Quorum sensing signalling and activated sludge microbial communities

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NANYANG
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**QUORUM SENSING SIGNALLING AND
ACTIVATED SLUDGE MICROBIAL
COMMUNITIES**

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**SCHOOL OF CIVIL AND ENVIRONMENTAL
ENGINEERING NANYANG TECHNOLOGICAL
UNIVERSITY**

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Quorum Sensing Signalling and Activated Sludge Microbial Communities

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ABSTRACT

Quorum sensing (QS) signalling has been extensively studied in the context of single-species populations. However, defining the relevance of QS in complex, multi-species microbial communities remains one of the greatest challenges for understanding the ecological role of QS in nature. Here, a long-term bioreactor ecology study was performed to address the links between QS, metabolic function, organization and composition of complex microbial consortia of the activated sludge system. The metabolic function of the community and the conversion of floccular biomass to highly structured granules were found to be non-random, but highly associated with *N*-acyl-homoserine-lactone mediated QS and with the engagement of multiple, phylogenetically distinct, community species. Importantly, these QS regulated behaviours were also found to be co-modulated by the biological degradation of signals, called quorum quenching. The discovery of the potential roles of QS in mixed microbial community function in this study provides an alternative to improve the operation of wastewater treatment communities.

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LIST OF SYMBOLS

°C	Degrees Celsius
AHL	Acyl Homoserine Lactone
AHS	Acyl Homoserine
C4-HSL	<i>N</i> -butyryl-DL-homoserine lactone
3OC6-HSL	<i>N</i> -(3-oxohexanoyl)-DL-homoserine lactone
C6-HSL	<i>N</i> -hexanoyl-DL-homoserine lactone
C7-HSL	<i>N</i> -heptanoyl-DL-homoserine lactone
3OC8-HSL	<i>N</i> -(3-oxooctanoyl)-L-homoserine lactone
C8-HSL	<i>N</i> -octanoyl-DL-homoserine lactone
3OC10-HSL	<i>N</i> -(3-oxodecanoyl)-L-homoserine lactone
C10-HSL	<i>N</i> -decanoyl-DL-homoserine lactone
3OC12-HSL	<i>N</i> -(3-oxododecanoyl)-L-homoserine lactone
C12-HSL	<i>N</i> -dodecanoyl-DL-homoserine lactone
3OHC12-HSL	<i>N</i> -(3-hydroxydodecanoyl)-DL-homoserine lactone
3OC14-HSL	<i>N</i> -(3-oxotetradecanoyl)-L-homoserine lactone
C14-HSL	<i>N</i> -tetradecanoyl-DL-homoserine lactone
AI-2	Autoinducer-2
Amp^R	Ampicilin Resistance
AOB	Ammonium Oxidizing Bacteria
APHA	American Public Health Association
BCA	Bicinchoninic Acid
BNR	Biological Nutrient Removal
CLSM	Confocal Laser Scanning Microscopy
COD	Chemical Oxygen Demand
d	Day
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DO	Dissolved Oxygen
EBPR	Enhanced Biological Phosphorus Removal
eDNA	Extracellular Deoxynucleic Acids
EPS	Extracellular Polymeric Substances
ESI	Electrospray Ionization
GAO	Glycogen Accumulating Organism
GFP	Green Fluorescent Protein
Gm^R	Gentamycin Resistance

LIST OF SYMBOLS

h	Hour
HPLC-MS/MS	High Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
HRT	Hydrolic Retention Time
Kan^R	Kanamycin Resistance
LOD	Limit of Detection
LOQ	Limit of Quantification
L	Liter
μL	Microliter
m/z	Mass to Charge Ratio
μm	Micrometer
min	Minute
MLSS	Mixed Liquor Suspended Solid
MLVSS	Mixed Liquor Volatile Suspended Solid
mM	Milimolar
nM	Nanomolar
nt	Nucleotide
μM	Micromolar
MRM	Multiple Reaction Monitoring
NOB	Nitrite Oxidizing Bacteria
OD	Optical Density
OTU	Operational Taxonomic Unit
PAO	Polyphosphate Accumulating Organism
PLC	Programmable Logic Controller
PS/PN	Polysaccharide/Protein Ratio
QQ	Quorum Quenching
QS	Quorum Sensing
rpm	Revolution Per Minute
rRNA	Ribosomal Ribonucleic Acids
s	Second
SBR	Sequencing Batch Reactor
SEM	Standard Error of the Mean
SND	Simultaneous Nitrification and Denitrification
SNDPR	Simultaneous Nitrification, Denitrification and Phosphorus Removal
Sp^R	Spectinomycin Resistance
SWW	Synthetic Wastewater

LIST OF SYMBOLS

SVI	Sludge Volumetric Index
Tc^R	Tetracyclin Resistance
TLC	Thin-layer Chromatography
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight/Volume
X-gal	5-bromo-4-chloro-3-indolyl b-D-galactopyranoside

LIST OF PUBLICATIONS

Publications

1. **Tan, C. H.**, Koh, K. S., Xie, C., Tay, M., Zhou, Y., Williams, R., Ng, W. J., Rice, S. A. and Kjelleberg, S. (2014), “The role of quorum sensing signaling in EPS production and the assembly of a sludge community into aerobic granules”, *The ISME Journal*, doi: 10.1038/ismej.2013.240.
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 8. Kjelleberg, S., **Tan, C. H.** and Rice, S. A., “Signalling controlled structure function of activated sludge microbial communities”, Conference presentation at 14th International Society for Microbial Ecology 2012, Copenhagen, Denmark (Oral presentation).
 9. **Tan, C. H.**, Koh, K. S., Zhou, Y., Rice, S. A., Kjelleberg, S., Ng, W. J., Zheng, P. and Zhan Z., “UHPLC-MS/MS method for quantitative analysis of N-acylhomoserine lactones from sludge samples”, Conference presentation at Separation Science 2011, Singapore (Poster presentation).
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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Quorum sensing (QS) signalling is defined as the ability of bacteria to coordinate social behaviour, by responding to small diffusible chemical molecules produced and released into the immediate environment by members of its own species, in a signal concentration dependent manner (Lazdunski *et al.*, 2004; Williams *et al.*, 2007). The signal concentration is related to the population cell density and the QS response is triggered only when a threshold signal concentration is reached, thus bacteria involved in QS signalling can effectively monitor their population size via the signalling molecules to ensure the appropriate regulation of gene expression (Williams *et al.*, 2007). Depending on the species of interest and the QS systems involved, density regulated genes vary from those encoding exoproducts, e.g. virulence factors, antibiotics and extracellular polysaccharides, to those that control motility, aggregation, biofilm formation and other processes (Williams *et al.*, 2007; West *et al.*, 2012). The best studied QS system utilizes *N*-acyl-homoserine-lactones (AHLs) as the signal compound. Over 100 species of Proteobacteria, Bacteroidetes and Cyanobacteria have been found to use AHLs for communication (Sharif *et al.*, 2008; Romero *et al.*, 2010; Ahlgren *et al.*, 2011). More recently, a species of methanogens, *Methanosaeta harundinacea* 6Ac, from the Archaea was also documented to employ the same class of signalling molecules for coordinating cell assembly and metabolic carbon flux (Zhang *et al.*, 2012), suggesting that AHL mediated QS signalling is widespread throughout the microbial domains of life.

A substantial understanding of QS signalling biology has been gained since the first proposed QS model in 1970s (Nealson *et al.*, 1970; Nealson, 1977). However, most of the research was based on studies using single-species populations that were either grown planktonically in well-defined laboratory conditions or in biofilm systems with continuous flowing liquid medium. Such conditions are in stark contrast to natural environments where the microbes were first isolated and this experimental approach leaves many uncertainties as to whether the population derived concept of QS signalling is relevant in the ecological context (Redfield, 2002; Dulla and Lindow, 2008). Unlike the laboratory model systems, microbes are rarely found as isolated

populations in nature. Instead, microbial communities containing hundreds to thousands of species are common and most, if not all, of these microbial communities exist in matrix encased biofilm structures (Kolter and Greenberg, 2006). Not only will the spatial distribution of QS microbes within the biofilm architecture impact on the signalling process, it has also been reported that many QS interfering organisms, such as signal degraders coexist with signal producers in complex communities and may thus influence QS signalling at the community level (d'Angelo-Picard *et al.*, 2005; Wang and Leadbetter, 2005). In addition, microbes in natural settings commonly experience dynamic environmental conditions, such as fluctuations in pH, temperature, nutrient concentration, oxygen level, humidity and hydrodynamics, all of which can potentially interfere with QS signalling in natural settings (Rice *et al.*, 2005; Boyer and Wisniewski-Dyé, 2009; Decho *et al.*, 2009). For example, high rates of diffusion and dilution of the AHL signal in the marine environment could prevent the signal from accumulating to a sufficient local concentration at which QS is induced (Redfield, 2002). Despite these challenges, it is now increasingly clear that QS occurs in and appears to be highly relevant for complex communities, whether they are biofilms on rocks in streams (McLean *et al.*, 1997), stromatolites (Decho *et al.*, 2009) or biofilms formed on hollow fibre membranes in bioreactors (Yeon *et al.*, 2009a). In some cases, QS signalling has also been proposed to mediate interspecies interactions in complex communities, further implying an important role for QS in most habitats (Riedel *et al.*, 2001; Dulla and Lindow, 2009; Elias and Banin, 2012). Given the recent advancement in technologies for sequencing and analytical techniques, we are now in a better position to further explore the complexity of QS signalling biology at the community level to address key questions, such as how QS may function to coordinate the community behaviour and the organisms involved, and how they manage to compete with non-QS or QS interfering organisms in a diverse range of natural and engineered ecosystems.

In this thesis, bioreactor systems inoculated with complex activated sludge communities were used as the experimental platform of study to determine the role of QS signalling at the community level. The activated sludge represents one of the most dynamic and species-rich ecosystems (Kjelleberg *et al.*, 2014). This complex ecosystem often accommodates thousands of microbial species, including bacteria,

eukaryotes and Archaea important for effective treatment of water and wastewater. In addition to the highly diverse nature of the sludge community composition, the use of a fully automated bioreactor has the advantage that the system can be operated to precisely control and monitor a number of parameters, including dissolved oxygen concentration, pH, temperature, nutrient level, as well as the formation of compact aggregates, called microbial granules. These metadata are essential to allow assessment of the simultaneous impact of a series of environmental factors on the production, accumulation, stability, maintenance and functional role of AHL signals in a single system, and hence provide a rare opportunity to further reveal the complexity of QS signalling biology in both natural and engineered environments.

1.2 OBJECTIVES AND SCOPES

Using a multi-pronged approach, involving meta-omics, analytical chemistry and microbiology, the potential roles of QS signalling in moderating the structure and function of the activated sludge community were investigated in this thesis. In particular, the relationship between AHL-based QS signalling and sludge community metabolism as well as microbial granulation were quantified. The specific objectives and scopes of the thesis chapters were:

Chapter 3: To determine the role of AHL mediated QS signalling in community metabolism in simultaneous nitrification, denitrification and phosphorus removal, via the nitrite shortcut at the floccular stage, and to identify the microbes engaged in both QS signalling and bioprocesses.

Chapter 4: To determine the role of AHL mediated QS signalling in the development of microbial granules and the mechanism involved, as well as to identify the microbes engaged in both granulation and QS signalling processes.

Chapter 5: To investigate how AHL mediated QS signalling can be modulated at the community level in relation to the coexistence of non-QS organisms, including the signal-degrading microbes or quorum quenchers.

1.3 CHAPTER SYNOPSES

Chapter 3 describes the dynamics of AHL mediated QS signalling in the floccular sludge community over an extended period of 574 days. The relationships between the long-term AHL expression, microbial species engaged and the sludge community energy metabolism in terms of nitrification and denitrification via the nitrite shortcut are presented. Parts of the results presented in this chapter have been submitted to *Nature Communications* (2013) for publication - “The interplay of quorum sensing and quenching in mixed microbial communities”.

Chapter 4 describes the developmental process of the highly structured microbial granules from the fluffy floccular sludge. Evidence for the potential role of AHL mediated QS signalling and extracellular polymeric substances as well as the involvement of specific microbial species associated with the QS signalling in the granulation process is presented. The results in this chapter have been published in *The ISME Journal* (2014) - “The role of quorum sensing signaling in EPS production and the assembly of a sludge community into aerobic granules”.

Chapter 5 describes the role of microbially mediated signal degradation activities or quorum quenching (QQ) in the coordination and regulation of AHL mediated QS signalling at the community level. Evidence of QQ activities in shaping of specific AHL signalling in complex communities is provided using both top-down and bottom-up research approaches. Parts of the results presented in this chapter have been submitted to *Nature Communications* (2013) for publication - “The interplay of quorum sensing and quenching in mixed microbial communities”.

Chapter 6 provides a general discussion on the implications and applications of current findings in this thesis, and suggests a number of possible directions for future research.

CHAPTER 2: LITERATURE REVIEW

Quorum sensing (QS) signalling has been such an intriguing area of research in part because it suggests that seemingly simple, unicellular bacteria are capable of multicellular behaviour. Such multicellular behaviour has been shown to be coordinated via the production and perception of small diffusible chemical molecules. To date, studies of QS regulated social behaviour and the associated QS mechanisms have been primarily conducted using single-species, planktonic population systems under defined laboratory conditions. While these population studies have yielded great insights into QS biology, they may not be generally representative of natural environments or the ecosystems in which the bacteria are naturally present. For example, bacteria predominantly exist as mixtures of phylogenetically diverse species called communities, where only a small fraction of the community might participate in QS. In addition, these microbial communities commonly exist as high density, matrix encased biofilm structures, rather than as freely suspended populations. It is currently unclear what effect such differences in microbial community composition, spatial organization as well as the prevailing environmental conditions may have on our understanding of the current, population based paradigm of QS regulation and whether that can accurately be applied to microbial communities.

A microbial population is defined as a group of cells belonging to a single species and the signalling event occurring within a single-species population is defined as intraspecies signalling (Diggle *et al.*, 2007). By contrast, a microbial community is composed of two or more species and can be comprised of hundreds to thousands of distinct species, including bacteria, eukaryotes and Archaea (Kolenbrander *et al.*, 2002). The latter is generally referred to as an ecosystem, where interspecies or interkingdom signalling, defined as the signalling across different species of organisms, is expected to occur (Diggle *et al.*, 2007; Williams, 2007). The present review summarizes the current understanding of the fundamental aspects of QS signalling at the population level and aims to demonstrate how this can be extended to investigate QS based regulation at the community level in natural and engineered ecosystems. The evidence for QS signalling in different ecosystems will be presented and the potential role for QS in those complex environments will be addressed. In particular, challenges

facing QS signalling in those habitats will be emphasized. Finally, the review will present the current state of the art for our understanding of laboratory bioreactor systems using activated sludge communities as a model ecosystem to address the relevance of QS signalling in an ecological context.

2.1 QUORUM SENSING SIGNALLING IN BACTERIA

2.1.1 The concept of QS signalling

QS signalling has been conceptualized as a function of signal production and perception to coordinate bacterial social behaviour in response to cell density (Straight and Kolter, 2009; West *et al.*, 2012). The hypothesis is that QS regulated behaviour, such as production of extracellular factors which are available to all population members, termed public goods, are most beneficial at high cell densities and that QS provides a mechanism to optimize the production of public goods at such cell densities (Darch *et al.*, 2012). Often, this is achieved via the positive feedback mechanism of QS, in which the uptake of signals from the extracellular environment results in an exponential increase in production of the signals by the population, a phenomenon known as autoinduction. This rapid amplification of signals at high cell densities ensures that the expression of multiple target genes is coordinated at the population level, and thus production of the associated public goods allows bacteria to rapidly respond and adapt to dynamic environmental conditions for survival (Lazdunski *et al.*, 2004).

2.1.2 QS signalling systems

Since the first discovery of the *Vibrio fischeri* LuxI/R QS system in 1977 (Nealson, 1977), QS research has progressed tremendously and a number of novel QS systems have been described in different bacterial species (Lazdunski *et al.*, 2004; Williams, 2007; Straight and Kolter, 2009). These QS systems can be generally categorized based on the signalling molecules employed, including *N*-acyl-homoserine-lactones (AHLs) (Pearson *et al.*, 1995), oligopeptides (Novick, 2003), autoinducer-2 (AI-2) (Pereira *et al.*, 2012), diffusible signalling factors (DSF; methyl dodecanoic acid) (Deng *et al.*, 2010), pseudomonas quinolone signal (PQS) (Pesci *et al.*, 1999), hydroxyl-palmitic acid methyl ester (PAME) (Flavier *et al.*, 1997), cyclic-di-peptides (Holden *et al.*, 1999), etc. While some of these QS systems are unique to particular taxonomic groups of

bacteria, such as PQS in *Pseudomonas aeruginosa* (Pesci *et al.*, 1999), many of the signalling systems appear to be conserved in a wide range of bacterial species, including oligopeptide based signalling system in Gram positive bacteria, AHL mediated QS in Gram negative bacteria and the AI-2 signalling system found in both Gram positive and Gram negative bacteria (Visick and Fuqua, 2005). In particular, the AI-2 has been described as a universal signal for interspecies communication as many different bacterial species have been found to carry the *luxS* gene coding for the AI-2 synthase (Winzer *et al.*, 2002). Despite the widespread nature of the AI-2 system, there remains a lack of consensus as to whether the AI-2 is a true signal in all bacteria. The AI-2 is a common metabolic by-product in the recycling process of *S*-adenosyl-L-methionine and is important in the detoxification of metabolic intermediates produced during methyl transferase reactions, and hence the AI-2 is not produced specifically for the signalling purpose (Diggle *et al.*, 2007). In addition, with the exception of *Vibrio* and the *Salmonella* spp., there are limited reports where a specific AI-2 receptor has been identified (Surette *et al.*, 1999). A dedicated receptor is one of the criteria that define a signalling pathway, where the receptor confers specificity in perception of the signal and is involved in the intracellular transduction of signal perception into a phenotypic response, via specific changes in gene expression (Keller and Surette, 2006).

In contrast to the AI-2 signal, both oligopeptide and AHL based QS signalling systems are recognized as intraspecies communication systems which facilitate cooperative behaviour within the population (Williams, 2007). The specificity of these intraspecies signalling systems is dependent on the chemical structures of the signalling molecules and the binding sites of the receptor proteins. In AHL mediated signalling systems, there are two central components – an ‘I’ protein, which enzymatically synthesizes the AHL signal, and an ‘R’ protein, which is the signal receptor and also the transcriptional regulator (Lazdunski *et al.*, 2004). In most cases, the I/R proteins are homologous to the LuxI/R proteins of *V. fischeri*, however, there are exceptions, such as the AHL synthases, LuxM in *Vibrio harveyi* (Bassler *et al.*, 1993), AinS in *V. fischeri* (Hanzelka *et al.*, 1999) and HdtS in *Pseudomonas fluorescens* F113 (Laue *et al.*, 2000). Unlike the AI-2 system which has only two different signal conformations generated by the LuxS (Bassler *et al.*, 1993), different AHL synthases and their homologs are capable of

producing a diverse array of structurally related signalling molecules. These AHL signals have conserved structures, composed of a homoserine lactone ring that is linked by an amide bond to an acyl chain (Figure 2-1) (Fuqua *et al.*, 2001). Naturally occurring AHLs exhibit variable acyl chain lengths, oxidation states and saturation levels. The acyl chain length varies from 4 to 18 carbons and often, the chain has even number of carbons, although AHLs with 5, 7 and 11 carbon acyl chains have also been identified (Morohoshi *et al.*, 2004; Chan *et al.*, 2011; Chong *et al.*, 2012). In addition, the carbon molecule at position 3 on the acyl chain can be substituted with a functional group composed of hydroxyl, carbonyl or hydrogen groups. The acyl chains may also be unsaturated at positions 5 or 7 for some of the longer acyl chains (Fuqua *et al.*, 2001). The large variety of potential AHLs, coupled with their specific binding to the LuxR receptors, may thus contribute to the high specificity of the signalling system necessary for intraspecies communication in bacteria of diverse origins (Diggle *et al.*, 2007).

Figure 2-1. The chemical structure of acyl homoserine lactone signal molecule. The R-group represents either the hydroxyl (OH), the carbonyl (O) or the hydrogen (H) functional group. The n can vary from 0 to 14, which corresponds to 4 to 18 carbons, respectively in a complete acyl chain. (From Fuqua *et al.*, 2001)

While the AHL mediated QS signalling may have been first discovered as an intraspecies communication system (Fuqua *et al.*, 2001), there is increasing evidence that such systems may also involve interspecies interactions in complex ecosystems (Dulla and Lindow, 2009; Hosni *et al.*, 2011). It has been reported that a single species may encode multiple pairs of functionally distinct *luxI/R* genes and each LuxI homolog protein may generate more than a single type of AHLs (De Kievit *et al.*, 2001; Case *et al.*, 2008). For example, *Yersinia pseudotuberculosis* contains two pairs of *luxI/R* homologs (Case *et al.*, 2008) and is capable of synthesizing at least 24 different AHLs (Ortori *et al.*, 2007). Although as many as 61 different AHL structural permutations are possible, more than 100 different Proteobacteria species have been reported to employ the AHL mediated signalling system (Ahlgren *et al.*, 2011) and the list of

microorganisms has recently expanded to include species from Bacteroidetes (Huang *et al.*, 2008; Romero *et al.*, 2010; Rashid *et al.*, 2011), Cyanobacteria (Romero *et al.*, 2008; Sharif *et al.*, 2008) and Archaea (Zhang *et al.*, 2012). It is apparent that the number of microbial species adopting the AHL based signalling system has surpassed the pool of AHL structures available in nature, suggesting considerable opportunity for cross talk between different species inhabiting in the same environmental niche. Indeed, several AHL signals, such as *N*-(3-oxohexanoyl)-L-homoserine lactone (3OC6-HSL) and *N*-(3-oxooctanoyl)-L-homoserine lactone (3OC8-HSL) have been commonly found to be employed by a wide range of bacterial species isolated from a single environment for QS signalling, further supporting the possibility of interspecies signalling (d'Angelo-Picard *et al.*, 2005; Dulla and Lindow, 2009; Hosni *et al.*, 2011; Chong *et al.*, 2012).

Despite the signal commonality, specificity of the signal receptor may also contribute to the possible engagement of AHLs in interspecies interactions. Although the LuxR receptors have been previously proposed to respond only to their cognate AHL(s) (Diggle *et al.*, 2007), some of the receptor homologs, such as TraR in *Agrobacterium tumefaciens* (Shaw *et al.*, 1997) and CviR in *Chromobacterium violaceum* (McClellan *et al.*, 1997) can bind to other non-cognate AHLs albeit at much lower affinities. Furthermore, the number of *luxR* genes in a given species is often unequal to the number of the *luxI* genes. Case *et al.* (2008) reported that 45 out of the 65 complete bacterial genomes had a high ratio of *luxR* to *luxI* genes. Given that each LuxR homolog responds to a particular AHL or a family of AHLs, the increased variety of LuxR homologs in a species expands their sensing capability. Similarly, incomplete AHL QS circuits (that is, with the *luxR* gene homologs only) have also been documented in some bacterial species, such as the SdiA receptor protein in *Escherichia coli* (Hughes *et al.*, 2010), *Salmonella typhimurium* (Dyszal *et al.*, 2010) and *Brucella melitensis* (Weeks *et al.*, 2010). A recent phylogenetic survey further revealed the presence of such partial QS circuits in 45 out of 512 complete bacterial genomes annotated, suggesting the widespread nature of the orphan *luxR* genes in diverse microbial taxa (Case *et al.*, 2008). These bacteria may therefore use the additional LuxR proteins to detect the neighbouring cells or to access the ecological conditions via the perception of the AHLs accumulated in the extracellular environment. *E. coli*, for

example, has been shown to respond to the endogenous AHLs produced by the microbiota in the cow rumen through the SdiA receptor, and thus may use the perception of the AHLs to sense the acidic environment leading to the timely expression of colonization factors that facilitate infection of the host (Hughes *et al.*, 2010). Based on the ability of the receptor to perceive multiple signals, the production of multiple receptors by a single organism, the expression of a receptor in the absence of an AHL synthase, as well as the limited number of AHL structures relative to the number of AHL active species, it is highly likely that AHLs participate in interspecies interactions, termed cross talk in complex ecosystems.

