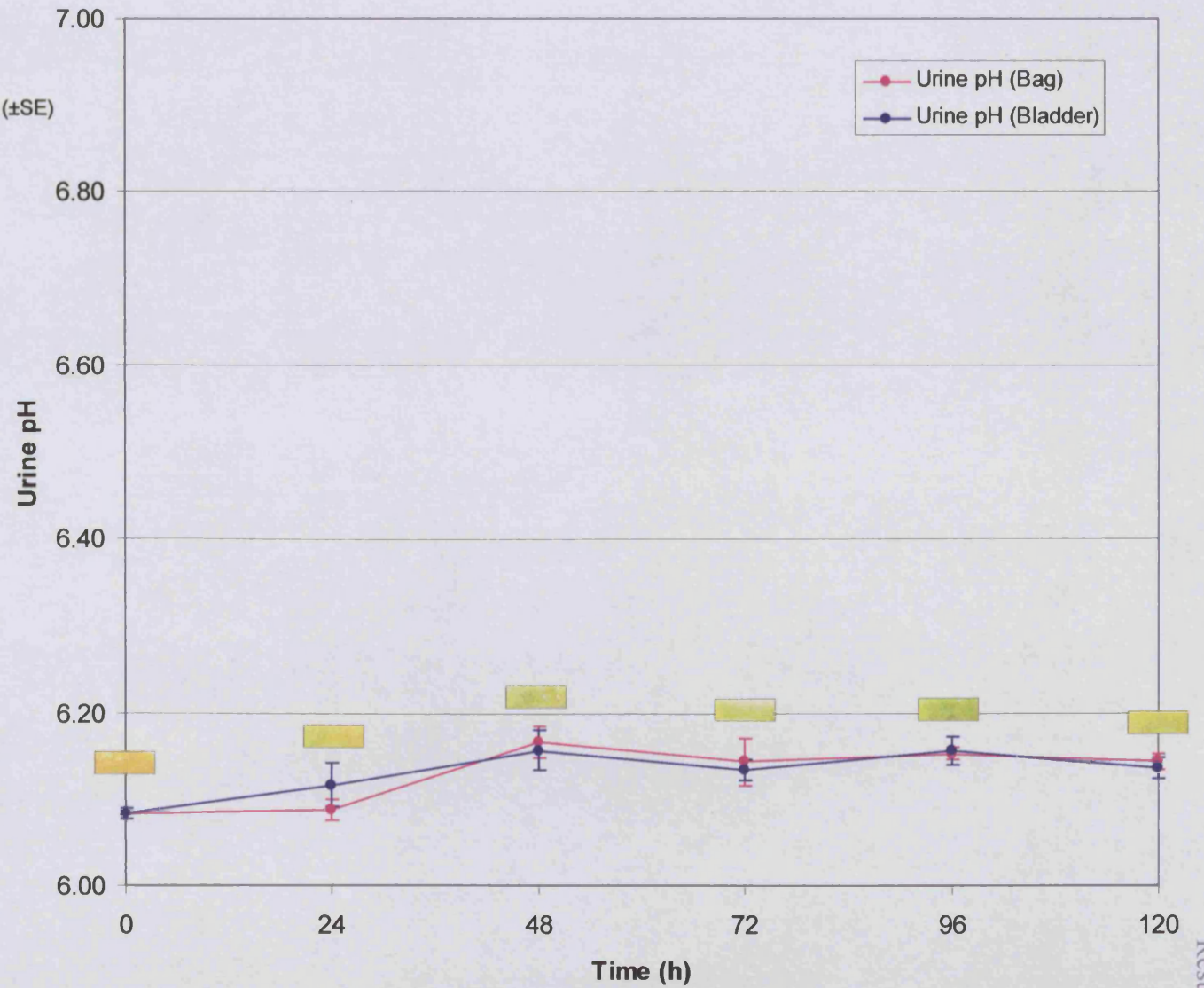



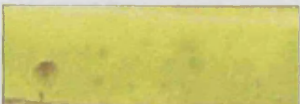




Figure 3.55: Urinary pH variation in the bladder and urine collection bag of models inoculated with *E. coli* RB13 over 120 h. The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated.

Time (h) Urine pH (Bag) (\pm SE)

120		6.17 (\pm 0.03)
96		6.16 (\pm 0.03)
72		6.17 (\pm 0.03)
48		6.12 (\pm 0.01)
24		6.11 (\pm 0.04)
0		6.11 (\pm 0.03)

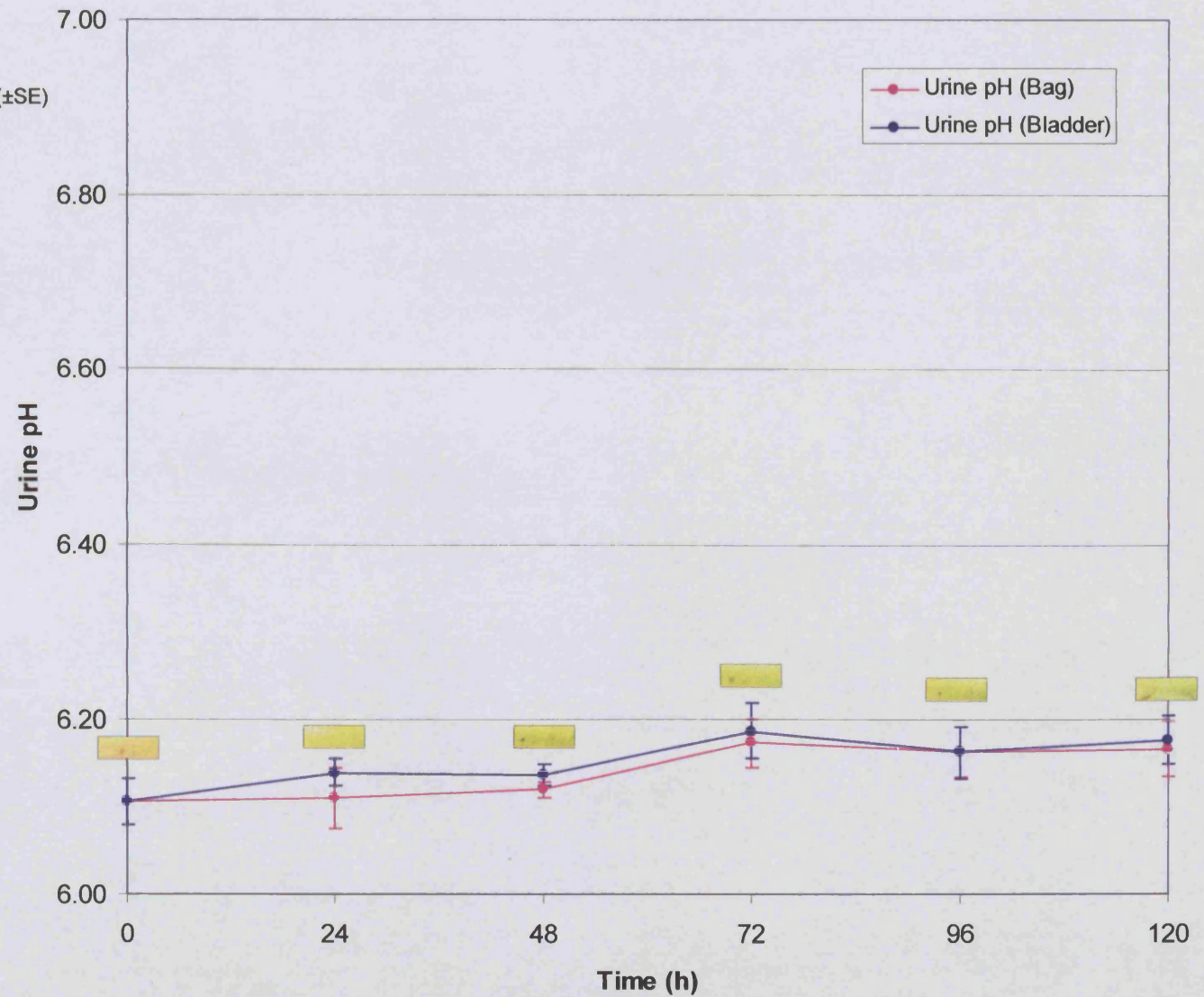








Figure 3.56: Urinary pH variation in the bladder and urine collection bag of models inoculated with *Ent. faecalis* RB5 over 120 h. The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated.

Time (h) Urine pH (Bag) (\pm SE)

120		6.89 (\pm 0.08)
96		6.90 (\pm 0.11)
72		6.85 (\pm 0.18)
48		6.90 (\pm 0.12)
24		6.23 (\pm 0.05)
0		6.10 (\pm 0.01)

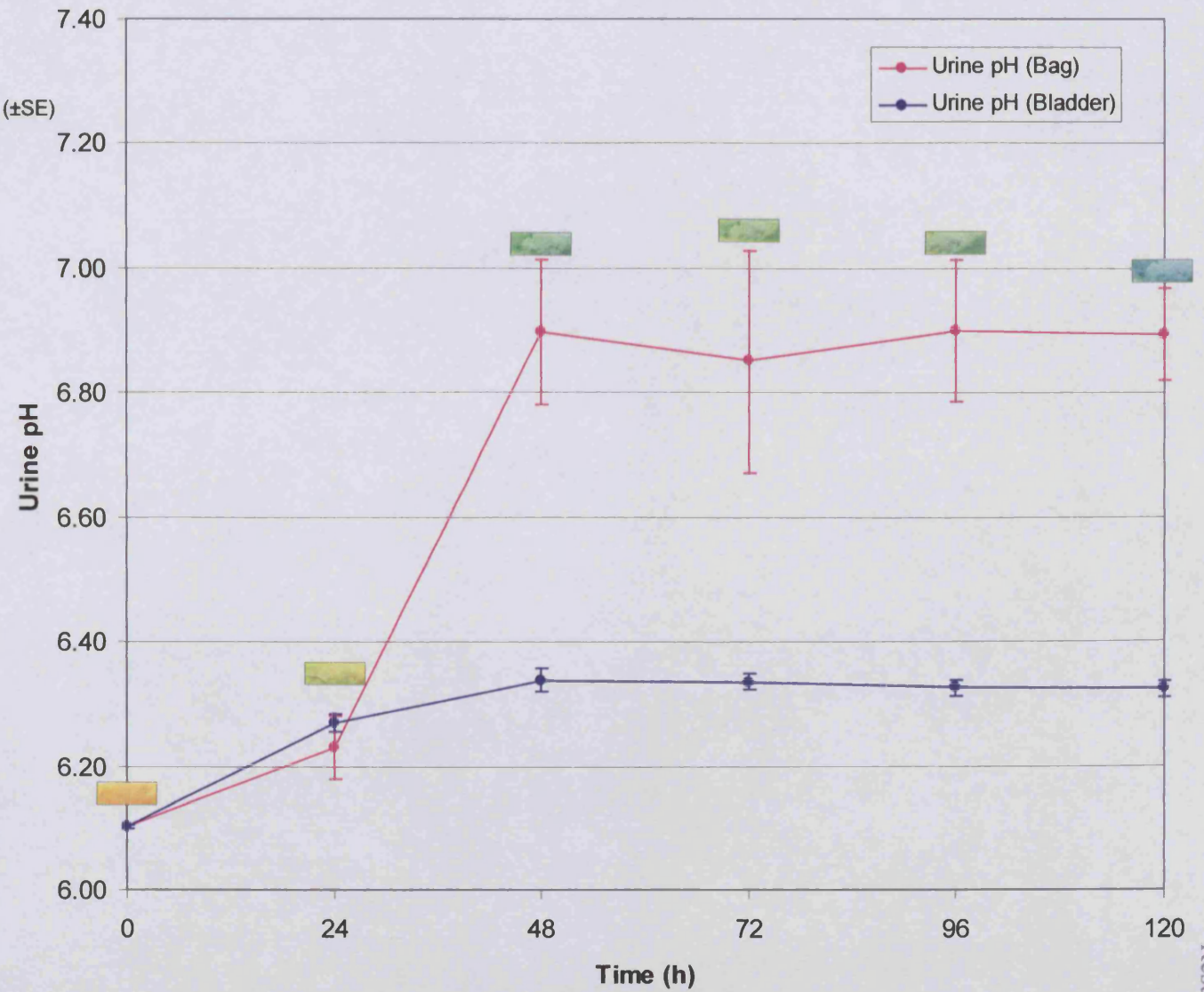


Figure 3.57: Urinary pH variation in the bladder and urine collection bag of models inoculated with *Ps. aeruginosa* RB16 over 120 h. The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated. The sensor was considered to be positive at 120 h.

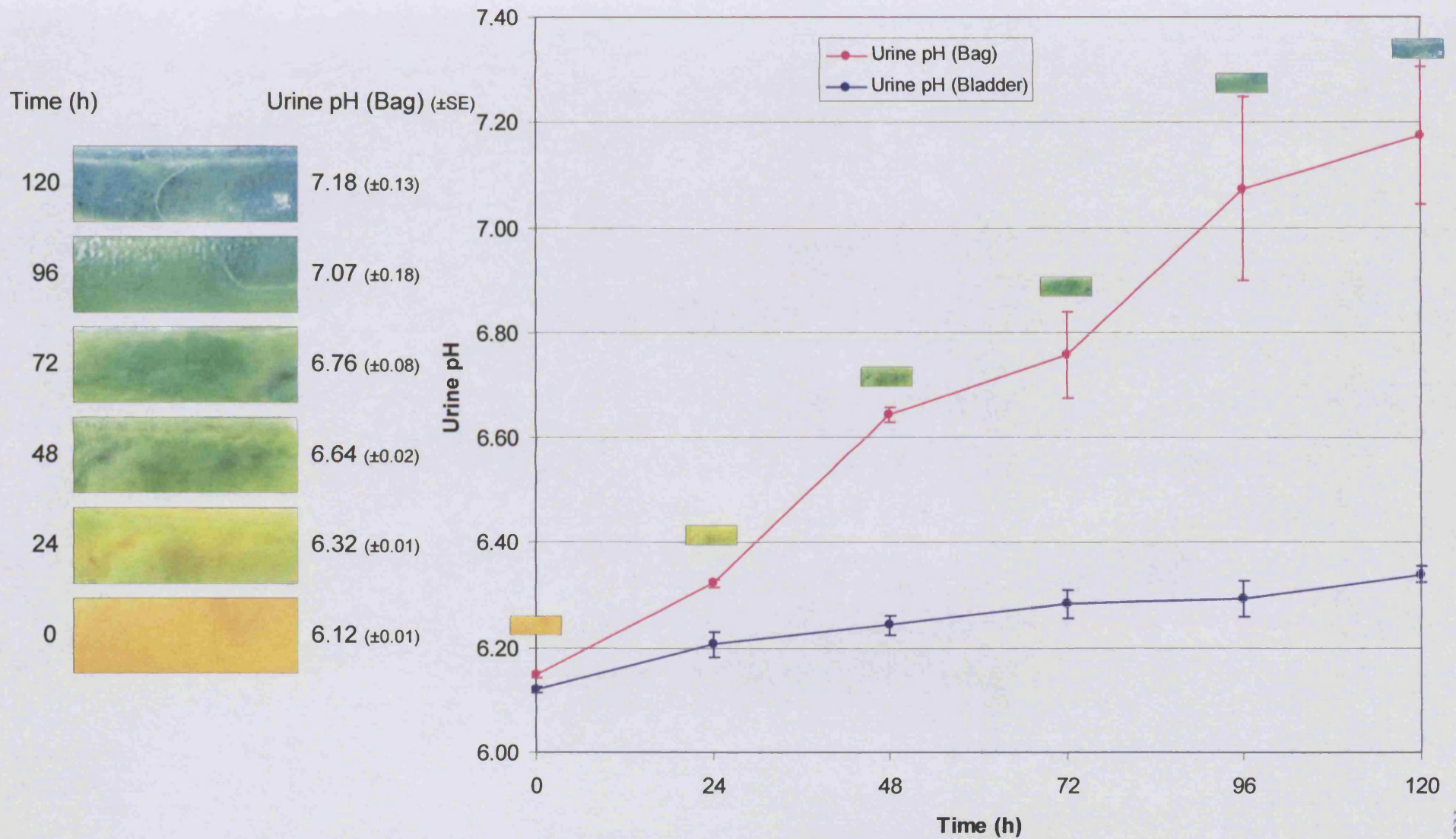








Figure 3.58: Urinary pH variation in the bladder and urine collection bag of models inoculated with *Pv. stuartii* RB14 over 120 h. The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated. The sensor was considered to be positive at 120 h.

