MODELING MICROBIAL SURVIVAL IN BUILDUP BIOFILM FOR COMPLEX MEDICAL DEVICES

by

Rosemarie Howie

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Medical Microbiology and Infectious Diseases

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

This research focused on the transmission of infectious agents by complex medical devices such as flexible endoscopes. It was hypothesized that the biofilm that forms in narrow lumen flexible endoscopes is a "buildup biofilm" (BBF) that develops as a result of cyclical exposure to wet/dry phases in the usage/processing protocol. BBF has a unique composition and microbial survival characteristics, compared to traditional biofilm formation (TBF) that forms when a surface is constantly bathed in fluid. This research investigated whether the BBF matrix presented a greater challenge to disinfectant efficacy and microbial eradication than TBF.

With use, the internal channels of flexible endoscopes are coated with patient secretions facilitating microbial adherence and biofilm formation. Scope reprocessing involves cleaning, disinfection, and drying. No data are currently available to detail microbial survival and transmissibility. However, some evidence suggests that over repeated use, patient-used scopes have a buildup of biofilm-like material.

The relative survivability of bacteria, mycobacteria, fungi and viruses when dried on a surface, in TBF, or within BBF is unknown and was addressed. This study included a relevant test medium and microorganisms mimicking conditions found in gastrointestinal (GI) /respiratory patient-used endoscopes. A unique modeling approach, adapting the MBEC (Minimum Biofilm Eradication Concentration) system for TBF, evaluated microbial survival in BBF formed by treatments with repetitive cycles of (1) drying, (2)

drying and disinfectant exposure, and (3) drying, disinfectant exposure and re-exposure to the test organism. The efficacy of two disinfectants, glutaraldehyde and accelerated hydrogen peroxide, on the survival of organisms was compared.

This project demonstrated that buildup was much more pronounced when a cross-linking agent was used as the disinfectant. The combination of an organic matrix and aldehyde disinfection quickly produced a protective BBF facilitating high levels of organism survival. In contrast, if an oxidizing agent was used for disinfection and organic levels were kept low, organism survival was not detected.

Notably, once established, the microbial load of BBF has a significantly faster rate of accumulation than in TBF. The implications are that as flexible endoscopes are repeatedly used and reprocessed, the assurance of effective high-level disinfection decreases, especially if a cross-linking agent such as glutaraldehyde is used.

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LIST OF ABBREVIATIONS

AER	Automated endoscope reprocessors
AHP	Accelerated hydrogen peroxide
ATP	Adenosine triphosphate
ATS	Artificial test soil
AOAC	Association of Analytical Communities
BA	Sheep blood agar
BBF	Buildup biofilm formation
BCIP	5-bromo-chloro-3-indoyl phosphate
CFU	Colony forming units
CJD	Creutzfeldt-Jacob Disease
CPE	Cytopathic effects
CTC	5-cyano-2,3-ditolyltetrazolium chloride
DAPI	4,6-diamidino-2-phenylindole dihydrochloride
DMEM	Delbecco's Minimum Essential Medium
ECM	Extracellular matrix
EPS	Exopolysaccharide
ERCP	Endoscopic retrograde cholangiopancreatography
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GI	Gastrointestinal
GLUT	Glutaraldehyde
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLD	High-level disinfection
HP	Hydrogen peroxide
LD	Limit of detection
LLD	Low-level disinfection
LPS	Lipopolysaccharide
MBEC	Minimum biofilm eradication concentration
MEC	Minimum effective concentration
MEM	Minimum Essential Medium
MRSA	Methicillin-resistant staphylococcus
MTT	diamethylthiazol-diphenyltetrazolium bromide
NBT	Nitro blue tetrazolium
ON	Overnight
PA	Peracetic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PI	Propidium iodide
PVC	Polyvinyl chloride

QCT	Quantitative carrier test
RF	Reduction factor
RLU	Relative light units
RO	Reverse osmosis
RT	Room temperature
SARS-CoV	Severe acute respiratory syndrome -associated coronovirus
SEM	Scanning electron microscopy
SHP	Standardized hydrogen peroxide
sPBS	Sterile phosphate buffered saline
SVHR	Sindbis virus
TBF	Traditional biofilm formation
TO	Thiazole orange
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TTC	Triphenyltetrazolium chloride
vCJD	Variant Creutzfeldt-Jacob Disease
VRE	Vancomycin-resistant enterococcus
XTT	Modified tetrazolium hydroxide

INTRODUCTION

1. General overview of medical device reprocessing and infection transmission issues

An increase in the number and seriousness of hospital-acquired infections can be expected due to: increasing numbers of immunocompromised patients, elderly and long-term hospitalized patients, coupled with increasing multiple-antibiotic resistant organisms, as well as biocide resistant organisms.[64] These infections are frequently surface-associated with respect to the hospital environment or medical devices and represent a significant cost to the healthcare system; estimated at $2 billion per year in the developed world.[64] Associated bacteremias are most commonly encountered in intensive-care units, haematology and oncology units with 50% associated with staphylococci, and 50% of *S.aureus* being methicillin-resistant.[64] Overall, microbial contamination on medical devices and healthcare surfaces contributes annually to as many as 90,000 deaths in the United States and up to 12,000 deaths in Canada.[24,74]

The incidence of hospital-related infections due to medical devices is about 5.5% of total hospital patients or two million patients per year in the U.S.A., resulting in significant additional medical costs in excess of $28,000 per patient.[60] Infection transmission is dependant on the device, infectious agent, and patient; and specifically related to the device-type, location (implant/external), construction (material, design); surgical considerations (technique, care, infection prevention); the infectious agent's virulence; and the patient's level of resistance.[60] Mechanisms of device-related infections include: (1) internal body access by environmental bacteria or those resident on the body surface;

(2) facilitation of bacterial adherence to a device when it is coated by host factors induced by local inflammation in response to the foreign material; (3) an immunocompromised patient; and (4) undetected biofilm formation in the device facilitating microbial protection, release of free bacteria into tissues or blood and/or emergence of antibiotic resistant bacteria.[60]

Medical devices can include instruments, apparatuses, implants, or associated components or accessories, used to diagnose, treat, or prevent disease or conditions in humans (or animals), but whose primary function is not dependent on reaction within the body.[19,47] Medical device infections can result from single-use devices such as implanted devices (e.g., cardiovascular, neural, ocular, or orthopaedic implants) or externalized devices such as catheters (e.g. cardiovascular, intravenous, urinary catheters). Bacterial access predominately occurs at implantation; most frequently by staphylococci.[60] Infection due to biofilm formation is the major reason for medical device removal. Mortality rates up to 70% due to infections occurring from heart valve implants and to 40% for open fracture implants have been reported.[60]

Nosocomial infection transmission can also occur from any reusable medical or surgical device. These devices are dependent on adequate processing including cleaning and disinfection or sterilization to prevent cross-contamination and possible infection transmission. Such devices were classified by E.H. Spaulding with respect to the level of risk of infection involved in usage thereby defining the appropriate level of disinfection.[11] Categories include: (1) Critical devices, which enter sterile body tissues or vascular areas,

e.g., surgical instruments, biopsy forceps, and require sterilization, i.e. destruction of all microorganisms (including bacterial spores); (2) Semi-critical medical devices, which contact mucous membranes, generally without penetration of sterile tissues, e.g. endoscopes, bronchoscopes, and require a minimum of high-level disinfection (HLD), destroying all vegetative microorganisms, mycobacteria, viruses, fungal spores (not all bacterial spores); (3) Noncritical devices, which generally do not contact the patient, but may contact intact skin, e.g., stethoscopes or patient carts, and require low-level disinfection (LLD), destroying most vegetative bacteria, fungi and some viruses (not mycobacteria or bacterial spores).[11, 83]

Within these categories are complex devices that are heat-sensitive and require the use of liquid disinfectants. These devices present the biggest risk in nosocomial infection transmission and flexible endoscopes are most problematic. Flexible endoscopes are valuable, indispensable analytical and surgical tools for prevention, diagnosis and treatment of disease. For example, colonoscopy is the gold standard for colon cancer screening. These scopes can be used between 300 –1100 times per year and utilization of these less invasive medical procedures is on the increase.[10] However, despite approved guidelines for reprocessing flexible endoscopes, transmission of infectious agents continues to occur. [1,10, 67, 87, 130] The difficulty in achieving complete disinfection is related to scope design complexity, heat sensitivity, and disinfection efficacy[20,109,116] Infection transmission is mainly attributed to breeches in reprocessing protocols and frequency of usage, but can also be related to cracks, crevices and the complex narrow lumen within these flexible endoscopes. These can be havens for bioburden and an accumulation of

organic material, sheltering organisms from the cleaning or disinfection process, or preventing adequate disinfection, ultimately resulting in reservoirs for disease transmission, and/or the presence of resistant organisms. In addition, the possibility for recontamination of the endoscope or accessories after adequate disinfection exists (e.g., from environmental sources). [5, 8, 10, 40, 95, 100, 109,140]

2. Flexible endoscopes

2.1 Endoscope design issues

The structure of currently used flexible endoscopes is complex. As described by Alvaredo et al.,[10] the flexible gastrointestinal (GI) scope consists of a control head, flexible shaft and movable tip. The head is connected to the light source by a cord with internal channels to transmit air, water and suction (Figure 1). The flexible bronchoscope is a similar design, but smaller and without water and air channels. The bendable long, hose-like shaft is equipped with an internal light source and fiber-optic lens (camera). Due to the design and material composition of flexible endoscopes, the device cannot withstand heat or many chemicals. The inability to steam sterilize these scopes has increased the time, cost and inefficacy of reprocessing, making mechanical cleaning crucial to achieving adequate disinfection levels.[10]

Narrow lumened flexible endoscopes, such as gastrointestinal scopes (colonoscopes, sigmoidoscopes), bronchoscopes, nasopharyngoscopes, etc are able to enter a variety of body cavities. These scopes are subjected to high levels of microbial contamination (bioburden) and protein/organic material resulting from the body cavities they must enter,

with patient-used bronchoscopes or colonoscopes being contaminated with up to 4.8 Log_{10}[40] and ~9 Log_{10} bacteria,[5] respectively.

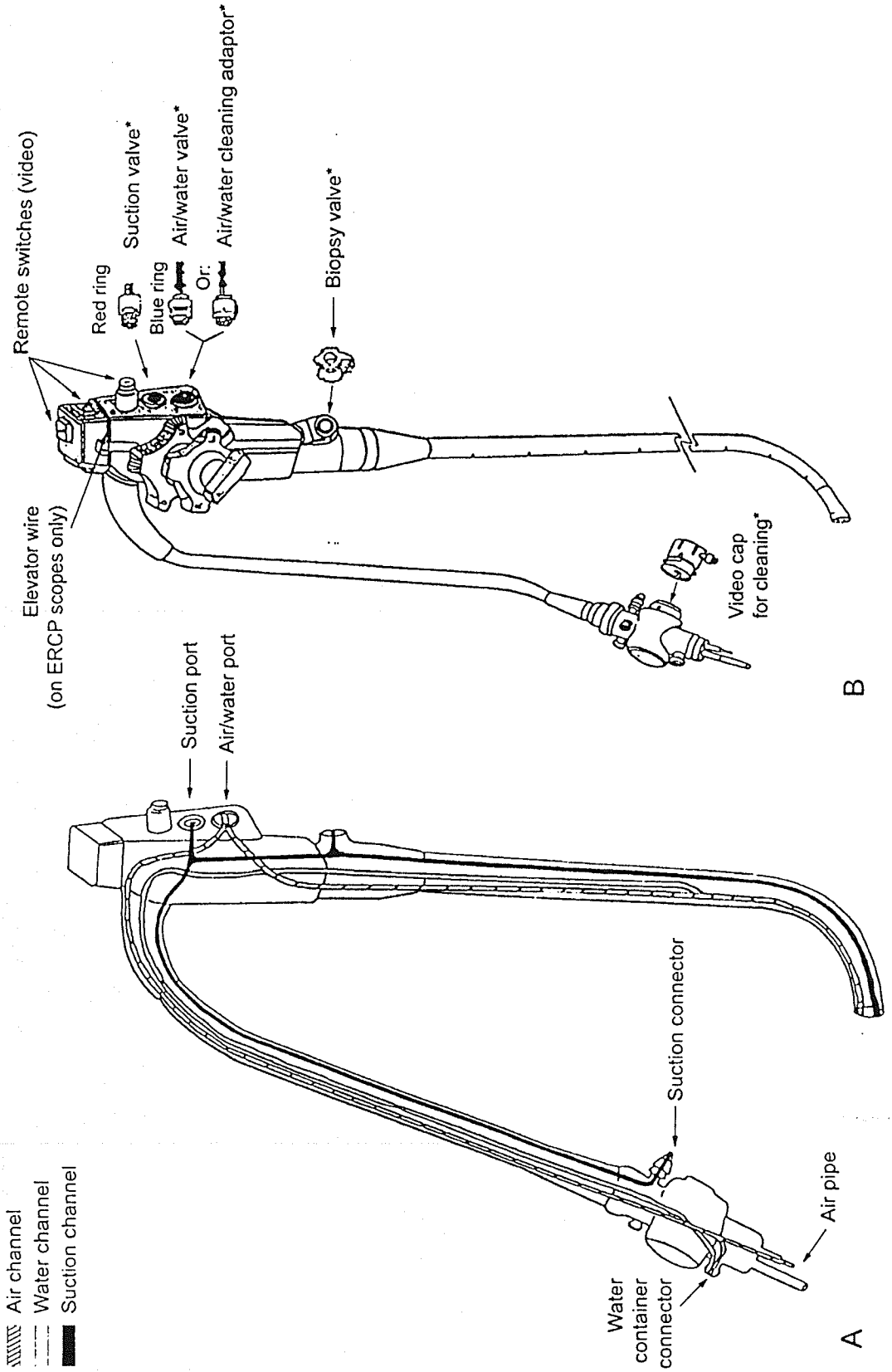
Cleaning is challenging due to the sharp angles, rough, porous or occluded surfaces resulting in many inaccessible sites capable of accumulating bioburden and organic material within the narrow lumened scope.[10] As well, the device is fragile, with honeycombed tubing, consisting of multiple small, long, cross-connected lumen, some with blind endings, making drying, microbiological sampling, and inspection difficult following cleaning.[5,10,116] Due to the high usage of these scopes, the structural integrity can be hindered, providing further havens for bioburden to escape removal, possible biofilm formation and reservoirs of pathogens and inadequate disinfection.[5, 8,10,95,100,140]

GI scope designs may have a greater propensity toward pathogen transmission.[8,20] In particular, side-viewing duodenoscopes having an elevator channel pose a significant challenge to disinfection efficacy. This is due to difficulty in cleaning this channel (refer to Section 2.1.1) and the associated endoscopic retrograde cholangiopancreatography (ERCP) procedure, which transgresses the sterile biliary tract, which is highly vulnerable to bacterial infection.[8,95] Accessories, such as valves, biopsy forceps, brushes, tubing, and water bottles must also be appropriately cleaned and disinfected (or sterilized) to ensure adequate reprocessing (if disposable items are not used).[10,83]

Figure 1. Parts of a Flexible Endoscope

The channel arrangement (A) and parts/switches (B) of flexible video colonoscope (e.g. Olympus brand for illustrative purposes) is shown. The parts indicated by an "*" are stored separately from the scope during storage. Figure from, Guidelines for Infection Prevention and Control in Endoscopy, Manitoba Advisory Committee on Infectious Diseases, September 2000.[83] Used with permission.

Figure 1: Parts of a Flexible Endoscope



2.1.1. Reprocessing: Manual versus Automated, Current guidelines

Current reprocessing guidelines for flexible endoscopes require thorough cleaning followed by a minimum of high-level disinfection as outlined below according to the recommendations of the American Society of Gastroenterology,[11] Ontario Ministry of Health,[106] and Manitoba Public Health Advisory Committee for Endoscopy.[83]

- Inspection for damage to the endoscope

This is accomplished by a leak test.[83] Air pressure is applied to the insertion tube of the endoscope. Resultant air bubbles indicate scope damage and render the scope unusable until repaired. If there are no leaks, the scope is transported to the cleaning area in a closed container.

- Cleaning

Prompt, meticulous cleaning is required immediately after use to remove organic soil (blood, feces, respiratory secretions) that may facilitate embedding of microbes and prevent disinfectant penetration or inactivate germicidal activity.[10] Enzymatic detergents are widely recommended for effective cleaning. Ideally before the scope can dry, all accessible channels are irrigated with detergent and tap water to loosen organic material; the air/water channel is cleared via forced air; detachable parts are removed and soaked in detergent; the outside of the insertion tube and all accessible channels are rigorously cleaned, and intricate inside channels are scrubbed with tiny brushes, using enzymatic detergents and followed with thorough rinsing with tap water. Endoscopes with an elevator channel must have this channel manually flushed with enzymatic detergent and rinsed. If manual cleaning cannot be performed immediately, the endoscope can be flushed and left soaking in enzymatic detergent solution. However, enzymatic detergents

must be adequately removed by rinsing, since residual amounts can contribute to the protein buildup on medical devices.[6]

- Sterilization or disinfection

Sterilization with ethylene oxide can be performed but requires long aeration; as such, liquid chemical disinfection is most commonly used to achieve high-level disinfection. Disinfection occurs by complete submersion in a tank or placement in an automated washer (see following details) using an aldehyde-based (e.g. glutaraldehyde, orthophthalaldehyde) or oxidizer-based agent (e.g. peracetic acid, hydrogen peroxide). Use of chemical agents registered with Health Canada or the Food and Drug Administration (FDA) as sterilant/disinfectants are appropriate as high-level disinfectants when used at the manufacturer's recommended concentration, time, and period of continuous use to ensure efficacy and avoid dilution or inactivation of the disinfectant.

- Rinsing

A final rinsing with sterile, filtered or tap water ensures removal of all disinfectant and eliminates any toxic effect on the next patient.

- Drying

If the endoscope was rinsed with tap water (or if scope is to be stored before next use) flushing with 70% -90% ethyl or isopropyl alcohol to dry is recommended followed by drying with compressed air. Final drying (with forced air and/or hand drying) is imperative to prevent recontamination by waterborne microorganisms. Hanging the scope vertically to dry can further prevent environmental contamination. All detachable parts are removed (valves are stored separately to prevent microbial overgrowth in channels).

Automated Endoscope Reprocessors (AER):

This equipment has benefits of automation (time and manpower), containment of vapors (which may be toxic), reduction of exposure to contaminated devices and disinfectants for personnel, and circulation of disinfectant during processing. The AER is used after leak testing and the manual cleaning process. Some AERs have washing cycles and all have disinfection cycles. Specific reprocessing protocols are available for different models. After the endoscope and components are placed in the AER, all endoscope channels are connected, ensuring all internal surfaces are exposed to HLD. Enzymatic detergents used for washing and disinfectants used for HLD are circulated through all channels. After disinfectant exposure, rinse cycles and alcohol cycles follow (or if necessary alcohol rinse is done manually), followed by drying (as described above).

Concerns regarding the use of AERs include:[11,83] (1) Design flaws in AERs have occurred and it is imperative to review FDA advisories; (2) Inadequate disinfection can occur if all endoscope channels are not exposed to equal pressure, if trapped air exists in channels, or if disinfectant is diluted with wash or rinse water during reprocessing; (3) the elevator channel in duodenoscopes can be ineffectively disinfected in many AERs and may need manual processing; (4) Microbial colonization may result from any residual water in the AER (e.g. hoses, reservoirs) possibly causing contamination in subsequent reprocessing cycles; (5) A compatibility check for a specific endoscope and AER model is imperative to ensure effective reprocessing (e.g. connecting systems).

Concerns regarding reprocessing efficacy for both manual and AER include:[11,83] (1) The sterilization or disinfection process is rendered ineffective if excessive organic material

or moisture remains on or in the endoscope after reprocessing. (2) Enzymatic detergents used for washing must be discarded after a single use since they are not microbicidal or able to slow microbial growth. (3) Endoscopy reprocessing is considered safe and effective for eliminating microorganisms only when performed as per recommended guidelines; then endoscopy can be performed on any patient with a confirmed or suspected infection of any pathogen (e.g. *Hepatitis B virus* (HBV), vancomycin-resistant enterococcus (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), *human immunodeficiency virus* (HIV), *Clostridium difficile,* prions).[95]

Quality control of the disinfection process to prevent microbial survival and overgrowth includes:[11,83] (1) Monitoring of the disinfectant to ensure the minimum effective concentration of the active ingredient is used (e.g, reusable high-level disinfectants must be stress tested over the recommended reuse time);[109] (2) Periodic sampling of endoscope channels to assure adequate reprocessing (recommended but not a strict requirement of guidance documents). Sampling is most effective after the weekend when the scope has been stored for the longest time between reprocessing and usage. Since microorganisms require moisture to replicate, channels should be dry to ensure no microbial replication occurs. Occasional environmental microorganisms may be found in stored endoscopes, but pose no risk in appropriately dried scopes.

2.1.2. Breeches in reprocessing

Transmission of microorganisms from flexible endoscopes is mainly attributed to breeches in reprocessing guidelines recommended by the manufacturer or guidance

document. Surveys have cited the major inadequacies relating to infection transmission from flexible endoscopes to include: inconsistent cleaning procedures with ineffective removal of soil; unmonitored and/or no verification of active ingredients in HLD disinfectants; inconsistent HLD practices; and inappropriately trained employees to handle complex medical devices.[109]

Compliance to accepted reprocessing guidelines has improved over the years, but a minority of endoscopy centers still have not conformed [11] and overall adherence has been cited as "poor".[40] All reported incidences of infection transmission have been attributed to improper cleaning and disinfection (except in the case of a design defect in certain bronchoscopes).[87] For example, two successive patients undergoing colonoscopy following a patient with active *hepatitis C virus* (HCV) infection were infected, with infection attributed to the absence of cleaning of the biopsy channel and inadequate HLD (exposure to 2% glutaraldehyde for 5 minutes rather than the required 20 to 45 minutes).[10] In other cases, efficacy levels of disinfectants in tanks were rarely checked despite continuous usage, e.g. levels of glutaraldehyde have been noted below 0.5%, well below the minimum effective concentration of 1% to 1.5%.[85,116]

Of all the steps in endoscope reprocessing, adequate cleaning is paramount. Challenges to cleaning include: (1) Blockage of channels with organic debris is especially difficult to clean manually, and may be missed by channel irrigators in AERs if flow continues in other channels.[11] (2) It is not always possible to clean scopes immediately after usage, e.g., scopes from late night/early morning use are sometimes left until the next day to

reprocess. (3) Interior surfaces of reprocessed flexible endoscope channels have revealed holes in the channels resulting in areas untouched by cleaning and heavy encrustations of patient material.[11] It is critical to physically remove bioburden, especially before it has dried. Residual organic matter may render the process ineffective, preventing adequate penetration of disinfectants[83] and/or contributing to the formation of biofilm, e.g., as confirmed by scanning electron microscopy of channels in endoscopes sent to repair service centers.[100] Current reprocessing guidelines do not take this into account, and were developed based on testing that often only evaluated a single challenge-reprocessing test.

Post-processing parameters, specifically drying of reprocessed endoscopes, is crucial in the prevention of environment-to-patient transmission,[8,95] especially involving Gram-negative bacteria and GI endoscopy (e.g., ERCP), and AERs. Infection transmission has been associated with water-borne microorganisms, e.g., *Pseudomonas aeruginosa* was transmitted to multiple patients and resulted in patient injury and death.[33,95] However, such transmission was prevented when 70% alcohol and forced air drying was applied to endoscope channels.

2.2. Agents used for cleaning and disinfection

2.2.1. Cleaning - Enzymatic detergents

An appropriate cleaning agent must break down and remove organic material and debris in a worst-case scenario without damage to the endoscope.[109] For flexible endoscopes, chemical detergents are not widely used due to potential damaging effects on the scopes. Therefore enzymatic detergents are most often recommended. Enzymatic detergents can

effectively break down blood, proteins (via proteases), fats (via lipases), and carbohydrates (via amylases). The detergent formulation may contain agents responsible for dissolving the organic matter; degrading of lipids and proteins; wetting (lowering surface tension for improved contact with the device and organic matter); emulsifying, dispersing and suspending matter, thereby preventing redeposition and sequestering, as well as preventing mineral precipitation.[109] Some detergents require minimum contact times. Most enzymatic detergents involved in endoscope reprocessing are not designed to kill microbes, nor do they claim to do so.[6] Studies of currently available enzymatic detergents used in endoscopy have shown variable and limited microbial killing ability.[6]

2.2.2. Biocides – mode of action, critical factors affecting efficacy

According to Russell,[114] the action of biocides on microorganisms involves: (1) cell surface adsorption, (2) interaction with outer cell layers, (3) cellular uptake, and (4) target site interaction. The resultant biocide (unlike antibiotic) action affects many microbe functions (via interaction with proteins, enzymes, and nucleic acids), is non-selective, and attacks most types of microorganisms.[114] However, biocide efficacy is affected by the contact time, concentration, pH, temperature, organic matrix, and specific organism type. The microbicide resistance of individual microorganisms may also be affected by the stage and conditions of their growth, e.g., stress adaptation responses following chemical stress (e.g., oxidative stress induced by peroxygens) or growth in low nutrient media; and the ability to form biofilm.[114, 125] According to Weber and Rutala,[145] overall microbial resistance to biocides occurs by mechanisms similar to antibiotic resistance and can be intrinsic or acquired. Biocide resistance may be due to biocide inactivation or

modification, target-site alteration, and altered intracellular concentration (e.g., decreased permeability or increased efflux of biocide). Biocide resistance to HLD by hydrogen peroxide, glutaraldehyde, chlorine, or alcohol has never been an acquired property via plasmids.[145] However, glutaraldehyde-resistant mutant strains of *Mycobacterium chelonae* have been associated with altered cell wall polysaccharides.[84]

2.2.3. Liquid chemical disinfectants for endoscopes

2.2.3.1. Introduction: HLD versus sterilization:

Although steam sterilization is the recommended method for reprocessing all reusable medical devices including flexible endoscopes, the heat-sensitivity of flexible endoscopes prohibits this practice. Low temperature plasma sterilization can be used on select scopes with lumen dimensions facilitating penetration. The gold standard for sterilization of flexible endoscopes is low temperature gas sterilization using ethylene oxide. This is also not widely used due to occupational safety and the lengthy processing time of 60 min to 120 min (prolonged by the necessity to off-gas ethylene oxide by aeration).[109] Sterility, however, is not an absolute requirement for reprocessing of flexible endoscopes. Reprocessed flexible endoscopes require a minimum of HLD, that is achieved at low temperatures with liquid chemical disinfectants which must: kill or inactivate all microorganisms (except high numbers of bacterial spores);[145] maintain microbicidal activity in the presence of organic matter; [10,145] and be compatible with the endoscope and be nontoxic to personnel.[10,11] HLD of flexible endoscopes can be achieved by aldehyde-based or oxidizer-based agents.[10, 87, 116] Currently used agents are discussed below. However, other agents (e.g., hypochlorite, quaternary ammonium compounds, and

phenolics) are not recommended due to inadequate microbicidal ability, toxicity, and/or device-damaging potential. New products and technologies include: improved enzymatic detergents, rapidly tuberculocidal low-temperature-acting glutaraldehyde-based solutions, low-temperature sterilization using hydrogen peroxide-based-plasma, and a sheath-based technology covering the insertion tube.[6,10,95]

2.2.3.2.Glutaraldehyde (GLUT)

An acid glutaraldehyde product, pH 3.0 to 6.3, used at concentrations $\geq$ 2%, is acceptable as a high-level disinfectant, however some solutions are corrosive to metal. An alkaline glutaraldehyde product, pH 7.5 to 8.5, is available at > 2.4.% concentration, but requires addition of activation reagents consisting of an alkaline buffer, surface-tension depressant, an anti-corrosive compound and a water-soluble dye.[83] This activated alkaline glutaraldehyde has enhanced microbicidal activity (sporocidal, fungicidal, virucidal and bactericidal) and material compatibility. GLUT is the most widely used[8] and cost-effective high-level disinfectant for endoscope reprocessing and is resistant to neutralization by organic soil.[10,83] Its disinfection ability is attributed to the alteration of RNA, DNA and protein synthesis in a broad range of microorganisms by alkylation of sulfhydryl, hydroxyl, carboxyl, and amino groups.[116] Interaction with amino acids allows GLUT to bind essential components in the bacterial cell envelope, e.g., peptidoglycan or teichoic acids for Gram-positive bacteria or lipoproteins in the outer cell components of Gram-negative bacteria, and can prevent the release of certain membrane bound enzymes (www.virox.com). GLUT's potent virucidal activity has been associated with capsid changes.[86] The efficacy of glutaraldehyde HLD for endoscope disinfection has been well

documented.[10, 115, 116,127] Exposure to ≥2% GLUT for 20 minutes at room temperature is the most commonly accepted time/concentration for HLD used in the process of endoscope disinfection in Canada and some European countries.[8, 20, 116] A minimum effective concentration (MEC) of 1% to 1.5% is required for HLD to be successful. GLUT deficiencies include toxicity, chemical irritation, protein fixation, polymerization,[30] as well as the development of microbial resistance by *Mycobacteria chelonae*,[53, 137] *P. aeruginosa*[26, 104] and *Salmonella enteritidis*.[41] As well after activation, shelf-life and stability is limited to 14 days (or 28 days if in-use dilution does not exceed 50%).[10,83]

Ortho-phthaldehyde may be a more desirable aldehyde-based disinfectant with greater stability, low-toxicity, no required activation, and a shorter required contact time of 5 minutes.[95] It has faster action against mycobacteria than GLUT, but is a weaker sporicide.[99] Although it presents fewer cross-linking issues than GLUT, it can stain proteins. It has also been associated with sensitization concerns.[99] Due to cost it is not commonly used and the majority of endoscope reprocessing is done with GLUT.

2.2.3.3. Hydrogen peroxide (HP)

Hydrogen peroxide is a rapid oxidizer, producing hydroxyl free radicals that attack essential cellular lipids, proteins, and DNA, as well as having the capability of removing organic debris.[83,116] HP can be used both as a surface and instrument disinfectant depending on formulation and concentration used. It is bactericidal, virucidal, fungicidal and sporicidal, capable of HLD at recommended concentrations and contact times.

Generally HP is more effective against Gram-positive than Gram-negative bacteria, but organisms producing catalase or other peroxidases may be more tolerant to HP.[86] Unaltered HP is relatively unstable and slow acting.[99] It is commercially stabilized, known as stabilized hydrogen peroxide (SHP). Newer formulations classified as high-level disinfectants are potent anti-microbial agents, e.g., 7.5 % HP with 0.85% phosphoric acid solution, with a minimum effective concentration of 6% HP.[83] This formulation may be incompatible with some endoscope models since it can cause corrosion of copper, zinc, brass and damage to rubber and plastic. Another formulation of 7.5% HP has accelerated anti-microbial activity due to addition of surfactants and sequestering agents, known as accelerated hydrogen peroxide (AHP). The mechanism of action of AHP is believed to occur by: (1) disruption of cell membrane permeability, inhibition of enzyme activity, denaturation of cellular proteins; (2) formation of a highly reactive hydroxyl radical which attacks membrane lipids, DNA and essential cell components; (3) bivalent cation sequestration which disrupts cell structure and function; and (4) interruption of species transport across the cellular membrane (www.virox.com). Most recently, a 2% AHP formulation has become available for HLD in 5 minutes.[99]

2.2.3.4.Peracetic acid (PA)

PA consists of a combination of acetic acid, hydrogen peroxide and water, with broad-spectrum antimicrobial activity.[83] It is a rapid oxidizer, highly biocidal even in the presence of organic soil, and more effective than HP or glutaraldehyde-based disinfectants, especially against bacterial spores. However, PA is associated with significant health concerns from direct contact or inhalation. Although this formulation

can be corrosive to endoscope models, it can be used in an AER system with a final concentration of 0.2% peracetic, a buffer and an anticorrosive agent.[10,83]

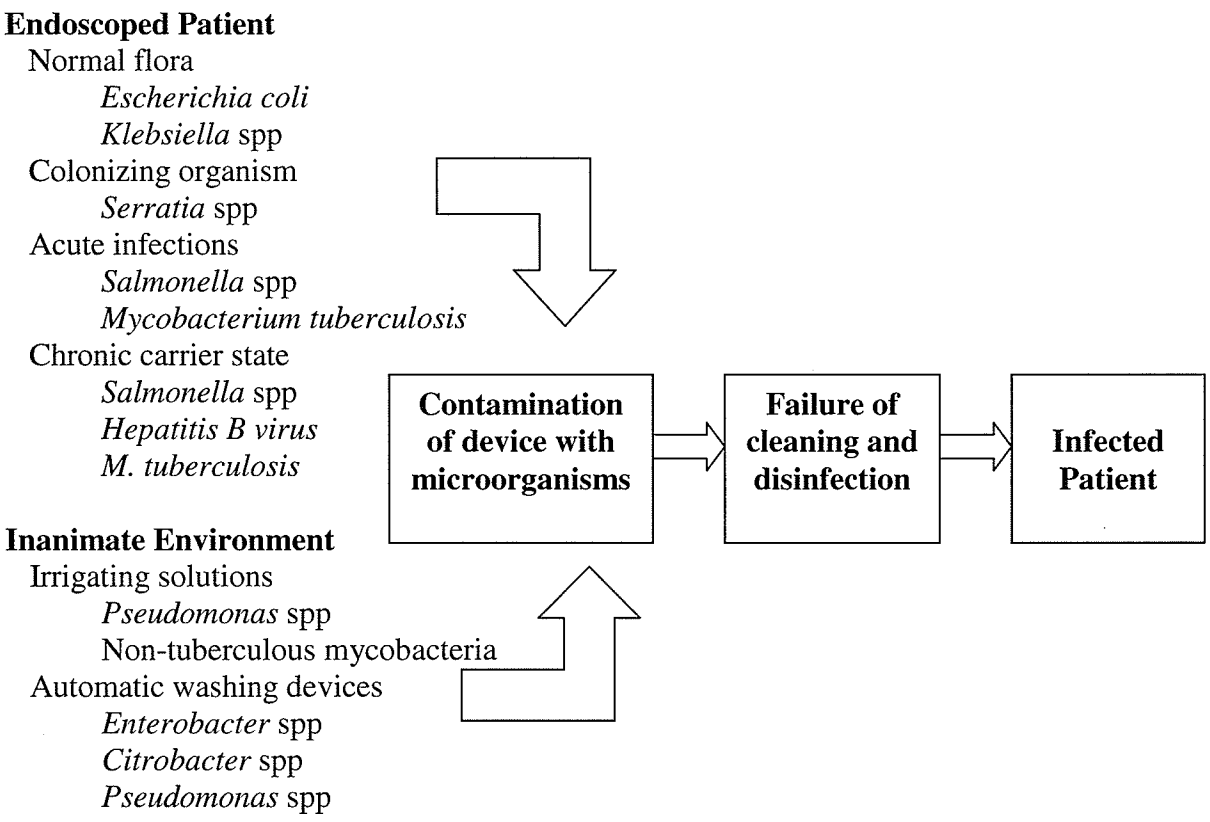
2.3. Infection transmission related to flexible endoscopes

Reprocessed endoscopes can be a reservoir to transmit infectious agents to patients. The source of the agent may be a previous patient, the environment (e.g. irrigating solutions or AERs) or handling during reprocessing (Figure 2).[10,129] Overall, the reported incidence of transmission of pathogens related to GI endoscopy or flexible bronchoscopy is a rare event, and all cases of infection transmission reported have been attributed to breeches in reprocessing protocol or as in 2003, to a design defect in certain bronchoscope models prohibiting effective cleaning and disinfection.[40] The most common breeches in reprocessing related to transmission include: inadequate cleaning, disinfectant exposure, rinsing and/or drying.[10] A significant factor in infection transmission from reprocessed flexible endoscopes is microbial survival associated with the accumulation of organic material and prevention of adequate disinfection.[40,100] An estimated 2.7% of procedures result in some sort of infection, translating to ~270,000 infections per year in the U.S.A.,[27] in spite of FDA and manufacturers recommendations for disinfection and sterilization. However, these statistics are considered to be an underestimation and the actual incidence of transmission is unknown due to a lack of well-designed prospective studies, and unreported or unrecognized infections.[11, 40, 87, 95]

Figure 2: Nosocomial transmission of microorganisms by endoscopes

Flow diagram shows major routes and sources of nosocomial transmission of microorganisms by endoscopes. (Figure and description, as in Spach et al.[129]) Used with permission

Figure 2: Nosocomial transmission of microorganisms by endoscopes



2.3.1. Infections transmitted by flexible endoscopes

An estimated ~34 million GI procedures are performed annually in the U.S.A.[95] The reported incidence of infection transmission is 1 in 10 million GI procedures, with a decreasing number of infections reported over time (281 cases from 1966 to 1982 compared to 35 cases in the last decade, all related to breeches in reprocessing).[95] Viral transmission related to GI endoscopy includes eight reported cases of HCV, with only two confirmed and related to inadequate disinfection. A large, significant study of HCV transmission reported no seroconversions following endoscopy, suggesting HCV transmission does not occur when reprocessing guidelines are followed.[32] Similar reports exist regarding HBV.[17,90,95] Potential GI endoscopic viral contaminants include *Severe Acute Respiratory Syndrome-associated coronavirus* (SARS-CoV) (found in feces and intestinal epithelial cells) and *avian Influenza A virus* (H5N1) (infecting respiratory and GI tracts of poultry and humans); but should not pose a risk with adequate reprocessing protocols.[95,107] Bacterial transmission via GI endoscopy has been dominated by Gram-negative bacilli. Since 1974, 48 cases of *Salmonella* species were reported (all related to breeches in reprocessing) but none after 1988 following the publication of standardized protocols.[95] *P. aeruginosa* is the most commonly reported organism attributed to a lack of drying of endoscopic channels and contamination of AERs and/or water supplies.[8,95] Classen[33] reported seven cases of *P. aeruginosa* bacteremia following endoscopy within 5 days.[95] Twelve cases of transmission of *Helicobacter pylori* have been reported; all due to inadequate reprocessing.[95] Candidal transmission has been reported in immunocompromised patients.(www.health.qld.gov.au/EndoscopeReprocessing/Module1 1htm)

In contrast, reports of contamination and infection for bronchoscopes have increased over time (6 cases from 1967-1979 compared to ~41 cases from 1990-2003) and involve a shift in contaminants from diverse bacteria (1970-1980) to environmental or commensal pathogens e.g., *P. aeruginosa*, *S. marcescens*, nontuberculous mycobacteria and environmental fungi.[40] This reflects a shift in contamination mechanisms from a lack of established infection control protocols and use of ineffective disinfectants or inadequate cleaning to contaminated AERs and breaches in established reprocessing protocols.[40] Mehta et al.[87] reported infection transmission related to bronchoscopy in the U.S.A. involving: 18 infections (with transmission causing significant illness) from 1975 to 2003, most commonly involving *P. aeruginosa*, *M. tuberculosis* and *S. marcescens* (related to one probable death), but also including *M. chelonae*, multi-drug resistant *M. tuberculosis*, and *Burkholderia pseudomallei*. In 2003, the first confirmed transmission of infection in spite of following reprocessing recommendations involved the transmission of *P. aeruginosa* affecting 33 patients (possibly causing 3 deaths) and was attributed to a defective scope design.[67,130] Three cases of *P. aeruginosa* infection have been related to contaminated AERs. Pseudo-infections (contaminating organisms in bronchoscopy specimens but no resultant clinical disease) are most commonly related to *P. aeruginosa*, *S. marcescens*, and mycobacteria (most prevalently *M. chelonae*). Transmission of viruses has not been related to flexible bronchoscopic procedures, but airborne transmission of enveloped influenza viruses and SARS-CoV pose a potential risk for healthcare workers.[40,87]

3. Biofilm in flexible endoscopes

3.1.Introduction

Biofilms are "complex communities of microorganisms attached to a surface or interface, enclosed in an exopolysaccharide matrix of microbial and host origin"[35] resulting in a three-dimensional structure distinguished by phenotypic characteristics maximizing growth and survival.[10,35,64] Microbial advantages within a biofilm include: (1) protection (from host defences, antimicrobial agents, desiccation, and fluid and mechanical forces), (2) nutrient acquisition (by trapping nutrients, and metabolic cooperation), (3) new traits (via plasmid/genetic transfer, expression of novel genes, and selective mutation), and (4) intercellular communication (e.g., quorum sensing).[64] Biofilm's reduced susceptibility to specific biocides varies with different species, but is related to: (1) limited access to bacterial cells, (2) chemical interactions between the biocide and biofilm, (3) cellular changes in micro-environments, e.g., nutrient- and oxygen limited cells, adoption of a quiescent state, (4) degradative enzymes effective at low levels of the biocide, (5) genetic exchange between cells, (6) quorum sensing, (7) persister cells (incapable of programmed cell death, feeding on nearby lysed cells) and enclaves of survivors, (8) adaptation/mutation, and (9) biocide efflux.[50,114]

Biofilms have predominately been associated with implant-related infections, an increased resistance to antibiotic treatment, and are responsible for at least 65% of all bacterial infections in humans.[35,36] Biofilm formation is particularly related to indwelling catheters (e.g. infection rates of 3%-5% for central venous catheters; up to 50% for urinary catheters)[64] and implanted medical devices (e.g. orthopaedic implants). Infections

result from normal flora introduced at the time of implant insertion. *Staphylococcus epidermidis* and *S. aureus* are most frequently responsible, and to a lesser extent, Enterococci , *E. coli*, *P. aeruginosa* and *C. albicans*.[60,64] Difficult elimination of biofilm necessitates device removal (e.g., 1% of hip implants).[64]

Biofilm formation occurs in flexible endoscopes, adhering to internal channels, as reported by repair companies and studies with scanning electron microscopy (SEM).[10, 100] Biofilms on endoscope tubing have mainly been due to microorganisms associated with water, e.g. *Mycobacteria spp.* and *Legionella*.[78,82] As well, the frequent, repeated use of endoscopes can cause microscopic damage to the inner tubing and the resultant rough surfaces may facilitate biofilm formation.[71,78,100] Flexible endoscopes used in the GI tract are difficult to adequately clean resulting in residual patient soil and microbial reservoirs with potential transmission.[8,94] Patient-derived materials (e.g. protein, hemoglobin, carbohydrate, viable organisms and endotoxins) gain access to difficult to clean areas, e.g., the biopsy channel and air/water and elevator channels, and to non-ported internal sections of accessory devices (e.g. the inner channel of biopsy forceps).[5, 8] A trans-Canada survey found residual soil in biopsy channels of reprocessed patient-ready flexible endoscopes to be pervasive, with only 24.4% of scopes reprocessed with GLUT having soil parameters below the benchmark level.[8] Microbes are the most difficult component to remove, demonstrating up to 4 Log_{10} increases following single-use testing and storage for 7 days.[9] Gradually, as scopes are progressively used, a buildup of residual patient material and organisms develops, potentially causing lumen occlusion, scope inflexibility, and loss of functionality.[5,8,100] Currently published data on

buildup in flexible endoscopes are limited to visual evaluation of the inner portion of the biopsy channel or viable counts of microbial levels from indirect sampling of intact lumen.[94, 100] However, little is published regarding the composition or time frame of accumulation.

