Towards and activated sludge floc formation model based on microbial colonisation of chitin

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Towards an activated sludge floc formation model based on microbial colonisation of chitin

By

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A thesis submitted in fulfilment of the requirements of the degree of Master of Philosophy

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AHL	Acyl homoserine lactone
AIP	Autoinducer peptide
AI-2	Autoinducer 2
Alum	Aluminium sulphate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BHL	N-Butanoyl-homoserine lactone
Blast	Basic local alignment search tool
BOD	Biological oxygen demand
C	Celsius
CBD	Chitin binding protein
CFB	Cytophaga-Flavobacteria-Bacteroidetes
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
C4-HSL	N-Butanoyl-homoserine lactone
C6-HSL	N-(3-oxo-hexanoyl)-homoserine lactone
C12-HSL	N-Dodecanoyl-homoserine lactone
DD	Degree of deacetylation
DGGE	Denaturant gradient gel electrophoresis
DHL	Decanoyl-homoserine lactone
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EPS	Extracellular polymeric substances
ETOH	Ethanol
FITC	Fluorescein isothiocyanate
xg	Gravitational constant

g	gram
GFP	Green fluorescent protein
GlcNAc	N-acetyl-D-glucosamine
$(GlcNAc)_2$	N,N'-diacetylchitobiose
h	hours
HHL	Hexanoyl-homoserine lactone
HMDS	Hexamethyldisilazane
HPLC	High pressure liquid chromatography
kV	Kilovolts
L	Litre
LFC	Laminar flow cell
LB	Luria bertini
M	Molar
MBG	Molecular biological grade
MBR	Membrane bioreactor
Mg	Milligram
Min	Minutes
ml	Millilitre
mM	MilliMolar
mm	Millimetre
MS	Mass spectrometry
MW	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanograms
NH_4OAc	Ammonium acetate

nM	nanoMolar
nm	nanometre
NSI	Nanoelectrospray Ionisation
OdDHL	N-Dodecanoyl-homoserine lactone
OHL	N-octanoyl-L-homoserine lactone
OHHL	N-(3-oxo-hexanoyl)-homoserine lactone
OOHL	3-oxo-octanoyl-homoserine lactone
OUT	Operational taxanomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain Reaction
Pmol	Picomolar
PTS	Phosphotransferase system
QS	Quorum sensing
Rcf	Relative centrifuge force
Rpm	rotations per minute
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
Sp.	Species
SS	Sludge supernatant
TAE	Tris Acetate EDTA
Tris	Tris*Hydroxymethyl*aminomethane
µm	Micrometre
µl	Microlitre
V	Volt
v/v	Volume per volume
w/v	Weight per volume
α	Alpha

β	Beta
β-GlcNAcidases	β-N-acetylglucosaminidase
γ	Gamma

Abstract

Chitin is one of the most abundant biopolymers on Earth. In this thesis, the colonisation of chitin by activated sludge bacteria was explored. Chitin was incubated with pure cultures of *Aeromonas hydrophila* GC1 isolated from activated sludge and with activated sludge itself. Stages of biofilm development were monitored by CLSM and SEM. Biomass accumulation was assessed by DNA yields. Denaturing Gradient Gel Electrophoresis and pyrosequencing were used to characterise bacterial communities colonising chitin. NSI-MS, TLC, and bioassays were used to detect AHL production on the surface of chitin. *Aeromonas* (pBB-luxR), a GFP based monitor strain, was also employed. Chitin degradation was monitored by SEM. Chitinase activity was detected using a colorimetric chitinase assay.

Results showed that cells attach to chitin after 24 hours of incubation. DNA yields revealed that biomass of *A. hydrophila* on chitin increases after 24 hours of incubation and decreases after 200 hours. Microscopy showed that integrity of the chitin becomes disrupted after 288 hours of being incubated in sludge. Results revealed that members of the *chitinophagaceae* family, of the *bacteroidetes* phylum, are the most abundant bacteria in sludge incubated with chitin. The monitor strain assay proved to be the most suitable method for AHL detection. AHLs were detected on chitin pieces at 24 hours, before cell attachment to chitin was observed. Chitinase activity was detected after 24 hours. These results supported the proposed model for chitin colonisation; where AHLs that bind and coat chitin are produced, followed by bacterial colonisation of the chitin surface and up-regulation of chitinase expression.

1.1 Introduction to wastewater treatment:

Wastewater treatment systems are one of the most significant inventions in modern history. Prior to the existence of wastewater treatment systems, the cosmopolitan streets of the most cultured cities in Europe were flooded with wastewater, pure drinking water was scarce, and the pathogens associated with wastewater often caused epidemics within these cities. Wastewater treatment plants allowed the separation of contaminated wastewater from potential drinking water, and allowed the possibility of reusing the produced effluent.

Wastewater consists of suspended solids, biodegradable organics, refractory organics, dissolved inorganics, nutrients, heavy metals, pollutants and microbiological pathogens [1]. The wastewater treatment process is concerned with the removal of these constituents and the production of an effluent that is safe to be released into the environment or reused.

1.1.1 The wastewater treatment process:

Wastewater systems involve preliminary, primary, secondary, and tertiary treatments. Preliminary treatments remove larger constituents of influent such as sticks, rocks, and grit. After larger particles are removed from the wastewater, primary treatments partially remove organic matter and suspended solids. Secondary treatment removes biodegradable organic matter reflected by biological oxygen demand (BOD) and chemical oxygen demand (COD) and suspended solids. In some treatment systems, nitrogen and phosphorus removal is also achieved in the secondary treatment stage [1]. Many different operations may be employed to achieve BOD, COD, and nutrient removal in secondary treatment. Of the different operations, activated sludge is the most commonly applied. Tertiary treatment involves additional nutrient removal and disinfection. Inorganic nutrients, trace chemicals, and pathogens are removed during the tertiary step [2]. Figure 1.1 illustrates the components in a typical activated sludge treatment plant

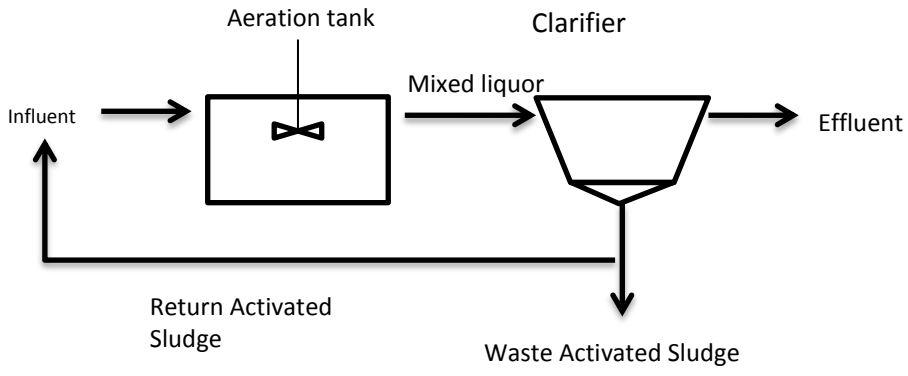


Figure 1.1: Conventional activated sludge treatment process.

1.1.1.1 Activated sludge process:

The activated sludge treatment process is one of the most efficient and widely used for biological treatment of municipal and industrial wastewater. The process was established in 1913 and requires the accumulation of microorganisms, organic material, and suspended solids into flocs, which settle and result in clean effluent [1]. The process has since been optimized to facilitate treatment of larger quantities of wastewater from both municipal and industrial sources.

The activated sludge process involves three main stages: reaction, separation of solids and liquids, and returning settled activated sludge [1]. After primary wastewater treatment, wastewater is pumped into an aerated reaction tank where the conditions that allow the microbes already present within the wastewater to flourish are provided. The activated sludge process relies primarily on the ability of microbes to form flocs, complex suspended solid aggregates [3] consisting of a consortium of microorganisms in addition to the water contaminants the microbes are required to degrade. The mixed liquor, or the mixture of activated sludge and influent wastewater, then passes into a sedimentation tank where the flocs settle and the treated water leaves the sedimentation tank as effluent. Without flocculation the sludge does not separate. The activated sludge which settles in sedimentation tanks is recycled as return activated sludge and used to treat more wastewater [4].

1.1.2 Microbiological Consortia of Activated sludge:

With respect to microbes, activated sludge typically consists of bacteria, archaea, viruses, fungi and protozoa [5]. The following diagram briefly illustrates the microbiological processes that occur within the activated sludge processes.

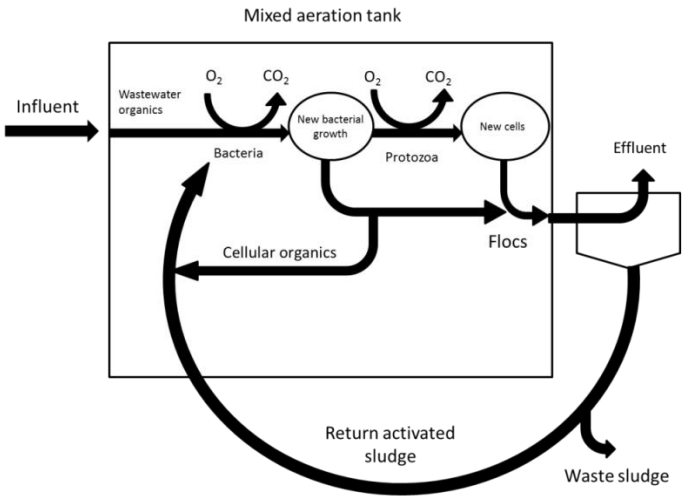


Figure 1.2: Microbiological processes in activated sludge. This diagram is adapted from [5].

The microbiological organisms in activated sludge are organised within flocs, a complex structure embedded in extracellular polymeric substances (EPS) [6]. The matrix may also contain organic and inorganic particles that have not been completely characterized. Some known components of the EPS, however, include polysaccharides, proteins, humic substances and nucleic acids [5, 7, 8].

Different bacterial species within the sludge population play different roles in the treatment process. Sludge communities typically include polymer degrading bacteria, nitrifying and denitrifying bacteria, dissimilatory iron-reducing bacteria, sulphur oxidisers and sulphate reducers [5]. Figure 1.3 shows the abundance of bacteria activated sludge samples obtained from different membrane bioreactors (MBRs).

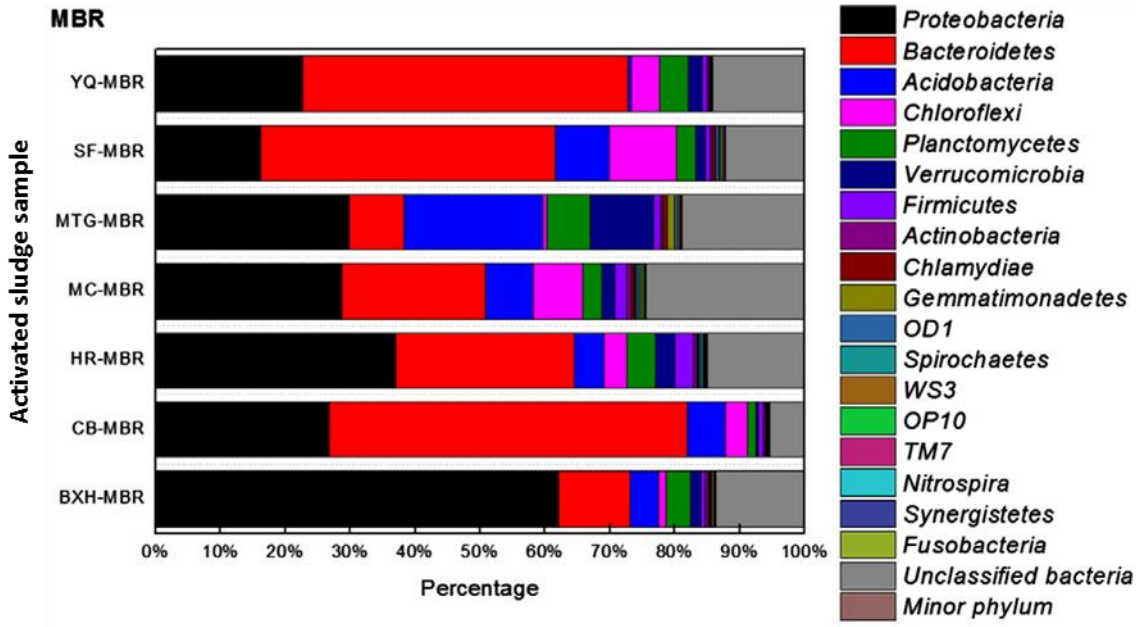


Figure 1.3: Bacterial community composition in activated sludge samples obtained from different MBRs in different wastewater treatment plants. After sample collection, DNA was extracted and prepared for pyrosequencing analysis. Abundance is presented in percentage, based on RDP Classifier classification. Adapted from [9].

In addition to the different bacterial species and their roles, protozoa play an important part in controlling the bacterial population within sludge. These microbes ingest cells through endocytosis and phagotrophy and influence the community distribution within activated sludge [5].

1.1.3 Operational problems and limitations of activated sludge:

Although some of the operational problems in activated sludge processes may be attributed to clarifier operation and other mechanical issues, the majority of complications are microbiological. The major problems that face activated sludge systems include, bulking sludge, rising sludge and foaming [1].

Bulking of sludge occurs when flocs have poor settling abilities, due to the growth of filamentous organisms such as *Beggiatoa* and *Thiothrix* [1]. Variations in the wastewater influent in temperature, pH and flow rate affect the growth of the filamentous microorganisms and bulking of sludge. Other factors such as low dissolved oxygen and nutrients encourage the growth of filamentous organisms over the growth of organisms that result in good settling flocs [1].

Rising sludge is mostly caused by denitrification. The nitrogen gas formed from the denitrification of nitrates and nitrites, gets trapped within flocs, which causes the sludge to rise instead of settle in the clarifier [1].

Foaming involves the formation of a brown layer of foam on top of the sludge. Foaming is caused mainly by filamentous bacteria, but has been reported to involve non-filamentous microorganisms as well [4]. Actinomycetes such as *Nocardia* and *Rhodococcus* species are involved in foaming. Excess fatty acids, nitrogen and phosphorus enhance the growth of foam causing bacteria within sludge [4]. The organisms cause hydrophobic flocs that create foam when they come into contact with and stabilize air bubbles [1].

Most of these problems can be overcome once they occur by manipulating mechanical parameters such as sludge retention time and clarifier operation, but these problems cannot be prevented from occurring. This is because the underlying microbiological processes that take place within activated sludge remain to be fully characterized and understood.

1.1.4 Commercial coagulants used in wastewater treatment systems:

As mentioned above, floc formation is an essential phenomena in the activated sludge process, and it entails biofilm formation around a surface. In wastewater treatment plants organic and inorganic coagulants, surfaces that encourage flocculation/coagulation of bacteria and waste within wastewater, are added [10]. Some inorganic additives include aluminium sulphate and ferric chloride; organic additives include non-ionic polymers and naturally occurring flocculants [10]. Once the solid particles have coagulated/flocculated, they can be removed. Furthermore, inefficient removal of particulate matter in the primary treatment step will have detrimental effects on the later phases of treatment [2].

The most commercial and widely used coagulant used in wastewater systems has been aluminium sulphate or alum [2]. Alum is cost-effective, efficient in its purpose, and easy to use [11]. However, there has been concern regarding the excessive use of alum as a coagulant as studies have shown that alum has negative health effects [12] with links to Alzheimer's disease [13]. This has resulted in increasing interest in the use of bioflocculants such as chitin and chitosan.

The study of bioflocculants and their impact on microbiological processes that occur in activated sludge treatments has been very limited, even when trying to address the issues that can occur within sludge treatment plants. Troubleshooting has taking an engineering approach rather than addressing activated sludge as a microbiological niche and understanding the processes that underlie it. The main microbiological phenomena discussed here are biofilm formation, flocculation and the regulation of these through intracellular communication, known as quorum sensing.

1.2 Biofilms:

Biofilms are complex structures composed of bacterial communities that have attached to surfaces or interfaces [14, 15]. The bacteria that make-up a biofilm can be heterogeneous [14] and are often called sessile communities [16].

In 1978, Costerton et al found that bacterial biofilms outnumbered planktonic cells on river rock surfaces, which led ultimately to a demonstration of the growth of bacterial pathogens on tissue surfaces [17]. The group observed that bacteria in biofilms influenced host disease symptoms and showed resistance to antimicrobial activity [17]. These discoveries uncovered the medical significance of biofilms and drew attention away from the pathogenesis of planktonic cells [17].

In the environment, most bacteria exist in biofilms and these can be found on many different surfaces. Biofilms have been found on rock surfaces [17], marine organisms [18-20], biomedical devices such as catheters [15] and contact lenses [21].

Biofilms confer resistance to antibiotics and biocides [22]. This has been shown in biofilms containing *Salmonella typhimurium* [23] and *Candida albicans* [24]. Cells deeply embedded in a biofilm become tolerant to certain biocides because they are protected by EPS and the layers of cells above [22]. The exposure of the top cells, however, to the biocides allows the cells within the biofilm to become resistant to the biocide [22].

1.2.1 The Biofilm life-cycle:

The process of biofilm formation has been studied extensively in many organisms. It involves attachment of planktonic cells to a surface, microcolony formation, biofilm maturation and formation of an EPS Matrix, cell death and finally detachment from the biofilm.

1.2.1.1 Attachment of Planktonic cells to a surface:

The first step in biofilm formation is the attachment of a bacterial cell to a surface [14]. The initial stages of biofilm formation have not yet been conclusively elucidated, but there are many opinions concerning what is involved and what influences these first steps. Pratt et al showed that flagella facilitate movement towards a surface and play an important role in the initial stages of biofilm development in *E. coli* [25]. In *Pseudomonas aeruginosa*, it has been shown that flagella and type IV pilli aid in bacterial surface attachment [26]. Type I pilli also play an important role in the surface attachment of *E. coli* [25]. Other studies have shown that many proteins are required for the early stages of biofilm formation, although the functions of all these proteins are yet to be characterised [26].

After coming in close contact with or moving towards a surface, bacterial attachment will take place [27]. The forces involved in this initial attachment phase include Van der Waals forces, electrostatic and hydrophobic interactions [28, 29]. This initial attachment is known as reversible attachment and is facilitated by covalent and hydrogen bonds [27, 30]. Reversible attachment is followed by irreversible attachment, where the attachment forces dramatically strengthen and removal of attached cells from a surface becomes difficult [27].

1.2.1.2 Micro-colony formation:

Once attachment to a surface has been established, bacterial aggregates or microcolonies are formed [31]. The bacteria in the microcolonies release proteins and enzymes which enable other bacterial species to utilize the surface [15]. Motility of the attached organisms also enables them to move across a surface, thus expanding the microcolonies surface area [25]. With new bacteria colonising the surface and the attached organisms multiplying, the microcolony matures into a biofilm. In *Pseudomonas aeruginosa*, these processes have been thoroughly described [31]. Already-present aggregates of *P. aeruginosa* can multiply and grow, resulting in a flat biofilm that undergoes further maturation and becomes a structured biofilm [32-34]. A bacterial stalk or attached non-motile population of bacteria can attract motile bacteria to attach onto the surface, possibly via the production of rhamnolipids [35].

1.2.1.3 EPS matrix formation and biofilm maturation:

As the biofilm develops, an extracellular polymeric matrix forms around it [14, 32]. This matrix is composed of proteins, nucleic acids, and exopolysaccharides [14, 36]. The bacterial communities within the biofilm release different proteins, nucleic acids and exopolysaccharides that cause changes to the biofilm [37].

The bacterial species within a biofilm vary, depending on environmental factors that cause certain species to proliferate and others to decline, such as a utilizable food source or environmental stress. Certain species will have the ability to express the necessary genes to survive the conditions within the biofilm. An example of stressful conditions in a biofilm is the lack of oxygen inside the biofilm; bacteria at the bottom of a biofilm have to be able to activate genes and express enzymes to adapt to the changed conditions and utilize other electron acceptors [38]. Other factors determine which species are more abundant in a biofilm, such as acylated homoserine lactones (AHLs) production. Davies et al showed that *P. aeruginosa* biofilms deficient in autoinducing signal molecules are sensitive to detergents, suggesting that the antimicrobial attributes of biofilms are influenced by cell-cell signalling [39].

1.2.1.4 Cell death within biofilms:

Cell death is an important feature of the biofilm life-cycle [16] and has been described in *Psuedoalteromonas tunicata* [40] and *P. aeruginosa* [41]. In *P. aeruginosa*, cell death can be caused by accumulation of reactive oxygen chemicals within the biofilm [41].

1.2.1.5 Detachment from the biofilm:

Once a mature biofilm has been formed, some of the bacteria differentiate into planktonic cells [42] that detach from the biofilm, disperse in the environment, and ultimately attach to and colonise a new surface, repeating the cycle [16]. Figure 1.4 represents the different stages in biofilm development.

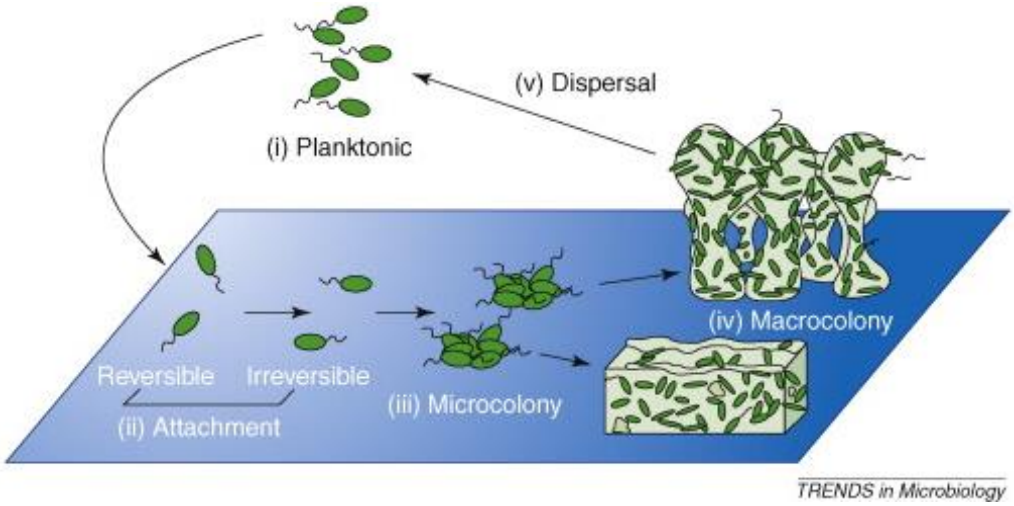


Figure 1.4: The biofilm life-cycle. Planktonic cells attach to a surface reversibly, then irreversibly to form micro-colonies. These micro-colonies mature into a biofilm which is engulfed in an EPS. The biofilm matures into macro-colonies, from which cells detach to search for a new surface to attach to. Taken from[38].

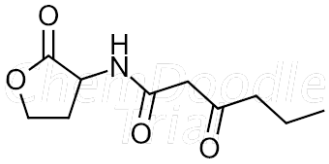
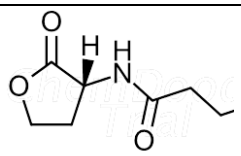
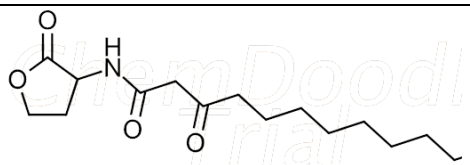
1.3 Quorum sensing:

Patterns of bacterial gene expression differ dramatically in biofilms compared to planktonic cells [22]. These phenotypes are expressed according to the requirements of the population within the biofilm and can be regulated by cell-cell signalling, or quorum sensing (QS) [43].

The term quorum sensing was coined in a pivotal review by Fuqua *et al*, and was used to describe a cell-density dependent system that bacteria use to orchestrate gene expression. Quorum sensing is mediated by the accumulation of diffusible signalling molecules in or around a bacterial population The concentration of the molecule reflects the cell density of a bacterial population and dictates if this density level is sufficient for population response [44-46]. These molecules are sometimes called autoinducing molecules and are produced in both Gram negative and positive bacteria [46, 47]. These molecules mediate phenomena such as swarming and twitching motility, and bioluminescence [48, 49].

In Gram negative bacteria, the diffusible autoinducing molecules are commonly Acyl homoserine lactones or AHLs. AHLs are composed of a homoserine lactone ring or HSL and an acyl side-chain, which can range from 4 to 18 carbon molecules. Some AHLs are illustrated in Table 1.1 [50, 51].

Table 1.1: Chemical structures of OHHL, BHL and OdDHL.

Name	Chemical structure	Abbreviation
N-(3-oxo-hexanoyl)-homoserine lactone		OHHL (C6-HSL)
N-Butanoyl-homoserine lactone		BHL (C4-HSL)
N-Dodecanoyl-homoserine lactone		OdDHL (C12-HSL)

1.3.1 The discovery of quorum sensing in *Vibrio fischeri:*

V. fischeri is a luminescent marine organism that colonises the squid *Euprymna scolopes* [46]. Nealson et al observed that luciferase, the enzyme responsible for bioluminescence, is produced during the latter portion of the logarithmic growth phase of bioluminescent bacterial cells, and they termed this phenomenon "autoinduction" [52]. This autoinduction was later found to be facilitated by an "autoinducer" that is released during later stages of bacterial growth [53-55].

The autoinducer promoting luciferase production in *V. fischeri* was isolated by Eberhard et al. The molecule was identified as N-(3-oxo-hexanoyl)-homoserine lactone OHHL [54]. The genes involved in autoinduction and luminescence in *V. fischeri* are termed *lux* genes, which comprises of *luxR,* and *luxI.*

1.3.2. Quorum sensing in Gram negative bacteria:

The production of AHLs is not specific to *V. fischeri*, or that the function of AHLs is limited to regulation of luminescence. AHL mediated gene expression regulates the production of extracellular enzymes such as chitinase in *Chromobacterium violaceum* [56] and *Serratia* sp. [57], and exoprotease in *Aeromonas hydrophila* [58].

