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Magnetic Particle Induction and Its Importance in Biofilm Research

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1. Introduction

Since the mid-twentieth century scientists have been aware that aquatic bacteria are more abundant as biofilms on solid surfaces than as suspended free cells (ZoBell, 1943). The last few decades have seen significant advancement in our understanding of the development of biofilms and the processes occurring within these colonies of adhered microorganisms (Coenye & Nelis, 2010; Hall-Stoodley et al., 2004). Two features in particular distinguish microorganisms in biofilms from their free-living counterparts. The first is their ability to produce a coherent extracellular polymeric matrix (containing polysaccharides, proteins and DNA) which results in firmer attachment to the surface (Costerton et al., 1987; Donlan & Costerton, 2002). The other is the coordinated behaviour of the cells embedded in this matrix due to communication by a process known as quorum sensing. Quorum sensing is the secretion and detection of inducer molecules that accumulate as a function of cell density. At a threshold population density the accumulated autoinducers bind to cellular receptors activating transcription of certain genes (Costerton & Lapin-Scott, 1995; Hall-Stoodley et al., 2004; Nadell et al., 2008; Sauer, 2003).
While the existence of a biofilm is beneficial in many settings, for example in waste water treatment plants where they play an essential role in flocculation and nutrient removal (Nicolella et al., 2000; Wagner & Loy, 2002), their presence can also be extremely harmful or costly. Biofilms are implicated in numerous diseases, including cystic fibrosis and tuberculosis (Lam et al., 1980; Singh et al., 2000); they also contaminate food, its packaging and the water distribution network thereby posing a serious threat to human health (Flemming, 2002; Kumar & Anand, 1998; LeChavalier et al., 1987). Microorganism colonization and extracellular polymeric substance (EPS) secretion on man-made structures such as heat exchangers and the hulls of ships can result in decreased performance and increased operating costs (Meesters et al., 2003; Schultz et al., 2011). As such, biofilms have become a priority subject in many research areas in recent years. Publications in the fields of biomedicine (Guo et al., 2008; Morton et al., 1998), waste water treatment (Liu & Fang, 2003; Pollard, 2010), ecology (Lubarsky et al., 2010; Yallop et al., 2000), food science (Carpentier &

Cerf, 1993) and biotechnology (Flemming & Wingender, 2001; Houghton & Quarmby, 1999) serve to highlight the wide ranging importance of biofilms and their secretions of EPS.
Technological developments originating in different fields will have translational value. We report here on the MagPI (**Mag**netic **P**article **I**nduction) System, one such development in the field of environmental science. The MagPI System uses magnetic induction of ferrous particles to quantify the adhesive capacity of a test surface. As the "stickiness" of surfaces can often be attributed to the presence of a biofilm the MagPI System can be used to evaluate biofilm formation and state of development. Previously, measurements of this process have been conducted using large laboratory scale systems that can be both expensive and labour intensive. A variety of relevant procedures and devices are presented (Table 1).
In this paper we will review the key phases in the development of the MagPI System, outline the procedures for use, review its current applications and highlight uses for this device that will be of relevance to biomedical sciences.

2. Technical aspects and development of the MagPI System

The MagPI System has been developed by a multidisciplinary team led by the University of St Andrews. Initially the goal of development was to produce a device that could sensitively measure the adhesive capacity of sediment surfaces. The adhesive capacity or retentive ability of the sediment surface is a proxy for bed stability. Several devices based on different approaches already exist to measure sediment stability, e.g. water flow [Sedflume (McNeil et al., 1996); SETEG (Haag et al., 2001)], water jets [CSM (Paterson, 1989)] and propellers [EROMES (Schuenemann & Kuehl, 1991)]. To measure sediment stability these devices require that bed failure occurs. The MagPI System is capable of repeatedly measuring changes in surface properties below the point of bed failure (incipient erosion) that are undetectable by these other devices. For example, changes in adhesion during early stages of biofilm formation. As such, its use will fill a gap in our knowledge of properties and behaviour of surfaces and sediments (Larson et al., 2009).

2.1 The electromagnet

In the early stages of construction commercially available magnets were tested for their suitability. However, common problems included too large a surface area to be useful in observing particle reaction to the increasing magnetic force or inadequate strength to uplift the test particles. As a result, electromagnets were specially constructed by coiling insulated copper wire around a ferrous alloy core (Figure 1). The wire gauge, core dimensions and the number of turns in the coil can be varied between models to create electromagnets with different ranges.

2.2 Ferrous test particles

The test particles (Figure 1) are composed of a mixture of ferrous materials mixed with fluorescent pigment to increase their visibility (Partrac Ltd., Glasgow, UK). After their production a spectrum of particle sizes exist (80-400 μm). Particles are homogenized by sieving them into different size classes. The targets for MagPI need not be confined to particles. Almost any target design can be envisaged as long as the target is attracted by a magnetic field. So far small metal discs (<1cm diameter) and larger metal spheres (c.f 1-3mm) have also been tested. The choice of target depends on the purpose of the study.

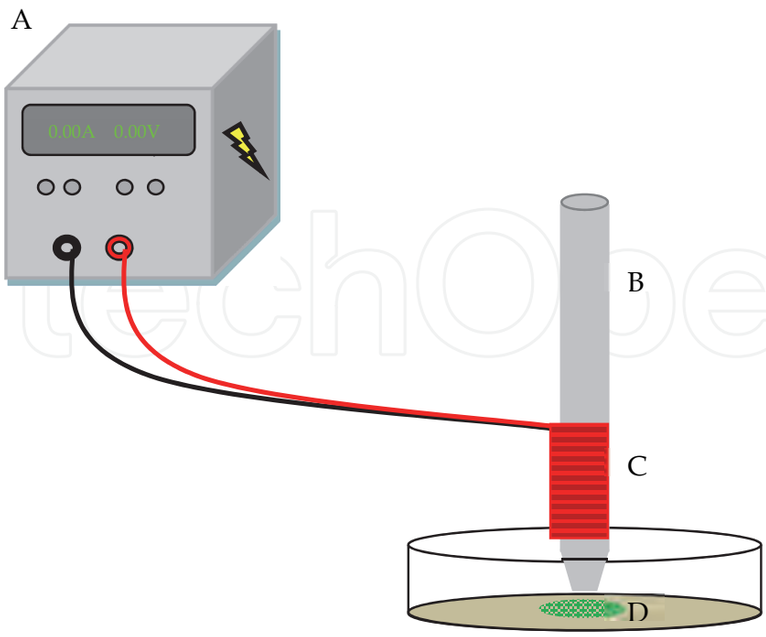

Fig. 1. Schematic diagram of the MagPI system. A) Voltage and current variable power supply. B) Aluminium rod. Acts as a heat sink and can be attached to a micromanipulator or clamp when MagPI system is in use to hold it at a set height above test surface. C) Ferrous alloy core around which copper wire is coiled. The core is waterproofed by coating it in a plastic film. D) Fluorescent ferrous test particles.

3. Standard operating procedure

The laboratory-based MagPI System consists of a variable electromagnet controlled by a power supply capable of producing fine scale increments of current and voltage and the specially designed magnetic particles (Figure 1). To ensure repeatable measurements are taken, magnetic particles of a known size and density must be consistently applied to the test surface in a relatively even single layer. The procedure followed when using the MagPI system in the laboratory is detailed below:

1. Magnetic particles of a known size and density are suspended in water.
2. The particle-water mixture is then drawn into a pipette or syringe. The suspended particles are allowed to settle to the tip of the pipette or syringe.
3. A couple of drops of the mixture are sufficient to distribute a single layer of particles to an area of c. 1 cm^2. This is ejected about 1 cm above the test surface into the overlying medium.
4. The time interval between particle application and retraction depends on the objective of the investigation. If the adhesive capacity of the test surface is in question then the measurement of magnetic force required to uplift the particles from the test surface should be taken immediately. This is the most reliable way to ensure repeatable measurements. Particles left on the surface a period of time before the uplift process begins will become incorporated into the biofilm to some extent. Thus, the strength required to retract the particles is also influenced by the ability of the developing biofilm to entrap particles. The MagPI probe (electromagnet) is lowered into position above the particles (Generally 5 – 10mm from the surface) using a micromanipulator.