3.2. Traditional Biofilm formation (TBF)

Biofilm can develop on any surface bathed in fluid and exposed to microorganisms.[55] An initial surface-conditioning phase generally occurs within minutes to hours as the surface is coated with body fluids containing ions, polysaccharides, lipids, and glycoproteins. Microbial attachment, growth and colonization of the surface lead to the formation of mature biofilm over days.[50,55,64] Microbial adherence, the most critical stage of biofilm formation, is affected by: (1) bacterial concentration, time, temperature, fluid environment, and the surface characteristics; (2) forces affecting adherence (e.g. Van der Waals, repulsive electrostatic, and hydrophobic forces); and (3) the liquid phase interactions.[55] The matrix consists of 2%-5% microbial cells, 2% exopolysaccharide (EPS), 95%-99% water and 2% supplementary components (RNA, DNA, proteins, and enzymes).[64] The highly hydrated EPS facilitates a 3-dimensional structure, adhesion, stabilization, and heterogeneity. Mature biofilm is an ecosystem of microorganisms with varying metabolic characteristics within mushroom-like formations of extracellular polymeric substances separated by channels for diffusion of nutrients and dilution of waste material and communication via quorum sensing.[50,55,64] Planktonic microorganisms move between the sessile columns of microbes. The ecosystem may be monomicrobic or interact with secondary colonizers. TBF results when microorganisms are allowed to

replicate and develop an ecosystem on a surface constantly bathed in fluid (e.g., on an indwelling urinary tract catheter, intravenous line, or a medical implant). (Figure 3)

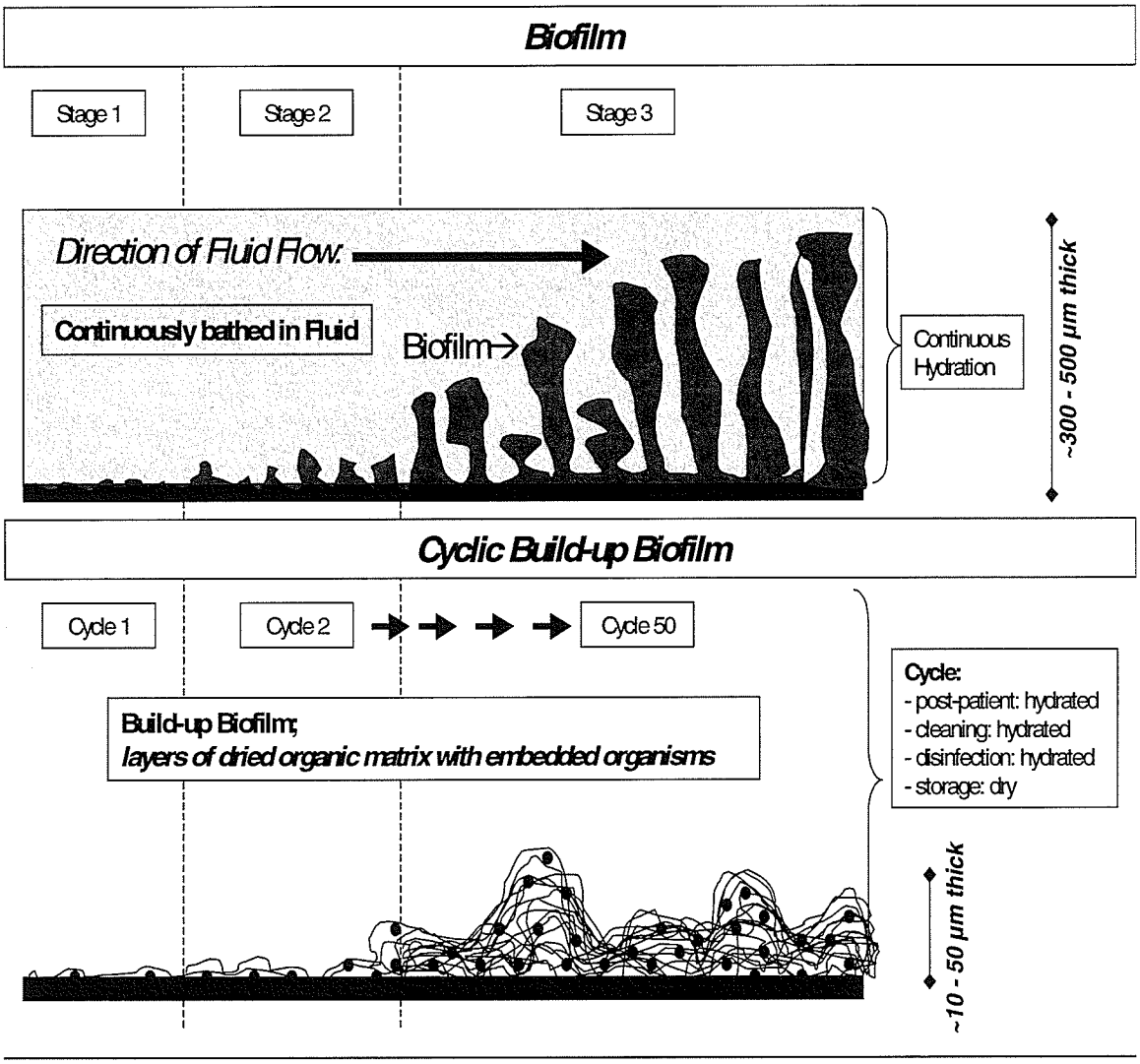
3.3.Buildup biofilm formation (BBF)

The buildup material in channels of flexible endoscopes represents a progressive buildup of organic material and/or biofilm formation that can develop within channels of reused flexible endoscopes (Figure 3). BBF has a similar initiation to TBF: channel lumen surfaces are exposed to patient secretions and various fluids and follow the same initial surface conditioning. However GI medical devices are exposed to high levels of microbes due to contact with the mucosal surface of the gut (whereas implants or intravenous lines are initially in a sterile environment). As well, the GI device can be used repeatedly during a day, exposed to a wet phase during use and reprocessing and a dry phase during storage (e.g. overnight, weekends, or times of non-use), resulting in a cycle of wet/dry exposure contrary to TBF formation. It is possible that during the dry phase, the liquid interface kinetics are eliminated in a cyclical fashion allowing for increased opportunity for short-range surface interactions, thereby facilitating BBF in reprocessed narrow lumen medical devices despite exposure to currently acceptable reprocessing protocols. Overall BBF appears as a two-phase process: (1) the lumen surface is conditioned with residual patient secretions and subsequent exposure to patient mucosal secretions and/or environmental sources facilitating microbial adherence, and (2) with repeated use over time there is subsequent BBF encrustation further facilitating microbial protection and survival. To date, the survivability of microorganisms within BBF is unknown.

Figure 3. Development of traditional biofilm compared to buildup biofilm

The cyclic buildup of organisms and organic material on continually reprocessed medical devices is different from the accumulation of biofilm in a constantly hydrated environment. (Figure and description from personal communication with University of Manitoba Biofilm Research Group, 2007).

Figure 3. Development of traditional biofilm compared to buildup biofilm



3.4.Impact of biofilm formation on reprocessing

The inability of reprocessing detergents and antimicrobial agents to adequately remove and/or inactivate endoscope-related biofilm formation is an important and relevant current issue.[78, 140] Disinfectant efficacy depends on the type of active ingredient (e.g., cross-linking versus oxidizing agent), the ability to penetrate the biofilm formation and contact cells, and inactivation of the agent by the extracellular matrix (ECM). Therefore, microbial survival and disease transmission may result from inadequate disinfectant/ sterilant penetration into biofilm and/or buildup material, as it has been demonstrated that neither manual nor automated protocols for scope disinfection can adequately clean/remove biological soil from difficult to clean areas.[8] It is possible for the buildup of sequestered material to gain access to a subsequent patient by breaking off and/or being transported by fluids or accessory devices passing through a channel. However the risk is low due to the low levels of microorganisms that are likely to survive disinfection coupled with the hardiness of the mucosal surface encountered by endoscopes.[8] Serious infection-related complications could include the retention of white blood cells carrying HIV by endoscope-related biofilm.[78,100] However, little is known regarding the protection of microorganisms from disinfection/sterilization in narrow lumen reusable medical devices (e.g. flexible endoscopes and GI accessory devices such as biopsy forceps).

4. Models available to evaluate biofilm formation

4.1. Static versus flow model

To ensure the greatest applicability of research data, a modeling system must be designed to simulate a variety of conditions found in the *in situ* environments. Therefore static

culture conditions are best suited to simulate like environments, e.g. urethral stent exposure to urinary stasis; while flow conditions are best suited to simulate once-through bacterial adhesion studies, i.e., bacterial biofilm development on a relevant substratum, as occurs on vascular devices or dental implants.[88] Differences between cell-cell signalling have been reported between biofilm developed under static versus flow conditions suggesting physiological differences[29,62] and highlighting the importance of using the most appropriate model to obtain the most applicable data. The flow models are most applicable to GI flexible endoscopes, which encounter the movement of patient, procedural, and reprocessing fluids facilitating possible bacterial adhesion and biofilm formation. Shear force is the basis for biofilm formation and flow models incorporate fluid movement by either pumping or agitation over glass beads, coupons/carriers (of glass, metal, polystyrene, or other relevant surface materials), or filter systems.[29] Experimental models of biofilm formation in medical devices can be divided into three categories: semibatch culture flow; continuous flow; and the Minimum Biofilm Eradication Concentration (MBEC) system.

4.2. Semibatch culture flow model

This system employs a semibatch culture biofilm development involving cultures of bacterial strains grown in nutrient broth in beakers with e.g. catheter sections or other relevant substratum materials added to each beaker for each treatment set, and biofilm formation facilitated by a rotary shaker, with colonized surfaces characterized directly or following physical/chemical treatments to assess antimicrobial efficacy.[88]

4.3. Continuous culture flow model

A second type of system is a continuous culture flow model using dynamic flow conditions that are pumped through tubing or over a stationary coupon via peristaltic pumps with recirculation or in a single-pass mode. Examples include the flow cell model, e.g. an endoscope lumen channel/carrier is inserted in a flow cell apparatus and soiled with bacterial culture under continuous flow conditions, rinsed and subsequently exposed to treatment.[88] As well the modified Robbins' device uses a continuous flow system controlled by pumps, which introduces the inoculum from a flask through a channel/line and over the lumen surface held in place by removable studs. This device can hold multiple lumens each forming equivalent biofilm formation.[29]

4.4.Biofilm loop system

A different flow model involves a biofilm loop system set in a water bath, consisting of three sections of connected tubing: one section of sterile tubing is fed through a peristaltic pump connected to a second section of tubing used as the sample loop, which itself is connected to a third section of tubing, which is directed back to the original bottle of sterile nutrient media containing the bacterial inoculum. The inoculated medium is allowed to circulate for 72h at 30°C to develop stabilized biofilm in the tubing loop[105, 112] (Figure 4). Multiple loop systems can be set up (e.g. 4 systems) for subsequent challenge with different disinfectants.[112] The advantage of this model is the ability to remove sampling sections from the sample loop and reattachment of loop system for further continued biofilm formation and testing. However, this model, as with the models described previously, represents biofilm development systems that are limited in the

range and/or number of tests which can be practically accomplished per system, as well as being labour intensive, requiring a complex set up involving pumps, flasks, and lines, and possible introduction of contamination. [29]

4.5. MBEC assay system

A fourth option, the MBEC assay system (also known as the Calgary Biofilm Device) addresses these issues and allows for the development of multiple equivalent biofilms and simultaneous and/or continued varied testing in a biosafety cabinet. The device consists of several formats, one with a 96-well microtitre plate capable of holding the inoculum and/or media and a lid with 96 pegs, on which surface biofilm can form uniformly (Figure 5). Biofilm is formed via the shear force created by a rocking/tilt table apparatus. Subsequently pegs may be broken off individually and tested or the entire pegged lid may be shifted to standard 96-well tray for further quantitative or qualitative testing. [28,29]

Extraction of bioburden from the test carrier:

In all the methods discussed cells may be recovered by standard methods to remove bioburden from the carrier by scraping with Teflon scrapers, vortexing, sonication (using cold reagents and minimum necessary pulse times), and shaking. If needed, adding 2mm sterile glass beads to suspensions followed by high-speed vortexing may disrupt aggregates of biofilm.[88] Verification and an assessment of extraction can be done by a qualitative assessment of microbial outgrowth of the extracted carrier in the appropriate medium or by *in situ* staining of the extracted carrier (e.g., using a live/dead stain). [78, 88]

Figure 4. Biofilm loop system

Biofilm formation within a simulated endoscope lumen (Figure and description as per Rook and McDonnell[112]): A section of sterile tubing (L1) is fed through a peristaltic pump and further joined by a connector ( ) to a sample loop (L2), and again to a third section of tubing (L3), which is directed back to the sterile medium bottle. Following inoculation of the medium with the test bacterial specimen, the inoculated medium circulates through the tubings for 72 h at 30°C for biofilm formation. The biofilm loop is harvested by aseptically removing the sample loop (L2) from the water bath and cutting positive samples from it. The biofilm loop can be further exposed to disinfectant by placement into a disinfectant solution (simulating a manual soak technique) or by replacing the media bottle with disinfectant solution and allowing the solution to flow through the tubing at a specified flow rate and time (simulating an AER process). Used with permission.

Figure 4. Biofilm loop system

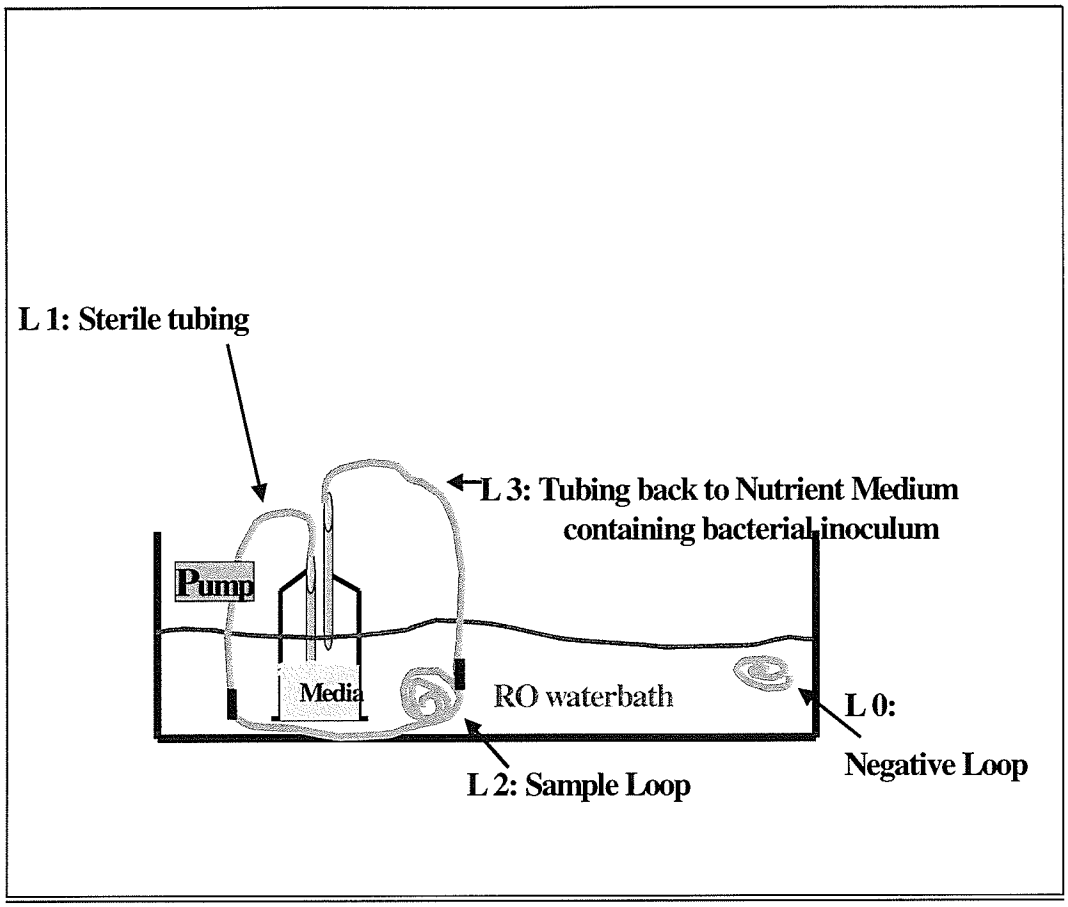
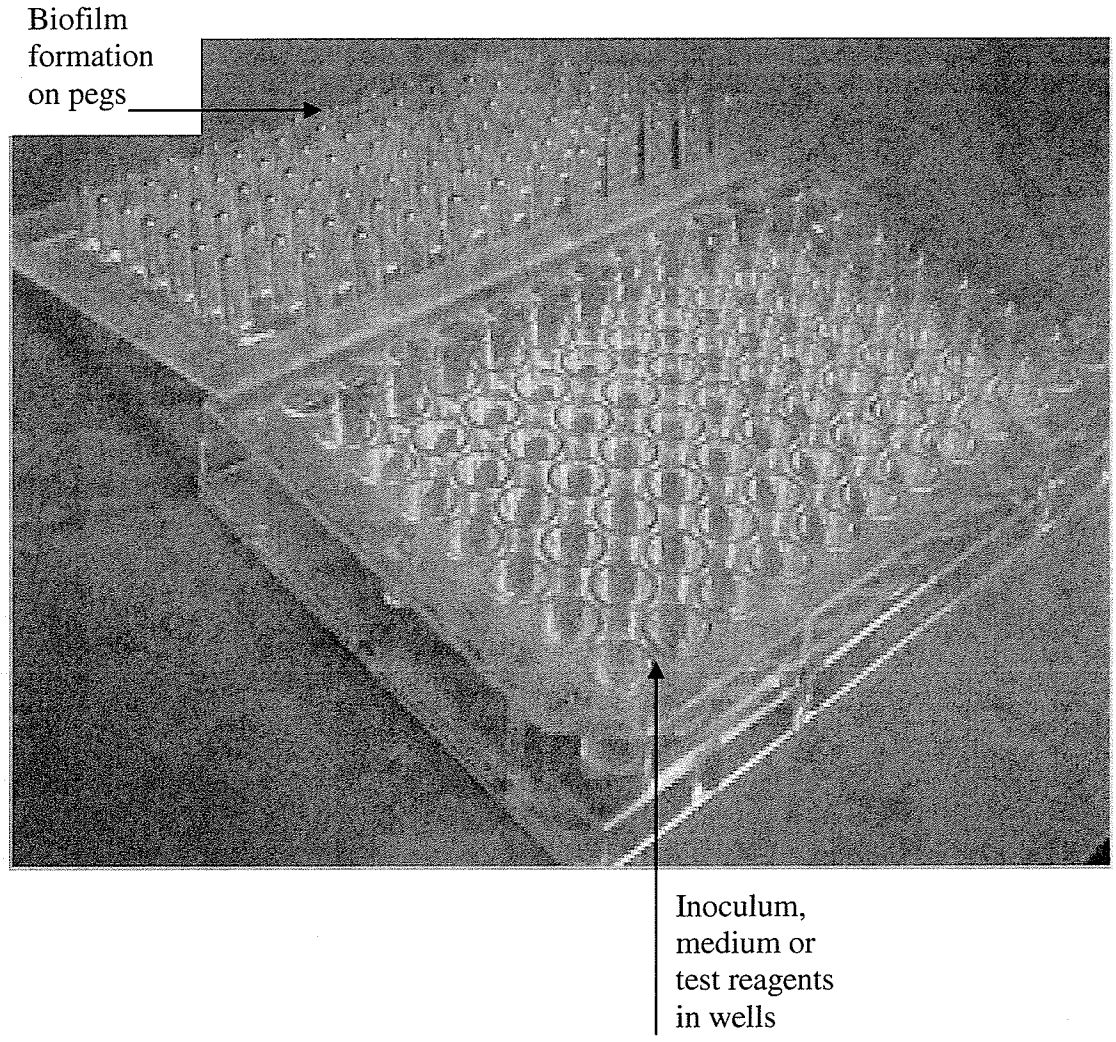


Figure 5. The MBEC assay system

The MBEC (Minimum Biofilm Eradication Concentration) assay system (also known as the Calgary biofilm device) facilitates biofilm formation and measurement of anti-microbial (antibiotic or biocide) sensitivity of bacterial biofilm. The system uses a 96 well microtitre plate format coupled with a 96-pegged lid. Bacterial adherence, colonization, and biofilm formation occur on the surface of the individual pegs. The wells supply solutions supporting bacterial growth (e.g. inoculum, nutrient media), rinses, and microbicide challenge.[98, 57] (Picture from www.innovotech.ca) Used with permission.

Figure 5. The MBEC assay system
(Picture from www.innovotech.ca)



4.6. Growth medium for biofilm formation

To date, a critical, inadequately addressed parameter in biofilm formation is the growth medium (soil). To most reliably model biofilm formation reflective of the specific environment encountered by a medical device and defining the resultant ecosystem, it is important to mimic in vivo/in-use conditions as closely as possible.[140] For example, EPS production in *S. epidermidis* was associated with the type of growth medium used;[81] and microorganisms dried in the presence of a specific cell culture medium survived plasma and ethylene oxide sterilization.[5] Biofilm forms under low nutrient conditions in the environment, and biofilm models have used comparable media.[22, 81, 110] However, the inner lumen surfaces of endoscopic devices are potentially exposed to high and complex levels of microbes and nutrients found in patient mucous secretions. Some biofilm models have used complex nutritional media (e.g., bovine ox bile[76]), but few in vitro models use composite nutritional soils. An artificial test soil (ATS) formulation has been developed based on data from patient-used narrow lumen medical devices.[5] ATS mimics the hemoglobin, carbohydrate, protein, endotoxin and/or bile levels present in material recovered from patient-used flexible narrow-lumen endoscopes used in colonoscopy, duodenoscopy, and bronchoscopy, based on worst-case types and amounts of physiological soil components. ATS, a scientifically valid test soil, has been used to assess the efficacy of cleaning detergents for medical devices[6] and simulated-use studies.[9]

5. Models used to evaluate disinfection efficacy for medical devices

5.1.Critical parameters

Disinfectant efficacy must be determined under well-defined conditions and result in broad-spectrum irreversible, lethal action against bacteria, bacterial spores, fungi, and viruses.[39] Generally test bacteria should undergo a minimum of two subcultures in the test medium. The test medium must be conducive to the growth of organisms and representative of the environment tested.[125] Since all pathogens are suspended in some type of body fluid and precleaning often only reduces soil loads, organisms should be suspended in an organic matrix mimicking potential pathogen protection.[125] Matrices relating to healthcare/medical device surfaces have included 5%-10% serum or a similar standardized formulation of albumin, mucin and Tryptone;[125] the ATS[6] and feces.[85]

Other factors affecting microbicidal performance and ensuring test reproducibility include: (1) Standardization of the inoculum (size, physiological state) with a titre based on the test procedure, avoiding the "inoculum effect" (i.e., the disinfectant's activity is reduced in the presence of proteins);[39] and (2) The test surface which could "hide" the pathogen or neutralize the disinfectant.[125] Current carrier tests use disposable glass penicylinders, stainless steel, plastic or polyvinyl chloride tubing carriers.[5,39,125] Microbicide application can be relevant since the volume per unit area of the inoculated surface can affect microbicidal potency.[121,125] (3) Factors such as temperature (generally recommended at room temperature (22°C – 25°C)), pH, and humidity, as well as concentration and time of contact, should be monitored.[39,125] (4) According to Cremieux et al., [39] a final disinfectant neutralization step is required to prevent carryover into

subcultures. Dilution alone may be sufficient, but it may be necessary to add neutralizing agents, such as phospholipids (e.g., lecithin) or nonionic surfactants (e.g. polysorbates).[133] Neutralizer toxicity must be tested within the system. Separation of microorganisms from the disinfectant solution can be done by washing (centrifugation, membrane filtration, or physical removal). Comparing test results to a recoverable bioburden control will control any procedural effects on microbial counts. Loss of microorganisms during disinfectant testing and bioburden recovery can be eliminated by the use of carriers in a closed system[121,124] (e.g., for viral work) and recovery via membrane filtration (e.g., for bacterial and yeast testing).

5.2.Test method categories

Tests for microbicide efficacy include suspension, carrier, simulated use, field use, and reuse stress testing.[125] Suspension tests are used to assess disinfectant efficacy in a solely fluid environment resulting in a general assessment of microbicidal activity under ideal conditions. Current Hard Surface Carrier Tests are generally used to test disinfection of surfaces and instruments using a quantitative approach comparing the initial population with the number of disinfectant survivors. The organism is applied and dried onto the carrier surface, followed by microbicide exposure for a required contact time, eluted from the carrier, and assayed for viability. Because disinfectant penetration is reduced, the hard surface carrier tests are more relevant, stringent, and confirmatory than suspension testing in evaluating formulations used in healthcare settings.[117,124,125] Quantitative carrier tests (QCT) can differ in testing conditions: QCT-1 tests are conducted under ideal killing conditions; QCT-2 tests are more stringent, mimicking field conditions, using a soil

(organic) load, restricted volumes of disinfectant, and uneven carrier surfaces.[125] Microbicide efficacy in reprocessed flexible endoscopes can be confirmed by simulated use testing (i.e., in vitro contamination of scopes with test organisms followed by reprocessing) and field use testing (performed on patient-used scopes with subsequent reprocessing).[125]

5.3.Assays used in disinfection models

Overall a combination of complementary methods to assess killing of microorganisms via a variety of parameters is most beneficial to predict and evaluate disinfectant efficacy.[148] Assays for the identification and enumeration of microorganisms can be classified by detection of viable or nonviable cells. Viability assays require organisms to multiply in a normal time frame facilitating detection at a minimum population level.[39] For bacteria and yeast, assessment of viability based on culture of surviving organisms, can be either qualitative or quantitative, with detection of growth in broth or on agar medium after incubation. Quantitative viability plate counts are based on growth and replication of each initial cell into a distinct colony under specified conditions. Qualitative outgrowth on a carrier placed into growth medium results in turbidity changes assessed visually or by absorbance reading indicative of viable bacteria.[39] Bordas found qualitative outgrowth was ~1 $\log_{10}$ more sensitive than solid cultures.[20] Limitations include: long incubation times required for detection (16 to 48 hours); a labour and material-intensive procedure (e.g., viability counts); and underestimation from bacterial clumping, inability to extract all the bioburden from the carrier, or the inability to revive/culture stressed survivors.

Measuring metabolic activity of bacteria can provided earlier detection of viability[39] and is independent of culturablity, but dependent on actively respiring bacteria (aerobic and facultative anaerobic) with functional membranes.[148] Limitations include: (1) Assays indicate total cell population,[39] not purity or identification (verified by viability plating or polymerase chain reaction (PCR));[64] and (2) A detection limit of ~3 $\log_{10}$ cfu/mL with an inability to evaluate early growth or low metabolic rates.[64] Tetrazolium assays using the redox dye 5-cyano-2,3-ditolyltetrazolium chloride (CTC)[15] quantify bacterial CTC reduction to a fluorescent formazan salt detected by epifluorescent illumination.[88,148] Similarly, triphenyltetrazolium chloride (TTC), diamethylthiazol-diphenyltetrazolium bromide (MTT)[15] or modified tetrazolium hydroxide (XTT)[108] demonstrate a linear relationship between reagent reduction and final product absorbance relating to the number of actively metabolizing bacteria.[39] The assays are used in situ, but biofilm may hinder reagent transport.[88] Adenosine triphosphate (ATP) bioluminescence, using a luciferine-luciferase system after a cell lysis step,[39] is based on luciferase's requirement for ATP in light production, and can detect viable bacteria, yeast and biofilm formation.[65]

Rapid quantitative enumeration can be done by imaging, e.g., epifluorescent microscopy directly or after carrier extraction, involving staining by fluorescent dyes excited in the ultraviolet range followed by epifluorescent characterization.[78,88] Various stains can be used to assess biofilm bacteria on medical implants or complex medical devices, e.g., acridine orange, a nucleic acid dye or DAPI (4,6-diamidino-2-phenylindole dihydrochloride), a nuclear and chromosome counterstain.[78,88,148] However, the ability to enumerate and distinguish live from dead cells provides further assessment of

microbicidal activity. In thiazole orange (TO) with propidium iodide (PI) staining for nucleic acid, PI can only enter compromised membranes (dead cells), but TO is variably permeant to all cells resulting in a green and red fluorescent signal in viable and dead cells, respectively.

Direct study of biofilm on endoscope channels is not clinically practical and indirect study may be challenged by difficult revival of biofilm bacteria in planktonic culture.[18] Although not indicative of viability, other methods have been used as a marker of biofilm in reprocessed endoscopes, including PCR detection of DNA, in situ imaging methods, e.g., SEM for surface morphology, and confocal microscopy (3-dimensional laser detail of internal/external characteristics using fluorescent molecular probes).[78, 88,140, 141]

Viral assessment requires reliable and sensitive cell culture systems to determine infectivity in quantitative or semi-quantitative assays for plaque forming ability or cytopathic effects respectively.[124] Assays require neutralization of a disinfectant's effects and the determination of possible toxicity or enhancement by the neutralizing agent.[39,125] Detection of HCV, HBV, and HIV by PCR from patient-ready endoscopes,[18] and SEM of biofilm have been used, but cannot distinguish between inactivated and viable virus.[100]

Reprocessing guidelines for endoscopes do not specify chemical analysis of the bioburden (i.e., organic and inorganic contaminants of the media and organisms) for carbohydrate, protein, hemoglobin, and endotoxin. However, these parameters can be related to the risk for infection transmission (e.g., indicating the presence of organisms

and/or the potential to harbour organisms).[8] Specifically, carbohydrate analyses can be related to biofilms, which contain large amounts of polysaccharide.[79] These analyses can be performed in situ or from extracted bioburden; however, the absorbance can only accurately be read on solubilized material (a possible source of underestimation).[5]

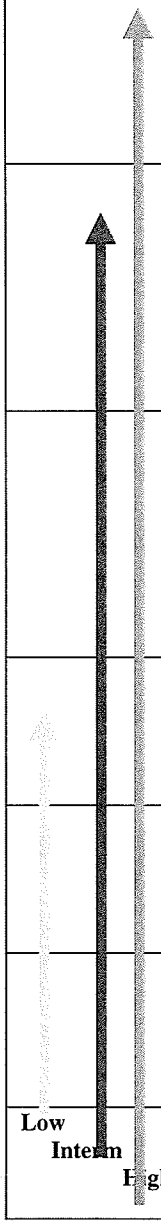
5.4.Microorganisms used in disinfection models

5.4.1. Introduction

The environmental survival abilities and resistance/susceptibility to biocides of prions, viruses and classes of microorganisms has been ranked from most to least susceptible as: prions, coccidian, bacterial spores, mycobacteria, non-enveloped viruses, fungi, Gram-negative bacteria , Gram-positive bacteria, and lipid enveloped viruses,[73,114] as shown in Table 1.[125]

Target organisms used in disinfection models should: (1) relate to human pathogens associated with the healthcare environment and/or a specific medical device; (2) demonstrate a resistance level reflective of the class of target microorganism; and (3) be cultured and tested under conditions reflective of the clinical setting.[121,125] Most relevant to nosocomical transmission are such resistant organisms as VRE, MRSA, and multidrug-resistant *P. aeruginosa* and *M. tuberculosis*, *Acinetobacter* species, extended spectrum B-lactamase-producing *E. coli*, and *K. pneumoniae*.[72,145] Surrogate microorganisms, representing a class of microorganism and human pathogen, can be used for microbicide testing, e.g., in cases of highly pathogenic or non-cultivable organisms[121,125] (Table 1).[125]

Table 1. The relative resistance/susceptibility of major groups of pathogens to microbicides* (Table and description reproduced with permission from *Australian Infection Control* vol 9 (3):84-100 (Syed Sattar). Copyright© Australian Infection Control Association 2004. Published by CSIRO PUBLISHING, Melbourne, Australia. http://www.publish.csiro.au/nid/242/issue/4562.htm)

Microbicide Level	Major classes of pathogens	Examples of pathogens	Examples of surrogates in testing	Examples of liquid microbicides and effective conc.(ppm)
	Spore-forming bacteria	*Bacillus anthracis* *Clostridium difficile*	*Bacillus subtilus* *Clostridium sporogenes*	Chlorine dioxide (1000) Peracetic acid AHP (20,000-70,000) Bleach (52,000) Acidified domestic bleach (5,200)
	Mycobacteria	*Mycobacterium tuberculosis* *M. avium*	*Mycobacterium terrae* *M. bovis*	75% (v/v) Ethanol Alkaline glutaraldehyde (20,000) Ortho-phthaladehyde (5,500) Domestic bleach (1000) AHP (5000)
	Non-enveloped (hydrophilic) viruses	*Hepatitis A* *Adenovirus* *Calicivirus*	*Polio-, rota-, rhinoviruses*	75% (v/v) Ethanol Alkaline glutaraldehyde (20,000) Ortho-phthaladehyde (5,500) Bleach (1000-3000) AHP (1000-5000) Phenolics (500-1000)
	Filamentous and non-filamentous fungi	*Stachybotris chartarum* *Crytococcus neoformans*	*Trichophyton mentagrophytes* *Candida albicans*	Quats (500-750) Domestic bleach (100-500) AHP (1000-5000)
	Vegetative bacteria (other than mycobacteria)	*Acinetobacter baumanii* *VRE*	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Quats (200-400) Domestic bleach (100-500) AHP (1000-5000)
	Enveloped (lipophilic) viruses	*Respiratory syncytial virus* *Haemorrhagic fever virus*	*Herpes* and *Influenza viruses*	Quats (200-400) Phenolics (300-500) Domestic bleach (50-100) AHP (1000-5000)
Low Interm High				

*The relative microbicide resistance/susceptibility is intended as a rough guide. Individual species of pathogens may be more or less resistant than others in their class depending on the stage and conditions of their growth, type of body fluid they are suspended in and the specific type and concentration of the microbicide being applied.

5.4.2. Mycobacteria

Intrinsic resistance to microbicides is attributed to a complex highly hydrophobic, lipid-rich, waxy cell wall consisting of a mycoylarabinogalactan-peptidoglycan skeleton, complex lipids, lipopolysaccharides and proteins limiting sufficient biocide penetration into the cell.[86,114] Glutaraldehyde resistance in a strain of *M. chelonae* is not related to uptake,[86] but rather to increased hydrophobicity and altered cell wall polysaccharides.[84]

For Association of Analytical Communities (AOAC) International tests, *M. bovis* is used as a confirmatory test for activity against *M.tuberculosis*, but it is relatively slow growing. Thus *M. terrae* is the new surrogate for determination of mycobactericidal activity.[59,125] Comparatively, *M. chelonae* demonstrates relatively lower resistance to several disinfectants such as GLUT and ortho-phthaladehyde (OPA). [52] A minimum of a 6 Log_{10} reduction in viability is required for claims of HLD against mycobacteria.

Mycobacteria are significant nosocomial pathogens associated with: (1) *M. tuberculosis* transmission directly (airborne) and indirectly, particularly via bronchoscopes;[1,40, 87, 147] (2) Multi-drug resistant strains of *M. tuberculosis*;[87,116,145] and (3) GLUT-resistant strains of *M. chelonae* from reprocessed endoscopes.[53] Contamination of reprocessed endoscopes with atypical mycobacteria is related to their ubiquitous presence in water,[96] biofilm formation,[54,82] and increasing association with immunocompromised patients.[63]

5.4.3. Fungus/yeast

Intrinsic properties relating to microbiocide resistance/susceptibility involve the rigid cell wall (composed of glucan and mannan) with pores generally too small for entry of large

molecules (molecular weight >700).[114] Biocide susceptibility of *Saccharomyces cerevisiae* (similar to *C. albicans*) has been reported to be linked to cell wall thickness, which increases in older cultures, while porosity decreases.[114] Species differences in environmental survival are related to different hydrophobicities,[136] but limited studies of environmental survival and/or disinfectant activity against fungal pathogens exist.[103,136]

Test organisms for determining fungicidal activity include molds or yeasts. AOAC testing has relied on *Trichophyton mentagrophytes*, *C. albicans* or *S. cerevisae*.[39, 125] Generally a minimum of a 4 Log_{10} reduction in viability is required for HLD of fungi.[39]

C. albicans is a significant nosocomial pathogen and a good surrogate candidate since it is: (1) the most common *Candida* clinical isolate;[136] (2) associated with nosocomial spread from contaminated medical devices or healthcare workers;[136] (3) associated with biofilm in indwelling medical devices (e.g. central venous catheters);[44] and (4) suitable for carrier testing methods.[39,125]

5.4.4. Gram-negative bacteria

Intrinsic properties relating to microbicide resistance/susceptibility involve the cell wall, (composed of a thin layer of peptidoglycans), an outer membrane and a periplasmic zone, resulting in overall greater resistance to biocides than Gram-positive bacteria.[114,148] The cell wall's outer membrane acts as a barrier to chemical entry, possibly aided by the inner, cytoplasmic membrane. Biocide chemistry plays an important role in entry: overall large hydrophilic entities do not enter well.[114] Plasmid-mediated resistance exists, but intrinsic resistance is a more significant factor.[86]

Target or surrogate organisms to determine bactericidal activity include: *P. aeuruginosa*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Proteus mirabilis*, *Proteus vulgaris*, *Acinetobacter baumannii* and *E. coli*.[39] For AOAC International tests *P. aeruginosa* is recommended.[125]

P.aeruginosa is a significant nosocomial pathogen and suitable surrogate since it is: (1) associated with cystic fibrosis lung (biofilm) infections and a plausible contaminant of bronchoscopy;[87,101] (2) associated with biofilm formation on indwelling lines and catheters;[44,60] (3) frequently associated with potable water, persistence of moisture in AERs and reprocessed endoscopes;[33, 67, 82, 106, 130, 132, 140] (4) capable of multi-drug resistance;[145] and (5) ranking high in resistance to many disinfectants, attributable to its LPS composition, cation content of the outer membrane, and small porin size.[86]

5.4.5. Gram-positive bacteria

Intrinsic properties relating to microbicide resistance/susceptibility involve the cell wall, composed of multilayered peptidoglycans and teichoic acids and proteins. The peptidoglycan layer is not protective against biocides, resulting in susceptibility.[148] Resistance of Gram-positive bacteria to chemical biocides is generally attributed to cell envelope plasticity and the thickness and level of peptidoglycan cross-linking.[86]

Target or surrogate organisms used to determine bactericidal activity have included: *S. aureus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus hirae*, and *Lactobacillus brevis*.[39] *S. aureus* is used in AOAC International testing for bactericidal

activity,[125] however enterococci are generally more resistant than staphylococci (although species variability exists).[86, 114]

Significant Gram-positive nosocomial pathogens include: [44,145] (1) MRSA, one of the most relevant nosocomial pathogens; (2) *S. aureus*, related to biofilm formation on indwelling medical devices (e.g., orthopaedic implants and intravascular catheters); (3) Enterococci, associated with biofilm formation in indwelling medical devices (e.g., central venous catheters, orthopaedic implants); and (4) *E. faecalis*, a biofilm-associated microbe colonizing the GI tract, with microbicide resistance implications for VRE.

5.4.6. Nonenveloped viruses

Nonenveloped (hydrophilic) viruses generally display a resistance to gastric acidity causing GI infections and a greater resistance to virucides compared to enveloped viruses. Intrinisic properties relating to microbiocide resistance/susceptibility of these viruses involve the absence of the lipid bilayer envelope, presenting a resistant external barrier (since 70% to 95% of the mass is protein); and many degrees of redundancy in viral symmetry and numbers of protein subunits (likely requiring multiple hits of virucide to achieve inactivation).[121] Generally, smaller nonenveloped viruses are more resistant to virucides than larger viruses attributable to simpler structure and greater capability of being protected by surface soiling or microtopography.[121]

Poliovirus, a small nonenveloped enterovirus, is considered the "gold standard" for germicidal testing of viruses.[59] However due to eradication of poliomyelitis, restricted

usage of even the vaccine strains is anticipated.[124] Examples of suggested surrogate viruses include human *adenovirus*, *feline calicivirus* (closely related to non-culturable *Norovirus*), and human *rotavirus*. Generally, reductions in viral titre of 2- to 4 Log_{10} are acceptable for hard surface carrier testing of virucides for all viruses.[124] For disinfectant efficacy testing, virucidal activity is based on results with nonenveloped viruses.[121]

Examples of human pathogens and associated diseases include: *adenoviruses* (respiratory tract infections), *Noroviruses* (gastroenteritis), *enteroviruses* (e.g. pneumonia, hand-foot-mouth disease, respiratory infections), *Hepatitis A virus* (infectious hepatitis), *polioviruses* (poliomyelitis), *rhinoviruses* (common cold), and *rotaviruses* (acute diarrhea).[121,124] Specific pathogen transmission related to endoscopes was described previously in the section, Infection transmission.

5.4.7. Enveloped viruses

Enveloped (hydrophobic or lipophilic) viruses are more sensitive to microbicides than nonenveloped viruses due to the viral envelope and the necessity to remain intact for infectivity. However, a range of susceptibility exists for these viruses.[121]

Testing of target organisms can be based on pathogenicity, e.g., HIV-1. Surrogate viruses alleviate difficulties in culturability of pathogens, e.g., HBV and HCV, (surrogates include duck HBV and *bovine viral diarrhea virus,* respectively);[122] or pathogenicity, e.g., SARS-CoV (surrogate, *Coronaviruses*). *Sindbis virus* (Alphavirus genus, *Togaviridae* family), similar in structure to flaviruses (e.g., HCV, West Nile fever) and

having a low risk of infectivity, is an optimal indicator for inactivation of enveloped viruses.[45]

Although enveloped viruses are generally more fragile to environmental conditions, blood-borne enveloped viruses including HBV and HIV have been associated with relatively longer environmental survival.[72] HBV and HCV are the most prevalent blood-borne pathogens, associated in a healthcare setting with injection devices, blood spills, and heat-sensitive medical devices (e.g., flexible endoscopes[17,134]). Sensitivity to chemical and physical agents can limit their survival and spread,[122] however, survival and transmission of HCV during colonscopy has been reported.[23] Specific pathogen transmission related to endoscopes was described previously, in Infection transmission.

6. Current research problem

6.1. Gaps in current biofilm models – need for cyclic buildup model

Studies on current endoscopy and accessory device reprocessing protocols and the associated clinical risks "emphasize that currently recommended reprocessing protocols have a lower than desirable margin of safety, and that failure is likely if the cleaning steps are not followed in meticulous detail".[38] This concern is supported by Vickery's studies showing >2% bacterial contamination rate in patient-ready endoscopes in spite of adherence to reprocessing guidelines.[140]

Current validation of reprocessing protocols for GI medical and accessory devices in the U.S.A. is based on the FDA requirement for a "worst-case" testing method. This

generally employs a new unused device that is soiled with organisms and/or an organic/inorganic challenge followed by an evaluation of the cleaning and disinfection/sterilization stage. Therefore, the impact of repeated soiling and reprocessing on cleaning and disinfection is not accounted for in the standard validation process. Yet published and confirmed device-related infectious disease transmission has been attributed to problems occurring in patient-used medical devices after multiple use and reprocessing and often associated with breeches in the manual reprocessing stages and /or prolonged reprocessing.[38,94] Overall, pathogen spread via flexible endoscopes has been attributed to the following factors: "failure to follow recommended guidelines for disinfection; organisms harboured in a site inaccessible to cleaning and disinfection; presence of resistant organisms; and recontamination of the endoscope or accessories after adequate disinfection."[40]

Little is known regarding the progressive buildup of material within the channels of flexible endoscopes, which can be used and reprocessed up to 1000 times per year. This project evaluated the risk of microbial survival within the buildup material, and focused on the role of biofilm formation in infection transmission from narrow lumen medical devices such as flexible endoscopes. An in vitro model for biofilm formation was developed which more accurately reflects the buildup of bioburden in patient-used flexible endoscopes. Furthermore, the model is based on repetitive microbial exposure and disinfection cycles mimicking in-use conditions. The model is used to characterize the kinetics of buildup biofilm that accumulates over time and to evaluate the microbial survival and disinfectant efficacy in the buildup biofilm that develops. The microbial

killing efficacy of two high-level disinfectants (2% glutaraldehyde and 7% accelerated hydrogen peroxide) was evaluated for a range of microorganisms including bacteria, mycobacteria, fungi, and viruses when in the biofilm matrix. Ultimately, this novel model will be instructive in defining the threshold level of buildup biofilm that results in disinfection failure. Such a model can suggest approaches that may prevent the threshold level of buildup from occurring and thereby reduce the risk of infection transmission from endoscopy.

6.2. Hypotheses

The biofilm which forms in narrow lumen flexible endoscopes is a BBF, and develops as a result of cyclical exposure to wet/dry phases in the usage/reprocessing protocol.

BBF has a unique composition and different microbial survival characteristics to TBF that forms in lumen constantly bathed in fluid.

BBF results in a matrix/biofilm formation representing a greater challenge to disinfectant penetration and microbial eradication than TBF.

To address these hypotheses an in vitro model was developed since currently no such model exists. This model of BBF, using ATS as the test medium, should more accurately reflect what occurs in patient-used narrow lumen medical devices compared to traditional in vitro TBF models using low-nutrient growth medium.

It is believed that by modeling BBF, a better understanding of disinfectant efficacy in flexible endoscope reprocessing and possible microbial transmission will be gained.

For example, the model will demonstrate the effects when BBF is repeatedly exposed to high-level disinfection. This is of concern, especially for aldehydes, which can cross-link and cause fixation of organic material, potentially forming a protective layer of fixed proteinacious material over embedded viable microorganisms.[8,100]

7. Research study approach

The relative survivability of bacteria, mycobacteria, fungi and viruses when dried on a surface, in TBF, or within BBF is unknown and will be addressed by this research study by incorporating a relevant test medium/soil and test microorganisms mimicking conditions found in GI/respiratory patient-used flexible endoscopes. Using these conditions, this study will reliably model biofilm formation in reusable narrow lumen medical devices to determine microbial survival. The efficacy of two common disinfectants, glutaraldehyde and hydrogen peroxide, on survival of organisms will be compared.

The objectives of this study were to determine:

- To what extent and under what conditions various microorganisms (e.g. bacteria, mycobacteria, fungi, and viruses) can survive on dried surfaces or within biofilm, in particular BBF, during dry storage.
- Whether BBF represents a greater challenge to disinfection efficacy than TBF.

This research study is the first to develop a model to facilitate studying microbial survival in reprocessed narrow lumen medical devices used in the GI tract that mimics repeated reprocessing and resultant buildup biofilm, and facilitates the evaluation of disinfection efficacy. The model provides scientifically valid results regarding microbial survival characteristics within the BBF, and the efficacy of different high-level disinfectants in combating microorganisms within BBF. The data from this research project has implications regarding the potential risk for infection transmission posed by BBF, i.e., if viable organisms survive in BBF, can they be transferred during the next patient-use when exposed to liquid secretions?

The relative survivability of bacteria, mycobacteria, fungi and viruses within BBF is unknown, and new device testing does not consider BBF. The development of a novel model addressing the formation of BBF for a variety of representative organisms and data generated from this study will have significant impact on conclusions regarding the adequacy of cleaning and disinfection of flexible endoscopes. Information generated from conditions mimicking those encountered by reusable flexible endoscopes can help define critical parameters resulting in disinfection failure, including: (1) the impact of organic matter on microbial survival and the importance of effective cleaning prior to disinfectant exposure; (2) critical buildup levels of biofilm formation capable of surviving accepted reprocessing protocols and facilitating infection transmission; (3) the efficacy of different high-level disinfectants formulations in combating microbial survival in TBF and BBF. Therefore, this model can help to identify approaches that prevent critical buildup and infection transmission from occurring, thereby facilitating the

development of improved reprocessing methods for existing narrow lumen medical devices. Furthermore, licensing review bodies (e.g., Health Canada, FDA) require American manufacturers of flexible endoscopes to provide a worst-case testing to validate processing protocols. This generally involves new, unused devices soiled with organisms and/or an organic/inorganic challenge to demonstrate acceptable microbial reduction. Therefore, this model will facilitate development of adequate reprocessing guidelines for existing and new technologies of reusable narrow lumen flexible endoscopes. Thus, results from this research study will provide data for an evidence-based approach on how to improve reprocessing and reduce device-related infections. Insights gained can be used to ensure that methods for device reprocessing represent the lowest risk possible for infectious disease transmission. Ultimately, this research can improve patient care and reduce infection transmission by narrow lumen flexible endoscopes.