1.3.3 Quorum sensing in Gram positive bacteria:

Gram positive bacteria use oligopeptides or autoinducer peptides (AIP) for cell-cell communication and histidine kinases as the receptors for these molecules [46]. Bacteria, such as *Staphylococcus aureus*, synthesise the oligopeptides in the form of precursor peptides and these are exported from the cell using the ATP-dependent ABC transport system [59].

The best discussed autoinducing molecule produced in Gram positive bacteria is AI-2, a furanosyl borate diester, which is transcribed by the *luxS* gene [59]. AI-2 regulates virulence [19] in Gram positive bacteria.

1.3.4 Quorum sensing in activated sludge:

Quorum sensing is a feature of bacterial communities in many different environments including the marine environment [55], plants [60] and mammalian hosts [31]. AHL production has also been shown to be a feature and a determinant of the composition of activated sludge communities [61]. Morgan-Sagastume et al used cross-feeding assays to analyse municipal activated sludge samples and found that *Aeromonas* and *Pseudomonas* bacterial species were responsible for AHL production [62].

Chong et al, 2012 tested strains isolated from activated sludge for AHLs activating LuxR, TraR, and CviR and discovered that AHLs are produced by *Aeromonas, Citrobacter, Acinetobacter, Klebsiella, Pseudomonas* and other strains [63]. These strains were also tested for extracellular enzyme activity. These enzymes include chitinase, lipase, cellulose and elastase. Some of the strains that tested positive for both AHL activity and extracellular enzyme production were further tested to discover any correlation between the two events. *Aeromonas hydrophila* GC1, showed upregulation in the production of extracellular enzymes, such as chitin, upon addition of exogenous AHL. It was concluded that AHLs regulate the production of extracellular enzymes in activated sludge bacteria [63].

1.4 Introduction to chitin:

Chitin is the second most abundant biopolymer in nature [64] after cellulose [65]. This polysaccharide is found in the cell walls of fungi and in the exoskeletons of insects and crustaceans [65, 66]. The degradation and utilization of chitin plays an important role in the turnover of carbon and nitrogen in the environment [67]. In addition to a strong presence in the environment, chitin and its derivatives have been in used in many industrial applications.

1.4.1 Chemical nature:

Chitin is composed of N-acetyl-D-glucosamine (GlcNAc) monomers [68] linked by β-1,4 glycosidic bonds [69] in linear chains. Chitin occurs in two main forms named α chitin and β chitin [70]. A third chitin form, γ chitin, is rarely found in the environment and is considered to be a variant of α chitin [71]. Chains in particles of β chitin are aligned in a parallel fashion, while the chains in α chitin are not [72], as depicted in figure 1.5 below. The folding of the chitin chains in this way make α chitin harder than β chitin[72], with the former being found in crabs and shrimps [73], and the latter being found in centric diatoms [74].

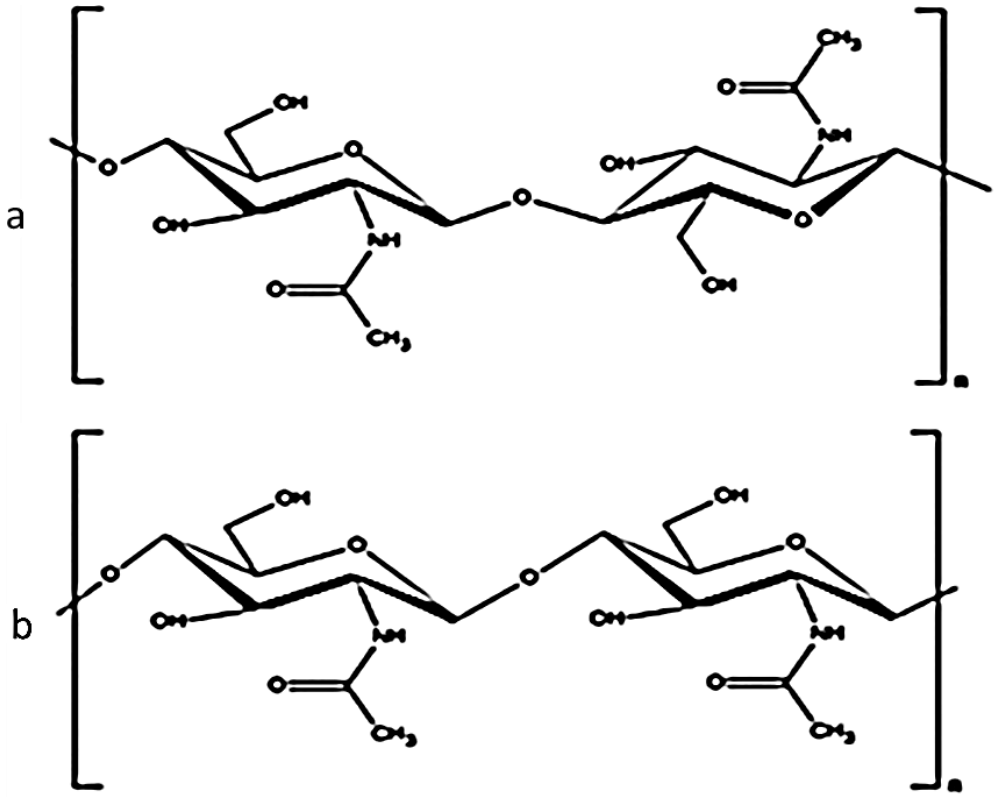


Figure 1.5: Chemical structures of α chitin (a) and β chitin (b). Taken from [75]

Chemical derivatives of chitin include chitosan, a partially deacetylated variant of chitin [71]. The deacetylation of chitin can be obtained by treating the chitin in alkaline solution or hydrolysing the chitin using chitin deacetylase [71]. The degree of deacetylation (DD) and molecular weight influence the solubility and other factors between chitosan preparations. The higher the degree of deacetylation, the higher the solubility of the chitosan [71].

1.4.2 Chitin in the environment:

1.4.2.1 Chitin detection:

Vibrio furnissii binds to and degrades chitin after it "senses" the chitin [76]. *V. furnissii*, a marine microorganism, senses chitin in the environment via chemotaxis [19]. Chitin oligosaccharides are leaked into the environment from injured or dead marine organisms and it is suspected that chitin-colonising bacteria release chitinases into the marine environment once one source of chitin has been exhausted. These chitinases come into contact with chitin in the environment, solubilize the chitin and release chitin oligosaccharides, creating a new gradient the bacteria can respond to [77].

1.4.2.2 Attachment to chitin and biofilm formation:

V. furnissii attachment to chitin was exhibited before the mechanism of bacterial sensing of their substrate was discussed. The bacteria bind to G1cNAc, mannose, and glucose via a broad specificity lectin that is expressed only when there is a sufficient amount of nutrients in the environment to sustain its expression [76]. Theoretically, the marine organism will stop expressing the lectin, detach itself from the sugar surface, and migrate to an environment with favourable conditions. More recent studies have shown that a chitin binding protein (CBD) is normally bound to ChiS, the chitin sensing gene, keeping it repressed [78]. When the CBD is induced by (GlcNAc)n, it releases ChiS, which in turn causes the production of chitinolytic genes, thus enabling chitin oligosaccharide utilization [83].

Electrostatic attraction plays an important role in the coagulation of particulate solids to chitosan, but it is not significant in the flocculation of bacterial suspensions to chitosan Strand et al found that chitosans with low charge density resulted in high zeta potentials for *E. coli*, which indicates that there are other features that dictate the attachment of bacteria to chitosan and chitin [79]. Chitosan with GlcNAc residues showed improved flocculation on chitosan of different bacterial species [80]. Similar to adsorption of solid particles, chitosan with lower DD showed more adsorption of *E. coli* [81].The optimal MW for bacterial adsorption is also different, as *E. coli* adsorbed better to low MW chitosans [79, 81].

1.4.2.3 Chitin utilisation and degradation:

The initial step of chitin degradation is the conversion of chitin into soluble oligosaccharides. Extracellular chitinases produced by microorganisms solubilizes chitin and converts it into oligosaccharides that are carried into the cell's periplasmic space [82]. Chitporin or ChiP, encoded by *chiP*, in *V. furnissii*, is suspected to be the porin responsible for transportation of chitin

oligosaccharides into the periplasmic space of the cells [83] and showed homology with porins in other bacterial species, such as *E. coli* and *B. pertussis* [83].

The major product of chitinase action on chitin is disaccharide N,N'-diacetylchitobiose $(GlcNAc)_2$ [84]. $(GlcNAc)_2$ can also be formed in the periplasmic space of the cell if chitinase activity results in higher oligosaccharides [84]. The higher oligosaccharides can be broken down by two periplasmic enzymes: the chitodextrinase EndoI enzyme and the β-N-acetylglucosaminidase (β-GlcNAcidases) ExoI enzyme [84].

ExoI hydrolyzes chitin oligosaccharides in the periplasmic space into $(GlcNAc)_2$ [84],which is then transported into the cytosol via the phosphoenolpyruvate:glycose (PTS) [76, 85, 86]. The enzyme IINag is responsible for translocation of G1cNAc and is also known as the GlcNAc permease [85].

The disaccharide $(GlcNAc)_2$ is transported into the cytoplasm by a phosphorylase (chbP) [87]. The transport of the dissacharide into the cytoplasm is important for subsequent processes in the catabolic pathway [87]. Once GlcNAc is converted to GlcNAc-6-P, it is deacylated by GlcNAc deacetylase, yielding acetate and glucosamine-6-P, which is in turn deaminated into fructose-6-P and NH_3 [76].

1.4.3 Chitin in industry:

The great applied potential of chitin and chitosan stems from their bio-renewability and biodegradability. Applications include wound dressing [88], cosmetics [89] and wastewater treatment [2].

1.4.3.1 Chitin and chitosan as coagulants:

Chitin and chitosan are used as a coagulant in wastewater treatment and the adsorption efficiencies of chitin and chitosan have been assessed for different substrates. Earlier studies looked at the adsorption efficiency of kaolin on chitosan [90, 91] Molecular weight (MW) and (DD) of the chitosan particles used affected the adsorption process [71]. More recent studies have assessed adsorption efficiency of bentonite suspensions to chitosan particles and have come to similar conclusions. Low doses of chitosan have been shown to be sufficient in the sedimentation of bentonite [92]. The study also observed that the higher the molecular weight of chitosan, the more efficient the coagulation process [92].

Chitosan has been shown to be efficient at a pH ranging from 4-7 [92]. Commercial use of chitosan at a higher pH results in precipitation [93] and requires higher chitosan doses to carry out the same

function [81]. Acidic conditions increase the solubility of chitosan, and therefore enhance the efficiency of the flocculation process [11, 93]

The use of chitosan over other coagulants is advantageous because chitosan is non-toxic [94] and biorenewable; chitosan facilitates more efficient microbial degradation of the colloidal elements as it is biodegradable [2, 95]; and does not affect the pH of treated water [2] The chemical make-up of chitosan also makes the overall process of adsorption more efficient: the –OH groups in chitosan decreases its hydrophobicity [96] and the presence of amine groups allows chitosan to bind to cationic metals [97]

1.5 Thesis objectives and summary:

Based on the results obtained by Chong et al, 2012 which suggested that bacteria within activated sludge flocs produce AHLs to regulate extracellular enzyme production, a conceptual model for the colonisation of surfaces, specifically the chitin surface, by activated sludge was conceived. The model hypothesises that activated sludge bacteria form a biofilm around the particle, creating a floc that aides in the wastewater treatment process. Upon biofilm formation, AHL mediated regulation of extracellular enzymes such as chitinases commences. A schematic of the conceptual model is presented in figure 1.6.

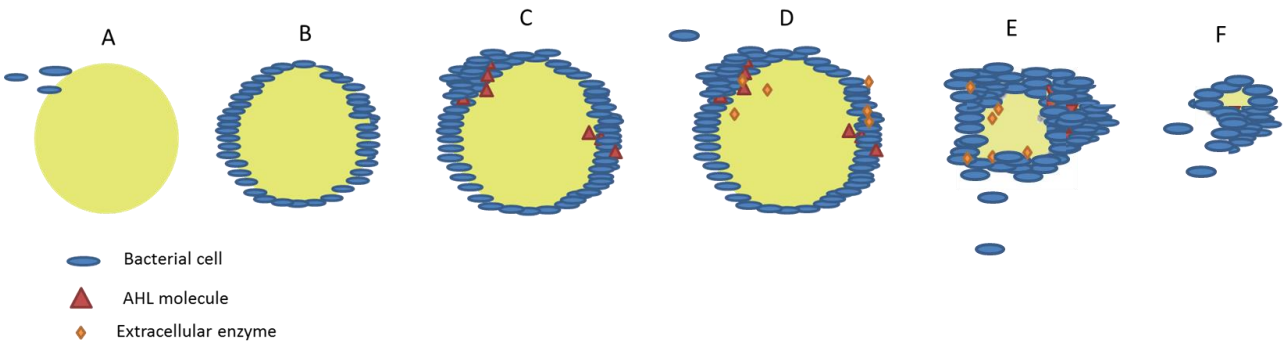


Figure 1.6: Stages of chitin colonisation by bacteria in activated sludge. Bacterial cells in sludge attach to the chitin surface (A). Further cell attachment to chitin takes place, followed by the formation of a biofilm around the chitin surface (B). During maturation of the biofilm AHL molecules are released by cells (C), and AHLs mediate the production of extracellular enzymes (such as chitinases) that can degrade the chitin particle (D). Chitin is degraded by the action of chitinases (E-F) and cells detach to colonise a new chitin surface.

This thesis aims to test the hypothesised model by addressing the following objectives:

1. To characterize the colonisation/biofilm cycle/flocculation of activated sludge bacteria onto the chitin surface.
2. To characterize the microbial profiles of the formed biofilms on chitin.
3. To determine the impact of the chitin surface on AHL mediated regulation and the timing at which AHL production by microbes attached to chitin takes place.

In chapter 2 of this thesis, *Aeromonas hydrophila* and activated sludge cultures were incubated with chitin. Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM) were used to monitor cell attachment and biofilm formation on chitin. Denaturant Gradient Gel Electrophoresis (DGGE) and pyrosequencing were used to identify the microbiological community profiles growing on chitin.

In chapter 3, AHL production and chitinase activity were tested in *A. hydrophila* and activated sludge. To test AHL production, AHLs were extracted from cultures and extracts were analysed using overlay assays and NSI-MS. A monitor strain, *Aeromonas* (pBB-luxR), was also used to monitor AHL production. Chitinase activity was tested using a chitinase assay. Ammonia production was also tested. SEM was used to monitor chitin consumption.

In Chapter 4, the results obtained are discussed in a broader context and future directions and considered.

Chapter 2: Colonisation of chitin by activated sludge and *Aeromonas hydrophila* GC1

2.1. Introduction:

Activated sludge relies on the formation and settling of flocs from wastewater for the production of clean effluent. The microorganisms that participate in the formation of these flocs include bacteria, protozoa, archaea, viruses and fungi [5]. Although floc formation is a crucial part of the activated sludge process, the microbiology underlying the phenomena remains relatively uncharacterized. Furthering our understanding of these microbiological phenomena may be key to improving the overall treatment process and eliminating some of the problems that commonly occur in activated sludge systems globally.

Chitin is a highly abundant polysaccharide found in the cell walls of fungi and in the exoskeletons of insects and crustaceans [76].The chitin present in cell walls has the ability to take up reactive dyes in wastewater [98]. Chitin and chitosans have been shown to adsorb cadmium, nickel and zinc from aqueous solutions [99]. However, there exists no mention in the literature of the characterization of colonization of these particles by activated sludge microbes. Very little is known about the fate of chitin in activated sludge. The goal associated with the experiments described in this chapter is to generate fundamental knowledge regarding the process of colonisation and degradation of chitin in activated sludge. The specific aims of the chapter are:

Aim 1 – To elucidate the different steps involved in *Aeromonas hydrophila* colonisation and biofilm formation on chitin particles including chronological attachment and spatial distribution on the surface using CLSM and DNA extraction yields.

Aim 2 - To describe the timing of cell attachment and biofilm formation on chitin in activated sludge through SEM

Aim 3 – To describe the diversity of bacterial species colonising chitin in activated sludge over time using DGGE fingerprinting, band sequencing and pyrosequencing of 16S rRNA amplicons.

Chapter two describes the culture conditions and approaches used to achieve the aims specified above. *A. hydrophila* GC1, an activated sludge isolate that has the ability to both produce chitinases that degrade chitin, and produce AHLs [63] was chosen for pure culture experiments. The findings of these approaches are presented and include community analysis of sludge and visual evidence of chitin colonization.

2.2 Materials and methods:

2.2.1 Bacterial strains:

A. hydrophila strain GC1, isolated from activated sludge by Chong et al, 2012 was used for all pure culture experiments. This strain tested positive for AHL production, as well as the production of several enzymes such as Lipase and chitinase [63]. This strain was chosen as a suitable candidate for experimentation because it encompasses the phenotypes this study is concerned with, mainly AHL and chitinase production.

2.2.2 Sample collection:

Four litres of activated sludge was collected from St. Marys wastewater treatment facility (St Marys, Sydney, Australia). The samples were collected from the aerobic unit process (aeration step) of a four stage Biological Nutrient Removal type treatment. Activated sludge samples (200 ml) were incubated in 1 L erlenmeyer flasks and incubated at room temperature at 150 rpm for 26 days. Four cultures were supplemented with 10 g of practical grade, coarse chitin flakes (Sigma-Aldrich, C9213). After the addition of chitin flakes, one of the cultures was autoclaved to obtain a sterile control. An activated sludge culture was set up without the addition of chitin flakes. These controls were established to monitor differences in chitinase concentration between cultures containing sludge and chitin, sterile cultures containing sludge and chitin, and cultures containing sludge.

2.2.3. Culture conditions:

Strains were cultured in Luria-Bertani broth (LB) which consists of 10 g of Bacto-Tryptone, 5 g of yeast extract and 10 g of NaCl in 1L. The pH was adjusted to 7.5 with 5 M NaOH before being autoclaved. Cultures were incubated at room temperature with shaking at 150 rpm unless otherwise stated. Coarse, practical grade chitin flakes from Sigma Aldrich were added to the culture at 0.1% w/v.

2.2.4Molecular analysis:

2.2.4.1 DNA extraction:

2.2.4.1.1 Pure culture extraction:

A phenol-chloroform extraction of nucleic acids was performed on chitin pieces incubated with *A. hydrophila* in LB media. 5-7 pieces of washed chitin pieces were placed in 2 ml eppendorf tubes with silica beads and 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1). The tubes were then placed in a Qiagen Tissue lyser II and bead-beaten for 5 minutes. The tubes were then centrifuged in an

eppendorf Centrifuge 5415 R at 16.1 xg (maximum speed) for 4 minutes at room temperature. The aqueous phase was transferred to a fresh eppendorf tube, and 0.5 mL of chloroform:isoamyl alcohol solution was added. The tubes were then centrifuged at maxmimum speed for 4 minutes at room temperature, and aqueous phase was transferred to a fresh eppendorf tube. The tubes were incubated at room temperature for 10 minutes after the addition of 7.5 M NH_4OAc (1/2 volume of aqueous phase) and isopropanol (2 volumes of the aqueous phase). The tubes were then centrifuged at maximum speed at 4^oC for 20 minutes. Supernatant was decanted and pellets were washed with 0.5 mL of 80% ethanol. After centrifuging tubes at max speed at 4^oC for 4 minutes, supernatant was decanted, pellets were air dried and resuspended in 30 µL of Molecular biological Grade (MBG) water.

2.2.4.1.2 Activated sludge extraction:

Samples of activated sludge and chitin flakes were taken from the culture at different time intervals and used for DNA extraction. To remove any microorganisms that were not irreversibly attached, chitin flakes were washed three times with activated sludge supernatant (prepared by filtering activated sludge through a 0.2 µm filter). Washed chitin samples (5-7 pieces of similar size) or activated sludge samples (3 mL) were mixed with 3 mL of lysis buffer in 15 mL falcon tubes. The Lysis buffer consisted of 40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose in 200 ml of water. The buffer was adjusted to pH 8 with 5 M NaOH. To the samples and lysis buffer, 100 µL of lysozyme from a 125 mg/L stock in filter sterilised TE buffer was added. SDS (20%) was also added (100µl) and samples were then shaken at 37^oC for 2 hours. Proteinase K (100 µl) (at a concentration of 0.5 mg/mL) was then added to each sample and samples were shaken at 55^oC for 2 hours. Samples were then stored overnight at -20^oC before removing 1 ml of clear solution into a new tube, supplementing with phenol chloroform:Isoamylalcohol and vortexing. The aqueous layer was removed and 7.5 M Ammonium acetate and chloroform were added to the samples and mixed. The aqueous layer was removed again and cold isopropanol was added to the samples before being stored overnight at -20^oC. Samples were then centrifuged and the supernatant was discarded before being washed with 300 µl of 80% cold ethanol. Ethanol was decanted from the tubes and tubes were left to dry. Tubes were then eluted with 30 µl of molecular biology grade (MBG) water.

2.2.4.2 Polymerase chain reactions:

Polymerase chain reactions (PCRs) were set up to amplify the 16S rRNA gene fragments in the DNA extracts. Each PCR tube contained 20 µl of Promega mastermix, 4 µl of the forward primer GC338F and reverse primer 530R [100], 1 µl of BSA, and 2 µl of DNA template. These primers were used to enable the use of the PCR product in Denaturing Gradient Gel Electrophoresis (DGGE). The PCR

protocol was as follows: The initial denaturation was at 95^{o}C for 2 minutes, denaturation was at 94^{o}C for 30 seconds, annealing at 61^{o}C for 30 seconds, extension at 72^{o}C for 30 sec. The cycle was repeated 35 times, at an elongation temperature of 72^{o}C for 10 min.

2.2.4.3 Denaturing gradient gel electrophoresis:

DGGE was carried out using the BioRad Dcode Universal Mutation Detection System using 30% and 60% Acrylamide gel gradient solutions. Samples of 20 µl (sample: loading dye, 1:5) were inserted into gel wells, and run for 16 hours at 75 V. The gel was then stained with 1000x Sybergold in TAE (1X) for 15 minutes and visualised using a BioRad Gel Documentation system. Bands of interest in the gel were excised using plastic band cutter tips (supplier) and placed in eppendorf tubes supplemented with MBG water. These samples were introduced into a new PCR using the same primers as in section 2.2.4.2 and separated again by DGGE to confirm purity before sequencing. Solutions of 250 ml are composed of 62.5 ml of 40% Bis acrylamide (1:37:5), 30 ml Deionised formamide in low (30%) solutions, 60 ml Deionised formamide in high (60%) solutions, 5 ml of 50x TAE buffer, 31.5 g of urea in 30% solutions, and 63 g of urea in 60% solutions [101]. The volume is made up with MilliQ water.

2.2.4.4 Sequencing:

Bands of interest from DGGE gels were put through a sequencing PCR using the GC338F forward primer in the following reaction: 96^{o}C for 10 seconds for denaturation, 50^{o}C for 5 seconds for annealing, 60^{o}C for 4 minutes for extension for 25 cycles. Each PCR tube contained 1 µl BigDye terminator V3.1 (Applied Biosystems), 20-50 ng PCR product, 3.2 pmol primer, and 3.5 µl 5x buffer (Applied Biosystems). The tubes were made up to 20 µl with MBG water. The PCR products were then cleaned up and precipitated by adding 5 µl of 125 mM EDTA and 60 µl of 100% ETOH to PCR products followed by vortexing and incubation at room temperature for 5 minutes to precipitate. Samples were then centrifuged at 16 xg for 20 minutes. Supernatant was decanted, and samples were washed with 70% ethanol twice before being aspirated, dried, and submitted for Sanger sequencing at the Ramaciotti Centre for Gene Function and Analysis at UNSW. Sequences were analysed using the NCBI database. A Nucleotide Blast (www.ncbi.nlm.nih.gov) search was conducted to determine the sequence with which the samples had most homology.

2.2.4.5. Pyrosequencing:

DNA extracts were subject to pyrosequencing at the Hawkesbury Institute for the Environment. The universal primer set 926 Forward and 1392 Reverse was used to carry out the amplification [102]. The

sequences were then analysed using Mothur [103]. Sequences were aligned, filtered. Chimeras were checked and the sequences were then classified into operational taxanomic units (OTUs).

2.2.5 Microscopy:

2.2.5.1 CLSM microscopy:

Samples were extracted from *A. hydrophila* cultures at different time intervals and taken for light and epiflourescence microscopy. Chitin pieces from cultures or activated sludge were washed with fresh LB media or sludge supernatant respectively, stained with 0.1% acridine orange [104]. Acridine orange binds to DNA with and has an absorption wavelength of 502 nm and emits at 526 nm and visualised under the FITC filter cube at 60X magnification using an Olympus BX51 flourescence microscope.

2.2.5.2 Scanning electron microscopy (SEM):

Chitin pieces were removed from cultures after 24, 48, 168 and 288 hours incubation and washed with filtered sludge supernatant to remove any non-attached cells. The chitin was then fixed with 2.5% v/v glutaraldehyde and phosphate buffer overnight. The chitin was then passed through ethanol concentration ranging from 35% to 100%. The chitin pieces were then placed in 50% and 100% Hexamethyldisilazane (HMDS) to dry the pieces. Gold sputtering was performed with an Emitech K550 sputter coater after placing the chitin pieces on aliminum stubs. These were then visualised using an ESEM Quanta 500 microscope. Supplied voltage was 20.0 kV [105].