Method	Basic procedure	Advantages	Limitations	References
Microscopy (e.g. epifluorescence, laser-scanning confocal, transmission electron, scanning electron)	Varies between different microscopic techniques. For some microscopic methods biofilms are treated with a fixing agent (e.g. formaldehyde, glutaraldehyde) and stained (e.g. with acridine orange, ruthenium red, safranin) prior to imaging.	Non-destructive High resolution images provide information of biofilm morphology, phylogeny and matrix structure and architecture	Labour intensive Require specialist training Costly Pre-treatment can alter specimen morphology Potential for underestimation of biofilm levels if thickness not measured Not quantitative	Lawrence et al., 2003; Morató et al., 2004; Perkins et al., 2006; Priester et al., 2007
Crystal Violet Method	Biofilm cells stained with crystal violet. The dye incorporated into sessile microorganisms is then solubilised and the absorbance of the solution measured.	Affordable Doesn't require specialist training	Time consuming High variations for a same result Efficiency of biofilm removal from surface unknown	Musk et al., 2005; Vesterlund et al., 2005
Flow Cytometry	Adhered microorganisms are removed from their surface (e.g. by sonication). Cells are suspended in a rapidly flowing stream of water that passes by an electronic detection apparatus.	Rapidly obtains and processes data Reveals heterogeneity of cells: numbers, size distribution, physiological and biochemical characteristics	Expensive Requires specialist training Efficiency of biofilm removal from surface unknown	Vives-Rego et al., 2000; Williams et al., 1999
BioFilm Ring Test®	A magnetic bead solution is added to bacterial cultures on a microtitre plate. After a period of incubation a magnet is used to assemble the non-immobilized beads to the bottom of the well. The resulting spot is quantified through specialized image algorithms.	Easy to operate Automated Sensitive to early stages of biofilm formation Results obtained quickly Repeatable	Cannot be used to quantify biofilm formation in nature No information on phylogeny or morphology obtained as with microscopy	Chavant et al., 2007
MagPI System	Magnetic beads are applied to the biofilm and exposed to an incrementally increasing magnetic force. The force at which the beads are recaptured from the surface can be taken as an indication of the extent of biofilm formation.	Easy to operate Inexpensive Can be used to measure biofilm formation on any surface Sensitive to early stages of biofilm formation Non-destructive Results obtained rapidly Repeatable	Not automated No information on phylogeny or morphology obtained as with microscopy	Larson et al., 2009

Table 1. Methods and procedures used for studying biofilms

5. The current to the probe is gradually increased (~ 0.2A increments). As the current increases so too does the magnetic force acting on the particles.

6. Four levels of particle response to the increasing magnetic field have been identified. The fourth level is the least subjective and should therefore be taken as the end point of the experiment (Figure 2).
7. Prior to repeat measurements being taken on the same test surface it is made certain that no particles from a previous measurement remain in the area to be tested.
8. It is advisable to calibrate at the start, during and at end of an experiment to account for changes in the coil resistance that would result in a loss of magnetic field strength.

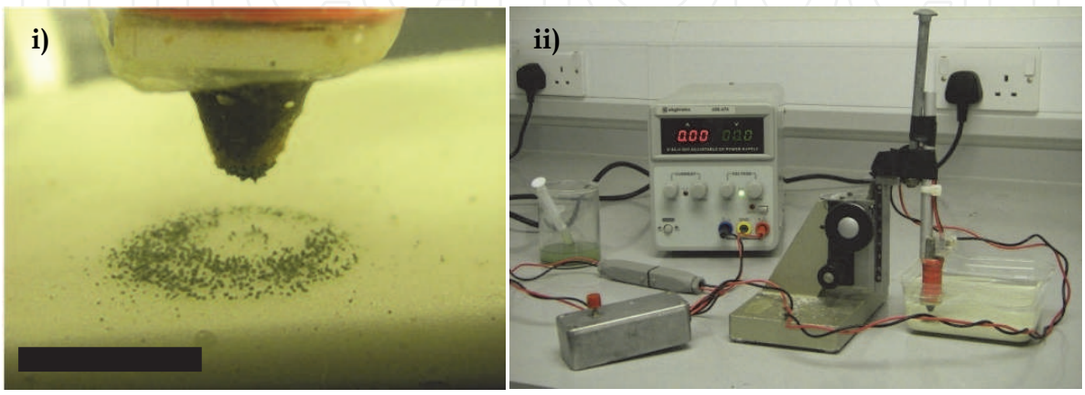

Fig. 2. **i)** Stage 4 of particle response to a magnetic field- complete removal of particles from the test surface below the electromagnet tip, particles can be seen adhering to the electromagnet. (Scale-bar: 1cm) **ii)** MagPI System set-up

4. Calibrations and magnetic force equation

Calibrations enable comparison of results obtained using different MagPI probes or in different laboratories or experiments. To calibrate the device the probe is placed at a set distance above a sensor connected to a Gauss meter. The voltage and current are increased incrementally (0.2V/ ~0.1A per increment) while all other factors remain constant. The magnetic flux density (MFD) for each voltage increase is measured by the Gauss meter in mTesla and recorded. Calibrations have been carried out with the probe submerged in both distilled water and seawater at distances of 5, 7 and 10mm between the probe and the Gauss meter sensor. Each calibration was carried out in triplicate.

No significant difference was found between the freshwater and distilled water calibrations (α=0.05). There was a strong linear relationship between voltage and MFD at all distances (r^2: 5mm = 0.99; 7mm = 0.99; 10mm = 0.99). When measuring the adhesive capacity of a test surface the voltage at which particles are uplifted from the surface (stage 4) is recorded and the MFD can later be calculated from the straight line equation obtained in calibrations (Figure 3).

The attractive magnet force acting on the particles at the point of uplift can be calculated according to the following equation:

Equation 1. $$F = B^2A / 2\mu_0$$

Where B is the MFD, A is the area of the magnetic pole facing the test surface and μ_0 is the permeability of free space (constant during measurements in the same medium).

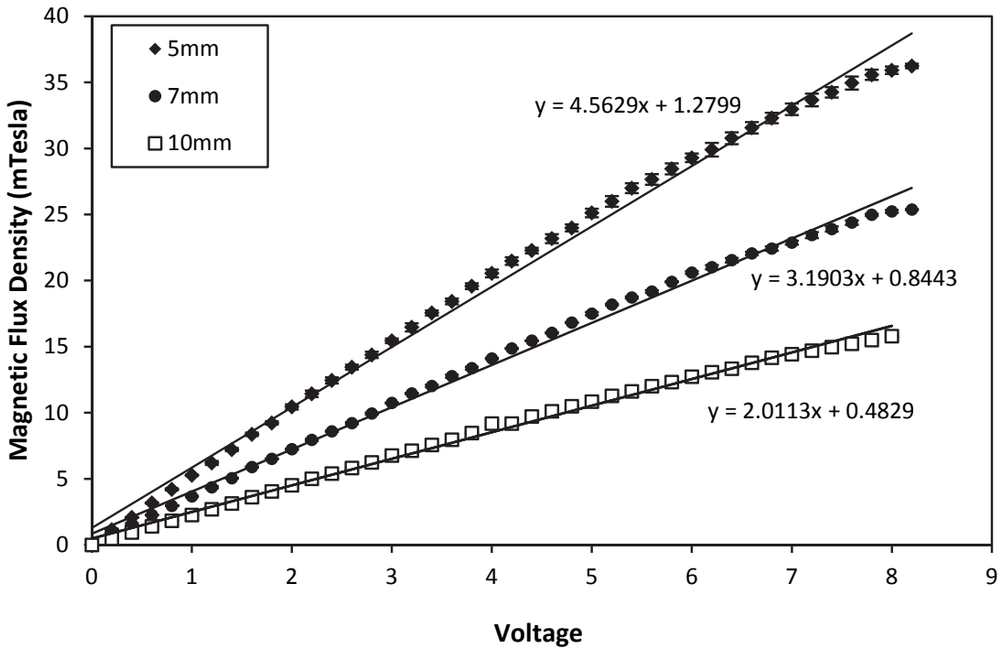

Fig. 3. Seawater calibration curve (n = 3, +/- standard error). Distances reported are MagPI probe from a sensor connected to a Gauss meter.