Time (h) Urine pH (Bag) (\pm SE)

120		6.52 (\pm 0.01)
96		6.53 (\pm 0.04)
72		6.61 (\pm 0.21)
48		6.33 (\pm 0.05)
24		6.14 (\pm 0.03)
0		6.10 (\pm 0.01)

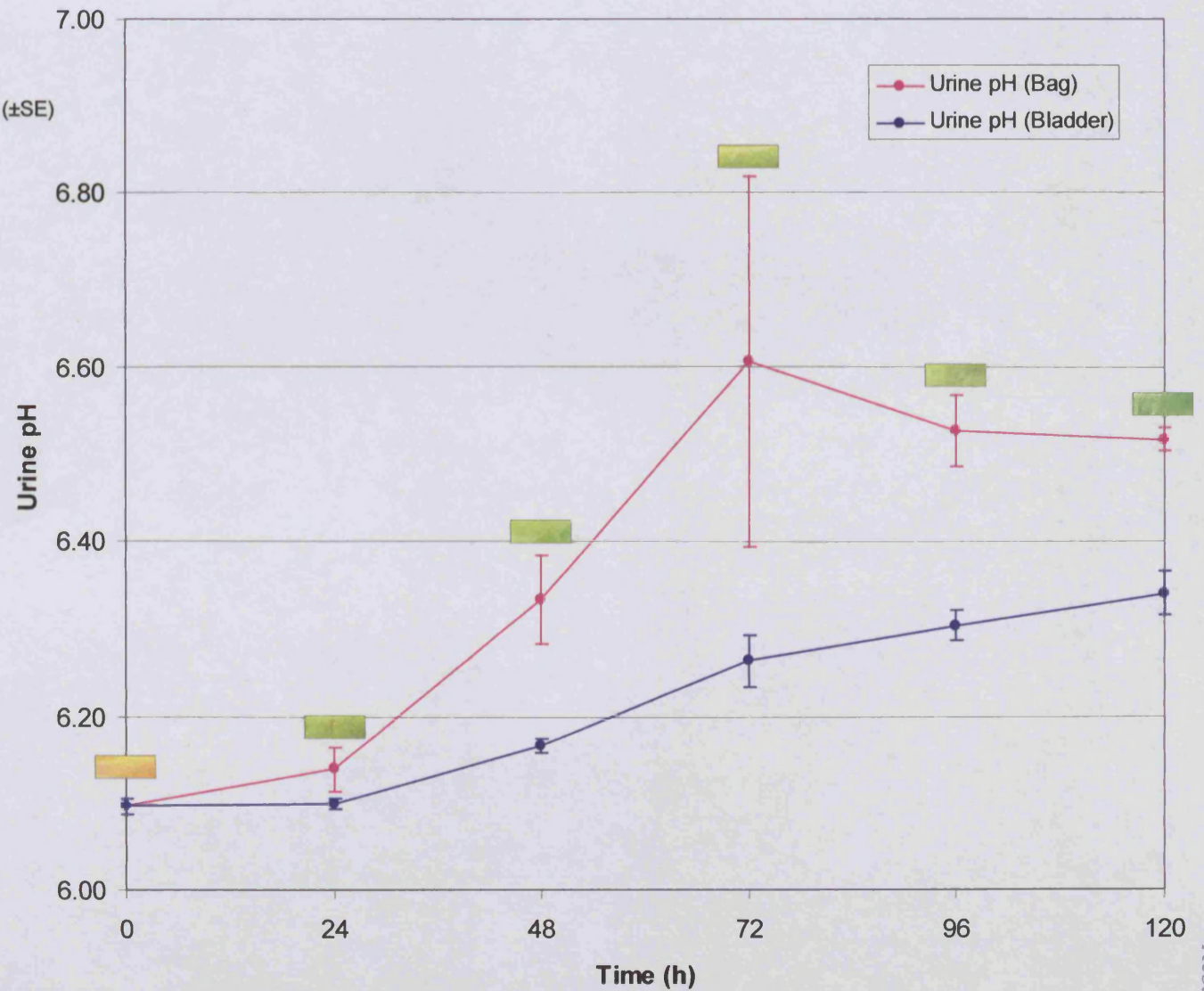


Figure 3.59: Urinary pH variation in the bladder and urine collection bag of models inoculated with *K. pneumoniae* SDM17 over 120 h. The colour variation of a pH sensor placed in the urine collection bag is also shown. *Each data point is the mean of 3 replicates, with the standard error indicated. The sensor was judged to be negative throughout the experimental period.

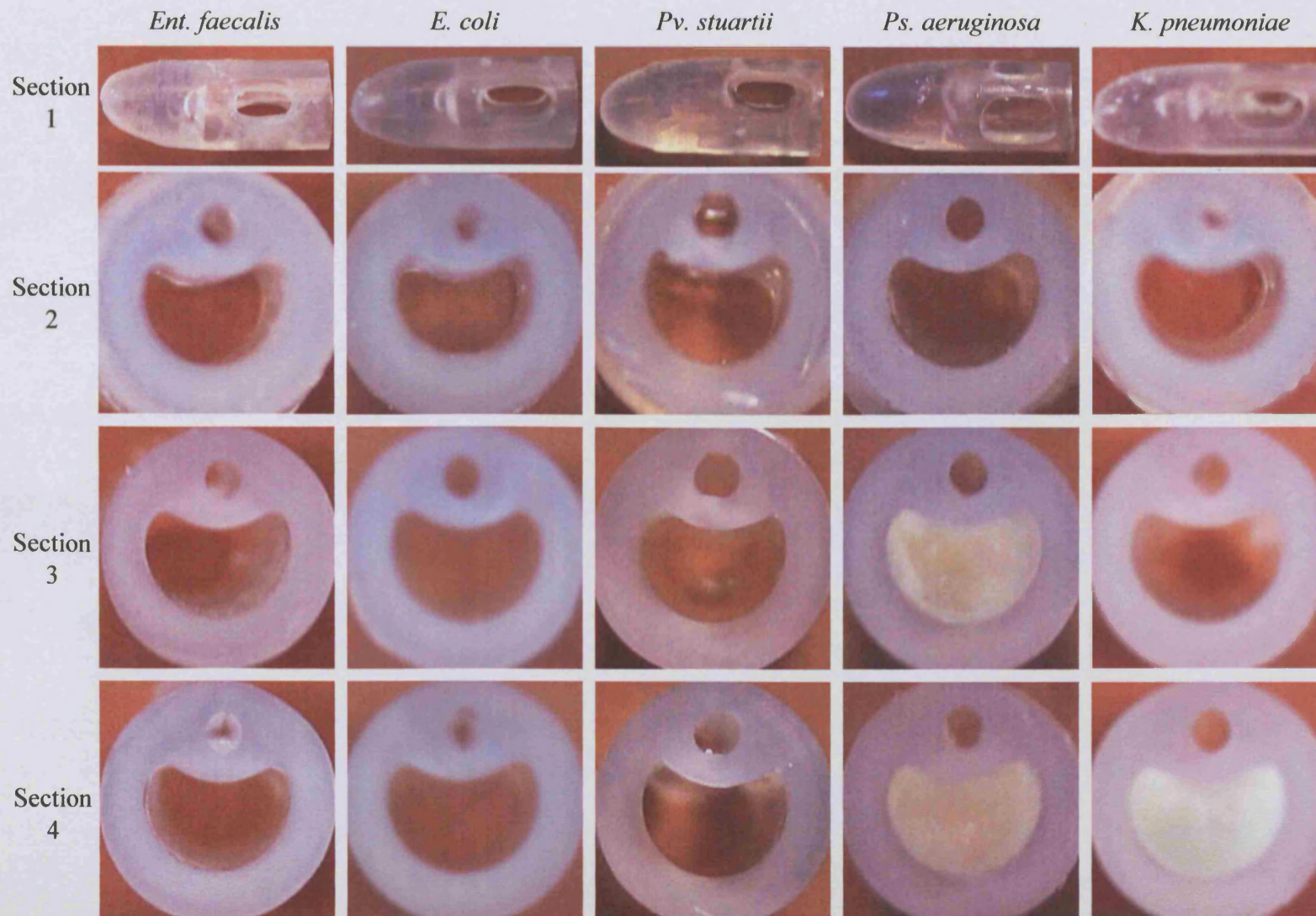


Figure 3.60: Digital photographs of catheters removed from the bladder model after 120 h.

3.6 The modulation of *Pr. mirabilis* induced catheter blockage in multi-species catheter communities.

The microbial communities isolated from the urine of long-term catheterised patients are often composed of several species (Clayton *et al.*, 1982; Warren *et al.*, 1982; Jewes *et al.*, 1988). A recent study by Mathur *et al.* (2006a) on a group of long-term catheterised patients with a history of *Proteus* infection found that the presence of *Pr. mirabilis* in the catheterised urinary tract did not necessarily lead to catheter blockage. The life-spans of the catheters ranged from 2 – 98 days and in some cases catheters did not block and were changed on schedule. In view of these observations, it was decided to examine the possibility that the other species present in the mixed communities infecting these patients can moderate the rate at which *Pr. mirabilis* blocks catheters. Communities from patients with *Pr. mirabilis* who did not experience frequent catheter blockage were reconstituted in the bladder model and blockage times compared to those of the *Pr. mirabilis* isolate alone.

The microbial communities used in these experiments were collected during the prospective study conducted by Mathur *et al.* (2006a). These authors had taken urine samples on a weekly basis, subjected them to routine bacteriological analysis and stored the isolates at -80 °C. It was thus possible to reconstitute the urinary flora (except for non-culturables) of these patients and examine their ability to block catheters *in vitro*. Experiments were conducted in bladder models on the reconstituted communities from three patients.

Bladder models were inoculated with 1 ml of a 4 h logarithmic culture in artificial urine of each isolate from the reconstituted community. Control models were inoculated with just the *Pr. mirabilis* isolate from each community (1 ml). At 24 h intervals, up to a maximum of 168 h, or at blockage if it occurred, urine samples were taken from the bladder chamber. The pHs of the urine samples were measured and the viable bacterial cell populations of each isolate determined. The extent of encrustation present on catheter sections was visualised using digital photography and LV-SEM. Statistical analysis (Two Sample t-test) was used to determine if there was any significant difference between the mean blockage times of the test and control catheters.

The data collected from models inoculated with the reconstituted community from patient 6 (*Pr. mirabilis* (AK) and *Ent. faecalis* (O)) are shown in Figures 3.61 & 3.62. Blockage occurred in all three replicates but the mean blockage time of the test catheters (55 h) was significantly greater ($P=0.037$) than that of the control (25 h). There was no significant difference between the mean urine pH initially ($P=0.659$), after 24 h ($P=0.120$) and at blockage ($P=0.270$) for the test and control models and this was also true for the mean numbers of *Pr. mirabilis* (AK) cells present initially ($P=0.613$), after 24 h ($P=0.413$) and at blockage ($P=0.136$).

Figure 3.63 shows the data from experiments using the reconstituted community from patient 9 (*Pr. mirabilis* (AO), *E. coli* (AM), *K. pneumoniae* (AN) and *Ent. faecalis* (AAC)). All of the catheters blocked and there was no significant difference ($P=0.643$) between the mean blockage times of the test (40 h) and control (35 h)

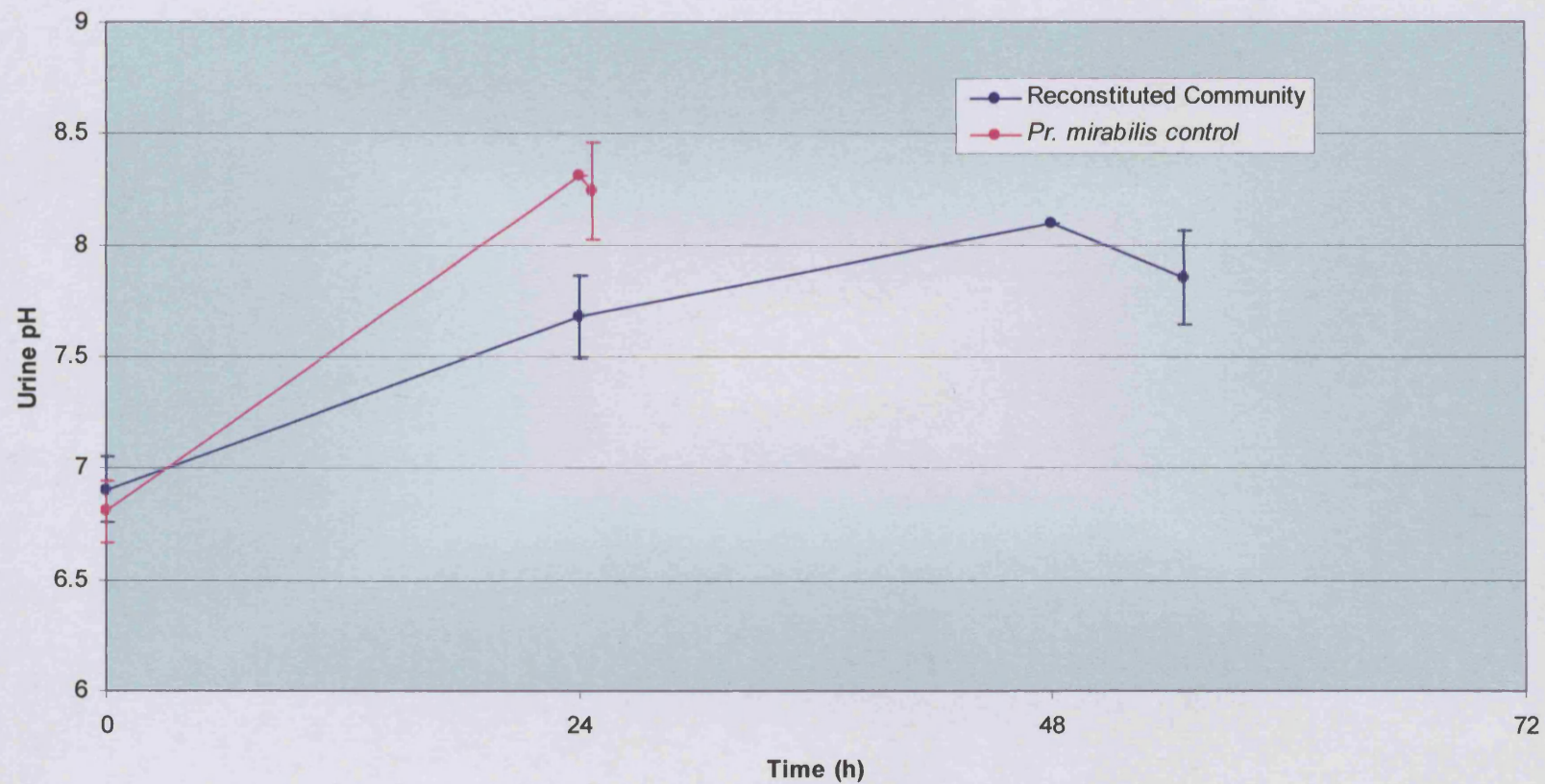


Figure 3.61: The pH of urine samples from models inoculated with a reconstituted community (Patient 6) consisting of *Pr. mirabilis* and *Ent. faecalis*, and a control inoculated with the *Pr. mirabilis* isolate. The means \pm SE were calculated from three replicated experiments.