2.2.6 Protein assay:

For both protein and chitinase assays, samples were prepared by bead-beating washed chitin pieces in a 2 m tube with 1.5 ml LB and silica beads to lyse cells and remove them from the chitin surface. Bead-beating was performed using the Qiagen Tissue Lyser II and tubes were then spun down for 10 min at 16 rcf. The supernatant was removed and used in chitinase and protein assays.

The BCA Protein Assay Reagent Kit (Pierce) was used to quantify protein from chitin pieces. The assay was done in a 96 well micro plate. Standards were prepared and assayed in triplicate in the assay, their concentration ranging from 0-2000 µg/ml. Standards and samples at volumes of 20 µl were added to the plate. Working reagent (200 µl) was added and the plate was covered and incubated at 37^{o}C for half an hour before being read using the Spectra MAX340 Plate Reader at 562 nm. The working reagent is prepared by mixing two reagents, A and B at a ratio of 50:1, A:B. Reagent A

contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartate. Reagent B contains 4% cupric sulfate.

2.3 Results

2.3.1. *Aeromonas hydrophila* GC1 attachment to chitin and biofilm formation (biomass accumulation)

To determine when cell attachment to chitin takes place, *A. hydrophila GC1* was grown in aerobic cultures containing LB media and ground chitin pieces. The media was replaced twice a day to remove planktonic cells and encourage colonisation of chitin in the cultures. Samples were taken throughout the experiment over 240 h and analysed by DNA extraction yield and microscopy.

Protein quantification yielded extremely high levels of protein at early time points in the cultures which do not coincide with the DNA yield results. At the zero time point, no colonisation of chitin had taken place, and a high level of protein was detected in the assay. This is an indication chitin interferes with the assay, yielding false positive results. The assay is sensitive to nitrogen (targets amine groups) which is present in chitin (ammonia) and this probably resulted in the unusually high protein yields. In addition to ammonia, it is possible that protein adsorbed to chitin also influenced the assay. It was concluded that protein quantification is not suitable for bacterial growth quantification on chitin.

To further observe the timing at which chitin colonisation takes place, DNA extractions were performed on chitin pieces taken from cultures at 0, 24, 72, 120, 168, and 216 hours. The DNA yield from chitin reflects the amount of biomass accumulating on the particle. Considerable variation was observed between replicates. The first replicate yielded most DNA (241 ng/µl) at 72 hours, the second at 120 hours (450 ng/µl), and the third at 168 hours (445 ng/µl). The second and third replicates yielded almost the same maximum amount of DNA, but at different time points, whereas the first replicate yielded a much lower maximum DNA concentration (Figure 2.1). The variation in DNA yields indicates that the process of colonisation is not homogenous or uniform and differs from one piece of chitin to another, which may be due to variations in composition of the chitin surface.

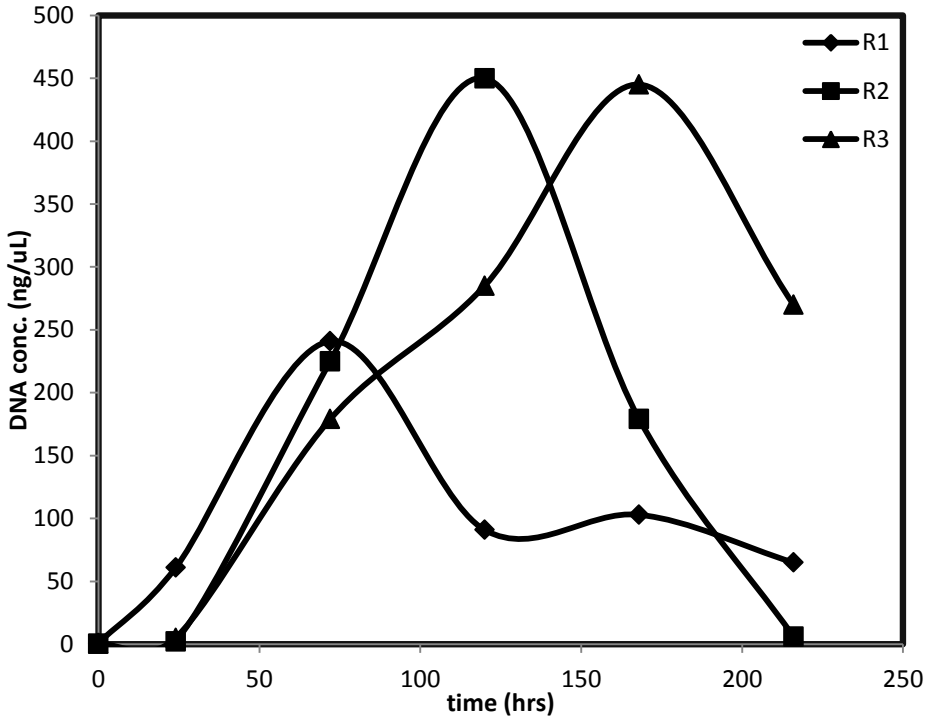


Figure 2.1: DNA yield from chitin pieces. Chitin pieces (5-7 pieces of similar size) were removed from triplicate cultures at 0, 24, 72, 120, 168, and 216 hours and subject to DNA extraction.

Figure 2.2 and Figure 2.3 illustrate the progression of chitin colonisation by strain GC1 at 168 and 240 hours. Phase contrast images of the samples were obtained. Epiflourescence and phase contrast images were combined enabling observation of the location of cells on the chitin surface (Figure 2.2 and 2.3). After 168 hours (7 days) of incubation, cells were attached to the peripheries of the chitin particle. Cell distribution on the chitin surface at 168 hours was uneven with single cells and cell aggregates attached to the surface. After 240 hours of incubation, cells were seen attached throughout the chitin surface (inner portions of chitin under microscope focus). At 288 hours a similar phenomenon was seen.

Based on microscopy observations presented (Figure 2.2), *A. hydrophila* GC1 requires a week (168h) to form microcolonies on chitin. DNA yields (Figure 2.1) show that attachment takes place as early as 24 hours, however; no cells were seen on chitin under the microscope (data not shown). However, even though aggregates were seen in different locations of a single chitin piece, other locations remained intact and free of colonisation even after a week – 10 days of incubation (Figure 2.2 288e, 288h).

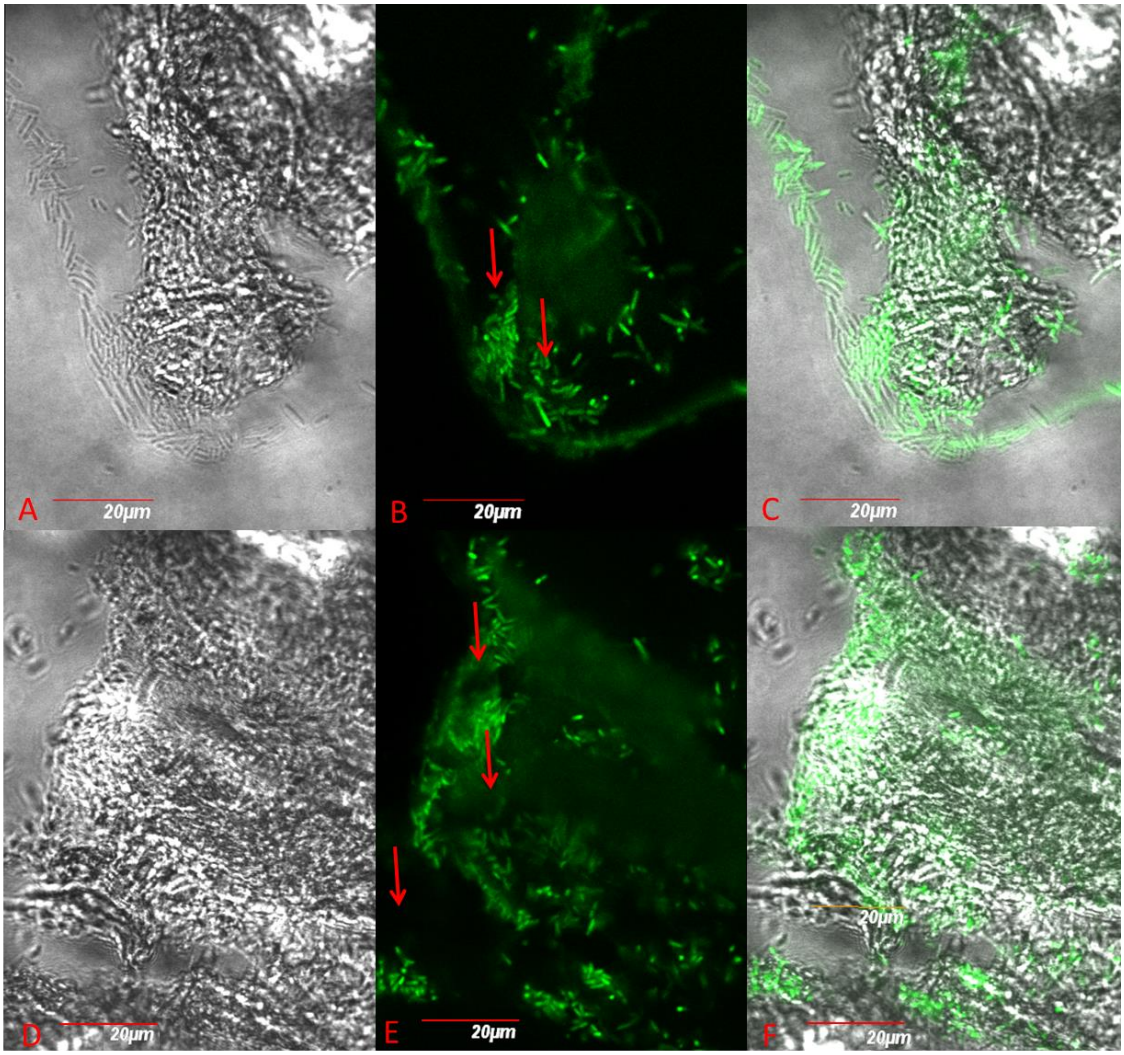


Figure 2.2: *A. hydrophila* GC1 attachment to chitin particles after 168 hours incubation. The figure is composed of two rows, each row, being an example of GC1 attachment to chitin. Each row contains 3 images of the same example; a phase contrast image, an epiflourescence image, and a combination of the two. Aggregates of cells can be seen on the chitin pieces, as indicated by the arrows. These images are representative of the total 6 examples (images) obtained.

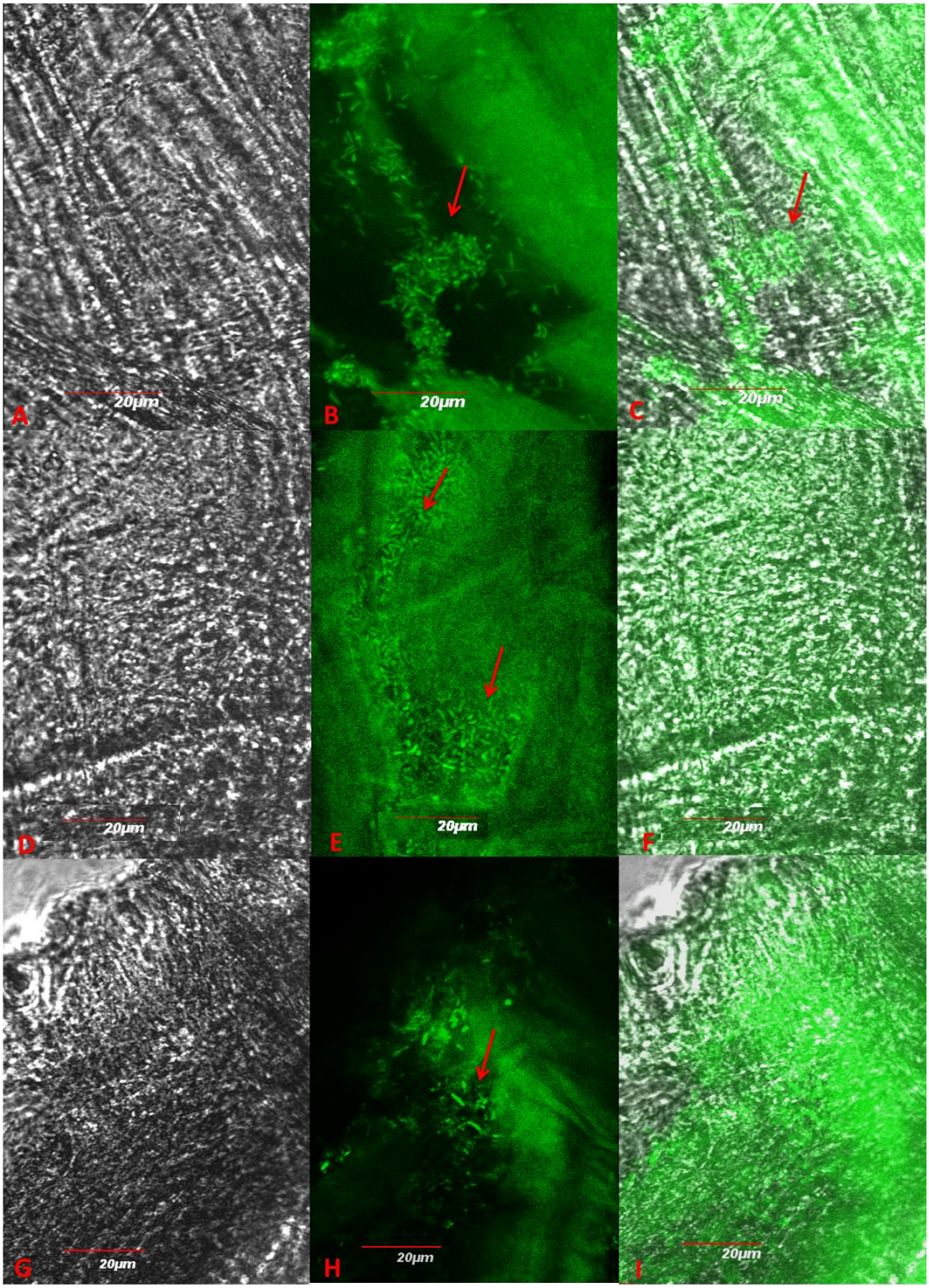


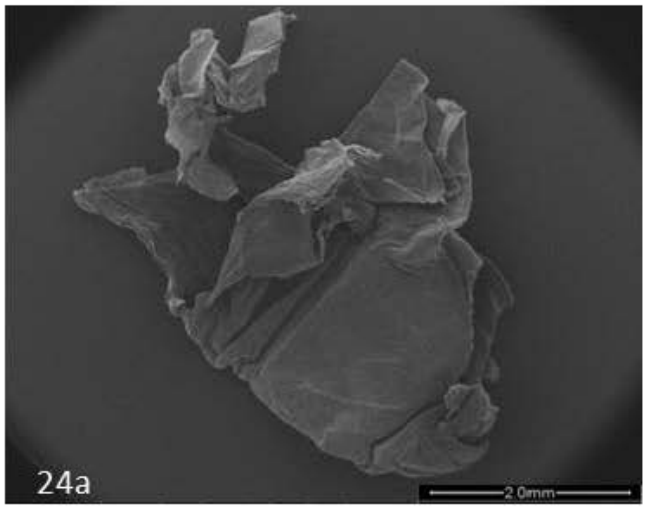
Figure 2.3: Colonisation of chitin by *A. hydrohpila* GC1. The figure shows 3 rows of images taken from chitin at 240 hours. The images belonging to one row represent the same image, with the first being phase contrast, the second being epiflourescence, and the third being a combination of the two. Arrows indicate the location of the cells on the chitin, which in contrast to Figure 2.2. are observed all throughout the chitin surfaces, not only the edges.

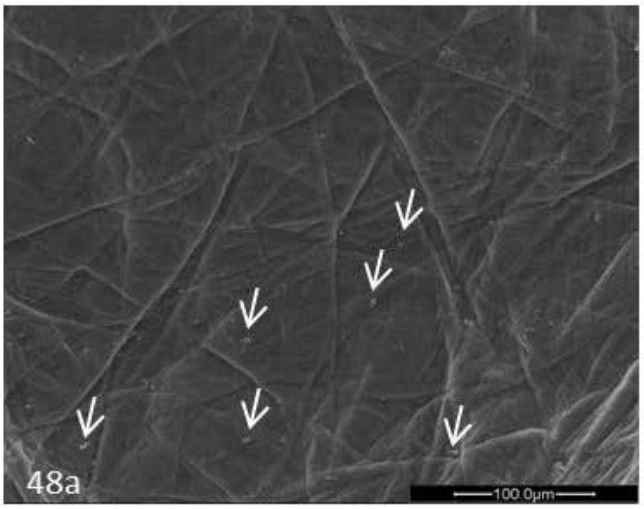
2.3.2. Cell attachment and biofilm formation on chitin particles in activated sludge:

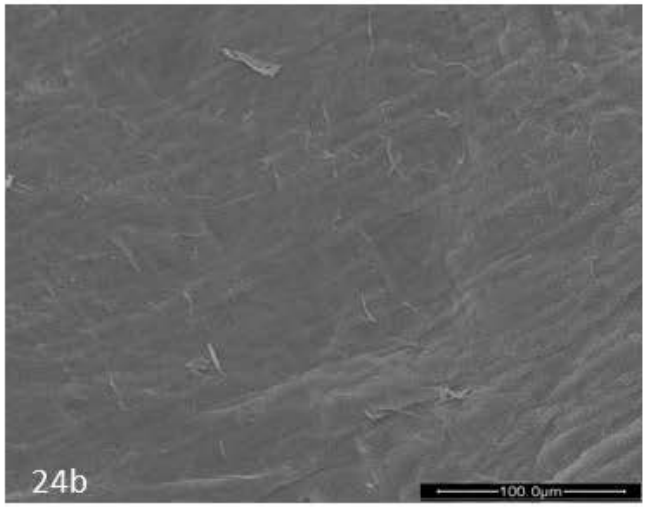
To determine if chitin colonisation occurred and followed similar processes of attachment and growth to biofilm formation models and to describe the timing, chitin flakes were incubated in activated sludge cultures for 624 hours (26 days). Samples were extracted from the cultures after 24, 48, 96, 120, 168, and 288 hours of incubation and analysed using Scanning Electron Microscopy (SEM). Figure 2.4 represents chitin colonization by organisms in activated sludge at 24 and 48 h. After 24 hours of incubation, few cells were seen attached to the chitin. At 48 hours, cells were unevenly scattered on the chitin surface, and clumped in small micro-colony-like structures. The morphologies of the cells on the chitin surface varied from different sized rods (24d, 48b and 48d in Figure 2.4) to coccoid shaped cells (48b and 48d in Figure 2.4) and spiral shaped cells (48d in Figure 2.4).

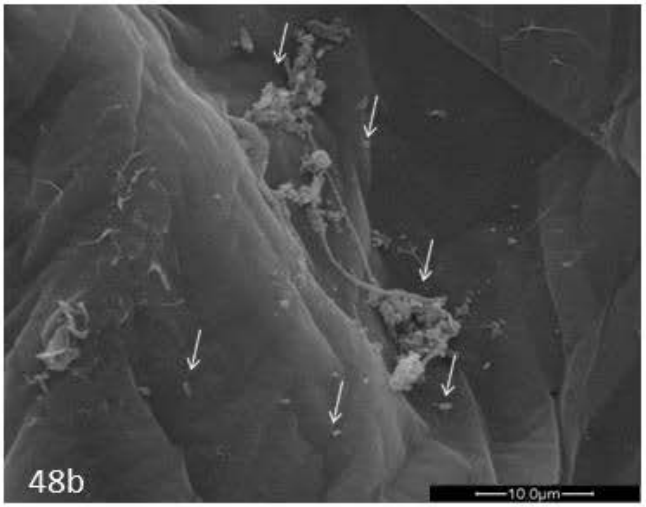
After one week of incubation, the number and size of visible micro-colonies increased. Micro-colony cells were engulfed in a semi-transparent layer (Figure 2.5:168d). Single cells were also attached and distributed unevenly on the chitin surface. At 168 h, an area of 0.1 mm^2 contains approximately five microcolonies consisting of 20-100 cells and approximately 50 microcolonies consisting of 5-20 cells. The remaining surface area contains approximately 5400 single cells with approximately 5-10 um distance between them. On average the cell density is estimated at 69×10^3 cells/mm^2.

After almost 2 weeks of incubation, micro-colony size had increased, cells and micro-colonies were extensively scattered throughout most of the surface, and cells were growing within crevices of the particle. The surface of the chitin was ruptured in some locations, indicating consumption of the chitin. Cell aggregates were seen within the ruptured surface of the chitin. Although many changes were seen in the chitin surface and the nature of colonisation of the surface, some parts remained completely intact. Figure 2.5:288f, for example, represents an area of 248 um^2. This area contains channels in the surface, an aggregate attached to the surface, and portion of the area that is intact.


24a
2.0mm


48a
100.0µm


24b
100.0µm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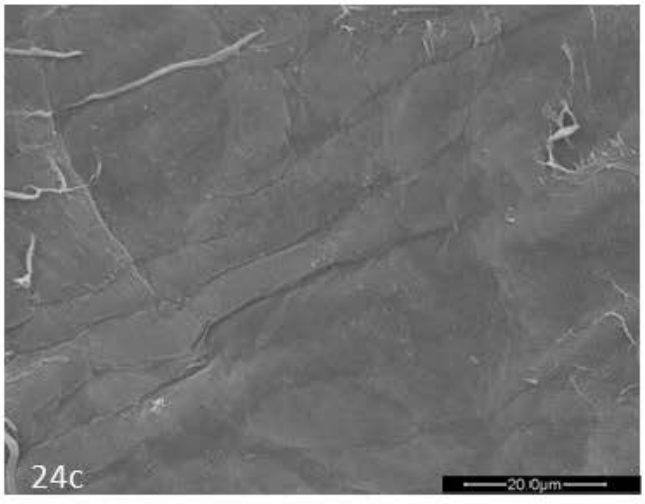
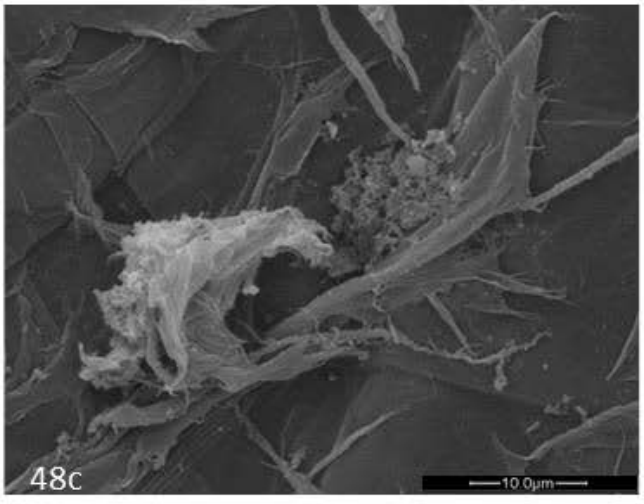
10.0µm


24c
20.0µm


48c
10.0µm

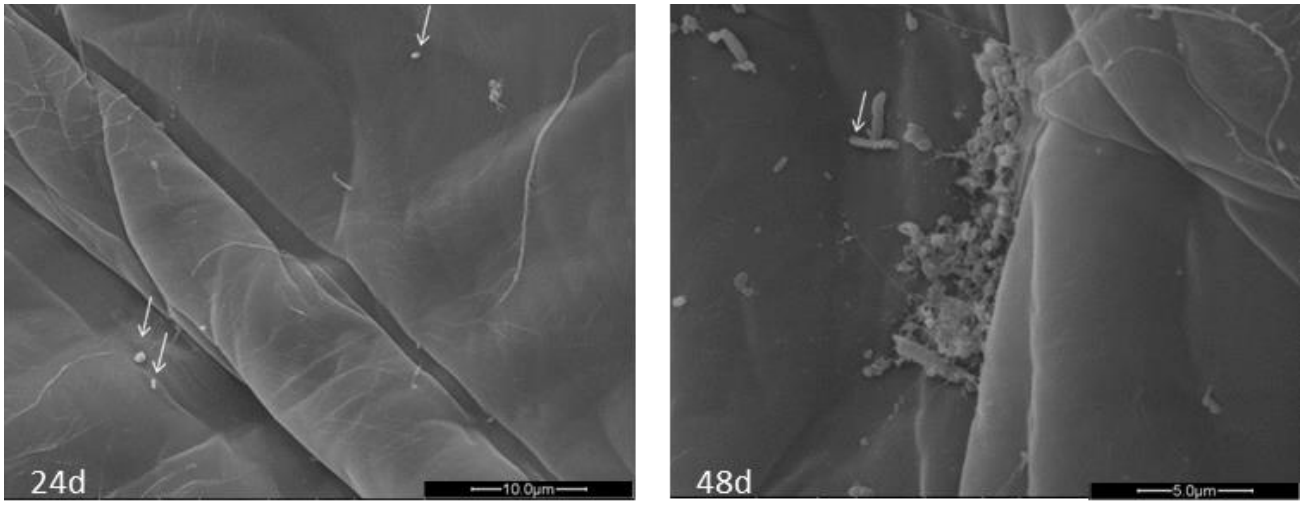
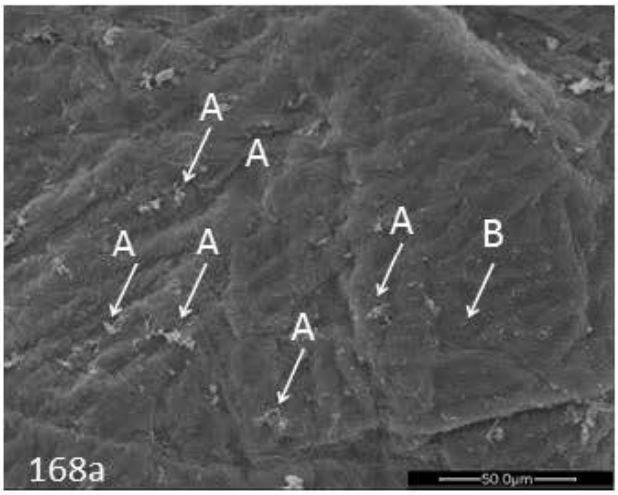
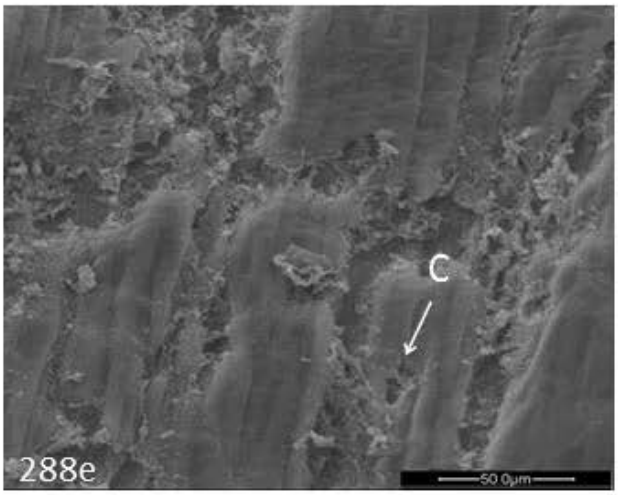
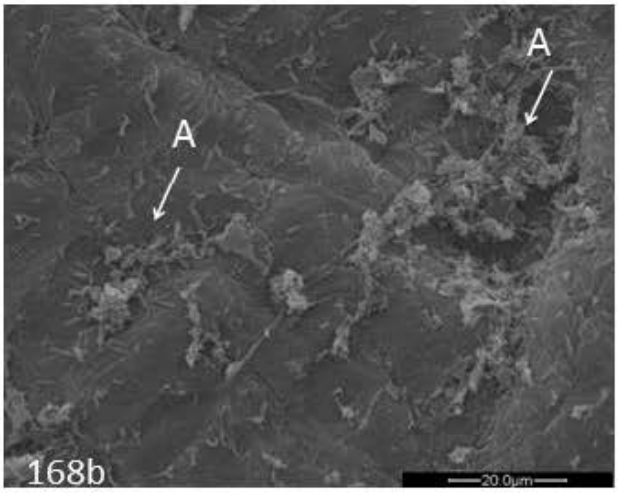


Figure 2.4: Cell attachment and biofilm formation on chitin in activated sludge. The left-hand side column represents 4 images taken from 24hours chitin sample, and the right-hand side column represents 4 images taken from a 48 hours chitin sample. Arrows indicate aggregates of cells; rod, coccoid, and spiral shaped cells.