5. Biofilms in aquatic systems

Biofilms are ubiquitous in benthic aquatic environments (Battin et al., 2003; Larson et al., 2009; Lubarsky et al., 2010) where they regulate a number of important ecosystem services such as nutrient cycling (Battin et al., 2003; Cyr & Morton, 2006), pollutant accumulation (Schlekat et al., 1998; Wolfaardt et al., 1998) and biodegradation (Battin et al., 2003). More recently the influence of benthic microbial assemblages on sediment stability has been proven (Decho, 2000; Gerbersdorf et al., 2008; Spears et al., 2007). Traditionally physico-chemical and biochemical processes were considered to be the most important drivers of sediment stability (Calles, 1983; McNeil & Lick, 2004). Microbial assemblages can enhance the stability of sediment in two ways, either directly, via the presence of physical mats (Dodds, 2003) or indirectly. Benthic microbes indirectly increase stability by secreting EPS which enhances adhesion and cohesion between the EPS molecules and sediment particles (Decho, 1994). Annular flume experiments have shown that the presence of a biofilm at the sediment surface significantly increases the energy required to erode the sediment compared to those sediments without biostabilisation (Droppo et al., 2001). These findings are transferable to the natural environment. Strong correlations between sediment stability, benthic algal biomass and EPS concentration have been observed in marine systems (Sutherland et al., 1998; Yallop et al., 2000). Although biostabilisation has also been observed in freshwater systems (Droppo et al., 2007; Gerbersdorf et al., 2007 & Spears et al, 2007) correlations between the aforementioned parameters are weaker. It is evident that under high electrolyte concentrations the effect of EPS on sediment stability is enhanced (Spears et al., 2008). This emphasizes the need for a device, such as the MagPI System, that is sensitive enough to discern subtle changes in sediment stability across freshwater environments

where low ionic concentrations generally place sediment stability below the range measurable by other devices (See section 2). Understanding the processes that control the erodibility and transport of sediments and their associated pollutants is vital for safeguarding the economic and ecological health of aquatic systems (Förstner at al., 2004; Westrich & Förstner, 2005; Wood & Armitage, 1999).

5.1 First applications of the MagPI System

The MagPI System was first introduced by Larson et al. (2009). Calibration data for an earlier prototype was presented and the ability of the MagPI System to precisely detect small differences in adhesion was demonstrated by measurements taken on a variety of abiotic and biotic test surfaces. Since then the MagPI System has contributed significantly to advancement in our understanding of biostabilisation. Until recently research into the biostabilisation of sediments focussed largely on the stabilising effect of benthic microalgae and their carbohydrate-rich EPS (Underwood & Paterson, 2003; Spears et al., 2008; Stal, 2003). The contribution of benthic bacteria was for the most part overlooked despite their omnipresence at sediment surfaces and their ability to produce copious amounts of EPS as recognized from medical (Costerton et al., 1999), biotechnology (Wang et al., 2006) and industrial investigations (Kumar & Anand, 1998). Studies in which the MagPI System has been used to measure sediment stability appear to show that the role of heterotrophic bacteria in biostabilisation far exceeds what was previously thought and may even surpass that of microalgae.

Gerbersdorf et al. (2009) investigated the biostabilisation potential of natural microbial assemblages on a non-cohesive substratum under conditions of nutrient limitation and repletion. Measurements of adhesion / stability obtained by the MagPI System and the Cohesive Strength Meter (CSM) were related to EPS (protein and carbohydrate), bacterial cell numbers, bacterial community composition, diatom biomass and diatom assemblage composition. The sensitivity of the MagPI System was highlighted by the inability of the CSM to determine differences in substratum stability between the control (no microorganisms and no nutrient addition) and early stages of the experimental treatments while the MagPI System indicated a significant increase in adhesive capacity as compared to the control even at this early stage in biofilm development. Nutrient addition appeared to profit the microalgae whereas bacteria dominated in nutrient-deplete cultures. The taxonomic shift between treatments resulted in differences in EPS composition which in turn moderated the biostabilisation capacity: microalgal dominated cultures were found to be less stable than those cultures where bacteria were prolific. Lubarsky et al. (2010) utilised the MagPI System in a comparison of pure bacterial cultures, axenic microalgal cultures and mixed assemblages grown on a non-cohesive substratum in an attempt to elucidate the individual stabilising capacity of the main biofilm components. Pure bacterial cultures had a significantly higher stabilisation potential compared to the microalgae. These results back-up the assertions of Gerbersdorf et al. (2008) that bacteria do play an important role in biostabilisation and can be regarded as “ecosystem engineers”. Mixed assemblages were more stable than either pure bacterial cultures or microalgae. However, the hypothesis of a synergistic relationship between the microalgae and bacteria in terms of stability was discounted and it was deemed more likely that in mixed microbial culture the combination of EPS components with different mechanical properties and characteristics accounted for the increase in stability.

6. Biofilms in medicine

In recent years there has been an alarming rise in the occurrence of multi-drug resistant microorganism infections (Fridkin & Gaynes, 1999; Gaynes & Edwards, 2005; Lessa et al., 2009; Livermore, 2000). Two bacterial strains are of particular concern: meticillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (Smith & Hunter, 2008). The persistence of these infectious microorganisms, despite measures to control them, is attributed to their existence as biofilms rather than as free-floating cells (Donlan & Costerton, 2002). Microorganisms incorporated into a biofilm have 10 to 1000 times more resistance to antimicrobial agents than planktonic microorganisms (Mah & O'Toole, 2001). An estimated 65 – 80% of infections in the developed world are biofilm related (Coenye & Nelis, 2010). It has been speculated that the emergence of biofilm diseases results from the success of vaccines and antibiotics against diseases originating from planktonic bacteria (Costerton, 2001).

Several mechanisms are considered to be responsible for sessile microorganisms' resistance to antibiotics. They include: i) the delayed or incomplete penetration of antimicrobial agents through the extracellular polymeric matrix in which cells are enclosed (Stewart, 1997; Suci et al., 1994), ii) slower growth rates and metabolism of sessile microorganisms compared to planktonic ones and hence slower uptake of antibiotics (Anwar et al., 1992; Evans et al., 1990) and iii) quorum sensing induction of a biofilm specific phenotype (Mah & O'Toole, 2001; Dagostino et al., 1991; Whiteley et al., 2000). For example, it has been suggested that in *Escherichia coli* biofilms the induction of the rpoS gene instigates a physiological response which in turn leads to antibiotic resistance (Adams & McLean, 1999). The disruption of quorum sensing systems is being investigated as a novel means of preventing or limiting biofilm pathogens (Dong & Zhang, 2005; Stickler et al., 1998). Of the numerous products seen to inhibit quorum sensing in *in vitro* systems a few (halogenated furanones, fungal compounds and garlic extract) have been tested with great success in animal models (Hentzer et al., 2003; Manefield et al., 2002; Rasmussen et al., 2005; Wu et al., 2000, 2004). However, the potential toxicity of furanones and patulins prevents their clinical use and garlic extract would have to be administered to humans in huge quantities to be comparable to the dosage used in mouse experiments (Rasmussen & Givskov, 2006).