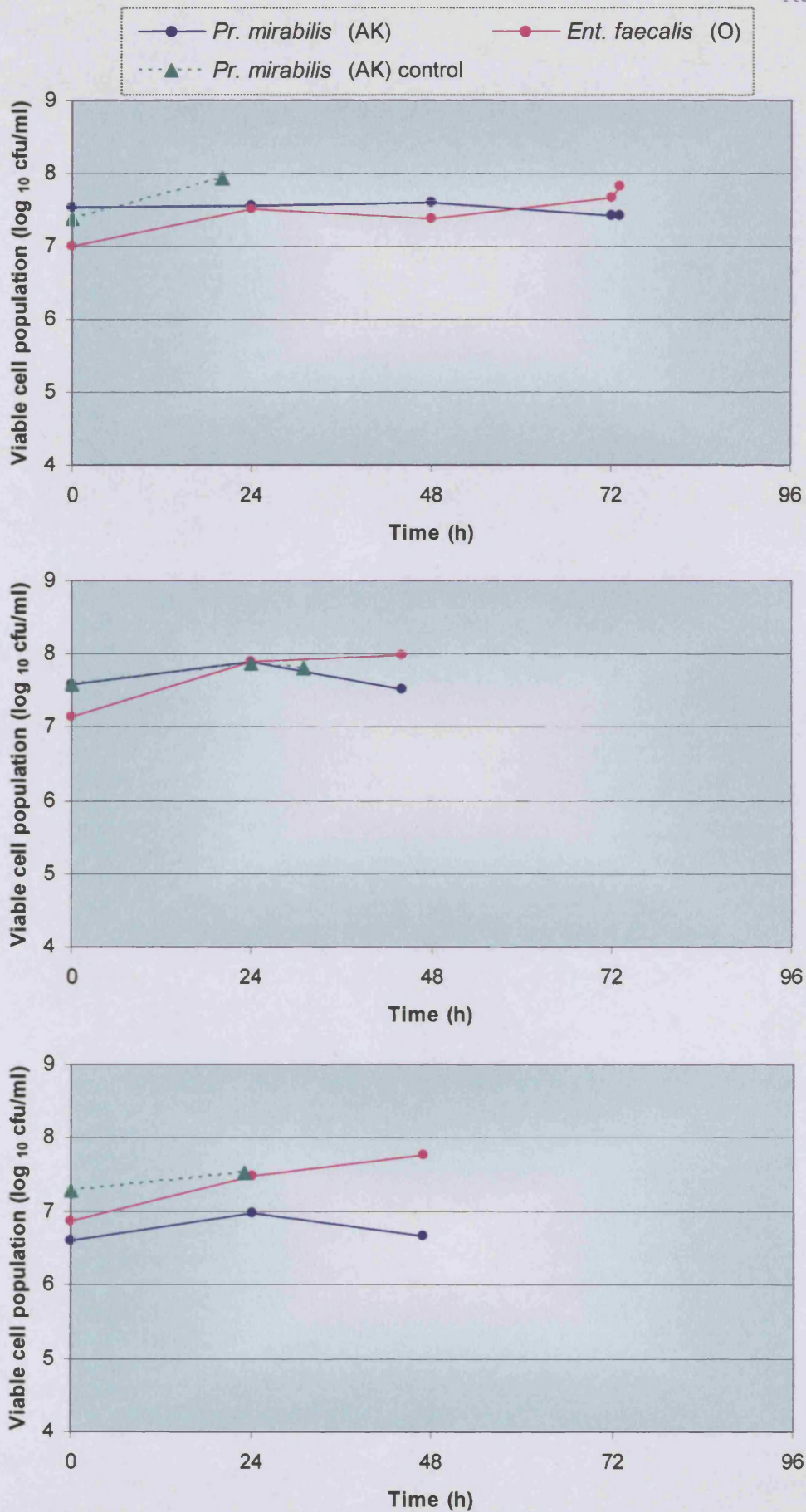


Figure 3.62: The viable cell populations (\log_{10} cfu/ml) of urine from models inoculated with a reconstituted community (Patient 6) consisting of *Pr. mirabilis* and *Ent. faecalis*, and a control inoculated with the *Pr. mirabilis* isolate alone. The data shown is for each individual replicate.

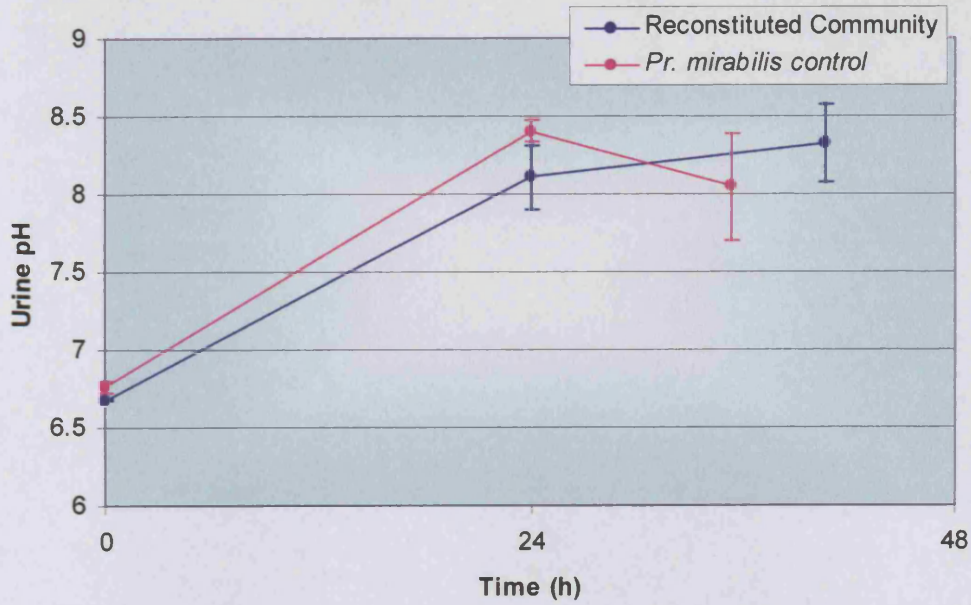


Figure 3.63a: The pH of urine samples from models inoculated with a reconstituted community (Patient 9) consisting of *Pr. mirabilis*, *E. coli*, *K. pneumoniae* and *Ent. faecalis* and a control inoculated with the *Pr. mirabilis* isolate. The means \pm SE were calculated from three replicated experiments.

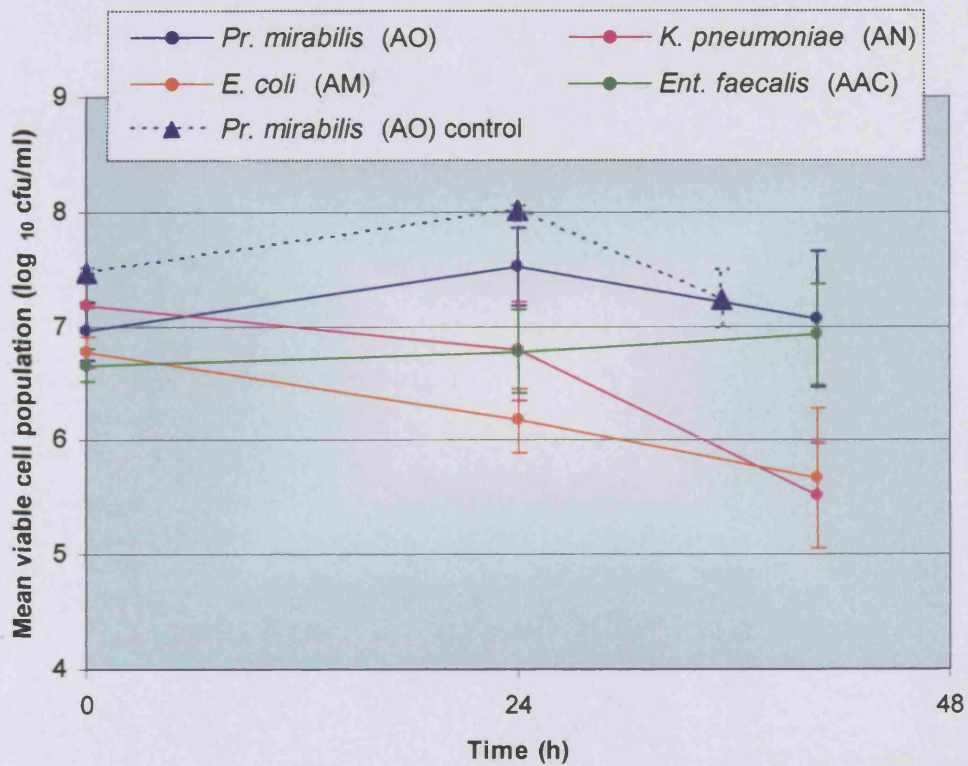


Figure 3.63b: The viable cell population (\log_{10} cfu/ml) of urine from models inoculated with a reconstituted catheter community (Patient 9) consisting of *Pr. mirabilis*, *E. coli*, *K. pneumoniae* and *Ent. faecalis* and a control inoculated with the *Pr. mirabilis* isolate. The means \pm SE were calculated from three replicated experiments.

models. There was no significant difference between the mean initial urine pH ($P=0.077$), mean urine pH at blockage ($P=0.540$) and the mean numbers of *Pr. mirabilis* (AO) cells present at blockage ($P=0.800$) for the test and control experiments. There was however, a significant difference ($P=0.019$) between the mean numbers of *Pr. mirabilis* cells present at the start of the experiment.

Figures 3.64 & 3.65 show the data collected for the reconstituted community from patient 19, consisting of *Pr. mirabilis* (AAAX), *Ps. aeruginosa* (AAAY), *E. coli* (AAAZ), and *K. oxytoca* (AAAAA). Blockage occurred in all of the control catheters (mean 40 h) and one of the test catheters (147 h) but two of the test catheters drained freely until the experiment was stopped (168 h). There was no significant difference between the mean initial urine pH ($P=0.398$) of the test and control models. The other three organisms however clearly affected the ability of the *Pr. mirabilis* isolate to produce alkaline conditions and by 24 h the mean pH of the urine in the control models (pH 7.68 ± 0.19) was significantly higher than that in the test models (pH 6.41 ± 0.02) ($P=0.021$). There was also a significant difference ($P=0.009$) between the mean final urine pH of the test models which did not block and the controls. Analysis of the viable cell count data showed no significant difference ($P=0.530$) between the mean initial *Pr. mirabilis* cfu/ml of the test and control catheters. By 24 h however, the mean viable cell count of *Pr. mirabilis* in the test models was significantly reduced compared to that in the controls ($P=0.004$). The inhibitory effect of the mixed community on the population of *Pr. mirabilis* seemed to persist throughout the experimental period. The other three species were obviously growing well in the models as each maintained cell populations around $10^7 - 10^8$ cfu/ml.

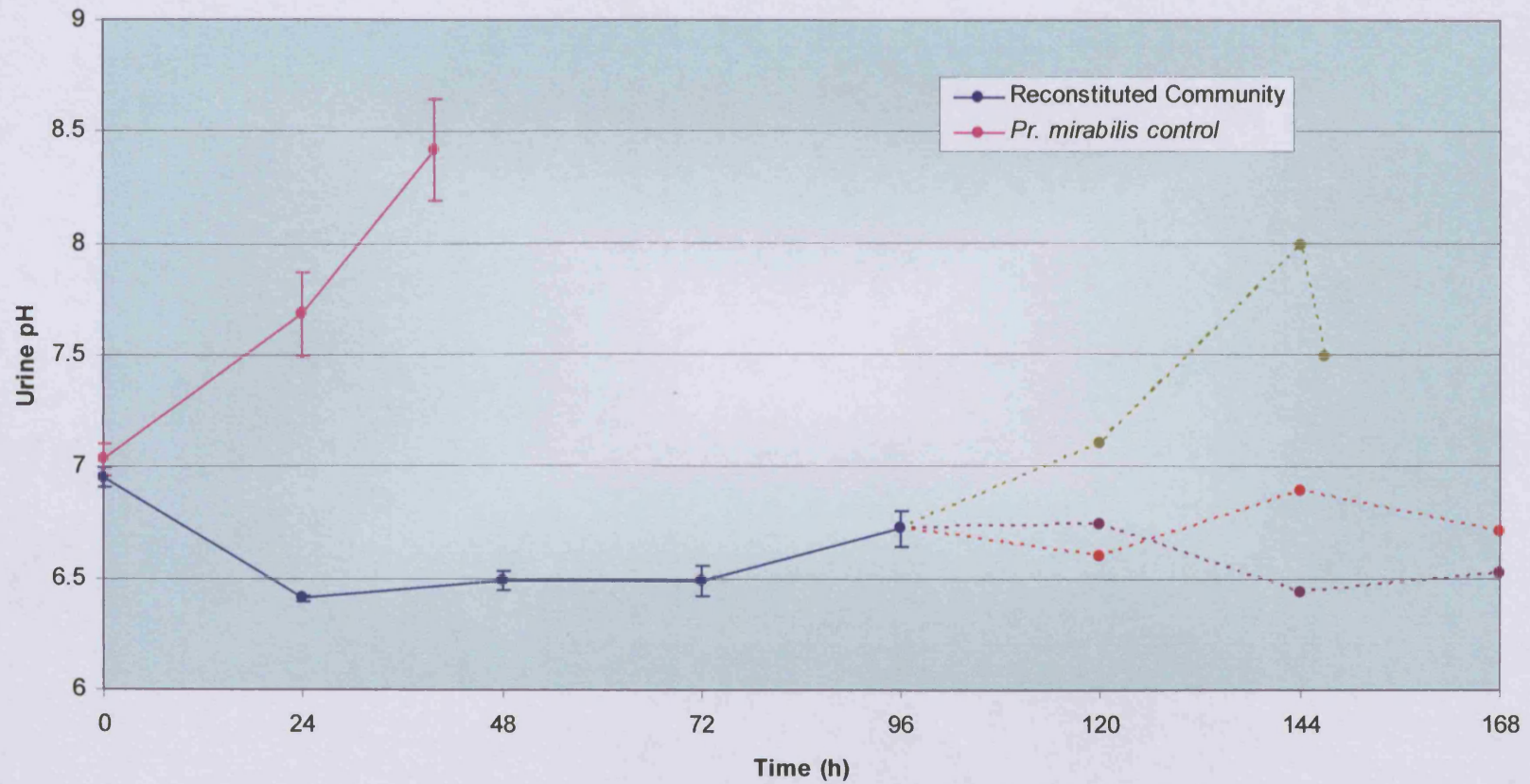


Figure 3.64: The mean pH of urine samples from models inoculated with a reconstituted catheter community (Patient 19) consisting of *Pr. mirabilis*, *Ps. aeruginosa*, *E. coli* and *K. oxytoca*, and a control inoculated with the *Pr. mirabilis* isolate alone.
 *The data for the reconstituted community is shown as the mean of three replicates (\pm SE) for data points up to 96 h and thereafter as the individual replicates (indicated \cdots for the first replicate, \cdots for the second replicate and \cdots for the third replicate)

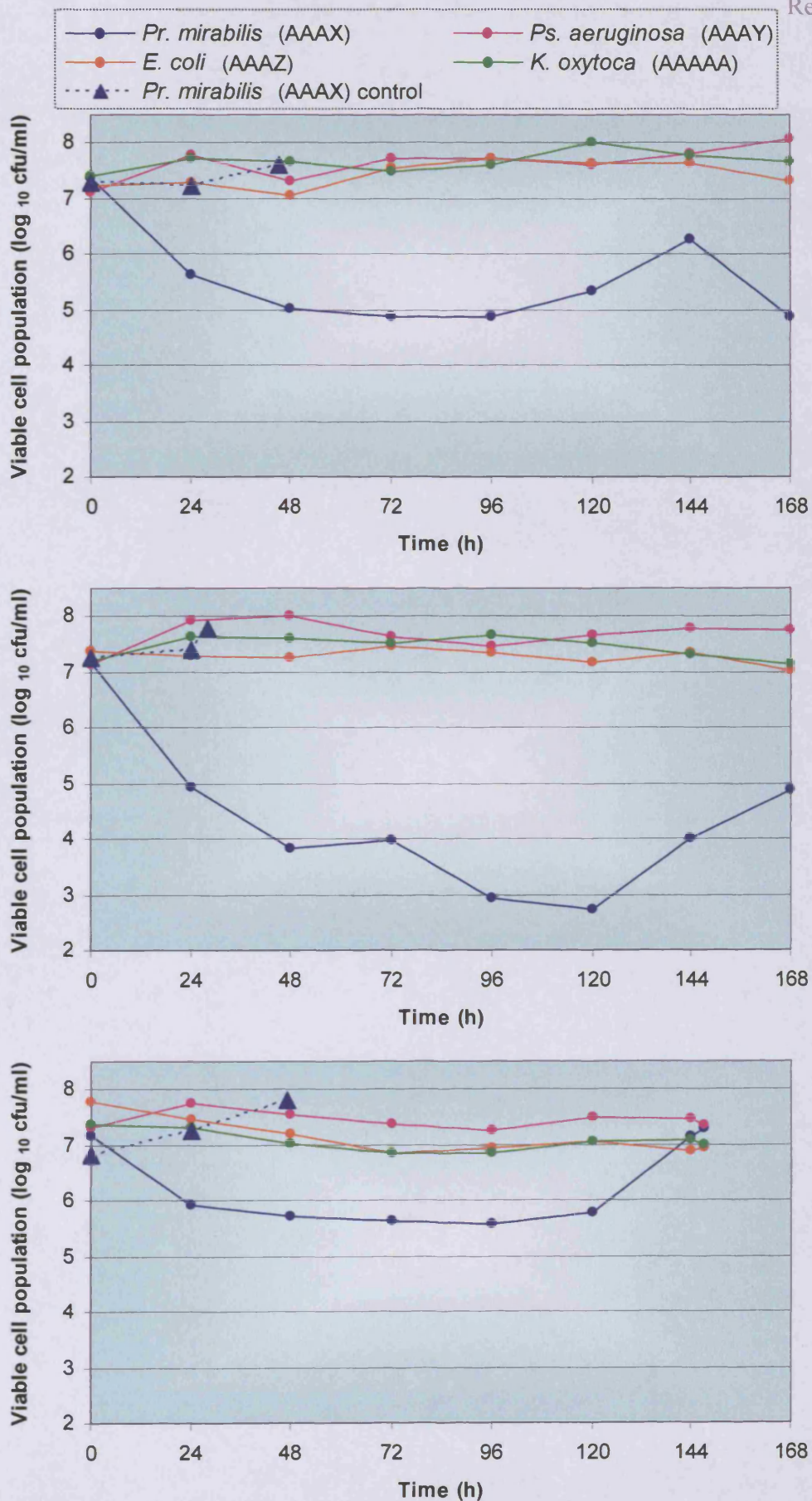


Figure 3.65: The viable cell population (log₁₀ cfu/ml) of urine from models inoculated with a reconstituted catheter community (Patient 19) consisting of *Pr. mirabilis*, *Ps. aeruginosa*, *E. coli* and *K. oxytoca*, and a control inoculated with the *Pr. mirabilis* isolate alone. The data shown is for each individual replicate.

