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A study of the factors that modulate the rate of crystalline *Proteus mirabilis* biofilm development on urinary catheters

Thesis presented for the degree of
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September 2006

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Acknowledgements

Thanks and acknowledgements go to:

My supervisor Dr Dave Stickler for all his help and expertise. For his encouragement to carry out research in this fascinating area of microbiology and for providing funding.

Mike Turner, Rob Young, and Ant Hann for all their help with various aspects of the electron microscopy. Mike O'Reilly for his help with the FAAS analyses and Joan Hubbard for being the star that she is. From always managing to do my mass of autoclaving, even at short notice, to putting up with the stench of the lab's waste on a daily occurrence!

Dr Simon Jackson and Mrs Joan Parton of the Department of Medical Microbiology, University Hospital, Wales for their expertise and guidance in teaching the endotoxin assay methods. Marcus Drake and Sunil Mathur of the BioMed Centre, Southmead Hospital, Bristol for collecting urine samples and catheters for me.

Past and present members of Lab 1.51: Gwenz, Mikey (PC Long), Gareth (Earl of Barry), Nora (chick), Shezza, Rob (Geldoff/Broominator/2Benches /Broomie/Richard), Steve (Steveybean), Zeliha, Nicola, and John, who have all made my time in Cardiff so enjoyable. From answering work related queries to accompanying me on very drunken nights out! And to all the other Post-grads, Post-docs and randoms I've met along the way in Cardiff, special mention to Spunky, Lady Harvey, Cat, Shooter, The Baron of Cwmbran, Siân, Gareth J, and the girlies from Park Place Gym!

A huge thank you to my mum, dad and Grandma who have helped out with various problems, be they computer or financial, and hounded me to "hurry up and finish". I finally did it!!!! Woo hoo! And to my lil' bro for "attempting" to proof-read my introduction but losing the will to live half way through.

And finally to the inhabitants of Pathogen City: Otis Marksmen, Kyle Oxymora, Mort and Morag Moraine, Morgan Ella, Provo Stuart, Olav Australia and Proton Ted. Thanks for making writing-up more bearable!

Publications and Presentations

Publications

- Stickler, D. J., J. C. Lear, N. S. Morris, **S. M. Macleod,** A. Downer, D. H. Cadd, and W. J. Feast. (2006). Observations on the adherence of *Proteus mirabilis* onto polymer surfaces. *J. Appl. Microbiol.* 100(5): 1028-1033.

Oral presentations

- **Macleod S. M.** (2006). The ability of other uropathogens to prevent catheter encrustation and blockage by *Proteus mirabilis*. Presented at the All-Wales Microbiology Meeting, Gregynog Hall, Wales: 20-22 March 2006.

Poster Presentations

- **Macleod, S. M.,** and D. J. Stickler. (2004). Q-205. Can *Proteus mirabilis* colonize *Pseudomonas aeruginosa* urinary catheter biofilms? Presented at the American Society for Microbiology 104th General Meeting, New Orleans, USA: 24-27 May 2004.

Abstract

Around 50% of patients enduring long-term catheterisation experience encrustation and blockage of their catheters. This problem stems from infection by urease producing bacterial species, in particular *Proteus mirabilis*. The urease enzyme hydrolyses urea to carbon dioxide and ammonia which elevates the urinary pH. Under these alkaline conditions crystals of calcium and magnesium phosphates form and the crystalline bacterial biofilm that develops on the catheter can eventually block the flow of urine through its lumen. Catheter blockage in this way can induce complications that put the health of the patient at serious risk. Currently there are no effective methods for controlling this process and little is known about the bacterial or host factors that might modulate the rate of catheter encrustation. The extent to which catheter biofilms contain potentially dangerous levels of endotoxin is also unknown. In view of the lack of information relating to these issues the objectives of this study were to: (a) gain an insight into the complexity of the urinary flora of patients undergoing long-term catheterisation; (b) examine the bacterial composition of catheter biofilms for evidence of antagonisms between *Pr. mirabilis* and other species; (c) test the effects of other uropathogens on the ability of *Pr. mirabilis* to produce catheter encrustations in laboratory models of the catheterised bladder; (d) examine the hypothesis that coaggregation between *Pr. mirabilis* and other species is involved in the formation of crystalline catheter biofilms; and (e) determine whether endotoxin can be found in catheter biofilms from patients undergoing long-term catheterisation.

Over a six-week period urine samples were analysed from five patients undergoing long-term catheterisation. The urinary flora was both polymicrobial and dynamic, commonly containing at least four bacterial species. The pH of the urine varied from week to week. The presence of *Pr. mirabilis* was always associated with alkaline urine (mean pH 8.66). The presence of other urease producing species such as *Pseudomonas aeruginosa* and *Morganella morganii* were not associated with highly alkaline urine. In the cases of the four patients who did not suffer from catheter blockages in the study period, the nucleation pH (pH_n) of their urine at week six was above the pH of their voided urine (pH_v). The only patient in which the pH_n was below the pH_v had a stable *Pr. mirabilis* infection and had two catheters block during the study period. A significant negative correlation was found between the urinary concentrations of calcium and magnesium and the nucleation pH value. Strategies to decrease the concentrations of these divalent cations will act to increase the nucleation pH and reduce the rate of crystal accumulation and mineralised bacterial biofilm development. To control catheter encrustation it will be essential to prevent the ability of *Pr. mirabilis* to elevate the pH of the urine above its nucleation pH.

Analysis of the data on 106 catheter biofilm communities from long-term hospital and community-dwelling catheterised individuals revealed that the overall incidence of *Pr. mirabilis* was 30.19%. Particularly when species such as *Klebsiella pneumoniae* were recovered from catheters, the percentage incidence of *Pr. mirabilis* was above this figure. In contrast, when species such as *Escherichia coli*, *Morg. morganii* or *Enterobacter cloacae* were present on a catheter, *Pr. mirabilis* was rarely or never found. An experimental approach, using laboratory models of the catheterised bladder, was used to investigate the interactions of *Pr. mirabilis* with the test

organisms *Et. cloacae*, *Morg. morganii*, *Kl. pneumoniae*, *E. coli*, and *Ps. aeruginosa* in more detail.

Experiments in laboratory models showed that super-infection of *Pr. mirabilis* after 24 h growth of one of each of the test species had little or no effect on the ability of *Pr. mirabilis* to encrust and block catheters. However, growth of *Et. cloacae*, *Morg. morganii*, *Kl. pneumoniae*, or *E. coli* for 72 h prior to *Pr. mirabilis* super-infection significantly delayed catheter blockage. When *Pr. mirabilis* was inoculated into models 72 h after *Et. cloacae* for example, the mean time to blockage was extended from 28.74 h to 60.73 h ($P \leq 0.01$). In all cases however, *Pr. mirabilis* was eventually able to generate alkaline urine, induce crystal formation, and block the catheters. Throughout the series of experiments in the bladder models none of the mono-cultures of the five test species proved capable of blocking catheters with crystalline biofilm. However, some signs of encrustation appeared on catheters in models that had been incubated with *Morg. morganii* for > 120 h.

A *Pr. mirabilis* from the urinary flora of a catheterised patient who had no signs of encrustation was capable of blocking catheters *in vitro*. Reconstituting a four-member bacterial community from this patient significantly slowed the rise in urinary pH and postponed blockage compared to models infected with the *Pr. mirabilis* alone (121.17 h vs. 44.36 h; $P \leq 0.05$).

There was no evidence of specific coaggregation between *Pr. mirabilis* and other uropathogens that might determine community structure in catheter biofilms. It was demonstrated that crystal formation occurred in alkaline conditions and produced macroscopic aggregation of crystals and cells.

Biofilms on sections of catheters received from patients were found to contain endotoxin levels ranging from 282.8 to 917.2 ng/4 cm length of catheter. Long-term catheterised patients are therefore potentially at risk of catheter-associated endotoxaemia and the serious complications that can follow.

This investigation has begun to explore some of the factors that might modulate the rate of catheter encrustation and blockage caused by *Pr. mirabilis*. The results from this study suggest that antagonistic interactions between *Pr. mirabilis* and other urinary tract organisms do exist. The identification of benign bacterial species that inhibit crystalline biofilm formation opens up the prospect of developing a novel biological interference strategy to control this complication of long-term catheterisation that undermines the health of so many elderly and disabled people.

Contents **Page**

List of Figures **Page**

List of Tables **Page**

List of abbreviations

Unit abbreviations

Acceleration due to gravity	*g*
Centimetres	cm
Charrière units	Ch
Colony forming units	cfu
Degrees centigrade	°C
Endotoxin units	EU
Grams	g
Hours	h
Litres	L
Microlitres	µl
Micrometres	µm
Milligrams	mg
Millilitres	ml
Millimetres	mm
Nanograms	ng
Nanometres	nm
Parts per million	ppm
Picograms	pg
Revolutions per minute	rpm
Seconds	s
Volume/volume	v/v
Weight/volume	w/v

Organism abbreviations

Actinomyces naeslundii	*A. naeslundii*
Blastamonas natatoria	*B. natatoria*
Citrobacter freundii	*C. freundii*
Citrobacter koseri	*C. koseri*
Escherichia coli	*E. coli*
Enterobacter cloacae	*Ent. cloacae*
Enterococcus durans	*Ent. durans*
Enterococcus faecalis	*Ent. faecalis*
Enterococcus faecium	*Ent. faecium*
Enterococcus hirae	*Ent. hirae*
Fusobacterium nucleatum	*F. nucleatum*
Gardnerella vaginalis	*G. vaginalis*
Klebsiella pneumoniae	*Kl. pneumoniae*
Klebsiella oxytoca	*Kl. oxytoca*
Micrococcus luteus	*M. luteus*
Micrococcus lylae	*M. lylae*
Morganella morganii	*Morg. morganii*
Proteus mirabilis	*Pr. mirabilis*
Proteus vulgaris	*Pr. vulgaris*
Providencia rettgeri	*Prov. rettgeri*

Organism abbreviations continued

Providencia stuartii	*Prov. stuartii*
Pseudomonas aeruginosa	*Ps. aeruginosa*
Serratia marcescens	*Ser. marcescens*
Staphylococcus aureus	*S. aureus*
Streptococcus sanguis	*Strep. sanguis*

Other abbreviations

Acylated homoserine lactones	AHLs
American Type Culture Collection	ATCC
Approximately	approx.
Calcium	Ca
Colistin Inositol Agar	CI Agar
Cystine Lactose Electrolyte Deficient Agar	CLED Agar
Early morning urine	EMU
Extracellular/exo Polysaccharide	EPS
Hexaammineruthenium trichloride	HexRT
High vacuum scanning electron microscopy	HV-SEM
Indwelling urethral catheter	IUC
Limulus Amoebocyte Lysate	LAL
Lipopolysaccharide	LPS
Low vacuum scanning electron microscopy	LV-SEM
Magnesium	Mg
Mid-afternoon urine	MAU
Midday urine	MDU
Nucleation pH derived from optical density	pH_n
Nucleation pH derived from flame atomic absorption spectroscopy	pH_n^{Ca}, pH_n^{Mg}
Odds ratio	OR
Scanning electron microscopy	SEM
Spinal cord injured	SCI
Standard error of the mean	SE
Sterile deionised water	SDW
Sterile double-deionised water	SDDW
Tryptone soya agar	TSA
Tryptone soya broth	TSB
Urinary tract infection	UTI
Voided urinary pH	pH_v
Xylose Lysine Desoxycholate medium	XLD medium

SECTION 1

Introduction

1.1 The management of bladder dysfunction

The quality of life of many elderly people is undermined by bladder dysfunction. The care of numerous patients disabled by spinal cord injury (SCI), strokes, and neuropathies such as multiple sclerosis is also complicated by the loss of the ability to control the release of urine from the bladder. The main manifestations are (a) the distressing and often stigmatising condition of urinary incontinence (UI) and (b) the potentially life threatening retention of urine in the bladder. Demographic analysis, showing that by 2030 around one third of the population of the UK will be over 60 (Greengoss *et al.*, 1997), indicates that the numbers of people suffering from these conditions is likely to increase.

It is estimated that between 2.5 and 4 million people are affected to some degree by urinary incontinence in the UK alone (Pollock, 2000). However, it is likely that a maximum of only 10% receive some type of professional help (Smith, 1982). A working party of The Royal College of Physicians (1995) reported that in the UK 40% of individuals resident in nursing homes and 25% of those in residential homes were incontinent. In addition, incontinence amongst the home dwelling population ranged from 5-20% for women and 3-10% for men depending on age. Other studies have reported on the high incidence of incontinence in particular groups of patients. For example, Coppola *et al.*, (2002) found that 48% of a population of elderly patients suffered from the problem and Jørgensen *et al.*, (2005) recorded that 17% of a group of community dwelling, long-term stroke survivors were incontinent.

There are significant costs associated with the treatment, care and management of UI. The annual financial burden of UI in the elderly ($\geq$ 65 years old) in the USA in 1995 was estimated by Wagner and Hu (1998) to be $26.3 billion (1995 US dollars). This approximation was based on direct costs including diagnostic and assessment procedures, treatment and outpatient care, and indirect costs such as

lost income and time spent by relatives and friends on behalf of the patient. In two similar studies Wilson *et al.*, (2001) estimated the direct costs of UI in all age groups in the USA to be approximately $16.3 billion per annum (1995 US dollars) whereas in the UK Pollock (2000), of the UK Continence Foundation, estimated the annual cost of UI to the NHS to be nearly £424 million.

As indicated by the report of The Royal College of Physicians (1995) "incontinence is a symptom with many causes". It should not be considered a normal process of getting old. Firstly an effort to correct any potentially reversible causes should be dealt with as the primary step in the treatment of UI (Ouslander and Schnelle, 1995; Connor and Lind 2001; Dash *et al.*, 2004). Behavioural techniques such as a voiding schedule, bladder retraining, and pelvic floor strengthening exercises can be attempted. If unsuccessful alone they can be combined with a variety of surgical or pharmacological interventions, or medical devices (Warren, 1991; Sarkar and Ritch, 2000). The management of bladder dysfunction in those patients whose incontinence fails to be solved depends on the circumstances of a given individual. A number of factors need to be considered when deciding on the optimum choice. These include hand function and manual dexterity, spasticity of the legs, cognitive function, the associated care system, the quality of life that will result, and the patient's own preference. The optimal approach can typically impart a normal quality of life for the individual (Royal College of Physicians, 1995).

There are essentially five choices of drainage systems. Exceptionally absorbent porous pads and pants, that need to be changed every 2-3 h, or condom drainage (men only), an external system linking a condom to a drainage bag via a connecting tube, may be appropriate in particular cases. Intermittent catheterisation, in which a sterile catheter is introduced into the bladder for a few minutes every 3-6 h, can be an effective way of achieving drainage. This allows

the bladder to expand and contract, which may help reduce contracture (fibrosis and shrinkage) and does not upset the bladder defence mechanisms (Nickel, 1991). A slight variation on this is intermittent self catheterisation (ISC) which the patient can perform themselves with clean, but not necessarily sterile, catheters (Lapides *et al.*, 1972). In patients with good manual dexterity and cognitive function this technique is very successful in maintaining urinary continence (Wyndaele and Maes, 1990). Finally there are indwelling catheters which are inserted into the bladder either suprapubically, through the lower anterior abdominal wall, or more commonly via the urethra.

1.2 Indwelling urethral catheters

Indwelling urethral catheters (IUCs) are hollow, flexible tubes that are passed, via the urethra, into the bladder. At the catheter tip is an eye-hole through which urine drains, via the lumen, into a collecting bag strapped to a leg or situated on a stand.

There are a number of conditions that justify the catheterisation of a patient. Urethral catheters offer relief of possible life endangering urine retention caused by blockage of the urethra; assist in the healing of the urethra and the adjacent tissues after urinary tract surgery; allow accurate determinations of urine yield in seriously ill individuals; and can supply a dry environment for long-term elderly or neurologically damaged (spinal cord injured or neurological diseased) incontinent patients (Beeson, 1958; Kunin and McCormack, 1966; Slade and Gillespie, 1985; Stickler, 1996; Leblebicioglu *et al.*, 2003).

Evidence of bladder catheterisation in Greece was found in the writings of Hippocrates which date back to around 400 BC (Moog *et al.*, 2005). Dr Fredrick E. B. Foley (1891-1966), a Minneapolis urologist, was the first to design and develop a soft, flexible, self-retaining catheter in the 1930s. These catheters had

two interior channels, an inflation line and a drainage lumen, and once in position the retention balloon at the catheter tip could be inflated in the bladder to secure the catheter in place. Although urological medicine has progressed a great deal since these early days the design of the urinary catheter has remained essentially the same. It is on this "Foley" design that modern catheters are still based.

Catheter sizes are measured in Charrière (Ch), French (F) or French gauge (Fg) units. These units relate to the external diameter. One Ch/F/Fg unit is roughly 0.33 mm, so a 14 Ch catheter has an external diameter of approximately 4.62 mm. A catheter with the smallest diameter that permits adequate drainage (usually sizes 12-14 Ch) should be used (Woollons, 1996). Using one any bigger than necessary may cause urethral damage, inflammation, and urine bypass (Kunin, 1987). Catheters are available with balloon volumes ranging from 5-30 ml in three different lengths (minimum lengths): male (38 cm), female (22 cm) and paediatric (Pomfret, 2000). Catheters are manufactured either from silicone (all-silicone catheters) or from latex. Nowadays latex catheters are often coated with silicone, hydrogel or Teflon.

IUCs are one of the most commonly used medical devices. Estimated numbers of catheters inserted annually in the US alone are over 30 million (Dariouche, 2001). In a prevalence study of catheter usage in 40 hospitals spanning eight European countries Jepsen *et al.*, (1982) reported that 11% of all hospitalised patients were undergoing indwelling urethral catheterisation. Kunin (1987) stated that IUCs were inserted into about 10% of all hospital patients. A decade of prevalence studies in a teaching hospital in Connecticut carried out between 1985 and 1995 by Weinstein *et al.*, (1999) monitored the rise in catheter use from 9 to 16%. It is not only in a hospital setting that catheterisation is common practice. Nearly 4% of patients receiving home care in Denmark (Zimakoff *et al.*, 1993) had indwelling catheters. In addition, nursing homes commonly have a considerable

proportion of catheterised residents. Around 7.5% of nursing home inhabitants in Maryland, USA (Warren *et al.*, 1989), 4.9% in Denmark (Zimakoff *et al.*, 1993) and 9% in three healthcare districts in England (McNulty *et al.*, 2003) had indwelling catheters.

Catheter-free care of the elderly with bladder dysfunction was trialled by Nordqvist *et al.*, (1984) between 1977 and 1978. Of the catheterised patients admitted to the test ward 94% had their catheters removed and most patients preferred the catheter-free nursing. Costs for antibiotics, laundry, and hygiene products were all reduced in the test ward compared to the control wards. At the follow-up evaluation 78% of those still living were still catheter-free, and of these patients 32% were continent. These results demonstrate what can be achieved when incontinence management is approached with a positive constructive attitude.

Urethral catheters are used to a wide extent throughout the world but are often inserted indiscriminately in patients where other methods of bladder drainage would be more appropriate. Inappropriate catheterisation without good reason, in particular for the control of urinary incontinence when other methods of bladder drainage may have been more suitable, was evident in 21% of the patients studied by Jain *et al.*, (1995) and 31% of patients analysed by Saint *et al.*, (2000). The latter study highlighted healthcare providers (physicians, residents, interns, and students) were oblivious to patient catheterisation 28% of the time. Appropriate catheter usage however, can allow patients to regain a degree of independence and provide some relief for an otherwise distressing and stigmatising condition.

1.3 Catheter-associated urinary tract infections

Catheters, under certain circumstances, are necessary devices for drainage of a dysfunctional bladder. However, catheterisation frequently results in urinary tract

infection (UTI) in many of the already infirm and compromised users. Many of the complications associated with the use of catheters stem from these infections. The large numbers of community-dwelling and hospital-dwelling residents that have an indwelling urethral catheter suggests that a sizeable proportion of the population are at risk of developing catheter-associated UTI (C-A UTI).

Indwelling urethral catheters are one of the principle causes of nosocomial- and community-contracted infections. Data from the first National Survey of Infection in English and Welsh hospitals in 1980 (Meers *et al.*, 1981) revealed that of the hospital acquired infections investigated 41% originated from the catheterised urinary tract. In addition, the overall rates of catheter-associated urinary tract infection in a multi-centre prevalence study (Jepson *et al.*, 1982) were 6.5% for nosocomially-acquired, and 6.1% for community-acquired infections. Looking specifically at general hospital wards these rates varied between 14% in Norway to 0% in Holland, Germany, and Austria. A highly significant correlation ($P < 0.005$) was shown to exist between catheter prevalence and the prevalence of UTI. In a large nationwide study Haley *et al.*, (1985) estimated that over 2 million nosocomial infections occurred over a 12 month period (1975-1976) amongst 6449 acute-care hospitals in the USA. Urinary tract infections represented 42% of these nosocomial infections with approximately 2.4 infections per 100 admissions.

The second National Prevalence Survey of Infection in Hospitals, performed throughout the British Isles between 1993 and 1994, reported that the urinary tract was the source of 23.2% of all hospital-acquired infections (Emmerson *et al.*, 1996). The percentage attributable to the catheterised urinary tract was not disclosed. The urinary tract also represented the third most common site for nosocomial acquired infections in 61 paediatric intensive care units between 1992 and 1997 (Richards *et al.*, 1999). Of these infections, 77% were associated with

the use of invasive urinary tract catheters. Furthermore, in a nationwide one day prevalence study in 29 Turkish hospitals by Leblebicioglu *et al.*, (2003) 222 nosocomial UTIs were recorded (overall prevalence 1.7%). Of these, 65.3% were related to the presence of a urinary catheter. Recently the National Nosocomial Infections Surveillance System Report (Cardo *et al.*, 2004) quoted rates of hospital-acquired, catheter-associated urinary tract infection of 3-6.7% depending on the type of ward.

Bacterial, host and device factors all interact during device-related infections (Darouiche, 2001). Indwelling urinary bladder catheters are partially implanted devices that provide a connection from the outside world to a normally sterile body cavity. Nickel (1991) described the catheter as "a bridge, along which bacteria can travel" and as Warren *et al.*, (1989) also highlighted, catheters are an appealing site for bacterial colonization, a detail that became more evident in the following years.

The insertion site around the urethral meatus is a highly contaminated area with a rich microbial flora. Introducing a catheter, or other surgical or prosthetic device, into the bladder allows bacteria to enter via a number of routes (Kunin, 1987; Nickel, 1991). Firstly, bacteria can be transferred from the lower urethra into the bladder during catheter insertion. Strict aseptic techniques using sterile catheters usually means contamination of the bladder via this route is uncommon and accounts for only around 2% of the infections (Nickel, 1991). Secondly, drainage bag contamination with exogenous organisms, due to regular tap opening and poor asepsis, permits bacteria access to the bladder via intra-luminal retrograde spread up through the drainage tube and catheter. Finally, endogenous organisms established around the urethral meatus or on the perineal skin can gain entry to the bladder by moving between the exterior surface of the catheter and the urethral mucosa (peri-catheter route).

The main protective mechanism of a healthy bladder against infection is its flushing capacity which efficiently eradicates small numbers of organisms. This defence is compromised in catheterised individuals. Due to the nature of the indwelling urethral catheter the bladder is unable to empty completely and a sump of urine permanently collects below the level of the eye-hole. Invading bacteria can thus quickly become established in the bladder. Stark and Maki (1984) showed that even small numbers of bacteria in the catheterised bladder would often proliferate to $>10^5$ organisms/ml within 48 h. They proposed that the detection of bacteria in the urine of a catheterised bladder (bacteriuria) at any level is indicative of early infection as it is likely to increase to large populations within a few days.

Cuthbert Dukes (1928) was one of the first people to make the link between catheterisation and infection. He observed that after rectal surgery the necessary drainage of the bladder by an indwelling catheter presented a significant risk of the patient acquiring a dangerous infection. He concluded that the catheter was the most likely route along which the bacteria travelled from contaminated wooden pegs used to seal the catheter ends. As a solution he designed the "St. Mark's Hospital Retained Catheter" that incorporated an antiseptic irrigation mechanism to allow the distal end of the catheter to be kept sealed, and hence sterile, with antiseptic. Dukes observed that if an open drainage system was employed after catheter insertion it was for only one or two days that his patients' urine remained infection free. In contrast, infection was prevented for several weeks if a closed drainage system was used.

Despite these major discoveries open drainage systems, in which catheters dripped urine into open containers, were commonly used until the 1960s. Miller *et al.*, (1960) carried out a clinical study into the effects of a closed drainage system in which catheters dripped into sealed, sterilised containers. They reported that

infections occurred in 73% of those with open drainage, whereas only 10% of those on closed drainage developed a UTI. In a subsequent study, Kunin and McCormack (1966) reported that over three quarters of 676 patients undergoing short-term catheterisation remained free from bacteriuria at the time of catheter withdrawal or hospital discharge when sterile, disposable, plastic, closed-drainage bag systems were used.

Kass and Schneiderman (1957) used *Serratia marcescens* to demonstrate infection via bacterial migration between the catheter and the urethral epithelia. Bacteriuria resulted within one to three days after application of a culture to the peri-urethral mucosa of three semi-comatose, catheterised patients. Garibaldi *et al.*, (1980) also highlighted the importance of the peri-catheter route of infection. Not only did bacteriuria occur significantly more often in patients with positive peri-urethral cultures than those with negative samples, but 85% had bacteriuria that developed from the same species as was first isolated from their meatal swab.

In his famous editorial entitled "The Case against the Catheter" Beeson (1958) emphasised the potential dangers to patients posed by the use of the indwelling catheter. He pointed out that serious disease could result from catheterisation and documented evidence that retrograde infection up the drainage tube from contaminated urine was the probable cause of the infections. Infected drainage bags also constitute "reservoirs of infection" with the potential for transfer to susceptible patients if good asepsis is neglected (Garibaldi *et al.*, 1974).

Attempts have been made to determine the relative importance of the two main routes of infection. Daifuku and Stamm (1984) for example performed a prospective study on patients from day one of catheterisation until the catheter was removed. They reported that 12 of 18 females had peri-urethral and 14 of 18 females had peri-anal skin contamination with the infecting organism before its

appearance in the urine. In men however, only five of 17 infections were preceded by skin colonization of either area. They concluded that in women the main route of infection was from the urethral meatus along the outside of the catheter, whereas in men the intra-luminal migration of organisms contaminating the drainage bag was the major route. In a similar but much larger study Tambyah *et al.*, (1999) reported no significant difference between men and women in this respect. Their data suggested that in 66% of cases the route of infection was along the outside of the catheter and in 34% it was intra-luminal. It is likely of course that the relative importance of each route will vary with the standard of catheter care (Gillespie *et al.*, 1983). The blockage of one route simply allowing the other to become more important.

The probability of infection depends on the length of time *in situ*, the longer the catheter is in place the greater the chance that infection will ensue. Cumulative incidence of bacteriuria in short-term catheterisation (between 2-10 days) was estimated to be 26% (Saint, 2000). During the first week of catheterisation the daily probability of infection was approximately 8% and half of the catheterised patients had bacteriuria by day 10 (Garibaldi *et al.*, 1974). Long-term catheterisation, defined as the catheter being in place for greater than 28 days (Slade and Gillespie, 1985), ultimately results in bacteriuria in all cases (Stickler and Zimakoff, 1994).

Urinary tract infections that manifest outside the hospital setting in the absence of catheters are commonly caused by *Escherichia coli*. Catheter-associated UTIs on the other hand often involve a wide range of different organisms. In the first week of catheterisation infections are usually by single species of bacteria, commonly *Staphylococcus epidermidis*, *Enterococcus faecalis* or *E. coli* (Bultitude and Eykin, 1973; Gillespie *et al.*, 1983; Tambyah *et al.*, 1999; Matsukawa *et al.*, 2005). Generally the longer the catheter remains in place the more likely that a

multi-species infection will develop (Jewes, *et al.*, 1988). Of the 1259 urine samples analysed in the study by Warren *et al.*, (1987) 95% had two or more species at densities $\geq 10^5$ cfu/ml. Complex mixed communities of organisms commonly develop in the urine of patients undergoing long-term catheterisation. Gram-negative nosocomial species including *E. coli*, *Providencia stuartii*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Morganella morganii*, and *Klebsiella* spp., eventually colonize the bladder urine and become stable members of the urinary flora (Clayton *et al.*, 1982; Warren *et al.*, 1982). These populations, which can contain up to seven species, are very difficult to eliminate from the catheterised bladder by antibiotic treatment whilst the catheter remains in place (Clayton *et al.*, 1982). It is common practice not to attempt antibiotic therapy unless there is evidence that the infection has spread to the bloodstream or kidneys (Kunin, 1987).

1.4 Other complications associated with indwelling urethral catheters

Bacteriuria in patients enduring long-term catheterisation is not associated with symptomatic infection in the majority of patients for most of the time. Many patients however become vulnerable at times to complications which can pose serious risks to health (Slade and Gillespie, 1985; Sticker and Zimakoff, 1994). In a prospective study of catheterised patients in a nursing home for example, Ouslander *et al.*, (1987) recorded an incidence of symptomatic UTI of 0.69 per 100 catheterised days. Eighty percent of the study population had at least one episode in the nine month study period. In a study of catheterised women in Maryland, Warren *et al.*, (1987) established a significant association between febrile episodes and death; 50% of the deaths occurred during bouts of fever which most likely originated from the urinary tract.

Platt and colleagues (1982) reported an almost three-fold increase in mortality in hospital patients due to acquired urinary tract infection during indwelling bladder

catheterisation. Similarly, a large-scale year long study conducted by Kunin *et al.*, (1992) investigated whether the use of urinary catheters in elderly nursing home patients had an independent effect on morbidity and mortality. Morbidity and mortality rates for catheterised patients were significantly higher ($P \leq 0.01$) than matched, non-catheterised patients. Patients who were catheterised for 76-100% of the study period were approximately three times more likely to be hospitalised, spent three times as many days in hospital, and were also three times more likely to be dead at the end of the year than non-catheterised patients.

1.4.1 Pyelonephritis

Infection can ascend from the bladder to the kidneys and cause complications, such as pyelonephritis, which can impair kidney function (Slade and Gillespie, 1985). A post-mortem study of 75 nursing home residents for example, showed that acute inflammation of renal tissue was present in 38% of patients who had indwelling catheters at the time of death, compared to only 5% in non-catheterised patients (Warren *et al.*, 1988). Catheterisation at death was the only factor statistically associated with inflammation of the kidneys.

1.4.2 Bacteraemia

Catheter related bacteraemia occurs when the presence of the same infecting organism is found in both the urine and the blood. Due to the considerable numbers of catheterised patients, infection of the urinary tract is a key cause of hospital-acquired bacteraemia (Kunin, 1997). Jepson *et al.*, (1982) reported that nosocomial bacteraemia was five times more widespread amongst patients with UTI than in those without, and almost three times higher in catheterised than non-catheterised patients. More recently, hospital-acquired bacteraemia originating from device-associated sources in 73 English hospitals was surveyed between 1997-2001 (Coello *et al.*, 2003). Eight percent of all bacteraemias in teaching hospitals and 10.6% in non-teaching hospitals were derived from catheter-

associated UTIs. *Proteus* species were the second most common blood isolate after *E. coli*, and species of *Enterobacter* and *Klebsiella* along with *Ps. aeruginosa* were also frequently recovered.

It should be recognised that in catheterised patients bacteraemia is usually symptom free (Slade and Gillespie, 1985). Whilst 20 of 197 catheter changes in a study population of home-dwelling catheterised patients resulted in bacteraemia on no occasion did any systemic symptoms develop (Jewes *et al.*, 1988). In a small proportion of patients however, bacteraemia can develop into symptomatic, life-threatening endocarditis, or septicaemia and septic shock (Slade and Gillespie, 1985). Leblebicioglu *et al.*, (2003) for example reported that the prevalence of septicaemia was significantly higher ($P \leq 0.001$) in catheterised compared to non-catheterised patients.

1.4.3 Kidney and bladder stones

Urinary calculus formation in the bladder and kidney is a frequent, serious occurrence in long-term catheterised patients. Kidney stones are the more problematic and can impede urine flow and lead to abscesses of the kidneys, pyelonephritis, and septicaemia (Stickler and Zimakoff, 1994). A retrospective study carried out by Larsen *et al.*, (1997) found a significantly higher ($P \leq 0.001$) incidence of bladder and kidney stones in catheterised male spinal injured patients in comparison to those that were indwelling catheter free. In the first year after injury, spinal cord injured (SCI) patients using indwelling or intermittent catheters had higher risks (around nine-fold and four-fold respectively) of bladder stone occurrence than those who were catheter free (Chen *et al.*, 2001). In subsequent years management by indwelling and intermittent catheters became even more significant.

Griffith *et al.*, (1976b) established that bacteria, particularly *Pr. mirabilis*, had a fundamental role in urinary stone formation. If urease-producing bacteria are present in the urine and contribute to their formation the calculi are known as "infection stones" (Clapham *et al.*, 1990). Of the stones treated in a clinic in Germany from 1991-2000 11.6% were infection stones (Bichler *et al.*, 2002). These stones vary in composition from hard, solid mineralised masses to less crystalline diffuse aggregations. Both types are combinations of bacteria, crystals, and organic material (Clapham *et al.*, 1990; Stickler and Zimakoff, 1994). Scanning electron microscopy analysis of infection calculi discovered bacteria throughout the stones from the core sections to the peripheries (Takeuchi *et al.*, 1984; McLean *et al.*, 1988).

The process of infection stone formation has been summarised by Clapham *et al.*, (1990) and Stickler and Zimakoff (1994). Bacterial urease from urea-splitting bacteria increases the local pH which triggers the precipitation mainly of crystals of magnesium ammonium phosphate (struvite) and amorphous calcium phosphates (carbonate- and hydroxy-apatite). Stones composed of struvite and/or apatite were nearly always correlated with infection by ureolytic organisms and could thus be considered infection stones (Schwartz and Stoller, 1999; Bichler *et al.*, 2002). In addition, the anionic polysaccharide capsule, a gelatinous organic substance, excreted by cells attracts calcium and magnesium cations. *In vitro* studies have established that the presence of *Pr. mirabilis* enhances struvite formation possibly due to the capsule's specific affinity for Mg^{2+} ions (Clapham *et al.*, 1990; Dumanski *et al.*, 1994). Bichler *et al.*, (2002) suggested that "struvite-apatite dust" forms peri-bacterially around urease positive bacteria. Deposition of the crystals can then occur on the bacterial surface from which growth can proceed. Also, intra-bacterial crystallisation can occur and following lysis of the cells the microliths already formed can become nidi for stone formation. Mature stones develop via continual bacterial growth, crystal formation and incorporation

of organic material. Damage to, and irritation of, the mucosa of the bladder and urethra caused by the ammonia liberated during urea hydrolysis is a further effect of ureolytic organism infection. Not only does this increase the receptivity of the urinary tract to bacterial adherence but it might also aid the formation of these stones (Hedelin, 2002).

Infection stones provide a refuge for bacteria deep within their core, giving protection against antimicrobials. This may well be responsible for the failure of antibacterial chemotherapies against infection stone disease (Kunin, 1987; Stickler and Zimakoff, 1994; Coker *et al.*, 2000). Furthermore, stones or insufficient stone removal after treatments such as shock wave lithotripsy often result in fragments becoming foci for re-infection and stone re-occurrence (Clapham *et al.*, 1990; Sabbuba *et al.*, 2004).

1.4.4 Catheter blockage by encrustation

Blockage of the eye-hole and/or lumen by encrustation is a major complication in patients with long-term IUCs. Of 450 catheterised patients studied in six months by Kohler-Ockmore and Feneley (1996) 509 complications required emergency treatment. The main problem was catheter blockage. During a more in-depth 12-week study of 54 of these patients there were 141 catheter blockages, 64 catheter changes and 189 cases of urine bypass. Roughly 50% of all long-term catheter users experience episodes of catheter blockage as a result of encrustation (Cools and van der Meer, 1986; Kunin *et al.*, 1987a; Getliffe, 1990; Getliffe, 1994a). A slightly higher figure was noted by Mobley and Warren (1987) when 67 catheter blockages in 23/32 female patients were recorded over 12 months. Catheter blockage can cause torment and distress especially in the community dwelling population where professional assistance is not instantly available (Getliffe and Mulhall, 1991; Stickler and Zimakoff, 1994).

Crystalline catheter encrustations are composed mainly of crystals of struvite (ammonium magnesium phosphate) and amorphous particles of apatite (calcium phosphates) (Hukins *et al.*, 1983; Hedelin *et al.*, 1984; Cox *et al.*, 1987, 1989; Hedelin *et al.*, 1991; Stickler *et al.*, 1993a, 1993b) in conjunction with bacterial biofilm (Cox *et al.*, 1989), host proteins (Hallson and Rose 1979; Kunin, 1987) and mucous (Kunin, 1987) i.e., they have a composition equivalent to urinary tract infection stones.

Catheter encrustation, which will be discussed in more detail in section 1.7.2, can encase the retention balloon and completely impede urine flow by blocking the eye-hole and the catheter lumen (Stickler and Zimakoff, 1994). Figure 1.1 shows low vacuum scanning electron micrographs of cross-sectional images of the lumens of all-silicone catheters: (a) as they look upon insertion into the bladder (sterile, and unused); (b) partly occluded; and (c) fully obstructed by encrustation.

The crystalline material can irritate bladder epithelia (Kunin, 1987) and may cause pain and damage to the urethra upon catheter removal (Getliffe and Mulhall, 1991; Stickler and Zimakoff, 1994). The painful retention of bacteriuric urine, due to catheter obstruction, can assist ascending infection of the urinary tract up to the kidneys which can result in severe, occasionally fatal, consequences including pyelonephritis, septicaemia and shock (Kunin, 1987). Leakage of urine around the outside of blocked catheters means that patients are effectively incontinent with all the associated difficulties of care and management. Fragments of the deposits can also be shed into the bladder and these can form the basis of future stones and infections (Clapham *et al.*, 1990).

Although catheter blockage is a common phenomenon some patients remain free from this complication. Kunin and colleagues (1987b) grouped nursing home patients as either "blockers"; those patients whose catheters became repeatedly,

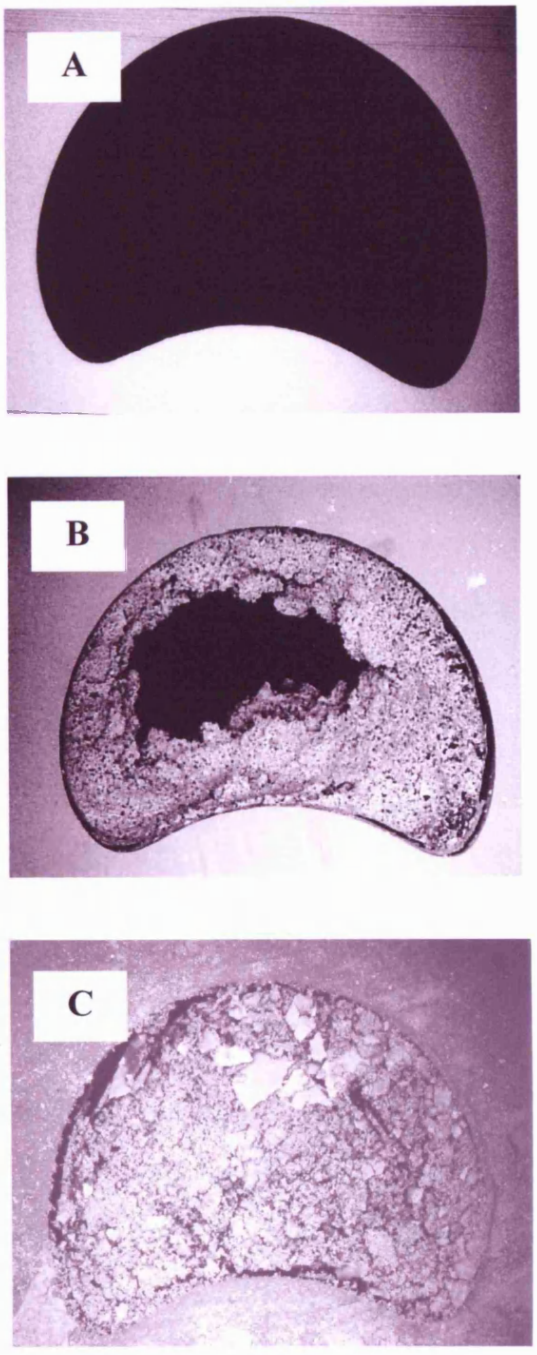


Figure 1.1 – Scanning electron micrographs of cross-sections of all-silicone catheter lumens

(A) As they look upon insertion into the bladder (sterile, and unused), (B) partly occluded by *Pr. mirabilis* generated encrustation and (C) fully obstructed by *Pr. mirabilis* generated encrustation.

completely or partially, obstructed by crystalline bacterial biofilm, or "non-blockers"; patients in whom the flow of urine was not impeded. Blockers often required catheter changes within a maximum of a few weeks whilst non-blockers frequently only needed routine catheter changes at scheduled 12 week intervals. In a subsequent study, Kunin (1989) found that age, daily living activities and mental state did not differ significantly between the two patient groups. Similarly, significant differences were not observed for urinary calcium, chloride, oxalate, potassium, protein, sodium, uric acid, osmolarity, and total urine volume. Blockers however, voided urine with increased alkalinity containing significantly less magnesium, phosphate, and urea and were more frequently colonized by *Pr. mirabilis* and *Prov. stuartii* than non-blockers. Numerous other studies have detected the occurrence and frequency of *Pr. mirabilis* in association with encrustations in the catheterised urinary tract (Bruce *et al.*, 1974; Mobley and Warren, 1987; Hedelin *et al.*, 1991; Stickler *et al.*, 1993a, 1993b). Stickler *et al.*, (1993a) also noted that infections with non-urease producing organisms seldom resulted in visible crystalline catheter encrustations.

1.4.5 Bladder cancer

Several studies on patients with spinal cord damage indicate that bladder catheterisation for over 10 years is related to an increased incidence of cancer of the bladder epithelium (bladder carcinoma) (Stickler and Zimakoff, 1994). During the period 1944 to 1966 Melzak (1966) observed 11 cases of bladder cancer in 3800 paraplegics at Stoke Mandeville Hospital. In all cases chronic UTI and bladder drainage by an indwelling suprapubic or urethral catheter were the only common factors. In the same spinal unit 25 cases of bladder carcinoma were found in medical records of 6744 SCI patients (El-Masri and Fellows, 1981). This rate was over 20-fold higher than expected. Twenty of the patients died within two years of diagnosis. A study by Groah *et al.*, (2002) found that SCI patients managed exclusively by an indwelling catheter (suprapubic or urethral) had a

significantly greater risk of bladder cancer than those using non-indwelling methods. They were also 25 times more likely to develop bladder cancer than the general US population.

Nitrosamines are carcinogenic compounds, formed from nitrites and amines, which have been found to produce tumours in various laboratory animals. They have been implicated in the formation of bladder cancer via the action of nitrate-reducing bacteria. In the urine of 97% of the catheterised paraplegics studied by Tricker *et al.*, (1991) and Stickler *et al.*, (1992) high levels of these nitrosamines were frequently found in conjunction with nitrate-reducing bacteria. In contrast the sterile urine of control subjects in both studies lacked both nitrites and nitrosamines. A different theory was proposed by Akaza *et al.*, (1984). These authors suggested that tumours could arise after continued exposure to substances not deemed to be carcinogenic themselves. Persistent stimulation of the urethral epithelium was all that was required to initiate carcinogenesis in murine models. As such, the presence of a long-term catheter in the urethra or persistent bacterial infection of the catheterised urinary tract could be such triggers.

1.5 Attempts to prevent urinary infection in patients undergoing long-term catheterisation

The only way to avoid catheter-associated UTI is to refrain from continuous bladder drainage. This is not always feasible and their long-term use is indispensable in many cases. For those individuals with no alternatives, recommendations to use IUCs as a last resort followed by prompt removal as the only way to reduce infection (Beeson, 1958; Jepson *et al.*, 1982; Kunin, 1987) must be particularly disheartening. Despite a great deal of effort over many years it has so far proved impossible to prevent urinary tract infections developing in those patients undergoing long-term bladder catheterisation. Many apparently

rational attempts to block the routes of infection have been tried but with little success (Stickler and Chawla, 1987; Kunin, 1997).

1.5.1 Disinfection of the drainage bag

The basis behind the idea of putting disinfectants in the drainage bag is twofold: (1) to prevent bacteria from retrograde ascension from the bag to the bladder; and (2) to prevent cross-infection between patients (Kunin, 1987). Various disinfectants have been tested but none have proven to be effective. For example, Gillespie *et al.*, (1983) showed in a controlled trial that addition of chlorhexidine to the drainage bags of men catheterised after surgery failed to cause a significant reduction in the incidence of UTI. Hydrogen peroxide instillations every 8h were also reported by Thompson *et al.*, (1984) to cause no decrease in the occurrence of bacteriuria.

1.5.2 Bladder washouts

The instillations of various antibacterial solutions into the bladder as a preventative measure for UTI have their basis back in the early 20th century. Dukes (1928) encouraged bladder irrigations with a warm oxycyanide of mercury solution as a method of UTI prevention. There remains much controversy and confusion over the use and efficacy of bladder washouts and instillations, nevertheless they are still often carried out routinely in those catheterised for the long-term (Roe, 1989; Kunin, 1997; Evans and Feneley, 2000). Generally the technique involves the instillation of 50-100 ml of antiseptic solution through the catheter into the bladder. The catheter is then clamped, and after 20-30 min the clamp is removed to allow the solution to drain out. Little clinical success has been achieved when bladder washouts have been used to prevent or treat C-A UTI.

Washouts with noxythiolin resulted in sterility of only 6% of the urine samples taken from elderly catheterised patients (Brocklehurst and Brocklehurst, 1978). When a kanamycin and colistin combination bladder washout was used on a catheterised SCI patient with *Kl. pneumoniae* bacteriuria the *Klebsiella* was eradicated but was replaced with a kanamycin resistant *Pr. mirabilis* (Clayton *et al.*, 1982). Davies *et al.*, (1987) observed no significant decrease in bacteriuria in elderly catheterised patients receiving twice daily washouts with chlorhexidine diacetate (0.02%) for three weeks. Stickler *et al.*, (1987a) also investigated the effects of chlorhexidine bladder washouts on the urinary flora of catheterised patients. They concluded that any positive effects observed were "minimal and temporary" and suggested that washouts with chlorhexidine should be discontinued. The efficacy of twice daily bladder washouts with mandelic acid on catheterised patients with *Proteus* or *Pseudomonas* spp., bacteriuria was investigated by Robertson and Norton (1990). *Pseudomonas* was cleared from the majority of patients (23/26) with a mean of 32 washouts whilst only 2/13 *Proteus* infections were eliminated with the same number of treatments.

Using laboratory models of the catheterised bladder Stickler *et al.*, (1987b) and King and Stickler (1991) replicated the bladder washout procedure *in vitro* and confirmed the poor clinical efficacy of solutions including chlorhexidine and noxythiolin. Stickler *et al.*, (1987b) suggested that even if initial treatments eliminated the organisms re-seeding of the urine, probably sourced from the antibiotic resistant biofilms that were growing on the bladder wall, occurred. Evidence to support such a theory was subsequently presented by Stickler and Hewett (1991).

Roe (1989) and Getliffe (1994a) reported that around 50% of the nurses questioned were recommending and/or were implementing the use of chlorhexidine as a bladder washout solution. A more recent study by Evans and

Feneley (2000) revealed that although around 42% of patients were still receiving bladder washouts, community nurses throughout three healthcare trusts in Bristol had taken notice of both clinical and *in vitro* evidence. The use of chlorhexidine bladder washouts as a means of preventing infection was no longer being advocated.

1.5.3 Systemic prophylactic antibiotics

Patients undergoing prophylactic antibiotic therapy acquired bacteriuria significantly less often than patients not receiving antibiotics (Garibaldi *et al.*, 1974). This beneficial effect however only lasted for the first four days of catheterisation. Antibiotic therapy in those catheterised for more than four days had no benefits and in fact predisposed the patients to bacteriuria with resistant species. In accordance Britt *et al.*, (1977) reported that although prophylaxis with cefazolin sodium decreased both the daily and overall incidence of UTI, upon hospital discharge rates of UTI were comparable in the placebo and treated groups. Antibiotic prophylaxis may have some use in those with short-term catheters, at risk from major complications, as it may postpone infection until the catheter is withdrawn. However, its use is contraindicated in those undergoing long-term catheterisation (Clayton *et al.*, 1982; Slade and Gillespie, 1985; Kunin, 1987; Nickel, 1991; Kunin, 1997).

1.5.4 Urethral meatus cleansing

Many infections have been shown to arise from the bacterial flora colonizing the skin around the urethral meatus (Garibaldi *et al.*, 1980; Daifuku and Stamm, 1984). It is not surprising that efforts have been made to prevent infection by applying antiseptics to this site. In general, the evidence suggests that such regimes are of little benefit. Burke *et al.*, (1981) for example showed that twice daily peri-meatal washing with a povidone-iodine solution followed by an application of a povidone-iodine ointment, or a once daily washing with a non-

antibacterial soap far from having a benefit lead to higher incidences of bacteriuria compared to those not given either treatment.

As part of a comprehensive policy to try and block the routes of infection in catheterised patients the Southampton Infection Control Team (1982) used daily washes with chlorhexidine to cleanse the peri-urethral skin. The antiseptic was also incorporated into a gel used to lubricate the passage of the catheter through the urethra and instilled into the drainage bag each time the urine was emptied. They also applied a cream containing chlorhexidine to the catheter insertion site daily. Although they reported an initial drop in the incidence of catheter-associated UTI an outbreak, involving over 90 patients, occurred with a chlorhexidine and other multi-drug resistant strain of *Pr. mirabilis* (Walker and Lowes, 1985; Dance *et al.*, 1987). The problem was only resolved when the policy was discontinued. The use of antibacterial agents in attempts to block all conceivable routes of infection in the management of long-term catheterised patients thus seems to be counterproductive.

1.5.5 Antimicrobial catheters

As indwelling urethral catheters provide the route via which bacteria gain entry to the bladder it seems logical to target bacteria as they make their way along the external and luminal surfaces. For a biocide to be successful as a polymer-linked anti-infective material it should meet a variety of requisites: (1) be effective against a broad spectrum of species; (2) keep its potency once incorporated into the polymer; (3) release effective concentrations for the entire time *in situ*; (4) be stable in the host; and (5) cause no undesirable side effects (Schierholz *et al.*, 1997). Whilst many efforts have been made to develop catheters that liberate antimicrobial agents relatively few studies have examined their effectiveness in clinical trials.

1.5.5.1 Antibiotic-based catheters

Despite concerns that have been expressed that the use of catheters impregnated with antibiotics could exacerbate the problem of multiple drug resistance by efflux of sub-lethal concentrations of antibiotics (Guggenbichler *et al.*, 1999; Rösch and Lugauer, 1999), several such catheters have been developed. For example, Cho *et al.*, (2001) produced a gentamicin-coated catheter and norfloxacin was used in devices produced by Park *et al.*, (2003). These are important drugs commonly used for the treatment of life-threatening infections that can complicate catheter-associated UTI. The development of resistance to these antibiotics would be particularly dangerous.

Darouiche *et al.*, (1997a) produced all-silicone catheters impregnated with two antibiotics; minocycline and rifampin. This combination had a broad antimicrobial spectrum and they argued that as neither agent was used to treat symptomatic UTIs their use in tandem was unlikely to result in resistance. These catheters were tested for efficacy in a clinical trial (Darouiche *et al.*, 1999). Patients who were assigned the impregnated catheters had significantly reduced rates of bacteriuria than those assigned the control all-silicone catheters at day 7 (15.2% vs. 39.7%) and at day 14 (58.5% vs. 83.55). However, these differences were attributable to a decrease in the rate of Gram-positive bacteriuria, rates of Gram-negative bacteriuria were not significantly different between the two groups (46.4% vs. 47.1%). The authors suggested that these catheters were probably not suitable for long-term catheterisation. In view of the limited effect of these catheters in only preventing Gram-positive bacteriuria in patients under-going short-term catheterisation these catheters were not further developed or marketed by the manufacturer.

The only antibiotic containing catheter that has been introduced into clinical practice is the Release Nitrofurazone Foley catheter produced by the Rochester

Medical Corporation (Stewartsville, Minnesota, USA). Nitrofurazone is commonly used for the treatment of UTI and has activity against a wide range of Gram-positive and Gram-negative bacteria. Important exceptions however, are *Ps. aeruginosa* and to some degree *Pr. mirabilis* (Johnson *et al.*, 1993b).

Three clinical trails have compared the efficacy of the nitrofurazone impregnated catheters with control all-silicone or silicone-coated latex catheters. A randomised, prospective study by Maki *et al.*, (1997) found a significantly lower rate of bacteriuria in short-term (1 to 7 days) catheterised patients assigned the antibacterial catheter (2.4%) compared to the control group (6.9%). When *Candida* infections were included however, rates of bacteriuria were not significantly different (4.7% vs. 8.0%). It should be noted that this study was not peer-reviewed and was only published as a conference abstract. A second trial was carried out in short-term catheterised postoperative and trauma patients (Al-Habdan *et al.*, 2003). The nitrofurazone catheter allocated group had a rate of bacteriuria significantly lower than that of the control group (0% and 12% respectively). A multi-centre study of short-term catheterised patients in five university hospitals in South Korea was performed by Lee *et al.*, (2004a). Of the patients in the control catheter group 22.4% had a catheter-associated UTI, whilst the incidence of infection in those assigned to the nitrofurazone catheter was 15.2%. These rates were not significantly different. Of the patients catheterised for 5-7 days however, the rates of bacteriuria were significantly decreased in the nitrofurazone group compared with the control group. Conclusions from the recent review by Johnson *et al.*, (2006) were that nitrofurazone catheters do seem to reduce asymptomatic bacteriuria in short-term use in particular groups of patients. There is no evidence however of the prevention of symptomatic UTI or efficacy in the long-term. It is unlikely these catheters will be of benefit to those catheterised for extended periods, circumstances in which nitrofurazone resistant species cause major complications.

1.5.5.2 Silver-based catheters

Silver containing materials are used in several prosthetic devices but their use in the urinary tract is controversial and questionable (Dariouche, 1999). Whilst silver has a broad spectrum of activity (Russell and Hugo, 1994) the activity of silver ions in urine may be neutralised by chloride ions (Schierholz *et al.*, 1998). Despite this, silver has been incorporated into urinary catheters as silver oxide, silver citrate, silver alloy and in its metallic form.

Riley *et al.*, (1995) tested a silicone catheter that was coated with 5% silver oxide on its outer surface in a large clinical trial of 1309 hospital patients undergoing short-term catheterisation. The study failed to detect any significant difference in the incidence of infection between patients with silver catheters (11.4%) and the control group who received silicone-coated latex catheters (12.9%). These results suggested that silver oxide catheters had no protective effect even in the short-term and they have not been developed any further. Kumon *et al.*, (2001) developed a catheter coating which contained silver citrate, lecithin and liquid silicone. Laboratory tests showed that the coating inhibited colonization by *E. coli* and *Ps. aeruginosa* and prevented migration of *E. coli* over its surface. Clinical trials are currently in progress but the results have so far not been reported. Bard Medical (Covington, Georgia, USA) has developed a latex-based catheter in which metallic silver is incorporated into a gold and palladium layer. This permits the slow release of Ag^+ into surrounding fluids for extended periods whilst an external hydrogel coating provides lubricity (Davenport and Keeley, 2005). This catheter has undergone extensive clinical trials and is now marketed as the Bardex® I.C. catheter.

Whilst some clinical trials have reported that these silver-based catheters reduce bacteriuria in patients catheterised for short periods (Saint *et al.*, 1998; Rupp *et al.*, 2004) a recent Cochrane review (Brosnahan *et al.*, 2004) concluded that

although the data suggests these catheters might reduce the risk of infection in the short-term, the evidence is weak and the trials are generally of poor quality. In addition, reviewing the results from these trials Trautner *et al.*, (2005a) and Johnson *et al.*, (2006) pointed out that there was no evidence that these catheters were at all effective in preventing infection in long-term catheterised patients. In addition resistance to silver might become a problem in the future.

1.6 Bacterial biofilms

A biofilm is a concentrated, structured assemblage of organisms (single, or multi-species) and macromolecules enclosed within a self-generated polysaccharide matrix that attaches generally to an abiotic or biotic surface (Costerton *et al.*, 1987; 1995). Bacteria exist in the planktonic (free-floating) state or the sessile (surface-bound, adherent) state. In the natural environment most bacteria are found within biofilms (Costerton and Lappin-Scott, 1989; Watnick and Kolter, 2000). This surface adherence seems to be a strategy adopted to remain within a favourable environment and to maximise population survival in situations where they would otherwise be rapidly eliminated or swept away (Watnick and Kolter, 2000). Biofilms can be thought of as complex, structured, differentiated populations that are constantly in a state of flux (Donlan, 2002). Watnick and Kolter (2000) likened a biofilm to an active city with regular movement of cells into and out of a neighbourhood, erection of new developments, and the dismantling of old buildings. It has become increasingly clear that the colonization of medical devices, such as catheters, by bacterial biofilms is an integral step in the pathogenesis of the infections that result from their use (Darouiche, 2001). An understanding of biofilm development is important if we are to devise effective strategies to prevent these infections.

Biofilm formation and maturation occurs via a number of steps. Figure 1.2 presents some of the main aspects of biofilm development. A conditioning layer

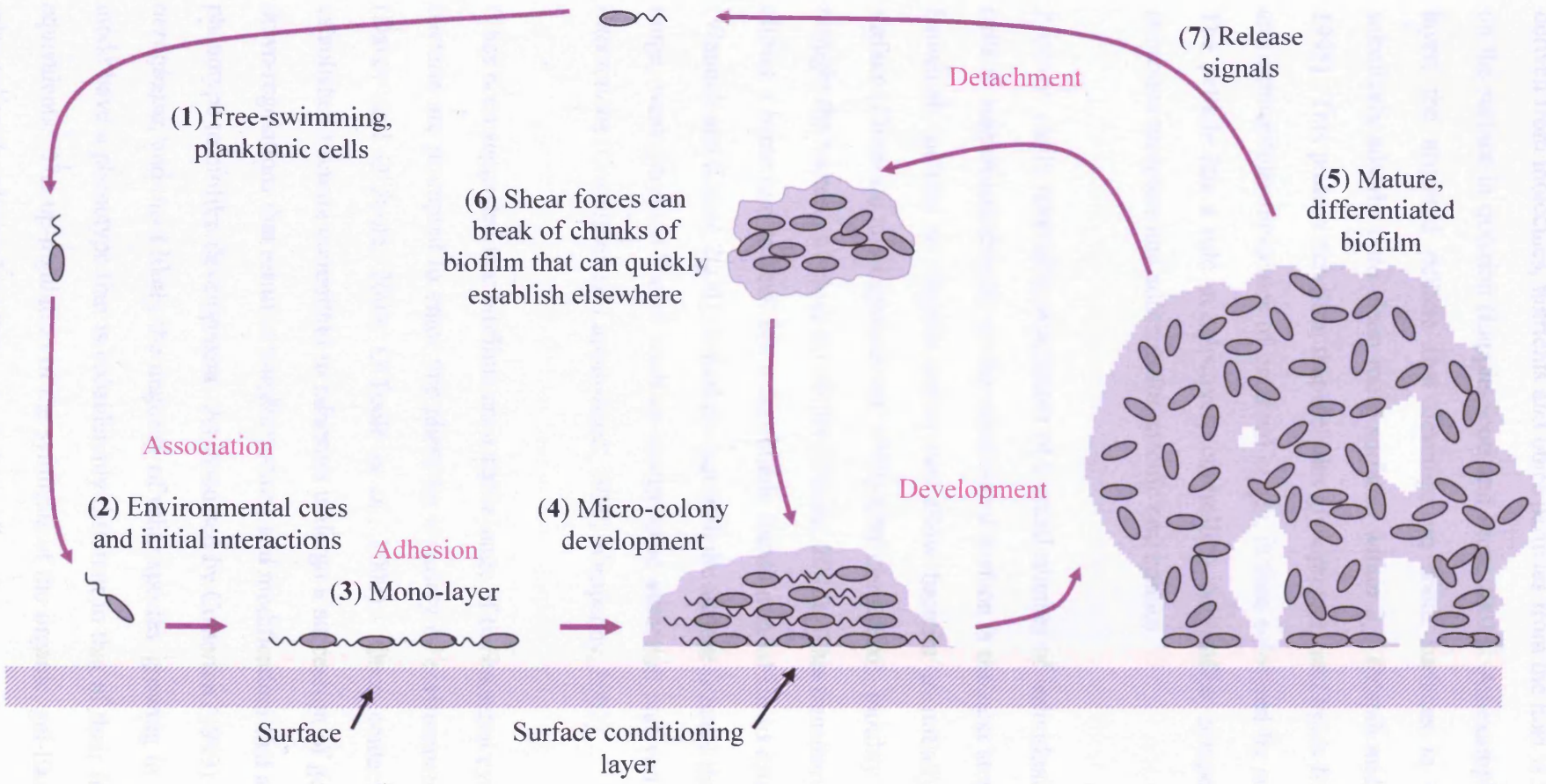


Figure 1.2 – Diagrammatic depiction of water channel penetrated Pseudomonad biofilm development (modified from Costerton *et al.*, 1987; O'Toole *et al.*, 2000).

Planktonic cells (1) that come into contact with a surface due to environmental signals or chance initially associate reversibly with a surface (2). Cells that commit to adhesion first form a mono-layer (3) and increase secretion of exo-polysaccharides. Through cell division and recruitment from the bulk liquid micro-colonies form (4) that reside within the polysaccharide matrix. Over time a mature, differentiated biofilm develops (5). Fragments can break off and establish elsewhere (6) whilst production of detachment signals can reverse cells to the planktonic phase (7). This allows cells to seek new, more favourable areas to colonize once biofilm conditions become adverse.

derived from molecules, nutrients and other particles from the host is first formed on the surface in question (Lappin-Scott and Bass, 2001). An example of such a layer, the acquired pellicle that develops on tooth surfaces in the mouth, selectively adsorbs onto clean tooth surfaces within 2 h (Marsh and Bradshaw, 1995). This protein rich film, that includes glycoproteins and lipids from the host and extracelluler molecules of bacterial origin, is then colonized by oral bacteria. The pellicle has a role in selectively controlling the biofilm composition as it possesses receptors and adhesins for specific oral species.

Primary, easily reversible attachment of a small number of individual planktonic cells in suspension directly to the conditioned surface is the next step. Gravity, Brownian motion, or flagella action can allow bacteria to initially contact a surface (Costerton and Lappin-Scott, 1989) after chemotaxis, motility or flow has brought the bacteria in close proximity (Dunne, 2002). This transitory adherence allows a bacterium to seek out a favourable location in which to establish itself (Watnick and Kolter, 2000). Initial contact with the surface is made through long-range, weak physical forces such as electrostatic attraction and van der Waals interactions (Costerton and Lappin-Scott, 1989; Wimpenny, 2000).

It has been suggested that biofilms are a stable stage of the bacterial cycle and that bacteria are prompted to enter this phase by a variety of environmental triggers (Davey and O'Toole, 2000; O'Toole *et al.*, 2000). Once contact has been established bacteria committed to adhesion undergo a succession of gene up- and down-regulations that result in transformations and modifications that adjusts their phenotype to biofilm development. As discussed by Costerton (1999) cells of *Ps. aeruginosa*, and most likely the majority of other species, growing in the biofilm mode have a phenotype that is considerably different to that of their free-floating equivalents. The up-regulation of the synthesis of the organic, gel-like "capsule" (also called the glycocalyx) that surrounds a cell and consists of secreted bacterial

exopolysaccharide (EPS) polymers is one such transformation. Experiments carried out by Davies *et al.*, (1993) were some of the earliest to show that surface association and attachment altered gene expression in *Ps. aeruginosa.* The expression of a gene involved with the synthesis of the EPS alginate (*algC*) became up-regulated three- to five-fold in cells newly attached to a Teflon surface compared to free-floating equivalents in liquid.

A long-standing, irreversible association is then formed with the surface (Dunne, 2002). This is achieved by means of molecular interactions via bacterial adhesins such as fimbriae, pili and proteins, hydrogen bonding and hydrophobic interactions (Gristina 1987; Costerton and Lappin-Scott, 1989). Once cells are bound in a monolayer, cell division results in sister cells that also reside within the glycocalyx matrix. As this process progresses microcolonies form and enlarge, moving ever closer together. With additional cells from the bulk fluid also attaching it leads to a gradual generation of a continuous bacterial biofilm that can contain not only numerous species of viable bacteria but water, proteins, minerals, nutrients, cell debris, host inflammatory proteins, and particulate matter (Sutherland, 2001; Donlan, 2002; Dunne, 2002). In most cases a biofilm has an overall negative charge and this anionic feature enables metal cations, such as Ca^{2+} and Mg^{2+}, from the surrounding liquid to bind or become trapped (McLean *et al.*, 1999), a feature particularly important in *Pr. mirabilis* biofilms which will be discussed in section 1.7.2.

Dispersal of cells from biofilms can occur in a number of ways: daughter cells can be cast from active microcolonies; quorum signals can cause shedding; and breakage of biofilm fragments can occur under high flow rates (Donlan, 2002). As such, depending on the manner of detachment the cells can either rapidly revert to the planktonic phenotype or preserve some of their biofilm characteristics. Detachment from the biofilm can occur by production of

exopolysaccharide lyases (Allison *et al.*, 1998), possibly under quorum signal control, which degrade the exopolysaccharide and release single cells to seek new surroundings. This can be in response to starvation, hostile environmental conditions, or aggressive adjacent cells (Allison *et al.*, 1998; Watnick and Kolter, 2000).

Scanning confocal laser microscopy (SCLM) was used to visualise hydrated biofilms by Lawrence *et al*, (1991). It became apparent that there were species specific biofilm architectures that ranged in the incorporation of extracellular materials and amount of space from 73-98%. Three basic types of biofilm architectures have been proposed: the dense confluent; the mosaic; and the water channel penetrated (Sutherland, 2001). All three varieties can form under different conditions with evidence suggesting that substrate concentration is related to structure (Wimpenny and Colasanti, 1997).

Each biofilm community is unique and although a biofilm structure can vary depending on the organism(s) present, its location, and the accessibility of nutrients, (Stickler, 1999; Sutherland, 2001) there seems to be some basic structural elements common to the majority of biofilms. Microcolonies, distinct exopolysaccharide-covered communities, are the basic subunits of the majority of biofilms and can be arranged in a horizontal and/or vertical manner. Within these microcolonies themselves exists a structured population and a degree of heterogeneity (Costerton and Lappin-Scott, 1989; Costerton *et al.*, 1999). They are often dissected by interstitial spaces or water channels that may have a role in conveying nutrients in, and waste products out of the biofilm, in addition to allowing the transfer of enzymes, metabolites, and other solutes between biofilm members (Sutherland, 2001). Donlan (2002) for example, presented an epifluorescent image of a 14 day old mixed species biofilm in a potable water reactor that showed both the heterogeneity of a mature biofilm and its perforation

by water channels. Mature biofilms examined by Lawrence *et al.*, (1991) showed no indication of discrete microcolonies suggesting that a reorganisation of cells during the development of some biofilms often occurs. Other biofilms frequently have mushroom-shaped surface projections of cells (Costerton, 1999) the purpose of which could be to allow fragments containing large numbers of cells easy dispersion when the need arises (Gristina, 1987).

Pratt and Kolter (1998) investigated the formation of biofilms by *E. coli*. They found that motility was of the utmost importance in *E. coli* biofilm formation. Non-motile cells or those with impaired motility adhered poorly to polyvinylchloride (PVC) and did so only in small clusters. It was suggested that motility might be required both to overcome repellent surface forces and to aid biofilm spread along a surface. Type I pili were imperative for stable attachment. Cells that had lost the capacity to form type I pili failed to attach to PVC at all.

Similarly *Ps. aeruginosa* surface attachment defective (*sad*) mutants, unable to form biofilms, were found to have malfunctions in flagella-mediated motility or type IV pili formation (O'Toole and Kolter, 1998). Phase-contrast, time-lapse microscopy was used to monitor biofilm formation. The wild-type cells had formed microcolonies dispersed over a confluent monolayer by eight hours. In contrast, non-motile cells failed to attach over the eight hours whilst pili lacking mutants only formed a diffuse monolayer and lacked the distinct microcolony formation of the wild-type. For *Ps. aeruginosa* at least it appears that flagella and/or motility are required for primary attachment and diffuse monolayer formation, whereas pili have a later role in biofilm confluency, microcolony development, and biofilm maturation.

One of the advantages of growing in a biofilm is the degree of protection from antibiotics afforded to those in the community. Biofilms populations are

inherently refractory to antimicrobials possibly due to two reasons: (1) cells deep within the biofilm may have a lowered metabolic activity; and (2) the structure of a biofilm may act as a perfusion barrier (Nickel and McLean, 1998). Bacteria in biofilms can often tolerate antimicrobial concentrations 1000-1500 times greater than those that eliminate planktonic cells (Costerton, 1999). Dunne (2002) suggested that the mechanism of antimicrobial resistance conferred by a biofilm is most likely a mixture of the two reasons above, in addition to the biofilm microenvironment affecting the activity of the therapeutic agent. Xu *et al.*, (2000) confirmed the heterogeneity of *Ps. aeruginosa* biofilms in terms of protein synthesis, respiratory activity and growth rate. They demonstrated that only the exterior one-fifth of the biofilm, adjacent to the biofilm/fluid interface, was metabolically functional. If bacteria within these biofilms are not effectively eradicated with antibiotics, as is often the case, released planktonic cells may instigate repeated acute infections or detached sections may generate new, disseminated origins of persistent infection (Costerton *et al.*, 1987; Costerton and Lappin-Scott, 1989).

The physical proximity of organisms in a biofilm is likely to facilitate metabolic and physiological collaborations between populations of cells (Costerton *et al.*, 1995). Interactions between bacteria in biofilms are mainly conveyed via bacterial substances that diffuse from one cell to another and include acylated homoserine lactones (AHLs), proteins, DNA/RNA, and metabolites (Watnick and Kolter, 2000). These transmissible components may have a number of roles including; varying protein expression of adjacent cells, regulating the species distribution within the biofilm, allowing transfer of genetic traits, and enticement and integration of bacteria into the biofilm (Watnick and Kolter, 2000).

Biofilms provide perfect opportunities for bacterial conjugation and the transfer of plasmids. The niche of a biofilm probably offers intimate cell-to-cell contact

which allows enhanced rates of conjugation (Donlan, 2002). Roberts *et al.*, (1999) demonstrated that the conjugative transposon Tn*5397*, that conferred tetracycline resistance, was transferred from a non-oral strain to an oral species within 6 h of its inoculation into a dental plaque microcosm. This observation emphasises the importance of conjugation in the rapid spread of antibiotic resistance.

AHLs are secreted Gram-negative signal molecules that accumulate in bacterial communities. When a specific cell density is reached, termed the quorum, the AHLs can react with cell surface receptors that in turn activate gene expression. As a result, a group of genes can be expressed simultaneously by a collective as a response to local cell densities (Fuqua *et al.*, 1996). The cell-to-cell communication systems in *Ps. aeruginosa* (the *lasR-lasI* and the *rhlR-rhlI* systems) that produce quorum signals were investigated for their participation in *Ps. aeruginosa* biofilm development by Davies *et al.*, (1998). After two weeks growth the wild-type strain produced a thick biofilm with distinctive mushroom-like microcolonies separated by water channels. Mutants lacking both of these systems had attached and proliferated like the wild-type but had an abnormal biofilm structure consisting of a thin, densely packed, continuous layer. In addition, the mutant biofilms had lost their resistance to the surfactant sodium dodecyl sulphate. One or both of these signalling systems appeared to be involved not in the early stages of biofilm adherence or proliferation but later in the differentiation of the biofilm. Further elucidation revealed that the *lasI* mutant, which lacked the *N*-(3-oxododecanoyl)-L-homoserine lactone formed similar biofilms to the double mutant. It was this compound that was required for normal biofilm differentiation, a property which could be re-induced by the addition of the AHL to the medium. The authors concluded that cell-to-cell communication via AHLs thus appears to have a role in the architectural development of biofilms in at least some species.

On the contrary, experiments by Stoodley *et al.*, (1999) found that whilst AHLs were of importance in the rates of colonization and detachment they had little purpose in determining *Ps. aeruginosa* biofilm structure. They suggested that structure was more influenced by the flow conditions. It is likely that a number of factors interrelate and help shape a biofilm. These include the properties of the surface to which the biofilm attaches, the nutrient levels in the surrounding medium, biofilm species associations (Stoodley *et al.*, 1997) in addition to AHLs and flow rates.

AHL activity in naturally occurring aquatic biofilms was first shown by McLean *et al.*, (1997). Subsequently Stickler *et al.*, (1998a) were the first to demonstrate that AHLs were produced by bacterial isolates from biofilms on urethral catheters taken from patients and by *Ps. aeruginosa* laboratory grown catheter biofilms.

1.6.1 Bacterial biofilms on indwelling urethral catheters

In a healthy host small numbers of infecting bacteria are normally eradicated by efficient defence systems. Problems arise when either the densities of organisms are overwhelming, defence mechanisms are damaged or weakened, tissues surfaces are injured, or a foreign body is present (Schierholz and Beuth, 2001). Bacterial biofilms on urinary catheters comprise enormous populations and are often responsible for infection associated complications in the care of catheterised patients. This section will briefly focus on bacterial biofilm formation on urinary catheters.

In vitro studies by McGovern *et al.*, (1997) revealed that within 24 h the surfaces of pieces of polyurethane incubated in urine became significantly more hydrophilic. As soon as a catheter is implanted into the bladder it is exposed to macromolecule rich urine. These large molecules form a surface-conditioning film within hours by adsorbing onto the catheter surface. This film might be

composed of, amongst other things, host-derived inflammatory response proteins, complement, fibrinogen, and fibronectin (Dunne, 2002) in addition to carbon, nitrogen, sodium, and calcium containing compounds (Reid *et al.*, 1992). Most catheters removed and examined within 3 days of being inserted into the bladder were found, by Ohkawa and associates (1990), to have acquired a fibrin containing film on their surfaces. The authors implied that this film was derived from host tissues, damaged by the catheter insertion, and had a part to play in biofilm formation by trapping bacteria and initiating biofilm formation. They observed bacteria embedded in a biofilm matrix and implied that materials originating from the host "such as fibrin and debris of the uroepithelial cells" were components of the biofilm in addition to bacterial secreted glycocalyx.

Many different species have been found in catheter biofilms and most long-term catheters are heavily colonized by polymicrobial communities (Ramsay *et al.*, 1989; Ganderton *et al.*, 1992). Whilst little is known about precisely how all the various species attach to the catheter, it is clear that in patients with bacteriuria, colonization and biofilm development can occur rapidly. For example, after only a three day period *in situ* one of the catheters examined by Ganderton *et al.*, (1992) was colonized by a 50 μm thick biofilm containing *E. coli*, a *Citrobacter* sp., and *Ps. aeruginosa*. Similarly Goto *et al.*, (1999) observed microcolonies of *Ps. aeruginosa* present on Teflon catheters after only 6 h, and a 20 μm thick biofilm present within 24 h. Nickel *et al.*, (1985a) used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to determine that bacteria colonized catheters in glycocalyx-covered biofilms.

Catheter surfaces possess especially appealing sites for colonization because they lack all of the defence mechanisms that healthy tissue surfaces possess (Morris *et al.*, 1999). None of the catheters types (latex, silicone elastomer, PVC, all-silicone or Teflon coated latex) inspected by Ramsay *et al.*, (1989) were biofilm

free. Catheter surfaces, particularly those of latex-based devices, have been shown to be irregular in nature (Axelsson *et al.*, 1977, Cox, 1990; Stickler *et al.*, 2003b). The rims of the catheter eye-holes are especially un-even. The ridges and crevices on these surfaces provide ideal sites for bacterial colonization in addition to trapping crystals as urine flows through the eye-hole. Crystalline biofilm development has been shown to be initiated at this site before spreading down the catheter lumen (Stickler *et al.*, 2003b).

Biofilms have been found throughout the entire catheter drainage system. Bacterial biofilms have been found on the taps of drainage bags and shown to have spread, in a retrograde fashion, through the drainage bag into the catheter (Nickel *et al.*, 1985a). In corroboration Stickler (1996) presented scanning electron micrographs of internal surfaces of the drainage bag tap, the drainage bag, the non-return valve, and the drainage tubing showing evident bacterial colonization in each image.

Organisms present in a biofilm are not necessarily found in the urine and *vice versa* (Nickel *et al.*, 1989; Ramsay *et al.*, 1989; Ohkawa *et al.*, 1990). This has implications for antibiotic therapy choice. Antibiotic therapy can eliminate cells in suspension but cells in the biofilm mode of growth are much more tolerant to the levels of antibiotics usually administered. Once antibiotic therapy is stopped re-seeding of the urine from the refractory biofilm population can result in quick recurrence of an infection. Ganderton *et al.*, (1992) reported that of the 13 patients without bacteriuria four had organisms recovered from their catheters. One patient who was receiving gentamicin treatment grew only low densities of *Ps. aeruginosa* from their urine. However, large numbers of *Ps. aeruginosa*, *Pr. mirabilis* and *Prov. stuartii* were isolated from the catheter, all of which registered as susceptible to gentamicin on conventional disk-diffusion testing.

Evidence of the antibiotic resistance of cells in catheter biofilms was demonstrated by Nickel *et al.*, (1985b) who observed the ineffectiveness of tobramycin on *Ps. aeruginosa* biofilms growing on catheter materials *in vitro*. Many cells survived exposure to concentrations > 1000 μg/ml whilst growing as a biofilm, whereas detached cells that had reverted to the planktonic stage were totally eliminated by a 50 μg/ml solution.

Some organisms present in catheter biofilms can raise the pH of the urine and the biofilm microenvironment via production of the urease enzyme. In response to the increased alkalinity minerals such as calcium and magnesium phosphates precipitate out of solution and deposit in the biofilm. *Pr. mirabilis*, *Morg. morganii*, *Ps. aeruginosa*, *Kl. pneumoniae* and *Pr. vulgaris* are common urease producing organisms however it is *Pr. mirabilis* that is the particularly problematic species (Kunin, 1989; Stickler *et al.*, 1993a).

1.7 *Proteus mirabilis* and its biofilms

1.7.1 *Proteus mirabilis*

Proteus mirabilis is a motile, Gram-negative, rod-shaped bacterium. It is a member of the enterobacteria and is prevalent in the gastrointestinal (GI) tracts of humans and animals, contaminated water, manure, and soil. It is an opportunistic pathogen that can cause pneumonias and wound infections but its predominant role is in urinary tract infections. Mobley and Belas (1995) reviewed the role of *Pr. mirabilis* in UTIs and a summary of the data they collated is presented in Table 1.1. As can be seen, the organism is not often a common cause of UTIs in normal adults. It is much more likely to be isolated from infections in patients with abnormal urinary tracts or those whose urinary tract is subject to catheterisation. In patients with indwelling bladder catheters *Pr. mirabilis* had been isolated in 44% of cases. *Pr. mirabilis* often colonizes the urinary tract via self-infection by endogenous strains in the lower GI tract (Mobley and Belas, 1995;

Type of UTI	Patient group	Percentage (%) UTIs caused by *Pr. mirabilis*
Acute cystitis	Women	3
Hospital UTI	Men and women	5
Recurrent UTI	Women	6
Recurrent UTI	Men	6
Complicated UTI	Men and women	9
Intermittent catheter	Men and women	15
Condom catheter	Men	25
Ileal conduit	Men and women	33
Long-term urethral catheter	Men and women	44

Table 1.1 – The percentage of urinary tract infections (UTIs) caused by *Pr. mirabilis*

Modified from Mobley and Belas (1995).

Mathur *et al.*, 2005). However *Proteus* bacteriuria may also develop from exogenous strains via cross-contamination between catheterised patients by medical and nursing staff (Nicolle, 2002).

The organism possesses a number of virulence factors including: peritrichous flagella, fimbrial adhesins, urease, haemolysin and an IgA-degrading protease, in addition to iron chelators (Coker *et al.*, 2000).

The genus *Proteus* was named by Gustav Hauser after the Greek mythological god of the ocean in Homer's Odyssey who had the ability to change forms to evade capture. *Pr. mirabilis* is a dimorphic organism that can transform itself from short, rod-shaped, vegetative swimmer cells to elongated, hyper-flagellated swarmer cells. This change can occur in response to contact with solid surfaces or in viscous environments (Mobley and Belas, 1995). The swarmer cells line up in rafts and move in a co-ordinated fashion (Williams and Schwarzhoff, 1978). A recent study by Jones *et al.*, (2004) used SEM to show that the flagella of cells in these rafts were intertwined in a helical manner. Mutants deficient in these elaborate structures failed to swarm.

Physiological changes such as an up-regulation of haemolysin and urease also occurs on transformation to swarmer cells (Allison *et al.*, 1992b). *Pr. mirabilis* demonstrates brief synchronized spurts of swarming on solid surfaces *in vitro* followed by relapses to a surface-bound vegetative form (consolidation). The swarming phase allows spread across a surface whereas the consolidation phase permits extensive multiplication before swarming is re-initiated by the outer edge of the colony (Rauprich *et al.*, 1996). Cyclical repetitions of this activity produce characteristic concentric rings of swarming and consolidation across surfaces such as agar (Figure 1.3).