6.1 Biofilms and infectious diseases

The chronic pneumonia that affects Cystic Fibrosis (CF) sufferers is one infection for which there is definitive proof of *P.aeruginosa* biofilm involvement. The well established morphological evidence is supplied by the recovery of cells from the airways of CF patients which are embedded in a thick matrix of extracellular material (Costerton et al., 1983; Lam et al., 1980). The hypothesis that the pathogen grows as a biofilm has more recently been reinforced by chemical data. Singh et al. (2000) present *in vitro* data showing that biofilm populations of *P.aeruginosa* produce more butyryl (C4) acyl homoseine lactone (AHL) than the oxyodecanoyl (C12) quorum signal whereas planktonic cells favour production of the C12 signal. Sputum examined directly from CF patients revealed that the ratio of the C4 to C12 signal was comparable to that found in the *in vitro* grown biofilms. Prior to these findings, infections could be classed as biofilm-related based solely on morphological evidence. The addition of this chemical data has established CF pneumonia as the definitive biofilm infection (Costerton, 2001).

CF itself is an autosomal recessive hereditary disease in which a net deficiency of water renders the respiratory mucous more viscous and as a result impairs the mucociliary clearance of inhaled particles from the airways leaving the patient vulnerable to bacterial infection (Donlan & Costerton, 2002). *S.aureus* and *Haemophilus influenzae* infections usually predispose airway colonisation by *P.aeruginosa* (Koch & Hoiby, 1993). Infections due to these strains can generally be controlled by antimicrobial agents. However, antipseudomonal therapy is less effective. By adolescence most Cystic Fibrosis patients will have become chronically infected with *P.aeruginosa* which can be severely debilitating and often fatal (Koch & Hoiby, 1993; Lykzak et al., 2002). The exact mechanisms by which the CF lung becomes colonised with *P.aeruginosa* biofilms are unknown. One hypothesis is that the thick respiratory mucus provides a matrix scaffold and creates a hypoxic environment in which *P.aeruginosa* are believed to thrive (Worlitzsch et al., 2002; Yoon et al., 2002). The second hypothesis is that airway inflammation enhances pseudomonal receptors on the respiratory epithelia to which *P.aeruginosa* can attach (Hall-Stoodley et al., 2004).
CF is just one example of a biofilm related infection. Other diseases in which infectious biofilms are implicated include native valve endocarditis where bacteria or fungi in the blood stream adhere to vascular endothelium and potentially lead to structural damage of the valve tissues (Donlan & Costerton, 2002); Otitis media, a common childhood ear infection (Hall-Stoodley et al., 2006) and Peritontitis, a disease affecting the supporting tissue of teeth (Schaudinn et al., 2009).

6.2 Biofilms on indwelling medical devices

Infectious biofilm formation in the human body is not restricted to biotic surfaces. Indwelling medical devices (e.g. prosthetic heart valves, contact lenses, intrauterine devices and urethral catheters) are susceptible to bacterial adhesion and the subsequent formation of a biofilm. Bacteria may originate from the skin of the patient, health care workers, tap water or other fluid to which the device is exposed (Donlan, 2001). The adhesion of microorganisms to urinary catheters is particularly problematic. Catheter associated urinary tract infections (CAUTI) are the most common hospital acquired infection (Desai et al., 2010). Urinary catheters are tubular, latex or silicon devices inserted into the bladder via the urethra for a variety of purposes including collection of urine during surgery, measuring urine output, prevention of urine retention and control of urinary incontinence (Schumm & Lam, 2008). Urinary catheters are used in enormous numbers in modern medicine. An investigation carried out across eight European countries showed that 11% of hospitalised patients were undergoing catheterisation (Jepsen et al., 1987).
The occurrence of urinary tract infection is related to the length of time a patient is subject to catheterisation. Of those patients undergoing short term catheterisation (up to 7 days) 10 to 50% acquire an infection (Haley et al. 1981; Mulhall et al. 1988) and virtually all patients undergoing long-term catheterisation (longer than 28 days) develop infections (Warren, 1991). While the acquired infections are generally asymptomatic, patients are at risk from a variety of complications that render them more vulnerable than non-catheterized patients. Platt et al. (1982) revealed in a study of hospitalised patients that the development of a urinary tract infection during catheterisation was associated with an almost threefold increase in mortality. Kidney and bladder stones, bladder cancer, bacteraemia and pyelonephritis are among the complications that can potentially afflict catheterised patients (Stickler & Zimakoff, 1994).

The scale of this problem puts the development of catheter surfaces that prevent biofilm formation at the forefront of medical research. The most common antimicrobial compounds in urinary catheters are silver and nitrofurazone. However, their effectiveness is variable between different studies. One review (Schumm & Lam, 2008) concluded that silver alloy catheters did decrease the occurrence of asymptomatic bacteriuria in patients undergoing both short term and long term catheterisation, although this decrease was less pronounced in those patients catheterised for over a week. Desai *et al*. (2010) found that nitrofurazone-impregnated catheters had only a minimal preventative effect in the earlier stages of *E.coli* and *E.faecalis* biofilm development (< 5 days) and that silver impregnation had a negligible effect. The incorporation of a silver releasing device between the drainage tube and catheter was found to provide an antibacterial barrier that could protect against bacterial colonisation for periods of at least 10 days in the bag and 8 days for the tubes and catheters (Stickler et al., 1994). The principle of this device is that as urine flows through it bactericidal silver ions will be released by dissolution.

6.3 The MagPI System in medical research

It is clear that health care providers and medical microbiologists still have some way to go in identifying strategies that will prevent biofilm related infections. The MagPI System could prove to be a very useful tool in these investigations due to its ability to detect early stage biofilm formation. It could, for example, be utilized in the laboratory based development of anti-biofilm coatings and materials for indwelling medical devices or to assess the effectiveness of quorum sensing disruptors and antibiotics on biofilm formation. It could also be used in more frontline actions against biofilm infection such as in hospital disinfection to identify bacterial colonization on equipment. In the case study below (Section 7.0) we have used the MagPI System to investigate the effect of antibiotics on biofilm development in aquatic systems. Although this study was not carried out on bacterial biofilms in a setting relevant to medical science it does demonstrate the straight forward approach of the MagPI system to measuring early-stage biofilm formation and highlights its transitional value between different scientific disciplines. Besides the uses for the MagPI System in sediment ecology research and medical and pharmaceutical research, we have identified numerous fields in which the MagPI System could be utilised (Table 2).

Biofilm Occurrence	Problem/ Effect	Mitigation Efforts	MagPI Potential Use	References
Teeth	Plaque formation and dental caries	Incorporation of antimicrobial agents (e.g. bisbiguandines, metal ions, phenols, quaternary ammonium compounds) into toothpaste and mouth rinses.	Development of anti-plaque products and research into the bacteriology of plaque biofilms.	Marsh, 2011; Rosan & Lamont, 2000
Water distribution network	Clogging of pipes, decrease in water velocity and carrying capacity, Increased corrosion and energy utilisation. Potential contamination by pathogens.	Chemical water treatment e.g. chlorination.	Monitoring biofilm formation. Developing new technologies and treatments.	LeChevalier et al.,1987; Lund & Ormerod, 1995; Momba et al. 2000
Ships' Hulls	Increased surface roughness increases frictional resistance and thus fuel consumption. Decreased top speed and range.	Anti-biofouling coatings which incorporate biocides e.g. Tributyl tin (use is regulated due to their toxicity to non-target marine species) or copper.	Development of non-toxic anti-fouling coatings.	Champ, 2003; Schultz et al., 2010