Digital photographs of catheters removed from the model at blockage or after 168 h are shown in Figure 3.66. Both the test and control catheters show heavy encrustation for models inoculated with the communities from patients 6 and 9. The control catheter for the reconstituted patient 19 community also shows heavy encrustation, but the test catheter shows an unusual biofilm formation which is distinct from any of those seen in the previous sections, appearing to consist of both crystalline and mucoid material.

LV-SEM of the test and control catheters for patients 6 and 19 are presented in Figure 3.67. The micrographs confirm that in the case of patient 6, the *Ent. faecalis* did not prevent catheter blockage by crystalline biofilm. The community of *Ps. aeruginosa*, *E. coli* and *K. oxytoca* from the urine of patient 19 however, clearly reduced the extent of crystalline material deposited on the catheter by *Pr. mirabilis*.

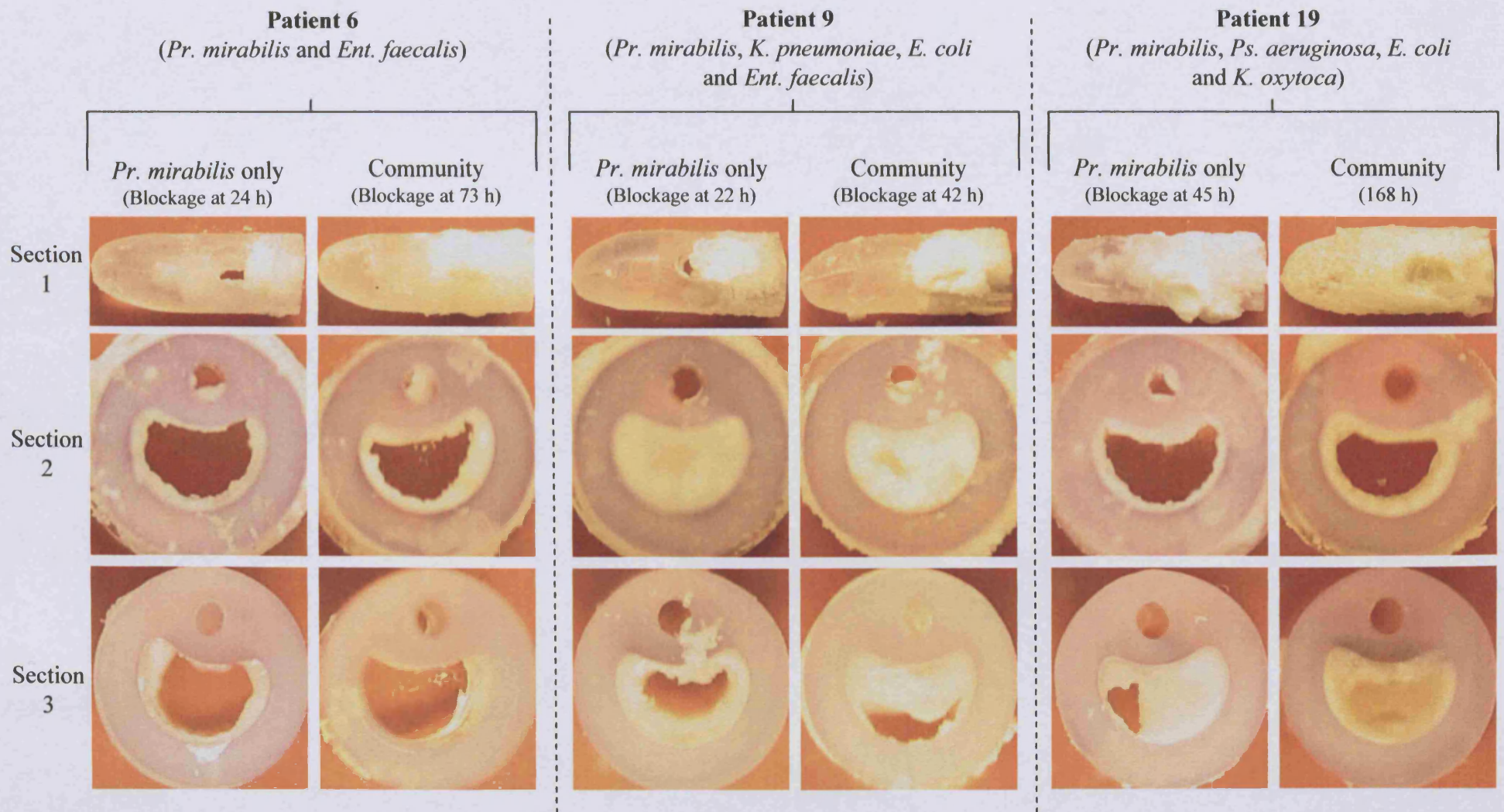


Figure 3.66: Comparison of encrustation after 168 h or at blockage for reconstituted catheter communities against the *Pr. mirabilis* isolate from that community.

Patient 6
(*Pr. mirabilis* and *Ent. faecalis*)

Patient 19
(*Pr. mirabilis*, *Ps. aeruginosa*, *E. coli* and *K. oxytoca*)

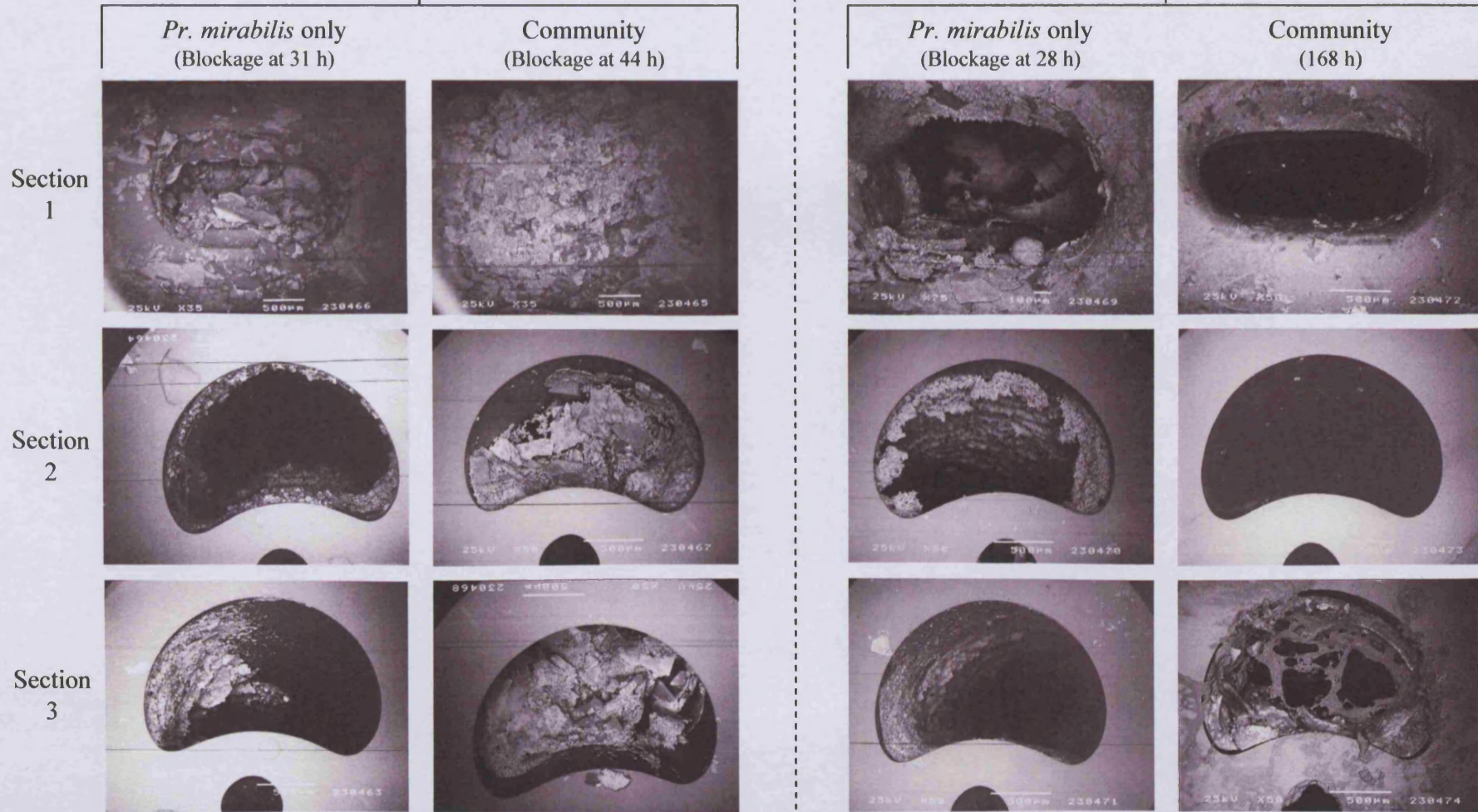


Figure 3.67: LV-SEM showing the encrustation on catheters at 168 h or blockage produced by reconstituted communities and the *Pr. mirabilis* isolate from those communities.

Section 4

Discussion

4. Discussion.

It is clear from the literature that a diverse bacterial flora develops in the urine of patients undergoing long-term catheterisation (Clayton *et al.*, 1982; Warren *et al.*, 1982; Muder *et al.*, 1992). The biofilms that colonize the catheters also reflect this diversity. When *Pr. mirabilis* is a component of the biofilm community then crystalline deposits will form and cause serious complications in catheter care (Stickler *et al.*, 1993b). The urease produced by this species is the driving force of the crystallization process. While several other urinary tract pathogens are capable of producing this enzyme, there is little epidemiological or experimental evidence to indicate whether or not they are involved in the encrustation and blockage of patient's catheters. If we are to devise a comprehensive strategy to deal with this troublesome complication it is important to identify all the urinary tract pathogens that are capable of producing crystalline biofilm in urine.

4.1. Which urease producing species are able to cause catheter encrustation and blockage?

Over the years successive workers in the catheter research group in the Cardiff School of Biosciences have built up a collection of isolates from biofilms on patients catheters. In an initial set of experiments the ability of isolates of nine urease producing species to encrust and block catheters were examined in the bladder model. The results presented in Figures 3.1 – 3.11 and Table 3.1 are summaries of the data collected from triplicate experiments on single isolates of each species.

On the basis of the final urine pH data, the times catheters took to block, encrustation rates, urease activity, digital photographs and HV-SEM images, the urease positive species tested can be divided into three groups:

Group 1: Rapid encrusters (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*).

These species were able to raise urinary pH above mean values of 8.2, produce relatively high urease activity, and cause the deposition of large quantities of Ca and Mg on catheters, leading to the blockage of catheters by crystalline biofilms consisting of apatite microcrystals and struvite (Figure 3.7) within mean times of 37 h (Table 3.1).

Group 2: Slow encrusters (*M. morgani*, *Staph. aureus* and *Staph. saprophyticus*).

These species were able to raise urinary pH to mean levels of 6.89 – 7.39, sufficient to cause the formation of some encrustation on the catheter surface after 96 h, but with final urine pHs significantly less than those of group 1 but significantly greater than the controls ($P < 0.05$). The crystals present in the biofilms are mainly large calcium phosphate crystals (Figures 3.8 & 3.9), with some microcrystals present in the *M. morgani* biofilm (Figure 3.8).

Group 3: Non-encrusters (*Pv. stuartii*, *Ps. aeruginosa* and *K. pneumoniae*).

These species were unable to raise urinary pH above mean levels of 6.45 which was insufficient to cause the formation of encrustation on the catheters (Figure 3.10), with final urine pHs not significantly different ($P > 0.05$) from those produced by the urease negative control but significantly less ($P < 0.05$) than those of group 2 and unable to

cause the hydrolysis of significantly more urea than the urease negative controls in the urease assay (Figure 3.4). Although both *Ps. aeruginosa* and *K. pneumoniae* were able to form thick mucoid biofilms (Figure 3.6) no evidence of encrustation was found (Figure 3.10).

In order to check that the isolates tested in the bladder models (Table 3.1) were representative, three additional urease positive catheter isolates of each species were examined for their ability to cause catheter blockage. For the *Pr. mirabilis* isolates Dienes typing was used to confirm that the isolates used were distinct strains (Figure 3.12). Experiments were performed once for each of the alternative isolates and the data for each species pooled (Table 3.2). For the isolates of the three species which were able to cause blockage (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*) there was no significant difference between the mean blockage times or final urine pHs of the original and pooled alternative isolates. With the exception of *Staph. aureus* (Figure 3.21) none of the other species tested showed a significant difference between the mean final urine pHs of the original and pooled alternative isolates. Digital photographs of catheters removed from the model show little difference between the original and alternative isolates for most of the species tested. The exception to this is again *Staph. aureus*, which while showing some encrustation on the original isolate showed none on any of the alternative isolates (Figure 3.17). Interestingly while the urine pH after 96 h was significantly higher for the original isolate than for the pooled alternative isolates ($P<0.001$) the mean viable cells present were significantly lower ($P<0.001$) (Figure 3.21). The results presented in Table 3.2 essentially confirm the classification of the species into the three categories. The only exception to this is

that the data for *Staph. aureus* suggest that most urease producing isolates of this species belong to group 3, the non-encrusters.

A number of urease positive isolates from species not commonly isolated from the catheterised urinary tract (*Ser. marcescens*, *K. oxytoca*, *Enterobacter cloacae*, *C. freundii* and *C. koseri*) were also tested in the model of the catheterised urinary tract. These species all performed in a similar way to the group 3 species (*Pv. stuartii*, *K. pneumoniae* and *Ps. aeruginosa*), being unable to raise the pH of the urine above pH 6.4 in the model, unable to block catheters and showing no signs of encrustation (Figures 3.22 – 3.25). This data supports the inclusion of these species in group 3 (non-encrusters).

The experiments performed in the bladder models used large inocula (10 ml of urine cultures containing approximately 10^8 cfu/ml). A relatively concentrated urine with a pH_n of 6.54 was supplied to the bladder chamber at a flow rate of 0.5 ml/min. These conditions were intended to simulate conditions which can apply particularly in elderly catheterised patients who have low fluid intakes and heavily infected urines. They can be considered as representing the worst case scenario. The experimental conditions were thus highly favourable to crystalline biofilm formation. Therefore if organisms fail to produce encrustation under these experimental conditions it is unlikely that they will do so in patients. Group 3 species (*Pv. stuartii*, *Ps. aeruginosa*, *K. pneumoniae*, *K. oxytoca*, *Ser. marcescens*, *Enterobacter cloacae*, *C. freundii* and *C. koseri*) are thus not likely to be appropriate targets for control strategies.

Those species classified as group 2 (*M. morgani*, *Staph. aureus* and *Staph.*

saprophyticus) were able to raise the pH of the urine (means 6.89 – 7.39) sufficiently to cause the formation of encrustation on the catheter surface in the model, but the pH increase was far less than for the group 1 species. The pH_n of the urine used in these experiments was 6.54 (Figure 3.3). This is somewhat lower than that found in the majority of patients during the studies of Choong *et al.* (1999; 2001) and Mathur *et al.* (2006a) who determined the mean pH_n of the majority of patients to be in the range 7.3 – 8.1. Given the moderate increases in urinary pH caused by infection with group 2 species, these patients would not be expected to show an increase in catheter blockage as a result of infection with these species. Choong *et al.* (1999; 2001) identified a small group of patients termed ‘urease negative blockers’ in which catheter blockage occurred without urease positive bacteriuria. The six patients (from 35) identified by Choong *et al.* (1999) and eight (from 64) by Choong *et al.* (2001) had mean pH_n s of 6.45 and 6.49 respectively. Mathur *et al.* (2006b) also showed that a small number of patients have very low pH_n levels. This small group of patients with low pH_n s might be expected to be vulnerable to catheter encrustation by group 2 species (as well as group 1 species) while the majority of patients are unlikely to be affected.