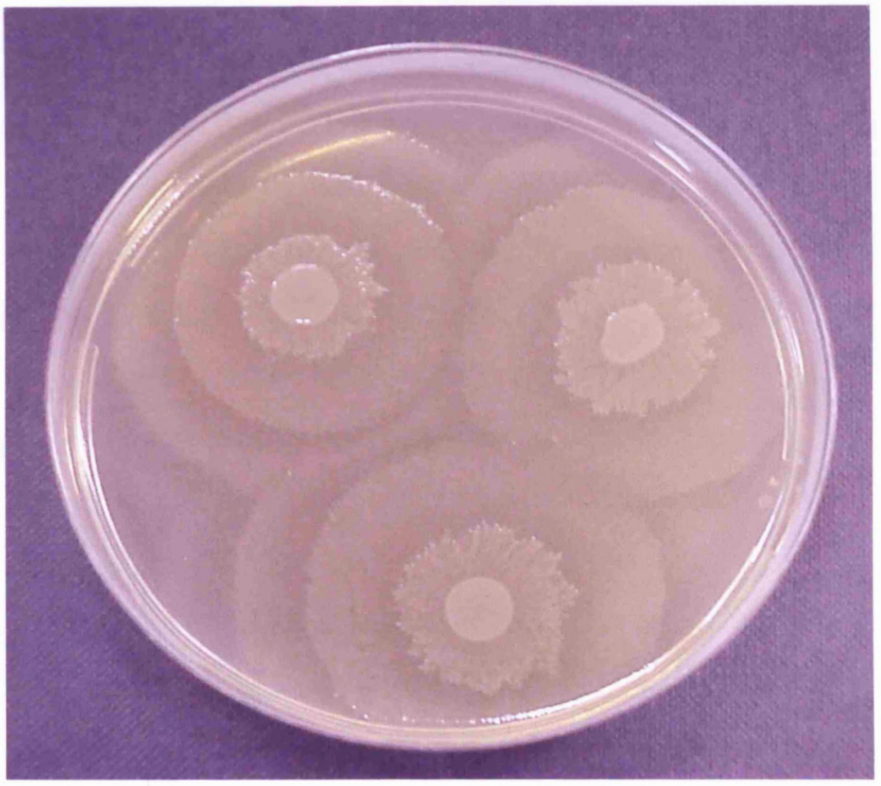


Figure 1.3 – The classic "bulls-eye" pattern of swarming and consolidation produced by *Pr. mirabilis* on Tryptone Soya Agar.

Three 10 µl aliquots of a 4 h culture of *Pr. mirabilis* B2 were spot inoculated onto the agar surface and incubated overnight at 37°C

Differentiation into swarmer cells is a response to environmental influences. The chemical signal glutamine induced differentiation and seemed to chemotactically direct swarming (Allison *et al.*, 1993). Furthermore the same authors found that differentiation was additionally promoted by surface contact. Contact with an indwelling catheter could thus provide the stimulus needed for swarming. Using simple *in vitro* models Stickler and Hughes (1999) established that *Pr. mirabilis* could also rapidly swarm over urinary catheters. They proposed that swarming may have functions in instigating catheter-associated infection and the spread of bacterial biofilm over the surface. Support for this hypothesis was recently provided by Jones *et al.*, (2004). They found that non-swarming mutants had lost their ability to migrate across sections of all-silicone catheters. Surface attachment however, was shown to be independent of swarming activity (Jones *et al.*, 2005a).

Mobley *et al.*, (1996) reported experiments in which non-catheterised mice were inoculated urethrally with a wild-type *Pr. mirabilis* and a flagella-deficient mutant. The data showed that significantly less bladder and kidney colonization was produced by the mutant, suggesting the importance of motility in the pathogenicity of *Pr. mirabilis* in the urinary tract.

Allison *et al.*, (1994) presented evidence that swarming has a role in the pathogenesis of pyelonephritis. Experiments in which the bladders of mice were inoculated with test organisms showed that in contrast to wild-type strains, motile but non-swarming mutants were unable to establish themselves in the bladder or ascend to the kidneys. Intravenous injection of these non-swarming strains into mice revealed much lower mortality rates compared to the wild-type. On the other hand, Zunino *et al.*, (1994) presented evidence which questioned the involvement of swarming in pathogenesis. They isolated a non-flagellated, non-swarming strain of *Pr. mirabilis* from a patient with symptomatic UTI.

Experiments in mouse models demonstrated that this strain was still effective in causing ascending infection of the urinary tract. More recently Jansen *et al.*, (2003) reported experiments in mice in which they found that swimmer cells were the predominant population throughout the urinary tract and that swarmer cells were rarely found.

Swarming may have a role in the invasion of tissue by *Pr. mirabilis*. *In vitro* tests with human uroepithelial cells demonstrated that non-motile, non-swarming mutants were totally non-invasive, whilst motile swarm-defective mutants had significantly lowered invasion abilities compared to the wild-type (Allison *et al.*, 1992a).

Once present in the bladder urine *Pr. mirabilis* induces production of fimbriae including mannose-resistant/*Proteus*-like fimbriae (MR/P) and *Pr. mirabilis* fimbriae (PMF) (Mobley and Belas, 1995) that have an affinity for distinct urinary tract mucosal cells (Coker *et al.*, 2000). Both fimbriae appear to contribute to bladder colonization (Bahrani *et al.*, 1994; Mobley *et al.*, 1994; Zunino *et al.*, 2001). In addition, evidence for both MR/P fimbriae (Bahrani *et al.*, 1994; Mobley *et al.*, 1994; Zunino *et al.*, 2001) and PMF fimbriae (Zunino *et al.*, 2003) have shown they both seem to be of some importance for upper urinary tract colonization.

The urease (urea amidohydrolase) produced *by Pr. mirabilis* is a cytoplasmic, nickel-requiring enzyme that hydrolyses urea to ammonia and carbon dioxide. Under the alkaline conditions that develop in urine polyvalent ions of Ca^{2+} and Mg^{2+} precipitate out of solution. This process is detailed in section 1.7.2. Swarming in the presence of urine, when urease activity is up-regulated might therefore quicken up the process of mineral deposition (Stickler and Hughes, 1999). Urease may have a function in nitrogen and/or carbon metabolism of the

cell, or be involved in pH regulation or the elimination of toxic concentrations of urea surrounding the bacterium (McLean *et al.*, 1988). The urease enzymes of *Pr. mirabilis* isolates investigated by Jones and Mobley (1987) had lower affinities for urea but significantly increased ($P < 0.001$) rates of hydrolysis (6- to 30-fold) than the other three ureolytic species tested. The concentrations of urea to which *Pr. mirabilis* is exposed to in the urinary tract, (up to 500 mM) implies that the enzyme's active sites would be fully saturated and operating at its maximum rate (Jones and Mobley, 1987; Mobley and Warren, 1987).

There is strong evidence from animal infection models that urease is a virulence factor involved in the pathogenicity of *Pr. mirabilis*. For example, urease negative mutants failed to establish themselves in the bladders and kidneys when inoculated into the lower urinary tracts of experimental mice (Jones *et al.*, 1990; Johnson *et al.*, 1993a). Furthermore, whilst 35% of the mice challenged with the wild-type urease-positive parent strain died, all the mice inoculated with the urease-negative mutant survived (Johnson *et al.*, 1993a).

1.7.2 *Pr. mirabilis* biofilms, encrustation and catheter blockage

In humans it is in the catheterised urinary tract that *Pr. mirabilis* becomes a particularly important pathogen. The complications it causes stem from its ability to produce crystalline biofilms that encrust and block indwelling urinary catheters. Cox *et al.*, (1989) showed via scanning electron microscopy that catheter encrustations were composed of crystals and bacteria. These observations have been confirmed since by Stickler *et al.*, (1993a, 1993b), Winters *et al.*, (1995), Stickler (1996) and Stickler *et al*, (1998b). Catheter encrustations thus bear a resemblance to infection-associated stones, throughout which bacterial cells can also be detected (Takeuchi *et al.*, 1984).

As can be seen from Figure 1.1 these crystalline biofilms can develop to such an extent that they can be visible to the naked eye and can generate problems with urine flow by partially or entirely blocking the luminal route through the catheter. Around 50% of patients with long-term catheters suffer from eye-hole and/or luminal blockage as a result of this encrustation (Cools and van der Meer, 1986; Kunin *et al.*, 1987a; Getliffe, 1990; Getliffe, 1994a).

Pr. mirabilis is the most commonly isolated organisms from the urine of patients suffering with regular catheter blockage (Mobley and Warren, 1987; Kunin, 1989; Kohler-Ockmore and Feneley, 1996). It is also the organism most frequently recovered from catheter encrustations and was not found in biofilms lacking crystalline material (Stickler *et al.*, 1993a). Stickler *et al.*, (1998b), using *in vitro* models of the catheterised bladder, revealed that only *Pr. mirabilis*, *Proteus vulgaris* and *Providencia rettgeri* could generate alkaline conditions and luminal crystalline biofilm formation. *Morg. morganii*, *Klebsiella pneumoniae* and *Ps. aeruginosa*, despite being designated as urease-positive strains by conventional tests, failed to raise the urinary pH and cause catheter encrustation over the 24 h test period.

1.7.2.1 The formation of crystalline biofilms

The main constituents of the encrustations that form on urinary catheters are crystalline magnesium ammonium phosphate ($MgNH_4PO_4.6H_2O$; struvite) and forms of calcium phosphates (apatites) including hydroxyapatite ($Ca_5(PO_4)_3OH$) and more often the poorly crystalline variety carbonate apatite ($Ca_5(PO_4,CO_3,OH)_3OH$) (Bruce *et al.*, 1974; Hukins *et al.*, 1983; Hedelin *et al.*, 1984; Cox *et al.*, 1987, 1989; Stickler *et al.*, 1993a, 1993b). Hedelin and colleagues (1984) found that a mix of struvite and calcium phosphates, or calcium phosphates alone, were the main constituents of over 90% of the encrustations. In order for struvite and apatite to form an alkaline environment is required which is

achieved by *Pr. mirabilis* via the action of its urease enzyme. Of the virulence factors possessed by *Pr. mirabilis* urease production is responsible for catheter encrustation.

Urease hydrolyses urea to ammonia and carbamate/carbamic acid. Normal human urine contains large quantities of urea excreted into the urine at normal rates of between 6-17 g/24 h (United States National Library of Medicine at http://www.nlm.nih.gov/medlineplus/ency/article/003605.htm). A large substrate concentration is therefore available for use. In addition, urine also contains numerous inorganic cations and anions such as Na^+, Ca^{2+}, Mg^{2+}, Cl^-, and $H_2PO_4^-$ (Clapham *et al.*, 1990).

Ammonia and carbon dioxide are formed via a carbamic acid intermediate by urease catalysed hydrolysis of urea (Hedelin *et al.*, 1985; McLean *et al.*, 1988; Clapham *et al.*, 1990; Burne and Chen, 2000):

(1) $CO(NH_2)_2 + H_2O \longrightarrow NH_3 + CO(NH_2)OH \longrightarrow 2NH_3 + CO_2$

The pH of normal urine is slightly acidic, around 6 (McLean *et al.*, 1988; Clapham *et al.*, 1990). At this pH both products equilibrate with water. The two ammonia molecules become protonated, to form ammonium and hydroxide ions, whilst the carbon dioxide initially forms hydrogen carbonate.

(2) $2NH_3 + 2H_20 \leftrightarrow 2NH_4^+ + 2OH^-$

(3) $CO_2 + H_20 \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$

H_2CO_3 is a weak acid and dissociates into a hydrogen carbonate ion poorly in urine of normal pH. The production of two OH^- ions to each H^+ ion raises the pH of the urine. As the pH increases the CO_2 reaction shifts to the right so at very high alkalinities a carbonate ion and $2H^+$ ions are produced:

(4) $H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$

The H^+ ions begin to mop up the OH^- with the result that the pH rise begins to slow and reaches a maximum of about 9.

The increase in pH generated by the bacterial urease is significant because it produces conditions in which the normally soluble building blocks of struvite and apatite become insoluble. Hedelin *et al.*, (1985) reported that precipitation of calcium phosphates begins around pH 6.8 followed by magnesium phosphate at pH 7.

There is evidence that the exopolysaccharide produced by *Pr. mirabilis* also plays a role in the formation of the crystalline biofilm. Clapham *et al.*, (1990) found that the gel-like glycocalyx matrix that is produced around the cells enhances struvite crystal formation. Dumanski *et al.*, (1994) reported that the anionic polysaccharide capsule of *Pr. mirabilis* had a unique ability to weakly attract magnesium ions from urine. It's weak binding capacity most likely concentrates Mg^{2+} ready for struvite formation but allows prompt release for crystal growth. Furthermore, it has been shown that crystals that form or become trapped in the *Proteus* biofilm are protected from dissolution by high flow rates of acidic urine (McLean *et al.*, 1991) conditions in which struvite normally dissolves readily (Griffith *et al.*, 1976a, 1976b; McLean *et al.*, 1991).

A simple synopsis of catheter encrustation was provided by Stickler (1996): (1) urinary tract invasion by urease-generating bacteria; (2) bacterial cells attach to the conditioning layer which is rapidly laid down on catheter surfaces in urine; (3) the adhered cells undergo multiplication surrounded by a polysaccharide matrix to form a biofilm; (4) urease hydrolysis of urea raises the urinary and biofilm pH; (5) calcium and magnesium phosphate crystallisation occurs in and on the biofilm as well as in the urine; (7) the continued development of the biofilm eventually blocks the flow of urine through the catheter.

There is strong evidence that the main factor involved in catheter encrustation is infection by urease-producing bacteria (Mobley and Warren, 1987; Kunin, 1989;

Stickler *et al.*, 1993a, 1998b). Little is known however about the factors that modulate the rate of crystalline biofilm formation. In some patients it might be that factors other than urease have a role in the process. For example, Choong *et al.*, (1999) re-examined the relationship between the pH of the urine voided by catheterised patients (pH_v) and the nucleation pH (pH_n) i.e. the pH at which crystalline material starts to precipitate from the urine. In a group of patients lacking urease-positive bacteriuria and designated as non-blockers, the mean pH_v was 5.97 compared to the mean pH_n value of 7.46, clearly explaining the lack of catheter encrustation in these cases. Urease positive organisms were isolated from the urine of most patients designated as blockers and in this group the mean pH values were pH_v 7.85 and pH_n 8.15. However, there was also a minority of patients designated as blockers who apparently were not infected by urease producers. In these cases the mean pH_n was unusually low (6.45) compared to the mean pH_v of 7.36 allowing precipitation at voided pH.

1.8 Attempts to control encrustation on urethral catheters

Over the years many attempts have been made to develop strategies to control the complication of catheter encrustation and blockage. There is little evidence however to suggest that any of them have been effective in preventing the clinical crises that result from catheter blockage (Kunin, 1997). Community nurses, who are often responsible for the care of catheterised patients, have expressed their uncertainty about how to deal with the problem (Roe, 1989; Capewell and Morris, 1993).

1.8.1 Irrigations with catheter maintenance solutions

A common attempt to decrease the extent of luminal catheter blockage by crystalline biofilm and debris relies on catheter washouts (Getliffe, 1990). Various "catheter maintenance solutions" are available and are in use today. The

idea is that these solutions physically disrupt and flush out the debris and/or dissolve the encrustation from the luminal surface.

There is some laboratory evidence that irrigations with acidic solutions reduce catheter encrustations. Washouts with either 1% mandelic acid or Suby G (a citric acid based solution) decreased the encrustation that had been caused by *Pr. mirabilis* infection of urine in *in vitro* models of the catheterised bladder (Getliffe, 1994b). In contrast, a 0.9% saline solution had little effect suggesting that a purely mechanical flushing was not adequate for reducing the amount of encrustation. Similar *in vitro* experiments demonstrated that 2 consecutive washouts with 50 ml Suby G was more successful at dissolving calcium and magnesium from luminal catheter biofilms than the 100 ml often used (Getliffe *et al.*, 2000). Suby G was the most commonly used solution in clinical practice recorded by Getliffe (1990, 1994a). This was despite no thoroughly controlled trials to establish its *in vivo* efficacy or its most suitable programme of administration.

Kennedy *et al.*, (1992) explored the clinical effectiveness of some of these solutions in a small cross-over clinical trial. The three washout solutions evaluated; 0.9% saline, Suby G (3.23% citric acid, 0.38% magnesium oxide, 0.7% sodium bicarbonate, 0.01% bisodium edetate) and Solution R (6% citric acid, 0.6% gluconolactone, 2.3% magnesium carbonate, 0.01% bisodium edentate) failed to reduce crystal formation. In addition, red blood cells and bladder epithelial cells were commonly observed in the returned washout indicating a degree of bladder mucosa irritation. It is unknown to what extent repetitive washouts could damage the bladder lining. Furthermore, incidences that break the closed drainage system, as during washout procedures, increase the risk of infection and should be performed with extreme caution, if at all.

1.8.2 Dietary acidification of the urine

Attempts have been made to control the rise in urinary pH that is responsible for crystal deposition by the ingestion of acidifying agents such as ascorbic acid. The early work of McDonald and Murphy (1959) had shown that when patients were given a daily dose of 2.5 g ascorbic acid their urinary pH could be lowered significantly. Subsequently however, it was found that if patients' urine contained urease producing organisms the ascorbic acid had no effect on the pH of the urine (Murphy *et al.*, 1965). The *in vitro* experiments of Bibby and Hukins (1993) demonstrated clearly that the addition of H^+ to urine that contained the enzyme urease was rapidly neutralised by increased hydrolysis of urea to ammonia. They calculated that to prevent the pH rise from the hydrolysis of urea in the urine produced in one day would require the addition of 2.7 L of 0.1 M hydrochloric acid! The strategy of dietary acidification of the urine alone is thus not a practical approach for encrustation prevention.

1.8.3 Catheter materials and coatings

Although some manufacturers claim that their catheters resist encrustation, the evidence in the literature suggests that this is not the case. Experiments conducted in laboratory models of the catheterised bladder infected with *Pr. mirabilis* demonstrated that all types of catheters currently available, including hydrogel/silver coated latex catheters, were vulnerable to encrustation (Morris *et al.*, 1997; Morris and Stickler, 1998a). All-silicone catheters took slightly longer to block than latex-based catheters but this could be explained by the marginally larger drainage lumen rather than encrustation resistance properties of the catheters themselves. A 14 Ch latex based catheter has a round drainage lumen of around 1.5 mm in diameter whereas an all-silicone catheter has a crescent shaped lumen with an approximate width of 2.5 mm (Figure 1.4). Clinical experience also shows that all types of catheters are vulnerable to this

complication (Hukins *et al.*, 1983; Bull *et al.*, 1991; Stickler *et al.*, 1993a; Kunin, 1997).

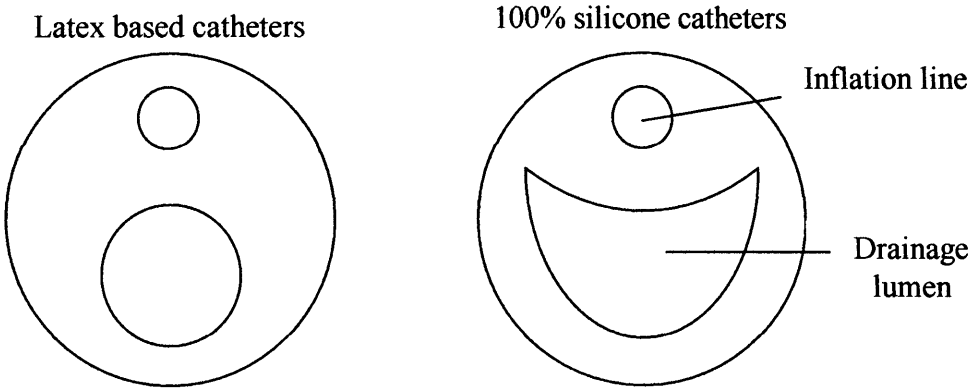


Figure 1.4 – An illustration of the varying lumen sizes of 2-way latex and silicone based urethral catheters

Recently Stickler *et al.*, (2006) reported that despite the initial inhibition of attachment conferred by strongly electron-donating surface coatings all of the surfaces tested in the parallel-plate flow-cell eventually became colonized *by Pr. mirabilis*. Once the urinary pH had reached alkaline levels (its nucleation pH) aggregates of bacteria and amorphous phosphates deposited onto the surfaces. Biofilm development proceeded from this point with further attachment of cells, crystals and aggregates. The authors concluded that future approaches to prevent the bacterial colonization and the formation of encrustations on urinary tract devices must not only consider the surface properties of the material but should also target the crystallisation of phosphates in the urine.

1.8.4 Urease inhibitors

Targeting the urease enzyme with inhibitors, rather than *Pr. mirabilis* itself, is an attractive approach for the treatment and prevention of catheter encrustation. It would avoid the use of antibiotics and the potential for selecting resistant strains. Urease inhibitors, namely acetohydroxamic acid (AHA; Trade name Lithostat) and N-[diaminophosphinyl]-4-flurobenzamide (fluorofamide), have been

investigated mainly as control measures for the formation of infection-associated kidney stones.

Acetohydroxamic acid was found to lower the urinary pH and ammonia concentrations in patients with staghorn renal calculi (Griffith *et al.*, 1978). In addition a clinical trial provided evidence that this compound was also effective at inhibiting the development of infection-induced renal struvite stones (Williams *et al.*, 1984). Patients in the small study conducted by Burns and Gauthier (1984) had significantly lowered amounts of catheter encrustation after oral AHA therapy compared to their pre-study levels. Despite its efficacy the use of AHA has been limited due to its toxicity. Deep-vein thrombosis, pulmonary embolism and tremors are all side effects that have been reported (Williams *et al.*, 1984). Fluorofamide was shown to inhibit struvite formation in the bladder of experimental rats (Millner *et al.*, 1982). The authors suggested that as the activity of fluorofamide was around 1000-fold greater than that of AHA and it was less toxic it may have a role in preventing urolithiasis in patients. In laboratory catheterised bladder models AHA and fluorofamide were shown to lack bactericidal activity but did successfully prevent the rise in urinary pH and significantly lowered the subsequent crystalline deposition caused by *Pr. mirabilis* (Morris and Stickler, 1998b). Morris and Stickler (1998b) concluded that if these inhibitors were to be used in clinical practice novel ways of introducing them directly into the bladder would need to be developed.

1.8.5 Fluid intake

Many catheterised patients, especially the elderly, drink very little and nurses often encourage them to increase their fluid intake (Kunin, 1987; Dowse, 2005). It would seem to be common sense that increasing a patient's fluid intake, so as to produce more urine, would keep more Ca^{2+} and Mg^{2+} in solution. There is clinical evidence to support such a policy. Burr and Nuseibeh (1997) for example

analysed the urine of 60 catheterised SCI patients and found that the urine of patients designated as "catheter blockers" was more concentrated than that of "non-blockers". They explained the importance of maintaining a regular fluid intake throughout the day. In this way periods when concentrated urine, in which crystallisation was likely to occur, could be avoided. The *in vitro* experiments by Suller *et al.*, (2005) showed that by simply diluting the urine it was possible to raise its nucleation pH and make crystal deposition less likely. These workers also showed that increasing the citrate content of the urine had a similar inhibitory effect on crystallisation of calcium and magnesium phosphates. Recent experiments, in laboratory models of the catheterised bladder, confirmed that diluting urine and increasing its citrate content both significantly reduced the rate of crystalline biofilm formation on catheters (Stickler and Morgan, 2006).

Many patients are advised to drink cranberry juice to try and prevent catheter blockage (Morris, 1999). The efficacy of this policy was examined in experiments in laboratory models by Morris and Stickler (2001). They found no significant difference in the amounts of calcium and magnesium deposited on catheters supplied with pooled human urine collected from volunteers with fluid intakes supplemented with 500 ml of either cranberry juice or water. They concluded that the important factor in preventing catheter encrustation was maintaining a good fluid intake. Whilst there has been no clinical trial to demonstrate that increasing fluid intake reduces catheter encrustation and blockage in patients, the evidence suggests that such a strategy would be effective. The difficulty with this policy is that many of the elderly and frail patients resist attempts to encourage them to drink more.

1.8.6 Preventative catheter changes

Norberg *et al.*, (1983) put forward the idea that the catheters of patients designated as blockers had characteristic life-spans. They suggested that the median time to

blockage from two to five observations could be used as a reliable predictor of catheter life. Characterisation of the catheter life-span of each patient would then allow a proactive catheter change to be made before a blockage event occurred. In this way the clinical crises induced by unexpected catheter blockage could be avoided (Getliffe, 1994a, 2003). However, catheters still block unexpectedly. Mathur *et al.*, (2006) for example, recently reported considerable variation in catheter life-span, even in patients who were all infected with *Pr. mirabilis*.

1.8.7 Use of catheter valves

A catheter valve is a small device that can be attached to the distal end of a catheter. Urine is held within the bladder and drained by opening the valve intermittently. This procedure may aid removal of encrustation by physically disrupting and flushing out any luminal crystalline biofilm. Sabbuba *et al.*, (2005) evaluated whether intermittent, valve-controlled release of urine from the catheterised bladder decreased the extent of encrustation that is seen when urine is allowed to drain continuously. Experiments in laboratory models using a manual valve revealed significant improvements in times to blockage when it was opened at 2 h or 4 h intervals during the day, followed by 16 h or 12 h of continuous overnight drainage. The use of an automatic valve opened at every 2 h or 4 h during the day and night further extended times to blockage. The most significant effect, increasing time to blockage from 44.7 h to 159.7 h, was seen when the automatic valve opened at 4 h intervals throughout the day and night. Whilst it would be worth examining this strategy in patients, it is recognised that it would only be applicable to a restricted group of patients with good manual dexterity, satisfactory cognitive function and relatively stable bladders.

1.8.8 Electrified catheters

The application of a direct electric current through silver electrodes placed in urine results in a phenomenon known as iontophoresis in which bactericidal silver

ions are released into the urine (Davis *et al.*, 1992). Chakravarti *et al.*, (2005) investigated the effects of iontophoresis on urethral catheter encrusting deposits of crystalline *Pr. mirabilis* biofilms. Two fine silver wire electrodes were attached to all-silicone catheters in such a way that the weak 150 μA current only flowed across the catheter eye-holes. Control all-silicone catheters and catheters that had wires affixed but no current supplied blocked between 22 and 48 h after *Pr. mirabilis* infection. Iontophoretic catheters however took a significantly longer to become blocked (mean of 133 h). The released silver ion concentration in the urine decreased as time elapsed and was paralleled with oxidation of the electrodes, an increase in viable cell number and an increase in pH, eventually culminating in blockage. For the time the electrodes were active they prevented the characteristic development of *Pr. mirabilis* infection of the catheterised urinary bladder. Such a system, though interesting, obviously requires further development if it is to be introduced into clinical practice.

1.8.9 The catheter balloon as a reservoir for antibacterial agents

Bibby *et al.*, (1995) proposed that inflation of the catheter balloon with an antimicrobial agent rather than water might control the encrustation of catheters by diffusion through the balloon into the bladder urine. They demonstrated that mandelic acid could indeed diffuse in low concentrations through the balloon. Mandelic acid is not very active against *Pr. mirabilis* however, being bactericidal in urine at concentrations around 1-3 mg/ml (Dr. Gareth Williams, Cardiff University, personal communication). In contrast the biocide triclosan is very active against *Pr. mirabilis*. The MIC of this agent against strains of *Pr. mirabilis* isolated from encrusted catheters was reported by Stickler (2002) to be around 0.5 μg/ml.

Stickler *et al.*, (2003a) and Jones *et al.*, (2005c) subsequently demonstrated in laboratory bladder models that inflating the catheter balloon with a solution of

triclosan prevented the rise in urinary pH despite heavy infection with *Pr. mirabilis*. This in turn allowed the catheters to drain unobstructed, throughout the 7-day experimental period. In comparison, catheters with their balloons inflated with water became blocked by encrusted deposits within around 24 h. Clinical trials are now required to assess this approach *in vivo*.

1.8.10 Biocontrol

Preventing catheter-associated urinary tract infections and catheter encrustation are difficult problems requiring ingenious solutions. Trautner and Dariouche (2004) suggested that antibacterial agents should be avoided as they will inevitably fail to suppress the abundant, diverse and often multi-resistant organisms that contaminate the catheterised urinary tract. As an alternative they proposed that colonization by benign organisms might prevent subsequent colonization by pathogens. If bacterial interference is to be used as a strategy against *Pr. mirabilis* it is necessary to know more about the ecology of catheter biofilms and the interactions between the species in these commonly multi-species communities.

1.9 Some associations and interactions between organisms forming biofilms

Coaggregation was first described nearly 40 years ago by Gibbons and Nygaard (1970). It is defined as the "process of adhesion between genetically distinct bacterial partners. It can take one of three forms: intergeneric, intrageneric (interspecies) or intraspecies (interstrain) partnerships. Autoaggregation is the process of adhesion between genetically identical cells. This could be a means of ensuring colonization and establishment of the species in question in a "self-centred" manner. Coaggregation occurs between cells in the liquid phase. It is a highly specific process mediated by adhesins on one organism binding to specific receptors on the surface of another.

Environmental parameters such as nutrient concentration, pH, and flow are often in a state of flux in many bacterial habitats. Bacteria that coaggregate into communities are more likely to endure these fluctuations (Rickard *et al.*, 2003a). These same authors concluded that coaggregation has a function in the development of some mixed species biofilms allowing cells to profit from the advantages of such physiological and metabolic co-operation. Coaggregation and autoaggregation have been shown to facilitate the development of biofilm communities between strains isolated from a range of environments including the oral cavity (Kolenbrander, 1989), freshwater (Rickard *et al.*, 2003b), and sewage sludge (Malik *et al.*, 2003). However, little is known about coaggregation between organisms that inhabit the urinary tract.

Burmølle *et al.*, (2006) recently examined synergistic interactions in biofilms isolated from the microbial surface community of *Ulva australis*, a marine alga. A combination of four isolates: *Acinetobacter lwoffii*, *Dokdonia dunghaensis*, *Microbacterium phyllosphaerae* and *Shewanella japonica* when grown together increased biofilm biomass by over 167% compared to that of the single isolates alone. In addition, cells in the mixed community were afforded enhanced protection from antimicrobial solutions of both tetracycline and hydrogen peroxide, and invasion by a biocide producing *Pseudoalteromonas tunicata.* These species gained fitness advantages from growing in a multi-species biofilm compared to growing individually.

1.10 Endotoxins

Lipopolysaccharide (LPS) molecules are major components of the outer cell membrane of all Gram-negative bacteria. They are important for the structure and function of these membranes, they mediate communication with the environment, and can act as potent toxins (endotoxins) when released from the cell. Release occurs mainly on break-up of the cell after death *in vivo* via antibiotic actions,

phagocytosis, or complement-mediated lysis (Jackson, 1994). Gram-negative endotoxins have been linked to a number of serious diseases such as ARDS (acute respiratory distress syndrome), sepsis and septic shock, and multiple organ failure (Barnett and Cosslett, 1998)

LPS has three components. The outermost part of the molecule is the O-specific polysaccharide. It is the hydrophilic section of LPS and is highly variable in its composition between species. This area is responsible for the immunological serotype and stimulates the manufacture of specific antibodies by the host. It also determines the colony morphology of the bacterial strain. Loss of the O-side chain via mutation results in "rough strains" which are less virulent, as opposed to "smooth stains" that possess the O-polysaccharide. In addition the O-specific polysaccharide has roles in adhesion to non-biological surfaces in at least some species (Williams and Fletcher, 1996). Adjacent to the O-side chain is a short chain of 7-/8-carbon sugars, often including the unique 2-keto-3-deoxyoctonic (KDO) acid. This comprises the outer and inner core (R) polysaccharide section which is structurally similar in related species. Losses in the core region produce what are known as "deep rough mutants". These mutants are sensitive to hydrophobic compounds and demonstrate that this area is important in membrane permeability. Bound to the core polysaccharide is the lipid A segment. Lipid A, constructed of a bi-phosphorylated glycolipid, is the hydrophobic part of the LPS molecule and is highly conserved amongst all Gram-negative bacteria. It is this component that is associated with the molecule's toxicity and triggers the host's innate immune system. It also acts as the membrane securing section of the LPS molecule.

Endotoxaemia, endotoxins in the blood, can result from, amongst other things, Gram-negative septicaemia, infectious foci or exogenous sources (Barnett and Cosslett, 1998). LPS causes a variety of non-specific, complex

pathophysiological responses irrespective of their origin due to the conserved nature of the lipid A section. The mechanism of action of LPS is not fully elucidated. It is known however, that it combines with an LPS-binding protein in the blood and this complex then binds with CD14 receptors on monocytes and macrophages. In response, the macrophages release a number of inflammatory mediators including cytokines which stimulate innate and specific immune responses. Both the complement and coagulation cascades can also be directly activated resulting in inflammation and inappropriate disseminated intravascular coagulation (DIC). The severe bombardment of inflammatory mediators can cause vasodilation, damage capillary endothelia, and cause local and systemic cell, generalised tissue and organ damage, which can lead to septic shock and death.

Frederick Bang (1956) was the first to discover that a Gram-negative infection of a *Vibrio* species led to lethal intravascular coagulation of *Limulus polyphemus* (the horseshoe crab). These initial observations form the basis of current endotoxin detection assays important in the diagnosis of bacterial infections of body fluids and other liquids.

1.10.1 Endotoxins and the urinary tract

An early study by Garibaldi *et al.*, (1973) showed a remarkable relationship between endotoxaemia and Gram-negative bacteriuria in 39 patients undergoing short-term indwelling bladder catheterisation. Of the 24 patients with bacteriuria 17 had at least one positive test for LPS and 16 of these had identifiable Gram-negative organisms isolated from their urine. The mean endotoxin concentration in the positive patients was 0.014 μg/ml.

Different species seem to be able to release endotoxins in varying quantities. Urine samples from long-term catheterised patients with *Enterobacteriaceae*

bacteriuria were found to contain concentrations of endotoxin around two- to three-fold higher than those infected with *Ps. aeruginosa* (Hurley and Tosolini, 1992).

Ascertaining the occurrence of UTI in patients receiving antibiotics for other reasons can be quite problematic because many of the causative organisms are eliminated. Endotoxins are released from both growing and dead, disintegrating bacteria and it is this fact that has stimulated the investigation into endotoxiuria, endotoxins in the urine, as a measure of UTI in those receiving antibiotic therapy. Berger *et al.*, (1996) demonstrated that the frequency of endotoxiuria in comparison to bacteriuria was not affected by the administration of antibiotics. They concluded that the diagnosis of a UTI in the presence of negative urine culture might be established via endotoxiuria detection. Boelke *et al.*, (2001) confirmed this observation in kidney transplant patients. They also showed that endotoxin assays typically indicated infections of the urinary tract a couple of days preceding urine cultures, functioning as an early specific indicator of UTI in this group of highly susceptible patients.

A study by Tanaka *et al.*, (1988) determined the levels of endotoxaemia in patients who had undergone treatment, such as ultrasonic lithotripsy, for urinary tract calculi. The three patients with infectious struvite stones all tested positive for endotoxaemia. It was surmised, but not confirmed, that the endotoxin responsible for the endotoxaemia in these patients came not only from the bacteria present in the urine but from the cells present within the stones that were released when the stones were disintegrated. It was not until 14 years later that McAleer *et al.*, (2002) presented the case of an 8 yr old boy who underwent percutaneous kidney stone lithotripsy for treatment of a staghorn calculus. He developed severe sepsis and died only 12 hr after the procedure. Endotoxin assays were performed on the stone fragments and yielded massive concentrations of 285,000 pg/g, whilst

urine bacteriology revealed the presence of *Pr. mirabilis*. This was the first report in which endotoxin was confirmed to have originated from a kidney stone.

An outbreak of severe fevers, mainly after transurethral resection of the prostate, prompted an investigation which was reported by Gephart (1984). It was found that some of the lots of Foley catheters and catheter lubricants that were used were heavily contaminated with endotoxin. It was suggested that this endotoxin might have been absorbed through the surgical wound into the circulatory system. In the presence of trauma to the urinary tract in patients enduring long-term catheterisation endotoxin, if present in catheter biofilms, could well gain access to the bloodstream and pose potentially serious risks to health.

1.11 Aims of the study

Catheter encrustation is clearly brought about by the development of crystalline *Pr. mirabilis* biofilms. Little is known however about the bacterial and host factors that might modulate the rate of catheter encrustation. Crystalline catheter biofilms commonly contain *Pr. mirabilis* in communities composed of several other species. The extent to which these other species might influence the rate of encrustation in unknown. If bacterial factors inhibiting crystalline biofilm formation could be identified it might be possible to develop a novel biological interference strategy to control this complication that undermines the health of so many elderly and disabled people. The extent to which *Pr. mirabilis* biofilms produce potentially dangerous levels of endotoxin in patient's bladders is also unknown. In view of the lack of information relating to these issues the aims of the work reported in this thesis were:

- To gain an insight into the complexity of the urinary flora of patients undergoing long-term catheterisation.

- To examine data on the bacterial composition of catheter biofilms for evidence of associations or antagonisms between *Pr. mirabilis* and other species.

- To test the effects of other uropathogens on the ability of *Pr. mirabilis* to produce catheter encrustations in laboratory models of the catheterised bladder.

- To examine the hypothesis that coaggregation between *Pr. mirabilis* and other species is involved in the formation of crystalline catheter biofilms.

- To determine whether endotoxin can be found in catheter biofilms from patients undergoing long-term catheterisation.

SECTION 2

Materials & Methods

2.1 Materials

2.1.1 Chemicals

The chemical agents used in this study, unless stated otherwise, were purchased from Fisher Scientific Ltd., (Loughborough, UK).

2.1.2 Bacteria

The urinary tract bacteria used throughout this study were clinical isolates from biofilms on catheters removed from patients enduring long-term catheterisation. They were donated for use by Dr Nicola Morris, Dr Steve Jones, Mr Sheridan Morgan, Mr Rob Broomfield (University of Wales, Cardiff, UK) and Mr. Sunil Mathur (Biomed Centre, Bristol Urological Institute, Southmead Hospital, Bristol, UK). Also, as part of the study, a collection of strains was isolated from the urine of chronically catheterised patients. Pure cultures of each isolate were Gram-stained, examined for indole and oxidase production, and then submitted to the appropriate commercial BBL^{TM} Crystal identification kit (Becton Dickinson, Oxford, UK). Once identified, or the identity confirmed, the strains were kept as stock cultures in a 5% volume/volume (v/v) glycerol solution, stored at -80°C in Nalge cryogenic vials (Nalge Europe Ltd., Hereford, UK) and for experimental purposes were subcultured weekly onto Cystine Lactose Electrolyte-Deficient (CLED) Agar. The identity of *Staphylococcus aureus* isolates was confirmed using the Mastastaph rapid latex agglutination test (Mast Diagnostics, Merseyside, UK).

Streptococcus sanguis GW2 and the American Type Culture Collection (ATCC) strain *Actinomyces naeslundii* 12104, kindly supplied by Dr Pauline Handley

(School of Biological Sciences, University of Manchester, UK) were oral cavity organisms used as positive controls in the coaggregation assays. A urease negative *Pr. mirabilis* (HI42320 *ure*$^-$) and its isogenic wild-type parent strain (HI4320 *wt*), which had been isolated from an elderly woman with urethral catheter-associated bacteriuria, were kindly donated by Professor Harry Mobley (Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, 48109 USA). These strains also had applications in the coaggregation assays.

2.1.3 Culture media

2.1.3.1 Agars

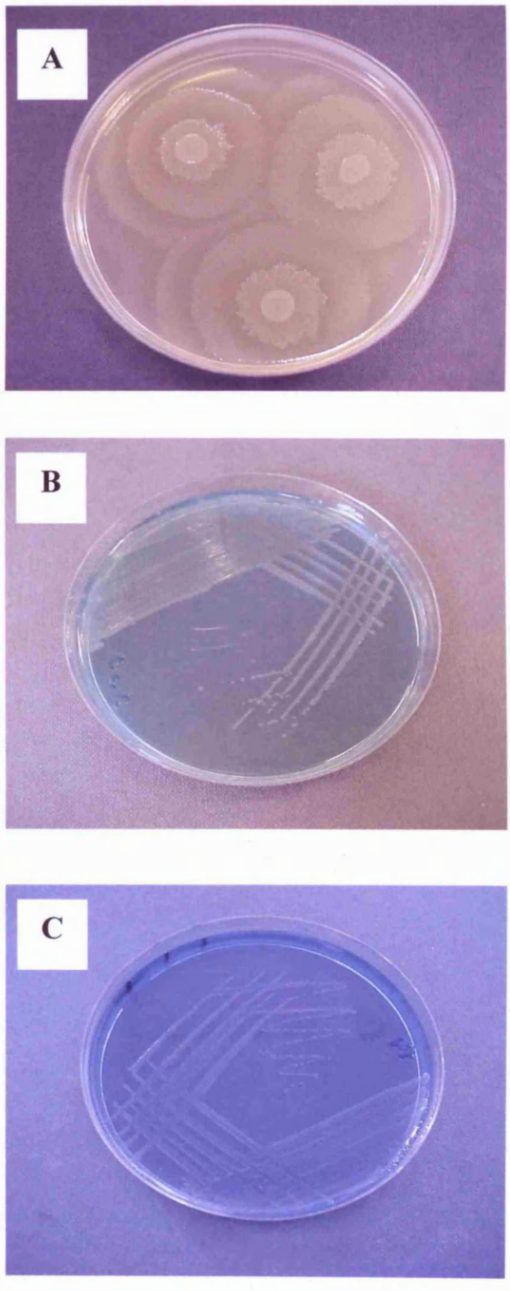
All the media used was purchased in the dehydrated form from Oxoid Ltd., (Basingstoke, UK) unless otherwise stated. Agars were prepared in 500 ml batches using deionised water, sterilised by autoclaving at 121°C for 15 min and allowed to cool to 50°C in a water bath (Grant Sub14 Water Bath, Cambridge, UL). They were then aseptically poured into sterile triple-vented Petri dishes, unless otherwise stated.

- Tryptone Soya Agar (TSA) is a general-purpose agar that supports the growth of a wide variety of aerobic and anaerobic organisms. It was used for the identification of swarming organisms during the clinical study (Figure 2.1A).

- Cystine Lactose Electrolyte-Deficient (CLED) Agar supports the growth of all urinary pathogens and confers good colony differentiation between species. The deficiency of electrolytes prevents swarming of *Pr. mirabilis*. (Figure 2.1B). This agar was used for viable cell counts of all samples that contained only one urinary organism and was the general growth media used to produce inocula of all urinary strains for experimental purposes.

Figure 2.1 - Swarming of *Pr. mirabilis* on TSA and its inhibition on CLED and CI Agars

(A) *Pr. mirabilis* spot inoculated onto TSA and incubated overnight at 37 °C. The characteristic "bulls-eye" concentric-ring pattern of swarming and consolidation can be clearly seen. The deficiency of electrolytes in **(B)** differential CLED Agar and **(C)** selective CI Agar prevent the swarming of *Pr. mirabilis* when inoculated onto these media.



- Colistin Inositol (CI) Agar, a selective, anti-swarming medium (Figure 2.1C) devised by Clayton *et al.*, (1982), was used for viable cell counts of *Pr. mirabilis* in all samples where *Pr. mirabilis* was present in conjunction with other organisms except *Morg. morganii* (Figure 2.2A). This medium contained 28 g/L Nutrient Agar, 10 g/L myo-Inositol (Sigma-Aldrich Chemical Company, Poole, UK), 0.1 g/L colistin methanesulphonate (Sigma-Aldrich) and 0.02 g/L bromothymol blue reagent (Sigma-Aldrich).

- Xylose Lysine Desoxycholate (XLD) medium is a differential medium and was used in this study for viable cell enumeration of mixed specimens of *Pr. mirabilis* and *Morg. morganii* (Figure 2.2B).

- Pseudomonas C-N Selective Agar (Pseudomonas Agar Base plus a cetrimide (200 mg/L) and sodium nalidixate (15 mg/L) selective supplement) was used for selective enumeration of *Ps. aeruginosa* in samples containing *Ps. aeruginosa* and *Pr. mirabilis* (Figure 2.2C).

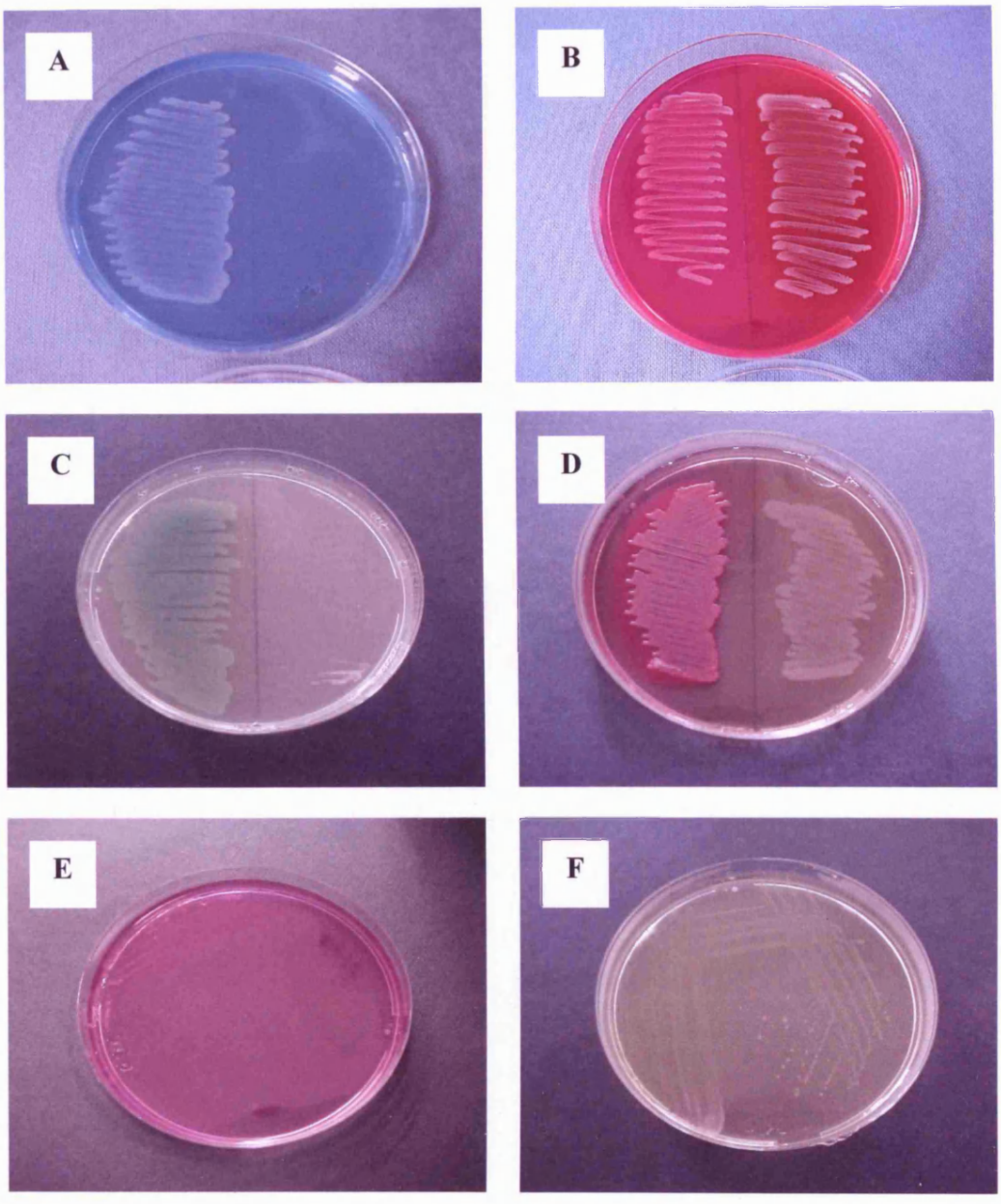
- MacConkey Agar is a differential medium for the detection and isolation of coliforms and other enteric pathogens. In this context it was employed for enumeration of viable cells in samples containing *E. coli* (red/pink colonies) and *Pr. mirabilis* (orange colonies) (Figure 2.2D).

- Urea Agar (Urea Agar Base containing 2% v/v urea) confirmed the presence of the urease enzyme in organisms determined to be urease positive using commercially available identification kits. The 95 ml batches were autoclaved for 20 min before aseptic addition of a 5 ml ampoule of 40% urea solution. A positive reaction for urease was indicated by production of a pink colour upon overnight incubation of a suspect organism (Figure 2.2E). A negative reaction resulted in the agar remaining orangey/yellow (Figure 2.2F).

- Chromogenic UTI medium permits presumptive identification and discrimination of all the significant UTI causing organisms. The incorporation of two chromogenic substrates and tryptophan results in different colour reactions depending on the species present. The chromogen X-glucoside is cleaved by β–glucosidase and results in enterococci colonies appearing blue.

Figure 2.2 – Examples of some urinary tract organisms on selective and differential media

(A) *Pr. mirabilis* (growth) and *Ps. aeruginosa* (no growth) on Colistin Inositol Agar, **(B)** *Morg. morganii* (pink) and *Pr. mirabilis* (red) growing on XLD medium, **(C)** *Ps. aeruginosa* (growth) and *Pr. mirabilis* (no growth) on Pseudomonas Selective Agar, **(D)** *E. coli* (red) and *Pr. mirabilis* (orange) growing on MacConkey Agar, **(E)** *Pr. mirabilis* HI4320 *wt* on Urea Agar, **(F)** *Pr. mirabilis* HI4320 *ure*$^{-}$ mutant on Urea Agar.



The β–galactosidase of *E. coli* cleaves the second substrate Red-galactoside producing pink colonies. Cleavage of both generates the purple colony colour of coliforms. The incorporation of tryptophan reveals the presence of tryptophan deaminase production in *Proteus*, *Morganella*, and *Providencia* species, the resultant colonies of which appear brown. Some commonly isolated urinary tract pathogens streaked onto Chromogenic UTI medium can be seen in Figure 2.3.

- Blood agar (Brain Heart Infusion Agar (47 g/L) supplemented with 0.3% weight/volume (w/v) yeast extract and 5% v/v defibrinated horse blood) was used to cultivate the two oral strains, *Strep. sanguis* and *A. naeslundii*.

2.1.3.2 Broths

Liquid growth media were sterilised by autoclaving at 121°C for 15 min

- Tryptone Soya Broth (TSB) is a multipurpose solution that was used to culture urinary tract organisms overnight (100 ml volumes) and was added as a supplement to artificial urine (1 g/L).

- Brain Heart Infusion (BHI) broth supplemented with 0.3% yeast extract, was prepared in 200 ml batches and functioned as a growth medium for overnight culturing of the oral pathogens *Strep. sanguis* GW2 and *A. naeslundii* ATCC 12104.

- Coaggregation Buffer was prepared in 1 L batches and contained 0.121 g/L Trizma® Base (Tris[hydroxymethyl]aminomethane) (Sigma-Aldrich), 0.020 g/L $MgCl_2$, 0.015 g/L $CaCl_2$ and 8.77 g/L NaCl. This buffer served as the re-suspension medium for the two oral cavity strains in preliminary coaggregation assays to allow the phenomenon to be visualised.

2.1.4 Artificial urine

Artificial urine, prepared the day before use, was used throughout the study and was based on that described by Griffith *et al.*, (1976a). The quantities of chemicals in Table 2.1 were added, in order, to deionised water. Once the urea

Figure 2.3 – Some commonly isolated urinary tract bacteria on Chromogenic UTI medium

An illustration of the variety of colours that are produced by different species on Chromogenic UTI medium: **(A)** *Pr. mirabilis*, **(B)** *Ent. faecalis*, **(C)** *Ps. aeruginosa*, **(D)** *E. coli*, **(E)** *Enterobacter cloacae* and **(F)** *Kl. pneumoniae*.

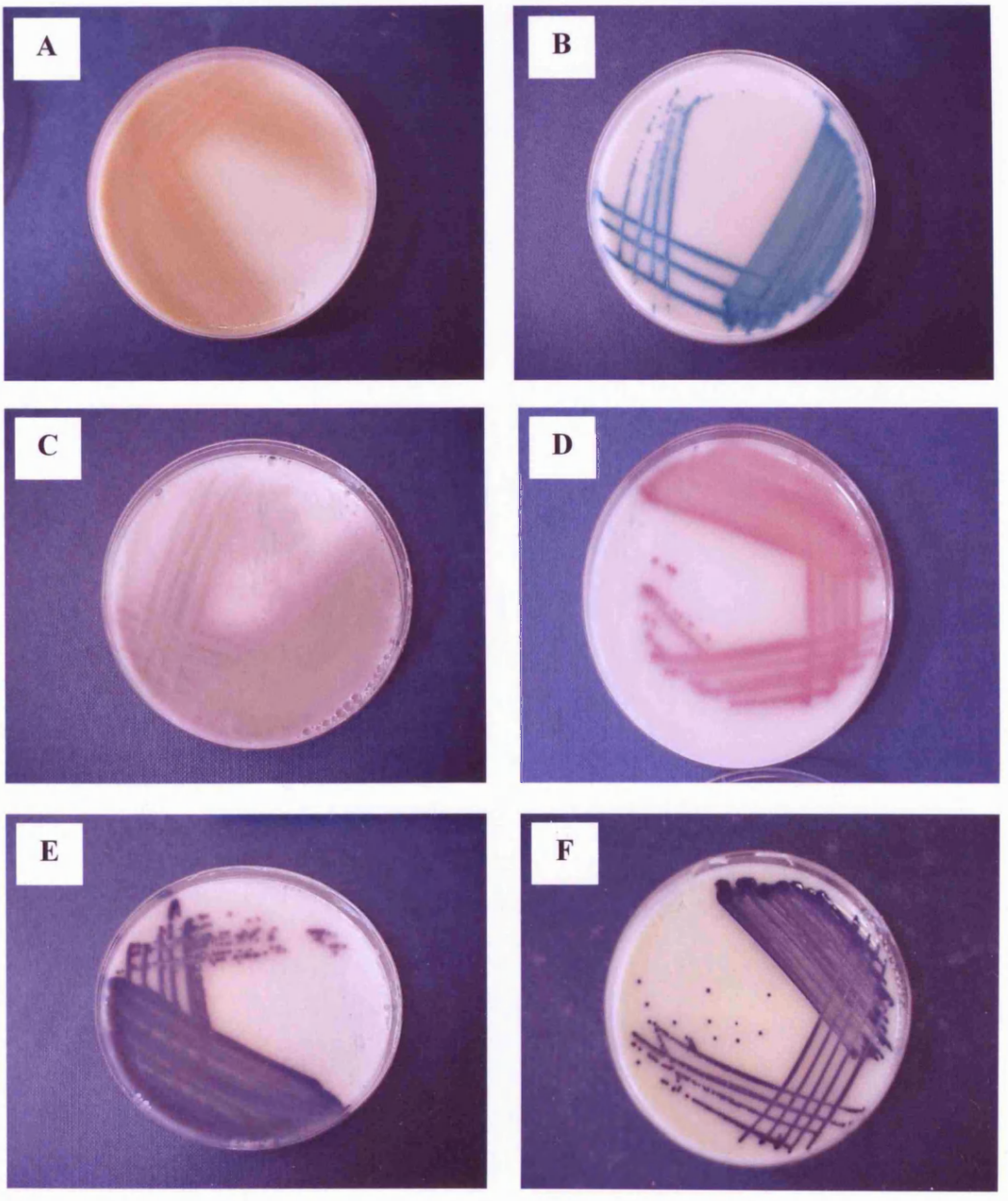


Table 2.1 – The chemical composition of artificial urine

A list of the amounts of dehydrated ingredients that were added to deionised water in the preparation of the artificial urine.

Chemical (Chemical Formula)	g/L
Sodium sulphate (Na_2SO4)	2.30
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.65
Magnesium chloride ($MgCl_2.6H_20$)	0.65
Sodium chloride (NaCl)	4.60
tri-Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	0.65
Sodium oxalate $(COONa)_2$	0.02
Potassium dihydrogen orthophosphate (KH_2PO_4)	2.80
Potassium chloride (KCl)	1.60
Ammonium chloride (NH_4Cl)	1.00
Urea ($NH_2CO.NH_2$)	25.00
pH adjustment to 6.10	
Gelatine	5.00
Filter sterilisation	
Tryptone Soya Broth	1.00

had dissolved the pH was adjusted to 6.10 using 3 M NaOH before addition of the gelatine. The solution was heated gently until the gelatine had dissolved. Sterilisation of the artificial urine was then achieved via membrane filtration using a Sartobran P® capsule filter (Sartorious, Goettingen, Germany) with a pore size range of 0.20-0.45 μm. A Watson-Marlow 505U peristaltic pump (Watson-Marlow Bredel Pumps Ltd., Falmouth, UK) was used to pump the urine through the filter into sterile 2 L or 5 L glass aspirators (Fisher, UK). Tryptone Soya Broth (TSB) was prepared separately and sterilised by autoclaving at 121°C for 15 min before addition to the sterile artificial urine just prior to experimental set-up.

2.1.5 Catheters

The all-silicone catheters used throughout the study were purchased from Bard Ltd., (Crawley, UK). These catheters were 2-way Foley catheters of a 14 Ch size (4.7 mm outer circumference), a standard male, 43 cm, length with a 10 ml inflatable retention balloon. These catheters were used because Morris *et al.*, (1997) reported that 14 Ch sized catheters were the most prevalent in long-term bladder management. Hydrogel-coated latex (Biocath) and silicone-coated latex (Silicone Elastomer) catheters, also purchased from Bard Ltd., were used in the endotoxin assays.

2.2 Methods

2.2.1 Viable cell counts

2.2.1.1 Spot inoculum method

In order to establish the number of viable cells in cultures and experimental samples the Miles and Misra technique (1938) was adopted for samples containing

single species, or for mixed species samples for which selective agars were available. Isotonic, ¼-strength Ringer's solution (Oxoid Ltd.,) was used to prepare serial dilutions of the suspensions, most commonly 10^{-1} to 10^{-6}. Triplicate aliquots (10 µl) of each dilution were spot inoculated onto a pre-poured, dry agar plate and left to dry. Each dilution series was prepared three times and inoculated onto three individual plates. Once dry the plates were inverted and incubated overnight at 37°C. The dilution that produced growth of 3-30 distinct colonies was used to calculate the viable cell count.

2.2.1.2 Spread plate method

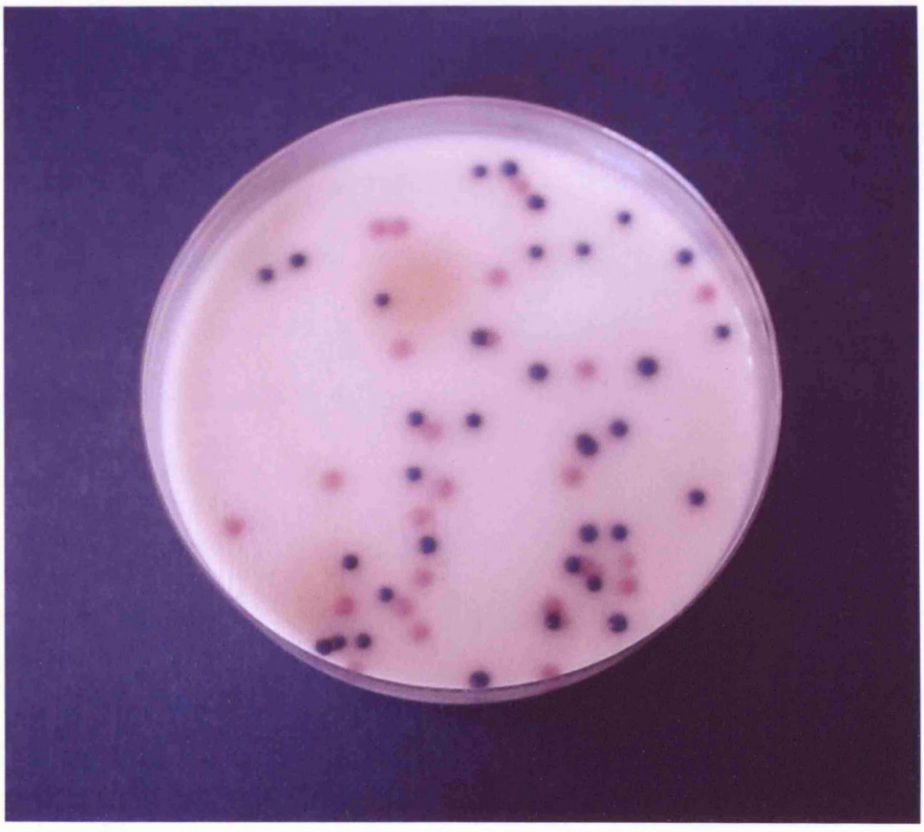
In those samples that had more than one organism for which selective agars did not exist for each species the spread plate protocol was followed. Again serial dilutions were made, but in contrast a 10 µl aliquot was pipetted onto the middle of an appropriate agar plate and spread over the entire surface using a sterile glass rod. Triplicate aliquots of each dilution were pipetted onto three separate plates. Each dilution series was prepared three times and inoculated onto 18 individual plates. Figure 2.4 illustrates a spread plate viable cell count on Chromogenic UTI medium.

2.2.2 pH determinations

All pHs were measured using a Fisherbrand Accumet® Basic AB15 bench pH meter (Fisher) standardised with a pH 7 phosphate buffer and a pH 10 borate buffer (Fisher). The glass electrode was immersed in the sample until the pH reading stabilised.

Figure 2.4 – A spread plate viable cell count on Chromogenic UTI medium

Chromogenic UTI medium allows enumeration of species from multi-species samples via the spread plate method. In this image three different species can be seen and easily counted; *Pr. mirabilis* (orangey brown), *E. coli* (pink) and *Kl. pneumoniae* (blue).



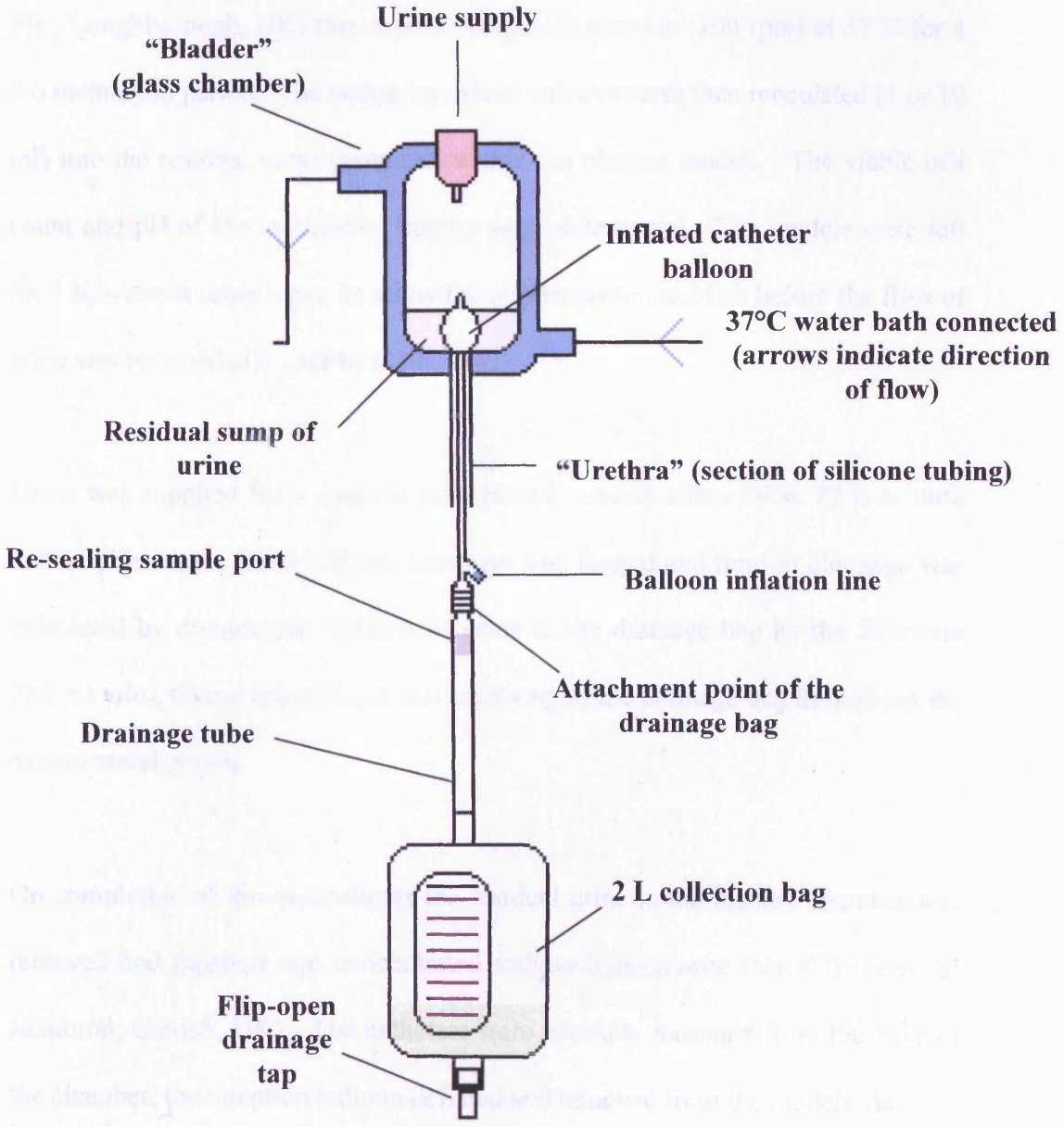
2.2.3 The laboratory model of the catheterised bladder

The simple laboratory model used in experimental work was devised by Stickler *et al.*, (1994) in order to investigate biofilm formation and its control in a reproducible, standardised system. The model (Figure 2.5) incorporated a 180 ml glass chamber surrounded by a water jacket. A Julabo 19 circulating water bath (Jencons Plc., Leighton Buzzard, UK) supplied water at 37°C to maintain the models at normal human body temperature.

All models were sterilised at 121°C for 15 min and the all-silicone catheters positioned in "the bladder" via aseptic insertion up through "the urethra", a section of silicone tubing attached to an aperture at the bottom of the model. The catheter balloon was then inflated by injection of 10 ml sterilised, deionised water which anchored the catheter in place and sealed the internal bladder compartment from the outside environment. Care was taken to maintain sterility, gloves were worn when handling the sterile apparatus and catheters, and as much of the experimental set-up as possible was performed within a laminar flow hood. Each catheter was then connected to a sterile 2 L bardia® bed bag (Bard Ltd.,) situated on a stand directly below the bench on which the models were situated. Models were positioned at the same height and distance apart and the drainage tubes of the drainage bags were sellotaped to the bench top. This was in an effort to standardise the conditions for each model and to allow unobstructed drainage of the overflow. Sterile silicone tubing, "the ureters", attached the aspirators of sterile urine, acting as "the kidneys", to the bladder models. This resulted in a fully closed system. Urine was then supplied to the bladders, from the aspirators, via a Sci-Q 323 10 channel peristaltic pump (Watson-Marlow). A residual volume

Figure 2.5 – Schematic representation of the laboratory model of the catheterised bladder

The catheter is inserted up through the "urethra", a length of silicone tubing attached to an aperture at the bottom of the glass chamber, and inflated with 10 ml of sterile water. The circulating water bath is connected which maintains the internal environment at 37°C. Urine is pumped into the bladder until a residual sump collects below the catheter eye-hole. Any excess drains via the catheter lumen, through the drainage tube and into a collection bag. To collect a sample for viable cell count and pH analysis the drainage tubing below the re-sealing port was clamped and the urine was aspirated from the port using a sterile needle and syringe.



(approx 15 ml) collected in each model just below the catheter eye-hole. Any surplus urine then drained, via the eyehole, through the catheter lumen into the drainage bag.

2.2.3.1 Basic protocol for operation of the bladder models

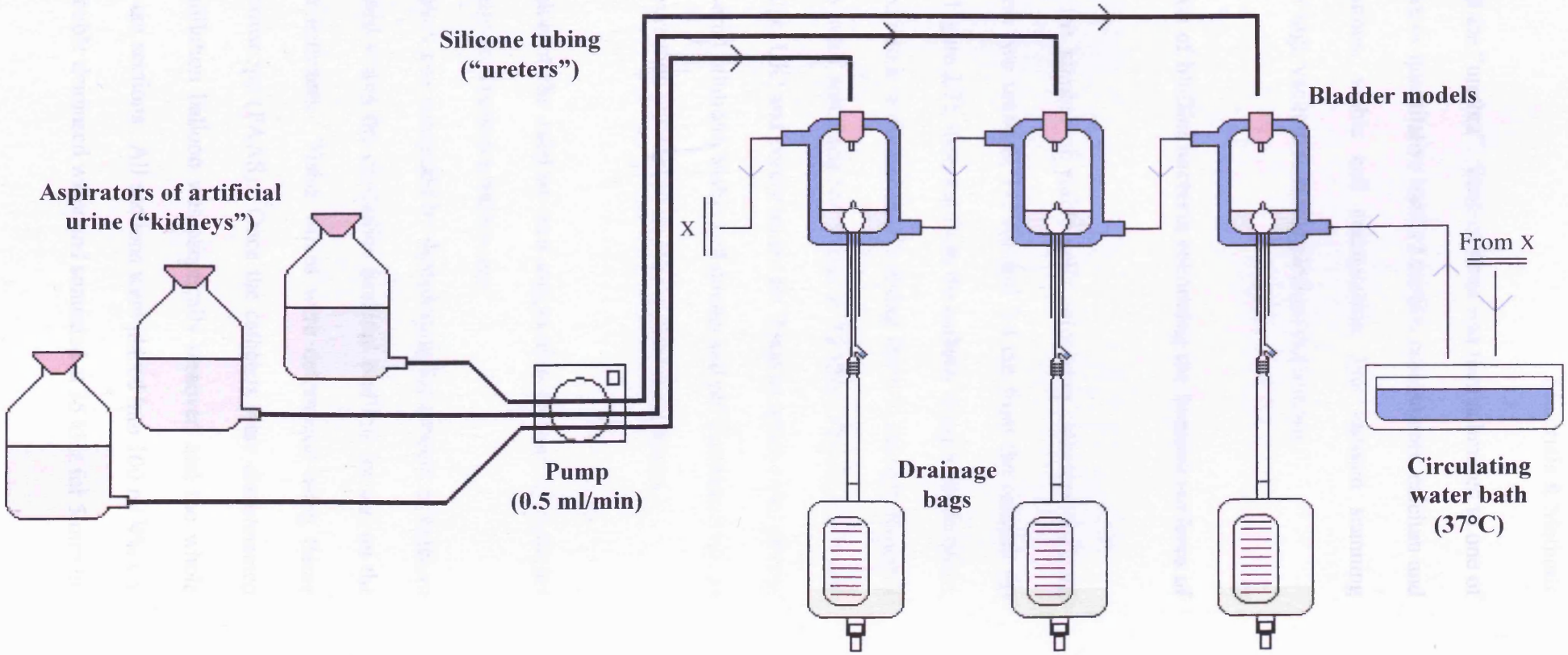
Sets of models were assembled as shown in Figure 2.6. The morning of each experiment test organisms were inoculated into flasks of artificial urine (100 ml). The flasks were placed within an orbital shaking incubator (Sanyo-Gallenkamp Plc., Loughborough, UK) that maintained gentle aeration (100 rpm) at 37°C for a 4 h incubation period. The young log-phase cultures were then inoculated (1 or 10 ml) into the residual urine contained within the bladder models. The viable cell count and pH of the inoculating culture were determined. The models were left for 1 h, without urine input, to allow the organisms to establish before the flow of urine was resumed at 0.5 ml/min.

Urine was supplied for a specific time period, usually either 24 h, 72 h or until catheter blockage. Each catheter blockage was logged and time to blockage was calculated by dividing the volume of urine in the drainage bag by the flow rate (0.5 ml/min), taking into account any emptying of the drainage bag throughout the experimental period.

On completion of the experiments the residual urine in the bladder chamber was removed and pipetted into concentrated sodium hypochlorite (NaOCl) (Newhall Janitorial, Cardiff, UK). The catheters were carefully loosened from the base of the chamber, the retention balloon deflated and removed from the models via

Figure 2.6 - Schematic representation of the catheterised bladder model set-up

Once the catheters had been inserted the models were connected together via a circulating water jacket. The catheters were attached to drainage bags situated on stands below the bench and sterile artificial urine was supplied from the "kidneys" by a peristaltic pump (0.5 ml/min). As urine was delivered to the models the overflow drained through the catheter eye-hole, via the lumen, into the drainage bag.



extraction back through the "urethra". Each catheter was then submitted to one of the following qualitative or quantitative luminal biofilm assessments; calcium and magnesium determinations, viable cell enumeration, low vacuum scanning electron microscopy, or high vacuum scanning electron microscopy.

2.2.4 Enumeration of biofilm bacteria colonizing the luminal surfaces of catheters

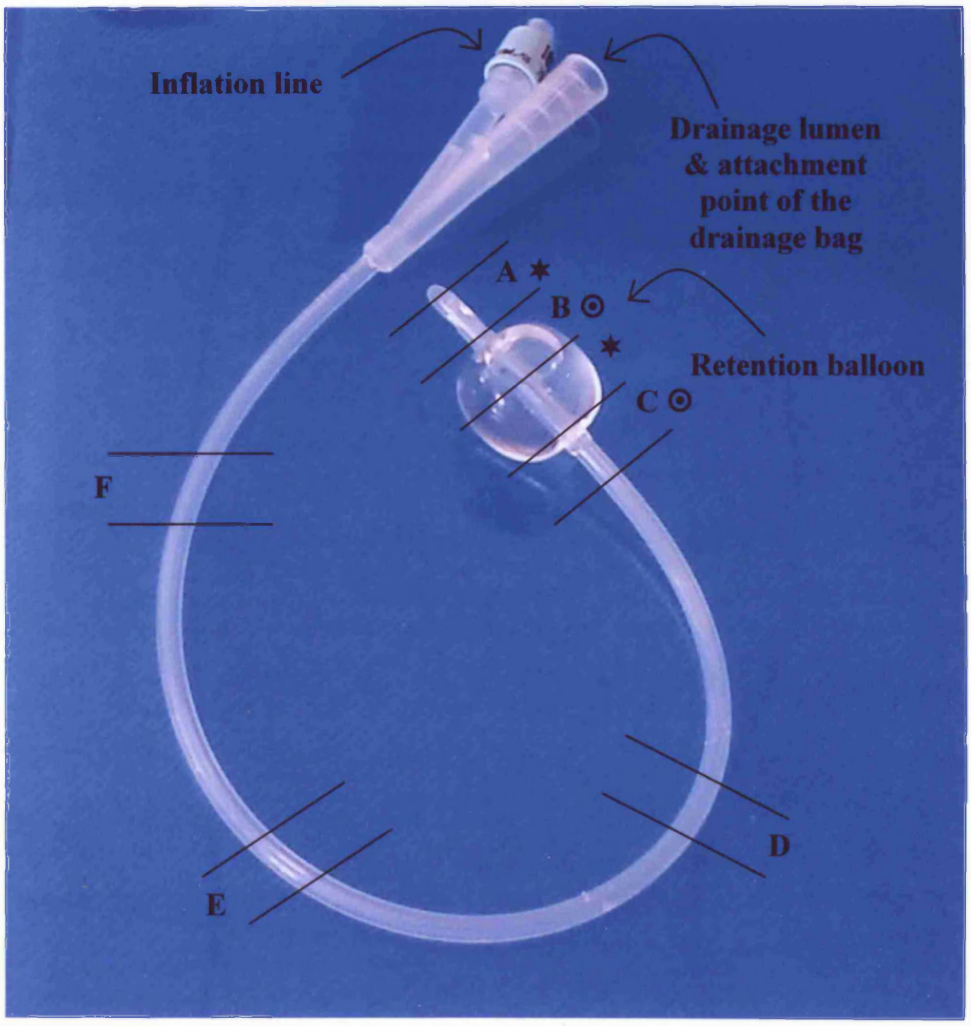
In order to determine the number of viable cells colonizing catheters removed from the *in vitro* models two sections, 1-2 cm and 3-4 cm from the catheter tip (sections marked ⊙ in Figure 2.7), were cut from the catheter using a sterile blade. Each section was placed into a sterile universal holding 10 ml ¼ strength Ringer's solution. The sections were sonicated for 5 min at 35 kHz (Transsonic Water Bath, Camlab, Cambridge, UK) and vortex mixed for 2 min to remove and disrupt any luminal biofilm. Serial dilutions, viable cell counts and pH measurements, as described previously, were then undertaken on the resultant suspensions.

2.2.5 Determination of the calcium and magnesium content of catheter biofilms by flame atomic absorption spectroscopy

The degree of encrustation was measured by determining the amount of calcium and magnesium deposited within the crystalline bacterial biofilms formed on the luminal surface of the catheters. These values were determined using flame atomic absorption spectroscopy (FAAS). Once the catheters were disconnected from the models the inflation balloon was aseptically removed and the whole catheter was cut into 1 cm sections. All sections were placed into 100 ml 4% v/v nitric acid (HNO_3) in double deionised water and sonicated at 35 kHz for 5 min to

Figure 2.7 – Position of the catheter sections used for electron microscopy and biofilm viable cell counts

The catheter shown below is a 14 Ch all-silicone catheter with the retention balloon inflated by injection of 10 ml sterile water. The sections marked ⊙ were used for biofilm viable cell counts whilst those denoted ✶ were used for high vacuum SEM. On separate occasions the six lettered 1 cm sections represent the segments used for low vacuum SEM.



A = 0-1 cm D = 9-10 cm

B = 1-2 cm E = 19-20 cm

C = 3-4 cm F = 29-30 cm

aid biofilm removal and disruption. Samples were stood for at least 48 h to allow the crystals to dissolve and kept at 4°C until analysed.

FAAS was undertaken on a SpectrAA-100 Spectrophotometer (Varian Inc., Oxford, UK) calibrated with 1000 ppm Spectrosol® standards obtained from VWR International (Poole, UK). These standards were diluted with 1% HNO_3 to provide ranges of calcium from 0.2-3.0 parts per million (ppm) and magnesium from 0.05-0.4 ppm. Dilutions (1:5, 1:10, 1:50, 1:100, 1:200, 1:400, 1:500) of each sample were prepared using 4% HNO_3 in order to obtain concentrations of each metal ion that would fall within the range of the two standard curves. Samples were aspirated into the flame (air-acetylene for magnesium and nitrous oxide-acetylene for calcium) and the concentrations of calcium, at 422.7 nm, and magnesium, at 285.2 nm, were measured in ppm.

To obtain the concentrations of calcium and magnesium in solution (μg/ml) per catheter a simple calculation was applied:

concentration (ppm) x dilution factor x 100

To be able to relate calcium and magnesium deposition from catheters run for varying lengths of time the concentrations per catheter were subsequently divided by the length of time each catheter was draining urine *in situ*. This further calculation demonstrated the average rate at which calcium and magnesium was deposited within the bacterial biofilm.

2.2.6 Electron microscopy methods

2.2.6.1 Low vacuum scanning electron microscopy

Low vacuum scanning microscopy (LV-SEM) was used to visualise the extent of crystalline biofilm around the catheter eye-hole and the gradient of encrustation

along the luminal channel. Catheters that were to be examined by LV-SEM were removed from the bladder models, taking care not to disrupt the bacterial biofilm. Each catheter was cut into six 1 cm sections (sections A-F in Figure 2.7) using a sterile blade. Adhesive carbon disks were used to mount the samples onto aluminium stubs. Sections B-F were mounted vertically to expose the lumen and the eye-hole section (section A) was laid on its side to expose the eye-hole. These samples needed no prior preparation and were viewed at 25 kv using the low vacuum setting of a JEOL 5200 scanning electron microscope (JEOL Technics Ltd., Tokyo, Japan). The resulting micrographs recorded the profile of encrustation from the catheter eyehole through to the distal end of the lumen.

2.2.6.2 Hexaammineruthenium trichloride fixation protocol and high vacuum scanning electron microscopy

All of the reagents used for the fixation of samples for high vacuum scanning electron microscopy (HV-SEM) were purchased from Agar Scientific Ltd., (Stansted, UK) except the hexaammineruthenium trichloride (HexRT) ($Ru(NH_3)_6Cl_3$) which was acquired from Johnson Matthey Materials Technology Ltd., (Hertfordshire, UK) In order to show detailed structures of hydrated specimens in a scanning electron microscope prior drying is required. Air drying causes damage to the specimen due to the surface tensions that arise, so critical point drying after chemical fixation and alcohol dehydration was used.

After careful removal from the bladder models 1 cm sections (sections marked ✱ in Figure 2.7) comprising the eye-hole segment (0-1 cm from the catheter tip) and the section 2-3 cm from the tip were cut from the catheters. Both sections were

sliced longitudinally to expose the luminal surface taking care not to compress the lumen and disturb the biofilm.

Primary chemical fixation of the isolated catheter pieces was accomplished by immersing the sections in fixative (2.5% glutaraldehyde in 0.05 M sodium cacodylate ($(CH_3)2AsO_2Na.3H_2O$) buffer plus 0.7% HexRT) made up 1 h before use to allow pH stabilisation. The samples were left overnight at room temperature and the following day the fixative was removed and replaced with fresh 0.05 M sodium cacodylate buffer for 15 min. Post-fixation of the samples was achieved, after buffer removal, by addition of 1% osmium tetroxide (OsO_4) in 0.05 M sodium cacodylate buffer for 1 h at room temperature. This was followed by two 5 min deionised water washes before specimen dehydration in an ascending ethanol solution series (15 min each of a 70% and 90% solution and 2x 15 min of a 100% solution). Sections were then critically point dried using a Balzers CPD 030 critical point dryer (Balzers Ltd., Milton Keynes, UK) with liquid carbon dioxide (CO_2) as the transition medium.

Once dried, samples were attached to aluminium stubs using quick-drying silver paint (silver in methyl isobutyl ketone) and left to set for at least 24 h before being gold sputtered (Edwards S150B Sputter Coater, BOCEdwards, Sussex, UK). Samples were then examined using a Philips XL-20 series scanning electron microscope (Philips Electronics, Eindhoven, Netherlands).