Biofilm Occurrence	Problem/ Effect	Mitigation Efforts	MagPI Potential Use	References
Food stuff and food packaging	Economic loss due to food spoilage. Serious hygiene problem- adherence of pathogenic microorganism poses threat to human health.	Coatings and paints with antimicrobial agents for factory floors, walls etc. Removal of surface roughness of machinery. Disinfection of factories. Inhibition of biofilm development on food contact surfaces by bioactive compounds (e.g. Nisin).	Monitoring biofilm development in industrial plants. Development of antimicrobial agents for food packaging.	Kumar & Anand, 1998
Fluid flow systems (Industrial system cooling processes)	Decreased efficiency of heat exchangers, corrosion and health risk to workers if there's pathogen contamination.	Chemical water treatment with biocides e.g. hypochlorite, chlorine dioxide, bromine, ozone. Biofiltration.	Development of non-toxic chemical treatments and new technologies. Monitoring biofilm development.	Flemming, 2002; Meester et al., 2003

Table 2. Examples of biofilm occurrence, their effect and ways in which the MagPI could be used to help overcome these problems

7. Case study: The effect of antibiotics on bacterial biostabilisation potential

7.1 Introduction

In recent years awareness of antibiotics as common contaminants of aquatic systems has increased significantly (Kümmerer, 2001, 2009; Santos et al., 2010; Segura et al., 2009). Antibiotics reportedly occur in wastewater treatment plant (WWTP) effluent and surface waters at concentrations ranging from ng l^{-1} to several µg l^{-1} (Costanzo et al., 2005; Hirsch et al., 1999). There are a number of routes via which antibiotics can reach aquatic systems (Figure 4). As an important group of pharmaceuticals antibiotics are used extensively to treat infectious diseases in humans. Following consumption antibiotics are subject to metabolic reactions, such as hydroxylation, cleavage or glucuronation. However, between 30 and 90% of the administered dosage of antibiotics is excreted from the body still in a biologically active form (Jjemba, 2006; Rang et al., 1999). Some of these compounds will later be released into aquatic systems in effluent from WWTPs. Several investigations have shown that residual pharmaceuticals are incompletely removed by waste water treatment procedures (Heberer, 2002; Ternes, 2002; Xu et al., 2007). Antibiotics are also used in huge quantities in animal husbandry and increasingly in aquaculture to protect the health of animals, enhance growth and promote nutritional efficiency (Sarmah et al., 2006). As a result antibiotics also enter surface and ground water after leaching from animal feed and excrement (Christian et al., 2003). Another major contributor of antibiotics to aquatic systems is pharmaceutical manufacturers. Holm et al. (1995) found that groundwater down gradient from landfill used by a pharmaceutical company contained a large variety of sulphonamides at concentrations up to 5mg l^{-1}. Another investigation revealed that antibiotics were occurring in the mg l^{-1} range in effluent from drug manufacturing in India (Larsson et al., 2007).

Antibiotics released into aquatic environments are a concern for several reasons, including: i) contamination of water used for irrigation, drinking or recreation, ii) promotion of bacterial resistance to antibiotics (Kümmerer, 2009), iii) disruption of sewage treatment facilities in which microorganisms perform waste water treatment functions (Gomez et al.,

1996; Campos et al., 2001) and iv) their potential to negatively impact important ecosystem services regulated by microorganisms e.g. denitrification, nitrogen-fixation and organic matter degradation (Costanzo et al., 2005; Hirsch et al., 1999).

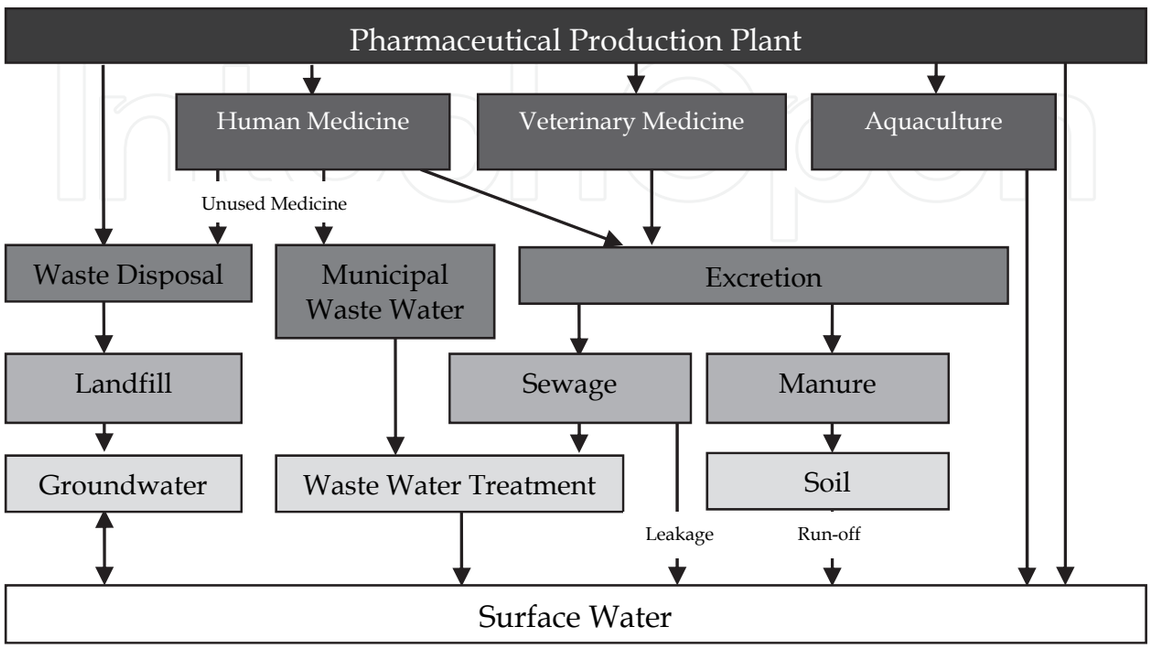

Fig. 4. Schematic diagram showing possible pathways of antibiotics into aquatic systems.

As previously discussed microbial consortia in aquatic systems drive a number of important processes in aquatic ecosystems (Section 5.0). One of these functions is biostabilisation whereby microorganisms living in biofilms at the sediment surface mediate the response of the sediment to erosive forces. Bacteria in biofilms are known to play an important role in sediment stabilisation (Gerbersdorf et al., 2009; Lubarsky et al., 2010). The objective of the present study was to investigate the biostabilisation potential of natural bacterial biofilms when exposed to environmentally relevant concentrations of antibiotics. Understanding the biostabilisation capacity of biofilms and its impairment by pollutants is important for successful sediment management in waterways and coastal zones.

Chloramphenicol, a bacteriostatic antibiotic, was selected for use in this investigation. It inhibits the growth and reproduction of certain bacteria by preventing peptide bond formation and thus disrupts the growth of peptide chains (Brosche & Backhaus, 2010). The use of chloramphenicol in human medicine is restricted due to its toxic properties (Forth at al., 1992) and its use has been completely banned in veterinary medicine since 1995 (BGW, 1996). However, chloramphenicol is still used extensively in aquaculture (Fierro & Olivia, 2009). Chloramphenicol occurs in surface water at relatively low concentrations compared to some other antibiotics, a maximum concentration of 0.06µg l^{-1} has been recorded (Hirsch et al., 1999). The concentrations used in this experiment (5, 10 and 50µg l^{-1}) are not environmentally relevant concentrations of chloramphenicol itself. They were chosen to represent concentrations of total antibiotics in surface waters. Hirsch et al. (1999) found that the concentrations of certain antibiotics in surface waters reached 1.7µg l^{-1}. The mean

concentration of antibiotics in WWTP effluent in the Thames catchment area has been estimated at 62µg l^{-1} . By convention the concentration of antibiotics in surface waters where no measurements exist is taken as 10% of the concentration in WWTP effluent (Hirsch et al., 1999; Singer et al., 2011). Thus, it would not be unrealistic that background total antibiotic concentrations of 5µgl^{-1}, as used in this investigation, exist in some waterways.
Over the course of the experiment the MagPI System was used to measure the adhesive capacity of the substratum, a proxy for sediment stability. It was hypothesised that the MagPI System would detect a negative effect on substratum stability as a result of antibiotic exposure and that this effect would become increasingly pronounced as antibiotic concentration increased.