2.1.3 QS regulated population behaviours

In general, QS signalling has been proposed to be an important mechanism to increase the fitness of a bacterial population through the regulation of diverse phenotypic traits that collectively contribute to distinct population social behaviour (Williams *et al.*, 2007). These phenotypic traits are often associated with the production of a variety of extracellular factors, commonly referred to as public goods (Sandoz *et al.*, 2007), such as exoenzymes, virulence factors, antibiotics, siderophore, extracellular polymeric substances (EPS), etc. (Table 2-1), that can be shared by all individuals within a population and hence support the view of QS as a cooperative trait (Darch *et al.*, 2012). In some cases, QS has also been shown to modulate the expression of intracellular enzymes, which are not shared among the individuals (Toyofuku *et al.*, 2007; Dandekar *et al.*, 2012). Such intracellular enzymes are hence termed private goods that usually benefit only the QS responders or the QS cooperators (Darch *et al.*, 2012). As such, the QS regulated private good behaviour has been proposed as an essential mechanism to maintain the integrity of QS systems in nature (Dandekar *et al.*, 2012).

Table 2-1. Examples of AHL mediated QS regulated phenotypes.

Phenotype	Organism	Reference
Biofilm development		
Formation/Aggregation	<i>Acinetobacter baumannii</i> , <i>Aeromonas hydrophila</i> , <i>Burkholderia cepacia</i> , <i>Burkholderia pseudomallei</i> , <i>Pantoea stewartii</i> , <i>P. aeruginosa</i> , <i>Pseudomonas putida</i> , <i>Y. pseudotuberculosis</i> , <i>Serratia marcescens</i>	(Davies <i>et al.</i> , 1998; Atkinson <i>et al.</i> , 1999; Huber <i>et al.</i> , 2001; Lynch <i>et al.</i> , 2002; Coulthurst <i>et al.</i> , 2006; Dubern <i>et al.</i> , 2006; Koutsoudis <i>et al.</i> , 2006; Labbate <i>et al.</i> , 2007; Khajanchi <i>et al.</i> , 2009; Gamage <i>et al.</i> , 2011; Bhargava <i>et al.</i> , 2012)
Dispersal	<i>Serratia liquefaciens</i> *, <i>Rhodobacter sphaeroides</i> , <i>Pseudomonas aureofaciens</i>	(Puskas <i>et al.</i> , 1997; Rice <i>et al.</i> , 2005; Maddula <i>et al.</i> , 2006)
Motility		
Swimming	<i>Pseudomonas syringae</i> , <i>Yersinia enterocolitica</i>	(Quiñones <i>et al.</i> , 2005; Atkinson <i>et al.</i> , 2006; Kanda <i>et al.</i> , 2011)
Swarming	<i>B. cepacia</i> , <i>Serratia liquefaciens</i> *, <i>Y. enterocolitica</i>	(Huber <i>et al.</i> , 2001; Atkinson <i>et al.</i> , 2006; Coulthurst <i>et al.</i> , 2006)
Sliding	<i>S. marcescens</i>	(Horng <i>et al.</i> , 2002)
Exoenzyme production		
Proteases	<i>A. hydrophila</i> , <i>B. pseudomallei</i> , <i>P. aeruginosa</i> , <i>P. aureofaciens</i> , <i>S. liquefaciens</i> *	(Eberl <i>et al.</i> , 1996; Pearson <i>et al.</i> , 1997; Zhang and Pierson, 2001; Valade <i>et al.</i> , 2004; Khajanchi <i>et al.</i> , 2009)
Nucleases	<i>S. marcescens</i>	(Horng <i>et al.</i> , 2002)
Antibiotic production	<i>Burkholderia thailandensis</i> , <i>Erwinia carotovora</i> , <i>P. aureofaciens</i> , <i>Serratia plymuthica</i>	(Thomson <i>et al.</i> , 2000; Maddula <i>et al.</i> , 2006; Liu <i>et al.</i> , 2007; Duerkop <i>et al.</i> , 2009)
Biosurfactant production	<i>S. liquefaciens</i> *, <i>S. marcescens</i>	(Lindum <i>et al.</i> , 1998; Wei <i>et al.</i> , 2004)
Extracellular polymeric substances (EPS) production	<i>P. stewartii</i> , <i>P. aeruginosa</i> , <i>Pseudomonas savastoni</i> , <i>P. syringae</i> , <i>Sinorhizobium meliloti</i>	(von Bodman <i>et al.</i> , 1998; Pellock <i>et al.</i> , 2002; Quiñones <i>et al.</i> , 2005; Allesen-Holm <i>et al.</i> , 2006; Herrera <i>et al.</i> , 2008; Hosni <i>et al.</i> , 2011)

Virulence factor production	<i>A. hydrophila</i> , <i>Agrobacterium vitis</i> , <i>B. cepacia</i> , <i>B. pseudomallei</i> , <i>Burkholderia mallei</i> , <i>E. carotovora</i> , <i>P. aeruginosa</i> , <i>P. savastanoi</i> , <i>P. syringae</i>	(Aguilar <i>et al.</i> , 2003; Smith and Iglewski, 2003; Hao <i>et al.</i> , 2005; Sjöblom <i>et al.</i> , 2006; Dulla and Lindow, 2009; Hosni <i>et al.</i> , 2011; Kozlova <i>et al.</i> , 2011; Majerczyk <i>et al.</i> , 2013)
Siderophore production	<i>B. cepacia</i> , <i>P. aeruginosa</i>	(Stintzi <i>et al.</i> , 1998; Aguilar <i>et al.</i> , 2003)
Pigment secretion	<i>C. violaceum</i> , <i>Serratia</i> sp.	(McClean <i>et al.</i> , 1997; Thomson <i>et al.</i> , 2000)
Plasmid conjugation/transfer	<i>A. tumefaciens</i> , <i>Rhizobium leguminosarum</i>	(Zhang <i>et al.</i> , 1993; McAnulla <i>et al.</i> , 2007)
Bioluminescence	<i>V. fischeri</i>	(Nealson <i>et al.</i> , 1970; Nealson, 1977)
Stress anticipation	<i>Burkholderia glumae</i> , <i>Burkholderia thailandensis</i> , <i>B. pseudomallei</i>	(Goo <i>et al.</i> , 2012)
Central metabolism	<i>P. aeruginosa</i> , <i>Gloeotheca</i> sp.	(Yoon <i>et al.</i> , 2002; Toyofuku <i>et al.</i> , 2007; Sharif <i>et al.</i> , 2008)

* *Serratia liquefaciens* MG1 was recently reclassified as *Serratia marcescens* MG1 (Rice *et al.*, 2005)

2.1.3.1 AHL mediated QS and bacterial central metabolism

Bacterial central metabolism, in terms of energy recovery and building of cellular components, such as proteins and nucleic acids, is vital to the growth and reproduction of an individual. These processes often depend on highly complex regulatory mechanisms and AHL mediated QS signalling was recently found to play a role in the control of central metabolic functions of some species, including *P. aeruginosa* (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007) and Cyanobacteria *Gloeotheca* sp. (Sharif *et al.*, 2008). In *P. aeruginosa*, QS has been shown to regulate energy metabolism via the denitrification pathway, in which nitrate and nitrite are used as the electron acceptors instead of oxygen during respiration (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007). Without a functional QS system, the viability of *P. aeruginosa* QS mutant decreased significantly compared to the wild-type strain under anoxic conditions, suggesting that QS protects the cells from metabolic intoxication during denitrification (Yoon *et al.*, 2002). Importantly, although the QS regulated intracellular enzymes are not shared among the population cells, all individuals benefit from the coordinated behaviour through protection from toxic metabolic by-products, suggesting that QS regulated private goods may also benefit more than the QS responders. QS signalling has also been shown to regulate carbohydrate and amino acid metabolisms in Cyanobacteria *Gloeotheca* sp. (Sharif *et al.*, 2008). Even though the detailed mechanism involved remains to be demonstrated, provision of exogenous AHLs to the Cyanobacteria culture was clearly shown to affect the expression of cellular enzymes, in particular those involved in the carbon dioxide fixation and nitrogen recovery pathways. It is therefore apparent that QS signalling has an additional role in direct control of bacterial cell growth, apart from the fitness associated behaviours that are typically reported in a wide range of bacterial species.

2.1.3.2 AHL mediated QS and biofilm development

It is now recognized that bacteria in nature predominantly exist as matrix encased communities, termed biofilms (Kolter and Greenberg, 2006). Microbial biofilms, as demonstrated in many single-species studies, generally develop in a step wise manner from initial surface attachment to microcolony formation, maturation and differentiation through to dispersal (Parsek and Greenberg, 2005). While much remains to be learned about the complex regulatory system underlying these processes, AHL

mediated QS has been shown to play important roles in various stages of biofilm development for a wide variety of bacteria (Table 2-1) (Puskas *et al.*, 1997; Allison *et al.*, 1998; Davies *et al.*, 1998; Atkinson *et al.*, 1999; Huber *et al.*, 2001; Lynch *et al.*, 2002; Hammer and Bassler, 2003; Labbate *et al.*, 2004; Rice *et al.*, 2005; Dubern *et al.*, 2006; Maddula *et al.*, 2006). Many of these QS driven biofilm development processes involve the control of bacterial motility and EPS production (Allison *et al.*, 1998; Davies *et al.*, 1998; Atkinson *et al.*, 1999; Allesen-Holm *et al.*, 2006; Koutsoudis *et al.*, 2006). For example, in *P. stewartii*, expression of EPS, the stewartan, is governed by the *esaIR* QS system which acts a repressor of EPS production (Minogue *et al.*, 2002; Koutsoudis *et al.*, 2006). In the absence of AHL at low cell densities, EPS production is suppressed by EsaR and this negative regulation of EPS appears to enhance the attachment of the wild type *P. stewartii* in the early stage of biofilm development (Koutsoudis *et al.*, 2006). In the *esaR* mutant, copious amounts of EPS are produced in the early stage of biofilm development, leading to the formation of amorphous, detached biofilms (flocs). In contrast, the AHL deficient strain (*esaI* mutant) displays robust adhesion and forms thick microbial mats without further maturing into structured biofilms, suggesting that the timely control of EPS production by QS signalling is essential for the development of biofilms by *P. stewartii* (Koutsoudis *et al.*, 2006). It was later shown that the biofilm maturation process was facilitated by controlling the swarming motility of the cells via the stewartan EPS in a QS dependent manner (Herrera *et al.*, 2008). Similarly, in *P. aeruginosa*, mutations in the *lasI* synthase resulted in loss of the 3OC12-HSL signal and consequently the formation of biofilms that were flat and undifferentiated in contrast to the highly structured, mature biofilms of the wild-type strain (Davies *et al.*, 1998). Allesen-Holm *et al.* (2006) further demonstrated that the formation of the highly structured biofilms requires the presence of extracellular DNA (eDNA), an EPS component secreted by the wild-type *P. aeruginosa*. The secretion of eDNA was later shown to be, in part, dependent on the *lasI/R* and *rhlIR* QS systems (Allesen-Holm *et al.*, 2006). In parallel with these findings, the role of AHL mediated QS signalling in biofilm formation (or aggregation) in *A. baumannii* (Bhargava *et al.*, 2012), *P. fluorescens* (Allison *et al.*, 1998), and *Y. pseudotuberculosis* (Atkinson *et al.*, 1999) as well as biofilm-specific differentiation and maturation in other model organisms, such as *S. marcescens* (Labbate *et al.*, 2004),

B. cepacia (Huber *et al.*, 2001) and *A. hydrophila* (Lynch *et al.*, 2002) have been established.

QS has also been demonstrated to be involved in biofilm dispersal. For example, the *cerI/R* QS system in *R. sphaeroides* was shown to negatively regulate cellular aggregation (Puskas *et al.*, 1997). Mutations in the AHL synthase, *cerI*, which directs synthesis of *N*-(7,8-*cis*-tetradecenoyl)-HSL, resulted in hyperaggregation of cells in liquid culture while the exogenous addition of AHLs dispersed the *cerI* mutant aggregates. Similar aggregation and aggregate dispersal effects were observed in *P. aureofaciens* after mutations of the *csaI* and *phzI* AHL synthases, and after addition of the respective cognate AHLs to the QS mutant cultures (Maddula *et al.*, 2006). In *Vibrio cholera*, it was further shown that QS negatively regulates production of *vps* exopolysaccharides, which are important for cellular aggregation, adhesion and biofilm formation (Hammer and Bassler, 2003). QS induction at high cell densities inhibits the production of *vps* and therefore leads to biofilm dispersal. The use of QS to disperse cells from the biofilms is thought to allow cells to escape from resource-depleted niches and subsequently to colonize new surfaces for a new cycle of biofilm development.

2.2 THE ECOLOGICAL RELEVANCE OF QS SIGNALLING

It is apparent that AHL mediated QS plays a key role in modulating diverse physiological processes in many microbial species. However, the impact of QS signalling in natural and engineered ecosystems where complex, multiple species coexist, remains largely unknown. The high microbial diversity in these ecosystems along with the highly dynamic environmental conditions makes the analysis and interpretation of QS technically challenging. For these reasons, the *in situ* role of QS signalling in complex environments has rarely been studied in detail thus far. Alternatively, researchers have attempted to address the relevance and the role of QS signalling in complex environments using simple, mixed-species systems where two defined microbial species are co-cultured and studied for their behaviours in connection to the QS signalling. While such information provides an indication of potential QS function, such studies may not truly reflect the ecological role of QS under natural environmental conditions.

2.2.1 QS signalling in simple, mixed-species systems

Riedel *et al.* (2001) were among the first to investigate the role of AHL signals in mediating interspecies interactions using a co-culture system composed of *P. aeruginosa* and *B. cepacia*. Both *in vivo* and *in vitro* examinations of the interactions suggest a unidirectional AHL based communication where *B. cepacia* was capable of perceiving AHLs produced by *P. aeruginosa* and resulting in mixed microcolony formation as well as virulence factor expression. The physical association between the two species, as controlled via the QS signalling, was proposed to be important for chronic infection in cystic fibrosis patients (Riedel *et al.*, 2001). Interestingly, such synergistic relationships are also common between *Pseudomonas* and other microbial species (Sibley *et al.*, 2008; Dulla and Lindow, 2009; Hosni *et al.*, 2011; Bhargava *et al.*, 2012). In *Pseudomonas savastanoi*, AHL mediated QS was shown to be important for plant colonization and communication with other non-pathogenic epiphytes, such as *Erwinia toletana* and *P. agglomerans* (Hosni *et al.*, 2011). Importantly, co-inoculation of *P. savastanoi* and *E. toletana* onto an olive plant was shown to enhance the disease symptoms and increase the growth of both species significantly as compared to the infection by either species alone or co-infection by QS mutant strains of the two (Hosni *et al.*, 2011). Similarly, using a fly infection model, Sibley *et al.* (2008) showed that one third of the oropharyngeal isolates (13) that were avirulent or beneficial to the host fly individually can enhance the pathogenicity of a microbial community with *P. aeruginosa*. Although it remains unclear if the enhanced pathogenicity was a synergistic effect mediated by AHL signalling between *P. aeruginosa* and the isolates, activation of both *lasIR* and *rhlIR* QS systems in *P. aeruginosa* during the co-infection was observed (Sibley *et al.*, 2008). In an even larger screening experiment, 77 out of 1,117 bacterial strains isolated from leaf surfaces were found to produce AHLs that could activate the QS regulated host infection by *P. syringae* in co-culture, providing further evidence that AHLs could mediate interspecies signalling in natural environments and suggests that cooperation occurs among the diverse microbial species (Dulla and Lindow, 2009).

Whilst high cell densities may promote QS dependent cooperation in complex communities, intensive competitions over resources in local populations may also occur at high cell densities, especially within biofilms (Elias and Banin, 2012; Rendueles and

Ghigo, 2012). In fact, antagonism is commonly observed among microbes from similar niches and may be mediated by multiple mechanisms including, the production of toxins, scavenger molecules and antimicrobial compounds (Rendueles and Ghigo, 2012). These mechanisms can be, in part, driven by QS (An *et al.*, 2006; Moons *et al.*, 2006; Chandler *et al.*, 2012). Notably, the QS conferred fitness is often the result of QS regulated production of antimicrobial compounds (Elias and Banin, 2012). Examples of such interactions have been clearly illustrated in co-culture of *Serratia plymuthica* and *E. coli* (Moons *et al.*, 2006), and between bacteria and fungi, e.g. *Burkholderia lata* and *Rhizoctonia solani* (Schmidt *et al.*, 2009). Interestingly, a direct bactericidal effect of 3OC12-HSL signals and its tetramic acid derivative on Gram positive bacteria (Kaufmann *et al.*, 2005), as well as a growth inhibitory role of *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-HSL (3OHC14-HSL) signal on certain strains of *Rhizobium leguminosarum* have also been observed (Schripsema *et al.*, 1996), suggesting that the AHL signals, in particular those with long acyl chains can act as the antimicrobial compounds by themselves. Using a computer simulation, Chandler *et al.* (2012) proposed an alternative role of QS signalling as a surveillance system in complex communities. The authors showed that some bacterial species may use their promiscuous signal receptors to eavesdrop on the presence of neighbouring cells and gain an additional competitive advantage, such as by early activation of QS regulated antimicrobial compound production to counter the growth of their competitors (Chandler *et al.*, 2012). On the other hand, a co-culture study using *P. aeruginosa* and *A. tumefaciens* reported that QS may increase fitness without the involvement of antimicrobial compounds (An *et al.*, 2006). Instead, QS controlled nutrient acquisition, and hence a faster growth rate, was proposed to enable the *P. aeruginosa* to outcompete *A. tumefaciens* in both planktonic and biofilm growth conditions (An *et al.*, 2006).

2.2.2 QS signalling in complex, mixed-species ecosystems

It is clear that studies using synthetic, mixed-species model systems have provided invaluable insights into the QS biology in microbial communities as discussed above. Based on such studies, it has been shown that QS can play important roles in either facilitating the establishment of stable communities, or in the exclusion of competitors. Despite being a convenient approach to test the QS hypothesis at the community level, these simple, dual-species models lack the required complexity to show how QS may

function in highly diverse, natural microbial consortia. In particular, it is now well-appreciated that AHL producers are ubiquitous in many natural and engineered ecosystems, including host associated cavities (Nealson, 1977; Morohoshi *et al.*, 2005; Borlee *et al.*, 2008; Hughes *et al.*, 2010), soil/rhizosphere environments (d'Angelo-Picard *et al.*, 2005), marine waters (Huang *et al.*, 2008; Tait *et al.*, 2009), microbial mats (Decho *et al.*, 2009) and wastewater treatment plants (Morgan-Sagastume *et al.*, 2005; Chong *et al.*, 2012), all of which have communities comprised of hundreds to thousands of species. These findings clearly indicate a possibility of QS signalling in natural habitats. A detailed understanding of QS regulated community behaviour in these environments involving complex communities would be thus critically needed.

Unlike the population based studies, genetic manipulation to determine the role of QS signalling is almost impossible in the context of complex communities. Alternatively, the effect of the exogenous addition of AHLs to such complex communities have been monitored and shown to coordinate community phenotypes analogous to the QS regulated behaviours at the population level. For example, Yeon *et al.* (2009a) showed that formation of highly complex, mixed-species sludge biofilms on a membrane bioreactor (MBR) was closely associated with the production of *N*-octanoyl-L-homoserine lactone (C8-HSL) signal. Exogenous supply of the C8-HSL signal to the sludge community was found to increase the EPS production and to accelerate the formation of biofilms, a community behaviour that could be impeded by the addition of AHL degrading enzymes to the MBR system, further supporting the role of QS in mediating complex community biofilm formation (Yeon *et al.*, 2009a; Yeon *et al.*, 2009b). In other studies, the exogenous addition of AHLs to the activated sludge was also found to alter the sludge community composition (Valle *et al.*, 2004), to improve phenol degradation (Valle *et al.*, 2004) and to increase exoenzyme production (Chong *et al.*, 2012). More recently, AHL mediated QS has also been implicated in controlling energy metabolism of a sludge community where the addition of AHLs was clearly shown to enhance the rate of anoxic ammonium oxidation by the community (De Clippeleir *et al.*, 2011). These findings thus imply that microbial communities in natural environments are capable of perceiving and responding to AHLs to control phenotypes which can be monitored at the community level.

2.3 THE INTERFERENCE OF QS SIGNALLING

Despite the responsiveness of complex communities to exogenous AHLs which are often supplied at high signal concentrations in the laboratory experiments, the effectiveness of QS signalling mediated by AHLs at native levels remains poorly studied. Not only is information limited on the endogenous signal concentrations in natural and engineered ecosystems, but also limited with respect to the stability and bioavailability of AHL signals over extended time spans. Indeed, in complex environments where multiple species coexist, many factors other than cell density have been identified that can influence the accumulation and perception of AHLs, and hence the efficiency of QS in modulating the physiological activities of bacteria in complex communities (Boyer and Wisniewski-Dyé, 2009).

2.3.1 Factors affecting signal accumulation and perception

The environmental factors interfering with the QS signalling process can generally be divided into two categories, physiochemical and the biological factors, depending on the nature of the interfering mechanisms and also the source of the interference.

2.3.1.1 Physiochemical interference

pH and temperature

Physical conditions, such as alkaline pH and high temperature have been reported to interfere with the AHL driven QS systems via a signal-degradation approach (Byers *et al.*, 2002; Yates *et al.*, 2002). It was shown that AHLs can be inactivated by a pH dependent lactonolysis process where the lactone rings were opened to form *N*-acyl-homoserine (AHS) molecules devoid of signaling properties (Byers *et al.*, 2002; Yates *et al.*, 2002). Whilst 70% of *N*-propionyl-homoserine lactone (C3-HSL) is inactivated at pH 6, *N*-butyryl-homoserine lactone (C4-HSL) is completely hydrolysed at pH 8 (Byers *et al.*, 2002). The rate of inactivation is clearly dependent on the nature of the signals where AHLs with short acyl chains (C4 to C6) are more susceptible to alkaline pH than the AHLs with medium to long acyl chains (> C8) (Yates *et al.*, 2002; Decho *et al.*, 2009). Comparatively, AHLs substituted with a carbonyl group were also found to be more sensitive to alkaline lactonolysis than their unsubstituted counterparts (Yates *et al.*, 2002). Moreover, it has been shown that the pH mediated AHL inactivation can be accelerated by elevated temperature. Increasing the temperature from 22°C to 37°C

was found to enhance the degradation rate 1.5 to 3.1 times, depending on the types of AHLs (Yates *et al.*, 2002). This lactonalysis process however is reversible. Yates *et al.* (2002) reported that the AHL molecules can be re-lactonased to the original, functional AHLs by acidifying the medium to less than pH 2 at 4°C for 48 h. One example of such pH mediated signalling interference in nature was presented recently by Decho *et al.* (2009) on a study of AHL accumulation in microbial mats. The authors showed that accretion of AHLs in microbial mats was dependent on the diel pH, which can rise from pH 6.8 during darkness to pH 9.4 during the daytime as a consequence of switching from net respiration to net photosynthesis. While large amounts of AHLs were extractable at night, the AHLs diminished almost completely during the day, suggesting a huge impact of environmental pH on the AHL mediated QS signalling in natural settings. Such fluctuations would be expected to have important implications for QS mediated control of phenotypes.

Diffusion and advection

QS signalling is mediated by diffusible chemical molecules such as AHLs, activation of the signalling system is therefore also a function of mass transfer in addition to the cell density factor (Redfield, 2002). This is particularly relevant in the environmental context since most bacteria, if not all, form biofilms that are encased within the highly adhesive EPS matrix, a diffusion barrier to the transportation of molecules especially those with hydrophobic nature. As such, signals may accumulate within the biofilms depending to the density of the biofilms, the medium hydrophobicity and also the nature of the signalling molecule (Boyer and Wisniewski-Dyé, 2009), which may essentially restrict the communication between cells at a distance. For example, Charlton *et al.* (2000) showed that the amount of 3OC12-HSL accumulating in *P. aeruginosa* biofilms was at least 600 times more concentrated than the 3OC12-HSL secreted into the supernatant, and hence the 3OC12-HSL may not be an effective signal for coordinating behaviours of population cells at far distances. By contrast, the C4-HSL, being less hydrophobic than the 3OC12-HSL counterpart, and hence having fewer interactions with the EPS matrix, has been proposed to be the principal signal mediating the QS processes in biofilm-related conditions (Singh *et al.*, 2000). While it remains to be demonstrated that AHL signals are present in the circulating liquid, accretion of AHLs within the biomass component of microbial mats (Decho *et al.*,

2009) and sludge biofilms in a MBR system (Yeon *et al.*, 2009a) were evidenced, consistent with the observations made in single-species *P. aeruginosa* biofilms. Despite the preferential accumulation of some AHLs in the biofilm matrix, some signals can diffuse into the supernatant and the signal diffusion can be mediated by the circulating fluids. This phenomenon, termed advection, will lead to signal washout and dilution (Horswill *et al.*, 2007). The effect of advection on QS signalling in nature has been elegantly demonstrated by Dulla and Lindow (2008) in a study of QS induction in *P. syringae* aggregates on both dry and wet leave surfaces. It was shown that on wet surfaces, analogous to periods of rain, the flowing fluid can easily disrupt the QS signalling of the epiphytic bacteria by diluting signals accumulated within cellular aggregates (Dulla and Lindow, 2008).