2.2.6.3 Vapour fixation

Vapour fixation was carried out in an enclosed square glass container. The fixative used was an 8% paraformaldehyde and 12.5% glutaraldehyde solution. A

20 ml volume was dispensed into the base of the container. Tape was used to suspend a Petri dish (devoid of its lid) in close proximity to, but above the level of the fixative in the base. The dish was used to hold the samples to be fixed. The container was sealed using Nescofilm (Azwell Inc., Osaka, Japan) to prevent evaporation of the fixative. Samples were left to fix for 24 h before drying in a dessicator (24 h). Once dry, the samples were carefully attached to aluminium stubs using quick-drying silver paint, left to set for at least 24 h and then gold sputtered. Samples were then viewed in a Philips XL-20 series scanning electron microscope.

2.2.7 Coaggregation methods

2.2.7.1 Visual coaggregation assays

Visual coaggregation of test organisms was examined using a modification of the method described by Cisar *et al.*, (1979). Two known coaggregators, the oral strains, *Actinomyces naeslundii* ATCC 12104 and *Streptococcus sanguis* GW2 (Dr. Pauline Handley, University of Manchester, personal communication) were used as positive controls throughout the assays.

Fresh plates of *A. naeslundii* ATCC 12104 and *Strep. sanguis* GW2 were used to inoculate separate flasks of BHI broth (100 ml). These were placed within an anaerobic GasPak™ Jar (Becton Dickinson) containing a 0.5% palladium catalyst, a BBL™ dry anaerobic indicator strip (Becton Dickinson), along with a BBL™ GasPak™ disposable anaerobic hydrogen and carbon dioxide generator envelope (Becton Dickinson) activated by addition of water (10 ml). The jars were statically incubated overnight at 37°C. Urinary strains were inoculated into

separate flasks of TSB (100 ml) and incubated overnight at 37°C with gentle aeration. All cells were harvested by centrifugation at 5000 x *g* for 10 min at 4°C (Avanti® J-E Centrifuge, Beckman Coulter UK Ltd., High Wycombe, UK) and washed three times in re-suspension medium. Oral strains were washed in coaggregation buffer in the initial experiments. In later experiments both the oral and urinary isolates were washed in artificial urine. Each strain was adjusted to an optical density of 1.5 at 650 nm using a Heλios γ UV-Visible Spectrometer (Unicam, Cambridge, UK). Viable cell counts were performed in order to ensure standardisation of cell density between samples and the pH of the suspensions were also measured.

Assays were set-up in sterilised test tubes: control single species assays (to monitor autoaggregation) consisted of 2 ml inocula and 2 ml re-suspension medium; dual mixed species combinations (to monitor coaggregation) consisted of 2 ml *Pr. mirabilis* HI4320 *wt/ure*$^-$ and 2 ml of the "test" organism (or 2 ml *Strep. sanguis* GW2 and 2 ml *A. naeslundii* ATCC 12104 in the positive coaggregation control); and a re-suspension medium (buffer or urine) negative control (4 ml). After a short period of mixing (10 s vortex and 20 s gentle roll between the palms of the hands) one of the semi-quantitative scores (Table 2.2) was subjectively assigned to each suspension after visually assessing the degree of coaggregation. Any auto-aggregation was scored using the same criteria and the score deducted from the coaggregation score. The scoring was completed every hour from the time of first mixing (t = 0) for 6 h and then once more at 24 h. At both 6 h and 24 h the pH was also measured. Between mixings the test tubes were statically incubated at 37°C.

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

Table 2.2 – Visual coaggregation scoring system

Preliminary experiments showed considerable coaggregation score variability between replicates at 24 h. Aggregation was no longer apparent in some cases and in others turbidity became visible for the first time. For this reason it was decided that the scores at 24 h would no longer be measured.

2.2.7.2 Filtration and high vacuum scanning electron microscopy of suspensions demonstrating auto- and coaggregation

The assays were performed as explained previously but were stopped at t = 4 (4 h after first mixing and scoring, when previous investigations had demonstrated all those combinations that auto- or coaggregated had done so by this time). Aliquots (1 ml) of those samples that displayed any auto- or coaggregation were filtered through 13 mm diameter, Nucleopore® polycarbonate membranes with a 10 μm pore size (Whatman®, Maidstone, UK, bought from Agar Scientific). The membranes were then submitted to vapour fixation, as described in section 2.2.6.3, to permit visualisation of any cell-cell or cell-crystal aggregates.

2.2.7.3 Viable cell counts of bacterial-crystal aggregates

In a separate set of experiments coaggregation assays were again stopped at t = 4. Aliquots (1 ml) were filtered through 13 mm diameter, Nucleopore® polycarbonate membranes with 10 μm pores. The membranes were then transferred to artificial urine (10 ml) and stood until the aggregates had dissolved. Viable cell counts of the suspensions were then determined.

2.2.8 Bacteriological analysis of urine samples obtained from patients with long-term catheters

The six week study monitored the weekly bacteriology of the urine of five long-term catheterised patients resident within two nursing homes in the Southmead area of Bristol, UK. The patients' catheters were clamped for 20 min to allow urine to pool at the re-sealing port. The first 2 ml of urine was discarded and the following volume that collected was aspirated into sterile universal containers. The specimens were labelled with the patient code and refrigerated (4°C) until collection and analysis. On return to the lab they were immediately cultured onto CLED agar, Chromogenic UTI medium and TSA, and the pH was measured. Each colony type was sub-cultured onto CLED agar. Once pure cultures were obtained identification of each bacterial isolate was performed as described in section 2.1.2.

2.2.9 Nucleation pH determination of urine

The methods used were based on that described by Choong *et al.*, (1999).

2.2.9.1 Optical density method

The pH of freshly voided urine samples were measured to determine the pH_v. The samples were then transferred to a water bath at 37°C for the remainder of the

experiment. Hydrochloric acid (8%) was used to decrease the pH to 6 and this was followed by a pH increase, up to pH 10, using 3 M NaOH. At every 0.2 increment the optical density was measured at 600 nm using a sample of the urine at pH 6 as the blank.

2.2.9.2 Flame atomic absorption spectroscopy method

Aliquots (2 ml) of urine were also removed at each pH increment to determine the concentration of calcium and magnesium in solution. To remove any crystalline or amorphous matter centrifugation of the samples was carried out at 3500 rpm for 3 min using a bench top centrifuge (Genofuge 16 M, Techne). The resultant supernatant was then diluted in 4% v/v nitric acid and submitted to FAAS as described in section 2.2.5.

The nucleation pH (pH_n), the pH at which calcium and magnesium phosphates began to precipitate from solution, was then determined from both the optical density data (pH_n) and the FAAS data (pH_n^{Ca}, pH_n^{Mg}). Least squares regression analysis as used by Choong *et al.*, (1999) was performed on each data line once plotted against pH. The intersection of the regression lines from the two straight-line elements of the data was considered to be the nucleation pH. An example of this method is given in Appendix A. Preliminary tests showed that the pH_n values generated from the simple, inexpensive optical density method approximated to those determined using the atomic absorption spectroscopy method. The mean pH_n values obtained from both methods were not significantly different ($P > 0.05$). Thus the optical density method was adopted as the method of choice for future pH_n determinations.

2.2.10 Determination of biofilm endotoxin using the Endotoxin Kinetic-QCL® assay

The Kinetic-QCL® test is a quantitative, chromogenic, Gram-negative endotoxin assay used to determine the amount of endotoxin present in an aqueous specimen. The sample to be tested is mixed with a co-lyophilised mixture of *Limulus* Amoebocyte Lysate (LAL) and a synthetic chromogenic substrate, incubated within a Kinetic-QCL® plate reader, and automatically examined for the production of a yellow colour, due to released p-Nitroaniline (pNa), over time. The "reaction time", the time taken for the colour to develop, is inversely proportional to the amount of endotoxin present. The endotoxin concentration of the test samples can then be calculated from a standard curve.

All reagents and equipment for the test was purchased from Cambrex Bio Science Inc., (Wokingham, UK) and throughout the assay Eppendorf BioPur® pipette tips, containing < 0.001 endotoxin units (EU)/ml, were used.

The lyophilised *E. coli* 055:B5 endotoxin, used to produce the standard curve, was reconstituted with the exact volume of pyrogen-free LAL reagent water (containing < 0.005 EU/ml) stated on the Certificate of Quality to yield a solution containing 50 EU/ml. The solution was vortexed mixed for 15 min and once hydrated was stored at 2-8°C for up to one month. Subsequent use of this stock required warming to room temperature and a 5 min vortex to ensure all endotoxin was in suspension. The reconstituted *E. coli* endotoxin stock was used to create a series of five dilutions spanning the range of 0.005-50 EU/ml. The dilutions were

prepared in endotoxin-free glass tubes (containing < 0.005 EU/ml) using 0.9 ml LAL reagent water.

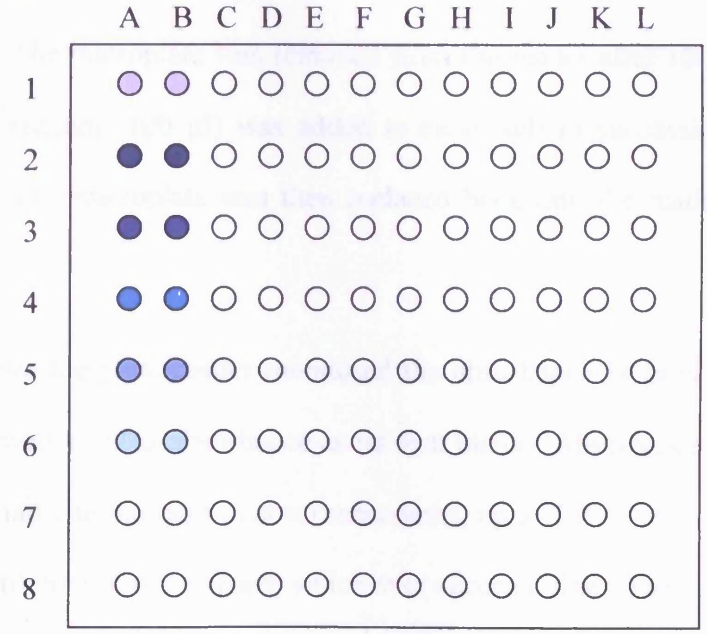
To check that un-used catheters were not sources of endotoxin, the tip section (top 4 cm) were cut from all-silicone, hydrogel-coated latex and silicone-coated latex catheters using sterile disposable scalpels (Swann-Morton, Sheffield, UK). These were further cut into 1 cm sections and suspended in 10 ml of sterile double-deionised water (SDDW) in sterile universal containers. The sections were then subjected to sonication for 5 min and vigorous agitation on a vortex mixer for 2 min. An all-silicone catheter from a laboratory bladder model infected with *Pr. mirabilis* was removed at catheter blockage and three catheters removed from long-term catheterised individuals were processed in the same way. Each 1 cm section was visually assessed for luminal encrustation and an aliquot of biofilm suspension was removed for bacteriological analysis.

Extracts of cultures, catheter biofilms, and other samples were centrifuged for 5 min at 3500 rpm to sediment any particulate matter. The supernatant, containing any free, active lipopolysaccharide, was decanted off. It was stored at 4°C if it was to be assayed within 48 h and frozen at -24°C if analysed after this time period. To allow the amount of endotoxin to fall within the range of the standards dilutions of the supernatants, usually 1:10-1:100,000, were prepared using LAL water as the diluent. Those samples that had an endotoxin level below that of the lowest standard were considered to be apyrogenic.

As illustrated in Figure 2.8, wells A1 and B1 of the 96-well, endotoxin-free microtitre plate were left empty as negative controls and duplicate aliquots (100

Figure 2.8 – The Kinetic-QCL™ assay microplate layout

Wells A1 and B1 were left empty to act as endotoxin negative controls. Wells A2 and B2, A3 and B3, A4 and B4, A5 and B5, A6 and B6 were filled the 50, 5, 0.5 0.05 and 0.005 EU/ml standards respectively. Test samples were then added to subsequent pairs of wells starting at A7 and B7 until all samples were distributed.



Wells	Endotoxin standard (EU/ml)
	0
	50
	5
	0.5
	0.05
	0.005

µl) of the endotoxin standards and test sample dilutions were dispensed into the remaining wells. Once the wells were filled with the various samples the plate was incubated at 37°C in the Kinetic-QCL reader for 10 min. Near the end of the pre-incubation period the co-lyophilised LAL/chromogenic substrate reagent was reconstituted in 2.6 ml LAL water and swirled thoroughly but gently to avoid foaming. The microplate was removed from the reader after 10 min had elapsed and LAL reagent (100 µl) was added to each well in succession as quickly as possible. The microplate was then replaced back into the reader and the assay initiated.

Every 2 min the plate reader monitored the absorbance of each well at 405 nm using the well's initial absorbance as its own blank. Absorbance was continually measured until an increase in absorbance units, up to 0.200, was reached by every well or until 90 min has elapsed, which ever occurred first. The time taken for this to arise was the "reaction time". The WinKQCL® LAL Endotoxin Detection software, by design, computes a log/log correlation of the reaction time of each of the five standards with its endotoxin concentration and produces a standard curve that should have a correlation coefficient in the range of -0.980 to -1.000. The endotoxin concentration of each of the test samples was determined, using the reaction times, from the standard curve and the ultimate result was adjusted to account for any sample dilution.

Inhibition can occur if material in the sample interferes with the assay. The consequence of this is that the reaction time is increased and a smaller amount of endotoxin than is actually present is measured. To ensure inhibition was not occurring in test samples a Positive Product Control (PPC) of a known endotoxin

concentration was used to "spike" the neat samples before they were assayed. The spike, 10 µl of the 0.5 EU/ml standard, resulting in a final concentration of 0.05 EU/ml once added to the appropriate wells before the pre-incubation stage. A spike was not added to any of the sample dilutions, any inhibition would be attenuated at these low concentrations. Spike recovery should be within ± 50% of the known concentration. If the level of recovery fell within this boundary the constituents of the sample were not affecting the amount of endotoxin measured.

2.2.11 Statistical analysis of experimental data

Unless otherwise stated all experiments were performed in triplicate and the mean value along with the standard error (SE) of the mean, if appropriate, is expressed. Minitab® statistical software release 13/14 (Minitab Inc, Pennsylvania, USA) was the computer package that was used for all statistical analyses. One-way analysis of variance (ANOVA) at the 95% level of significance and the associated Tukey-Kramer pair-wise comparison of means test, if appropriate, were the preferred statistical tests for all experiments. In order for the ANOVA results to be accepted a number of assumptions needed to be satisfied. If the data failed to comply with the normal distribution of residuals and did not have equal variances transformation was attempted. If transformation was unsuccessful in meeting these requirements then the non-parametric Kruskal-Wallis test was performed at the 95% significance level. For all statistical tests, a P value ≤ 0.05 was considered significant, a P value ≤ 0.010 was considered highly significant and a P value of ≤ 0.001 was considered very highly significant. A P value > 0.05 indicated no significant difference.

SECTION 3

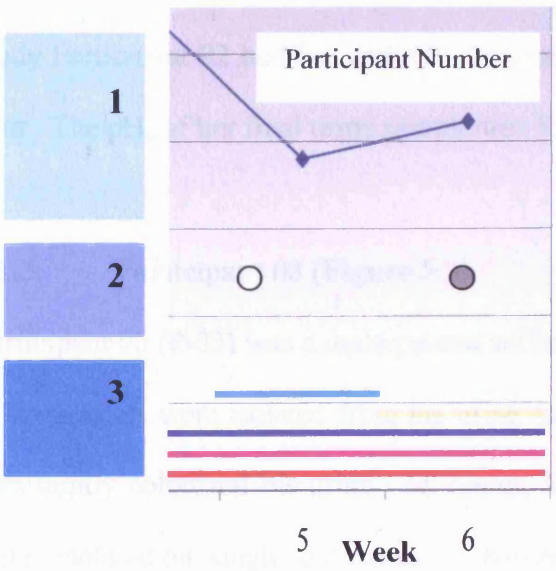
Results

3.1 Clinical study of long-term catheterised patients.

A small-scale clinical study was carried out over a six-week period on five long-term urethral catheterised patients resident in two nursing homes in the Southmead area of Bristol, UK. The purpose of this preliminary study was to gain an insight into the complexity of the urinary flora of catheterised patients. In addition, it produced further clinical isolates for the laboratory collection.

On a weekly basis urine samples were collected from the catheter sampling ports. The voided pH (pH_v) of each urine sample was measured and the bacterial flora isolated and identified. The final urine sample from each patient was also used to determine the nucleation pH (pH_n) of the urine at that time. Examples of pH_n determinations using optical density data are presented in Appendix A. Instances of catheter change were also recorded.

A summary of the results from the five patients are presented in Figures 3.1-3.5. A key is provided below each figure. Each figure is composed of 3 sections:



Section 1 shows the pH_v measurements as measured on the y-axis

Section 2 if present, has circles indicating catheter changes during the study. Catheters are designated as being changed due to blockage (grey-filled) or a scheduled routine change (un-filled).

Section 3 contains coloured lines representing the bacterial species isolated from the urine each week.

3.1.1 Participant 01 (Figure 3.1)

Participant 01 (P-01) was a female spinal cord injured patient. Over the six weeks four different species were isolated from her urine. *Kl. pneumoniae* and *Citrobacter koseri* were recovered throughout the entire study period. *E. coli* was present at the beginning of the study but disappeared from week four onwards. *Micrococcus luteus* was recovered from the urine in weeks three and four. The pH_v of the urine was consistently acidic ranging between 5.23 and 6.36. There were no instances of catheter blockage. No catheter changes, routine or otherwise, occurred during the six weeks. The urinary pH_n at week six was 6.67 compared to the pH_v of 5.23.

3.1.2 Participant 02 (Figure 3.2)

Participant 02 (P-02) was a female bedridden, spinal cord injured patient. The urine was consistently colonized by a three member community comprising of *Pr. mirabilis*, *Ps. aeruginosa* and *Ent. faecalis*. *M. luteus*, *Proteus vulgaris* and *Gardnerella vaginalis* were isolated from single samples. The pH_v of the urine was highly alkaline (mean 8.72 ± 0.11) throughout the six weeks. During the study Participant 02 had her catheter changed due to blockage in weeks two and four. The pH_n of her final urine sample was 8.13.

3.1.3 Participant 03 (Figure 3.3)

Participant 03 (P-03) was a male patient suffering from multiple sclerosis. A total of six species were isolated from his urine samples. *Kl. pneumoniae* and *E. coli* persistently colonized his urine. *M. luteus*, *Micrococcus lylae*, and *Ent. facaelis* were isolated on single occasions. *C. koseri* was isolated intermittently. The

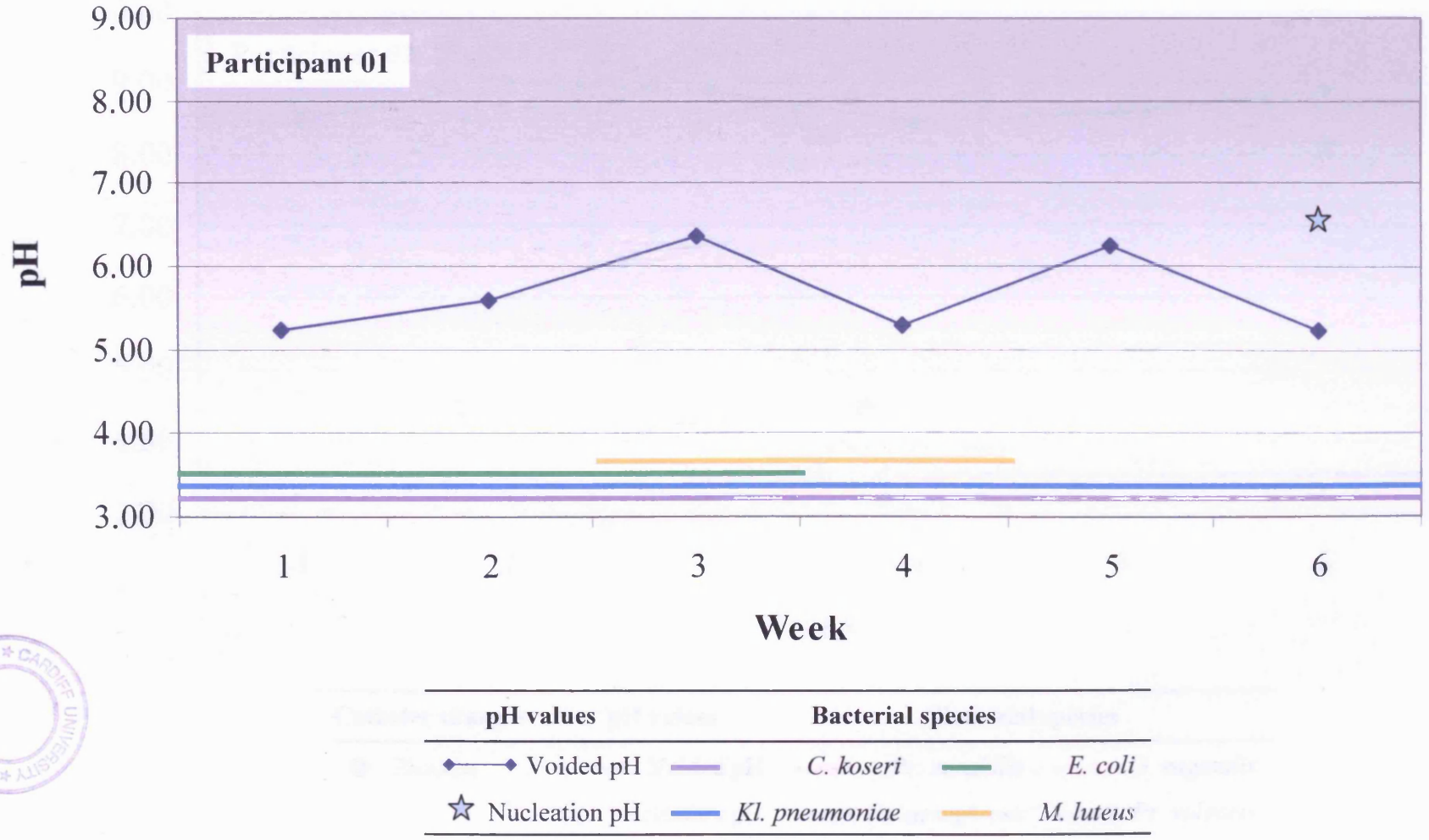


Figure 3.1 The voided urinary pH and urinary flora over the six-week study period for Participant 01

The nucleation pH of sample six was determined to be 6.67.

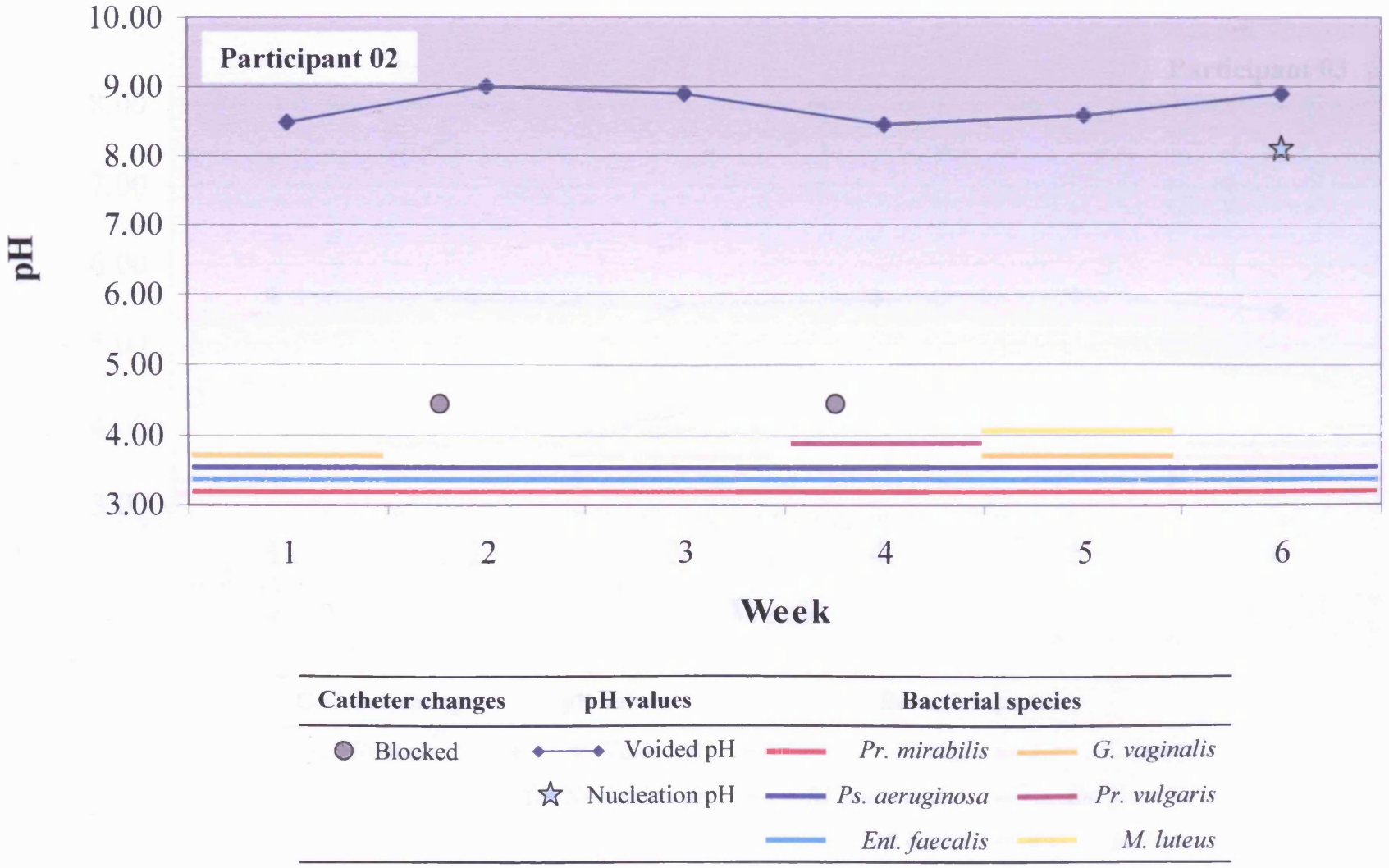


Figure 3.2 The voided urinary pH, urinary flora and catheter changes over the six-week study period for Participant 02

The nucleation pH of sample six was determined to be 8.13.

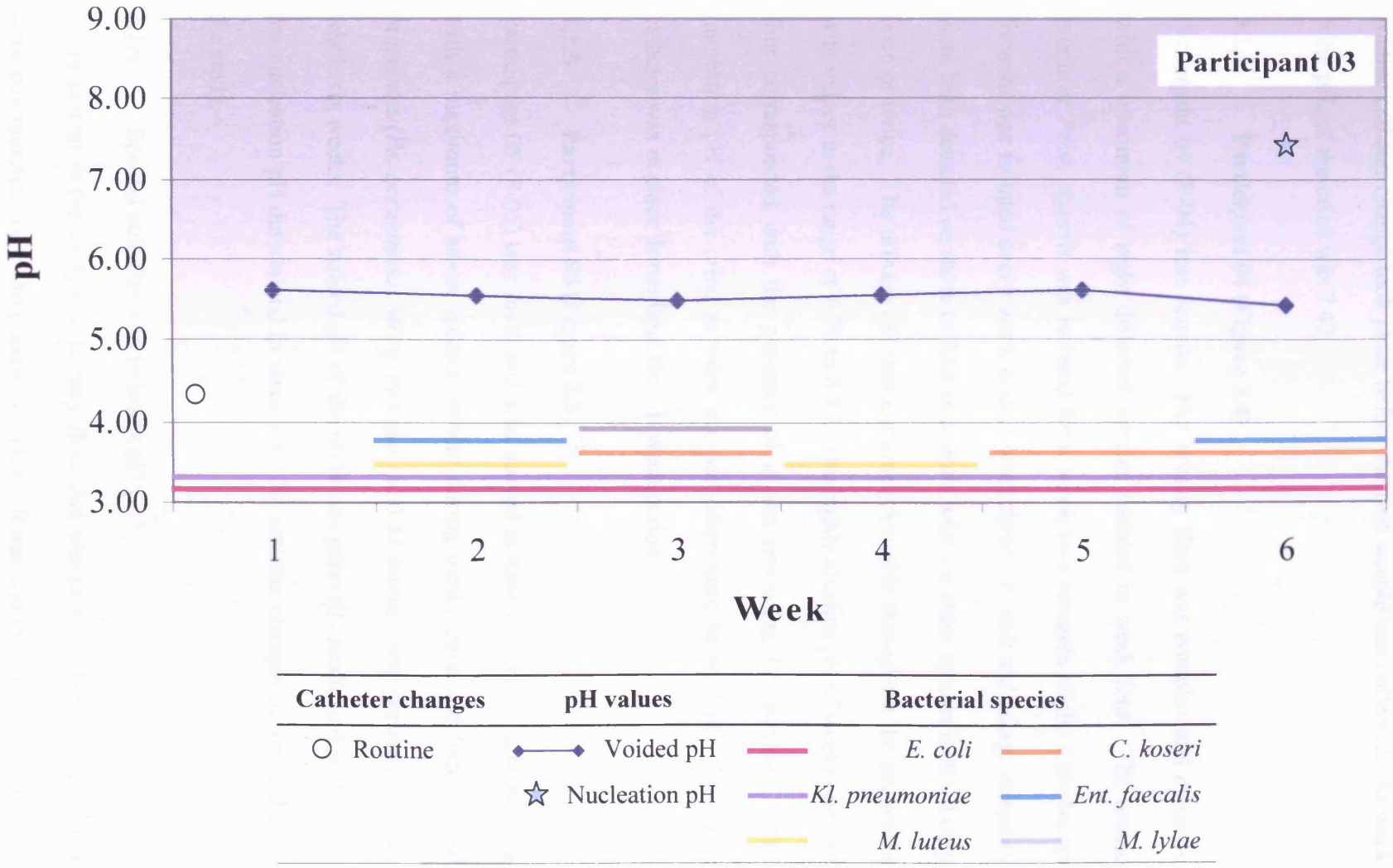


Figure 3.3 The voided urinary pH, urinary flora and catheter changes over the six-week study period for Participant 03

The nucleation pH of sample six was determined to be 7.42.

urine samples were consistently acidic with a mean pH of 5.54 ± 0.03. One routine catheter change took place before the first sample was collected. At week 6 the pH_n of the urine was 7.42.

3.1.4 Participant 04 (Figure 3.4)

Participant 04 (P-04) was female. Her urinary flora was complex and dynamic with a maximum of eight different species isolated in week four. The urease producer *Prov. stuartii* was isolated from week two onwards whilst *Citrobacter freundii* was isolated every week except week three. *E. coli* and *Morg. morganii* were both detected on three or four occasions with the other species isolated only once or twice. The urinary pH was extremely variable throughout the six weeks with values in the range of 6.70 to 8.35. The highly alkaline pH of weeks one and four corresponded with the presence of urease producing *Pr. mirabilis*. The nucleation pH of the urine at week six was determined to be 7.48. The same catheter was in place throughout the six-week period.

3.1.5 Participant 05 (Figure 3.5)

Participant 05 (P-05) was male and also proved to have a complex urinary flora with a maximum of seven species present during weeks three and four. Three organisms (*Ps. aeruginosa*, *Morg. morganii* and *M. luteus*) were persistent for the whole six weeks. The voided pH of the urine was generally acidic and well below the nucleation pH determined in week six. No catheter changes occurred during the study.

3.1.6 Species stability and urinary pH

Every patient in the study had urinary flora that was multi-species in nature and some communities were particularly complex. It was common to have a mean of

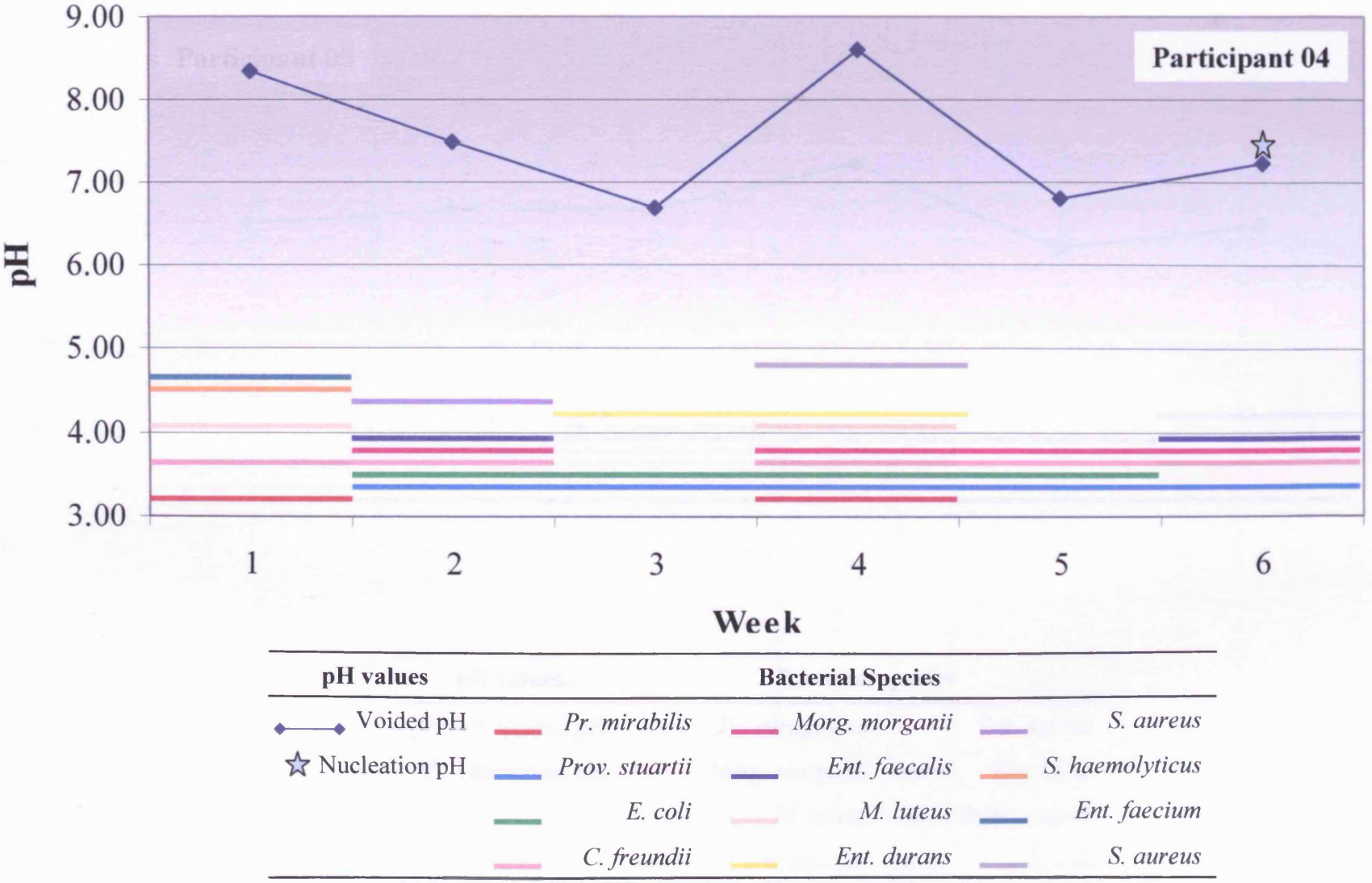


Figure 3.4 The voided urinary pH and urinary flora over the six-week study period for Participant 04

The nucleation pH of sample six was determined to be 7.48.

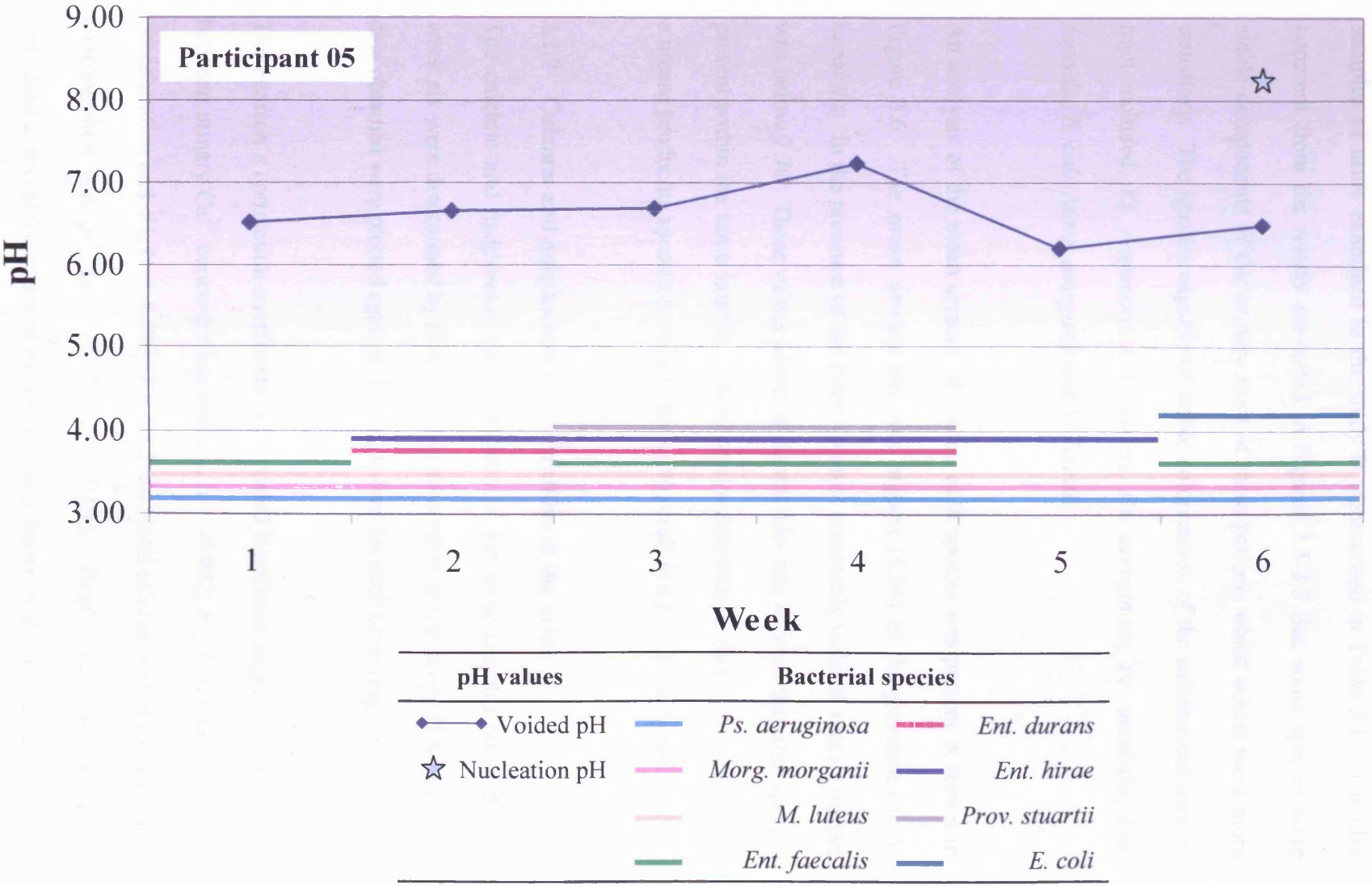


Figure 3.5 The voided urinary pH and urinary flora over the six-week study period for Participant 05

The nucleation pH of sample six was determined to be 8.16.

4 species isolated from weekly samples. The incidence of each species in the 30 samples of urine examined in the study is summarised in Table 3.1. It is also apparent from the results presented in Figures 3.1-3.5 that some species were stable components of the urinary flora of these patients whilst others were more transitory. The species capable of stable colonization of the catheterised urinary tract included; *Kl. pneumoniae*, *C. koseri*, *Ps. aeruginosa*, *Pr. mirabilis*, *Ent. faecalis*, *E. coli*, *Morg. morganii* and *M. luteus*.

An analysis of the mean urinary pH when each species was present is shown in Figure 3.6. The mean urinary pH was highest (8.66) in the presence of *Pr. mirabilis*. In the presence of the other 10 most commonly isolated species the pH was below 7.70. These values however do not take into account the other species present within the same sample. These results demonstrate that an infection with a urease producing species does not inevitably result in a high alkaline pH.

3.1.7 Calcium and magnesium concentrations in the urine

The calcium and magnesium concentrations in the urine samples taken during week six were determined by flame atomic absorption spectroscopy (FAAS). The data obtained were plotted against the pH_n values for each urine (Figures 3.7-3.9).

The Pearson's correlation coefficient (r) revealed significant negative correlations between urinary Ca^{2+} concentration and pH_n ($r = -0.982$; $P \leq 0.01$), urinary Mg^{2+} concentration and pH_n ($r = -0.893$; $P \leq 0.05$) and total calcium and magnesium ion concentration and pH_n ($r = -0.972$; $P \leq 0.01$). Regression equations were generated to reveal the extent of the associations between the ion concentrations

Species	**Incidence (%)**
Ent. faecalis	11.48
E. coli	11.48
M. luteus	10.66
Ps. aeruginosa	9.84
Kl. pneumoniae	9.84
Morg. morganii	8.20
Pr. mirabilis	7.38
C. koseri	7.38
Prov. stuartii	5.74
Ent. durans	4.10
C. freundii	4.10
Ent. hirae	3.28
G. vaginalis	2.46
S. haemolyticus	0.82
S. aureus	0.82
Pr. vulgaris	0.82
M. lylae	0.82
Ent. faecium	0.82

Table 3.1 – Incidence (%) of each species isolated from the urine of five long-term catheterised patients.

A total of 30 urine samples were tested.

Urease positive organisms are highlighted in pink.

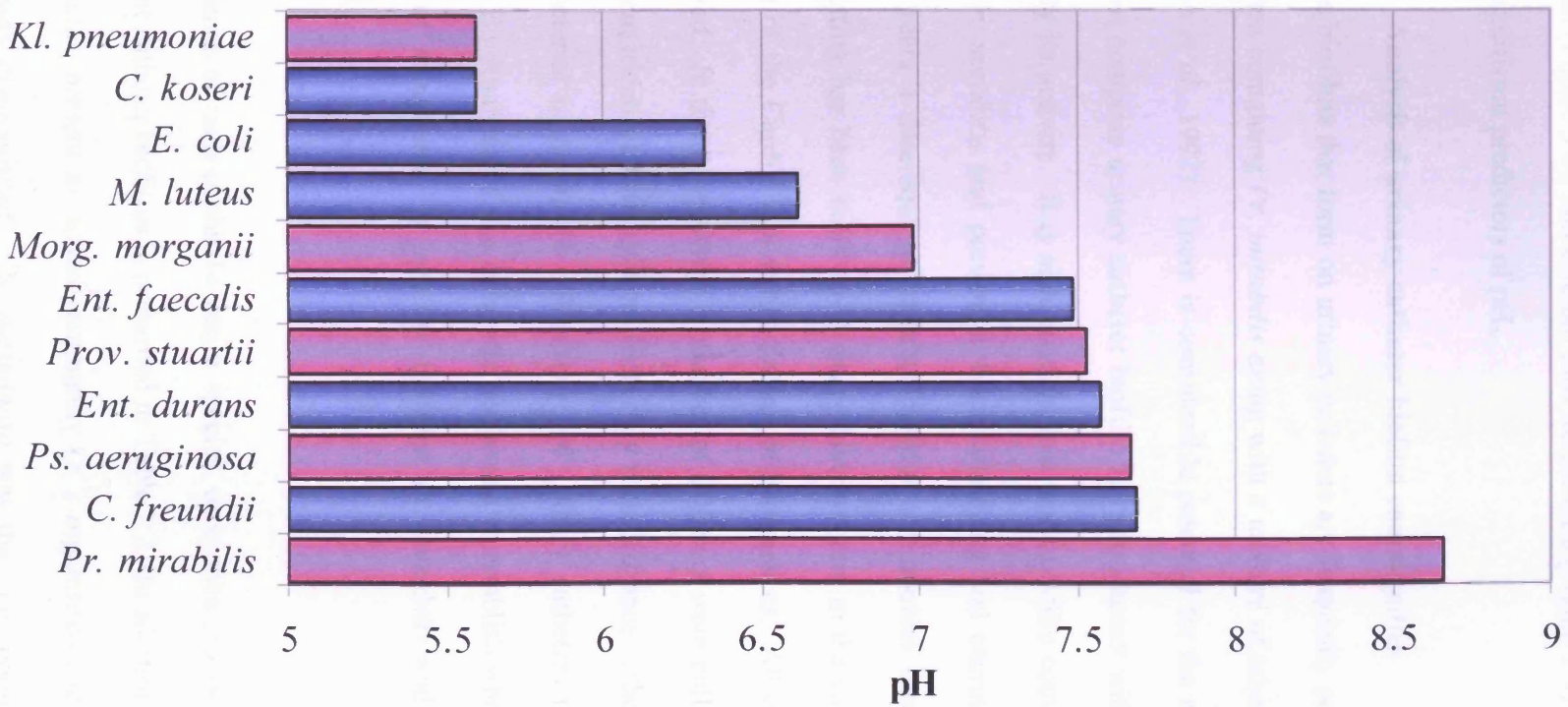


Figure 3.6 – Mean pH of the urine when the 11 most commonly isolated species were present.

and the pH_n. What can be seen from the data is that calcium (Figure 3.7), magnesium (Figure 3.8), and calcium plus magnesium (Figure 3.9) concentrations were all significant predictors of pH_n.

3.2 Analysis of urinary catheter biofilm communities

Crystalline biofilms that form on urinary catheters are frequently polymicrobial in nature, often containing *Pr. mirabilis* along with a mixture of other uropathogens (Ganderton *et al.*, 1992). There is considerable potential for the numerous other species that comprise urinary catheter biofilm flora to interact with *Pr. mirabilis* and modify its activity. It is also possible that some biofilm communities could exclude *Pr. mirabilis* and prevent it from colonizing and encrusting catheters. Over the years a data-base of catheter biofilm communities from the *in vivo*, clinical setting has been built up by successive workers in the catheter research laboratory of the Cardiff University School of Biosciences. All of the raw data was collated. In the cases where a number of catheters were collected from the same patient identical communities were only included once. The resultant data on the bacterial communities colonizing 106 urinary catheters is presented in Appendix B. Analysis of this data was performed to establish whether there was evidence of antagonism or synergy between *Pr. mirabilis* and other biofilm organisms.

The incidence of each of the 14 major species, or species groups, isolated from 106 patient catheter biofilms is presented in Table 3.2. In addition, the incidence of the species present as mixed-community ($\geq$ 2 organisms) and mono-species biofilms was distinguished. *Ps. aeruginosa* was the most commonly isolated organism overall whilst *Ent. faecalis*, *Pr. mirabilis*, and *E. coli* were also

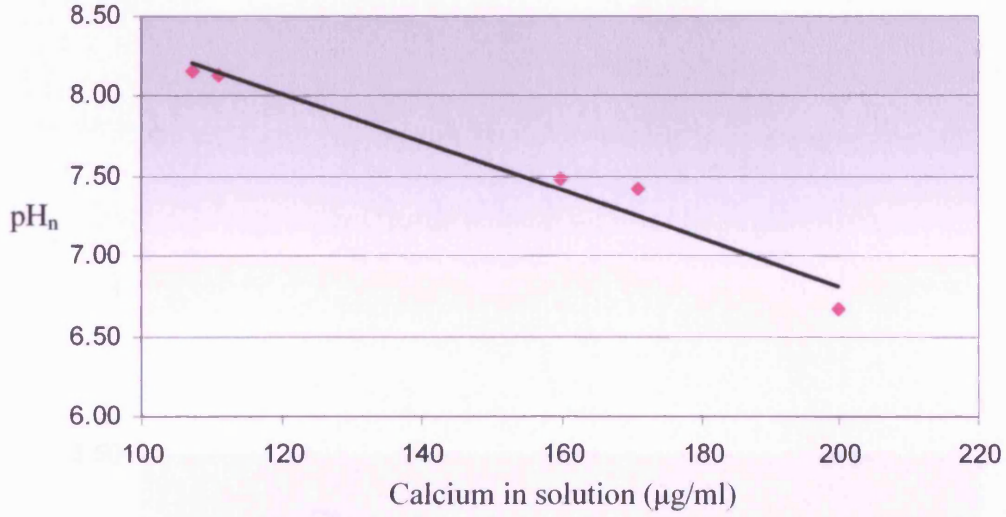


Figure 3.7 – Linear regression to show the relationship between voided urinary [Ca^{2+}] and the nucleation pH of urine from the study participants

Regression equation: $pH_n = 9.83 - 0.0151\ [Ca^{2+}]$

$r^2 = 96.3\%$ $P < 0.01$

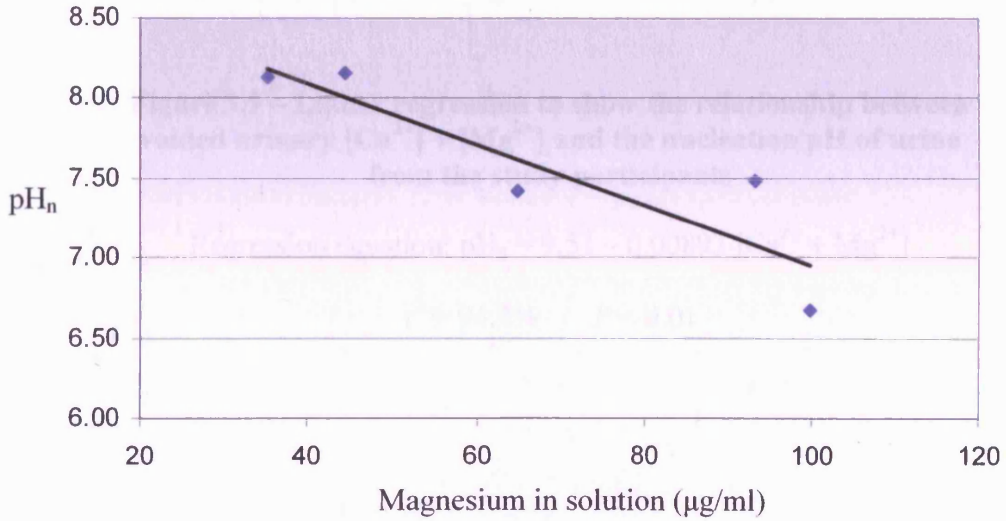


Figure 3.8 – Linear regression to show the relationship between voided urinary [Mg^{2+}] and the nucleation pH of urine from the study participants

Regression equation: $pH_n = 8.87 - 0.0192\ [Mg^{2+}]$

$r^2 = 79.7\%$ $P < 0.05$

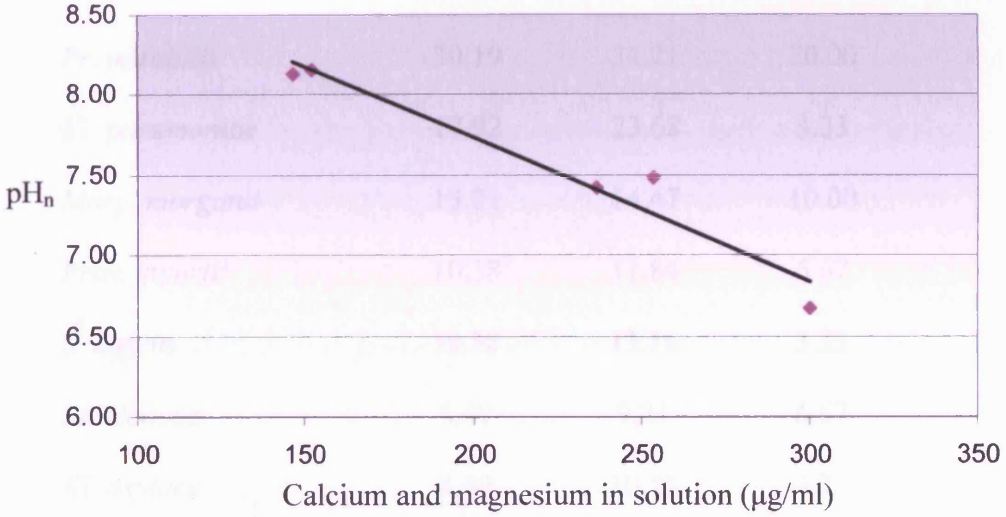


Figure 3.9 – Linear regression to show the relationship between voided urinary $[Ca^{2+}] + [Mg^{2+}]$ and the nucleation pH of urine from the study participants

Regression equation: $pH_n = 9.51 - 0.00893\ [Ca^{2+} + Mg^{2+}]$

$r^2 = 94.4\%$ $P < 0.01$

Species	Incidence (%)		
	All[a] catheter biofilms	**Mixed[b] species biofilms**	**Single[c] species biofilms**
Ps. aeruginosa	35.85	40.79	23.33
Ent. faecalis	33.96	44.74	6.67
E. coli	31.13	40.79	6.67
Pr. mirabilis	30.19	34.21	20.00
Kl. pneumoniae	17.92	23.68	3.33
Morg. morganii	13.21	14.47	10.00
Prov. stuartii	10.38	11.84	6.67
S. aureus	10.38	13.16	3.33
Et. cloacae	8.49	9.21	6.67
Kl. oxytoca	8.49	10.53	3.33
Other *Providencia* spp.	4.72	5.26	3.33
CNS	4.72	5.26	3.33
Citrobacter spp.	3.77	5.26	0.00
Pr. vulgaris	2.83	2.63	3.33

Table 3.2 – The incidence of species isolated from 106 urinary catheter biofilms

[a] 106 cases, [b] mixed species denotes ≥ 2 organisms; 76 cases, [c] 30 cases

CNS Coagulase negative staphylococci

Species isolated on ≤ 2 occasions from all biofilms were not included in the analysis.

frequently isolated. *Ps. aeruginosa* was the sole biofilm isolate on 7 of the 30 single species colonized catheters. Perhaps not unsurprisingly, *Pr. mirabilis* was the next most common organism isolated on six occasions. The alkaline microenvironment that *Pr. mirabilis* generates might well be inhabitable for many other species. The incidence of the other 12 species and species groups ranged from 10% to 0%.

A brief analysis of the co-occurrence of all the main catheter biofilm species was also undertaken. The results presented in Table 3.3 gives an overall view of the number of times pairs of species were isolated from the same biofilm. For example of the 11 catheters that were colonized by *Prov. stuartii* seven of them were also colonized by *Ps. aeruginosa*. In contrast however, *Ps. aeruginosa* was never isolated from a biofilm that had either *Et. cloacae*, coagulase-negative staphylococci, or *Pr. vulgaris* as biofilm members. This table demonstrates the complex nature of urinary catheter biofilms and gives an insight into possible synergisms and antagonisms that may regulate biofilm development.

3.2.1 The incidence of *Pr. mirabilis* in the presence of other common catheter biofilm species

Table 3.4 shows the incidence of catheter colonization by *Pr. mirabilis* in the presence of the 10 most common other catheterised urinary tract species, ranked on the basis of their frequency of co-colonization with *Proteus*. *Pr. mirabilis* was recovered from nearly half of the catheters also colonized by *Prov. stuartii*. It was also often isolated from catheters colonized by *Kl. pneumoniae* or *Ent. faecalis*. In contrast, at the other end of the spectrum *Morg. morganii* was associated with a

Organism	*Ps. aeruginosa*	*Ent. faecalis*	*E. coli*	*Pr. mirabilis*	*Kl. pneum*	*Morg. morganii*	*Prov. stuartii*	*S. aureus*	*Et. cloacae*	*Kl. oxytoca*	Other *Prov*	CNS	*Citro* spp	*Pr. vulgaris*
Ps. aeruginosa	38	14	9	9	8	7	7	4	0	1	1	0	1	0
	Ent. faecalis	36	12	14	8	4	3	4	3	2	0	0	2	0
		E. coli	33	6	5	5	2	2	3	5	0	0	3	1
			Pr. mirabilis	32	8	1	5	3	0	2	1	0	0	0
				Kl. pneum	19	0	1	1	2	0	2	0	0	1
					Morg. morganii	14	1	0	0	0	0	0	0	0
						Prov. stuartii	11	0	0	0	0	0	0	0
							S. aureus	11	2	1	0	0	1	0
								Et. cloacae	9	2	0	1	0	0
									Kl. oxytoca	9	0	1	0	0
										Other *Prov*	5	1	0	1
											CNS	5	0	0
												***Citro* spp.**	4	0
													Pr. vulgaris	3

Table 3.3 – Species co-colonization of 106 urinary catheter biofilms

Blue cells indicate the numbers of catheters colonized by each of the common uropathogens whilst yellow cells indicate the number of catheters also colonized by another specific organism.
Kl. pneum = *Kl. pneumoniae*; *Citro* = *Citrobacter*; *Prov* = *Providencia* spp.

Species present in the catheter biofilm	Number of catheters colonized	Number of catheters also colonized by *Pr. mirabilis*	% Incidence of *Pr. mirabilis*	% change[a] in *Pr. mirabilis* incidence
Prov. stuartii	11	5	45.45	15.26
Kl. pneumoniae	19	8	42.11	11.92
Ent. faecalis	36	14	38.89	8.70
S. aureus	11	3	27.27	-2.92
Ps. aeruginosa	38	9	23.68	-6.51
Kl. oxytoca	9	2	22.22	-7.97
Other *Providencia* spp.	5	1	20.00	-10.19
E. coli	33	6	18.18	-12.01
Morg. morganii	14	1	7.14	-23.05
Et. cloacae	9	0	0.00	-30.19

Table 3.4 – The incidence and percentage change in incidence of *Pr. mirabilis* in 106 urinary catheter biofilms in the presence of the other most common species

76 multi-species (≥2 organisms) and 30 single-species biofilms.

[a] Percentage change was calculated using the overall incidence of *Pr. mirabilis* from the 106 catheter biofilms = 30.19%.

Pr. mirabilis incidence of 7.14%, whilst *Pr. mirabilis* was not recovered from catheters that also had *Enterobacter cloacae* as a biofilm community member.

3.2.2 Chi square analysis

It can be assumed that if "species X" has antagonistic influences towards *Pr. mirabilis* then *Pr. mirabilis* is likely to occur less often than expected when "species X" is present as a biofilm community member. Similarly, if "species X" has synergistic interactions with *Pr. mirabilis* it might well be isolated on more occasions than expected.

The Chi-square Goodness of Fit test was used to determine whether the number of catheters colonized by *Pr. mirabilis* in the presence of each of the other main species deviated significantly from what was expected. To generate the expected numbers it was assumed that *Pr. mirabilis* had no affinity for, or no aversion to, growing with or without any other species. The number of catheters colonized by *Ent. faecalis* for example, was 36. Therefore the expected number of catheters colonized by *Pr. mirabilis* would be 18, whilst the expected number of catheters not colonized by *Pr. mirabilis* would also be 18. The results in Table 3.5 show that four species: *Ps. aeruginosa*, *E. coli*, *Morg. morganii*, and *Et. cloacae* caused a significant deviation in the frequency of *Pr. mirabilis* from what would be expected if this species was equally likely to colonize, or not colonize, a given catheter. The presence of these four species caused a significant decrease (Table 3.4) in the frequency of *Pr. mirabilis* from what was expected.

3.2.3 Odds Ratios

Odds ratios are used as measures of association i.e. to show a statistical relationship between two binary features. Table 3.6 illustrates a common table

Species	Chi square value	*P* value	Significant difference?
Prov. stuartii	0.0909	> 0.05	×
Kl. pneumoniae	0.4737	> 0.05	×
Ent. faecalis	1.778	> 0.05	×
S. aureus	2.273	> 0.05	×
Ps. aeruginosa	10.53	≤ 0.01	✓
Kl. oxytoca	2.778	> 0.05	×
Other *Providencia* spp.	1.800	> 0.05	×
E. coli	13.36	≤ 0.001	✓
Morg. morganii	10.29	≤ 0.01	✓
Et. cloacae	9.000	≤ 0.001	✓

Table 3.5 – Chi square results used to determine if the numbers of *Pr. mirabilis* deviated significantly from expected on catheters also colonized by other common urinary tract species

Critical value of chi squared = 3.841

constructed in order to determine odds ratios for pairs of organisms. Variable A in each case was *Pr. mirabilis*: A^- = *Pr. mirabilis* absence, A^+ = *Pr. mirabilis* presence. Variable B was the chosen partner organism: B^- = partner organism absence, B^+ = partner organism presence. Constants w through to z were the numbers of occasions each organism was isolated in each circumstance from the clinical communities recovered from the 106 catheter biofilms.

	B^+	B^-	Row total
A^+	w	x	w + x
A^-	y	z	y + z
Column total	w + y	x + z	w + x + y + z

Table 3.6 – A generic odds ratio data table

The odds that *Pr. mirabilis* presence (A^+) was associated with the presence of the second organism (B^+) was calculated using equation 1:

Equation 1 w/x

And similarly, the odds that the absence of *Pr. mirabilis* (A^-) was related to the presence of the partner organism (B^+) was determined by equation 2:

Equation 2 y/z

The ratio of the two odds, the odds ratio (OR), was simply:

Equation 3 (w/x) / (y/z)

If the OR = 1 this implied the event was just as likely in both groups. If the OR was > 1 this indicated the event was more likely in the first row and less likely in

the second row, and if OR was < 1 this denoted the event was less likely in the first row and more expected in the second row. The strength of the association increases as the OR increases in divergence from 1. The ratios were used to observe whether *Pr. mirabilis* was more or less likely to occur in conjunction with certain other species or in their absence. The results may therefore provide indications as to pair-wise inhibitions and antagonisms, or potential beneficial associations and synergisms.

The ratios generated from the catheter biofilm species data (Appendix C) can be seen in Table 3.7. Again these results suggest that *Pr. mirabilis* was more likely to be found in conjunction with *Prov. stuartii*, *Kl. pneumoniae* and *Ent. faecalis*, which all have odds ratios > 1. The other seven species had odds ratios of < 1, implying that *Pr. mirabilis* was less likely to be found in combination with them. It is thus possible that these species may actively prevent or exclude *Pr. mirabilis* from catheter biofilms.

These simple approaches provided a rationale for testing a range of organisms for their effects on *Pr. mirabilis* catheter biofilm development in the *in vitro* bladder models. As a result of these analyses five species were chosen to investigate their interactions with *Pr. mirabilis* in laboratory experiments: *Et. cloacae*, *Morg. morganii*, *E. coli*, *Ps. aeruginosa*, and *Kl. pneumoniae*.

3.3 Investigations into the development of mixed community bacterial biofilms on urinary catheters

Previous work investigating the formation and structure of crystalline bacterial biofilms has concentrated on their development by pure cultures of *Pr. mirabilis*

Association with *Pr. mirabilis*	Odds Ratio
Prov. stuartii	2.11
Kl. pneumoniae	1.94
Ent. faecalis	1.86
S. aureus	0.83
Ps. aeruginosa	0.61
Kl. oxytoca	0.70
Other *Providencia* spp.	0.50
E. coli	0.40
Morg. morganii	0.14
Et. cloacae	0.00

Table 3.7 – The odds ratios generated for the associations between *Pr. mirabilis* and the other common urinary catheter biofilm species

in models of the catheterised bladder (Morris *et al.*, 1997; Morris and Stickler 1998a; Winters *et al.*, 1995). While these single species biofilms do form on patients catheters, in long-term catheterised patients the urine and catheters are more commonly contaminated by polymicrobial communities (Clayton *et al.*, 1982; Ganderton *et al.*, 1992; Kohler-Ockmore and Feneley, 1996; Ohkawa *et al.*, 1990; Stickler, 1996; Warren *et al*, 1987). It is also well known that the rate at which catheters block in patients can vary from a few days to many weeks. The basic hypothesis to be tested in this section is that the presence of other species in urine will modulate or perhaps even prevent the development of crystalline *Pr. mirabilis* biofilms. In the initial set of experiments this hypothesis has been tested in experiments in which pairs of organisms (*Pr. mirabilis* and another species of urinary tract pathogen) were simultaneously introduced into the bladder model. In subsequent experiments, species were allowed to establish themselves in the bladder models for 24 or 72 h before *Pr. mirabilis* was introduced.

3.3.1 The simultaneous inoculation of bladder models with pairs of organisms

In these experiments, sets of three models were assembled in parallel. Cultures of the test organisms, grown in artificial urine for 4 h at 37°C, were used as inocula. *Pr. mirabilis* B2 (1 ml) was introduced into the first model, the "test" species (1 ml) was inoculated into the second, and further 1 ml inocula of both species were simultaneously introduced, i.e. co-inoculated, into the third. After 1 h to allow the organisms to establish themselves in the bladder chambers, urine was supplied to the models at 0.5 ml/min. The experiments were run until the two models infected with *Pr. mirabilis* had blocked. The urinary pH and the numbers of viable

cells/ml of each species present in the urine were determined at 0, 1 and every 24 h after that until blockage or stoppage.

These experiments were performed in quadruplicate and in each case the time to catheter blockage was recorded. At the end of the experiments, the urine supply was turned off and the catheters removed from the models. In three of the replicate experiments the extent of catheter encrustation was determined by analysis of the amounts of calcium and magnesium deposited per catheter. The rate of deposition was expressed as the amount of calcium and/or magnesium/catheter/h. Visual assessment of the biofilm formation by LV-SEM was performed on the catheters from the fourth replicate experiment.

3.3.1.1 Biofilm formation in models co-inoculated with *Pr. mirabilis* B2 and *Et. cloacae* RB19

The first species to be tested was *Et. cloacae*. This organism can often be recovered from the urine of catheterised patients (Ganderton *et al.*, 1992; Jepson *et al.*, 1982; Ohkawa *et al.*, 1990; Ramsay *et al.*, 1989). It is not a urease producer and has not been directly implicated in catheter encrustation. In the present study *Et. cloacae* was isolated from 8.49% of the catheter biofilms (Table 3.2). On the occasions it was present the incidence of *Pr. mirabilis* was zero (Table 3.4). These two species were never found as part of the same biofilm community. This may suggest a level of antagonism towards *Pr. mirabilis* that results in its exclusion from the biofilm environment. The results of experiments with *Pr. mirabilis* B2 and *Et. cloacae* RB19 are presented in Figures 3.10 to 3.12 and Table 3.8.

The models inoculated with *Et. cloacae* alone were still draining freely at the times both of the other models had blocked. The control *Pr. mirabilis* infected models all blocked within 19 h (mean 18.67 ± 0.12 h). Introducing *Pr. mirabilis* and *Et. cloacae* into the bladder models as a co-inoculation had the effect of increasing the mean time to blockage by a factor of about 1.65 (mean 30.76 ± 6.05 h). Analysis of the data showed that the assumptions of ANOVA could not be met, even after numerous attempts at transformation. The Kruskal-Wallis test was therefore used to compare the median times to catheter blockage (18.58 h and 31.3 h respectively), and it demonstrated that co-inoculation of *Pr. mirabilis* with *Et cloacae* significantly increased the time to catheter blockage ($P \leq 0.05$).

The results of the chemical estimation of the rates of deposition of calcium and magnesium on the catheters are presented in Figure 3.10. No significant differences ($P > 0.05$) were detected in the mean rates of calcium, magnesium, or total calcium and magnesium deposition on catheters from control *Pr. mirabilis* infected models and the co-infected models. Very little encrustation was recovered on the catheters from the models infected with a mono-culture of *Et. cloacae*, and these rates of encrustation proved to be significantly lower than those calculated for catheters from the other two types of model ($P \leq 0.001$; $\log_{10}$ Mg, $\log_{10}$ total encrustation, and $\sqrt{}$Ca data transformations were required).

The results of the viable cell counts and pH determinations on the residual urine at the beginning and end of the experimental period are shown in Table 3.8. The results indicate that at the end of the experiment the urine contained around 10^7 cfu/ml of *Pr. mirabilis* whilst *Et. cloacae* maintained population densities around 10^7 - 10^8 cfu/ml. It can be seen that in the presence of *Et. cloacae* the urine

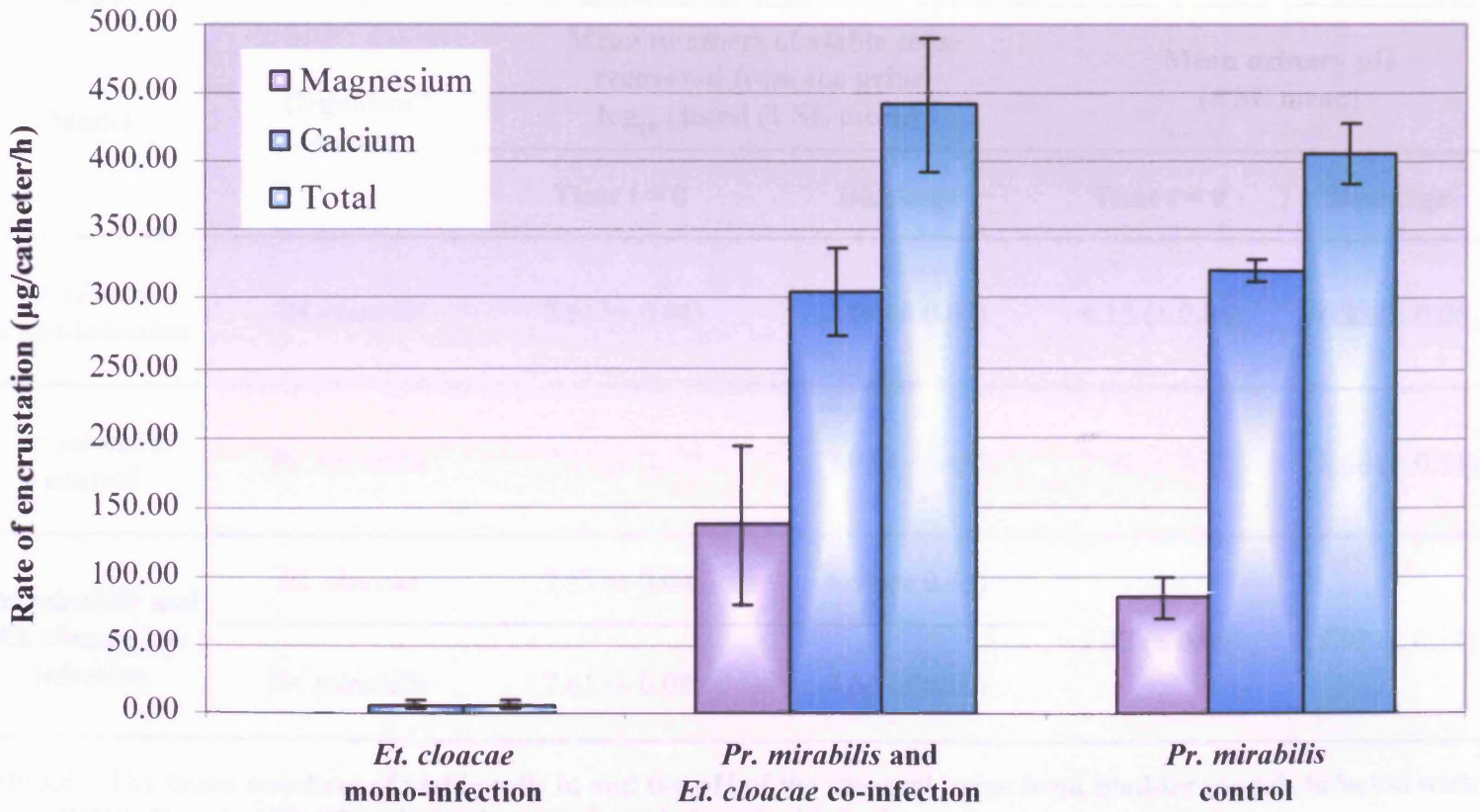


Figure 3.10 – The effect of co-inoculation of *Et. cloacae* RB19 on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except *Et. cloacae* mono-infections which were stopped when both others had blocked. The rate of calcium and magnesium deposition (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

Model	Organism counted	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)		Mean urinary pH (± SE mean)	
		Time t = 0	Blockage*	Time t = 0	Blockage*
***Et. cloacae* mono-infection**	*Et. cloacae*	7.61 (± 0.04)	7.99 (± 0.07)	6.18 (± 0.06)	6.22 (± 0.06)
***Pr. mirabilis* control**	*Pr. mirabilis*	7.53 (± 0.12)	7.05 (± 0.19)	7.49 (± 0.15)	8.66 (± 0.11)
***Pr. mirabilis* and *Et. cloacae* co-infection**	*Et. cloacae*	7.55 (± 0.04)	6.84 (± 0.45)	7.32 (± 0.09)	8.92 (± 0.11)
	Pr. mirabilis	7.61 (± 0.08)	7.65 (± 0.10)		

Table 3.8 – The mean numbers of viable cells in and the pH of the residual urine from bladder models infected with *Et. cloacae* RB19, *Pr. mirabilis* B2 and simultaneously co-infected with both species

The results are the means of four replicate experiments. Experiments were run until blockage of both models containing *Pr. mirabilis*.

* In the case of the *Et. cloacae* mono-infection, the urine was sampled at blockage of both *Pr. mirabilis* containing models.

The mean numbers of organisms inoculated into the models (2.34×10^8 cfu/ml *Et. cloacae*) and (2.63×10^8 cfu/ml *Pr. mirabilis*) were not significantly different ($P > 0.05$).

remained acidic throughout the experiment. *Pr. mirabilis* however, produced alkaline conditions (pH ranging from 8.32 - 9.04) in both the presence and absence of *Et. cloacae*. The mean pH of the urine from control *Pr. mirabilis* models was not significantly different to that of *Pr. mirabilis* and *Et. cloacae* co-inoculation models at experiment end (Kruskal-Wallis Test $P > 0.05$).

LV-SEM was carried out on catheters from the fourth replicate experiment. The micrographs showing the profile of luminal encrustation down each catheter are presented in Figure 3.11. The eye-hole sections of both catheters infected with *Pr. mirabilis* were blocked by crystalline material (Images 1A and 2A). The majority of the encrustation in the *Pr. mirabilis* control catheter was confined to the eye-hole section. The simultaneous co-inoculation of *Pr. mirabilis* and *Et. cloacae* had the effect of partially or totally occluding the lumen up to 10 cm from the catheter tip. The catheter inoculated with *Et. cloacae* alone was encrustation free down the entire length of the catheter (Images 3A-E).

It can be seen from Figure 3.12 that *Et. cloacae* deposited little crystalline material in the urine. In contrast, in both the *Pr. mirabilis* control and the co-inoculation model visible deposition of insoluble material had occurred.

3.3.1.2 Biofilm formation in models co-inoculated with *Pr. mirabilis* B2 and *Morg. morganii* SM18

The next species to be tested was *Morg. morganii*. *Morg. morganii* is a urease producer that has been commonly found in the urinary flora of patients undergoing long-term catheterisation (Ganderton *et al.*, 1992; Kunin, 1989; Mobley and Warren, 1987; Ohkawa *et al.*, 1990). Mobley and Warren (1987)

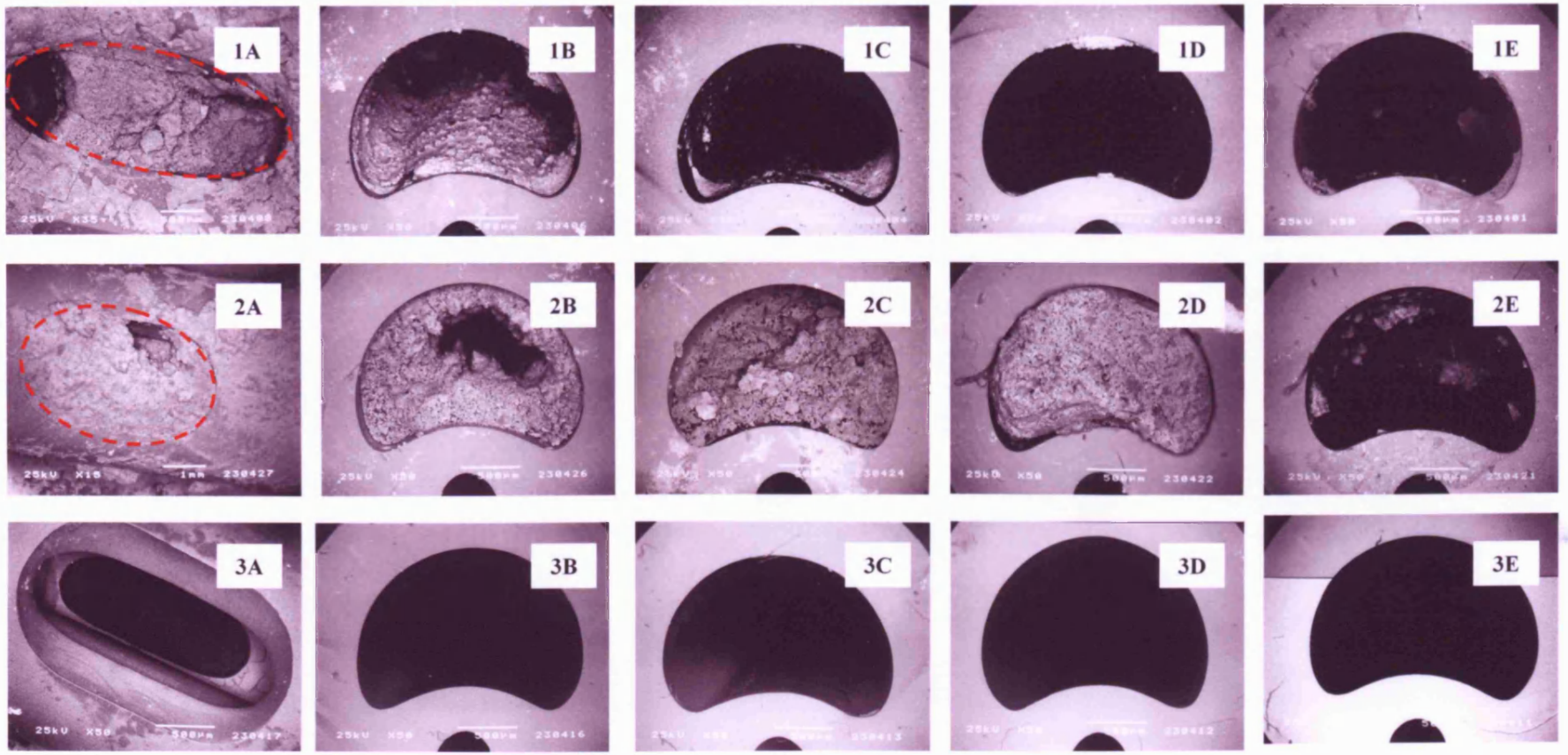


Figure 3.11 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 and *Et. cloacae* RB19 co-infection and (3) *Et. cloacae* RB19

Catheters were removed at blockage except the catheter from the pure *Et. cloacae* infected model that was removed when both *Pr. mirabilis* containing models had blocked.

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

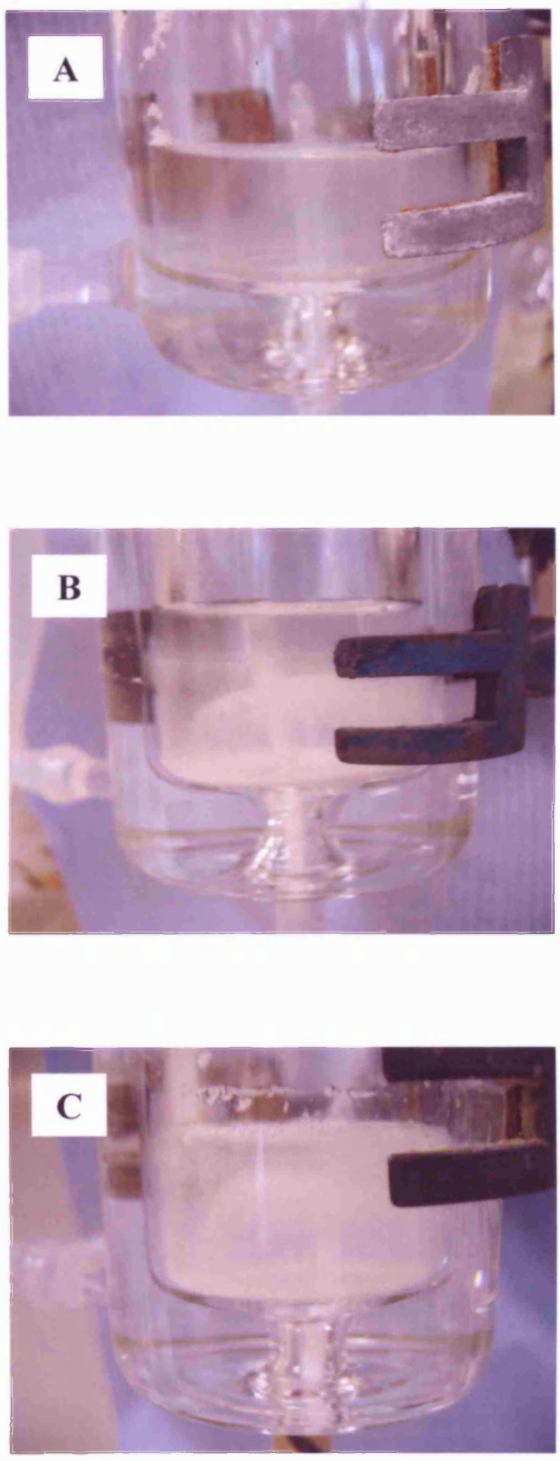


Figure 3.12 – Appearance of the residual urine in models inoculated with *Pr. mirabilis* B2 and *Et. cloacae* RB19

Image (A) *Et. cloacae* mono-infection at 24 h, (B) *Et. cloacae* and *Pr. mirabilis* co-inoculation at 24 h (blockage) and (C) *Pr. mirabilis* control at 19 h (blockage).

however, reported that unlike *Pr. mirabilis* it was identified significantly more often in urine specimens from patients whose catheters were unobstructed than from patients whose catheters were blocked. These authors suggested that *Morg. morganii* had a complex association with catheter encrustation and may, in some way, protect the catheter from obstruction. The data presented in Table 3.4 indicates that *Pr. mirabilis* is rarely found as part of the catheter biofilm flora when *Morg. morganii* is present. This might also indicate antagonism between the species. The results of the experiments with *Pr. mirabilis* B2 and *Morg. morganii* SM18 are presented in Figures 3.13 and 3.14 and Table 3.9.