MATERIALS AND METHODS

1. Microorganisms and culture

Test organisms in this research project were a representative range of microorganisms that could be associated with contamination of complex medical devices and/or healthcare environments (Table 2). Bacterial organisms were passaged on tryptic soy agar (TSA) (Oxoid, Toronto, Canada) medium at 35 °C following incubation for 24 to 48 hours. Mycobacterial and fungal organisms were sub-cultured on TSA supplemented with 5% whole sheep blood (BA) (Oxoid, Toronto, Canada) and incubated at 30 °C for 72 hours and 120 hours for *C. albicans* and *M. chelonae* respectively. Stock cultures were maintained in skim milk at –70 °C. All bacterial cultures were subcultured three times before experimentation.

Reovirus was prepared in murine L929 cells (ATCC #2) and amplified to the second passage in Minimum Essential Medium (MEM, (Gibco)) modified with sodium bicarbonate (22g/10L) and HEPES (12g/10L), pH 7.2; further supplemented with 2.5% fetal calf serum (Intergen), 2.5% VSP agammaglobulin serum, 2mM L-glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin and 1 ug/mL amphotericin-B. Plaque purification of *Reovirus* was according to Hazelton and Coombs.[58] *Sindbis virus* (SVHR) stocks were prepared in BHK-21 cells grown in monolayers in Delbecco Minimum Essential Medium (DMEM) with 5% FCS, 0.5% L-glutamine and 1% glucose and

Table 2: Microorganisms tested. All types and strains of microorganisms are listed with the origin of each and a study designation to differentiate multiple strains if necessary

Microorganisms	Source	Study designation
Acid fast bacteria		
Mycobacterium chelonae -glutaraldehyde sensitive strain	ATCC 19977	*M. chelonae*^{S}
Mycobacterium chelonae -glutaraldehyde resistant strain	United Kingdom (Griffiths 1997)	*M. chelonae*^{R}
Gram-negative bacteria		
Pseudomonas aeruginosa	ATCC 15442	*P. aeruginosa*
Gram-positive bacteria		
Enterococcus faecalis	ATCC 29212	*E. faecalis*
Yeast		
Candida albicans	ATCC 14053	*C. albicans*
Nonenveloped virus		
Reovirus Serotype: Type 3 Dearing (T3D)	Laboratory stock (Dr. K. Coombs)	*Reovirus*
Enveloped virus		
Sindbis virus -heat resistant strain	Laboratory stock (Dr. K. Coombs)	SVHR

harvested and clarified by centrifugation for 20 min at 1500 x g. Viruses were stored as frozen stocks at –70 °C and cell lines were stored in liquid nitrogen.

2. Test Disinfectants

Glutaraldehyde (Metricide™, Sybron Canada, Oakville, Canada) at a stock concentration of 2.6% (w/v) was tested for HLD as per manufacturer's recommendations. Accelerated hydrogen peroxide (PerCept, Virox, Mississauga, Canada) was evaluated at a 7% v/v for HLD concentration, as well at a 1:16 use-dilution as per manufacturer's recommendations for surface disinfection. Various other concentrations were evaluated to establish the concentration where breakthrough growth occurred. Stabilized hydrogen peroxide (3% SHP (v/v), PerDiem, Virox, Mississauga, Canada) was used at the manufacturer's suggested use-dilution of 1:64. Any dilution of disinfectants was done using sterile tap water, prepared immediately before each test and was not reused. Table 3 summarizes the various disinfectants and exposure conditions used.

3. Test soil

For this study, the test soil was defined as the organic and inorganic matrix in which the test microorganisms were suspended, either for seeding of carriers or feeding during biofilm formation. Developmental biofilm studies comparing the Biofilm Loop and MBEC systems used sterile 1% tryptic soy broth (TSB) (Sigma, St. Louis, Missouri, U.S.A.). However, to provide an organic challenge that mimicked medical device exposure in the body, an artificial test soil (ATS) was used in all subsequent biofilm

Table 3: Disinfectants and exposure conditions

Disinfectant	Disinfectant Exposure conditions: Concentration	Time (min)
Glutaraldehyde (GLUT)		
	2.6% [1]	1 – 20 (2 min intervals)
	0.1%	1 – 20 (2 min intervals)
Accelerated Hydrogen peroxide (AHP)		
	7% [1]	1- 20 (2 min intervals)
	0.5% [2]	1- 20 (2 min intervals)
	0.05%	1- 20 (2 min intervals)
Stabilized Hydrogen Peroxide (SHP)		
	3% [3]	10
	0.05% [4]	1 and 5

1. High-level disinfection as per manufacturer's recommended concentration and time:
   Glutaraldehyde: 2.6% (w/v) concentration for 20 min
   Accelerated Hydrogen Peroxide (PerCept): 7% (v/v) concentration for 20 min
2. Surface disinfection as per manufacturer's recommended dilution and time:
   Accelerated Hydrogen Peroxide (PerCept):
   used at 1:16 dilution (0.5% v/v final concentration) for 5 min
3. Low-level disinfection as per manufacturer's recommended concentration and time:
   Stabilized Hydrogen Peroxide (PerDiem): 3% (v/v) concentration for 10 min
4. Surface cleaning as per manufacturer's recommended dilution:
   Stabilized Hydrogen Peroxide (PerDiem):
   used at 1:64 dilution (0.05% v/v final concentration)

studies. ATS is composed of protein, carbohydrate, endotoxin and hemoglobin at worst-case levels as detected in patient-used flexible endoscopes.[5,6] This test soil formulation represented the highest organic component used in the study. For comparative survivability studies, test soils representing low and intermediate organic levels were also included. The test soils without organic content included: 0.01M phosphate-buffered saline, pH of 7.5 (PBS) (for environmental survival studies) or tap water (for biofilm studies). Also, a soil of intermediate organic content, i.e., an enzymatic detergent was also included in the biofilm studies. In a series of experiments representing the complete reprocessing scheme of endoscopes, an enzymatic detergent (Pentazyme (Case Medical Inc., Ridgefield, New Jersey, USA)) was used as a source of nutrition (feed) during biofilm formation. This enzyme detergent represented an intermediate level of organic matter. A description of organic loads for each test soil is presented in Table 4.

4. Surface carrier method

4.1. Surface carrier inoculation and quantitation

4.1.1. Test method and quantitation

A 1-cm piece of polyvinyl chloride (PVC) (Nalgene, Rochester, New York, U.S.A) sterile tubing with an inner diameter of 3 mm, mimicking the plastic surface often found in medical devices, was used as the test carrier. The test carrier was inoculated by placing 50 uL of the test suspension onto the carrier's inner surface using a micropipette, targeting a final inoculum of ~10^6 CFU/carrier (for bacteria and yeast),[6] or plaque

Table 4: Test soil analyses

	Test Soils[1]			
	ATS[2]	**Pentazyme[3]**	**sPBS[4]**	**sTAP[5]**
Component analysis (ug/mL)				
Protein[7]	11,800	18	<LD[6]	<LD
Carbohydrate[8]	2450	102	<LD	<LD
Hemoglobin[9]	880	<LD	<LD	<LD

[1] All soils were subjected to sterility checks and only used when sterility was confirmed.
[2] ATS: Artificial test soil was formulated as a 1X solution and new batches were prepared monthly, and filter sterilized using a 0.22um PES filter. Chemical analyses were conducted to ensure levels within the expected formulation stipulations (patented proprietary information not shown). Thereafter ATS was aliquoted under sterile conditions and stored at 4°C
[3] Pentazyme: enzymatic detergent, use dilution 1:125 in tap water
[4] sPBS: sterile (autoclaved) 0.01M phosphate-buffered saline, pH 7.5
[5] sTAP: sterile (autoclaved) tap water
[6] LD: assay limit of detection
[7] Protein was quantitated using the standard Bradford Protein microassay method with a bovine serum albumin standard (LD: 40 ug/mL)
[8] Carbohydrate was quantitated using the phenol-sulfuric acid method[79] microassay with glucose standard (LD: 800 ug/mL)
[9] Hemoglobin was quantitated using the tetramethyl-benzidine microassay[5] with a hemoglobin standard (LD: 150 ug/mL)

forming units (pfu)/carrier (for *Reovirus*) and cytopathic effects (CPE infective units)/carrier (for *Sindbis virus*). Inoculated carriers were dried overnight (ON) at room temperature (RT). All inoculations, drying, and subsequent testing were done in a Class II B3 biosafety cabinet.

For bacteria and yeast, each test inoculum was initially established by the direct colony suspension method. Therefore, organisms grown from TSA or BA pure cultures in exponential growth phase were used to prepare a suspension in sterile 0.01M phosphate - buffered saline, pH 7.5 (sPBS) equivalent to a 0.5 McFarland turbidity standard defined by spectrophotometry (530 nm) to approximate 1 x 10^8 CFU/mL. This was validated by viable counts resulting from serial dilution and spread plate quantitation on the appropriate medium. Each test suspension was finally suspended in the ATS, by transferring 1 mL of the suspension (approximately 1 x 10^8 CFU) to individual microfuge tubes and centrifuged at 16,000 x g for 5 minutes at 4°C. Following removal of the supernate, the pellet was resuspended in the ATS. To confirm the resultant bacterial inoculum counts in ATS, serial 1:10 dilutions in PBS were made with 100 uL of dilutions ranging from 10^{-3} to 10^{-6}, followed by spread plate quantitation under the appropriate conditions for each test organism as outlined earlier (Section 1).

Quantitative assessment of bacterial and fungal survival in the eluted sample was determined. Briefly, samples were serially diluted 1:10 in sterile TSB and 0.1 mL of each dilution was inoculated on the agar medium using the spread plate method. Viability was evaluated at 35 °C after 48 hours for vegetative bacteria, and at 30 °C for 72 hours and

120 hours for *C. albicans* and *M. chelonae* respectively. The limit of detection was 10 CFU/carrier.

Viruses were aliquoted and kept at –20°C in test media. Aliquots were thawed, appropriately diluted in ATS and used to inoculate carriers, which were held at RT for varying lengths of time. Quantitation of *Reovirus* titre was based on a standard quantitative plaque assay[58] using monolayers of the L929 cells in 6-well plates, inoculation with serial 10-fold dilutions of viral samples in gel/saline, and calculation of virus titre comparing control and test samples. A semi-quantitative assay of the cytopathic effects induced by *Sindbis virus* was used to determine viral titre. Half-$\log_{10}$ dilutions of SVHR samples were prepared in media and inoculated on confluent BHK 21 cell monolayers in 96-well plates. After 3 days infection at 37°C in 5% CO_2, cell monolayers were observed for CPE by fixation with paraformaldehyde and staining with 0.1% Crystal Violet. The limit of detection for virus was 10 PFU (*Reovirus*) or CPE (*Sindbis virus*) infective units per carrier.

Immediately after inoculation of the carriers and before drying, all microorganisms were eluted from 3 carriers to determine recoverable bioburden (recorded as test day –1). Immediately after drying ON, another 3 carriers were eluted to determine viable bioburden (recorded as test day 0). Following drying ON at RT, all desiccated carriers were left at RT enclosed in sterile petri dishes inside the biosafety cabinet and tested for viability at various times to 30 days (test days 1 to 30). Elution of all organisms from the test carrier was performed by aseptic transfer of the carrier to a sterile tube containing 1

mL sterile TSB (Sigma, St. Louis, Missouri, U.S.A) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York, U.S.A) (sPBS was used for viruses) followed by 2 min shaking, two sonications for 5 sec, and 3 min vortexing.

The bioburden reduction factor (RF) was calculated using the following equation:

$$RF = Log_{10} RB - Log_{10} T$$

Where:

T = viable count post treatment

RB = recoverable bioburden from positive control

4.1.2. Qualitative and semi-quantitative assessment of bacterial viability

Qualitative assessment of bacterial and fungal viability was also determined by outgrowth of test and control carriers in 1 mL sterile TSB-10% FBS with incubation at 35 °C for 10 days. Daily assessment for turbidity indicating bacterial growth was made. If turbidity resulted, identification was verified by re-plating a 100 uL sample. To eliminate the possibility of low, undetectable concentrations of residual viable organisms, a terminal subculture was done on all non-turbid media as well (data not shown).

In addition, microscopy with a live/dead viability stain (catalogue number 34943, BD Cell Viability Kit, BD Biosciences, San Jose, CA, U.S.A.) containing thiazole orange and propidium iodide was used to evaluate bacterial survival on the carriers as per manufacturer's directions, with an incubation time of 45 minutes in the dark. However, the sPBS diluent was supplemented with 0.01% Tween 20 (catalogue number P2287,

Sigma-Aldrich, St. Louis, MO, U.S.A.) and 1mM EDTA (J.T.Baker, Toronto, ON, Canada) for better dye penetration (e.g., reducing the lipopolysaccharide (LPS) layer in gram-negative organisms). Visualization by epifluorescence using a FITC filter facilitated enumeration of live versus dead organisms within several fields of view to a total of 1000 counted organisms. The resulting percentage of live organisms/carrier was used as a semi-quantitative assessment of viability/carrier.

4.2. Disinfectant exposure

4.2.1. Survival to optimal versus suboptimal disinfectant exposure

Inoculated carriers (as described previously) were exposed to disinfectants, Glutaraldehyde (GLUT) and Accelerated hydrogen peroxide (AHP) as described in Table 3 and various conditions were assessed to determine breakthrough survival at suboptimal disinfectant exposure.[6] Test carriers were exposed to disinfectant challenge by complete immersion in 1 mL of the disinfectant to be evaluated. Disinfectant dilutions were made in sterile tap water. After exposure, the disinfectant was gently aspirated using a sterile Pasteur pipette with minimum disturbance to the inoculated surface. Thereafter the carrier was gently rinsed 3 times in 1 mL sPBS. Following the final rinse, the test carrier was aseptically removed and placed into 1 mL of 10% FBS-TSB neutralizing agent and eluted from the carriers (as described below).[6] Quantification was based on viable counts of colony forming units (CFU), plaque forming units (PFU), or cytopathic effect (CPE) infective units (as stated earlier).

4.2.2. Accelerated hydrogen peroxide (AHP) and Stabilized hydrogen peroxide (SHP)

To determine the effect of hydrogen peroxide formulation on microbial kill, different formulations of HP surface cleaning agents, i.e. AHP and SHP, were compared at the breakthrough concentration and time for AHP (i.e., 0.05% AHP with 1 min exposure time) as well as at 5 min contact time (the manufacturer's recommended time for surface disinfection with 0.5% AHP) according to disinfectant exposure methods described earlier (Table 3).

4.3. Controls

Controls in the study included:

(i) an inoculum control (microorganisms in sPBS or ATS prior to inoculation of carrier);

(ii) a recoverable bioburden (positive control) accounting for the fluid effect of the test protocol (i.e., carriers inoculated and processed as per test carriers, with the exception of PBS replacing the disinfectant);

(iii) an organic medium control (test carriers inoculated with ATS alone);

(iv) a negative carrier control (no organism/no soil).

All data is presented as a mean and standard deviation of triplicate tests from three trials (i.e. 9 replicates in total).

4.4. Neutralization

4.4.1. Neutralization method

To prevent the possibility of residual effects of the test disinfectant on the microorganisms after experimental exposure and an overestimation of disinfectant

efficacy,[117,124,133] immediately following exposure to a disinfectant, the disinfectant was aspirated and the carrier was rinsed 3 times in 1 mL sPBS and the residual organisms on the carrier were eluted by sonication in 1 mL neutralization solution of 10% FBS in TSB for bacterial studies;[6] or incubation in 1 mL of the neutralization solution for 10min, followed by carrier removal and elution in 1 mL sPBS for viral studies. Both neutralization/elution protocols were equally effective for recoverable bioburden of bacteria (data not shown).

4.4.2. Confirmation of neutralization of the disinfectants

Dried carriers inoculated with each microorganism were challenged with each specific test disinfectant (at the conditions specified for HLD) and the neutralizing agent. Viability per carrier (quantitation described earlier) was determined to demonstrate neutralization efficacy (i.e., prevention of any carryover effect of killing after disinfectant removal) and neutralization effect (possible toxicity or enhancement) of the neutralizing agent by comparison of the following test and control protocols:[133]

A. Positive control (untreated): Recoverable bioburden

Carrier→PBS→rinse→TSB/elute

B. Neutralizer 10% FBS (no HLD):

Carrier→PBS→rinse→neutralizing agent/elute

C. 2.6% GLUT or 7% AHP (HLD) diluted/neutralized with 10% FBS:

Carrier→[1:100 GLUT or AHP / Neutralizing agent]→rinse→TSB/elute

D. HLD (GLUT or AHP) neutralized with 10% FBS as in test method:

Carrier→HLD→rinse→neutralizing agent/elute

4.4.3. Toxicity and Interference with viability assays in viral studies

The method of Sattar[124] was adapted (as per www.virox.com, June 2003) to ensure that any possible residual disinfectant in the neutralizing agent did not inhibit or enhance viral effects on cell culture. Therefore, 100 uL of a 1:100 dilution of the disinfectant with neutralizer was added to half the wells of a twelve-well plate; the remaining half received cell culture medium only. Following a 30-minute incubation, cells were analyzed by microscopy for toxic effects. Thereafter, all cells were washed with medium and virus added as per the PFU or CPE assay. In a similar assay, non-toxicity to cells in the CPE assay was confirmed for ATS and sPBS (the biofilm and recovery medium, respectively).

5. Traditional Biofilm (TBF)

5.1. Biofilm loop model

Formulation of biofilm within a simulated endoscope lumen was achieved by means of a biofilm loop model based on the method of Pineau et al.[105] and with modifications as per Rook and McDonnell[112] (described below and in Figure 4, Introduction). The test organisms, *E. faecalis* and *P.aeruginosa* were used individually for biofilm development, which occurred within flexible Tygon tubing (I.D. 6.4 mm, Nalgene, Rochester, New York, U.S.A). The model was contained in a water bath containing RO water (without antibacterials and replaced for each experiment). The model was constructed by feeding a segment of sterile tubing (L1) originating from a 1-liter bottle of sterile nutrient medium (1% TSB) through an adjustable peristaltic pump. Tubing (L1) was further attached to a sample loop (L2), and again to a third segment of tubing (L3) returning to the bottle of sterile nutrient medium. The nutrient broth was inoculated with the test organism for a

final concentration of ~3 CFU/mL. Thereafter the medium was circulated at a flow rate of ~5 mL/min for 72 hours at 30°C to form a stabilized biofilm within the tubing.

Biofilm was recovered after 72 hours development as per Rook and McDonnell.[112] Segments of the biofilm loop were harvested aseptically by removing the sample loop (L2) from the water bath, cutting ~12 cm lengths from the tubing (L2), and wiping the tubing exterior with 70% isopropyl alcohol solution for decontamination. Biofilm loop segments were further cut into 2 cm sections, placed in sterile Petri dishes, and cut again in half lengthwise. Bioburden recovery was achieved by elution of the organisms from the tubing segment, i.e., by rinsing the segment with sPBS to remove non-adherent bacteria and aseptic transfer of the carrier to a sterile tube containing 1 mL of sPBS followed by 2 min shaking, sonication for 5 min, and 1 min vortexing. Viability was evaluated using the spread plate method as described earlier.

5.2. MBEC model

Traditional biofilm formation of the test organisms, *E. faecalis, P. aeruginosa, C. albicans* or *M. chelonae*, were formed in the MBEC system (formerly MBEC Biofilms Technology, now Innovotech Ltd, Calgary, Alberta) under sterile conditions in a Class II B3 biosafety cabinet. The MBEC device consisted of a sterile 96 well microtitre plate and 96 peg lid (refer to Introduction, Section 4.5 and Figure 5). Biofilm formation (and related controls) were established on and recovered from the pegs as per the manufacturer's recommendations and method of Ceri et al.[28] and Harrison et al.[57] with some minor alterations. Early comparative studies with the Biofilm Loop system used

sterile 1% TSB as the medium for biofilm inoculation and feeding. However, for buildup biofilm studies, biofilm formation was modified to simulate endoscope-reprocessing conditions using a nutrient medium of ATS for inoculating (seeding) pegs and feeding biofilm formation at RT.

5.2.1. MBEC peg inoculation and biofilm initiation

According to the method of Ceri et al.[28] and Harrison et al.,[57] the inoculum per test organism was established from a 1.0 McFarland Standard and resuspended in growth medium (ATS) at ~3.0 x 10^8 CFU/mL in a biosafety cabinet (similar to Section 4.1.1). This suspension was further diluted 1/30 in ATS (1 mL suspension added to 29 mL ATS), resulting in ~10^7 CFU of test organism per millilitre of ATS medium. The initial cell number in the inoculum was verified by plating 10-fold serial dilutions of the inoculum. on the appropriate agar plates. Biofilm formation was established at room temperature in high nutrient conditions mimicking the environmental conditions seen by a GI endoscope (i.e., high organism loads seeded and grown in ATS).

In a biosafety cabinet, the sterile MBEC device was opened, and the pegged lid was set aside with undisturbed peg tips facing upward. An inoculum of 150 uL was added per well. A subset of wells received ATS medium without the test organism (the negative inoculum control). The pegged lid was placed on the corresponding microtitre plate wells containing the test inoculum. The inoculated MBEC system (lid/plate) was placed on a rocking table set at RT with an incline of ~10° and ~3 full rocks per minute. Pegs were colonized (seeded) for 24 hours to establish biofilm formation on all pegs per plate.[28,57]

5.2.2. Biofilm feeding

According to the method of Ceri et al.[28] and Harrison et al.,[57] after 24 hours, the MBEC device was placed in the biosafety cabinet. A sterile 96-well flat bottom microtitre plate was filled with 200 uL sPBS per well with a multichannel pipetter and used to rinse and remove non-adherent organisms from the biofilm formation. Another microtitre plate (the "feed" plate) was filled with 200 uL ATS per well, and used as the medium for continued biofilm growth (or alternate test mediums, e.g., sterile tap water, as required per study).

Under sterile conditions the peg lid was removed and placed into the fresh rinse plate and placed (closed) on the rocker table for 1 minute at moderate speed to remove non-adherent bacteria. Thereafter, in the biosafety cabinet, the peg lid was transferred to the "feed" plate. The closed MBEC device was placed on the rocker table as previously described for 24 hours. After biofilm development over 48 hours, 3 replicate positive and negative sample pegs were removed and recovered as a growth control to verify the number and type of organism that had formed in the biofilm.[57] Nutrient medium was replenished every 24 hours (referred to as feeding the biofilm) over the time course of the study. In this study, such biofilm formation was called traditional biofilm.

5.2.3. Biofilm recovery from MBEC pegs

At specified times, biofilm was removed from the pegs under sterile conditions from individual pegs.[57,28] For biofilm recovery, a 96-well microtitre plate was prepared by adding 200 uL of sPBS per well as a rinse plate, used as described above. Alternately, when small numbers of pegs were recovered, rinsing took place in 500 uL sPBS per

sterile microcentrifuge tube. Individual pegs were broken off the lid by placing a sterile haemostat at the lid/peg interface and breaking the peg off. Pegs were briefly rinsed in sPBS, 3 times for 20 seconds, to remove nonadherent material.

The rinsed pegs were transferred to recovery tubes containing sPBS in either a volume of 500 uL in a microfuge tube or in 1 mL in a 2 mL snap-cap test tube (Simport, Quebec, Canada). Tubes were shaken on a shaker table at high speed for 2 min, sonicated at 50/60 Hertz using a Bransonic 1200 Ultrasonic cleaner (Branson Canada, Pickering ON) for 5 minutes and vortexed for 1 min. Different sterile haemostats were used for each organism and for positive and negative pegs. As well, haemostats were rinsed with alcohol and dried between test peg removal (although no biofilm was expected at the peg/lid interface).

5.2.4. Controls in TBF

The inoculum was determined by removing a sample of the inoculum for quantitative viability counting by plating serial dilutions on the appropriate medium. Controls consisted of MBEC wells inoculated with medium only (negative inoculum control) to assess contamination and cross-contamination, and generally one test organism per plate was used to avoid cross-contamination. Preliminary studies used positive and negative control pegs per plate and were conducted to ensure strains of organisms used in the research study had the ability to form consistent biofilm in the MBEC system and that negative pegs remained negative throughout the course of study, i.e, a minimum of 30 days. To determine this, variability in eluted biofilm viability counts from random sample

pegs from various locations within a plate and between plates was compared.[57,98] To ensure the reliability of biofilm test results, each MBEC test plate contained positive and negative controls in addition to test pegs. For disinfectant challenge testing of biofilm, recoverable biofilm was determined by replacement of disinfectant treatments with sPBS to accurately take into account the fluid effect throughout the protocol where applicable (similar to carrier studies). There were a minimum of 9 replicates of all positive, negative, and test pegs for each test condition. In addition viability was assessed by a variety of assays (as described below).

For validation of the biofilm recovery method, sample positive and negative pegs were examined by the live/dead staining method after the rinsing to confirm the biofilm formation had not been disturbed and cross-contamination had not occurred (on negative pegs), and after bioburden removal from the peg to determine recoverability by the sonication process. Positive and negative pegs after recovery were also assessed for residual viability by the qualitative outgrowth method (in 10% FBS in TSB after incubation at 35 C for 10 days) as well as by undergoing a second elution with final viability determination by CFU counting.

5.2.5. Standardization of TBF

Biofilm growth curves for each of the test organisms grown in the traditional manner of continuous hydration over 30 days were established using the method described above. Preliminary studies using a live/dead stain (details in section 5.3.2) revealed these biofilm conditions would produce uniform formation covering 2/3 of the peg from the tip

upward. Culture conditions were established so that pegs were colonized with ~10^4 to 10^6 bacteria for all test organisms within 24 hours[98] and biofilm formation established within 48 hours for all test organisms.

5.3. Viablity assay methods

5.3.1. Viability assays

Quantitative assessment (viable counts) of recovered viable biofilm bacteria was achieved using serial 1:10 dilutions in sPBS in conjunction with the spread plate technique on TSA or BA, as outlined earlier (Section 1). Colony morphology was also examined as an indicator of culture purity, as was gram staining for verification at various times. Limit of detection for the viability assay was 10 CFU/peg.

Qualitative assessment of viability per peg was achieved by a direct outgrowth method, where pegs were aseptically removed from the MBEC system as described above and placed into sterile tubes containing 1 mL 10% FBS in TSB as described in Section 4.1.2, with and without an additional peg elution step in the closed system (i.e., mixing for 2 min, sonication for 5 min, and vortexing for 1 min) prior to incubation. Turbidity was the indicator for viability. Both positive and negative cultures were verified by spread plate methods. The limit of detection for qualitative outgrowth was 1 CFU/peg.

5.3.2. Live/dead viability stain

Pegs were aseptically broken from the lid and subjected to the live/dead staining method in the biosafety cabinet as described earlier. Microscopic examination allowed for the

assessment of biofilm formation on the pegs throughout the period of growth, as well as a semi-quantitative assessment of viability/peg (as described in Section 4.1.2). The limit of detection by this assay was 3- to 4 Log_{10}/peg for all test organisms.

5.3.3. Metabolic ATP bioluminescence assay

A bioluminescence assay for quantitative determination of ATP using firefly luciferase with the substrate D-luciferin was performed using an ATP determination kit (catalogue number A-22066, Molecular Probes, Eugene, OR, USA). Luminescence was detected by a standard luminometer and measured in relative light units (RLU). A luminometer that measured ATP from tubes (rather than a microtitre plate) was available and a 200 uL volume was used. A standard curve of ATP luminescence was prepared according to the manufacturer's directions. The ATP assay for bacterial and yeast test organisms was performed in two parts, (a) extraction of ATP with DMSO for 1 minute and (b) ATP reaction with luciferin/luciferase reaction solution for 1 minute. As a preliminary step to studying ATP levels in biofilm, bacterial suspensions of the test organisms (from 0.5 McFarland samples in sPBS with bacterial concentrations of approximately 10^8 CFU/mL) were serially diluted and tested at various concentrations. As per Gracia et al.,[51] DMSO (catalogue number D2650, Sigma-Aldrich, St. Louis, MO, U.S.A.) was used as the extractant of cellular ATP at a ratio of bacterial suspension/DMSO of 1/9 (v/v). The ATP in the experimental samples was determined from the standard curve. Verification of ATP extraction efficiency was based on placing a sample aliquot on the appropriate medium with no detection of viable cells remaining in the sample.[51] Three bioluminescence tests were required per trial: (1) the ATP standard; (2) the sample in the

absence of the luciferin-luciferase reagents (background light emission); and (3) light emission of the sample in the presence of the luciferin-luciferase reagents. For each test organism, the ATP level was related to CFU by comparison of duplicate test samples with overall linearity between approximately 4 Log_{10} to 8 Log_{10}, indicating the limit of detection of the ATP assay. All tests were done with 9 replicates in total.

The ATP assay was applied to assess biofilm formation of the test organisms from either the Loop or MBEC system according to Gracia et al.[51] However since a luminometer for detection of microplate cultures was not available, final luminescence determination was done in sterile 1 mL glass tubes, with 200uL total volume. Biofilm was prepared for the test organisms as described in Sections 5.1 and 5.2. Under sterile conditions, samples of either 1 cm Loop tubing or MBEC pegs were removed and rinsed in sPBS to eliminate non-adherent bacteria. For each system, biofilm bacteria were eluted as described in Section 5.1 and 5.2 and subjected to DMSO extraction as described above for bacteria in suspension. Since a microtitre luminometer reader was not available, this assay was not continued for BBF studies using the MBEC system, as in situ testing was not possible.

5.3.4. Metabolic redox assay

A modified tetrazolium salt reduction assay was used as an indicator of metabolic activity using chromogenic indicators, nitro blue tetrazolium (NBT) as an electron transfer agent and co-precipitate for 5-bromo-chloro-3-indoyl phosphate (BCIP) (catalogue numbers N-6547 B-6492 respectively, Molecular Probes, Eugene, OR, USA). The resulting dark

blue precipitate localized at the site of alkaline phosphatase was related to measurement of the cellular oxidative burst.

Stock solutions of NBT and BCIP were prepared as per the manufacturer's recommendations. NBT stock solution was prepared by mixing 20 mg NBT in 100mL of 5mM $MgCl_2$, 100 mM Tris, pH 9.7. BCIP was prepared by mixing 20 mg BCIP in 1 mL sterile filtered water. These stock solutions were stored at 4°C, protected from light for a maximum of 14 days. The redox assay was performed in two parts: (1) reaction with the test reagent and formation of an insoluble formazan precipitate; and (2) cell lysis with DMSO to release and solublize the blue precipitate for the terminal absorbance reading. On the day of testing, the redox reagent was prepared by bringing both solutions to room temperature and mixing NBT to BCIP at a ratio of 100:1 with the addition of 0.2% Tween 20 (catalogue number P2287, Sigma-Aldrich, St. Louis, MO, U.S.A.) and 1mM EDTA (J.T.Baker, Toronto, ON, Canada). The solution was filter sterilized through a 0.22 μm pore size filter. Cell lysis was achieved by addition of DMSO (as in ATP assay).

The protocol developed for the redox assay was adapted from the manufacturer's MTT Cell Proliferation Assay (catalogue number V-13154, Molecular Probes, Eugene, OR, USA) and involved adding 100 uL of the test reagent to the test organisms under sterile conditions, with incubation in the dark at 35°C for 18 hours. Thereafter an additional 100 uL of 1mM DMSO was added, and mixed for 10 minutes at 35°C. Resultant optical density readings of the colorometric change per reaction tube were done with a standard spectrophotometer set at 540 nm. Final absorbance readings were calculated by

subtraction of negative samples (media only, no inoculum) from test readings. Standard curves of metabolic activity for each test organism were produced from serial dilutions of test organisms suspended in medium, demonstrating a linear relationship between duplicate samples used to assess metabolic level determined by the redox reaction compared to viable colony forming units detected as previously described. Overall linearity was established in the range of approximately 3- to 4-Log_{10} to 9 Log_{10} for test organisms, indicating the limit of detection of the reduction assay. All tests were done with 9 replicates.

For biofilm studies, a microtitre protocol of the redox assay was performed under sterile conditions on biofilm formed on MBEC pegs for all test organisms as per the MBEC protocol outlined in Section 5.2. The redox assay was the only method of viability testing done in situ (on the MBEC pegs). To initiate the assay, the biofilm medium was removed and pegs were rinsed 3 times with sPBS to remove non-adherent organisms as previously described. The MBEC lid with colonized pegs was placed pegs-up to air dry for 5 minutes in the biosafety cabinet to dry excess PBS/peg and prevent dilution of test reagents. In this time wells of a new, sterile microtitre plate were filled with 200ul fresh medium to which 20 uL of NBT-BCIP working reagent was added and shaken gently, manually to mix the reagents. The colonized lid pegs were placed in the reagent microtitre plates, which were shaken for 2 minutes on the rocker table and incubated at 35°C for 18 hours. Thereafter 50 uL of 1mM DMSO was added to each well, the plate was manually shaken, the colonized lid pins replaced on the reagent plate, and incubated for 10 minutes at 35°C. The resultant absorbance was read in a standard microtitre plate

reader at 540 nm. Final metabolic activity was calculated by subtraction of background absorbance from the same test media in the absence of test organisms.

Standard tests involved control negative pegs in media (no inoculum, with sample pegs exposed to various treatments as defined by the experiments where applicable), defining the background reactivity; positive control pegs with biofilm (with sPBS replacing any disinfectant treatment where applicable); and test pegs colonized with biofilm and subjected to various treatments.

Confirmation of biofilm formation on sample positive and negative pegs was confirmed by live/dead staining as well as viability counts from eluted pegs, both methods as described earlier. Initial studies of the redox assay on biofilm formed in the MBEC system established correlation between the number of biofilm bacteria (and yeast) as determined by viability counts and the colorimetric change resulting in the redox assay directly reflecting metabolic activity.

5.4. Organic matrix analyses

5.4.1. Protein assay

Protein content was measured by the standard Bradford Protein microassay method with absorbance readings compared to a standard curve with bovine serum albumin diluted in sterile RO (reverse osmosis) water ranging from 0-40 ug/mL. The assay was performed in situ for biofilm formation on the MBEC pegs, measuring the organic matrix to which the test organisms had contributed.

5.4.2. Carbohydrate assay

Carbohydrate concentrations were determined by the phenol-sulfuric acid method[79] with test absorbance readings compared to a standard curve for glucose (dilutions in distilled water ranging from 0-800 ug/mL). Due to the nature of the reagents, the assay could not be done directly on the MBEC pegs, and biofilm was recovered (eluted from carrier or pegs) prior to measurement.

6. Buildup biofilm (BBF) model development

6.1. Cyclic BBF model approach

Biofilm formation was established by the MBEC system (as described in Section 5.2 for TBF) for 48 hours by each test organism, *E. faecalis, P. aeruginosa, M. chelonae*^{R} and *C. albicans*. However, to model the buildup of material in patient-used flexible endoscopes over time and measure survivability of test organisms within this buildup material, the biofilm formed was exposed to repetitive cycles of treatments representing stages in the reprocessing protocol. This modeling approach was designed to evaluate microbial survivability in BBF formed by treatments that were repetitive cycles of (1) drying, (2) drying and disinfectant exposure, (3) drying, disinfectant exposure and re-exposure to the test organism. Evaluation of survivability to each of these cyclical treatments provided the opportunity to assess the impact of individual stages as well as the cumulative effect of different stages of endoscope reprocessing on microbial survival. Furthermore, each cyclical treatment could be related to possible bioburden conditions within reprocessed flexible endoscopes.

6.1.1. Treatment cycle times

Treatment cycles of 2- and 3- days were compared to mimic overnight and weekend reprocessing conditions respectively. For 2-day cycle protocols, inoculated pegs were exposed to nutrient media (feed) or treatment on alternating days. In the 3-day cycle protocol inoculated pegs were fed for two days with fresh nutrient medium and exposed to treatment on the third day. All treatment cycles were compared to TBF formed in continuous hydrated conditions throughout the test period. All test sampling was done immediately pre- and post- treatment cycle on triplicate sample pegs. Generally for all methods described, the 2-day protocol will be referenced for simplicity (since the 3-day protocol was similar in methodology and results).

6.1.2. Treatment parameters

The MBEC pegs were consistently inoculated (seeded) in the ATS. Nutrient media used for feeding the biofilm growth was ATS (representing a high organic matrix), sterile tap water (representing a low organic matrix, as found in scope rinsing), or an enzymatic detergent (representing a moderate organic matrix, as found in scope cleaning).

For all treatments, drying was done ON (12 to 18 hours) at RT in a biosafety cabinet. Disinfectant challenge was: HLD with two commonly used disinfectants with different chemical bases, GLUT and AHP (for 20 minute exposure times at RT as per manufacturer's recommendations); and LLD with AHP (for 5minute exposure time at RT as per manufacturer's recommendations) to examine the effects of dilution and suboptimal concentration of disinfectants on survivability within various biofilm

formations. Following disinfectant exposure, pegs were neutralized using 10% FBS in TSB (for 10 minutes for HLD, and 5 minutes for LLD) to eliminate the possibility of disinfectant carryover to the recovery medium (as detailed in Section 2).

6.1.3. BBF controls

As for TBF, MBEC plates for BBF were generally inoculated with a single test organism to avoid cross-contamination. As well, each plate contained negative inoculum control pegs with exposure to test medium only. For all the BBF cycles, concurrent TBF growth curves for each test organism were prepared as described in Section 5.2. Recoverable TBF bioburden was used as a positive control defining the numbers of CFU per peg as biofilm development progressed over time, without interruption. Any change in growth detected by quantitative viability counts and qualitative outgrowth (and live/dead staining) outside the standard deviation in TBF from the standard growth curves established in preliminary studies resulted in discarding all plates inoculated for that trial. The TBF control was formed in MBEC plates at the same time, with the same inoculum samples, supplied with the same nutrient media lot, and with sample pegs removed and challenged with the same disinfectant lot and working solution at the same specified test times and conditions as for BBF. All test results were from a minimum of 9 replicates resulting from a minimum of triplicate replications from 3 separate trials.

6.2. Assay methods

Survivability of test organisms was measured by quantitative and qualitative viability assays described in Section 5.3.1 and by the metabolic reduction assay as per Section

5.3.4. To microscopically examine biofilm formation on pegs, pegs (on the lid or aseptically removed as per Section 5.2 and placed in microcentrifuge tubes) were rinsed with sPBS, air dried for 5 minutes, and stained with the live/dead stain (as per Section 4.1.2). If pegs were removed from the lid, then it was necessary to examine pegs after placement in a sterile petri dish with pegs placed horizontally with minimal disruption to the biofilm or in microtitre plate wells with pegs placed vertically tip upward.

6.3 Cyclic BBF model – cyclical drying

The drying phase represented a worst-case scenario, e.g., where the device was left without attention overnight prior to reprocessing. It is also indicative of the storage phase. The formation of BBF from repetitive exposure to episodes of drying alone could mimic discreet locations or blind spots in the narrow tubing of flexible endoscopes capable of harbouring bioburden and evading disinfectant challenge in spite of continual reprocessing procedures. Sample pegs removed at the end of each cycle of drying and exposed to disinfectant challenge defined the survivability within such BBF over time.

6.3.1. Overall BBF cyclic drying protocol

Initial investigation of microbial survival in BBF focused on BBF resulting from repetitive exposure of drying treatment alone on all test pegs over a 30-day period. Sample pegs were evaluated for viability pre-treatment (drying), post-treatment (drying), and post-treatment following exposure to disinfectant challenge. Concurrent to each BBF trial, TBF was formed on separate MBEC plates, with sample pegs removed and challenged with disinfectant at the same time as BBF pegs. All BBF and TBF plates

required in a trial were equally inoculated at the same time with biofilm formation continued over the same time period. BBF treatment (in this case, drying) occurred at every specified cycle time. Therefore although some pegs and plates were depleted as the trial continued (since pegs were removed for sampling) per cycle, those remaining received a continued, cumulative effect of the treatment.

6.3.2. Initiation of BBF

The initiation of biofilm formation for BBF was the same as for TBF over the initial 48 hours of development (described in Section 5.2.1 and 5.2.2). To monitor the possibility of contamination or cross-contamination due to the cycling procedure, negative pegs (exposed to medium only) were located throughout the plate in sufficient numbers (resulting in a minimum of 3 negative pegs per test cycle).

6.3.3. Cycling treatment (drying)

After biofilm formation was established (Day 2), BBF differed by TBF by cyclic exposure of the biofilm formation to episodes of drying between times of feeding. Therefore, in contrast to TBF, rather than directly refeeding biofilm every 24 hours, starting on Day 2 (the initiation of BBF cycle 1) and on alternate days thereafter (2-day cycle) for 30 days (15 cycles), BBF pegs were dried at RT/ON in the biosafety cabinet. This was accomplished by removing the MBEC lid with pegs, rinsing the pegs in sPBS (as described in Section 5.2.2) and placing the lid inverted in the biosafety cabinet, pegs upward, to dry ON. Four sets of sample pegs were removed with a sterile haemostat (as described in Section 5.2.3): One set (3 replicate pegs) underwent biofilm recovery

immediately after drying. A second set of triplicate pegs were exposed to high-level disinfectant challenge of glutaraldehyde by removing pegs aseptically with a sterile haemostat and placement into a microcentrifuge containing 500 uL of disinfectant. Following exposure, pegs were aseptically transferred by haemostat to a new microcentrifuge tube containing 500 uL sPBS and rinsed for 10 seconds, similarly transferred to new microcentrifuge tube containing 500 uL neutralizer (10% FBS in TSB) for 10 minutes to eliminate carryover of disinfectant into the recovery medium and finally transferred to 500 uL sPBS in a microcentrifuge tube for recovery (as described earlier, Section 5.2.3.). Third and fourth sets of triplicate pegs were similarly challenged with high-level accelerated hydrogen peroxide and low-level accelerated hydrogen peroxide respectively. The process for BBF formed by cyclic drying and control TBF is summarized in Table 5 (a,b).

When larger numbers of similarly treated pegs were tested, rinsing, disinfectant challenge and neutralization could be done in situ on the pegs by placing the lid pegs into a new microtitre plate with corresponding wells containing 200 uL per well of reagents (or medium only for wells corresponding to pegs not exposed to challenge).

After sample pegs were removed for testing, the remaining MBEC peg lids in the trial were each transferred to a new, sterile microtitre plate with 200uL fresh medium/well. The MBEC systems were replaced on the rocker table for biofilm formation (as previously described) for 24 hours at RT.

Thereafter, the MBEC system was returned to the biosafety cabinet, the positively and negatively inoculated pegs were again sampled pre-treatment (drying), and all remaining pegs in the trial were again dried ON/RT (as previously described), marking the initiation of BBF cycle 2 on Day 4. Again, after drying sample pegs were removed and exposed to disinfectant challenge (as previously described). All sample pegs were assessed for viability. Numbers of samples were sufficient for triplicate samples for each assay (quantitative counting, qualitative outgrowth and metabolic activity (done in situ on a separate plate) for each test period, as well as live/dead staining at various time points).

6.4 Cyclic BBF model – cyclical drying and disinfectant challenge

BBF formed from repetitive exposure of biofilm to drying and disinfectant challenge could represent persistent bioburden within the tubing of flexible endoscopes that is repeatedly exposed to drying and disinfectant challenge only during continual cycles of reprocessing. Removal of sample pegs throughout this BBF cycling process defined the survivability of the test organisms under the most challenging BBF conditions over time.

The same test organisms, biofilm initiation, and cycling time over 30 days were incorporated as described for the cyclic drying experiment (Section 6.3, 6.3.1, 6.3.2) with seeding in ATS, and growth medium of ATS compared to sTAP. However all pegs were exposed to repetitive cycles of drying and disinfectant challenge, with sample pegs removed and evaluated before and immediately after each dry/disinfectant cycle. In contrast to the protocol for cyclic drying alone, after MBEC pegs were dried in the biosafety cabinet, they were directly transferred to a new microtitre plate designated for

disinfectant challenge with GLUT/HLD, AHP/HLD, or AHP/LLD, followed by rinsing and neutralization, as described previously (Sections 6.1.2-6.3.3). Cycles consisted of alternating days of treatment (drying/disinfectant challenge) and exposure to growth medium. The process for BBF formed by repetitive cycles of drying and disinfectant challenge is summarized in Table 5 (c).

6.5 Cyclic BBF model – cyclical drying, disinfectant challenge and organism re-exposure

BBF formed from repetitive exposure of biofilm to drying, disinfectant challenge followed by re-exposure to test organism in ATS (reseeding) on all MBEC pegs was designed to mimic internal locations in the tubing of flexible endoscopes where bioburden was not removed, biofilm had formed, but was exposed to drying and disinfection as per each reprocessing cycle. To more closely resemble environments in the reprocessing scheme, biofilm growth was in enzymatic detergent (as used in the cleaning process) compared to sTAP (similar to rinsing stages). As well repetitive re-exposure to bioburden (as would be encountered for each new endoscopic procedure) finalized the cycle. The modeling approach reflected the progressive effect of reprocessing stages, representing the entire reprocessing scheme.