The mean urinary pHs reached by the group 1 species in this series of experiments were above 8.2. This is greater than the mean urinary pH_n of patients recorded by both Choong *et al.* (1999; 2001) and Mathur *et al.* (2006a) (mean pH_n range 7.3 – 8.1). As a result infection with any of these species is likely to lead to the formation of encrustation and catheter blockage in most patients. Some patients however have an extremely high pH_n , and as a result do not experience catheter blockages even when infected with group 1 species, as observed by Mathur *et al.* (2006a).

The results presented in this section are generally consistent with the laboratory and clinical data available in the literature. The association of *Pr. mirabilis* with catheter encrustation and blockage is well documented (Mobley and Warren, 1987; Kunin, 1989; Stickler *et al.* 1993b). The other species classified as group 1, *Pr. vulgaris* and *Pv. rettgeri*, have not been significantly associated with the formation of catheter encrustation in clinical studies (Mobley and Warren, 1987; Kunin, 1989), although Stickler *et al.* (1998a) found that their encrusting ability was similar to that of *Pr. mirabilis* when tested *in vitro*. Mobley and Warren (1987) found that *Pr. vulgaris* and *Pv. rettgeri* were present in 1 % and 4 % of urine samples respectively during their study while *Pr. mirabilis* was present in 58 %.

The results of the urease assay (Figure 3.4) show that the isolates tested of these three species were able to produce relatively high rates of urea hydrolysis in artificial urine, and there was no significant difference between the group 1 species in the time taken to cause catheter blockage, urine pH at blockage or rates of calcium and magnesium encrustation (Table 3.1, Figures 3.1 & 3.2). Images of the blocked catheters also showed no differences in the manner of encrustation (Figures 3.5 & 3.7). Why then has no clinical association been found between catheter encrustation and *Pr. vulgaris* or *Pv. rettgeri*? The answer to this question is most likely to be due to the fact that these two species are isolated far less frequently from the catheterised urinary tract than *Pr. mirabilis* (Mobley and Warren, 1987). Senior *et al.* (1979) reported that *Pr. mirabilis* was responsible for 96.5 % of urinary tract infections by the *Proteeae* tribe (*Proteus*, *Providencia* and *Morganella* sp.) members while *Pr. vulgaris* and *Pv. rettgeri* accounted for less than 2 %. The isolate collection available in the Cardiff

catheter laboratory from patient catheters over a number of years also reflects this. While the collection contains many isolates of *Pr. mirabilis* and several other species such as *Ps. aeruginosa*, there were only four isolates each of *Pr. vulgaris* and *Pv. rettgeri*. Senior and Leslie (1986) speculated that the low number of infections with *Pr. vulgaris* was related to its infrequent occurrence as a part of the gut microflora, which is often the source of bacteria infecting the urinary tract (Daifuku and Stamm, 1984). Any attempts to control the formation of encrustation would be wise to examine its effects on these two species however, since it is conceivable that a strategy which is effective against *Pr. mirabilis* but not *Pr. vulgaris* or *Pv. rettgeri* may simply select for them.

Those organisms classified as group 2 (*M. morgani*, *Staph. aureus* and *Staph. saprophyticus*) were all capable of causing some encrustation after 96 h. This group is less clear-cut than either group 1 or group 3, with some variation in the data collected between the species. All three species shared an ability to raise the urine pH above the nucleation pH of the urine and to cause the deposition of encrustation consisting of large calcium phosphate crystals on the catheter surface (Figure 3.8). Some apatite microcrystals were also present in the *M. morgani* biofilm (Figure 3.8). Stickler *et al.* (1998a) did not find any evidence of encrustation with *M. morgani* 40 h after inoculation in the model. This finding is consistent with the results reported here however, as the pH of the urine in the models inoculated with *M. morgani* did not rise above the pH_n (Figure 3.3) until after 48 h incubation (Figure 3.1).

The differences seen between the *M. morgani* and *Staph. aureus* isolates in the calcium encrustation rate data (Figure 3.2) are likely to be due to the relative

differences between the calcium pH_n of the artificial urine (6.54) and the final urine pH of the models. For *M. morganii* the difference is 0.85 of a pH unit (7.39) while for the *Staph. aureus* isolate it is 0.35 (6.89). The calcium precipitation profile (Figure 3.3) shows that more calcium will precipitate in the *M. morganii* model compared to the *Staph. aureus* model. Relatively little encrustation was apparent in the *Staph. aureus* biofilm compared to that of *M. morganii* (Figures 3.5 & 3.6). The occurrence of microcrystals in the *M. morganii* biofilm in addition to large calcium phosphate crystals (Figure 3.8) may also be linked to the greater difference between the final and nucleation pHs.

The urease assay data also show differences between the *M. morganii* and *Staph. aureus* isolates. This is most likely to be due to the mode of regulation of urease synthesis in these species. Urease production in *M. morganii* is constitutive (Rosenstein *et al.*, 1981) and so high expression of urease would be expected (Figure 3.4). However there is some evidence that urease production in *Staph. aureus* is upregulated in biofilms (Resch *et al.*, 2005). This would mean that an assay of urease production in suspension, such as the one used in this study, would be likely to under represent the expression of urease in a biofilm. The data from the bladder models, showing urine pH exceeding that of the group 3 organisms, would seem to suggest that this is the case although the assay appears to have accurately predicted biofilm urease expression for the other (Gram-negative) species tested.

There is some clinical data which suggests that *M. morganii* may serve to protect catheters from encrustation (Mobley and Warren, 1987). Other studies however have found no such association (Kunin, 1989). Both of these studies concentrated on

patients experiencing rapid catheter blockage, while the data collected in this study suggests that *M. morgani* (in common with *Staph. aureus* and *Staph. saprophyticus*) is only likely to encrust catheters slowly and in patients whose urine has a low pH_n. The analysis used in the studies by Mobley and Warren (1987) and Kunin (1989) make it unlikely that such a link would be detected. The *Staph. aureus* isolate tested was unusual in its ability to raise urinary pH when compared to other urease positive *Staph. aureus* isolates in our collection (Figures 3.17 & 3.21). This combined with its relatively low occurrence in CAUTI (Mobley and Warren, 1987; Kunin, 1989) means that it is also unlikely to be associated with catheter blockage clinically. The low occurrence of *Staph. saprophyticus* in CAUTI, exemplified by the fact that despite the large collection of isolates from catheters in our lab the one isolate found was from a patient with a urostomy, also means it is unlikely to be associated clinically with catheter blockage.

Those organisms classified as group 3 (non-encrusting) include a number of urease positive organisms which have not been associated with catheter encrustation, both in clinical and laboratory studies, such as *K. pneumoniae*, *K. oxytoca* and *Ps. aeruginosa* (Mobley and Warren, 1987; Kunin 1989; Stickler *et al.*, 1998a). These organisms control urease synthesis using the nitrogen regulatory system and as a result would not be expected to produce significant quantities of urease in the nitrogen rich environment of the urinary tract (Totten *et al.*, 1990; Collins *et al.*, 1993). Although not subjected to as rigorous testing as *K. pneumoniae*, *Ps. aeruginosa* and *Pv. stuartii* the bladder model and urease assay data collected for the remaining species in this group (*K. oxytoca*, *Ser. marcescens*, *Enterobacter cloacae*, *C. freundii* and *C. koseri*) strongly indicates that they should be included (Figures 3.4 & 3.22 – 3.25). The

classification of these species as urease positive may cause confusion when comparisons are made between patients with urease positive and urease negative bacteriuria, such as in the study by Choong *et al.* (2001).

While the majority of the group 3 species were unable to form thick biofilms on the catheters in the model, *Ps. aeruginosa* and *K. pneumoniae* formed thick mucoid biofilms (Figure 3.6). Despite this the flow of urine through the catheters was not impeded, indicating that these biofilms are extremely hydrated and likely to possess the water channels present in the Costerton biofilm model (Costerton *et al.*, 1995) which is based on the observation of *Ps. aeruginosa* biofilms. *K. oxytoca* also formed thick biofilms (further down the catheter than shown in Figure 3.23) which were similar to those formed by *K. pneumoniae*.

The inclusion of *Pv. stuartii* in this group is something of a surprise, given both the published data on urease production for this organism (D'Oriazo and Collins, 1993a; D'Oriazo and Collins, 1993b) and its reported clinical association with the formation of catheter encrustation (Kunin, 1989). D'Oriazo and Collins (1993a; 1993b) found that the plasmid borne urease of *Pv. stuartii* was induced by the presence of urea and was similar to that of *Pr. mirabilis*. Bladder models inoculated with four different isolates of *Pv. stuartii* in this study however, were unable to raise urine pH and catheters removed from the models showed no sign of encrustation (Figure 3.19). The isolate tested in the urease assay was also unable to produce significantly more urea hydrolysis than the urease negative control (Figure 3.4). It is possible that the regulation of urease synthesis in these isolates may be related to nitrogen starvation, as with *K. pneumoniae*, or that the induced urease operon is unable to produce

sufficient urease to cause the hydrolysis of significant quantities of urea. Macleod (2006) found that *Pv. stuartii* often occurred together with *Pr. mirabilis* in catheter biofilms. This association with *Pr. mirabilis* may be the reason for the observation by Kunin (1989) that *Pv. stuartii* is associated with catheter blockage, rather than *Pv. stuartii* itself forming the encrustation.

Even under conditions highly favourable to crystalline biofilm formation group 3 species (*Pv. stuartii*, *Ps. aeruginosa*, *K. pneumoniae*, *K. oxytoca*, *Ser. marcescens*, *Enterobacter cloacae*, *C. freundii* and *C. koseri*) were incapable of causing catheter encrustation. Group 2 species (*M. morgani*, *Staph. aureus* and *Staph. saprophyticus*) were able to cause slow encrustation under these conditions, but are only likely to cause encrustation in a small sub-set of patients with a low fluid intake and pH_n of less than 7.3. The three species capable of rapid encrustation were *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*. They were capable of raising the urinary pH to mean values of around 8.3 – 8.4 and therefore would be capable of encrusting catheters even in urine with a pH_n of up to 8.2. This group should be the target for strategies to control catheter encrustation.

4.2 The development of crystalline biofilm on silver-hydrogel and nitrofurazone catheters.

The ability of the antimicrobial catheters recently introduced into clinical practice to prevent CAUTI is the subject of some controversy (Trautner *et al.*, 2005a; Johnson *et al.*, 2006). Little work has been undertaken into their effectiveness at preventing the formation of crystalline biofilms which commonly occur during long-term

catheterisation (Cools and Van der Meer, 1986). The aim of this part of the study was to assess the ability of two antimicrobial catheters (silver-hydrogel coated latex, Bard IC catheter and the nitrofurazone impregnated all-silicone catheter, Rochester Medical NF catheter) to resist the formation of encrustation in the bladder model by five of the species previously identified as able to form encrustation on all-silicone catheters. All-silicone catheters were used as controls.

The three species previously classified as group 1 (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*) were all able to rapidly cause catheter blockage by crystalline biofilm on both the silver-hydrogel and nitrofurazone catheters (Table 3.3). There was no significant difference between the mean blockage times of the three species on all-silicone and nitrofurazone catheters. *Pr. mirabilis* and *Pv. rettgeri* had mean blockage times which were significantly faster than that of *Pr. vulgaris* on the silver-hydrogel catheters. No significant difference was seen between the mean times to blockage for each particular species on the three catheter types. Visual assessment of the crystalline biofilms using digital photography, LV-SEM and HV-SEM showed little difference between either the three species or the three catheter types, with extensive crystalline biofilms seen on all the catheters examined (Figures 3.28 – 3.30 & 3.32 – 3.34). The three species all showed urinary pHs > 8.0 throughout the experiment (Figure 3.26) and each species grew well in the urine in the models irrespective of the catheter type (Figure 3.27). All three group 1 species also showed relatively high MICs of nitrofurazone (Table 3.4), particularly *Pv. rettgeri* (64 – 128 µg/ml).

For the group 2 species tested (*M. morgani* and *Staph. aureus*) only *M. morgani* was able to cause catheter blockage in the all-silicone and nitrofurazone catheters, while

neither species caused blockage in the silver-hydrogel catheters (Table 3.3). The mean blockage times of the *M. morgani* infected models were significantly greater than those of the group 1 species for both the all-silicone and nitrofurazone catheters. There was no significant difference between the mean blockage times recorded for *M. morgani* on the all-silicone and nitrofurazone catheters. Visual assessment of the crystalline biofilms formed by *M. morgani* using digital photography, LV-SEM and HV-SEM showed that both the nitrofurazone and silver-hydrogel catheters were heavily encrusted (Figures 3.28 & 3.29, 3.31 – 3.33 & 3.35). The encrustation consisted of apatite microcrystals, with some large calcium phosphate crystals present on the silver-hydrogel catheter. In contrast the crystalline biofilms formed by *Staph. aureus* on both antimicrobial catheters were far less extensive, with little encrustation present. Examination of the *Staph. aureus* biofilms using HV-SEM showed the presence of both apatite microcrystals and large calcium phosphate crystals.

Both *M. morgani* and *Staph. aureus* showed a consistent pattern of mean urinary viable cell counts and mean urinary pH during the experiments irrespective of the catheter type (Figures 3.26 & 3.27). The pH of the urine infected with *Staph. aureus* generally remained stable between 6.5 – 7.0 for the 168 h of the experimental period. *M. morgani* however, after the first 48 h, was able to produce a gradual increase in the urinary pH to around 7.5 in the presence of all three catheter types. The *M. morgani* mean urinary viable cell counts fell in the first 24 h from an initial cfu/ml of above 10^8 to below 10^7 , before recovering steadily back to around 10^8 cfu/ml. The numbers of *Staph. aureus* cells present show a steady fall from around 10^8 cfu/ml initially to below 10^7 cfu/ml after 168 h.

The MICs of nitrofurazone for the *M. morgani* isolates were relatively high (32 – 64 µg/ml) and comparable with those of the group 1 species (Table 3.4). The MICs of nitrofurazone for the *Staph. aureus* isolates however were lower (8 – 16 µg/ml) and similar to that of the *Staph. aureus* control. The single isolate of *Staph. saprophyticus* tested was quite sensitive to nitrofurazone (MIC 4 µg/ml).

This section of the study shows that both the silver-hydrogel and nitrofurazone catheters tested are not effective in extending catheter lifespan *in vitro* in comparison to all-silicone controls for those species capable of causing rapid catheter blockage (group 1 – *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*). The antimicrobial catheters had no observable effect on the urinary pH, mean numbers of viable cells recovered from the urine or the nature of the crystalline biofilms generated when compared to the all-silicone controls for these species (Figures 3.26 – 3.30 & 3.32 – 3.34).

The data presented in Table 3.3 shows that the silver-hydrogel were consistently the first to block. Previous work in the bladder models inoculated with *Pr. mirabilis* had shown that latex based catheters, including the silver-hydrogel variety, blocked more rapidly than all-silicone devices (Morris *et al.*, 1997; Morris and Stickler, 1998a). These authors suggested that the thicker walls and narrower central channels of the latex catheters make them particularly vulnerable to rapid blockage by crystalline biofilm. Kunin *et al.* (1987b) also reported that in their clinical experience silicone catheters were significantly less likely to be encrusted or blocked than latex catheters. The reported MICs of silver for bacteria are subject to some variation due to a lack of standardisation and difficulties relating to the bioavailability of silver in some media (Silver, 2003). The data available for *Pr. mirabilis* is reported for isolates termed

'resistant' to silver, with the MIC of silver being between 27 and 65 $\mu\text{g/ml}$ (Hendry and Stewart, 1979; Filali *et al.*, 2000). Slawson *et al.* (1994) reported that a 'sensitive' strain of *Ps. stutzeri* had a MIC of silver of 5.4 $\mu\text{g/ml}$. The *in vitro* work conducted by Chakravarti *et al.* (2005) using a catheter fitted with silver electrodes which was shown to release silver ions at 0.25 – 0.1 $\mu\text{g/ml}$ into the urine and significantly delayed catheter encrustation demonstrates that silver can be effective in extending catheter lifespan when challenged by *Pr. mirabilis*. The fact that *Pr. mirabilis* persisted, albeit in significantly reduced numbers, indicates that this level of silver release may be below the MIC of silver for this isolate. Morgan (personal communication, 2007) determined the levels of silver ions present in human urine from bladder models using silver-hydrogel catheters to be lower than in the experiments performed by Chakravarti *et al.* (2005) at 0.01 – 0.02 $\mu\text{g/ml}$. The data presented in Figures 3.26 & 3.27 indicates that this lower quantity of silver ions released into the urine by the silver-hydrogel catheters is not sufficient to affect the growth or activity of any of the group 1 species.