A
A
A
A
A
B
A
168a
50.0µm


C
288e
50.0µm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A
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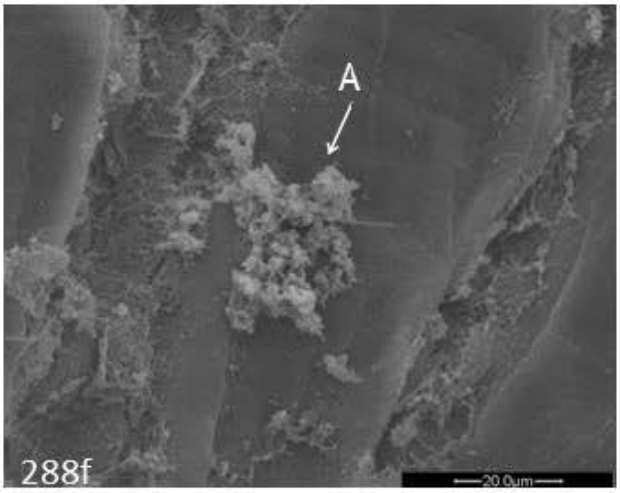
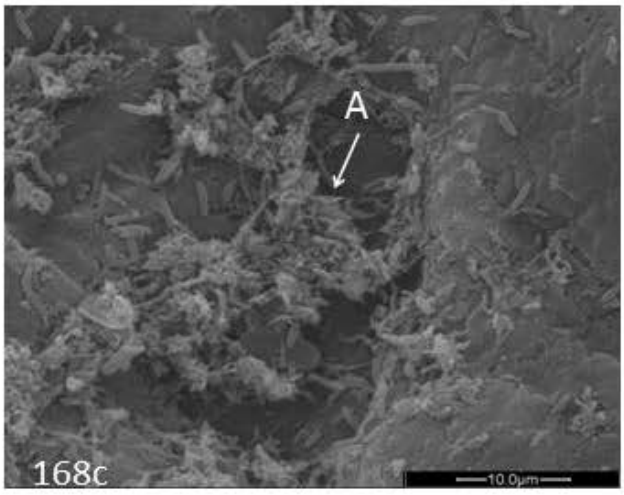
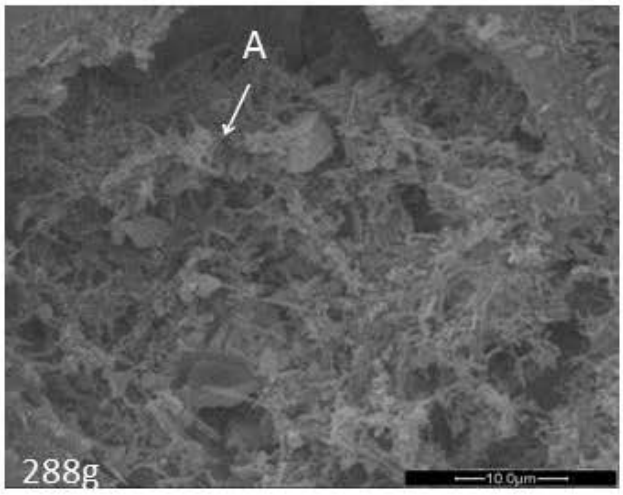

A
288f
20.0µm


A
168c
10.0µm


A
288g
10.0µm

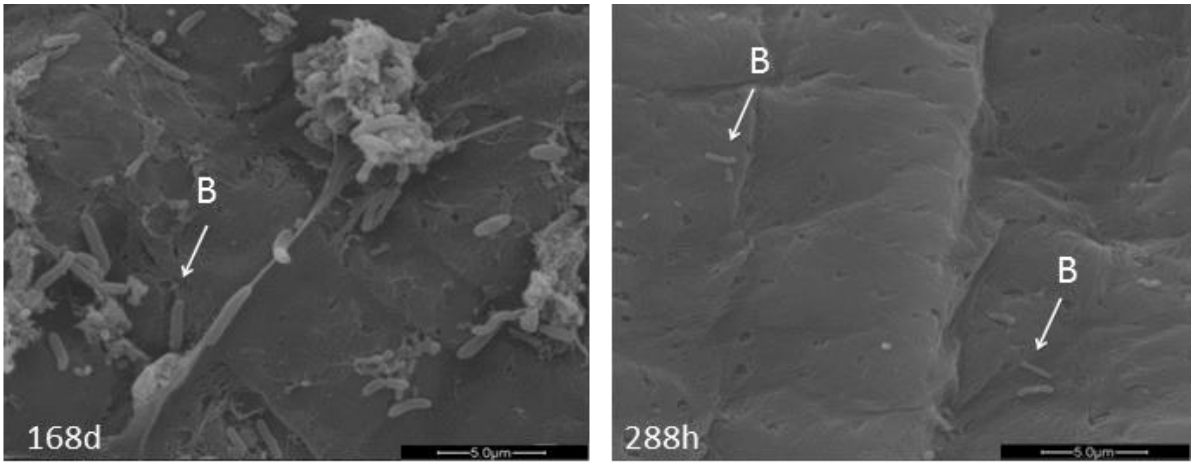


Figure 2.5: Colonisation/ micro-colony formation on chitin by activated sludge cells at 168 and 288 hours. Voltage input was 20 kV. Arrows labelled A indicate cells aggregates, arrows labelled B indicate single cells, and arrows labelled C indicate disruption of the chitin surface.

2.3.3 Determination of biofilm community composition on chitin particles:

Samples extracted from the cultures at different time points were used for community analysis of the cultures by DGGE and band sequencing Figure 2.6 compares DGGE profiles derived from DNA extracted from chitin pieces and the bulk sludge over time. Band-cutting was performed on bands that were more abundant or intense on the chitin piece extracts, or on bands that were present on chitin but not found in sludge and vice versa.

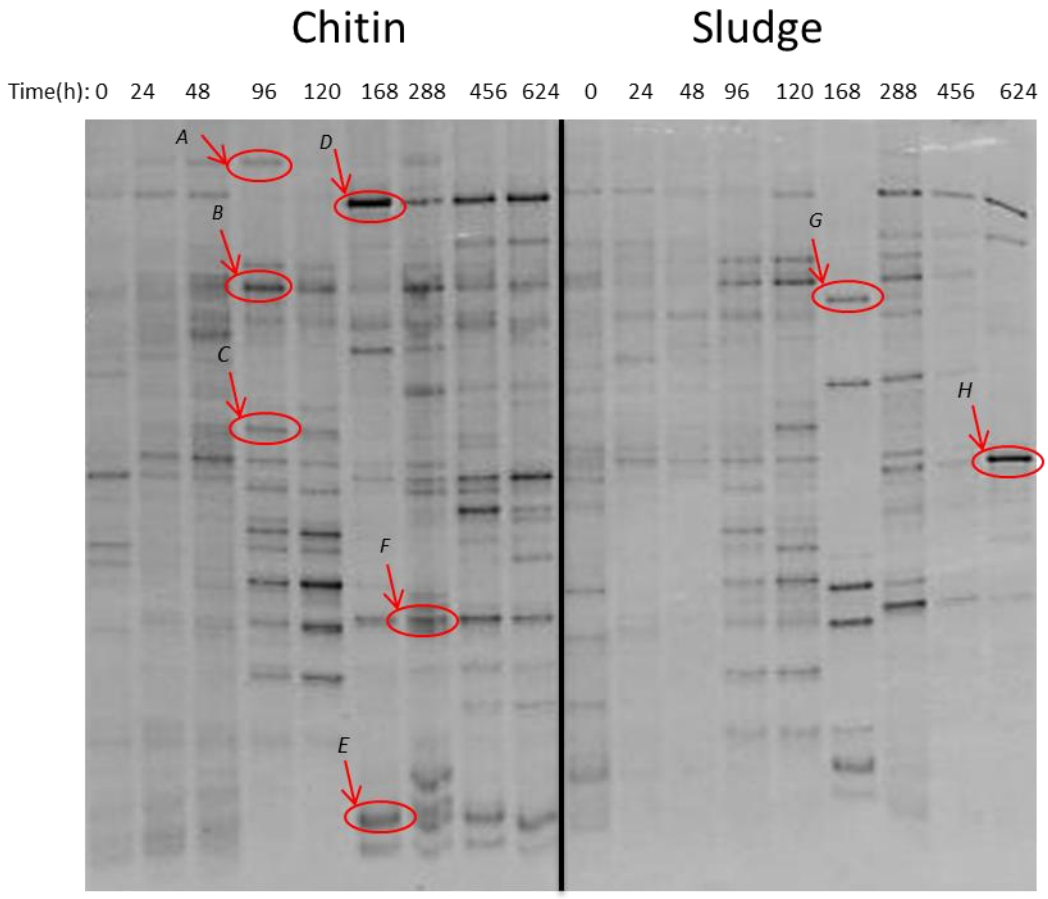


Figure 2.6: DGGE fingerprints of bacterial communities on chitin flakes incubated in activated sludge (left) and in the activated sludge itself (right) over 624 hours. Both chitin and sludge extracts came from the same culture. The bands that were excised from the gel are circled in red. The arrows and letters indicate band identifiers.

Bands A and E in Figure 2.6 represent bacteria that were found in the chitin DNA extracts, but were not seen in the sludge. According to the NCBI blast search, these bands represent *Sphingobacteria* and a *Sanguibacter/Cellulomonas/Streptomyces* sp., respectively. Band G, which represents a *Bacteriodetes* bacterium or *Sphingobacteriaceae* bacterium or *Flavbacterium*, was present in the sludge DNA and not found in the chitin DNA extracts. Band B (Ferruginibacter sp/Sphingobacteriales bacterium/Bacteriodetes bacterium, *Chitinophagaceae* bacterium); C (Chryseobacterium sp./Candidatus Amoebinatus sp/ Bacteriodetes bacterium); D (*Chloroflexi bacterium/ TM7 bacterium/ epsilon proteobacterium.*, and H (*Bergeyella zoohelcum)* were found in both extracts, but were more intense in chitin extracts. Band F (*Arthrobacter sp/ Leucobacter sp/ Clavibacter michiganensis)* was present in the sludge from 96 to 168 hours, but then appeared on the chitin pieces from 168 hours onward. Table 2.1 provides a list of the closest relatives to the sequences of the bands cut out from the gel. Up to 90% of the closest relatives from blast searches have been previously found in sludge.

Table 2.1: Blast hits for DGGE gel bands. The table lists the first blast results for which a phylogenetic assignment has been made. For A and H, the only hit other than uncultured bacterial strains was the match listed in the table. The 2nd, 3rd and 4th columns list the accession numbers, E value and maximum identity value as provided by the NCBI match list.

Band label	Species	Acession number	E value	Max. Identity (%)
A	*Sphingobacterium*	FJ756565.1	6e-64	97
B	*Ferruginibacter sp.*	JQ288691.1	1e-59	99
	Sphingobacteriales bacterium	JQ723671.1	1e-59	99
	Bacteroidetes bacterium	JN695872.1	3e-61	99
	Uncultured Chitinophagaceae bacterium	JF703357.1	2e-63	99
C	*Chryseobacterium sp*	JQ684227.1	7e-68	99
	Candidatus Amoebinatus sp.	AY526713.1	7e-68	99
	Bacteroidetes bacterium	AY726972.1	7e-68	99
D	*Chloroflexi bacterium*	JQ919719.1	2e-62	99
	TM7 bacterium	FJ629383.1	2e-62	99
	epsilon proteobacterium	AY154391.1	9e-62	98
E	*Sanguibacter sp*	GQ246705.1	3e-56	99
	Cellulomonas sp	JQ178193.1	3e-56	99
	Streptomyces sp	FJ418894.1	3e-56	99
F	*Arthrobacter sp*	JX228214.1	5e-39	90
	Leucobacter sp	JN713459.1	5e-39	89
	Clavibacter michiganensis	JX122180.1	2e-38	89
G	*Bacteroidetes bacterium*	JF985682.1	9e-67	99
	Sphingobacteriaceae bacterium	GU300373.1	7e-58	95
	Flavobacterium sp	JN032396.1	3e-57	96
H	*Bergeyella zoohelcum*	JN713353.1	3e-61	96

In addition to DGGE, pyrosequencing was performed on DNA extracts from chitin incubated in sludge, and the sludge from those same cultures. Samples that were pyrosequenced corresponded to time points (in hours): 0, 24, 96, 168, 456 in chitin, and 0, 24, 96, 456, and 624 in sludge from the cultures. The sequenced were analysed using Mothur and the results of the analysis, based on abundance of bacteria in OTUs, are presented in figures 2.7 and 2.8. In both chitin and sludge, the *Chitinophagceae* family was the most abundant, followed by *Flavobacteiaceae*, *Saprospiracea*e, and *Sanguibacteraceae*. *Chitinophagaceae*, on chitin pieces, increased from 14% of the community at 24 hours to 65% of the community at 288 hours. After 456 h, it had slightly decreased to 40%. In sludge, *Chitinophagaceae* was 42% after 456 hours. In chitin *Flavobacteriaceae* increased from 13% to 18%, while in sludge it increased from 17% to 19% at 456 hours, but then decreased to 6% after 624 hours. In both chitin and sludge, the *Saprospiraceae* family was about 9-11% at the initial and final time points, but reached down to 1% in chitin and 2% in sludge at 96 hours. *Sanguibacter* became abundant in chitin and sludge at later stages in the experiment, reaching 2% in chitin at 456 hours, and 14% in sludge at 624 hours. The *Neisseriaceae* family remained constant throughout the experiment in both sludge and chitin, at approximately 1-3%.

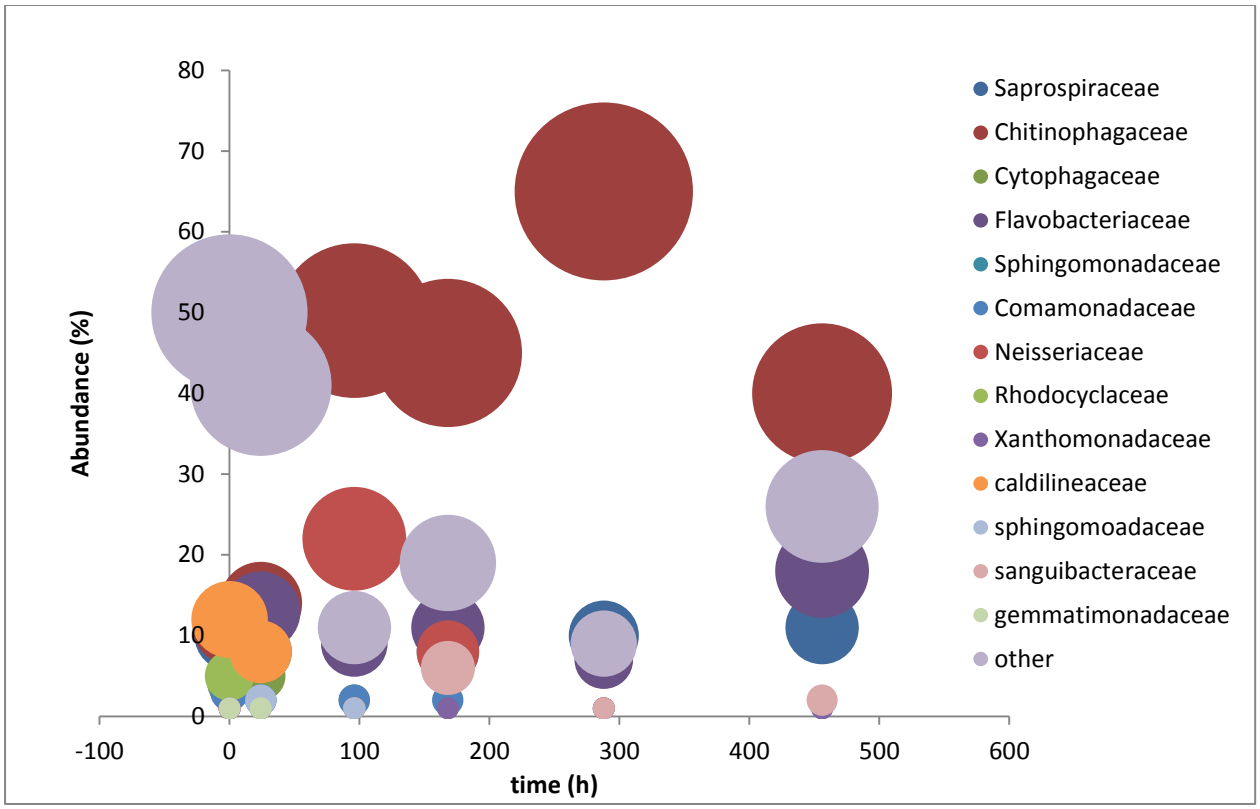


Figure 2.7 Abundance of different bacterial families in chitin incubated with sludge. The horizontal (x) axis represent the time (h) at which the original sample was removed from the cultures and had DNA extracted from.

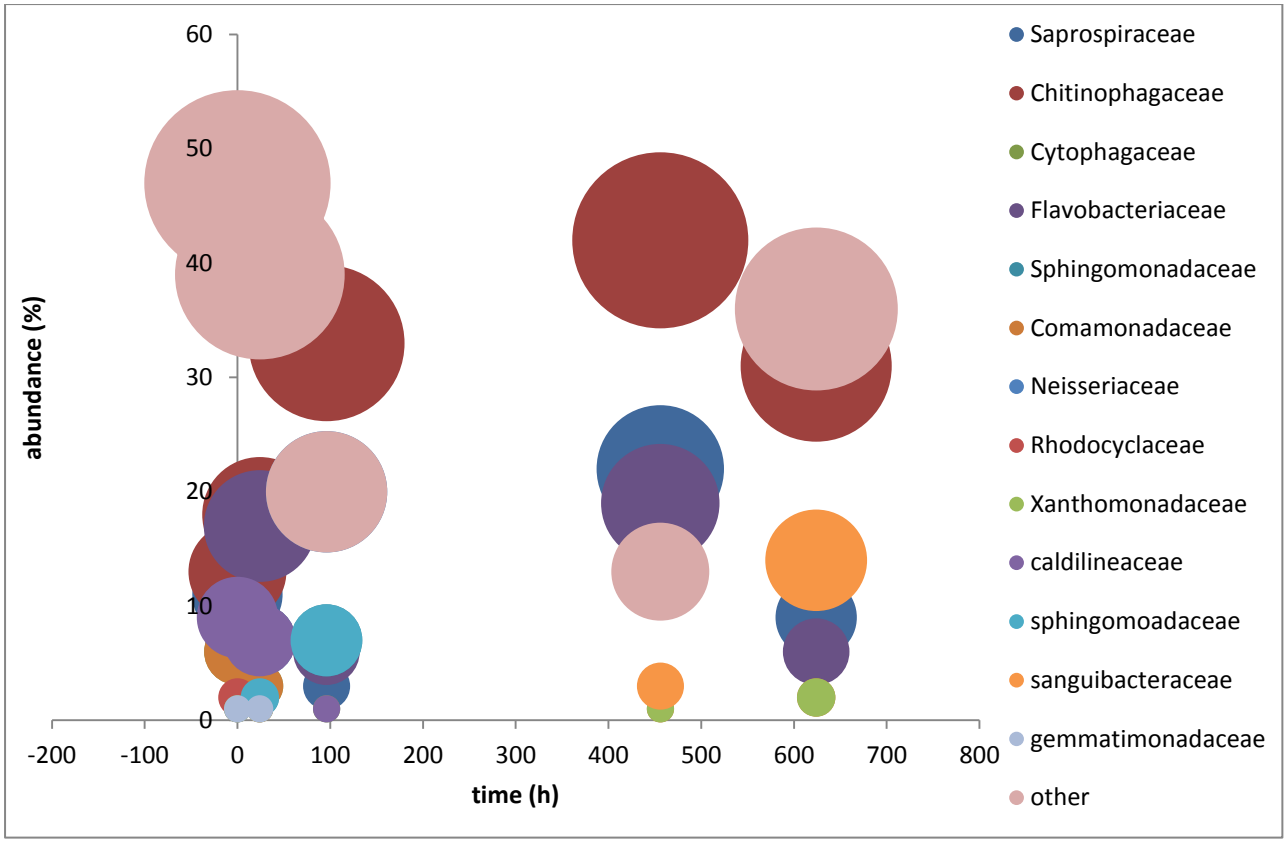


Figure 2.8 Abundance of different bacterial families in Sludge (in the presence of chitin). The horizontal (x) axis represent the time (h) at which the original sample was removed from the cultures and had DNA extracted from.

2.4 Discussion:

The first aim this study was to elucidate the steps and the timing of the steps involved in the colonisation of chitin by *A. hydrophila*. DNA extraction yields revealed that biomass began accumulating on chitin after 24 hours. Biomass continued to accumulate until after 120 hours (in two of the three replicates). After 168 hours, DNA yields began to decrease. The decrease in biomass after 120 hours could indicate detachment of cells from the chitin surface or the production of DNAses, which could degrade extracellular DNA. Similar studies conducted on *Psuedoalteromonas* strain S91 revealed that cells detach from chitin surface after 130 hours of inoculation into a Laminar flow cell (LFC) [106]. Detached *Psuedoalteromonas* cells proliferate in the bulk aqueous phase (as seen in this study) and then find another chitin surface to colonise [88]. This is also similar to the findings of Margolis et al, where *F. novicida* attached to and formed aggregates on chitin within a week [59]. These aggregates became more elaborate and are distributed throughout the chitin surface. Following from the model of the biofilm life-cycle, it is likely that a cell from an aggregate on chitin detaches and attaches to another site on the same or a neighbouring chitin piece. In the present study, the media was removed twice a day. The removal of media limits the reattachment of planktonic cells onto the chitin surface, and enables analysis of the initial cells that attached to chitin and proliferated. This is probably the reason that no increase of biomass or repetition of the DNA yield curve is seen after 200 hours. Based on the DNA yields and microscopy images obtained in this study, the biofilm life-cycle of *A. hydrophila* appears to follow the process described in section 1.2.1, with cells initially attaching to chitin (after 24 h), proliferating on the chitin surface and forming aggregates (168 h), and then detaching from the surface into the bulk aqueous phase (after 200 h).

CLSM images obtained of *A. hydrophila* colonisation of chitin show that the colonisation is heterogeneous; there are locations on a single piece of chitin that are colonised next to areas that are intact and have no cells attached. It is likely that more of the chitin would have been colonised over time had the media not been removed twice a day. It is also possible that detached cells do not reattach to another piece of chitin until the nutrients within the media are exhausted; this process is again hindered because of the changing of the media.

In addition to understanding the steps involved in chitin colonisation by an *A. hydrophila* activated sludge strain, the study aimed to monitor chitin colonisation in activated sludge. SEM images indicate that, similarly to *A. hydrophila,* activated sludge bacteria begin to attach to the chitin surface between 24 and 48 hours. Cells attach to different locations of the chitin, both on portions where the surface is flat and on locations where there a folds or crevices in the surface. SEM images revealed that at 48

hours cells attached to the surface as single cells or in aggregates of approximately 5 to 30 cells. After a week of incubation (168 hours), the presence of cells becomes widespread across the surface, however a homogenous mat of cells is not formed on the surface, rather, the cells exist as single cells or as clumps unevenly distributed on the surface This is different to colonisation of *Vibrio cholera* WT to squid pen chitin, where at 96 h a mat of cells was seen on the chitin [107]. Squid pen is composed, however, of β-chitin, which is considered "soft" chitin because of the monomer chains arrangement (section 1.4.1). Results presented here suggest that α-chitin is more resistant to colonisation. After 288 hours (12 days), chitin colonisation starts to affect the integrity of the chitin surface. Cells start to grow within channels and crevices in the chitin - a likely consequence of chitinolytic attack. The excessive colonisation of the chitin particle is also an indication that the chitin did not disrupt the C:N:P ratio in the sludge cultures and that the phosphate in the cultures was not limiting.