The same test organisms, biofilm initiation, and cycling time over 30 days were used as described for the cyclic drying/disinfectant experiment with seeding in ATS (Section 6.4), but growth medium consisted of enzymatic detergent (rather than ATS) compared to sTAP. Following disinfectant challenge and neutralization, triplicate sample pegs were removed and recovered as previously described. Thereafter all other pegs were

immediately transferred to a new microtitre plate containing 150 uL of the test inoculum in ATS (as for the initial biofilm inoculation) and the MBEC system was placed on the rocker table for biofilm growth (as per Section 6.3.2). After 24 hours, the reseeded pegs were rinsed and transferred to fresh medium, e.g, enzymatic detergent (200 uL/well in a new microtitre plate). The MBEC system was replaced on the rocker table for biofilm growth for 24 hours. Thereafter a new cycle began. Cycles consisted of alternating days of treatment (all pegs dried/disinfectant challenged/reseeded) and growth in enzymatic detergent media. The process for BBF formed by repetitive cycles of complete treatment (drying, disinfectant challenge, and bioburden re-exposure (re-seeding) is summarized in Table 5 (d).

7. Outgrowth testing

7.1 Introduction

Viability assays used previous to this time were unable to detect survival of low levels of organisms, especially early in BBF (of cyclic drying and disinfect challenge). To better understand BBF (and TBF) results, it was necessary to devise a method to recover surviving organisms in challenged biofilm. Proof of such existence throughout the cycling treatments would be further evidence for survival of microorganisms within BBF as opposed to possible sporadic contamination. A qualitative indirect outgrowth testing method was developed to aid in revival and recovery of organisms in BBF to answer the question: If low levels of organisms existed in BBF, could they be detected? Two test organisms, *E. faecalis* and *P. aeruginosa*, were included in this study.

Table 5. Summarization of Buildup biofilm (BBF) protocols

(a) Traditional biofilm

Step	Protocol
1	**Seed** (inoculum in ATS or ATS alone) all pegs for 24 h (positive + negative inoculum/plate) → Remove inoculum, rinse sPBS
2	**Feed** (fresh media: ATS or sTAP) all pegs for 24 h
3	After 48h, remove/rinse/recover 3 replicate positive + negative samples (positive BFF growth control + negative contamination control)
4	**Re-feed (fresh media, ATS or sTAP) all pegs every 24 h**
	Test schedule for sample pegs removed from MBEC plate: On alternate days starting at Day 2 to Day 30 (15 test times): Sample 3 replicate positive + negative pegs for BFF Challenge 3 replicate positive+negative pegs with Dry/HLD-GLUT Challenge 3 replicate positive+negative pegs with Dry/HLD-AHP Challenge 3 replicate positive+negative pegs with Dry/LLD-AHP (continual effect of hydration, single exposure to Dry / HLD or LLD

(b) Buildup biofilm: BBF formed by cyclic treatment of drying

Step	Protocol
1	**Seed** (inoculum in ATS or ATS alone) all pegs for 24 h (positive + negative inoculum/plate) → Remove inoculum, rinse sPBS
2	**Feed** (fresh media, ATS or sTAP) all pegs for 24 h
3	After 48h: (Pre-treatment samples) a) remove/rinse/recover 3 replicate positive + negative samples (positive BFF growth control + negative contamination control)
4	**Cycling begins: At 48h dry all pegs** (after pre-drying samples removed)
5	**On alternate days expose pegs to either (1) feeding (ATS or sTAP); or (2) drying, of all pegs remaining in trial**
	Test schedule on sample pegs removed from MBEC plate: On alternate days starting at Day 2 to Day 30 (15 test times or test cycles), After drying: Sample 3 replicate positive+negative pegs for BFF (effect of drying only) Challenge 3 replicate positive+negative pegs with HLD-GLUT (cumulative effect of drying, single exposure to HLD GLUT) Challenge 3 replicate positive+negative pegs with HLD-AHP (cumulative effect of drying, single exposure to HLD AHP) Challenge 3 replicate positive+negative pegs with LLD-AHP (cumulative effect of drying, single exposure to LLD-AHP)

(c) <u>BBF formed by cyclic treatment of drying and disinfectant challenge</u>

Step	Protocol
1	**Seed** (inoculum in ATS or ATS alone) all pegs for 24 h (positive + negative inoculum/plate) → Remove inoculum, rinse sPBS
2	**Feed** (fresh media: ATS or sTAP) all pegs for 24 h
3	After 48h: (Pre-treatment samples) a) remove/rinse/recover 3 replicate positive + negative samples (positive BFF growth control + negative contamination control)
4	**Cycling begins: At 48h dry all pegs** (after pre-drying samples removed) followed by disinfectant challenge of all pegs
5	**On alternate days expose pegs to either (1) feeding (ATS or sTAP); or (2) drying/disinfectant challenge of all pegs remaining in trial**
	<u>Test schedule on sample pegs removed from MBEC plate</u>: On alternate days starting at Day 2 to Day 30 (15 test times or test cycles), Sample 3 replicate positive+negative pegs for BFF (pre-cycle sample) After drying/disinfectant challenge per cycle: Test sample of 3 replicate positive+negative pegs with HLD-GLUT (cumulative effect of drying/HLD GLUT) Test sample of 3 replicate positive+negative pegs with HLD-AHP (cumulative effect of drying/HLD AHP) Test sample of 3 replicate positive+negative pegs with LLD-AHP (cumulative effect of drying/LLD-AHP)

(d) <u>BBF formed by the complete cyclic treatment: drying, disinfectant challenge, and bioburden re-exposure (re-seeding)</u>

Step	Protocol
1	**Seed** (inoculum in ATS or ATS alone) all pegs for 24 h (positive + negative inoculum/plate) → Remove inoculum, rinse sPBS
2	**Feed** (fresh media, enzymatic detergent or sTAP) all pegs for 24 h
3	After 48h: (Pre-treatment cycles) a) remove/rinse/recover 3 replicate positive + negative samples (positive BFF growth control + negative contamination control)
4	**Cycling begins: At 48h dry all pegs** (after pre-drying samples removed) followed by disinfectant challenge of all pins and re-seeding)
5	**On alternate days expose pegs to either: (1) Feeding (enzymatic detergent or sTAP); or (2) drying/disinfectant challenge /sample pegs removed/ reseeding* of all pegs remaining in trial**
	<u>Test schedule on sample pegs removed from MBEC plate</u>: On alternate days starting at Day 2 to Day 30 (15 test times or test cycles), Sample 3 replicate positive+negative pegs for BFF (pre-cycle sample) After drying/disinfectant challenge per cycle: Test sample of 3 replicate positive+negative pegs with HLD-GLUT (cumulative effect of drying/HLD GLUT/reseeding) Test sample of 3 replicate positive+negative pegs with HLD-AHP (cumulative effect of drying/HLD AHP/reseeding) Test sample of 3 replicate positive+negative pegs with LLD-AHP (cumulative effect of drying/LLD-AHP/reseeding)
	*RESEED (test organism in ATS or ATS alone (negative inoculum)) (cumulative effect of re-exposure to bioburden)

7.2. Assay methods

7.2.1. Indirect outgrowth analysis method

The test protocol involved 30 days of biofilm formation either as TBF compared to BBF formed by the greatest HLD challenge, i.e., cyclic drying and HLD, as well as by the complete BBF protocol (as described in Sections 5.2.1-5.2.4, 6.4 and 6.5 respectively). Organisms were tested with and without disinfectant challenge of HLD with GLUT and AHP. Initial biofilm inoculation and formation on MBEC pegs was as per the standard protocol. Following HLD (neutralization and rinsing, as per Section 6.4), sample pegs were sterilely placed in 10%FBS in TBS in sterile tubes that remained unopened at 35°C for 5 days (similar to the qualitative direct outgrowth test). These tubes were subjected to the standard elution protocol (shaking for 2 minutes, sonication for 5 minutes, vortexing for 1 minute) however the tubes remained closed. The tubes with pegs were re-incubated for 25 days at 35°C. Turbidity indicated positive growth and organisms were further blind-subbed on TSA regardless of turbidity for verification of positive or negative identification.

Concurrent to removing test pegs for the indirect qualitative outgrowth test, replicate sample pegs were removed and directly examined for viability by the quantitative viability counting method and direct qualitative outgrowth test as described in Section 5.3.1 and compared with the indirect outgrowth test. All testing was done on triplicate pegs in three separate trials (9 replicates in total). Controls were as stated in Section 6.1.3.

8. Viral survival in BBF model

A study was conducted to address the question whether a virus could survive and be recovered from biofilm formation in reprocessed endoscopes, as viral transmission has resulted from endoscopy.[17,90,95] Biofilm formation in the MBEC system followed the standard procedures previously described. Biofilm formation of *E. faecalis*, *P. aeruginosa* and *C. albicans* were studied. BBF from cyclic drying/HLD (as per Section 6.4), as well as BBF from cyclic drying/HLD/reseeding (as per Section 6.5) were compared with a positive biofilm control (i.e, TBF, as per Sections 5.2.1 –5.2.4). Once biofilm formation was established, *Reovirus* was introduced into the initial feeding medium. Viral recovery from biofilm was achieved by elution of the biofilm from the MBEC pegs (as for all MBEC studies), with further microfiltration for separation of the bacterial and viral components.

8.1. Introduction of *Reovirus*

Biofilm formation was initiated for each microorganism (as per the standard protocol). After 24 hour biofilm initiation (pre-cycling for BBF), each MBEC peg was rinsed in sPBS and fresh wells refilled with 200 uL of virus (as a 1/20 dilution of stock virus in ATS, with an expected titre of ~ 5 x 10^7 CPE/mL). Control wells on the MBEC plate received nutrient medium only (no virus) and were used to monitor biofilm development (BBF and TBF, as previously described) over time. BBF was formed by cyclic drying/HLD or cyclic drying/HLD/reseeding and incorporated either glutaraldehyde or accelerated hydrogen peroxide disinfectants. Reseeding in BBF included re-inoculation with *Reovirus* (similar to the initial viral inoculation). BBFs were formed simultaneously

with TBF over a 20-day period. The titre of the viral inoculum was confirmed in a semi-quantitative CPE assay (similar to *Sindbis virus* detection assay as per Section 4.1.1) using half-log dilutions of virus samples prepared in media and inoculated on confluent monolayers of the L929 cells.

8.2 Recovery and enumeration of *Reovirus*

At various cycle points pegs were removed, rinsed, neutralized and extracted (as previously described). Test points occurred on Day 2 pre-cycling (to establish that the virus was in the biofilm as opposed to the medium alone) and post-cycle (post dry/HLD, but pre-reseeding where applicable) on Days 3, 6, 10 and 20. Extracts from triplicate pegs were aseptically combined. Viral recovery was achieved by microfiltration through a 0.2 um membrane separating bacteria (on the membrane) from virus (in the filtrate). For every millilitre of extraction material passed through the filter, an equal amount of sPBS was passed to rinse the filter and free any trapped virus. All samples before and after filtration were kept on ice until viral and bacterial recovery assays were performed. The filtrate was evaluated for viral recoverability and survival by the CPE assay (similar to *Sindbis virus* detection assay as per Section 4.1.1). However, filtrate samples were tested without dilution and CPE results were noted as either positive or negative cytopathic effect. All test microtitre plates for the CPE assay included wells inoculated with cell maintenance medium only (cell viability control); extracted and filtered TBF and BBF without viral spike (control for cell toxicity); virus diluted 1/20 in ATS (positive virus control); extracted and filtered BBF with virus (test biofilm sample); extracted and filtered TBF with virus (positive biofilm control). In addition, the filtrate was tested for

bacterial contamination by the spread plate technique on BA plates. All test conditions were repeated over 3 trials with triplicate samples per trial.

9. Statistical Analysis

For biofilm formation on MBEC pegs, the following statistical analyses were performed as suggested by Harrison et al.[57] including determination of mean, standard deviation and one-way analysis of variance (ANOVA) comparing the mean viable cell counts of pooled rows within and between plates. These analyses were used to verify that the described method for biofolm inoculation and formation of each test organism produced equivalent biofilm growth on different rows within and between the MBEC plates over the test period of 30 days. For comparison of TBF and BBF and the resultant bioburden following HLD challenge, the Student's t-test was used.

RESULTS

Biofilm formation and disinfection efficacy for complex medical devices was modeled to examine the possibility of microbial survival under a variety of plausible conditions. The initial studies used a surface carrier methodology to examine survival of a range of microorganisms (bacteria, mycobacteria, fungi, enveloped and nonenveloped viruses) when dried onto surfaces and the impact of an organic matrix and suboptimal disinfectant exposure. Subsequent studies examined the same parameters when microorganisms were in a biofilm formed using either the Biofilm loop or the MBEC peg model. Resulting data using the MBEC system illustrated similarities and differences in survivability in traditional biofilm and buildup biofilm resulting from repetitive cycles of exposure to conditions mimicking endoscopic reprocessing stages.

1. Survival of microorganisms that were dried onto surfaces

There are no published direct comparisons of survival of viruses to bacteria, yeast and mycobacteria when dried in the presence of the same organic matrix. These studies examined the survival of microorganisms dried onto surfaces to provide an insight into the impact on nosocomial infection transmission under these conditions. A quantitative carrier test facilitated assessment of such survival characteristics.

Comparative survival of microorganisms dried in an organic medium (ATS) or in the absence of an organic medium (PBS) is shown in Figure 6: Nonenveloped virus versus enveloped virus is shown in Figure 6(a); Bacteria and yeast are shown in Figure 6(b); and

M. chelonae glutaradehyde resistant and sensitive strains are shown in Figure 6(c). A positive control of each virus held at –20° C resulted in no change in titre over 30 days (data not shown). Negative controls were run with no detectable microorganisms over the test period. Generally, organism survival in an organic mileux was ~1 Log_{10} higher than survival in PBS for all organisms. Overall, organic content had the greatest impact on survival for *Sindbis virus* (~ 3 Log_{10} difference in overnight drying alone) and the least impact on mycobacteria, particularly the glutaraldehyde sensitive strain.

Data from live/dead staining and qualitative outgrowth testing (Table 6) supported quantitative testing showing the viability of bacteria and yeast dried on surface carriers for 30 days in an organic medium. An exception was noted for *M. chelonae*^{R}, as no growth was detected by quantitative assay; however, survival of viable organisms on Day 30 was detected by live/dead staining and qualitative outgrowth techniques (suggesting prolonged viability of some organisms but little to no replication). Data from live/dead staining supported the impact of organic material on survival in the dried state. Drying in PBS resulted in greater loss of viability early in the test period and ultimately lower numbers of survivors reaching the limit of detection sooner in PBS than when dried in ATS. *P. aeruginosa* was an exception due to the profound effect of drying on organism survival noted immediately after drying overnight.

Figure 6. Impact of organic material on survival

(a) Viruses

Reovirus suspended either in an organic medium (ATS) (-●-) or in the absence of an organic medium (PBS) (--○--) and SVHR suspended in an organic medium (ATS) (-■-) or in the absence of an organic medium (PBS) (-□-), were dried onto the surface carrier and evaluated over 30 days at RT for survival as described in Methods. Day –1 represents recoverable bioburden from seeded carriers prior to drying, Day 0 represents viable bioburden after drying ON and marks the initiation of the 30-day viability-testing period following desiccation. *Reovirus* was quantified by plaque forming units (PFU) and SVHR as per cytopathic effects assay (CPE, infective units) as described in Materials and Methods. Results are presented as a mean and standard deviation from 9 replicates.

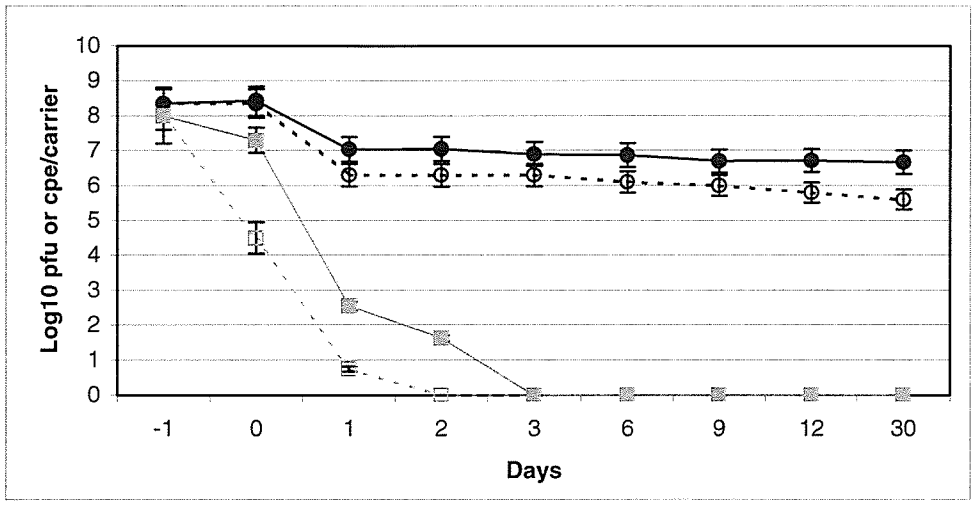
(b) Vegetative bacteria and yeast

Organisms, *E. faecalis* suspended in ATS (-•-) or PBS (--○--); *P. aeruginosa* suspended in ATS ( )and PBS ( ); and *C. albicans* suspended in ATS (-▲-) or PBS (--△--), dried onto the surface carrier, and evaluated over 30 days at RT for survival as described in Methods. Day –1 represents recoverable bioburden from seeded carriers prior to drying, Day 0 represents viable bioburden after drying ON and marks the initiation of the 30-day viability-testing period following desiccation. Results are presented as a mean and standard deviation from 9 replicates.

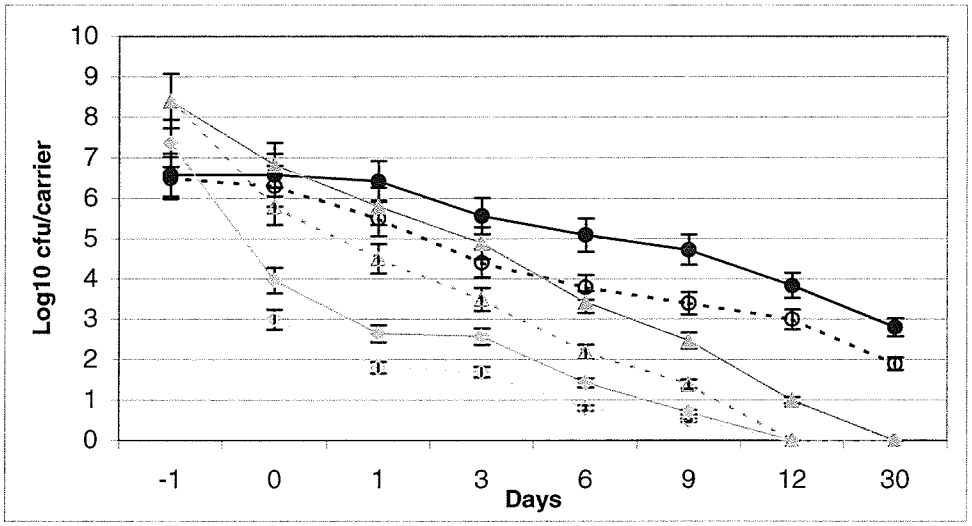
(c) Mycobacteria

M. chelonae^{S} suspended in ATS (-■–) or PBS (--□--) and *M. chelonae*^{R} suspended in ATS(-◆-) or PBS (--◊--) were dried onto the surface carrier, and evaluated over 30 days at RT for survival as described in Methods. Day –1 represents recoverable bioburden from seeded carriers prior to drying, Day 0 represents viable bioburden after drying ON and marks the initiation of the 30-day viability-testing period following desiccation. Results are presented as a mean and standard deviation from 9 replicates.

(a) Viruses



(b) Vegetative bacteria and yeast



(c) Mycobacteria

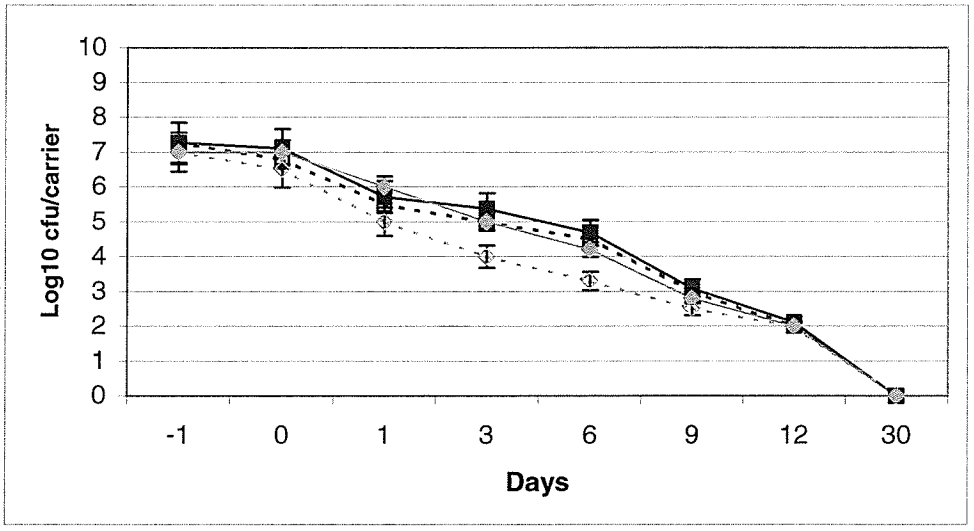


Table 6. Viability of bacteria and yeast dried on surface carriers for 30 days in an organic medium: live/dead staining and qualitative outgrowth compared to quantitative viability counts (average 9 tests +/- SD)

Microorganism: Day of testing	Quantitative viability[1] Log_{10} cfu/carrier (± SD)	TOPI viability stain[2] cfu/carrier (%live)	Qualitative outgrowth[3] # Positive/#Tested
***E. faecalis*:**			
Day-1[4]	$3.72 \times 10^6 \pm 1.9 \times 10^5$	3.65×10^6 (98%)	9/9
Day 0[5]	$3.16 \times 10^6 \pm 2.8 \times 10^5$	2.79×10^6 (75%)	9/9
Day 1[6]	$2.57 \times 10^6 \pm 3.3 \times 10^5$	1.12×10^6 (30%)	9/9
Day 3	$3.63 \times 10^5 \pm 4.6 \times 10^4$	1.86×10^5 (5%)	9/9
Day 6	$1.23 \times 10^5 \pm 2.0 \times 10^4$	7.44×10^4 (2%)	9/9
Day 9	$5.25 \times 10^4 \pm 5.5 \times 10^3$	5.58×10^4 (1.5%)	9/9
Day 12	$6.92 \times 10^3 \pm 9.4 \times 10^2$	4.46×10^4 (1.2%)	9/9
Day 30	$6.46 \times 10^2 \pm 7.9 \times 10^1$	2.98×10^4 (0.8%)	9/9
***P. aeruginosa*:**			
Day -1	$2.30 \times 10^7 \pm 3.5 \times 10^6$	1.86×10^7 (90%)	9/9
Day 0	$9.30 \times 10^4 \pm 1.1 \times 10^4$	1.15×10^5 (0.5%)	9/9
Day 1	$4.47 \times 10^2 \pm 9.5 \times 10^1$	<LD	9/9
Day 3	$3.80 \times 10^2 \pm 6.6 \times 10^1$	<LD	9/9
Day 6	$2.70 \times 10^1 \pm 4.8 \times 10^1$	<LD	7/9
Day 9	$5.0 \times 10^0 \pm 4 \times 10^0$	<LD	5/9
Day 12	<LD	<LD	0/9
Day 30	<LD	<LD	0/9
***C. albicans*:**			
Day -1	$2.57 \times 10^8 \pm 1.8 \times 10^7$	2.44×10^8 (95%)	9/9
Day 0	$6.61 \times 10^6 \pm 4.5 \times 10^5$	2.06×10^6 (0.8%)	9/9
Day 1	$6.31 \times 10^5 \pm 8.3 \times 10^4$	7.71×10^5 (0.3%)	9/9
Day 3	$7.76 \times 10^5 \pm 8.0 \times 10^4$	5.14×10^5 (0.2%)	9/9
Day 6	$2.69 \times 10^3 \pm 5.1 \times 10^2$	<LD	8/9
Day 9	$3.02 \times 10^2 \pm 8.4 \times 10^1$	<LD	5/9
Day 12	$2.69 \times 10^1 \pm 6.8 \times 10^1$	<LD	3/9
Day 30	<LD	<LD	0/9
***M. chelonae*[S]:**			
Day -1	$1.86 \times 10^7 \pm 1.8 \times 10^6$	1.49×10^7 (80%)	9/9
Day 0	$1.26 \times 10^7 \pm 3.6 \times 10^6$	9.30×10^6 (50%)	9/9
Day 1	$5.13 \times 10^5 \pm 4.2 \times 10^4$	5.58×10^6 (30%)	9/9
Day 3	$2.40 \times 10^5 \pm 7.0 \times 10^4$	9.30×10^5 (5%)	9/9
Day 6	$4.79 \times 10^4 \pm 7.5 \times 10^3$	9.30×10^4 (0.5%)	9/9
Day 9	$1.23 \times 10^3 \pm 9.6 \times 10^1$	1.86×10^4 (0.1%)	4/9
Day 12	$1.29 \times 10^2 \pm 9.9 \times 10^1$	3.72×10^4 (0.2%)	4/9
Day 30	<LD	<LD	0/9
***M. chelonae*[R]:**			
Day -1	$1.58 \times 10^7 \pm 2.9 \times 10^6$	1.42×10^7 (90%)	9/9

Day 0	$3.65 \times 10^6 \pm 4.8 \times 10^5$	9.48×10^6 (60%)	9/9
Day 1	$3.55 \times 10^6 \pm 7.2 \times 10^5$	3.16×10^6 (20%)	9/9
Day 3	$5.80 \times 10^5 \pm 2.4 \times 10^4$	1.58×10^5 (1%)	9/9
Day 6	$4.42 \times 10^4 \pm 6.8 \times 10^3$	1.58×10^4 (0.1%)	9/9
Day 9	$1.10 \times 10^4 \pm 3.5 \times 10^3$	1.58×10^4 (0.1%)	6/9
Day 12	$7.55 \times 10^3 \pm 8.5 \times 10^2$	3.16×10^4 (0.2%)	5/9
Day 30	<LD	1.58×10^4 (0.1%)	2/9

[1] Limit of detection (LD) for quantitative elution is 10 CFU/carrier
[2] Limit of detection (LD) for viability determination by TOPI is ~5×10^3 CFU/carrier (actual calculation is 0.1% of inoculum)
TOPI viability calculated by: % viability (determined by TOPI) x inoculum/carrier
SD for TOPI data $\leq$ 10%
[3] Limit of detection (LD) for qualitative outgrowth is 1 CFU/carrier
[4] Maximum recoverable bioburden
[5] Dried ON
[6] Survival in dried state after drying ON
All negative controls (carrier alone or carrier with soil only) showed no growth (<LD) for all test methods

2. Microbial killing efficacy of disinfectants

To accurately evaluate the efficacy of different disinfectants studied it was imperative to arrest the disinfectant's microbicidal activity at the end of the specified contact time.[117,124] An effective neutralizer must inhibit biocidal action and must not be toxic itself (or in combination with any agent) to the challenge organisms.[113,133] Sutton et al.[133] outlined the steps to validation of microbial recovery from disinfectants, which included neutralization efficacy and toxicity for a variety of agents (e.g., glycine, thiosulphate). Furthermore, studies have shown that addition of a neutralizer and /or dilution of the microorganism-microbicide mixture are effective, especially for viral studies.[117,124] In this study, neutralization was achieved by physical removal of the disinfectant by aspiration and dilution of any residual disinfectant through serial rinses in PBS and final exposure to a chemical neutralizer. Various chemical neutralizers (e.g., fetal bovine serum and glycine) used in this application have been successfully used in similar studies.[117] By comparing the surviving viable organisms following disinfectant exposure alone or with disinfectant diluted in neutralizing agent, the neutralization step was seen to be effective in inhibiting biocidal activity in the test protocol. The efficacy of neutralization is further supported by the fact that a 1:100 dilution of disinfectant in neutralizer was tested for confirmation. Results showed that neutralization neither inhibited nor enhanced viability for any of the microorganisms tested.

2.1. Confirmation of neutralization efficacy

As shown in Table 7, the neutralization protocol effectively inactivated GLUT and AHP facilitating quantitation of all microorganisms tested. Viability results were comparable

in the Positive control (PBS buffer solution, representing the maximum recoverable bioburden) to the survivors following neutralization of GLUT or AHP. As well, exposure to the neutralizing solution (10% FBS) in the absence of any HLD demonstrated an absence of toxic or enhancing effects of the neutralizing agent. When test carriers were exposed to the HLD without neutralization, no survivors were detected above the limit of detection, except in the case of glutaraldehyde exposure to *M.chelonae*^{R}, as expected. Results represent the average of 9 replicates (± SD) and the limit of detection (LD) was 10 CFU, PFU or CPE (infective units) per carrier for GLUT and AHP disinfection. Similar results were seen with SHP (data not shown).

Neutralization protocols using 10% FBS in TSB with 3% glycine following glutaraldehyde exposure and 0.1% sodium thiosulfate following hydrogen peroxide exposure were compared to 10% FBS in TSB alone (data not shown). All protocols showed effective neutralization, but because 10% FBS in TSB could be used for both GLUT and AHP, this method was used for all subsequent disinfectant exposure studies.

2.2. Toxicity and Interference with viability assays in viral studies

The neutralization agent and recovery media did not cause any visible cytotoxic effects to either cell line used for viral viability assays nor any notable interference in those assays.

Table 7. Validation of Neutralization method used for Glutaraldehyde (GLUT) and Accelerated Hydrogen Peroxide (AHP)

	Viable count after exposure to: (results represent average CFU (PFU or CPE*)/carrier) (9 replicates ± SD)			
Organism	PBS buffer solution[1]	Neutralizer solution[2]	Neutralizer solution plus GLUT[3]	Neutralizer solution plus AHP[4]
E. faecalis	$7.9 \times 10^6 \pm 2.5 \times 10^6$	$7.4 \times 10^6 \pm 1.1 \times 10^6$	$6.4 \times 10^6 \pm 2.3 \times 10^6$	$7.4 \times 10^6 \pm 1.6 \times 10^6$
P. aeruginosa	$7.6 \times 10^5 \pm 1.5 \times 10^5$	$6.0 \times 10^5 \pm 3.0 \times 10^5$	$4.6 \times 10^5 \pm 1.2 \times 10^5$	$4.4 \times 10^5 \pm 1.5 \times 10^5$
C. albicans	$3.9 \times 10^6 \pm 5.5 \times 10^5$	$4.5 \times 10^6 \pm 8.8 \times 10^5$	$5.0 \times 10^6 \pm 3.1 \times 10^6$	$3.9 \times 10^6 \pm 1.6 \times 10^6$
M. chelonae^S	$5.6 \times 10^6 \pm 5.3 \times 10^5$	$6.0 \times 10^6 \pm 8.6 \times 10^5$	$4.7 \times 10^6 \pm 2.9 \times 10^6$	$5.7 \times 10^6 \pm 1.1 \times 10^6$
M. chelonae^R	$2.1 \times 10^6 \pm 2.5 \times 10^5$	$2.0 \times 10^6 \pm 3.2 \times 10^5$	$2.0 \times 10^6 \pm 2.1 \times 10^5$	$1.9 \times 10^6 \pm 4.2 \times 10^5$
Reovirus	$4.4 \times 10^6 \pm 6.8 \times 10^5$	$4.0 \times 10^6 \pm 5.5 \times 10^5$	$3.8 \times 10^6 \pm 1.1 \times 10^6$	$3.9 \times 10^6 \pm 9.4 \times 10^5$
Sindbis virus	$4.7 \times 10^6 \pm 3.0 \times 10^6$	$3.0 \times 10^6 \pm 7.3 \times 10^5$	$2.2 \times 10^6 \pm 1.2 \times 10^6$	$3.1 \times 10^6 \pm 1.7 \times 10^6$

*CPE: infective units as determined by the CPE assay

All test carriers were inoculated and dried as per the Materials and Method:
[1] The inoculated carriers were exposed to sPBS only for 20 minutes and then carriers were eluted. This represents the recoverable bioburden.
[2] The inoculated carriers were exposed to sPBS for 20 minutes and then exposed to neutralizer solution (10% FBS in TSB) and eluted. This represents the effect of the neutralizer solution on the viability of the test organism.
[3] The 2.6% glutaraldehyde solution was mixed with neutralizer solution first and then the inoculated carrier was exposed to this solution for 20 minutes and then eluted for quantitation. This represents the efficacy of the neutralizer solution to inactivate 2.6% glutaraldehyde.
[4] The 7% AHP solution was mixed with neutralizer solution first and then the inoculated carrier was exposed to this solution for 20 minutes and then eluted for quantitation. This represents the efficacy of the neutralizer solution to inactivate 7% AHP.

Statistical analysis comparing means confirmed that there were no statistically significant differences between recoverable bioburden, the bioburden exposed to the neutralizer alone or the bioburden exposed to neutralized glutaraldehyde or neutralized AHP. This confirms that neutralization of the disinfectant was effective.

2.3. Efficacy of GLUT disinfectant

Various concentrations of GLUT were evaluated to determine killing efficacy. The microbial killing efficacy at the manufacturer's recommended concentration for HLD (2.6% GLUT for 20 min at RT) is shown in Figure 7. Effective kill ability at HLD concentration was seen for all test organisms, except *M. chelonae*^{R}. Evaluation of various concentrations were tested from 2.6% to 0.01% GLUT to determine the "breakthrough" concentration at which microorganisms survived. Breakthrough growth was defined as > 1 Log_{10} survival of vegetative bacteria after 1 min exposure to the disinfectant (under the specified test conditions). Breakthrough growth for all vegetative bacteria occurred at a 0.1% GLUT concentration and is shown in Figure 8.

2.4. Efficacy of AHP disinfectant

The results shown on Figure 9 indicate that 7% AHP at RT for 20 min (manufacturer's recommended condition for HLD) is effective at killing all organisms evaluated. At the manufacturers recommended concentration for surface disinfection (0.5% AHP for 5min at RT) there was microbial survival as shown in Figure 10. Evaluation of various concentrations of AHP were tested from 0.5% to 0.01% AHP to determine the "breakthrough" concentration at which microorganisms survived. Breakthrough was again defined as ≥ 1 Log_{10} survival of vegetative bacteria after 1 min exposure to the disinfectant. Breakthrough survival of vegetative bacteria after 1 min exposure to the disinfectant occurred at a 0.05% AHP, as shown in Figure 11.

Figure 7. Exposure to 2.6% Glutaraldehyde disinfectant over time

Organisms, *M. chelonae*^{R} (-◇-); *M. chelonae*^{S} (-■-); *Reovirus* (--O--); *C. albicans* (-▲-); *P. aeruginosa* ( ); *E. faecalis* (-●-); *SVHR* (-□-), were suspended in an organic medium (ATS), dried onto the surface carrier, and exposed to 2.6% Glutaraldehyde with increasing exposure times to 20 min finally representing high-level disinfectant conditions. Bacteria and yeast were quantified by colony forming units (CFU), *Reovirus* by plaque forming units (PFU) and *SVHR* as per cytopathic effects assay (CPE infective units) as described in Materials and Methods.

Figure 8. Exposure to 0.1% Glutaraldehyde disinfectant over time

Organisms, *M. chelonae*^{R} (-◇-); *M. chelonae*^{S} (-■-); *C. albicans* (-▲-); *Reovirus*(--O--); *P. aeruginosa* ( ); *E. faecalis* (-●-); *SVHR* (-□-), were suspended in an organic medium (ATS), dried onto the surface carrier, and exposed to 0.1% Glutaraldehyde with increasing exposure times to 20 min. At this concentration with 1 min exposure time, breakthrough survival of vegetative bacteria occurred. Bacteria and yeast were quantified by colony forming units (CFU), *Reovirus* by plaque forming units (PFU) and *SVHR* as per cytopathic effects assay (CPE infective units) as described in Materials and Methods.

Figure 7. Exposure to 2.6% Glutaraldehyde

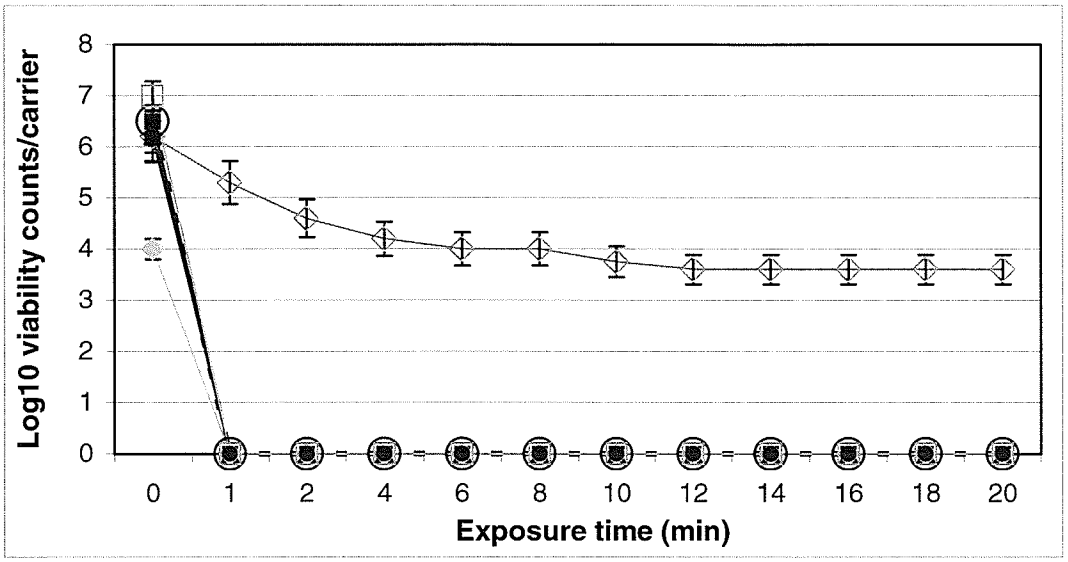


Figure 8. Exposure to 0.1% Glutaraldehyde

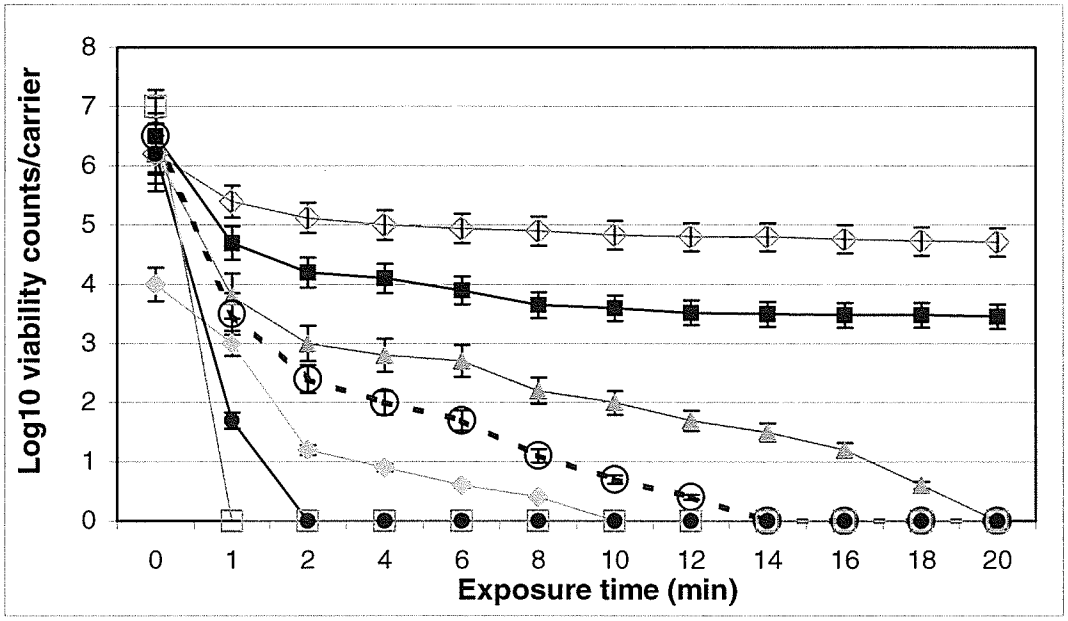


Figure 9. Exposure to 7% Accelerated hydrogen peroxide disinfectant over time

Organisms, *M. chelonae*^{R} (-◇-); *M. chelonae*^{S} (-■-); *C. albicans* (-▲-); *Reovirus*(--**O**--); *P. aeruginosa* ( ); *E. faecalis* (-●-); *SVHR* (-□-), were suspended in an organic medium (ATS), dried onto the surface carrier, and exposed to 7% AHP with increasing exposure times to 20 min finally representing high-level disinfectant conditions. Bacteria and yeast were quantified by colony forming units (CFU), *Reovirus* by plaque forming units (PFU) and *SVHR* as per cytopathic effects assay (CPE infective units) as described in Materials and Methods.

Figure 10. Exposure to 0.5% Accelerated hydrogen peroxide disinfectant over time

Organisms, *M. chelonae*^{R} (-◇-); *M. chelonae*^{S} (-■-); *C. albicans* (-▲-); *Reovirus*(--**O**--); *P. aeruginosa* ( ); *E. faecalis* (-●-); *SVHR* (-□-), were suspended in an organic medium (ATS), dried onto the surface carrier, and exposed to 0.5% AHP with increasing exposure times to 20 min. AHP is recommended as a surface disinfectant at this concentration with 5 min exposure time. Bacteria and yeast were quantified by colony forming units (CFU), *Reovirus* by plaque forming units (PFU) and *SVHR* as per cytopathic effects assay (CPE infective units) as described in Materials and Methods.

Figure 11. Exposure to 0.05% Accelerated hydrogen peroxide (AHP) disinfectant over time

Organisms *M. chelonae*^{R} (-◇-); *M. chelonae*^{S} (-■-); *C. albicans* (-▲-); *Reovirus* (--**O**--); *P. aeruginosa* ( ); *E. faecalis* (-●-); *SVHR* (-□-), were suspended in an organic medium (ATS), dried onto the surface carrier, and exposed to 0.05% AHP with increasing exposure times to 20 min. At this concentration with 1 min exposure time, breakthrough survival of vegetative bacteria occurred. Bacteria and yeast were quantified by colony forming units (CFU), *Reovirus* by plaque forming units (PFU) and *SVHR* as per cytopathic effects assay (CPE infective units) as described in Materials and Methods.