2.3.1.2 Biological interference

AHL mimics and inhibitors

Since the first discovery of halogenated furanones from the red macroalga *Delisea pulchra* (Givskov *et al.*, 1996), many other natural compounds derived from bacteria, algae, plants, fungi and animals have been documented to be capable of interfering with the AHL mediated QS signalling (Boyer and Wisniewski-Dyé, 2009). In general, there are two key mechanisms in which these diverse natural compounds can be involved in the QS interference. The first is by acting as a QS antagonist to compete with the AHL for receptor binding and consequently, inhibiting the QS induction. Compounds, such as halogenated furanones, phenethylamide (from the Gram positive bacteria *Halobacillus salinus*), L-canavanine (from the plant *Medicago sativa*), penicillic acid and patulin (from the fungi *Penicillium*) are categorized in this group (Manefield *et al.*, 1999; Teplitski *et al.*, 2000; Manefield *et al.*, 2002; Keshavan *et al.*, 2005; Rasmussen *et al.*, 2005; Teasdale *et al.*, 2009). The second method for interfering with QS is by behaving as an AHL mimic, which binds to the receptor and activates the QS response. Diketopiperazines secreted by a diverse range of bacterial species, including *Proteus mirabilis*, *Citrobacter freundii*, *Enterobacter agglomerans* and *Pseudomonas putida*, as well as interferon- γ from animal host immune system, are among the AHL mimics identified thus far. These compounds have been verified to be able to trigger a QS response that is usually driven by the AHL signals (Holden *et al.*, 1999; Wu *et al.*, 2005). Although the physiological role of most AHL mimics and inhibitors is yet to be

established, their activities provide important evidence for interspecies or even interkingdom cross talk and suggest the potential to modulate AHL mediated QS in natural environments.

AHL inactivating enzymes

AHLs in natural environments are often subjected to inactivation or degradation by a broad range of organisms, including both prokaryotes and eukaryotes (Czajkowski and Jafra, 2009). This enzyme mediated signal degradation phenomenon is commonly referred to as quorum quenching (QQ) (Dong and Zhang, 2005). Since the first report of QQ from *Bacillus* sp. in 2001, there are now more than 20 genera of bacteria that have been isolated and characterized that are capable of degrading AHLs enzymatically (Dong *et al.*, 2001; Boyer and Wisniewski-Dyé, 2009; Uroz *et al.*, 2009). More recently, the list of QQ organisms has been expanded to include fungi, plants and animals, suggesting that QQ is widespread in nature (Delalande *et al.*, 2005; Yang *et al.*, 2005; Uroz and Heinonsalo, 2008; Kalia *et al.*, 2011). In complex communities, QQ activities have also been found to prominently occur in a diverse range of natural habitats, including the soil, rhizosphere, and marine waters (d'Angelo-Picard *et al.*, 2005; Wang and Leadbetter, 2005; Hmelo and Van Mooy, 2009; Tait *et al.*, 2009). Importantly, the presence of AHL signals has also been determined in several of these habitats, suggesting a high possibility for the co-occurrence of QS signalling and microbially mediated QQ activities within a single environment (Wang and Leadbetter, 2005; Decho *et al.*, 2009).

2.3.2 Microbially mediated quorum quenching in complex, mixed-species ecosystems

2.3.2.1 The QQ mechanisms

According to the prediction by Dong and Zhang (2005), based on the chemical structure of AHLs, there may be at least four types of enzymes that can inactivate AHLs, namely the lactonase, decarboxylase, acylase and deaminase. Indeed, two out of four of the enzymes envisaged have been discovered with high frequencies while a new type of QQ enzyme, an oxidoreductase, was recently documented as well (Figure 2-2) (Uroz *et al.*, 2009). These enzymes are involved in lactone hydrolysis, amidohydrolysis and oxidoreduction.

Figure 2-2. AHL degradation pathways and degradation products. Broadly, AHLs can be inactivated by two different pathways: degradation pathways (1. lactonase hydrolysis mediated by AHL lactonases; 2. amidohydrolysis mediated by AHL acylases) and modification pathways (3. oxido-reduction mediated by oxido-reductase; 4. oxidation mediated by oxidase). (From Uroz *et al.*, 2009).

Lactone hydrolysis is mediated by AHL lactonases which hydrolyse the lactone rings of AHLs and lead to the formation of acyl homoserine (AHS) molecules (Figure 2-2). This process is identical to the alkaline pH mediated lactonolysis. As such, the reaction can be reversed by acidification of the medium. AHL lactonases (AiiA family) were first identified in several species of *Bacillus* (Dong *et al.*, 2001; Dong *et al.*, 2002; Thomas *et al.*, 2005; Liu *et al.*, 2008; Chan *et al.*, 2010). These AiiA lactonases were characterized as broad range QQ enzymes since they can hydrolyze all AHLs, ranging from C₄ to C₁₄, with or without a substitution at the C₃ position (Wang *et al.*, 2004). Numerous homologs of AiiA lactonases have subsequently been identified in other bacterial species, including the AhlK in *Klebsiella pneumoniae*, the AttM and AiiB in *A. tumefaciens*, and the AhlD in *Arthrobacter* (Zhang and Wang, 2002; Park *et al.*, 2003; Haudecoeur *et al.*, 2009). A second family of AHL lactonases, the QsdA, was found in *Rhodococcus erythropolis* (Uroz *et al.*, 2008). Although QsdA is not related to the AiiA family and is specific to the genus *Rhodococcus*, it displays broad range AHL QQ activity that is typical of the AiiA lactonases. While all of the bacteria derived lactonases catalyse the QQ activities intracellularly, extracellular lactonases have been documented in mammalian serum. These mammalian enzymes were identified as paraoxonases (Billecke *et al.*, 2000; Yang *et al.*, 2005). Unlike AiiA and QsdA which specifically target AHLs only, paraoxonases catalyse the hydrolysis of a wide range of molecules containing the lactone rings and ester bonds, suggesting that the paraoxonase enzymes have multiple roles in the mammalian system.

Aminohydrolysis, as suggested by the name, involves hydrolysis of the amide bond of AHLs. This results in the release of the homoserine lactone ring and the corresponding fatty acid chain (Figure 2-2). Unlike lactonolysis, this degradation process is irreversible and catalyzed only by the AHL acylases (Dong and Zhang, 2005). To date, there are six genes that have been identified to encode acylases, including *aiiD* (*Ralstonia* sp.), *aiiC* (*Anabaena* sp.), *ahlM* (*Streptomyces* sp.), *aac* (*Shewanella* sp.), *pvdQ* and *quiP* (*P. aeruginosa*) (Huang *et al.*, 2003; Lin *et al.*, 2003; Park *et al.*, 2005; Huang *et al.*, 2006; Romero *et al.*, 2008; Chen *et al.*, 2009). Similar to the bacterial AHL lactonases, all of the AHL acylases, except for *AhlM*, catalyse the reaction intracellularly (Park *et al.*, 2005). However, different from the AHL lactonases, AHL acylases degrade long-chain AHLs more efficiently than short-chain ones. For enzymes such as *AhlM*, *AaC*, *PvdQ* and *QuiP*, AHLs with acyl chains shorter than eight carbons are not typically degraded (Huang *et al.*, 2003; Park *et al.*, 2005; Huang *et al.*, 2006; Chen *et al.*, 2009). In contrast, porcine kidney acylases isolated from the mammalian system, were found to mediate degradation of AHLs with acyl chains of four to eight carbons only, suggesting different evolutionary origins for the bacterial and the eukaryotic acylases (Xu *et al.*, 2003).

Apart from degrading AHLs by lactonases and acylases, some bacteria may modify the AHLs (Figure 2-2). One mechanism is via oxidation or reduction of the important functional groups on the AHLs. The first AHL oxido-reductase identified in *R. erythropolis* was found to reduce the carbonyl group at the C3 position of oxo substituted AHLs to the corresponding 3-hydroxyl derivatives, rendering the AHLs inactive (Uroz *et al.*, 2005). The second AHL oxido-reductase is a P450 monooxygenase from *Bacillus megaterium*. This enzyme targets only the long-chain AHLs ($\geq$ C12) by oxidizing the acyl chain at certain carbon positions (Chowdhary *et al.*, 2007). These modifications can lead to structural changes of the AHLs and hence affect the interaction between the AHL and its cognate receptor protein and thus inhibit QS function.

2.3.2.2 The ecological significance of QQ

While much has been learned about the mechanisms of QQ in single-species laboratory studies, the role of QQ activities in natural habitats remains largely unknown. Since

some QS bacteria also encode QQ enzymes, it is commonly speculated that QQ enzymes regulate the turnover of AHLs to allow the system to ‘reset’ at some stage after QS has been activated (Roche *et al.*, 2004; Wang *et al.*, 2007a; Uroz *et al.*, 2009). Alternatively, QQ enzymes may have evolved to facilitate the use of AHLs as a source of carbon and nitrogen. In fact, this has been shown for several AHL degrading bacteria, including *Variovorax paradoxus*, *P. aeruginosa* and *B. cereus* that can utilize the degraded products of AHLs for growth (Leadbetter and Greenberg, 2001; Huang *et al.*, 2003; Chan *et al.*, 2009; Chan *et al.*, 2010). It has also been suggested that QQ enzymes may play an important role in the competition with QS populations co-inhabiting same environment (Roche *et al.*, 2004). For example, *E. corotovora* produces carbapenem antibiotics under the regulation of *cerI/R* QS signaling system, presumably to inhibit the growth of competitors (Manefield *et al.*, 2001). Any bacteria that can break down the QS signalling system will effectively prevent antibiotic production by *E. corotovora* and hence, their likelihood of survival. It is therefore possible that in complex communities, such as an activated sludge, microbes adopt different strategies to modulate their interactions with the neighbours through their QS and/or QQ activities to optimize resource accumulation and survival rate.

2.4 THE ACTIVATED SLUDGE MODEL ECOSYSTEM

The activated sludge represents one of the most dynamic and species-rich ecosystems studied to date (Kjelleberg *et al.*, 2014). This complex ecosystem often accommodates thousands of microbial species from all three domains of life important for effective treatment of water and wastewater. Despite the high complexity, the activated sludge can be an ideal model ecosystem to address the physiological and ecological role of QS signalling and QQ activities in complex environments.

2.4.1 Biological nutrient removal (BNR)

Biological treatment of wastewater is one of the most important biotechnologies developed and utilizes microorganisms to degrade a wide range of organic substances and to recycle essential elements such as carbon, nitrogen and phosphorus (Daims *et al.*, 2006). The treatment process is conventionally mediated by a highly diverse microbial community, growing as suspended floccular biomass, with appropriate mixing and aeration, termed an activated sludge (Metcalf and Eddy, 1991). While

constant aeration improves degradation of the organic compounds in wastewater by heterotrophs, complete removal of key nutrients, i.e. nitrogen and phosphorus, requires more complicated operating conditions, such as alternating aerobic-anoxic or anaerobic-aerobic stages, and the presence of unique groups of microorganisms such as nitrifiers, denitrifiers and polyphosphate-accumulating organisms (PAOs) (Seviour and Nielsen, 2010). The composition and specific activities of the microbial community will ultimately determine the efficiency and efficacy of the nutrient removal processes in a wastewater treatment plant (Wagner *et al.*, 2002). Despite the huge microbial diversity, the treatment processes are primarily driven by the prokaryotes (Seviour and Nielsen, 2010), which are also the key contributors to the biomass of the activated sludge community with an estimated $1-10 \times 10^{12}$ bacteria/g VSS (Nielsen and Nielsen, 2002). Both Gram positive and Gram negative bacterial species are present in the sludge community with the later being the dominant (Metcalf and Eddy, 1991; Wagner and Loy, 2002). A collective survey by Wagner *et al.* (2002) based on 16S ribosomal ribonucleic acid (rRNA) gene library analyses of eight activated sludge and biofilm ecosystems, further revealed that sequences affiliated with the Proteobacteria, Bacteroidetes and Actinobacteria were most frequently retrieved in all the libraries, and the Proteobacteria was often the most abundant phyla in each library (Wagner *et al.*, 2002). Depending on the nature of wastewater treated, the proportion of Proteobacteria can account for up to 92 % of the entire microbial community (Snaird *et al.*, 1997), suggesting that this phylum of bacteria is particularly important in the wastewater treatment processes (Wagner *et al.*, 2002). In fact, most of the bacterial species known to be involved in the BNR belong to the phylum Proteobacteria.

2.4.1.1 Nitrogen removal

In the conventional BNR system, nitrogen elimination is achieved via a two-stage process with aerobic nitrification converting ammonium to nitrite and nitrate, followed by anoxic denitrification, where nitrate is gradually reduced to nitrogen gas (Metcalf and Eddy, 1991). Some studies have shown that these two processes can occur concurrently within a single bioreactor under low dissolved oxygen conditions through the so-called simultaneous nitrification-denitrification (SND) process (Bertanza, 1997; Keller *et al.*, 1997), where ammonium is partially oxidized to nitrite, which is then subjected to denitrification for subsequent reduction to nitrogen gas (Figure 2-3) (van

Loosdrecht and Jetten, 1998). Regardless of the conventional pathway or the SND with nitrite shortcut pathway, the success of the above-mentioned processes is heavily dependent on the selection for or against specific functional groups of bacteria which generally include the ammonium oxidizing bacteria (AOB), the nitrite oxidizing bacteria (NOB) and the denitrifiers. It had been traditionally held that *Nitrosomonas europaea* (β -Proteobacteria) and *Nitrobacter* species (α -Proteobacteria) were the major AOB and NOB, respectively since they could be isolated from most of the nitrifying activated sludge and biofilms (Daims *et al.*, 2006). However, recent studies, based on culture independent methods, suggest that the AOB and NOB can be much more diverse than previously thought. In some cases, *Nitrococcus mobilis* (γ -Proteobacteria) was found to be the major AOB (Moussa *et al.*, 2006), while nitrifiers closely related to the genus *Nitrospira*, and not *Nitrobacter*, were identified by *in situ* techniques as the most important NOB in wastewater treatment plants (Daims *et al.*, 2001). The denitrifier community can be even more complex than the nitrifiers (Zumft, 1997). To date, more than 50 genera of bacteria have been classified as denitrifiers, with members of the genus *Pseudomonas* (γ -Proteobacteria) being the most common and widely distributed (Seviour and Nielsen, 2010).

Figure 2-3. Total nitrogen removal can be achieved via the conventional pathway (solid arrows) or the SND with nitrite shortcut pathway (broken arrows). The arrows in blue and red represent the nitrification and denitrification processes, respectively.

2.4.1.2 Phosphorus removal

Biological depletion of phosphorus is achieved via a process termed the enhanced biological phosphorus removal (EBPR), which mainly relies on the activities of the PAOs, a group of bacteria that are capable of accumulating polyphosphate intracellularly (Seviour and Nielsen, 2010). This process usually occurs under alternating anaerobic-aerobic conditions that favour the enrichment of PAOs, and the unintended glycogen accumulating organisms (GAOs). The GAOs, i.e. *Candidatus Competibacter phosphatis* (γ -Proteobacteria) and members related to the genus *Defluviicoccus* (α -Proteobacteria), which share similar physiologies with the PAOs but do not accumulate polyphosphate intracellularly, are believed to compete with the PAOs for carbon source during the anaerobic feeding phase (Crocetti *et al.*, 2002; Meyer *et al.*, 2006). As such, many studies have shown that when the GAOs dominate over the PAOs in the EBPR plants, the EBPR systems typically fail (Crocetti *et al.*, 2002; Daims *et al.*, 2006). The GAOs are phylogenetically diverse, and the same is true of the PAOs, which are represented by a large family of organisms that are also believed to coexist in many EBPR plants to mediate phosphate removal (Blackall *et al.*, 2002). The first and the most common PAOs identified in the EBPR plants were the uncultivated β -Proteobacterium, *Candidatus Accumulibacter phosphatis* (Hesseltmann *et al.*, 1999; Seviour and Nielsen, 2010). Additional PAO candidates include Actinobacteria (Liu *et al.*, 2001), and Proteobacteria of alpha (Kawaharasaki *et al.*, 1999), beta, i.e. *Rhodocyclus* related organisms (Kong *et al.*, 2004), and gamma subdivisions (Liu *et al.*, 2001). Interestingly, a novel group of PAOs capable of simultaneous uptake of phosphorus and reduction of nitrate/nitrite by the internal carbon source was recently documented in several anaerobic-anoxic EBPR systems (Kern-Jespersen *et al.*, 1994; Oehmen *et al.*, 2010; Zhou *et al.*, 2010). Due to this combined function, they have been denoted as the denitrifying PAOs (DPAOs).

2.4.1.3 Simultaneous nitrogen and phosphorus removal

In recent years, increasing efforts have been made to integrate the SND and the EBPR systems to achieve the most economical and sustainable process for wastewater nutrient removal (Zeng *et al.*, 2003; Zeng *et al.*, 2004; Kishida *et al.*, 2006; Yilmaz *et al.*, 2008; Zhou *et al.*, 2010). Correspondingly, a combined treatment process termed the simultaneous nitrification, denitrification and phosphorus removal (SNDPR) was

developed that uses the floccular sludge (Zeng *et al.*, 2003). However, it was found that removal of nitrate and nitrite in the SNDPR process was unsatisfactory as a consequence of incomplete coupling between the nitrification and denitrification processes, which usually occurs if aerobic/anoxic zones in the microbial aggregates are not suitably formed (Meyer *et al.*, 2005). To resolve this issue, it was proposed to use granular sludge in the SNDPR process (de Kreuk *et al.*, 2005). Granules are larger and denser forms of the microbial flocs used in the conventional activated sludge systems (Liu, 2008). The dense and compact features of granules magnify oxygen mass transfer limitation leading to the creation of aerobic/anoxic zones within the granules that are necessary for a reliable SNDPR system (de Kreuk *et al.*, 2005; Kishida *et al.*, 2006). Lemaire *et al.* (2008) have successfully used granular sludge to operate and maintain the SNDPR functions for more than 450 days in a laboratory scale sequencing batch reactor (SBR). Further examination of the community structure of the granules revealed the dominance of PAOs and GAOs with distinct spatial distributions, where the PAOs were located chiefly in the outermost 200 μm region of the granule while the GAOs were mainly found in the granular core (Lemaire *et al.*, 2008).

2.4.2 Microbial granulation

Granular sludge is not only inherently very different from the floccular sludge in terms of physical appearance, but also in terms of functional characteristics. Apart from the difference in density, microbial granules exhibit excellent settleability, high biomass retention, capability to withstand high loading rates and tolerance to toxicity and environmental fluctuations relative to floccular biomass (Liu and Tay, 2004; Tay *et al.*, 2004; Jiang *et al.*, 2006). These unique features of microbial granules not only provide the granular community with the capacity to treat an increased range of wastewaters with assorted constituents, including toxic organics (e.g phenol, pyridine, etc.), heavy metals, dyes and particulate matter, but also enable significant reduction of the plant operational cost and enhancement of treatment efficiency (Xu *et al.*, 2004; Jiang *et al.*, 2006; Adav *et al.*, 2008). Indeed, the robustness and versatility of microbial granulation has gained this novel biotechnology an increasing popularity in the wastewater treatment community as one of the most promising, next generation biotechnologies for wastewater treatment processes.

2.4.2.1 The granulation model

According to a model described by Tay and colleagues (2001b), microbial granulation is a gradual process of transformation from the fluffy floccular sludge to the compact aggregates to the formation of granular sludge and finally to the development of mature microbial granules under specific operating conditions (Figure 2-4). The granulation process can vary from weeks to months depending on the types of carbon substrates and also the specific operating parameters applied (Tay *et al.*, 2001b; Barr *et al.*, 2010). While the floccular sludge is typically characterized by having an average particle size of 50 μm in diameter and a sludge volumetric index at 30 min sedimentation (SVI₃₀) of 100 to 150 mL g^{-1} (Gonzalez-Gil and Holliger, 2011), microbial granules in most studies were found to have a minimum particle size of 100 μm and a SVI at 5 min sedimentation (SVI₅) of 50 mL g^{-1} or below (Barr *et al.*, 2010). Interestingly, the formation of microbial granules is generally coupled with increase expression of EPS and cellular hydrophobicity (Tay *et al.*, 2001c; Yang *et al.*, 2004; McSwain *et al.*, 2005; Sheng *et al.*, 2010). Indeed, granules share some features with surface associated biofilms and suggests there may be some commonalities in the regulation and development of both.

Figure 2-4. Images of sludge morphology at different stages of granulation: floccular sludge (A), compact aggregates (B), granular sludge (C) and mature granules (D) in a sequencing batch reactor fed with acetate as substrate (Tay *et al.*, 2001b).

2.4.2.2 Factors contributing to the formation and development of microbial granules

The conversion of floccular sludge into compact aggregates, the initiation of microbial granulation, is often the most critical and time consuming step that determines the successful development of microbial granules. Several factors contributing to the initiation process have been proposed, including the formation of scaffolds for microbial cell attachment (Beun *et al.*, 1999; Tay *et al.*, 2001b) and increased expression of EPS to enhance the adhesion between cells (McSwain *et al.*, 2005; Sheng *et al.*, 2010). Based on microscopic observations, Beun *et al.* (1999) were the first to propose the growth of filamentous fungi as a prerequisite for microbial granulation. During the start-up period, fungi dominated the sludge community and formed mycelial pellets with a compact structure under intensive hydrodynamic shear force. These pellets appeared to function as an immobilized matrix providing some surfaces to which bacteria could adhere and grow into colonies and eventually develop into compact aggregates under a continuous selection pressure for heavy particles. Similarly, filamentous bacteria was also observed to mediate the initiation of granulation in the same fashion (Tay *et al.*, 2001b).

Apart from the scaffold hypothesis, Liu and Tay (2002) proposed that additional forces are required to maintain stable multicellular contacts (bacterial attachment to the cellular scaffolds) for successful granule formation. Often, this could be achieved via the secretion of EPS by the bacterial cells (Liu, 2008). EPS are sticky materials that consist of variable proportions of extracellular polysaccharides, proteins, lipids, humics-like substances and nucleic acids (Liu, 2008; Flemming and Wingender, 2010; Sheng *et al.*, 2010). These sticky macromolecules can assist cell-cell adhesion and effectively cross-link bacteria into microbial aggregates as in the case of microbial granules. For example, it has been shown that metabolic blocking of extracellular polysaccharide synthesis can inhibit the formation of microbial granules, while accumulation of EPS promotes the microbial aggregation (Yang *et al.*, 2004; Sheng *et al.*, 2010). Tay and colleagues also demonstrated that development of microbial granules was coupled with a significant increase in EPS expressed as the ratio of polysaccharides (PS) to proteins (PN) (Tay *et al.*, 2001a; Tay *et al.*, 2001c). Floccular sludge maintained at low superficial gas velocities had an average PS/PN ratio of 3

throughout the study, while granules formed under high superficial gas velocities had the PS/PN ratio increased from 3 to 15. Interestingly, a decrease in the PS/PN ratio, indicating a decrease of extracellular polysaccharides (the PS/PN ratio dropped to about 3), was also detected along with the disappearance of microbial granules. Collectively, these results suggest a critical role of extracellular polysaccharides in the formation, development and maintenance of microbial granules. A subsequent starvation study further confirmed that the integrity of mature granules was maintained by beta-linked polysaccharides, the non-biodegradable extracellular polysaccharides located in the outer shell of the granules (Wang *et al.*, 2007b).