Control models inoculated with *Morg. morganii* were still draining freely at the times both *Pr. mirabilis* containing models had blocked. Catheters from control *Pr. mirabilis* infected models had all become blocked by 31 h (mean 22.32 ± 3.01 h). Simultaneous co-inoculation of *Pr. mirabilis* and *Morg. morganii* into the catheterised bladder models resulted in a mean catheter blockage time of 30.69 ± 7.46 h. These mean values were not significantly different when analysed using ANOVA ($P > 0.05$).

Rates of deposition of calcium and magnesium on the catheters, determined by atomic absorption spectroscopy, are displayed in Figure 3.13. One-way ANOVA revealed no significant differences ($P > 0.05$) in the mean rates of calcium, magnesium, and total calcium and magnesium deposition on catheters from *Pr. mirabilis* control models and *Pr. mirabilis* and *Morg. morganii* co-inoculated models. Very small amounts of calcium and magnesium were deposited on catheters from control *Morg. morganii* infected models. These rates of

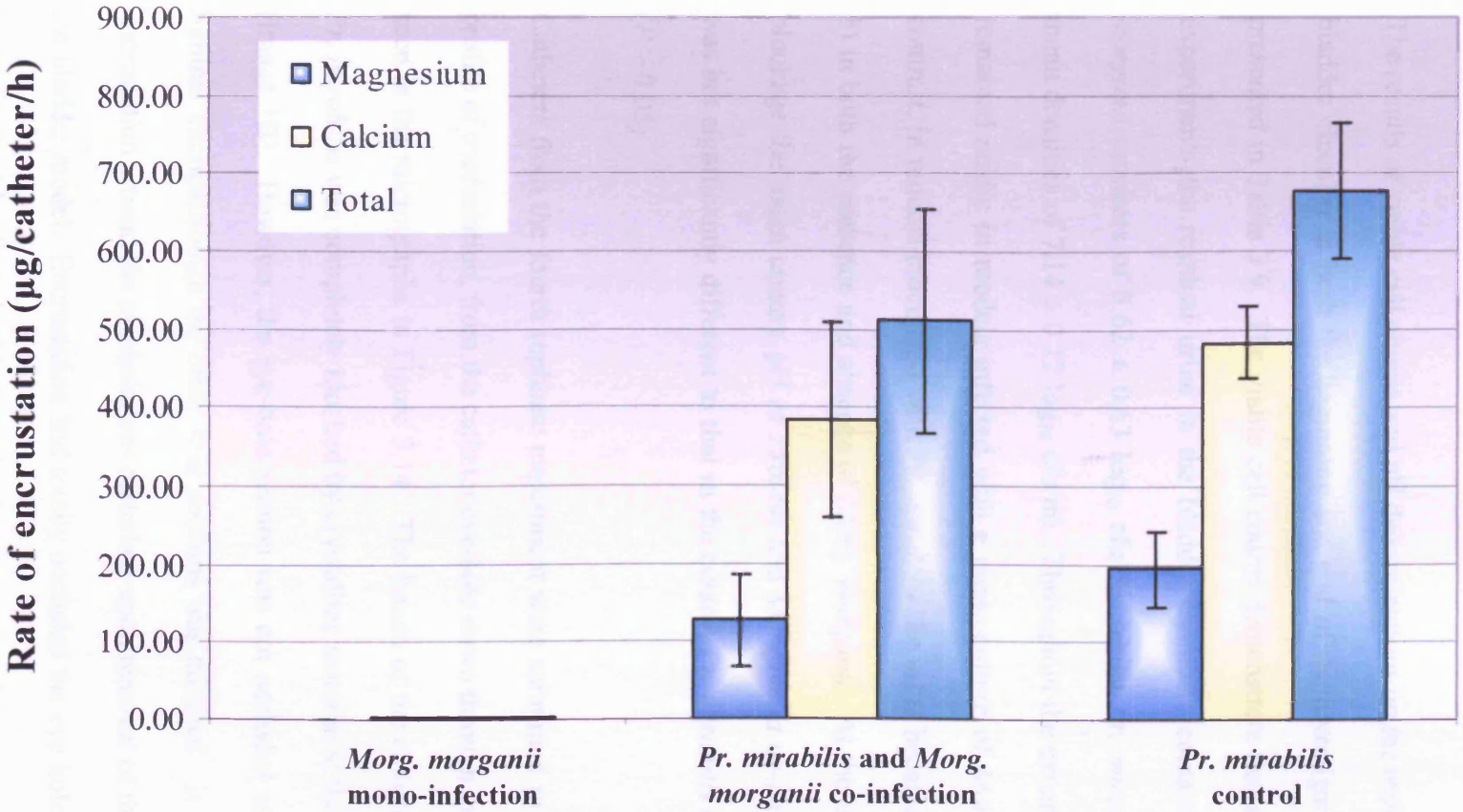


Figure 3.13 –The effect of co-inoculation of *Morg. morganii* SM18 on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except *Morg. morganii* mono-infections which were stopped when both others had blocked. The rate of calcium and magnesium deposition (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean

encrustation were significantly lower than those determined for the two *Pr. mirabilis* containing models ($P \leq 0.001$; $\log_{10}$ transformation).

The results of viable cell counts and pH determinations on the residual urine in the bladder chamber at both the beginning and end of the investigational period are presented in Table 3.9. The viable cell counts demonstrate that at the end of the experiments the residual urine in the bladder chambers contained mean *Morg. morganii* numbers of 5.62 ± 0.63 $\log_{10}$ cfu/ml, whilst *Pr. mirabilis* maintained mean densities of 7.14 ± 0.12 $\log_{10}$ cfu/ml. Throughout the experiments the urine remained acidic in models infected with a mono-culture of *Morg. morganii*. In contrast, in models inoculated with *Pr. mirabilis* the urine became alkaline (pH > 8) in both the presence and absence of *Morg. morganii*. At the times of catheter blockage the mean urinary pH of *Proteus* and *Morganella* co-inoculated models was not significantly different to that in the control *Pr. mirabilis* infected models ($P > 0.05$).

Catheters from the fourth replicate experiment were submitted to LV-SEM. The profile of encrustation, from the catheter eye-hole down through the lumen, can be seen in the micrographs in Figure 3.14. The lumen of the catheter infected with *Pr. mirabilis* was completely blocked by crystalline material at the 1-2 cm section (Image 1B). However, the eye-hole section was not occluded and the extent of luminal encrustation in the other four sections was minimal. It is possible that encrustation around the eye-hole was disturbed upon removal of the catheter from the bladder model. Encrustation had totally occluded the eye-hole of the catheter from the model infected with the mixed community of *Morg. morganii* and *Pr. mirabilis*. Heavy encrustation was seen up until section C (3-4 cm from the

Model	Organism counted	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)		Mean urinary pH (± SE mean)	
		Time t = 0	Blockage*	Time t = 0	Blockage*
Morg. morganii **mono-infection**	***Morg. morganii***	7.38 (± 0.16)	7.79 (± 0.24)	6.17 (± 0.06)	6.44 (± 0.04)
Pr. mirabilis **control**	***Pr. mirabilis***	7.49 (± 0.11)	7.43 (± 0.18)	7.49 (± 0.21)	8.58 (± 0.17)
Pr. mirabilis **and** ***Morg. morganii*** **co-infection**	***Morg. morganii***	7.23 (± 0.08)	5.62 (± 0.63)	7.73 (± 0.40)	8.84 (± 0.06)
	Pr. mirabilis	7.31 (± 0.03)	7.13 (± 0.12)		

Table 3.9 – The mean numbers of viable cells in and the pH of the residual urine from bladder models infected with *Morg. morganii* SM18, *Pr. mirabilis* B2 and simultaneously co-infected with both species

The results are the means of four replicate experiments. Experiments were run until blockage of both models containing *Pr. mirabilis*.

* In the case of the *Morg. morganii* mono-infection the urine was sampled at blockage of after both *Pr. mirabilis* containing models.

The mean numbers of organisms inoculated into the models (2.29×10^8 cfu/ml *Morg. morganii*) and (3.02×10^8 cfu/ml *Pr. mirabilis*) were not significantly different ($P > 0.05$).

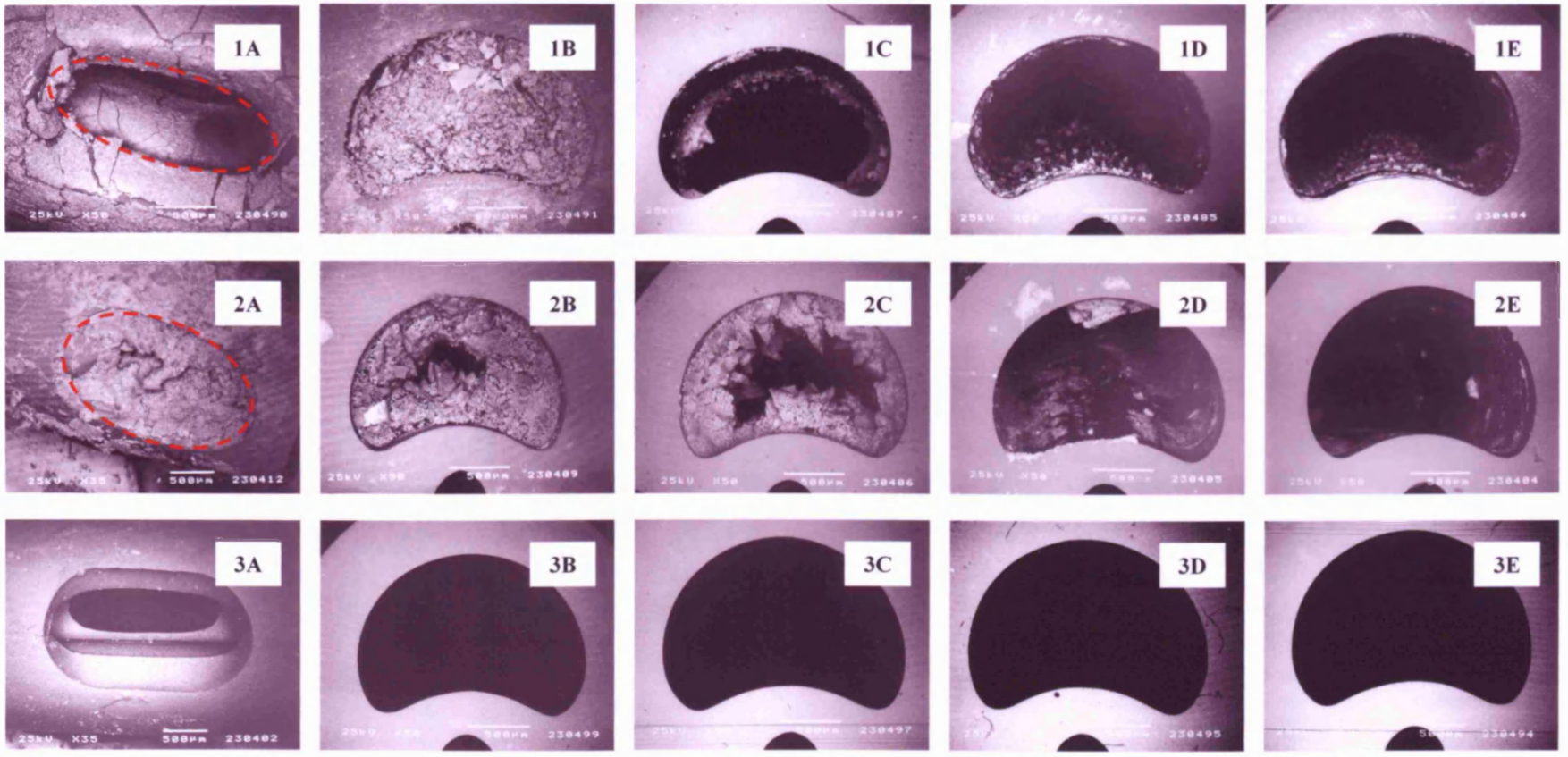


Figure 3.14 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 and *Morg. morganii* SM18 co-infection and (3) *Morg. morganii* SM18

Catheters were removed at blockage except the catheter from the pure *Morg. morganii* infected model that was removed when both *Pr. mirabilis* containing models had blocked.

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

catheter tip) with minimal amounts deposited towards the middle and distal regions of the catheter. The catheter from the model inoculated purely with *Morg. morganii* was free from encrustation along the complete length of the catheter (Images 3A-E).

The residual urine in the bladder chambers of each of the three model types at 24 h was comparable to those shown in Figure 3.12. The *Morg. morganii* control showed no signs of deposited crystalline material in the residual urine. In contrast, substantial deposition of crystalline material was visible in models infected with *Pr. mirabilis* alone and the mixed bacterial community.

3.3.1.3 Biofilm formation in models co-inoculated with *Pr. mirabilis* B2 and *E. coli* SM1

The third species to be tested was *E. coli*. *E. coli* is the most common pathogen of the urinary tract in uncomplicated infections (Farrell *et al.*, 2003; Gupta *et al.*, 1999; Leblebicioglu *et al.*, 2003; Lee *et al.*, 2004b). It is also frequently isolated from the urine of catheterised patients (Clayton *et al.*, 1982; Ohkawa *et al.*, 1990; Ramsay *et al.*, 1989; Wazait *et al.*, 2003). The results presented in Table 3.2 show that *E. coli* was recovered from 40.79% of the multi-species catheter biofilms examined. It does not produce urease and is not associated with catheter encrustation (Mobley and Warren, 1987). Table 3.4 reveals that when *E. coli* was a component of the biofilm community, the incidence of *Pr. mirabilis* was reduced by 12.01%. The results of the experiments with *Pr. mirabilis* B2 and *E. coli* SM1 are presented in Figures 3.15 and 3.16 and Table 3.10.

Control *Pr. mirabilis* inoculated models blocked at a mean time of 19.73 ± 2.88 h. Introducing *E. coli* into the bladder models together with *Pr. mirabilis* resulted in

a mean catheter blockage time of 23.59 ± 1.59 h. These values were not significantly different ($P > 0.05$; $\log_{10}$ transformation). Catheters in the control *E. coli* models were draining freely when the catheters from both *Pr. mirabilis* infected models blocked.

Atomic absorption spectroscopy analysis of the amounts of calcium and magnesium deposited upon catheters from each model type can be seen in Figure 3.15. Models incorporating *Pr. mirabilis* had extensive amounts of encrustation (> 300 μg/catheter/h). No significant difference was found between the mean rates of calcium, magnesium and total encrustation ($P > 0.05$) deposited on catheters in the models inoculated with the mixed community compared with the *Pr. mirabilis* control. Minute amounts of total encrustation were recovered from catheters from the *E. coli* controls (< 2.50 μg/catheter/h). This quantity was significantly lower than that on catheters from both *Pr. mirabilis* containing models ($P \leq 0.001$; $\log_{10}$ transformation).

Table 3.10 states the viable cell count data and the urinary pH results at the beginning and end of the experimental periods. The urinary pH of control *E. coli* infected models remained acidic, below 6.20. Each model type containing *Pr. mirabilis* raised the pH to alkaline levels by the end of the experiments (range 7.40 - 9.05). Comparing the mean pH of the residual urine in control *Pr. mirabilis* infected models with that of co-infected models indicated no significant difference ($P > 0.05$; pH^3 transformation).

LV-SEM was used to visualise the profile of luminal encrustation on catheters removed from the fourth replicate experiment (Figure 3.16). Heavy encrustation

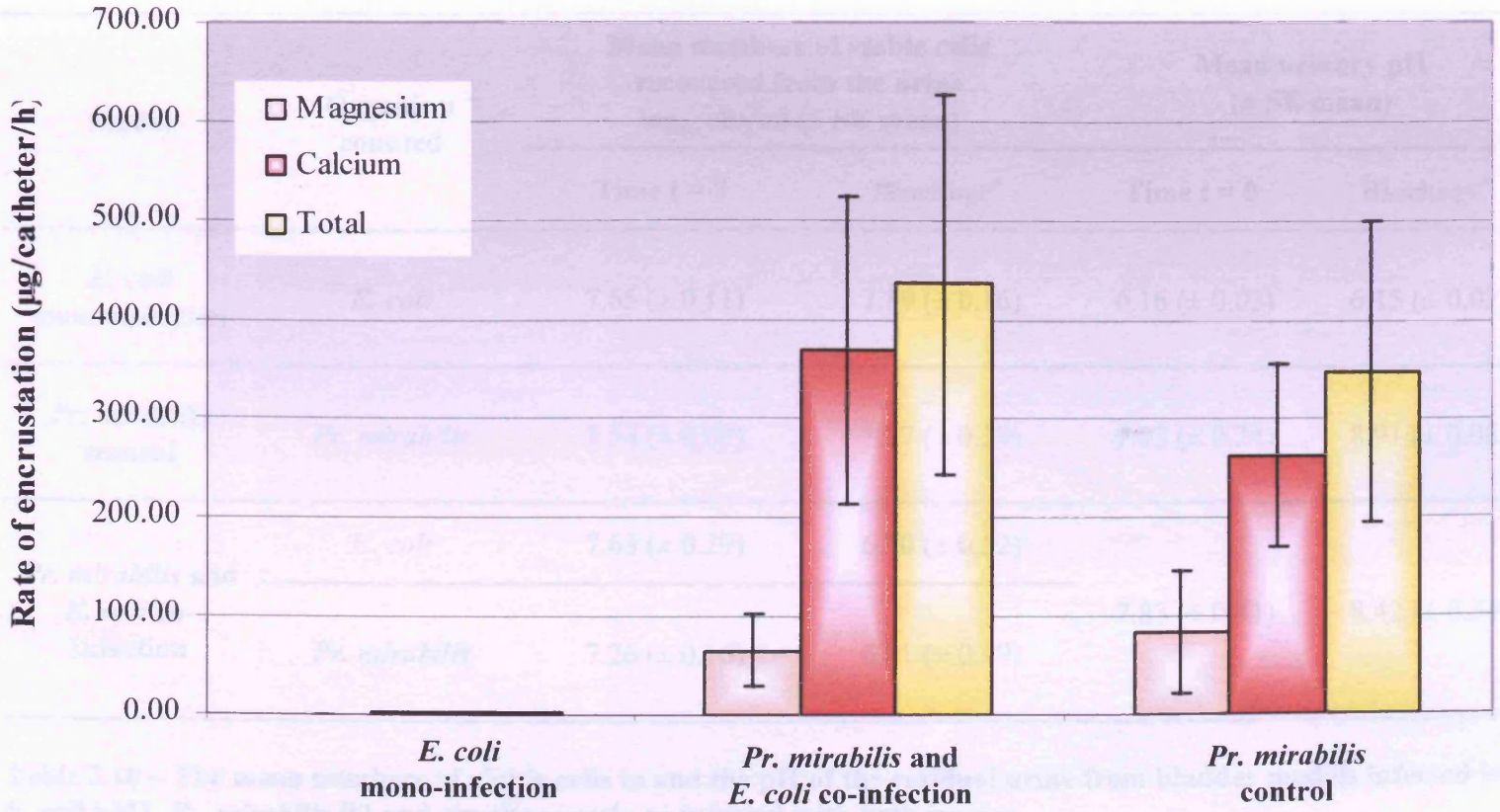


Figure 3.15 – The effect of co-inoculation of *E. coli* SM1 on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except the *E. coli* mono-infections which were stopped when both others had blocked. The rate of calcium and magnesium deposition (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

Model	Organism counted	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)		Mean urinary pH (± SE mean)	
		Time t = 0	Blockage*	Time t = 0	Blockage*
***E. coli* mono-infection**	*E. coli*	7.65 (± 0.11)	7.89 (± 0.16)	6.16 (± 0.03)	6.15 (± 0.02)
***Pr. mirabilis* control**	*Pr. mirabilis*	7.54 (± 0.03)	7.97 (± 0.29)	7.93 (± 0.24)	8.91 (± 0.08)
***Pr. mirabilis* and *E. coli* co-infection**	*E. coli*	7.63 (± 0.29)	6.50 (± 0.52)	7.83 (± 0.41)	8.42 (± 0.34)
	Pr. mirabilis	7.26 (± 0.16)	6.81 (± 0.49)		

Table 3.10 – The mean numbers of viable cells in and the pH of the residual urine from bladder models infected with E. *coli* SM1, *Pr. mirabilis* B2 and simultaneously co-infected with both species

The results are the means of four replicate experiments. Experiments were run until blockage of both models containing *Pr. mirabilis*.

* In the case of the *E. coli* control, the urine was sampled at blockage of both *Pr. mirabilis* containing models.

The mean numbers of organisms inoculated into the models (3.55×10^8 cfu/ml *E. coli*) and (3.63×10^8 cfu/ml *Pr. mirabilis*) were not significantly different ($P > 0.05$).

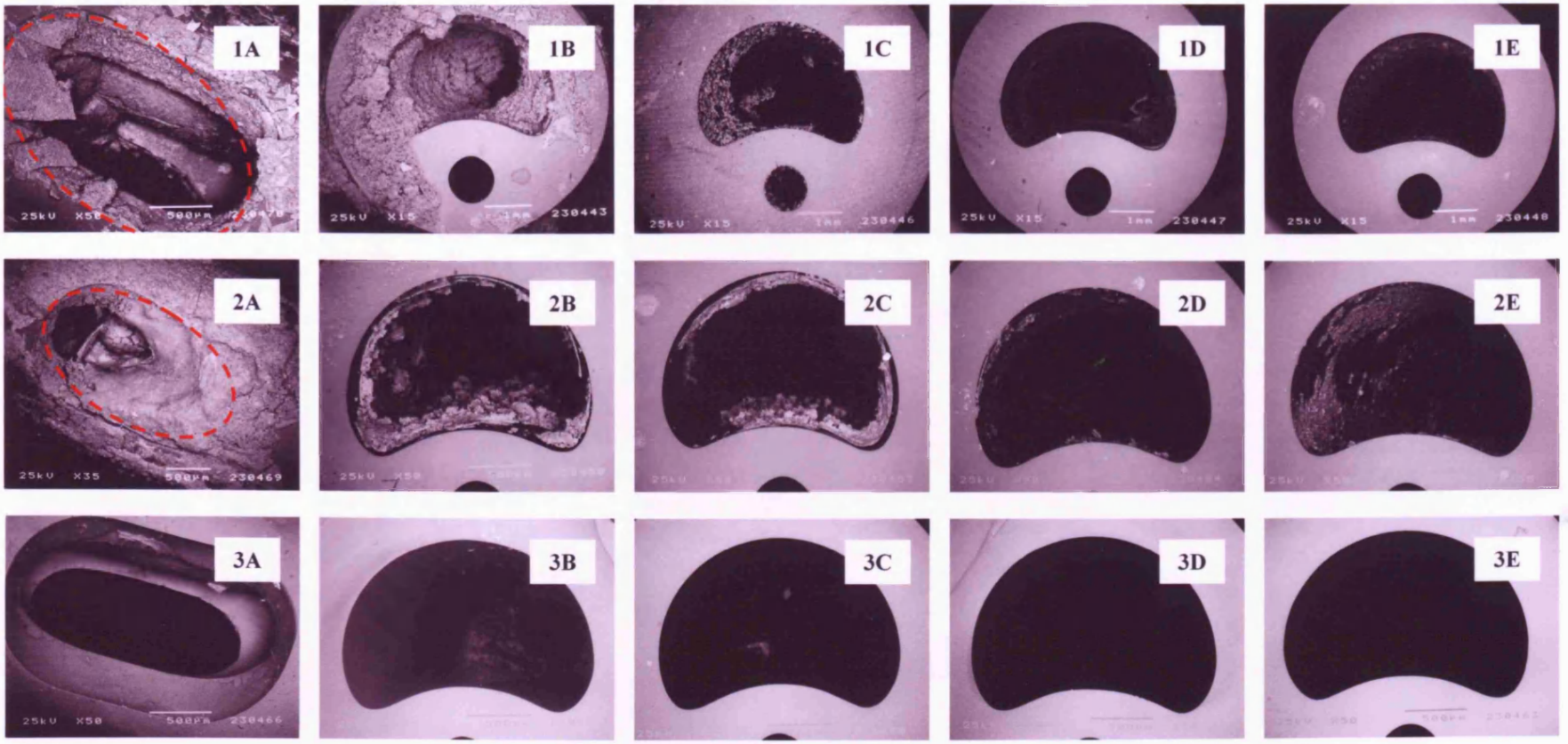


Figure 3.16 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 and *E. coli* SM1 co-infection and (3) *E. coli* SM1

Catheters were removed at blockage except the catheter from the pure *E. coli* infected model that was removed when both *Pr. mirabilis* containing models had blocked.

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

was visible on catheters removed from the models inoculated with *Pr. mirabilis* alone and from the model co-inoculated with the two species. The catheter removed from the *E. coli* control model was free from encrustation for its entire length.

The residual urine held within the glass bladders showed similar crystalline deposition as presented in Figure 3.12. At 24 h, the urine in the *E. coli* inoculated control model showed very little turbidity compared to that in the two *Pr. mirabilis* containing models.

3.3.1.4 Biofilm formation in models co-inoculated with *Pr. mirabilis* B2 and *Ps. aeruginosa* SM15

The fourth species to be tested was *Ps. aeruginosa*. This species is often a component of the urinary flora of catheterised patients (Clayton *et al.*, 1982; Ganderton *et al.*, 1992; Ohkawa *et al.*, 1990; Wazait *et al.*, 2003). *Ps. aeruginosa* is a urease producer and was found by Kohler-Ockmore and Feneley (1996) to be more commonly isolated from the urine of patients who had problems with their catheters becoming blocked by bacterial biofilm than from those that had few problems. The data presented in Table 3.4 shows that in the presence of *Ps. aeruginosa* the incidence of *Pr. mirabilis* in catheter biofilms was reduced to around 24%. The results of the experiments with *Pr. mirabilis* B2 and *Ps. aeruginosa* SM15 are presented in Figures 3.17 and 3.18 and Table 3.11.

The mean time to catheter blockage of control *Pr. mirabilis* infected models was 23.34 ± 4.29 h. Introducing *Pr. mirabilis* and *Ps. aeruginosa* as a co-inoculation into the bladder models increased the mean time to catheter blockage (41.43 ±

8.13 h). This increase, of approximately 18 h, was deemed to be significant when analysed using ANOVA ($P \leq 0.05$; $1/h^2$ transformation). *Ps. aeruginosa* infected control models were still draining freely at the time both *Pr. mirabilis* containing models had blocked.

Calcium and magnesium deposition rates, as determined by atomic absorption spectroscopy are presented in Figure 3.17. One-way ANOVA indicated no significant differences in the mean rates of calcium, magnesium and total deposition ($P > 0.05$) on catheters from control *Pr. mirabilis* infected models and those from models co-infected with *Pr. mirabilis* and *Ps. aeruginosa*. A mean of < 20 μg total encrustation/catheter/h was recovered from catheters from control *Ps. aeruginosa* infected models. This value was significantly lower ($P \leq 0.01$; $\log_{10}$ transformation) than the corresponding values for both *Pr. mirabilis* containing models.

The results of viable cell counts and pH determinations on the residual urine at both the start and end of each experiment are shown in Table 3.11. In models that were co-infected, *Ps. aeruginosa* was present at around 10^7 cfu/ml at the time of catheter blockage, whilst *Pr. mirabilis* densities reached approximately 10^8 cfu/ml. The pH of the urine in the *Ps. aeruginosa* control models remained acidic (range 6.10 – 6.68) for the duration of the experiments. In both the presence and absence of *Ps. aeruginosa*, *Pr. mirabilis* generated highly alkaline conditions (mean pH 8.85 ± 0.05 and 8.95 ± 0.17 respectively). These mean values were not significantly different ($P > 0.05$).

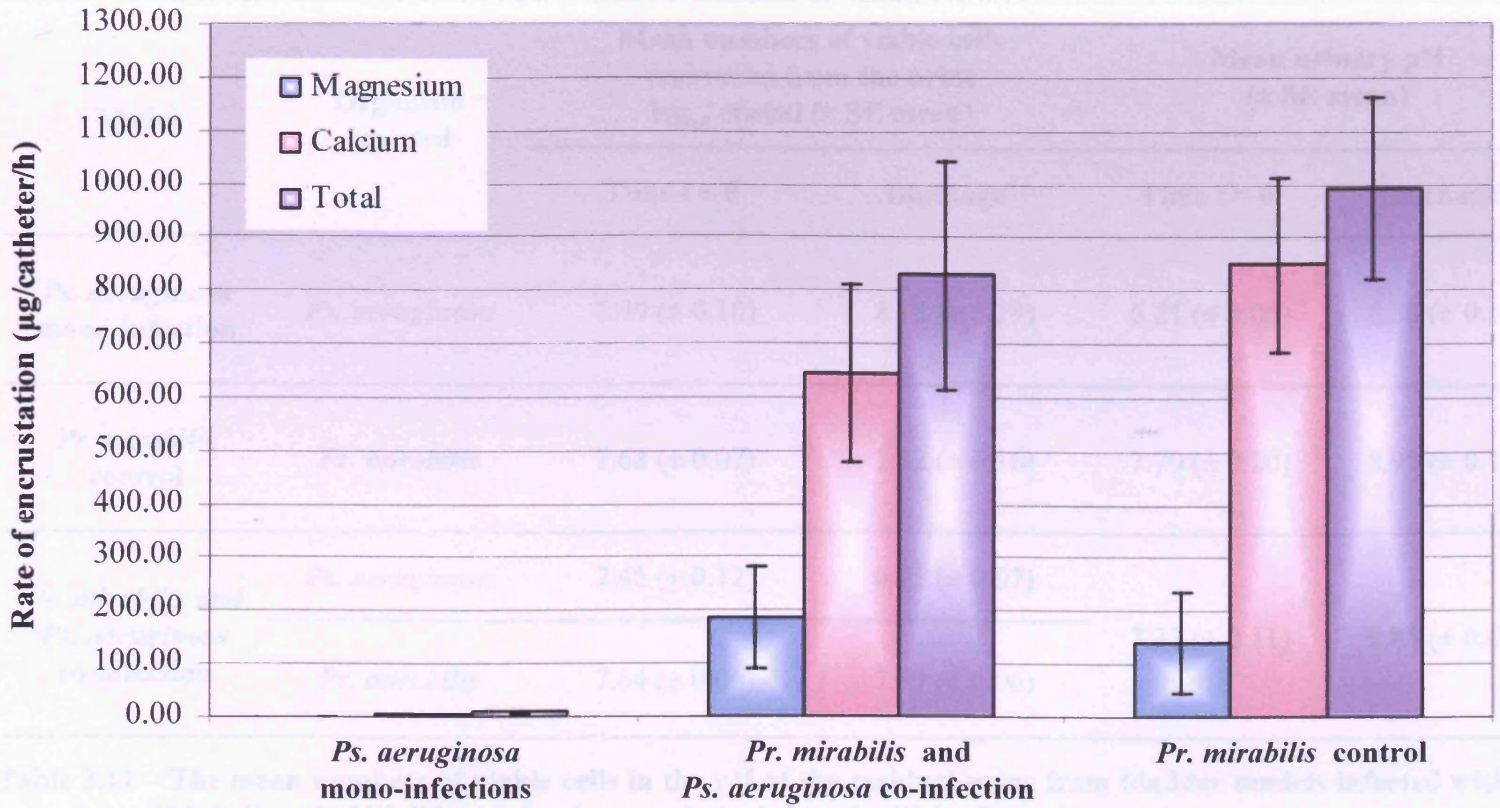


Figure 3.17 – The effect of co-inoculation of *Ps. aeruginosa* SM15 on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except the *Ps. aeruginosa* mono-infections which were stopped when both others had blocked. The rate of calcium and magnesium deposition (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

Model	Organism counted	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)		Mean urinary pH (± SE mean)	
		Time t = 0	Blockage*	Time t = 0	Blockage*
Ps. aeruginosa **mono-infection**	***Ps. aeruginosa***	7.49 (± 0.10)	8.15 (± 0.29)	6.21 (± 0.05)	6.38 (± 0.10)
Pr. mirabilis **control**	***Pr. mirabilis***	7.68 (± 0.07)	7.32 (± 0.16)	7.70 (± 0.20)	8.95 (± 0.17)
Pr. mirabilis **and** ***Ps. aeruginosa*** **co-infection**	***Ps. aeruginosa***	7.46 (± 0.12)	6.75 (± 0.07)	7.33 (± 0.11)	8.85 (± 0.05)
	Pr. mirabilis	7.64 (± 0.06)	7.99 (± 0.06)		

Table 3.11 – The mean numbers of viable cells in the pH of the residual urine from bladder models infected with *Ps. aeruginosa* SM15, *Pr. mirabilis* B2 and simultaneously co-infected with both species

The results are the means of four replicate experiments. Experiments were run until blockage of both models containing *Pr. mirabilis*.

* In the case of the *Ps. aeruginosa* control, the urine was sampled at blockage of both *Pr. mirabilis* containing models.

The mean numbers of organisms inoculated into the models (3.09×10^8 cfu/ml *Ps. aeruginosa*) and (3.16×10^8 cfu/ml *Pr. mirabilis*) were not significantly different ($P > 0.05$).

Catheters from the fourth replicate were examined using LV-SEM. Micrographs showing the extent of luminal encrustation down through each catheter are presented in Figure 3.18. The eye-hole sections of the catheters from models co-infected with *Pr. mirabilis* and *Ps. aeruginosa*, and those inoculated with *Pr. mirabilis* alone were totally occluded by crystalline bacterial biofilm (Images 1A and 2A) Both catheters from the two models containing *Pr. mirabilis* also had significant levels of luminal deposition in section B (1-2 cm from the catheter tip). Encrustation amounts diminished as the distance from the eye-hole increased. The catheter from the model inoculated with a mono-culture of *Ps. aeruginosa* was free from crystalline encrustation along its entire length (Images 3A-E).

In the case of the *Ps. aeruginosa* control only small amounts of insoluble material had deposited within the bladder chamber by 48 h. In contrast, extensive amounts of crystalline material were observed in the residual urine of both the *Pr. mirabilis* control and the mixed community infected bladders. The images obtained from these experiments are similar to those shown in Figure 3.12.

3.3.1.5 Biofilm formation in models co-inoculated with *Pr. mirabilis* B2 and *Kl. pneumoniae* SDM3

The fifth and final species to be tested was *Kl. pneumoniae*. This species is regularly a member of the urinary and biofilm flora of catheterised patients (Clayton *et al.*, 1982; Fawcett *et al.*, 1986; Kunin, 1989; Meers *et al.*, 1981). *Kl. pneumoniae* is a urease producer. It is of interest because the analysis presented in Table 3.4 indicates that it is commonly associated with *Pr. mirabilis* in catheter biofilms. However, Kunin (1989) reported that it was inversely associated with

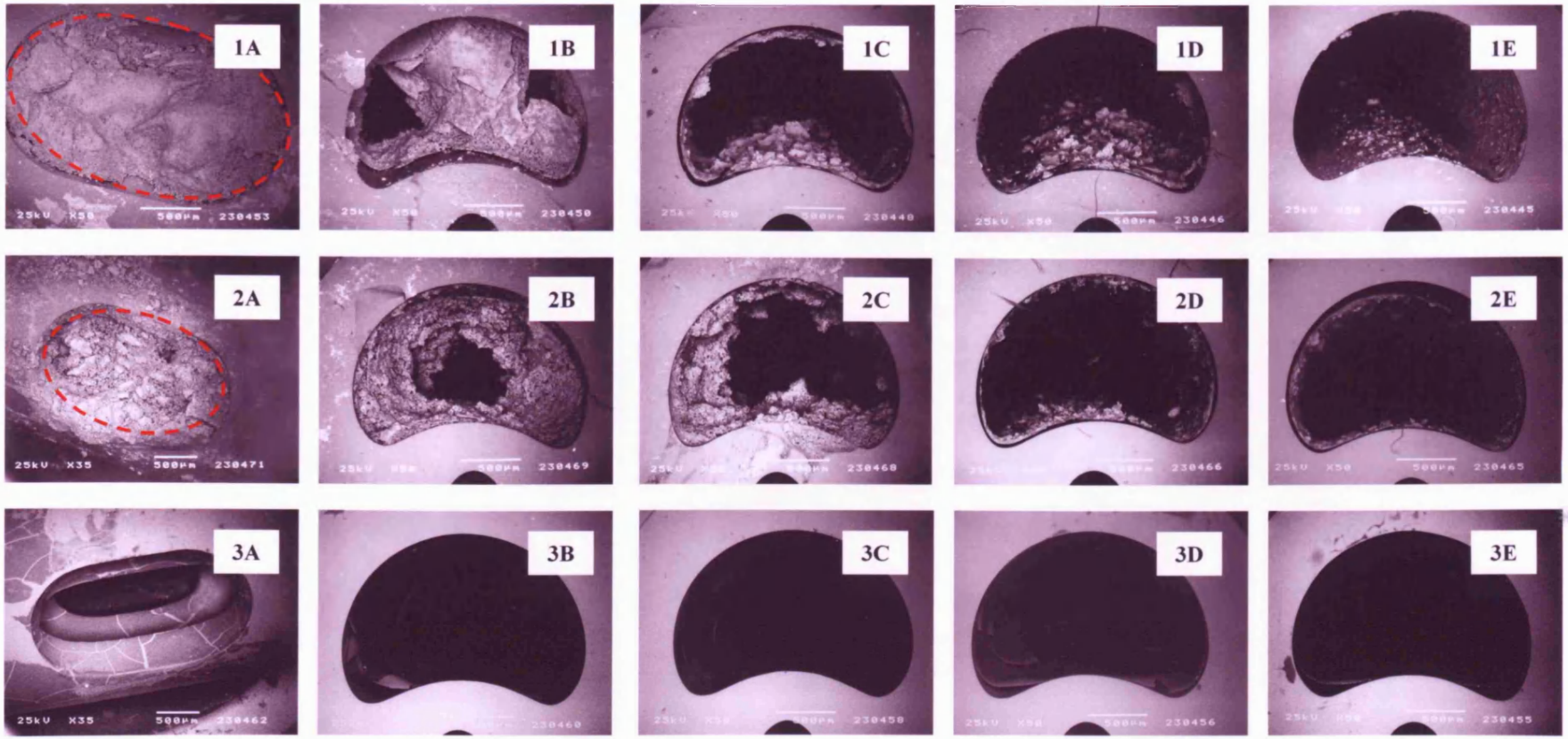


Figure 3.18 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 and *Ps. aeruginosa* SM15 co-infection and (3) *Ps. aeruginosa* SM15

Catheters were removed at blockage except the catheter from the pure *Ps. aeruginosa* infected model that was removed when both *Pr. mirabilis* containing models had blocked.

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

catheter blockage. The results of the experiments with *Pr. mirabilis* B2 and *Kl. pneumoniae* SDM3 are presented in Figures 3.19 and 3.20 and Table 3.12.

Catheters in the control *Kl. pneumoniae* infected models drained freely for the entirety of the experimental period. Catheters from control *Pr. mirabilis* infected models all blocked within 25 h (mean 24.81 ± 2.55 h). Co-inoculation of *Pr. mirabilis* and *Kl. pneumoniae* into the bladder models did not significantly increase the mean time to catheter blockage (32.50 ± 6.98 h; $P > 0.05$; 1/h transformation).

Rates of calcium, magnesium and total deposition on catheters can be seen in Figure 3.19. One-way ANOVA indicated no significant difference ($P > 0.05$) between the mean rates of calcium, magnesium, and total encrustation deposited in *Pr. mirabilis* control models, and *Pr. mirabilis* and *Kl. pneumoniae* co-inoculation models. Very small amounts of encrustation were recovered from catheters from the control *Kl. pneumoniae* models. Highly significant differences were indicated between the mean rates of encrustation ($\log_{10}$ transformations) from the *Kl. pneumoniae* control models and the two *Pr. mirabilis* containing models ($P \leq 0.001$).

The numbers of viable cells in the residual urine and the urinary pH at the beginning and end of the experiments are shown in Table 3.12. At the end of the experimental period the co-inoculation models contained roughly 10^4 cfu/ml of *Kl. pneumoniae* whereas *Pr. mirabilis* cells were present at densities around 10^7 cfu/ml. The pH results demonstrate that in the *Kl. pneumoniae* control the urine

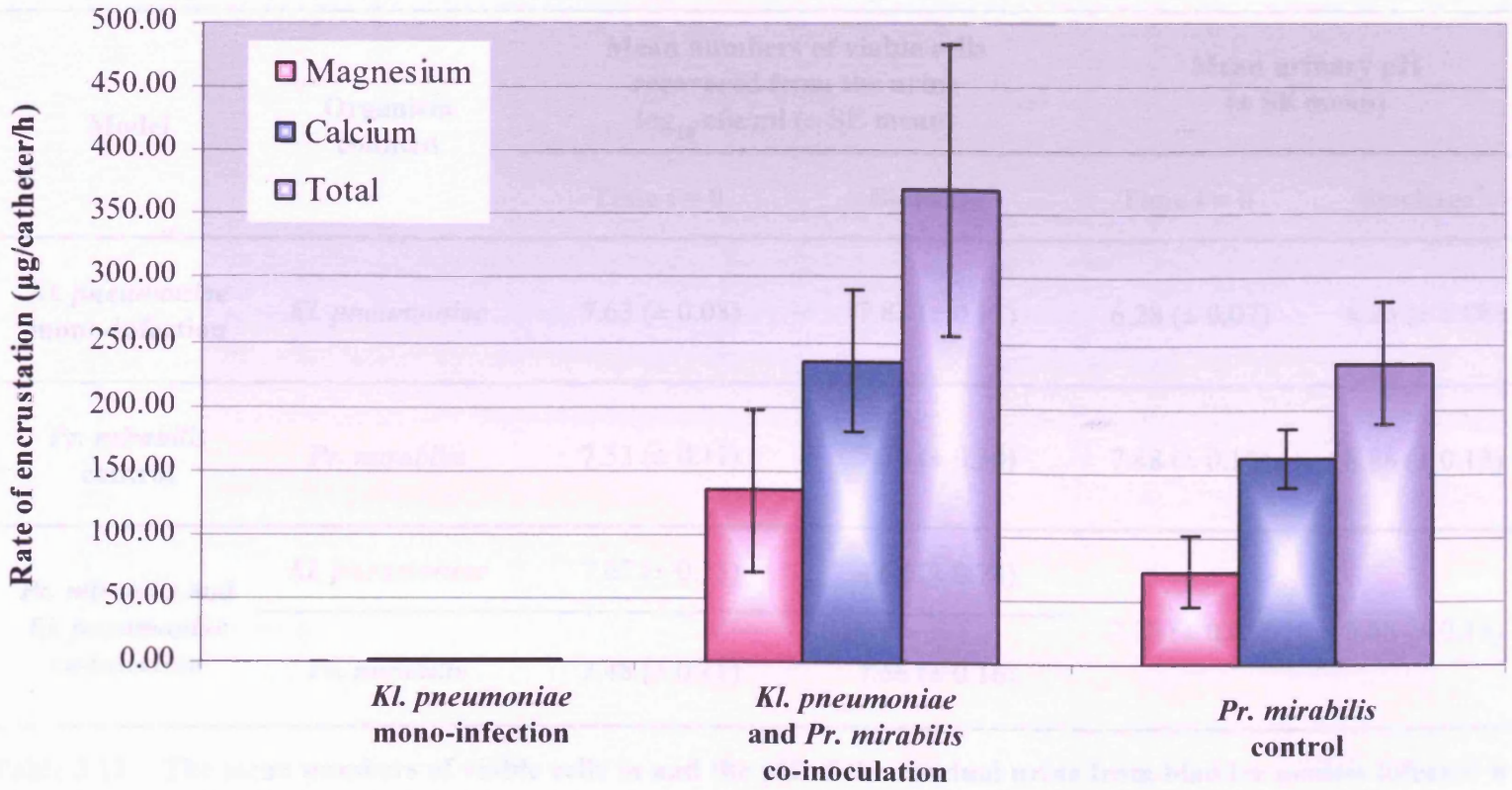


Figure 3.19 – The effect of co-inoculation of *Kl. pneumoniae* SDM3 on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except the *Kl. pneumoniae* mono-infections which were stopped when both others had blocked. The rate of calcium and magnesium deposition (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

Model	Organism counted	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)		Mean urinary pH (± SE mean)	
		Time t = 0	Blockage*	Time t = 0	Blockage*
***Kl. pneumoniae* mono-infection**	*Kl. pneumoniae*	7.63 (± 0.08)	7.83 (± 0.11)	6.28 (± 0.07)	6.26 (± 0.08)
***Pr. mirabilis* control**	*Pr. mirabilis*	7.53 (± 0.11)	7.70 (± 0.34)	7.48 (± 0.16)	8.88 (± 0.13)
***Pr. mirabilis* and *Kl. pneumoniae* co-infection**	*Kl. pneumoniae*	7.63 (± 0.13)	4.62 (± 0.78)	7.33 (± 0.08)	8.68 (± 0.14)
	Pr. mirabilis	7.48 (± 0.11)	7.66 (± 0.16)		

Table 3.12 – The mean numbers of viable cells in and the pH of the residual urine from bladder models infected with *Kl. pneumoniae* SDM3, *Pr. mirabilis* B2 and simultaneously co-infected with both species

The results are the means of four replicate experiments. Experiments were run until blockage of both models containing *Pr. mirabilis*.

* In the case of the *Kl. pneumoniae* control, the urine was sampled at blockage of both *Pr. mirabilis* containing models.

The mean numbers of organisms inoculated into the models (2.81 x 10^8 cfu/ml *Kl. pneumoniae*) and (3.02 x 10^8 cfu/ml *Pr. mirabilis*) were not significantly different ($P > 0.05$).

remained acidic for the duration of the experimental period. In both models infected with *Pr. mirabilis* the urine was highly alkaline at catheter blockage even in the presence of *Kl. pneumoniae*. The mean end urinary pH of the *Pr. mirabilis* and *Kl pneumoniae* co-inoculation models was not significantly different to that of the control *Pr. mirabilis* models ($P > 0.05$).

LV-SEM micrographs seen in Figure 3.20 show the deposition of crystalline material down through the lumens of catheters from the fourth replicate. The catheters from both *Pr. mirabilis* containing models had eyeholes that were totally or partially obstructed by encrustation (Images 1A and 2A). Considerable amounts of material were also present on sections B and C of the catheter from the control *Pr. mirabilis* model. The majority of the deposition along the co-inoculation catheter occurred within the first 2 cm. The catheter from the control *Kl. pneumoniae* infected model showed very little indications of encrustation (Images 3A-E).

The residual urine in the bladder chambers of each of the three model types at 24h was comparable to that shown in Figure 3.12. The *Kl. pneumoniae* control showed no signs of deposited crystalline material in the residual urine. In contrast, substantial deposition of crystalline material was visible in models infected with *Pr. mirabilis* and the two-member community.

3.3.2 *Pr. mirabilis* super-infection of juvenile (24 h) biofilms

The four species, *Et. cloacae*, *Morg. morganii*, *E. coli*, and *Ps. aeruginosa* that from the epidemiological data presented in Table 3.4 decreased the incidence of

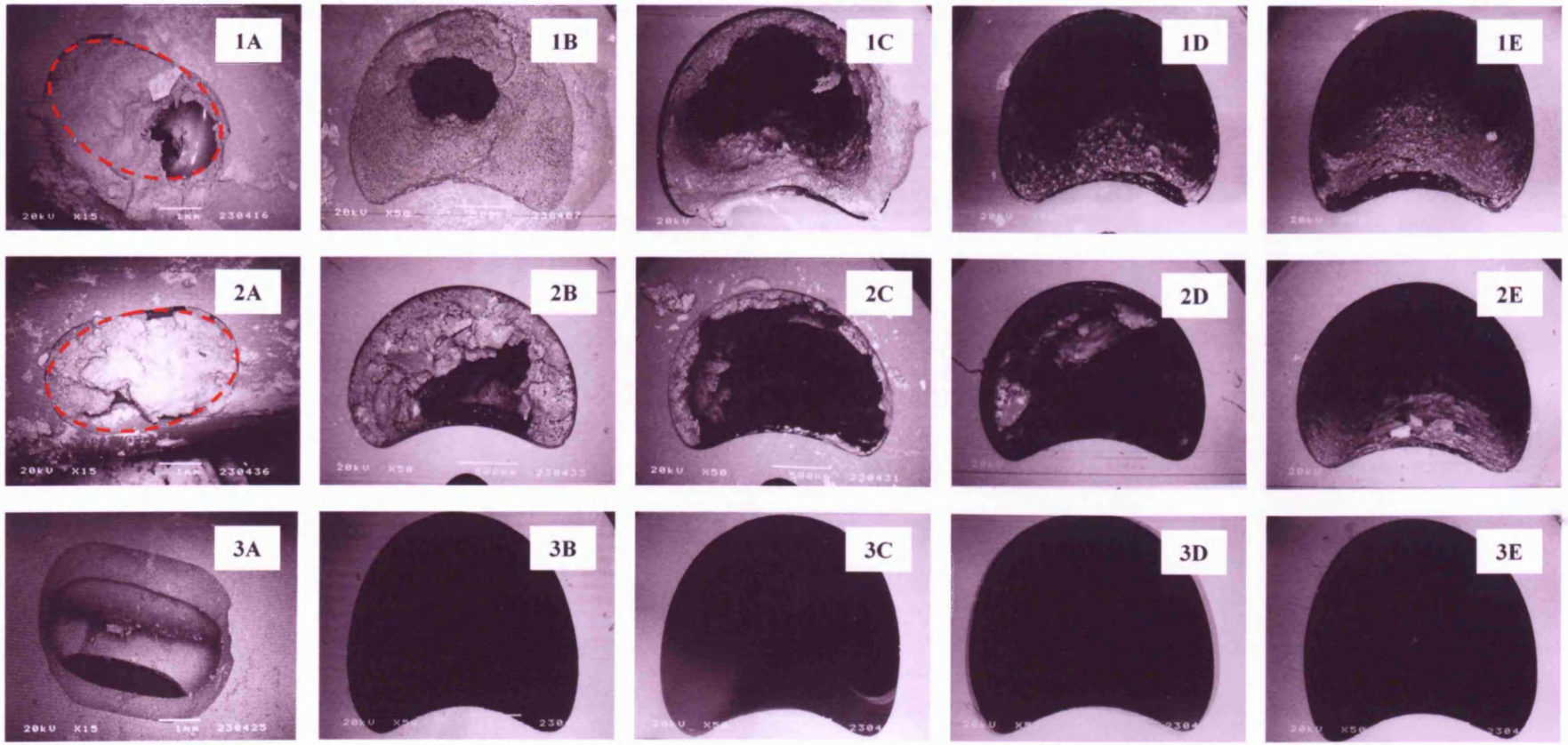


Figure 3.20 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 and *Kl. pneumoniae* SDM3 co-infection and (3) *Kl. pneumoniae* SDM3

Catheters were removed at blockage except the catheter from the pure *Kl. pneumoniae* infected model that was removed when both *Pr. mirabilis* containing models had blocked.

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

Pr. mirabilis, might have an ability to prevent or reduce *Pr. mirabilis* colonization of the urine after 24 h growth. Experiments were performed to investigate whether these species, if already present in the catheterised urinary tract in the form of a pure, young 24 h biofilm, resisted super-infection by *Pr. mirabilis*. In addition *Kl. pneumoniae* was investigated to see whether a 24 h biofilm promoted *Pr. mirabilis* colonization.

In these experiments, sets of four models were assembled in parallel. Cultures of the organisms, grown in artificial urine for 4 h at 37°C, were used as inocula. The non-*Proteus* species (10 ml) was inoculated into three bladder models (models 1, 2, and 3) on day one of the experimental period. After 1 h to allow the organisms to establish themselves in the bladder chambers, urine was supplied to the models at 0.5 ml/min. At 24 h model 1 was dismantled. Model 3 had its urine supply disconnected and was super-infected with *Pr. mirabilis* B2 (1 ml). At the same time as super-infection, a *Pr. mirabilis* control model (model 4) was also inoculated (1 ml). Both of these models were left for 1 h before the urine supply was resumed and sustained until both catheters had blocked. Model 2 was supplied with urine continuously and was allowed to drain until both *Pr. mirabilis* containing models had blocked. The urinary pH and the numbers of viable cells/ml of each species present in the urine were determined at 0, 1 and every 24 h after that until blockage or stoppage.

These experiments were performed in quadruplicate and in each case the time to catheter blockage was recorded. At the end of the experiments, the urine supply was turned off and the catheters removed from the models. In three of the

replicate experiments the extent of catheter encrustation was determined by analysis of the amounts of calcium and magnesium deposited per catheter using flame atomic absorption spectroscopy. The rate of deposition was expressed as the amount (μg) of calcium and/or magnesium/catheter/h. Visual assessment of the biofilm formation by LV-SEM was performed on the catheters from the fourth replicate experiment. In addition, digital images of the residual urine in the bladder chambers at various time points were also taken.

3.3.2.1 Crystalline biofilm formation in models super-infected with *Pr. mirabilis* B2 after 24 h *Et. cloacae* RB19 biofilm development

The analysis of the data on the incidence of species colonizing 106 catheters obtained from patients (Tables 3.4) suggests that biofilms containing *Et. cloacae* never become colonized by *Pr. mirabilis*. To explore whether this might be due to an ability of *Et. cloacae* biofilms to resist colonization by *Pr. mirabilis*, experiments were performed in which 24 h *Et. cloacae* biofilms were challenged with *Pr. mirabilis*. The results of super-infection experiments with *Pr. mirabilis* B2 and *Et. cloacae* RB19 are shown in Figures 3.21 to 3.23 and Table 3.13.

The mean time to catheter blockage from control *Pr. mirabilis* models (26.9 ± 4.59 h) was not significantly different ($P > 0.05$) to that of catheters from models inoculated with *Pr. mirabilis* after a 24 h *Et. cloacae* infection (45.34 ± 10.91 h). The presence of a 24 h *Et. cloacae* biofilm did not significantly increase the time *Pr. mirabilis* took to block each catheter. Pure *Et. cloacae* infected models drained freely for the entirety of the experiments.

Analysis of the rate of calcium, magnesium and total calcium and magnesium deposition from triplicate experiments (Figure 3.21) revealed that the rate of catheter encrustation by *Pr. mirabilis* was not significantly reduced ($P > 0.05$) by the presence of a young *Et. cloacae* community. Catheters from both the 24 h *Et. cloacae* infected models and the *Et. cloacae* infected models that were stopped at the end of the experiment, once catheters from the mixed community had blocked, had mineral deposition rates significantly lower ($P \leq 0.001$; $\log_{10}$ transformation) than both models containing *Pr. mirabilis*.

Table 3.13 presents data for the viable cell counts from, and pH measurements of, the residual urine from the bladder chambers. Urine in the two models inoculated with pure *Et. cloacae* remained acidic throughout the experiments. In the super-infection models (models 3) the initial pH at t = 0 after *Et. cloacae* introduction was also acidic. At catheter blockage after subsequent *Pr. mirabilis* infection however, the pH had risen to a mean of 8.76. This value was not significantly different to that of the *Pr. mirabilis* control ($P > 0.05$). There was no significant decrease ($P > 0.05$) in the numbers of *Pr. mirabilis* or *Et. cloacae* ($\log_{10}$ cfu/ml^3 transformation) cells in super-infection models (7.47 ± 0.26 vs. 7.02 ± 0.71 $\log_{10}$ cfu/ml) compared to the respective controls (7.45 ± 0.21 vs. 8.15 ± 0.13 $\log_{10}$ cfu/ml). These results suggest that no antagonism was occurring between these two species.

The electron micrographs presented in Figure 3.22 show cross-sections of catheters from the fourth replicate. These images corroborate the calcium and magnesium data. Catheters removed from control models infected with *Et. cloacae* at the end of the experimental period demonstrate little visible signs of

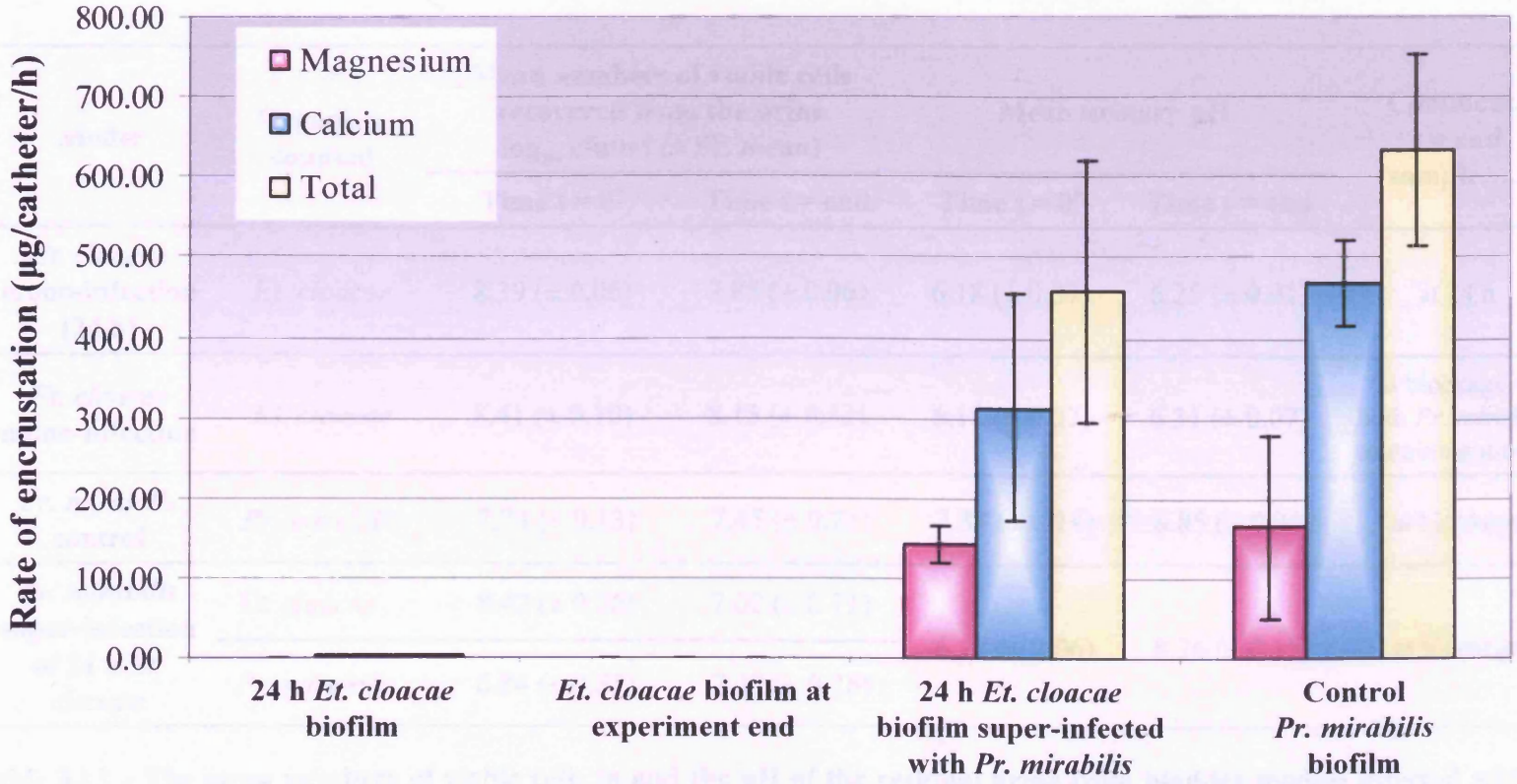


Figure 3.21 – The effect of 24 h *Et. cloacae* RB19 biofilm on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except pure *Et. cloacae* infected models which were stopped at either 24 h or when catheters from both *Pr. mirabilis* containing models had blocked. The rate of deposition of calcium and/or magnesium (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

Model	Organism counted	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)		Mean urinary pH		Comments t = end sample.....
		Time t = 0*	Time t = end	Time t = 0*	Time t = end	
***Et. cloacae* mono-infection (24 h)**	***Et. cloacae***	8.39 (± 0.06)	7.85 (± 0.06)	6.18 (± 0.07)	6.25 (± 0.01)	at 24 h
***Et. cloacae* mono-infection**	***Et. cloacae***	8.41 (± 0.10)	8.15 (± 0.13)	6.18 (± 0.07)	6.31 (± 0.07)	at blockage of both *Pr. mirabilis* containing models
***Pr. mirabilis* control**	***Pr. mirabilis***	7.74 (± 0.13)	7.45 (± 0.21)	7.87 (± 0.14)	8.85 (± 0.26)	at blockage
Pr. mirabilis* super-infection of 24 h *Et. cloacae	***Et. cloacae***	8.42 (± 0.06)	7.02 (± 0.71)	6.17 (± 0.06)	8.76 (± 0.15)	at blockage
	Pr. mirabilis	6.84 (± 0.85)	7.47 (± 0.26)			

Table 3.13 – The mean numbers of viable cells in and the pH of the residual urine from bladder models infected with *Et. cloacae* RB19, *Pr. mirabilis* B2 and *Pr. mirabilis* super-infection of 24 h *Et. cloacae*

The results shown are the mean values of four replicate experiments. Experiments were run until blockage of both models containing *Pr. mirabilis*.

* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 25 h after *Et. cloacae* inoculation.

The mean numbers of organisms inoculated into the models (3.31×10^8 cfu/ml *Et. cloacae*) and (3.39×10^8 cfu/ml *Pr. mirabilis*) were not significantly different ($P > 0.05$).

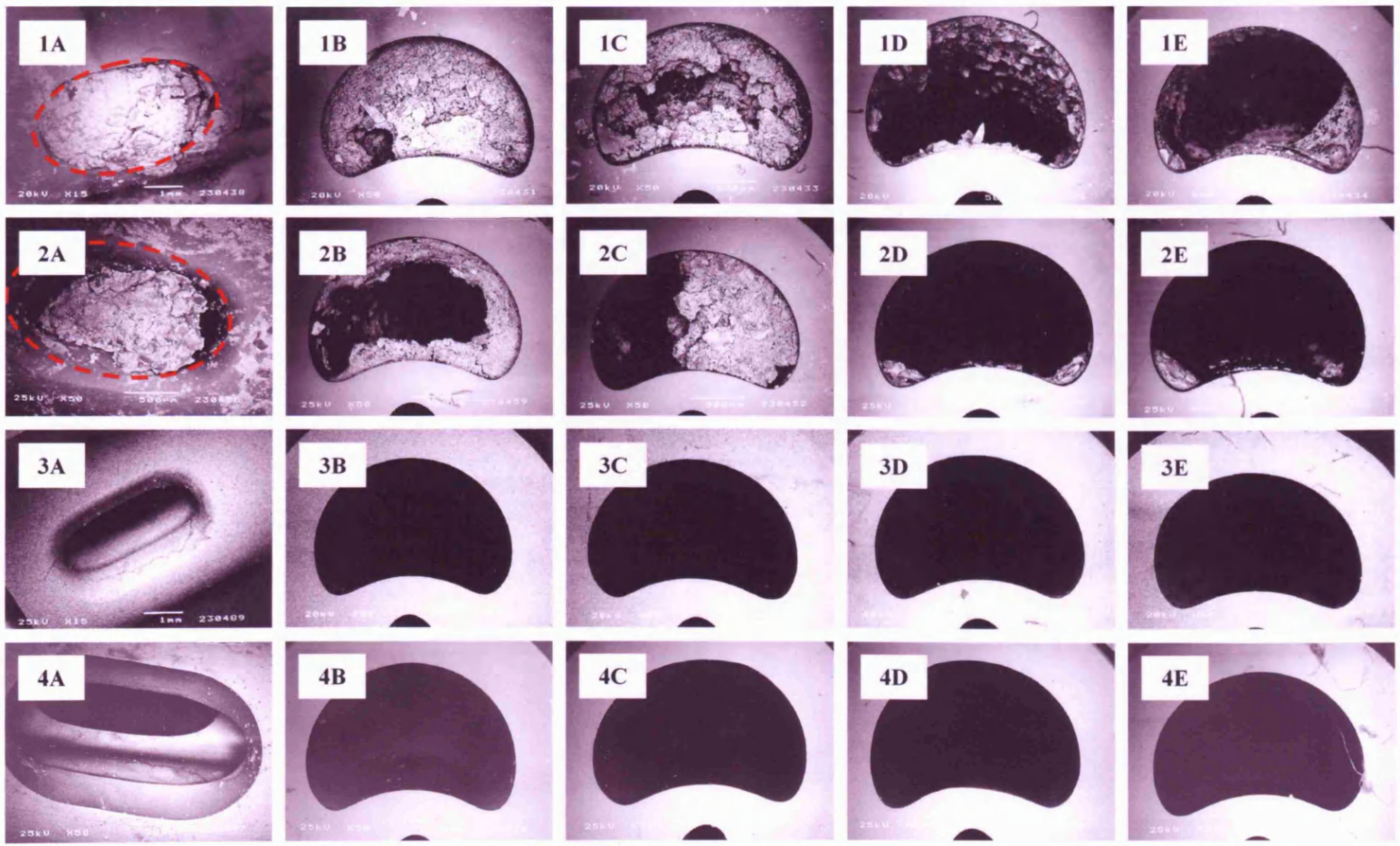


Figure 3.22 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 after 24 h *Et. cloacae* RB19 biofilm development, (3) *Et. cloacae* RB19 (24 h) and (4) *Et. cloacae* RB19

Catheters were removed at blockage except the catheters from the pure *Et. cloacae* infected models that were either removed at 24 h (3) or upon blockage of both *Pr. mirabilis* containing models (4).

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

luminal crystalline precipitate. In addition, high calcium and magnesium sedimentation rates were accompanied by eye-hole and luminal obstruction in the other two catheters.

Digital images of the turbidity of the urine due to crystal formation and sedimentation can be seen in Figure 3.23. *Pr. mirabilis* had caused the formation of a large amount of precipitate within 24 h. Interestingly, the presence of a 24 h established *Et. cloacae* community prevented precipitation for 24 h after *Pr. mirabilis* introduction. By 48 h however, a significant quantity of crystalline matter had settled to the bottom of the bladder chamber. Urine in *Et. cloacae* control models remained crystal free for the duration of the experiments.

3.3.2.2 Crystalline biofilm formation in models super-infected with *Pr. mirabilis* B2 after 24 h *Morg. morganii* SM18 biofilm development

The epidemiological data presented in Table 3.4 reveals that biofilms containing *Morg, morganii* rarely become colonized by *Pr. mirabilis*. This suggests that there might be antagonism between *Pr. mirabilis* and *Morg. morganii*. Perhaps young biofilms of *Morg, morganii* are able to prevent *Pr. mirabilis* colonization of the urine. The results of the experiments in which 24 h *Morg. morganii* SM18 biofilms were super-infected with *Pr. mirabilis* B2 are presented in Figures 3.24 to 3.26 and Table 3.14.

The control *Pr. mirabilis* catheters blocked at a mean time of 29.38 h (± 4.37 h) compared to a mean of 37.32 h (± 0.76) for the super-infection model catheters. This difference in mean time to blockage (TTB) however was non-significant ($P >$

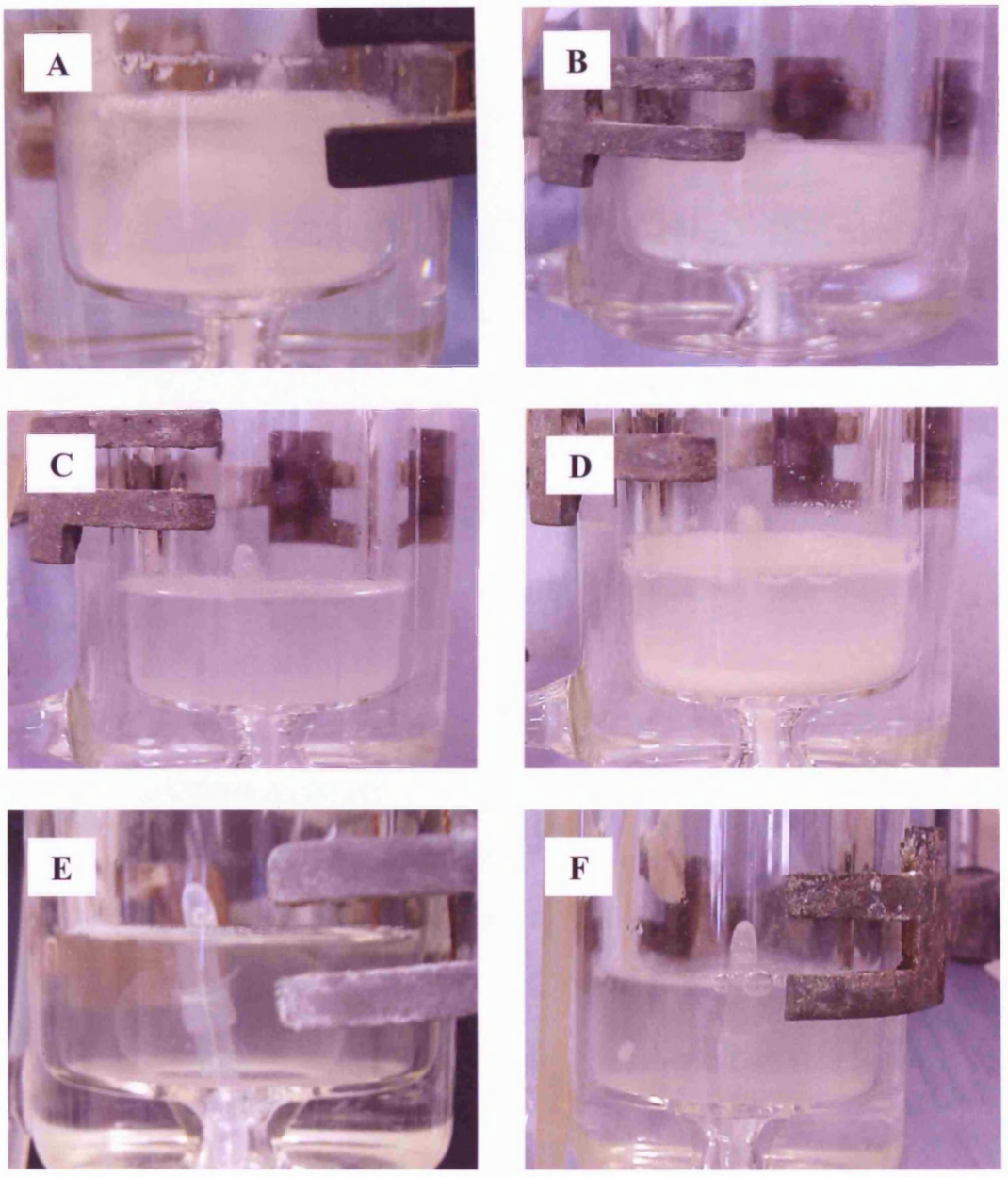


Figure 3.23 – The effect of a 24 h *Et. cloacae* RB19 biofilm on the extent of urinary crystalline precipitation caused by *Pr. mirabilis* B2

(A) *Pr. mirabilis* at 24 h, (B) *Pr. mirabilis* at 42 h (blockage occurred at 38.93 h), (C) 24 h *Et. cloacae* 24 h after *Pr. mirabilis* super-infection, (D) 24 h *Et. cloacae* 48 h after *Pr. mirabilis* super-infection, (E) *Et. cloacae* at 48 h and (F) *Et. cloacae* at 120 h.

Inocula sizes were 1ml *Pr. mirabilis* and 10 ml *Et. cloacae.*

0.05; TTB[4] transformation). Catheters from pure *Morg. morganii* infected models did not block.

The effects of a 24 h *Morg. morganii* community on the rate of encrustation as determined by calcium and magnesium analysis is illustrated in Figure 3.24. Both *Pr. mirabilis* containing models had high total deposition rates with calcium accumulation over 8 times more than that of magnesium. Interestingly, super-infection of a 24 h *Morg. morganii* community by *Pr. mirabilis* had the effect of increasing the calcium, and therefore the total encrustation, rate by over 200 μg/h. However, both models incorporating *Pr. mirabilis* had calcium, magnesium and summed calcium and magnesium deposition rates that were not significantly different ($P > 0.05$).

The high rates generated in the presence of *Pr. mirabilis* and the much lower deposition that occurred during pure *Morg. morganii* infection were confirmed by the low-vacuum scanning electron micrographs. Figure 3.25 shows heavy encrustation especially around the eye-holes of the two catheters from *Pr. mirabilis* containing models (Images 1A and 2A). The catheter from the super-infection model also had extensive crystalline material down most of the lumen, whilst little encrustation was evident on the catheter that had pure *Morg. morganii* infected urine draining through it for the duration of the experiment.

The effect of 24 h established *Morg. morganii* on the extent of crystalline precipitation caused by *Pr. mirabilis* was visualised by the urine turbidity throughout the experiments (Figure 3.26). In contrast to the heavily turbid urine of the *Pr. mirabilis* control 24 h after inoculation (Figure 3.26A), a young *Morg.*

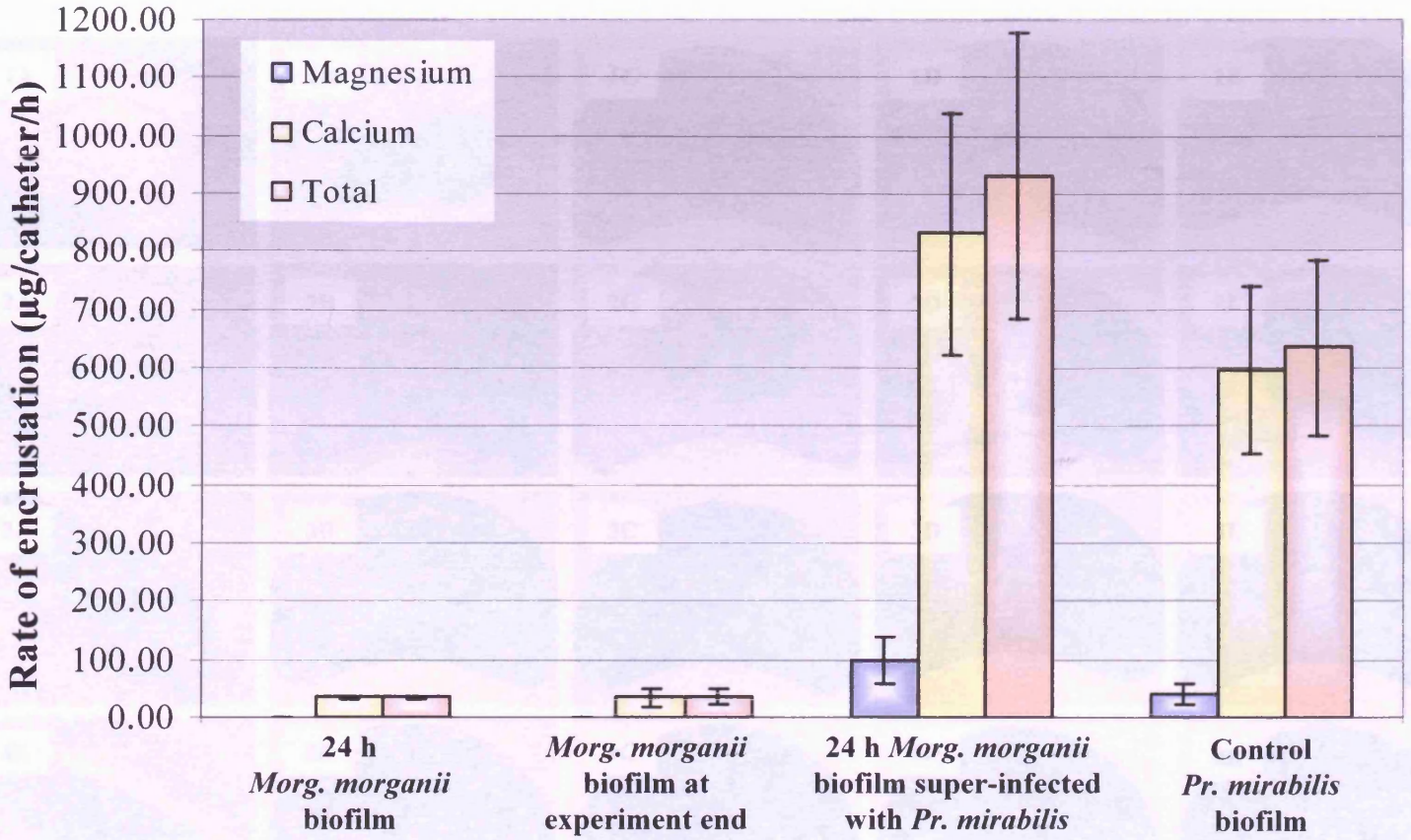


Figure 3.24 – The effect of 24 h *Morg. morganii* SM18 biofilm on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except pure *Morg. morganii* infected models which were stopped at either 24 h or when catheters from both *Pr. mirabilis* containing models had blocked. The rate of deposition of calcium and/or magnesium (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

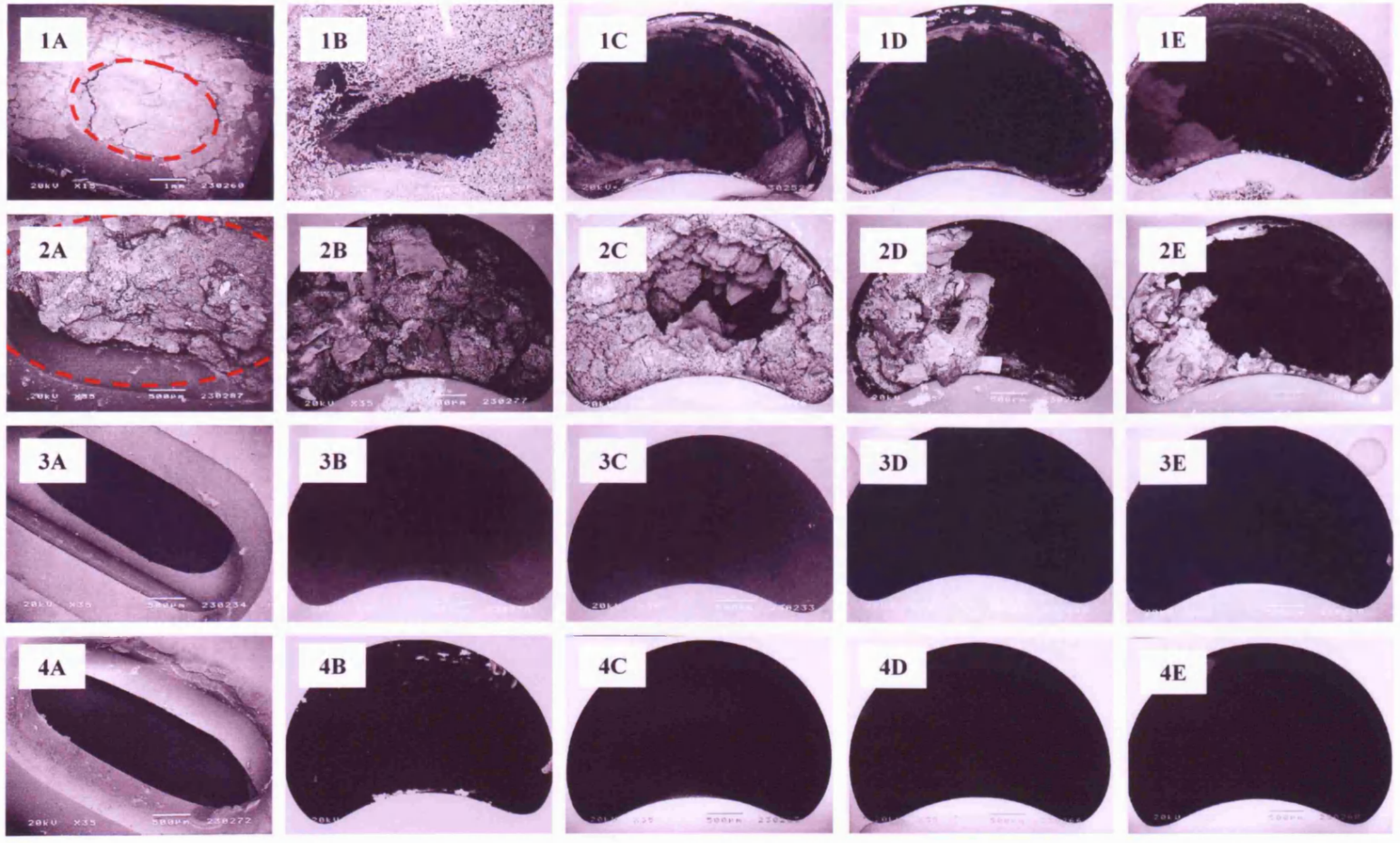


Figure 3.25 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 after 24 h *Morg. morganii* SM18 biofilm development, (3) *Morg. morganii* SM18 (24 h) and (4) *Morg. morganii* SM18

Catheters were removed at blockage except the catheters from the pure *Morg. morganii* infected models that were removed either at 24 h (3) or when both *Pr. mirabilis* containing models had blocked (4).