The results from the experiments using nitrofurazone catheters also show that they are not effective in delaying catheter blockage by the group 1 species (Table 3.3). As with the silver-hydrogel catheters there was no difference between the mean numbers of bacteria recovered or the urinary pH between the nitrofurazone catheters and the all-silicone controls (Figures 3.26 & 3.27). This is not surprising when the relatively high MICs of nitrofurazone for the group 1 species are taken into account (Table 3.4). The MIC of nitrofurazone for *Pr. mirabilis* found in this study (64 $\mu\text{g/ml}$, Table 3.4) is in agreement with that found by Johnson *et al.* (1993) (32 – 64 $\mu\text{g/ml}$). Johnson *et al.* (1993) reported that sections of nitrofurazone catheter were not able to cause

inhibition zones in lawns of the majority of *Proteus* sp. isolates tested on agar plates. Inhibition zones were produced however, against several other commonly occurring urinary tract pathogens such as *E. coli*. This raises an important question – would the use of nitrofurazone catheters in long-term catheterisation select for species which cause catheter encrustation and blockage? If so the use of these catheters would be potentially dangerous for the patients involved, given the complications associated with catheter blockage.

The data presented in Table 3.3 demonstrates that *M. morganii* was able to cause catheter blockage in both the nitrofurazone and all-silicone control catheters, clearly a reflection of the relative resistance of this organism to nitrofurazone (Table 3.4). Blockage did not occur within 168 h in any of the experiments using silver-hydrogel catheters. The presence of extensive encrustation (Figures 3.28 & 3.29) and the mean urinary pH being well above the pH_n at 168 h (Figure 3.26) however, both indicate that catheter blockage was imminent. The nature of the crystalline biofilms also seems relatively unaffected by the catheter type, with no evidence of struvite crystals on any of the catheters (Figures 3.31 & 3.35). It is interesting that the latex based silver-hydrogel catheters performed better than both silicone based catheters, given the reduced lumen size of latex based catheters. While this may suggest that this isolate is more sensitive to silver than the group 1 species tested there is little evidence to support this in Figure 3.27b.

The *Staph. aureus* isolate tested was unable to cause blockage in any of the catheters tested after 168 h (Table 3.3). Although *Staph. aureus* was the organism most sensitive to nitrofurazone (Table 3.4) there was no evidence that the concentration of

antibacterial agent diffusing from the catheter into the urine had any adverse effect on the population present in the bladder chamber (Figure 3.27).

This section of the study provides strong evidence that the antimicrobial catheters tested (silver-hydrogel and nitrofurazone) are unlikely to be able to either prevent or delay catheter blockage by *Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*. Given the lack of convincing evidence for their effectiveness in preventing bacteriuria even in short-term catheterisation (Brosnahan *et al.*, 2004; Trautner *et al.*, 2005a; Johnson *et al.*, 2006) the use of these catheters in long-term catheterisation is unnecessary, costly, and in the case of nitrofurazone catheters potentially counter productive.

4.3 The control of catheter encrustation by the delivery of antimicrobial agents through the retention balloon.

Previous workers have shown in studies in the bladder model that the retention balloon of catheters could be used to deliver the biocide triclosan to the residual urine and prevent encrustation by *Pr. mirabilis*. At concentrations of 1 – 10 mg/ml triclosan in the balloon, the rise in urinary pH was prevented, the bacterial populations in the urine significantly reduced and crystalline biofilm formation inhibited (Stickler *et al.*, 2003a; Jones *et al.*, 2005; Jones *et al.*, 2006). Unfortunately the formulations used in these experiments were not suitable for use in clinical studies because of difficulties in stability and sterilization. Williams (2006) found that solutions containing up to 3 mg/ml of triclosan could be prepared in 0.1 M sodium carbonate and that these solutions were stable and could be sterilized by membrane filtration. He further demonstrated that inflating the balloon with 3 mg/ml triclosan in 0.1 M

sodium carbonate was effective in preventing encrustation by *Pr. mirabilis*. The results presented in Table 3.5 confirm this activity and that this formulation was also effective against *Pr. vulgaris*. In both these cases in contrast to the controls the numbers of viable cells recovered from the urine fell steeply within 24 h. The pH of the urine in the bladder chamber fell below that of its pH_n (6.5) and the catheters drained freely for the 7 day experimental period (Figures 3.36 & 3.37). The effect on *Pv. rettgeri* was minimal however, with no significant difference between the blockage times or urine pHs recorded for the test and control models (Table 3.5, Figure 3.38). Both *Proteus* sp. tested had MICs of triclosan of $\leq 0.2 \mu\text{g/ml}$ while the MIC of *Pv. rettgeri* isolates was $64 \mu\text{g/ml}$ (Table 3.6).

Jones *et al.*, (2006) demonstrated that triclosan (10 mg/ml in 5% polyethylene glycol) in the retention balloon was able to inhibit biofilm formation on catheters by *Pr. mirabilis*, *Pv. stuartii*, *K. pneumoniae* and *Staph. aureus*. These organisms were also shown to have MICs of triclosan ranging from 0.2 – 0.6 $\mu\text{g/ml}$. In contrast species with MICs of $> 100 \mu\text{g/ml}$ (*Ser. marcescens*, *M. morgani* and *Ps. aeruginosa*) were able to produce extensive biofilms on catheters inflated with the triclosan formulation. The results presented in Table 3.5 and Figures 3.36 – 3.38 adds *Pr. vulgaris* to the list of organisms that should be inhibited by triclosan and *Pv. rettgeri* to the list of those that will resist the treatment.

The picture that is emerging from these laboratory studies is that triclosan at 3 mg/ml in the catheter balloon should be effective in preventing encrustations by *Pr. mirabilis* and *Pr. vulgaris* but will have no effect on encrustation produced by *Pv. rettgeri* or *M. morgani*. An important consideration is how does this picture relate to the incidence

of these species in the catheterised urinary tract? Mobley and Warren (1987) recorded the frequency of the occurrence of urease positive bacteria in urine samples. *Pr. mirabilis* and *M. morgani* were the most commonly occurring, being present in 58 and 54 % of 1,135 samples respectively, with *Pv. rettgeri* (4 %) and *Pr. vulgaris* (1 %) occurring far less frequently. Kunin (1989) found a similar pattern, with *Pr. mirabilis* being present in 34 % of 214 urine samples and *M. morgani* in 12 %. *Pv. rettgeri* and *Pr. vulgaris* were not isolated at all in this study. Ohkawa *et al.* (1990) and Matsukawa *et al.* (2005) examined the relationship between organisms present in urine samples and catheter biofilms in short-term catheterisation. In both studies the occurrence of all four species was relatively low. Ohkawa *et al.* (1990) found that for 57 paired cultures of urine and catheter surface *M. morgani* was present in 5 (9 %) urine cultures, 3 (5 %) catheters and once (2 %) on both. *Pr. mirabilis* and *Pr. vulgaris* were present less often, being isolated in 2 (4 %) urine cultures each, 5 (9 %) and 4 (7 %) catheters respectively and on both twice (4 %) each. *Pv. rettgeri* was not isolated. Matsukawa *et al.* (2005) also found that these species occurred infrequently, with *M. morgani* and *Pr. vulgaris* only occurring twice each and *Pv. rettgeri* once, with *Pr. mirabilis* not occurring at all. Macleod (2006) examined the bacterial flora of 106 catheter biofilms (both crystalline and non-crystalline) and reported that the numbers (%) of catheters colonized by these species was *Pr. mirabilis* 31 (30.2 %), *Pr. vulgaris* 3 (2.8 %), *Pv. rettgeri* 5 (4.7 %) and *M. morgani* 14 (13.2 %).

The evidence thus suggests that the triclosan strategy should prevent encrustation in most patients prone to this complication. Encrustation induced by *Pv. rettgeri* and *M. morgani* is likely to continue however if such a control strategy is used. It should be noted that encrustation by *M. morgani* will be a slow process and will only occur in

patients with low urinary pH_n values (< 7.3). There is of course the possibility that the widespread use of the triclosan strategy could select for the intrinsically resistant species. In addition acquired resistance to triclosan could also develop in sensitive species such as *Pr. mirabilis*.

Triclosan is widely used as an antibacterial agent in soaps, handwashes, deodorants, cosmetics, toothpaste and mouthwashes. There has been concern expressed that its overexploitation might result in the selection of resistant organisms (Levy, 2001; Schweizer, 2001). Despite extensive use for over 30 years however there has been little sign that its use in clinical, domestic or industrial situations has led to significant resistance. An important current application of triclosan is in the disinfection of patients colonized with MRSA (Coia *et al.*, 2006). There have been some reports of increased MICs of triclosan in MRSA up to 4 µg/ml (Cookson *et al.*, 1991; Brenwald and Fraise, 2003; Bayston *et al.*, 2007). Strains of bacteria exhibiting this level of resistance are still killed by 'in use' concentrations which are generally well in excess of the MIC (Lear *et al.*, 2002).

Jones *et al.* (2006) found that the amount of triclosan released into the urine from catheter balloons inflated with 10 mg/ml triclosan in 5 % aqueous polyethylene glycol over 48 h remained quite stable at approximately 0.1 µg/ml. This concentration of triclosan is close to its MIC for *Pr. mirabilis* (Table 3.6). Any increase in the MICs of triclosan found in *Pr. mirabilis* would thus be of concern in this context.

Experiments by Jones (2005) on triclosan resistant mutants created in the laboratory showed that some of the mutants were able to produce crystalline biofilm and block

catheters had been inflated with triclosan (10 mg/ml). These mutants had MICs of triclosan of 40 µg/ml compared to the parent strains in which the MIC was around 0.1 µg/ml. The development of similar resistance in clinical situations would render the triclosan strategy ineffective. For this reason the clinical use of the triclosan strategy should be limited strictly to those situations in which catheter blockage is being caused by sensitive species (*Pr. mirabilis* or *Pr. vulgaris*) and not used as a general anti-infection strategy for long-term catheterised patients. In addition there are some indications that triclosan resistance may also lead to antibiotic resistance (Cookson *et al.*, 1991). Some authors have also suggested that the occurrence of stable low-level triclosan resistant mutants in the environment might encourage the preferential survival of mutants resistant to other antimicrobial agents (Heath *et al.*, 1998; McMurry *et al.*, 1998). Other authors have cast doubts on this, but nonetheless emphasize that the use of triclosan should be limited to clinically useful situations (Gilbert and McBain, 2001; Russell, 2003). In view of this speculation it will be important in any subsequent clinical trial of the triclosan strategy to perform careful monitoring of urine samples to check for changes in the bacterial flora and its sensitivity to triclosan and antibiotics.

The catheter balloon has also been used to deliver antibacterials to the bladder by Carlsson *et al.* (2005). These authors developed a formulation which generated nitric oxide in the balloon. This agent then diffused through the balloon to the surrounding medium. A batch culture model of the catheterised urinary tract was used in these experiments. It consisted of long-necked 50 ml flasks containing 25 ml of urine inoculated with *E. coli* to a final population density of 10^6 cfu/ml. The urinary catheter was then inserted into the flask and the balloon inflated with the nitric oxide

generating solution. The solution (10 mM ascorbic acid 5 mM sodium nitrite in physiological saline at pH 2.5) was produced by mixing equal quantities of pH 2.5 20 mM ascorbic acid in physiological saline and pH 2.5 10 mM sodium nitrite in physiological saline immediately prior to inflation of the catheter balloon. Once the balloon had been inflated the catheter was pulled up to seal the neck of the flask. The flask was then inverted and incubated at 37 °C for 24 h.

The data presented in Figure 3.39 show that when the mixture used by Carlsson *et al.* (2005) was used to inflate catheter balloons in models there was no inhibition of *Pr. mirabilis* crystalline biofilm formation. The results of triplicate experiments showed no differences between the mean urinary pHs, mean urinary viable cell numbers and mean times to catheter blockage in the test and control models (Figure 3.39). Visual examination of catheters removed from the models at blockage also showed no differences between the test and control catheters, with all of the catheters showing large amounts of encrustation (Figure 3.40).

Nitric oxide has been shown to be important in the intracellular killing of microorganisms by macrophages (MacMicking *et al.*, 1997). The acidification of inorganic nitrite results in the production of a variety of reactive nitrogen species (RNSs) such as nitric oxide (NO) (Weitzberg and Lundberg, 1998). Carlsson *et al.* (2005) speculated that the combination of nitrite and ascorbic acid in acidic conditions with which they inflated the catheter balloon resulted in the formation of RNSs in the surrounding urine, which accounted for the antibacterial effect observed. They attributed the bactericidal effects observed to NO on the basis that (1) only small uncharged molecules such as NO would be expected to pass through the silicone

balloon membrane freely, (2) the use of ascorbic acid strongly favours the formation of NO from acidified nitrate over the formation of other RNSs and (3) that the NO donor DETE NONOate, which is believed to be a pure NO donor with minimal formation of other RNSs (Feelisch, 1998), also had bacteriostatic effects. They also suggested that once NO has passed through the membrane it might be able to form other uncharacterised compounds with antimicrobial activity. From the data provided by Carlsson *et al.* (2005) it can be seen that the formation of NO from the solution in the balloon is rapid, and that the amount of NO in the urine quickly became undetectable (within 3 h). This pulse of NO production was suggested by the authors to be responsible for the bactericidal effects observed. The longer lasting effects were attributed to the formation of more long-lived antibacterial compounds in the urine.

Why then was this strategy not effective in extending the life-span of catheters when challenged by *Pr. mirabilis*? Given the fact that NO is active against such a wide range of pathogens including fungi, protozoa and bacteria as part of the human immune response (Fang, 1997) it seems unlikely that resistance to NO the cause. The model system used by Carlsson *et al.* (2005) was a batch culture model. In the catheterised urinary tract the urine in the bladder is being constantly replaced by urine from the kidneys and is a continuous flow system. The pulse of NO generated by the solution used by Carlsson *et al.* (2005) must as a result quickly eliminate the infecting bacteria since the production of NO occurs over such a short time period. The formation of more long-lived antibacterial compounds in the urine by NO would not be relevant since they would also be washed out with the urine from the bladder. Observation of the models in the early stages of incubation suggested that the NO strategy had an initial effect. The urine in the test models was distinctly less turbid

than in the controls. On further incubation however, the turbidity of the culture in the test urine soon matched that in the controls. This series of experiments shows that even with a modest flow rate of 0.5 ml/min of urine the strategy was unable to eliminate the infecting bacteria and as a result had no effect on the time taken for the catheters to block. If this strategy is to be effective it will be necessary to prolong the generation of NO in the catheter balloon.

4.4 The effectiveness of an integrated sensor/modulator strategy for the control of catheter encrustation.

The simple bromothymol blue / cellulose acetate sensor developed by Stickler *et al.* (2006a) is capable of signalling the early stages of catheter encrustation. Placed in the urine drainage bag it can warn patients, carers or nurses in good time that the catheter needs to be replaced (Stickler *et al.*, 2006c). In this way the use of the sensor could avoid the clinical crises induced by catheter blockage. The sensor would be of even more value if there was an effective strategy that could be deployed to inhibit encrustation when the problem is signalled. It was decided therefore to test the sensor / modulator concept using triclosan as a means of blocking crystalline biofilm development.

The 0.1 M Na₂CO₃ solution used as a solvent for triclosan in Section 3.3 has a pH of 11. If the catheter balloon were to burst inside the bladder the release of 10 ml of this alkaline solution would be of concern. Other triclosan containing solutions have been investigated for their effect on *Pr. mirabilis* biofilm formation *in vitro* (Williams, 2006). Williams (2006) found that a diluted solution of Hy-shield handcream was

effective against *Pr. mirabilis* when tested in the bladder model and at this stage the preparation was being considered for use in the clinical trials of the strategy. It was decided therefore to use the Hy-shield handcream for the triclosan intervention, with triclosan in Na₂CO₃ as a comparison.