In general, excessive amount of EPS are produced by the bacterial cells under stress conditions as a protective measure (Seviour and Nielsen, 2010). Such stressful conditions are also applied in the operation of the SBR where enhanced production of EPS in microbial granules was noted to be closely associated with high stress level. The operation of SBR often consists of four steps in sequence, i.e. filling, reacting, settling and discharging (Liu, 2008). The operating parameters in each specific step can be manipulated to create dissimilar levels of stress conditions for granule formation. Hydrodynamic shear force, periodic feast-famine periods and a combination of short settling time, high volume exchange ratio and short discharge time are among those parameters identified key to induce the gene expression for EPS production (Table 2-2) (Adav *et al.*, 2008; Liu, 2008). Although other operating parameters may not exhibit a similar influence on the EPS production, they may nonetheless affect other aspects of the granulation, such as microbial diversity, microbial activities, physical appearance of granule, etc.

Table 2-2. List of SBR operating parameters and their impacts on microbial granulation.

SBR parameters	Condition examined	Critical for granulation?	Influence on other aspects
Substrate composition	Glucose, sucrose, phenol, etc.	No. Formation of granule is independent of type of feed.	Microbial structure of mature granules Microbial diversity
Substrate loading	2.5 to 15 kg COD m ⁻³ d ⁻¹	No. However, the rate of granulation will be affected.	Physical strength of mature granules Size of granules
Hydrodynamic	0.3 to 3.6 cm s ⁻¹	Important but no concrete	Compactness of

shear force		evidence as the primary inducer for granulation.	mature granules Physical structure of mature granules
Feast-famine regimen	Periodic	Favors granulation but no concrete evidence as the primary inducer for granulation.	Cell surface hydrophobicity EPS production
Dissolved oxygen	0.7 to 6.0 mg L ⁻¹	No.	Microbial activities
Reactor configuration		High H/D ratio favours for circular hydraulic attrition but no concrete evidence as the primary inducer for granulation.	Physical structure of mature granules
Solid retention time	3 to 40 d	No	Microbial activities
Cycle time	30 min to 24 h	Not a critical factor but too long or too short will inhibit granulation.	Size of granules
Settling time	2 to 20 min	Yes. Major hydraulic selection pressure.	EPS production Cell surface hydrophobicity
Exchange ratio	20 to 80%	Yes.	EPS production
Discharge time	5 to 20 min	Yes.	EPS production

Adapted from Liu Yu, 2008.

2.5 SUMMARY

Interactions between microbial species and their environment are crucial to the structure and function of microbial communities in nature. Understanding of the interaction dynamics is therefore a key step to unravel the complexity of microbial communities and is of significant importance for better control and manipulation of the community behaviour. In many cases, QS signalling has been proposed or demonstrated to be important for interspecies interactions in simple dual-species model systems (Elias and Banin, 2012). The relevance of QS signalling in the context of complex communities where the microbial diversity is constantly changing in relation to the temporal and spatial scale remains unclear. While accretion of QS signalling molecules in different natural habitats has been evidenced and many of the local communities were shown to respond to high concentrations of exogenous signals, the

principle and the physiological role of QS signalling in many of the respective communities have been inadequately addressed. In particular, the impact of many environmental factors, such as mass transfer in the context of structured biofilms, the coexistence of signal-quenching organisms, the variable nutrient supply over time and space on the effectiveness of QS signalling in natural environments remains to be explored. In this thesis, the highly complex activated sludge community cultivated in bioreactors was used as a model to investigate the ecological role of AHL mediated QS signalling under simulated environmental conditions on a long-term basis. Using these highly automated bioreactor model systems, it is possible to assess the simultaneous impact of a series of environmental factors on the production, accumulation, stability and function of AHLs. In particular, the relevance of AHL mediated QS signalling in community energy metabolism and structural assembly, as well as potential regulation of QS signalling by community QQ activities was investigated.

CHAPTER 3: QS SIGNALLING AND COMMUNITY METABOLISM

3.1 INTRODUCTION

In population studies, where single-species systems are used, quorum sensing (QS) signalling has been commonly shown to synchronize microbial social activities, including biofilm formation, virulence factor expression, and nutrient acquisition (Davies *et al.*, 1998; Smith and Iglewski, 2003; Studer *et al.*, 2008). More recently, the role of QS signalling has been shown to be involved in the regulation of central metabolism (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007; Sharif *et al.*, 2008). For example, the Cyanobacteria *Gloeotheca* was reported to rely on *N*-acyl-homoserine-lactone (AHL) mediated regulation to control carbohydrate and amino acid metabolism (Sharif *et al.*, 2008) where addition of exogenous AHLs was found to induce expression of genes involved in carbon dioxide fixation and amino acid synthesis in *Gloeotheca* sp. Similarly, activation of QS has also been demonstrated in *Pseudomonas aeruginosa* to modulate the levels of denitrification metabolites, such as nitrite and nitric oxide under anoxic conditions (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007). These findings suggest that QS signalling is not only important for biofilm development or virulence, but is also critical in regulating metabolic functions of microbial populations.

While the significance of QS has been clearly exemplified in many bacteria based population studies, the understanding of QS in complex microbial communities where multiple species coexist is relatively limited. Nevertheless, there is increasing evidence that AHL mediated QS is ubiquitous in natural and engineered ecosystems, including microbial mats, wastewater treatment plants, membrane bioreactors or in the rumen (Decho *et al.*, 2009; Yeon *et al.*, 2009a; Hughes *et al.*, 2010; Chong *et al.*, 2012). For example, a diverse array of AHLs, ranging from 4-14 carbons, were found to accumulate within microbial mats and their concentrations fluctuated according to the diel pH (Decho *et al.*, 2009). While attempts to investigate the functions of AHLs in these complex community systems have been made through exogenous addition of signals, the intrinsic role of AHL mediated QS in these systems remain poorly understood (Valle *et al.*, 2004; De Clippeleir *et al.*, 2011; Chong *et al.*, 2012). Further, many fundamental questions, such as signal concentration dynamics, signal threshold

for gene activation and the identity of signal producers and responders in complex microbial communities remain to be fully addressed.

Using a floccular sludge system, involving more than 500 different bacterial species as a model mixed microbial system, a long-term study, i.e. 82 weeks (574 days), was undertaken to examine the role of AHL mediated QS signalling in community metabolism. In particular, the study addressed the potential function of AHL mediated QS signalling in regulating simultaneous nitrification, denitrification and phosphorus removal (SNDPR) process, in which oxygen, nitrate and nitrite are used as the electron acceptor for energy recovery while phosphorus (orthophosphate) is stored as polyphosphate. The results showed that AHL mediated QS signalling was closely linked to nitrite accumulation, involved multiple community members and this QS signalling system employed picomolar to nanomolar concentrations of AHLs.

3.2 MATERIALS AND METHODS

3.2.1 Reactor setup and operation

A sequencing batch reactor (SBR; 10 x 76.4 cm, diameter x height) was operated as described by Zhou *et al.* (2010). Briefly, the SBR was seeded with floccular sludge from a water reclamation plant (Ulu Pandan, Singapore) that had been operated for municipal wastewater treatment. The SBR sludge culture was fed with synthetic wastewater (SWW) comprised of 150 mg COD L⁻¹ acetate and 50 mg COD L⁻¹ propionate as carbon source, 20 mg L⁻¹ ammonium, 10 mg L⁻¹ orthophosphate and trace nutrient elements (Smolders *et al.*, 1994). The SBR had a final working volume of 4 L and was operated at room temperature, c.a. 22°C ± 1°C. The operation of the SBR involved a 5 h cycle with two continuous stages of anaerobic, aerobic and anoxic periods (Zhou *et al.*, 2010). A total volume of 1 L SWW was fed per cycle and resulted in a hydraulic retention time (HRT) of 20 h. Bioreactor pH was maintained between pH 6.7 to 8.2 under the control of a programmable logic controller (PLC) via dosing of either 9.12 g L⁻¹ HCl or 10.0 g L⁻¹ NaOH. During the first 16 weeks of operation, dissolved oxygen (DO) was supplied at 2.0 to 3.0 mg L⁻¹ during the aerobic period. The DO level was subsequently reduced to and maintained at 1.0 to 1.5 mg L⁻¹ from week 16 onwards to achieve the nitrite shortcut (Zhou *et al.*, 2010). Nitrogen gas (N₂) was sparged intermittently at the anaerobic and anoxic periods to remove residual oxygen, through the PLC control.

3.2.2 Cycle study and sampling for analysis

System performance of the SBR was monitored via weekly cycle studies. In each cycle study (5 h), bioreactor mixed liquor was sampled at the end of each single phase (i.e. feeding, anaerobic, aerobic and anoxic). The mixed liquor was filtered to remove the biomass. The filtered supernatant was used to determine the concentration of ammonium (NH₄⁺), nitrite (NO₂⁻), nitrate (NO₃⁻) and orthophosphate (PO₄³⁻) in accordance to the APHA standard engineering methods (Eaton *et al.*, 2005). The highest concentration of nitrite and nitrate determined during the cycle were used to calculate the maximum nitrite to nitrate ratio. The concentration of sludge biomass, i.e. mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were also determined at the end of the cycle study according to the APHA methods (Eaton *et al.*, 2005). For consistency, the sludge biomass reported in the text

was based on the MLVSS unless otherwise specified. In each cycle study, samples for RNA and AHL analysis were collected. For RNA samples, 1 mL aliquots of uniformly mixed sludge samples were collected at the end of the anoxic period (2nd stage). The sludge biomass was immediately harvested at 8,000 x g for 5 min at 4°C and stored at -80°C for subsequent analysis. Similarly, 50 mL aliquots of treated effluents were kept at -80°C immediately after sampling for later extraction of AHLs.

3.2.3 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 3-1. *Agrobacterium tumefaciens* A136, *Chromobacterium violaceum* CV026, *Escherichia coli* JM109, *Escherichia coli* JBA357 and *Pseudomonas aeruginosa* MH602 were routinely maintained in Luria-Bertani medium supplemented with 5% w/v sodium chloride (LB5) and 1.5% w/v agar, unless otherwise indicated. Antibiotics were supplemented into the growth medium whenever necessary: tetracycline (4.5 µg mL⁻¹), spectinomycin (50 µg mL⁻¹), kanamycin (50 µg mL⁻¹), ampicillin (100 µg mL⁻¹) or gentamycin (40 µg mL⁻¹). Cultures on agar plates were incubated at 30°C for 24-48 h while the liquid cultures were incubated with constant agitation on a shaker at 200 rpm at 30°C for 24 h.

Table 3-1. Bacterial strains used in this study.

Strain	Genotype and/or phenotype ^a	Reference
<i>A. tumefaciens</i> A136	AHL biosensor, carrying pCF218 plasmid with <i>traR</i> , and pCF372 plasmid with <i>P_{traI}-lacZ</i> ; Tc ^R , Sp ^R	(Fuqua and Winans, 1996)
<i>C. violaceum</i> CV026	AHL biosensor, a double mini-Tn5 mutant from <i>C. violaceum</i> ATCC 31532; Kan ^R	(McClellan <i>et al.</i> , 1997)
<i>E. coli</i> JM109	<i>rec A1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ (lac-proAB) mcrA [F' traD36 proAB lacIq lacZ ΔM15]</i>	(Yanisch-Perron <i>et al.</i> , 1985)
<i>E. coli</i> JBA357	AHL biosensor, carrying pJBA89 plasmid with <i>luxR-P_{luxI}-RBSII-gfp</i> ; Amp ^R	(Andersen <i>et al.</i> , 2001)
<i>P. aeruginosa</i> MH602	AHL producing strain, carrying a pMH602- <i>gfp</i> plasmid; Gm ^R	(Hentzer <i>et al.</i> , 2002)

^a Abbreviations: Tc^R, tetracycline resistance; Sp^R, spectinomycin resistance; Kan^R, kanamycin resistance; Amp^R, ampicillin resistance; Gm^R, gentamycin resistance; *gfp*, green fluorescent protein.

3.2.4 *N*-Acyl-homoserine-lactones (AHLs)

Synthetic AHLs (Sigma-Aldrich, Singapore) used in this study are listed in Table 3-2. The synthetic AHLs were prepared freshly in 100% methanol (HPLC grade, Sigma Aldrich) to achieve a primary stock concentration of 1.0 g L⁻¹. The primary stocks were further diluted to achieve their respective final concentrations, individually or in combination, where appropriate using methanol:water (1:1 v/v) to serve as standards for subsequent thin-layer chromatography (TLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described in Sections 3.2.7, 3.2.8 and 3.2.9. For experiments involving addition of AHL signals to the cultures, the synthetic AHLs were freshly prepared in dimethyl sulfoxide (DMSO) to achieve a primary stock concentration of 100 mM. The primary stocks were further diluted in DMSO or culture media to achieve their respective working concentrations.

Table 3-2. List of *N*-acyl-homoserine-lactones (AHLs).

AHL Signal	Abbreviation
<i>N</i> -butyryl-DL-homoserine lactone	C4-HSL
<i>N</i> -(3-oxohexanoyl)-DL-homoserine lactone	3OC6-HSL
<i>N</i> -hexanoyl-DL-homoserine lactone	C6-HSL
<i>N</i> -heptanoyl-DL-homoserine lactone	C7-HSL
<i>N</i> -(3-oxooctanoyl)-L-homoserine lactone	3OC8-HSL
<i>N</i> -octanoyl-DL-homoserine lactone	C8-HSL
<i>N</i> -(3-oxodecanoyl)-L-homoserine lactone	3OC10-HSL
<i>N</i> -decanoyl-DL-homoserine lactone	C10-HSL
<i>N</i> -(3-oxododecanoyl)-L-homoserine lactone	3OC12-HSL
<i>N</i> -dodecanoyl-DL-homoserine lactone	C12-HSL
<i>N</i> -(3-hydroxydodecanoyl)-DL-homoserine lactone	3OHC12-HSL
<i>N</i> -(3-oxotetradecanoyl)-L-homoserine lactone	3OC14-HSL
<i>N</i> -tetradecanoyl-DL-homoserine lactone	C14-HSL

3.2.5 AHL detection bioassays

3.2.5.1 *A. tumefaciens* A136 agar based filter bioassay

An agar based AHL detection assay, using *A. tumefaciens* A136 as the biosensor, was adopted to detect the presence of cultivable AHL producing bacterial populations in the sludge community. Briefly, an overnight culture of *A. tumefaciens* A136 in LB5 was diluted 1:50 with fresh minimal salt medium (ABT) (Clark and Maaloe, 1967) and incubated at 30 °C with constant shaking at 200 rpm for 24 h. The indicator agar plates were prepared by mixing 50 mL of the 24 h culture with 100 mL of 1.2% w/v molten ABT agar and 50 µg mL⁻¹ of 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-

gal). The indicator agar plates were allowed to set and dry for 1 h. Ten milliliters of sludge samples were filtered through the 0.45 μm sterile filters (Pall Corp.) and the biomass retentates were placed onto the indicator agar plates. Heat inactivated sludge samples were included as negative controls, while an overnight culture of *P. aeruginosa* MH602 was used as the positive control. The indicator agar plates were incubated at 30 °C for 24-36 h.

3.2.5.2 *E. coli* JBA357 96 well microtitre plate bioassay

For detection of AHLs produced *in situ*, a 96 well microtitre plate bioassay was adopted using the biosensor *E. coli* JBA357 which expresses the green fluorescent protein (GFP) upon detecting AHLs. The overnight culture of *E. coli* JBA357 was first diluted 1:5 (v/v) with fresh LB5 medium and 100 μL of the diluted biosensor culture were added to 100 μL of sludge sample in a 96 well plate. The culture plate was subsequently incubated at room temperature, c.a. $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with constant shaking at 200 rpm for 4 h prior to the visualization of green fluorescent cells using confocal laser scanning microscopy (CLSM) (LSM710, Carl Zeiss) at an excitation/emission wavelength of 488 nm/522-535 nm. Further, the GFP expression by the biosensor, as a result of the presence of AHLs, was quantified at an OD_{488} using a microplate reader (Tecan Infinite M200). A heat inactivated sludge sample was included as the negative control. SWW to which synthetic AHL, 3OC6-HSL, was added at 10 nM was used as a positive control for the *in situ* AHL detection assay.

3.2.6 Extraction of AHLs

Extraction of AHLs from the treated effluent samples (bulk liquid/sludge supernatants) was performed as described by Shaw *et al.* (1997). Fifty milliliters of the sludge supernatants were extracted twice with 100 mL of dichloromethane (DCM). The DCM extract was allowed to dry using rotary evaporator and stored at -80°C until further analysis by TLC or LC-MS/MS, as described in Sections 3.2.7 and 3.2.8, respectively. The extraction efficiency for each AHL was calculated based on the recovery of 13 standard AHLs (Table 3-2) added at $5 \mu\text{g L}^{-1}$ and $50 \mu\text{g L}^{-1}$, to the heat inactivated sludge supernatant sample matrix using LC-MS/MS. In general, the recovery rates for all the AHLs ranged from 50% to 80%. The extraction efficiency for individual AHLs was factored into the subsequent quantification of the native AHLs present in each

sample. For all of the extraction experiments, a minimum of three independent extractions for each sample were conducted.

3.2.7 Characterization of AHLs by thin-layer chromatography (TLC)

The DCM extract was resuspended in 50 μL of methanol:water (1:1 v/v). Ten microliters of the sample extract were applied to C_{18} reversed-phase TLC plates (200 mm layer; Merck) and developed with methanol:water (60:40 v/v) (Shaw *et al.*, 1997). The plates were allowed to dry by evaporating the solvent after development but prior to the overlay of the biosensor consisting of either the *A. tumefaciens* A136 or the *C. violaceum* CV026 in molten agar. For the *A. tumefaciens* A136 overlay, overnight culture of the biosensor was diluted 1:50 (v/v) in fresh ABT medium with 0.6% w/v agar and 50 $\mu\text{g mL}^{-1}$ of X-gal. The culture was mixed thoroughly and immediately spread over the developed plate to produce an indicator overlay with a final thickness of 3 mm in a sterile plastic container. After the agar solidified, the culture plate was incubated at 30°C for 24 h. Similarly, the *C. violaceum* CV026 overlay was prepared by seeding fresh 0.6% w/v molten LB5 agar with 1:100 (v/v) overnight culture of the biosensor. Synthetic AHLs were included as standards where appropriate.

3.2.8 Detection of AHLs by mass spectrometry (MS)

The DCM extract was subjected to further analysis by LC-MS/MS (LCMS8030, Shimadzu Inc.). Briefly, 10 μL of the sample extract were chromatographed using a C_{18} reversed-phase column (Shim-pack XR-ODS column: 2 x 100 mm, 2.2 μm) at a flow rate of 0.3 mL min^{-1} . The mobile phase consisted of solvent A (25 mM ammonium formate with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). A gradient elution method was utilized which started at 5% solvent B for 0.5 min, followed by a linear gradient from 5 to 40% solvent B over 0.5 min, then to 95% solvent B over the next 5 min and remained isocratic at 95% solvent B for 1.5 min. The column was reequilibrated at 5% solvent B for 1 min. The AHL compounds separated by the LC system were ionized by electrospray ionization (ESI) under positive mode and detected using multiple reaction monitoring (MRM) approach (Morin *et al.*, 2003; Decho *et al.*, 2009). Matrix-matched MRM experiments were performed for all 13 standard AHLs (Table 3-2). MRM experiments were conducted with Q1 set to monitor for the precursor ion and Q3 for the specific transition ions according to the optimized

parameters for each AHL (Table A-1). The identity of each putative AHL in the DCM extract was confirmed based on specific LC retention time, appearance of the precursor ion m/z and two transition ions, as well as relative intensity of the two transition ions in comparison to the individual standard AHL. In addition, a full scan, ranging from m/z 100 to 350, coupled with precursor ion scan mode was conducted to provide further confirmation of the identity of putative AHLs in the each sample extract with respective to the standard AHLs (Decho *et al.*, 2009). Duplicate injections were performed for each sample extract. Triplicate blank injections were performed between sample injections to prevent sample carryover.

3.2.9 Quantification of AHLs by MS

For quantification of native AHLs present in the supernatant of sludge sample (or treated effluents), matrix-matched standard curves, ranging from $0.5 \mu\text{g L}^{-1}$ to $200 \mu\text{g L}^{-1}$, for all 13 standard AHLs were constructed using the optimized MRM method as described above. Two transition ions, characteristic of the respective AHLs, were used in the MRM approach and the transition ion with highest intensity was used to construct the standard curve (Table A-1). The analyte peak areas were integrated using a quantification software package (LabSolutions software, Shimadzu Inc.) and each integrated peak was inspected manually. The limits of detection (LOD) and quantification (LOQ) for each AHL were calculated with a signal-to-noise ratio of 3.3 and 10, respectively (Decho *et al.*, 2009). Correspondingly, the amount of each putative AHL in the sample extract was quantified based on the standard curve of the respective AHL with the extraction efficiency being factored in the calculation as described above (Section 3.2.6).

3.2.10 RNA extraction for meta-transcriptomic studies

Total RNA was extracted from sludge biomass using the FastRNA Pro Soil-Direct kit (MP Biomedicals) according to the manufacturer's guidelines. The extracted RNA was subjected to an additional DNA removal step using the TURBO DNA-free kit (Applied Biosystems) to remove any residual genomic DNA present in the RNA sample. The quality of RNA sample was examined using the Agilent 2100 bioanalyser with Agilent RNA 6000 kit and reported as the RNA integrity number (RIN). The quantity of total RNA and residual DNA were measured using Quant-iT RiboGreen RNA and

PicoGreen DNA assays (Invitrogen), respectively. Two hundred nanograms of total RNA were used for complementary DNA (cDNA) library preparation according to the manufacturer's instructions (Illumina Inc.). Each cDNA library was ligated with a unique adaptor sequence for sample multiplexing. A total of 16 different cDNA libraries were pooled and sequenced using the Miseq System (Illumina Inc.) as per the manufacturer's guidelines.

3.2.11 Sequence analysis

Total RNA sequencing data was analyzed using a fast tag-based method developed here. Briefly, a universal primer (CGACRRCCATGCANACCT) for the 16S rRNA hypervariable region (V6) was used to scan each sequencing read to obtain all 33 nt tag sequences downstream of the primer. The universal primer matches 94% of 16S rRNA sequences in the RDP database (Claesson *et al.*, 2010). Each different V6 tag was used as a signature sequence to represent one operational taxonomic unit (OTU). To eliminate sequencing error derived V6 tags, only V6 tags that were observed in at least 2 different reads were kept for analysis. To further filter for possible PCR duplication artefacts where abnormally high number of identical reads arise, V6 tags were removed where any single type of sequencing read accounted for $\geq 50\%$ of all reads covering that V6 tag.

3.2.12 Statistical analysis

All statistical analyses were performed using Prism version 5 for Windows (GraphPad Software Inc.). For correlation studies, Pearson correlation indexes were calculated and the resulting matrixes were clustered using an unsupervised hierarchical clustering method based on Euclidean distance with complete linkage as coded by Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and visualized using Java TreeView (<http://jtreeview.sourceforge.net>).

3.3 RESULTS

3.3.1 System performance of a floccular sludge community in a sequencing batch reactor (SBR)

AHL mediated QS signalling has recently been indicated to play a key role in energy metabolism, such as denitrification in *P. aeruginosa* (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007) and anaerobic ammonium oxidation in sludge communities (De Clippeleir *et al.*, 2011). In this study, the role of AHL mediated QS in community metabolism involving simultaneous nitrification, denitrification and phosphorus removal (SNDPR) via the nitrite pathway was investigated. The study system involved inoculation of an activated sludge consortium from a water reclamation plant (Ulu Pandan, Singapore) into a sequencing batch reactor (SBR) which was maintained under conditions selecting for the SNDPR functions for 82 weeks at room temperature, c.a. $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (Figure 3-1). To enrich for the community specialized in removing nitrogen via nitrite instead of the conventional nitrate pathway, dissolved oxygen (DO) that was initially supplied at a concentration of 2.0 to 3.0 mg L⁻¹ was reduced to 1.0 to 1.5 mg L⁻¹ from week 16 onwards. An active nitrite pathway was indicated by a high ratio of maximum nitrite to nitrate measurement ($\text{NO}_2^-:\text{NO}_3^-$) (Figure 3-1: A). The ratio increased steadily from an average of 1.1 prior to week 16 to a ratio of 116.7 at week 30. The high ratio (> 10) was maintained for the subsequent 33 weeks before it decreased to less than 1 at week 67. Removal of total nitrogen, in terms of ammonium, nitrite and nitrate, was also monitored (Figure 3-1: B). Stable removal of nitrogen at an average of 75% was achieved although there were periods where the nitrogen removal was low. There was no statistically significant difference for the efficiency of nitrogen removal during the high (Week 1 to 16; $n = 12$; 75.5%) versus the low (Week 17 to 82; $n = 32$; 74.1%) DO supply period (Unpaired t-test, $p = 0.7344$), nor there was a difference among the first nitrate dominant period ($\text{NO}_2^-:\text{NO}_3^- < 1$; Week 1 to 16; $n = 12$; 75.5%), the nitrite dominant period ($\text{NO}_2^-:\text{NO}_3^- > 1$; Week 17 to 63; $n = 24$; 70.3%) and the second nitrate dominant period ($\text{NO}_2^-:\text{NO}_3^- < 1$; Week 64 to 82; $n = 7$; 84.7%) (One way-ANOVA; $p = 0.1387$). For the phosphorus removal, two distinct cycles of low followed by high and stable removal efficiencies were recorded (Figure 3-1: C). The first cycle spanned weeks 1 to 46 in which an average removal efficiency as low as 15.7% was observed prior to week 15, while almost 100% of phosphorus supplied was removed subsequently during weeks 15-46. The second cycle which started from week 47

appeared to remove on average 21.3% of phosphorus from weeks 47 to 60, followed by almost complete removal of phosphorus thereafter. Sludge biomass or MLVSS was maintained within a range of 2,500 to 3,000 mg L⁻¹ throughout the experiment; while the sludge particle size was found to fluctuate between an average size of 80 to 110 µm. Microscopic analysis of the biomass structure indicated a consistent appearance of the flocculent biomass, similar to the activated sludge inoculum (data not shown).