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

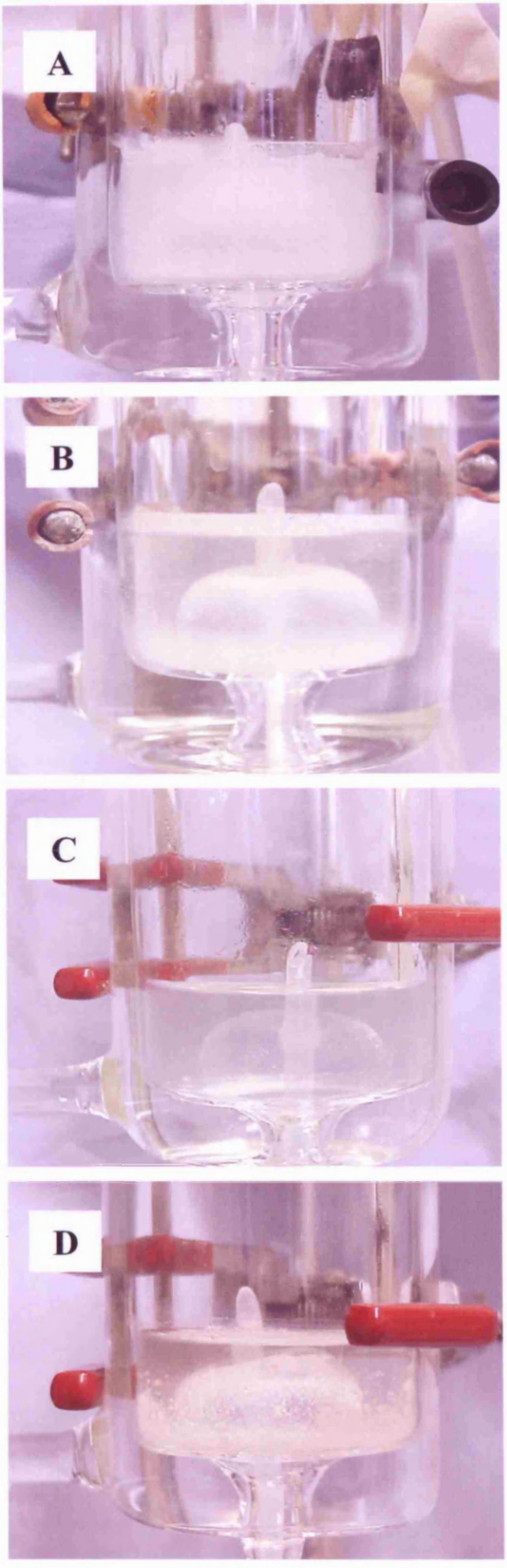


Figure 3.26 – The effect of a 24 h *Morg. morganii* SM18 biofilm on the extent of urinary crystalline precipitation caused by *Pr. mirabilis* B2

(A) *Pr. mirabilis* at 24 h, (B) 24 h *Morg. morganii* 24 h after *Pr. mirabilis* super-infection, (C) *Morg. morganii* at 48 h and (D) *Morg. morganii* at 96 h.

Inocula sizes were 1 ml *Pr. mirabilis* and 10 ml *Morg. morganii*.

morganii community seemed to somewhat reduce the amount of precipitate produced by *Pr. mirabilis* at the corresponding time period (Figure 3.26B). Unlike *Et. cloacae* the urine in pure *Morg. morganii* models gradually accumulated insoluble material within the bladder compartment. At 96 h this precipitate was easily visible but still to a much lesser extent than that seen in the *Pr. mirabilis* control at 24 h.

The data presented in Table 3.14 shows that the numbers of viable cells in the urine of models infected with *Morg. morganii* alone remained relatively stable over the duration of the experiments. In contrast, super-infection with *Pr. mirabilis* produced a significant reduction ($P \leq 0.01$) in the mean numbers of *Morg. morganii* in the urine from 7.78 $\log_{10}$ cfu/ml at t = 0 to 6.41 log10 cfu/ml at catheter blockage. The mean numbers of *Pr. mirabilis* cells recovered from the residual urine at the end of the experiment from the super-infection community (6.80 ± 0.23 $\log_{10}$ cfu/ml) and the control *Pr. mirabilis* models (7.46 ± 0.28 $\log_{10}$ cfu/ml) were significant different ($P > 0.05$). The results of the pH measurements in Table 3.14 reveal that even though *Morg. morganii* SM18 was a urease producer the mean urinary pH was acidic both at the start and end of the experiments. In comparison, by the end of the experiments the *Pr. mirabilis* super-infection models and those inoculated with a mono-culture of *Pr. mirabilis* had mean pH values of 8.90 and 8.83 respectively.

3.3.2.3 Crystalline biofilm formation in models super-infected with *Pr. mirabilis* B2 after 24 h biofilm development of *E. coli* SM1, *Ps. aeruginosa* SM15, or *Kl. pneumoniae* SDM3

The data presented in Table 3.4 reveals that if *E. coli* or *Ps. aeruginosa* are present in a catheter biofilm the incidence of *Pr. mirabilis* is reduced (decreases of

Model	Organism counted	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)		Mean urinary pH		Comments t = end sample…..
		Time t = 0*	Time t = end	Time t = 0*	Time t = end	
Morg. morganii **mono-infection (24h)**	*Morg. morganii*	7.71 (± 0.16)	7.58 (± 0.16)	6.31(± 0.04)	6.35 (± 0.06)	at 24 h
Morg. morganii **mono-infection**	*Morg. morganii*	7.70 (± 0.06)	7.57 (± 0.10)	6.27 (± 0.03)	6.79 (± 0.09)	at blockage of both *Pr. mirabilis* containing models
Pr. mirabilis **control**	*Pr. mirabilis*	6.89 (± 0.08)	7.46 (± 0.28)	7.12 (± 0.31)	8.83 (± 0.04)	at blockage
Pr. mirabilis **super-infection of 24 h** *Morg. morganii*	*Morg. morganii*	7.78 (± 0.10)	6.41 (± 0.28)	6.30 (± 0.03)	8.90 (± 0.13)	at blockage
	Pr. mirabilis	6.63 (± 0.13)	6.80 (± 0.23)			

Table 3.14 – The mean numbers of viable cells in and the pH of the residual urine from bladder models infected with *Morg. morganii* SM18, *Pr. mirabilis* B2 and *Pr. mirabilis* super-infection of 24 h *Morg. morganii*

The results shown are the mean values of four replicate experiments. Experiments were run until blockage of both models containing *Pr. mirabilis*.

* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 25 h after *Morg. morganii* inoculation.

The mean numbers of organisms inoculated into the models (1.66×10^8 cfu/ml *Morg. morganii*) and (2.34×10^8 cfu/ml *Pr. mirabilis*) were not significantly different ($P > 0.05$).

12.01% and 6.51% respectively). These results suggest that perhaps these two species have some antagonistic influences towards *Pr. mirabilis*. In contrast, *Kl. pneumoniae* was one of three species whose occurrence in catheter biofilms was associated with an increased incidence of *Pr. mirabilis*. *Kl. pneumoniae* might therefore, offer some sort of beneficial interactions to *Pr. mirabilis*. Results of *Pr. mirabilis* super-infection of juvenile (24 h) biofilms of *E. coli* SM1, *Ps. aeruginosa* SM15, and *Kl. pneumoniae* SDM3 are presented in Tables 3.15 to 3.17 and Figures 3.27 to 3.35.

Table 3.15 shows the mean times to catheter blockage of super-infection experiments of 24 h developed catheter biofilms of *E. coli*, *Ps. aeruginosa*, and *Kl. pneumoniae*. Catheters from pure *E. coli*, *Ps. aeruginosa*, and *Kl. pneumoniae* infected models all drained continuously for the duration of the experiments. The mean time to blockage of catheters from control *Pr. mirabilis* models (27.23 ± 5.06 h) was increased by nearly 20 h when an *E. coli* biofilm had been allowed to develop on the catheter for 24 h. Similarly the presence of a 24 h grown *Ps. aeruginosa* or *Kl. pneumoniae* biofilm increased the mean times to catheter blockage by approximately 10 h and 5 h respectively. Statistical analysis indicated no significant differences ($P > 0.05$) between the mean blockage times of the *Pr. mirabilis* controls and the super-infections of either a 24 h developed *E. coli*, *Ps. aeruginosa*, or *Kl. pneumoniae* (TTB^3 transformation) biofilm.

The amounts of calcium and magnesium that had accumulated around the eye-hole and on the luminal surfaces of catheters were quantified and the results presented in Figures 3.27 to 3.29. Two of the three variables (calcium and total

Species X	Mean time to catheter blockage h (± SE mean)	
	Super-infection of 24 h species X biofilms with *Pr. mirabilis*	***Pr. mirabilis* controls**
E. coli SM1	45.43 (± 5.06)	27.23 (± 5.06)
Ps. aeruginosa SM15	43.19 (± 7.41)	33.37 (± 3.88)
Kl. pneumoniae SDM3	30.57 (± 1.31)	24.50 (± 1.31)

Table 3.15 – The mean times to catheter blockage of *Pr. mirabilis* B2 super-infections of 24 h developed catheter biofilms of *E. coli* SM1, *Ps. aeruginosa* SM15, and *Kl. pneumoniae* SDM3, and the respective *Pr. mirabilis* controls

Experiments were run until blockage of both *Pr. mirabilis* containing catheters. Values are the means of 4 replicate experiments.

No significant difference ($P > 0.05$) was indicated between the mean times to catheter blockage of each super-infection compared to the respective *Pr. mirabilis* control.

encrustation rates) were enhanced when *Pr. mirabilis* was super-infected into a 24 h *Ps. aeruginosa* population in comparison to the *Pr. mirabilis* controls (Figure 3.27). On the other hand magnesium rates were decreased by nearly 40 µg/h. Minimal deposition occurred on catheters from *E. coli* controls whereas there were high mean hourly rates (> 450 µg/h) on catheters from models containing *Pr. mirabilis* (Figure 3.28). Similarly *Pr. mirabilis* super-infection of 24 h *Kl. pneumoniae* (Figure 3.29) had mean rates of total calcium and magnesium deposition of > 350 µg/h in contrast to negligible amounts on catheters from pure *Kl. pneumoniae* infected models. The differences in mean deposition rates between *Pr. mirabilis* super-infections of 24 h *Ps. aeruginosa*, *E. coli*, and *Kl. pneumoniae* compared to the *Pr. mirabilis* controls were all non-significant ($P > 0.05$) when analysed using ANOVA.

The mean numbers of organisms inoculated into the models were not significantly different (2.51×10^8 cfu/ml *Ps. aeruginosa* and 2.40×10^8 cfu/ml *Pr. mirabilis*; $P > 0.05$), (1.48×10^8 cfu/ml *Kl. pneumoniae* and 1.55×10^8 *Pr. mirabilis*; Kruskal-Wallis $P > 0.05$) and (2.95×10^8 cfu/ml *E. coli* and 2.19×10^8 cfu/ml *Pr. mirabilis*; $P > 0.05$). Viable cell counts were carried out at various time points and the mean data from the start and end of the experiments can be seen in Table 3.16. The mean numbers of viable *Pr. mirabilis* cells obtained from the urine of super-infections of 24 h *E. coli* and *Ps. aeruginosa* decreased in comparison to the pure *Pr. mirabilis* models. Conversely, the presence of a 24 h *Kl. pneumoniae* community increased the numbers of *Proteus* bacilli. These changes in *Pr. mirabilis* figures however were all non-significantly different ($P > 0.05$; Kruskal-Wallis $P > 0.05$ for the *Ps. aeruginosa* data).

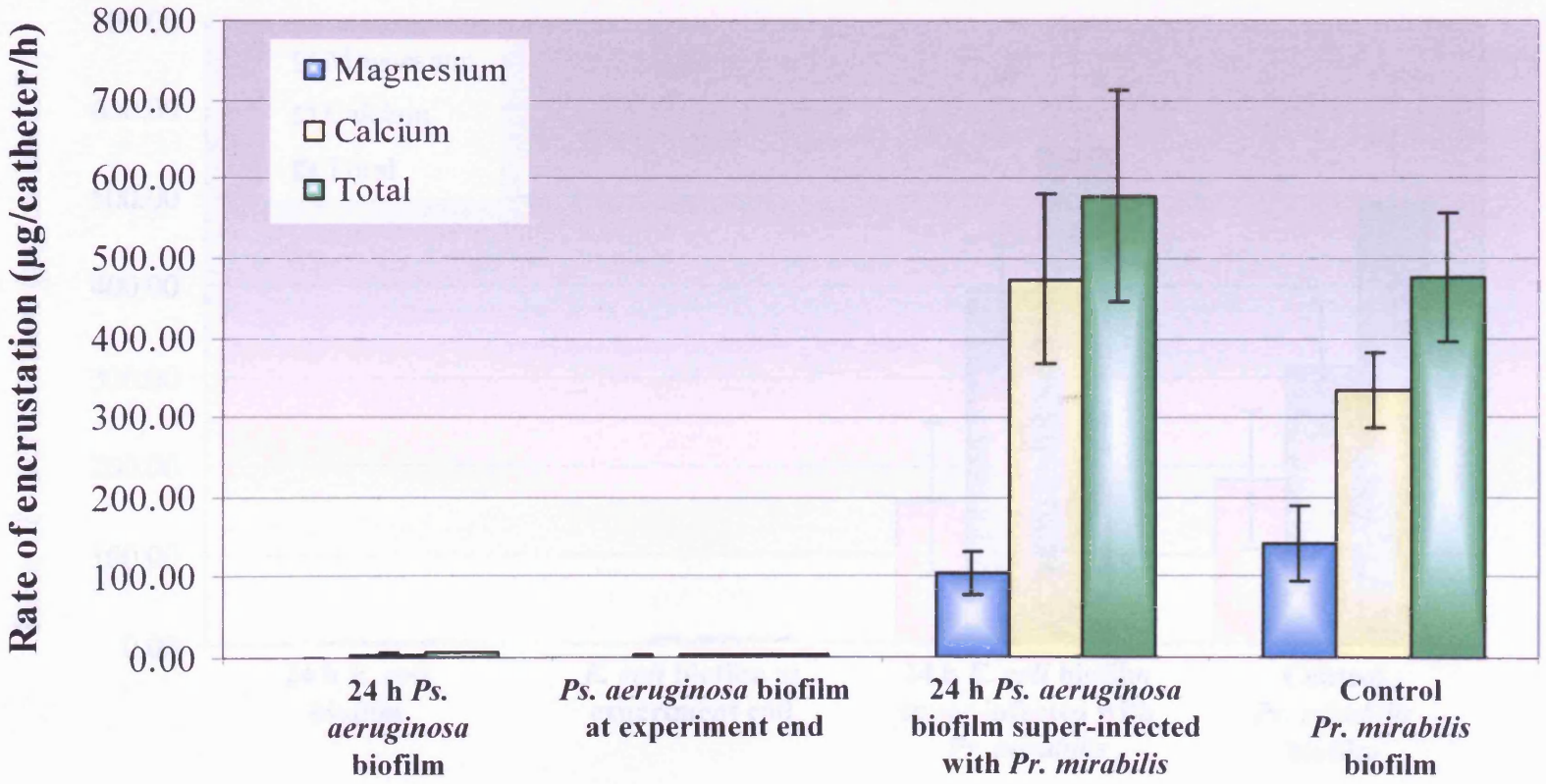


Figure 3.27 – The effect of 24 h *Ps. aeruginosa* SM15 biofilm on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except pure *Ps. aeruginosa* infected models which were stopped at either 24 h or when catheters from both *Pr. mirabilis* containing models had blocked. The rate of deposition of calcium and/or magnesium (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

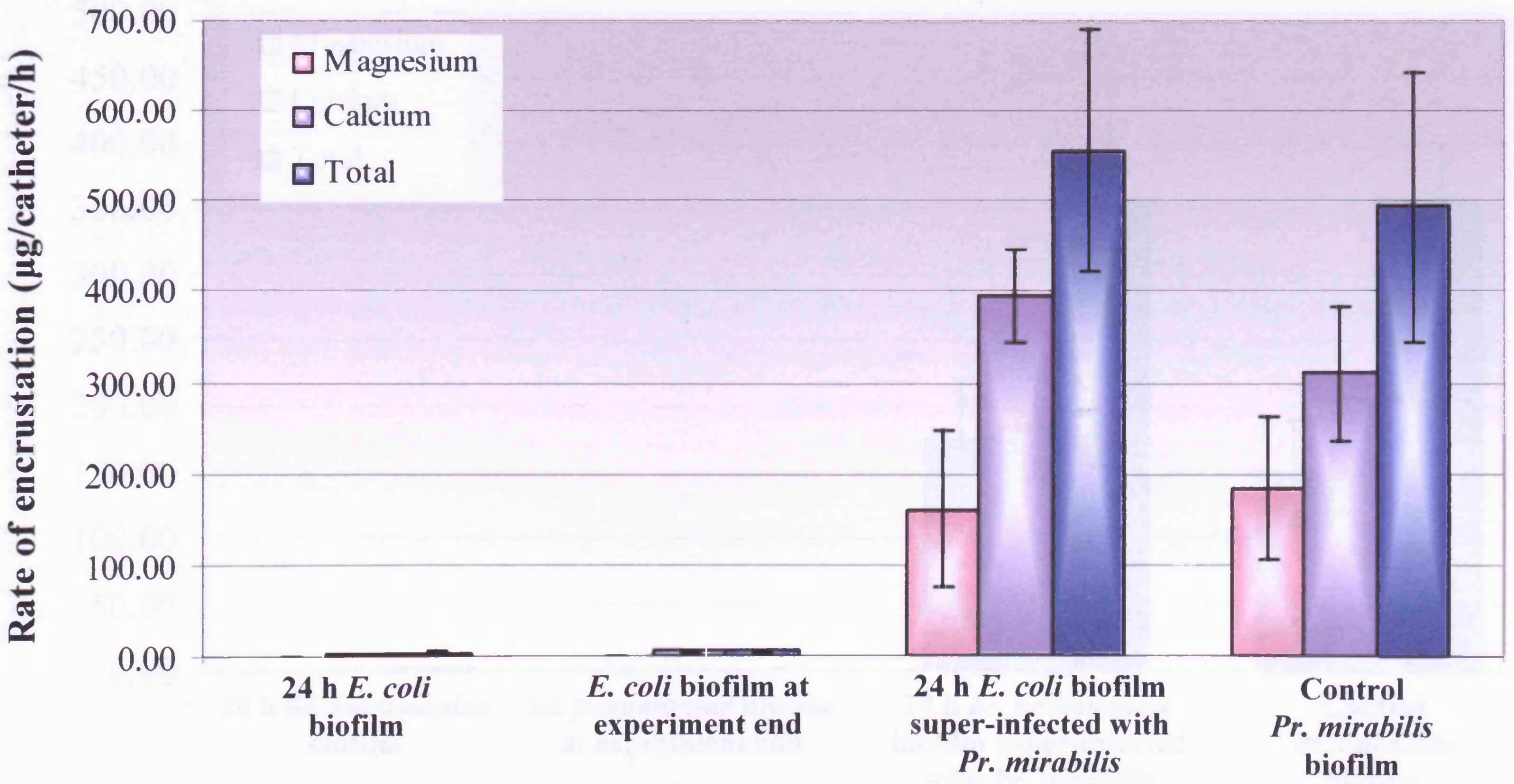


Figure 3.28 – The effect of 24 h *E. coli* SM1 biofilm on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except pure *E. coli* infected models which were stopped at either 24 h or when catheters from both *Pr. mirabilis* containing models had blocked. The rate of deposition of calcium and/or magnesium (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

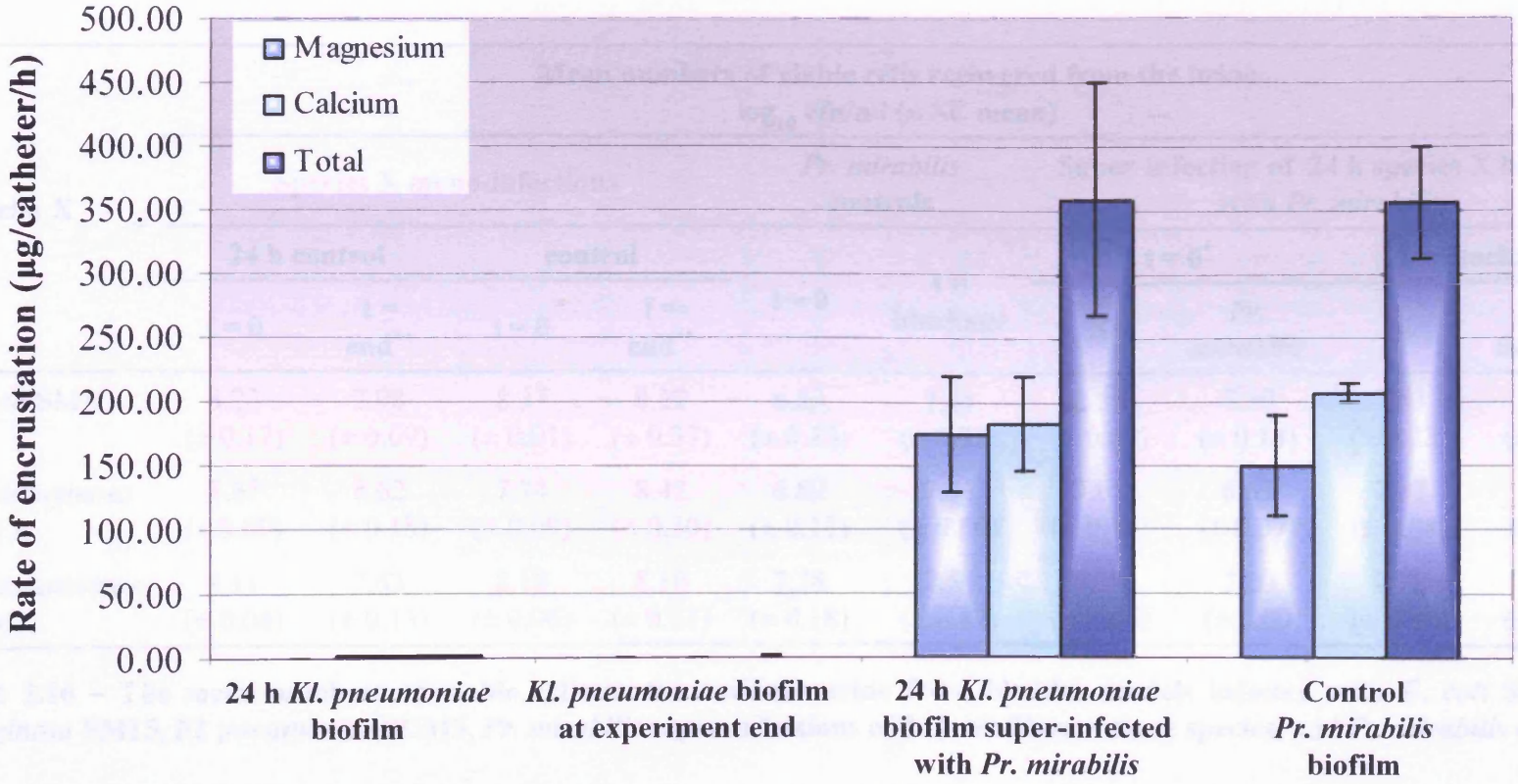


Figure 3.29 – The effect of 24 h *Kl. pneumoniae* SDM3 biofilm on the rate of catheter encrustation by *Pr. mirabilis* B2

Models were run until catheter blockage except pure *Kl. pneumoniae* infected models which were stopped at either 24 h or when catheters from both *Pr. mirabilis* containing models had blocked. The rate of deposition of calcium and/or magnesium (µg/catheter/h) was determined after removal from the models.

The results are the means of three replicate experiments. Error bars = standard error of the mean.

Species X	Mean numbers of viable cells recovered from the urine log_{10} cfu/ml (± SE mean)									
	Species X mono-infections				*Pr. mirabilis* controls		Super-infection of 24 h species X biofilms with *Pr. mirabilis*			
	24 h control		control				t = 0*		t = blockage	
	t = 0	t = end**	t = 0	t = end**	t = 0	t = blockage	X	*Pr. mirabilis*	X	*Pr. mirabilis*
E. coli SM1	8.22 (± 0.17)	7.98 (± 0.09)	8.37 (± 0.01)	8.22 (± 0.37)	6.89 (± 0.33)	7.41 (± 0.32)	8.34 (± 0.05)	7.30 (± 0.14)	7.63 (± 0.23)	6.88 (± 0.28)
Ps. aeruginosa SM15	7.67 (± 0.09)	8.62 (± 0.18)	7.74 (± 0.09)	8.42 (± 0.30)	6.82 (± 0.11)	7.51 (± 0.50)	7.65 (± 0.15)	6.63 (± 0.07)	7.17 (± 0.08)	7.23 (±0.33)
Kl. pneumoniae SDM3	8.11 (± 0.04)	7.63 (± 0.13)	8.18 (± 0.06)	8.10 (± 0.11)	7.38 (± 0.18)	7.54 (± 0.17)	8.18 (± 0.06)	7.20 (± 0.09	4.78 (± 0.96)	8.00 (± 0.11)

Table 3.16 – The mean numbers of viable cells in the residual urine from bladder models infected with *E. coli* SM1, *Ps. aeruginosa* SM15, *Kl. pneumoniae* SDM3, *Pr. mirabilis* super-infections of 24 h biofilms of these species, and *Pr. mirabilis* controls

Values are the means of 4 replicate experiments. Experiments were run until blockage of both *Pr. mirabilis* containing models.
* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 25 h after species X inoculation.
** In the case of the pure species X infected control models the urine was sampled either at 24 h or upon blockage of both *Pr. mirabilis* containing models.

In each case no significant difference ($P > 0.05$) was indicated between the mean numbers of *Pr. mirabilis* cells recovered from the urine of control *Pr. mirabilis* and super-infection models.

Measurements of pH were recorded at the same time points as viable cell counts were taken and are shown in Table 3.17. Urine samples from pure *E. coli*, *Ps. aeruginosa*, and *Kl. pneumoniae* infected models were consistently acidic throughout the experimental period despite the latter two organisms being urease positive. All models containing *Pr. mirabilis* had high alkaline end pHs ranging from 7.93 – 8.98. The mean pH at blockage of all three super-infections were not significantly different to the mean urinary pH of pure *Pr. mirabilis* infected models ($P > 0.05$; Kruskal-Wallis $P > 0.05$ for the *Kl. pneumoniae* data).

Catheters from the fourth replicate were examined using LV-SEM. In Figure 3.30 heavy or moderate encrustation was evident on each section of the two catheters that had *Pr. mirabilis* infected urine draining through it. In comparison the catheters that had *Kl. pneumoniae* flowing through them for the duration of the experiment showed no indications of crystal accumulation. These EM results are corroborated by images of the urine within the bladder chambers at various time points (Figure 3.31). The urine in the *Pr. mirabilis* control was heavily saturated with precipitate within 24 h. Although *Kl. pneumoniae* managed to stave off crystallisation for 24 h the presence of *Pr. mirabilis* ultimately resulted in urine containing large quantities of crystals and catheter blockage by a crystalline bacterial biofilm.

Blockage of both catheters in Figure 3.32 seemed to have occurred around the eye-hole section. Furthermore, the catheter from the super-infection model of *E. coli* SM1 had very heavy encrustation up to at least 20 cm from the catheter tip (Image 2E). The extensive precipitation in those models infected with *Pr.*

Species X	Mean urinary pH (± SE mean)							
	Species X mono-infections				*Pr. mirabilis* controls		Super-infection of 24 h species X biofilms with *Pr. mirabilis*	
	24 h control		Control					
	t = 0	t = end*	t = 0	t = end*	t = 0	t = blockage	t = 0	t = blockage
E. coli SM1	6.19 (± 0.02)	6.13 (± 0.02)	6.20 (± 0.01)	6.17 (± 0.04)	7.90 (± 0.42)	8.73 (± 0.17)	6.20 (± 0.02)	7.93 (± 0.34)
Ps. aeruginosa SM15	6.24 (± 0.04)	6.24 (± 0.02)	6.25 (± 0.02)	6.30 (± 0.03)	6.72 (± 0.04)	8.32 (± 0.23)	6.25 (± 0.01)	8.31 (± 0.29)
Kl. pneumoniae SDM3	6.81 (± 0.18)	6.16 (± 0.05)	6.76 (± 0.15)	6.34 (± 0.08)	7.29 (± 0.38)	8.98 (± 0.10)	6.77 (± 0.16)	8.91 (± 0.20)

Table 3.17 –The mean pH of the residual urine from bladder models infected with *E. coli* SM1, *Ps. aeruginosa* SM15, *Kl. pneumoniae* SDM3, *Pr. mirabilis* super-infections of 24 h biofilms of these species, and *Pr. mirabilis* controls

Values are the means of 4 replicate experiments. Experiments were run until blockage of both *Pr. mirabilis* containing models.
* In the case of the pure species X infected models the urine was sampled after both *Pr. mirabilis* containing models had blocked

In each case no significant difference ($P > 0.05$) was indicated between the mean urinary pH at blockage of *Pr. mirabilis* control and super-infections models.

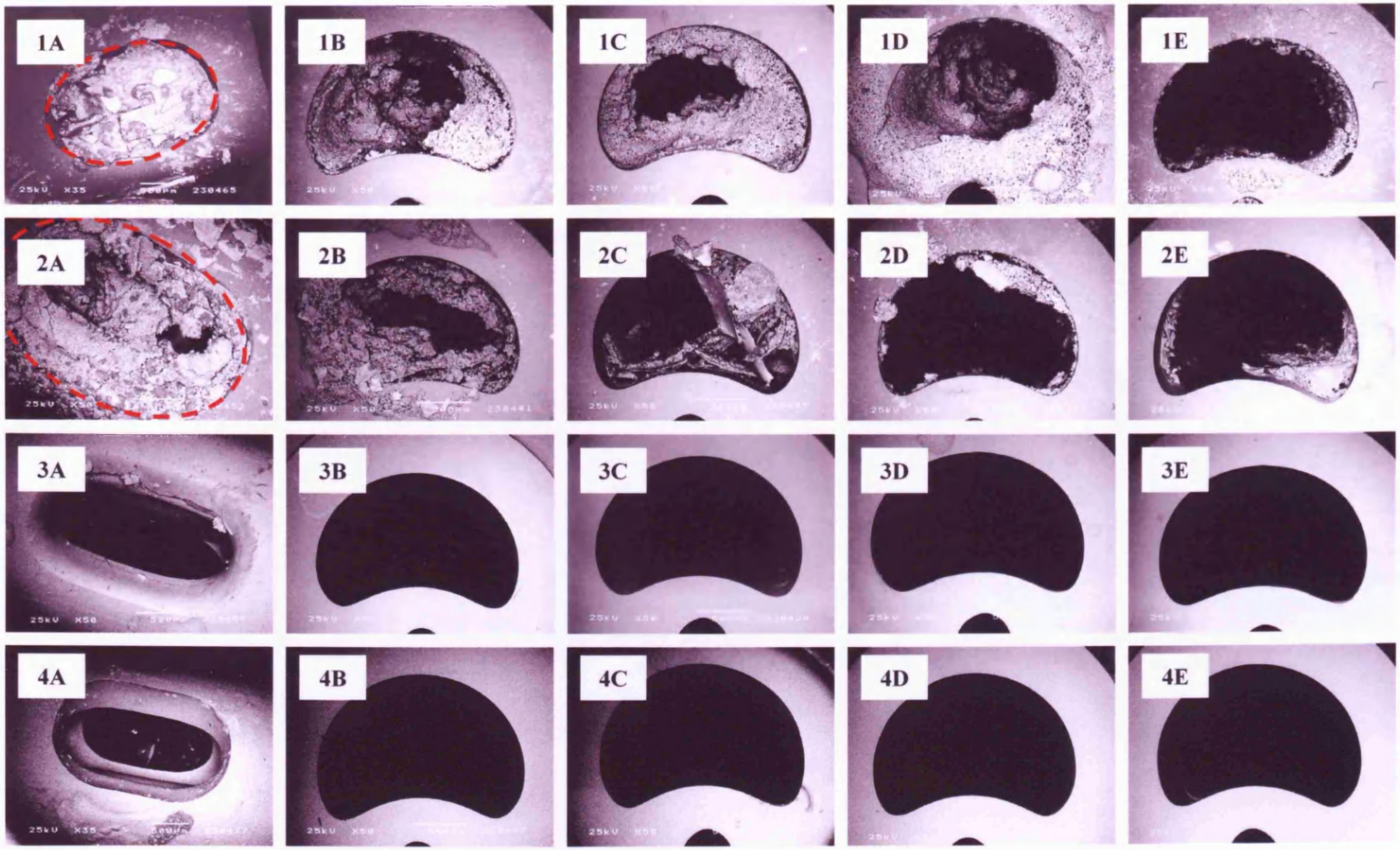


Figure 3.30 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 after 24 h *Kl. pneumoniae* SDM3 biofilm development, (3) *Kl. pneumoniae* SDM3 (24 h) (4) *Kl. pneumoniae* SDM3

Catheters were removed at blockage except the catheters from the pure *Kl. pneumoniae* infected models that were removed at either 24 h (3) or when both *Pr. mirabilis* containing models had blocked (4).

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

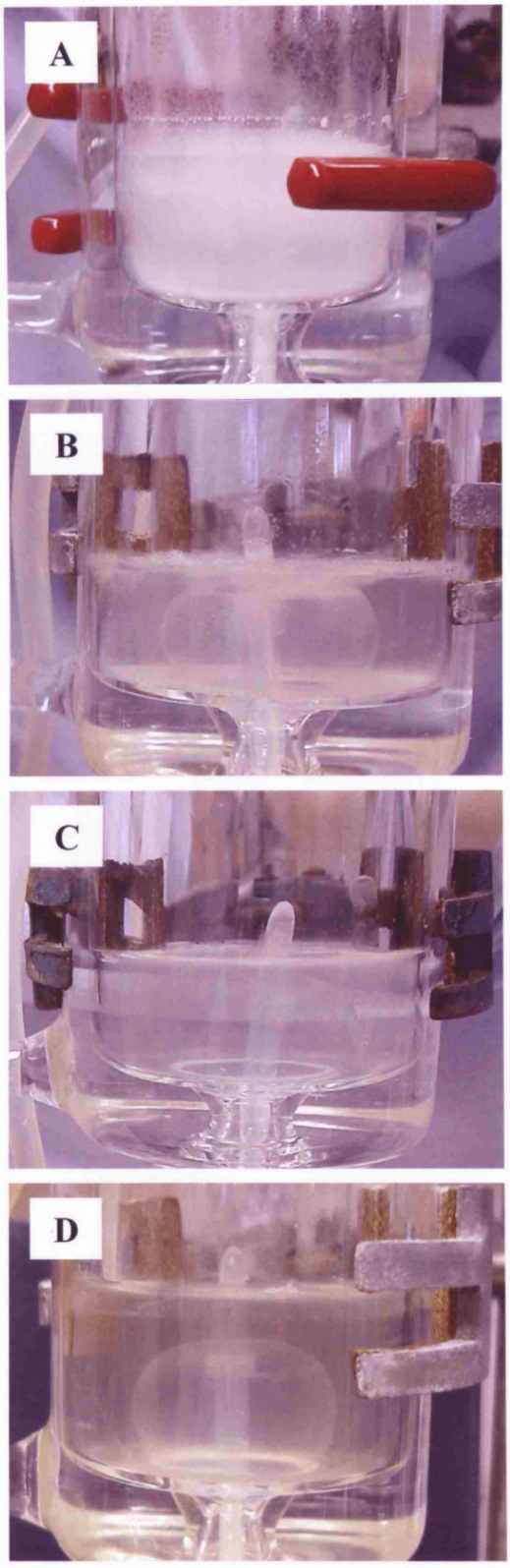


Figure 3.31 –The effect of a 24 h *Kl. pneumoniae* SDM3 biofilm on the extent of urinary crystalline precipitation caused by *Pr. mirabilis* B2

(A) *Pr. mirabilis* B2 at 24 h, (B) 24 h *Kl. pneumoniae* 24 h after super-infection with *Pr. mirabilis*, (C) *Kl. pneumoniae* at 48 h and (D) *Kl. pneumoniae* at 96 h.

Inocula sizes were 1 ml *Pr. mirabilis* and 10 ml *Kl. pneumoniae.*

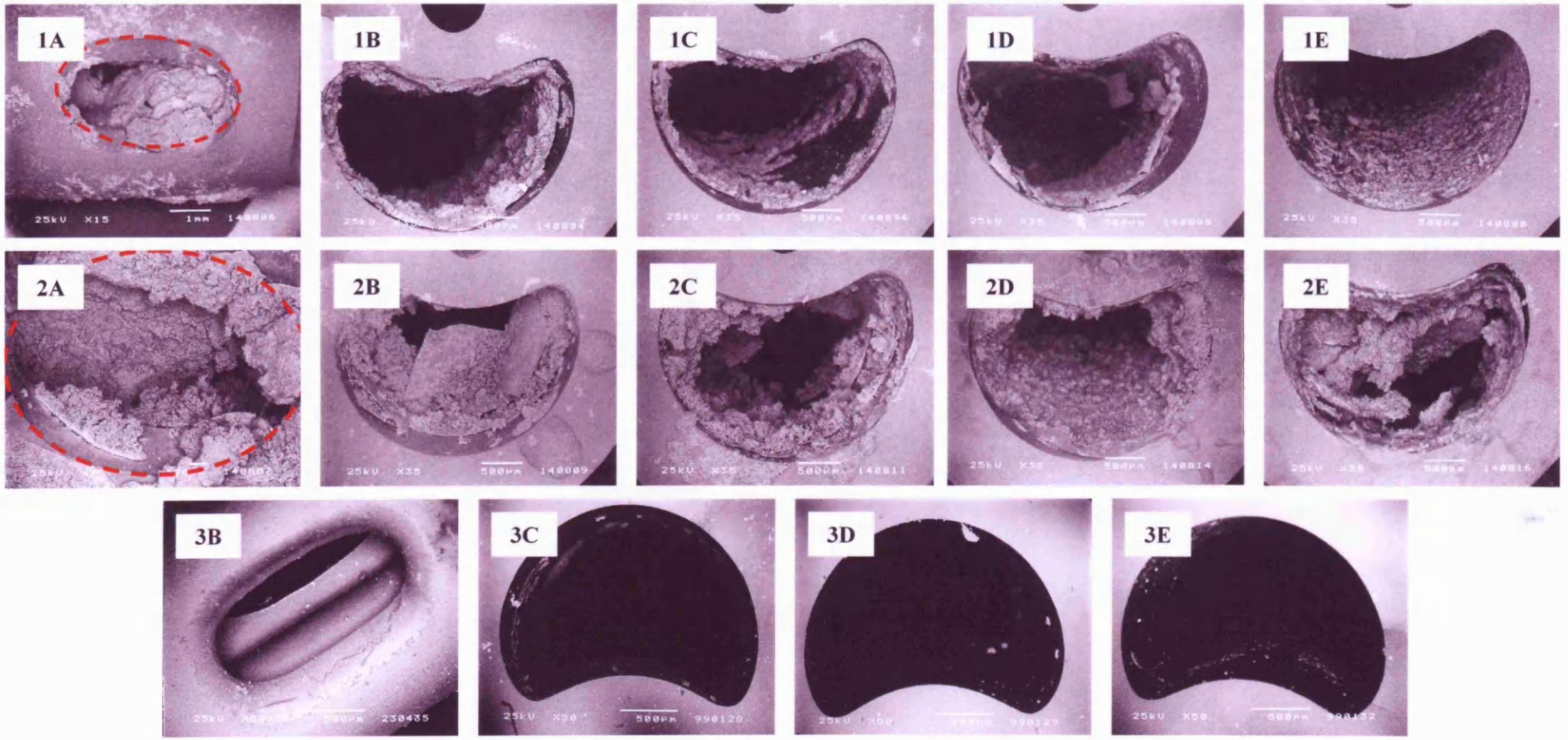


Figure 3.32 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 after 24 h *E. coli* SM1 biofilm development and (3) *E. coli* SM1

Catheters were removed at blockage except the catheter from the pure *E. coli* infected model that was removed when both *Pr. mirabilis* containing models had blocked.

The position of sections A-E are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-hole.

mirabilis was reduced when *E. coli* was present. Twenty-four hours after *Pr. mirabilis* infection deposits can be seen mainly on the top of the balloon and the base of the bladder chamber (Figure 3.33). However by 43 h, for the replicate shown, the extent of crystal formation was enough to prevent any further drainage of urine. Conversely, the urine in the *E. coli* controls remained free from precipitate and free from encrustation.

The catheter from the control *Ps. aeruginosa* inoculated model that had been *in vitro* for 24 h, and the one still draining freely at the end of the test period were encrustation free down the entire catheter length (Figure 3.34 Images 3A-E and 4A-E). A non-crystalline biofilm is evident in some sections especially where it was sloughed away from the luminal wall. A degree of encrustation was visible on each section down through to the distal end of catheters removed from the *Pr. mirabilis* control and the *Pr. mirabilis* super-infection after *Ps. aeruginosa* biofilm development for 24 h (Figure 3.34 Images 1B-E, 2A-E). The turbidity of the urine relating to crystal generation is evident in both models containing *Pr. mirabilis* (Figure 3.35 A and B). There was not an easily visible difference in the extent of precipitation in both models containing *Pr. mirabilis* 24 h after its inoculation. Figures 3.35 C and D show the lack of insoluble material accumulation in a *Ps. aeruginosa* control despite being a urease positive strain.

3.3.3 *Pr. mirabilis* super-infection of mature (72 h) biofilms

More mature biofilms of other species might of course have a greater ability to resist colonization by *Pr. mirabilis*. Experiments were therefore performed in models that had been "infected" with one of the four "test" species (*Et. cloacae*, *Morg. morganii*, *E. coli*, or *Ps. aeruginosa*) and subsequently "super-infected"

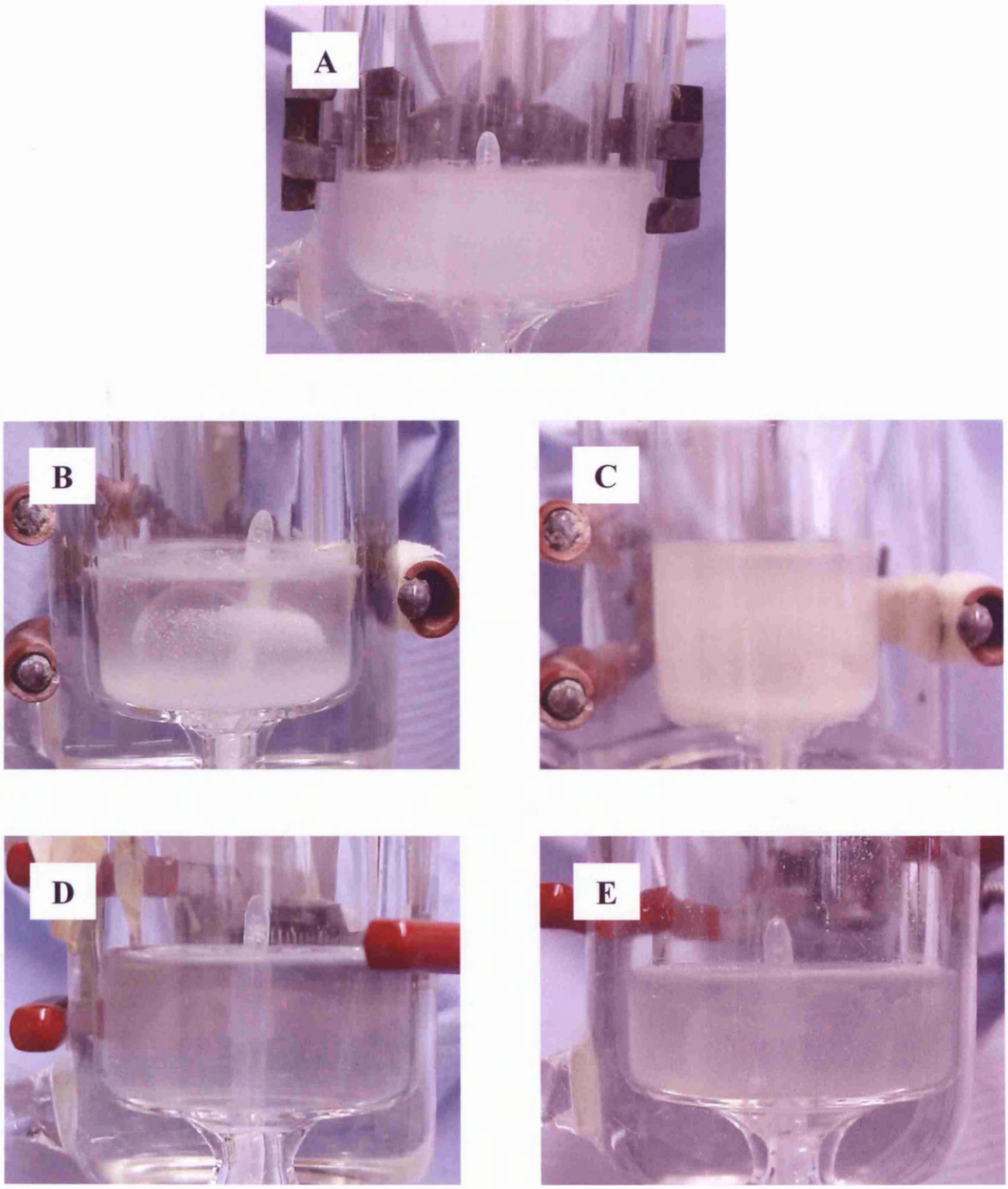


Figure 3.33 – The effect of a 24 h *E. coli* SM1 biofilm on the extent of urinary crystalline precipitation caused by *Pr. mirabilis* B2

(A) *Pr. mirabilis* B2 at 24 h, (B) 24 h *E. coli* 24 h after *Pr. mirabilis* super-infection, (C) 24 h *E. coli* 43 h after *Pr. mirabilis* super-infection (blockage), (D) *E. coli* at 48 h and (E) *E. coli* at 96 h.

Inocula sizes were 1ml *Pr. mirabilis* and 10 ml *E. coli*.

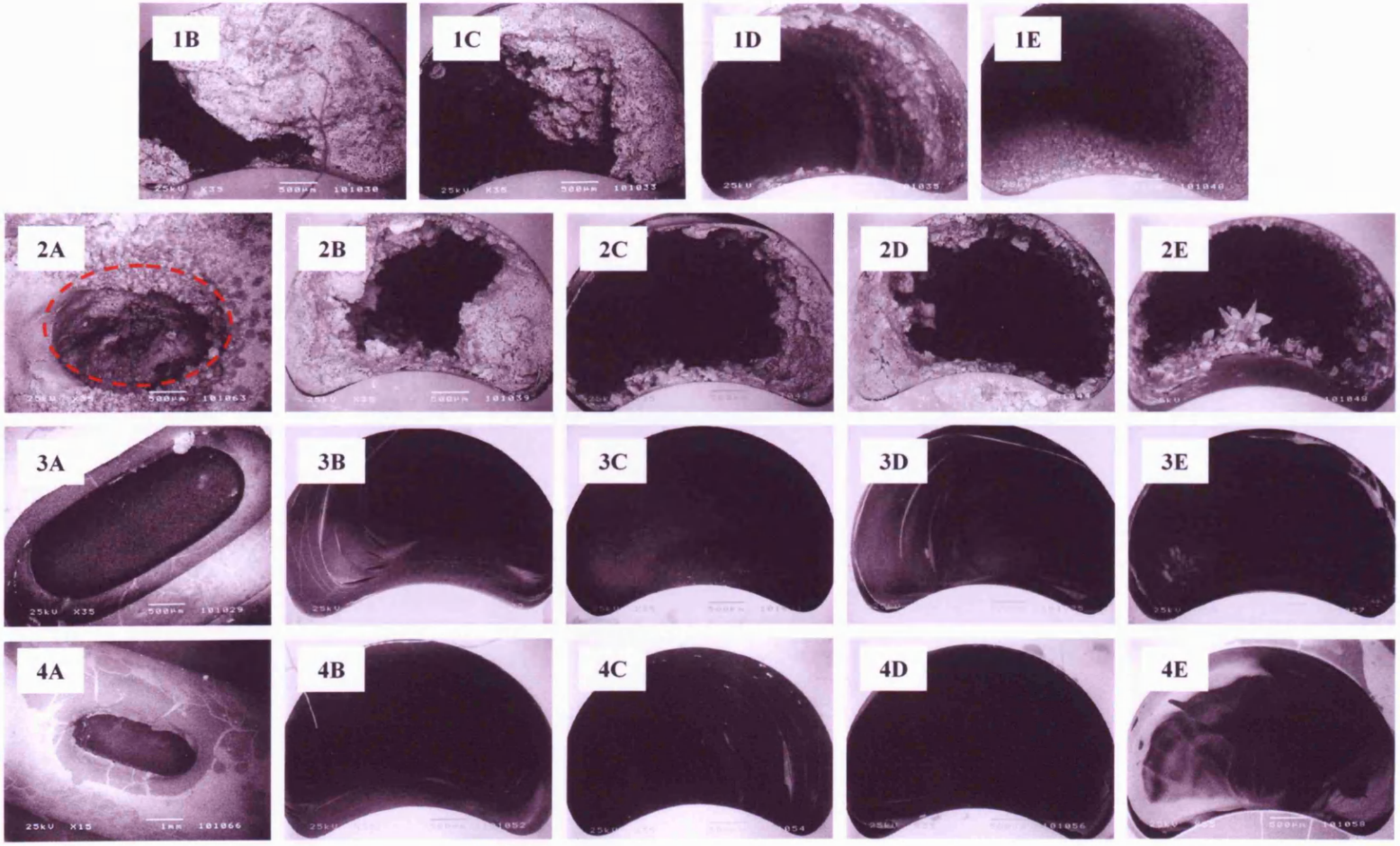


Figure 3.34 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* B2, (2) *Pr. mirabilis* B2 after 24 h *Ps. aeruginosa* SM15 biofilm development, (3) *Ps. aeruginosa* SM15 (24 h) and (4) *Ps. aeruginosa* SM15

Catheters were removed at blockage except the catheters from the pure *Ps. aeruginosa* infected models that were removed either at 24 h (3) or when both *Pr. mirabilis* containing models had blocked (4).

The position of sections A-E are indicated in Figure 2.7. Dashed red line indicate the position of the catheter eye-hole.

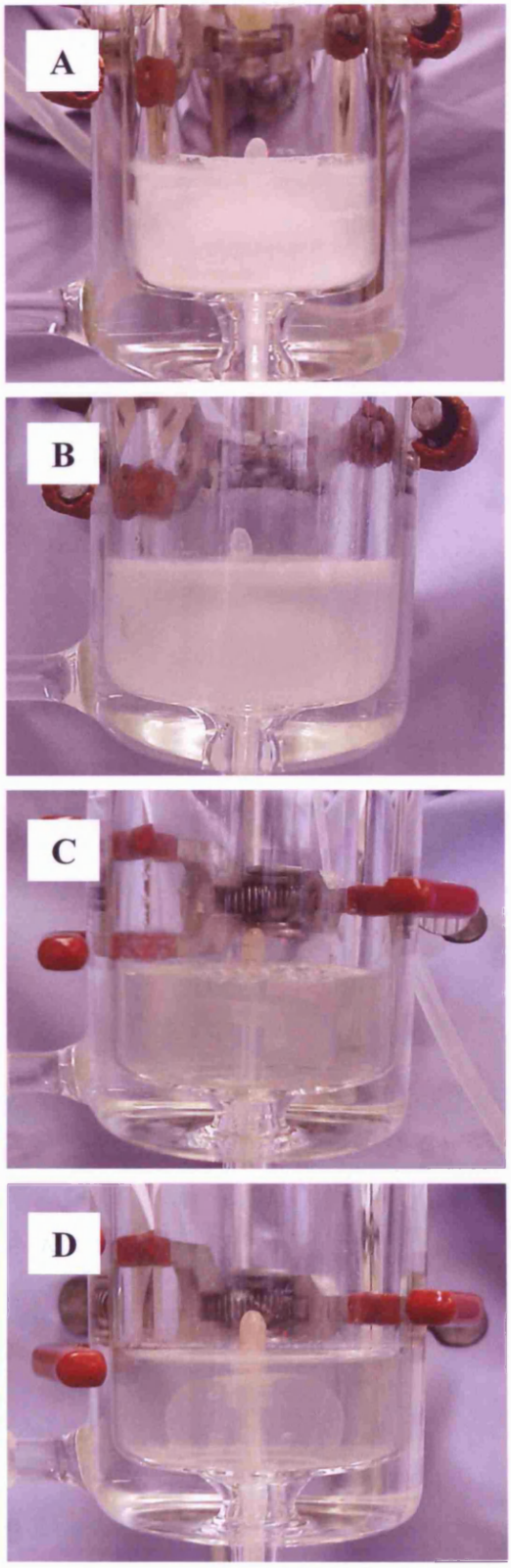


Figure 3.35 – The effect of a 24 h *Ps. aeruginosa* SM15 biofilm on the extent of urinary crystalline precipitation caused by *Pr. mirabilis* B2

(A) *Pr. mirabilis* at 24 h, (B) 24 h *Ps. aeruginosa* 24 h after super-infection with *Pr. mirabilis*, (C) *Ps. aeruginosa* at 48 h and (D) *Ps. aeruginosa* at 72 h.

Inocula sizes were 1 ml *Pr. mirabilis* and 10 ml *Ps. aeruginosa.*

with *Pr. mirabilis* after 72 h. Seventy-two hour *Kl. pneumoniae* biofilms were also super-infected with *Pr. mirabilis* to investigate the potential synergism between these two species that is implied from the data in Table 3.4. In addition, the ability of the five urinary tract pathogens to themselves form biofilms on all-silicone catheters was also assessed.

In these experiments, sets of four models were assembled in parallel. Cultures of the organisms, grown in artificial urine for 4 h at 37°C, were used as inocula. The test species (10 ml) was inoculated into three bladder models (models 1, 2 and 3) on day one of the experimental period. After 1 h to allow the organisms to establish themselves in the bladder chambers, urine was supplied to the models at 0.5 ml/min. At 72 h model 1 was dismantled. Model 3 had its urine supply disconnected and was super-infected with *Pr. mirabilis* B2 (1 ml). At the same time as super-infection a *Pr. mirabilis* control model (model 4) was also inoculated (1 ml). Both of these models were left for 1 h before the urine supply was resumed and sustained until both catheters had blocked. Model 2 was supplied with urine continuously and was allowed to drain until catheters from both *Pr. mirabilis* containing models had blocked. The urinary pH and the numbers of viable cells/ml of each species present in the urine were determined at 0, 1 and every 24 h after that until blockage or stoppage.

These experiments were performed in quadruplicate and in each case the time to catheter blockage was recorded. At the end of the experiments, the urine supply was turned off and the catheters removed from the models. In three of the replicate experiments the extent of catheter encrustation was determined by

analysis of the amounts of calcium and magnesium deposited per catheter. The rate of deposition was expressed as the amount (μg) of calcium and/or magnesium per catheter per h. Visual assessment of the biofilm formation by LV-SEM was performed on the catheters from the fourth replicate experiment. In addition, images of the residual urine in the bladder chambers at various time points were digitally captured.

In a further set of experiments the numbers of viable cells colonizing the catheter surfaces were enumerated. Sets of three models were prepared for each test species. One model was inoculated with the test organism and incubated for 72 h. A second model was inoculated with *Pr. mirabilis* and run until the catheter blocked. The third model was inoculated with the test species, incubated for 72 h, super-infected with *Pr. mirabilis* and incubated until blockage of the *Pr. mirabilis* control. The inocula were as described above. In each case the catheters were removed from the model and sectioned into two 1 cm portions (as shown in Figure 2.7). Each section was placed into 10 ml Ringer's solution and submitted to sonication (5 min) and a vortex mix (2 min). The numbers of viable cells in the resulting suspensions were determined by incubation on appropriate media for 24 h at 37°C. The numbers of viable cells colonizing each catheter was expressed as $\log_{10}$ cfu/cm. These experiments were performed in triplicate. Furthermore, high vacuum SEM was used to visualise the biofilms.

3.3.3.1 The effect of mature biofilms of other species on the ability of *Pr. mirabilis* to block catheters.

The effects of a 72 h established population of each of the five uropathogenic species on time to blockage are shown in Figure 3.36. For the purpose of the

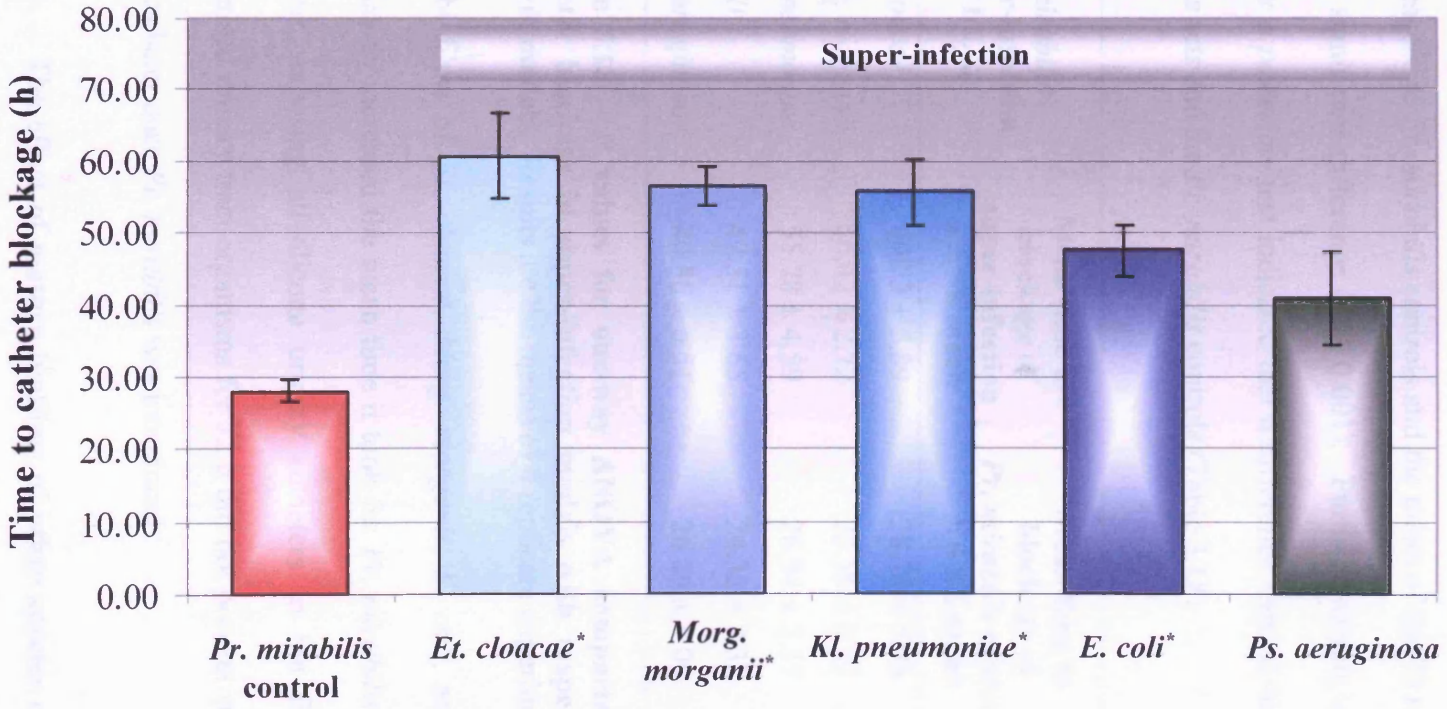


Figure 3.36 – The mean times *Pr. mirabilis* took to block catheters that were colonized by 72 h biofilms of other species

Urine grown cultures (4 h) of each organism were used as inocula. Control models were inoculated with *Pr. mirabilis* (1 ml). Test models were inoculated with one of the other species (10 ml), incubated for 72 h, and then super-infected with *Pr. mirabilis* (1 ml).

The results shown are the means of four replicate experiments except the *Pr. mirabilis* control (mean of 20 replicates). Error bars = standard error of the mean.

* The mean time to blockage of catheters from the *Et. cloacae*, *Morg. morganii*, *Kl. pneumoniae*, and *E. coli* super-infection models were significantly higher ($P \leq 0.05$) than that of their own respective *Pr. mirabilis* controls (data not shown) and the mean of the 20 replicates.

figure the time to blockage of the four *Pr. mirabilis* controls from each experimental set were grouped together to provide a mean of 20 replicates. One-way ANOVA comparing mean times to blockage of the super-infections with both their respective *Pr. mirabilis* controls and the mean of the 20 replicates revealed a highly significant difference ($P \leq 0.001$). Further analysis utilising the Tukey-Kramer *a posteriori* test indicated that a difference existed between only four of the data sets and the *Pr. mirabilis* controls (Table 3.18).

***Pr. mirabilis* super-infection of 72 h .....**	**Mean time to blockage of super-infection h ± SE mean**	**Mean time to blockage of *Pr. mirabilis* controls h ± SE mean**	**ANOVA *P* value**
Et. cloacae	60.73 ± 5.89	28.74 ± 5.98	0.009
Morg. morganii	56.42 ± 2.72	33.56 ± 0.57	0.000
Kl. pneumoniae	55.78 ± 4.59	26.94 ± 2.37	0.001
E. coli	47.51 ± 3.65	24.32 ± 2.37	0.002
Ps. aeruginosa	40.86 ± 6.51	26.86 ± 4.09	0.119

Table 3.18 – *P* values for one-way ANOVA comparing mean times to catheter blockage in super-infection models with respective *Pr. mirabilis* control models. Results are the means of 4 replicate experiments.

72 h biofilms of *Et. cloacae*, *Morg. morganii*, *E. coli*, and *Kl. pneumoniae* significantly increased the mean time it took for *Pr. mirabilis* to block catheters. However, exposing all-silicone urinary catheters to the five other common catheterised urinary tract organisms for 72 h did not prevent subsequent blockage of the catheter once *Pr. mirabilis* was introduced.

3.3.3.2 The effect of mature biofilms of other species on the numbers of viable *Pr. mirabilis* cells and the urinary pH

Samples were aspirated through the catheter sampling ports at t = 0 h, 1 h and every 24 h after that until blockage or stoppage at the end of the experiment. The

mean viable cell counts of all the inoculating cultures were not significantly different when analysed using ANOVA ($P > 0.05$; cell count^6 transformation). Numbers of viable cells recovered from the residual urine at the start and end of the experiments are shown in Tables 3.19 to 3.23.

The mean viable cell counts of *Pr. mirabilis* from the urine at t = 0 h were not significantly reduced in the presence of 72 h *Et. cloacae* compared to the *Pr. mirabilis* controls, as presented in Table 3.19 ($P > 0.05$). Similarly, at blockage *Pr. mirabilis* numbers were comparable in both *Pr. mirabilis* containing models. In contrast, at catheter blockage mean numbers of *Et. cloacae* had significantly decreased ($P \leq 0.05$), from 2.45 x 10^8 cfu/ml to 6.79 x 10^7 cfu/ml in super-infection models, whereas numbers in *Et. cloacae* controls remained similar throughout ($P > 0.05$). As shown in Table 3.20, in the presence of 72 h *Morg. morganii* the initial numbers of *Pr. mirabilis* were reduced ($P \leq 0.05$) in comparison to the *Pr. mirabilis* control (8.91 x 10^6 and 7.76 x 10^5 cfu/ml respectively). By the end of the experiments mean numbers of *Pr. mirabilis* in the super-infection models had recovered somewhat, yet they were still significantly different to numbers from control *Pr. mirabilis* infected models ($P \leq 0.05$). Viable cell counts of *Morg. morganii* were also significantly decreased ($P \leq 0.05$) at catheter blockage after *Pr. mirabilis* infection compared to numbers in *Morganella* mono-culture infected models.

Analysis of the data shown in Table 3.21 indicated that at catheter blockage mean numbers of *Pr. mirabilis* from the urine of super-infection models were comparable to numbers from control *Pr. mirabilis* infected models ($P > 0.05$).

Model	Mean numbers of viable cells recovered from the residual urine log_{10} cfu/ml (± SE mean)		Comments (t = end sample...)
	Time t = 0*	Time t = end	
Pr. mirabilis control	7.37 (± 0.16)	7.72 (± 0.17)	at blockage
Et. cloacae 72 h mon-infection	8.43 (± 0.08)	8.36 (± 0.02)	at 72 h
Et. cloacae mono-infection	8.37 (± 0.05)	8.77 (± 0.21)	at blockage of both *Pr. mirabilis* containing models
Super-infection models			
Pr. mirabilis	7.01 (± 0.43)	7.10 (± 0.22)	at blockage
Et. cloacae	8.39 (± 0.12)	7.83 (± 0.15)**	

Table 3.19 – The mean numbers of viable cells in the residual urine from bladder models infected with *Pr. mirabilis* B2, *Et. cloacae* RB19, and *Pr. mirabilis* super-infection of 72 h *Et. cloacae*

The results shown are the means of four replicate experiments.
* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 73 h after *Et. cloacae* infection.
** Numbers of viable *Et. cloacae* cells at catheter blockage were reduced ($P \leq 0.05$) in super-infection models compared to pure *Et. cloacae* controls.

The mean numbers of viable cells inoculated into the models (3.18×10^8 cfu/ml *Pr. mirabilis*) and (2.57×10^8 cfu/ml *Et. cloacae*) were not significantly different ($P > 0.05$).

Model	Mean numbers of viable cells recovered from the residual urine log_{10} cfu/ml (± SE mean)		Comments (t = end sample...)
	Time t = 0*	Time t = end	
Pr. mirabilis control	6.95 (± 0.22)	8.09 (± 0.06)	at blockage
Morg. morganii 72 h mono-infection	7.60 (± 0.10)	8.25 (± 0.23)	at 72 h
Morg. morganii mono-infection	7.74 (± 0.09)	8.07 (± 0.13)	at blockage of both *Pr. mirabilis* containing models
Super-infection models			
Pr. mirabilis	5.89 (± 0.32)	7.62 (± 0.13)**	at blockage
Morg. morganii	7.35 (± 0.10)	6.82 (± 0.26)***	

Table 3.20 – The mean numbers of viable cells in the residual urine from bladder models infected with *Pr. mirabilis* B2, *Morg. morganii* SM18, and *Pr. mirabilis* super-infection of 72 h *Morg. morganii*

The results shown are the means of four replicate experiments.
* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 73 h after *Morg. morganii* infection.
** Numbers of viable *Pr. mirabilis* cells at catheter blockage were reduced ($P \leq 0.05$) in super-infection models compared to pure *Pr. mirabilis* controls.
*** Numbers of viable *Morg. morganii* cells at catheter blockage were reduced ($P \leq 0.05$) in super-infection models compared to pure *Morg. morganii* controls.

The mean numbers of viable cells inoculated into the models (2.65×10^8 cfu/ml *Pr. mirabilis*) and (1.78×10^8 cfu/ml *Morg. morganii*) were not significantly different ($P > 0.05$)

Model	Mean numbers of viable cells recovered from the residual urine $\log_{10}$ cfu/ml (± SE mean)		Comments (t = end sample...)
	Time t = 0*	Time t = end	
Pr. mirabilis control	7.52 (± 0.13)	7.26 (± 0.29)	at blockage
E. coli 72 h mono-infection	8.39 (± 0.02)	7.50 (± 0.32)	at 72 h
E. coli mono-infection	8.36 (± 0.10)	8.25 (± 0.16)	at blockage of both *Pr. mirabilis* containing models
Super-infection models			
Pr. mirabilis	7.22 (± 0.07)	7.78 (± 0.21)	at blockage
E. coli	8.42 (± 0.03)	6.77 (± 0.61)	

Table 3.21 – The mean numbers of viable cells in the residual urine from bladder models infected with *Pr. mirabilis* B2, *E. coli* SM1, and *Pr. mirabilis* super-infection of 72 h *E. coli*

The results shown are the means of four replicate experiments.
* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 73 h after *E. coli* infection.

The mean numbers of viable cells inoculated into the models (2.66×10^8 cfu/ml *Pr. mirabilis*) and (3.67×10^8 cfu/ml *E. coli*) were not significantly different ($P > 0.05$).

After *Pr. mirabilis* super-infection numbers of *E. coli* decreased significantly from 2.40 x 10^8 to 1.82 x 10^7 cfu/ml (Kruskal-Wallis $P \leq 0.05$) but this decrease was equivalent to that seen in the pure *E. coli* models ($P > 0.05$).

At catheter blockage, mean numbers of *Ps. aeruginosa* from the urine of super-infection models were non-significantly different to that from the beginning of the experiment ($P > 0.05$; Table 3.22). In contrast, mean *Pr. mirabilis* densities from super-infection models significantly increased ($P \leq 0.05$) from 1.10 x 10^6 to 3.72 x 10^7 cfu/ml. Mean numbers of *Pr. mirabilis* were not significantly different between 72 h *Kl. pneumoniae* super-infected models and control *Pr. mirabilis* models at catheter blockage ($P > 0.05$; Table 3.23). However, mean *Kl. pneumoniae* densities in the super-infection models decreased from 2.40 x 10^8 cfu/ml at t = 0 h to 3.31 x 10^7 cfu/ml at catheter blockage. This decrease was statistically significant ($P \leq 0.05$) but was comparable to that observed in the pure *Kl. pneumoniae* models ($P > 0.05$).

Analysis of the data in Figure 3.37 revealed that 24 h after *Pr. mirabilis* inoculation all five super-infection models had a mean pH significantly below that of the 20 grouped *Pr. mirabilis* controls ($P \leq 0.001$). At 48 h after *Pr. mirabilis* infection (Figure 3.38) only super-infections of *Et. cloacae*, *Morg. morganii*, and *Kl. pneumoniae* had a mean urinary pH below both the *Pr. mirabilis* controls ($P \leq 0.01$). Figure 3.39 displays the mean end urinary pH measurements. At catheter blockage super-infections of both 72 h *Et. cloacae* RB19 and *Kl. pneumoniae* SDM3 still had mean urinary pH values that were significantly lower than the pure *Pr. mirabilis* inoculated models (Kruskal-Wallis $P \leq 0.05$).

Model	Mean numbers of viable cells recovered from the residual urine log_{10} cfu/ml (± SE mean)		Comments (t = end sample...)
	Time t = 0*	Time t = end	
Pr. mirabilis control	6.33 (± 0.20)	7.25 (± 0.23)	at blockage
Ps. aeruginosa 72 h mono-infection	7.39 (± 0.14)	8.93 (± 0.31)	at 72 h
Ps. aeruginosa mono-infection	7.44 (± 0.13)	7.73 (± 0.13)	at blockage of both *Pr. mirabilis* containing models
Super-infection models			
Pr. mirabilis	6.04 (± 0.31)	7.57 (± 0.34)	at blockage
Ps. aeruginosa	7.51 (± 0.12)	6.45 (± 0.46)	

Table 3.22 – The mean numbers of viable cells in the residual urine from bladder models infected with *Pr. mirabilis* B2, *Ps. aeruginosa* SM15, and *Pr. mirabilis* super-infection of 72 h *Ps. aeruginosa*

The results shown are the means of four replicate experiments.
* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 73 h after *Ps. aeruginosa* infection.

The mean numbers of viable cells inoculated into the models (3.04×10^8 cfu/ml *Pr. mirabilis*) and (2.79×10^8 cfu/ml *Ps. aeruginosa*) were not significantly different ($P > 0.05$).

Model	Mean numbers of viable cells recovered from the residual urine log_{10} cfu/ml (± SE mean)		Comments (t = end sample...)
	Time t = 0*	Time t = end	
Pr. mirabilis control	7.19 (± 0.26)	7.30 ± (0.40)	at blockage
Kl. pneumoniae 72 h mono-infection	8.30 (± 0.09)	7.67 ± (0.05)	at 72 h
Kl. pneumoniae mono-infection	8.31(± 0.12)	7.90 ± (0.19)	at blockage of both *Pr. mirabilis* containing models
Super-infection models			
Pr. mirabilis	6.89 (± 0.30)	6.93 ± (0.24)	at blockage
Kl. pneumoniae	8.38 (± 0.13)	7.52 ± (0.20)	

Table 3.23 – The mean numbers of viable cells in the residual urine from bladder models infected with *Pr. mirabilis* B2, *Kl. pneumoniae* SDM3, and *Pr. mirabilis* super-infection of 72 h *Kl. pneumoniae*

The results shown are the means of four replicate experiments.
* In the case of the super-infection models t = 0 for *Pr. mirabilis* was 73 h after *Kl. pneumoniae* infection.

The mean numbers of viable cells inoculated into the models (1.45×10^8 cfu/ml *Pr. mirabilis*) and (2.40×10^8 cfu/ml *Kl. pneumoniae*) were not significantly different ($P > 0.05$).

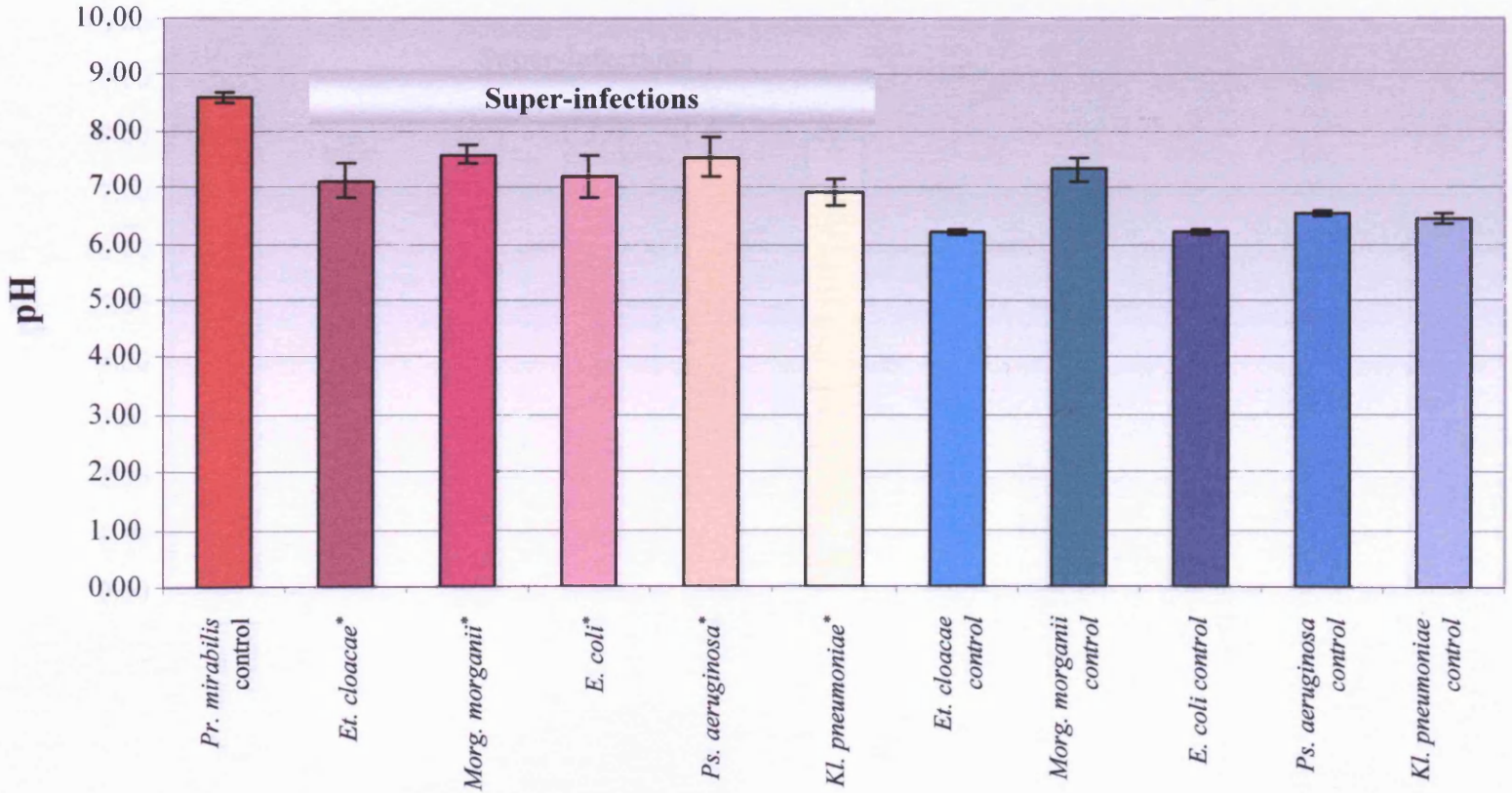


Figure 3.37 – Mean pH of the residual urine at 24 h (or blockage) after *Pr. mirabilis* inoculation (super-infection models and *Pr. mirabilis* controls) or 96 h (non-*Proteus* controls)

Super-infection models were inoculated with *Pr. mirabilis* after catheter colonization by 72 h biofilms of other species

Results are the means of four replicate experiments except the *Pr. mirabilis* control (mean of 20 replicates). Error bars = standard error of the mean. * All super-infection models had a mean urinary pH at 24 h post-*Proteus* infection that was significantly lower than their respective *Pr. mirabilis* controls ($P < 0.05$; data not shown) and the 20 grouped *Pr. mirabilis* controls ($P < 0.001$).

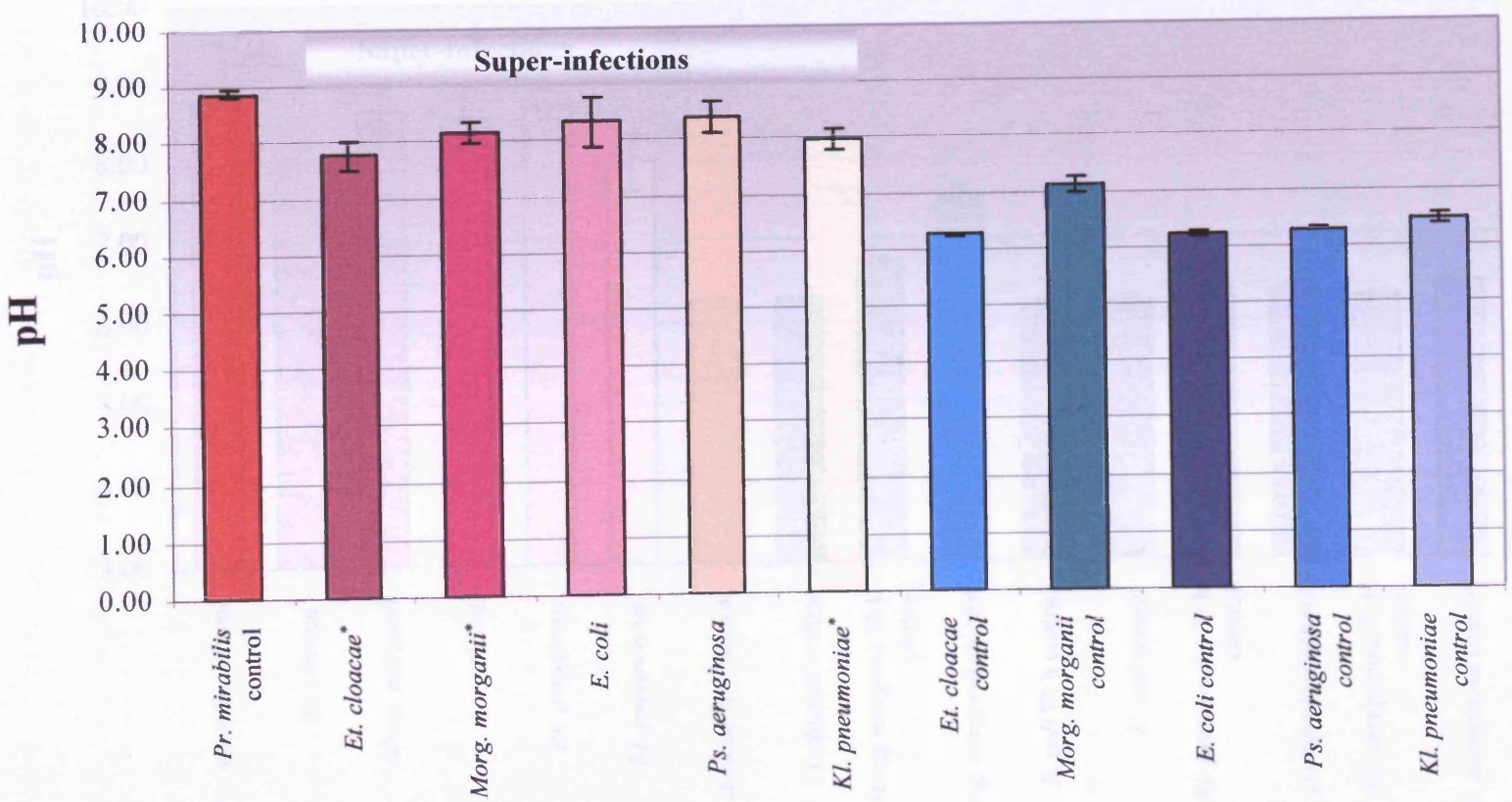


Figure 3.38 – Mean pH of the residual urine at 48 h (or blockage) after *Pr. mirabilis* inoculation (super-infection models and *Pr. mirabilis* controls) or 120 h (non-*Proteus* controls)

Super-infection models were inoculated with *Pr. mirabilis* after catheter colonization by 72 h biofilms of other species

Results are the means of four replicate experiments except the *Pr. mirabilis* control (mean of 20 replicates). Error bars = standard error of the mean. * Super-infections of *Et. cloacae*, *Morg. morganii* and *Kl. pneumoniae* had a mean urinary pH at 48 h post-*Proteus* infection that was significantly lower than their respective *Pr. mirabilis* controls ($P < 0.05$; data not shown) and the 20 grouped *Pr. mirabilis* controls ($P < 0.01$).