In the previous study using Hy-shield (Williams, 2006) catheters were placed in the models, inflated with the antiseptic (diluted to 3 mg/ml triclosan) and then the residual urine in the bladder chamber was inoculated with *Pr. mirabilis*. Under these circumstances encrustation was prevented and the catheters drained freely for the experimental period. The results presented in Figure 3.42 show that when the introduction of the Hy-shield was delayed until the sensor signalled, the intervention was less successful at inhibiting encrustation. In two of the replicated experiments the test catheters blocked soon after their respective controls. In the third replicate the triclosan treated catheter drained for 96 h post-intervention period but by 108 h the pH of the urine in the bladder had risen to 8.5 suggesting that encrustation was occurring on this catheter. The results presented in Figure 3.43 suggest that the Hy-shield was slightly more effective against *Pr. vulgaris*. While in one of the replicate experiments the treated catheters blocked, it drained for 92 h compared to 29 h for the control. In the other two replicates, the catheter drained freely for 112 and 117 h and the pH of the urine was maintained at about 6.5 throughout the experimental period. As expected in models inoculated with *Pv. rettgeri* the test and control catheters all blocked (Figure 3.44). There was no significant difference between the blockage times of the test and control catheters.

In the models inoculated with *Pr. mirabilis* where the balloon was inflated with 3 mg/ml triclosan in Na₂CO₃, blockage did not occur within 96 h post triclosan intervention for any of the test models (Figure 3.45). All control models blocked within 30 h of inoculation. The pH of the urine in the test models was maintained at or just below pH 6.5. These results indicate that triclosan in Na₂CO₃ is more effective than Hy-shield. Hy-shield cream has a complex formulation and it is possible that some of the ingredients inactivate or cause a reduction of the diffusion of triclosan through the membrane of the catheter balloon.

The sensors used in this study were able to signal the rise in urine pH caused by the infecting urease producing organisms (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*) (Figures 3.42 – 3.45). Encrustation was observed on catheters removed from models stopped when the sensor became blue (Figures 3.46 – 3.50), confirming the observations of Stickler *et al.* (2006a) that the sensor signalled the early stages of catheter encrustation. The scanning electron micrographs (Figures 3.48 – 3.50) also show that the sensors became colonized by crystalline biofilm, though not as heavily as the catheters.

The urine in the collection bags remained alkaline even when the bladder urine pH was reduced (Figures 3.42 – 3.45). Thus the sensors continued to give a positive signal for the duration of the experiments. It is clear therefore that for the sensor to accurately reflect the pH of urine in the bladder after triclosan intervention both the collection bag and sensor need to be replaced. This raises the question of whether the catheter will have to be changed when the triclosan strategy is initiated. The results presented in Figures 3.42 and 3.43 show that if Hy-shield is to be used to supply

triclosan then it would probably be prudent to remove the catheter that had started to encrust and replace it with one inflated with the antiseptic solutions. In the case of the triclosan in Na_2CO_3 this would probably not be necessary.

Overall the data presented in Section 3.4 shows that a combined sensor/modulator strategy can be effective in preventing catheter blockage by *Proteus* sp. (but not by *Pv. rettgeri*) *in vitro*.

4.5 The response of the pH sensor to slow-encrusting and non-encrusting bacterial species.

In clinical practice the sensor will of course be exposed to the wide range of bacterial species that infect the catheterised urinary tract. It is of interest therefore to investigate its response to slow-encrusting and non-encrusting bacterial species.

The results of the 168 h long experiments with the three slow encrusting species (Figures 3.51 – 3.54) show that catheter blockage by crystalline biofilm was induced by *M. morgani* and *Staph. saprophyticus* but not by *Staph. aureus*. The colour shown by the sensors reflected the pH of the urine in the collection bags well during the experiments. For the models inoculated with *M. morgani* and *Staph. saprophyticus* the mean times between the sensor becoming positive and catheter blockage were 37.3 h (± 2.85 h) and 41.0 h (± 15.0 h) respectively. For the *Staph. aureus* inoculated models the sensors in two of the three replicates became positive during the experiment, although the signal intensity reduced as the urinary pH in the collection bag fell towards the end of the experiment. One sensor eventually became negative

again. Digital photographs taken of catheters removed from the model (Figure 3.54) show the extensive crystalline biofilms on the blocked *M. morganii* and *Staph. saprophyticus* catheters. The catheter removed from the *Staph. aureus* infected model after 168 h shows that while it had not blocked it was heavily encrusted. These three species were designated as slow encrusters as a result of experiments performed in the bladder model over 96 h (Section 3.1). The extensive encrustation and catheter blockage observed in the 168 h long experiments confirm this classification.

The data collected from experiments with urease negative *E. coli* and *Ent. faecalis* (Figures 3.55 & 3.56) show that the urine pH remained low (< 6.2) in both the bladder and collection bag for these species. This was reflected in the lack of colour change shown by the sensors, which remained yellow for the duration of the experiments. No encrustation can be seen in digital images of catheters removed from models inoculated with these species after 120 h (Figure 3.60).

For models inoculated with *Ps. aeruginosa*, *Pv. stuartii* and *K. pneumoniae* the urine pH in the bladder chamber did not exceed 6.4 during the experiment (Figures 3.57 – 3.59). The urine pH in the collection bag however was higher, and this was reflected in the colour of the sensor. The sensors of one of the three replicates of both the *Ps. aeruginosa* and *Pv. stuartii* infected models became positive during the experiments. None of the sensors in the *K. pneumoniae* infected models became positive, although colour change from yellow to pale green was observed. Digital photography showed no encrustation on catheters removed from models infected with these three species after 120 h, though thick mucoid biofilm was present on some catheter sections from *Ps. aeruginosa* and *K. pneumoniae* infected models (Figure 3.60).

The data for the slow-encrusting species show that the sensor is capable of providing early warning of blockage in *M. morganii* and *Staph. saprophyticus* infected models. The results for *Staph. aureus* were not so clear cut. The sensor gave a positive signal at 72 h reflecting an increase in the pH of the urine in the bag to 8 and a pH of 7 in the urine in the bladder. The intensity of the signal reduced with time however as the pH of the urine dropped to 7.3 in the bag and 6.7 in the bladder. The catheters were heavily encrusted and would therefore probably have blocked if the experiment had been extended. In this case however, it is less convincing to state that the sensor gave a clear warning of catheter encrustation.

The response of the sensors to *Ps. aeruginosa* and *Pv. stuartii* was also interesting in that they gave positive signals and yet the catheters drained freely and no evidence of crystalline biofilm was seen. These reactions have therefore to be considered as false positives. The results with all the test species show that the pH of the urine in the bag was consistently higher than that of the urine in the bladder. This is understandable as the storage of the urine in the bag for periods of up to 18 h prior to emptying allows the urease producing bacteria to generate more ammonia. It was decided to place the sensor in the drainage bag rather than between the catheter and drainage tube as it was shown to produce a more rapid response to *Pr. mirabilis* at that site (Stickler *et al.*, 2006a). The results presented in Figures 3.51 – 3.60 suggest that perhaps the specificity of the sensor would be improved if it was located in a connector between the catheter and drainage tube. At this site it would register the pH of urine flowing from the bladder and thus in the cases of *Ps. aeruginosa* and *Pv. stuartii* should not give a signal. It is interesting however that when the sensor was tested in the urine

bags of patients (Stickler *et al.*, 2006c) it did not give positive signals in the presence of *Ps. aeruginosa*, *Pv. stuartii* or *K. pneumoniae*. It might also be interesting to see how a sensor at the catheter / drainage tube junction responded to *Staph. aureus*.

4.6 Are other urinary tract pathogens capable of modulating crystalline biofilm formation by *Pr. mirabilis*?

Mathur *et al.* (2006a) showed that in patients infected with *Pr. mirabilis* the rates at which catheters block vary considerably. In their study mixed communities of organisms were isolated from the urines of all the patients. The availability of the collection of organisms from these patients made it possible to investigate the effect of the bacterial communities on the ability of the *Pr. mirabilis* strains to encrust catheters in the laboratory model.

In the case of patient 6, while the *Pr. mirabilis* and *Ent. faecalis* were colonizing the urine, the catheter took 13 weeks to block with encrustation (Mathur *et al.*, 2006a). It could be the case that the *Pr. mirabilis* strain was a poor urease producer and not capable of generating the alkaline conditions necessary for rapid encrustation. The results presented in Figure 3.61 however, demonstrate that in the laboratory model this organism was perfectly capable of raising the urinary pH to 8.3 and blocking catheters in 25 h. In the presence of the *Ent. faecalis* the rate of encrustation was reduced and the catheters took significantly longer to block (25 h vs 55 h) ($P=0.037$). Although *Ent. faecalis* had little effect on the growth of *Pr. mirabilis* (Figure 3.62) and there was no significant difference between the mean urinary pHs at blockage ($P=0.27$) it did appear that the rise in urinary pH in the mixed community was slower

than in the pure culture of *Pr. mirabilis*. It is possible therefore that the *Ent. faecalis* in some way inhibited the production or activity of the *Pr. mirabilis* urease enzyme.

Consideration of some important clinical factors makes it difficult to accept that the delay in catheter blockage by *Ent. faecalis* observed in these experiments could explain the observation that in the patient the catheter took 13 weeks to block. The patient concerned was a 101 year old woman who was fitted with a percutaneous endoscopic gastronomy tube after a stroke. The pH_v of her urine was consistently high (7.5 – 8.0) throughout the lifetime of the catheter, testimony to the urease activity of the *Pr. mirabilis*. The pH_n of her urine however was even higher, fluctuating around pH 9. The probable reason for such dilute urine (unusual in an elderly catheterised patient) was that being fed with a gastronomy tube ensured a good fluid intake. It seems likely therefore that in the case of patient 6, the dilute urine with its high pH_n was the main reason for the very slow rate of catheter encrustation.

Patient 9 had urine which was colonized by *Pr. mirabilis*, *E. coli*, *K. pneumoniae* and *Ent. faecalis*. This patient did not experience catheter blockage for the duration of the study period (14 weeks) (Mathur, 2007). Control models inoculated with the *Pr. mirabilis* isolate alone quickly blocked (mean 35 h) demonstrating its ability to generate alkaline urine and form crystalline biofilm (Figure 3.63a). There was no significant difference between the mean blockage times of catheters in models inoculated with the *Pr. mirabilis* isolate alone (35 h) and the community (40 h) ($P=0.643$). The presence of other species of bacteria appeared to have little effect on the growth of the *Pr. mirabilis* or its production of urease. Conversely the numbers of

both the *E. coli* and *K. pneumoniae* isolates present were reduced from their initial population by the end of the experiment (Figure 3.63b).

Given the lack of an observable effect by the community in reducing the rate of encrustation by *Pr. mirabilis* in the model it seems unlikely that the isolates present in the community from this patient are linked to the lack of encrustation seen on the patients catheters. The pH_v of this patient's urine varied from pH 6 to 7.5 although it was generally around pH 7. The pH_n of the urine varied from pH 7 to 8.5 and was consistently higher than the pH_v , with the pH_n usually 1 – 2 pH units higher than the pH_v . This elevated pH_n is most likely to be due to relatively dilute urine. Patient 9 was living in her own home during this study, implying that she was relatively competent. As a result it may be reasonable to assume that she was able to provide herself with sufficient fluid to maintain dilute urine during the study.

Patient 19 did not experience catheter blockage during the study (13 weeks) (Mathur *et al.*, 2006a). His urine was colonized by *Pr. mirabilis*, *Ps. aeruginosa*, *E. coli*, and *K. oxytoca* for the majority of the study. While the *Pr. mirabilis* isolate was able to quickly block catheters in the model when tested alone (mean 40 h), only one of the models infected with the entire community had sufficient crystalline biofilm to cause catheter blockage (after 147 h) with neither of the remaining catheters blocking after 168 h. The urine pH in the control models was significantly higher than that of the test models after 24 h ($P=0.021$) and this corresponded to a reduction in the numbers of *Pr. mirabilis* present in the test models (Figures 3.64 & 3.65). While the numbers of *Pr. mirabilis* were reduced the urine pH remained below 7.0 in the test models. When *Pr. mirabilis* was able to recover back to the numbers present at the start of the

experiment in one of the test models the urinary pH rose and blockage quickly occurred (Figures 3.64 & 3.65). The other members of the community appeared to be able to inhibit the growth of *Pr. mirabilis* effectively while growing well themselves for the majority of the experiment (Figure 3.65). It should be noted that in the patient 19 test catheters through which urine flowed freely for 168 h some encrustation was present despite the reduction in *Pr. mirabilis* numbers (Figure 3.67). The biofilm present on the unblocked catheters after 168 h had an unusual form which was distinct from any of those seen previously in this study, appearing to consist of both crystalline and mucoid material (Figure 3.66).

Patient 19 was able to maintain a wide safety margin between urinary pH_v and pH_n for the duration of the study, in common with patients 6 & 9. While patients 6 & 9 had urinary pH_v s that were in the range 6.5 – 7.5, as might be expected in the case of a *Pr. mirabilis* infection, the urinary pH_v from patient 19 had a mean value of 4.77. This would suggest that the *Pr. mirabilis* isolate was having very little effect on urinary pH. Since this isolate had no problems in raising urinary pH and causing catheter blockage in the laboratory model it seems reasonable to assume that the effect seen in the models infected with the community may be occurring in the urine of this patient, with a restriction of the growth of *Pr. mirabilis* by the other species.

Bacterial interference with non-pathogenic bacteria has shown some success in preventing the establishment of infection by pathogenic strains. Darouiche *et al.* (2005) found that the deliberate establishment of a non-pathogenic *E. coli* strain on patient's catheters reduced the occurrence of the development of symptomatic UTI during the subsequent year by half. Unfortunately a subsequent trial found that the

presence of *Proteus* sp. in a patients urine had a deleterious effect on the *E. coli* biofilm and significantly reduced the numbers of *E. coli* present, indicating that the use of *E. coli* in this way would be unlikely to be effective against encrustation by *Proteus* sp. (Trautner *et al.*, 2007).

The communities from patients 9 & 19 involve several similar organisms. Both have an *E. coli* isolate and a *Klebsiella* sp. isolate (*K. pneumoniae* for patient 9 and *K. oxytoca* for patient 19). In addition the patient 19 community also has a *Ps. aeruginosa* isolate. The presence of the *Ps. aeruginosa* isolate would appear to be the main difference between the communities from these two patients. *Ps. aeruginosa* isolates, in common with both *K. pneumoniae* and *K. oxytoca*, have been observed to form unusually thick mucoid biofilms in previous experiments (Section 4.1), with those formed by *Ps. aeruginosa* being slightly more extensive than either *Klebsiella* sp. It seems likely that some property of the *Ps. aeruginosa* biofilm phenotype is affecting the growth of the *Pr. mirabilis* isolate, while not affecting the growth of the other two species which show consistently high numbers in all three replicates (Figure 3.65).

Sabbuba *et al.* (2002) noted the effectiveness of *Ps. aeruginosa* in reducing the swarming of *Pr. mirabilis* over sections of urinary catheter. It is possible that the apparent inhibition of *Pr. mirabilis* seen in the patient 19 community is due to the same or a related mechanism to the inhibition of swarming by *Ps. aeruginosa*. It is interesting to note that the species other than *Pr. mirabilis* from the communities reconstituted from patients 9 and 19 which were tested along with *Ps. aeruginosa* by

Sabbuba *et al.* (2002) (*E. coli*, *K. pneumoniae* and *Ent. faecalis*) had little or no effect on the swarming of *Pr. mirabilis*.

It would be useful to test the patient 19 *Ps. aeruginosa*, *E. coli* and *K. oxytoca* isolates individually against the *Pr. mirabilis* isolate to determine if the inhibitory effect is due to the actions of the community or a particular isolate. After determining the source of the inhibition the isolate(s) could then be tested against other *Pr. mirabilis* isolates and additional isolates of the same species tested for their activity against *Pr. mirabilis*. Prospective studies similar to that conducted by Mathur *et al.* (2006a) which searched specifically for patients with *Proteus* infection who do not experience catheter blockage would also be useful in identifying other candidates for use in this type of bacterial interference.

Section 5

General Discussion

and

Future work

5. General Discussion and Future Work.

Pr. mirabilis has been consistently identified as the species responsible for the majority of episodes of catheter blockage by crystalline biofilm (Mobley and Warren, 1987; Kunin, 1989; Stickler *et al.* 1993b; Stickler *et al.*, 1998a). Although this study demonstrates that *Pr. vulgaris* and *Pv. rettgeri* are capable of causing rapid encrustation in the bladder model, both of these species occur relatively infrequently in the catheterised urinary tract (Mobley and Warren, 1987; Kunin, 1989). Species identified as slow encrusters (*M. morgani*, *Staph. aureus* and *Staph. saprophyticus*) are also only likely to cause encrustation in a minority of patients whose urine has a low pH_n. As a result strategies to combat catheter encrustation must focus on *Pr. mirabilis* since resolving problems caused by this species would benefit the vast majority of patients affected.