Figure 3-1. Long-term system performance of the floccular sludge community over a period of 82 weeks. Maximum ratio of nitrite (NO_2^-) to nitrate (NO_3^-) (A), total nitrogen removal including ammonium (NH_4^+), NO_2^- and NO_3^- (B) and phosphorus removal (C), were determined as per cycle study on a weekly basis (Section 3.2.2). The dotted line (Week 16) separates the low dissolved oxygen (DO) supplied period, 1.0–1.5 mg L⁻¹, and the high DO supplied period, 2.0–3.0 mg L⁻¹. Details of cycle study analysis for particular weeks can be found in Figure A-1.

3.3.2 AHLs are produced by the floccular sludge community

QS activity was assessed by first determining if there were AHL producing bacteria in the floccular sludge community using the *A. tumefaciens* A136 agar based filter bioassay. In the presence of AHLs, the *A. tumefaciens* A136 biosensor expresses β -galactosidase that breaks down the X-gal substrate resulting in the production of a blue-colored product that can be easily visualized as a zone of blue pigmentation in the agar medium. When the retentate of the SNDPR sludge was placed onto the indicator plate, a zone of blue pigmentation in the agar medium appeared around the filter containing the sludge biomass, suggesting the presence of abundant QS organisms in the sludge community (Figure 3-2: A-i). Because many bacteria naturally produce β -galactosidase, a negative control was used, where the biosensor strain was left out of the indicator plate. Under these conditions, degradation of the X-gal was only observed in association with the bacterial biomass but not in the agar medium around the filter (Figure 3-2: A-ii). Thus, the zone of induction in the agar medium around the filter with the live SNDPR sludge is likely to be due to diffusion of the AHLs from the microbial colonies into the agar around the filter (Figure 3-2: A-i). None of the heat inactivated samples developed any form of blue coloration (Figure 3-2: B-i-ii).

Figure 3-2 (previous page). Agar based detection of diffusible AHLs, released by the floccular sludge community, using the *A. tumefaciens* A136 biosensor. A filter containing the sludge biomass retentate was placed on an ABT indicator plate with (A-i) or without the biosensor strain (A-ii) and the culture plates were further incubated at 30°C for 24 h prior to inspection. Heat inactivated sludge samples were included as negative controls (B-i to B-ii). Perception of AHLs by the biosensor is indicated by the appearance of blue color zone within the indicator agar plate as shown by the white arrow (A-i).

The AHL biosensor strain *E. coli* JBA357 was used to determine if AHLs were present and could be detected *in situ* in the floccular sludge community (Figure 3-3). In the presence of AHLs in the environment, the biosensor expressed the green fluorescent protein (GFP), which was visualized by confocal laser scanning microscopy (CLSM). When added to the sludge community, clusters of green fluorescent cells around the biomass were observed, indicating activation of the biosensor strain by AHLs (Figure 3-3: A). In contrast, the heat killed sludge sample did not activate the *E. coli* JBA357 biosensor (Figure 3-3: B). When the fluorescent intensity was quantified using a microplate reader, the level of fluorescence induced by the AHLs in the active sludge sample increased 3 to 20 fold (Figure A-2). To verify that AHLs were present in both sludge supernatant and within sludge biomass, samples were extracted followed by analysis using thin layer-chromatography (TLC) combined with the *A. tumefaciens* A136 biosensor. The putative AHL species present in the extracted sample was identified in comparison to the AHL standards which were separated based on the unique mobility of each AHL species on the TLC plate. It was found that both supernatant and biomass extract contained 3OC8-HSL during two consecutive weeks of bioreactor operation (Figure 3-4), suggesting that the floccular sludge community was actively engaged in AHL mediated QS signalling. However, the identity of the compound(s) that migrated more slowly on the TLC plate than the 3OC8-HSL observed in most samples (L1 to L5) remains unknown.

Figure 3-3. *In situ* detection of AHLs, released by the floccular sludge community, using the *E. coli* JBA357 biosensor. Co-incubation of the live sludge sample (A), or the heat inactivated sludge sample (B) with the biosensor at room temperature for 4 h with a constant shaking at 200 rpm prior to inspection by confocal laser scanning microscope (CLSM). The heat inactivated sludge sample served as a control for background fluorescence of the sludge biomass (auto-fluorescence). Additional controls for the bioassay included the addition of exogenous 3OC6-HSL at 10 nM to the biosensor as a positive control for the induction of the biosensor strain (C) and the presence of the biosensor alone as a negative control (D). Perception of AHLs by the biosensor is indicated by the appearance of green fluorescent cells. Images of green fluorescent cells were captured at 488 nm (i), unstained cells were visualized by

differential interference contrast (DIC) (ii) and the superimposed image of the two is shown in the right-end panel (iii). The black arrow indicates the sludge biomass. Magnification, x200; Scale bar, 100 μm .

Figure 3-4. TLC analysis of AHLs. Samples were chromatographed on a C18 reverse phase thin-layer plate, developed with methanol:water (60:40, v/v) and the spots were visualized with *A. tumefaciens* A136 biosensor. Standard AHLs: 2.5 pmol 3OC10-HSL (S1), 0.25 pmol 3OC8-HSL (S2) and 1.0 pmol 3OC6-HSL (S3). Samples are dichloromethane extracts of the following: 200 ml of sludge supernatant at week 17 (L1), 50 ml of sludge supernatant at weeks 17 (L2) and 18 (L4), and the sludge biomass, derived from 50 mL of sludge suspension, at weeks 17 (L3) and 18 (L5). The letter 'a' indicates the presence of 3OC8-HSL in the respective sample.

3.3.3 The levels of AHLs positively correlate with the nitrite concentrations

To further examine the relationship between the native AHLs and the community metabolism, a statistical approach was adopted to determine the correlation between the AHL expression and the system performance on a temporal basis. Since the approach requires precise and accurate measurement of the AHL concentration over time, the AHL analysis was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using standard curves and mass spectra of 13 different synthetic AHLs as quantitative references (Figure A-3). The experimentally determined extraction efficiency, which for each of the 13 standards ranged between 50 and 80%, was factored into the calculation of AHL concentration. AHLs ranging from C4 to C8 were detected in the supernatants sampled over 82 weeks while AHLs with 10 carbons or more were not resolved within the detection limit of the LC-MS/MS (0.2 pmol g⁻¹ to 2 pmol g⁻¹ after sample concentration and biomass normalization) (Figure 3-5). AHLs

that were the most commonly detected in this system over time were identified to be 3OC6-HSL (Figure 3-5: C) and 3OC8-HSL (Figure 3-5: E), consistent with the TLC results (Figure A-4). The amount of 3OC6-HSL and 3OC8-HSL detected in the sludge supernatant, after normalisation to sludge biomass, was estimated to be in a range of 1 to 100 pmol g⁻¹ (Figure 3-5: C and E) or 1 to 300 pM equivalent in concentrations (Figure A-5: C and E). Interestingly, the concentrations of these dominant AHLs and a lower abundant species, C8-HSL (Figure 3-5: D), were consistent from weeks 16 to 65 (2 to 50 pmol g⁻¹), which overlapped with the nitrite-prevailing period ($\text{NO}_2^-/\text{NO}_3^- > 1$; weeks 17 to 63) (Figure 3-1: A). When the relationship between AHL production and system performance was compared using the Pearson correlation method, both 3OC6-HSL and 3OC8-HSL appeared to have strong, positive correlation coefficients of 0.8192 ($p = 0.0002$) and 0.8062 ($p = 0.0003$), respectively, with the maximum nitrite to nitrate ratio (Figure 3-6). Other correlations, including AHLs versus total phosphorus removal, either did not reach a significant correlation threshold (± 0.75) or did not appear to be statistically significant ($p > 0.05$). Collectively, the correlation study suggests that specific AHLs may mediate the nitrite shortcut pathway, possibly involving enrichment of unique community members that are functionally important in both AHL signalling and nitrite pathway within the sludge biofilms.

Figure 3-5. LC-MS/MS profiling of AHLs released by the floccular sludge community into the supernatant over a period of 82 weeks. Samples extracted from 50 ml of sludge supernatants (treated effluents), which were collected on different bioreactor operational weeks, were analysed using MRM approach. The identity and quantity of individual AHLs in each sample, such as C4-HSL (A), C6-HSL (B), 3OC6-HSL (C), C8-HSL (D), and 3OC8-HSL (E), were determined based on comparison to standard curves generated for 13 different AHLs. The combined total amount of AHLs present is represented in (F). The data are normalized by the respective sample biomass. All results are shown as Mean $\pm$ SEM, three independent extractions per sample point.

Figure 3-6. The correlation between the expression of specific AHLs in the floccular sludge community with nitrogen removal via the nitrite shortcut. The relationship between the expression of individual AHL and the SNDPR system performance over time, in terms of total phosphorus and nitrogen removal, as well as nitrite to nitrate ratio ($\text{NO}_2^-:\text{NO}_3^-$), were calculated based on Pearson correlation and the correlation matrix was subsequently clustered using unsupervised hierarchical clustering based on Euclidean distance with complete linkage method (A). Individual relationship between the maximum nitrite to nitrate ratio and the expression of 3OC6-HSL (B) as well as 3OC8-HSL (C) are shown.

3.3.4 The correlation between community composition, AHL production and nitrite accumulation

A meta-rRNA sequence analysis was performed to characterize the changes in the sludge community composition over time. Analysis of a total of 156,163 16S rRNA gene sequences (using the V6 tag) (Table A-2) revealed a distinct community shift from high to low diversity when the dissolved oxygen (DO) level decreased from 2.0-3.0 mg L⁻¹ to 1.0-1.5 mg L⁻¹, as indicated by the Shannon-Weaver index (Figure A-6). The index decreased from an average of 18.4 prior to week 16 to an average of 10.1 from week 30 onwards and was relatively stable for the remainder of the experimental period. The relationship between the abundance of the top 50 most dominant community members with the AHL profiles (Figure 3-5: A-E) and the maximum nitrite to nitrate ratio (Figure 3-1: A) were determined using the Pearson correlation

coefficient method. Based on the Euclidean distance of classification, these correlations were grouped into three distinct clusters (Figure 3-7). Members in Cluster 1 showed strong, positive correlations with at least one of the AHLs, suggesting they may be involved in QS signalling, e.g. signal production. Interestingly, members in this cluster were also positively associated with the high nitrite to nitrate ratio, indicating a close relationship between QS signalling and nitrite metabolism of a particular species of microorganisms, including members of *Candidatus Competibacter* (Tag 3) and *Denitratisoma* (Tag 10). While only around 20% of total active community residents were accounted for by Cluster 1, an even smaller proportion of community members (<10%) were grouped into Cluster 2. Members in Cluster 2 were weakly correlated with the AHLs, although they may be correlated to nitrite metabolism. In contrast, most of the Cluster 3 members demonstrated strong, negative correlations with at least one of the AHLs and importantly, most were also negatively associated with the nitrite accumulation and accounted for more than 70% of the total reactor community. This negative correlation suggests that these organisms were either inhibited by the QS and nitrite producing community members or possibly that these organisms were responsible for the loss of nitrite function or QS. The latter possibility is supported by the recent identification of groups of bacteria that produce AHL degrading enzymes, or quorum quenching enzymes (Uroz *et al.*, 2009). The potential role of quorum quenching in mediating community level behaviour will be further addressed in Chapter 5.

Figure 3-7. Unsupervised clustering of the relationship of the top 50 community members with the expression of AHLs and nitrite to nitrate ratio ($\text{NO}_2^-:\text{NO}_3^-$). The relationships between the abundance of top 50 most abundant community members (each represented by a unique V6 tag) and the concentration profile of AHLs and maximum nitrite to nitrate ratio over a time series of 82 weeks (16 time points in total) were calculated based on the Pearson correlation. The correlation matrix was subjected to unsupervised hierarchical clustering based on the Euclidean distance with complete linkage method. “***” represents a V6 tag that is not able to be annotated based on the taxonomy classification pipeline stated in Materials and Methods. Instead, the closest relative with sequence identity matching more than 97% is shown where it is applicable.

3.4 DISCUSSION

Quorum sensing (QS) signalling has been an intriguing area of research in part because it suggests social behaviour in bacteria, where phenotypes are coordinated at the population or community level through the production and reception of diffusible signals. These concepts have played a key role in the development of sociomicrobiology which attempts to study group behaviour of microorganisms (Parsek and Greenberg, 2005). While it is clear that QS organisms are part of larger microbial communities in the environment, such as in biofilms formed on river rocks (McLean *et al.*, 1997) and floccular sludge in wastewater treatment plants (Morgan-Sagastume *et al.*, 2005; Chong *et al.*, 2012), studying QS and its role in driving specific community behaviour has been challenging due to technical limitations and hence, the vast literature on QS is primarily based on mono-culture, laboratory based systems. By building on technological advances in sequencing and analytical techniques, this work has begun to explore QS at the community level to address key questions on how QS systems function in complex environments and whether they are indeed effective mediators of community behaviour, specifically community metabolism in this case.

It was demonstrated here that a variety of AHLs ranging from 4 to 8 carbons (Figure 3-5) were consistently found in a floccular sludge bioreactor system undergoing simultaneous nitrification, denitrification and phosphorus removal (SNDPR) over an extended period of 82 weeks (Figure 3-1). Importantly, the expression of AHLs were found to link to the nitrification/denitrification process via the nitrite pathway (Figure 3-6). Elevated levels of AHL species, such as 3OC6-HSL and 3OC8-HSL were strongly associated with the switch from the nitrate-dominant period to the nitrite-prevailing period. Even though the dominance of nitrite may be initiated by a lowered DO concentration (e.g. $1.0 - 1.5 \text{ mg L}^{-1}$ on week 16 of the operation time), it was clearly shown that the levels of nitrite in the bioreactor environment fluctuated according to the concentrations of AHLs thereafter, instead of the DO levels, suggesting that AHL mediated QS may be involved in the nitrite shortcut during the nitrogen removal process (Figures 3-1 and 3-6). In other wastewater treatment systems, AHL mediated QS has also been, directly or indirectly, demonstrated or postulated to play a role in bioprocesses (Valle *et al.*, 2004; De Clippeleir *et al.*, 2011; Chong *et al.*, 2012). For example, sludge communities were found to respond swiftly to the

exogenously added AHL signals by enhancing phenol degradation (Valle *et al.*, 2004), increasing chitinase activities (Chong *et al.*, 2012), elevating anoxic ammonium oxidation rate (De Clippeleir *et al.*, 2011) and modifying the community composition (Valle *et al.*, 2004). Moreover, activation of the QS response has also been reported in pure culture of *P. aeruginosa* to regulate the expression of genes involved in the denitrification process to modulate the levels of nitrate, nitrite and other associated denitrification metabolites (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007). The strong, positive correlation between the long-term expression of AHLs and nitrite production observed in this study, suggests that QS mediated regulation of the nitrification/denitrification process may also be relevant in this experimental floccular system (Figure 3-6), although further confirmation of the role and the mechanism of QS in regulating nitrite production would be necessary. Together, these observations suggest that QS signalling is prevalent in the complex community systems and is not merely a population-specific property.

In population based QS, the signal concentration is the key determinant in synchronizing gene expression among signal responders (Williams *et al.*, 2007). Depending on the microbial species and the target genes involved, signal thresholds for coordinated gene activation or repression can vary from 1 to 1,000 nM, while the total signals accumulated in the liquid environment can be as high as 10 μ M in pure culture systems (Pearson *et al.*, 1995; Schaefer *et al.*, 2008). In contrast, AHLs that had accumulated in the bulk liquid medium of the bioreactor were present at unexpectedly low concentrations (Figure A-5). Quantitative analysis using LC-MS/MS revealed the levels of AHLs in the bioreactor ranged from 1 to 300 pM. Despite the low concentrations observed in this system, the amount of signals, per gram of sludge biomass, strongly correlated to the levels of nitrite accumulated in the sludge environment over time (Figure 3-6). Using a fluorescent bioreporter strain, it was further shown that the *in situ* levels of AHLs in the sludge community were sufficient to induce QS response in the bioreporter, clearly demonstrating that low signal concentrations are physiologically and biologically relevant in this model ecosystem (Figure 3-3). Consistent with these observations, two novel QS systems, one based on the aryl-homoserine-lactone signal in *Bradyrhizobium* BTAil (Ahlgren *et al.*, 2011) and another based on the branched acyl-homoserine-lactone signal in *Bradyrhizobium*

japonicum (Lindemann *et al.*, 2011), have recently been reported to respond to signal concentrations as low as 10 pM. Moreover, *Nitrosomonas europaea*, an important ammonium oxidizing bacteria (AOB) commonly found in water and wastewater treatment plants, has been documented to synthesize AHLs at sub-nanomolar to low nanomolar concentrations, i.e. 0.4 to 2.2 nM, in a chemostat system (Burton *et al.*, 2005), while the endogenous levels of AHLs were reported to be less than 20 pM in soil (Wang and Leadbetter, 2005). While the roles of the AHLs in most natural environments remain unclear, these findings suggest that sub-nanomolar concentrations of extracellular AHLs maybe common in nature and are probably important for QS regulated community behaviour.

Alternatively, the signals that accumulate in sludge biofilms may matter more than signals released into the bulk liquid medium in coordinating community behaviour, and is related to a concept termed diffusion sensing or efficiency sensing (Redfield, 2002; Hense *et al.*, 2007; Dulla and Lindow, 2008). AHLs may accumulate within biofilms, where the bacterial cells are present at high densities, as a result of constrained liquid flow and restricted signal diffusion due to the physical barrier imposed by both biofilm cells and the associated extracellular polymeric substances (EPS) matrix. Accordingly, QS induction may occur within microenvironments found in the biofilm structures when the local signal concentrations reach the necessary threshold concentrations. For example, 3OC12-HSL has been documented to be present at least 600 fold in excess of the concentration in the bulk liquid medium, in flow-cell *P. aeruginosa* biofilms under conditions where the hydrodynamic shear force is sufficiently low (Charlton *et al.*, 2000). Spatial analysis revealed maximal expression of QS regulated genes near the substratum of the biofilms but decreased with the increasing biofilm height where the biofilm cells were gradually exposed to higher medium flow rate (De Kievit *et al.*, 2001). These observations suggest that QS signalling is active and functional in specific microenvironments and that localized signalling is proposed to be one of the dominant forms of communication in nature where microorganisms predominantly exist in highly structured communities (Kolter and Greenberg, 2006). Indeed, accumulation of AHLs in complex microbial biofilms, such as microbial mats has been reported recently (Decho *et al.*, 2009). Although the functional role of those signals remains to be explored, the high levels of signals accumulated in the mat biofilms suggest that QS is

likely to be biologically relevant to those communities. In this study, it was shown that AHLs were also present in the floccular sludge biofilms and the species of AHLs identified in the sludge biomass were identical to those secreted into the bulk liquid medium (Figure 3-4). Even though it was not possible to precisely quantify the amount of signals present in the sludge biomass extracts using LC-MS/MS due to the high complexity of the sample matrix, the signal concentration extracted from the floccular sludge biomass was estimated based on the TLC results (Figure 3-4). The dominant signal, 3OC8-HSL, was found to be present in the floccular biomass at > 20 nM, which was at least 100 fold higher than the signal concentration in the bulk liquid at the same time point. This observation of higher signal concentration in sludge biofilms was further supported by clustering of AHL activated biosensors around the sludge biomass when *in situ* AHL production was examined (Figure 3-3: A). Thus, it is highly likely that QS induction would be active in the floccular biomass tested here, and may be involved in the regulation of nitrite accumulation.

Nitrite and other downstream by-products of denitrification, such as nitric oxide (NO) are toxic to microbial cells when they accumulate to high concentrations (Fang, 1997). Thus, the levels of the metabolites must be constantly maintained at the sublethal concentrations for the survival of the community. In *P. aeruginosa*, it has been clearly demonstrated that activation of the QS response, mediated by C4-HSL and 3OC12-HSL, is essential for the growth of biofilms in anoxic conditions where nitrate and nitrite were used as the electron acceptors for energy recovery (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007). AHL mediated QS was found to negatively regulate expression of genes involved in the denitrification process, such as reductases for nitrate, nitrite, nitric oxide and nitrous oxide, and thus, controlling the rate of denitrification. In the absence of such QS regulatory systems, *P. aeruginosa* QS mutant biofilms were not able to survive anoxic growth conditions, suggesting the important role of QS signalling in cellular metabolism and survival of the cells (Yoon *et al.*, 2002). Accordingly, it is proposed here that community members of diverse origins may behave cooperatively via QS signalling to regulate the concentrations of nitrite in complex environment to protect the community from intoxication by the accumulated denitrification metabolites. Indeed, important AOBs, such as *N. europaea* and *Nitrospira multiformis*, which are commonly involved in nitrite metabolism in water

and wastewater treatment plants (Arp *et al.*, 2007), have been documented to either produce AHLs (Burton *et al.*, 2005) or to encode AHL syntheses in their genomes (Chain *et al.*, 2003; Norton *et al.*, 2008). Although the link between AHLs and nitrite metabolism in these AOBs remains to be illustrated, addition of exogenous AHLs has been shown to accelerate recovery of *N. europaea* from starvation and also its capability to oxidize ammonium to nitrite (Batchelor *et al.*, 1997). Clearly, these findings suggest a potential for QS to be involved in the nitrite metabolism in microorganisms commonly found in natural environments, consistent with our observation for the complex microbial community in the bioreactor system. Nevertheless, despite the positive correlation between AHL expression and nitrite production, it remains to be demonstrated mechanistically how AHL based QS could be involved in the regulation of nitrite accumulation in this complex ecosystem and if the low extracellular signal concentrations were sufficient to activate the QS control system.

Based on meta-rRNA sequencing, the abundance of multiple active bacterial species was found to be closely associated with QS signalling and nitrite metabolism (Figure 3-7: Cluster 1). These include members from genera *Candidatus competibacter* and *Denitratisoma*, which have been previously reported to be denitrifiers (Fahrbach *et al.*, 2006; Lemaire *et al.*, 2008). Here, it was shown that both *Candidatus competibacter* and *Denitratisoma* were likely contributing to the AHL production or may be responding positively to the AHL signals in the sludge community, and that AHL mediated QS is closely related to the regulation of community nitrite metabolism. However, the QS role of these organisms in nitrite metabolism remains to be experimentally verified. In addition, approximately 50% of the Cluster 1 organisms were taxonomically unclassified, indicating that many of the potential AHL producers/responders and nitrifiers/denitrifiers are yet to be cultured and characterized in natural or engineered environments. Consistent with these findings, a recent meta-omic study, in which almost complete community sequencing was achieved on a highly diverse, full-scale nitrifying/denitrifying plant, reported that the nitrification/denitrification processes were driven chiefly by community members that are yet to be classified taxonomically (Kjelleberg *et al.*, 2014). On the other hand, more than 70% of the community members were found to be negatively associated with the

expression of AHLs and nitrite accumulation (Figure 3-7: Cluster 3). These microorganisms were from diverse phylogenetic groups, such as Proteobacteria, Bacteroidetes, Planctomycetes, and other unclassified taxonomic groups. While the decrease in abundance of diverse microbial species may be in part explained by the accumulation of toxic nitrite (and NO) in the environment or competition between different microbial species, it may also reflect a negative response of microorganisms to the accumulation of AHLs, including members that degrade AHLs, as part of the community signalling regulation or competition strategy. Although much remains unknown about the AHL degrading (quenching) populations and their impact on the QS signalling in this floccular sludge community, coexistence of diverse species of AHL producers and quenchers has been previously reported in the soil communities (d'Angelo-Picard *et al.*, 2005; Cirou *et al.*, 2007). We therefore hypothesized that the extent of negative correlations between community members and AHL expression may indicate the co-occurrence of AHL quenchers. This hypothesis is explored in Chapter 5.