Figure 9. Exposure to 7% Accelerated hydrogen peroxide

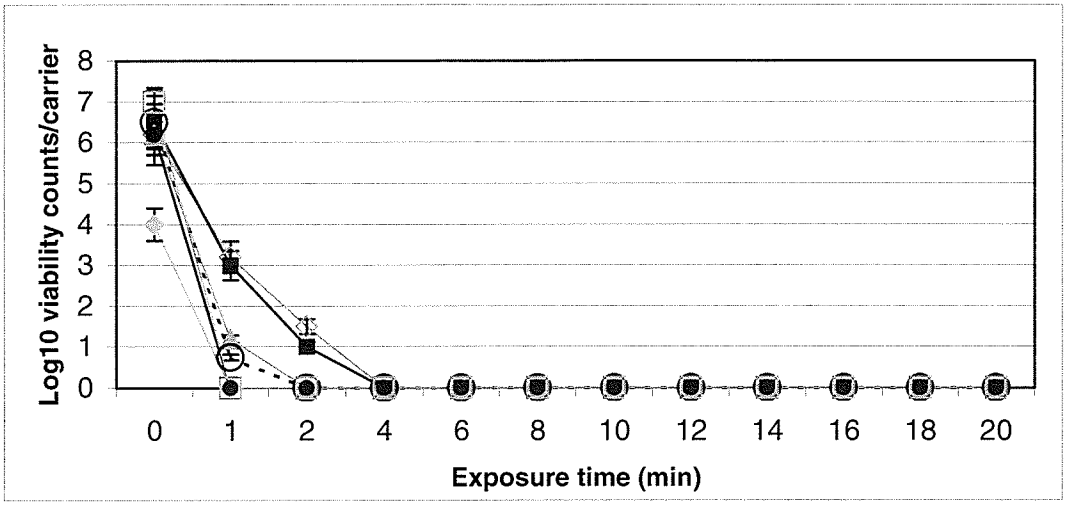


Figure 10. Exposure to 0.5% Accelerated hydrogen peroxide

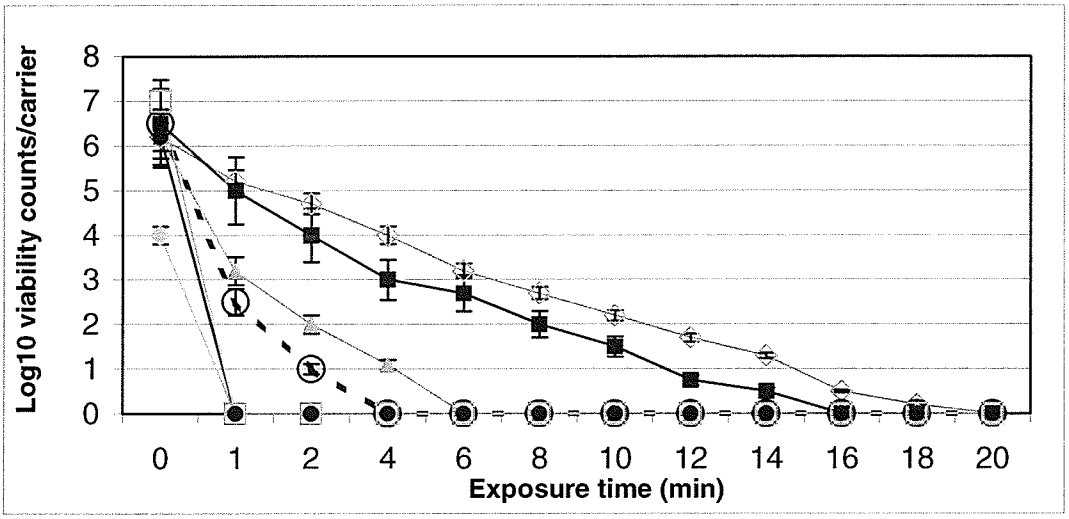
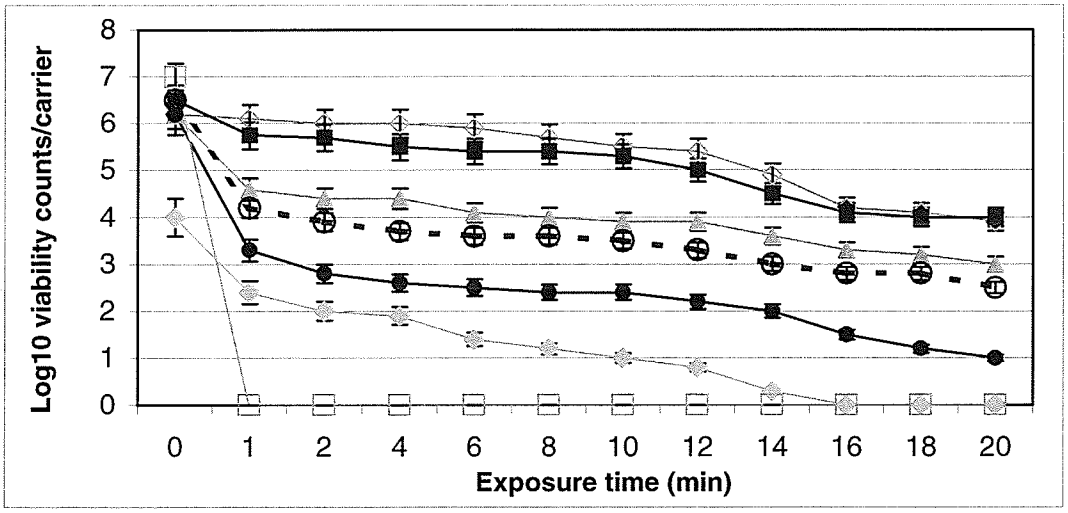


Figure 11. Exposure to 0.05% Accelerated hydrogen peroxide



2.5. Effect of a formulation on efficacy of microbial killing

Since formulation can affect a disinfectant's efficacy, two formulations of HP, stabilized HP (SHP) and accelerated and stabilized HP (AHP), were both evaluated at a dilution of 0.05%. Differences in efficacy as a result of HP formulation are shown in Table 8. When SHP was used at the manufacturer's recommended 3% concentration and 10 min time for disinfection it was an effective low-level disinfectant (LLD) for vegetative bacteria and yeast tested (data not shown).

3. Comparison of Biofilm loop model to MBEC model system

Two different biofilm models offering advantages in modeling microbial survival in buildup biofilm for complex medical devices were compared. Criteria for selection of a single model for BBF studies included the ability to produce multiple equivalent biofilm formation and assay results. Endpoint analyses included quantitative viability counts, metabolic (ATP and redox) activity, and protein and carbohydrate levels, measured at various time points throughout biofilm development. The overall goal was to select a single model with an assay system most capable of defining microbial survival in buildup biofilm.

A comparison of metabolic rate determined by redox activity was made in the MBEC and biofilm loop systems to ensure the absorbance values resulting from the redox assay had a linear relationship and accurately reflected viability counts. Figure 12(a) demonstrates a linear relationship between viability counts and metabolic activity determined by the redox assay over the range of ~2 Log_{10} (for *P. aeruginosa*) or 3 Log_{10} (for *E. faecalis*) to

Table 8: Effect of formulation on efficacy of microbial killing

Organism	Recoverable bioburden (Log_{10})	Log_{10}+reduction in viable counts after exposure to disinfectant			
		0.05% SHP*		0.05% AHP**	
		1min	5min	1min	5min
E. faecalis	7.06	0.04	1.10	2.40	3.30
P. aeruginosa	6.09	0.36	1.24	1.50	2.50
C. albicans	7.05	0.20	0.75	1.00	1.60
M. chelonae[S]	7.37	0.14	0.51	0.50	0.90

A stabilized HP formulation (SHP) at 0.05% was compared to an accelerated HP formulation (AHP) also at 0.05%. All results are the average of 9 experiments. Statistical analyses using the Student's t-test demonstrated significant differences ($p<0.0001$) between 0.05% AHP and 0.05% SHP, both at 1 min and 5 min exposure times.

+ Log_{10} reduction = [Log_{10} recoverable bioburden] – [Log_{10} viable organisms after testing]
* PerDiem: 3% SHP diluted 1:64
**PerCept: 7% AHP diluted 1:128

7 Log_{10} overall. Similar experiments were done comparing ATP activity and the redox assay. The results showed a good correlation between the two assays, verifying the reproducibility of the redox assay (data not shown). The redox assay was used for all buildup biofilm studies due to its reliability and flexible format (e.g., microtitre format).

Carbohydrate and protein content in the biofilm's organic matrix, which the organisms secrete and also contribute to, was measured. Figure 12(b) compares viability counts to protein levels in biofilm formation by *P. aeruginosa* in the biofilm loop and MBEC model systems. In both systems, carbohydrate levels were also determined (data not shown) and showed a similar trend to protein analysis, increasing to a maximum early in biofilm formation and plateauing as biofilm matured (data not shown). The same trends occurred when *E. faecalis* was used to form biofilm in both the MBEC and Loop systems (data not shown). Carbohydrate and protein level tests were most useful in detailing TBF, however less applicable in BBF studies; therefore, those test results are not shown.

Initial studies with the biofilm loop system had established a baseline of biofilm growth over time for each organism tested. However, since results were comparable between the biofilm loop and MBEC systems, the MBEC system was adopted for further studies due to its efficient microtitre format facilitating high throughput of samples.

Figure 12(c) compared the redox assay to quantitative viability counts in early biofilm formation from days 1 to 9 in the MBEC system. In agreement with the previous results for *P. aeruginosa* and *E. faecalis* (Figure 12a), there was a linear relationship between the

two methods used to evaluate viability. It was apparent the redox assay could be used for all organisms except *M. chelonae*^{S}, where low penetration of the reagent was probably responsible for the non-reactivity of the reagent. Based on these results, it was decided that subsequent experiments would use the viability and redox assay to assess microbial survival in various types of biofilm for all organisms except *M. chelonae*^{S}, where only the viability assay would be used. The data suggests the limit of detection by the redox assay as: ~2 Log_{10} for *P. aeruginosa*, ~3 Log_{10} for *E. faecalis* and *C. albicans*, and ~4 Log_{10} for *M. chelonae*^{R}.

4. Impact of organic load on biofilm formation: TBF compared to BBF

Controls for biofilm formation on MBEC pegs were performed for every plate used. The negative control consisted of an MBEC peg with growth medium (either ATS, water, or enzymatic detergent). The negative controls were included on all plates and used to monitor the possibility of bacterial contamination occurring throughout the test period. All MBEC trays contained positive controls of biofilm formation to demonstrate maximum viability. If any questionable results were discovered in controls, plates were discarded. All MBEC results stated are in relation to verified negative and positive pegs per test plate to assure valid test results. As well results represent a minimum of 3 replicates in 3 trials (totalling 9 replicates) with standard deviations stated.

Figure 12. Comparison of Biofilm loop model to MBEC model system

(a) Comparison of quantitative viability counts and metabolic activity

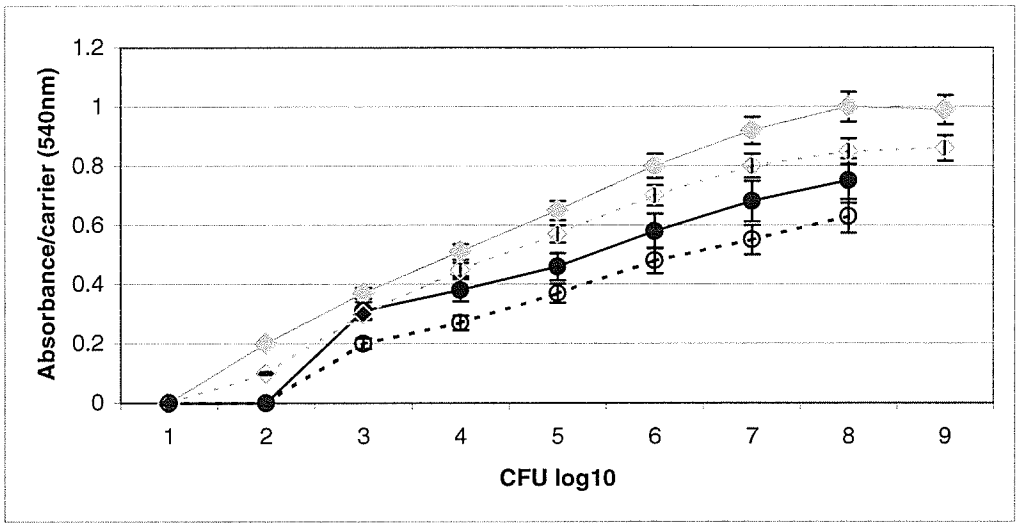
Biofilm formation of *P. aeruginosa* in the MBEC system (-◈-) and in the Loop system (--◇--) and *E. faecalis* in the MBEC system (-●-) or the Loop system (--○--) was evaluated by the redox assay and quantitative viability counts. (Data represents 9 replicates; SD is < 10%)

(b) Comparison of quantitative viability counts(□) and protein levels (- ◇-) in biofilm formation of *P. aeruginosa* (Data represents 9 replicates. Statistical analyses using the Student's t-test to compare resultant viability counts in the Loop and MBEC systems demonstrated no significant differences between the two models, $p<0.01$)

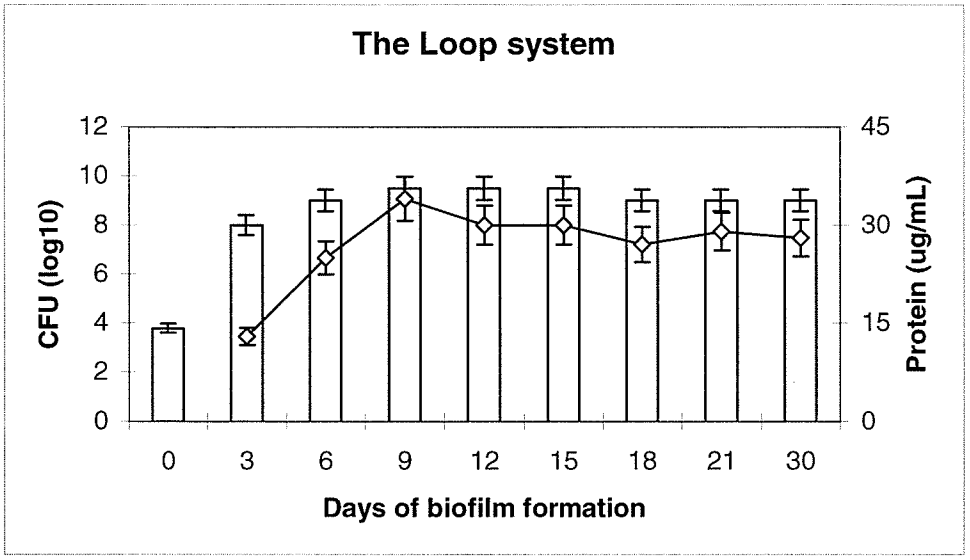
(c) Comparison of redox assay to quantitative viability counts for all test organisms using the MBEC system

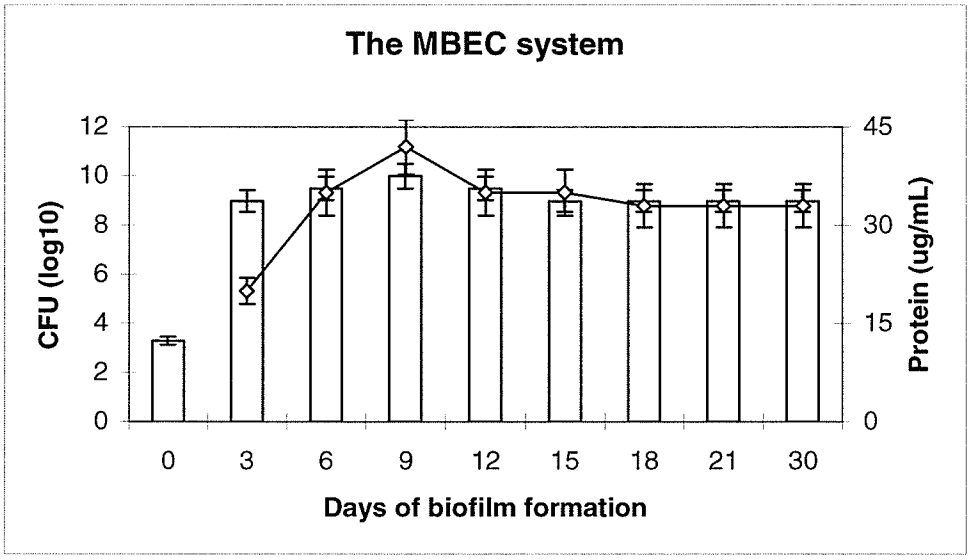
Viability of *C. albicans* (-▲-), *M. chelonae*^{R} (-◇-), *M. chelonae*^{S} (-■-), *P. aeruginosa* (-◈-), and *E. faecalis* (-●-) was assessed by metabolic activity using the redox assay and by quantitative counts following biofilm formation in the MBEC system for 9 days.

(a) Comparison of quantitative viability counts and metabolic activity

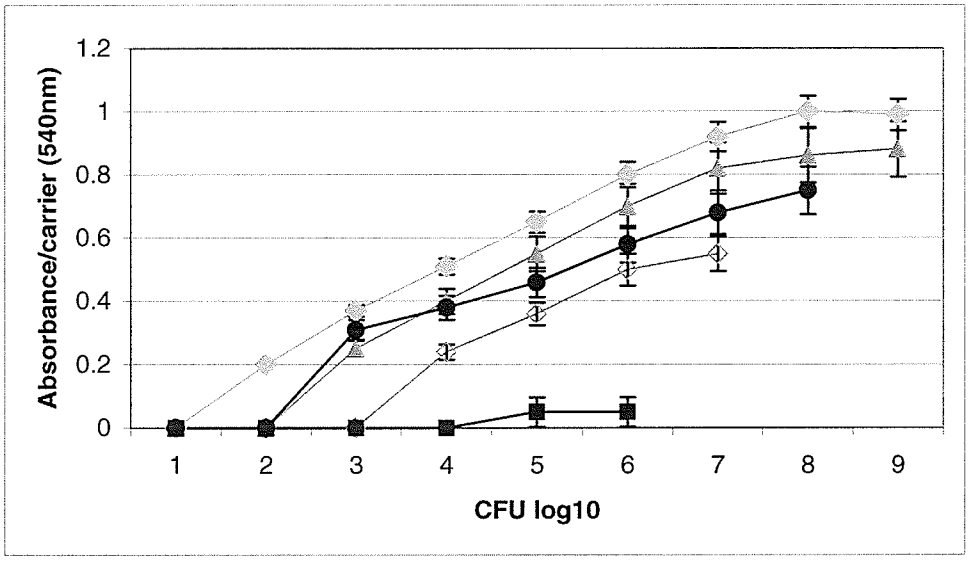


(b) Comparison of quantitative viability counts and protein levels in biofilm formation of *P. aeruginosa*





(c) Comparison of redox assay to quantitative viability counts for all test organisms using the MBEC system



Figures 13 to 20 detail TBF and repetitive cycles of BBF in high and low nutrient medium over 30 days for *E. faecalis, P. aeruginosa, C. albicans* and *M. chelonae*^{R}. Throughout the test period, sample pegs were challenged with GLUT and AHP. Although 2- and 3-day repetitive cycles were performed, only data from 2-day cycling is presented since both demonstrated similar results. For each test organism, a summary of breakthrough survival in days (for TBF) and cycle number (for each set of BBF conditions) are found in Tables 9 to 12.

4.1. Traditional biofilm formation on MBEC pegs

To determine the efficiency of HLD on microorganisms in biofilm formed on MBEC pegs, the 2-day cycling protocol was used. At various timepoints sample pegs with biofilm were exposed to disinfectant challenge. The results were defined by quantitative viability counting methods and found in Figures 13, 15, 17, 19, section (a) and Tables 9 to 12. Figures 14, 16, 18, 20, section (a) are the same TBF patterns but with AHP challenge rather than GLUT.

4.1.1. TBF Kinetics:

For each organism TBF is the biofilm formation initially described and this was considered the positive, hydrated control in all BBF trials. Some organisms formed biofilm more vigorously under the test conditions, e.g. *P. aeruginosa* (Figure 15a) and *C. albicans* (Figure 17a) producing organism loads of ~9 Log_{10} and 7.5 Log_{10} within 3 days formation in high nutrient medium respectively, followed by *E. faecalis* (Figure 13a),

and least vigorous formation by *M. chelonae* resulting in ~4 Log_{10} within 3 days (Figure 19a). For all TBF, the sooner the biofilm achieved maximum organism load, the sooner breakthrough survival to disinfectant challenge occurred.

The TBF that was formed with low nutrient medium (sterile tap water) compared to growth in ATS resulted in a significant reduction in the maximum biofilm formation for all test organisms of ~ 4 Log_{10} ($p<0.0001$). Since *M. chelonae*^{S} formed biofilm at the slowest and lowest levels, with least reaction with the metabolic activity reagents (data not shown), *M. chelonae*^{R} was chosen for all TBF/BBF comparative studies.

When TBF was formed with the enzymatic detergent as the organic source, early TBF had an organism load intermediate to growth in ATS and water. However, generally over the test time period of 30 days, organism levels in enzymatic detergent increased to approximate those seen in ATS-fed biofilm formation.

4.1.2. Impact of disinfectant challenge on survivability of organisms in TBF:

Organisms that formed biofilm more rapidly resulted in higher organism loads per peg, and survived disinfectant challenge (GLUT or AHP, HLD) at an earlier stage. The time to breakthrough was shorter especially in high nutrient medium. Although growth was limited in low nutrient medium, breakthrough survival to GLUT in TBF was also achieved over time. In all cases, initial breakthrough survival when exposed to HLD was at a very low level that was below the limit of detection by viability counts. Avid biofilm formers, *P. aeruginosa* and *C. albicans*, showed breakthrough survival to GLUT by Day

6 and Day 9, respectively, when TBF was formed in ATS. The breakthrough survival was slower when biofilm formation was in water, i.e., Day 18 and Day 24, respectively (Figure 15a, Table 10 and Figure 17a, Table 11, respectively). Intermediate times for breakthrough survival resulted when TBF was formed in enzymatic detergent (data not shown). However, *E. faecalis*, which did not form biofilm as effectively, survived GLUT challenge by Day 15 when the biofilm was formed in ATS, and Day 30 when it was formed in water (Figure 13a, Table 9). *P. aeruginosa* demonstrated the best survival in ATS following GLUT challenge, with an initial detectibility level of ~2 Log_{10} and a final level of survivors at the end of the 30 day test period of ~6 Log_{10} (Figure 15a). A similar trend for these organisms grown in high nutrient media was seen when AHP was used as the HLD challenge. As expected, *M. chelonae*^{R} was resistant to HLD using GLUT throughout the test period. However, this strain of *M. chelonae*^{R} was not fully resistant to GLUT, but the level of survivability following GLUT challenge increased over time from an initial ~1 Log_{10} reduction in survival to no detectable killing within ~3 days under high nutrient conditions and ~9 to 15 days under low nutrient conditions (Figure 19a).

Breakthrough survival to GLUT under HLD conditions occurred sooner in TBF development and ultimately with higher numbers of survivors than when AHP was used for the HLD challenge. For all organisms tested, there was no survival to AHP if the TBF was formed under low nutrient conditions (Table 9 –12, Figures 13-20, section (a)).

Challenge with AHP under LLD conditions (i.e., 0.5% AHP concentration and 5 minute exposure time) compared to HLD conditions, resulted in earlier breakthrough survival

and higher surviving organism loads in TBF formed in ATS for all organisms (Table 9 - 12, data for organism loads not shown). For TBF formed under low nutrient conditions, AHP at LLD conditions was effective for killing *E. faecalis* and *P. aeruginosa* with no survival detected by viability counts (Table 9 and 10, respectively) or by direct qualitative outgrowth (data not shown). This was similar to the HLD results. However, despite low nutrient conditions, both *C. albicans* and *M. chelonae*^{R} survived AHP LLD conditions, detected by Day 24 and 21 respectively (Table 11 and 12, respectively).

4.2. Buildup biofilm on MBEC pegs (Quantitative Viability Assay)

4.2.1. BBF formed by repetitive drying:

Microbial survivability was tested in BBF resulting from repetitive exposure of drying treatment alone on all test pegs over a 30-day period. One set of sample pegs was removed after each cycle of drying to detect the impact of cumulative cycles of drying on survivability within BBF. A second set of sample pegs was also removed at the end of each cycle of drying and further exposed to a single disinfectant challenge. The quantitative viability assay defined the survivability within such BBF over time.

4.2.2. Repetitive drying - impact on survivability:

The impact of repetitive drying alone (without disinfectant challenge) on survivability was examined. A similar pattern for all test organisms resulted from exposure to drying alone when compared to the positive hydrated control (Figures 13, 15, 17, 19, section (b), yellow compared to blue bars, equivalent to Figures 14, 16, 18, 20 for drying alone). The detrimental effect of drying (reduced organism load) was most evident early in BBF

(especially for *P. aeruginosa*) but not detectable in the later BBF stages (mature BBF). Overall the effects of drying were more pronounced when BBF was formed in low nutrient medium (water) compared to high nutrient medium (ATS).

4.2.3. Repetitive drying and single HLD exposure - impact on survivability:

The impact of repetitive drying on sample pegs removed at the end of each cycle and further challenged with a single exposure to HLD was examined. Survival to GLUT in BBF formed in ATS was achieved early in BBF formation for all test organisms, however when formed in low nutrient medium, survival to GLUT occurred in later cycles of drying (e.g., 6 and 12 additional cycles for *P. aeruginosa* and *E. faecalis* respectively) (Figures 13, 15, 17, 19, section (b), red and blue columns, Tables 9 - 12).

Comparatively, when BBF formed in ATS was challenged with AHP HLD, breakthrough survival occurred in later cycles (requiring at least twice the number of cycles) and in lower numbers for all test organisms (e.g., ~3 Log_{10} fewer survivors for *P. aeruginosa*). However, organisms did not survive AHP challenge when cycling was done under low nutrient conditions (Figures 14, 16, 18, 20, section (b), Tables 9 – 12).

Generally, BBF formed by repetitive drying cycles resulted in earlier breakthrough times than with TBF or all other BBF cycling conditions tested in this project, with the ultimate numbers of survivors similar to levels found in TBF after HLD challenge (except for AHP HLD which resulted in the absence of detectable bioburden in all forms of biofilm grown in low nutrient conditions) (Tables 9-12, Figures 13-20, section (a) and (b)).

4.3. BBF formed by repetitive drying and repetitive HLD :

BBF was formed on MBEC pegs under conditions of repetitive cycles of drying immediately followed by HLD. The cycles of drying with HLD exposure were repeated over 30 days. The survivability of microorganisms within the BBF at the end of each cycle (after drying and HLD) was determined. This treatment cycling scheme represented the most challenging conditions for microbial survival.

4.3.1. Repetitive drying and repetitive HLD – impact on survivability

When repetitive cycles of drying and HLD formed BBF, breakthrough survival to HLD only occurred with GLUT and only under high nutrient conditions for all test organisms (Figures 13, 15, 17, section (c)) except for *M. chelonae*^{R}, which also survived when BBF was formed under low nutrient conditions (Figure 19, section (c)). Survival under these BBF conditions was only detectable in the latter stages of cycling and resulted in relatively low levels of survivors, ≤ 2 Log_{10} by the end of the test period for all test organisms (except for *M. chelonae*^{R}). However no test organisms including *M. chelonae*^{R} survived AHP HLD challenge in this type of BBF, regardless of formation in high or low nutrient environments (Figures 14, 16, 18, 20, section (c)).

4.4. BBF formed by complete repetitive cycles: repetitive drying, repetitive HLD and repetitive bioburden exposure:

BBF was formed on MBEC pegs under conditions of repetitive cycles of drying, HLD and bioburden exposure. These cycles were repeated over 30 days with survivability within BBF examined after each cycle. All data presented represent BBF seeded in ATS

and formed in either enzymatic detergent or water with cyclic exposure to treatment conditions as stated above. No data for BBF formed in enzymatic detergent and directly challenged with HLD (without reseeding) is presented (except in cycle 1 where testing occurred following HLD challenge but prior to reseeding).

4.4.1. Repetitive drying, repetitive HLD, repetitive bioburden exposure – impact on survivability

Cycles mimicking a complete endoscope reprocessing scheme resulted in survivability to GLUT or AHP challenge when BBF was formed in the presence of an enzymatic detergent with repetitive cycles of drying, HLD, and reseeding with bioburden. Detectable survival by quantitative viable counts occurred earlier in the cycling process with GLUT than with AHP. When this BBF cycle was formed in the presence of low nutrient medium such as water (rather than enzymatic detergent) there was survival to the GLUT challenge, but no detectable survival to the AHP challenge (Table 9 –12). The only difference between this BBF formed in water and BBF formed by repetitive cycles of drying and HLD in a water medium was the cyclical re-exposure to bioburden in ATS (reseeding). Therefore cyclical reseeding with growth in low nutrient medium facilitated organism survival to GLUT HLD, detected in low levels for all test organisms in later cycles (except *M. chelonae*^{R} which was by nature resistant to GLUT) (Figures 13, 15, 17, 19, comparing section (d) to (c); Tables 9-12). In contrast, there was no survival to AHP HLD in any BBF (or TBF) formed in water (Tables 9-12).

4.5. Overall comparability of TBF and various BBF conditions:

Tables 9-12 summarize the breakthrough survival to various disinfectant challenges of test organisms grown under all tested conditions for biofilm formation. Overall BBF grown in ATS and formed by repetitive cycles of drying alone demonstrated the earliest detectable survival of test organisms to HLD challenge and achieved higher survivor loads earlier than in other biofilm formations when survival occurred following either GLUT or AHP HLD (Figures 13-20). BBF resulting from repetitive cycles of drying and HLD demonstrated the lowest survivability (Figures 13-20) and longest times to detect survival for all test organisms. This survival was only seen when the BBF was grown in ATS and challenged with GLUT. The time period to detectable survival was similar in TBF and BBF resulting from repetitive complete cycles (involving enzymatic detergent, drying, HLD and reseeding of bioburden) for all organisms except *P. aeruginosa,* which required longer times for detectable survival in complete cyclic BBF than for TBF.

Table 13 summarizes the rate of survival in TBF and BBF grown under high nutrient conditions (i.e., ATS for TBF and enzymatic detergent for BBF). Overall, BBF demonstrated a significantly greater increase in survivability once survivors were detectable particularly following GLUT challenge ($p<0.0001$) for all organisms except *M. chelonae*^{R} , which was by nature resistant to GLUT and did not form biofilm avidly.

4.6. Supporting evidence for quantitative viability results in TBF and BBF (Figure 21)

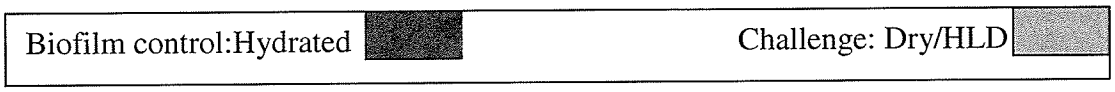
Measurement of metabolic rate by determination of redox potential as well as direct qualitative outgrowth of pegs was also employed to analyze TBF and BBF formation in

the different nutrient mediums and following disinfectant challenge. Results were in agreement with the quantitative viability counts for all test organisms. An example is shown in Figure 21, detailing biofilm formation in high nutrient medium of *E. faecalis* with respect to (1) TBF, without, and after drying and GLUT HLD on sample pegs, and (2) BBF formed by cyclic drying, with sample pegs challenged by GLUT HLD, and its impact on metabolic rate and ability for qualitative outgrowth. Following the same trend as the resultant viability counts (previously described in Figure 13a), in TBF without HLD challenge, metabolic rates rose in early TBF and levelled in mature TBF over time. Qualitative outgrowth testing also confirmed viability throughout the test period. When TBF was challenged with drying and HLD, direct qualitative outgrowth confirmed the survival of viable organisms over time. However, as with quantitative viability counts, survivability was not detected in early TBF, but was detected by Day 6 onward by qualitative outgrowth testing compared to Day 15 by CFU counts (Figure 13a). However, the metabolic rate was not detectable in these HLD challenged organisms, attributable to the low number of survivors and limit of detection of the redox assay (~2- to 4 Log_{10} depending on the specific test organism).

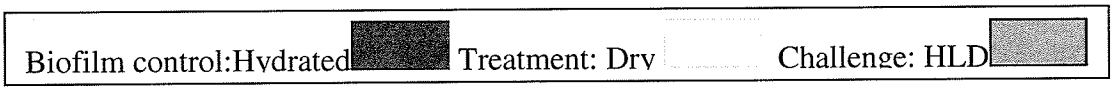
In BBF (as a result of cyclic drying) of *E. faecalis*, viability following GLUT HLD challenge was detectable by Day 6 (Cycle 3) by direct qualitative outgrowth methods (Figure 21 (c)) and by quantitative counts (Figure 13b), but not until Day 10 (Cycle 5) by metabolic rate. This was attributable to the lower limit of detection by the qualitative outgrowth (1 CFU/peg) or quantitative counting (10 CFU/peg) methods of analysis compared to the metabolic assay.

Figure 13. ***E. faecalis*** **challenged with Glutaraldehyde HLD**

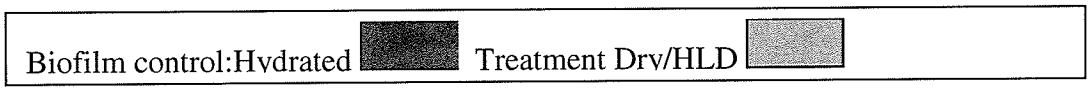
(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)



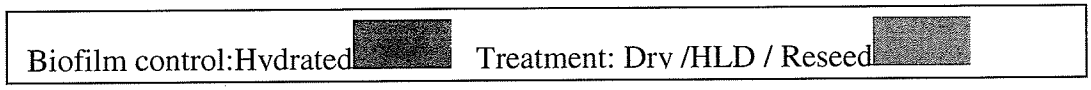
b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)



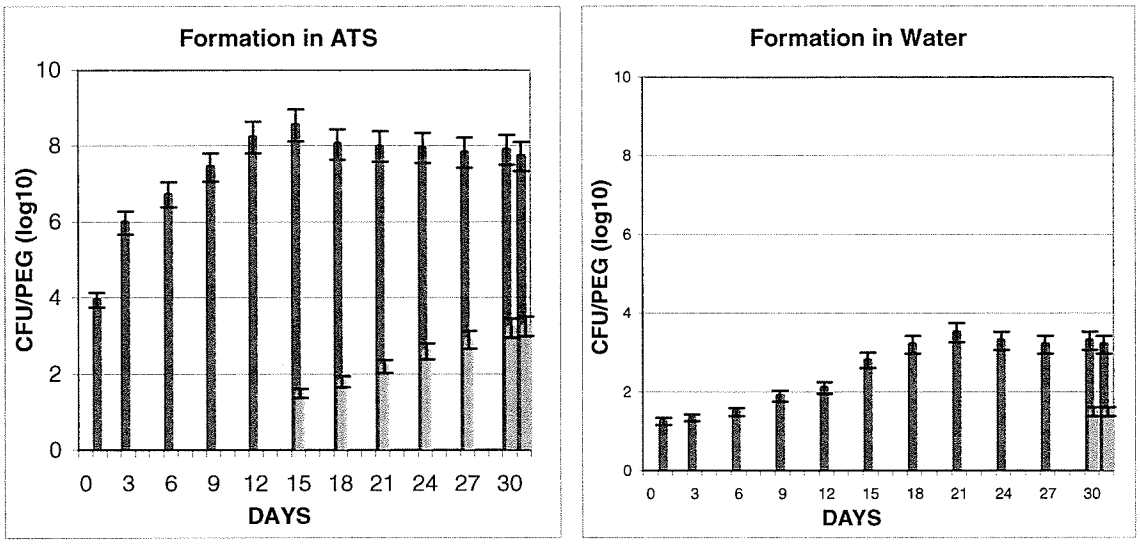
c) BBF: Repetitive Cycles of Drying and HLD



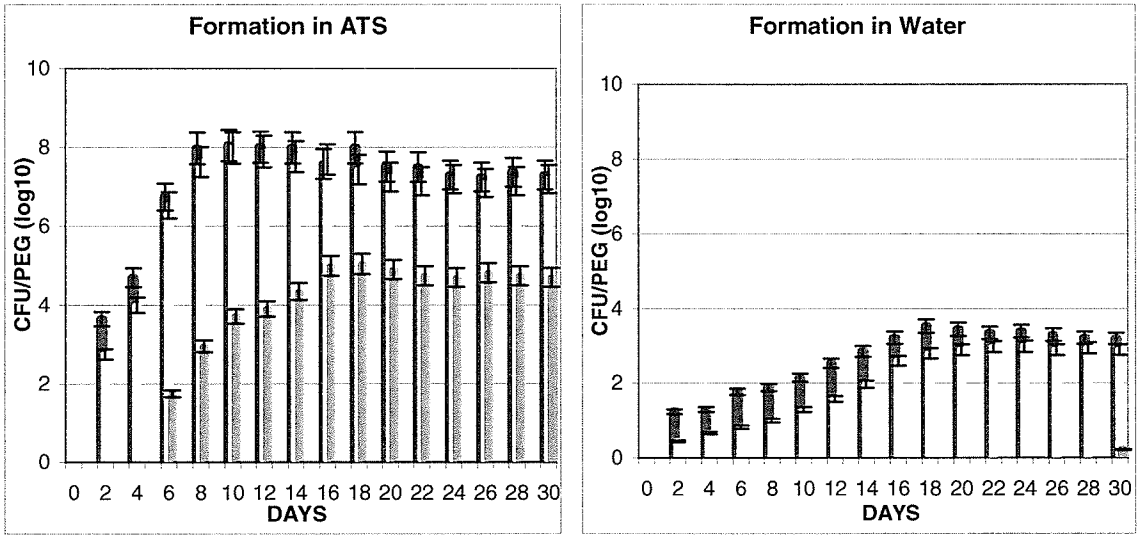
d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



(a) Traditional biofilm formation

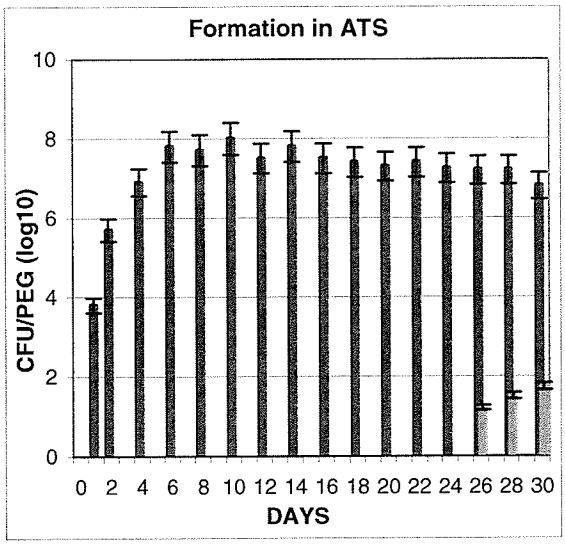


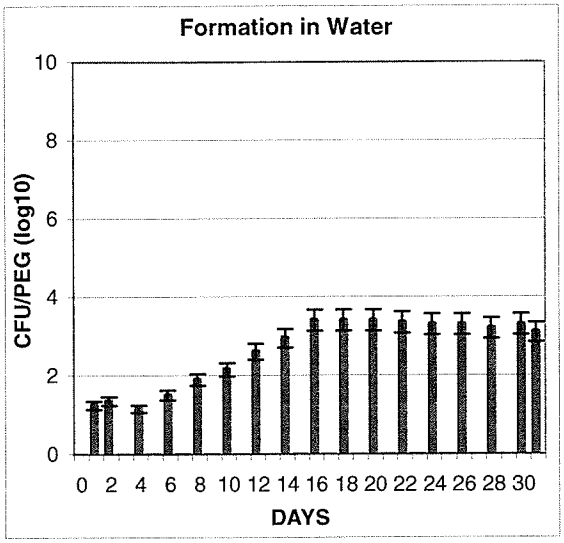
b) BBF: Repetitive Cycles of Drying



Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

c) BBF: Repetitive Cycles of Drying and HLD

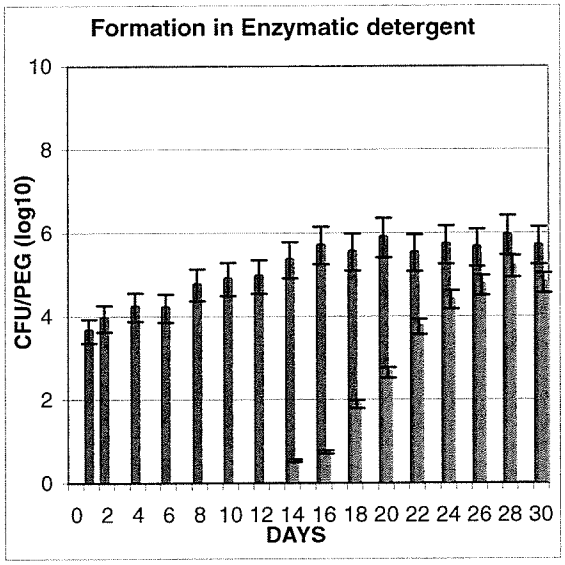


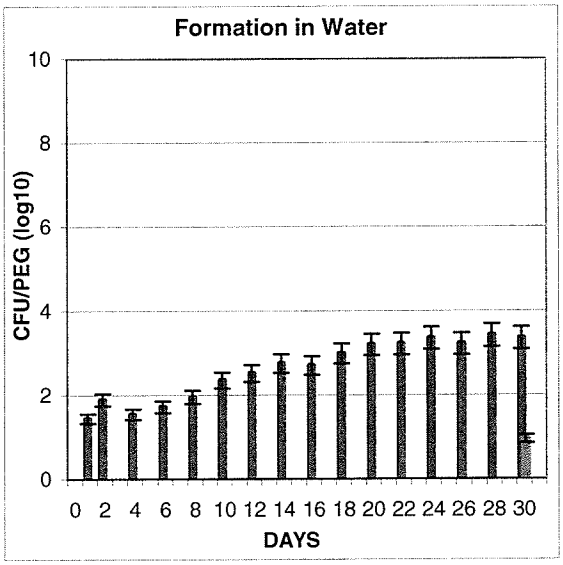


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



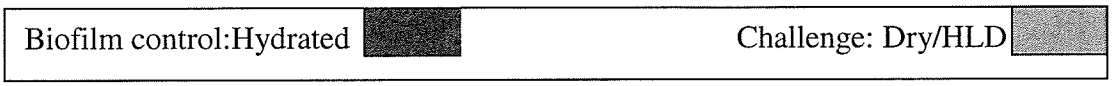


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

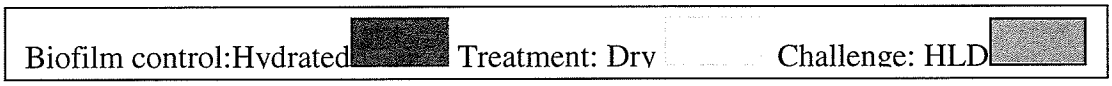
Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 14. ***E. faecalis*** **challenged with Accelerated HP- HLD**

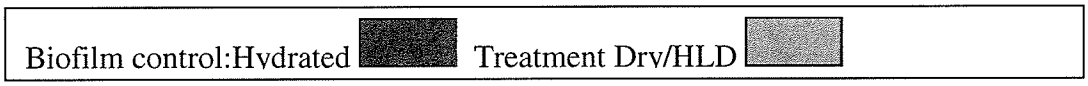
(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)



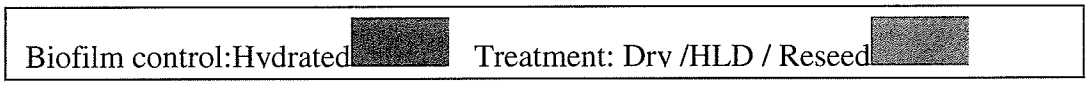
b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)



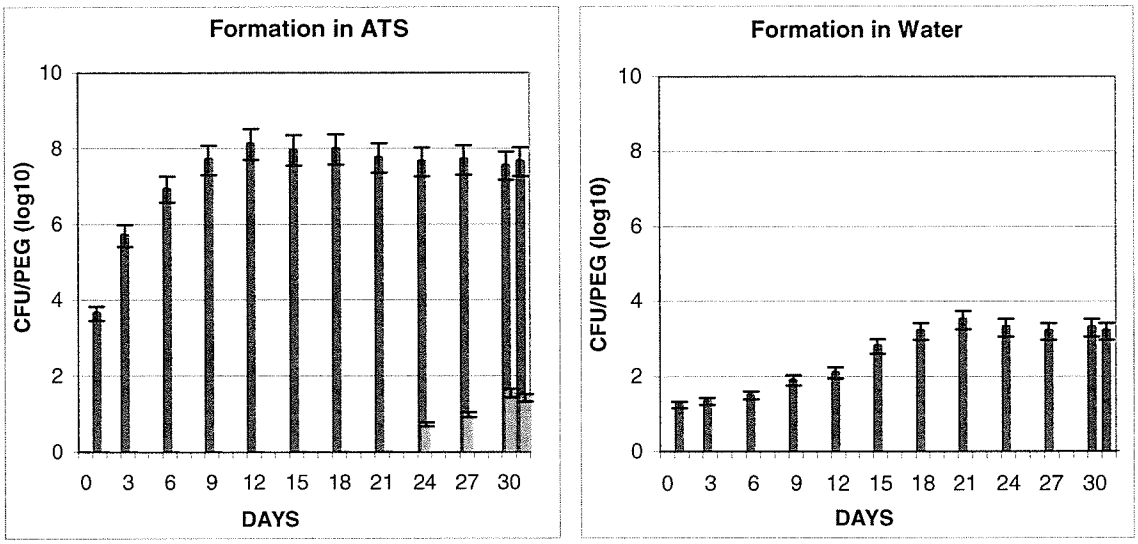
c) BBF: Repetitive Cycles of Drying and HLD



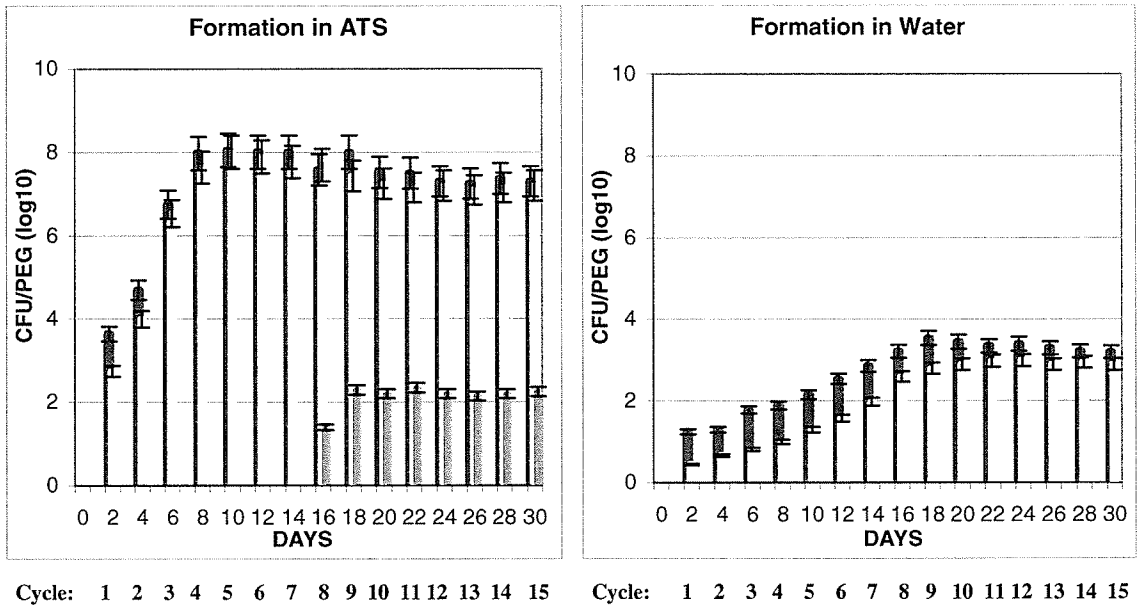
d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