The third aim of this study was to describe the diversity of the bacterial species colonising chitin in sludge. All of the family groups listed in Figures 2.7 and 2.8 belong to the *Bacteroidetes* phylum, with the exception of *Nisseriaceae,* which belong to the *Proteobacteria,* and *Caldilineaceae* which belongs to the *Chloroflexi*. Groups belonging to the *Bacteroidetes* phylum are known to degrade a range of biopolymers [108]. *Ferruginibacter,* which was identified by DGGE and sequencing has recently been found to belong to the *Chitinophagaceae* family, which was the most abundant on both chitin and in the sludge. [109]. The main genera in this family that were present were *Ferruginibacter, Terrimonas and Filimonas. Terrimonas* strains have been isolated from bulking sludge [110] and freshwater [111] but have not shown chitin degrading activity or AHL production [112]. *Ferruginibacter* has been previously found in wastewater plants [113] , but has not been shown to produce AHLs or chitinases [109]. *Filimonas* has not been shown to produce AHLs or chitinases. *Flavobacteria*, from the *Flavobacteriaceae* family, have been shown to utilize chitin. The main genus present in the *Nisseriaceae* group was *Chitinilyticum,* strains of which have been isolated from pond water and are able to grow aerobically and anaerobically in the presence of chitin, which they utilise as a carbon source [114]. A *Chloroflexi* bacterium was also detected in the DGGE profile. The *Sanguibateraceae* family becomes more abundant in sludge (14%) after 624 hours, while it comprises only 2% of the community on chitin pieces at 456 hours. Such results suggest that members of the *Sanguibateraceae* family do not attach to chitin, but utilise oligomers released from chitin as a result of chitinolytic activity in the sludge. The pyrosequences are consistent with the DGGE profiles and indicate that bacteria belonging to the *Bacteroidetes* phylum are present in the sludge at the start of the experiment, but highlight which families and genera within those families become more abundant when supplemented with chitin.

DNA yields and CLSM revealed that *A. hydrophila* attaches to chitin after 24 hours of incubation with chitin, continues to colonise the surface up until 168 hours. After 200 hours, *A. hydrophila* cells detach from the chitin surface into the bulk aqueous phase, possible to colonise a new surface. Similarly, activated sludge cells attach to chitin after 24 hours of incubation. Activated sludge cells continue to accumulate on the chitin surface and by 288 hours, start to grow in crevices in the particle. The main bacterial phyla that colonise chitin in sludge are *Bacteroides*, with bacteria from the *chitinophagaceae* family being the major colonisers. The most abundant genus is *Terrimonas*.

Chapter 3 AHL production and chitin consumption in activated sludge communities colonising chitin

3.1 Introduction:

AHL production, as discussed in section 1.3.3, has been observed in activated sludge, but the range of roles AHLs may have in activated sludge communities remains to be elucidated. Studies report that the production of AHLs by activated sludge communities may be involved in the formation of biofilms and biofouling of membranes in membrane bioreactors [115] and the regulation of enzymes such as chitinase .AHL production by sludge bacteria colonising chitin has not been studied before, even though chitin is used in some wastewater treatments.

Aeromonas species are chitinolytic bacteria commonly isolated from soil, marine and freshwater environments [1, 8]. They have been shown to produce both endo-chitinases [13] and exo-chitinases [2-3]. Membrane bound endo-chitinases first cleave the chitin substrate, which results in the release of chitin oligomers. The oligomers are then degraded by exo-chitinases produced by the *Aeromonas sp*. Although QS phenotypes have been reported in *A. hydrophila* before, the possibility of chitinase production in *Aeromonas* strains being a QS regulated phenotype has not been thoroughly explored. Defined phenotypes such as exoprotease production [4] and more complex phenotypes such as biofilm formation [5] are regulated by Quorum sensing in *A. hydrophila.* Studies conducted by Chong et al, 2012 show that *Aeromonas* strains isolated from sludge produce AHLs and chitinase and that the production of chitinase was upregulated by the addition of exogenous AHLs [63].

This chapter tests the findings of the work previously done by Chong et al, 2012 in activated sludge directly colonising chitin, and explores the correlation between AHL production and chitinase activity in chitin particles colonised by *A. hydrophila* and activated sludge with the following specific aims:

1. To test different methods of AHL detection and to determine the most suitable method for this study.
2. To explore the impact of chitin addition to activated sludge on AHL and chitinase production.
3. To correlate the timing of AHL production and chitinase production

The experimental approaches presented in this chapter were conducted to fulfil the aims specified above and include various methods of AHL detection, including bioassays, NSI, MS and TLC. The most suitable method for detection of AHLs was the bioassay using *Aeromonas* (pBB-luxR) [63]. The bioassay yielded clear and conclusive results regarding the timing of AHL production, and the ability of chitin incubated in sludge, to bind AHLs. Chitinase assays were performed to monitor the

production of chitinase. Evidence for the consumption of chitin is presented in the form of SEM images.

3.2 Materials and Methods

3.2.1 AHL detection:

3.2.1.1 Cross streaking assay/overlay assay using *Chromobacterium violacein* CV026:

Chromobacterium violacein CV026 was grown overnight in the presence of 25 µg/ml kanamycin in LB media. LB agar was prepared with 1.2% w/v and added to the overnight culture at proportion of 1:2, culture: agar. The mixture was then poured into sterile petri dishes and left to solidify for 10 minutes. Washed pieces of chitin were placed on the solidified agar plates and incubated at 30^{o}C overnight.

Similarly for the cross streaking assay, CV026 was grown overnight and LB agar plates were prepared. Chitin pieces were placed on the agar plates and CV026 was streaked onto the streak near the chitin plate. These plates were also incubated overnight at 30^{o}C. For both the overlay and cross streaking assays using CV026, positive controls were prepared by using 2 AHL standards, 20 nM OHHL and 20 nM C4-HSL. Standards were dissolved in methanol. Methanol was used as a negative control.

3.2.1.2 AHL extraction by ethyl acetate:

To examine AHL production sludge samples (20 ml) were taken at 0, 72, 96, 120, 336, and 408 h and centrifuged at 8000 rcf for 8 minutes. The supernatant was filtered through a 0.22 µm filter. The filtered supernatant was then transferred into a glass vial and extracted with ethyl acetate acidified with 0.1% formic acid. The mixture was shaken vigorously and the aqueous phase transferred to a 50 ml glass Schott bottle. This process was repeated three times. Ethyl acetate was left to evaporate in the laboratory fume hood. After evaporation 2 ml of ethyl acetate were added to the bottles before removing into a clean 2 ml HPLC vial. This ethyl acetate was also allowed to evaporate before finally reconstituting with 200 µl acidified methanol (0.1% formic acid).

3.2.1.3 AHL detection using thin layer chromatography (TLC):

Aluminium backed, normal phase Silica TLC plates (Merck Millipore, 105553) were spotted with 15 ul of sample extracted from sludge cultures at different time points and the standards of 20 mM OHHL, 20 mM BHL, 1 mM OdDHL, and 1 nM OHL. The plates were then developed with a mixture

of 60:40 Methanol:MilliQ water and dried at room temperature for 15 minutes. The plates were then placed in glass petri dishes with salt agar. The salt agar is MilliQ water with 0.5% NaCl w/v and 1.2% (w/v) agar. The TLC plate was then overlaid with an overnight *Argobacterium tumefaciens* A136 culture mixed with 1.2% LB agar at a proportion of 1:2, culture:agar. The plate was then incubated at 30˚C overnight. A136 cultures were supplemented with x-gal and 4.5 μg/ml tetracycline. Images of the plates were taken using a Canon PowerShot SX120 IS camera.

3.2.1.4. Nanoelectrospray ionisation(NSI) mass spectrometry (MS):

An Orbitrap LTQ XL (Thermo Fisher Scientific, San Jose Ca, USA) ion trap mass spectrometer equipped with a NSI source for NSI-MS was used for Nanospray detection of AHLs from extracted samples. After calibration and optimization of the instrument, samples were run and then analysed using the Qual Browser feature in Xcaliber 2.1 (Thermo Fisher Scientific). The following standards were used before running the samples: BHL, HHL, OHHL, OHL, OOHL and, DHL. Methanol was used as a blank.

3.2.1.5 AHL production bioassay using *Aeromonas* (pBB-luxR):

Grace Chong isolated an *Aeromonas* strain from activated sludge and tested it negative for AHL production. The isolate was chosen to be a GFP-based monitor strain [63]. In the resultant monitor strain, *Aeromonas* (pBB-luxR), LuxR induces the expression of Green Fluorescent Protein (GFP) in the presence of AHLs [6]. *Aeromonas* (pBB-luxR) was grown overnight in LB broth at 150 rpm, and then diluted in LB at a 1 in 5 dilution for half an hour. Culture samples were then incubated with the monitor strain at 30^{o}C for 4 hours, before being taken for visualisation under an Olympus BX51 fluorescence microscope.

As controls, fresh chitin pieces were incubated in different AHLs at different concentrations, and then incubated with the monitor strain. OHHL, BHL (C4-HSL) and OdDHL (C12-HSL) were incubated with fresh chitin pieces for 24 hours in sludge supernatant or PBS. The chitin pieces were then washed three times, with sludge supernatant and PBS. For chitin pieces that had been washed with sludge supernatant, the assay was performed in sludge supernatant (monitor strain grown overnight in LB was centrifuged and resuspended in (SS); for chitin washed in PBS the assay was performed in LB. After 4 hours of incubation with the monitor strain, chitin pieces were on an Olympus BX51 fluorescence microscope.

For the assays, chitin pieces were placed in 2 ml eppendorf tubes. Monitor stain (in LB or SS) was added to the tubes at a volume of 500 µl-1000 µl. The tubes were incubated for 4 h at 30^{o}C and 150 rpm.

3.2.2 Chitin consumption:

3.2.2.1 SEM microscopy:

SEM samples were prepared as described in 2.2.5.2.

3.2.2.2 Chitinase Assay:

A colorimetric assay (Sigma-Aldrich, CS0980) that relies on the hydrolysis of the assay substrate 4-Nitrophenyl N-acetyl-β-D-glucosaminide and the liberation of p-Nitrophenyl was used to quantify chitinase activity. Sodium carbonate is added to stop the reaction and ionize p-nitrophenyl into the yellow p-nitrophenylate ion. The absorbance of p-nitrophenylate is measured at 405 nm. For chitinases produced from cells bound to chitin, chitin pieces were extracted from cultures, bead beaten in the Qiagen Tissue Lyser II, and spun down. The supernatant was used in chitinase assays. For the detection of membrane-bound chitinases from cells not bound to chitin (in bulk aqueous phase of cultures), samples were spun down, the supernatant was decanted, and the pellet was resuspended in fresh media (sludge supernatant). This was then used in the assays. For detection of extracellular chitinase, samples were spun down, and the supernatant was used in the chitinase assay. An aliquot (50 µl) of the supernatant was added to 50 µl of the chitinase assay substrate (4-Nitrophenyl N-acetyl-β-D-glucosaminide) in a 96 well-plate which was incubated at 30^{o}C for 30 minutes. After incubation, stop solution was added and the absorbance was measured at 405 nm using a Spectra MAX340 Plate Reader.

3.3 Results

3.3.1 Production of AHLs by GC1 growing on chitin particles:

As mentioned previously, one of the aims of this study was to monitor the timing of AHL production by *A. hydrophila* on chitin flakes. AHLs were extracted from *A. hydrophila* growing on chitin and processed using NSI-MS. No AHLs were detected by NSI-MS. A similar result was obtained using TLC. Although *A. hydrophila* GC1 is known to produce BHL, HHL, OHL and dDHL [63] a simple overlay assay using *C. violacein* CV026 was performed to test if chitin associated GC1 cells have the capacity to produce AHLs when exposed to agar plates. Figure 3.1 presents the results of this bioassay. The reporter strain showed the strongest response to chitin pieces removed from the culture at 24 h. The experiment was conducted in triplicate and similar phenotypic responses were seen from CV026 to all triplicates. This result suggests chitin colonising cells can produce AHLs but does not unambiguously demonstrate AHL production on chitin.

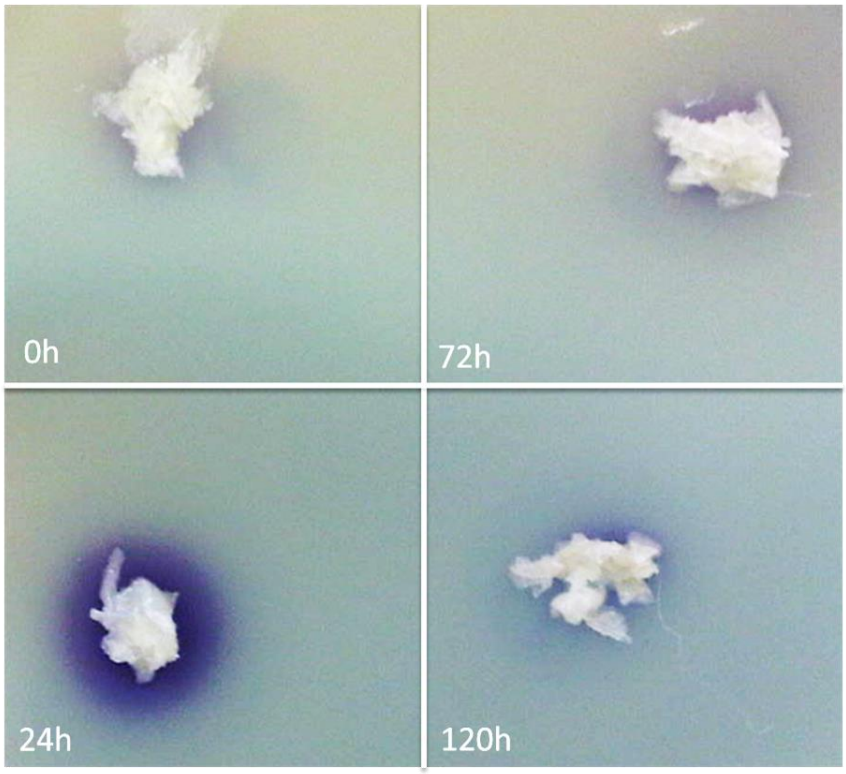
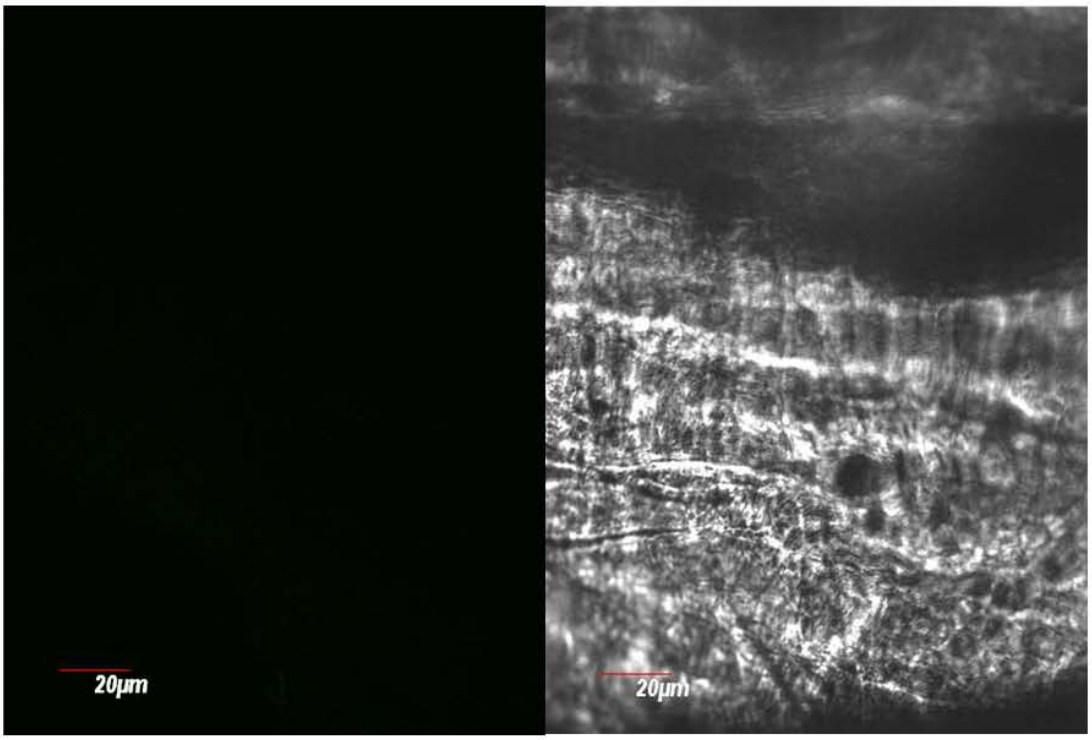


Figure 3.1: Overlay assay using *C. violacein* CV026. Overnight cultures of the reporter strain were overlaid with Chitin pieces extracted from *A. hydrophila* cultures at 0, 24, 72, and 120 hours.

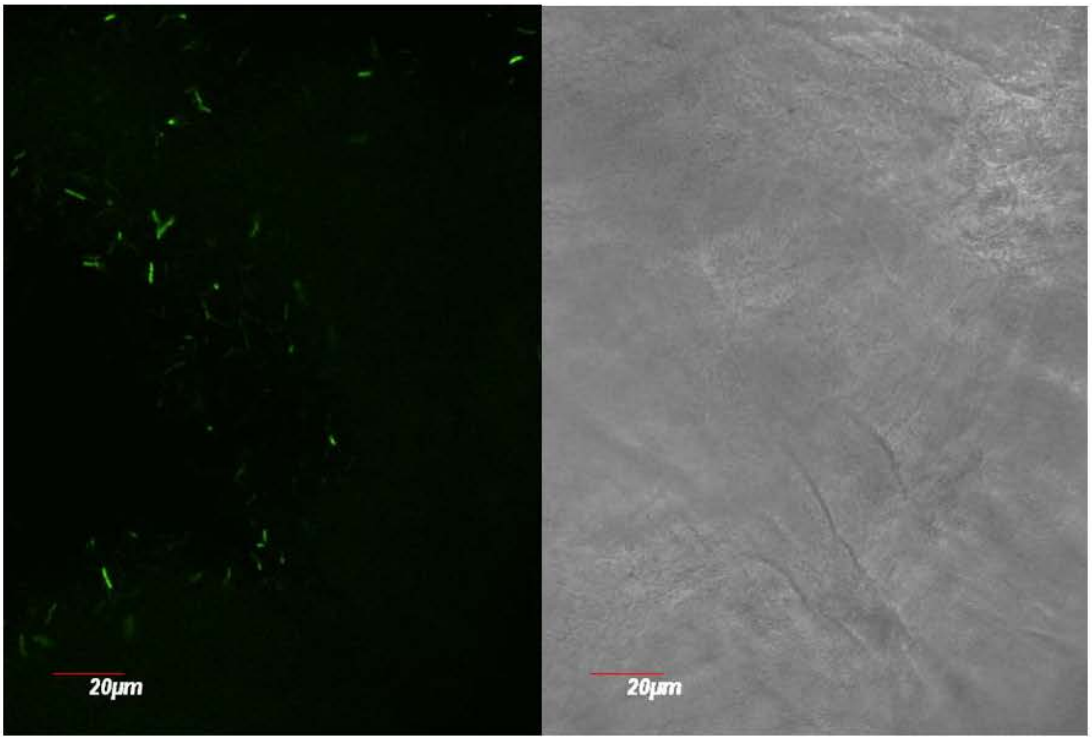
With MS and TLC approaches unable to detect AHLs extracted from colonised chitin pieces a bioassay using the monitor strain *Aeromonas* (pBB-luxR) was performed to determine whether AHLs are produced by *A. hydrophila* GC1 attached to chitin. Figure 3.2 shows that GFP production was

induced in *Aeromonas* (pBB-luxR) at 24, 96 and 120 h indicating that AHLs or molecules with similar function were present on the chitin surface at these time points. The monitor strain did not fluoresce in the bulk aqueous phase. It only showed fluorescence when attached to the chitin surface.

0 h



24 h



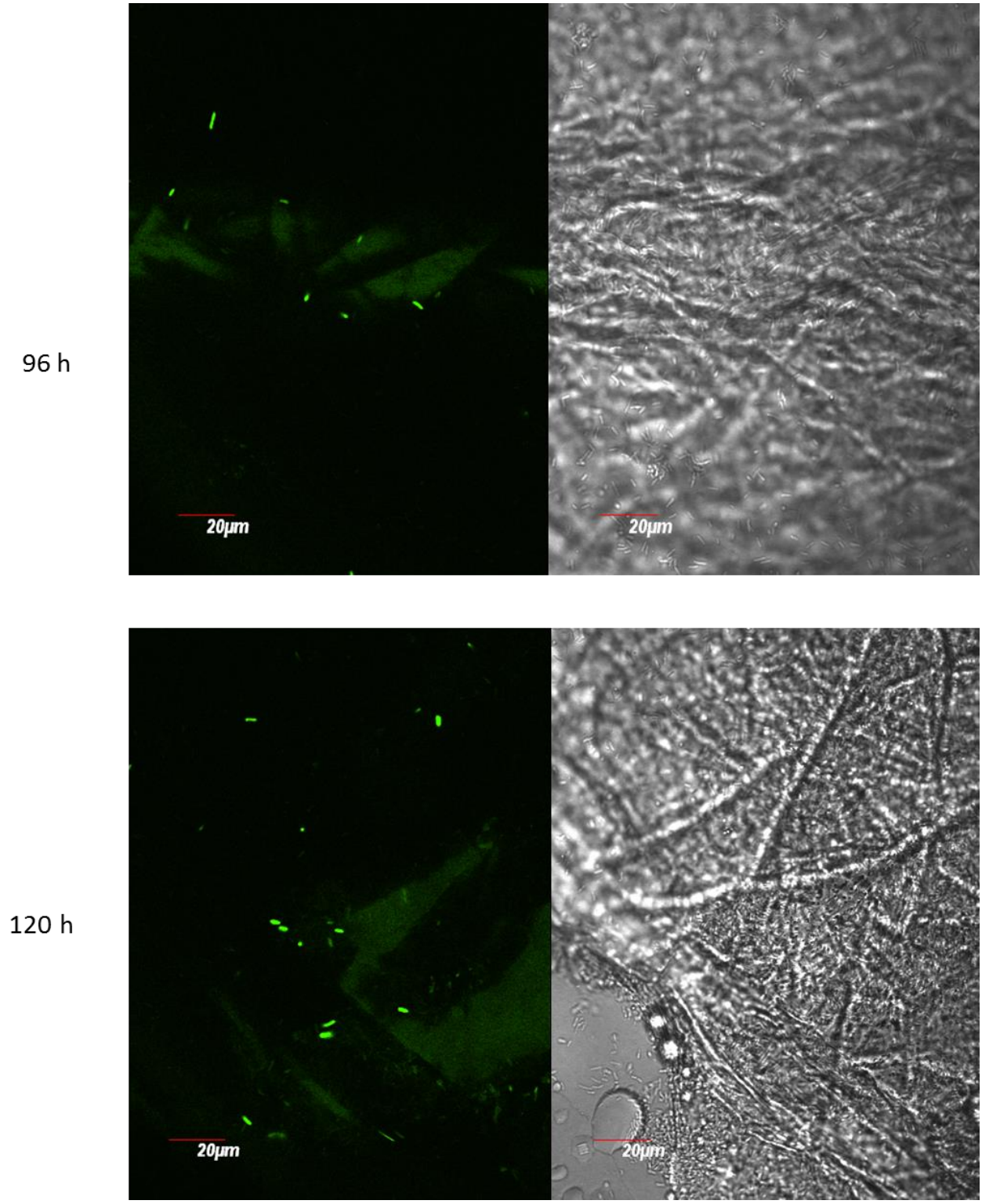


Figure 3.2: *Aeromonas* (pBB-luxR) assay performed on chitin pieces incubated in pure *A. hydrophila* GC1 cultures. Chitin pieces were removed from the cultures at 0, 24, 96, and 120 h. The time point at which chitin was removed from cultures is shown on the left-hand side of the images. The left hand side image is an epifluorescence image and the right hand side image is a phase contrast image. All images were taken at an input of 600 V.

3.3.2. Detection of AHL production by activated sludge incubated with chitin:

In an attempt to detect AHLs in activated sludge in response to the addition of chitin flakes TLC using an *Agrobacterium tumefaciens* A136 overlay method and Nanoelectrospray Ionisation Mass Spectrometry (NSI-MS) were employed. NSI-MS was again unsuccessful with no peaks referring to AHLs being detected. TLC plates overlaid with *A. tumefaciens* A136 detected 3 compounds with faint AHL-like activity in extracts derived from sludge samples taken at 96 and 120 hours in the presence of chitin (Figure. 3.3). This suggests the sludge was producing AHLs at those time points. No compounds with AHL-like activity were detected at other time points.