7.2 Materials and methods

7.2.1 Bacterial cultures

Surface sediment (20mm depth) from the intertidal mud flats of the Eden Estuary (Scotland, 56°22'N, 2°51'W) was mixed with the same volume of 1 µm filtered seawater and sonicated (Ultrasonic bath XB2 50 – 60 Hz) for 10 min to enhance detachment of the bacteria from the sediment, followed by two 10 min periods of centrifugation at 1500 rpm (Mistral E, Sanyo rotor 43122-105). The pellet (sediment fraction) was separated from the supernatant (containing bacterial fraction). The supernatant was centrifuged again, this time at 17000 rpm (Sorval, RC5B/C) for 10 min to obtain a microbial pellet. The resultant supernatant was discarded and the pellet with its associated bacteria was resuspended and passed through a 1.6 µm filter. The filter size was chosen to exclude the smallest expected microalgae from the estuarine sediment. Autoclaved standard nutrient broth (Fluka, peptone 15g l^{-1}, yeast extract 3g l^{-1}, sodium chloride 6g l^{-1}, D (+) glucose 1g l^{-1}) was added to the filtered supernatant (5:1). The bacterial stock cultures were left to establish under constant aeration and temperature (15°C) in the dark for one week prior to the experiment beginning.

7.2.2 Experimental set-up

Glass incubation chambers (L: 105mm, W: 105mm, H: 55mm) were filled to c. 1cm depth with 270 µm glass beads to provide a substratum for biofilm formation. The chambers were filled with 300ml autoclaved seawater (control) that had been spiked with defined concentrations of the antibiotic chloramphenicol (treatments). For the treatments a stock solution of chloramphenicol was prepared followed by dilution with autoclaved seawater (35psu) to the desired concentrations of 5 (T1), 10 (T2) and 50 (T3) µg l^{-1}. The glass chambers, including those for the control, were inoculated with 10ml of the bacterial stock solution to initiate biofilm growth. Four replicates were established for each of the treatments and the control. All incubation chambers were gently aerated and kept at a constant temperature (15°C) in a dark room over the experimental period of 6 days.

7.2.3 Sampling strategy

Sampling was carried out on days 0, 2, and 6. Samples for EPS protein analysis and low-temperature scanning electron microscopy (LTSEM, Figure 7) were obtained using a mini corer (cut-off 2ml syringe) and frozen immediately in liquid nitrogen and stored at -80°C until required for analysis.

7.2.4 Substratum stability

The adhesive capacity, a proxy for bed stability, of the biofilms growing on the glass beads was measured on sampling days by magnetic particle induction (MagPI System). Fluorescent particles of size range 150 - 250μm were applied to the test surface as outlined in Section 3. This particle size range was chosen to best represent the grain size of the substratum. The MagPI probe was set 7mm above the surface of the glass beads. The following equation was used to calculate the magnetic flux density (MFD) at total particle clearance.

Equation 2. $y = 3.1903x + 0.8443$

Obtained from the 7mm seawater calibration (Figure 3), where y is the MFD and x is the voltage at particle uplift from the test surface.

7.2.5 EPS extraction and colloidal protein analysis

Cores were placed in safety-lock Eppendorf caps with 2ml of distilled water and rotated for 1.5 hours by a horizontal mixer (Denley Spiramix 5) at room temperature. After centrifugation at 5000rpm (Mistral 3000E Sanyo, rotor 43122-105) for 15 minutes the supernatant was analysed for protein following the modified Lowry procedure (Raunkjaer et al., 1994). Protein concentration was measured by spectrophotometer at 750nm wavelength (BUCK Scientific, CECIL CE3021, UK) and protein concentrations were calculated according to BSA standard (Albumin from bovine serum: Sigma, cat no A 4503-10g) with results reported in $\mu g\ ml^{-1}$.

7.2.6 Statistical analysis

All statistical analysis was conducted using Minitab version 16 (Minitab, Coventry, UK). Substratum stability (mTesla) variation over time and between treatments was assessed using two-way analysis of variance (ANOVA: significance level $P < 0.05$). The unbalanced data set of colloidal protein concentration was tested for variation over time and between treatments by general linear modelling (significance level $P < 0.05$). One-way ANOVA (significance level $P < 0.05$) was applied followed by Tukey's post hoc test to determine which treatments showed a significant difference in adhesive capacity and protein concentration and also to determine for which treatments there was a time effect on the adhesive capacity and protein concentration.

7.3 Results

7.3.1 Substratum stability

A two-way ANOVA indicated significant variation in the response of sediment stability to both time ($P< 0.001$) and treatment ($P< 0.001$). For measurements of adhesive capacity taken directly after experimental set-up (day 0), there was no significant difference ($P= 0.484$) between treatments. On experiment days 2 and 6 there was a strong treatment effect (day 2 $P< 0.001$; day 6 $P= 0.001$). For both days, the mean adhesive capacity of the control was found to be significantly higher than any of the treatments. Statistical testing each day did not reveal a significant difference between the three treatments (Figure 5). One-way ANOVA determined a time effect only for the control ($P= 0.001$) and treatment 2 ($P= 0.012$). For the control measurements of adhesive capacity differed significantly between day 0 and

day 2 as well as day 0 and day 6 but no significant difference was found between days 2 and 6. For treatment 2, the adhesive capacity measurements on day 6 differed significantly from day 2 and day 0 but days 0 and 2 of the experiment were not significantly different to each other.

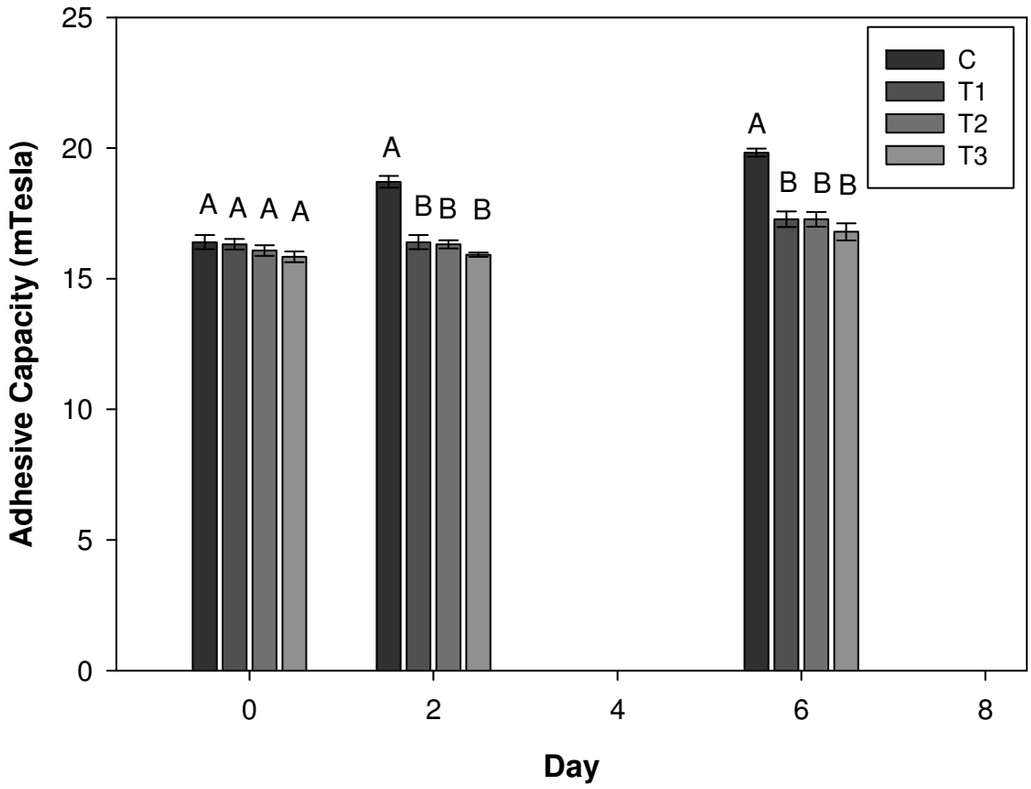

Fig. 5. Mean response (n=4, +/- standard error) of substratum adhesive capacity (proxy for sediment stability) to antibiotic exposure over the experimental period. C= control, T1= low antibiotic concentration (5 µg l^{-1}), T2= medium antibiotic concentration (10 µg l^{-1}), T3= high antibiotic concentration (50 µg l^{-1}). Where treatments do not share a letter denotes a significant difference on that day.