In this study, it has been shown that specific AHLs were consistently present in a complex activated sludge community over 574 days. In contrast to the commonly reported nanomolar to micromolar signal concentrations in pure culture laboratory systems, the AHLs were found to be present at picomolar levels in the bulk liquid medium of the bioreactor system while more than 20 nM of signals can be attained in the sludge biofilms. Despite the low signal concentrations in the bulk liquid, the levels of extracellular AHLs were strongly and positively correlated with nitrite production as well as the abundance of a diverse group of microorganisms in the bioreactor. While further experiments, including AHL add-back to the sludge community would be necessary to confirm the regulatory role of QS in this complex ecosystem, the current findings suggest that AHL based QS signalling can be potentially manipulated to modulate the specific pathway of community nitrogen metabolism during the process of nutrient removal. The nitrite shortcut, instead of the conventional nitrate pathway, is often preferred as it reduces the cost of operation in terms of dissolved oxygen and carbon source supplied, while controlling the amount of sludge biomass generated. Therefore, this study not only provides an insight into the complexity of community QS signalling but also a potential application to improve water and wastewater treatment processes, and sustainability of such systems.

CHAPTER 4 : QS SIGNALLING AND COMMUNITY ASSEMBLY

4.1 INTRODUCTION

Bacteria in natural habitats predominantly exist as matrix encased species-rich communities, either as surface associated biofilms or surface independent floccular aggregates. This is in contrast to their planktonic, single-species population counterparts which are commonly studied under laboratory conditions. The formation and dispersal of structured bacterial biofilms or aggregates occur in response to a range of environmental cues and signals, such as changes in nutrient concentrations, oxygen, temperature, as well as chemicals and predatory stresses (Bassler *et al.*, 1993; Matz *et al.*, 2004; McDougald *et al.*, 2011; Mitri *et al.*, 2011). In many cases, the signal transduction pathways, the associated changes in gene expression and the involvement of second messenger systems have also begun to be unravelled (Barraud *et al.*, 2009; Massie *et al.*, 2012; Petrova and Sauer, 2012). For example, it is now well appreciated that many bacteria rely on a regulatory system based on the production, secretion and response to signalling molecules, termed quorum sensing (QS), as a mechanism to control biofilm development for bacterial populations and this form of control is also postulated to occur within bacterial communities (Williams *et al.*, 2007). The *N*-acyl-homoserine-lactone (AHL) mediated quorum sensing system is one of the best studied bacterial QS systems and has been shown to regulate virulence factor expression, competition and biofilm formation in many different microorganisms and indeed has recently been found also in Archaea (Davies *et al.*, 1998; An *et al.*, 2006; Deng *et al.*, 2012; Zhang *et al.*, 2012).

QS research has made significant advances in defining the molecular pathways involved in AHL signalling and activation of QS regulated genes as well as the integration of AHL mediated QS with other global regulators to ensure the proper timing of gene expression (Williams *et al.*, 2007). While such studies have focused on populations of bacteria in the laboratory, bacteria in natural and man-made environments mainly exist as highly diverse and complex communities (Kolter and Greenberg, 2006; Werner *et al.*, 2011; Smith *et al.*, 2013). These communities are unlike those of population derived assemblages, with community members

differentially experiencing fluctuations in a wide range of parameters, including pH, temperature, nutrient concentration, oxygen level, and diffusion/dilution of signals (Boyer and Wisniewski-Dyé, 2009; Decho *et al.*, 2009; Yeon *et al.*, 2009a). Despite these challenges, it is now increasingly clear that QS occurs in and appears to be highly relevant for complex communities, whether they are biofilms on rocks in streams (McLean *et al.*, 1997), stromatolites (Decho *et al.*, 2009) or biofilms formed on hollow fibre membranes in bioreactors (Yeon *et al.*, 2009a). For example, the concentration of AHLs in a microbial mat was shown to vary with the diel cycle, driven by pH changes as a function of community metabolism and photosynthesis (Decho *et al.*, 2009). However, the role of QS signalling in microbial communities remains poorly understood both in terms of how the signals may impact on community assembly and the function of or interaction between the community members.

A microbial granulation system was used in this study as a model ecosystem for complex microbial community assembly. Granulation is an emerging nutrient removal biotechnology which enables decentralised low energy, cost effective and sustainable water treatment (Liu and Tay, 2004). The granulation reactor system has many advantages as a model ecosystem in that key process parameters, including dissolved oxygen concentrations, pH, temperature, carbon, nitrogen and phosphorus concentrations, as well as the conversion of the floccular biomass into dense, highly structured granules, can be precisely controlled and quantified. Acquiring this metadata set allows for the simultaneous and integrated investigation of environmental factors impacting on the production, accumulation, maintenance and functional role of AHL signals in the microbial community. Using this model ecosystem and a combination of analytical chemistry, metatranscriptomics and ecology study approaches, we have begun to explore the relationship between AHL based QS signalling and the assembly and morphogenesis of a highly complex granular sludge community. The results showed that the conversion of floccular biomass to highly structured granules and maintenance of intact, mature granules were strongly and positively correlated with *N*-acyl-homoserine-lactone (AHL) mediated QS.

4.2 MATERIALS AND METHODS

4.2.1 Reactor setup and operation

A sequencing batch reactor (SBR; 6 x 200 cm, diameter x height) was seeded with 3,000 mg L⁻¹ MLVSS of floccular sludge biomass from a bioreactor undergoing stable SNDPR performance as described in Section 3.2.1, with a final working volume of 4 L at room temperature c.a. 22 °C ± 1 °C. The SBR sludge culture was fed with synthetic wastewater (SWW) comprised of 150 mg COD L⁻¹ acetate and 50 mg COD L⁻¹ propionate as carbon source, 20 mg L⁻¹ ammonium, 10 mg L⁻¹ orthophosphate and trace nutrient elements (Smolders *et al.*, 1994; Zhou *et al.*, 2010), similar to the feeding regime applied to the floccular bioreactor (Section 3.2.1). The operation of the SBR involved a 6 h cycle comprised of two different stages: Stage I - feeding (8 min), anaerobic (60 min), aerobic (80 min at day 0 and gradually increased to 95 min by the end of week 5) and anoxic (40 min at day 0 and gradually increased to 50 min by the end of week 5); Stage II - feeding (2 min), anaerobic (30 min), aerobic (40 min at day 0 and gradually increased to 70 min by the end of week 5) and anoxic (30 min). Each cycle was completed with a settling stage (60 min at day 0 and gradually decreased to 5 min by the end of week 5) and a 10 min decanting stage. The settling time was maintained at 5 min per cycle from week 6 onwards. A total volume of 2 L SWW was fed to the bioreactor during the feeding period of each cycle and 2 L of treated effluents were discharged at the end of the cycle, resulting in a hydraulic retention time (HRT) of 12 h. The pH was monitored online and maintained at a range of pH 6.7 to pH 8.2 under the control of programmable logic controller (PLC) via dosing of either 9.12 g L⁻¹ HCl or 10.0 g L⁻¹ NaOH. Nitrogen gas (N₂) was sparged intermittently at a flow rate of 3.0 L min⁻¹ into the bioreactor throughout the operation except for the settling and decanting stages to provide complete mixing and hydrodynamic shear force for granulation. Air was supplied at a flow rate of 3.0 L min⁻¹ during the aerobic stage to achieve dissolved oxygen (DO) concentration of 3.0 - 4.0 mg L⁻¹, through the control of PLC.

4.2.2 Cycle study and sampling for analysis

A cycle study was conducted on a weekly basis throughout the course of the bioreactor operation to monitor system performance and granulation. In each cycle study (6 h), bioreactor mixed liquor was sampled at the beginning and at the end of the study. The

mixed liquor was filtered to remove the biomass. The filtered supernatant was used to determine the concentration of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and orthophosphate (PO_4^{3-}) in accordance to the APHA standard engineering methods (Eaton *et al.*, 2005). The concentration of sludge biomass, i.e. mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were also determined at the end of the cycle study according to the APHA methods (Eaton *et al.*, 2005). Unless stated otherwise, all the following sludge biomass will therefore refer to the measurement of MLVSS. Sludge particle size distribution was determined by a laser particle size analysis (Malvern Mastersizer Series 2600, Malvern). The morphology of the sludge particles was observed using a dissecting microscope (Olympus SZX2-ILL) equipped with a CCD camera (Q-Imaging). Sludge volumetric index (SVI), which measures the compactness of sludge biomass, was determined with some modifications (Eaton *et al.*, 2005). Instead of measuring the volume occupied by 1 L of uniformly mixed sludge suspension that had settled for 30 min in a standard 1 L graduated cylinder, the settling time was adjusted to 5 min (Liu, 2008). In every cycle study, 1 mL aliquots of uniformly mixed sludge samples were collected at the end of the anoxic period (Stage II). The sludge biomass was harvested at 8,000 x g for 5 min and stored at -80°C immediately for subsequent RNA and extracellular polymeric substances (EPS) analysis. Similarly, 50 mL aliquots of treated effluents were kept at -80°C immediately after sampling for later extraction of AHLs.

4.2.3 N-Acyl-homoserine-lactones (AHLs)

Synthetic AHLs (Sigma-Aldrich) used in this study are listed in Table 4-1. The synthetic AHLs were prepared freshly in 100% methanol (HPLC grade, Sigma Aldrich) to achieve a primary stock concentration of 1 g L⁻¹. The primary stocks were further diluted to achieve their respective final concentrations, individually or in combination, where appropriate using methanol:water (1:1 v/v) to serve as standards for subsequent liquid chromatography-mass spectrometry (LC-MS/MS) analysis as described in Sections 4.2.6 and 4.2.7. For experiments involving addition of AHL signals to the cultures, the synthetic AHLs were freshly prepared in dimethyl sulfoxide (DMSO) to achieve a primary stock concentration of 100 mM. The primary stocks were further diluted in DMSO or culture media to achieve their respective working concentrations.

Table 4-1. List of *N*-acyl-homoserine-lactones (AHLs).

AHL Signal	Abbreviation
<i>N</i> -butyryl-DL-homoserine lactone	C4-HSL
<i>N</i> -(3-oxohexanoyl)-DL-homoserine lactone	3OC6-HSL
<i>N</i> -hexanoyl-DL-homoserine lactone	C6-HSL
<i>N</i> -heptanoyl-DL-homoserine lactone	C7-HSL
<i>N</i> -(3-oxooctanoyl)-L-homoserine lactone	3OC8-HSL
<i>N</i> -octanoyl-DL-homoserine lactone	C8-HSL
<i>N</i> -(3-oxodecanoyl)-L-homoserine lactone	3OC10-HSL
<i>N</i> -decanoyl-DL-homoserine lactone	C10-HSL
<i>N</i> -(3-oxododecanoyl)-L-homoserine lactone	3OC12-HSL
<i>N</i> -dodecanoyl-DL-homoserine lactone	C12-HSL
<i>N</i> -(3-hydroxydodecanoyl)-DL-homoserine lactone	3OHC12-HSL
<i>N</i> -(3-oxotetradecanoyl)-L-homoserine lactone	3OC14-HSL
<i>N</i> -tetradecanoyl-DL-homoserine lactone	C14-HSL

4.2.4 AHL detection bioassay

For detection of AHLs produced *in situ*, a 96 well microtitre plate bioassay was adopted using the biosensor *Escherichia coli* JBA357 which expresses the green fluorescent protein (GFP) upon detecting AHLs (Andersen *et al.*, 2001). An overnight culture of *E. coli* JBA357 was first diluted 1:5 (v/v) with fresh Luria-Bertani (LB5) medium and 100 μ L of the diluted biosensor culture were added to 100 μ L of sludge sample in a 96 well plate. The culture plate was subsequently incubated at room temperature, c.a. $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with constant shaking at 200 rpm for 4 h prior to the visualization of green fluorescent cells using confocal laser scanning microscopy (CLSM) (LSM710, Carl Zeiss) at an excitation/emission wavelength of 488 nm/522-535 nm. Further, the GFP expression by the biosensor, as a result of the presence of AHLs, was quantified at an OD₄₈₈ using a microplate reader (Tecan Infinite M200). Heat inactivated sludge sample was included as the negative control. SWW to which synthetic AHL, 3OC6-HSL, was added at 10 nM was used as a positive control for the *in situ* AHL detection assay.

4.2.5 Extraction of AHLs

Extraction of AHLs from the treated effluent samples (bulk liquid/sludge supernatants) was performed as described by Shaw *et al.* (1997). Fifty milliliters of the sludge supernatants were extracted twice with 100 mL of dichloromethane (DCM). The DCM extract was allowed to dry using rotary evaporator and stored at -80°C until further

analysis by LC-MS/MS, as described in Sections 4.2.6 and 4.2.7, respectively. The extraction efficiency for each AHL was calculated based on the recovery of 13 standard AHLs (Table 4-1) added at 5 $\mu\text{g L}^{-1}$ and 50 $\mu\text{g L}^{-1}$, to the heat inactivated sludge supernatant sample matrix using LC-MS/MS. In general, the recovery rates for all the AHLs ranged from 50% to 80%. The extraction efficiency for individual AHLs was factored into the subsequent quantification of the native AHLs present in each sample. For all of the extraction experiments, a minimum of three independent extractions for each sample were conducted.

4.2.6 Detection of AHLs by mass spectrometry (MS)

The DCM extract was subjected to analysis by LC-MS/MS (LCMS8030, Shimadzu Inc.). Briefly, 10 μL of the sample extract were chromatographed using a C_{18} reversed-phase column (Shim-pack XR-ODS column: 2 x 100 mm, 2.2 μm) at a flow rate of 0.3 mL min^{-1} . The mobile phase consisted of solvent A (25 mM ammonium formate with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). A gradient elution method was utilized which started at 5% solvent B for 0.5 min, followed by a linear gradient from 5 to 40% solvent B over 0.5 min, then to 95% solvent B over the next 5 min and remained isocratic at 95% solvent B for 1.5 min. The column was reequilibrated at 5% solvent B for 1 min. The AHL compounds separated by the LC system were ionized by electrospray ionization (ESI) under positive mode and detected using multiple reaction monitoring (MRM) approach (Morin *et al.*, 2003; Decho *et al.*, 2009). Matrix matched MRM experiments were performed for all 13 standard AHLs (Table 4-1). MRM experiments were conducted with Q1 set to monitor for the precursor ion and Q3 for the specific transition ions according to the optimized parameters for each AHL (Table A-1). The identity of each putative AHL in the DCM extract was confirmed based on specific LC retention time, appearance of the precursor ion m/z and two transition ions, as well as relative intensity of the two transition ions in comparison to the individual standard AHL. In addition, a full scan, ranging from m/z 100 to 350, coupled with precursor ion scan mode was conducted to provide further confirmation of the identity of putative AHLs in the each sample extract with respective to the standard AHLs (Decho *et al.*, 2009). Duplicate injections were performed for each sample extract. Triplicate blank injections were performed between sample injections to prevent sample carryover.

4.2.7 Quantification of AHLs by MS

For quantification of native AHLs present in the supernatant of sludge samples (or treated effluents), matrix-matched standard curves, ranging from 0.5 $\mu\text{g L}^{-1}$ to 200 $\mu\text{g L}^{-1}$, for all 13 standard AHLs were constructed using the optimized MRM method as described above. Two transition ions, characteristic of the respective AHLs, were used in the MRM approach and the transition ion with highest intensity was used to construct the standard curve (Table A-1). The analyte peak areas were integrated using a quantification software package (LabSolutions software, Shimadzu Inc.) and each integrated peak was inspected manually. The limits of detection (LOD) and quantification (LOQ) for each AHL were calculated with a signal-to-noise ratio of 3.3 and 10, respectively (Decho *et al.*, 2009). Correspondingly, the amount of each putative AHL in the sample extract was quantified based on the standard curve of the respective AHLs with the extraction efficiencies being factored in the calculation as described above (Section 4.2.6).

4.2.8 Extraction and quantification of extracellular polymeric substances (EPS)

Extraction of EPS from the sludge biomass was performed according to the formaldehyde/NaOH method with some modifications (Adav and Lee, 2007). Briefly, 1 mL of uniformly mixed sludge suspension was harvested at 8,000 $\times g$ for 5 min. The sludge biomass was ground gently with a clean glass rod to disrupt the granular particles and subjected to freeze-drying for 24 h. The dried biomass of individual samples was recorded. The sludge samples were resuspended in 1 mL of 0.9% NaCl (w/v) solution at pH 7.2 and fixed with 0.6% formaldehyde (v/v) for 1 h at 4 °C. Four hundred microliters of 1 M NaOH were added to the sludge suspensions for 3 h at 4 °C prior to harvesting EPS at 17,000 $\times g$ for 15 min at 4 °C. The extracted samples were stored at -80 °C until further analysis. The major constituents of EPS, including extracellular polysaccharides (PS) and extracellular proteins (PN) were quantified independently. The PS was measured using the phenol-sulfuric assay with glucose as the standard (Dubois *et al.*, 1956) while the PN was quantified by the bicinchoninic acid assay (BCA) with bovine serum albumin as the standard (Smith *et al.*, 1985). For

all the extraction experiments, a minimum of three independent extractions for each were conducted.

4.2.9 AHL add-back study

Two liters of floccular sludge biomass were harvested from the floccular bioreactor (Section 3.2.1) for the AHL add-back study. The sludge biomass was washed 3 times with 0.9% NaCl at pH 7.2 prior to the exposure of fresh SWW. The sludge suspension was evenly distributed to 39 conical flasks with 40 mL each. Forty microliters of AHLs, i.e. C6-HSL, 3OC6-HSL, C8-HSL, 3OC8-HSL, C12-HSL and 3OC12-HSL, were added immediately to the individual flasks at a final concentration of 100 nM or 5,000 nM each. A DMSO solvent control was included as the negative control. Three replicates per treatment were performed. The culture flasks were incubated at 100 rpm for 1 h at room temperature. Two aliquots of 1 mL uniformly mixed sludge suspension were sampled from each flask at time 0 and 1 h post addition of AHLs. The sludge biomass was harvested at 8,000 x g for 5 min and snap-frozen with liquid nitrogen prior to the storage at -80°C for further EPS analysis as described in Section 4.2.8. Measurements of MLSS and MLVSS were taken for each flask at the beginning and the end of the experiment.

4.2.10 RNA extraction for meta-transcriptomic studies

Total RNA was extracted from sludge biomass using the FastRNA Pro Soil-Direct kit (MP Biomedicals) according to the manufacturer's guidelines. The extracted RNA was subjected to an additional DNA removal step using the TURBO DNA-free kit (Applied Biosystems) to remove any residual genomic DNA present in the RNA sample. The quality of the RNA samples was examined using the Agilent 2100 bioanalyser with Agilent RNA 6000 kit and reported as the RNA integrity number (RIN). The quantity of total RNA and residual DNA were measured using Quant-iT RiboGreen RNA and PicoGreen DNA assays (Invitrogen), respectively. Two hundred nanograms of total RNA were used for complimentary DNA (cDNA) library preparation according to the manufacturer's instructions (Illumina Inc.). Each cDNA library was ligated with a unique adaptor sequence for sample multiplexing. A total of 16 different cDNA libraries were pooled and sequenced using the Miseq System (Illumina Inc.) as per the manufacturer's guidelines.

4.2.11 Sequence analysis

Total RNA sequencing data was analyzed using a fast tag-based method developed here. Briefly, a universal primer (CGACRRCCATGCANACCT) for the 16S rRNA hypervariable region (V6) was used to scan each sequencing read to obtain all 33 nt tag sequences downstream of the primer. The universal primer matches 94% of 16S rRNA sequences in the RDP database (Claesson *et al.*, 2010). Each different V6 tag was used as a signature sequence to represent one operational taxonomic unit (OTU). To eliminate sequencing error derived V6 tags, only V6 tags that were observed in at least 2 different reads were kept for analysis. To further filter for possible PCR duplication artefacts where abnormally high number of identical reads arise, V6 tags were removed where any single type of sequencing read accounted for $\geq 50\%$ of all reads covering that V6 tag.

4.2.12 Bacterial and fungal isolation, identification and characterization

Floccular sludge biomass from the SNDPR bioreactor was harvested between weeks 40-44 (Section 3.3.1) to seed the granular bioreactor (Section 4.2.1) and to isolate microorganisms in pure culture. A total of 330 isolates, including bacteria and fungi were isolated using five different culture media, i.e. Nutrient broth, R2A, *Pseudomonas* isolation medium, Synthetic Waste Water (SWW) (Smolders *et al.*, 1994; Zhou *et al.*, 2010) and ABT (Clark and Maaloe, 1967; Chong *et al.*, 2012)(Section 5.2.3). All the isolates were identified by rDNA gene sequencing (Section 5.2.4). Identified isolates were subsequently examined for their ability to produce and/or degrade AHLs according to the bioassays as described in Sections 5.2.5 and 5.2.6. For standardization and compatibility of the bioassays, all isolates were cultured and maintained in Luria-Bertani medium supplemented with 5% w/v sodium chloride (LB5) at room temperature for detection of AHL production and degradation activities unless stated otherwise.

4.2.13 Statistical analysis

All statistical analyses were performed using Prism version 5 for Windows (GraphPad Software Inc.). For correlation studies, Pearson correlation indexes were calculated and the resulting matrixes were clustered using an unsupervised hierarchical clustering

method based on Euclidean distance with complete linkage as coded by Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and visualized using Java TreeView (<http://jtreeview.sourceforge.net>).

4.3 RESULTS

4.3.1 Development of a complex, functional granular sludge community

This study was designed to determine the role of QS in the function of an activated sludge community, specifically granulation and nutrient removal, in a sequencing batch reactor (SBR). Granules are defined as compact aggregates that have a minimum particle size of 100 μm in diameter and have a sludge volumetric index (SVI_5) of 50 mL g^{-1} and below, an index that is used to measure the density (compactness) of the biomass (Barr *et al.*, 2010). Samples were collected weekly to monitor granulation as quantified by SVI_5 and particle size. By comparing the measurements and the stereoscopic images of the biomass, five distinct phases of granule development were defined (Figure 4-1). In the early inoculation stage, the biomass was characterised as a floccular, loosely associated microbial community with a mean particle diameter of 60 μm (50th percentile distribution; Phase I, Weeks 0-5). During this period, the bioreactor settling time was decreased from 60 min to 5 min to select for high density sludge biomass, where the community experienced a 40% loss of biomass as MLVSS decreased from 3,000 mg L^{-1} to 1,820 mg L^{-1} and, a 70% reduction in the biomass volume, as determined by the decrease of SVI_5 from 300 mL g^{-1} to 106 mL g^{-1} . By week 8, compact aggregates were observed in the bioreactor with an apparent increase of particle size from 60 μm to 180 μm and a marked decline in SVI_5 to less than 50 mL g^{-1} , suggesting the transition of the microbial community from the floccular phase into the initiation phase of granulation (Phase II, Weeks 5-8). This was followed by a rapid expansion in the size of the aggregates, reaching a maximum diameter of 1,400 μm (90th percentile distribution) at the maturation phase by week 15 (Phase III, Weeks 8-15) prior to the development into the maintenance phase (Phase IV, Weeks 15-20), where the particle size of the granules was at least 500 μm in size (10th percentile distribution). The life-cycle ended with the disintegration of the mature granules at week 20, where the biomass dispersed from the mature structures to revert to floccular biomass as displayed by the marked decline in mean particle size, to 200 μm (50th percentile distribution) and an increase in the SVI_5 index to 89 mL g^{-1} (Phase V, Weeks 20-22).