Biofilms cause problems in a wide variety of situations, from implanted devices to the hulls of ships. A number of attempts have been made to produce materials which inhibit the initial adhesion of bacteria to surfaces. In implanted devices a major problem with this strategy is that the device often becomes coated by serum proteins, providing an ideal surface for bacterial adherence and biofilm formation (Gristina, 1987). Surfaces which antibacterial agents are either attached to or released from have been widely utilised to combat biofilm associated infection in implanted devices including heart valves, prosthetic joint replacements and venous catheters. Silver has been commonly used in this role, with varying effectiveness (Darouiche, 1999; Kjaergard *et al.*, 1999). Antoci *et al.* (2007) found that covalently bonding vancomycin to the surface of a titanium alloy was effective at preventing bacterial

colonization of the surface by *Staph. aureus in vitro*. It remains to be seen whether this approach will be sufficient to overcome the problem of coating by serum proteins *in vivo*. Antifouling surfaces or coatings have been used to prevent the growth of organisms on ships hulls since the Napoleonic wars, where copper was used to prevent fouling on warships. Many of the strategies used were highly toxic but recently legislation has required the use of minimally or non-toxic (to the marine environment) coatings (Wigglesworth-Cooksey *et al.*, 2007). This has led to the development of alternative methods for the prevention of fouling including the adaptation of antifouling strategies used by marine organisms. The most interesting of these is the use of halogenated furanones from the red alga *Delisea pulchra* (de Nys *et al.*, 1995). Synthetic halogenated furanones have been shown to be effective in disrupting biofilm formation in *Ps. aeruginosa* by interfering with quorum sensing (Hentzer *et al.*, 2002). This disruption is due to the furanones competing with the AHLs used by Gram-negative bacteria in cell to cell communication. Unfortunately due to their high reactivity these halogenated furanones are likely to be too toxic for use in humans (Hentzer and Givskov, 2003). They do however highlight the potential of this approach in disrupting biofilm formation on implanted devices.

Although these strategies might prove effective against most biofilm associated problems, the formation of catheter encrustation by *Pr. mirabilis* presents a different challenge. It is a physically and chemically driven process where the contribution of *Pr. mirabilis* is mainly to produce urease which raises the urinary pH and causes the precipitation of calcium and magnesium phosphates. Once the urinary pH_v exceeds the pH_n crystal formation occurs in the urine. These crystals aggregate with *Pr. mirabilis* and are deposited on the catheter surface, eventually causing blockage of the

catheter lumen. As a result in order to prevent catheter blockage by *Pr. mirabilis* the urinary pH_n must be maintained above the pH_v . There are two ways in which this may be accomplished. Restricting either the growth or urease production of *Pr. mirabilis* would prevent the rise in urinary pH_v . This can be achieved by the use of antimicrobials released into the urine, as shown by the use of triclosan (Jones *et al.*, 2005; Jones *et al.*, 2006), or by urease inhibitors (Morris and Stickler, 1998b). Resveratrol, a naturally occurring polyphenol found in red wine, grapes and peanuts has been found to inhibit urease production and swarming as well as several other virulence factors in *Pr. mirabilis* (Wang *et al.*, 2006). Studies on the cytotoxicity of resveratrol have found that it may be suitable for use clinically, unlike other urease inhibitors such as acetohydroxamic acid (Babich *et al.*, 2000). The second option is to raise urinary pH_n to levels where it exceeds the pH_v caused by *Pr. mirabilis* infection. This can be achieved by increasing the fluid and intake of citrate, a metal ion chelator, which dilutes the urine and raises the urinary pH_n (Suller *et al.*, 2005; Stickler and Morgan, 2006).

The simple cellulose acetate / bromothymol blue sensor developed by Stickler *et al.* (2006a; 2006c) provides a constant indication of urinary pH in the catheter bag. The placement of the sensor at the catheter / drainage tube junction would allow direct monitoring of urinary pH_v . This might improve the specificity of the signal, since the urine in the collection bag may have elevated urinary pH relative to the pH_v due to the action of urease while the urine is stored in the bag. The development of an inexpensive sensor able to detect both urinary pH_v and pH_n would be extremely useful in the management of catheter blockage. This would give health care staff and patients

warning of imminent blockage and allow the targeted application of the triclosan or any other moderation strategy.

Experiments examining the response of the sensor to urease producing species other than rapid encrusters found that while the sensor performed well in predicting blockage by slow encrusting species a number of false positive signals were observed with non-encrusting species. Although this was not reflected by the clinical data (Stickler *et al.*, 2006c) it may be that the placement of the sensor at the catheter / drainage tube junction might improve the specificity of the sensor under these circumstances. This could be determined in experiments in the laboratory model.

The intrinsic resistance of *Pv. rettgeri* to triclosan, while not likely to be a problem initially due to its infrequent occurrence, may become a significant problem if the use of the triclosan strategy clinically causes its selection. As a result it would be prudent to investigate the possibility of using a similar method to deliver antimicrobials to which *Pv. rettgeri* is sensitive to the catheterised urinary tract using the bladder model. A combination of triclosan with any agents found to be effective against *Pv. rettgeri* would provide a strategy effective against all of the bacterial species capable of causing rapid encrustation. Williams (2006) found that nalidixic acid was able to diffuse from the catheter balloon in sufficient quantities to significantly extend the mean time to blockage of catheters by *Pr. mirabilis*. Unfortunately there is wide variation in the activity of this drug against *Pv. rettgeri*, the MIC ranging from 2 – 256 µg/ml (Hawkey and Hawkey, 1984). Research into agents more consistently active against *Pv. rettgeri* which are deliverable via the catheter balloon should be conducted.

The use of nitric oxide by Carlsson *et al.* (2005), while not found to be effective in the bladder model, is an interesting attempt to use a component of the human immune response to combat CAUTI. The production of NO in the catheter balloon did initially reduce the turbidity of the urine in the bladder model, suggesting that it can affect the growth of *Pr. mirabilis*. The short duration of the NO release however meant that it did not reduce the time to blockage of the catheters. It would be interesting to test the ability of a NO producing solution able to release NO over a longer period of time to extend catheter lifespan in the bladder model.

An alternative use for NO may be in combining it with the triclosan strategy. Triclosan does not immediately diffuse from the catheter balloon after inflation, taking up to 4 h to reach maximum levels (Jones, 2005). The quick release of NO by the solution used by Carlsson *et al.* (2005) might be able to cover this gap, with a combined solution providing consistent antimicrobial activity in the urine. This could be tested in principle in the bladder model.

The use of antimicrobial strategies to eliminate *Pr. mirabilis* colonization of the catheterised urinary tract raises the question of what happens next. Will *Pr. mirabilis* inevitably re-colonize the urine of these patients after successful elimination? Sabbuba *et al.* (2004) found that infection stones from the bladders of long-term catheterised patients experiencing catheter encrustation contained the same strain of *Pr. mirabilis* as in the catheter encrustation. It is possible that bacteria in these stones may be protected from the action of antimicrobials that were effective in eliminating *Pr. mirabilis* from the catheter and urine. Preventing the rapid reoccurrence of *Pr. mirabilis* colonization may as a result require the removal of these stones. Any

studies into the effectiveness of antimicrobial strategies in eliminating *Pr. mirabilis* would need to take this into account when considering the long-term management of patients.

Although the use of antibacterial agents does show promise in controlling the formation of encrustation it would be far better in the long-term to find ways of reducing encrustation by other means. This would avoid the problems of resistance to the antimicrobial, important given the relatively small numbers of new antimicrobials coming into clinical use, and also any adverse reactions to the antimicrobial that might be experienced by the patient. Mathur *et al.* (2006a) observed that patients with a high urinary pH_n were far less likely to experience catheter blockage by encrustation. Stickler and Morgan (2006) demonstrated that the dilution of urine and addition of citrate combine to raise the pH_n of urine significantly. This strategy avoids the use of antimicrobials and so bypasses the problem of resistance. It is a strategy which should be effective against all of the urease producing species since it raises the pH_n , thereby blocking a fundamental part of the bacterially induced encrustation process. Stickler and Morgan (2006) showed that this strategy was effective against *Pr. mirabilis*. Experiments in the bladder model would confirm its effectiveness against other bacterial species capable of causing encrustation. Increasing fluid intake is a simple measure that could be used effectively on the majority of patients and it would be interesting to see its effect on the occurrence of catheter encrustation in a clinical trial.

Some patients are chronically colonized by *Pr. mirabilis* while others are not. This raises the question of why this should be. Is there a reason other than that they have

not been exposed to it? Mathur *et al.* (2005) found that the isolate of *Pr. mirabilis* colonizing the catheterised urinary tract is often identical to that found in the patients gut microflora. It would be interesting to examine the extent to which catheterised patients without *Pr. mirabilis* as part of their urinary flora have it as a part of their gut microflora. It may be that for some patients with an already established community colonizing their urine that this community serves to prevent the establishment of other species such as *Pr. mirabilis*. This also could be tested in experiments in the bladder model.

A recent study by Laube *et al.* (2007) found that a diamond-like carbon coating (DLC) was effective in preventing the formation of encrustation on ureteral stents in patients who had previously experienced problems with stent encrustation. This material has the additional advantage of being relatively low friction, which may mean that its inclusion in urinary catheters would cause less trauma to the urethral mucosa on insertion. Unfortunately *Pr. mirabilis* was not one of the bacteria isolated from patients during this study so its effectiveness against this species is unknown. It would be interesting to examine the effect of urinary catheters coated with DLC on the formation of crystalline biofilm by *Pr. mirabilis* in the bladder model.

The data presented on the effect of catheter communities on the formation of encrustation shows the potential for bacterial interference in combating catheter blockage. Bacterial interference using a non-pathogenic strain of *E. coli* has shown some success in reducing the occurrence of symptomatic UTI (Darouiche *et al.*, 2005). Unfortunately the use of this strain was not effective against *Proteus* sp. (Trautner *et al.*, 2007). Trautner *et al.* (2005b) demonstrated that a colicin expressing

strain of *E. coli* was able to prevent the establishment of a colicin susceptible strain of *E. coli* on catheters *in vitro*. They proposed that the creation of a non-pathogenic bacterial strain able to produce a broad spectrum of bacteriocins active against uropathogens would potentially be effective in preventing the establishment of these species on urinary catheters when used in the manner of Darouiche *et al.* (2005). This is an interesting application of bacterial interference although the use of genetically modified organisms in a medical context may face strong opposition from regulatory authorities. The experiences of Darouiche *et al.* (2005) and Trautner *et al.* (2007) show that it is possible, at least in *E. coli*, to use a non-pathogenic strain to prevent or restrict the establishment of pathogenic strains. It would be interesting to see if this approach could be effective for *Pr. mirabilis*. Different strains of *Pr. mirabilis* are quite antagonistic towards each other when swarming and this is the basis of Dienes typing. Laboratory experiments could be performed to determine whether the presence of an established urease-negative strain of *Pr. mirabilis* might be effective in preventing the colonization of catheters by urease-positive strains of *Pr. mirabilis* of different Dienes type.

The inhibitory effect of the catheter community (from patient 19) on the formation of encrustation by *Pr. mirabilis* requires more study. It is necessary to identify whether one of the species present in this community is responsible for the inhibition of *Pr. mirabilis* or if the effect is due to the whole community. Once the source of the inhibition is identified it may be possible to determine the specific factors responsible. This could potentially lead to the use of a bacterial strain in the manner of Darouiche *et al.* (2005) but targeted specifically at preventing *Pr. mirabilis* colonization. Clinical studies similar to that performed by Mathur *et al.* (2006a) looking

specifically for patients with *Proteus* infection but with a low urinary pH who are not experiencing catheter blockage might produce further suitable candidate communities for bacterial interference studies. In addition the collection of communities from patients without *Proteus* infection would allow their testing *in vitro* to determine if the communities were preventing the establishment of *Proteus* on their catheters.

It is important that any strategy used for the control of encrustation does not have serious side effects. If antimicrobial strategies such as using triclosan are unable to completely eliminate *Pr. mirabilis* from the urinary tract then may be necessary to extend their use as a long-term moderator rather than just for short-term crisis management. Strategies involving alteration of urinary composition such as the use of high citrate drinks are inherently long-term since they do not attempt to remove the *Pr. mirabilis* colonization. The use of either of these methodologies would need to be closely monitored in order to detect potential problems.

The insight we now have about the mechanisms of catheter encrustation make it very clear that we have to be more ingenious than simply putting antimicrobials in catheters if we are to solve this problem that complicates the care of so many elderly and disabled people.

Section 6

Conclusions

6. Conclusions

1. Three species (*Pr. mirabilis*, *Pr. vulgaris* and *Pv. rettgeri*) were identified as rapid catheter encrusters. They were able to raise the urinary pH in the model to > 8 , causing the formation of encrustation and blockage. Assays of urease activity showed that these species were able to cause far more urea hydrolysis than the majority of urease producing species tested. As *Pr. vulgaris* and *Pv. rettgeri* are rarely found in the catheterised urinary tract, strategies to control the formation of catheter encrustation should focus on *Pr. mirabilis*.

2. *M. morgani*, *Staph. aureus* and *Staph. saprophyticus* are able to raise the mean urinary pH from 6.1 to 6.89 – 7.39 over 96 h and cause the slow formation of crystalline biofilm on urinary catheters. Crystalline biofilm is only likely to be formed by these species in a minority of cases in patients whose urine has a consistently low pH_n.

3. A number of commonly occurring species identified as urease positive in standard diagnostic tests were found to be non-encrusting in the model (*Pv. stuartii*, *Ps. aeruginosa*, *K. pneumoniae*, *K. oxytoca*, *Ser. marcescens*, *Enterobacter cloacae*, *C. freundii* and *C. koseri*). These species were not able to raise the mean urinary pH above mean levels of 6.45 and did not form crystalline biofilm. Urease assays on these species in artificial urine found that they were unable to cause the hydrolysis of significantly more urea than the urease negative controls. These species are not appropriate targets for control strategies.

4. Comparison of the effectiveness of antibacterial catheters (silver-hydrogel and nitrofurazone) with all-silicone controls showed no significant differences in time to blockage, mean urinary pH or bacterial populations when challenged by *Pr. mirabilis*, *Pr. vulgaris* or *Pv. rettgeri*. These antibacterial catheters are thus vulnerable to encrustation by crystalline biofilm.

5. The use of a 3 mg/ml triclosan in 0.1 M sodium carbonate solution to inflate catheter balloons was effective in preventing catheter blockage by *Pr. mirabilis* and *Pr. vulgaris* for > 168 h in the model. Both *Proteus* sp. tested had MICs of triclosan of ≤ 0.2 $\mu\text{g/ml}$. This strategy was not effective against *Pv. rettgeri*, which showed no significant difference in mean blockage time between the test and control catheters. The MIC of triclosan of *Pv. rettgeri* isolates was 64 $\mu\text{g/ml}$.

6. The sensor / modulator strategy in which the cellulose acetate – bromothymol blue sensor signals that the urinary pH has risen and that the catheter balloon should be inflated with triclosan, was successful in preventing catheter blockage by *Pr. mirabilis* and *Pr. vulgaris* but not *Pv. rettgeri*. The change in the sensor from yellow to blue signals that encrustation has started to build up on the catheter. When triclosan at 3 mg/ml in 0.1 M sodium carbonate is then introduced into the balloon the further development of the crystalline biofilm is arrested and the catheter drains freely.

7. The pH sensor was effective in providing early warning of blockage by the slow encrusting species *M. morgani* and *Staph. saprophyticus*. For *Staph. aureus* however the reaction of the sensor was less clear-cut.

8. Experiments examining the reaction of the sensor to non-encrusting species in the model system showed that while it generally remained negative, some false positive signals were observed for *Ps. aeruginosa* and *Pv. stuartii*.

9. Inflation of the catheter balloon with a nitric oxide producing solution was not effective in preventing encrustation and blockage of catheters in the model by *Pr. mirabilis*. There was no significant difference between the mean catheter blockage times in the test and control models.

10. Organisms present with *Pr. mirabilis* in the mixed urinary flora of the catheterised urinary tract can have an inhibitory effect on the ability of the *Pr. mirabilis* strain to encrust catheters.

Section 7

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7. References

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