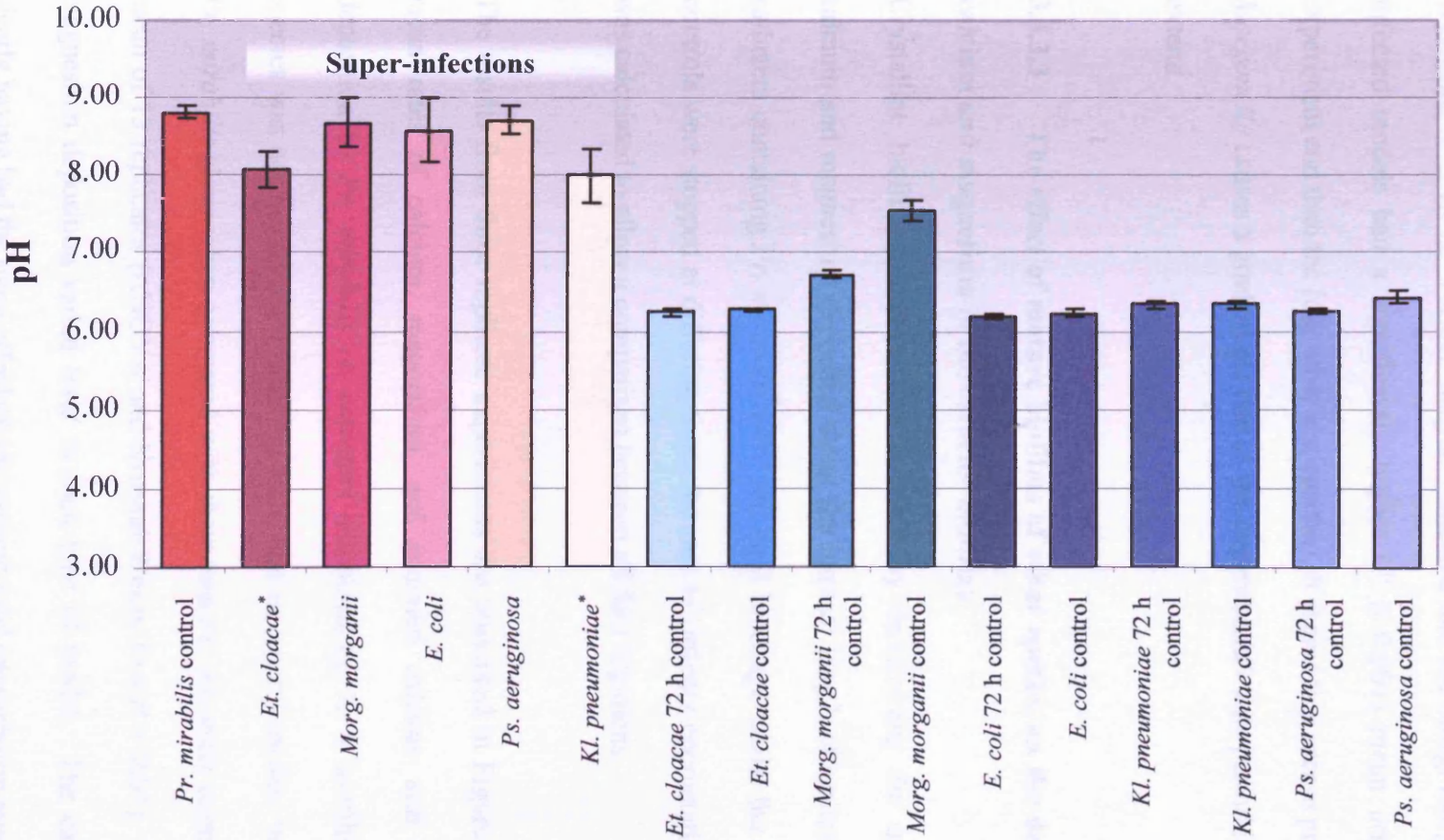


Figure 3.39 – Mean pH of the residual urine at experiment end of super-infection models (*Pr. mirabilis* super-infection of catheters colonized by 72 h biofilms of other species) and respective controls. All models containing *Pr. mirabilis* were run until catheter blockage. Test organisms were run for either 72 h or stopped upon blockage of both *Pr. mirabilis* containing models.

Results are the means of four replicate experiments except the *Pr. mirabilis* control (mean of 20 replicates). Error bars = standard error of the mean. * Super-infections of 72 h *Et. cloacae* and *Kl. pneumoniae* had mean urinary pHs at blockage that were significantly lower than the 20 *Pr. mirabilis* replicate controls ($P < 0.05$).

The pH values from all the non-*Proteus* infected models were all significantly lower than the *Pr. mirabilis* controls despite three of them being urease producing organisms ($P \leq 0.01$). Further analysis indicated that the *Morg. morganii* mono-infected models had a significantly higher ($P \leq 0.001$) mean urinary pH at experiment end than the four other test species. It seems that urease production by *Morganella* causes a gradual pH rise as the experimental time period is allowed to extend.

3.3.3.3 The effect of mature biofilms of other species on the deposition of calcium and magnesium in the catheter biofilms.

Crystalline biofilm formation was assessed by determining the quantities of calcium and magnesium deposited along the luminal length of catheters. As the catheters containing *Pr. mirabilis* were run until blockage whilst the test species controls were stopped at differing times the rate of catheter encrustation per hour was calculated to allow a comparison between all four regimens.

The results from three replicate experiments are presented in Figure 3.40. The mean rates of calcium, magnesium, and summed calcium and magnesium deposition by *Pr. mirabilis* on catheters colonized by 72 h biofilms of other species was not significantly different from that in control models infected with *Pr. mirabilis* alone when compared with their own *Pr. mirabilis* controls and the mean of 15 replicates (ANOVA and Kruskal-Wallis Test $P > 0.05$). The rate of magnesium deposition varied little in each type of model. The calcium rates clearly having had the main effect on the varying total encrustation amounts.

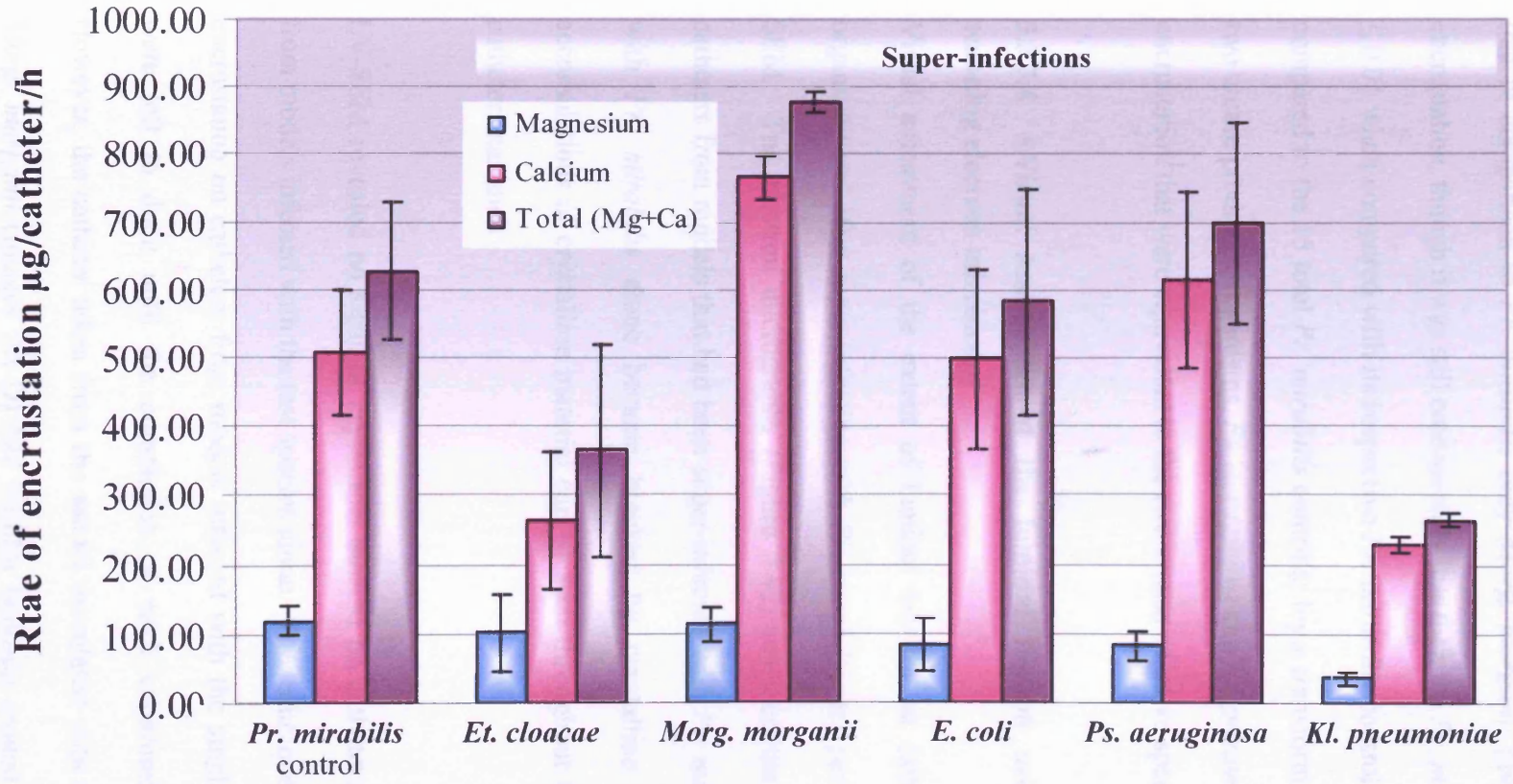


Figure 3.40 – The rates of encrustation by *Pr. mirabilis* B2 on catheters colonized by 72 h biofilms of other species

Control models were inoculated with *Pr. mirabilis* and super-infection models were inoculated with *Pr. mirabilis* after catheter colonization of the test organisms for 72 h.

Rates for the super-infection models were calculated using the time after *Pr. mirabilis* inoculation until blockage. The results presented are the means of three replicate experiments except *Pr. mirabilis* (mean of 15 replicates). Error bars = standard error of the mean.

All super-infection models had mean or median total encrustation rates that were non significantly different (ANOVA and Kruskal-Wallis $P > 0.05$) to that of the total 15 *Pr. mirabilis* replicates (shown) and their own *Pr. mirabilis* controls (data not shown).

The ability of the five non-*Proteus* species to cause luminal calcium and magnesium encrustation was also determined. The results in Figure 3.41 show that in comparison to *Pr. mirabilis* only *Morg. morganii* produced appreciable encrustation, though it was still considerably less than the *Pr. mirabilis* controls ($P \leq 0.01$ when compared with its respective *Pr. mirabilis* controls; $P \leq 0.001$ when compared to the 15 total *Pr. mirabilis* controls; $\log_{10}$ transformations). The other two urease producing organisms, *Ps. aeruginosa* and *Kl. pneumoniae*, had rates of encrustation that were equivalent to the two urease negative species ($P > 0.05$).

3.3.3.4 Visual assessment of the luminal biofilm using low vacuum scanning electron microscopy

Visual assessment of the extent of luminal biofilm on catheters by the test organisms and after super-infection with *Pr. mirabilis* was performed using LV-SEM. The electron micrographs (Figure 3.42) revealed that in all cases the catheters from models that had been super-infected with *Pr. mirabilis* or infected with *Pr. mirabilis* alone became blocked by crystalline biofilm. Dense accumulations of crystalline material can be seen throughout the lumen of each catheter examined.

LV-SEM revealed no signs of crystalline biofilm on catheters removed at 72 h from models infected with the test species alone. The technique also showed little encrustation on catheters from models infected with the single test species that were left to drain until the completion of each experiment (Figure 3.43). However, the catheter taken from the model inoculated with a mono-culture of *Morg. morganii* (Images 2A-D) had biofilm build-up around the eye-hole and very tightly packed slabs of encrustation, particularly visible in section B. In

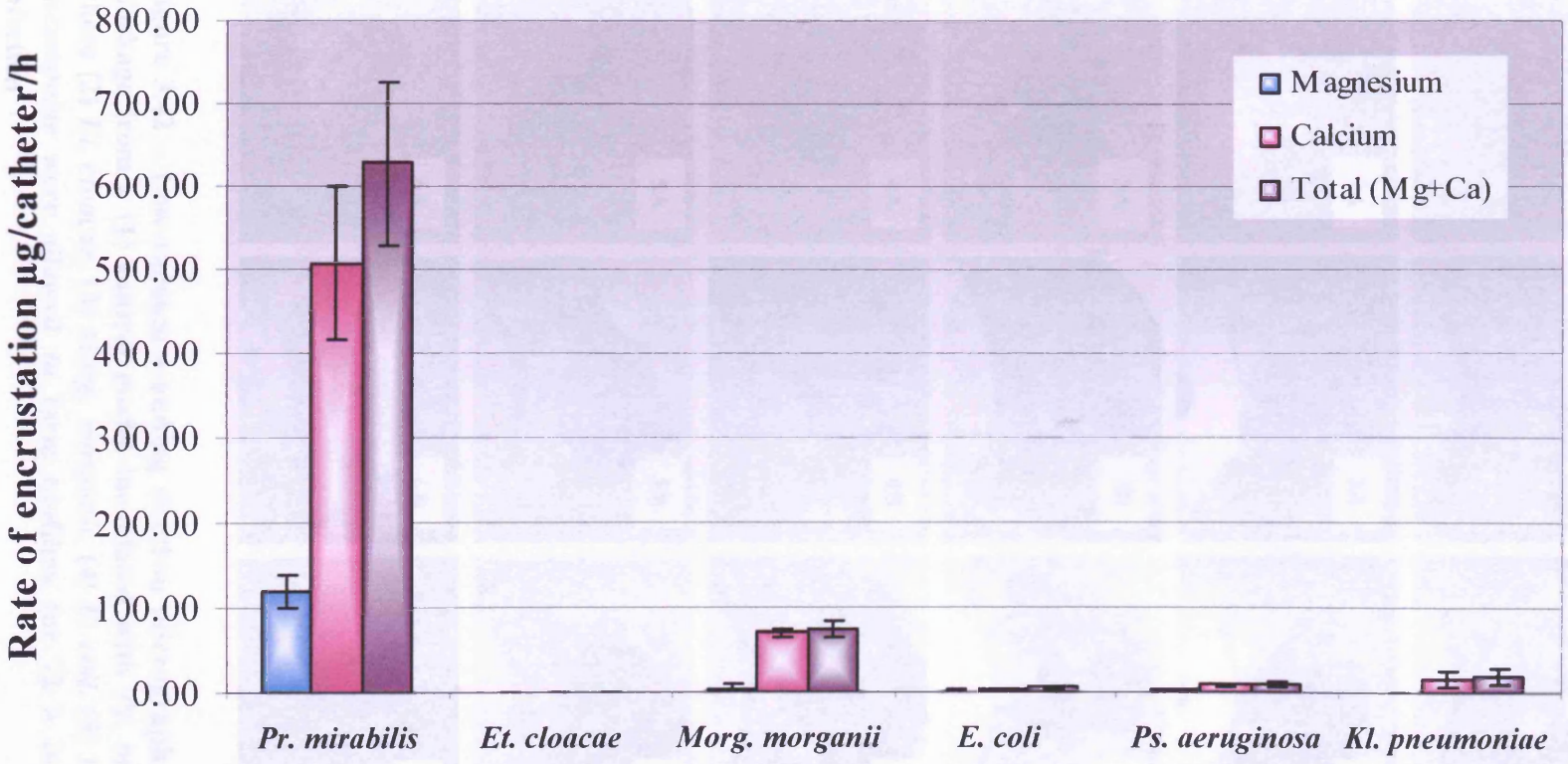


Figure 3.41 – The rates of catheter encrustation by various urinary tract pathogens

Models were inoculated with 10 ml of the respective organism except the *Pr. mirabilis* controls which were inoculated with 1 ml (4 h urine cultures at 37° C).

The results are the means of three replicate experiments. Error bars = standard error of the mean.

Highly significant differences are indicated ($P \leq 0.001$) between the mean rates of magnesium, calcium and summed magnesium and calcium deposition between the five test species controls in comparison to the 15 *Pr. mirabilis* controls.

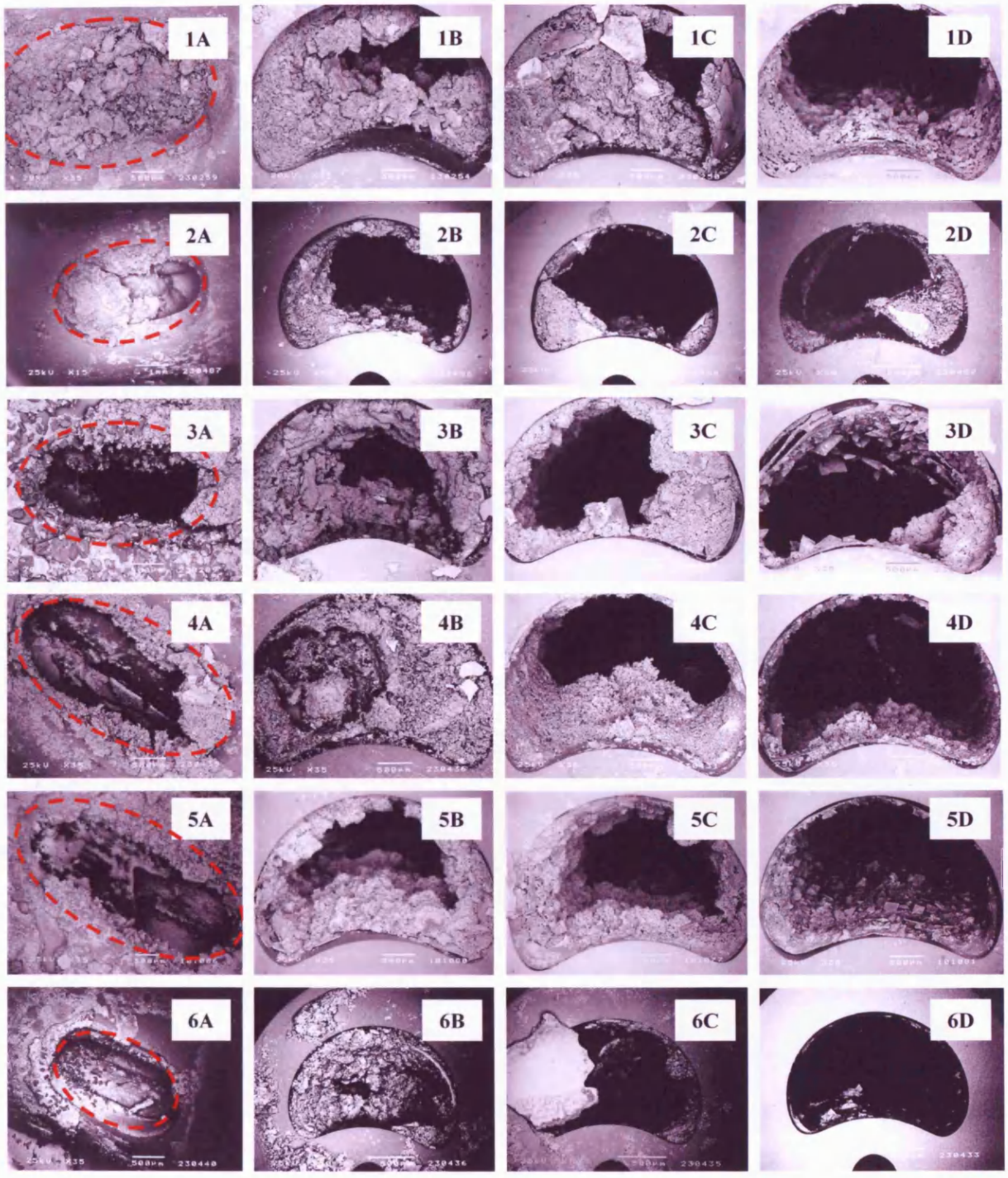


Figure 3.42 – Low-vacuum scanning electron micrographs of catheters removed at blockage from a (1) control model inoculated with *Pr. mirabilis*, and from models where (2) *Et. cloacae*, (3) *Morg. morganii*, (4) *E. coli*, (5) *Ps. aeruginosa*, and (6) *Kl. pneumoniae* were allowed to form biofilms for 72 h before *Pr. mirabilis* super-infection

The position of the sections A-D are indicated in Figure 2.7. Dashed red lines indicate the position of the catheter eye-holes.

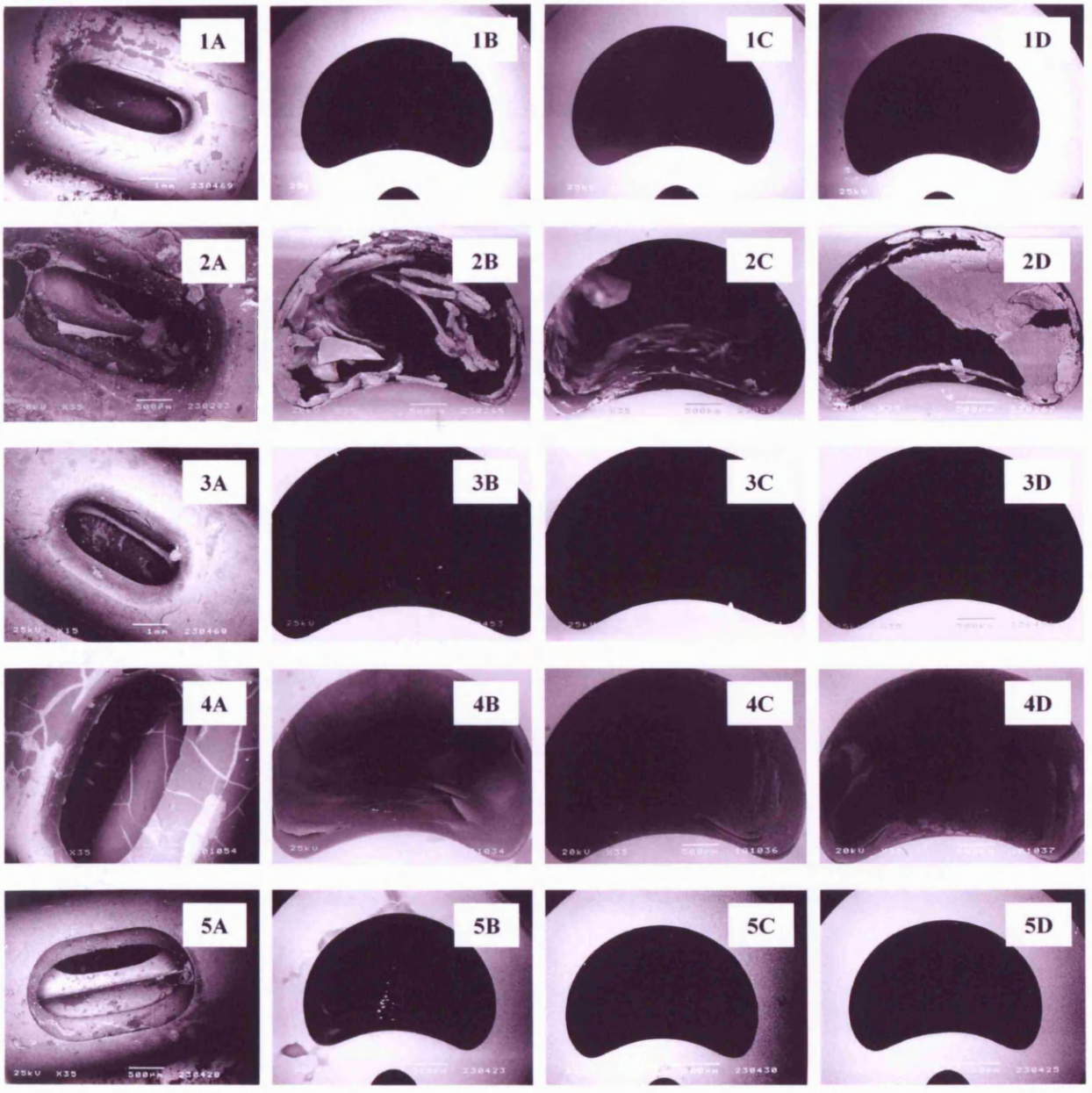


Figure 3.43 – Low-vacuum scanning electron micrographs of catheters removed from models infected with mono-cultures of (1) *Et. cloacae*, (2) *Morg. morganii*, (3) *E. coli*, (4) *Ps. aeruginosa*, and (5) *Kl. pneumoniae* at the end of each experiment upon blockage of both *Pr. mirabilis* containing models

The position of the sections A-D are indicated in Figure 2.7.

addition, a non-crystalline biofilm was evident sloughing away from the luminal wall on the catheter removed from the pure *Ps. aeruginosa* infected model (Images 4B-D).

3.3.3.5 The ability of *Pr. mirabilis* to colonize 72 h biofilms of other species

At the end of the experiments to test the ability of *Pr. mirabilis* to encrust catheters that were colonized by 72 h biofilms of other species, catheters were removed from the models and the numbers of the various organisms colonizing the luminal surfaces enumerated. The pH of the cell suspensions after biofilm disruption were also recorded.

The biofilm viable cell counts for the catheters removed from control models infected with *Pr. mirabilis*, models incubated with the test organism for 72 h before super-infection with *Pr. mirabilis*, and the 72 h incubated pure test organism models are presented in Tables 3.24 to 3.28. The catheters taken from the test organism infected models at 72 h were used to establish the numbers of attached cells prior to *Pr. mirabilis* inoculation. Super-infection models were stopped at blockage of the *Pr. mirabilis* control. This allowed the numbers of *Pr. mirabilis* incorporated into the 72 h developed biofilms to be compared to the numbers of the organism when allowed to form mono-species biofilms. Mean viable cell counts of the inoculating cultures were non-significantly different in each case ($P > 0.05$). Statistical analysis of the mean *Pr. mirabilis* numbers colonizing catheters from *Pr. mirabilis* mono-culture infected models and super-infection models revealed that only a 72 h *Et. cloacae* biofilm significantly reduced the mean numbers of *Pr. mirabilis* colonizing the 1-2 cm catheter section

Model	Number of cells recovered from the catheter log_{10} cfu/cm (± SE mean)	
	1-2 cm section	**3-4 cm section**
Et. cloacae mono-infection at 72 h	7.93 (± 0.22)	7.65 (± 0.35)
Pr. mirabilis control at blockage	7.58 (± 0.14)	7.46 (± 0.22)
Super-infection models at blockage of *Pr. mirabilis* control		
Et. cloacae	8.12 (± 0.28)	7.71 (± 0.11)
Pr. mirabilis	6.05 (± 0.53)*	6.62 (± 0.56)

Table 3.24 – The ability of *Pr. mirabilis* to colonize 72 h *Et. cloacae* biofilms

Results are the means of three replicate experiments.

The mean numbers of viable cells inoculated into the models (3.18×10^8 cfu/ml *Pr. mirabilis*) and 2.57×10^8 cfu/ml *Et. cloacae*) were not significantly different ($P > 0.05$).

* Numbers of *Pr. mirabilis* recovered from catheters from models that had been super-infected with *Pr. mirabilis* after 72 h biofilm formation by *Et. cloacae* were significantly lower ($P \leq 0.05$) than *Pr. mirabilis* controls.

Model	Number of cells recovered from the catheter log_{10} cfu/cm (± SE mean)	
	1-2 cm section	**3-4 cm section**
Morg. morganii mono-infection at 72 h	7.29 (± 0.26)	7.03 (± 0.53)
Pr. mirabilis control at blockage	7.30 (± 0.30)	6.49 (± 0.60)
Super-infection models at blockage of *Pr. mirabilis* control		
Morg. morganii	7.33 (± 0.55)	6.02 (± 0.31)
Pr. mirabilis	7.37 (± 0.46)	6.36 (± 0.39)

Table 3.25 – The ability of *Pr. mirabilis* to colonize 72 h *Morg. morganii* biofilms

Results are the means of three replicate experiments.

The mean numbers of viable cells inoculated into the models (2.65×10^8 cfu/ml *Pr. mirabilis*) and 1.78×10^8 cfu/ml *Morg. morganii*) were not significantly different ($P > 0.05$).

No significant difference was indicated ($P > 0.05$) between numbers of both organisms recovered from catheters removed from super-infection models and respective controls.

Model	Number of cells recovered from the catheter log_{10} cfu/cm (± SE mean)	
	1-2 cm section	**3-4 cm section**
E. coli mono-infection at 72 h	8.03 (± 0.18)	7.49 (± 0.33)
Pr. mirabilis control at blockage	7.52 (± 0.18)	7.29 (± 0.52)
Super-infection models at blockage of *Pr. mirabilis* control		
E. coli	6.24 (± 0.86)	6.23 (± 0.53)
Pr. mirabilis	6.87 (± 0.56)	6.63 (± 0.76)

Table 3.26 – The ability of *Pr. mirabilis* to colonize 72 h *E. coli* biofilms

Results are the means of three replicate experiments.

The mean numbers of viable cells inoculated into the models (2.66×10^8 cfu/ml *Pr. mirabilis*) and 3.67×10^8 cfu/ml *E. coli*) were not significantly different ($P > 0.05$).

No significant difference was indicated ($P > 0.05$) between numbers of both organisms recovered from catheters removed from super-infection models and respective controls.

Model	Number of cells recovered from the catheter log_{10} cfu/cm (± SE mean)	
	1-2 cm section	**3-4 cm section**
Ps. aeruginosa mono-infection at 72 h	8.75 (± 0.12)	9.05 (± 0.08)
Pr. mirabilis control at blockage	6.70 (± 0.40)	7.17 (± 0.83)
Super-infection models at blockage of *Pr. mirabilis* control		
Ps. aeruginosa	6.13 (± 0.78)*	6.22 (± 0.51)*
Pr. mirabilis	6.39 (± 0.72)	6.76 (± 0.40)

Table 3.27 – The ability of *Pr. mirabilis* to colonize 72 h *Ps. aeruginosa* biofilms

Results are the means of three replicate experiments.

The mean numbers of viable cells inoculated into the models (3.04×10^8 cfu/ml *Pr. mirabilis*) and 2.79×10^8 cfu/ml *Ps. aeruginosa*) were not significantly different ($P > 0.05$).

* Numbers of *Ps. aeruginosa* recovered from catheters from models that had been super-infected with *Pr. mirabilis* were significantly lower ($P \leq 0.05$) than from catheters removed from pure *Ps. aeruginosa* infected models.

Model	Number of cells recovered from the catheter log_{10} cfu/cm (± SE mean)	
	1-2 cm section	**3-4 cm section**
Kl. pneumoniae mono-infection at 72 h	6.99 (± 0.05)	7.25 (± 0.87)
Pr. mirabilis control at blockage	6.60 (± 0.41)	6.04 (± 0.55)
Super-infection models at blockage of *Pr. mirabilis* control		
Kl. pneumoniae	6.77 (± 0.16)	6.03 (± 0.42)
Pr. mirabilis	6.52 (± 0.49)	6.30 (± 0.47)

Table 3.28 – The ability of *Pr. mirabilis* to colonize 72 h *Kl. pneumoniae* biofilms

Results are the means of three replicate experiments

The mean numbers of viable cells inoculated into the models (1.45×10^8 cfu/ml *Pr. mirabilis*) and 2.40×10^8 cfu/ml *Kl. pneumoniae*) were not significantly different ($P > 0.05$).

No significant difference was indicated ($P > 0.05$) between numbers of both organisms recovered from catheters removed from super-infection models and respective controls.

($P \leq 0.05$). In addition, *Ps. aeruginosa* was the only organism to have significantly lower ($P \leq 0.01$) numbers of attached cells on both sections of catheters from the super-infection models compared to catheters from control models inoculated with *Ps. aeruginosa.* With the exception of the biofilm generated by super-infection of *Et. cloacae*, the catheter biofilm suspensions from all of the models that had been infected by *Pr. mirabilis* had a pH > 8 (Table 3.29).

3.3.3.6 Observations on the bacterial colonization of catheters using high vacuum scanning electron microscopy

Sections of catheters removed from infected models were examined by high-vacuum SEM after fixation with hexaammineruthenium trichloride and critical point dehydration to visualise bacterial colonization. Two sections of each catheter were prepared, the 0-1 cm section (the eyehole region) and the 2-3 cm from the catheter tip section.

Figures 3.44 to 3.46 show scanning electron micrographs of sections from catheters subjected to 72 h biofilm development of the five test organisms. Dense bacterial biofilms were evident on all catheters. These images established the extent of bacterial attachment prior to *Pr. mirabilis* inoculation and confirmed the lack of crystalline material within these biofilms.

Catheters removed from models infected with *Pr. mirabilis* alone were observed after catheter blockage. Some of the images obtained are presented in Figure 3.47. An extensive and heavily crystalline biofilm was observed on the luminal

Model	Mean pH of suspension (± SE mean)	
	1-2 cm section	3-4 cm section
E. coli mono-infection	6.75 (± 0.17)	6.70 (± 0.20)
Ps. aeruginosa mono-infection	6.93 (± 0.06)	7.02 (± 0.08)
Et. cloacae mono-infection	7.19 (± 0.12)	7.16 (± 0.10)
Et. cloacae super-infection	7.28 (± 0.06)*	7.25 (± 0.13)**
Kl. pneumoniae mono-infection	7.44 (± 0.21)	7.68 (± 0.40)
Morg, morganii mono-infection	7.74 (± 0.22)	7.57 (± 0.13)
E. coli super-infection	8.15 (± 0.11)*	7.24 (± 0.38)**
Kl. pneumoniae super-infection	8.33 (± 0.53)	8.08 (± 0.49)**
Morg. morganii super-infection	8.57 (± 0.32)	8.26 (± 0.43)**
Ps. aeruginosa super-infection	9.32 (± 0.08)	9.32 (± 0.07)***
Pr. mirabilis control	9.10 (± 0.10)	9.05 (± 0.11)

Table 3.29 – The mean pH of the suspensions generated from the disruption of the biofilm on two catheter sections taken from models super-infected with *Pr. mirabilis* at 72 h and respective controls

Control catheters were removed at 72 h except *Pr. mirabilis* controls which were removed upon catheter blockage. Catheters from super-infection models were removed when the corresponding *Pr. mirabilis* control catheter had blocked.

The results presented are the mean of 3 replicate experiments except the *Pr. mirabilis* control (mean of 15 replicates).

* Super-infections of 72 h *Et. cloacae* and *E. coli* produced a mean 1-2 cm biofilm pH that was significantly lower than the *Pr. mirabilis* control ($P \leq 0.05$).
** All super-infections except *Ps. aeruginosa* had significantly lower 3-4 cm biofilm pH compared to the *Pr. mirabilis* control ($P \leq 0.05$).
*** Super-infection of a 72 h biofilm of *Ps. aeruginosa* significantly increased the mean 3-4 cm pH compared to the *Pr. mirabilis* control ($P \leq 0.05$).

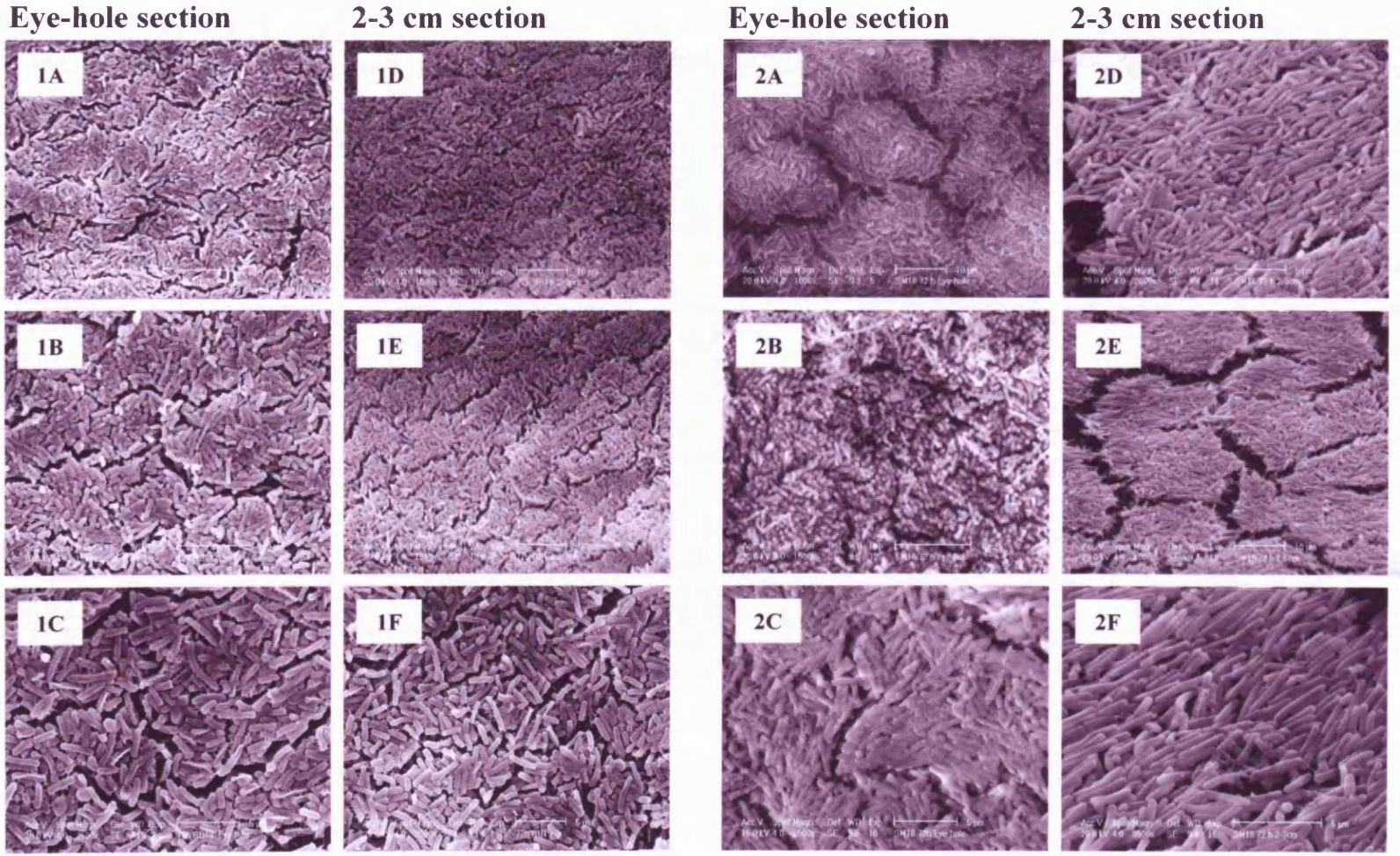


Figure 3.44 – High vacuum SEM images displaying the bacterial biofilm on the luminal surface of two catheter sections removed at 72 h from models inoculated with (1) *Et. cloacae* RB19 and (2) *Morg. morganii* SM18

A thick lawn of bacilli covered the luminal surface of each catheter. No crystalline encrustation was observed.

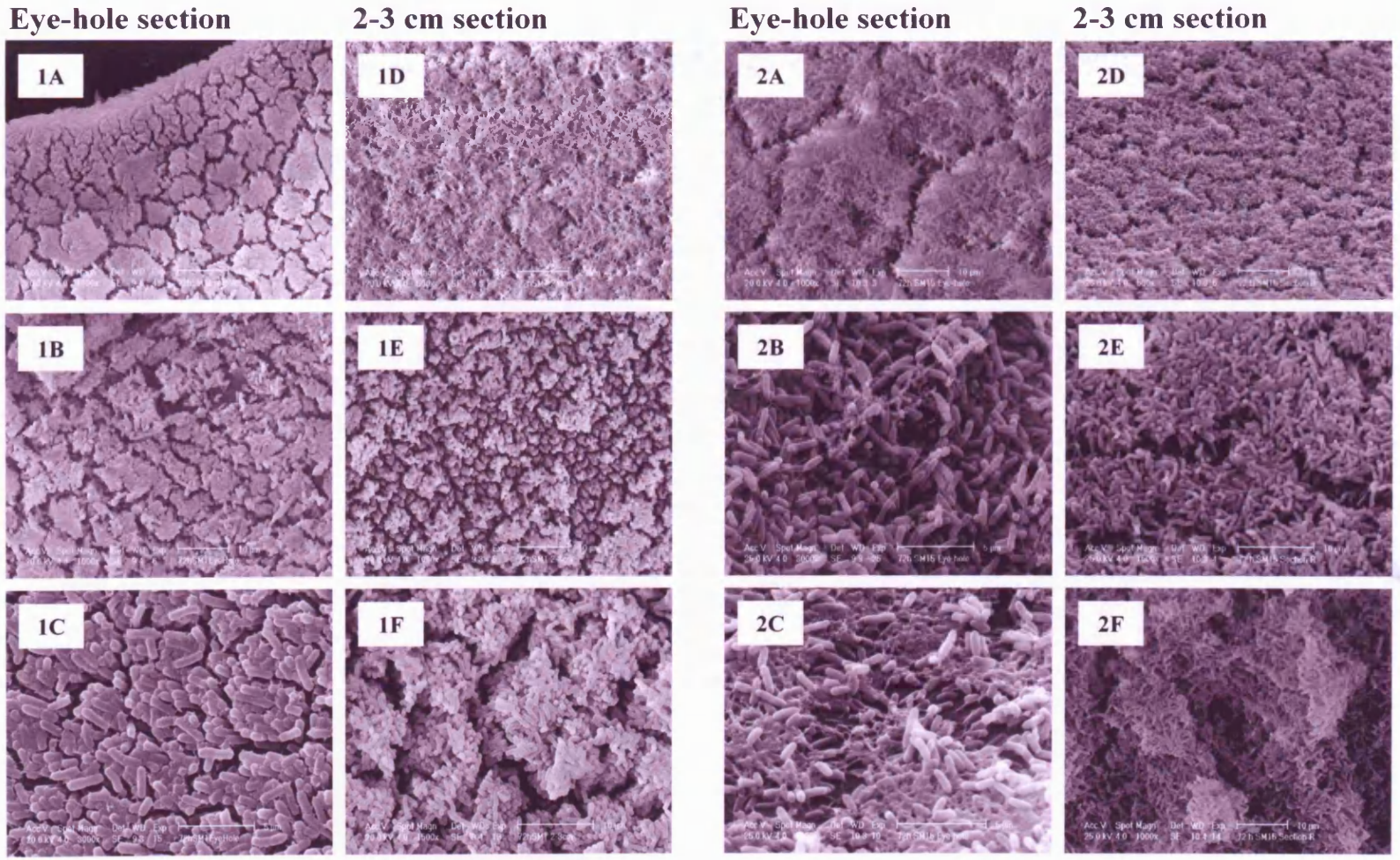
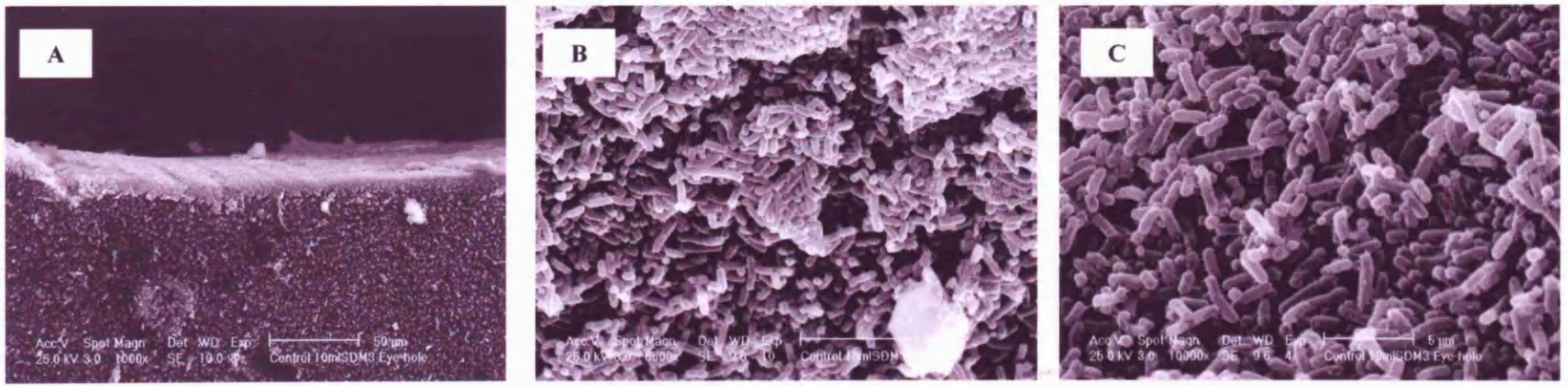


Figure 3.45 – High vacuum SEM images displaying the bacterial biofilm on the luminal surface of two catheter sections removed at 72 h from models inoculated with (1) *E. coli* SM1 and (2) *Ps. aeruginosa* SM15

No crystalline material was observed but a thick biofilm of bacterial cells covered both sections from both catheters.

Eye-hole section



2-3 cm section

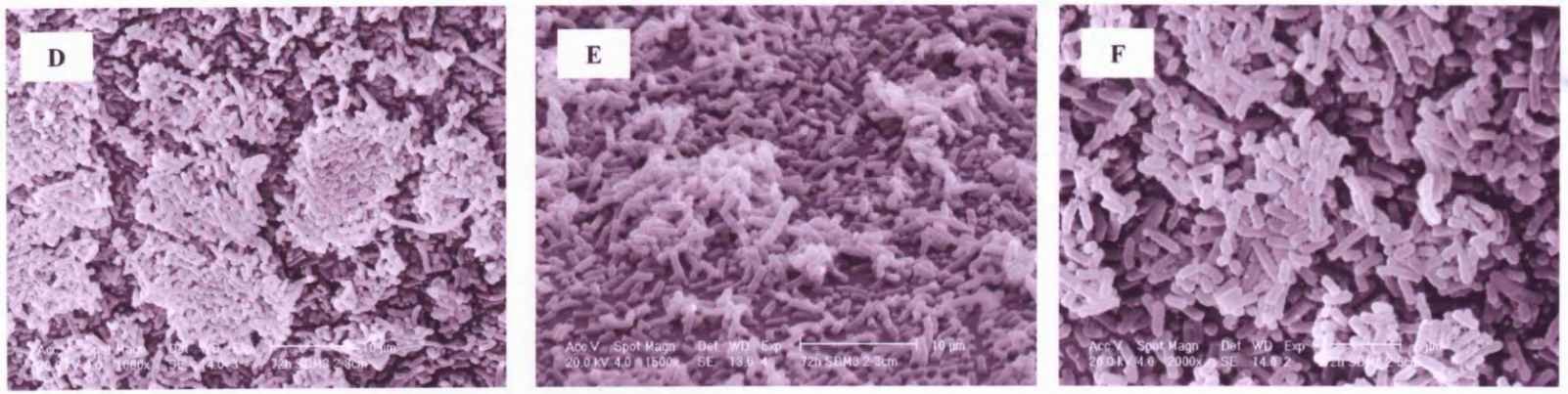
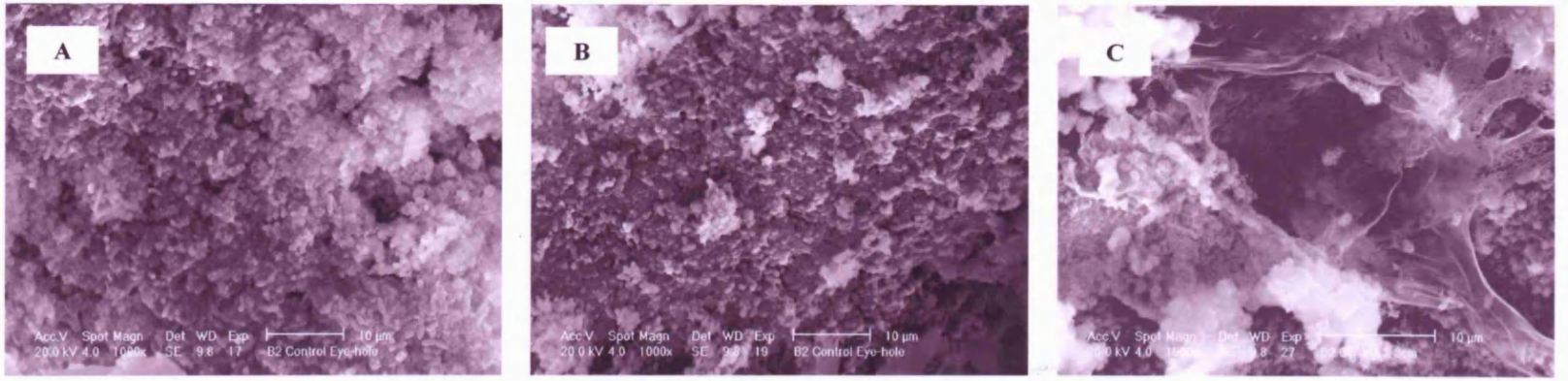


Figure 3.46 – High vacuum SEM images displaying the bacterial biofilm on the luminal surface of two catheter sections removed at 72 h from models inoculated with *Kl. pneumoniae* SDM3

A thick layer of *Klebsiella* was seen on all sections with a few crystals visible, as in image 1B.

Eye-hole section



2-3 cm section

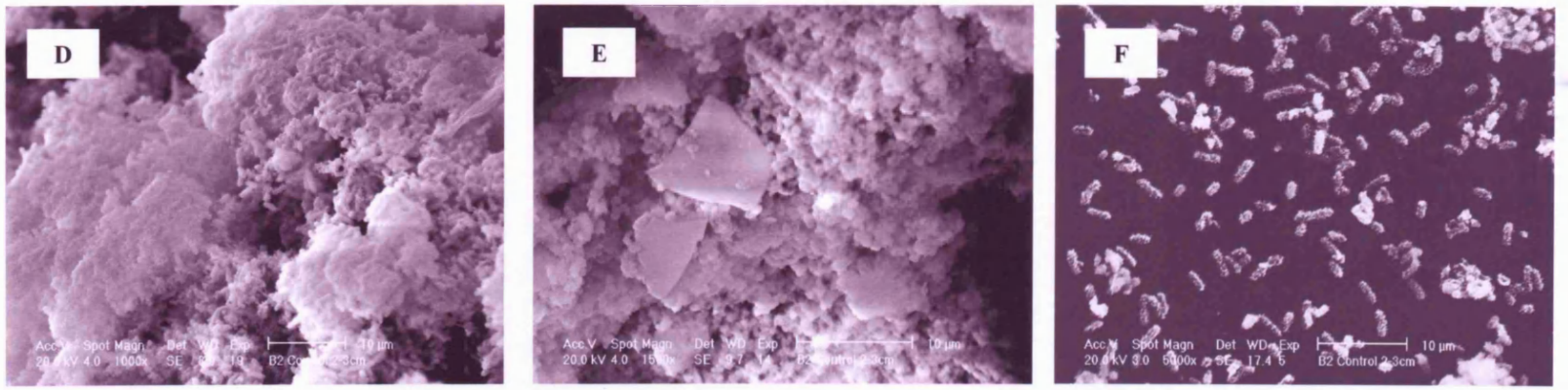


Figure 3.47 – High vacuum SEM images displaying the bacterial biofilm on the luminal surface of two catheter sections removed at catheter blockage from models inoculated with *Pr. mirabilis* B2

Substantial numbers of *Pr. mirabilis* cells were visible. Bacteria can be seen associated with amorphous material in image D and colonizing large regular shaped crystals in E. Some areas of the 2-3 cm section were more sparsely covered which allowed better resolution of individual *Pr. mirabilis* bacilli (F).

surfaces of these catheters. The image in 2C of a scattering of single *Pr. mirabilis* bacilli allowed closer inspection of their outer surface. Rather than having a smooth surface numerous hair-like protrusions were visible.

The electron micrographs in Figures 3.48 to 3.50 are of catheters from super-infection models that were removed and observed once the respective *Pr. mirabilis* control catheters had blocked. In each case large numbers of bacilli formed dense bacterial biofilms. Crystalline material was also visible on some of these sections.

3.3.3.7 The effect of 72 h established uropathogens on the formation of urinary crystalline precipitate by *Pr. mirabilis*

The images in Figure 3.51 are of the residual urine in the bladder chambers of models inoculated with the five non-*Proteus* species 96 h after the start of the experiments. The *Morg. morganii* infected model showed signs of insoluble crystal sedimentation whereas the other four had urine that was crystalline precipitate free. The images presented in Figure 3.52 are of the urine in a *Pr. mirabilis* control model at 24 h and of the five super-infection models 24 h after *Pr. mirabilis* introduction (i.e., 96 h from the start of the experiment). *Et. cloacae* RB19, and to a lesser extent *E. coli* SM1, seemed to reduce the amount of precipitation caused by *Pr. mirabilis* B2 24 h after its inoculation.

3.3.4 An *in vitro* investigation of a *Pr. mirabilis* containing community from a patient whose catheters were not blocking.

A recent paper by Mathur *et al.*, (2006) reported a study of catheter encrustation in a group of patients who were infected with *Pr. mirabilis*. A number of interesting

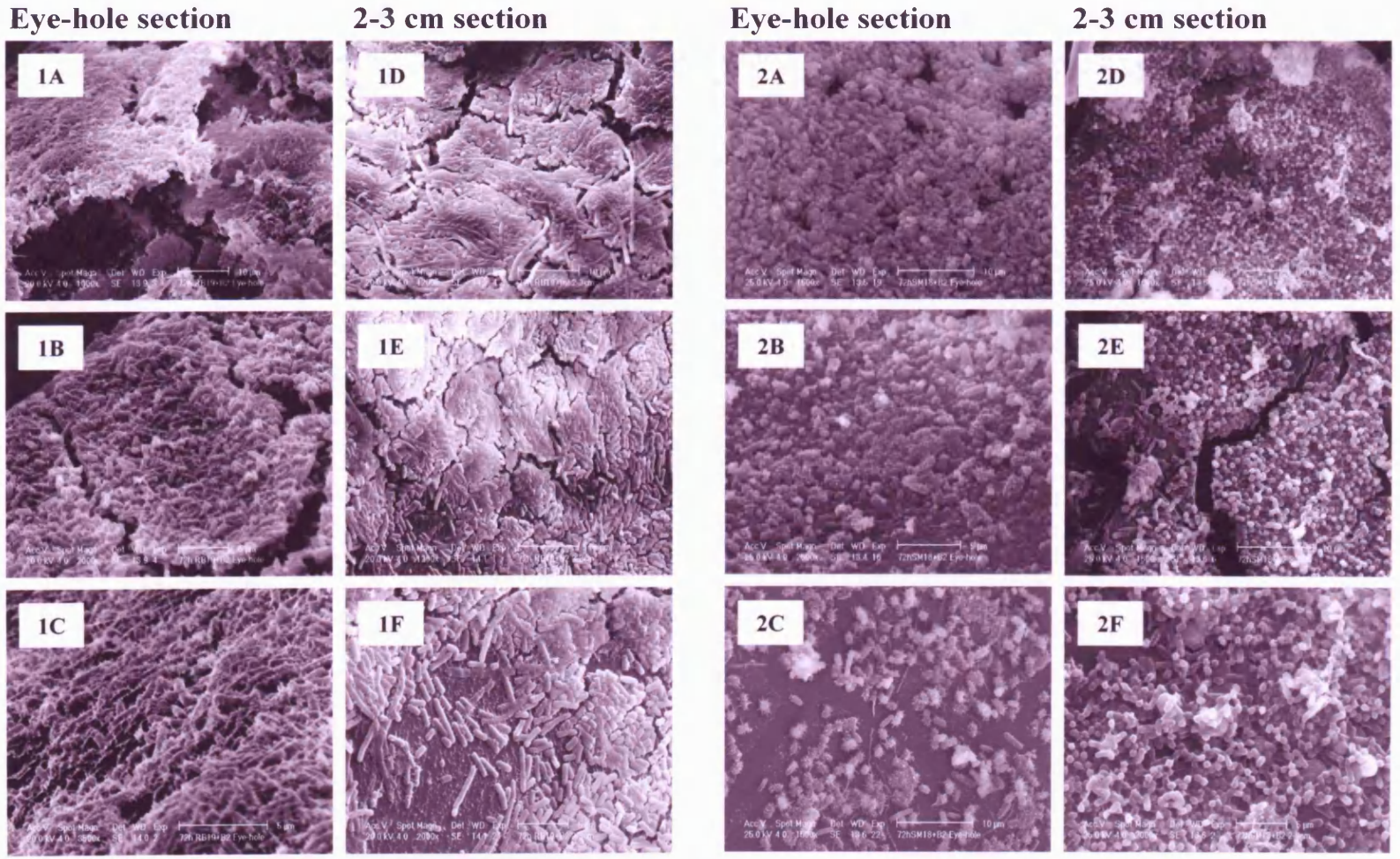


Figure 3.48 – High vacuum SEM images displaying the bacterial biofilm on the luminal surface of two catheter sections removed, once the respective *Pr. mirabilis* control catheters had blocked, from models inoculated with *Pr. mirabilis* B2 after 72 h (1) *Et. cloacae* RB19 and (2) *Morg. morganii* SM18 biofilm development

Lawns of cells are evident in each image. *Pr. mirabilis* swarmer cells can be clearly seen as part of the biofilm in image 1D. Amorphous crystalline material is evident on some sections. Images 2A-C show the highly detailed surface structures of *Pr. mirabilis* cells in comparison to the smoother *Morg. morganii* bacilli.

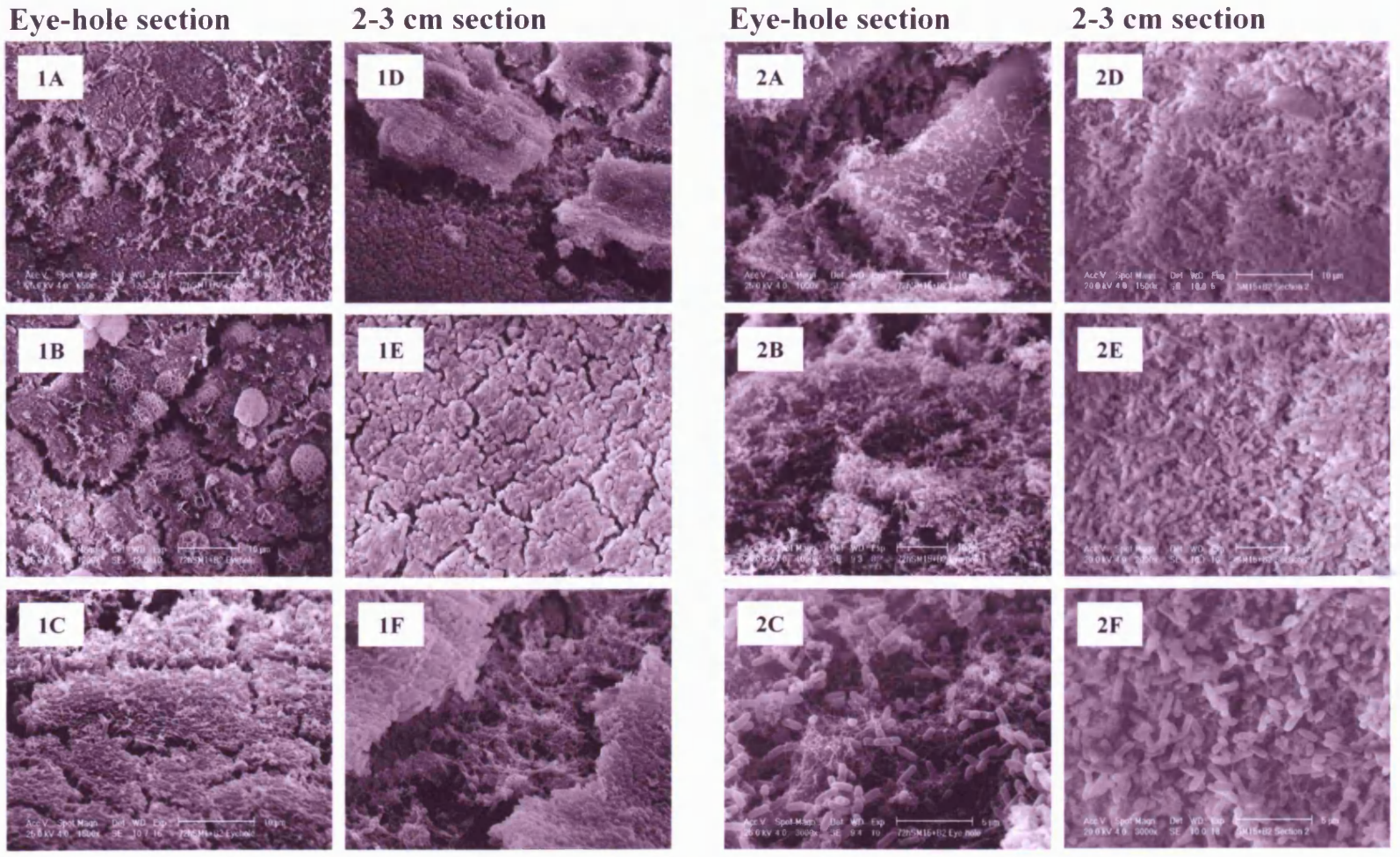
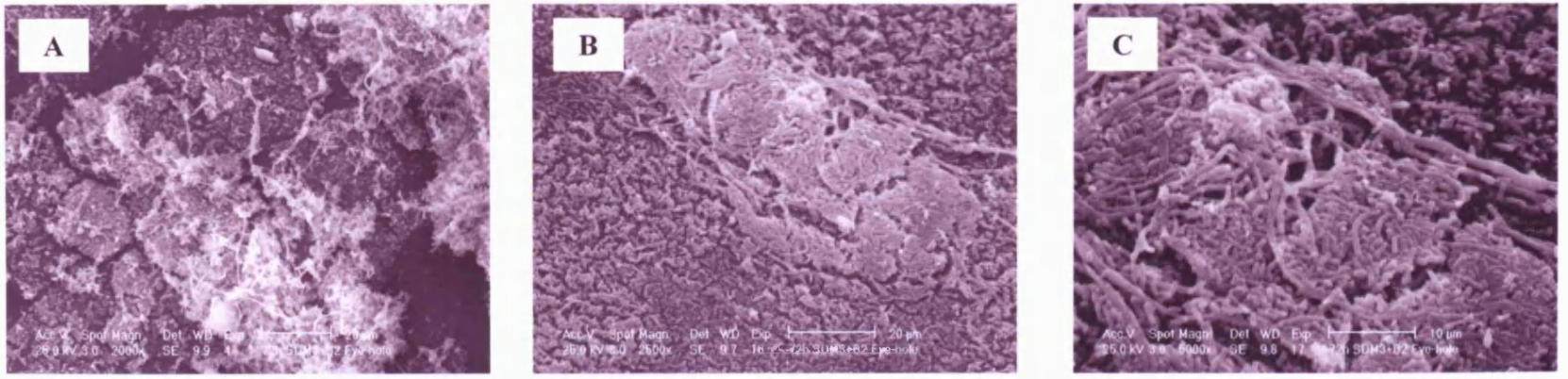


Figure 3.49 – High vacuum SEM images displaying the bacterial biofilm on the luminal surface of two catheter sections removed, once the respective *Pr. mirabilis* control catheters had blocked, from models inoculated with *Pr. mirabilis* B2 after 72 h (1) *E. coli* SM1 and (2) *Ps. aeruginosa* SM15 biofilm development

An extensive bacterial biofilm can be seen on both catheters. The sponge-like structures seen in 1B are most likely an artefact of the fixing process. Colonization by bacilli of large crystals characteristic of struvite can be seen in 2A.

Eye-hole section



2-3 cm section

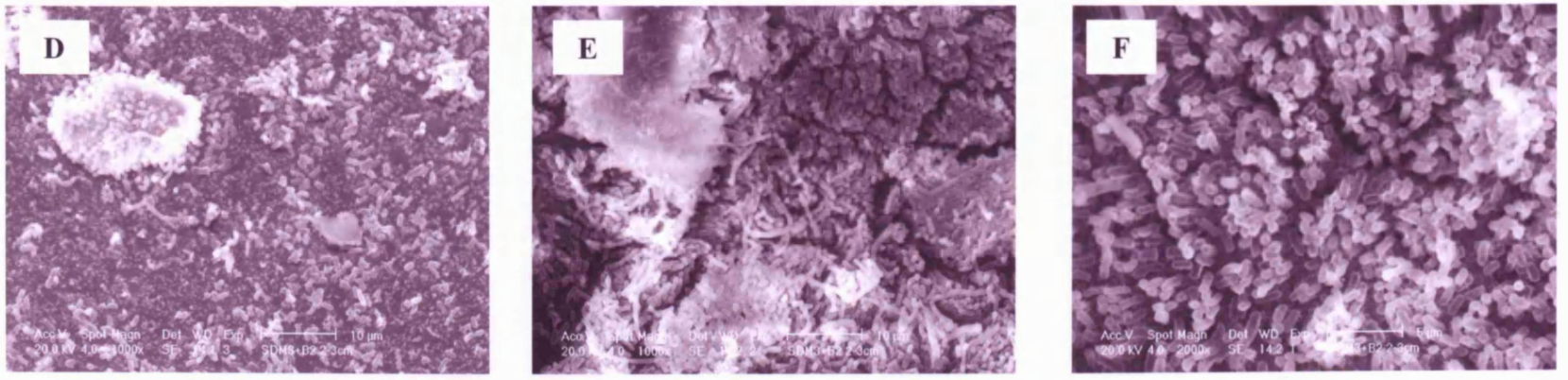


Figure 3.50 – High vacuum SEM images displaying the bacterial biofilm on the luminal surface of two catheter sections removed, once the respective *Pr. mirabilis* control catheters had blocked, from models inoculated with *Pr. mirabilis* B2 after 72 h *Kl. pneumoniae* SDM3 biofilm development

Elongated *Pr. mirabilis* swarmer cells can be seen as part of the biofilm (A-C).

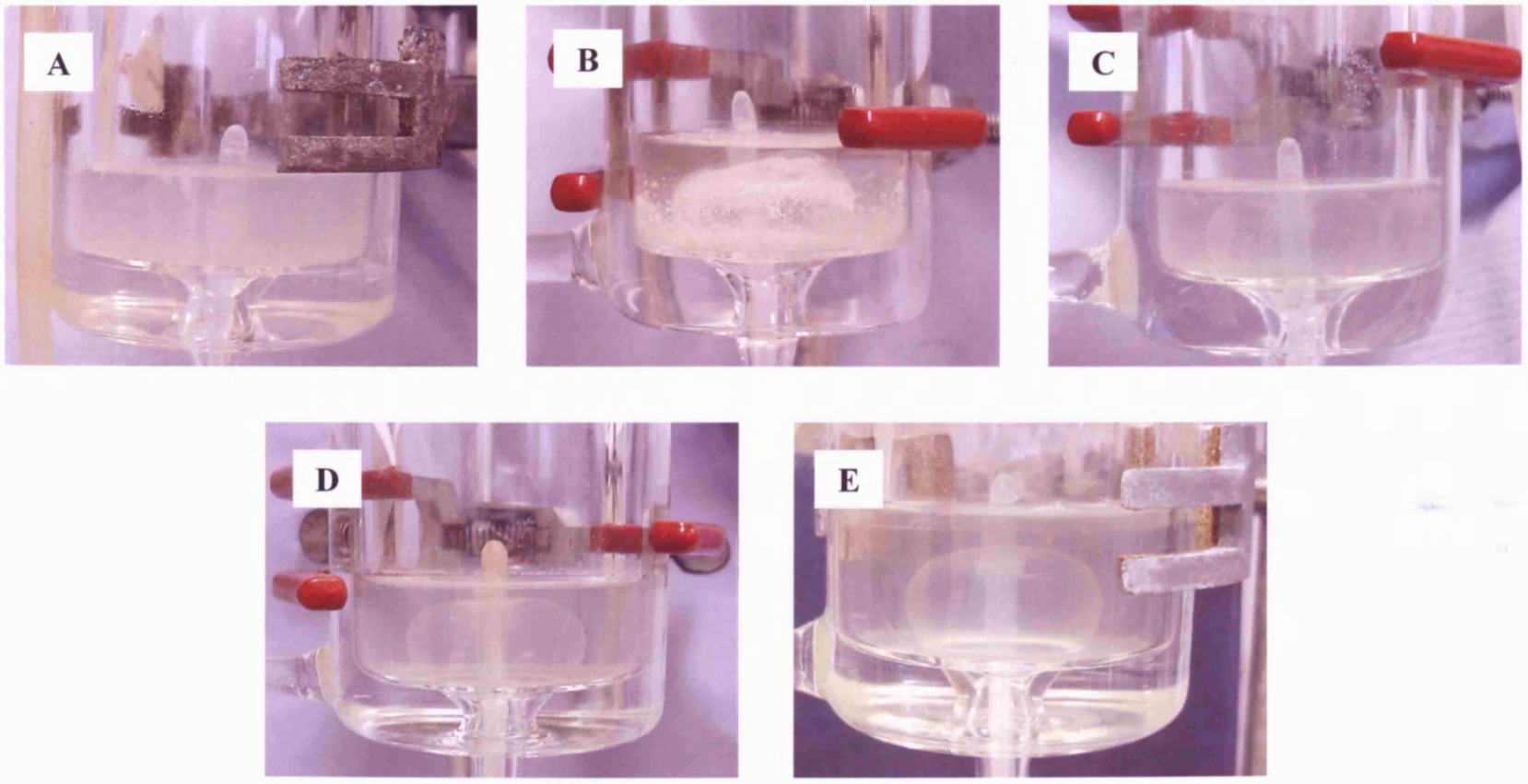


Figure 3.51 – The extent of urinary crystalline precipitation in models inoculated with five non-*Proteus* uropathogens at 96 h

(A) *Et. cloacae* RB19, (B) *Morg. morganii* SM18, (C) *E. coli* SM1, (D) *Ps. aeruginosa* SM15 and (E) *Kl. pneumoniae* SDM3

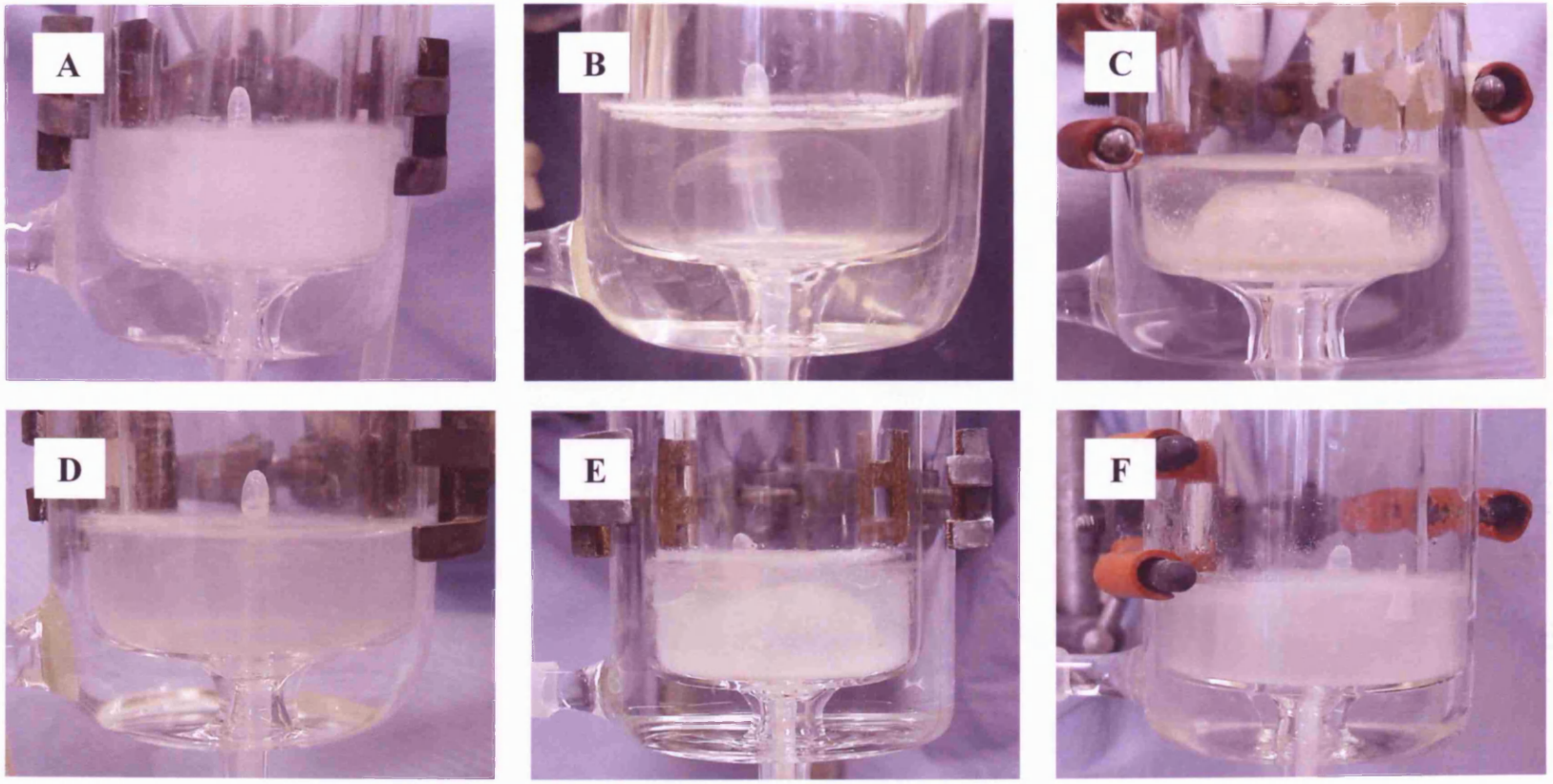


Figure 3.52 – The effect of 72 h biofilm growth of five different uropathogens on the extent of urinary crystalline precipitation by *Pr. mirabilis* 24 h after its super-infection

(A) *Pr. mirabilis* B2 control at 24 h, (B) *Et. cloacae* RB19, (C) *Morg. morganii* SM18, (D) *E. coli* SM1, (E) *Ps. aeruginosa* SM15 and (F) *Kl. pneumoniae* SDM3

bacterial communities were highlighted by the investigation. Data from the study, presented in Figure 3.53 was from a patient who despite having *Pr. mirabilis* as a component of his urinary flora had no problems with catheter encrustation. In addition to *Pr. mirabilis*, *Kl. pneumoniae* (re-identified as *Kl. oxytoca*) and *Ps. aeruginosa* were persistent during the 13 week period. *E. coli* was also isolated for 9 of the 13 weeks. It can be seen that the urinary pH was consistently much lower than the nucleation pH. His catheters drained freely with no signs of encrustation when changed at scheduled 8 week intervals.

The identification and availability of a *Pr. mirabilis* containing urinary community which did not produce encrustation and blockage of a patient's catheter presented an opportunity to study the factors that might inhibit crystalline biofilm formation. Experiments were thus carried out to examine whether the strain of *Pr. mirabilis* concerned was incapable of producing alkaline urine and catheter encrustation and/or whether the other members of the bacterial community modified its activity.

Models were assembled in parallel in sets of two. Models were infected with either a pure culture of *Pr. mirabilis* AAAX or a mixed community containing *Pr. mirabilis* AAAX, *Ps. aeruginosa* AAAY, *E. coli* AAAZ, and *Kl. oxytoca* AAAAA (1 ml of 4 h urine cultures of each organism were used as inocula). All models were supplied with urine until the catheters became blocked. The pH and viable cell counts from the residual urine in the bladder chamber were recorded at 0 h (upon resumption of the urine supply), 1 h and every 24 h after that until blockage. Time to catheter blockage was recorded from triplicate experiments

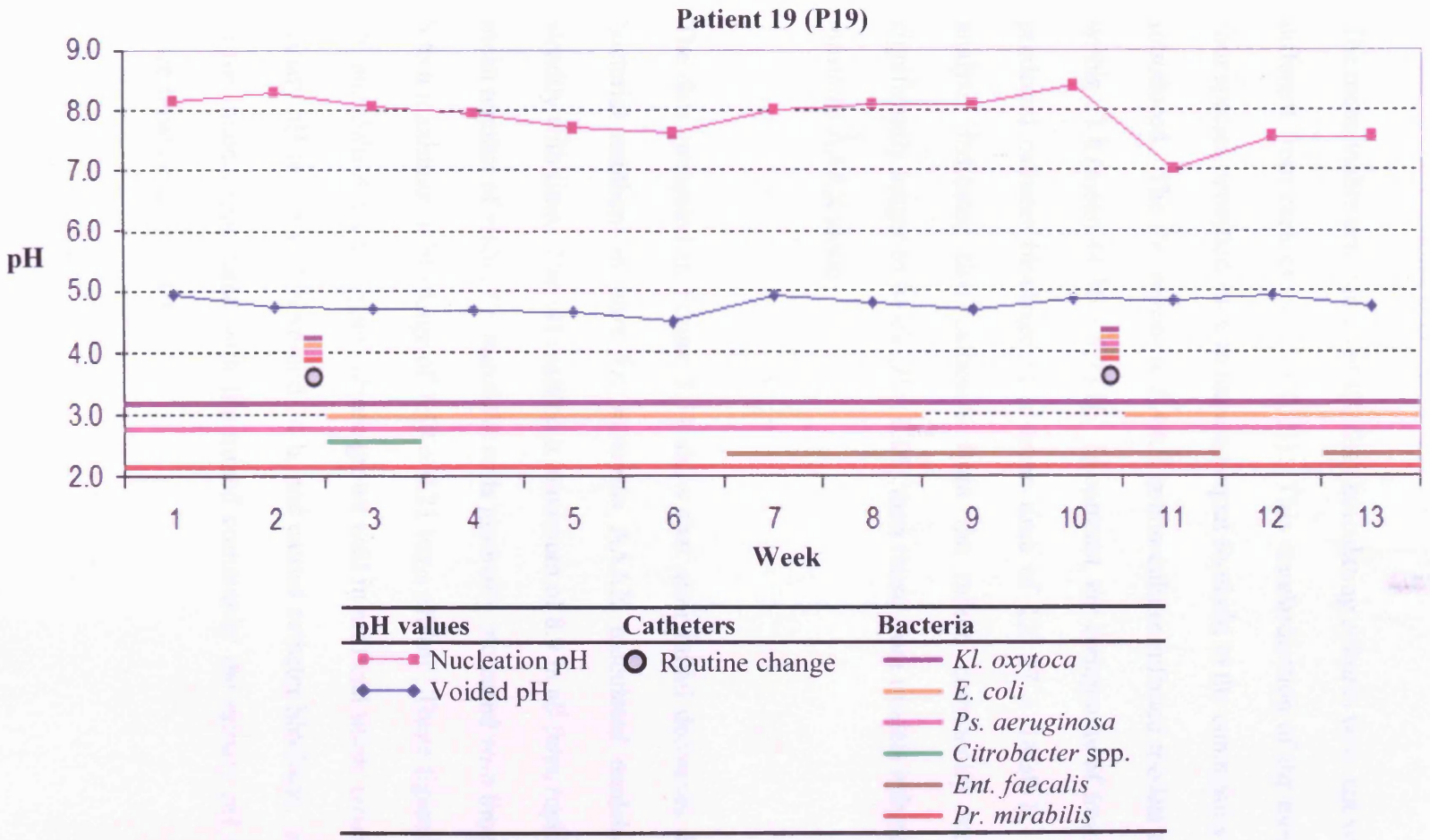


Figure 3.53 – Summary data for Patient 19 (P19) modified from Mathur *et al.*, (2006)

Nucleation pH, voided pH, catheter changes and organisms isolated from the urine and the catheter biofilms throughout the 13-week study period.

and the two blocked catheters from the third replicate were examined by LV-SEM.

The mean viable cell counts of the four inoculating cultures were not significantly different from each other ($P > 0.05$). This standardisation of the inocula of the four species permitted each to have an equal foothold in the community when first introduced. The *Pr. mirabilis* AAAX mono-culture infected models all blocked within 53 h (mean 44.36 ± 5.73 h). In contrast, the community of four organisms produced catheter blockage at a mean time of 121.17 ± 18.38 h. Statistical analysis indicated that catheters from the mixed community models took significantly longer to block ($P \leq 0.05$) than those from models infected with *Pr. mirabilis* AAAX alone.

The data presented in Figure 3.54 show that after initial decreases the pH and bacterial numbers in pure *Pr. mirabilis* AAAX inoculated models increased steadily with time. The pH reached a maximum of 8.9 in all three replicates. The mean number of viable *Pr. mirabilis* cells gradually increased with time after t = 1 h to a maximum at blockage of 7.58 ± 0.21 $\log_{10}$ cfu/ml. These figures show that *Pr. mirabilis* AAAX in pure culture grows well in artificial urine, raises the mean urinary pH to > 8 within around 24 h, and causes catheter blockage. In contrast, in the models inoculated with the mixed community the urinary pH rose much more slowly (Table 3.30).

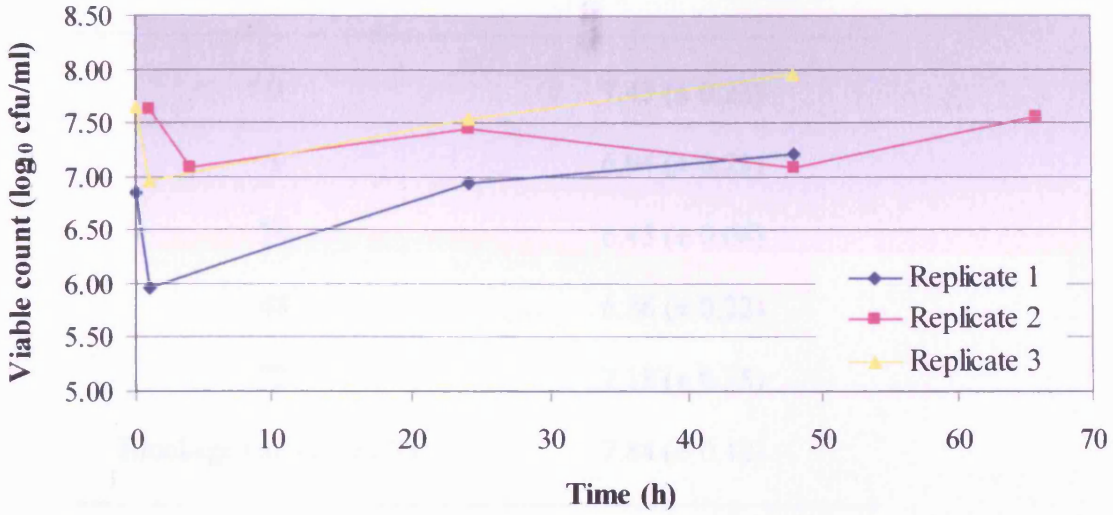


Figure 3.54a – Viable cell counts

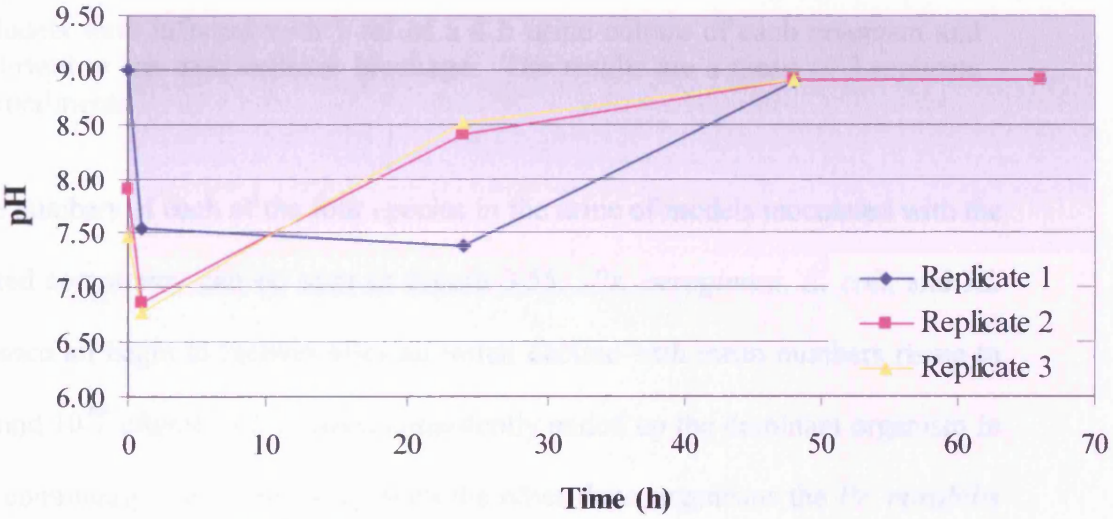


Figure 3.54b – Urinary pH

Figure 3.54 – Viable cell counts and urinary pH results from the residual urine of models inoculated with *Pr. mirabilis* AAAX

Models were infected with *Pr. mirabilis* AAAX (1 ml of a 4 h urine culture) and allowed to run until catheter blockage.