Figure 4-1. Developmental process of granular sludge community in a sequencing batch reactor based on microscopic visualisation of structures (A), particle size distribution and sludge volumetric index at 5 min (SVI_5) (B), and sludge biomass concentration (C). Magnification in A, $\times 15$ (bar, $500 \mu\text{m}$). In B, 10th percentile (open circles), 50th percentile (open squares) and 90th percentile (open triangles) indicate that 10%, 50% and 90% of total particles were below the corresponding size distribution, respectively; while SVI_5 (solid triangles) measures the compactness of the sludge particles. Concentration of sludge biomass, in terms of MLSS (open squares) and MLVSS (solid squares), was also determined (C). The dotted lines separate the different developmental phases of granulation, i.e. Phases I to V.

In addition to the structural changes of sludge particles during the granulation process, relatively high nutrient removal efficiencies were achieved with 99% of soluble COD, 84% of total nitrogen and 85% of total phosphate being removed on average throughout the study period (Figure A-7). Removal of total nitrogen was fairly constant with a minimum 70% of total nitrogen removed for most of the time points investigated (Figure A-7: A). In contrast, removal of total phosphate was erratic prior to the aggregate expansion/maturation phase but was consistent thereafter (Figure A-7: B). Hence, a functional microbial granulation system with SNDPR performance was established as a model of study for subsequent investigation of the mechanisms involved in the granulation process.

4.3.2 The levels of AHLs correlate with the development of granular sludge community

To determine the role of QS in the developmental process of granular sludge community, the production of AHLs by the sludge community was assessed over the entire course of the study. Similar to the findings in the floccular sludge community (Chapter 3: Figure 3-3), *in situ* AHLs generated by the granular sludge community were found to activate the *E. coli* JBA357 biosensor, suggesting that AHL mediated QS was functioning at physiologically relevant concentrations during the granulation process (Figure 4-2). To quantitatively describe the profile of the AHLs produced in this complex, dynamic community, sludge supernatant extracts were analysed by LC-MS/MS using standard curves and the mass spectra of 13 different synthetic AHLs as quantitative references (Figure A-3). The experimentally determined extraction efficiency, for each of the 13 standards, between 50 and 80%, was factored into the AHL quantification. Consistent with previous findings for the floccular sludge community (Chapter 3: Figure 3-5), only AHLs with short to medium acyl chains, ranging from 4 to 8 carbons, were detected in the granular SBR supernatants sampled over 22 weeks (Figure 4-3). AHLs with 10 carbons or more were not resolved within the detection limit of the LC-MS/MS (0.2 to 2 pmol g⁻¹ after sample concentration and biomass normalization). 3OC8-HSL was the most abundant signal, at a concentration 5 to 30 fold higher than those of the other AHLs. The amount of 3OC8-HSL detected in the sludge supernatant, after normalisation to sludge biomass, was estimated to be in the range of 6 to 325 pmol g⁻¹ (Fig. 4-3: F), or 10 to 210 pM equivalent in concentration (Figure A-8: F), far lower than the nanomolar to micromolar concentrations typically reported for pure culture population based systems (Pearson *et al.*, 1995; Schaefer *et al.*, 2008). Nevertheless, the amount of specific AHLs released, per gram of sludge biomass, closely correlated with distinct stages of granule development. For example, most of the AHLs detected, from C4 to C8, increased 10 to 100 fold in concentration in Phase II compared to Phase I (Figure 4-3), corresponding to the initiation of granule formation (Figures 4-1). When the relationship between AHLs and granulation at different developmental phases was determined using the Pearson correlation method, all of these signals were strongly and positively correlated with sludge particle size (as a granulation measure) at the transition from Phase I to II ($r > 0.8567$, $p < 0.0032$) (Figure 4-4). Some AHLs, e.g. C4-HSL and C6-HSL,

continued to increase through Phase III (Figure 4-3: A and B); while other AHLs, e.g. 3OC6-HSL, C7-HSL, C8-HSL, and 3OC8-HSL, decreased 5 to 10 fold during the same phase (Figure 4-3: C-G). A second rise in all AHL levels was observed at the maintenance phase (Phase IV) and the levels of AHLs declined or were below the detection limit at the time when the granules started to disperse (Phase V) (Figure 4-3). The amounts of C6-HSL and C8-HSL, in particular, were strongly and positively correlated with the sludge particle size during the transition between Phases IV and V (C6-HSL: $r = 0.7983$, $p = 0.0314$; C8-HSL: $r = 0.7670$, $p = 0.0442$). Thus, high concentrations of AHLs were associated with granule formation and low levels were associated with granule collapse.

Figure 4-2 (next page). *In situ* detection of AHLs, released by the granular sludge community using the *E. coli* JBA357 biosensor. Co-incubation of the sludge sample (A and B), or the heat inactivated sludge sample (C) with the biosensor at room temperature for 4 h with constant shaking at 200 rpm prior to inspection by confocal laser scanning microscope (CLSM). The heat inactivated sludge sample served as a background fluorescence control for the sludge biomass (auto-fluorescence). Additional controls for the bioassay included the addition of exogenous 3OC6-HSL at 10 nM to the biosensor, a positive control (D), and the presence of the biosensor alone as a negative control (E). Images of green fluorescent cells were captured at 488 nm (i), the total unstained cells were visualized by differential interference contrast (DIC) (ii) and the superimposed image of the two is shown in the right-end panel (iii). The black arrow indicates the granular sludge biomass. Magnification: A, x160; B, x400; C, x120; D-E, x200; Scale bar, 100 μm .

Figure 4-3. LC-MS/MS profiling of AHLs released by the sludge community during granulation. Samples extracted from 50 ml of sludge supernatants, which were collected on different bioreactor operational weeks, were analysed using the MRM approach. The identity and quantity of individual AHLs present in each sample, such as C4-HSL (A), C6-HSL (B), 3OC6-HSL (C), C7-HSL (D), C8-HSL (E) and 3OC8-HSL (F), were assessed in comparison with the MRM profiles of 13 standard AHLs. The data presented above were normalized to the respective sample biomass. All results are shown as the Mean $\pm$ SEM, with three independent extractions per sample point. The dotted lines separate the different developmental phases of granulation, i.e. Phases I to V, while n.d. represents 'not determined'.

Figure 4-4. The correlation of AHL expression with the developmental phases of granulation. The relationship between individual AHLs and sludge particle size at specific phase transitions, was calculated using a Pearson correlation coefficient. The correlation matrixes were grouped using unsupervised hierarchical clustering based on Euclidean distance with complete linkage method.

4.3.3 Expression of extracellular polymeric substances (EPS) correlates with granule formation and AHL expression

One of the key features of biofilm formation is the production of EPS which has been proposed to provide structure to the biofilms and is important for many properties of biofilms, including its viscoelastic features as well as for binding and sequestering antimicrobial compounds (Kolter and Greenberg, 2006; Flemming and Wingender, 2010). In granulation, the production of specific polysaccharides, i.e. granulan, has been shown to be a key contributor to the conversion of floccular biomass into granules (Seviour *et al.*, 2012). Therefore, the protein and polysaccharide content of the EPS were quantified (Flemming and Wingender, 2010) to correlate changes in EPS production during granulation (Figures 4-5 and 4-6). Both protein and polysaccharide components of the EPS increased in concentration (from 60 to 120 mg g⁻¹ and from 10 to 35 mg g⁻¹, respectively) as the floccular biomass began to transform into a granular community (Phases I-II). The amount of protein showed a slight decline in Phase III and remained relatively stable thereafter (100 mg g⁻¹) (Figure 4-5: B). In contrast, the polysaccharide concentration declined during Phases IV and V, returning to the initial concentration observed for the floccular sludge (10 mg g⁻¹) (Figure 4-5: A). Consistent with the polysaccharide profile, the polysaccharide to protein ratio (PS/PN) increased

during Phase I-II transition and decreased during Phase IV-V transition (Figure 4-5: C). For the latter transition, the PS/PN ratio decreased from 0.25 to 0.12, a ratio which was comparable to the EPS composition of the floccular biomass. When analysed by the Pearson correlation method, it was clearly observed that the PS/PN ratio strongly and positively correlated with the sludge particle size, in particular during the Phase I-II (floccular to granular phase; $r = 0.8950$, $p = 0.0011$) and Phase IV-V (granular to floccular phase; $r = 0.8357$, $p = 0.0192$) transitions (Figure 4-6: A). Not only did the EPS content correlate with the development and dispersal of microbial granules, but it was observed that the PS/PN ratio also correlated with AHL accumulation in the bioreactor (Figure 4-6: B). Specifically, a strong, positive correlation between all AHLs and the PS/PN ratio was observed at the transition period between Phases I and II ($r > 0.7899$, $p < 0.0113$), while C8-HSL was the only signal that significantly correlated with the PS/PN ratio at the transition between Phases IV and V ($r = 0.7921$, $p = 0.0337$) (Figure 4-6: B).

Figure 4-5. Chemical profiling of EPS produced by the sludge community during granulation. Total EPS was extracted from the sludge biomass at different developmental time points, and individual components of EPS, i.e. extracellular polysaccharides-PS (A) and extracellular proteins-PN (B), were quantified independently. Each EPS component was normalized to the respective sample biomass. The ratio of extracellular polysaccharide to protein (PS/PN) expressed is depicted (C). All results are shown as the Mean $\pm$ SEM, with three independent extractions per sample point. The dotted lines separate the different developmental phases of granulation, i.e. Phases I to V.

Figure 4-6. The correlation of EPS concentration with granulation (A) and AHLs (B) at different developmental phases. The relationship between the ratio of extracellular polysaccharide to protein (PS/PN) and sludge particle size (a granulation measure: d0.5 or 50th percentile distribution), as well as the individual AHLs, at specific phase transitions, were calculated based on Pearson correlation. The PS/PN ratio was used to represent expression of EPS. The correlation matrixes were clustered using unsupervised hierarchical clustering based on Euclidean distance with complete linkage method.

4.3.4 Add-back of specific AHLs drives EPS production by the floccular sludge community

The exogenous addition of 3OC6-HSL, 3OC8-HSL and 3OC12-HSL, as well as the unsubstituted C6-HSL, significantly increased the production of either extracellular polysaccharides or proteins or both by the floccular sludge community, while C8-HSL and C12-HSL had no effect on the total EPS production after 1 h of incubation (Figure 4-7: A and B). Moreover, the production of extracellular polysaccharides was found to be induced in a signal concentration-dependent manner. A marked increase in polysaccharides (14% to 36%) was observed within 1 h of incubation with specific AHL species, such as C6-HSL, 3OC6-HSL, 3OC8-HSL and 3OC12-HSL (Figure 4-7: A). Addition of these AHLs also resulted in an increase in the PS/PN ratio of the floccular sludge from 0.21 to 0.24 (Figure 4-7: C), consistent with the PS/PN ratio

observed for the granular sludge (Figure 4-5: C). The presence of these AHLs *in situ* (Figure 4-3) and their association with EPS induction (Figure 4-6: B) could suggest they play a role in regulating EPS production during granulation.

Figure 4-7. The impact of AHLs on the floccular sludge on EPS production. Six different synthetic AHLs were individually added to the microcosms, each at two different concentrations, 100 nM (open bars) and 5,000 nM (solid bars), to the flasks containing floccular sludge for 1 h with constant shaking at 100 rpm. DMSO was used as a solvent control (gray bar). Expression of extracellular polysaccharides-PS (A) and

extracellular proteins-PN (B) were assessed 1 h post incubation. Each EPS component was normalised to the respective sample biomass. The ratio of extracellular polysaccharide to protein (PS/PN) expressed is depicted (C). Results are shown as the Mean $\pm$ SEM, with three independent replicates per treatment. Two-way ANOVA was performed and Bonferroni post-tests were conducted to compare each treatment to the negative control where significant differences are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.5 Community composition correlates with AHL production and granulation

Analysis of 67,611 16S rRNA gene sequences obtained from a meta-rRNA sequencing study (Table A-3) using the Shannon-Weaver index, a measure of community richness and evenness, revealed a distinct community shift from high to low diversity when the floccular biomass (Phase I) was first transformed into compact aggregates (Phase II), after which the community regained the high diversity during the maintenance phase (Phase IV) (Figure A-9). As the bioreactor was inoculated once only with no further introduction of fresh sludge, fluctuations in community diversity over time reflect dynamic changes in abundance of particular microbial species due to selection and adaptation, rather than changes in absolute community richness. To relate the changes in microbial species abundance and community evenness to granulation and AHL production, Pearson correlation coefficients were calculated by comparing the top 50 most abundant community members with the concentration of individual AHL signalling molecules as well as the stage of granulation. The microbial community members were divided into three clusters based on their correlation with AHL concentration and granulation (Figure 4-8). Community members in Cluster 1 showed no correlation to AHLs nor granulation. In Cluster 2, more than 50% of the total number of active species demonstrated a strong, positive correlation with at least one AHL as well as with granulation. Organisms in this group, for example those belonging to *Nitrosomonas*, *Burkholderiales*, and *Xanthomonadaceae*, have previously been shown to be AHL producers (Burton *et al.*, 2005; d'Angelo-Picard *et al.*, 2005; Suárez-Moreno *et al.*, 2010). Furthermore, a *Xanthomonadaceae* bacterium (Tag 6) was isolated from the granular sludge and we demonstrated its production of AHLs, ranging from C6 to C10, with C7-HSL being the dominant signal (Figure A-10: A and C). Other community members in Cluster 2 included *Candidatus Accumulibacter* and *Candidatus Competibacter*, which were previously shown to be important for granulation in SNDPR bioreactors (Seviour *et al.*, 2011). Here, it is shown that

members of *Candidatus Accumulibacter* and *Candidatus Competibacter* were strongly and positively associated with QS signalling during the granulation process. Microorganisms that grouped together in Cluster 3 were negatively associated with granulation as well as AHL concentration. Interestingly, a Cluster 3 member, a *Comamonadaceae* bacterium (Tag 17), was isolated and found to be an AHL signal degrader (Figure A-10: B and D).

Figure 4-8 (previous page). Unsupervised clustering of the top 50 community members in relation to the expression of AHLs and granulation. The relationship between the abundance of top 50 most dominant community members (each represented by a unique V6 tag), and concentration profile of AHLs as well as the granulation measures over a time series of 22 weeks (16 time points in total) were calculated based on Pearson correlation coefficient. The correlation matrixes were grouped using unsupervised hierarchical clustering based on the Euclidean distance with complete linkage method. Granulation determinants assessed included particle size distribution (d0.1, 10th percentile; d0.5, 50th percentile; d0.9, 90th percentile) and sludge volumetric index (SVI₅). “***” represents a V6 tag that is not able to be annotated based on the taxonomy classification pipeline stated in Materials and Methods. Instead, the closest relative with sequence identity matching more than 97% is shown where it is applicable.

4.4 DISCUSSION

It was previously shown that AHLs were expressed dynamically in the floccular sludge system over an extended period of 82 weeks and that AHL mediated QS signalling was closely associated with the community metabolism in the nitrification/denitrification process specifically via the nitrite pathway (Chapter 3). In this study, the role of QS signalling in the structural assembly of the sludge community in which the floccular sludge was transformed into compact granular biomass under defined bioreactor operating conditions was addressed. The consistent and reproducible development of granular biomass is a key objective for the wastewater industry because the unique properties of granules will lead to significant improvements in treatment time as well as economic and social gains in the associated reduced footprint of the wastewater treatment plants (Yilmaz *et al.*, 2008). Thus, the study of QS in the granulation process is not only of interest in understanding the role of QS on microbial communities, but also has potential practical outcomes for the wastewater treatment industry.

This study has demonstrated that AHLs, ranging from 4 to 8 carbons, were present in the microbial community during the entire granulation life cycle (Figure 4-3). Quantitative analysis of AHLs in the bulk liquid of the granulation system revealed that the concentrations of AHLs ranged from 1 to 210 pM (Figure A-8), which is 5 to 200 times lower than those found for many pure culture systems (Pearson *et al.*, 1995; Burton *et al.*, 2005; Schaefer *et al.*, 2008) and is consistent with the range of AHL concentrations detected in the floccular sludge system described in Chapter 3 (Figure A-5). Despite the low signal concentrations, the AHLs were physiologically relevant and could be detected *in situ* using a fluorescent bioreporter strain (Figure 4-2). Consistent with our observation on the biological relevance of low AHL concentrations, two novel QS systems using aryl-homoserine-lactone (Ahlgren *et al.*, 2011) and branched acyl-homoserine-lactone (Lindemann *et al.*, 2011), have recently been reported to respond to signal concentrations as low as 10 pM. Moreover, it was also demonstrated that endogenous levels of AHLs were less than 20 pM in soil (Wang and Leadbetter, 2005), suggesting that the concentration of extracellular signals in natural environments may be lower than is typically observed for microbial population cultures in the laboratory. While the role of QS remains largely unknown in most natural and engineered habitats, this study has clearly established a strong, positive

correlation between AHL based QS and the morphogenesis of microbial granules (Figure 4-4). Elevated levels of specific AHLs in the bulk liquid were strongly associated with the initiation of granulation, while signal concentrations decreased markedly during the disintegration phase (Figures 4-1, 4-3 and 4-4). Similar patterns and profiles of AHL production in relation to granulation were observed in two additional, independent replicates that investigated the initiation of granulation, indicating the QS response in relation to the timing of granulation is reproducible (data not shown). It is thus tempting to speculate that QS signalling may play a key role in the granulation process by mediating the communication between different sludge particles for cellular aggregation or are involved in the regulation of community behaviour associated with granular physiology.

It is also possible that the low signal concentrations in the bulk liquid may represent an equilibrium concentration, where the signals, which primarily partition to the biofilm, diffuse out of the sludge biomass into the bulk liquid. It has been proposed that diffusion rates and local concentrations of signals matter more than bulk AHL concentrations, a concept termed diffusion sensing or efficiency sensing (Redfield, 2002; Hense *et al.*, 2007; Dulla and Lindow, 2008). If the local AHL concentrations around the QS organisms can reach a threshold level before subsequently being diluted away into the bulk liquid, then the community may still be able to efficiently employ QS. In the previous chapter, it was shown that the dominant AHL, i.e. 3OC8-HSL, was present in the floccular biomass at > 20 nM, which was at least 100 fold higher than the signal concentration in the bulk liquid at the same time point (Chapter 3). Given that there was a 10 to 100 fold increase in signal levels (per gram of biomass) in the bulk liquid when the floccular biomass was first transformed into the compact aggregates (Figure 4-3), it is reasonable to expect that much higher signal concentrations would be present in the granular sludge biofilms. Using those data, and the data presented here for the concentration of AHLs in the bulk liquid, the 3OC8-HSL is estimated to be 300 nM in the granular sludge biomass. In support of this, a higher proportion of AHL activated biosensors was observed to cluster around the granular sludge biomass relative to those activated in the sludge supernatant when *in situ* AHL production was examined (Figure 4-2). These observations are consistent with a previous report showing that AHLs preferentially partition to the biofilm, reaching concentrations 600

fold in excess of the signal concentration in the surrounding bulk liquid medium (Charlton *et al.*, 2000). Thus, it is highly likely that QS induction would be active in the floccular and granular biomass tested here, and may be important in the development of microbial granules.

Previous studies have shown that AHL mediated QS is important for biofilm development and in some species, this is mediated partly through regulation of EPS production (Davies *et al.*, 1998; Koutsoudis *et al.*, 2006). Additionally, the development of granules has been associated with the production of the polysaccharide component granulan (Seviour *et al.*, 2011). It was interesting to note that in this study, EPS production also showed a strong, positive correlation with AHL concentration and granulation, suggesting some interrelationship of these processes (Figure 4-6). While it is not possible to disentangle cause and effect from these data, the increased EPS production upon the exogenous addition of AHLs to microcosms of floccular sludge may support the hypothesis that the AHLs which were most abundant in the bioreactor community (3OC6-HSL, 3OC8-HSL and C6-HSL) are involved in the regulation of EPS production (Figure 4-3 and 4-7). The increased EPS production is unlikely to simply be due to either selection from species that produce EPS, since the experiments were conducted over a short time frame (1 h) or a consequence of the community utilizing the AHLs as a nutritional source given that the EPS expression was only induced by specific AHLs (Figure 4-7). Although the amount of EPS induced by specific AHLs in the batch culture was not as extensive as observed in the natural granulation course, the EPS induction was statistically significant ($p < 0.0001$; Two-Way ANOVA) and likely to be of biological important. The low level of EPS induction observed in these experiments may be in part explained by the short exposure time despite a relatively high signal dosage. Alternatively, this may suggest that the EPS production during granulation is in part driven by AHL signalling in combination with other unknown environmental factors.

Based on meta-rRNA sequencing, the abundance of more than 50% of the community members were positively correlated with AHL concentration across the 23 week experiment (Figure 4-8: Cluster 2). Many of the genera identified here have previously been shown to be involved in AHL mediated QS (Burton *et al.*, 2005; d'Angelo-Picard

et al., 2005; Suárez-Moreno *et al.*, 2010), and AHL production for several of these community members, e.g. Tag 6 (*Lysobacter brunescens*) and Tag 14 (*Stenotrophomonas* sp.) from the family *Xanthomonadaceae*, were confirmed following their isolation (Figure A-10: A and C). While not all of the bacteria identified in Cluster 2 may be direct contributors to the QS signal pool, they nonetheless likely play important roles in the expression of QS dependent phenotypes. For example, it has been shown that *Escherichia coli*, which does not produce AHLs, can respond to AHLs via the orphan AHL receptor SdiA, leading to specific changes in gene expression (Hughes *et al.*, 2010). Moreover, a phylogenetic survey by Case *et al.* (2008) demonstrated that 45 out of 512 complete genomes contained incomplete QS circuits, with homologs of the AHL receptor gene *luxR* but not the AHL synthase gene *luxI*. In our system, it was observed that several members of two dominant genera in the community, i.e. *Candidatus Competibacter* and *Candidatus Accumulator*, previously demonstrated to be essential for granulation through the production of granular associated with the EPS (Seviour *et al.*, 2011), were positively correlated with QS and granulation (Cluster 2), suggesting that they may participate in, or benefit from, QS even though they have not yet been demonstrated to produce AHL signals or carry AHL receptor homologs.

While it is not yet possible to determine if QS signalling was a direct cause to granulation, the observation here that the three processes, EPS production, community composition changes and AHL concentrations, are all strongly correlated with each other does suggest they are important in the granulation process. This study has shown that QS signalling is strongly correlated with the conversion of a floccular biofilm into granular biomass and that this restructuring is also positively correlated with an increase in EPS production and a specific community species composition. This combination of observations suggests that QS may play a role in restructuring of the community, potentially through the regulation of EPS. Here, AHL production may lead to induction of EPS synthesis and hence granulation or the changes in QS signals may be a consequence of community selection during the granulation process. It is clear either way that AHL mediated signalling and community are linked here and hence QS is relevant for complex microbial communities. Signal concentrations were at least 100 times higher in the biomass, i.e. floccular sludge (Chapter 3) and were in the ranges

previously shown to induce bioreporter strains. This would imply that QS is most relevant within the structured floccular or granular community, although there is still a possibility for interfloccular or intergranular communication via the levels of low extracellular AHLs. We submit that the dynamic physiological conditions experienced by the microbial community in the granular biofilm reactor would not be dissimilar from environmental conditions experienced by many other complex communities in a wide range of natural and bioprocess systems, such as microbial mats, anaerobic digesters, membrane bioreactors, and river streams. It is therefore likely that QS signalling plays important functional roles and mediates cooperative microbial community behaviour in particular within biofilm structures.

CHAPTER 5: MODULATION OF COMMUNITY SIGNALLING

5.1 INTRODUCTION

In most natural and engineered ecosystems, bacteria predominantly reside in highly structured and coordinated communities, in a surface-dependent or independent manner, commonly known as microbial biofilms (Davey and O'toole, 2000). These microbial consortia are typically comprised of hundreds to thousands of bacterial species with varying metabolic capabilities and collectively constitute a unique mode of life with distinctive community properties relative to the planktonic cells (Kolter and Greenberg, 2006; Elias and Banin, 2012). The overall structure and function of complex biofilms is typically driven by interactions between the different microbial species, which can either be synergistic via metabolic cooperation or antagonistic through competition for resources (Davey and O'toole, 2000; Elias and Banin, 2012). Understanding the interspecies dynamics within biofilms may therefore be important in order to control and regulate the ecosystem functions, for example the engineering of highly stable and sustainable microbial communities for water and wastewater treatment by promoting synergistic relationships among community members, or the treatment of biofilm related diseases by enhancing competitive fitness of commensal microbiota in the host.