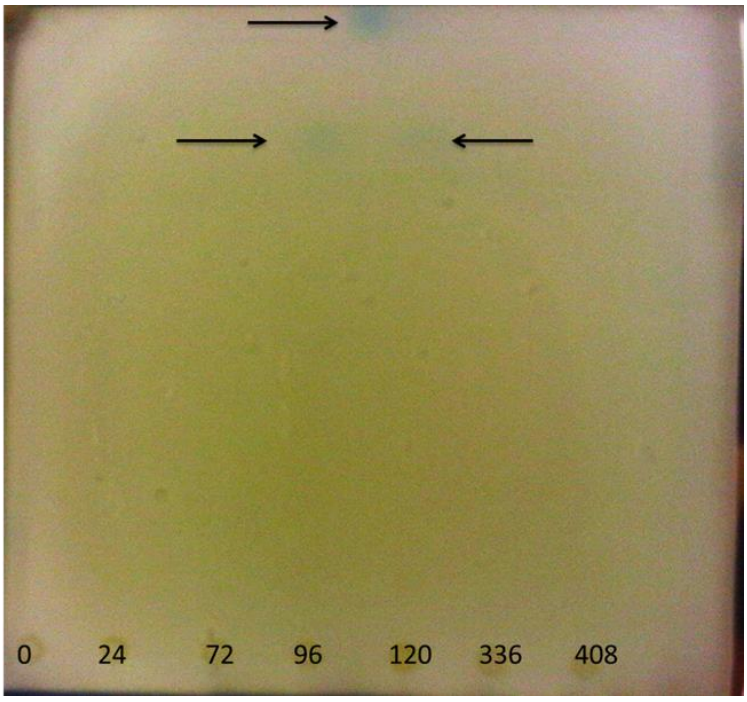


Figure 3.3: TLC plate using *A. tumefaciens* A136. The time points (0-408 h) are listed at the bottom of the figure.

To test for AHL production on chitin colonised by sludge species, chitin pieces were incubated in activated sludge to allow colonisation and subsequently rinsed and incubated in the presence of the monitor strain *Aeromonas* (pBB-luxR) in LB or sludge supernatant. The monitor strain was tested for differences in sensitivity in LB or supernatant by incubating with 0 nM, 50 nM and 100 nM OHHL. Figure 3.4 shows the monitor strain incubated with the different concentrations of OHHL in both LB and sludge supernatant. Testing of the monitor strain in sludge supernatant was performed to assess whether the media impeded the assay and to ensure that it would detect AHLs and produce GFP in the chitin pieces incubated in sludge. The monitor strain was responsive to OHHL in both media, though it was more sensitive in LB.

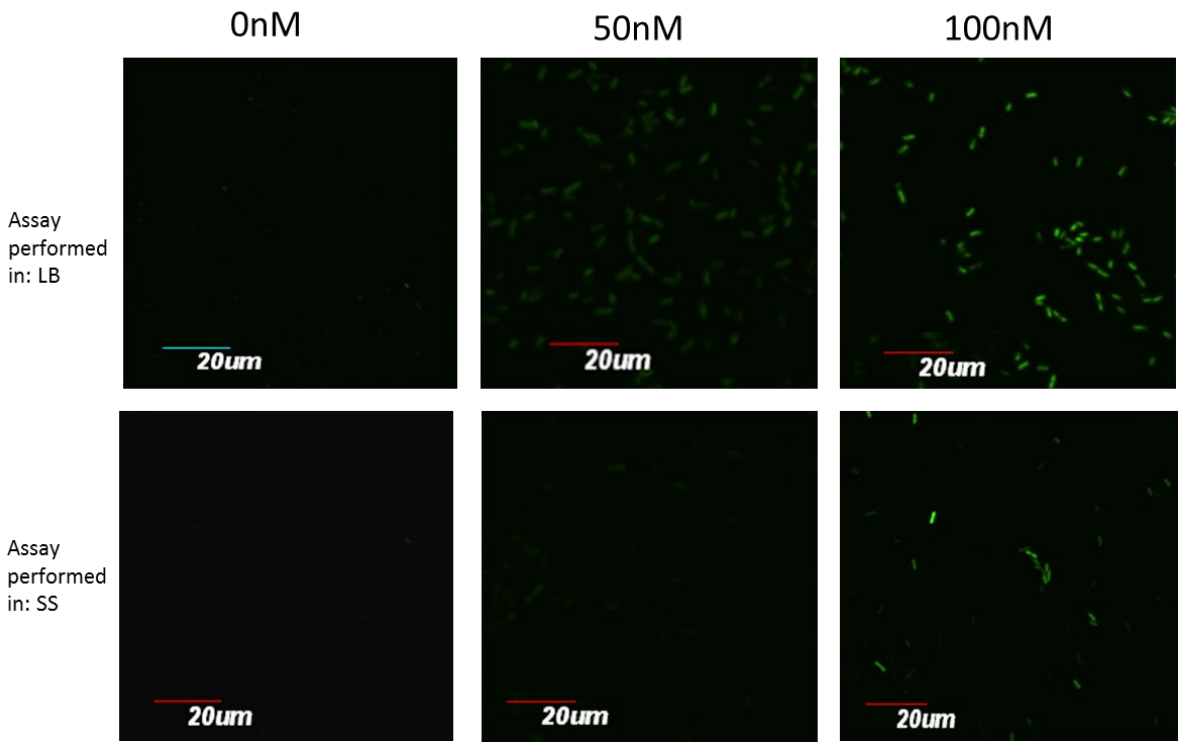


Figure 3.4: *Aeromonas* (pBB-luxR) incubated with 0, 50, and 100 nM OHHL in Luria Bertini media (LB) and Sludge supernatant (SS). The first row of images refers to the monitor strain incubated in LB media while the second row refers to monitor strain incubated in sludge supernatant. The first column of images shows the monitor strain incubated with 0nM OHHL, the second column 50 nM OHHL, and the third column 100 nM OHHL. All images were taken with a voltage input of 600 V.

Figure 3.5 and Figure 3.6 show activation of the AHL monitor strain on chitin pieces after colonisation in activated sludge with the assay carried out in LB and sludge supernatant respectively. At 0 hours incubation in sludge, no GFP was visualised on chitin. AHLs were detected on chitin pieces after 24, 72, 96, and 120 h incubation in sludge with the assay carried out in LB. AHLs were detected on chitin pieces after a 24 and 96 h incubation in sludge, but not after 72 and 120 h when the assay was carried out in sludge supernatant. In all cases the activity was associated with the chitin surface and not present in the bulk aqueous phase.

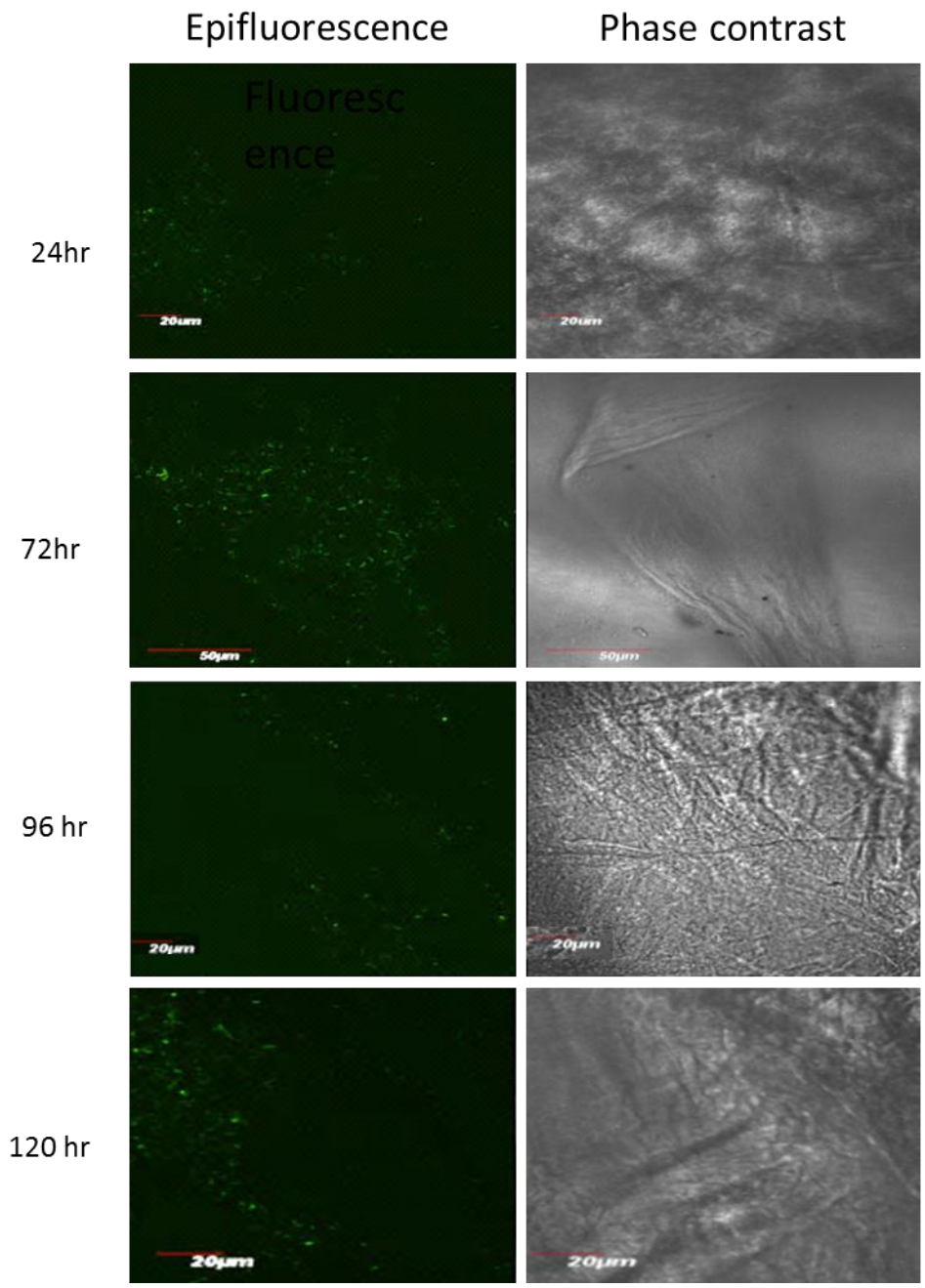


Figure 3.5: GFP production by *Aeromonas* (pBB-luxR) on chitin pieces incubated in activated sludge. The assay was performed in LB broth. Each row represents a single image taken of chitin, with the first being a fluorescence image and the second being a phase contrast image. Images were taken of the chitin at 24 hours (1st row), 72 hours (2nd row), 96 hours (3rd row) and 120 hours (4th row). All images were taken with a voltage input of 600 V.

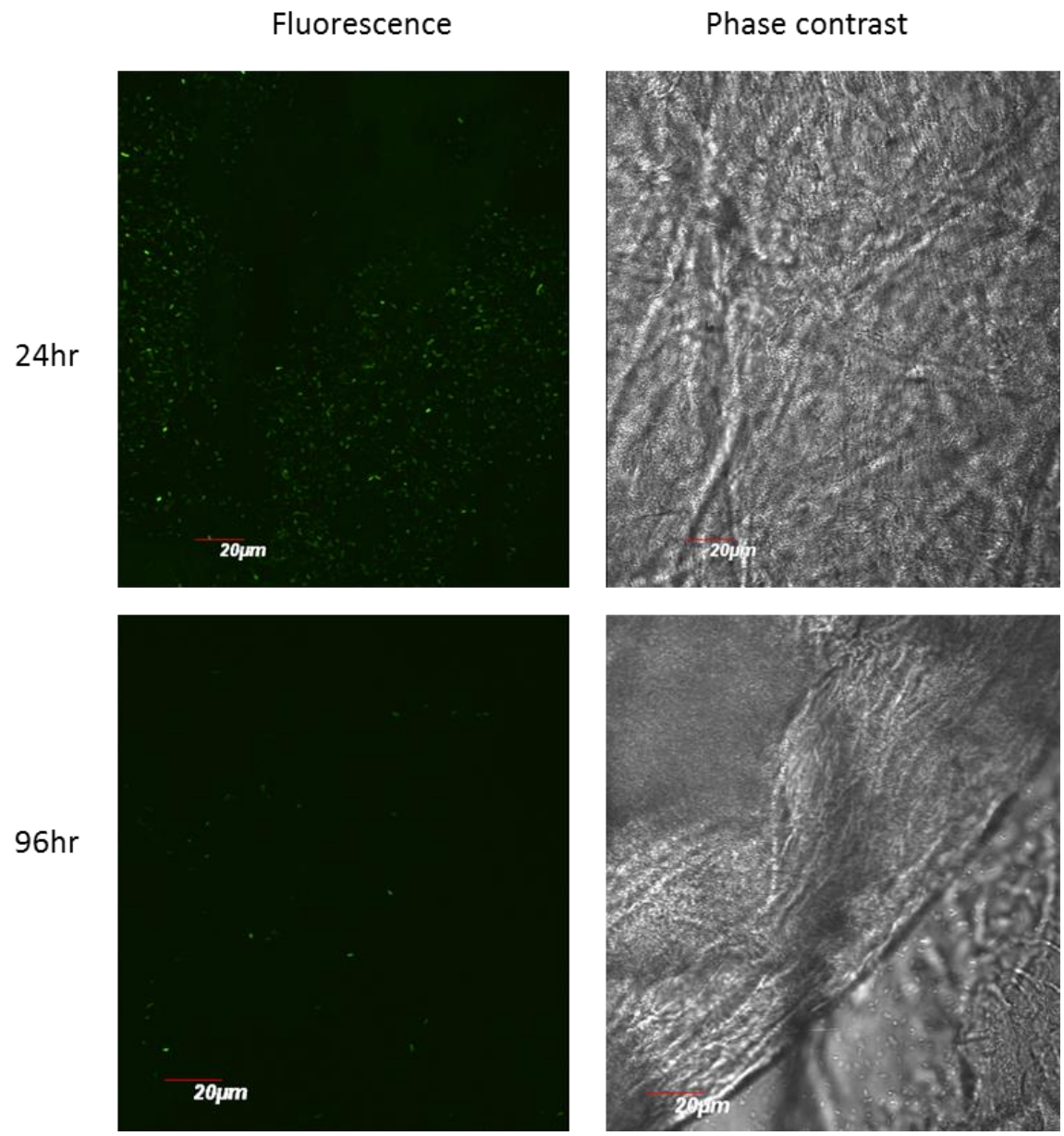


Figure 3.6: GFP production by *Aeromonas* (pBB-luxR) on chitin pieces incubated in sludge supernatant (SS). Each row represents a single image taken of chitin, with the first being a fluorescence image and the second being a phase contrast image. Images were taken of the chitin at 24 hours (1st row) and 96 hours (2nd row). All images were taken with a voltage input of 600V.

The differences in results obtained from both media led to questions concerning the impact that growing *Aeromonas* (pBB-luxR) in different media might have. Hence, a growth curve (Appendix 1) was constructed for the monitor strain over a six hour period in both LB media and SS. The results show that the growth and hence activity of *Aeromonas* (pBB-luxR) is significantly higher in LB than it is in SS. In addition to differences in cell numbers of the monitor strain in different media, it is

possible that activated sludge supernatant contains lactonases that degrade AHLs and hence affect the assay.

The detection of AHLs on chitin pieces after 24 h of incubation in activated sludge was surprising given the lack of biomass observed by SEM at this time point (Figure 2.4). This led to the hypothesis that AHLs in sludge stick to chitin prior to surface colonisation. To test this chitin pieces were incubated in the presence of 50-100 nM OHHL, and BHL in sludge supernatant of PBS and subsequently rinsed thoroughly and subject to the AHL bioassay as above. Figure 3.7 reveals activation of the monitor strain by the chitin surface after exposure to OHHL and BHL. This confirms that short chain AHLs bind to chitin and suggests AHL binding to chitin may facilitate colonisation of the chitin surface.

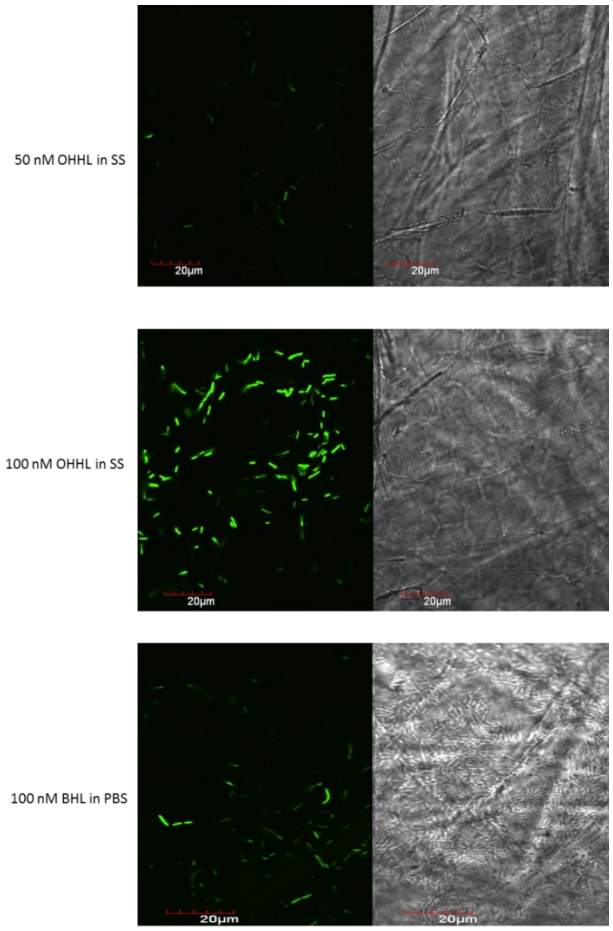


Figure 3.7: GFP production by *Aeromonas* (pBB-luxR) on chitin pieces incubated with different AHLs. The figure shows four images taken in epiflourescence (left hand side) and phase (right hand side). The concentration of AHLs and media in which the assay was performed is depicted on the left hand side of the images. All images were taken at an input of 600 V.

3.3.3. Detection of cell-bound and extracellular chitinase activity in pure *A. hydrophila* cultures:

To observe if *A. hydrophila* GC1 was producing chitinases, chitinase assays were performed. *A. hydrophila* GC1 was incubated in LB media and supplemented with chitin at room temperature, with no shaking. Chitin pieces were removed from the cultures at different time points. These chitin pieces were bead beaten as described in section 3.2.2.2, and the resultant supernatant was used as the sample for chitinase assays. Figure 3.8 shows that there is an increase in chitinase production after 0 hour, increased up until 120 hours of incubation, where it reached a plateau.

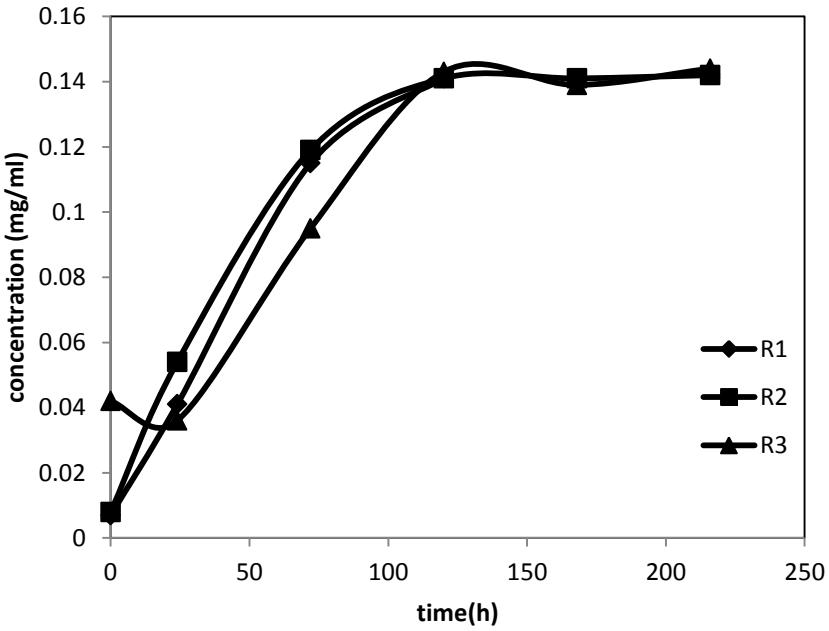


Figure 3.8: Chitinase production from *A. hydrophila* cells associated with chitin. Chitin pieces were extracted from cultures at 0, 24, 72, 120, 168, and 216 h. The figure presents the chitinase production curves from the triplicate cultures set up for the experiment (R1, R2, and R3).

3.3.4 Detection of chitin degradation:

SEM imaging of chitin pieces incubated in activated sludge generate clear visual evidence of penetration of the chitin surface by sludge microbes. After 48 h cells were inside of pores and folds of the heterogeneous surface of the chitin particle. After 288 hours, SEM images revealed holes and channel-like structures on the chitin surface, with cells living inside and around these holes and channels. These structures were seen throughout the examined chitin pieces, however, there were still patches of the surface that remained intact and un-colonised by cells after 288 hours. These images

provide a clear indication that the bacteria attached to the surface of chitin were consuming the chitin, or at least, cleaving and penetrating into the particle (Figure 3.9).

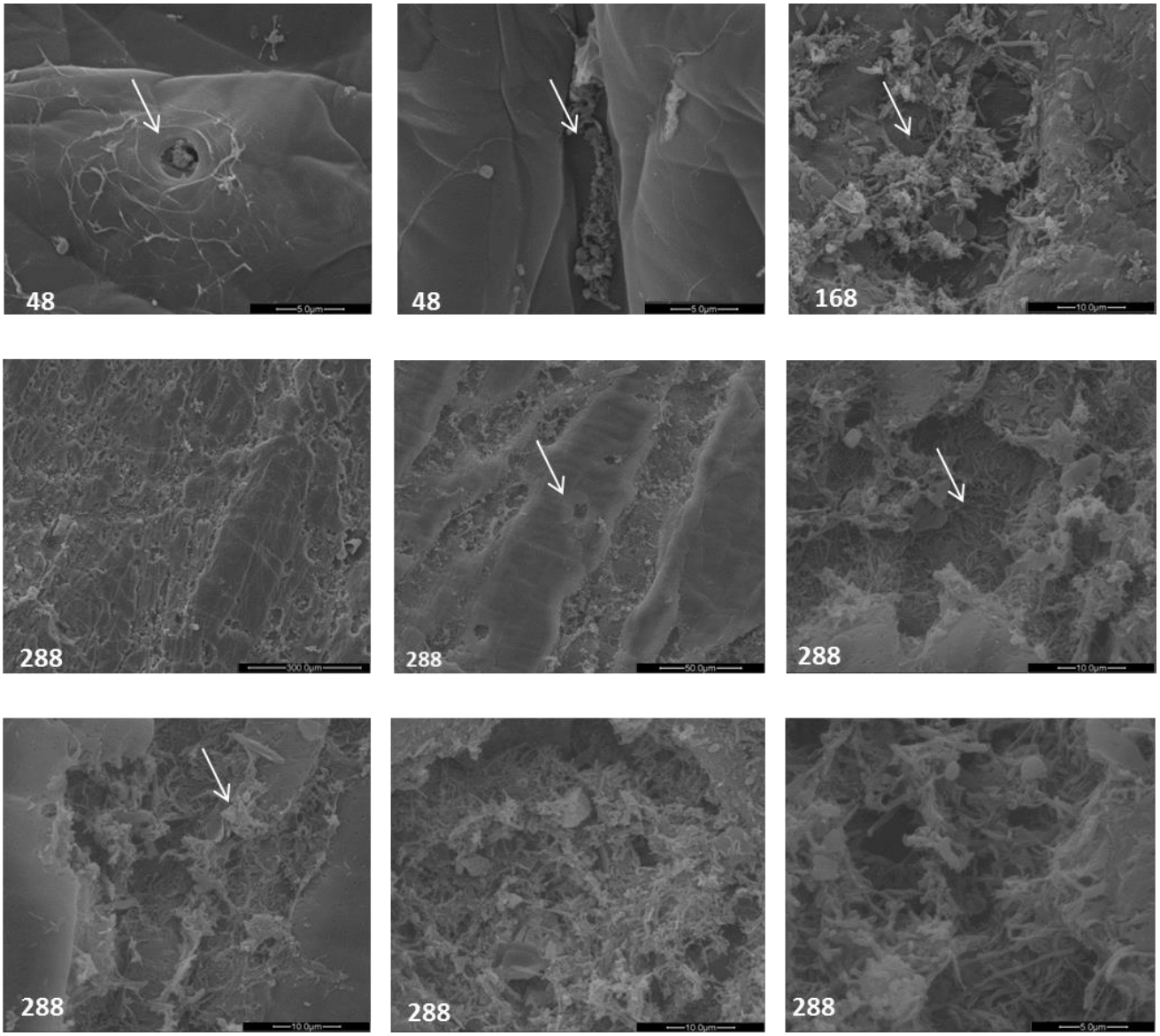


Figure 3.9: SEM images depicting the consumption of chitin. The numbers on the left hand bottom corners of the images represent the time at which the chitin pieces were sampled from the cultures in hours. These range from 48 hours to 288 hours (the numbers on the left-hand corner of the images).

3.3.5 Detection of cell-bound and extracellular chitinase activity in activated sludge cultures:

Chitinase assays were performed on sludge samples incubated with chitin over time to quantify extracellular chitinase production, and membrane-bound chitinase production from cells growing on chitin particles and cells growing in the bulk sludge. Cell associated chitinase was produced on the chitin particles and in sludge, the latter being at higher levels than the former. On chitin particles, low

levels of chitinase were detected, and the concentration of chitinase peaked at 96 hours (Figure 3.11). Cell associated chitinase followed a bimodal pattern, with an initial surge in production at 6 hours, then decreased rapidly until 24 hours. Chitinase production then gradually increased after 24 hours, peaking at 150 hours. Extracellular chitinase activity was not observed before 96 hours. However, a surge of chitinase production occurred between 96 and 120 hours. This increase in chitinase production was not seen in sludge where no chitin was added (Figure 3.10), which shows that the addition of chitin to activated sludge stimulated production of extracellular chitinase. Chitinase concentrations decreased after 120 hours until the experiment was terminated after 2 weeks.