7.3.2 Colloidal protein concentration

There was a significant response to time ($P< 0.001$) but not treatment ($P= 0.087$) in colloidal protein concentration. On days 0 and 2 of the experiment no significant difference was found between treatments (Day 0 $P= 0.319$; Day 2 $P= 0.401$). One-way ANOVA of protein concentration on day 6 revealed a significant variation between treatments ($P= 0.03$). T1 (low antibiotic concentration) was significantly higher than the control (C). Neither T1 nor C was significantly different from T2 and T3 on day 6 of the experiment (Figure 6). Statistical testing revealed a time effect only for T1 ($P= 0.001$) and T2 ($P= 0.012$). For T1, the protein concentration differed significantly between day 0 and day 6 and no significant difference was found between days 2 and day 6 or day 2 and day 0. For treatment 2, the protein concentration on day 6 differed significantly from days 0 and 2 but protein concentration on days 0 and 2 were not significantly different.

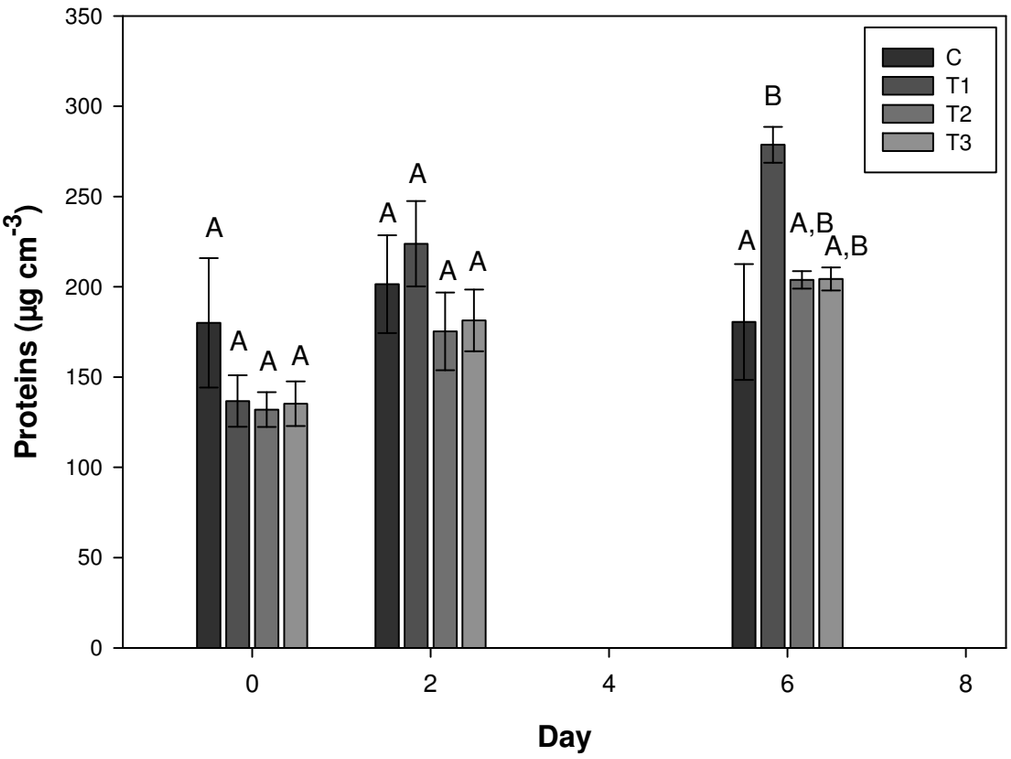

Fig. 6. Mean response (n=4, +/- standard error) of colloidal protein concentration to antibiotic exposure over the experimental period. C= control, T1= low antibiotic concentration (5 µg l^{-1}), T2= medium antibiotic concentration (10 µg l^{-1}), T3= high antibiotic concentration (50 µg l^{-1}). Where treatments do not share a letter denotes a significant difference on that day.

7.4 Discussion

If the results for the adhesive capacity measured by magnetic particle induction are taken as a proxy for biofilm formation then it would appear that biofilm development was significantly higher on the substratum surface of the control when compared to all treatments. No significant time effect on the adhesive capacity was found for treatments 1 and 3. As the substratum was composed wholly of non-cohesive glass beads the binding force observed in the control and in treatment 2 (on day 6 only) must have been due to bacterial adhesion and EPS secretion. The control had a significantly higher adhesive capacity than each of the treatments on days 2 and 6 of the experiment. This suggests that the biostabilisation potential of bacteria is affected by antibiotics at concentrations likely to be found in natural surface waters. In the event of an influenza pandemic the amount of antibiotics reaching surface waters is predicted to increase. Singer et al. (2011) project a mean total antibiotic concentration of 15µg l^{-1} and a maximum concentration of 80µg l^{-1} in the Thames catchment area in the event of a severe pandemic. Our results for the adhesive capacity of treatment 3 (50 µg l^{-1}) suggest that the biostabilisation potential of bacteria in aquatic systems would be significantly affected in the event of a severe influenza pandemic.

To-date the majority of studies addressing the effects of pharmaceuticals on aquatic microorganisms have been conducted using concentrations greater than those observed in the environment (Halling-Sorensen, 2001; Kümmerer et al., 2000; Pomati et al., 2004). Of the investigations conducted using environmentally relevant concentrations of antibiotics there has been a strong indication that antibiotics in aquatic ecosystems have the potential to influence biotic processes (Costanzo et al., 2005). Schreiber and Szewzyk (2008) conducted an experiment using environmentally relevant concentrations (0.5 – 50µg l^{-1}) of antibiotics. They found that antibiotic exposure enhanced, inhibited or had no influence on the initial adhesion of bacteria to a surface. The effect was dependent on the selected pharmaceutical, the bacterial strain and the adhesion surface as well as antibiotic concentration. In aquatic systems there are a myriad of antibiotics present all of which function differently. In addition biofilms are not composed solely of bacteria, other microorganisms, microalgae for example, may also be present in surface sediment biofilms. Our results highlight the need for investigations into the effect of pharmaceuticals at concentrations occurring in surface waters on biostabilisation as well as other important ecosystem services conducted by microorganisms in aquatic ecosystems.