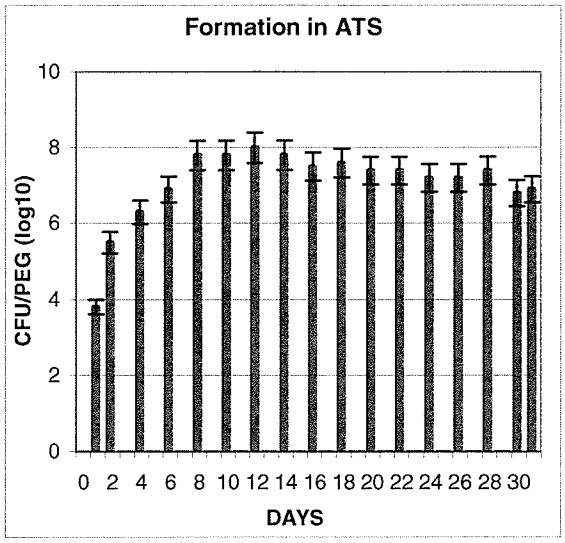
(a) Traditional biofilm formation

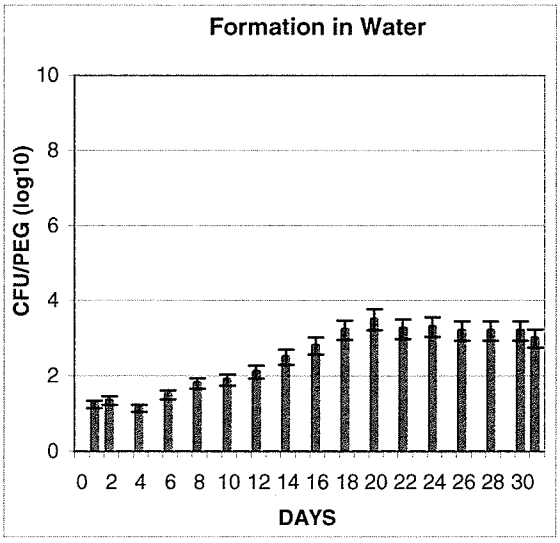


(b) BBF: Repetitive Cycles of drying



c) BBF: Repetitive Cycles of Drying and HLD

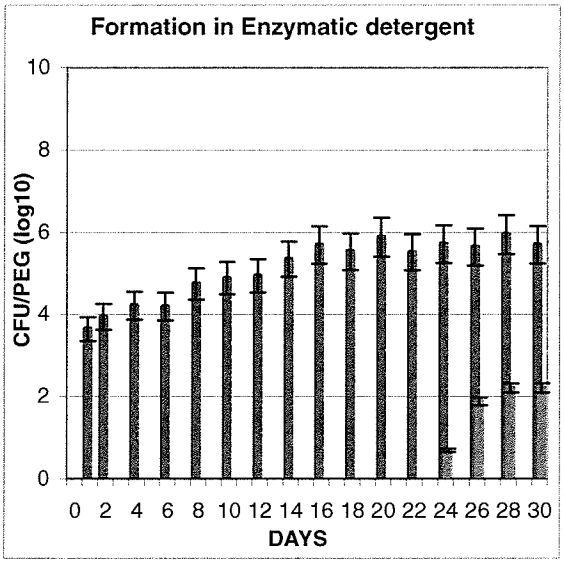


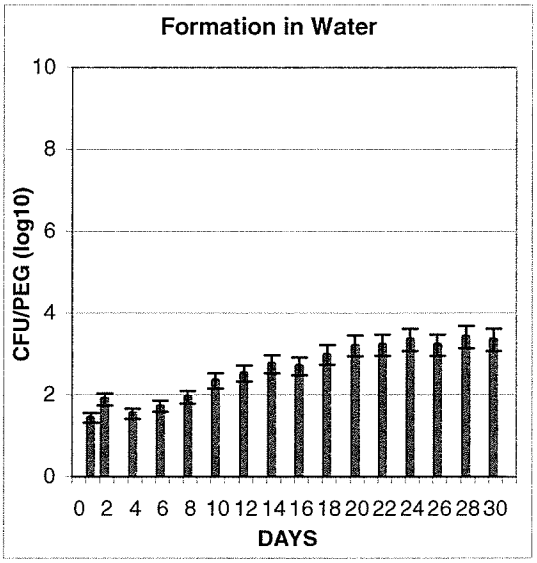


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



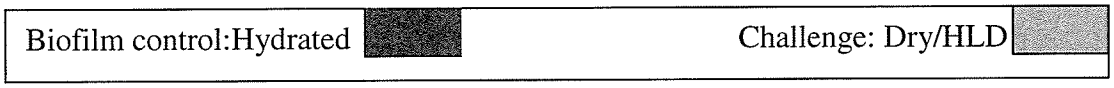


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

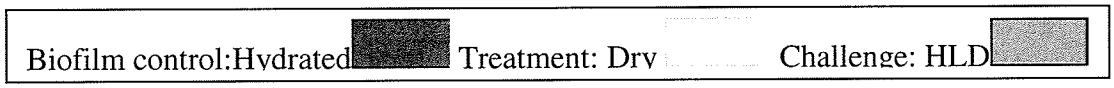
Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 15. *P. aeruginosa* challenged with Glutaraldehyde HLD

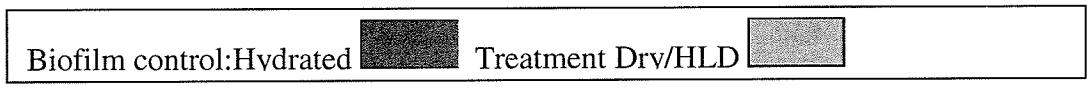
(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)



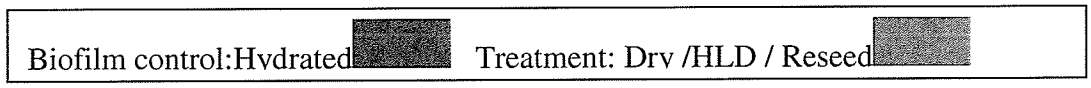
b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)



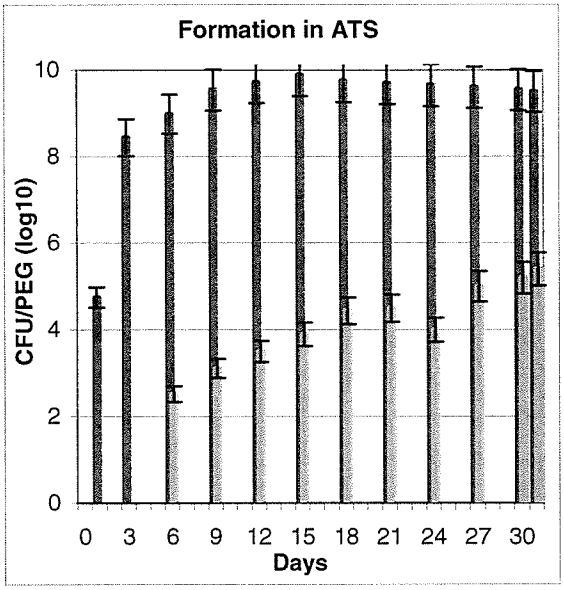
c) BBF: Repetitive Cycles of Drying and HLD

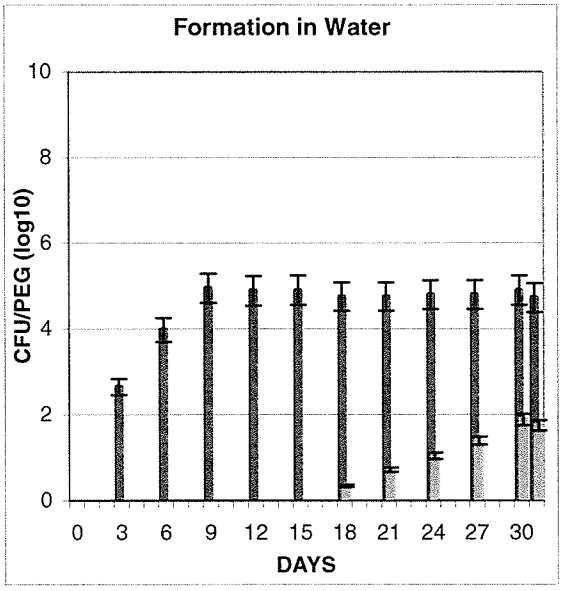


d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden

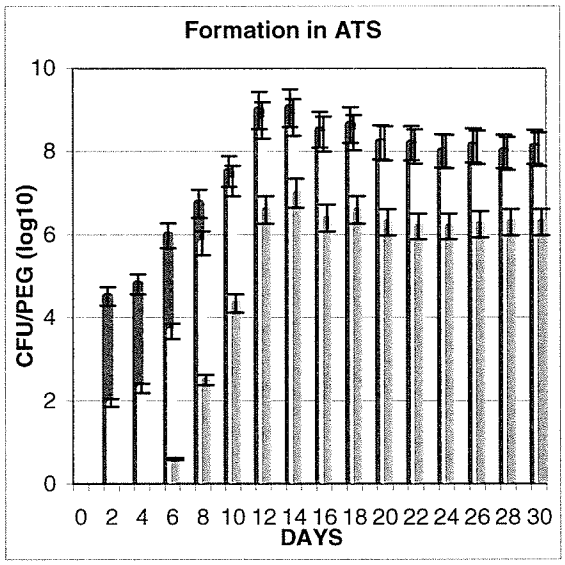


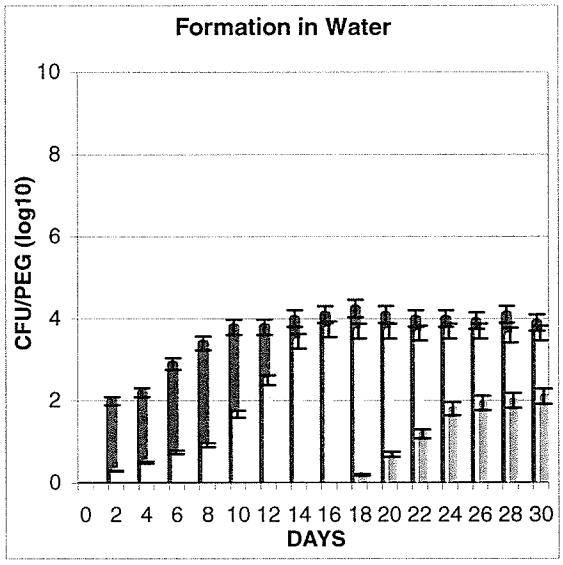
(a) Traditional biofilm formation





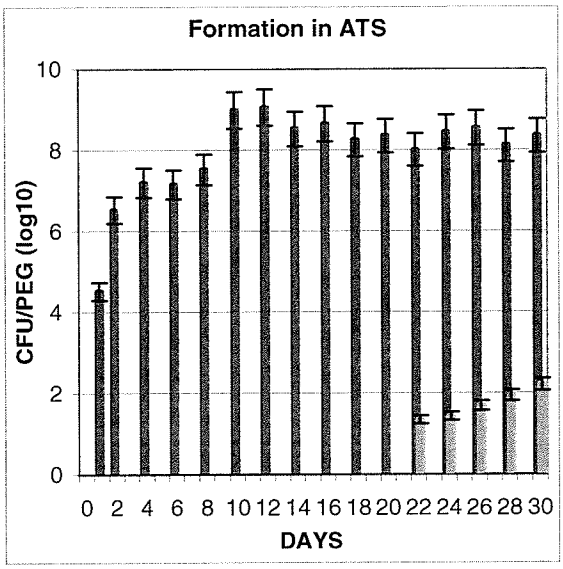
b) BBF: Repetitive Cycles of Drying



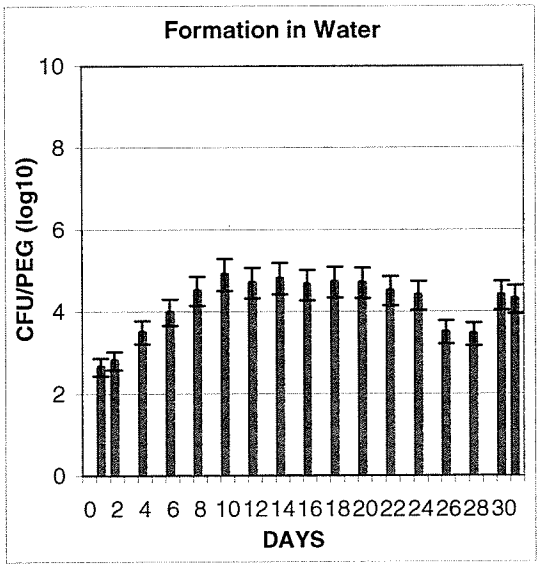


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

c) BBF: Repetitive Cycles of Drying and HLD

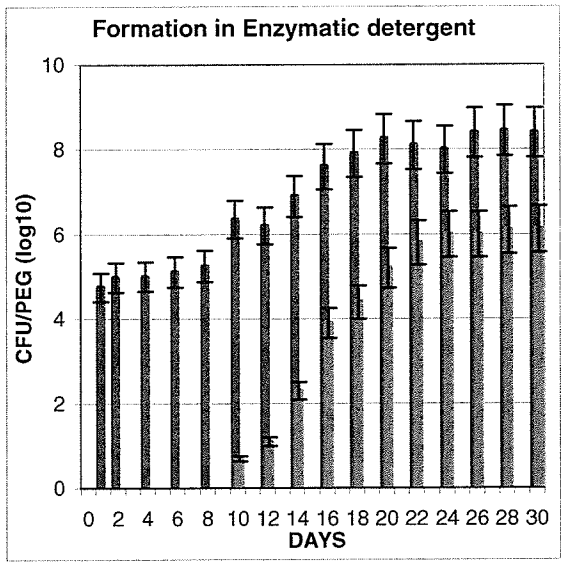


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

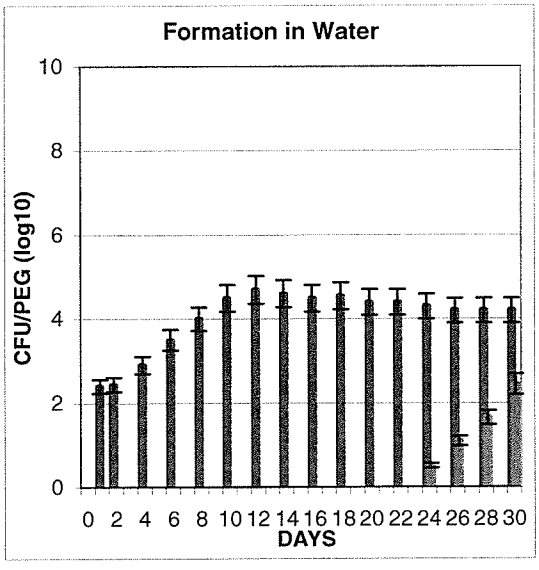


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

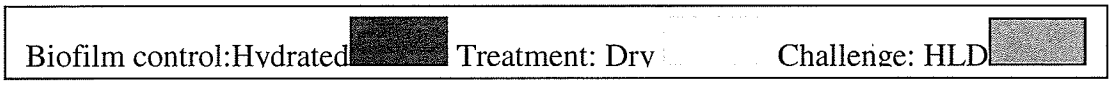


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

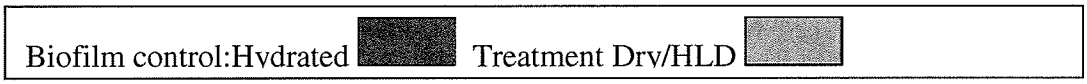
Figure 16. ***P. aeruginosa*** **following Accelerated HP- HLD**

(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)

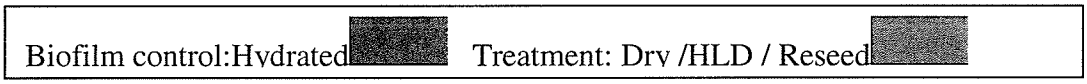
b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)



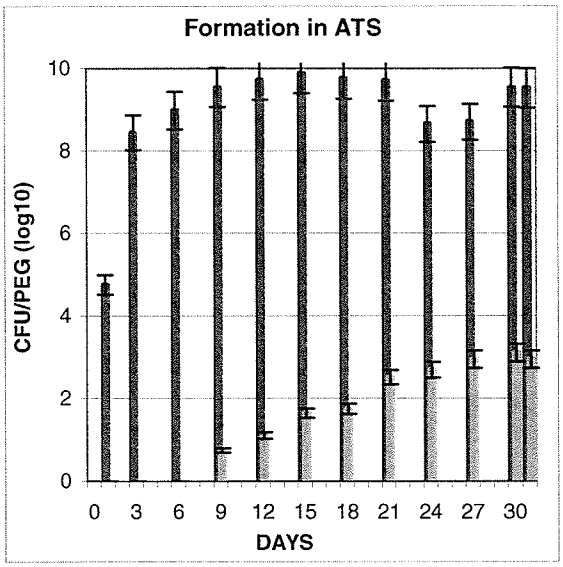
c) BBF: Repetitive Cycles of Drying and HLD

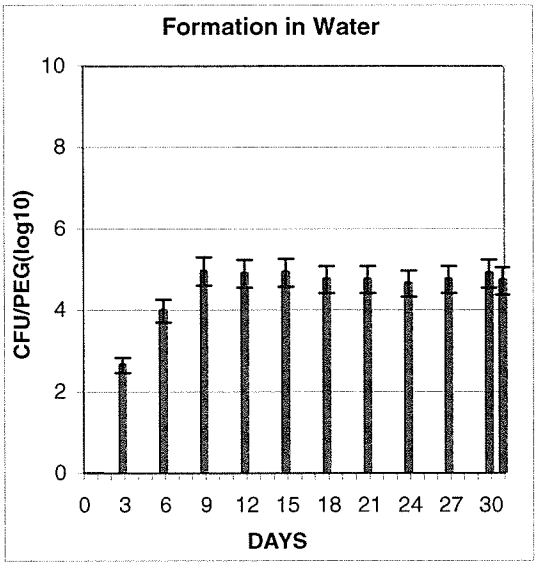


d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden

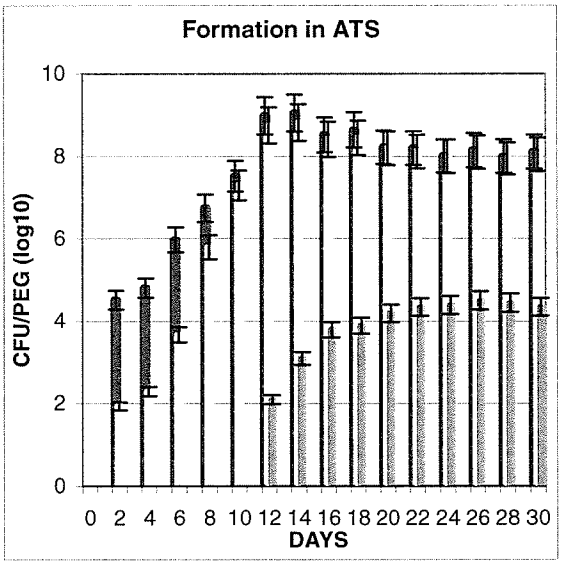


a) Traditional biofilm formation

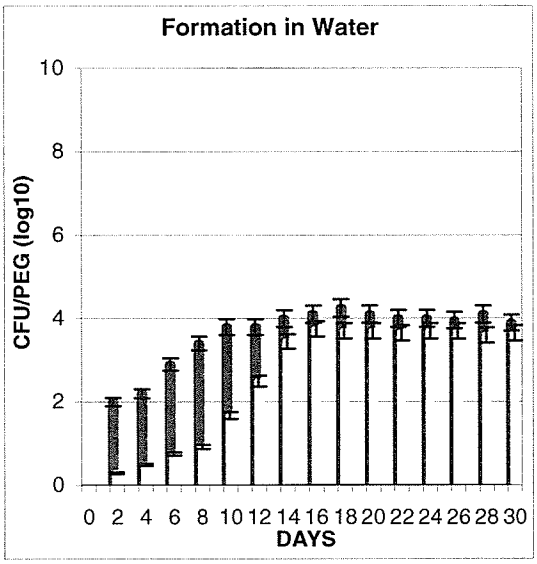




b) BBF: Repetitive Cycles of Drying

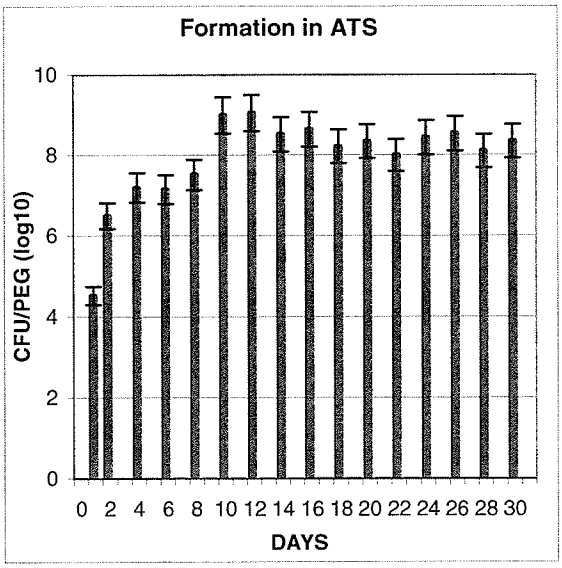


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

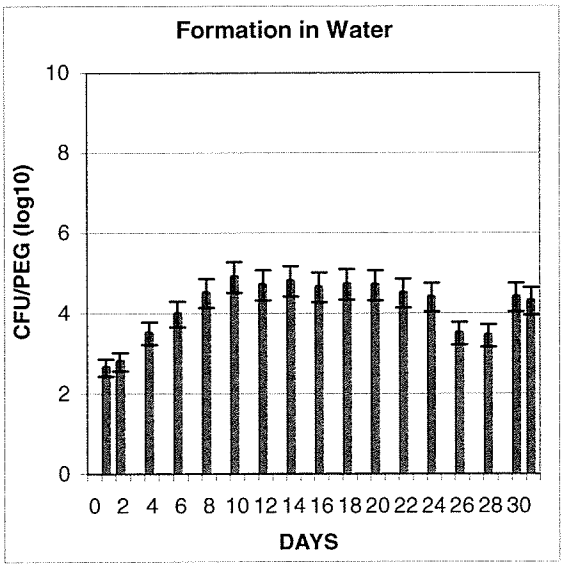


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

c) BBF: Repetitive Cycles of Drying and HLD

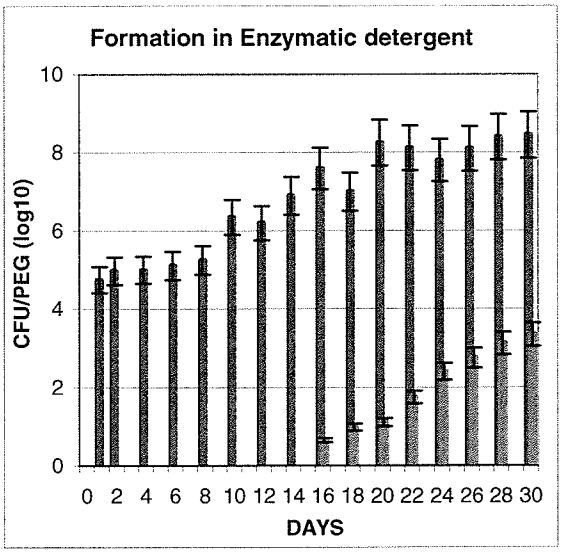


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

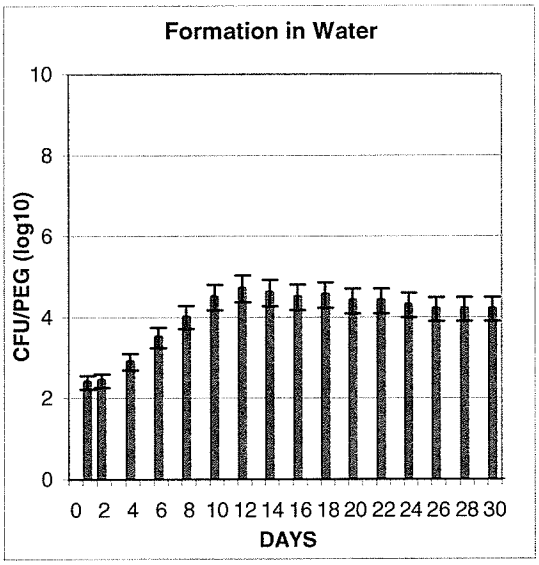


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

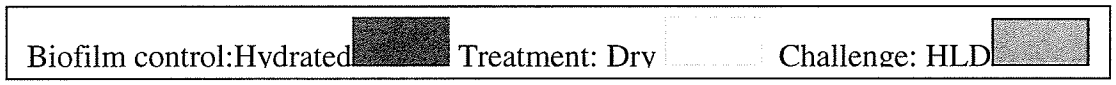


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 17. *C. albicans* challenged with Glutaraldehyde HLD

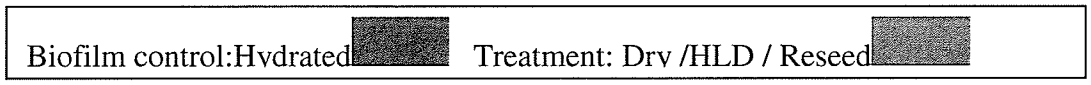
(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)

b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)

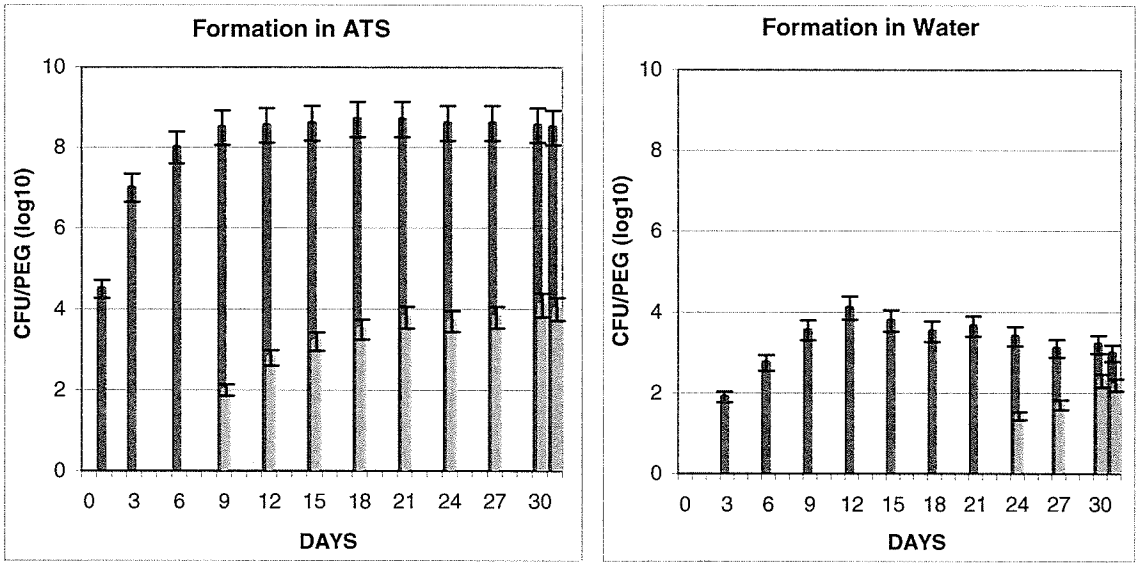


c) BBF: Repetitive Cycles of Drying and HLD

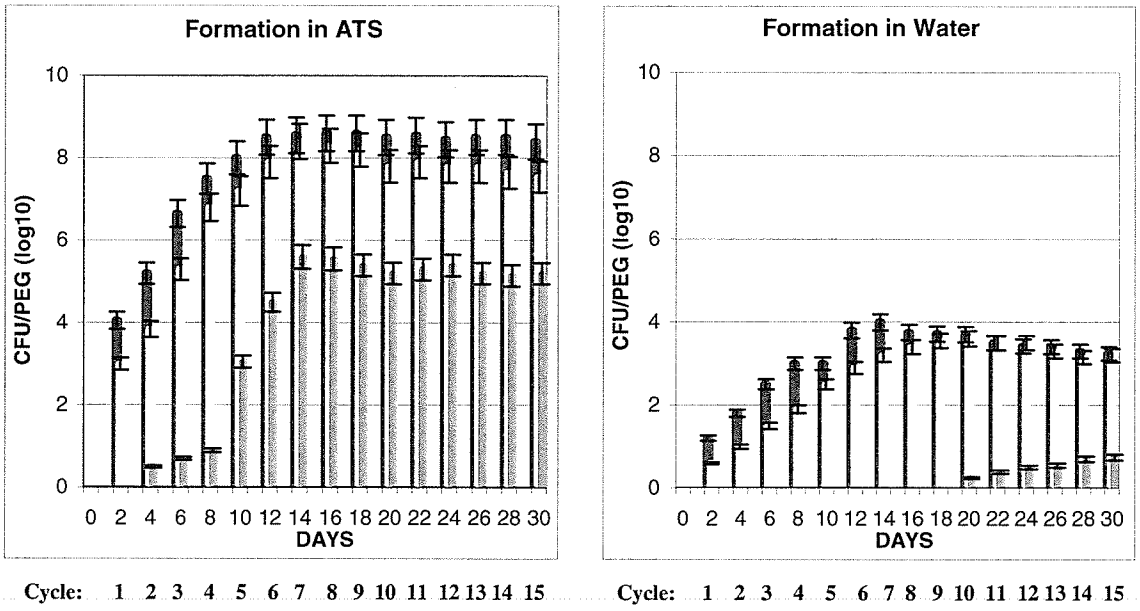
d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



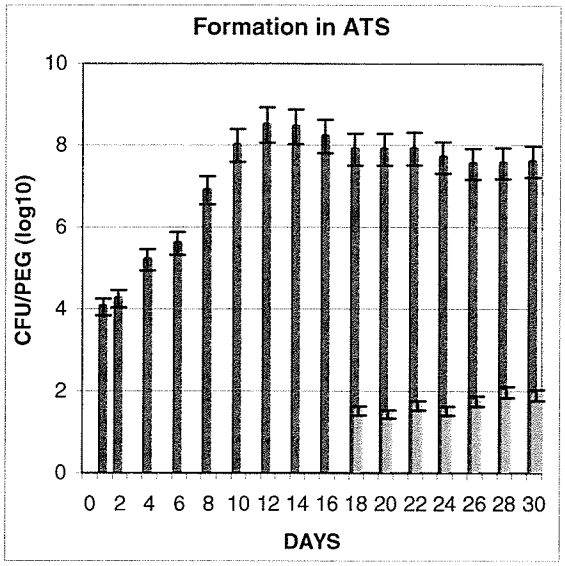
(a) Traditional biofilm formation



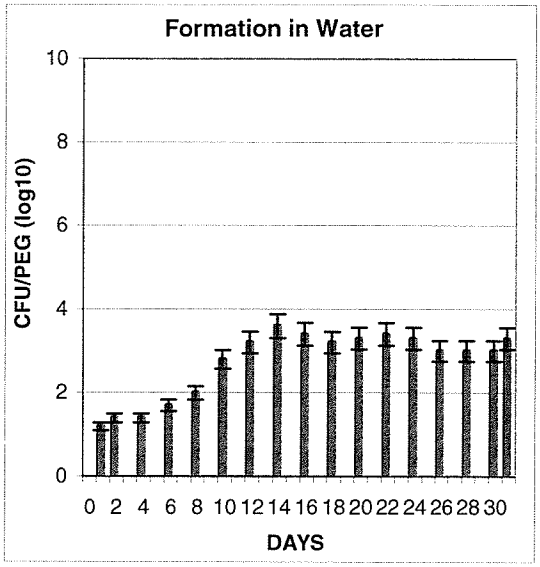
b) BBF: Repetitive Cycles of Drying



c) BBF: Repetitive Cycles of Drying and HLD

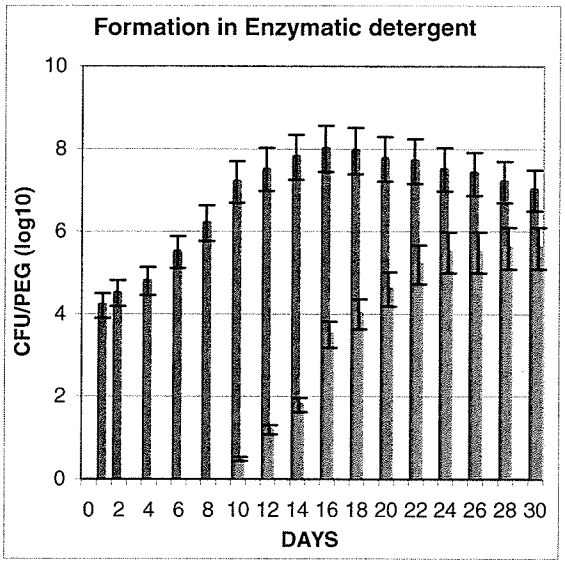


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

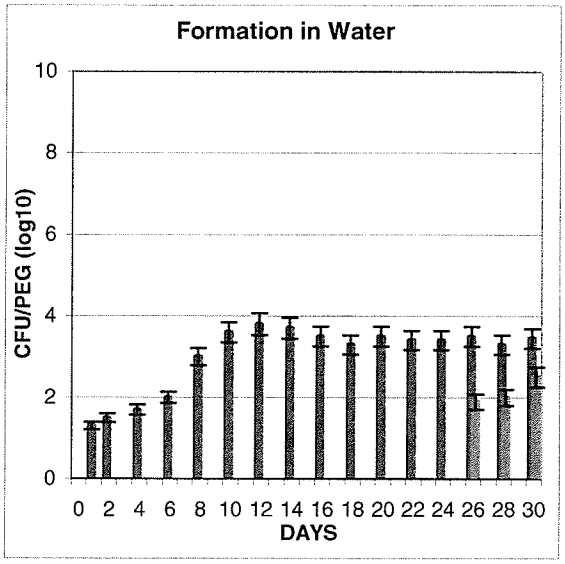


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



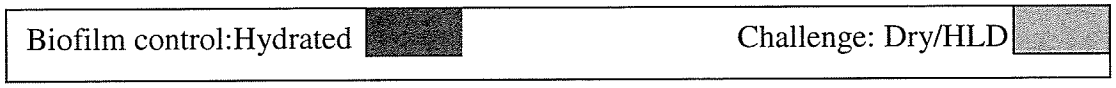
Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



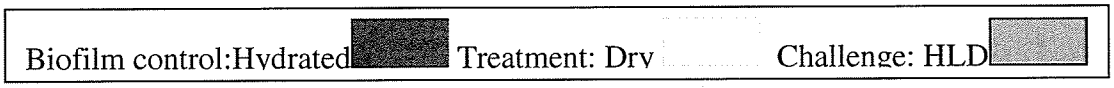
Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 18. ***C. albicans*** **following Accelerated HP- HLD**

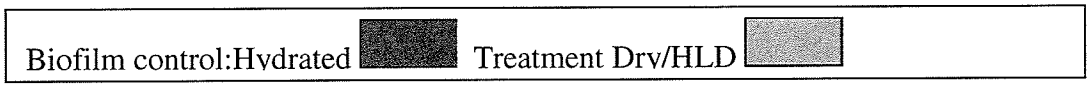
(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)



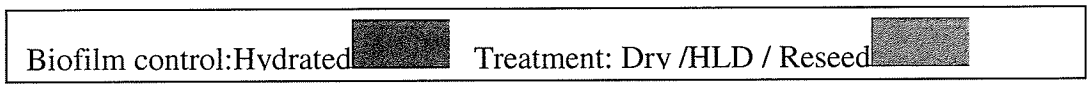
b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)



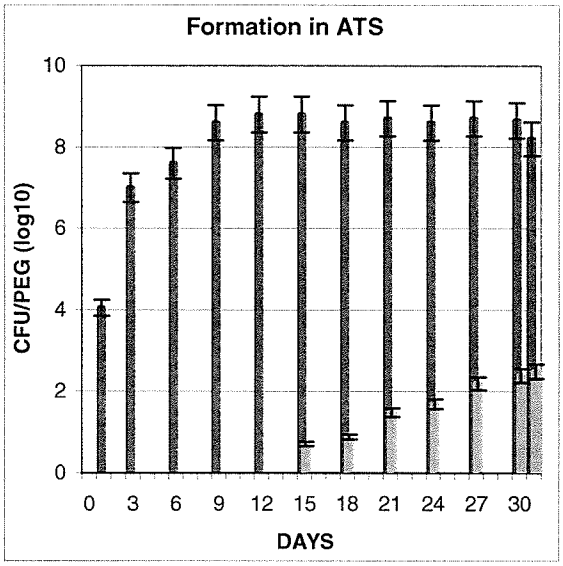
c) BBF: Repetitive Cycles of Drying and HLD

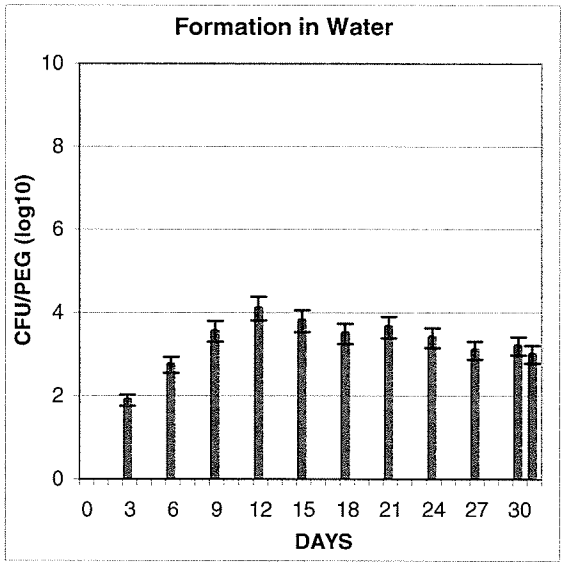


d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden

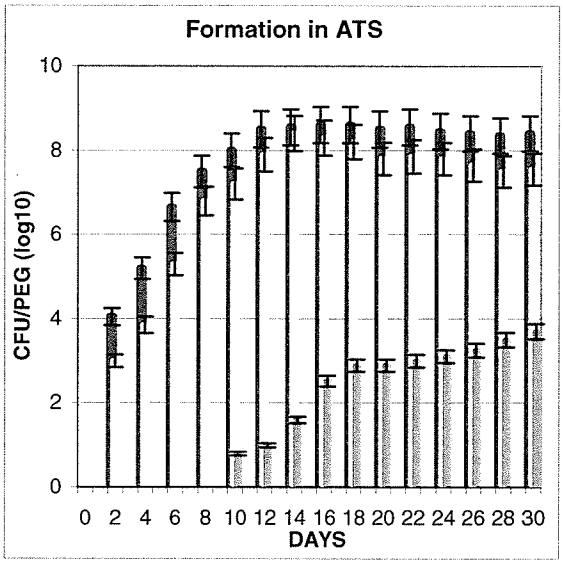


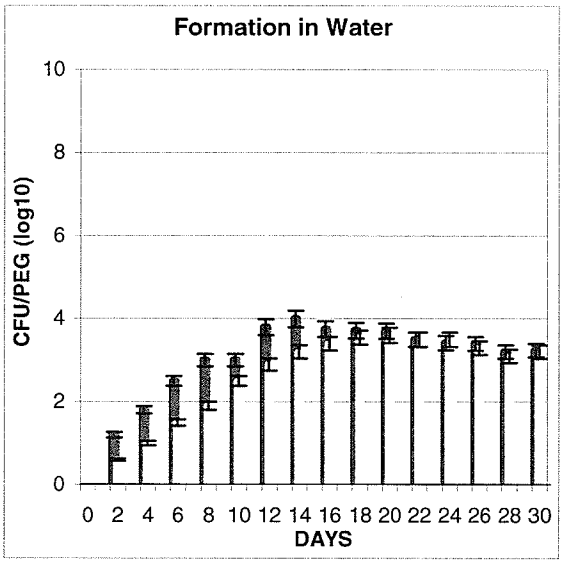
a) Traditional biofilm formation





b) BBF: Repetitive Cycles of Drying

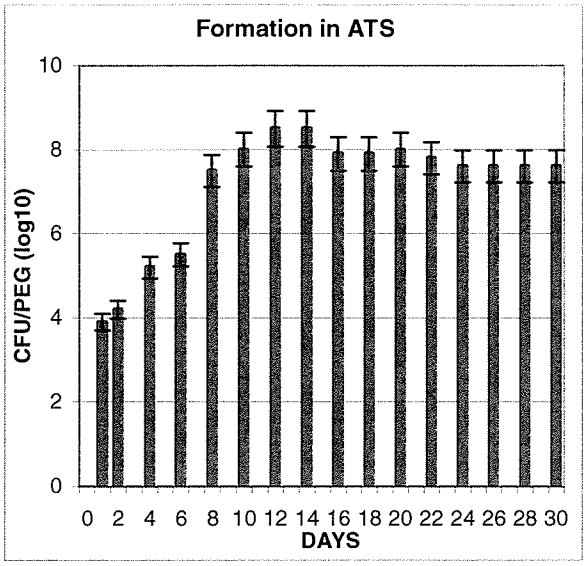




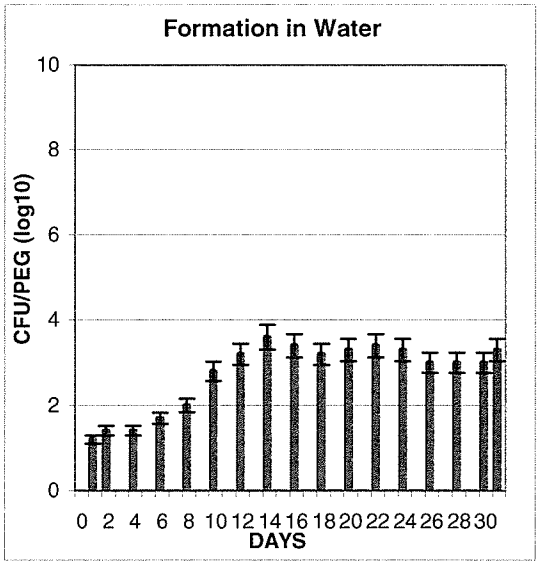
Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

c) BBF: Repetitive Cycles of Drying and HLD

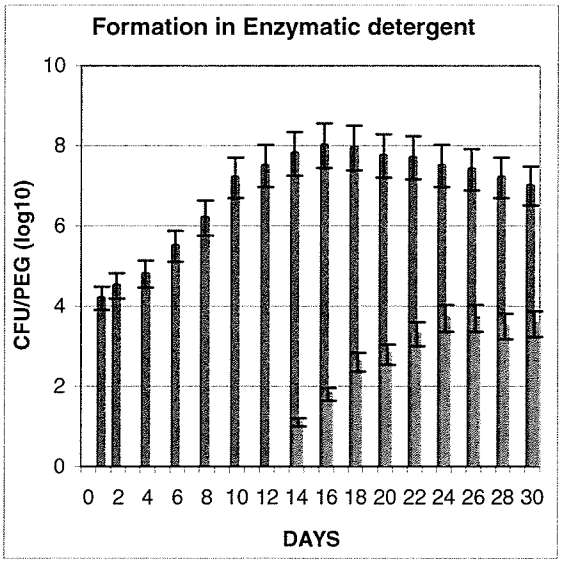


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

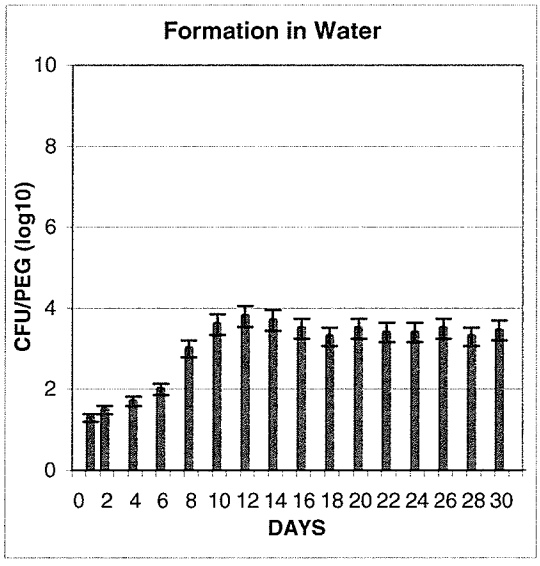


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

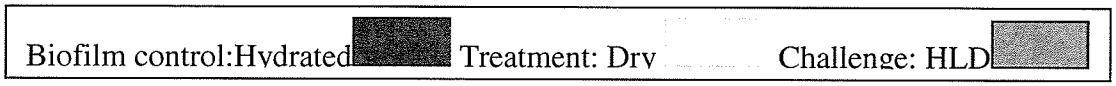


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

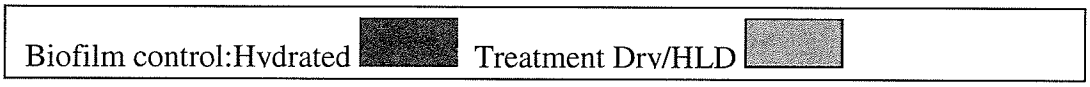
Figure 19. *M. chelonae*[R] challenged with Glutaraldehyde HLD

(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)

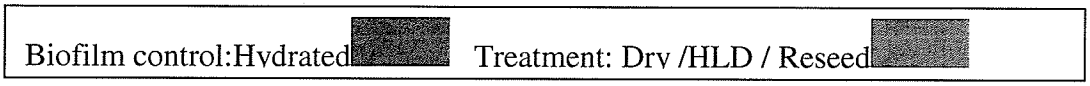
b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)