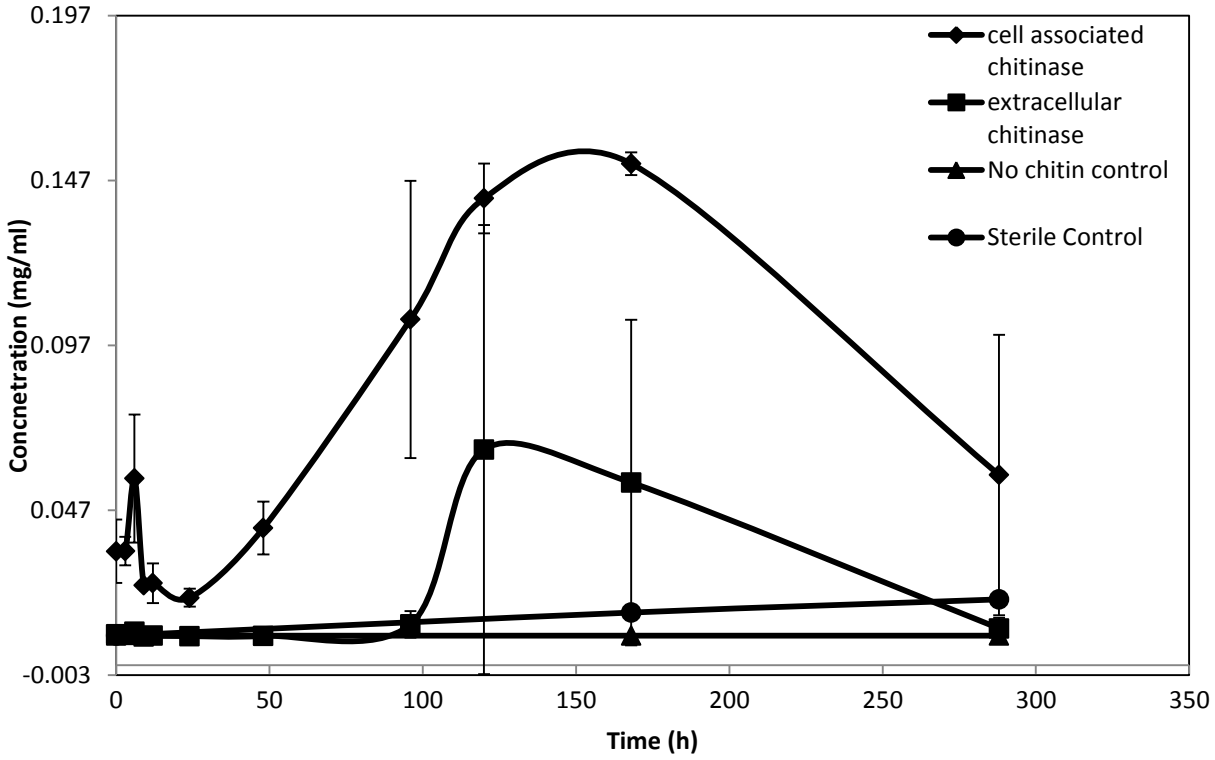


Figure 3.10: Chitinase activity in activated sludge cultures with and without chitin supplementation. Activated sludge cultures with no added chitin were tested for extracellular chitinase production. Error bars represent standard deviation.

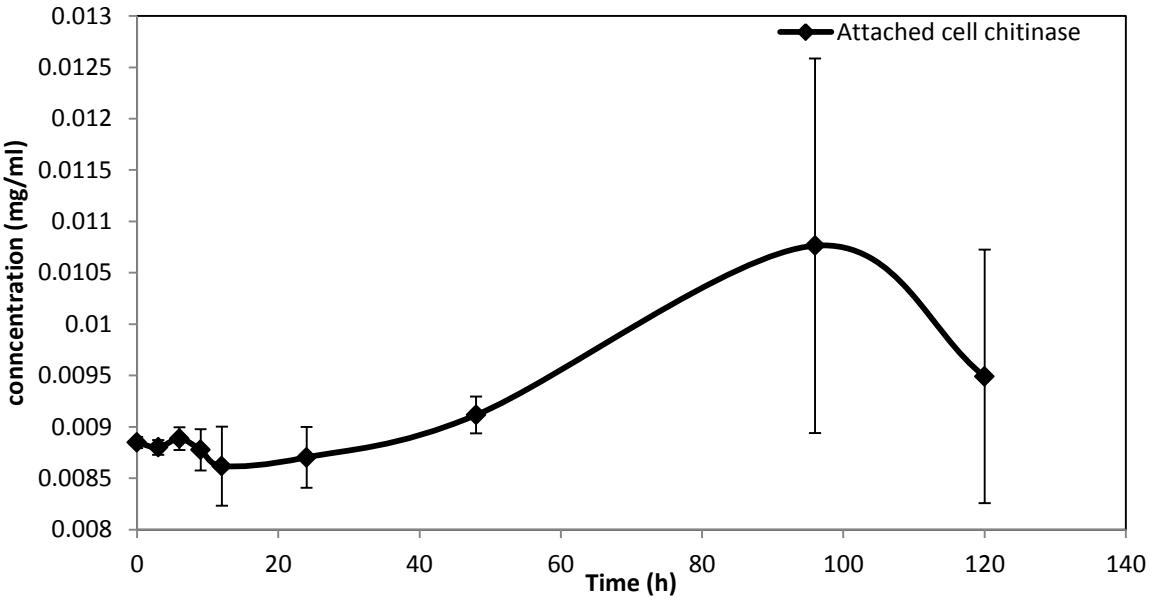


Figure 3.11: Chitinase activity in cells attached to chitin in activated sludge. Error bars represent standard deviation.

3.3 Discussion:

In this chapter AHL bioassays, SEM and chitinase assays and were used to investigate the relationship between AHL production, chitin colonisation and chitin degradation in a model bacterium and in activated sludge. In pure cultures of *A. hydrophila*, AHLs were detectable after 24 hours of incubation, while chitinase activity was observed after 0 hours, gradually increasing up until 120 hours. On chitin pieces incubated in sludge, AHLs were also detectable at 24 hours. Cell associated chitinase in the bulk aqueous phase was detected after 24 hours, while extracellular chitinase in the bulk aqueous phase was detected after 96 hours. On chitin pieces, chitinase was detectable after 24 hours. Cell associated chitinases and chitinases produced by cells attached to chitin were produced at an earlier time than extracellular chitinase. This suggests that cells attached to chitin and cells in the bulk phase may produce cell associated chitinases which cleave it, releasing oligomers into the bulk aqueous phase. Cells may then produce extracellular chitinase to further degrade the oligomers to obtain a nutrient source.

As stated in the introduction to this chapter, one of the aims of the study was to identify the most suitable method for AHL detection in cultures. The use of the monitor strain *Aeromonas* (pBB-luxR) proved to be the method that yielded the most consistent and conclusive results. Similar biosensor based detection techniques have been used to detect AHLs in wastewater such as the whole-cell pigment based system for the quantification of N-butyryl homoserine lactone [116]. In the study, *Psuedomonas aeruginosa* was selected to be the AHL biosensor [116]. The other methods used in this study were not efficient in detecting AHLs. It is likely that AHLs were not produced in the culture at high enough concentrations to be detected by the TLC and NSI/MS technologies used. It is also highly likely that the method of extraction of AHLs is not suitable to be performed on chitin, especially since we have established in this study that AHLs stick to chitin. This conclusion can be made since *A. tumafaciens* has been shown to respond to AHL quantities as low as 30 pmol [117] and NSI MS methods can detect nM concentrations of AHLs. It might be necessary to develop a method of extraction from chitin that is more effective in removing attached AHLs from the chitin surface.

The results presented in this chapter show that in a period of only 24 hours, chitin has the ability to bind AHLs produced in sludge (Figures 3.5 and 3.6). This observation has not been made before although it is known that chitin can bind dyes[118] and metals [98]. We can make this conclusion because the SEM images obtained show that bacterial colonisation of chitin at 24 hours is minimal, and the concentration of cells is not high enough for AHL production (Figure 2.4). The AHLs stimulating GFP production in the monitor strain at 24 hours must be produced by the bacteria in

sludge. The chitin surface now becomes a surface that can not only be utilised as an energy source, but is also a surface coated in AHLs. The ability of chitin to bind AHLs is supported by the observation that AHLs were not observed in the bulk phase. Bacteria that can colonise chitin and utilise AHLs as an energy source (quorum quenchers) may have an advantage in this environment, as well as bacteria that can colonise chitin and use AHLs to regulate biofilm formation and the production of certain enzymes.

A trend in chitinase production was established in pure cultures and activated sludge. Chitinase levels in *A. hydrophila* cells attached to chitin peaked at 120 hours. Chitinase production in activated sludge, whether it be membrane bound chitinase produced by cells in the sludge or cells attached to chitin or extracellular chitinase, also peaked at around 120 hours. A study performed on bacteria isolated from lake water showed a peak in chitinase production at 96 hours of incubation with colloidal chitin, and 120 hours for *Bacillus pabulli Kl* [119]. The result is similar to what was obtained in this study, however, bacteria normally produce chitinase to utilise colloidal and suspended [120] chitin earlier. The variation in chitinase activity in different forms of chitin could arise from the different ways of processing the chitin and the original form of the chitin (α-chitin or β-chitin) [121]. In this experiment, it is difficult to form a direct link between chitinase, which reaches high concentrations after 48 hours and AHL production because AHLs bind to chitin. It cannot be concluded that the cells attached to the chitin are producing the AHLs. AHLs produced by bacteria in the sludge can attach to chitin and may promote chitinase production in cells attached to chitin, as chitinase production increased after 24 hours, and AHLs were already attached to chitin at that time. However, the rise in chitinase production at around 120 h is consistent in chitinase in the sludge and chitinase produced by cells attached to chitin.

This study provides strong evidence that chitin has the ability to bind short chain AHLs, in solutions containing only AHLs, pure bacterial cultures, and activated sludge cultures. The binding of AHLs to chitin may provide an added advantage to certain bacteria in activated sludge and may enable one opportunistic organism to exploit other microbes colonising chitin. The results presented in this chapter add to our knowledge and understanding of the role of chitin in the environment.

Chapter 4: General discussion and concluding remarks

This study sought to collect fundamental data regarding biofilm formation on chitin by activated sludge organisms, and gain insight into the roles of AHLs and chitinases in this process. It was discovered that in pure cultures of *Aeromonas hydrophila* isolated from activated sludge, the biofilm/flocculation cycle on chitin, from initial attachment to dispersal, is completed in 200 h (8 days or approximately a week). Initial attachment of *A. hydrophila* begins after 24 hours of incubation, and multiplication of attached cells take place, resulting in microcolonies at 168 h throughout the chitin surface. After 200 h, DNA yields decrease, which suggests that cells may detach from microcolonies and colonise the chitin surface in other locations. Other studies experimenting with growth of chitin often report faster cycles, where chitin is colonised rapidly (in 24 hours) by *A. hydrophila*, however in these cases, the chitin has been modified (by the addition of concentrated HCl which transforms chitin flakes into a homogenous suspension) to make it a more readily utilisable surface [120]. However other studies are congruent with observations made here. For example, *F. novicida* has been shown to require a week to form aggregates or microcolonies on the chitin surface [59].

A similar colonisation pattern occurs when chitin is incubated in activated sludge. Cell attachment takes place after 24 hours, microcolonies are visible after 168 hours, and cells within the microcolonies begin to utilise the chitin, resulting in channels and crevices in the chitin surface by 288 hours. In addition to establishing a time line for colonisation of coarse grade α-chitin by *A. hydrophila,* the study also discovered that the process of colonisation is not homogenous from one chitin piece to another, or even on different locations of the same chitin piece. This might be due to differences in the composition of the chitin surface, or the presence of AHLs on the surface. Microscopy images presented in this study show that different locations of chitin can be at different stages of the biofilm cycle, where one location of chitin may be disintegrating due to chitinolytic activity, another location may be colonised by small colonies or individual cells, and another location may be completely intact with no cells present. These results suggest that the colonisation of chitin is a continual process sludge where a single surface of chitin is not exhausted simultaneously in a short period of time.

The activated sludge collected in this study, was composed primarily of bacteria that belong to the *Bacteroidetes* phylum. This is common in the activated sludge of municipal and industrial wastewater treatment plants [113, 122] and filamentous candidates within this phylum often cause bulking in sludge [123] This study has revealed that when activated sludge is supplemented with chitin, bacteria belonging to the *Chitinophagaceae* family become the most abundant in the sludge. Although members of this family have not been shown to produce AHLs, quorum sensing systems have been

found in *Bacteroides fragilis*, which belongs to the *Cytophaga-Flavobacteria-Bacteroidetes* group. Nine LuxR orthologues were found and the strain was sensitive to C6-HSL, which increased its antibiotic resitance. Also, chitin degradation has been shown in *Chitinophaga oryziterrae* [124]. Hence, it can be hypothesised that the members of the *chitinphagaceae* family found in this study attach and colonise chitin, and utilise it as an energy source.

One of the major advantages of this study is that bacteria are exposed to chitin, but not removed from the environmental sample and placed in artificial media. Artificial media creates bias towards cultivable bacteria, which are often in the *Proteobacteria* group. Although *Aeromonas* strains are present in sludge and produce AHLs and chitinases, they were found to represent only 0.03% of the community before exposure to chitin and were absent from chitin microbial profiles after incubation. It is possible that *Aeromonas* strains are exploited by parasitic bacteria, as they are by *Psuedomonas aeruginosa.* When incubated with chitin, *P. aeruginosa* releases pyocyanin after *A. hydorphila* has proliferated in the co-culture, leading to a decrease in *A. hydrophila* viability. The *P. aeruginosa* then uses the oligomers in the culture, which result from *A. hydrophila* chtinolytic activity, as an energy source [120].

Aeromonas strains and other members of the *Proteobacteria* group are also capable of quorum sensing, however the families in these were present at a relative abundance less than 1%. Even though the abundance of these families was low, it is possible that they produce AHLs in sludge at concentrations that impact of sludge ecology. The observation that AHLs concentrate at the chitin surface lends credence to this supposition.

To explore the relationship between AHL concentration and chitin colonisation, experiments were conducted to identify the stage in chitin biofilm formation when AHL concentrations became biologically relevant (ie. high enough to stimulate an AHL dependent biosensor). The expectation was that AHL accumulation would follow cell surface attachment and microcolony formation. Surprisingly, the AHL concentration on the chitin surface was high enough to activate an AHL biosensor prior to the appearance of cells on the chitin surface, suggesting AHLs produced by cells not associated with chitin can bind to the chitin surface. This was subsequently tested directly by exogenous AHL addition with results confirming that AHLs bind chitin and are biologically available at the surface.

Although this is the first example of AHLs binding to chitin, it is well known that chitin has the ability to adsorb different compounds. Chitin adsorbs humic acid (which is present in activated sludge flocs)

[125], melanoidins [126], metals such as nickel and zinc [99] dyes such as indigo carmine dye [118, 127] and even uranium [128]. AHLs can now be added to this list.

The discovery that AHLs produced in sludge or added exogenously in sludge supernatant bind to chitin dramatically alters our understanding of the relationship between quorum sensing and chitin colonisation in activated sludge. Firstly, the presence of chitin is likely to decrease AHL concentrations in the bulk aqueous phase and possibly in sludge flocs through sequestration to the chitin surface. In effect chitin is likely to attract AHL dependent activities in activated sludge away from other potential AHL microenvironment 'hot spots'.

Secondly, attachment of AHL responsive cells to AHL coated chitin is likely to have an immediate impact on AHL regulated phenotypes. This provides for the first time a reasonable explanation as to why surface colonisation phenotypes (ie. attachment) are regulated by AHL mediated gene expression [39]. As an example, it is expected that bacteria encoding AHL dependent expression of membrane bound chitinases that function to anchor cells to chitin, will experience rapid expression of such chitin binding proteins when they detect AHLs on the chitin surface. Thus it is hypothesised that it will be common in species that pioneer chitin colonisation to regulate membrane bound chitinase genes through AHL mediated gene expression. As a second example, it is known that AHL concentration regulates extracellular DNA release in *Pseudomonas aeruginosa* [129] so it is possible that bacterial cells attaching to AHL coated chitin may release DNA that can act as a scaffold for cell attachment and biofilm development [130].

Thirdly, bacteria possessing acylases or hydrolases (so called quorum quenching bacteria) that can degrade AHLs and use them as a carbon and energy source [131] may be attracted to AHL coated chitin surfaces. This in turn may impact on AHL concentrations on chitin and influence subsequent colonisation. It is hypothesised that the community pioneering chitin colonisation may also be enriched in quorum quenching bacteria.

Following on from this work, further studies need to be conducted to test the impact of AHL-coated chitin on colonisation and degradation of chitin. Experiments can be done where chitin is coated in AHL and then incubated in pure cultures. Chitinase production can be tested in these cultures. Comparative experiments can be set up where AHL-coated chitin and non-coated chitin is the sole energy source for microorganisms to assess if AHLs on coated chitin provide an advantage for the bacteria. Also, LuxI-homologue knockout mutants that normally use AHL mediated gene expression to regulate chitinase can be incubated with AHL-coated chitin to see if the AHLs restore chitinase activity in these mutants.

In conclusion, the colonisation pattern of chitin by an activated sludge strain and activated sludge was characterized. The chitin surface was discovered to be different to other colonisable surface as it has the ability to bind AHLs. This phenomenon has not been reported before and may well change our understanding of how surfaces can interact with the AHLs produced by the colonising and surrounding microorganisms. AHL coated chitin may provide a more favourable surface to certain members of the sludge community. Further experimentation is required to elucidate if the cells in the sludge attach directly to the chitin surface or AHLs on the surface. The microbial profiles of the bacteria that colonise chitin in sludge were identified. The main phylum that colonises chitin was *Bacteriodetes*, with the family *Chitinophagaceae* being the most abundant in that phylum. *A. hydrophila* colonises and utilises chitin, and completes the colonisation cycle in 200 hours in pure culture. It is not, however, present as a coloniser of chitin in activated sludge. Chitinase production reaches a peak at 120 hours and the effect of this chitinolytic activity is visible after 288 hours of colonisation, where the chitin surface becomes compromised. Chitin's ability to bind AHLs may also present an advantage to organisms that produce AHL regulated enzymes like chitinase. However, more studies are required to confirm this. This study, combining the need to shift to biorenewable and non-toxic coagulants in wastewater application and form a deeper understanding of the flocculation process in activated sludge, aimed to portray a realistic model of the microbiology involved in the colonisation of chitin. Figure 4.1 is a schematic of the newly proposed model based on the results obtained throughout this study. With the findings of this study, and further research and experimentation, we will acquire a better understanding of flocculation in sludge as an environmental niche, and will hence be able to manipulate wastewater systems to improve their overall efficiency and performance.

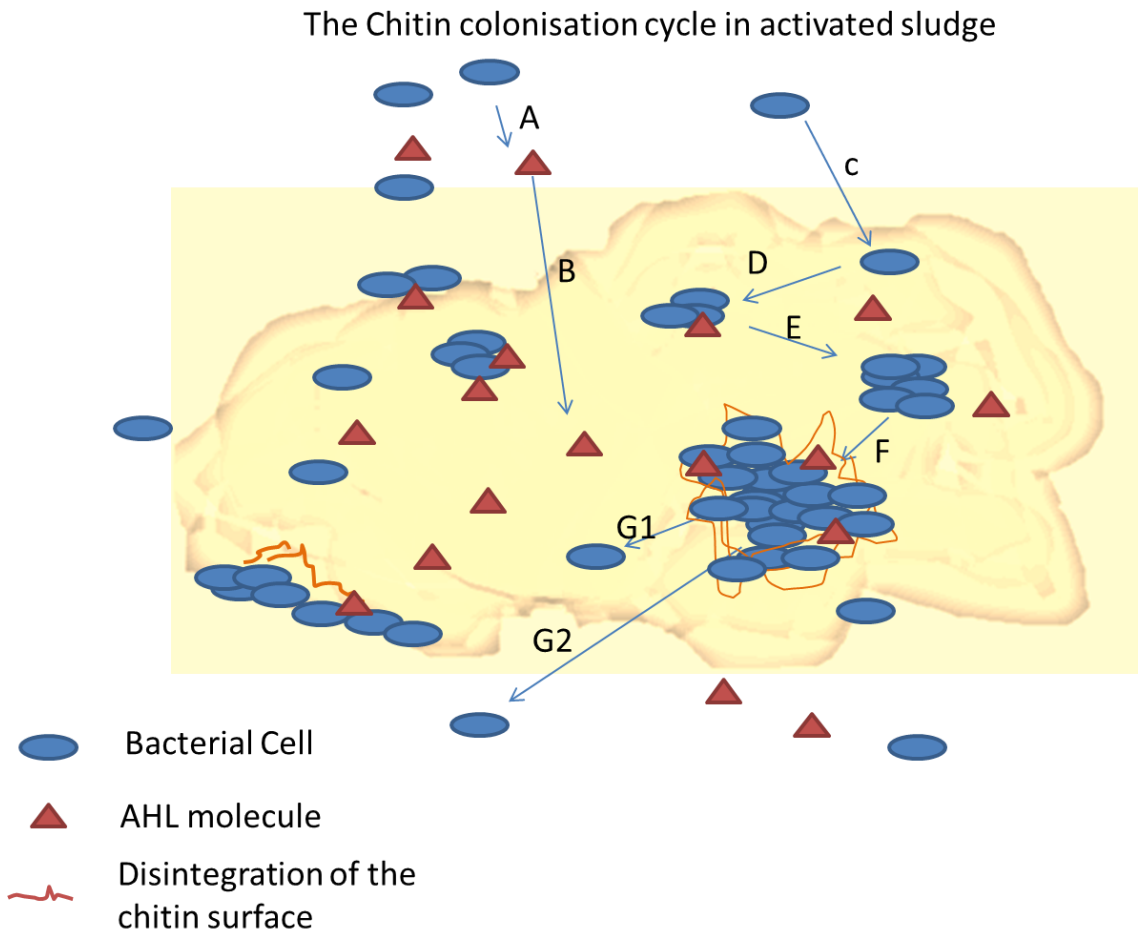


Figure 4.1: The chitin colonisation/utilisation cycle by bacteria in activated sludge. Cells in the Bulk phase release AHLs into the bulk (A) which migrate toward and bind chitin (B). Cells in the bulk phase attach to chitin (C) and form aggregates (D). These aggregates become microcolonies (E). The microcolonies continue to grow and chitinolytic activity begins to affect the integrity of the chitin surface (F). Cells in the microcolonies detach, either to recolonise another location on the chitin (G1) or into the bulk phase (G2). Different locations of the chitin will be undergoing different stages of the colonisation cycle, with cells attaching all throughout the surface, and on the peripheries.

References

1. Tchobanoglous, G., F.L. Burton, and H.D. Stensel, *Metcalf and Eddy Inc-Waste Water Enginneering Treatment and Reuse.* 2003. **Fourth Edition, Tata McGraw Hill Publishing Company Limited, New Delhi, India**

2. Renault, F., et al., *Chitosan for coagulation/flocculation processes – An eco-friendly approach.* European Polymer Journal, 2009. **45**(5): p. 1337-1348.
3. Jin, B., B.-M. Wilén, and P. Lant, *A comprehensive insight into floc characteristics and their impact on compressibility and settleability of activated sludge.* Chemical Engineering Journal, 2003. **95**(1–3): p. 221-234.
4. Soddell, J.A. and R.J. Seviour, *Microbiology of foaming in activated sludge plants.* Journal of Applied Microbiology, 1990. **69**(2): p. 145-176.
5. Seviour, R. and P. Nielsen H, *Microbial Ecology of Activated Sludge.* IWA publishing, 2010: p. 688.
6. Gómez-Suárez, C., et al., *Influence of extracellular polymeric substances on deposition and redeposition of Pseudomonas aeruginosa to surfaces.* Microbiology, 2002. **148**(4): p. 1161-1169.
7. Comte, S., G. Guibaud, and M. Baudu, *Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties: Part I. Comparison of the efficiency of eight EPS extraction methods.* Enzyme and Microbial Technology, 2006. **38**(1–2): p. 237-245.
8. McSwain, B.S., et al., *Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge.* Applied and Environmental Microbiology, 2005. **71**(2): p. 1051-1057.
9. Hu, M., et al., *Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis.* Bioresource Technology, 2012. **117**(0): p. 72-79.
10. Stechemesser, H. and B. Dobias,

Coagulation and flocculation. Surfactant science series

Taylor & Francis, Boca Raton

2005.

11. Huang, C., S. Chen, and J. Ruhsing Pan, *Optimal condition for modification of chitosan: a biopolymer for coagulation of colloidal particles.* Water Research, 2000. **34**(3): p. 1057-1062.
12. Driscoll, C.T. and R.D. Letterman, *Factors regulating residual aluminium concentrations in treated waters.* Environmetrics, 1995. **6**(3): p. 287-305.
13. McLachlan, D.R.C., *Aluminium and the risk for alzheimer's disease.* Environmetrics, 1995. **6**(3): p. 233-275.
14. Costerton, J.W. and Z. Lewandowski, *Microbial Biofilms.* Annu. Rev. Microbiol, 1995. **49**: p. 711-745.
15. Donlan, R.M. and J.W. Costerton, *Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms.* Clinical Microbiology Reviews, 2002. **15**(2): p. 167-193.
16. Webb, J.S., M. Givskov, and S. Kjelleberg, *Bacterial biofilms: prokaryotic adventures in multicellularity.* Current Opinion in Microbiology, 2003. **6**(6): p. 578-585.
17. Costerton, J.W., G.G. Geesey, and K.J. Chen, *How Bacteria Stick.* Sci. Am., 1978. **238**(1): p. 86-95.
18. Bassler, B.L., et al., *Chitin utilization by marine bacteria. Chemotaxis to chitin oligosaccharides by Vibrio furnissii.* Journal of Biological Chemistry, 1991. **266**(36): p. 24268-75.
19. Yu, C., B.L. Bassler, and S. Roseman, *Chemotaxis of the marine bacterium Vibrio furnissii to sugars. A potential mechanism for initiating the chitin catabolic cascade.* Journal of Biological Chemistry, 1993. **268**(13): p. 9405-9.
20. Kirchner, M., *Microbial colonization of copepod body surface and chitin degradation in the Sea.* Helgol Meeresunters, 1995. **49**: p. 201-212.