As previously discussed (Section 5.0) EPS production by microorganisms adhered to the sediment surface is thought to significantly increase its stability (Underwood & Paterson, 2003). Traditionally, microalgae and their polysaccharide-rich EPS were considered to be the principal binding force (Underwood & Paterson, 2003). However, recent work suggests that biofilm bacteria and bacterial EPS which is estimated to contain up to 60% protein (Flemming & Wingender, 2001) are more important for biostabilisation than previously considered and that a synergistic effect between EPS protein and EPS carbohydrate might strengthen their binding forces (Gerbersdorf et al., 2008; Lubarsky et al., 2010). In spite of this, no correlation was found ($\alpha = 0.05$) between substratum adhesiveness and colloidal protein. Adhesive capacity results imply that there is no biofilm formation for treatment 1 and 3 but that biofilm formation was not inhibited in treatment 2 or in the control. However, if we take protein concentration rather than the MagPI System measurements as an indication of biofilm formation then it would appear that there was no biofilm formation in the control as there is no significant time effect on protein concentration. Both adhesive capacity results and protein concentration suggest the development of biofilms in treatment 2. A time effect on the protein concentration of treatment 1 was also observed and the protein concentration was found to be significantly higher for treatment 1 than for the control on the final day of the experiment. The higher colloidal protein concentration observed in treatment 1 on the final day of the experiment may be the product of a stress response by the bacteria to antibiotic exposure. Studies have shown that at subinhibitory levels some antibiotics stimulate EPS production by certain bacteria (Rachid et al., 2000). The lack of correlation between protein concentration and adhesive capacity in this experiment may indicate that proteins do not actually play a very important role in biostabilisation in this experimental system. Alternatively, exposure of the bacterial cultures to chloramphenicol may not necessarily affect the quantity of the EPS as much as the quality. It is possible that the higher molecular weight fraction of EPS protein is responsible for the binding characteristics that have been observed in natural bacterial assemblages (Lubarsky et al., 2010). Inhibition of peptide bond formation by chloramphenicol may result in the excretion of only small molecular weight protein molecules which have no influence on sediment stability.

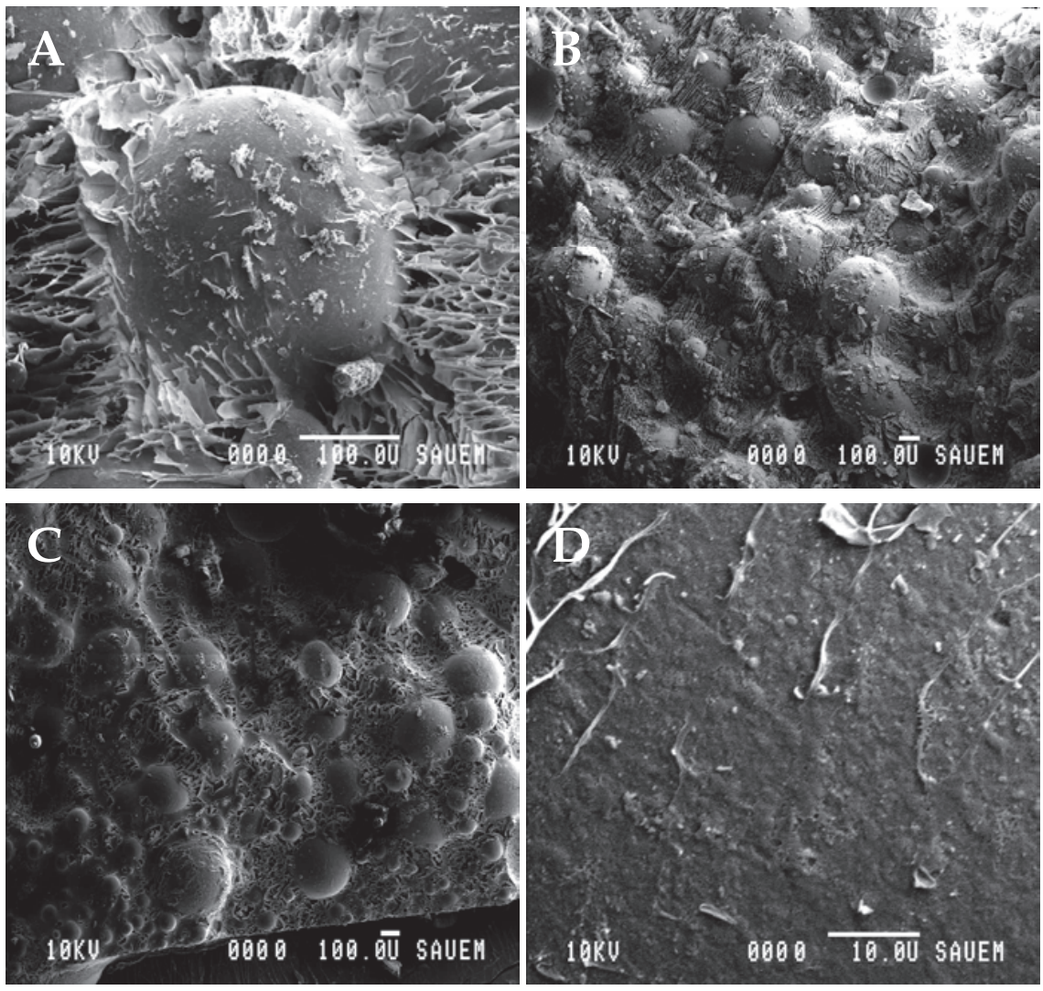

Fig. 7. Low- temperature scanning electronmicroscopy (LTSEM) images of biofilms on glass beads. **A**: Day 0, control. Low organic matter cover, honeycomb structure around bead is water; **B:** Day 6, T3; **C:** Day 6, T2; **D:** Close-up of biofilm on glass bead surface, Day 6, T1.

7.5 Conclusion

The adhesive capacity results for this experiment successfully demonstrate the ability of the MagPI System to determine subtle changes in surface adhesion as a result of biofilm formation. The stability of the non-cohesive glass bead substratum was significantly increased during the experimental period for the control. Although there was a detrimental effect on biostabilisation as a result of the bacteria being exposed to antibiotics in the treatments the effect was not as hypothesised; the adhesive capacity was not found to decrease with increasing chloramphenicol concentration. It must be considered that this experiment targeted only one group of biofilm microorganisms and used a single compound. As such these findings cannot be taken as conclusive proof that the levels of antibiotics found in our waterways are having a damaging effect on the sediment stabilisation potential of biofilms. They do however highlight the need for further investigations using a mixture of antibiotics at environmentally relevant concentrations and

varied microbial assemblages. Future work should also calibrate MagPI System measurements of adhesive capacity against biological variables other than EPS protein concentration, for example bacterial cell numbers or EPS carbohydrate concentration.

8. Summary

Biofilms have become an important research topic across numerous scientific disciplines in recent years. While their presence can be desirable or beneficial in some situations, it can be incredibly harmful or costly in others. Bacterial biofilms can be particularly harmful to human health and pose a serious challenge in modern medicine. The last decade has seen a significant increase in the occurrence of multi-drug resistant microorganism infections. The persistence of these infectious microorganisms is attributed to their existence as biofilms rather than as free-floating cells. It is thought that microorganisms in biofilms have 10 to 100 times more resistance to antibiotics than their planktonic counterparts. Biofilm research in medical science, as well as in many other fields, has previously been conducted using time-consuming procedures or large laboratory scale systems that can be both expensive and labour intensive. The MagPI System presented in this paper is an alternative method for biofilm detection. It uses magnetic induction of ferrous particles to quantify the adhesive capacity of a test surface. As the "stickiness" of surfaces can often be attributed to the presence and growth phase of a biofilm the MagPI System can be used to evaluate biofilm formation and state of development. This system has already been used with much success in the field of sediment ecology and we propose its use across a number of other fields where research questions require a measure of adhesion or extent of biofilm formation. The MagPI System may be especially useful in medical science. It could, for example, be used in the development of anti-microbial indwelling medical devices, to evaluate the effect of antibiotics on biofilm formation or in the disinfection of healthcare facilities. In summary, the MagPI System combines a highly variable system, of logistic ease and relatively low cost providing a means of repeatedly and non-destructively quantifying the adhesive capacity of a test surface as a result of biofilm formation.

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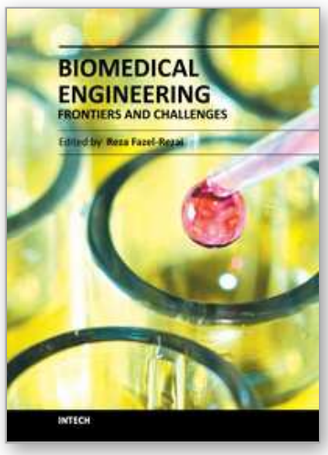

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