Time (h)	Mean pH (± SE mean)
0	7.43 (± 0.23)
1	6.94 (± 0.23)
24	6.45 (± 0.06)
48	6.86 (± 0.22)
72	7.18 (± 0.35)
Blockage (mean 121.71)	7.84 (± 0.12)

Table 3.30 – Mean pH results from the residual urine of models infected with the mixed community containing *Pr. mirabilis* AAAX, *Ps. aeruginosa* AAAY, *E. coli*, AAAZ, and *Kl. oxytoca* AAAAA

Models were infected with 1 ml of a 4 h urine culture of each organism and allowed to run until catheter blockage. The results are a mean of 3 replicate experiments.

The numbers of each of the four species in the urine of models inoculated with the mixed community can be seen in Figure 3.55. *Ps. aeruginosa*, *E. coli*, and *Kl. oxytoca* all begin to recover after an initial decline with mean numbers rising to around 10^{7-8} cfu/ml. *Kl. oxytoca* consistently ended up the dominant organism in the community. In combination with the other three organisms the *Pr. mirabilis* strain fared poorly. Mean viable cell counts of *Pr. mirabilis* from mixed community models (2.04×10^6 cfu/ml) were significantly lower ($P \leq 0.05$) than from *Pr. mirabilis* AAAX mono-culture infected models (3.80×10^7 cfu/ml).

The digital images presented in Figures 3.56 confirm the slower deposition of crystalline material in urine inoculated with the mixed community compared to that inoculated with *Pr. mirabilis* alone.

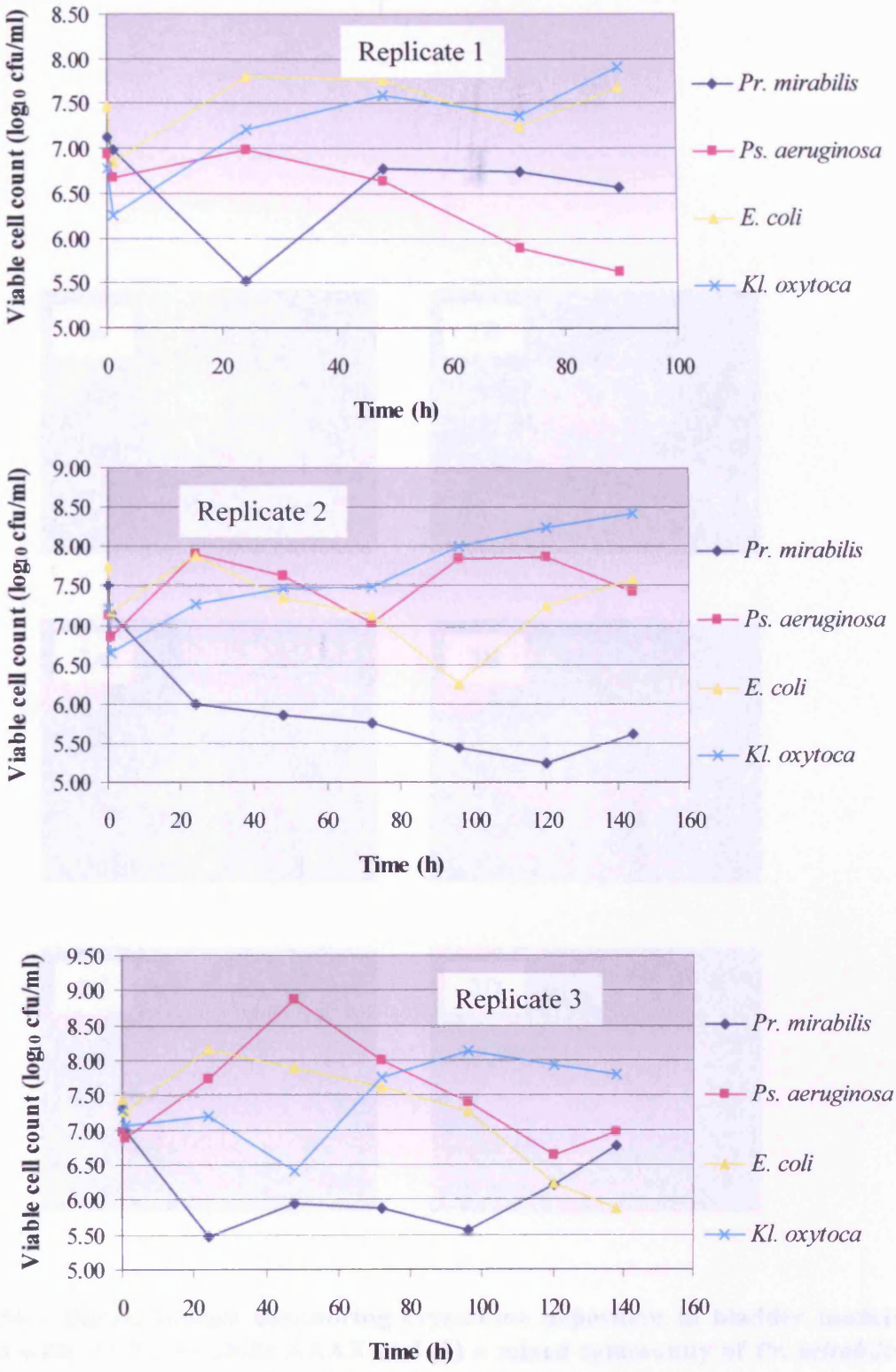


Figure 3.55 – Viable cell counts from the residual urine of models infected with the mixed community containing *Pr. mirabilis* AAAX, *Ps. aeruginosa* AAAY, *E. coli* AAAZ and *Kl. oxytoca* AAAAA.

Models were infected with 1 ml of a 4 h urine culture of each organism and allowed to run until catheter blockage.

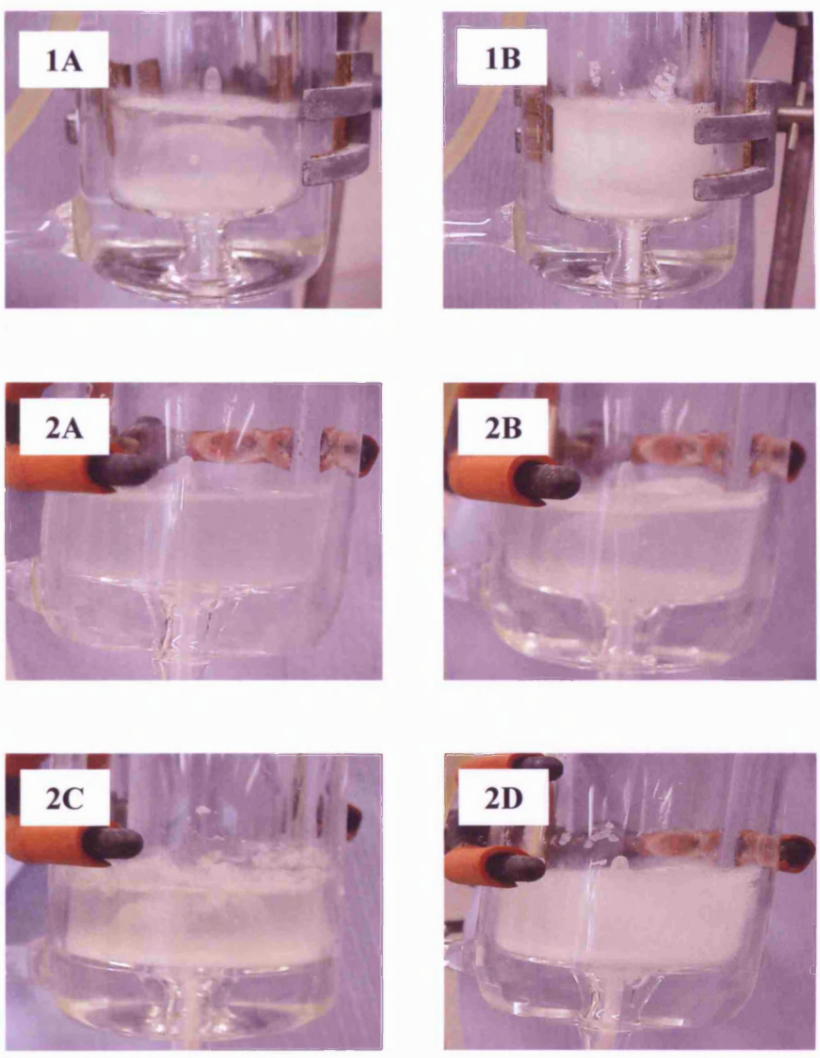


Figure 3.56 - Digital images monitoring crystalline deposition in bladder models inoculated with (1) *Pr. mirabilis* AAAX and (2) a mixed community of *Pr. mirabilis* AAAX, *Ps. aeruginosa* AAAY, *E. coli* AAAZ, and *Kl. oxytoca* AAAAA.

Image (A) 24 h, (B) 48 h, (C) 72 h, (D) 120 h after the urine supply was resumed. The catheter blocked at 48 h (model 1) and 135.87 h (model 2).

LV-SEM revealed distinct differences in the nature of the material blocking the catheters from the two sets of models (Figure 3.57). The pure *Pr. mirabilis* population produced a more dense, highly crystalline biofilm (1A-E) than the mixed community (2A-E), and seemed to block the catheter lumen rather than the eye-hole.

3.4 Coaggregation in the formation of catheter biofilms

In the human mouth many species of bacteria mouth clump together to from aggregates. On their surfaces they have polymers that can mediate precise cell-to-cell binding between genetically distinct organisms. This phenomenon enables bacteria to coaggregate with specific sets of partners. As a result, during formation, succession, and maturation of dental plaque biofilm defined matrices of multi-species communities can build up (Gibbons and Nygaard, 1970; Kolenbrander *et al.*, 1999). The physical proximity of organisms can facilitate metabolic and physiological collaborations between populations of cells (Costerton, *et al.*, 1995). The extent to which the coaggregation phenomenon occurs in the development of the multi-species biofilms that colonize long-term indwelling catheters is unknown.

3.4.1 Coaggregation of control strains in artificial urine

The coaggregation of oral bacteria is commonly tested in deionised water (Rickard *et al*, 1999; Rickard *et al*, 2004) or special "coaggregation buffers" (Kolenbrander and Phucus 1984; Kolenbrander *et al.*, 1990). If coaggregation is to play a role in the formation of urinary catheter biofilms, it will need to occur in urine. Preliminary experiments were therefore performed with the control strains in both coaggregation buffer and artificial urine over a 6 h period. The mean

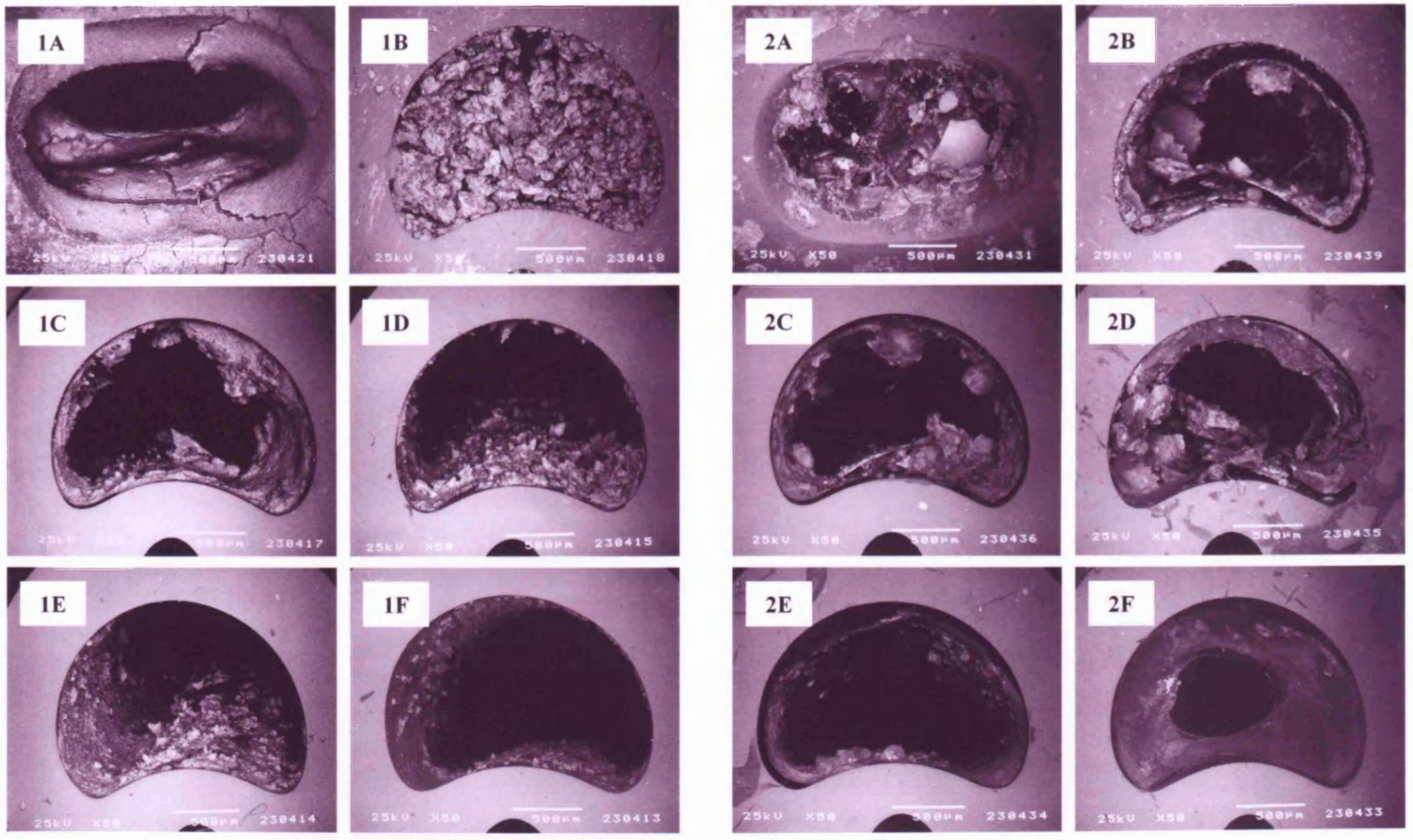


Figure 3.57 – Low-vacuum scanning electron micrographs of catheters removed from models infected with (1) *Pr. mirabilis* AAAX and (2) a mixed community of *Pr. mirabilis* AAAX, *Ps. aeruginosa* AAAY, *E. coli* AAAZ and *Kl. oxytoca* AAAAA

Catheters were removed at blockage. The position of sections A-F are indicated in Figure 2.7.

results are presented in Tables 3.31 and 3.32 and confirmed that *A. naeslundii* ATCC 12104 and *Strep. sanguis* GW2 coaggregated well in urine and thus could be used as controls in subsequent tests with urinary catheter isolates. These experiments also demonstrated that the artificial urine was non-inhibitory to the coaggregation process. In all cases both the single species and the re-suspension medium controls showed no signs of aggregation. An example of coaggregation of the oral strains in artificial urine is shown in Figure 3.58.

3.4.2 Do urinary catheter biofilm isolates autoaggregate in artificial urine?

As a further set of controls, tests were performed to check for autoaggregation of 13 species isolated from urinary catheter biofilms. This involved setting up mixtures of culture (2 ml) and artificial urine (2 ml). In addition to monitoring aggregation every hour for 6 h the pH of the suspension at 6 h was also measured. The mean results from triplicate experiments (unless stated otherwise) are shown in Table 3.33. No significant difference existed between the median numbers of viable cells in each inoculum (Kruskal-Wallis $P > 0.05$).

Six organisms showed some degree of autoaggregation. Aggregation was first seen at 1 h for *Pr. mirabilis*, *Prov. rettgeri*, *Prov. stuartii*,and *Morg. morganii* at 3 h for *Kl. pneumoniae* and not until 4 h for *Kl. oxytoca*.

A highly significant difference ($P \leq 0.001$) was indicated between the mean end pH of the assay suspensions. Two distinct groups of strains were apparent, one group generated alkaline urine and in the other group the urine remained acidic.

Organisms	Time (h)						
	0	1	2	3	4	5	6
A. naeslundii ATCC 12104 control	0	0	0	0	0	0	0
Strep. sanguis GW2 control	0	0	0	0	0	0	0
ATCC 12104 + GW2 +ve control	3	4	4	4	4	4	4
Coaggregation buffer control	0	0	0	0	0	0	0

Table 3.31 – Mean visual coaggregation scores of *A. naeslundii* ATCC 12104 and *Strep. sanguis* GW2 in coaggregation buffer

Mean of three replicate experiments. Positive results are indicated in red.

Organisms	Time (h)						
	0	1	2	3	4	5	6
A. naeslundii ATCC 12104 control	0	0	0	0	0	0	0
Strep. sanguis GW2 control	0	0	0	0	0	0	0
ATCC 12104 + GW2 +ve control	2	3	4	4	4	4	4
Urine control	0	0	0	0	0	0	0

Table 3.32 – Mean visual coaggregation scores of *A. naeslundii* ATCC 12104 and *Strep. sanguis* GW2 in artificial urine

Mean of three replicate experiments. Positive results are indicated in red.

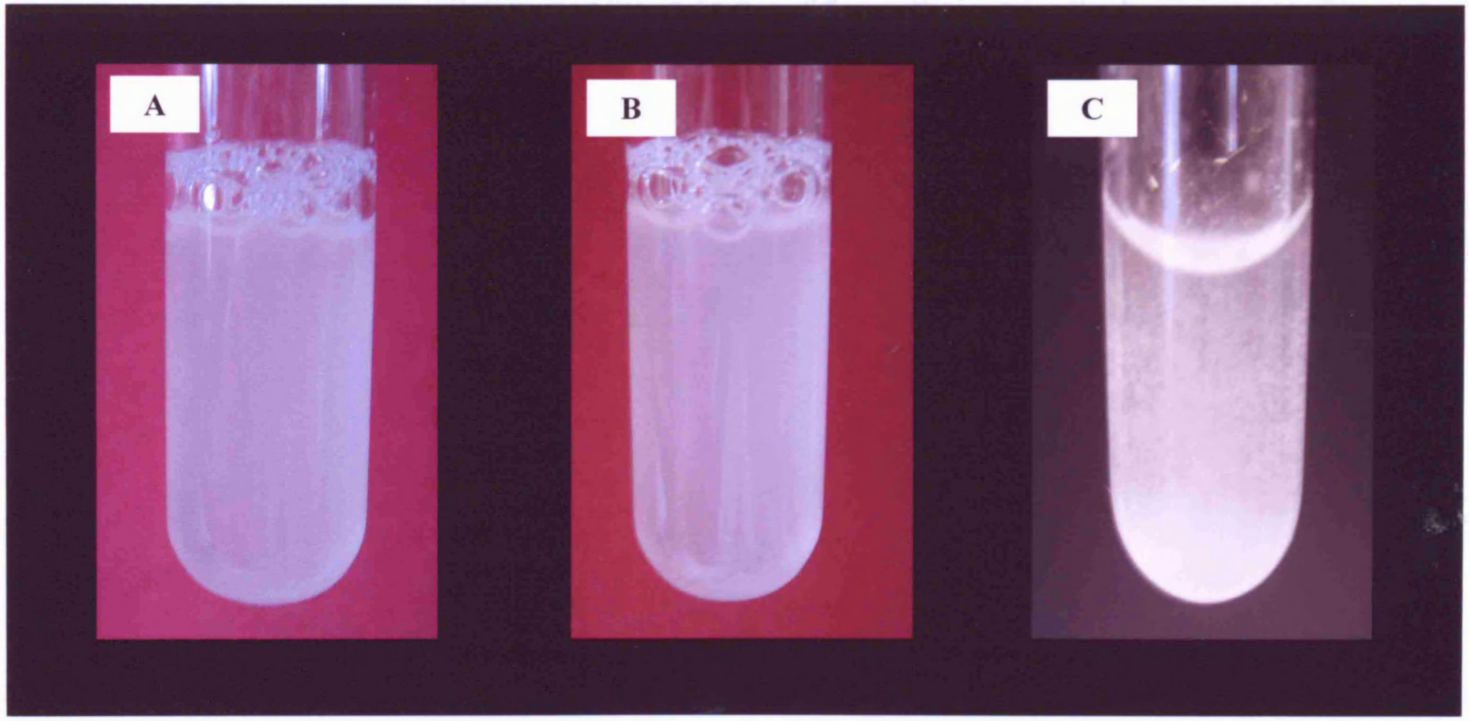


Figure 3.58 – *A. naeslundii* ATCC 12104 and *Strep. sanguis* GW2 visual coaggregation reaction in artificial urine at 1 h

Image (A) *A. naeslundii* ATCC 12104 control – coaggregation score 0, (B) *Strep. sanguis* GW2 control – coaggregation score 0 and (C) *A. naeslundii* and *Strep. sanguis* positive control. Bacterial flocs can be seen settling to the bottom of the test tube (coaggregation score +3)

Species	Time (h)							
	0	1	2	3	4	5	6	6 h pH
Strep. sanguis GW2 [a]	0	0	0	0	0	0	0	6.36
A. naeslundii ATCC 12104 [a]	0	0	0	0	0	0	0	6.41
GW2 + 12104 +ve [a]	3	3	3	4	4	4	4	6.69
Pr. mirabilis HI4320 *wt* [b]	0	2	2	1	1	1	1	9.10
Prov. rettgeri SDM1	0	1	2	2	2	2	1	9.07
Kl. pneumoniae SDM3	0	0	0	1	1	1	1	9.06
C. koseri SDM4	0	0	0	0	0	0	0	6.62
Ps. aeruginosa SDM5	0	0	0	0	0	0	0	7.44
S. aureus SDM6	0	0	0	0	0	0	0	6.42
E. coli SDM8	0	0	0	0	0	0	0	6.46
Ent. faecalis SDM13	0	0	0	0	0	0	0	6.66
Prov. stuartii RB14	0	1	1	1	1	1	1	8.94
Morg. morganii RB15	0	1	1	1	1	1	1	9.00
Et. cloacae RB19	0	0	0	0	0	0	0	6.44
Kl. oxytoca NSM44	0	0	0	0	1	1	2	8.58
S. haemolyticus SM145	0	0	0	0	0	0	0	6.26
Urine control [a]	0	0	0	0	0	0	0	6.22

Table 3.33 – Mean visual autoaggregation scores of urinary catheter biofilm species in artificial urine and the pH of the suspension at 6 h. Results are the means of six replicate experiments unless stated otherwise ([a] mean of 24 replicates; [b] mean of 12 replicates). Positive coaggregation scores are indicated in red. Alkaline pH is indicated in blue.

Interestingly, autoaggregation was only observed within suspensions that had become alkaline. These suspensions corresponded to urease producing organisms. All non-urease producing isolates investigated, and the urine control, had acidic pH at 6 h and showed no signs of aggregation. *Ps. aeruginosa* SDM5 was a urease-producing strain yet it failed to demonstrate any autoaggregation throughout the 6 h period. At 6 h the mean pH of suspensions of *Ps. aeruginosa* SDM5 was only slightly alkaline.

3.4.3 Does a urease-producing *Pr. mirabilis* coaggregate with other urinary catheter biofilm species in artificial urine?

In the next set of experiments visual coaggregation between *Pr. mirabilis* HI4320 *wt* and the 12 other urinary tract isolates was investigated. The results from replicate experiments are displayed in Table 3.34. Statistical analysis on the viable cell counts showed no significant difference (Kruskal-Wallis $P > 0.05$) between the median numbers of cells in each of the inocula.

All combinations showed apparent coaggregation with *Pr. mirabilis* at 1 h (at the +1/+2 levels) with some degree of coaggregation visible for the duration of the experimental period. All suspensions were alkaline at 6 h due to each combination containing the urease-producing *Pr. mirabilis*. The urine control remained acidic and aggregation free throughout the experiments.

Due to the autoaggregation profile of *Pr. mirabilis* HI4320 *wt* and some of the other strains over the 6 h period (Table 3.33) an adjusted score table was constructed that compensated for this phenomenon. Any coaggregation score

Species combination (*Pr. mirabilis* HI4320 *wt* + ...)	Time (h)							6 h pH
	0	1	2	3	4	5	6	
Pr. mirabilis HI4320 *wt* control [a]	0	2	2	1	1	1	1	9.10
Prov. rettgeri	0	2	2	1	1	1	1	9.28
Kl. pneumoniae	0	2	2	1	1	1	1	9.13
C. koseri	0	1	1	1	1	1	1	9.27
Ps. aeruginosa	0	1	1	1	1	1	1	9.14
S. aureus	0	2	2	1	1	1	1	9.02
E. coli	0	1	1	1	1	1	1	9.17
Ent. faecalis	0	2	2	2	2	2	2	9.13
Prov. stuartii	0	2	2	2	1	1	1	9.10
Morg. morganii	0	1	1	1	1	1	1	9.12
Et. cloacae	0	2	2	1	1	1	1	9.01
Kl. oxytoca	0	2	2	1	1	1	1	9.06
S. haemolyticus	0	2	1	1	1	1	1	9.09
Urine control [a]	0	0	0	0	0	0	0	6.19

Table 3.34 – Mean visual coaggregation scores of urinary catheter biofilm species with *Pr. mirabilis* HI4320 *wt* in artificial urine and the pH of the suspension at 6 h. Results are the means of three replicate experiments unless stated otherwise ([a] mean of 12 replicates). Positive coaggregation scores are indicated in red. Alkaline pH is indicated in blue.

between a pair of organisms had the corresponding autoaggregation scores deducted from it. The resulting adjusted scores can be seen in Table 3.35. Any positive scores after adjustment might suggest true coaggregation. Only one combination had positive results after adjustment. *Pr. mirabilis* and *Ent. faecalis* consistently had a +1 score between 3 h and 6 h indicating that perhaps a genuine coaggregation was occurring between these two species.

3.4.4 Does autoaggregation and coaggregation occur when phosphate crystals are not formed in the urine?

The hypothesis for this section of the investigation was that there are no specific interactions between *Pr. mirabilis* and other urinary organisms that lead to coaggregation. The composition of the bacterial biofilm communities that colonize urinary catheters is simply determined by the aggregation of whatever species are present in the urine. This aggregation is brought about by the crystals that form when the urease activity of *Pr. mirabilis* causes the pH of the urine to rise above the nucleation pH. The following experiments were designed to test this idea.

To investigate whether aggregation seen in previous experiments was brought about by the formation of crystals, the coaggregation assays were repeated with an isogenic urease negative mutant, *Pr. mirabilis* HI4320 *ure*$^-$ (Jones *et al.*, 1990), in place of the parent wild-type strain. The lack of the urease enzyme in this strain would mean that the pH in many of the assay tubes would remain acidic and therefore crystalline matter would not form. Any aggregation scores in these assays would thus suggest true coaggregation.

Species combination (*Pr. mirabilis* HI4320 *wt* + ...)	Time (h)							6 h pH
	0	1	2	3	4	5	6	
Prov. rettgeri	0	0	0	0	0	0	0	**9.28**
Kl. pneumoniae	0	0	0	0	0	0	0	**9.13**
C. koseri	0	0	0	0	0	0	0	**9.27**
Ps. aeruginosa	0	0	0	0	0	0	0	**9.14**
S. aureus	0	0	0	0	0	0	0	**9.02**
E. coli	0	0	0	0	0	0	0	**9.17**
Ent. faecalis	0	0	0	1	1	1	1	**9.13**
Prov. stuartii	0	0	0	0	0	0	0	**9.10**
Morg. morganii	0	0	0	0	0	0	0	**9.12**
Et. cloacae	0	0	0	0	0	0	0	**9.01**
Kl. oxytoca	0	0	0	0	0	0	0	**9.06**
S. haemolyticus	0	0	0	0	0	0	0	**9.09**

Table 3.35 – Mean visual coaggregation scores of urinary catheter biofilm species with *Pr. mirabilis* HI4320 *wt* in artificial urine adjusted to compensate for autoaggregation of both partners. The pH of the suspension at 6 h is also shown. Results are the means of three replicate experiments. Positive coaggregation scores are indicated in red. Alkaline pH is indicated in blue.

The results are presented in Table 3.36. This time all assays between the urease-negative *Pr. mirabilis* and a urease-negative partner organism had acidic mean pH values at 6 h and exhibited no signs of coaggregation. It can also be seen that five of the 12 organisms (*Prov. rettgeri*, *Kl. pneumoniae*, *Prov. stuartii*, *Morg. morganii*, and *Kl. oxytoca*) showed a degree of coaggregation with *Pr. mirabilis* HI4320 *ure*$^-$. All five were urease producing organisms and again all had alkaline 6 h pH values. Again *Ps. aeruginosa* had a low pH at 6 h and showed no degree of coaggregation with the urease-negative *Pr. mirabilis*.

To compensate for the autoaggregation of the five urease-positive species (Table 3.33) the coaggregation scores were adjusted by deducting the autoaggregation score from the coaggregation score. The results of which are presented in Table 3.37. Only two of the combinations retained positive scores after adjustment, (1) *Pr. mirabilis* and *Prov. stuartii* and (2) *Pr. mirabilis* and *Morg. morganii*. These results were only low scoring but implied a level of coaggregation was perhaps occurring between these species.

3.4.5 Is the formation of crystalline material responsible for autoaggregation and/ or coaggregation?

Two simple sets of experiments were devised to further investigate crystal formation mediated coaggregation. Assays were initiated as described previously but the experimental period was shortened to 4 h. Those suspensions showing coaggregation at 4 h were diluted with artificial urine (1:2) and filtered (1 ml) through 10 μm polycarbonate membranes. The membranes were vapour fixed for

Species combination (*Pr. mirabilis* HI4320 *ure*$^-$ + ...)	Time (h)							6 h pH
	0	1	2	3	4	5	6	
Pr. mirabilis HI4320 *ure*$^-$ control [a]	0	0	0	0	0	0	0	6.74
Prov. rettgeri	0	1	2	2	2	1	1	8.95
Kl. pneumoniae	0	0	1	1	1	1	1	8.97
C. koseri	0	0	0	0	0	0	0	6.71
Ps. aeruginosa	0	0	0	0	0	0	0	6.83
S. aureus	0	0	0	0	0	0	0	6.96
E. coli	0	0	0	0	0	0	0	6.67
Ent. faecalis	0	0	0	0	0	0	0	6.80
Prov. stuartii	0	2	2	2	1	1	1	9.06
Morg. morganii	0	0	0	1	2	2	2	8.94
Et. cloacae	0	0	0	0	0	0	0	6.73
Kl. oxytoca	0	0	0	0	0	1	2	8.73
S. haemolyticus	0	0	0	0	0	0	0	6.50
Urine control [a]	0	0	0	0	0	0	0	6.24

Table 3.36 – Mean visual coaggregation scores of urinary catheter biofilm species with *Pr. mirabilis* HI4320 *ure*$^-$ mutant in artificial urine and the pH of the suspension at 6 h. Results are the means of three replicate experiments unless stated otherwise ([a] mean of 12 replicates). Positive coaggregation scores are indicated in red. Alkaline pH is indicated in blue.

Species combination (*Pr. mirabilis* HI4320 *ure*$^-$ + ...)	Time (h)							6 h pH
	0	1	2	3	4	5	6	
Prov. rettgeri	0	0	0	0	0	0	0	8.95
Kl. pneumoniae	0	0	1	0	0	0	0	8.97
Prov. stuartii	0	1	1	1	0	0	0	9.06
Morg. morganii	0	0	0	0	1	1	1	8.94
Kl. oxytoca	0	0	0	0	0	0	0	8.73

Table 3.37 – Mean visual coaggregation scores of urinary catheter biofilm species with *Pr. mirabilis* HI4320 *ure*$^-$ mutant in artificial urine adjusted to compensate for autoaggregation of the urease producing partner. The pH of the suspension at 6 h is also shown. Results are the means of three replicate experiments. Positive coaggregation scores are indicated in red. Alkaline pH is indicated in blue

24 h, dried within a dessicator overnight, gold sputtered, and viewed in a Phillips XL-20 scanning electron microscope under high vacuum.

High vacuum scanning electron microscope images of some membrane surfaces are shown in Figures 3.59 to 3.63. Three combinations; *Pr. mirabilis* and *Ps. aeruginosa*, *Pr. mirabilis* and *E. coli*, and *Pr. mirabilis* and *Ent. faecalis*, and the respective controls can be seen. Regular shaped crystals, most likely magnesium ammonium phosphate, were present in each combination containing *Pr. mirabilis* HI4320 *wt* along with what looks to be amorphous calcium phosphate and in some cases bacterial cells (Figures 3.59 to 3.62). In contrast the three non-*Pr. mirabilis* controls showed no signs of crystalline material (Figure 3.63). On the membranes through which *Ps. aeruginosa* and *E. coli* controls were filtered bacterial cells were seen in large isolated patches (Images A and B respectively). However, only single cells or pairs were sparsely seen on membranes from the *Ent. faecalis* control (C1 and C2).

In the second set of experiments coaggregation assays were again performed for 4 h. At this time, aliquots (1 ml) of the suspension were filtered through 10 μm polycarbonate membranes. The membranes were then transferred to artificial urine (10 ml) in sterile universal containers until any aggregates had dissolved. Viable cell counts were then performed on this suspension. If calcium phosphate formation had caused bacterial aggregation then the proportions of the original inocula recovered from the suspensions would be significantly higher than the urease-negative control (a suspension of *E. coli* SDM8 was filtered to assess the proportion of cells that were trapped on the surface of the membranes in the absence of alkalinity and crystalline precipitation).

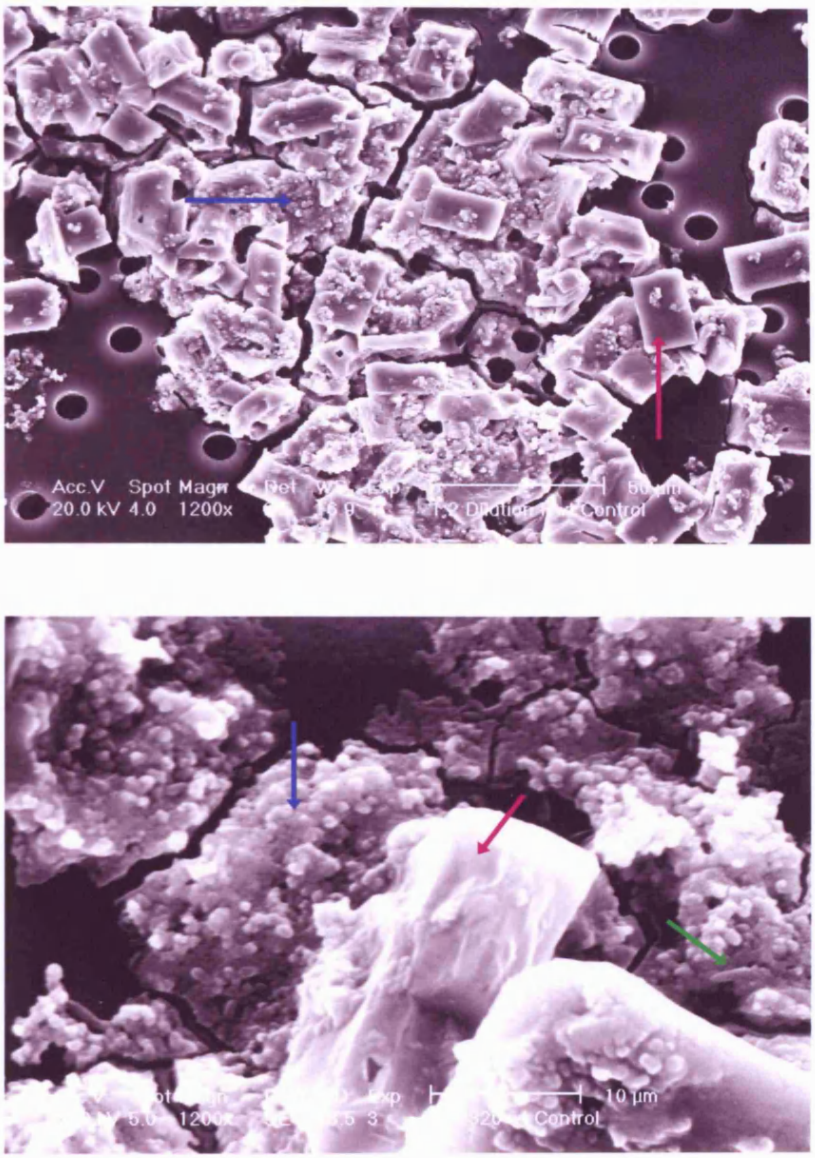


Figure 3.59 – High vacuum SEM images of a diluted, filtered suspension of *Pr. mirabilis* HI4320 *wt* control

Crystals and amorphous particle aggregates (indicated by pink and blue arrows respectively) can be seen in both images. Bacterial cells are indicated by green arrows

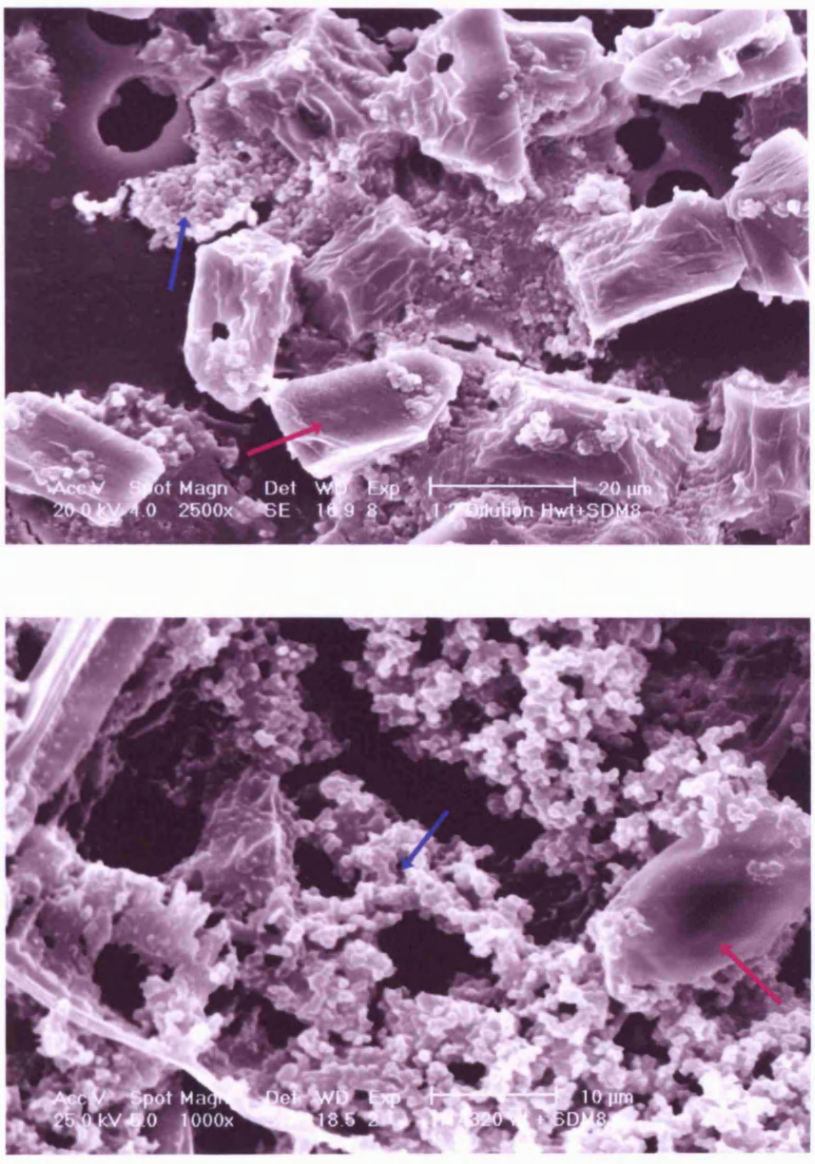


Figure 3.60 – High vacuum SEM images of a diluted, filtered suspension of *E. coli* SM8 and *Pr. mirabilis* HI4320

Masses of crystals and amorphous particle aggregates (indicated by pink and blue arrows respectively) were observed

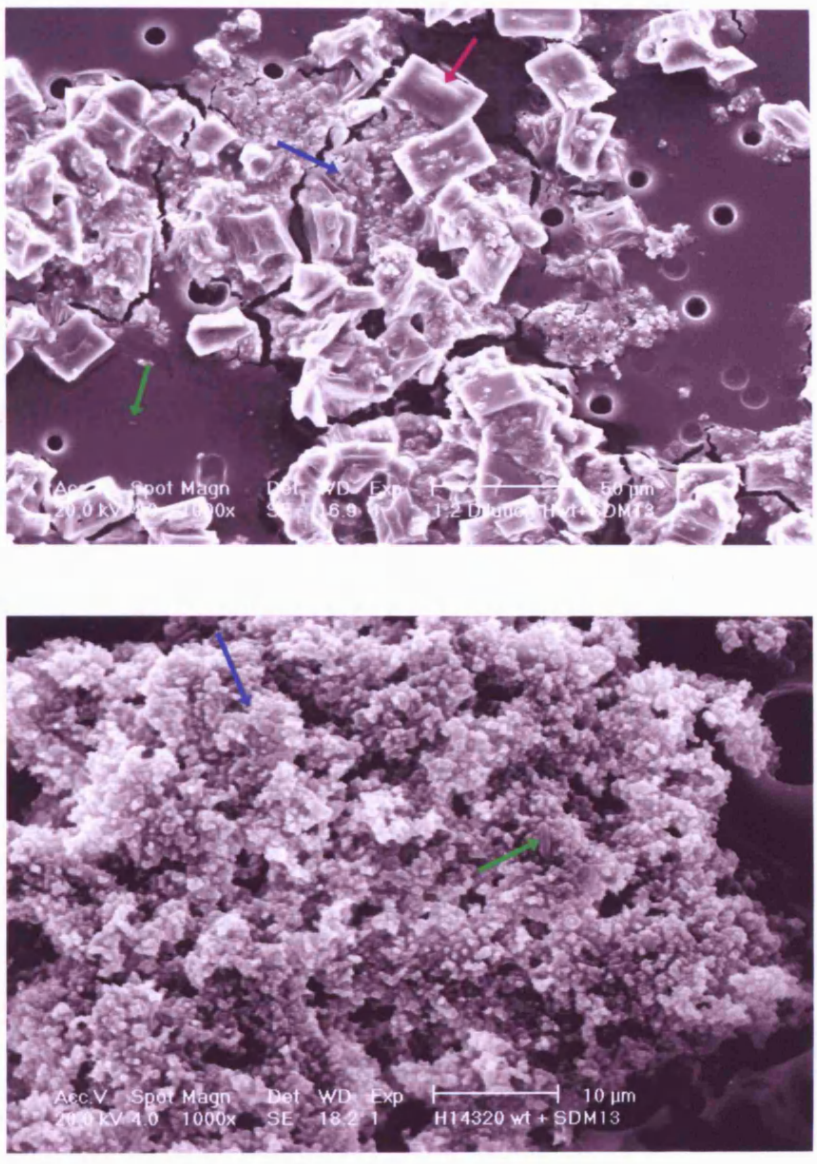


Figure 3.61 – High vacuum SEM images of a diluted, filtered suspension of *Ent. faecalis* SDM13 and *Pr. mirabilis* HI4320

Crystals and amorphous particle aggregates (indicated by pink and blue arrows respectively) can be seen. Bacterial cells, cocci and rods, are indicated by green arrows

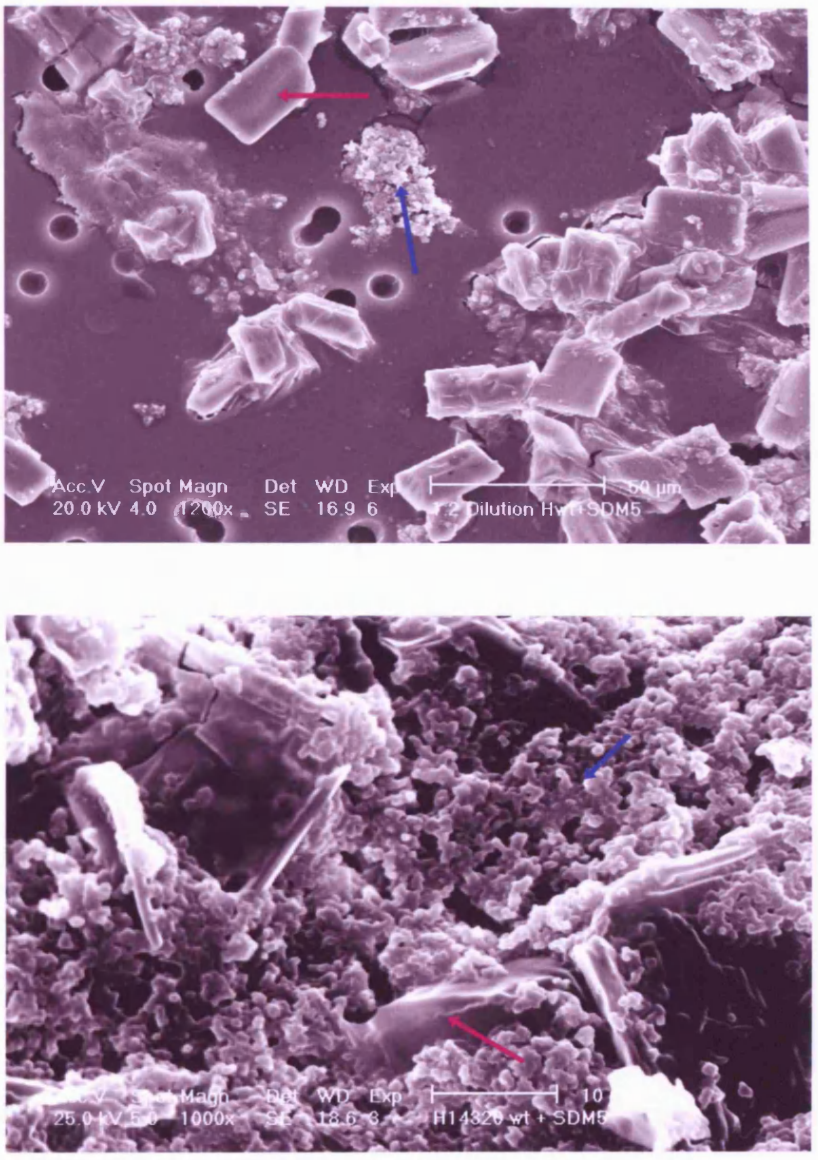


Figure 3.62 – High vacuum SEM images of a diluted, filtered suspension of *Ps. aeruginosa* SDM5 and *Pr. mirabilis* HI4320

Amorphous particle and tabular crystal aggregates (indicated by blue and pink arrows respectively) were seen

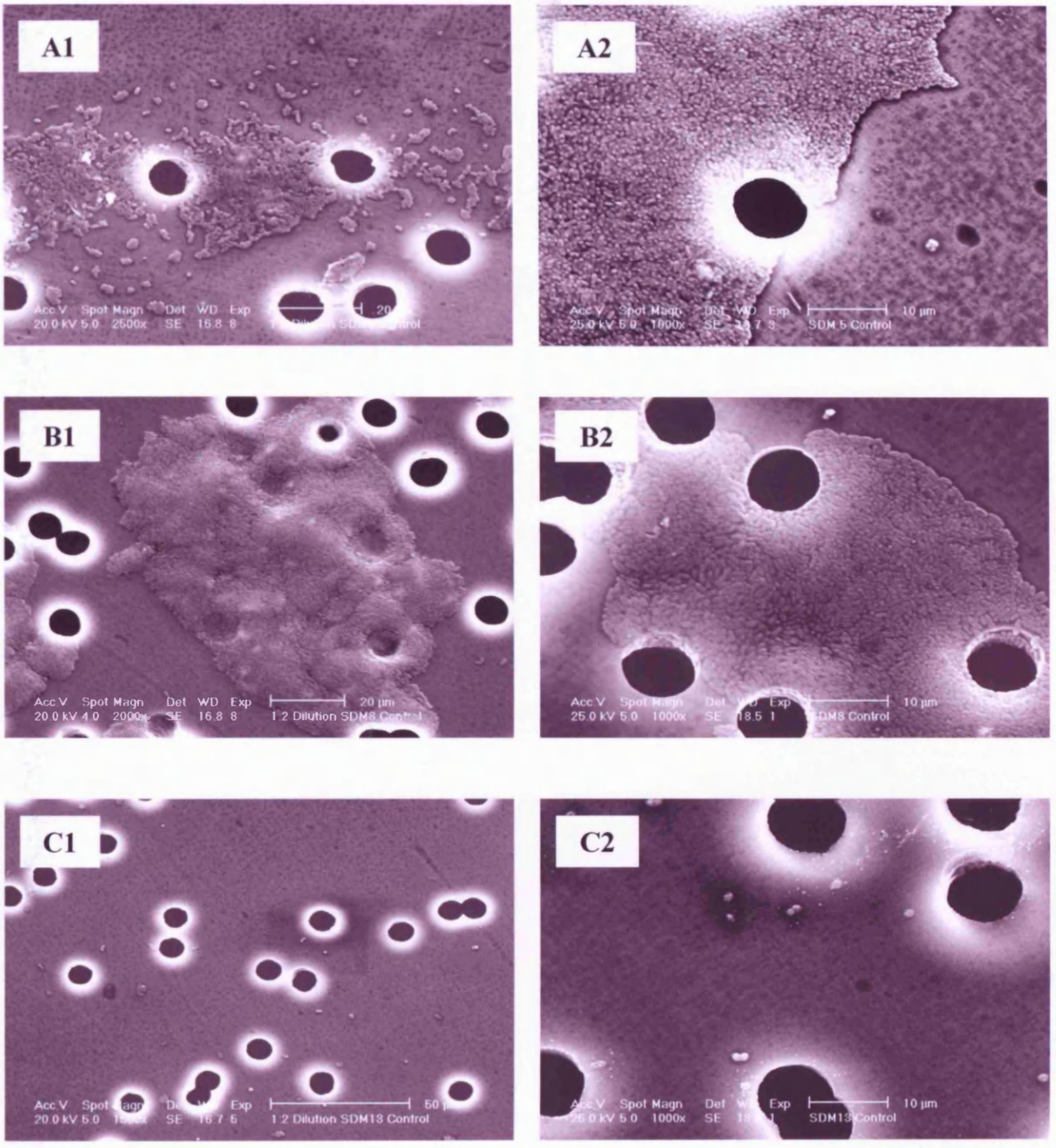


Figure 3.63 – High vacuum SEM images of diluted, filtered suspensions of *Ps. aeruginosa* SDM5 (A1 and A2), *E. coli* SDM8 (B1 and B2), and *Ent. faecalis* SDM13 (C1 and C2)

Isolated patches of cells were observed when pure *Ps. aeruginosa* and *E. coli* were filtered, in contrast to only sparse pairs of *Ent. faecalis* cocci.

Table 3.38 displays the mean proportions of the original inocula of each species that were retained on the membranes. Pair-wise analyses were performed using either the Kruskal-Wallis test or one-way ANOVA. The six urease negative organisms had proportions of original inocula retained on the membranes that were significantly greater than the *E. coli* control ($P \leq 0.05$). There were no significant differences ($P > 0.05$) between the proportions of retained urease-positive organisms after 4 h coaggregation with *Pr. mirabilis* in comparison to *Pr. mirabilis* alone. These results suggest that cells had become aggregated with the phosphates that had formed in the urine.

3.5 Catheter biofilms as a source of endotoxin

An early study by Garibaldi *et al.*, (1973) had shown a striking association between endotoxaemia and Gram-negative bacteriuria in patients undergoing indwelling bladder catheterisation. As infection-associated stones and the crystalline material that encrusts catheters are produced by urease producing Gram-negative organisms they are both potential sources of endotoxin. McAleer *et al.*, (2002) reported the case of an 8 yr old boy who underwent percutaneous nephrolithotripsy and died 12 h later having developed severe sepsis. Assays on fragments of the kidney stone yielded massive levels of endotoxin (approx. 285 ng/g) and bacteriological analysis revealed *Pr. mirabilis* in the urine. The boy was the youngest reported patient with presumed endotoxin-induced septic shock and this case was apparently the first in which the endotoxin source was shown to be an infection-associated kidney stone. The aim of this section of the investigation was to test whether endotoxin could be found in catheter biofilms.

Species combination (*Pr. mirabilis* HI4320 *wt* + ...)	Mean viable cell count of inoculum (cfu/ml)	Mean number of viable cells recovered from the membrane (cfu/ml)	Mean proportions (%) of original inocula recovered from the membranes
Prov. rettgeri SDM1	2.13×10^9	7.99×10^7	3.75
Kl. pneumoniae SDM3	2.73×10^9	2.33×10^7	0.85
C. koseri SDM4	4.67×10^9	9.70×10^7	2.08
Ps. aeruginosa SDM5	7.76×10^9	1.33×10^8	1.71
S. aureus SDM6	1.82×10^9	1.76×10^7	0.97
E. coli SDM8	4.11×10^9	6.15×10^7	1.50
Ent. faecalis SDM13	2.45×10^9	3.70×10^7	1.51
Prov. stuartii RB14 [a]	4.91×10^9	4.73×10^7	0.96
Morg. morganii RB15	5.03×10^9	8.96×10^7	1.78
Et. cloacae RB19	2.10×10^9	4.74×10^7	2.23
Kl. oxytoca NSM44	3.04×10^9	3.50×10^7	1.15
S. haemolyticus SM145	1.20×10^9	3.77×10^7	3.14
E. coli SDM8 control	7.83×10^9	1.01×10^7	0.13
Pr. mirabilis HI4320 *wt* control [b]	5.72×10^9	1.27×10^8	2.22

Table 3.38 – Proportions of original inocula aggregated by phosphates after 4 h coaggregation with *Pr. mirabilis* HI4320 *wt*

Aliquots (1 ml) were filtered through 10 um polycarbonate membranes. Collected material was then allowed to dissolve in 10 ml artificial urine and the numbers of viable cells determined.

No significant difference was indicated between the mean viable cell counts of the inocula ($P > 0.05$). Results are the means of three replicate experiments unless stated otherwise ([a] mean of 2 reps; [b] mean of 12 reps)

All six urease-negative species had significantly more cells retained after 4 h coaggregation with *Pr. mirabilis* than the control *E. coli* ($P \leq 0.05$). All of the species had proportions of retained cells not significantly different to the *Pr. mirabilis* control.

3.5.1 Detection of endotoxin using the Kinetic-QCL® Endotoxin Detection Assay

Human urine was collected from a healthy individual. An aliquot was filtered through a 0.2 μm pore size, non-pyrogenic Minisart® membrane filter and both filtered and non-filtered samples were assayed. Cultures of five common Gram-negative urinary tract pathogens growing in artificial urine (4 h at 37 °C) were analysed. Cultures were prepared for analysis by centrifugation at 3500 rpm for 5 min. The supernatant was then removed and along with a sample of the artificial urine were both analysed. In each assay LAL Reagent water and SDDW were also tested for the presence of endotoxin.

The data for the standard curves of the five assays are shown in Appendix D. The level of detection for two of the five assays was 0.05 EU/ml whilst the others had a detection threshold of 0.005 EU/ml

The results presented in Table 3.39 indicate that the urinary cultures of Gram-negative bacteria contained substantial amounts of free endotoxin which could not have come from any of the reagents used in the assay. The amounts of endotoxin recovered from the fresh all-silicone catheters were barely detectable in the assay. Slightly higher concentrations were recovered from the latex-based catheters. In contrast, considerable amounts of endotoxin were found on sections of catheters that had been colonized by bacterial biofilms both *in vitro* and *in vivo*.

Catheters from the *in vivo* setting were removed from long-term catheterised patients at the BioMed Centre's Catheter Clinic, Southmead Hospital, Bristol. Bacteriological analysis of these catheters revealed mixed species communities.

Sample	Endotoxin concentration (ng/ml)
Unfiltered healthy human urine	< 0.005
Filtered healthy human urine	< 0.005
Sterile double-deionised water[a]	< 0.0005
LAL Reagent water[a]	< 0.005
Artificial urine[b]	1.152
Sterile hydrogel coated latex catheter section[a]	1.004*
Sterile all-silicone catheter section[a]	0.0740*
Sterile silicone coated latex catheter section[a]	0.0415*
Pr. mirabilis B2 culture	645.1
Pr. mirabilis AAAX culture	357.3
E. coli culture	191.0
Morg. morganii culture	154.5
Ps. aeruginosa culture	116.4
All-silicone catheter section encrusted with a *Pr. mirabilis* biofilm[a*]	1856**
Patient A catheter section*	917.2**
Patient B catheter section*	480.6**
Patient C catheter section*	282.8**

Table 3.39 – Results of the Kinetic-QCL® endotoxin detection assays

In each case one replicate experiment was carried out unless stated otherwise ([a] mean of 3 replicates, [b] mean of 2 replicates)

* Catheter sections comprised from the catheter tip to 4 cm below

** Units for these results are ng/4 cm catheter section. Cultures were grown for 4 h at 37°C in artificial urine

The hydrogel-coated latex catheter from Patient A was colonized by a three membered community consisting of *Ps. aeruginosa*, *Ent. faecalis* and *M. luteus*. The hydrogel-coated latex catheter removed from Patient B was colonized by *Ps. aeruginosa* and *Ent. faecalis*, and the biofilm on the all-silicone catheter from Patient C contained both *Micrococcus sedentarius* and *Ent. faecalis*. All three catheters were free from visible luminal encrustation. In contrast the pure *Pr. mirabilis* biofilm generated *in vitro* had extensive luminal encrustation up to 3 cm from the catheter tip.

SECTION 4

Discussion

4 Discussion

There is both strong epidemiological (Mobley and Warren, 1987; Kunin, 1989; Stickler *et al.*, 1993a; Kohler-Ockmore and Feneley, 1996) and experimental (Stickler *et al.*, 1998b; Jones *et al.*, 2005b) evidence that *Pr. mirabilis* is the main cause of catheter encrustation. Laboratory studies on the development and control of crystalline biofilms on urinary catheters have historically involved experiments with pure cultures of *Pr. mirabilis* (Winters *et al.*, 1995; Morris *et al.*, 1997; Morris *et al.*, 1998a, 1998b; Stickler *et al.*, 2002; Stickler *et al.*, 2003a, 2003b; Jones *et al.*, 2005c; Sabbuba *et al.*, 2005). As such, this had led to a good understanding of the essential features of the process. Clinical studies however, have revealed that catheter-associated urinary tract infections in long-term catheterised patients are usually by mixed communities. *Pr. mirabilis* is commonly found as part of a mixed urinary flora rather than as a pure culture (Bultitude and Eykin, 1973; Clayton *et al.*, 1982; Warren *et al.*, 1982; Gillespie *et al.*, 1983; Warren *et al.*, 1987; Matsukawa *et al.*, 2005).

It has also become clear that in patients infected with *Pr. mirabilis* the rate at which their catheters encrust and block varies considerably (2 days to 14 weeks) (Mathur *et al.*, 2006). We have little insight into the factors controlling the rate of crystalline biofilm formation. It could be that other organisms in the urinary flora modulate the rate of the process. If some other species slows down or inhibits the formation of encrustation it opens up the prospect of biological, rather than chemical, interference as a control strategy. To examine the possibilities and develop these novel ideas we need to know more about the interactions between organisms in the urine of long-term catheterised patients and in the biofilms that

form on their catheters. The plan was thus to examine the ecology of catheter biofilm and urinary communities by epidemiological and experimental approaches.

4.1 The clinical study

The main objective of the clinical study was to gain first-hand experience of the nature of the bacterial communities that colonize the urine of long-term catheterised patients. There were no preliminary selection criteria for these patients and no prior knowledge of their urinary microbiology. It was also not known whether they were catheter "blockers" or "non-blockers". Study participants were fitted with either size 14 Ch 100% silicone catheters (P-01, P-04 and P-05) or size 14 Ch silicone-coated latex catheters (P-02 and P-03). Even from such a small-scale study it was very clear that complex and dynamic populations of organisms are involved in these infections (Figures 3.1 to 3.5).

Mono-species infections were not seen in any of the patients. One sample was found to contain eight species (Figure 3.4). The modal and mean number of species per sample was four. These species rich communities are characteristic of the urine and catheter biofilms of long-term catheterised patients (Clayton *et al.*, 1982; Warren *et al.*, 1982; Kunin *et al.*, 1987a; Jewes *et al.*, 1988; Ganderton *et al.*, 1992).

Some species were stable colonizers whilst others were transitory components of the urinary flora. Participant 01 had stable urinary co-colonization by *C. koseri* and *Kl. pneumoniae* with more transient visits by *E. coli* and *M. luteus*. A stable three-member community of *Pr. mirabilis*, *Ent. faecalis*, and *Ps. aeruginosa* was

resident in the urine of P-02 with three other species making fleeting one week appearances. Participant 03 had *E. coli* and *Kl. pneumoniae* isolated every week. *M. luteus*, *C. koseri*, *Ent. faecalis*, and *M. lylae* were isolated sporadically. Participant 04 was the only patient not to have any species that persisted throughout the six weeks, although once *Prov. stuartii* had colonized the urine it was then isolated consistently. Twelve different species in total were isolated from this patient. *Ps. aeruginosa*, *Morg. morganii*, and *M. luteus* were stable components of the flora of P-05 with five other species isolated intermittently. Clayton *et al.*, (1982) also found that species such as *Kl. pneumoniae*, *E. coli*, *Ps. aeruginosa*, and *Pr. mirabilis* persisted in spinal-injured patients for long periods. More recently Sabbuba *et al.*, (2003) reported on the considerable stability of *Pr. mirabilis* in the catheterised urinary tract. Over 121 days the same genotype of *Proteus* was isolated from the urine of a patient despite eight catheter changes.

Table 3.1 was constructed in order to get an overall view of the incidence of each species. The two most common species were both urease negative, *E. coli* and *Ent. faecalis* (11.48%). These findings were similar to those of the multi-centre survey completed by Jepson *et al.*, (1982) and those reported by Wazait *et al.*, (2003). Of the urease producing organisms *Ps. aeruginosa* and *Kl. pneumoniae* were the most prevalent (9.84%). The urease producing *Pr. mirabilis* was 7th in the overall prevalence ranking.

Examining the individual summary graphs for each participant (Figures 3.1 to 3.5) it can also be seen that there was considerable weekly variation in voided pH both within patients and between patients. Of the five study participants three

(Participants 01, 03, 05) had an acidic mean pH, two of which (P-01 and P-03) were below 6.00.

Bacteriological analysis demonstrated that all the patients had at least one urease positive species isolated from each weekly urine sample. An infection with urease producing organisms however, did not necessarily produce an alkaline pH or induce catheter blockage, as seen in Figure 3.5 for example. In this patient the voided pH was generally acidic except for week four when it increased to a mildly alkaline value of 7.22. This was despite consistent infection with as least 2 urease producing organisms, *Ps. aeruginosa* and *Morg. morganii*. These clinical observations support the conclusions of Stickler *et al.*, (1998b) from *in vitro* experiments; that these urease positive species are not capable of producing catheter blocking crystalline biofilm. Colonization with *Pr. mirabilis* on the other hand always raised the pH to highly alkaline (> 8.5) levels (Figures 3.2 and 3.4). These were therefore "classic" *Pr. mirabilis* infections in respect to pH rather than cases such as those occasionally seen by Mathur *et al.*, (2006) where a few patients with *Pr. mirabilis* bacteriuria were voiding acidic urine.

The data in Figure 3.6 suggests that when *Ent. faecalis*, *Prov. stuartii*, *Ent. durans*, *Ps. aeruginosa*, *C. freundii*, or *Pr. mirabilis* were present the mean pH was alkaline. The urease-negative species in this list could not have been responsible for raising the urinary pH, however they were species commonly found in communities along with *Pr. mirabilis*. The other five species had mean acidic pH values as they were generally not present in the same samples as *Pr. mirabilis*. It is interesting to speculate that these species: *Kl. pneumoniae*, *C.*

koseri, *E. coli*, *M. luteus*, and *Morg. morganii* had some sort of protective effect against infection by *Pr. mirabilis*. Mobley and Warren (1987) observed that *Morg. morganii* was the most common species found in samples from patients without blockage problems and suggested a possible antagonistic interaction between these species. The clinical study by Kunin (1989) found that *Kl. pneumoniae* was isolated significantly more often from non-blockers in contrast to *Pr. mirabilis*, which was recovered in significantly greater frequency from blockers.

4.1.1 Catheter life

The time between an individual's catheter change is known as a "catheter life". Norberg *et al.*, (1983) performed an investigation into the catheter lives of 20 geriatric inpatients of a Swedish hospital. The scheduled catheter life of the silicone-coated latex catheters was 30 days. The 20 study patients however had problems attributed to catheter blockage or self-removal which resulted in shortened catheter lives. For unknown reasons catheter lifespans varied extensively both between patients and within the same patient. In the majority of patients the distribution of catheter lives was wide. Those who tended to have short lifespans intermittently having very long ones and *vice versa*. The authors suggested that the median time from 3 to 5 observations could be used as a more reliable prediction of blockage time.

In Norberg's study (1983) the median catheter lifespan was nine days. In the current study there were only three catheter changes between the five participants during the six weeks, and one of these was a scheduled routine replacement at the

beginning of the study. *Pr. mirabilis* was present in the urine of Participant 02 throughout the study. Unsurprisingly P-02 had two catheter changes due to blockage during the six weeks (Figure 3.2). The period between the two catheters of P-02 becoming blocked was 13 days. The other four patients can be considered to have single catheter lifespans of > 42 days, though without a longer observation period it is not known whether these times were "normal" for each patient. The absence of blockage events in four of the five patients however is not necessarily indicative of non-blocker status. Mathur *et al.*, (2006) observed that some of their study participants had median catheter lifespans of > 42 days despite *Pr. mirabilis* bacteriuria. They argued against using the classic blocker/non-blocker criteria of blockage in < 30/> 30 days respectively, and instead suggested the use of the terms rapid and slow encrusters in those colonized by *Pr. mirabilis*. It is feasible that P-02 had regular problems with catheter encrustation and can thus be defined as a rapid encruster. In contrast in P-04 for example, the sporadic presence of *Pr. mirabilis*, although it caused temporary elevations in pH, caused no catheter blockage events (Figure 3.4). It is possible that this was due to its transitory existence and/or because this patient was a slow encruster.

The type of catheter differed between Participants 02 and 04, the former possessing a silicone-coated latex catheter (silicone elastomer) and the latter a 100% silicone catheter. Although Morris *et al.*, (1997) found that all of the 18 commercially available catheters became blocked by crystalline *Pr. mirabilis* biofilm, those manufactured from silicone took considerably longer to block, a feature also observed by Kunin *et al.*, (1987b). Both groups of authors considered that this attribute was likely due to the larger lumen size of all-silicone catheters.

The internal lumen diameter was found to have a significant positive correlation with time to blockage (Morris *et al.*, 1997). So as well as P-04 perhaps being a slow encruster the larger lumen size of the all-silicone catheter might have conferred an additional benefit.

4.1.2 The nucleation pH of the urine

Choong *et al.*, (2001) reported that the relationship between the pH at which crystals form in the urine, the nucleation pH (pH_n), and the pH of the voided urine (pH_v) is critical for catheterised patients. If the pH_v of urine exceeds the pH_n then catheter encrustation will occur. In patients not prone to this complication, the pH_v was normally well below the pH_n (they quoted mean values of 6.26 for pH_v compared to 7.66 for pH_n). The safety margin between these values ensured that crystal formation did not occur. In patients considered to be blockers they reported a mean pH_v value of 7.85, elevated by urease activity, which was above the mean pH_n of 7.58. In these cases the patients were storing and voiding urine that had already reached the critical nucleation pH. Mathur *et al.*, (2006) went on to show that the difference between pH_v and pH_n also influenced the rate at which catheters blocked in patients infected with *Pr. mirabilis*.

It cannot be assumed that because the week six nucleation pH was higher or lower than the voided pH of that week that it was consistently this way throughout the study period. It is now known that an individual's pH_n, along with the pH_v can change from week-to-week and can vary from day-to-day and even throughout the day (Suller *et al.*, 2005; Mathur *et al.*, 2006; Appendix A). It is interesting however, that the pH_v of the urine samples obtained at week six from patients 01,

03, 04 and 05 were all below the pH_n i.e., the safety margin was positive (Figures 3.1 and 3.3 to 3.5). None of these patients suffered catheter blockage during the study. Only in the case of P-02 (Figure 3.2) was the safety margin negative (i.e., the pH_v above the pH_n) and this patient had two catheters block over the 6-week period.

It has been suggested that the presence of bacterial cells may provide an environment in which crystal nucleation and development more readily occurs (Clapham *et al.*, 1990). Suller *et al.*, (2005) examined whether the addition of bacterial cells decreased the nucleation pH by promoting precipitation. Cells of *E. coli*, *Ent. faecalis*, *Pr. mirabilis*, and *Ps. aeruginosa* at varying concentrations had very little effect on the pH_n. This implies that the pH_n is an intrinsic feature of the urine itself rather than a factor of the bacterial flora present within it.

4.1.3 Calcium and magnesium concentrations in relation to nucleation pH

Getliffe (1994a) and Choong *et al.*, (1999) found no differences in urinary calcium or magnesium concentrations of catheter blockers in comparison to non-blockers. Significant differences in urinary $[Ca^{2+}]$ were however found by Kunin *et al*, (1987a) and Burr and Nuseibeh (1997).

The calcium and magnesium concentrations from the five study participants were analysed for their relation to the pH_n values at week six (Figures 3.7 to 3.9). The significant negative correlation between the concentrations of these divalent metal ions and the pH_n values indicates that simply diluting the urine should reduce encrustation. The concentration of calcium had a greater effect on the nucleation

pH than the magnesium or the total calcium plus magnesium concentrations. As calcium containing apatite precipitates at a lower pH than magnesium containing struvite (Hedelin *et al.*, 1985) it might be expected to have more of an influence on the pH at which crystal precipitation occurs.

From the limited data collected, the initial concentrations of calcium and magnesium thus seem to be two important factors that influence the pH_n value in long-term catheterised patients with diverse but rich urinary flora. Strategies to decrease the urinary concentrations of calcium and magnesium via increased fluid intake or citrate supplementation (Suller *et al.*, 2005; Stickler and Morgan, 2006) will act to increase the pH_n and reduce the rate of crystal accumulation and mineralised bacterial biofilm development. These approaches may well have a clinical, prophylactic use in those individuals catheterised for extended periods.

4.2 Ecological evidence of interactions between species in catheter biofilms

About half of the patients undergoing long-term catheterisation will avoid infection by *Pr. mirabilis* and the complications of catheter encrustation (Cools and van der Meer, 1986; Kunin *et al.*, 1987a, 1987b; Getliffe, 1990; Kohler-Ockmore and Feneley 1996). It could be that the catheterised urinary tract of some patients are just not exposed to the challenge of this species. *Pr. mirabilis* is a common human faecal organism however and genotyping has shown that the strains of *Pr. mirabilis* colonizing the urine of catheterised patients are often identical to those resident in the faecal flora (Mathur *et al.*, 2005). *Pr. mirabilis* is not normally amongst the primary colonizers of the catheterised urinary tract, it

usually infects patients who already have an established urinary flora (Bultitude and Eykin, 1973; Clayton *et al.*, 1982; Warren *et al.*, 1982; Gillespie *et al.*, 1983; Tambyah *et al.*, 1999; Matsukawa *et al.*, 2005). A possible alternative explanation therefore, is that some patients remain encrustation free because their resident urinary and/or biofilm flora is antagonistic to *Pr. mirabilis* and prevents it from establishing in the catheterised bladder. Some organisms might well be valuable in the fight against *Pr. mirabilis* colonization.

There is some evidence in the literature suggesting interactions between organisms in the urine of catheterised patients. Mobley and Warren (1987) reported a study which involved the bacteriological analysis of 1135 weekly urine samples from 32 chronically catheterised patients. Whilst *Pr. mirabilis* was strongly associated with catheter blockage, *Morg. morganii* was found more often in samples from patients who had not suffered any catheter obstructions. They suggested that *Morg. morganii* in some way inhibited catheter encrustation in spite of high numbers of *Pr. mirabilis* by interfering or modulating its effects. In a similar study Kunin (1989) found that *Kl. pneumoniae* was generally not found in the urine of catheter blockers who were infected with *Pr. mirabilis*.

The availability of data on the nature of the flora of catheter biofilms from the work of previous researchers at the catheter lab at Cardiff University facilitated an examination of possible interactions between organisms in the biofilms rather than just the urine. The catheters had been collected from long-term catheterised patients in hospital and community care in a variety of locations in South Wales and the South West of England. They had been collected over a number of years

so the collated information was therefore temporally and spatially diverse. Repeat catheters from individual patients with identical bacterial flora were not included in the analysis.

The summary of the collated data (Tables 3.2) reveals that the species in the mixed community biofilms were characteristic of those found in the urine of individuals catheterised for extended periods (Clayton *et al.*, 1982; Warren *et al.*, 1982; Stickler *et al.*, 1988). *Ps. aeruginosa* was the commonest species isolated overall (Table 3.2). These results are consistent with those of Stickler *et al.*, (1988). Over the period of one year these workers obtained regular urine samples from 21 catheterised patients and reported that *Ps. aeruginosa* was the most frequently isolated organism especially during periods of antibiotic therapy. They attributed this to the organism's ability to adhere to silicone and Teflon catheter material to a significantly greater degree than the eight other species tested in *in vitro* experiments. In addition they suggested that one of the reasons for its high incidence was its resistance to many antibiotics. Of the three species shown by Stickler *et al.*, (1998b) to be able to cause significant crystalline encrustation in laboratory models of the catheterised bladder *Pr. mirabilis* had the highest incidence in this study (Table 3.2) confirming its importance in the catheterised urinary tract.

The analysis of the co-occurrences of the various species in the biofilms, presented in Table 3.3, indicates several interesting associations. For example, when *Pr. stuartii* was found in a catheter biofilm it was often (7 out of 11 times) found together with *Ps. aeruginosa*. On the other hand when *Et. cloacae* was isolated,

Ps. aeruginosa was never found. Examination of the data in relation to *Pr. mirabilis* was of course the primary interest. These data were extracted from Table 3.3, the species ranked on the basis of their frequency of co-incidence with *Pr. mirabilis* and presented in Table 3.4.

The overall incidence of *Pr. mirabilis* on the 106 catheters was 30.19% (Table 3.2). The data presented in Table 3.4 illustrates that particularly when *Prov. stuartii*, *Kl. pneumoniae*, or *Ent. faecalis* were recovered from catheters, the percentage incidence of *Pr. mirabilis* was clearly above this figure. In contrast, when species such as *E. coli*, *Morg. morganii*, or *Et. cloacae* were present on a catheter *Pr. mirabilis* was rarely or never found.

The Chi square analysis performed on the data (Table 3.5) suggests that for four of the species analysed co-colonization of *Pr. mirabilis* in the catheter biofilm was significantly different from its expected frequency on those catheters. *Pr. mirabilis* was found significantly less often than expected (Table 3.4) on catheters that were also colonized by *Ps. aeruginosa* ($P \leq 0.01$), *E. coli* ($P \leq 0.001$), *Morg. morganii* ($P \leq 0.01$), or *Et. cloacae* ($P \leq 0.001$).

Analysis of the associations between species by calculation of the odds ratios (Table 3.7) ranked *Prov. stuartii*, *Kl. pneumoniae*, and *Ent. faecalis* as being the species most likely to be found in conjunction with *Pr. mirabilis*. *Morg. morganii* and *Et. cloacae* were again identified as the species least likely to be found colonizing catheters with *Pr. mirabilis*.

The results of the analysis of the biofilm flora are interesting in relation to some earlier experimental studies by Sabbuba *et al.*, (2002). These workers tested, in a simple laboratory model, the ability of *Pr. mirabilis* to migrate over catheter surfaces in the presence of other urinary tract pathogens. They found that swarmer cells of *Pr. mirabilis* could migrate over catheters through populations of *E. coli*, *Kl. pneumoniae*, *Ent. faecalis*, and *S. aureus*. There was no sign of migration inhibition by these species. In the cases of *Kl. pneumoniae* and *S. aureus*, the *Pr. mirabilis* swarmer cells were even capable of transporting these non-motile organisms over the catheter surfaces. Isolates of *Serratia marcescens* and *Ps. aeruginosa* however, significantly inhibited surface migration of *Pr. mirabilis*. In view of the results presented in Tables 3.4, 3.5 and 3.7 it would be interesting to test the ability of *Morg. morganii* and *Et. cloacae* to inhibit the migration of *Pr. mirabilis*. If this occurred it would suggest that colonization of the catheter insertion site by these species could prevent the movement of *Pr. mirabilis* along the outside of the catheter into the bladder.

The results of the ecological approaches to the analysis of the catheter biofilms (Tables 3.4, 3.5 and 3.7) do suggest that there might be interactions between *Pr. mirabilis* and other species. It was decided to examine these possibilities in more detail using an experimental approach. The interactions of *Pr. mirabilis* with *Et. cloacae*, *Morg. morganii*, *Ps. aeruginosa*, and *Kl. pneumoniae* were investigated in the laboratory models of the catheterised bladder. In addition *E. coli* was also selected because of its recent use as a biological control organism against infection in the catheterised urinary tract (Darouiche *et al.*, 2001, 2005).

4.3 Investigations into the development of mixed community bacterial biofilms on urethral catheters

The simple laboratory models of the catheterised bladder used in the experiments reported in section 3.3 have been exploited to study the development of crystalline biofilm formation on various types of catheters (Morris *et al.*, 1997; Stickler *et al.*, 2003b). They have also been used extensively to test novel strategies for controlling and preventing catheter encrustation (Morris and Stickler, 1998b; Morris and Stickler 2001; Jones *et al.*, 2005b, 2005c; Sabbuba *et al.*, 2005; Stickler and Morgan, 2006). Whilst they clearly do not exactly reproduce the clinical situation, they allow comparative studies to be performed under controlled, standardised conditions. The effects of interventions can be assessed in experiments using defined populations of test organisms, with urine of a standard composition being supplied to the bladder chamber at set flow rates. The flow rate used in the experiments in this study (0.5 ml/min, i.e. 750 ml/day) being chosen to simulate that typically produced by many elderly catheterised patients who have characteristically low fluid intakes.

Many experiments have been performed in these, and other similar models, using pure cultures of various uropathogens (Harkes *et al.*, 1992; Nickel *et al.*, 1992; Darouiche *et al.*, 1997b; Morris *et al.*, 1997; Morris and Stickler, 1998b; Gaonkar *et al.*, 2003; Stickler *et al.*, 2003b; Jones *et al.*, 2005b, 2005c). However, few if any have examined biofilm formation by the sorts of mixed bacterial communities commonly infecting the urine of patients undergoing long-term catheterisation. The experiments reported in section 3.3 examined the formation of biofilms in models inoculated with pairs of organisms. *Pr. mirabilis* and one of a series of

five other species commonly found in the urine of catheterised patients were used as inocula. The test organisms were chosen because there was some epidemiological evidence that they might interact in some way with *Pr. mirabilis*. The objectives of the experiments were to establish whether any of the other species reduced the ability of *Pr. mirabilis* to produce alkaline urine and encrust catheters with crystalline biofilm. In the first set of experiments the pairs of species were inoculated into the bladder chambers simultaneously.

4.3.1 The simultaneous inoculation of bladder models with pairs of organisms

It is clear from the results presented in section 3.3.1 that the simultaneous co-inoculation of *Pr. mirabilis* with other urinary species had no major influences on its ability to encrust catheters. In all cases the two-member communities blocked catheters within mean times of 42 h. A summary of the mean times the various communities took to block catheters is presented in Table 4.1.

Pr. mirabilis **+ test species**	**Mean time to catheter blockage in co-inoculated models h ± SE mean**	**Mean time to catheter blockage of *Pr. mirabilis* controls h ± SE mean**
Et. cloacae	30.76 ± 6.05*	18.67 ± 0.12
Morg. morganii	30.69 ± 7.46	22.32 ± 3.01
E. coli	23.59 ± 1.59	19.73 ± 2.88
Ps. aeruginosa	41.43 ± 8.13*	23.34 ± 4.29
Kl. pneumoniae	32.50 ± 6.98	24.81 ± 2.55

Table 4.1 – Summary table of the mean times to catheter blockage from the simultaneous co-inoculation experiments

Results are the means of four replicate experiments. * Only co-inoculation with *Et. cloacae* or *Ps. aeruginosa* significantly increased time to catheter blockage ($P \leq 0.05$) compared to the *Pr. mirabilis* controls.

The mean values were all greater than the corresponding *Pr. mirabilis* controls, but only in the cases of *Et. cloacae* and *Ps. aeruginosa* were these differences significant ($P \leq 0.05$). All the models inoculated with mono-cultures of the test organisms drained freely for the duration of the experiments.