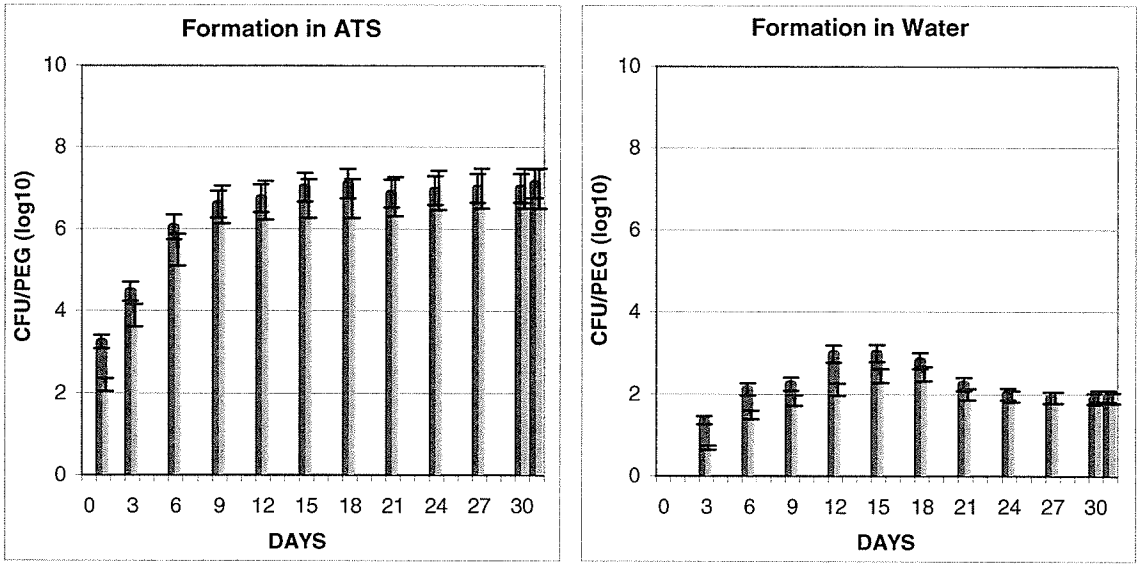
c) BBF: Repetitive Cycles of Drying and HLD



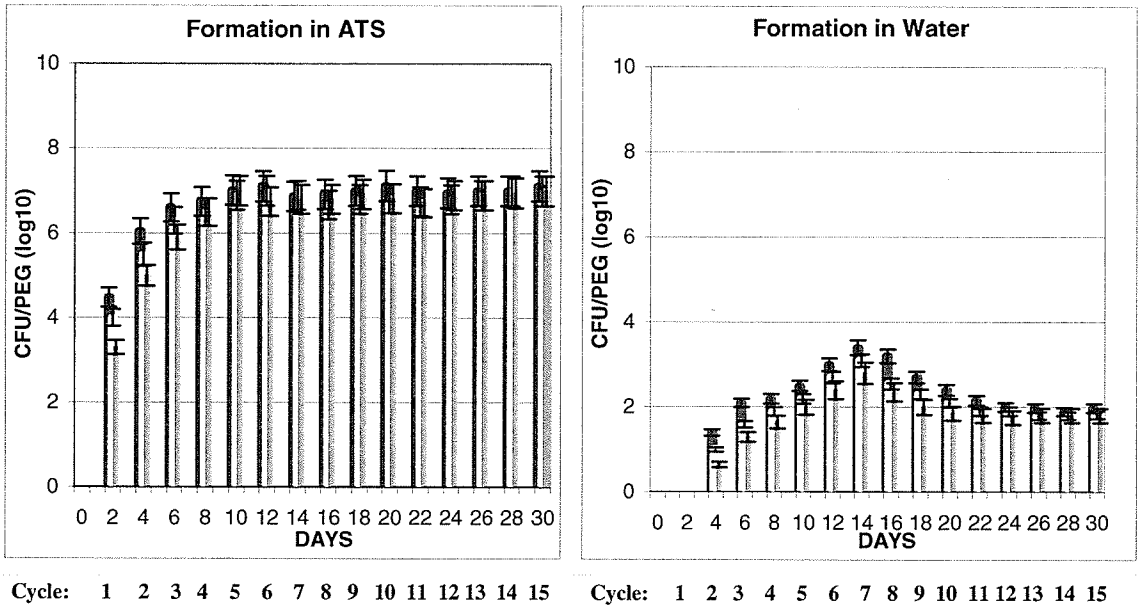
d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



(a) Traditional biofilm formation



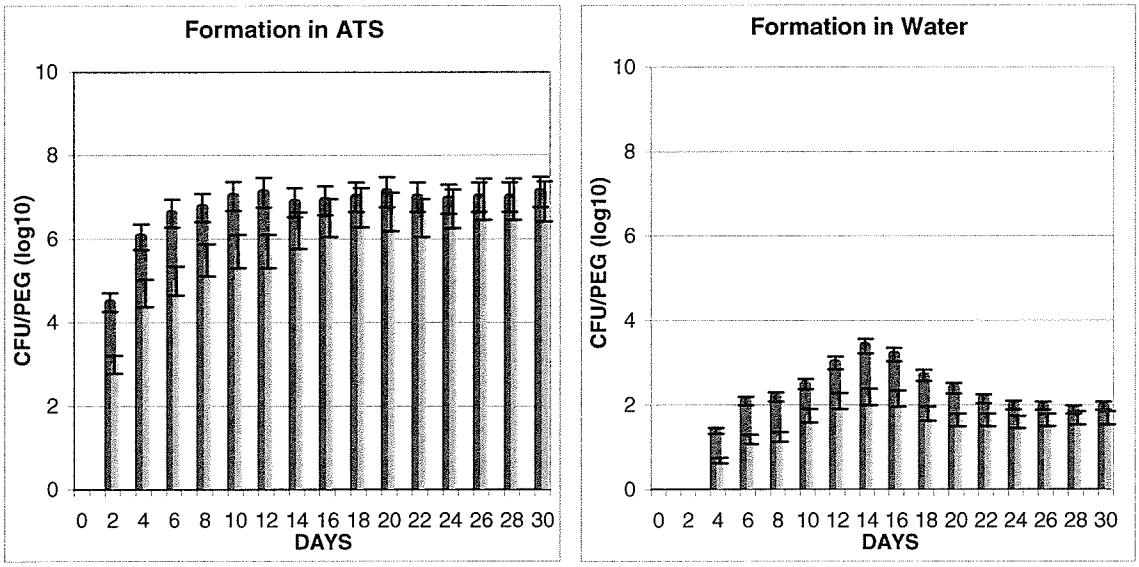
b) BBF: Repetitive Cycles of Drying



Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

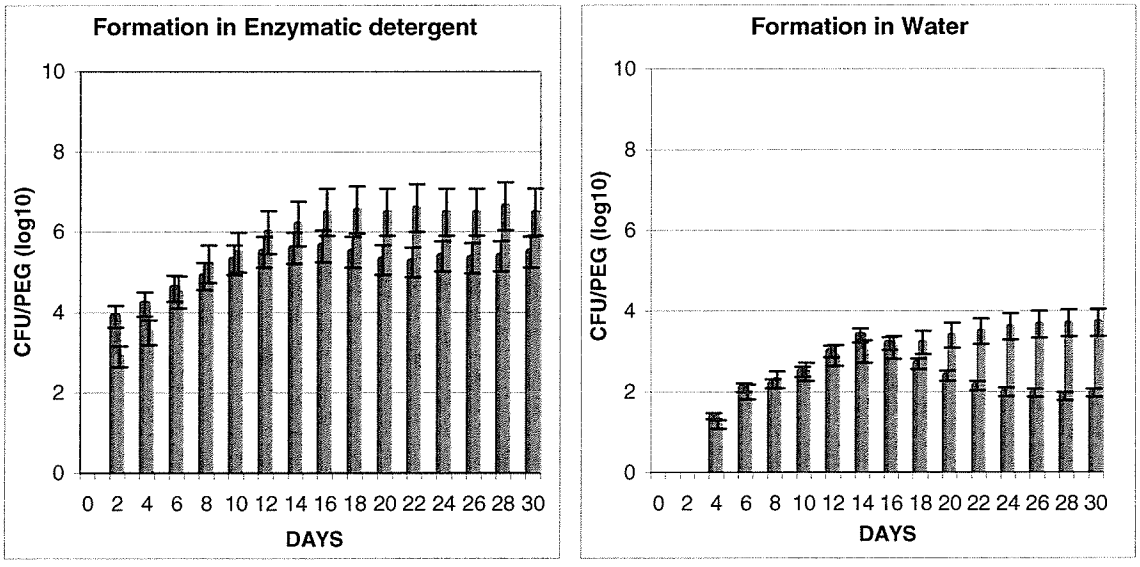
Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

c) BBF: Repetitive Cycles of Drying and HLD



Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

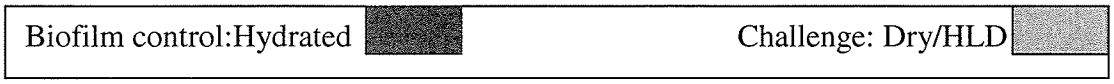
d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden



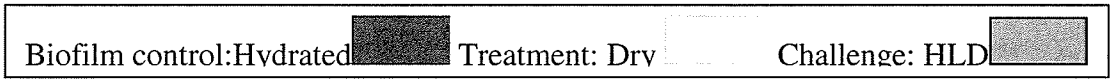
Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 20. *M. chelonae*^{R} challenged with AHP HLD

(a) Traditional biofilm formation (continuously grown in medium with sample pegs removed and challenged with drying and HLD)



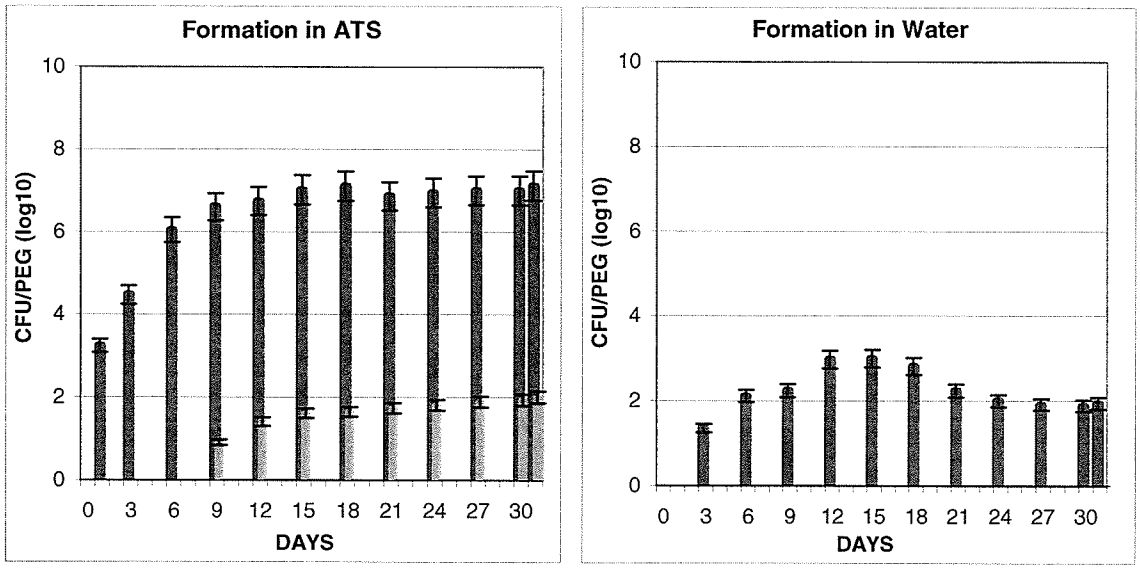
b) BBF: Repetitive Cycles of Drying (sample pegs removed, challenged with HLD)



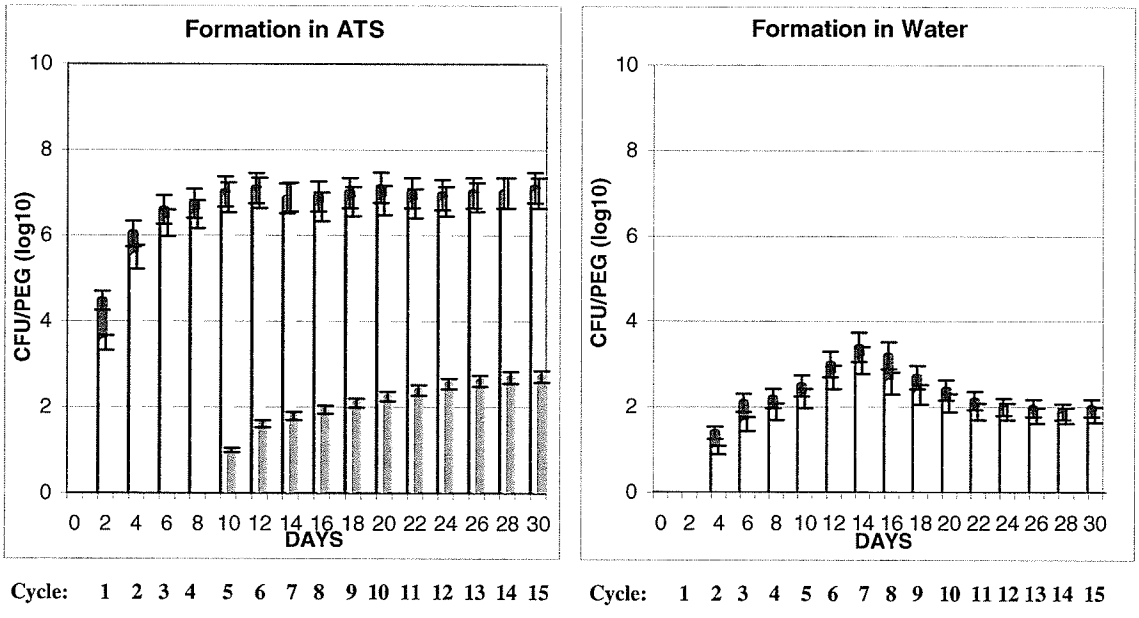
c) BBF: Repetitive Cycles of Drying and HLD

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden

(a) Traditional biofilm formation



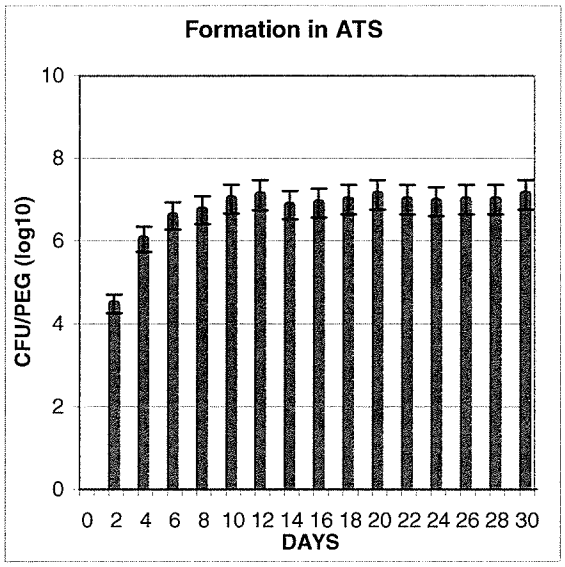
b) BBF: Repetitive Cycles of Drying

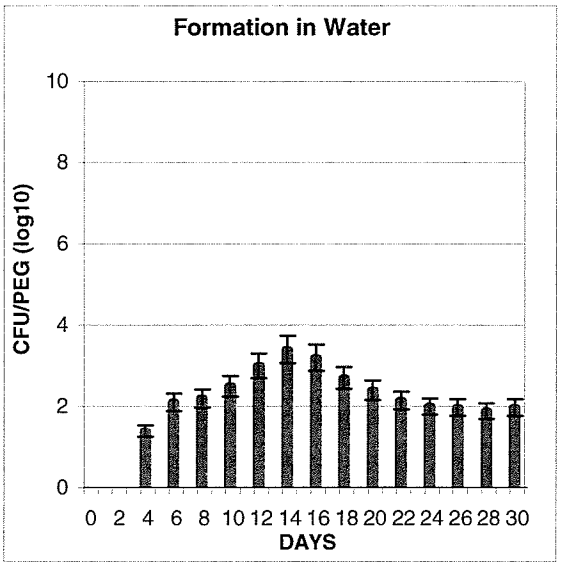


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

c) BBF: Repetitive Cycles of Drying and HLD

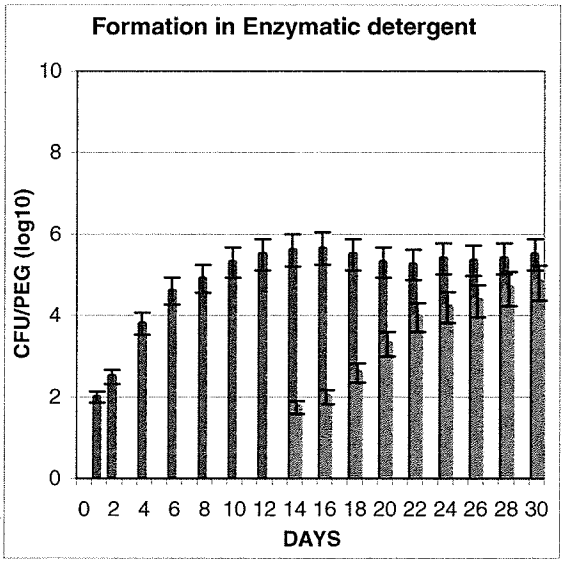


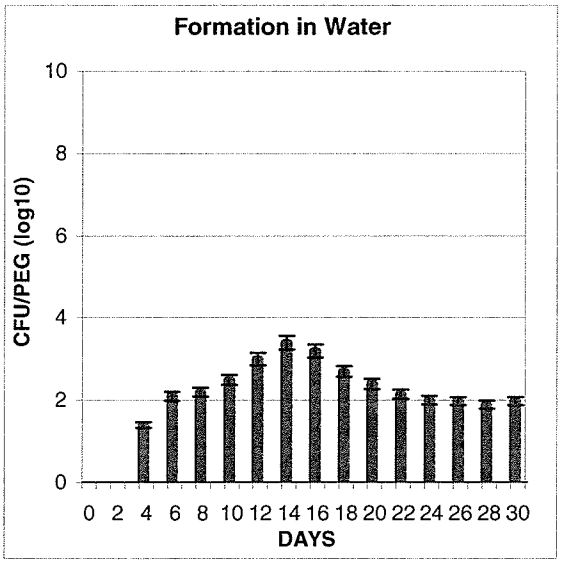


Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

d) BBF: Repetitive complete cycles of drying, HLD, reseeding of bioburden





Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Cycle: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Tables 9-12. Summary of Initiation of breakthrough survival in various biofilm formations [results are an average of 9 replicates; SD $\leq$ 10%]

The day or cycle when breakthrough growth was first detected is colour coded for each type of biofilm formation:

For Traditional biofilm the initial day of breakthrough is coded in: BLUE
For Buildup biofilm the initial cycle of breakthrough is coded in:

YELLOW -for Cyclic Drying
RED -for Cyclic Drying/HLD
GREEN -for Cyclic Drying/HLD/Reseeding

[1] High nutrient media (ATS or Enzymatic detergent)
[2] NBD: no breakthrough detected; limit of detection for viability counting is 10 cfu/peg
[3] Low nutrient media (Water)

Table 9. ***E. faecalis*:**
Initiation of breakthrough survival in various biofilm formations

(A) Biofilm formation in high nutrient media[1]

HLD challenge: GLUT

TBF-day	**0—2—4—6—8—10—12—14—15—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10 —11—12—13—14—15**	

HLD challenge: AHP

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	**NBD**[2]

LLD challenge: AHP

TBF-day	**0—2 3 4—6 —8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	

(B) Biofilm formation in low nutrient media[3]

HLD challenge: GLUT

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7— 8 — 9—10—11—12—13— 14—15 15**	**NBD**

HLD challenge: AHP

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	**NBD**
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	**NBD** **NBD** **NBD**

LLD challenge: AHP

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	**NBD**
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	**NBD** **NBD** **NBD**

Table 10. ***P. aeruginosa*:**
Initiation of breakthrough survival in various biofilm formations

(A) Biofilm formation in high nutrient media[1]

HLD challenge: GLUT

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15**	

HLD challenge: AHP

TBF-day	**0—2—4—6—8 9 10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15**	**NBD[2]**

LLD challenge: AHP

TBF-day	**0—2 3 4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15**	

(B) Biofilm formation in low nutrient media[3]

HLD challenge: GLUT

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15**	**NBD**

HLD challenge: AHP

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	**NBD**
BBF-cycle	**0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15**	**NBD** **NBD** **NBD**

LLD challenge: AHP

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	**NBD**
BBF-cycle	**0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15**	**NBD** **NBD** **NBD**

Table 11. *C. albicans*:
Initiation of breakthrough survival in various biofilm formations

(A) Biofilm formation in high nutrient media[1]

HLD challenge: GLUT

TBF-day 0—2—4—6—8—9—10—12—14—16—18—20—22—24—26—28—30

BBF-cycle 0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15

HLD challenge: AHP

TBF-day 0—2—4—6—8—10—12—14—15—16—18—20—22—24—26—28—30

BBF-cycle 0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15 NBD[2]

LLD challenge: AHP

TBF-day 0—1—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30

BBF-cycle 0—1—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15

(B) Biofilm formation in low nutrient media[3]

HLD challenge: GLUT

TBF-day 0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30

BBF-cycle 0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15 NBD

HLD challenge: AHP

TBF-day 0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30 **NBD**

BBF-cycle 0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15 NBD
NBD
NBD

LLD challenge: AHP

TBF-day 0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30

BBF-cycle 0—1—2—3—4—5—6—7—8—9—10—11—12—13—14—15 NBD

Table 12. *M. chelonae*^{R}:
Initiation of breakthrough survival in various biofilm formations

(A) Biofilm formation in high nutrient media[1]

HLD challenge: GLUT

TBF-day	**0-1-2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0-1 1 1 -2—3—4— 5 — 6 — 7— 8 — 9—10—11—12—13— 14 —15**	

HLD challenge: AHP

TBF-day	**0—2—4—6—8 9 10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	**NBD**[2]

LLD challenge: AHP

TBF-day	**0-1-2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0- 1 1 -2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	

(B) Biofilm formation in low nutrient media[3]

HLD challenge: GLUT

TBF-day	**0-1-23-4—6—8—10—12—14—16—18—20—22—24—26—28—30**	
BBF-cycle	**0 —1-2 2 2-3—4— 5 — 6 — 7— 8 — 9—10—11—12—13— 14—15**	

HLD challenge: AHP

TBF-day	**0—2—4—6—8—10—12—14—16—18—20—22—24—26—28—30**	**NBD**
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	NBD NBD NBD

LLD challenge: AHP

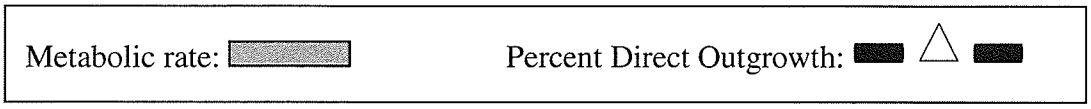
TBF-day	**0—2—4—6—8—10—12—14—16—18—2021 22—24—26—28—30**	
BBF-cycle	**0—1—2—3—4— 5 — 6 — 7 — 8 — 9—10—11—12—13— 14—15**	**NBD**

Table 13. Rate of biofilm survival in TBF[1] and BBF[1] following HLD with GLUT and AHP

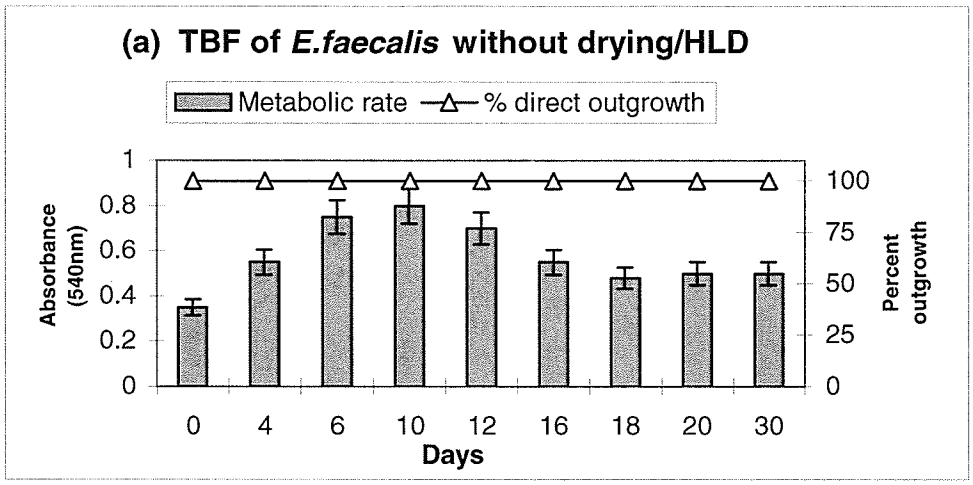
	Rate of survival [2]			
Organism	**TBF Treatment: GLUT**	**BBF Treatment: GLUT**	**TBF Treatment: AHP**	**BBF Treatment: AHP**
E. faecalis	2	**10**[3]	2.3	3.1
P. aeruginosa	2	**8.6**[3]	3.4	4.9
C. albicans	2	**9.3**[3]	3.4	3.8
M. chelonae[R]	2.1	2.2	2	2.4

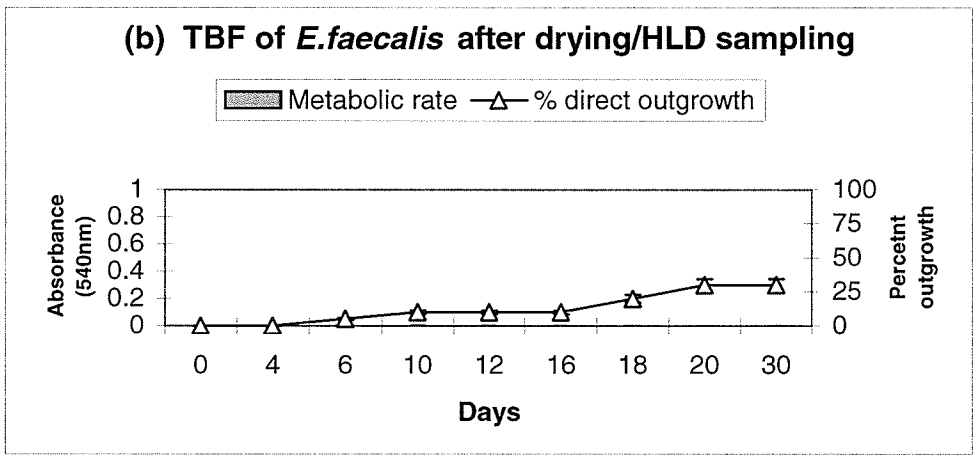
[1] TBF and BBF (complete cycling) grown under high nutrient conditions
[2] Rate of survival was calculated by the ratio of:
Maximum Log_{10} CFU / Initial breakthrough Log_{10}CFU
[3] BBF demonstrated significantly greater increase in survival once survivors were detectable, particularly following GLUT challenge for *E. faecalis*, *P. aeruginosa* and *C. albicans* (Student's t-test, $p<0.0001$).

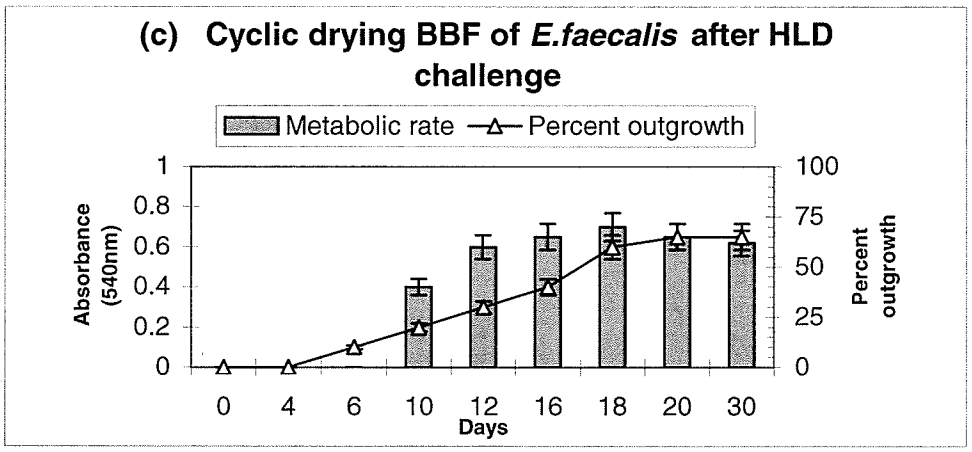
Figure 21. Metabolic rate[1] and viability[2] of *E. faecalis* in TBF and BBF



(a) TBF (without drying/HLD)
(b) TBF after drying/HLD sampling
(c) BBF formed by repetitive cycles of drying.
All biofilm was formed in ATS.
[1]Metabolic rate is measured in the redox assay by absorbance reading at 540 nm.
[2]Viability measured by direct qualitative outgrowth, calculated as percent outgrowth by:
Number positive outgrowths / Number of pegs tested x 100


(a) TBF of E.faecalis without drying/HLD
Metabolic rate
% direct outgrowth
Absorbance (540nm)
Percent outgrowth
1
0.8
0.6
0.4
0.2
0
100
75
50
25
0
0 4 6 10 12 16 18 20 30
Days


(b) TBF of E.faecalis after drying/HLD sampling
Metabolic rate
% direct outgrowth
Absorbance (540nm)
Percetnt outgrowth
1
0.8
0.6
0.4
0.2
0
100
75
50
25
0
0 4 6 10 12 16 18 20 30
Days


(c) Cyclic drying BBF of E.faecalis after HLD challenge
Metabolic rate
Percent outgrowth
Absorbance (540nm)
Percent outgrowth
1
0.8
0.6
0.4
0.2
0
100
75
50
25
0
0 4 6 10 12 16 18 20 30
Days

5. Determination of survivability and recoverability of viable organisms in TBF and BBF following HLD

The qualitative indirect outgrowth testing method was used to establish the detectibility and recoverability of damaged and/or low levels of viable organisms embedded within TBF or BBF. This protocol encouraged the release of embedded organisms by sonication coupled with recovery and growth in enriched media over 30 days (a similar time period to the cycling protocol).

Results from the indirect qualitative outgrowth test for TBF or BBF (e.g., cyclic drying/HLD) formed in ATS and challenged by GLUT were compared to replicate pegs where bioburden was directly removed and viability assessed by the quantitative viability test (without the enhanced recovery protocol) (Table 14). Results from the indirect outgrowth method verify microbial survival to GLUT HLD in TBF and BBF. However the indirect qualitative outgrowth method detected survivability earlier in both TBF and BBF than quantitative counting (e.g. for *E. faecalis* and *P. aeruginosa* survival was detected by testing on Day 3 by outgrowth testing only). Results in BBF also demonstrated survival of organisms throughout the BBF cycling period, including in the earlier cycles where organisms could not be detected by quantitative counting methods. Outgrowth results also indicated that as TBF matured, survivability increased (e.g. the number of positive pegs increased and the average time to detect viability decreased). However as more cycles of BBF occurred, survivability or recoverability decreased (e.g., the number of positive pegs decreased, requiring a longer time to detect viability compared to TBF). A similar trend as seen in BBF from repetitive cycles of drying and

HLD was seen in BBF resulting from complete cycles, but higher loads of surviving organisms resulted (data not shown).

When similarly challenged with AHP HLD, survival was detected in mature TBF for *E. faecalis* and *P. aeruginosa* by indirect qualitative outgrowth and quantitative counts (although *E.faecalis* required longer maturation time before survival resulted). Similar to the GLUT challenge, as TBF matured, survivability to AHP increased. However, viability was not detectable in BBF formed by repetitive cycles of drying and AHP HLD by any of the test/recovery methods. Overall, survivability was seen less frequently in BBF compared to TBF when challenged with AHP HLD.

Overall, the results indicated that the indirect qualitative outgrowth was a more sensitive method to detect the presence of viable organisms than the quantitative viability counting method. Even when BBF was formed under the most challenging conditions (repetitive cycles of drying and HLD), this method facilitated the detection of microbial survival.

6. Recoverability of *Reovirus* from BBF formed by repetitive cycles of drying and HLD compared to BBF from complete cycles

This study addressed the possible survivability and recoverability of a virus from biofilm formation. *Reovirus* was introduced into two types of BBF conditions: (1) BBF resulting from repetitive cycles of drying and HLD with growth facilitated by ATS, and (2) BBF resulting from complete repetitive cycles (repetitive drying, repetitive HLD, repetitive bioburden exposure with growth facilitated in an enzymatic detergent).

Table 14. Indirect qualitative outgrowth testing for survival of organisms embedded in TBF or BBF[1] following HLD[2] compared to quantitative viability[3] results

HLD: 2.6% GLUT

Microorganism: BFF	Average CFU/peg[4] (Log_{10})	Growth[5] # Positive/#Tested	Average time to detection (days)
E. faecalis:			
TBF – Day 3	< LD[6]	2/9	8
TBF – Day 15	1.8	2/9	1
TBF – Day 30	2.9	4/9	1
BBF – Day 3	< LD	2/9	30
BBF – Day 15	< LD	1/9	10
BBF – Day 30	1.75	1/9	1
P. aeruginosa:			
TBF – Day 3	<LD	3/9	4
TBF – Day 15	3.7	7/9	1
TBF – Day 30	4.8	9/9	1
BBF – Day 3	< LD	4/9	20
BBF – Day 15	< LD	2/9	10
BBF – Day 30	1.9	2/9	1

HLD: 7% AHP

Microorganism: BFF	Average CFU/peg[4] (Log_{10})	Growth[5] # Positive/#Tested	Average time to detection (days)
E. faecalis:			
TBF – Day 3	< LD[6]	0/9	-
TBF – Day 15	< LD	0/9	-
TBF – Day 30	1.6	**2/9**	1
BBF – Day 3	< LD	0/9	-
BBF – Day 15	< LD	0/9	-
BBF – Day 30	< LD	0/9	-
P. aeruginosa:			
TBF – Day 3	<LD	0/9	-
TBF – Day 15	1.6	**4/9**	1
TBF – Day 30	2.5	**4/9**	1
BBF – Day 3	< LD	0/9	-
BBF – Day 15	< LD	0/9	-
BBF – Day 30	< LD	0/9	-

[1] 3-day cyclical drying/HLD in ATS medium
[2] HLD for cyclic BBF pegs and sample pegs of TBF (formed in ATS)
[3] Quantitative viability testing on replicate pegs (without enhanced recovery protocol)
[4] Average of 9 pegs
[5] Indirect qualitative outgrowth (enhanced recovery protocol):
TBF or BBF peg placed in 10% FBS-TSB, shake/sonicate/vortex (as per extraction method), then continue to incubate at 35°C / 30d
[6] LD: limit of detection is 10 CFU/peg for quantitative viability counts

To measure viral presence and recoverability in the biofilm, preliminary tests were undertaken, which established the viral titre in a CPE assay by using a TCID50 method, similar to the CPE assay described earlier for *Sindbis virus*. The resultant titre was 7 x 10^7 CPE infective units/mL. The biofilm medium, ATS, and the extraction medium (sPBS) were tested and found to be non-toxic to the cells in the assay, confirming results from the earlier carrier surface survival experiments. The viability of L929 cells throughout the study was confirmed in wells receiving cell culture medium only. Studies to determine the efficacy of the viral recovery method revealed that ~50% of viral load was recoverable from the viral spiked biofilm following extraction from the MBEC pegs and microfiltration.

Biofilm formations in ATS by *E. faecalis, P. aeruginosa*, and *C. albicans* were compared over a period of 20 days. A viral spike of ~5 x 10^7 CPE infective units/mL was added to the test biofilm as described in Materials and Methods. Survivability and recoverability of *Reovirus* from biofilm was investigated using TBF (with no drying or HLD) as a positive control. The CPE assay for viral recovery from biofilm was qualitative, indicating either a positive or negative recovery of virus. Under these testing conditions, the positive biofilm control resulted in recovery of the virus from the TBF of all organisms tested at various time points to 20 days.

When BBF formed by repetitive cycles of drying and GLUT HLD was tested for viral recovery, no viral recovery was achieved with any of the organisms tested.

However there was sporadic recovery of virus in BBF of complete cycles (involving drying, HLD and reseeding of bacteria and virus) when challenged with GLUT HLD, but not following AHP HLD. Table 15 shows an example of the results for BBF formed by *P. aeruginosa*. However recovery from GLUT HLD was only seen in BBF formed by *P. aeruginosa* and *C. albicans*, and not *E. faecalis* (results of *C. albicans*, and *E. faecalis* are not shown).

7. Statistical analysis

MBEC statistical analyses showed no significant variation in counts regardless of peg location on or between plates, indicating equivalent biofilm formation. Resulting statistical determinations using ANOVA for each test organism were: *E. faecalis*, $p = 0.252$; *P. aeruginosa*, $p = 0.153$; *C. albicans*, $p = 0.893$; and *M. chelonae*^{R}, $p = 0.665$. Comparison of the maximum bioburden resulting in TBF and BBF following either GLUT or AHP challenge resulted in significant statistical differences ($p<0.0001$) using the Student's t-test for all organisms in the following comparisons: TBF compared to BBF challenged with either GLUT or AHP; GLUT compared to AHP challenge of either BBF or TBF; High compared to low nutrient conditions for TBF or BBF, challenged with either GLUT or AHP; BBF formed under high nutrient conditions compared to TBF formed under low nutrient conditions, when challenged with GLUT (i.e., BBF modeling of patient-use and reprocessing compared to TBF resulting from patient-use and wet storage); BBF formed after 10 cycles compared to TBF after a single round of testing, with formation under high nutrient conditions and exposure to GLUT (comparing the kill ability of GLUT in BBF over time to one round of testing in TBF).

Table 15: Recovery of *Reovirus* from BBF[1] complete cycles

	VIABLE *REOVIRUS* DETECTED (Number of positive viral recoveries / number of trials)			
HLD Challenge	**Positive control[2] Day 3**	**Positive control Day 6**	**Positive control Day 10**	**Positive control Day 20**
No HLD	3/3	3/3	3/3	3/3
	BBF Cycle 1	**BBF Cycle 3**	**BBF Cycle 5**	**BBF Cycle 10**
GLUT	1/3	1/3	0/3	1/3
AHP	0/3	0/3	0/3	0/3

[1] BBF resulting from 2-day complete cycles of dry/HLD/reseeding
BBF was generated by *P. aeruginosa.*
Reseeding was with both *P. aeruginosa* and *Reovirus.*
Testing was done post cycle HLD/drying, but pre reseeding

[2] Positive control was continuous biofilm formation (TBF) over 20 days without any challenge of drying or HLD.

DISCUSSION

1. Overview

Cross contamination between surfaces, equipment, and patients, either directly or indirectly, is a leading cause of infection transmission in the healthcare setting.[1, 6, 13, 21, 53, 61, 74, 86, 92, 101,114,122, 129,137,138,141,146] Therefore it is important to understand microbial survival so that an effective means of preventing nosocomial transmission can be developed.

This research focused on the transmission of infectious agents by complex medical devices such as flexible endoscopes. The project hypothesized that the biofilm that forms in narrow lumen flexible endoscopes is a buildup biofilm (BBF) that develops as a result of cyclical exposure to wet/dry phases in the usage/processing protocol. Furthermore, it was hypothesized that the BBF has a unique composition and different microbial survival characteristics compared to traditional biofilm formation (TBF) that forms when a surface is constantly bathed in fluid. Specifically, this research wanted to determine if BBF results in a matrix/biofilm formation representing a greater challenge to disinfectant penetration and microbial eradication than TBF.

As complex medical devices such as flexible endoscopes are used on patients, the internal channels of the scope are coated with patient secretions facilitating microbial adherence and biofilm formation. Thereafter, scopes are reprocessed involving cleaning, disinfection, and drying. No data is currently available to detail what occurs with respect to microbial survival and transmissibility. However, there is some evidence that flexible

endoscopes undergoing repeated rounds of patient-use and exposure to reprocessing accumulate a buildup of bioburden and biofilm.[10,94,100] A unique contribution of this research was the development of a model with the capability to not only evaluate TBF in this situation, but more significantly to evaluate BBF. Using the MBEC model system, this project has demonstrated that buildup is much more pronounced when a cross-linking agent is used as the disinfectant. The combination of an organic matrix and aldehyde disinfection quickly produces a protective BBF that facilitates high levels of organism survival. The data showed that if an oxidizing agent was used for disinfection and if organic levels were kept low, organism survival did not occur. AHP was used as an example of an oxidizing agent. Other oxidizing agents, such as peracetic acid, which are more commonly used agents in endoscope reprocessing, were not specifically evaluated in this project. However, the expectation is that the findings would likely mimic those for the AHP formulation.

The data demonstrated that the impact of repeated rounds of reprocessing on buildup in endoscope channels is no longer merely a theoretical speculation. A key finding in the research was that once established, the microbial load of BBF has a faster rate of accumulation than in TBF in dried bioburden. Therefore, the chance of organisms coming off and being transmitted is much greater once BBF has developed. The implications are that as flexible endoscopes are repeatedly used and reprocessed, the load of bioburden increases, as does the risk of transmission of pathogens.

2. The biofilm modeling system

A novel application of the MBEC system was developed allowing researchers to better investigate repeated rounds of use and reprocessing of flexible endoscopes. Many models of biofilm formation exist, but do not address the repeated use that is inherent for reusable complex medical devices. A number of approaches for modeling biofilm development traditionally include carriers with dried bioburden, microtitre plate wells with adherent bacterial monolayers,[89] tubing with biofilm[140] and the MBEC system for TBF.[28]

Most notably in this project, the novel use of the MBEC system as an in-vitro model of BBF, using repetitive cycles of reprocessing stages (i.e., repetitive cycles of exposure to organic sources (e.g., ATS, enzymatic detergents, etc.), drying and/or disinfectant challenge and/or bioburden) has provided a more accurate model of what occurs in patient-used narrow-lumened devices compared to TBF. The BBF model was designed to mimic the composition and kinetic formation of BBF found in patient-used GI (and respiratory) endoscopes. Because the MBEC system can be used for TBF as well as BBF, the focus of this work was on differences between these biofilm formations.

2.1. Model development

The MBEC system was found to produce similar trends and levels of biofilm formation as the biofilm loop system. Both systems provided little variation and good reproducibility as noted in other published studies for biofilm loop type models.[140] The MBEC system facilitated direct comparisons of different test microorganisms under a

variety of biofilm conditions. In agreement with other reports,[28, 57, 98] the MBEC system was: a rapid, reproducible system; capable of high-throughput microbicide susceptibility testing of microbial biofilm; and applicable for a wide variety of organisms. The MBEC system is most often used to study bacterial biofilms and comparative susceptibilities to antibiotics. The minimum biofilm eradication concentration has been proposed as the standard for chemotherapeutic drug and biocide testing.[28, 57] In this work, the effect of biofilm formation on disinfectant efficacy was studied, similar to other published research.[57] However, in this application the MBEC value (i.e., the lowest dilution of antimicrobial at which bacteria fail to regrow) was not investigated. Rather, the day or cycle of biofilm formation where viability was first detected (i.e., breakthrough survival) following disinfectant challenge and increase in microbial load over time was assessed. The development of biofilm at room temperature was studied rather than at 35°C, as this is the appropriate temperature for medical device reprocessing. Biofilm formed more slowly at this temperature, therefore to ensure maturity of the biofilm, the studies were carried out over 30 days rather than 24 to 48 hours as in other MBEC studies.[57]

When looking at biofilm (TBF or BBF) accurate quantitation of organisms depends on appropriate harvesting from the surface. This research used a combination of vortexing, shaking and sonication, which was considered applicable to both TBF and BBF. The removal of biofilm was verified by live/dead staining of eluted surfaces, where no detection of live cells translated to less than 3 Log_{10} viable organisms (limit of detection); by direct qualitative outgrowth testing, which indicated no or low numbers of bacteria remained attached to the surface (due to length of time for turbidity to result); and by

repeating the recovery process on MBEC pegs, to verify significant numbers of microorganisms did not remain on the surfaces after the recovery process.

To accurately detect microbes in the MBEC model (TBF and BBF), it was important to incorporate multiple methods of assessment[148] since viability may be masked or underestimated in certain circumstances. For example, biofilm cells recovered from surfaces may exhibit poor growth on agar plates potentially resulting in false culture negative results.[36] In this work, multiple methods were used to assess viability including: viable plate counts, metabolic rate (redox) and direct and indirect qualitative outgrowth in growth medium. Comparison based studies demonstrated that an indirect method of qualitative outgrowth was most sensitive in the detection of viable organisms present in test samples, particularly following HLD challenge (as shown in Figure 21 and Table 14). That method facilitated the release and recovery of HLD-damaged but viable microorganisms even if they were trapped in the matrix. In contrast, direct qualitative outgrowth is based on cultivation of accessible organisms. Overall, the testing methods confirmed the reliability of using multiple methods of quantitation in TBF and BBF. Although not used in this study, a single test method of CTC-DAPI staining has been reported to be a good indicator of killing ability of surface-associated bacteria since it incorporates cultivation, image analysis, and metabolic measurement.[148]

3. Biofilm formation relevant to flexible endoscope channels

Flexible endoscopes are repeatedly used and reprocessed up to thousands of times per year, with a resultant continual re-exposure to cycles of wet and dry phases. It is expected

that following patient-use, reprocessing will effectively eliminate all residual bioburden (microbes and organic matter from patient secretions). However it is now known that there is an accumulation of buildup material as a result of continual cycles of patient-use and reprocessing. This buildup of material in the narrow lumen of patient-used endoscopes that are reprocessed and stored dry would have a unique composition and microbial proliferation, biofilm formation and survival characteristics compared to biofilm formed under conditions of undisturbed continual hydration.[8, 94] The microbial survivability within this BBF has not been well understood to date. Therefore the study of buildup biofilm was considered in this research, as the most representative of conditions found in reprocessed endoscopes compared to studies reflective of a single round of reprocessing. The MBEC model of BBF represented a worst-case scenario, i.e. assuming biofilm formation had formed on the medical device.