21. McLaughlin, B., et al., *Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis.* Journal of Applied Microbiology, 1998. **84**(5): p. 827-838.
22. Bridier, A., et al., *Resistance of bacterial biofilms to disinfectants: a review.* Biofouling, 2011. **27**(9): p. 1017-1032.
23. HS, W., et al., *Comparative susceptibility of planktonic and 3-day-old Salmonella Typhimurium biofilms to disinfectants.* J Appl Bacteriol, 2010. **108**: p. 2222-2228.
24. JE, N., et al., *Reduced biocide susceptibility in Candida albicans biofilms.* Antimicrob Agents Chemother, 2008. **52**: p. 3411-3413.
25. LA, P. and K. R, *Genetic analysis of E. coli biofilm formation: roles of flagella, motility, chemotaxis and type I Pili.* Molecular Micrbiology, 1998. **30**: p. 285-293.
26. GA, O.T. and K. R, *The initiation of biofilm formationin Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis.* Molecular Micrbiology, 1998. **28**: p. 449-461.
27. Palmer, J., S. Flint, and J. Brooks, *Bacterial cell attachment, the beginning of a biofilm.* Journal of Industrial Microbiology & Biotechnology, 2007. **34**(9): p. 577-588.
28. P, G., et al., *Surface characteristics and adhesion of E. coli and Staphylococcus epidermidis.* J Appl Bacteriol, 1991. **71**: p. 72-77.
29. MCM, V.L., et al., *Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion.* Applied & Environmental Microbiology, 1987. **53**: p. 1898-1901.
30. CG, K. and A. SK, *Significance of microbial biofilms in food industry: a review.* Int J Foof Microbiol, 1998. **42**: p. 9-27.
31. Kirisits, M.J. and M.R. Parsek, *Does Pseudomonas aeruginosa use intercellular signalling to build biofilm communities?* Cell Microbiol, 2006. **8**(12): p. 1841-9.
32. Lawrence, J.R., et al., *Optical sectioning of microbial biofilms.* J Bacteriol, 1991. **173**(20): p. 6558-67.
33. Klausen, M., et al., *Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants.* Mol Microbiol, 2003. **48**(6): p. 1511-24.
34. Klausen, M., et al., *Involvement of bacterial migration in the development of complex multicellular structures in Pseudomonas aeruginosa biofilms.* Mol Microbiol, 2003. **50**(1): p. 61-8.
35. Lequette, Y. and E.P. Greenberg, *Timing and Localization of Rhamnolipid Synthesis Gene Expression in Pseudomonas aeruginosa Biofilms.* Journal of Bacteriology, 2005. **187**(1): p. 37-44.
36. Branda, S.S., et al., *Biofilms: the matrix revisited.* Trends in Microbiology, 2005. **13**(1): p. 20-26.
37. Sauer, K., et al., *Pseudomonas aeruginosa Displays Multiple Phenotypes during Development as a Biofilm.* Journal of Bacteriology, 2002. **184**(4): p. 1140-1154.
38. Monds, R.D. and G.A. O'Toole, *The developmental model of microbial biofilms: ten years of a paradigm up for review.* Trends in Microbiology, 2009. **17**(2): p. 73-87.
39. Davies, D.G., et al., *The involvement of cell-to-cell signals in the development of a bacterial biofilm.* Science, 1998. **280**(5361): p. 295-8.
40. Mai-Prochnow, A., et al., *Biofilm development and cell death in the marine bacterium Pseudoalteromonas tunicata.* Applied & Environmental Microbiology, 2004. **70**(6): p. 3232-8.
41. Webb, J.S., et al., *Cell Death in Pseudomonas aeruginosa Biofilm Development.* Journal of Bacteriology, 2003. **185**(15): p. 4585-4592.
42. Stoodley, P., et al., *Biofilms as complex differentiated communities.* Annu Rev Microbiol, 2002. **56**: p. 187-209.
43. MR, P. and G. EP, *Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: a signaling mechanism involved in associations with higher organisms.* Proc Natl Acad Sci, 2000. **97**: p. 8789-8793.
44. Fuqua, W.C., S.C. Winans, and E.P. Greenberg, *Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators.* Bacteriol. 1994, 1994. **176**(2): p. 269-275.
45. Williams, P., et al., *Quorum sensing and the population-dependent control of virulence.* Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 2000. **355**(1397): p. 667-680.
46. Miller, M.B. and B.L. Bassler, *Quorum Sensing in Bacteria.* Annu Rev Microbiol, 2001. **55**: p. 165-199.

47. Federle, M.J. and B.L. Bassler, *Interspecies communication in bacteria.* The Journal of Clinical Investigation, 2003. **112**(9): p. 1291-1299.
48. Fuqua, W.C., S.C. Winans, and E.P. Greenberg, *CENSUS AND CONSENSUS INBACTERIAL ECOSYSTEMS: The*

LuxR-LuxI Family of Quorum-Sensing transcriptional Regulators. Annu Rev Microbiol, 1996. **50**: p. 727-751.
49. Salmond, G.P., et al., *The bacterial 'enigma': cracking the code of cell-cell communication.* Molecular Microbiology, 1995. **16**(4): p. 615-24.
50. Marketon, M.M., et al., *Characterization of the Sinorhizobium meliloti sinR/sinI Locus and the Production of Novel N-Acyl Homoserine Lactones.* Journal of Bacteriology, 2002. **184**(20): p. 5686-5695.
51. Kumari, A., P. Pasini, and S. Daunert, *Detection of bacterial quorum sensing <i>N</i>-acyl homoserine lactones in clinical samples.* Analytical and Bioanalytical Chemistry, 2008. **391**(5): p. 1619-1627.
52. Nealson, K.H., T. Platt, and J.W. Hastings, *Cellular control of the synthesis and activity of the bacterial luminescent system.* Journal of Bacteriology, 1970. **104**(1): p. 313-22.
53. Eberhard, A., *Inhibition and activation of bacterial luciferase synthesis.* Journal of Bacteriology, 1972. **109**(3): p. 1101-5.
54. Eberhard, A., et al., *Structural identification of autoinducer of Photobacterium fischeri luciferase.* Biochemistry, 1981. **20**(9): p. 2444-9.
55. Nealson, K.H., *Autoinduction of bacterial luciferase.* Archives of Microbiology, 1977. **112**(1): p. 73-79.
56. Chernin, L.S., et al., *Chitinolytic Activity in Chromobacterium violaceum: Substrate Analysis and Regulation by Quorum Sensing.* Journal of Bacteriology, 1998. **180**(17): p. 4435-4441.
57. Christensen, A.B., et al., *Quorum-sensing-directed protein expression in Serratia proteamaculans B5a.* Microbiology, 2003. **149**(2): p. 471-483.
58. Swift, S., et al., *Quorum Sensing-Dependent Regulation and Blockade of Exoprotease Production in Aeromonas hydrophila.* Infection and Immunity, 1999. **67**(10): p. 5192-5199.
59. Xavier, K.B. and B.L. Bassler, *LuxS quorum sensing: more than just a numbers game.* Current Opinion in Microbiology, 2003. **6**(2): p. 191-197.
60. Jones, S., et al., *The lux autoinducer regulates the production of exoenzyme virulence determinants in Erwinia carotovora and Pseudomonas aeruginosa.* EMBO Journal, 1993. **12**(6): p. 2477-82.
61. Valle, A., et al., *N-acyl-l-homoserine lactones (AHLs) affect microbial community composition and function in activated sludge.* Environmental Microbiology, 2004. **6**(4): p. 424-433.
62. Morgan-Sagastume, F., et al., *Production of acylated homoserine lactones by Aeromonas and Pseudomonas strains isolated from municipal activated sludge.* Can J Microbiol, 2005. **51**(11): p. 924-33.
63. Chong, G., et al., *The presence and role of bacterial quorum sensing in activated sludge.* Microbial Biotechnology, 2012. **5**(5): p. 621-633.
64. Margolis, J.J., et al., *Contributions of Francisella tularensis subsp. novicida chitinases and Sec secretion system to biofilm formation on chitin.* Applied & Environmental Microbiology, 2010. **76**(2): p. 596-608.
65. Meibom, K.L., et al., *The Vibrio cholerae chitin utilization program.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(8): p. 2524-2529.
66. Tharanathan, R.N. and F.S. Kittur, *Chitin--the undisputed biomolecule of great potential.* Crit Rev Food Sci Nutr, 2003. **43**(1): p. 61-87.
67. McCreath, K.J. and G.W. Gooday, *A rapid and sensitive microassay for determination of chitinolytic activity.* Journal of Microbiological Methods, 1992. **14**(4): p. 229-237.
68. Dweltz, N.E., *The structure of chitin.* Biochimica et Biophysica Acta, 1960. **44**(0): p. 416-435.
69. Minke, R. and J. Blackwell, *The structure of α-chitin.* Journal of Molecular Biology, 1978. **120**(2): p. 167-181.
70. RUDALL, K.M. and W. and KENCHINGTON, *THE CHITIN SYSTEM.* Biological Reviews, 1973. **48**: p. 597-633.

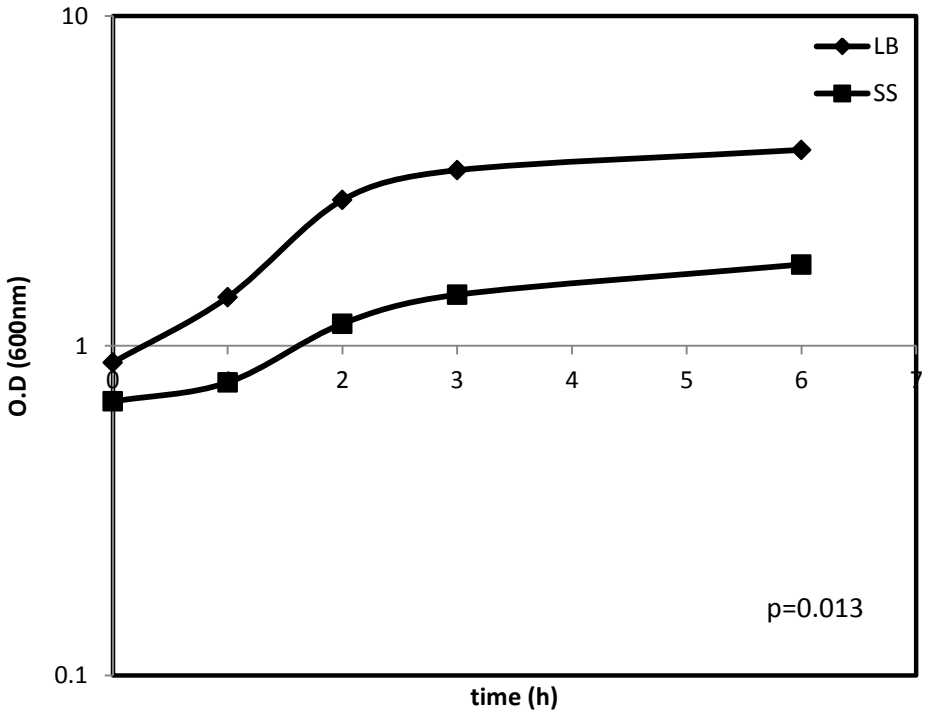
71. Rinaudo, M., *Chitin and chitosan: Properties and applications.* Progress in Polymer Science, 2006. **31**(7): p. 603-632.
72. Pillai, C.K.S., W. Paul, and C.P. Sharma, *Chitin and chitosan polymers: Chemistry, solubility and fiber formation.* Progress in Polymer Science, 2009. **34**(7): p. 641-678.
73. Matsumiya, M., et al., *Substrate specificity of chitinases from two species of fish, greenling, Hexagrammos otakii, and common mackerel, Scomber japonicus, and the insect, tobacco hornworm, Manduca sexta.* Biosci Biotechnol Biochem, 2006. **70**(4): p. 971-9.
74. Herth, W., *The site of β-chitin fibril formation in centric diatoms. II. The chitin-forming cytoplasmic structures.* Journal of Ultrastructure Research, 1979. **68**(1): p. 16-27.
75. Oh, H. and K. Nam, *Invited paper: Application of chitin and Chitosan toward electrochemical hybrid device.* Electronic Materials Letters, 2011. **7**(1): p. 13-16.
76. Yu, C., et al., *Chitin utilization by marine bacteria. A physiological function for bacterial adhesion to immobilized carbohydrates.* Journal of Biological Chemistry, 1991. **266**(36): p. 24260-7.
77. Keyhani, N.O. and S. Roseman, *Physiological aspects of chitin catabolism in marine bacteria.* Biochimica et Biophysica Acta (BBA) - General Subjects, 1999. **1473**(1): p. 108-122.
78. Li, X. and S. Roseman, *The chitinolytic cascade in Vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(2): p. 627-631.
79. Strand, S.P., K.M. Vårum, and K. Østgaard, *Interactions between chitosans and bacterial suspensions: adsorption and flocculation.* Colloids and Surfaces B: Biointerfaces, 2003. **27**(1): p. 71-81.
80. Strand, S.P., T. Nordengen, and K. Østgaard, *Efficiency of chitosans applied for flocculation of different bacteria.* Water Research, 2002. **36**(19): p. 4745-4752.
81. Strand, S.P., et al., *Electrophoretic Light Scattering Studies of Chitosans with Different Degrees of N-acetylation.* Biomacromolecules, 2001. **2**(4): p. 1310-1314.
82. Bassler, B.L., et al., *Chitin utilization by marine bacteria. Degradation and catabolism of chitin oligosaccharides by Vibrio furnissii.* Journal of Biological Chemistry, 1991. **266**(36): p. 24276-86.
83. Keyhani, N.O., X.-B. Li, and S. Roseman, *Chitin Catabolism in the Marine Bacterium Vibrio furnissii : IDENTIFICATION AND MOLECULAR CLONING OF A CHITOPORIN.* Journal of Biological Chemistry, 2000. **275**(42): p. 33068-33076.
84. Keyhani, N.O. and S. Roseman, *The Chitin Catabolic Cascade in the Marine Bacterium Vibrio furnissii: MOLECULAR CLONING, ISOLATION, AND CHARACTERIZATION OF A PERIPLASMIC CHITODEXTRINASE.* Journal of Biological Chemistry, 1996. **271**(52): p. 33414-33424.
85. Bouma, C.L. and S. Roseman, *Sugar Transport by the Marine Chitinolytic Bacterium Vibrio furnissii: MOLECULAR CLONING AND ANALYSIS OF THE GLUCOSE AND N-ACETYLGLUCOSAMINE PERMEASES.* Journal of Biological Chemistry, 1996. **271**(52): p. 33457-33467.
86. Keyhani, N.O., et al., *The Chitin Catabolic Cascade in the Marine Bacterium Vibrio furnissii: CHARACTERIZATION OF AN N,N'-DIACETYL-CHITOBIOSE TRANSPORT SYSTEM.* Journal of Biological Chemistry, 1996. **271**(52): p. 33409-33413.
87. Park, J.K., N.O. Keyhani, and S. Roseman, *Chitin Catabolism in the Marine Bacterium Vibrio furnissii : IDENTIFICATION, MOLECULAR CLONING, AND CHARACTERIZATION OF AN,N'-DIACETYLCHITOBIOSE PHOSPHORYLASE.* Journal of Biological Chemistry, 2000. **275**(42): p. 33077-33083.
88. Yusof, N.L.B.M., et al., *Flexible chitin films as potential wound-dressing materials: Wound model studies.* Journal of Biomedical Materials Research Part A, 2003. **66A**(2): p. 224-232.
89. Muzzarelli, R., et al., *Em Encyclopedia of Polymers Science Engineering.* Wiley, New York, 1985. **3**: p. 340-440.
90. Demarger-Andre, S. and A. Domard, *Chitosan behaviours in a dispersion of undecylenic acid.* Carbohydrate polymers, 1993. **22**(2): p. 117-126.
91. Domard, A. and A. Cartier, *Preparation, separation and characterization of the D-glucosamine oligomer series.* Chitin and Chitosan, 1989: p. 383-387.
92. Roussy, J., et al., *Influence of chitosan characteristics on the coagulation and the flocculation of bentonite suspensions.* Water Research, 2005. **39**(14): p. 3247-3258.

93. Rojas-Reyna, R., et al., *Flocculation efficiency of modified water soluble chitosan versus commonly used commercial polyelectrolytes.* Carbohydrate Polymers, 2010. **81**(2): p. 317-322.
94. Bratby, J., *Coagulation and flocculation in water and wastewater treatment*. 2006: International Water Assn.
95. Huang, C. and Y. Chen, *Coagulation of colloidal particles in water by chitosan.* Journal of Chemical Technology & Biotechnology, 1996. **66**(3): p. 227-232.
96. Sorlier, P., et al., *Relation between the Degree of Acetylation and the Electrostatic Properties of Chitin and Chitosan.* Biomacromolecules, 2001. **2**(3): p. 765-772.
97. Rhazi, M., et al., *Influence of the nature of the metal ions on the complexation with chitosan.: Application to the treatment of liquid waste.* European Polymer Journal, 2002. **38**(8): p. 1523-1530.
98. Zhou, D., et al., *Cellulose/chitin beads for adsorption of heavy metals in aqueous solution.* Water Research, 2004. **38**(11): p. 2643-2650.
99. Becker, T., M. Schlaak, and H. Strasdeit, *Adsorption of nickel(II), zinc(II) and cadmium(II) by new chitosan derivatives.* Reactive and Functional Polymers, 2000. **44**(3): p. 289-298.
100. Houlden, A., et al., *Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops.* FEMS Microbiology Ecology, 2008. **65**(2): p. 193-201.
101. Nakatsu, C.H., V. Torsvik, and L. Øvreås, *Soil Community Analysis Using DGGE of 16S rDNA Polymerase Chain Reaction Products Indiana Agric. Exp. Stn. Purdue Univ. #16101.* Soil Sci. Soc. Am. J., 2000. **64**(4): p. 1382-1388.
102. Engelbrektson, A., et al., *Experimental factors affecting PCR-based estimates of microbial species richness and evenness.* ISME J, 2010. **4**(5): p. 642-647.
103. Schloss, P.D., et al., *Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities.* Applied and Environmental Microbiology, 2009. **75**(23): p. 7537-7541.
104. Tamplin, M.L., et al., *Attachment of Vibrio cholerae serogroup O1 to zooplankton and phytoplankton of Bangladesh waters.* Applied and Environmental Microbiology, 1990. **56**(6): p. 1977-1980.
105. Hazrin-Chong, N.H. and M. Manefield, *An alternative SEM drying method using hexamethyldisilazane (HMDS) for microbial cell attachment studies on sub-bituminous coal.* Journal of Microbiological Methods, 2012. **90**(2): p. 96-99.
106. Baty, A.M., et al., *Spatial and Temporal Variations in Chitinolytic Gene Expression and Bacterial Biomass Production during Chitin Degradation.* Applied and Environmental Microbiology, 2000. **66**(8): p. 3574-3585.
107. Reguera, G. and R. Kolter, *Virulence and the Environment: a Novel Role for Vibrio cholerae Toxin-Coregulated Pili in Biofilm Formation on Chitin.* Journal of Bacteriology, 2005. **187**(10): p. 3551-3555.
108. Shiratori, H., et al., *Filimonas lacunae gen. nov., sp. nov., a member of the phylum Bacteroidetes isolated from fresh water.* International Journal of Systematic and Evolutionary Microbiology, 2009. **59**(5): p. 1137-1142.
109. Lim, J.H., S.-H. Baek, and S.-T. Lee, *Ferruginibacter alkalilentus gen. nov., sp. nov. and Ferruginibacter lapsinanis sp. nov., novel members of the family 'Chitinophagaceae' in the phylum Bacteroidetes, isolated from freshwater sediment.* International Journal of Systematic and Evolutionary Microbiology, 2009. **59**(10): p. 2394-2399.
110. Jin, D., et al., *Terrimonas pekingensis sp. nov., isolated from bulking sludge, and emended description of the genus Terrimonas and Terrimonas ferruginea, T. lutea and T. aquatica.* International Journal of Systematic and Evolutionary Microbiology, 2012.
111. Sheu, S.-Y., et al., *Terrimonas aquatica sp. nov., isolated from a freshwater spring.* International Journal of Systematic and Evolutionary Microbiology, 2010. **60**(12): p. 2705-2709.
112. Xie, C.-H. and A. Yokota, *Reclassification of [Flavobacterium] ferrugineum as Terrimonas ferruginea gen. nov., comb. nov., and description of Terrimonas lutea sp. nov., isolated from soil.* International Journal of Systematic and Evolutionary Microbiology, 2006. **56**(5): p. 1117-1121.
113. Wang, X., et al., *Pyrosequencing Analysis of Bacterial Diversity in 14 Wastewater Treatment Systems in China.* Applied and Environmental Microbiology, 2012. **78**(19): p. 7042-7047.

114. Chang, S.-C., et al., *Chitinilyticum litopenaei sp. nov., isolated from a freshwater shrimp pond, and emended description of the genus Chitinilyticum.* International Journal of Systematic and Evolutionary Microbiology, 2009. **59**(11): p. 2651-2655.
115. Yeon, K.-M., et al., *Quorum Sensing: A New Biofouling Control Paradigm in a Membrane Bioreactor for Advanced Wastewater Treatment.* Environmental Science & Technology, 2008. **43**(2): p. 380-385.
116. Yong, Y.-C. and J.-J. Zhong, *A genetically engineered whole-cell pigment-based bacterial biosensing system for quantification of< i> N</i>-butyryl homoserine lactone quorum sensing signal.* Biosensors and Bioelectronics, 2009. **25**(1): p. 41-47.
117. Ravn, L., et al., *Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics.* Journal of Microbiological Methods, 2001. **44**(3): p. 239-251.
118. Prado, A.G.S., et al., *Comparative adsorption studies of indigo carmine dye on chitin and chitosan.* Journal of Colloid and Interface Science, 2004. **277**(1): p. 43-47.
119. Donderski, W. and M. Trzebiatowska, *Chitinase activity production by planktonic, benthic and epiphytic bacteria inhabiting the Moty Bay of the Jeziorak Lake (Poland).* Polish Journal of Environmental Studies, 1999. **8**: p. 215-220.
120. Jagmann, N., H.-P. Brachvogel, and B. Philipp, *Parasitic growth of Pseudomonas aeruginosa in co-culture with the chitinolytic bacterium Aeromonas hydrophila.* Environmental Microbiology, 2010. **12**(6): p. 1787-1802.
121. Svitil, A.L., et al., *Chitin Degradation Proteins Produced by the Marine Bacterium Vibrio harveyi Growing on Different Forms of Chitin.* Applied and environmental microbiology, 1997. **63**(2): p. 408-413.
122. Du, C., et al., *Bacterial diversity in activated sludge from a consecutively aerated submerged membrane bioreactor treating domestic wastewater.* Journal of Environmental Sciences, 2008. **20**(10): p. 1210-1217.
123. Kragelund, C., et al., *Identity, abundance and ecophysiology of filamentous Chloroflexi species present in activated sludge treatment plants.* FEMS Microbiology Ecology, 2007. **59**(3): p. 671-682.
124. Chung, E.J., et al., *Chitinophaga oryziterrae sp. nov., isolated from the rhizosphere soil of rice (Oryza sativa L.).* International Journal of Systematic and Evolutionary Microbiology, 2012.
125. Ngah, W.S.W. and A. Musa, *Adsorption of humic acid onto chitin and chitosan.* Journal of Applied Polymer Science, 1998. **69**(12): p. 2305-2310.
126. Dolphen, R. and P. Thiravetyan, *Adsorption of melanoidins by chitin nanofibers.* Chemical Engineering Journal, 2011. **166**(3): p. 890-895.
127. Gulnaz, O., et al., *Sorption of basic dyes from aqueous solution by activated sludge.* Journal of Hazardous Materials, 2004. **108**(3): p. 183-188.
128. Tsezos, M., *The role of chitin in uranium adsorption by R. arrhizus.* Biotechnology and Bioengineering, 1983. **25**(8): p. 2025-2040.
129. Allesen-Holm, M., et al., *A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms.* Molecular Microbiology, 2006. **59**(4): p. 1114-1128.
130. Das, T. and M. Manefield, *Pyocyanin Promotes Extracellular DNA Release in <italic>Pseudomonas aeruginosa</italic>.* PLoS ONE, 2012. **7**(10): p. e46718.
131. Chan, K.-G., et al., *Characterization of N-acylhomoserine lactone-degrading bacteria associated with the Zingiber officinale (ginger) rhizosphere: Co-existence of quorum quenching and quorum sensing in Acinetobacter and Burkholderia.* BMC Microbiology, 2011. **11**(1): p. 51.

Appendices

Appendix 1:



Appendix 1: Growth curve of *Aeromonas* (pBB-luxR) in LB broth and Sludge Supernatant (SS). After growing the monitor strain in LB overnight, cells were then diluted in LB (1:5) and incubated for half an hour. Then, cells were either incubated in LB or spun down and resuspended in SS. The growth of

the monitor strain in the two media was monitored for a period of 6 hours. A student t-test was performed and the p value represented indicates significant difference between the two growth curves.