Rates of calcium and/or magnesium deposition (μg/catheter/h) on catheters from all models that were inoculated with *Pr. mirabilis*, either alone or as a combination were high (Figures 3.10, 3.13, 3.15, 3.17 and 3.19). Total calcium and magnesium rates were commonly > 400 μg/h. No differences ($P > 0.05$ existed between the rates in *Pr. mirabilis* controls and co-infection experiments. The rates of deposition on catheters from models inoculated with the five non-*Proteus* test organisms alone were all negligible. *Et. cloacae* and *E. coli* were both urease-negative organisms and were expected not to generate luminal encrustation. *Morg. morganii* SM18, *Ps. aeruginosa* SM15, and *Kl. pneumoniae* SDM3 all registered as urease-positive in standard laboratory tests. However, at the end of the experiments the mean rates of total calcium and magnesium accumulation were only 3.80, 7.92, and 1.68 μg/catheter/h respectively. These findings correspond with the observations of Stickler *et al.*, (1998b), who reported that over the course of 24 h in the bladder models, three isolates each of *Morg. morganii*, *Kl. pneumoniae*, and *Ps. aeruginosa* produced little encrustation compared to *Pr. mirabilis*.

The data presented in Tables 3.8 to 3.12 show that the viable cell counts of the five test species all decreased on incubation with *Pr. mirabilis*. The mean viable cell counts at blockage were lower in the urine containing the mixed cultures than

in the corresponding cultures of the five test species growing alone ($P \leq 0.05$). These results suggest that if *Pr. mirabilis* is present in mixed urinary infections in the catheterised bladder, over time it will become the dominant organism. Similarly, Fletcher *et al.*, (1994) hypothesised that *E. coli* colonization of urine and catheter surfaces *in vitro* would be affected by *Prov. stuartii* when inoculated together due to competition for attachment or antagonistic interactions. They found that the urease-negative *Prov. stuartii* was dominant in both locations but both organisms persisted. In contrast, Anderson *et al.*, (1979) reported species survival in mixed populations was a feature of bacterial growth rate. Using a laboratory model of the normal bladder, supplied with pooled human urine, they showed that mixed cultures of fast growing *E. coli* combined with a slower growing species such as *Pr. mirabilis* became nearly pure *E. coli* cultures within 24 h. This evidently does not happen in the catheterised urinary tract.

The pattern of results for the urinary pH was similar with each pair of organisms tested (Tables 3.8 to 3.12). Each of the five test species growing alone maintained an acidic pH from experiment start to finish. In contrast, the pH rose well above pH 8 in all the *Pr. mirabilis* control and mixed culture models. There were no statistical differences between values produced in each mixed culture and the corresponding *Pr. mirabilis* controls ($P > 0.05$). In view of these results it was not surprising that the low-vacuum scanning electron micrographs shown in Figures 3.11, 3.14, 3.16, 3.18 and 3.20 revealed that catheters from models infected with mono-cultures of *Et. cloacae*, *Morg. morganii*, *E. coli*, *Ps. aeruginosa*, or *Kl. pneumoniae* were all free from crystalline material. Extensive encrustation around the eye-holes and to some extent throughout the entire drainage lumen

however, was evident on catheters removed from all *Pr. mirabilis* infected bladder models. These and the previous findings correspond with those observed by Stickler *et al.*, (1993a, 1998b).

These simultaneous co-inoculation experiments simulated situations in which new, dual-species infections were initiated. Such infections might occur early on in the catheterisation period. The results suggest that if a mixed infection comprising of equal numbers of *Pr. mirabilis* and another species colonize the catheterised urinary tract *Pr. mirabilis* will out-compete the other organism. Calcium and magnesium phosphates will then form in the urine and on the catheter, and catheter blockage will rapidly follow.

4.3.2 *Pr. mirabilis* super-infection of juvenile (24 h) biofilms

By the time *Pr. mirabilis* colonizes the catheterised urinary tract on many occasions it might be expected that other organisms have already begun to establish themselves. This situation might pose a greater challenge for *Pr. mirabilis*. Allowing the five test organisms to develop biofilms for 24 h before *Pr. mirabilis* super-infection however, did not increase significantly the mean time taken for the drainage lumen to become blocked by encrustation. The mean range of values of 30.57 ± 1.13 h to 45.43 ± 10.91 h was not significantly different to that of the *Pr. mirabilis* controls (range 24.5 ± 1.31 h to 33.37 ± 3.88 h; $P > 0.05$).

Quantification of the amounts of calcium and magnesium that had accumulated on catheters (Figures 3.21, 3.24 and 3.27-3.29) showed results that were similar to those obtained from the co-inoculation experiments. All catheters from pure test

organism infected models, those removed at 24 h and those removed at the end of the experiments, had very low levels of calcium and magnesium recovered from their luminal surfaces. In no case did the presence of a 24 h biofilm significantly affect the rate of calcium and magnesium deposition ($P > 0.05$).

The cell densities of *Pr. mirabilis* in the urine at the end of all experiments were equivalent in both the *Pr. mirabilis* controls and the 24 h super-infection models. Young 24 h biofilms of *Et. cloacae*, *Morg. morganii*, *E. coli*, *Ps. aeruginosa*, or *Kl. pneumoniae* failed to cause significant changes in the number of viable *Proteus* cells (Tables 3.13, 3.14 and 3.16).

Figures 3.22, 3.25, 3.30, 3.32 and 3.34 show that at the end of the experiments the five test organisms, despite *Ps. aeruginosa*, *Morg. morganii*, and *Kl. pneumoniae* being urease-producers, were free from encrustation. Blockage of the eyehole or within the first few centimetres of the lumen occurred in most catheters from models that had been infected with *Pr. mirabilis*. Substantial crystalline material was also visible up to 10 and sometimes 20 cm below the eyehole. Morris *et al.*, (1997), in their experiments with laboratory bladder models, found that the majority of encrustation was deposited in the 10 cm of lumen below the eye-hole regardless of the type of catheter. Similar observations were made by Kunin *et al.*, (1987a). If the tip section immediately below the eye-hole was only partially blocked there was encrustation of a similar amount throughout the rest of the lumen. However if the tip section was completely obstructed the extent of encrustation decreased down through the lumen as the distance from the tip increased.

Within 24 h the urine in models containing *Pr. mirabilis* alone consistently had urine that was heavily turbid, indicating the precipitation of large amounts of phosphates. With the exception of *Morg. morganii* SM18, the urine in the bladder chambers of models infected with the four other test organisms alone remained free from crystalline precipitate (Figures 3.23, 3.31, 3.33 and 3.35). *Morg. morganii* gradually accumulated insoluble material within the bladder compartment (Figure 3.26). At 96 h this precipitate was easily visible but still to a much lesser extent than that in the *Pr. mirabilis* controls at 24 h. As the *Morg. morganii* control was stopped once both *Pr. mirabilis* containing models had blocked it is not known whether, if allowed to continue, the catheters in these models would have eventually blocked themselves.

Tables 3.13, 3.14 and 3.17 show that the pH of the urine at catheter blockage in all the models containing *Pr. mirabilis* rose to > 8.3 except when an 24 h *E coli* biofilm was present (pH 7.93 ± 0.34). The highest mean urinary pH of the five test organisms at the end of the experiments was generated by *Morg. morganii* (6.79 ± 0.09; Table 3.14). In two previous studies urease-producing *Morg. morganii* was not capable of generating alkaline conditions or creating crystalline biofilm over periods of 24 or 48 h in *in vitro* bladder models (Stickler *et al.*, 1998b; Jones *et al.*, 2005b). Evidence presented in this study however, suggests that this organism can raise the pH to levels at which calcium and magnesium phosphates begin to precipitate over extended periods of time.

The results from section 3.3.2 clearly show that in models in which the five other urinary tract pathogens have been growing for 24 h and producing young biofilms

on the catheters, super-infection with *Pr. mirabilis* rapidly induces alkaline urine and crystalline biofilm formation. There were no signs that any of the 24 h biofilms had any inhibitory effects on the ability of *Pr. mirabilis* to encrust and block catheters.

4.3.3 *Pr. mirabilis* super-infection of mature (72 h) biofilms

In a final attempt to investigate whether *Et. cloacae*, *Morg. morganii*, *E. coli*, *Ps. aeruginosa*, or *Kl. pneumoniae* could modulate the extent of encrusting biofilm caused by *Pr. mirabilis* the five test organisms were allowed to develop biofilms for 72 h before super-infection with *Pr. mirabilis*. The data presented in Figure 3.36 shows that super-infection with *Pr. mirabilis* under these conditions still resulted, in all cases, in catheter blockage. The analysis of the data however, (Table 3.18) indicated that except for *Ps. aeruginosa*, the other four uropathogens significantly delayed the mean times the catheter took to block.

Et. cloacae 72 h biofilms had the most pronounced effect on *Pr. mirabilis*, extending the mean time to catheter blockage to over 60 h. Throughout the experiments the urinary pH in the *Et. cloacae* and *Pr. mirabilis* mixed models was significantly lower ($P \leq 0.05$) than in the *Pr. mirabilis* controls (Figure 3.37 to 3.39). It was also interesting that there was no significant difference between the mean urinary viable cell counts of *Pr. mirabilis* at blockage in the control and super-infection models (Table 3.19). The mean viable numbers of *Et. cloacae* in the super-infection models (7.83 ± 0.15 $\log_{10}$ cfu/ml) was however, significantly lower than in the control model (8.77 ± 0.21 $\log_{10}$ cfu/ml; $P \leq 0.05$). These results

support the hypothesis that there is a degree of antagonism between these organisms.

In the case of *Morg. morganii*, the mean time to catheter blockage was extended to over 56 h. The pH of the urine rose more slowly in the super-infection models than in the control *Pr. mirabilis* (Table 3.37 and 3.38), but at blockage there was no significant difference between the mean pH values (Tables 3.39). The pH data from the *Morg. morganii* control models show that over the longer incubation period of these experiments, this organism was capable of producing alkaline urine (mean pH 7.54 ± 0.13). The mean numbers of viable cells of both species were significantly lower ($P \leq 0.05$; Table 3.20) in the super-infected models than in the respective mono-species infected models, suggesting that they didn't grow as well together as they did apart.

It took *Pr. mirabilis* a mean time of over 55 h to block catheters already colonized by 72 h biofilms of *Kl. pneumoniae*. At catheter blockage, mean numbers of *Pr. mirabilis* (6.93 ± 0.24 log10 cfu/ml) and *Kl. pneumoniae* (7.52 ± 0.20 log10 cfu/ml) in the mixed species models were not significantly different to those in their respective controls ($P > 0.05$, Table 3.23). These species seemed to grow just as well together as they did separately. Interestingly, at catheter blockage the mean urinary pH in the *Klebsiella* super-infection models (pH 8.00 ± 0.34) was still significantly lower than the mean pH of 8.82 ± 0.08 of the *Pr. mirabilis* controls ($P \leq 0.05$; Figure 3.29).

Seventy-two hour biofilms of *E. coli* also caused a significant increase in the time taken for *Pr. mirabilis* to block catheters (from 24.32 ± 2.37 h to 47.51 ± 3.65 h).

Neither species had significant changes in cell number when grown as a mixed culture compared to their controls ($P > 0.05$; Table 3.21). Although the urinary pH rose more slowly in super-infection models than in models inoculated with a mono-culture of *Pr. mirabilis*, by the time of catheter blockage mean values were not significantly different ($P > 0.05$; Figure 3.29).

Ps. aeruginosa 72 h biofilms did not significantly increase the time to catheter blockage caused by super-infected *Pr. mirabilis* ($P > 0.05$; Figure 3.36 and Table 3.18). Once again, the pH in the super-infection models increased at a slower rate than in the pure *Pr. mirabilis* models (Figures 3.27 to 2.29) but by catheter blockage the differences were not significant ($P > 0.05$). At the end of the experiments numbers of viable *Pseudomonas* and *Proteus* cells recovered in the urine were equivalent in both mono-culture infected and super-infected models ($P > 0.05$; Table 3.22). These results suggest that *Pr. mirabilis* and *Ps. aeruginosa* grow equally well together as apart.

It has to be said that the values of the data on the rate of deposition of calcium and magnesium on the catheters (Figure 3.40) was probably undermined by the variability obtained by replicate experiments. This can be seen for example in the case of super-infection of *Et. cloacae*. The mean value of the rate of encrustation (approx. 370 μg/catheter/h) would appear to be considerably lower than that from the *Pr. mirabilis* control (around 630 μg/catheter/h). The standard errors of these mean values (99.84 and 157.27 μg/catheter/h respectively) and the statistical analysis however, revealed no significant difference between the mean values ($P > 0.05$). It is likely that the variation in the data was due to variability in the

amounts of encrusted material that were lost from catheters as they were removed from the models and prepared for chemical analysis.

The micrographs of the catheters removed at the end of the experiments from the models that had been inoculated with the mono-cultures of *Et. cloacae*, *E. coli*, *Kl. pneumoniae*, or *Ps. aeruginosa* showed no signs of crystalline biofilm (Figure 3.43). However, there were some signs of crystalline material around the eye-hole and in the lumen of the catheter from the model infected with *Morg. morganii* (Images 2A-D). It was much less obvious than that on the catheters from all models infected with *Pr. mirabilis* (Figure 4.42) but suggests that *Morg. morganii* could be a slow encruster.

The experiments reported in Figures 3.44 to 3.46 confirmed that over the 72 h incubation period, the test organisms all established substantial biofilms on the catheters. It is also clear that *Pr. mirabilis* was able to colonize these biofilms (Tables 3.24 to 3.28). Fletcher *et al.*, (1994) reported that when either a urease-negative *Prov. stuartii* or *E. coli* was allowed to become established on catheter surfaces *in vitro* before infection with the other, the initial colonizer failed to prevent subsequent colonization by the secondary colonizer.

Catheters removed from super-infection models at blockage of the *Pr. mirabilis* control (Figures 3.48 to 3.50) show that the extent of crystalline material in each case was considerably less than that seen on the catheter colonized by *Pr. mirabilis* alone (Figure 3.47). Comparing LV and HV-SEM images (Figures 3.42 and 3.47) of catheters from *Pr mirabilis* infected models it is apparent that a large

proportion of encrusted material had been washed away due to the nature of the high vacuum specimen preparation process. Fixation, washing, dehydration, and critical point drying have disrupted much of the encrustation that was present on the catheter surface to reveal the bacterial layer beneath. In the super-infection experiments *Pr. mirabilis* was not attaching to the catheter directly but to an already established biofilm. As such, any crystalline material that formed may have been more prone to disruption or dissolution during the fixing process.

The control *Pr. mirabilis* infected model showed marked insoluble matter throughout the residual urine within 24 h (Figure 3.52 Image A). In contrast, to varying degrees all 72 h biofilms of the other species slowed this precipitation. Deposition of the crystalline material mainly at the bottom of the bladder chamber and at the urine/air interface was evident in the *Morg. morganii* super-infection experiments (Figure 3.52 Image C) and was also seen when *Morganella* was growing alone (Figure 3.51). What significance this has for catheter blockage is unknown.

The overall conclusions from the results presented in section 3.3 must be that only when the other test species have established themselves in the urine and catheter biofilms for 72 h do they significantly impair the ability of *Pr. mirabilis* to produce alkaline urine and form crystalline biofilms. These effects however seem to be transient and in all cases *Pr. mirabilis* was eventually able to raise the urinary pH, colonize the biofilm, induce crystal formation, and block the catheters.

4.3.4. An *in vitro* investigation of a *Pr. mirabilis* containing community from a patient whose catheters were not blocking.

Recently a prospective study was completed on 20 long-term catheterised patients all colonized with *Pr. mirabilis* (Mathur *et al.* 2006). Over several months weekly bacteriological analysis of each participant's urine was performed and both the voided pH and the nucleation pH were determined. Any catheters that were removed throughout the period were examined for encrustation and cultured to determine the biofilm flora. One patient who was particularly interesting was Patient 19. Patient 19 was a fairly independent, home-dwelling male who had been catheterised for several years because of bladder outlet obstruction caused by prostate enlargement (Mr Sunil Mathur, Bristol BioMed Centre, personal communication). He had had no previous problems with catheter encrustation and suffered no blockage events during the 13 weeks of the study. Despite colonization with *Pr. mirabilis*, at cell densities comparable to those in other patients, he had a consistently acidic voided urinary pH (mean 4.77), a consistently high nucleation pH (mean 7.87) and therefore a consistently wide, positive safety margin (mean 3.11) (Figure 3.53). It can clearly be seen this is why crystallisation did not occur in this patient's urine and thus afforded protection from encrustation.

In addition to *Pr. mirabilis* (designated AAAX), Patient 19 also had *Kl. pneumoniae* (re-identified as *Kl. oxytoca* AAAAA) and *Ps. aeruginosa* AAAY isolated each week whilst *E. coli* AAAZ was recovered from 9 of the 13 urine samples. The identification and availability of such a stable, interesting community provoked an investigation using the laboratory catheterised bladder

models. The objectives of these investigations were to determine: (1) if *Pr. mirabilis* AAAX was an impotent strain and simply failed to cause significant crystal deposition leading to luminal build-up and eventual blockage of the catheter; or (2) if *Pr. mirabilis* AAAX was a fully functioning strain that had its actions modified by the other members of the community.

The urinary pH in the mixed community models (Table 3.30) increased much more slowly than in the pure *Pr. mirabilis* AAAX inoculated models (Figure 3.54b). At 24 h, whilst the urine in the models inoculated with the mono-culture of *Pr. mirabilis* had reached a mean pH of 8.10, the urine inoculated with the mixed community had a significantly lower pH of only 6.45 ($P \leq 0.05$). The urinary pH of replicate three was still acidic at 96 h and of replicate two at 120 h. This explains the noticeably extended mean time to catheter blockage of the mixed community compared to that of *Pr. mirabilis* alone (121.17 h vs. 53 h; $P \leq 0.05$).

Images in Figure 3.56 corroborate the slower deposition of crystalline material in the urine of the reconstituted, four-member community. Furthermore, Figure 3.57 depicts the variation in both the type of encrustation and the location of blockage seen with the two infection scenarios. Clapham *et al.*, (1990) reported that the presence of *Pr. mirabilis* cells enhanced X-shaped struvite crystal formation whereas in the absence of *Proteus* a more tabular variety was formed. The significantly lower density of *Pr. mirabilis* AAAX in the clinical community compared to when it was allowed to grow alone might have had an effect on the crystal morphology and rate of crystal formation.

Figures 3.54 and 3.57 clearly show there was nothing intrinsically impotent about *Pr. mirabilis* AAAX. It was as effective as other *Pr. mirabilis* strains at causing catheter blockage as shown earlier in this study and by other authors including Morris *et al.*, (1997) and Jones *et al.*, (2005c).

Urea hydrolysis by bacterial urease is the initiator of catheter encrustation. Variations in the urease activity of *Pr. mirabilis* isolates could explain the widely differing scales of encrustation seen by Mathur *et al.*, (2006) in patients all infected with this species. A poor urease activity of *Pr. mirabilis* AAAX might explain the lack of alkaline pH, phosphate precipitation and catheter encrustation seen in this patient. Surprisingly, it has subsequently been established that the urease activity of *Pr. mirabilis* AAAX is normal, 0.36 μmol urea hydrolysed/mg protein/minute (Mr. S. Mathur, personal communication). This was higher than isolates from rapid heavy encrusters who had regular catheter blockages within 28 days. What then is fascinating is why, despite infection with an efficient urease producing *Pr. mirabilis*, the urinary pH was not higher.

The significantly lower ($P \leq 0.05$) mean viable cell counts of *Pr. mirabilis* AAAX (2.04×10^6 cfu/ml) in the mixed community compared to pure AAAX models was similar to that found in the patients urine by Mathur *et al.*, (2006). These workers did not however determine the cell densities of the other community members. In the current study the other three species reached numbers around 10^7 - 10^8 cfu/ml at blockage and *Kl. oxytoca* was consistently the dominant organism. Determining the mean numbers of organisms at various time periods throughout

the experiments it was found that *E. coli* was dominant at 24 h, *Ps. aeruginosa* was dominant at 48 h and from 72 h until blockage *Kl. oxytoca* dominated.

This combination of clinical isolates did seem to provide some degree of antagonism towards *Pr. mirabilis* AAAX although it was only transient. It would have been interesting to have performed super-infection experiments with this community. Allowing the *Ps. aeruginosa*, *E. coli*, and *Kl. oxytoca* to become established for 24/72 h might have provided significant protection from the *Pr. mirabilis*.

Whilst the results from the bladder model experiments suggest that the other species significantly modulate the rate at which the *Pr. mirabilis* strain encrusts and blocks catheters, they cannot account completely for the inhibition of catheter encrustation observed in the patient. It is likely therefore, that some host urinary factors are responsible for the situation observed *in vivo*. A high fluid intake may reduce encrustation by lowering the calcium and magnesium concentrations and raising the nucleation pH (Suller *et al.*, 2005; Stickler and Morgan, 2006). Similarly citrate-therapy or ingestion of citrate-containing drinks (Wang *et al.*, 1993; Wang *et al.*, 1994; Suller *et al.*, 2005; Stickler and Morgan, 2006) might have the same effect. However, these factors cannot account for the exceptionally low voided pH which was observed in this patient even in the presence of *Pr. mirabilis*.

Mathur *et al.*, (2006) had shown that the patient was infected with *Pr. mirabilis* throughout the full 13 week study period. The strain produced normal levels of

the urease enzyme and in mono-culture in the laboratory models was clearly capable of generating alkaline urine and encrusting catheters. In the patient however, the pH of the urine did not rise above 5.0. Therefore it is likely that the urine of this patient contained a factor which inhibited the activity of the urease enzyme.

4.4 Coaggregation in the formation of biofilms

4.4.1 Coaggregation between oral bacteria

The results in Tables 3.31 and 3.32 confirmed that the two oral strains *A. naeslundii* and *Strep. sanguis* were coaggregators. In addition, using artificial urine as the coaggregation suspension medium had a minor effect on the coaggregation scores at 0 and 1 h but at all subsequent time points the scores were identical. Coaggregation has been extensively studied in the formation of plaque in the oral cavity. Members of the plaque community are located in a particular spatial arrangement as both a structural and functional community (Wimpenny, 2000). Cell-to-cell attachment via coaggregation has a major role to play in the species succession and formation of mature dental plaque (Gibbons and Nygaard, 1970). Over 700 bacterial strains covering 15 genera have been found to be involved in plaque coaggregation and each strain or strain group has very specific partners (Gibbons and Nygaard, 1970; Kolenbrander, 1989). For example, intergeneric coaggregation between the early colonizer *Actinomyces naeslundii* strain 1 occurred specifically with early colonizers *Streptococcus sanguis* strains 31 and 34 but not with other *Strep. sanguis* strains or any other streptococci (Gibbons and Nygaard, 1970).

Intrageneric (interspecies) coaggregation is very infrequent except in the streptococci (Kolenbrander *et al.*, 1990). Intrageneric coaggregation might be of importance to streptococci and other primary colonizers and may help explain their predominance on freshly cleaned tooth surfaces. Two non-coaggregating cells can be joined via a "bridging organism" that is a common partner to both to form multigeneric coaggregates (Kolenbrander, 1989). For example, *Fusobacterium* spp., do not coaggregate with each other yet they extensively coaggregate with most of the other common oral genera. Many early colonizers readily coaggregate with *Fusobacterium nucleatum*, and whilst later colonizers such as *Capnocytophaga* and *Treponema* often do not "recognise" early colonizers they do adhere to the developing layers via coaggregation with *F. nucleatum* (Kolenbrander *et al.*, 1993). In regards to plaque formation therefore, it appears that coaggregation results in a temporal and spatial distribution of species.

The surface molecules that are responsible for coaggregation between most oral species are protein adhesins on one cell and complementary carbohydrate receptors on the partner cell (Cisar *et al.*, 1979; Kolenbrander *et al.*, 1993; Rickard *et al.*, 1999). As such, the viability of the coaggregating cells is not imperative as interactions are simply reliant on cell surface molecules.

High shear forces are at work in the oral cavity. A non-coaggregating cell would be rapidly swept away and it seems likely there is a high selection pressure for bacteria to express the aggregative phenotype constitutively (Gilbert and Rickard,

2004). Recently it has been shown that aquatic bacteria also possess such abilities.

4.4.2 Coaggregation between aquatic bacteria

Biofilms are commonplace in aquatic environments. Buswell and colleagues (1997) looked at the role of coaggregation in biofilm samples taken from an aquatic biofilm model. Each of the 19 different isolates characterised exhibited some level of coaggregation with at least one other isolate. Coaggregation initiation began at varying times for different pairs and in some cases the degree of coaggregation increased with time. Some maximum coaggregations also occurred more quickly than others. This may have a role in community succession equivalent to that occurring during plaque development. Some species that coaggregate quickly could be early colonizers and slower coaggregators may be later biofilm colonizers. These investigators found a higher proportion of coaggregations using a spectrophotometric method but the results were more variable than the only slightly less sensitive visual assay.

In a similar study Rickard *et al.*, (2003b) compared the proportion of coaggregating partners in a freshwater biofilm to that of the surrounding planktonic population. Of the biofilm strains 97% coaggregated with at least one other isolate. On the contrary, only 53% of the surrounding bulk liquid strains coaggregated. Intergeneric coaggregation was common between strains from both locations. Intrageneric coaggregation was somewhat less common and only appeared between biofilm isolates. The authors suggested that the bacteria found in the biofilm had been integrated selectively. Autoaggregating strains were also

more prevailing in the biofilm community. This is likely to be a self-centred method of ensuring incorporation of genetically identical cells.

In another study by Rickard *et al.*, (2002) each of the 19 freshwater biofilm isolates differed in the number and identity of other strains with which they coaggregated. *Blastamonas natatoria* strain 2.1 coaggregated with all 18 other isolates and was described as a "promiscuous coaggregator", acting in the same way as *F. nucleatum* in oral biofilms. This ability may confer colonization and biofilm integration advantages as it can coaggregate with any other species already present.

Aquatic bacterial coaggregation is switched on or off depending on the growth phase i.e. a pattern of appearance then disappearance of the phenomenon is seen. As suggested by Rickard *et al.*, (2004) this cyclical event might have a role in allowing transfer of aquatic species from a parent biofilm via loss of coaggregation into a new developing biofilm via expression of coaggregation. In aquatic environment models, well-developed sumptuous biofilms were generated in conditions of high shear force (Rickard *et al.*, 2003b). This is in accordance with the notion that under such conditions selection pressures encourage coaggregation.

The coaggregation ability of *B. natatoria* 2.1 and *Micrococcus luteus* 2.13 were assessed under three different environments: growth in liquid culture, agar growth and growth in an artificial hydrogel biofilm matrix by Rickard *et al.*, (2004). What became apparent was that cells growing in a biofilm were still able to

express adhesins and receptors as efficiently as planktonic equivalents. Once a cell became part of a biofilm its receptors and adhesins were still available to other cells. Protein adhesin-saccharide receptor interactions have been revealed to also be the mediators of aquatic biofilm coaggregation with some strains able to express multiple receptors, adhesins or both (Rickard *et al.*, 1999; Rickard *et al.*, 2000). This could be a reason why some strains do not coaggregate i.e., two strains have to possess complementary receptors and adhesins to be able to coaggregate.

4.4.3 Do urinary catheter biofilm isolates autoaggregate in artificial urine?

In this study early log-phase cultures of 14 species of common Gram-negative and Gram-positive catheter biofilm species were assessed for their ability to autoaggregate in artificial urine (Table 3.33). Cisar *et al.*, (1979) and Gibbons and Nygaard (1970) both demonstrated that lag, log and stationary phases of growth had no effect on coaggregation between oral strains. *Pr. mirabilis* and five other species had positive autoaggregation scores at some point during the experimental period.

The strain of *Pr. mirabilis* used in this study (HI4320 *wt*) is known to produce mannose-resistant *Proteus*-like (MR/P) fimbriae (Bahrani *et al.*, 1994). These fimbriae are important for colonization of the urinary tract (Jansen *et al.*, 2004). Mutants with MR/P continuously switched on (MR/P ON) were observed to autoaggregate and formed biofilms at an enhanced rate compared to both the wild-type strain and MR/P OFF mutants. The ability of *Pr. mirabilis* to autoaggregate and rapidly establish a biofilm would thus be advantageous in environments such

as the urinary tract and may explain why the majority of the strains isolated from this site express these fimbriae (Jansen *et al.*, 2004).

It is important to note that all of the strains that showed some degree of aggregation in the assays were all urease producers and generated alkaline conditions (pH > 8.5) in the urine within six hours (Table 3.33). The granular turbidity seen in these tests could therefore have simply been due to the precipitation of crystalline material rather than autoaggregation of bacterial cells.

4.4.4 Does a urease-producing *Pr. mirabilis* coaggregate with other urinary catheter biofilm species in artificial urine?

Urease-producing *Pr. mirabilis* HI4320 was next investigated for its ability to coaggregate with the 12 other urinary tract pathogens (Table 3.34). The pH of the suspension medium has been shown not to effect oral coaggregation over the wide range of pH 5 to 10 (Kinder and Holt, 1989; Kolenbrander, 1989). If this is also true for urogenital coaggregation then the phenomenon would not be inhibited by the high alkaline urinary pH generated by *Pr. mirabilis* and the other urease-producing organisms. The degree of aggregation in the solution containing both species is recognised to be the level of autoaggregation plus the level of coaggregation. Significant, true coaggregation is apparent when the reaction score of both species exceeds that of the controls at a specific time point. As such the autoaggregation scores are taken from the score observed from the assay containing both species. After the scores were adjusted for autoaggregation (Table 3.35) only one combination: *Ent. faecalis* and *Pr. mirabilis* retained a positive score at the +1 level.

4.4.5 Does autoaggregation and coaggregation occur when crystalline material is not formed in the urine?

With only one possible minor coaggregation reaction occurring between *Pr. mirabilis* and *Ent. faecalis* it seemed likely that coaggregation was not a major factor in the development of *Pr. mirabilis* containing catheter biofilms. The hypothesis that was investigated in the next set of experiments was that the aggregation seen in Tables 3.33 to 3.35 was mediated by the precipitation of phosphates rather than via specific cell-cell interactions. The precipitation that occurred when the nucleation pH of the urine was reached would thus cause the aggregation and deposition of whatever species were present in the urine.

Use of a urease-negative isogenic mutant of the wild-type (*Pr. mirabilis* HI4320 *ure*$^-$) allowed the study of coaggregation without the confounding problem of increasing alkalinity and precipitation of phosphates in many of the assay tubes (Table 3.36). Once phosphate formation was prevented all the signs of the autoaggregative properties of *Pr. mirabilis* disappeared. This suggests that the aggregation observed previously was due to the formation of insoluble material. MR/P fimbriae are known to mediate autoaggregation in *Pr. mirabilis* (Jansen *et al.*, 2004). The fact that autoaggregation properties were not seen in *Pr. mirabilis* HI4320 *ure*$^-$ might be because the culture conditions in this study (aerated overnight cultures in TSB) were not optimum for MR/P fimbriae expression. Three 48 h passages of *Pr. mirabilis* in nutrient broth incubated statically at 37°C provide i*n vitro* conditions that favour MR/P fimbriae expression (Bahrani *et al.*, 1994).

Five of the tubes containing urease-positive species that showed aggregation at the +1/+2 levels had a 6 h pH > 8.5. Only combinations of *Pr. mirabilis* and *Prov. stuartii* and *Pr. mirabilis* and *Morg. morganii* retained a consistent positive set of scores once they had been adjusted for autoaggregation (Table 3.37). Without controlling the rise in pH in the tubes containing the five urease-positive species it is impossible to determine whether the auto- and coaggregation that is seen is simply formation of calcium and magnesium phosphates. It would have been worthwhile to have repeated the assays in dilute urine with an increased nucleation pH or in urine containing a urease inhibitor such as flurofamide. Both approaches would have prevented crystal nucleation. In addition phase-contrast microscopy of the aggregates might have established whether they were composed of cell-cell or cell-crystal clusters.

4.4.6 Is the formation of crystalline material responsible for autoaggregation and/or coaggregation?

The SEM images presented in Figures 3.59 to 3.62 are of pairs of organisms that had been incubated in urine for 4 h and then filtered through 10 μm membranes to trap aggregated material. It can be seen that when urease-producing *Pr. mirabilis* was present there were crystals, indicative of magnesium phosphate, and amorphous particles, typical of calcium phosphate, in each of the combinations. In some images a few bacterial cells can also be seen. Images in Figure 3.63 confirm the lack of crystalline material in autoaggregation assays tubes containing a urease negative species. Viable cell counts performed on the residue collected on the membranes (Table 3.38) provided further evidence that the precipitated phosphates aggregated with the cells in suspension. The turbidity seen in the

auto- and coaggregation assays was thus mediated by crystal formation and this produced bacterial aggregation.

Winters *et al.*, (1995) used transmission electron microscopy to observe the structure and analyse the components of encrusted *Pr. mirabilis* biofilms generated in catheterised bladder models. TEM revealed struvite-like crystals present within the biofilm matrix and also in direct contact with the catheter surface. X-ray micro-analysis and scanning transmission X-ray microscopy showed that aggregates of calcium phosphate were dispersed throughout the matrix associated with bacterial cells. There was also evidence that apatite formation was occurring around the bacterial cells.

The recent study by Stickler *et al.*, (2006a) used a parallel-plate flow chamber system coupled to image capture and analysis equipment to examine the adhesion of *Pr. mirabilis* onto various surfaces. A strongly electron-donating agarose hydrogel was the least attractive to cells of a urease-producing strain of *Pr. mirabilis* suspended in buffer (pH 7.4). When urine cultures of the test organisms were used similar low bacterial adhesion occurred for the first 3 h. At 4 h aggregates of cells and amorphous matter appeared both in the urine and on the hydrogel surface. Experiments with urine cultures of *Pr. mirabilis* HI4320 *wt* and an isogenic urease-negative mutant (*Pr. mirabilis* HI4320 *ure*$^-$) revealed that over the 6 h experimental period the pH of the urine in the wild-type culture increased to 8.5. Once the pH reached 8.2 (around 180 min) cells of the wild-type began to aggregate with amorphous material that had formed in the urine. These aggregates were then deposited onto the hydrogel surface and crystalline biofilm formation was initiated. In the case of the mutant the pH of the culture did not

rise above the nucleation pH of the urine, crystals did not form and cells failed to attach within the 360 min period.

Calcium phosphate has been shown to aggregate with cells in suspension from a wide range of bacterial species (Berry and Siragusa, 1997). The evidence suggests therefore, that in catheterised patients infected with urease-producing bacteria capable of elevating the urinary pH above the pH_n, the crystalline material that is formed will aggregate and coaggregate with whatever species are contaminating the urine. The macroscopic material produced will then be deposited on the catheter surface and initiate crystalline biofilm formation even on surfaces that resist colonization by cells alone. This has important implications for the development of strategies to prevent catheter encrustation. It suggests that selecting materials for catheter manufacture on the basis of surface properties likely to resist adherence of bacterial cells will not be effective. It seems that to stop colonization of catheters in urinary tracts infected by *Pr. mirabilis*, it will be crucial to prevent the crystallisation of struvite and apatite in the urine.

4.5 Catheter biofilms as a source of endotoxin

Endotoxins generated in the urinary tract can gain access to the bloodstream and induce the catastrophic effects of septicaemia and endotoxin shock (O'Keefe *et al.*, 1993; McAleer *et al.*, 2002). There is also evidence that endotoxin can have local pathological effects in the urinary tract. Endotoxin has been shown in primate models to inhibit ureteral peristalsis and promote ascension of bacteria up to the kidneys (Roberts, 1992). If this is the case in humans then pyelonephritis and septicaemia would be possible successors. The instillation of *E. coli* LPS

(100 μg/ml) into uncatheterised bladders of murine models produced an increase in nerve growth factor within 90 min and histological changes including oedema and haemorrhage within 24 h (Bjorling *et al.*, 2001). Gargan *et al.*, (1993) investigated the effects of *E. coli* growth metabolites and endotoxin on the functions of neutrophils in urine. Addition of *E. coli* cells plus 10 μg/ml endotoxin did not significantly affect PMNL killing. Increasing endotoxin concentrations up to 200 μg/ml corresponded with a steady decrease in killing. Phagocytosis however was not affected. The authors indicated that as long as the organisms were phagocytosed effectively intracellular killing might not be essential for bacterial elimination as PMNLs shed into the urine would be cleared upon voiding. In the catheterised bladder complete voiding is not possible and the result of decreased intracellular killing in high concentrations of endotoxin probably supports bacterial persistence.

Procedures and manipulations to remove urinary tract stones have been shown to be correlated with bacteraemia and/or endotoxaemia and/or sepsis and septic shock. Tanaka *et al.*, (1988) inferred that the source of the endotoxin that was released into the blood after urinary tract stone lithotripsy was not only from bacteriuria but from intercalculi cells released on stone break-up. Bacteraemia was found after surgery in 20/117 patients studied by Rao *et al.*, (1991) who underwent various stone treatment procedures. There was an association between the organisms cultured from the blood and those cultured from the stones. Additionally endotoxins in the blood were detected significantly more frequently in patients who had pre-operative bacteriuria (20/38) compared with those with sterile urine (17/79). Post-operative bacteriuria was not measured in this study.

Nine cases of sepsis that occurred within 6 h of percutaneous or endoscopic urinary tract stone manipulation were observed by O'Keefe *et al.*, (1993) over 45 months. Six of the nine patients subsequently died. Blood and urine cultures were negative in three patients.

In three groups of uncatheterised patients with varying sizes and numbers of renal calculi significant increases in urinary endotoxin and bacteriuria were recorded after extracorporeal shock wave lithotripsy (ESWL) treatment (Li *et al.*, 2001). Urine cultures prior to treatment were negative and endotoxin concentrations were around 24 ng/ml in all patients. Stone fragmentation by ESWL seemed to release both bacteria and endotoxin into the urine. A year later McAleer *et al.*, (2002) reported the case of an 8 year old boy who had developed sepsis after percutaneous nephrolithotripsy (PCNL) of a staghorn calculus and died 12 h after surgery. The stone was an infection stone predominantly composed of apatite, struvite and *Pr. mirabilis* cells. Endotoxin assays on the stone fragments yielded high concentrations (> 285 ng/g). This was the first report that established that urinary tract infection stones were large sources of endotoxin.

Garibaldi *et al.*, (1973) used the simple *Limulus* gel-clot assay to show that endotoxin could often be found in the plasma of patients undergoing short-term indwelling catheterisation. Of the 20 patients who had at least one positive endotoxin test, 17 were bacteriuric with organisms such as *Proteus* species and *E. coli*. A mean endotoxin concentration of 0.013 μg/ml plasma was measured with a maximum of 0.04 μg/ml.

Hurley and Tosolini (1992) measured the endotoxin concentrations of urine samples from long-term catheterised patients in a spinal rehabilitation unit. It was found that, for a given cell number, the concentrations of endotoxin were roughly 0.4 $\log_{10}$ units lower in those with *Ps. aeruginosa* bacteriuria than in those with a pure enteric bacterial infection (*E. coli*, *Klebsiella* spp., *Proteus* spp., or *Providencia* spp.). Although only one replicate was performed the results from the current study indicate that *Ps. aeruginosa* did release a lower amount of endotoxin than all four species of enterobacteria. These differences might be due to variable rates of endotoxin release per cell or the release of endotoxin of various potencies (Mattsby-Baltzer *et al.*, 1991).

Crystalline catheter biofilms have a composition equivalent to urinary tract stones (Hukins *et al.*, 1983; Hedelin *et al.*, 1984; Cox *et al.*, 1987; Kunin, 1987; Cox *et al.*, 1989; Stickler *et al.*, 1993a, 1993b). It was therefore suspected that they could also be major sources of endotoxin.

Unfiltered and filtered human urine free from bacterial contamination were also free from detectable levels of endotoxin (Table 3.38). Reagents for the assay, the sterile double-deionised water and LAL reagent water, were encouragingly also endotoxin free. The artificial urine had a mean detectable level of endotoxin. As the artificial urine was prepared in a sterile but non-pyrogen-free environment it is possible that residual environmental endotoxin is responsible for these results.

Sections of sterile unused all-silicone, hydrogel-coated latex (Biocath) and silicone-coated latex (Silicone elastomer) catheters demonstrated levels of

endotoxin around 0.04 ng/ml, 1 ng/ml and 0.07 ng/ml respectively (Table 3.38). Although some of this could be attributed to contamination from the collection procedure it is also possible that the endotoxin concentrations measured on the three catheter types may have been introduced during the manufacturing procedures. Silicone catheters are produced by extrusion whilst latex catheters are created using dip moulding. Neither manufacturing process is performed under sterile conditions. The catheters are only rendered sterile (by irradiation or ethylene oxide gas) after production and whilst this kills any viable bacteria present on the surfaces endotoxin, which is not removed by normal sterilisation procedures, remains. Catheters although they are required to be sterile do not have to be free from endotoxin. In contrast, and perhaps somewhat surprisingly, bladder and catheter irrigation fluids have to state that they are endotoxin-free (Gephart, 1984).

Sterile catheter endotoxin extraction in this study was only performed for 7 min at room temperature. It is likely that under *in vivo* conditions endotoxin, if present, will elute at higher rates and/or for longer periods. For example Gephart (1984) reported on an outbreak of severe fevers following prostate surgery. After investigation it was found that some of the Foley catheters and catheter insertion lubricants were heavily contaminated with endotoxin. Catheter concentrations ranged from 120 ng/ml following an intralumenal rinse with endotoxin free water to 49,150 ng/ml from catheters that had been incubated in a 40°C shaking water bath for 30 min. Pyrogenicity also occurred when extracts from two silicone-elastomer Foley catheters were injected into rabbits (Haishima *et al.*, 2001).

Catheter contamination with bacterial LPS was again found to be the source of the pyrogenicity.

Even after the mean value of endotoxin measured in the artificial urine (1.152 ng/ml) was subtracted, the 4 h artificial urine cultures of the five Gram-negative urinary pathogens contained substantial levels of endotoxin between 116.4-645.1 ng/ml. The additional free endotoxin must have been generated by the bacterial population. Examining the pH of the cultures in Table 4.2 it can be seen that both *Pr. mirabilis* cultures generated pH values > 8 which correspond to the highest endotoxin levels. It is possible that the high pH resulted in a greater number of lysed cells and the higher the levels of released endotoxin.

Culture organism	pH at 4 h
Pr mirabilis B2	8.66
Pr. mirabilis AAAX	8.32
Morg. morganii RB15	6.36
Ps. aeruginosa SMD5	6.35
E. coli SDM8	6.23

Table 4.2 – pH values of the 4 h artificial urine cultures used in the Kinetic-QCL® endotoxin assays

It would have been interesting to measure the pH after a 24 h and 48 h incubation time and take urine samples at varying time periods from the bladder chamber of laboratory models inoculated with each organism. In this way the change in endotoxin concentrations as time progressed could be followed in parallel with pH, viable cell numbers and biofilm development.

The *Pr. mirabilis* biofilm that was generated *in vitro* over 24 h using a catheterised bladder model and the three biofilms from long-term catheterised patients yielded considerable amounts of endotoxin (Table 3.38). Long-term catheterised patients are potentially at risk of catheter-associated endotoxaemia. Patient C is interesting because although only Gram-positive *M. luteus* and *Ent. faecalis* were isolated from the biofilm the endotoxin recovered could be indicative of a previous Gram-negative infection that had been recently cleared. Jorgensen *et al.*, (1973) and Jorgensen (1979) reported that organism viability was not essential for detection by the *Limulus* assay. Gentamicin killed *E. coli* and *Kl. pneumoniae* cultures still registered as positive for endotoxin. Similarly two patients who had negative urine cultures but had endotoxin levels 350 ≥ pg/ml had a final dose of Loracarbef administered only 5 h before samples were taken (Matsumoto *et al.*, 1991). It would have been useful to find out whether Patient C had indeed undergone antibiotic therapy prior to catheter removal.

Cells were not lysed during this investigation but merely disrupted from their biofilm with a vortex and sonication procedure found previously to be suitable for both biofilm removal and cell survival (Dr. Liese Ganderton, Cardiff University, personal communication). Therefore only free endotoxin was measured. It is likely that the total endotoxin concentration of the biofilms were considerably higher. Intact, undisturbed biofilms are likely to harbour dead cells and free endotoxin deep within the polysaccharide matrix. Flushing of the catheter and bladder during washouts with catheter maintenance solutions that physically and/or chemically disrupts the biofilm, or biofilm disturbance upon catheter removal, might liberate large concentrations of endotoxin. Additionally

antibiotics administered either for symptomatic UTI or for other foci of infection may kill bacteria colonizing the catheter and release endotoxin in this way. Dofferhoff *et al.*, (1995) used murine models of *E. coli* sepsis to show that although aztreonam, ceftazidime and imipenem antibiotics and a placebo did not cause significant differences in the concentrations of blood endotoxin they did have an effect on IL-6 and TNF production. High IL-6 concentrations being associated with increased mortality. In contrast, five different types of antibiotics (ciprofloxacin, ceftazidime, imipenem meropenem and tobramycin) had varying effects on the amount of endotoxin release from *E. coli* cells *in vitro* (Trautmann *et al.*, 1998).

Holland *et al.*, (2000) found evidence of endotoxin in other biofilms. These workers hypothesised that endotoxin released from biofilms growing in the instrument steriliser reservoirs was the cause of an outbreak of keratitis (corneal infection) in their Vancouver eye clinic in 1998. *Burkholderia pickettii* was isolated from one of the steriliser reservoirs and samples from the same site were positive for endotoxin. They suggested that bacteria from the biofilm would be released into the reservoir and once passed into the autoclave would be killed during the sterilisation cycle. The bacterial endotoxin however, only destroyed at temperatures around 250°C for 30 min (Kinetic-QCL™ LAL Test Instruction Manual, Cambrex Bio Science) would remain and deposit on the sterile instruments. It could then be transferred to the corneal flap during the surgical procedure. Endotoxin was also recovered from an eye of a patient who went on to develop keratitis. Measures to remove the biofilm from the reservoir resulted in a significantly lower rate of infection.

The presence of endotoxin on urethral catheters is potentially dangerous. Gephart (1984) suggested that the large surgical wound produced during prostate surgery provided a site through which endotoxin was likely adsorbed. The long-term presence of an IUC can damage and irritate the bladder and urethral epithelia (Slade and Gillespie, 1985). This may also result in sites through which endotoxin generated by the colonizing bacterial community can gain direct access to the blood stream.

A more detailed experimental and clinical study is needed to examine whether *Pr. mirabilis* biofilms contain more free endotoxin than biofilm communities containing other species.

4.6 General discussion and future work

Recently urologists at Southmead Hospital in Bristol set up a special clinic for patients suffering from complications associated with the long-term use of indwelling bladder catheters. Within 12 months they had received over 100 referrals from local general practitioners. By far the most common problem experienced in the clinic were those caused by encrustation and blockage of their catheters (Mr. S. Mathur, personal communication). Although we know that these problems are brought about by *Pr. mirabilis*, it is clear that even now, we can do little to prevent infection by this organism. Antibacterial agents have failed to protect the chronically catheterised bladder. Convincing arguments have been put forward by Trautner and Darouiche (2004) that the catheterised urinary tract is so vulnerable to contamination from large populations of faecal organisms, that it is fruitless to use antimicrobials to try and maintain sterile urine. Indeed attempts to

do so will only promote the selection of resistant organisms. They suggested that biocontrol by bacterial interference should be considered as a potentially more attractive alternative.

4.6.1 What else can be done? Prospects for biocontrol of crystalline biofilm formation

Bacterial interference or biocontrol, the ability of a non-pathogenic microorganism to protect against infection by pathogenic strains is a naturally occurring phenomenon that is likely to have a role in the balance between the healthy and diseased state. The close proximity of cells within a biofilm results in, amongst other things, stiff competition for binding sites and nutrients. In addition, anti-microbial or antagonistic compounds excreted by some species such as biosurfactants, bacteriocins, and antibiotics might eliminate heterologous cells nearby (Sutherland, 2001). For example, Tait and Sutherland (1999) performed experiments which demonstrated that a soluble anti-microbial agent produced by a strain of *Ps. aeruginosa* when added to biofilms of *Et. cloacae* growing on glass beads reduced the numbers of adhered *Enterobacter* cells by 50%. In addition after 8 h of growth *Ps. aeruginosa* was easily able to colonize *Et. cloacae* biofilms but the converse was not possible. Artificial inoculation with a biocontrol agent may thus have potential to prevent urinary tract infection and/or biofilm formation on implanted urethral catheters by uropathogens.

Over 20 years ago Chan *et al.*, (1985) showed that a strain of *Lactobacillus* from a healthy woman competitively excluded to some degree a variety of uropathogens from subsequently binding to uroepithelial cells *in vitro*. Several studies since

then have focussed on the abilities of *Lactobacillus* spp., to act as biocontrol agents to prevent infection of the urinary tract. Initial findings by Reid and co-workers (1994) suggested that insertion of freeze-dried *Lactobacillus casei* var. *rhamnosus* into the vagina could be used to control infection of the urinary tract. This study prompted the further investigation of direct intravaginal implantation of *Lactobacillus* spp. The UTI rates in 49 women given either a combination of two lactobacilli or *Lactobacillus* growth factor via a vaginal suppository decreased from the previous year by 73% and 79% respectively whilst viable lactobacilli counts increased with therapy (referenced in Reid *et al.*, 2001a).

An oral probiotic consisting of two biosurfactant-producing *Lactobacillus* species was investigated for delivery to the vagina after intake in women with almost monthly non-catheter related UTIs and/or yeast vaginitis and/or bacterial vaginosis (Reid *et al.*, 2001a). After one week of a 14 day therapy period the strains had colonized the vaginas of all 10 patients. One patient had asymptomatic enterococci eliminated from her bladder and vagina within 7 days and after 16 months of daily ingestion of the probiotic remained infection free.

The mechanism of interference that allows some lactobacilli to be effective stems from its own adherence in the urogenital tract inhibiting pathogen colonization by blocking receptors (Boris and Barbés, 2000) and/or through steric hindrance (Chan *et al.*, 1985). Lactobacilli can also coaggregate with potential uropathogens which may prove to be important in maintaining a healthy state (Reid *et al.*, 1990; Boris *et al.*, 1998). In addition antimicrobial by-products of the metabolism of *Lactobacillus* spp., which can be antagonistic to urogenital pathogens include the

generation of acids, bacteriocins and biosurfactants (McGroarty and Reid, 1988; Boris and Barbés, 2000; Fraga *et al.*, 2005). As biocontrol does not inevitably cause the death of the pathogenic organism there is likely to be less selective pressure for the development of resistance (Reid *et al.*, 2001b).

Pr. mirabilis and *Proteus vulgaris* were among the species that did not coaggregate with any of the lactobacilli tested by Reid *et al.*, (1988). This would limit the usefulness of these species as a biocontrol strategy for preventing *Pr. mirabilis* infection and biofilm development. Recently however, specific control of *Pr. mirabilis* by an indigenous *Lactobacillus murinus* strain Lb02 isolated from a female mouse was assessed both *in vitro* and using murine ascending UTI models by Fraga *et al.*, (2005). The strain demonstrated antibacterial activity, related to acid production, against *Pr. mirabilis* and *S. aureus* strains *in vitro*. In uncatheterised mouse models a preventative dose administered transurethrally prior to infection significantly decreased the concentration of *Pr. mirabilis* in the bladder and kidneys compared to control mice who received a dose of PBS. In another set of experiments four treatment doses of Lb02 after *Pr. mirabilis* infection significantly decreased the number of cells in the bladder but not the kidneys compared to control, non-treated mice. In both sets of experiments Lb02 could not be recovered from either the bladder or kidneys of treated mice at the end of the 7-day experimental period.

It seems therefore, that intravaginal inoculation of lactobacilli has been reported to have beneficial effects in reducing symptomatic UTI in non-catheterised females. Unfortunately, these lactobacilli are not likely candidates for the prevention of

catheter-associated UTI as they seem to be unable to colonize the bladder. Bruce and Reid (1988) for example, found that they could not be re-isolated from the urine some 48 h after direct inoculation into the bladder.

Instead of using whole lactobacilli cells preliminary work by Velraeds *et al.*, (1996) adsorbed biosurfactants produced by *Lactobacillus acidophilus* RC14 and *Lactobacillus fermentum* B54 (surlactins) onto glass overnight. Biosurfactants are compounds of microbial origin that demonstrate surface and emulsifying activities and as a result may influence bacterial adhesion and detachment. An attractive feature of these biosurfactants is that only very small quantities are needed to initiate an effect. Using a parallel-plate flow-cell system adhesion of *Ent. faecalis* was inhibited by adsorbed biosurfactants from *L. acidophilus* RC14 and *L. fermentum* B54. It must be noted that these experiments were performed using glass, a material that would not be found in the catheterised urinary tract but it does demonstrate the anti-adhesive actions of these biosurfactants against at least one pathogenic organism. However, more recently the coating of vinyl catheters with a *Bacillus subtilis* excreted surfactant (surfactin) solution prior to infection *in vitro* reduced the amount of biofilm formed by *Pr. mirabilis*, *E. coli*, *Salmonella typhimurium* and *Salmonella enterica* (Mireles *et al.*, 2001).

The possibility of bacterial interference as a means of UTI prevention in SCI patients using a variety of bladder management regimes was explored in a pilot study by Darouiche *et al.*, (2001). The test strain used in the study, *E. coli* 83972, had been isolated from the urine of a girl with stable asymptomatic bacteriuria and was not able to express the virulence factors typical of pyelonephritic *E. coli*. Bladder instillation of *E. coli* 83972 resulted in colonization of 30 patients for ≥ 1

month and almost 50% of the 44 patients had long-term bladder colonization (≥ 3 months) with the strain. Co-colonization with other organisms was significantly ($P < 0.001$) less likely in the presence of *E. coli* 83972 than in its absence. Only two of the 30 successfully colonized patients acquired symptomatic UTI. Genera such as *Pseudomonas*, *Enterobacter* and *Morganella* colonized for less than 3 months whereas *Klebsiella* and *Enterococcus* colonized for longer. Those patients who became colonized by E. coli 83972 experienced symptomatic UTI rates that were significantly lower ($P \leq 0.001$) than they had previously.

More recently Darouiche and his team (2005) carried out a prospective, randomised, double-blind, placebo-controlled trial which monitored the ability of *E. coli* 83972 to prevent UTI in spine injured patients. They reported that those patients whose bladders were successfully colonized by the avirulent strain were half as likely ($P \leq 0.01$) than those non-colonized patients to develop symptomatic UTI in the following year. The authors admitted that they were not sure of the exact mechanism by which their avirulent *E. coli* exerts its protective effects. They suggested that it might be due to obstruction of binding sites on the bladder mucosa or the secretion of factors antagonistic to other uropathogens.

Trautner *et al.*, (2002, 2003) expanded the use of this *E. coli* strain and investigated the role of bacterial interference *in vitro* in relation to bladder catheter biofilms. Sections of hydrogel coated latex catheters were incubated in cultures of *E. coli* 83972 for 24 h. These sections, and control sections incubated in sterile both, were then subsequently exposed, for 30 min *in vitro*, to a selection of uropathogens. The pre-inoculated sections significantly reduced colonization by

pathogenic strains of *Ent. faecalis*, *E. coli*, *Prov. stuartii* and *Candida albicans* compared to the control sections. Considering its importance in the catheterised urinary tract it is surprising that *Pr. mirabilis* was not tested in this study. In addition, although these *in vitro* experiments are promising it is hard to imagine how a 30 min incubation time relates to the extended periods of bacterial challenge that occur *in vivo*. A pilot trial is in currently in progress to assess the efficacy of these *E. coli* colonised urinary catheters in SCI catheterised patients (Trautner *et al.*, 2005). We await these results with interest.

Bacteriocins are natural antibiotics produced by bacteria which are lethal to closely related strains only. Bacteriocins frequently produced by *Enterobacter* spp., have been shown to inhibit growth of other *Enterobacter* strains and *Klebsiella* isolates but not *Pr. mirabilis* (Bauernfeind and Petermüller, 1984). It would have been interesting to have determined whether *Et. cloacae* RB19 did produce a bacteriocin active against the *Pr. mirabilis* B2 strain used in this study. Those produced by *E. coli* and some related *Enterobacteriaceae* are called colicins. In addition to their work with *E. coli* 83972 Trautner and team (2005a) showed *in vitro* that catheter surface colonized by a colicin-expressing strain of *E. coli* (K-12) completely inhibited successive colonization by a colicin-susceptible *E. coli* strain. As noted by the authors the current species specificity of colicins limits their clinical use. The goal would be to create a genetically-modified avirulent *E. coli* that expresses a variety of bacteriocins against a wide range of urinary tract pathogens.

Bacterial interference to modulate the rate of formation of crystalline biofilms on catheters is an attractive alternative strategy to those based on antibacterial

chemicals. An organism, or community of organisms, capable of inhibiting the activities of *Pr. mirabilis* could be used to colonize patients urine and thus prevent catheter blockage. The results reported in this thesis suggest that *Pr. mirabilis* is going to be a difficult organism to control. It was only possible to identify bacterial populations that had relatively minor and transient effects on the ability of *Pr. mirabilis* to encrust and block catheters. The insight gained into the formation of crystalline biofilms in this study and the recent work of others in the field however, has identified the crucial issue for a control strategy. To control catheter encrustation it will be essential to prevent the ability of *Pr. mirabilis* to elevate the pH of the urine above its nucleation pH.

4.6.2 Future work

One isolate of only five species were chosen to investigate in the catheterised bladder models in this study. These species were chosen because of the suggestions of their possible interactions with *Pr. mirabilis*. Due to the complex nature of catheter biofilm and urine communities it is possible that *Pr. mirabilis* might be antagonised by one of the numerous other species that are often found in these populations. Experiments with a wider range of isolates and species should be undertaken. Similarly, various combinations of two, three, four, or more species might be more effective. A more detailed investigation into the associations between the various species might reveal further potential interactions. Such combinations could then be investigated using the *in vitro* catheterised bladder models.

Patient 19 and his urinary and biofilm flora are particularly interesting. The transient inhibition seen *in vitro* might be due to one or two of the three other

organisms in particular. Identification of the particular species responsible would allow experiments to be performed using higher bacterial loads of these organisms. Similarly, super-infection experiments with young and mature established biofilms of *Ps. aeruginosa* AAAY, *E. coli* AAAZ, and *Kl. oxytoca* AAAAA might provide a more formidable challenge for *Pr. mirabilis* AAAX. Another fascinating experiment to perform would be to collect urine from Patient 19. Once filtered, it could be supplied to the catheterised bladder models to investigate whether it indeed possesses any anti-urease and/or antibacterial activity.

In future investigations it will be important to concentrate on long-term catheterised patients who do not encrust. A more detailed clinical study to identify other patients colonized with *Pr. mirabilis* but who suffer from no catheter encrustation or blockage problems might provide clues as to candidate communities with possible interference abilities.

Although from this study it seems unlikely that coaggregation is important in the formation of catheter biofilms further exploration of this phenomenon might reveal otherwise. Coaggregation studies could be performed with a wider selection of strains and comparisons made between biofilm with urinary isolates. Uropathogen coaggregation may also be optimum at specific growth periods. Assays could be carried out with cells harvested at exponential, early-stationary, mid-stationary and late-stationary phases. In all cases the assays could be performed in dilute urine. This would raise the nucleation pH so that even in the presence of urease producers crystal formation would be prevented.

Coadhesion experiments, using the parallel-plate flow chamber, a green fluorescent protein (GFP) expressing strain of *Pr. mirabilis* (*Pr. mirabilis* HI4320 pBAC001) and epifluorescent microscopy, would allow a unique insight into the way *Pr. mirabilis* integrates into the biofilms of already established species or competes with other species during co-infection.

Further, more detailed, studies are also required to assess the presence and concentrations of endotoxin in patients' catheter biofilms and to identify any communities that generate high, potentially dangerous levels.

4.7 Concluding remarks

The established importance of crystalline biofilms of *Pr. mirabilis* in association with indwelling catheters of the urinary tract deserves more attention. It is important to prevent crystalline biofilm formation in the bladder because any new technological device that is developed, whether a catheter or other indwelling prosthesis, will still have to withstand the process of encrustation.

Considering the massive annual expenditure that the management and treatment of urinary incontinence, and in particular catheter-associated infections, costs health authorities worldwide there is still little priority given to such an important topic. At present there are no successful and clinically efficacious procedures available for the control of the crystalline biofilms that so often form on long-term IUCs. Current attempts may temporarily increase the life-span of a catheter but none provide a lasting result.

The multi-factorial nature of biomaterial-associated biofilms means that their prevention and control is an extremely complicated task. It is important to note

that it is unlikely one strategy alone will completely solve the problem. Bacteria provide intimidating adversaries. They have the ability to adapt quickly and have managed to circumvent almost all of our attempts to prevent colonization of IUCs to date. Novel, and perhaps ingenious, methods for the prevention of the crystalline, catheter-encrusting biofilms produced by *Pr. mirabilis* may need to be simple approaches that can be applied in the home, residential care or hospital setting if needed. Continual input, from a multi-disciplinary team of scientists, will be required if we are to stand any chance of combating these infections.

This study aimed to investigate some of the patient and bacterial factors that might have a role to play in the formation of these problematic biofilms. Further elucidation of these factors may provide an insight into areas of weakness that could be exploited in future prevention strategies. The efforts to improve the health and well-being of such a large number of disabled and elderly people must continue.

4.8 Conclusions

1. The urinary flora of long-term catheterised patients was both polymicrobial and dynamic. Weekly urine samples often contained four different bacterial species. *Ent. faecalis* and *E. coli* were the most commonly isolated species with an incidence of 11.48%. *Ps. aeruginosa* was the most commonly isolated urease-producing species (9.84%) whilst the incidence of *Pr. mirabilis* was 7.38%.

2. The pH of the patients' urine varied from week to week. The presence of *Pr. mirabilis* was always associated with alkaline urine (mean pH 8.66). Other urease producing organisms such as *Ps. aeruginosa*, *Morg. morganii*, and *Kl. pneumoniae* were not associated with a highly alkaline pH.

3. In the cases of the four patients who did not block their catheters in the six-week study period, the pH_n of their urine at week six was above the pH_v. The only patient in which the pH_n was below the pH_v had two catheter blockages over the six weeks. These observations would seem to support the conclusions of other workers that the difference between the pH_v and pH_n is a critical factor in determining catheter encrustation.

4. The data collated from 106 urinary catheter biofilms revealed that 76 were multi-species and 30 were single species in nature. *Ps. aeruginosa* was the most commonly isolated species overall whilst *Pr. mirabilis* was isolated from 30% of the catheters.

5. Analysis of the co-occurrences of various species in catheter biofilms suggested some interesting interactions between organisms. *Pr. mirabilis* was commonly found in the presence of *Prov. stuartii*, *Kl. pneumoniae* or *Ent.*

faecalis. In contrast when *Et. cloacae* or *Morg. morganii* were present *Pr. mirabilis* was rarely or never found.

6. Simultaneous inoculation of the catheterised bladder models with *Et. cloacae*, *Morg. morganii*, *E. coli*, *Ps. aeruginosa*, or *Kl. pneumoniae* together with *Pr. mirabilis* had little or no effect on the ability of *Pr. mirabilis* to encrust and block catheters.

7. The growth of the five test species in the models for 24 h prior to super-infection with *Pr. mirabilis* did not significantly affect the time *Pr. mirabilis* took to block the catheters.

8. When *Et. cloacae*, *Morg. morganii*, *Kl. pneumoniae*, or *E. coli* were grown as mono-cultures in the bladder models for 72 h prior to super-infection with *Pr. mirabilis* they significantly delayed the production of alkaline urine and catheter blockage. In all cases however, *Pr. mirabilis* was eventually able to generate alkaline urine, induce crystal formation, colonize the extensive biofilms formed by the test organisms and block the catheters.

9. Throughout the series of experiments in the bladder models none of the mono-cultures of the five test species proved capable of blocking catheters with crystalline biofilm. However, some signs of encrustation appeared on catheters in models that had been incubated with *Morg. morganii* for > 120 h.

10. The strain of *Pr. mirabilis* present together with *Kl. oxytoca*, *Ps. aeruginosa* and *E. coli* in the urine of a patient who did not suffer from the complication of catheter encrustation was shown to be perfectly capable of encrusting catheters. When models were simultaneously inoculated with the four-

member community, whilst the catheters eventually blocked with crystalline biofilm, the rise in urinary pH and the times to blockage were significantly slower than in the models infected with the *Pr. mirabilis* alone. Although the bacterial community slowed the formation of crystalline biofilm, the patients' urine must also contain inhibitory factors that prevented his catheters from blocking.

11. The macroscopic aggregation of material seen when *Pr. mirabilis* and another species were inoculated together in urine, was due to the precipitation of calcium and magnesium phosphates which then aggregated with the bacteria present in the test. There was no evidence of specific coaggregation between *Pr. mirabilis* and other urinary species that might determine the composition of catheter biofilm communities.

12. The insight gained into the formation of crystalline biofilms in this study, and the recent work of others in the field, has identified the crucial issue for a control strategy. To control catheter encrustation it will be essential to prevent the ability of *Pr. mirabilis* to elevate the pH of the urine above its nucleation pH.

13. Urine cultures of common Gram-negative uropathogens produced endotoxin concentrations ranging from 116.4 ng/ml for *Ps. aeruginosa* to 645.1 ng/ml for *Pr. mirabilis* B2.

14. Sections of catheters removed from long-term catheterised patients colonized with mixed species biofilms contained high levels of endotoxin ranging from 282.8 to 917.2 ng/4 cm length of catheter.

SECTION 5

Appendices

Appendix A

Nucleation pH (pH_n)

Nucleation pH determination methods and
results from nucleation pH investigations

The nucleation pH (pH_n)

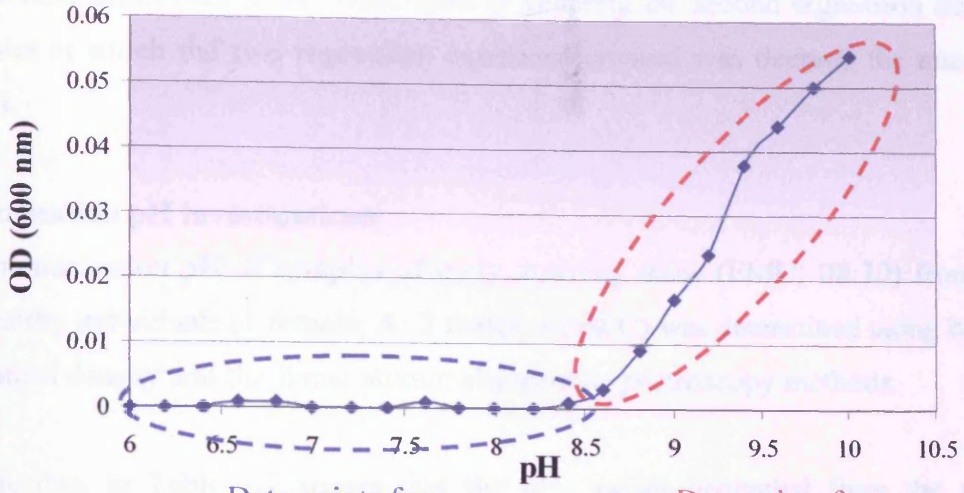
As the pH of the urine was increased [Ca^{2+}] and [Mg^{2+}] initially decreased gradually. At a specific pH, the pH_n, any further pH rise resulted in a considerable reduction of these ions in solution. The pH at which this occurred differed slightly depending on whether calcium or magnesium was being monitored. The decrease in ion concentration was paralleled by an increase in optical density as amorphous salts were formed and began to precipitate. Precipitates in equivalent experiments performed by Langley and Fry (1997) were analysed and found to be calcium and magnesium phosphates. As such, it was assumed that the decrease of calcium and magnesium ions in solution and the increase in optical density observed in this study could be attributed to the formation of these products.

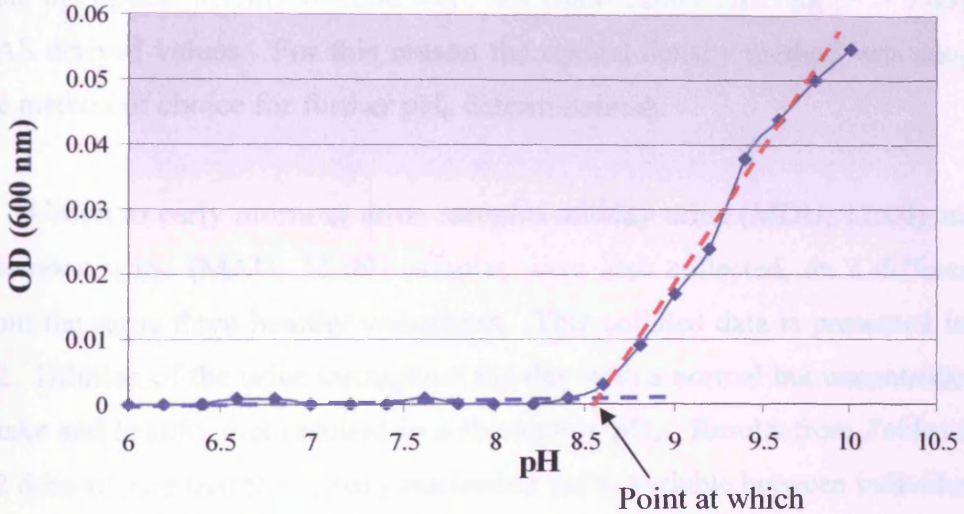
Optical density derived pH_n values

Simple scatter plots of the optical density raw data against pH were produced initially to observe the distribution of the data points. The data were then grouped into the two sets that were going to be used to generate the regression equations. The first regression line (RG1) was determined using the basal readings (around 0.000-0.003 in most cases). The first value to differ considerably from the basal readings, and from which point the optical density continued to rise, was taken as the start of the next data set. Those values in the straight-line element of the next set of data points were used to generate the second regression line (RG2). The point at which the two regression equations crossed was deemed the nucleation pH. This was calculated by solving the equations.

Example

All the points for regression line 1 were basal readings of 0.000-0.002. The reading at pH 8.6 was the first value to differ considerably from the basal readings and was the start of the rise in OD, this value was taken as the beginning of the second data set. As all of the remaining data points approximated to a straight line they were all used to generate the second regression equation.





RG1 OD = -0.00285 + 0.000457 pH

RG2 OD = -0.340 + 0.0398 pH

Nucleation pH (pH_n^{OD}) = 8.57

Atomic absorption spectroscopy derived pH_n values

Similarly, simple scatter plots of the raw data (calcium or magnesium concentrations in solution against pH) were produced initially. The data were then grouped into the two sets that were going to be used to generate the regression equations. The first regression line was determined using the straight line section of the initial readings. The first value to differ considerably from the initial readings and from which point the ion concentration continued to fall was

taken as the start of the next data set. Those values in the straight-line element of the next set of data points were used to generate the second regression line. The point at which the two regression equations crossed was deemed the nucleation pH.

Nucleation pH investigations

The nucleation pH of samples of early morning urine (EMU; 08:30) from three healthy individuals (1 female, A; 2 males B and C) was determined using both the optical density and the flame atomic absorption spectroscopy methods.

The data in Table A1 shows that the pH_n values generated from the simple, inexpensive optical density method closely approximate to those obtained from the atomic absorption spectroscopy technique. The mean pH_n values obtained from the optical density method were not significantly different ($P > 0.05$) to the AAS derived values. For this reason the optical density method was adopted as the method of choice for further pH_n determinations.

In addition to early morning urine samples midday urine (MDU; 12:00) and mid-afternoon urine (MAU; 15:00) samples were also collected, on a different day, from the same three healthy volunteers. This collated data is presented in Table A2. Dilution of the urine throughout the day with a normal but uncontrolled fluid intake and healthy diet resulted in a fluctuating pH_n. Results from Tables A1 and A2 demonstrate that the urinary nucleation pH is variable between individuals and shows variation on different days and at different times of the day.

Volunteer	Urine sample	Replicate	Voided urinary pH	FAAS derived nucleation pH		OD derived nucleation pH
				pH_n^{Ca}	pH_n^{Mg}	pH_n
A	EMU	1	6.41	8.71	8.74	8.71
		2	6.4	7.45	9.01	7.62
		3	6.71	8.04	8.16	8.11
		Mean	6.51	8.07	8.64	8.15
B	EMU	1	6.52	8.04	7.80	7.60
		2	6.81	8.00	8.62	8.61
		3	6.28	8.58	8.67	8.74
		Mean	6.54	8.21	8.36	8.32
C	EMU	1	6.08	8.50	8.55	8.48
		2	6.31	8.22	8.44	8.55
		3	5.93	8.23	8.60	8.84
		Mean	6.11	8.32	8.53	8.62

Table A1 – The nucleation pH of early morning urine (EMU) samples from three healthy volunteers determined from both flame atomic absorption spectroscopy (FAAS) and optical density (OD) methods

Volunteer	Urine sample	Replicate	Voided urinary pH	pH_n	Urine sample	Replicate	Voided urinary pH	pH_n
A	MDU	1	6.31	7.93	MAU	1	6.25	8.38
		2	6.44	8.27		2	5.94	8.72
		3	6.09	7.75		3	6.01	8.09
		Mean	6.27	7.98		Mean	6.07	8.40
B	MDU	1	6.51	7.52	MAU	1	6.37	8.00
		2	6.64	7.47		2	6.40	7.88
		3	6.20	7.90		3	6.05	7.68
		Mean	6.45	7.63		Mean	6.27	7.85
C	MDU	1	6.20	7.67	MAU	1	5.89	7.98
		2	6.09	7.67		2	5.74	7.76
		3	6.07	7.46		3	6.99	8.13
		Mean	6.12	7.60		Mean	6.21	7.96

Table A2 – The nucleation pH of midday urine (MDU) and mid-afternoon urine (MAU) samples collected on the same day from three healthy volunteers determined using the optical density (OD) method.

Appendix B

Bacterial communities isolated from 106 urinary catheters

Repeat catheters from the same patient with identical bacterial communities were not included

Species	Catheter number																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Pr. mirabilis		1			1						1			1									
Ps. aeruginosa	1								1	1							1						1
E. coli																			1			1	
Ent. faecalis			1	1																			
S. aureus																1							
Kl. pneumoniae															1								
Kl oxytoca																							
Morg. morganii							1																
Et. cloacae								1				1											
Prov. stuartii						1												1					
Pr. vulgaris																				1			
Ser. marcescens																							
CNS													1										
Other *Providencia* spp.																					1		
Citrobacter spp.																							
M. luteus																							
Acinetobacter spp.																							
Other *Enterococcus* spp.																							
Candida spp																							
Aerococcus spp.																							
Flavimonas spp.																							

Species	Catheter number																					
	24	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**
Pr. mirabilis	1				1					1	1		1	1					1		1	1	
Ps. aeruginosa			1	1				1		1						1	1	1	1				1
E. coli											1	1			1	1	1			1			1
Ent. faecalis								1			1												
S. aureus													1	1	1								
Kl. pneumoniae																			1			1	
Kl oxytoca		1																		1			
Morg. morganii						1	1					1				1	1	1					
Et. cloacae																							
Prov. stuartii										1									1		1		1
Pr. vulgaris																							
Ser. marcescens																							
CNS									2														
Other *Providencia* spp.																						1	
Citrobacter spp.																							
M. luteus																							
Acinetobacter spp.																							
Other *Enterococcus* spp.																							
Candida spp																							
Aerococcus spp.																							
Flavimonas spp.																							

Species	Catheter number																						
	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
Pr. mirabilis							1						1	1				1			1	1	
Ps. aeruginosa	1	1	1	1		1		1		1			1	1	1	1	1	1					
E. coli			1	1						1	1					1			1	1		1	1
Ent. faecalis						1	1				1		1		1	1	1	1	1	1	1	1	
S. aureus								1															
Kl. pneumoniae				1	1	1			1	1				1					1				1
Kl oxytoca																					1	1	
Morg. morganii	1	1													1								
Et. cloacae									1											1			
Prov. stuartii			1												1		1	1					
Pr. vulgaris					1																		
Ser. marcescens																							
CNS																							
Other *Providencia* spp.					1																		
Citrobacter spp.								1			1												
M. luteus												1											
Acinetobacter spp.									1														
Other *Enterococcus* spp.						1			1														
Candida spp																							
Aerococcus spp.														1									
Flavimonas spp.												1											

Species	Catheter number																					
	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
Pr. mirabilis		1		1	1				1	1	1		1	1	1	1	1				1		
Ps. aeruginosa	1	1				1	1	1					1			1					1	1	
E. coli			1	1	1		1											1	1		1		1
Ent. faecalis	1	1	1	1	1		1	1			1	1	1		1	1	1			1			
S. aureus						1	1	1	1			1											
Kl. pneumoniae	1	1												1	1	1	1		1				
Kl oxytoca						1																	1
Morg. morganii			1	1																		1	
Et. cloacae												1											1
Prov. stuartii										1													
Pr. vulgaris																		1					
Ser. marcescens									1														
CNS																							
Other *Providencia* spp.																							
Citrobacter spp.																							
M. luteus																							
Acinetobacter spp.																							
Other *Enterococcus* spp.																							
Candida spp																				1			
Aerococcus spp.																							
Flavimonas spp.																							

Species	Catheter number													
	93	94	95	96	97	98	99	100	101	102	103	104	105	106
Pr. mirabilis												1		
Ps. aeruginosa									1	1				
E. coli	1				1		1	1			1	1	1	
Ent. faecalis				1		1		1		1	1			1
S. aureus						1								
Kl. pneumoniae						1								
Kl oxytoca	1	1			1									
Morg. morganii				1										
Et. cloacae		1				1	1							
Prov. stuartii														
Pr. vulgaris														
Ser. marcescens														
CNS		1	1											
Other *Providencia* spp.			1						1					
Citrobacter spp.											1		1	
M. luteus														
Acinetobacter spp.														
Other *Enterococcus* spp.														
Candida spp														1
Aerococcus spp.														
Flavimonas spp.														

Appendix C

Odds ratio tables

Odds ratio tables generated to calculate the associations between *Pr. mirabilis* and the 10 other most common catheter biofilm species

Odds ratios are highlighted in purple

	Et. cloacae present	*Et. cloacae* absent	Row total	Ratio
Pr. mirabilis present	0	32	32	0.00
Pr. mirabilis absent	9	65	74	0.14
Column total	9	97	106	**0.00**

	Morg. morganii present	*Morg. morganii* absent	Row total	Ratio
Pr. mirabilis present	1	33	32	0.03
Pr. mirabilis absent	13	61	74	0.21
Column ttal	14	92	106	**0.14**

	E. coli present	*E. coli* absent	Row total	Ratio
Pr. mirabilis present	6	26	32	0.23
Pr. mirabilis absent	27	47	74	0.57
Column total	33	73	106	**0.40**

	Other *Providencia* spp. present	Other *Providencia* spp. absent	Row total	Ratio
Pr. mirabilis present	1	31	32	0.03
Pr. mirabilis absent	4	70	74	0.06
Column total	5	101	106	**0.50**

	Kl. oxytoca present	*Kl. oxytoca* absent	Row total	Ratio
Pr. mirabilis present	2	30	32	0.07
Pr. mirabilis absent	7	67	74	0.10
Column total	9	97	106	**0.70**

	Ps. aeruginosa present	*Ps. aeruginosa* absent	Row total	Ratio
Pr. mirabilis present	9	23	32	0.39
Pr. mirabilis absent	29	45	74	0.64
Column total	38	68	106	**0.61**

	S. aureus present	*S. aureus* absent	Row total	Ratio
Pr. mirabilis present	3	29	32	0.10
Pr. mirabilis absent	8	66	74	0.12
Column total	11	95	106	**0.83**

	Ent. faecalis present	*Ent. faecalis* absent	Row total	Ratio
Pr. mirabilis present	14	18	32	0.78
Pr. mirabilis absent	22	52	74	0.42
Column total	36	70	106	**1.86**

	Kl. pneumoniae present	*Kl. pneumoniae* absent	Row total	Ratio
Pr. mirabilis present	8	24	32	0.33
Pr. mirabilis absent	11	63	74	0.17
Column total	19	87	106	**1.94**

	Prov. stuartii present	*Prov. stuartii* absent	Row total	Ratio
Pr. mirabilis present	5	27	32	0.19
Pr. mirabilis absent	6	68	74	0.09
Column total	11	95	106	**2.11**

Appendix D

Standard curve data produced for the Kinetic-QCL® endotoxin assays

Thresholds of sensitivity are highlighted in blue

Concentration (EU/ml)	Average Reaction Time (s)	Back Prediction
Blank	> 3450	< 0.05
0.005	> 3450	< 0.05
0.05	2476	0.0432
0.5	1441	0.572
5	883	5.935
50	584	42.65

Assay 1
Correlation co-efficient (r) = -0.998

Concentration (EU/ml)	Average Reaction Time (s)	Back Prediction
Blank	> 3900	< 0.05
0.005	> 3900	< 0.05
0.05	2752	0.0411
0.5	1582	0.6228
5	1004	5.822
50	672	41.98

Assay 2
Correlation co-efficient (r) = -0.997

Concentration (EU/ml)	Average Reaction Time (s)	Back Prediction
Blank	> 4650	< 0.005
0.005	4337	0.0046
0.05	2544	0.0468
0.5	1403	0.6212
5	851	5.434
50	529	47.83

Assay 3
Correlation co-efficient (r) = -0.999

Concentration (EU/ml)	Average Reaction Time (s)	Back Prediction
Blank	> 4350	< 0.005
0.005	2387	0.0209
0.05	2210	0.0547
0.5	1531	0.3873
5	811	6.375
50	517	46.3

Assay 4
Correlation co-efficient (r) = -0.997

Concentration (EU/ml)	Average Reaction Time (s)	Back Prediction
Blank	> 3750	< 0.005
0.005	3553	0.0061
0.05	2429	0.0368
0.5	1384	0.5273
5	855	5.154
50	526	51.38

Assay 5
Correlation co-efficient (r) = -0.999

SECTION 6

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