In this research study, buildup biofilm resulting from repetitive cycles of individual stages and cumulative stages of reprocessing were studied; with the complete repetitive cycles (repetitive drying, repetitive HLD and repetitive bioburden exposure) being the most likely simulation of residual biofilm found in reprocessed scopes. It was hypothesized that the buildup of patient soil in GI flexible endoscopes resulted in a buildup biofilm presenting a greater challenge to disinfection penetration than either dried patient soil alone or traditional biofilm formation. The MBEC BBF model (and data generated from it) was a novel attempt to address the consequences of microbial survival in reprocessed narrow-lumen medical devices. This model has provided relevant data

regarding the kinetics of BBF over time compared to TBF and the resultant protective characteristics of each biofilm type.

TBF forms under conditions of continual hydration. Therefore, TBF associated with endoscopes has predominately been associated with water sources, involving *Legionella* spp, *Mycobacteria* spp and *P. aeruginosa*, in particular with endoscope washer-disinfectors (AERs) or inadequate drying following reprocessing [2, 40, 95] In particular, persistence of *P. aeruginosa* has been associated with transmission from defective bronchoscopes despite undergoing three cycles of reprocessing;[67, 130] and is the most reported organism related to GI endoscopy infection transmission.[95] Supporting data from this research study, also demonstrated *P. aeruginosa* as the most vigorous biofilm former of all the test organisms, closely followed by *C. albicans* (Section (a) of Figures 13 to 20), with corresponding survival to HLD occurring earliest in biofilm formation. Both *P. aeruginosa* and *C. albicans* have been associated with clinical biofilms, e.g., implant infections,[64] recovered from dental unit water systems,[143] and are considered difficult pathogens to eliminate by the endoscopic disinfection process by the World Gastroenterology Organization.(http://www.worldgastroenterololgy.org/globalguidelines) In fact, it has been reported that biofilm infection by *C. albicans* on indwelling medical devices are escalating.[70]

4. Drying alone facilitates organism survival

4.1. Effect of drying of bioburden on nosocomial transmission

Crevices, joints, surface pores and entrapped patient-soil in working channels of the scope and in the coiled sheath (e.g., of the biopsy forceps) challenge effective cleaning and disinfection in flexible endoscopes.[109] Dried soil is most difficult to remove, facilitating microbial reservoirs and biofilm formation.[10, 100,109] There are few studies that compare the survival of enveloped and nonenveloped viruses to bacteria, yeast and mycobacterial when dried on a surface. Therefore, microbial survival on PVC surfaces under conditions associated with nosocomial transmission[72, 116] was studied.

4.2. The effect of an organic matrix

The study supported previous and published data[72,135] demonstrating that survival of vegetative bacteria and viruses was enhanced ~1 Log_{10} when in an organic matrix compared to survival without this matrix (Figure 6). This emphasizes that cleaning of environmental surfaces or medical devices is important to reduce the level of residual organic material that could provide protection to microbes. As reported for bronchoscopes, ineffective cleaning has been linked to viral infection transmission.[40]

4.3. Persistent reservoirs of microorganisms

Nosocomial transmission is facilitated by persistent reservoirs of pathogens. The data from this study supported the potential for nonenveloped viruses and *Enterococci* to be persistent reservoirs especially in an organic matrix. Specifically, the nonenveloped virus, *Reovirus*, and *E. faecalis* demonstrated prolonged environmental persistence when dried

in an organic matrix; *Reovirus* survived over 30 days with a ~2 Log_{10} reduction, and *E. faecalis* survived with a ~4 Log_{10} reduction factor (Figures 6 a,b).

Prolonged survival of *Reovirus* supports the potential of such nonenveloped gastrointestinal viruses to create environmental reservoirs that can persist and lead to infection transmission in humans.[138] Mammalian reoviruses are prototypes for the family *Reoviridae*, which includes rotavirus associated with infectious gastroenteritis. Rotavirus diarrhea is a leading cause of hospitalization for young children in industrialized countries and responsible for 6% of all diarrheal episodes and 20% of associated deaths in children in developing countries.[13] The data for *Reovirus* supports similar reports of long-term survival on dry inanimate surfaces of human nonenveloped GI viruses (*rotavirus, HAV, poliovirus, Norovirus* or *Calicivirus*) from several days to approximately 2 months.[13,72,135]

The results for survival of enterococci support previous reports of prolonged environmental survival for enterococci and staphylococci.[61, 92, 114, 146] In a review of persistence of nosocomial pathogens, *Enterococcus* spp. persisted for months in the environment,[72] and healthcare surfaces are commonly contaminated with such hardy bacteria.[74] Implications are far-reaching since Kramer et al.[72] stated that Gram-positive bacteria such as VRE and MRSA are among the microorganisms showing strongest evidence for the environment's role in infection transmission during outbreaks; and experimental evidence shows that *E. faecalis, E. faecium,* and *S. aureus* have similar

prolonged survival characteristics with no difference in survival between susceptible and multi-antibiotic resistant strains.[72, ,92, 146]

The data in this study supported published data that showed both *M. chelonae*, (glutaraldehyde resistant and sensitive strains), as well as *C. albicans* represent persistent microbial reservoirs, surviving several days (Figure 6) to months.[72,96] Persistence of mycobacterial species in the environment can facilitate transmission via aerosols as well as medical devices; e.g., a highly drug resistant strain of *M. tuberculosis* has been associated with persistence in contaminated bronchoscopes and subsequent transmission to consecutive patients for 17 days.[1] The prolonged survival characteristics for the isolate of *C. albicans* used in this study corroborates findings found for clinical isolates[136] and provides evidence as to why *C. albicans* is the most common clinical Candida isolate and the most important nosocomial fungal pathogen.[72]

Results from this study confirm that persistent reservoirs of *P. aeruginosa* stem from moist environments,[2, 6, 40, 61, 72, 101] since overnight drying itself reduced the viability dramatically. Furthermore, the data showed that *P. aeruginosa* continued to survive at low levels in the dried state for 9 days, consistent with other studies.[72] This is significant since this organism is a major concern as a hospital-acquired pathogen,[67,130] associated with multi-drug resistance strains,[145] and a 300-fold increase in resistance of disinfection when dried onto surfaces compared to in suspension.[118]

Nosocomial relevance of enveloped viruses is more related to prevalence (e.g., HBV and HCV)[122] and pathogenesis (HIV), rather than persistence. In this study, SVHR survived only to day 2. Although short-lived, persistence of enveloped viruses may have clinical relevance. SVHR is often chosen to model HCV, which has been reported to survive in an endoscope with subsequent transmission to three successive patients within a few days.[23] Other blood-borne enveloped viruses, e.g. HIV, have persisted on inanimate surfaces for 1 to 3 days [91] to more than 1 week for HBV[72] (also associated with transmission from inadequately disinfected gastroendoscopes[17,90]).

4.4. Dried bioburden challenged with recommended disinfectant conditions

The data demonstrated that both GLUT and AHP at the manufacturer's recommended use-dilution for HLD are effective for all microorganisms evaluated. The data suggested that if HLD is performed properly the risk of pathogen transmission related to reprocessed GI (or respiratory) endoscopes is extremely low. [87,95] Other studies have revealed similar results for vegetative bacteria;[12,142] yeast;[12,16] enveloped viruses;[20,95] nonenveloped viruses;[16, 80, 85, 117] and *M. chelonae*[S.87, 144] This supports the plausible efficacy of HLD for MRSA, VRE, and multi-drug resistant *M. tuberculosis*, which have been reported as equally susceptible to AHP,[126](www.virox.com., Sept.2004) and GLUT[116,145] as their non-resistant counterparts. Also, although *Reovirus* survived drying on surfaces longer than other organisms, suggesting difficult eradication, its elimination by disinfectants was similar to that of yeast in this study.

However, the glutaraldehyde resistant strain of *M. chelonae* was resistant to GLUT even at the recommended concentration for HLD, consistent with other studies.[53,131, 139,144] The data from this study supports previous reports suggesting that AHP may be effective against *M. chelonae*^{R} when used at the recommended concentration for HLD.[116, 120,123,126]

5. Relative protection that TBF and BBF provide against HLD

It has been reported repeatedly[11,94] that endoscopes reprocessed in accordance with reprocessing and infection control guidelines present "virtually no risk of transmission of patient-borne or environmental microorganisms."[95] However, this research data establishes that microbial survival to HLD can occur in both TBF and BBF, with microbial loads increasing most quickly in BBF. (The data discussed below will refer to BBF resulting from complete reprocessing cycles, unless otherwise stated).

When biofilm formation was allowed to continue unchallenged, the TBF kinetic profiles for all organisms in this study (Section (a) of Figures 13 to 20) demonstrated characteristics showing: a) a pattern where organisms achieved a threshold (maximum) number and plateaued at that level over time. Comparable biofilm profiles using the MBEC system have been reported for similar organisms.[14,28, 57] The kinetics of TBF in the MBEC model implies the following biofilm formation scheme. The initial exposure of the MBEC peg to bioburden facilitated biofilm initiation (microbial attachment, replication, production of the extracellular matrix (ECM)). Thereafter, development was dependent on nutrient conditions. Higher nutrient content resulted in greater microbial load in a growing biofilm. Under low nutrient conditions, biofilm growth rate was lower,

as noted in similar work.[49] However, once the biofilm matured, even in high nutrient conditions, nutrient diffusion was apparently limited through the extracellular matrix/biofilm formation thereby limiting microbial replication and ECM production, until ultimately the nutrient supply to biofilm cells was lowered to the point of preventing cell replication and ECM production entirely.

An enzymatic detergent was used as an organic source for biofilm formation to investigate the possible implications in endoscope reprocessing. Its role is to remove patient soil (organic matter, microorganisms) by physical action, to perhaps slow microbial growth, but not necessarily to kill organisms. In this study, the cycling process did not include any physical brushing, however exposure was not static (due to the rocking motion of the MBEC biofilm formation process). Published studies have reported varying efficacies of different enzymatic detergents depending on formulation,[6] as well as the inability of such solutions to sufficiently reduce viable bacterial load or remove bacterial exopolysaccharide.[140] In this study the enzymatic detergent was used at the manufacturer's use-dilution, with contact time overnight compared to the recommended 5 minutes. Results demonstrated the ability of organisms to replicate under these conditions. Therefore, the practice (although not recommended in reprocessing guidelines) of leaving patient-used scopes in enzymatic detergent overnight or over the weekend can serve to increase rather than reduce microbial load and protein buildup, thereby hindering the efficacy of the disinfection process.

When challenged by HLD, both TBF and BBF facilitated organism survival and the maximum bioburden plateau and breakthrough times were reflective of the organic content in the media. Ultimately TBF and BBF had a negative effect on the penetration and efficacy of anti-microbial treatments, similar to publications discussing TBF.[102] All organisms showed that if survival to HLD challenge occurred, it occurred in mature TBF, not in early TBF, i.e., the higher and more stable the organism load in biofilm, the higher the chance of organism survival and detectibility. Microorganisms in mature TBF can persist in a dormant stage, possibly affecting chemical disinfectant efficacy (as is known for antibiotic efficacy on TBF).[102] In TBF, avid biofilm formers (e.g., *P. aeruginosa* and *C. albicans*) demonstrated the earliest survival to HLD challenge. However, TBF formed by the MBEC system was protective for all test organisms (including those not associated with water sources, e.g., *E. faecalis*). *M. chelonae*^{R} demonstrated the lowest numbers of organisms in biofilm (i.e., the slowest biofilm development) compared to the other test organisms. However, over time survival to AHP was seen and although *M. chelonae*^{R} was by nature resistant to GLUT challenge, GLUT resistance increased (more survivors resulted) during biofilm formation as well. As expected, when TBF or BBF were challenged with a more dilute version of disinfectant (e.g., LLD of AHP), survival generally occurred sooner.

However, TBF and BBF differ in the kinetics of the breakthrough survival and the resultant levels of survivors. The addition of a bioburden re-exposure step in the cycles in combination with drying and disinfection had a significant and profound effect in increasing microbial survivability in spite of repeated HLD challenge. This was most

evident with GLUT challenge for all microorganisms tested (to a lesser extent by *M. chelonae*^{R} due the inherent tolerance to GLUT) (Table 13). Although it may require a longer time for organisms within this BBF to be detected compared to TBF, BBF eventually surpassed the organism loads in TBF over the same time period of 30 days. For example, *E. faecalis* had final organism loads of ~ 3 Log_{10} in TBF over 30 days compared to 5 Log_{10} in BBF over the same time period (15 cycles). As TBF matured, survivability increased gradually and plateaued; whereas BBF demonstrated steeply escalating numbers of microorganisms within the BBF. BBF involving GLUT HLD presented the greatest challenge to disinfectant eradication of microorganisms. AHP was more effective in penetrating and preventing biofilm than GLUT. These data provide a possible explanation for published reports stating residual levels of organisms persist even when proper reprocessing is followed.[8, 140, 143]

5.1. Impact of an organic matrix in TBF and BBF

All formations of biofilm tested demonstrated the same results: an organic matrix significantly enhanced microbial growth and reduced the efficacy of the disinfectant challenge (GLUT and AHP), protecting and facilitating increased survivability for all test organisms. As in other studies, nutrient availability significantly increases microbial replication and ECM production; whereas decreased growth rates result when biofilm organisms are grown in nutrient-limited environments.[49] Results from this study suggest that in vitro models of microbial survival in TBF formed in low nutrient medium would produce significantly different results than models of BBF formed in high nutrient medium.

The protective effect of organic matter in biofilm and in reprocessed flexible endoscopes is an important issue in infection transmission.[6, 10,100] The ability of organic material to inactivate or bind to disinfectants is associated with limited penetration of the disinfectant into the biofilm formation. The result is inactivation and dilution of the disinfectant as it penetrates into the biofilm structure. The data presented in this study demonstrated that the presence of organic material in TBF or BBF significantly limits HLD efficacy. The data suggests that deep within the biofilm structure, organisms are protected by the ECM and organic material and are not affected by exposure to disinfectant challenge.

5.2. Efficacy of GLUT compared to AHP

Although both AHP and GLUT are recommended high-level disinfectants for endoscope reprocessing, [10, 40, 87, 116] the benefits of HP chemistry in reduction of bioburden has been cited in published reports.[6, 10] This evaluation of TBF and BBF provides the first biofilm related data to support the significant differences in efficacy.

GLUT is a widely used, broad-spectrum high-level disinfectant.[10, 87, 95,116] However, the data from this research suggests that GLUT represents a worst-case scenario for endoscope reprocessing: (1) Breakthrough survival was detected sooner with GLUT challenge than with AHP consistently for all biofilm organisms; and (2) Survival to GLUT challenge occurred irrespective of the organic levels in the biofilm, whereas AHP consistently eliminated any detectable viability in all biofilm formations in water (analogous to reprocessing rinses or inadequate drying) (Tables 9 to 12). GLUT is a cross-linking agent and this action is prohibitive to efficient biofilm penetration. GLUT

likely effectively kills external organisms in the biofilm structure, fixing them to the surface. As more cycles of use/reprocessing occur, GLUT penetration into an expanding biofilm buildup is limited causing a dilution of disinfectant and efficacy with increasing rounds of reprocessing. Resultant biofilm bacteria are ultimately protected by the cross-linking action.[7, 8] Bisset et al.[18] demonstrated microbial survival to GLUT disinfection of patient-used endoscopes with a corresponding increase in "outgrowth" with increasing rounds of endoscope use and reprocessing. Alfa et al.[7] demonstrated that microbial overgrowth in tubing of perfusion equipment used for esophageal/rectal motility testing was not controllable with GLUT disinfection, and overgrowth prevention and elimination required tube replacement, disinfection with an oxidizing agent and dry storage.

An important consideration regarding GLUT disinfection of endoscopes is its efficacy against *Mycobacterium* species,[116, 137] associated with contamination of water supplies,[37] water tanks and AERs,[54] bronchoscope contamination resulting from suboptimal disinfectant exposure[56] or defective bronchoscope parts,[69, 87, 96] and the emergence of GLUT-resistant strains of atypical mycobacteria with possible resistance to multiple antibiotics.[96,139] This research supports the serious threat of nosocomial infection transmission posed by *M. chelonae*^{R}. Its limited inactivation by GLUT HLD challenge was augmented to 100% tolerance as biofilm developed over time.

In contrast AHP was a significantly more effective disinfectant than GLUT: (1) Against *M. chelonae*^{R} (as seen with other disinfectant types, e.g., ethanol[96]); (2) Demonstrating superior ability to reduce bioburden in TBF and BBF (capable of attacking cellular lipids,

proteins, DNA [115] and reducing the ECM of biofilm); (3) Only AHP was capable of eliminating detectable BBF organisms resulting from repetitive cycling of drying and HLD in a high organic environment (without reseeding) (Figures 14, 16, 18, 20, section (c); Tables 9 to 12); (4) When TBF and BBF were challenged with AHP, no recovery of *Reovirus* resulted in contrast to GLUT HLD challenge (Table 15); and (5) When AHP was used at the use-dilution and time for surface disinfection (LLD rather than at the recommended HLD concentration and time), AHP effectively eliminated all viable vegetative bacteria in TBF or BBF grown under low nutrient conditions. Breakthrough survival to AHP in TBF and BBF related to high levels of bioburden (> 6 Log_{10}) and maturity of the biofilm. For example in TBF and BBF of *E. faecalis*, survival to AHP challenge was detected on Day 24 with surviving organism levels reaching ~2 Log_{10} by the end of the test period. In contrast, survival to GLUT challenge generally resulted ~ 10 days sooner with higher final microbial loads. Other researchers have reported such superior effectiveness in peroxide-based disinfectants.[7, 148] Microbicides with similar oxidizing chemistries that are used in endoscope reprocessing (e.g. peracetic acid) would be expected to show similar superior effectiveness.[141, 142]

5.3. BBF's protective qualities relate to type of cyclic reprocessing buildup

BBF formed by complete repetitive cycling demonstrated the greatest escalation of microbial survival following breakthrough. However, BBF formed as a result of repeated cycles of drying alone, formed the most protective environment of all types of biofilm tested and for all microorganisms tested. It was apparent, especially for *P. aeruginosa*, that as the biofilm formation progressed (organisms replicated and were increasingly

encased and protected by the ECM), any detrimental effects of drying on survivability were eliminated as cycling continued (Section (b) of Figures 13 to 20). However the resultant bioburden was compacted over repetitive cycles, with an encrustation of organic matter (viable and nonviable organisms in soil). This BBF demonstrated earliest breakthrough survival to HLD for all biofilms and approximately 2 Log_{10} higher resultant organism loads than TBF, likely attributable to reduced penetration of the disinfectant. AHP was most effective in killing microorganisms, although breakthrough survival resulted for all test organisms challenged with AHP HLD (albeit with a longer time required and lower resultant numbers of organisms over the test period compared to GLUT challenge). The clinical relevance of such BBF was demonstrated in the case of a defective bronchoscope design that prohibited adequate cleaning and disinfection, in spite of following accepted reprocessing protocols. The end result was transmission of *P. aeruginosa* affecting 33 patients and 3 suspected deaths.[67, 130]

The least protective of BBF conditions tested resulted from repetitive cycles of drying and HLD challenge. Cycling with repetitive challenges of drying plus repetitive AHP HLD, was effective in eliminating all test organisms (Section (c) of Figures 14, 16, 18, 20) in BBF, regardless of the nutrient environment within which the BBF was formed. Confirmation of AHP efficacy and the absence (or nonrecoverability) of viable organisms in this BBF were demonstrated with the indirect outgrowth method (Table 14). However, BBF formed as a result of repeated cycles of drying with repeated GLUT challenge, facilitated survival of all test organisms; but growth was slow and low levels of organisms ($\leq$ 2 Log_{10}) resulted at the end of the test period. As expected, *M. chelonae*^{R},

demonstrated similar enhanced survival to GLUT challenge in all biofilm formations due to an inherent resistance (as discussed with TBF). This type of cycling supports the reported efficacy of endoscopic reprocessing procedures when recommended reprocessing protocols are meticulously followed (facilitating the removal of all microorganisms by cleaning and disinfectant) as well as publications reporting the persistence of organisms at or below 2 Log_{10} in spite of following reprocessing protocols.[8, 18, 143]

5.4. Unique survival and recovery for BBF compared to TBF

Results using the MBEC model to evaluate the efficacy of disinfectant penetration and microbial survival demonstrated that organisms persist in BBF and TBF and are recoverable following GLUT HLD (and in mature TBF following AHP challenge). **However, studies to verify the existence and persistence of viable organisms in biofilm following HLD challenge suggested TBF and BBF provide unique survival opportunities.** In TBF, organisms develop then maintain biofilm formation over time, as demonstrated by an initial increase in numbers of viable organisms and metabolic activity followed by a levelling and reduction in replication and metabolic processes. When challenged by GLUT HLD at various times during formation there was an increase in tolerance to HLD as the biofilm matured in high nutrient conditions, as seen by an increase in the total number of survivors on an increasing number of positive MBEC pegs (Table 14). Therefore the longer TBF is allowed to develop, the greater the chances of microbes surviving and being recovered. In TBF, microbial recovery from AHP was also possible (representing the only clear example where mature TBF offered greater

protection than BBF). In support, in a study of TBF relating to dental unit water systems, total viable counts of water-borne test organisms were eliminated by disinfectant challenge (of both aldehyde and HP chemistries) after 14 days growth in water, but all the biofilm was not removed from the tubing as evidenced by microscopic imaging.[143]

In contrast, BBF (resulting from repetitive cycles of drying/HLD) had fewer pegs demonstrating survival as the number of cycles increased and required longer times for detectibility (Table 14). Data with complete BBF cycling demonstrated significantly higher microbial loads compared to TBF formed in the same time period (Figures 13-20; Table 13). This suggests that surviving organisms remained trapped or embedded due to compounded cycles of GLUT cross-linking the bioburden; however, once microbial survival is established in reprocessed endoscopes, microbial loads escalate quickly.

The physiological relevance of nonrecoverable organisms is questionable, however it does not necessarily imply sterility. The possibility of BBF coming off during reprocessing exists with metabolically dormant organisms becoming replicative in the optimal environment of a new host. Similar analogies cannot be made with AHP; under the most challenging BBF conditions no viable organisms survived or were recoverable (Table 14).

5.5. Limits of tolerance of microbial contamination

The standard of 200 CFU/mL has been suggested as a limit of tolerance of microbial contamination (e.g., in potable water, dental lines, endoscopes) by the Centres for

Diseases Control and Prevention as well as the American Dental Association.[143] In a Canadian-wide survey of patient-ready duodenoscopes (ERCP scopes), microbial overgrowth (>200 CFU/mL) was found in 30% of the scope samples, a significant decrease in microbial load from previous studies attributable to effective drying following reprocessing.[8] In that study, all samples with higher loads (>200 CFU/mL) were predominantly Gram-negative rods. Therefore the contamination was likely either introduced externally (i.e., from the environment during storage) or water- associated (introduced during reprocessing), or had persisted in buildup material in the channels of the scope (i.e., the biopsy channel).[8] The opportunity for microbial survival in endoscope channels to exceed the limits of tolerance is supported by the data from the MBEC peg model of TBF and BBF, particularly following GLUT challenge in BBF formed by the complete reprocessing protocol (e.g., up to ~6 Log_{10}/peg for *P. aeruginosa*). However, ultimately infection transmission is determined by the pathogenicity of the organism and susceptibility of the patient, in addition to the number of microbes.

5.6. Recovery of *Reovirus* from TBF and BBF

Although most viruses are readily neutralized with disinfectants, as suggested by the initial investigations in this research and reported by others,[40] there is a theoretical risk of transmission of viruses harboured in biofilm and organic residue in endoscopes. This risk increases dramatically if inadequate cleaning of endoscopes occurs. Consequently viral transmission from flexible endoscopes has occurred, mainly attributable to breeches in reprocessing, e.g., *hepatitis B* and *hepatitis C* in GI endoscopes.[40] Since it was demonstrated that organisms in TBF or BBF can survive HLD or rounds of HLD

(respectively), this further supported the possibility of viral survival within the biofilm. In this study, the consistent recovery of *Reovirus* from TBF and sporadic recovery from BBF of complete cycles following GLUT HLD (not AHP HLD) implies the plausibility of viral transmission when avid biofilm forming organisms contaminate an endoscope and a scope is reprocessed with GLUT. This data also correlated well with the viral survival data over 30 days in the initial research work. However, virus could not be recovered from BBF of repeated cycles of drying and GLUT HLD (without re-exposure to organisms). Whether this was a true reflection of eradication of *Reovirus* or nonrecoverable virus is not known. Methods of viral detection in endoscope simulation studies, such as SEM[140] or nucleic acid detection[18] were not employed since detection indicates presence but does not distinguish between inactivated and viable virus.

5.7. Overall survival of microorganisms in TBF and BBF and possible transmission

The presence of soil and biofilm in patient-ready endoscopes introduces the possibility of decontamination failure and infection transmission as biofilm formation protects organisms from the environment and chemical activation.[95,100,140] This protection is also extended to microorganisms harboured in organic debris and biofilm, e.g., viruses.[40] Buildup biofilm, especially when formed by repeated exposure to cross-linking of proteins by GLUT HLD, can trap microorganisms during cyclic reprocessing. These trapped organisms exist in a relatively dormant state, which further hinders effective eradication by chemical disinfectants. The resultant situation facilitates the possibility of infection transmission from patient to patient. For example, if viable organisms trapped within BBF become dislodged from the inner channels of the scope or removed by

accessory devices (e.g., biopsy forceps) transmission can occur.[8] The data from this research project indicate the clinical relevance of biofilm buildup within reprocessed endoscopes and lends support to the possibility of infection transmission by reprocessed endoscopes.

6. Impact of this research on reprocessing of flexible endoscopes

Available data regarding disinfection efficacy in the presence of soil trapped in flexible endoscopes is limited. The question of whether there is time for biofilm formation to develop in a colonoscopic procedure of 20 to 30 minutes is questionable.[35] However multiple cycles of usage and reprocessing in a day facilitates conditions conducive to biofilm formation: during patient-use an influx of blood/water and feces (supplying nutrients and organisms) occurs initiating biofilm formation; over time these can be trapped within the EPS; while during reprocessing exposure to hydration occurs. [100] At the end of the day, although the scope is stored dry, the EPS can likely withstand the dehydration time and organism presence is likely despite following current reprocessing guidelines.[100] As well, cases of inadequate cleaning (the absence of which was reported in 30% of AER procedures in a US survey),[94] contaminated water supply, or wet storage, all strongly contribute to biofilm formation. Therefore TBF (associated with patient-use and wet storage) and BBF (associated with patient-use and adequate reprocessing) can occur in scopes. However, when reprocessing guidelines are properly followed and the scope is stored dry, TBF is less likely to occur. Studies of BBF compared to TBF indicated that TBF and BBF are both protective, but BBF had unique, superior survival characteristics to TBF. The data from this study demonstrated for the first time that when BBF is formed

under conditions most representative of endoscope use and reprocessing (i.e., exposure to high nutrient conditions, GLUT HLD and repeated cycles of complete reprocessing) a significantly more protective environment resulted for organisms compared to TBF.

The data from this study supports the value of using the BBF for research and development of improved endoscope reprocessing methods. For example, data from the MBEC model of GI scopes with patient-use and proper reprocessing (e.g., BBF of *P. aeruginosa* formed under high nutrient conditions and challenged with GLUT (Figure 15d)) resulted in microbial survival of ~6 Log_{10} CFU/peg within 10 cycles compared to < 1 Log_{10} CFU/peg within 20 days in the model of a scope with patient-use and wet storage (e.g., TBF of *P. aeruginosa* formed under low nutrient conditions and challenged with GLUT (Figure 15a)). In addition, guidelines for reprocessing and studies of cleaning agents or biocides for efficacy on biofilm[140] are generally based on single-use testing and TBF, which may be easier to remove than BBF. Data from this research comparing the efficacy of GLUT on early TBF compared to BBF over time suggest that young TBF may not be indicative of the kill ability in BBF over time.

Data from this study highlights the possibility that non-detectibility of viable organisms may not imply efficacy of HLD, particularly in BBF formed by repeated exposure to a cross-linking agent. The indirect outgrowth method demonstrated that organism survival in the matrix is possible, particularly following GLUT challenge. The determination of the absence of residual microorganisms following reprocessing is dependent on the sensitivity of the detection method.[20] Improved reprocessing including sonication to

dislodge and remove fixed bioburden could facilitate removal of such trapped organisms.[36] More studies in this area are necessary.

An organic matrix in TBF or BBF enhanced growth rate and reduced disinfectant efficacy. Enzymatic detergents serve as an organic source facilitating TBF and BBF. It supports the need to thoroughly rinse after the cleaning phase. The use of a detergent with proven capability of microbial killing may by a useful alternative in ensuring disinfection efficacy.[6, 140]

The mode of action of a specific disinfectant is important. Oxidizing chemicals like AHP are a better killer regardless of the matrix compared to a cross-linking agent like GLUT which resulted in greater buildup and reduced penetration and killing. (Although GLUT was effective in single use situations in the presence of organic matter (as seen in the initial studies with dried bioburden). A novel formulation of 2% AHP capable of HLD in 5 minutes has shown promising results for endoscope reprocessing.[99] Although GLUT appeared less effective than AHP in disinfection efficacy of biofilm, other types of disinfectant also have demonstrated reduced biocide efficacy in biofilm, e.g., *Salmonella enteritidis* biofilm grown in vitro was protected from challenge by trisodium phosphate.[71] Other chemical formulations should be investigated with respect to TBF and BBF since little data exists. Candidates, not neutralized by organic material and with the ability to remove (rather than fix) organic material and debris, could include peracetic acid since several reports state it does not fix soil (however one report has shown blood fixation);[94] or more recently introduced substitutes for glutaraldehyde, e.g, 0.55% ortho-

phthaldehyde (OPA) or 0.1% to 0.3% peracids. As well, alternate technologies,[10] such as electrolyzed water have been suggested for endoscope reprocessing.[94]

7. Prevention of critical buildup and infection transmission

For optimal disinfection efficacy to penetrate bioburden and kill microbes in the channels of flexible endoscopes, prior cleaning of patient-used endoscopes should reduce the buildup of organic material in the narrow lumen.[6] Thereafter, to maintain a contaminant-free scope requires dry storage, rendering reprocessing after storage and before reuse unnecessary. [94] The existence of soil and biofilm in reprocessed flexible endoscopes indicates an inadequacy in the current reprocessing protocols (i.e., cleaning, disinfection, storage),[100] and there are currently no accepted protocols or mechanisms to verify adequacy of cleaning and HLD at the time of endoscope use.[94] Current guidelines for reprocessing and testing of new biocides are based on a single use testing. Yet flexible endoscopes, continually used and reprocessed, are associated with microbial survival and transmission.[100] Problems encountered with endoscopes occur after the scopes have been used for a while.[18,94,109] As demonstrated in this research, HLD with GLUT and AHP is very effective on microorganisms dried in soil onto carriers when done according to manufacturers recommendations. However efficacy is reduced when biofilm formation occurs and over time there is survivability of microorganisms. BBF formed from repeated cycles of complete reprocessing stages presents the greatest threat for infection transmission, as the residual viable loads are often far greater than the 200 CFU limit recommended as the acceptable cut-off. The data from the study of BBF challenges published reports suggesting that patient-ready endoscopes pose a very low risk to

healthy patients,[18] and supports the need for validation of the reprocessing protocol (i.e., cleaning and HLD) and using multiple rounds of testing rather than the one round used currently.

The cleaning step is crucial to disinfectant efficacy in endoscope reprocessing. In this study, a common enzymatic detergent was used in the model, which demonstrated an inability to remove or reduce TBF or BBF. Although other studies have evaluated the efficacy of various types of cleaners (with and without enzymes) to remove biofilm on simulated endoscope channels,[140] results from this study suggest that the effect on BBF should be investigated to ensure effective biofilm removal in reprocessed endoscopes. This is particularly critical in the air and water channels of flexible endoscopes (that cannot be mechanically cleaned and rely on chemical means) and damaged endoscope linings to ensure adequate disinfection.[4,140,100] Therefore changes in the design of flexible endoscopes to reduce complexity and porosity, as well novel approaches such as the incorporation of "anti-biofilm" agents (e.g., transition metal catalysts or acylated homoserine lactone molecules)[82] could be beneficial.

Why is infection transmission such a rarely reported event when the model suggests that organisms can persist in reprocessed endoscopes and transmission is possible? Most reports implicate a lack of tracking, reporting and association of infections acquired after a length of time following the procedure. However a more common reason is that the mucous membrane is an effective barrier when patients are exposed to reprocessed endoscopes. As well, prophylactic antibiotics are important factors.

Data from this research project suggests that the potential is real for patient-to-patient transmission of pathogens in BBF and TBF during endoscopy as well as environment-to-patient transmission of Gram-negative bacteria and mycobacteria. It is believed that the novel model used in this research project mimicking BBF in narrow lumen of flexible endoscopes more accurately depicted what occurs in patient-used narrow lumen medical devices compared to in vitro TBF models, especially those using low nutrient medium. Results from such models would predict significantly lower survivability than seen with BBF model using high nutrient medium.

Specific requirements necessary to ensure the efficacy of the disinfection process suggested by this project are: (1) Removal of soil and bioburden, which can harbour organisms and facilitate biofilm formation. Cleaning agents must degrade and remove soil efficiently and effectively. The ability to remove soils (e.g., feces, saliva, blood, gastric contents, bile, urine) depends on whether the soil is moist or dried. Cleaning flexible endoscopes should occur immediately after each procedure. Allowing the soil to dry changes the profile of the bioburden and the survivability of the associated microorganisms.[109] (2) Ensuring the quality of water and identifying the types and quantity of possible contaminants. Potable water is commonly contaminated with *Pseudomonas* spp., *Escherichia coli*, fecal coliforms, *Giardia lamblia*, heterotrophic bacteria, *Legionella* spp., and viruses.[109] (3) Suppression of residual biofilm by use of a disinfectant with oxidizing ability to weaken adhesion between ECM cells facilitating removal and increasing effective disinfectant concentration. (4) Limiting nutrient concentration and sources encountered in the reprocessing protocol.

8. Limitations and future consideration in modeling BBF

The possibility of developing a reproducible in-vitro model of biofilm buildup in narrow lumen medical devices is plagued by the inherent variability in the patient-use and reprocessing of these medical devices and the resultant cumulative residual soil buildup. The incorporation of a worst-case soil/medium and microorganisms representative of the environment tested has aided in making the study results more relevant. However a comparison of data from the in vitro model to the actual buildup that occurs over time in patient-used flexible endoscopes in centers using various disinfectant methods would assist in validating the model results.

The efficacy of GI endoscopy disinfectant regimes are generally tested in vitro initially and subsequently assessed by in-use testing. In vitro testing is generally best applicable in determining effective concentration and contact time, while in-use testing is most applicable in testing comparative disinfection regimes.[20] Bordas[20] found significant differences between disinfectant efficacy for results from in vitro testing compared to in-use testing for GI flexible endoscopes, suggesting that true disinfectant efficacy requires longer times or greater concentrations than suggested by in vitro tests. This research study and relevant BBF modeling system addressed this paradox and results supported the findings of Bordas[20] by demonstrating that the model of buildup of patient soil in reprocessed GI flexible endoscopes resulted in a buildup biofilm which presented a greater challenge to disinfection than either dried patient soil alone or traditional biofilm formation (particularly early TBF).

The model system for TBF and BBF is monomicrobic and would presumably have a simpler structure than biofilm/bioburden developed in situ having a greater level of complexity and types of organisms.[18,64,79] Specific test organisms were chosen based on their ability to form biofilm. Variation between specific species was not considered. However, all organisms that were examined formed biofilm to some degree. Although clinical isolates were not tested, they would likely be capable of biofilm formation in the test system, and it would be useful to evaluate such strains. Since the carrier surface type (e.g. chemical composition, surface charge, porosity) can influence the attachment[125] and biofilm formation,[64,140] to increase the applicability of the in vitro model results, the same lumen channel material as used in flexible endoscopes was used as the carrier material in the initial dessication studies and for the flow model tubing in the initial biofilm studies, which in turn had demonstrated comparable biofilm formation to the MBEC model subsequently used in the study of BBF. However BBF studies were not repeated on endoscope channel material since MBEC pegs are not available in this material.

Some organism types were not included in this study. This was due to absence of reported cases of transmission via flexible endoscopes and intrinsic resistance characteristics rendering these organisms beyond the capability of HLD, requiring sterilization. These included: bacterial spore-formers, e.g. *Clostridium difficile* as HLD does not require the destruction of high numbers of spores and no reported cases of transmission exist;[10] Cryptosporidia, where absence of cases is attributed to advances in cleaning and the organisms' susceptibility to drying; and prions, e.g., Creutzfeldt-Jacob Disease (CJD), since flexible endoscopes do not contact associated tissues or secretions

and cleaning is considered sufficient to reduce potential inocula. (However, variant CJD (vCJD) can be detected in lymphoid tissue such as tonsils, appendix, ileum and rectum. Although this tissue is considered 50% less infective than CNS tissue,[10, 94] known scope contamination results in removal of the scope from further patient-use.

The data from this research indicated an increased risk of pathogen transmission due to the repetitive buildup of biofilm, in particular when a cross-linking agent is used as the disinfectant, stressing the possible health risks when BBF (and TBF) is allowed to form. The data supports current concerns regarding the exposure of low concentrations or activities of biocides to organisms embedded within biofilm and the selection of tolerant bacteria,[140] particularly in BBF. Although cross-resistance between biocides and antibiotics is currently under debate, with clinical evidence being equivocal,[125] such strains exist, with experimental evidence suggesting a common efflux mechanism.[77] For example, an association of GLUT tolerance with antibiotic resistance in *M. chelonae*^{R} is known. [96] This supports the importance of using disinfectant formulations capable of penetration and elimination of biofilm and the need for further investigations in this area.

9. Related factors affecting microbial survival and disinfection efficacy

9.1. Dried bioburden challenged with suboptimal disinfectant conditions

Both AHP and GLUT can be reused for reprocessing endoscopes over a period of days, which introduces the possibility of dilution, evaporation, breakdown and/or neutralization from accumulated proteins.[85,123] GLUT concentrations used in endoscope reprocessing have been reported below the accepted minimum effective concentration (MEC) of 1% -

1.5% to below 0.5%;[85,116] and ~7% AHP levels falling to ~6% after 14 days of reuse.[123] Therefore disinfectant concentrations below the HLD level were evaluated to determine the level of dilution where there would be inefficient kill (i.e., breakthrough survival).

The data (Figures 8, 11) indicated that if the disinfectants were diluted, than a significant number and variety of organisms survived and only the enveloped virus was eliminated. The results provide a probable explanation for reports of persistence and transmission of pathogens in the clinical setting when similar suboptimal endoscopic reprocessing has occurred.[40, 87,95] The enveloped virus, SVHR, was more susceptible to germicides than nonenveloped viruses and vegetative bacteria in this study, as suggested by other reports.[121,122,145] This supports reports that HIV can be readily neutralized by disinfection, and HIV transmission by a contaminated endoscope has never been reported.[40,95] However the potential for transmission of enveloped viruses exists[32,87,95] (HIV-RNA has been isolated from bronchoscopes used on HIV-infected patients[87]; and HBV[17, 90] and HCV,[23] were transmitted by contaminated gastroendoscopes, attributed to inadequate reprocessing).

The breakthrough survival for vegetative bacteria occurred at a much lower dilution of GLUT (i.e. a 1:25 dilution, 0.1% GLUT concentration) than for AHP (a 1:128 dilution, 0.05% concentration) (Figures 8, 11). The data expands on the findings of Cole et al.[34] who reported that even minor dilution of GLUT greatly reduced the killing ability of this HLD and underscores recommendations that GLUT should not be used at a concentration below 2%. The data also underscores the importance of quality assurance monitoring of

concentration especially for GLUT, since dilution and spontaneous polymerization of aldehydes limit germicide activity and may occur before the maximum reuse period.[87]

9.2. Surface disinfection

Chemical disinfection of contaminated healthcare surfaces (and non-critical medical devices, e.g. patient carts) should reduce the likelihood of disease transmission, requiring LLD (which must destroy most vegetative bacteria, fungi and some viruses).[19] A variety of LLD products are registered with Health Canada and the United States Environmental Protection Agency. This study investigated hydrogen peroxide-based products, AHP and SHP. The recommended use-dilution of AHP as a surface disinfectant for bacteria and viruses of 1:16 with 5 min contact time has been found to be effective by other researchers,[120] and in this study, exceeded the manufacturers recommendations (e.g., virucidal and bactericidal objectives were achieved within 1min). Therefore, one can expect pathogens of greatest concern, MRSA and VRE, to be effectively eliminated by surface disinfection with AHP.

9.3. The effect of a disinfectant's formulation

The data (Table 8) clearly demonstrated that disinfection conditions are not interchangeable between different microbicides even if the active chemical used is the same.[125, 129] The results illustrated that formulation has a profound effect on killing ability over time, as previously reported.[11,95] The AHP formulation is more effective (the reduction factor is higher by up to 2 Log_{10}) than the SHP formulation when used at the same time and concentration. Rutala et al.[117] has indicated that surface disinfectants will

dry within 1 min. The data demonstrate that at the 0.05% recommended use-dilution for SHP killing ability alone cannot be relied on to effectively eradiate microorganisms from surfaces. This again stresses the importance of adequate physical action to ensure microbes are removed during the cleaning process.

10. Conclusions impacting flexible endoscopes and nosocomial transmission

This study has shown that an organic matrix facilitates microbial survival and creates environmental reservoirs. In particular, *Reovirus*, a representative nonenveloped gastrointestinal virus, and *E. faecalis*, a Gram-positive enterococci, were capable of prolonged environmental persistence. However both could be readily eliminated in dried bioburden by high-level disinfectants such as GLUT and AHP and by surface disinfection with AHP when used under recommended conditions. Of conditions tested, only AHP could effectively eliminate *M. chelonae*^{R}. Suboptimal disinfectant exposure resulted in the survival of a significant number and variety of microorganisms, with the exception of the enveloped test virus. These findings support the importance of strict adherence to recommended guidelines for endoscope reprocessing to prevent microbial persistence and transmission, as well as biofilm formation.

This critical buildup of dried bioburden is made more robust by biofilm formation (TBF and BBF), found on the internal channels of endoscopes [10, 18, 100, 140] and attributable to inadequate bioburden removal or inactivation of biofilm formation in spite of following acceptable reprocessing guidelines. This is believed to facilitate the persistence of microorganisms, tolerance to disinfectant challenge, and possible infection transmission.

[10,40,71,78,100] Data from this study has provided scientific evidence supporting this possibility. The magnitude of relevance is reflected in the example of colonoscopy, now the gold standard for detection and prevention of colorectal cancer, affecting 147,000 new cases per year in the US[94] with increasing national and global screening programs. As well, endoscopy is a valuable tool to monitor very ill (and likely immunocompromised) patients.[18] These patients represent the greatest risk in infection transmission from endoscopy by suffering the greatest threat from invading pathogens as well as harbouring and possibly contaminating endoscopes with the greatest numbers and variety of microbes,[18] including nosocomial superbugs, MRSA, VRE and *C. difficile*.

Results from this novel research of modeling biofilm formation and disinfection efficacy for complex medical devices have suggested an increased ability for microbial survival from dried bioburden (e.g., inadequately cleaned surfaces) to TBF (e.g., in AERs or conditions of inadequate drying) and ultimately to BBF (e.g. occluded locations within the scope channels) for a variety of microorganisms (including avid and non-avid biofilm formers and viruses). The cross-linking agent, GLUT, most commonly used in endoscope reprocessing facilitated the greatest protective environment and ultimately the greatest reservoir of microrganisms compared to an oxidizing agent (AHP). However, when biofilm is protected in an organic environment repeatedly exposed to nutrients and microorgansims, organism survival can result in spite of the oxidizing chemistry. It has been suggested that strategies focusing on decreasing biofilm and viability in endoscope disinfection will have a significant part in reducing the risk of pathogen transmission.[10] Data from this study suggest that optimum conditions to prevent disinfection failure

include the use of an oxidizing agent as the disinfectant and keeping organic levels low with effective cleaning. The implications are that as flexible endoscopes are repeatedly used and reprocessed, HLD assurance decreases, particularly when a cross-linking agent like GLUT is used. The BBF model is highly relevant to published reports on flexible endoscopes and infection transmission describing: organic matter, biofilm formation and microbial contamination problems occurring only after "multiple cleaning and disinfectant cycles over the life of the instrument" [94,100] (i.e., BBF); the persistence of residual levels of organisms in scope channels in spite of reprocessing;[18,100] and the difficulty in relating in vitro experiments to disinfection efficacy in GI scope testing.[20] The adaptation of the MBEC system to model not only TBF but also BBF has provided a novel model to more accurately assess disinfection efficacy for reprocessed endoscopes and AERs. This research suggests that the MBEC model would be beneficial in the assessment of novel microbicides for reprocessing (e.g., electrolysed acid water),[75, 95] which should be tested with respect to BBF.

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