In many cases, interspecies interactions may involve communication via small diffusible signalling molecules, a mechanism generally termed quorum sensing (QS) (Elias and Banin, 2012). Many bacteria employ QS to synchronize population behaviour, including, but not limited to, biofilm formation, exoenzyme production and virulence factor secretion, to optimize population growth and survival in different environments (Shapiro, 1998; Williams *et al.*, 2007). The *N*-acyl-homoserine-lactone (AHL) mediated QS system is one of the most well-characterized bacterial communication systems and is present in approximately 10% of Proteobacteria isolated from various ecological niches (Valle *et al.*, 2004; d'Angelo-Picard *et al.*, 2005; Cirou *et al.*, 2007). As a consequence of multiple community members sharing the same classes of signalling molecules, cross talk or communication among bacteria of different species or even between organisms from different domains is possible

(Pierson *et al.*, 1998; Riedel *et al.*, 2001; Dulla and Lindow, 2009; Hosni *et al.*, 2011). For example, *Burkholderia cepacia* was reported to perceive the AHLs released by *Pseudomonas aeruginosa* in co-culture, leading to the formation of mixed microcolonies, in contrast to the formation of separated, non-mixed microcolonies in the absence of QS activity (Riedel *et al.*, 2001). In addition, there is increasing evidence that AHLs are present at biologically relevant concentrations in a diverse range of environments, including sputum from cystic fibrosis patients (Singh *et al.*, 2000), microbial mats (Decho *et al.*, 2009), host rumen (Hughes *et al.*, 2010) and wastewater treatment plants (De Clippeleir *et al.*, 2011; Chong *et al.*, 2012). More recently, the AHL mediated QS signalling was also found to be closely linked to the community nitrogen metabolism in a floccular sludge system (Chapter 3) as well as the conversion of fluffy floccular sludge into highly structured microbial granules (Chapter 4), with more than 500 different bacterial species engaged. These observations thus strongly suggest that signal cross talk is prevalent and important within microbial communities; however, it is unclear how such interactions can be coordinated among the large number of species commonly present to achieve their communal behaviour.

In the previous chapters, it was shown that the accumulation of specific AHL signals in either the floccular (Chapter 3: Section 3.3.4) or the granular sludge system (Chapter 4: Section 4.3.5) was strongly correlated with the presence of multiple, specific microbial species in the communities, including species that degrade or quench AHLs. It is therefore hypothesized that AHL degrading activities or quorum quenching (QQ) may play a role in modulating QS signalling and thus, the interactions among members of diverse phylogenies to synchronize community behaviour. The present study addresses this hypothesis by investigating the relationship between QS and QQ in complex microbial communities using the floccular sludge system as a model of study.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 5-1. *Agrobacterium tumefaciens* A136, *Chromobacterium violaceum* CV026, *Escherichia coli* JM109, *Escherichia coli* JBA357 and *Pseudomonas aeruginosa* MH602 were routinely maintained in Luria-Bertani medium supplemented with 5% w/v sodium chloride (LB5) and 1.5% w/v agar, unless otherwise indicated. Antibiotics were supplemented into the growth medium whenever necessary: tetracycline (4.5 $\mu\text{g mL}^{-1}$), spectinomycin (50 $\mu\text{g mL}^{-1}$), kanamycin (50 $\mu\text{g mL}^{-1}$), ampicillin (100 $\mu\text{g mL}^{-1}$) or gentamycin (40 $\mu\text{g mL}^{-1}$). Cultures on agar plates were incubated at 30°C for 24-48 h while the liquid cultures were incubated with constant agitation on a shaker at 200 rpm at 30°C for 24 h.

Table 5-1. Bacterial strains used in this study.

Strain	Genotype and/or phenotype ^a	Reference
<i>A. tumefaciens</i> A136	AHL biosensor, carrying pCF218 plasmid with <i>traR</i> , and pCF372 plasmid with <i>P_{traI}-lacZ</i> ; Tc ^R , Sp ^R	(Fuqua and Winans, 1996)
<i>C. violaceum</i> CV026	AHL biosensor a double mini-Tn5 mutant from <i>Chrobacterium violaceum</i> ATCC 31532; Kan ^R	(McClellan <i>et al.</i> , 1997)
<i>E. coli</i> JM109	<i>rec A1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ (lac-proAB) mcrA [F' traD36 proAB lacIq lacZ ΔM15]</i>	(Yanisch-Perron <i>et al.</i> , 1985)
<i>E. coli</i> JBA357	AHL biosensor, carrying pJBA89 plasmid with <i>luxR-P_{luxI}-RBSII-gfp</i> ; Amp ^R	(Andersen <i>et al.</i> , 2001)
<i>P. aeruginosa</i> MH602	AHL producing strain, carrying a pMH602- <i>gfp</i> plasmid; Gm ^R	(Hentzer <i>et al.</i> , 2002)

^a Abbreviations: Tc^R, tetracycline resistance; Sp^R, spectinomycin resistance; Kan^R, kanamycin resistance; Amp^R, ampicillin resistance; Gm^R, gentamycin resistance; *gfp*, green fluorescent protein.

5.2.2 N-Acyl-homoserine-lactones (AHLs)

Synthetic AHLs (Sigma-Aldrich) used in this study are listed in Table 5-2. The synthetic AHLs were prepared freshly in 100% methanol (HPLC grade, Sigma Aldrich) to achieve a primary stock concentration of 1 g L⁻¹. The primary stocks were further diluted to achieve their respective final concentrations, individually or in combination, where appropriate using methanol:water (1:1 v/v) to serve as standards for subsequent liquid chromatography-mass spectrometry (LC-MS/MS) analysis as described in

Section 5.2.5.2. For experiments involving addition of AHL signals to the cultures, the synthetic AHLs were freshly prepared in dimethyl sulfoxide (DMSO) to achieve a primary stock concentration of 100 mM. The primary stocks were further diluted in DMSO or culture media to achieve their respective working concentrations.

Table 5-2. List of *N*-acyl-homoserine-lactones (AHLs).

AHL Signal	Abbreviation
<i>N</i> -butyryl-DL-homoserine lactone	C4-HSL
<i>N</i> -(3-oxohexanoyl)-DL-homoserine lactone	3OC6-HSL
<i>N</i> -hexanoyl-DL-homoserine lactone	C6-HSL
<i>N</i> -heptanoyl-DL-homoserine lactone	C7-HSL
<i>N</i> -(3-oxooctanoyl)-L-homoserine lactone	3OC8-HSL
<i>N</i> -octanoyl-DL-homoserine lactone	C8-HSL
<i>N</i> -(3-oxodecanoyl)-L-homoserine lactone	3OC10-HSL
<i>N</i> -decanoyl-DL-homoserine lactone	C10-HSL
<i>N</i> -(3-oxododecanoyl)-L-homoserine lactone	3OC12-HSL
<i>N</i> -dodecanoyl-DL-homoserine lactone	C12-HSL
<i>N</i> -(3-hydroxydodecanoyl)-DL-homoserine lactone	3OHC12-HSL
<i>N</i> -(3-oxotetradecanoyl)-L-homoserine lactone	3OC14-HSL
<i>N</i> -tetradecanoyl-DL-homoserine lactone	C14-HSL

5.2.3 Bacterial and fungal isolation from floccular sludge community

A total of five independent isolation experiments were performed from week 40 to week 44 of the floccular bioreactor operation (Section 3.3.1). Each time, 20 mL of uniformly mixed sludge samples were collected from the bioreactor at the end of an operational cycle. The sludge samples were vortexed vigorously for 5 min to disperse the floccular cells. The cell suspension was serially diluted and appropriate dilutions were spread onto the following culture media: Nutrient broth, R2A, *Pseudomonas* isolation medium, Synthetic Waste Water (SWW) (Smolders *et al.*, 1994; Zhou *et al.*, 2010) and ABT (Clark and Maaloe, 1967; Chong *et al.*, 2012) supplemented with 1.5% w/v agar each. All culture media were purchased from Becton, Dickinson and Company, unless stated otherwise. Plates were incubated at room temperature, c.a. $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, for 5 d to isolate total cultivable bacteria and fungi. A total of 330 isolates with distinct colony morphologies were randomly collected from the culture plates. Each isolate was restreaked at least 3 times on the respective culture media to ensure purity prior to identification by rRNA gene sequencing. Identified isolates were subsequently examined for their ability to produce and/or degrade AHLs according to

the bioassays as described in Sections 5.2.5 and 5.2.6. For standardization and compatibility of the bioassays, all isolates were cultured and maintained in Luria-Bertani medium supplemented with 5% w/v sodium chloride (LB5) at room temperature for detection of AHL production and degradation activities unless stated otherwise.

5.2.4 Identification of bacterial and fungal isolates by DNA sequencing

The purified isolates were identified by sequencing of their ribosomal DNA sequences. For bacterial identification, universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG) and 1492R (5'-ACGGTTACCTTGTTACGACTT) targeting the 16S rRNA gene were used (Lane, 1991), while primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) amplifying the internal transcribed spacer (ITS) region of eukaryotes was used to identify isolates of fungal origin (Gardes *et al.*, 1991). Polymerase chain reactions (PCR) were performed in a final volume of 50 μ L containing 2 mM MgCl₂, 1x AmpliTaq PCR buffer, 12 pmol of each primer, 0.4 μ M dNTPs, 1 unit of AmpliTaq polymerase and 1 μ L of cell extract. PCR cycling conditions, for both bacterial and fungal-specific primer sets, were 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were analysed by gel electrophoresis and purified using QIAquick PCR purification kit (Qiagen). The purified PCR products were subjected to DNA sequencing using the respective primers. Briefly, the sequencing reactions were performed using 50 ng of the purified PCR products in a 20 μ L reaction containing 1 μ L Big Dye Terminator™, 3.2 pmol of primer and 1x buffer. The cycling conditions, used for the sequencing reactions were denaturation at 96°C for 1 min and followed by 30 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The sequencing products were purified using butanol purification method as described (Tillett and Neilan, 1999). The purified sequencing products were analysed by ABTBiotech, Singapore. The sequences were inspected and assembled using DNA Baser sequence assembler v3.5.2 (Heracle BioSoft SRL) and further inspected using DNASTAR Lasergene 10.1 (DNASTAR Inc.). Potential chimera sequences were examined using Greengenes (<http://greengenes.lbl.gov>). Homology sequence analysis was performed using online sequence similarity search tools such as BLAST (Basic Local Alignment Search Tool)

provided by the National Centre for Biotechnology Information (NCBI), Greengenes (http://greengenes.lbl.gov/cgi-bin/JD_Tutorial/nph-16S.cgi) and SILVA (<http://www.arb-silva.de>). The isolate sequences obtained in this study are available in GenBank database under accession number KC252636 - KC252965.

5.2.5 AHL detection assays

5.2.5.1 Bacterial isolates AHL production assessed by bioassays

AHL production by individual isolates was assessed using three different AHL biosensors including *A. tumefaciens* A136 (Fuqua and Winans, 1996), *C. violaceum* CV026 (McClellan *et al.*, 1997) and *E. coli* JBA357 (Andersen *et al.*, 2001). For the *A. tumefaciens* A136 bioassay, ABT indicator agar plate with 50 µg mL⁻¹ of 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal) was prepared while LB5 agar plate was used for the *C. violaceum* CV026 bioassay. Ten microliters of overnight cultures of biosensors were spotted onto the respective indicator plates while 10 µL of overnight cultures of isolates were placed 5 mm apart from the biosensor spot. The indicator plates were incubated at 30°C for 48 h. For detection of AHLs produced *in situ*, a 96 well plate bioassay was adopted using the biosensor *E. coli* JBA357 which fluoresces green upon detecting AHLs. The overnight culture of *E. coli* JBA357 was first diluted 1:5 (v/v) with fresh LB5 medium and 100 µL of the diluted biosensor culture were added to 100 µL of isolate cultures in a 96 well plate. The culture plate was subsequently incubated at room temperature, c.a. 22°C ± 1°C, with constant shaking at 200 rpm for 4 h prior to the visualization of green fluorescent cells using confocal laser scanning microscope (LSM710, Carl Zeiss) at an excitation/emission wavelength of 488 nm/522-535 nm. Positive controls, e.g. *P. aeruginosa* MH602 and fresh culture medium amended with 3OC6-HSL, as well as negative controls, e.g. *E. coli* JM109 and fresh culture medium, were included in each experiment.

5.2.5.2 Extraction and detection of AHLs by mass spectrometer (MS)

Extraction of AHLs from the overnight cultures of isolates was performed as described by Shaw *et al.* (1997). Briefly, 50 ml of overnight cultures were centrifuged at 8,000 x g for 10 min and the cell pellets were removed. The supernatants were extracted twice with two equal volumes of dichloromethane (DCM). The DCM extracts were concentrated by rotary evaporation, and resuspended in 50 µL of methanol: water (1:1

v/v) prior to analysis by LC-MS/MS (LCMS8030, Shimadzu Inc.). Briefly, 10 μ L of the sample extract were chromatographed using a C₁₈ reversed-phase column (Shim-pack XR-ODS column: 2 x 100 mm, 2.2 μ m) at a flow rate of 0.3 mL min⁻¹. The mobile phase consisted of solvent A (25 mM ammonium formate with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). A gradient elution method was utilized which started at 5% solvent B for 0.5 min, followed by a linear gradient from 5 to 40% solvent B over 0.5 min, then to 95% solvent B over the next 5 min and remained isocratic at 95% solvent B for 1.5 min. The column was reequilibrated at 5% solvent B for 1 min. The AHL compounds separated by the LC system were ionized by electrospray ionization (ESI) under positive mode and detected using multiple reaction monitoring (MRM) approach (Morin *et al.*, 2003; Decho *et al.*, 2009). Matrix matched MRM experiments were performed for all 13 standard AHLs (Table 5-2). MRM experiments were conducted with Q1 set to monitor for the precursor ion and Q3 for the specific transition ions according to the optimized parameters for each AHL (Table A-1). The identity of each putative AHL in the DCM extract was confirmed based on specific LC retention time, appearance of the precursor ion *m/z* and two transition ions, as well as relative intensity of the two transition ions in comparison to the individual standard AHL. In addition, a full scan, ranging from *m/z* 100 to 350, coupled with precursor ion scan mode was conducted to provide further confirmation of the identity of putative AHLs in the each sample extract with respective to the standard AHLs (Decho *et al.*, 2009). Duplicate injections were performed for each sample extract. Triplicate blank injections were performed between sample injections to prevent sample carryover.

5.2.6 AHL inactivation assays

5.2.6.1 *In situ* AHL inactivation assessed by the *A. tumefaciens* A136 agar based spot bioassay

Synthetic AHLs dissolved in DMSO solvent were added to the sludge samples to achieve a final concentration of 5 μ M. The mixtures were incubated at room temperature, c.a. 22°C $\pm$ 1°C, with constant shaking at 200 rpm. Aliquots of samples were collected from the mixtures at different time points, i.e. 0, 1, 4, and 12 h, and the sludge biomass was removed by centrifugation at 8,000 x *g* for 10 min. The supernatant was transferred into a 96 well microtiter plate and subjected to UV exposure for 30 min

to eliminate any remaining microbial cells (Dong *et al.*, 2000; Zhang *et al.*, 2007). Residual AHLs in the supernatant was measured using the *A. tumefaciens* A136 agar based spot bioassay as illustrated previously (Dong *et al.*, 2000; Zhang *et al.*, 2007). Briefly, six agar bars with a width of 1 cm each were aseptically carved from the ABT agar plates (1.5%) supplemented with 50 $\mu\text{g mL}^{-1}$ of X-gal. Five microliters of samples were spotted at one end of the agar bars. Approximately 0.5 μL of overnight culture of the *A. tumefaciens* A136 biosensor was spotted at progressively further distances from the loaded samples. The plates were incubated at 30°C for 24 h. Residual AHLs were estimated in relative to the controls. Positive and negative controls included AHLs added to SWW at pH 10.2, and SWW at pH 6.7 and heat inactivated sludge samples. AHLs are known to be inactivated (quenched) by hydrolysis at alkali pH e.g. pH 10.2 (Byers *et al.*, 2002), while minimal signal inactivation was expected for AHLs added to the SWW at pH 6.7 and the heat inactivated sludge samples. AHLs added to the heat inactivated sludge samples were used as the physical sorption controls as well. The pH of each sample was closely monitored throughout the study.

5.2.6.2 *In situ* AHL inactivation kinetics assessed by the MS

The kinetics of AHL inactivation mediated by the sludge community was determined. Synthetic AHLs dissolved in DMSO were added in combination to the sludge samples to achieve final concentrations of 5 μM for each AHL and incubated at room temperature c.a. 22°C $\pm$ 1°C, with constant shaking (200 rpm). Aliquots of samples were collected from the mixtures at different time points, i.e. 0, 1, 2, 4, 8 and 24 h, and the sludge biomass was removed by centrifugation at 8,000 $\times g$ for 10 min. Residual AHLs in the supernatant was measured by LC-MS/MS as described above (Section 5.2.5.2). Negative controls, including heat inactivated sludge and SWW media were included. The pH of each sample was closely monitored throughout the study and ranged between pH 6.7 and 6.9. The effect of pH on the integrity of AHLs was also assessed by adding synthetic AHLs to SWW at different pH values ranging from pH 6.7 to pH 8.3. AHL signals may adsorb onto the sludge surfaces and become unavailable for extraction and detection rather than being inactivated *per se*. The sorption values of AHLs were determined by comparing the residual AHLs in the SWW media and heat inactivated sludge controls, added immediately as well as after 1 h of incubation. The sorption of 5 μM short to medium-chain AHLs (C4-C8) to the heat

inactivated sludge was found to be experimentally insignificant while the sorption of the long-chain AHLs (C10-C12) to the heat inactivated sludge was determined to be 10-30%.

5.2.6.3 Spatial AHL inactivation assessed by the *E. coli* JBA357 96 well plate bioassay

QQ activities present in sludge biomass and sludge supernatant were separately tested. Briefly, 10 mL of sludge samples were collected and centrifuged at 8,000 x g for 10 min. The sludge pellets were washed with SWW at pH 6.7 while the sludge supernatants were filtered through the 0.2 µm filters (Sartorius). QQ activities in both samples were subsequently assessed by the assay described above. 3OC6-HSL was used as the AHL reference in this particular case. Residual 3OC6-HSL in the each sample was measured using the *E. coli* JBA357 96 well plate AHL detection bioassay described in Section 5.2.5.1, with minor modifications. Instead of CLSM (LSM710, Carl Zeiss) visualization, the green fluorescent intensity was quantified using a microplate reader (Tecan Infinite M200) at excitation and emission wavelengths of 488 nm and 530 nm, respectively. A standard curve was constructed to estimate the amount of 3OC6-HSL present. Negative controls, i.e. heat inactivated sludge biomass and sludge supernatant, were included.

5.2.6.4 Enzyme mediated AHL inactivation assessed by the *E. coli* JBA357 96 well plate bioassay

To assess enzyme mediated AHL degradation, total proteins were first extracted from the sludge samples by sonication as described previously (Zhang *et al.*, 2007). After sonication, cell debris were removed by centrifugation at 16,000 x g for 20 min at 4°C. The supernatant fraction containing total protein extract was analyzed using the Bichichoninic acid (BCA) assay (Thermo Scientific Pierce). Enzyme mediated signal inactivation was quantified according to the procedure described above except that no UV treatment was employed. One milligram of the total protein extract was used for each quenching reaction. The quenching kinetics of the total protein extract was assessed based on the 3OC6-HSL. Residual 3OC6-HSL at 0, 30, 60 and 120 min, was measured using the *E. coli* JBA357 96 well plate AHL detection bioassay described in Section 5.2.5.1. A heat inactivated protein extract was included as the negative control.

5.2.6.5 Bacterial isolates mediated AHL inactivation assessed by the *A. tumefaciens* A136 agar based spot bioassay

For screening of QQ activities among the isolates, AHLs with short (3OC6-HSL), medium (3OC8-HSL) and long (3OC12-HSL) acyl chains were tested. Synthetic AHLs were added to the overnight culture of isolates to achieve a final concentration of 5 μ M. The mixtures were incubated at room temperature, c.a. $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with constant shaking at 200 rpm for 2 h. After centrifugation and UV sterilization, residual AHLs were quantified using the *A. tumefaciens* A136 agar based spot bioassay as described in Section 5.2.6.1. *E. coli* JM109 was used as the negative control. The pH of the cultures was recorded at the end of the experiment and was always between pH 6.9 to 7.2.

5.2.7 Determination of lactonase activities

Five microliters of 1 M HCl were added to 50 μ L of UV treated QQ samples to achieve a final pH of less than 2.0 (Yates *et al.*, 2002). The mixtures were incubated at 4°C for 48 h and residual AHLs were quantified by the *A. tumefaciens* A136 agar based spot bioassay (Section 5.2.6.1). A blank control with 5 μ L of miliQ water added instead of 1M HCl was included. Positive controls for the acidification process were prepared by incubating AHLs in SWW at pH 10.2, resulting in the hydrolysis of the lactone rings, similar to the lactonase activity. The alkali treated samples were subsequently acidified to less than pH 2.0 to induce lactone ring closure.

5.2.8 Phylogeny analysis

Representative isolate sequences of bacterial origin with unique QS and QQ profiles were selected for phylogenetic tree construction. The sequences were aligned by ClustalW Multiple alignment package provided by the BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) and a consensus region covering all the sequences was selected for further analysis. The aligned sequences were subjected to phylogenetic tree construction using the neighbour-joining method (Saitou and Nei, 1987) provided by the MEGA4 (<http://www.megasoftware.net/mega4/mega.html>). Maximum likelihood bootstrap analyses were carried out with 1,000 replicates.

5.2.9 Statistical analysis

All statistical analyses were performed using the Prism version 5 for Windows (GraphPad Software Inc.).

5.3 RESULTS

5.3.1 Floccular sludge community preferentially degrades AHLs with long acyl chains.

Previous work (Chapter 3: Sections 3.3.2 and 3.3.3) demonstrated the presence of AHL mediated QS in the SNDPR floccular sludge system over an operational period of 82 weeks. To determine if AHL specific QQ activity was present in this model system, synthetic AHLs were added individually or as a mixture to the floccular sludge, harvested at different time points of bioreactor operation (Chapter 3: Figure 3-1), to characterize the rates of signal degradation. In general, the AHLs were readily inactivated at different kinetic rates (Figure A-12), and the signal half-life was inversely proportional to the length of the acyl chain, regardless of the substitution at C3 position (Figure 5-1). While the half-lives for short-chain signals C6-HSL and 3OC6-HSL were 1.95 ± 0.45 h and 2.57 ± 0.29 h, respectively; the half-lives of the long-chain signals C12-HSL and 3OC12-HSL were significantly shorter at 0.79 ± 0.08 h and 0.66 ± 0.06 h, respectively. This loss of signal was dependent on biological activity, where the half-lives of the AHLs in the presence of live sludge were substantially lower than when incubated in the presence of heat inactivated sludge. For example, the signal half-lives for 3OC6-HSL, 3OC8-HSL, 3OC10-HSL, and 3OC12-HSL were 10, 21, 27 and 53 fold shorter, respectively, in the presence of live sludge compared to heat inactivated controls (Figure 5-1B). The short-chain AHLs, e.g. C4-HSL, were degraded faster than the long-chain signals in the presence of the heat inactivated biomass (Figure 5-1), consistent with signal auto-degradation kinetics in media control at pH 6.7 (Figure A-13). The pH was consistently determined to be at pH 6.8 ± 0.1 for both live and heat inactivated sludge samples during the batch experiments.

Because the bioreactor pH may fluctuate between pH 6.7 to 8.2 during the operation (Figure A-11) and the alkaline pH may mediate AHL degradation (Yates *et al.*, 2002; Decho *et al.*, 2009), the kinetics of AHL inactivation in different pH conditions were investigated. The half-lives for all AHLs decreased as the media pH increased (Figure A-13). Nevertheless, the half-lives for all AHLs were at least 3 to 10 fold longer than when incubated in the presence of live sludge, even if the media pH was raised to 8.3. In addition, AHLs added to the sludge may adsorb to the sludge surfaces and become

unavailable for extraction and detection, rather than being inactivated. Therefore, the proportion of AHLs adsorbed to the sludge was determined directly in heat inactivated sludge. Approximately 10-30% of the long-chain AHLs were determined to be lost due to adsorption to the biomass with the remaining 70-90% of signal loss attributed to biological activity (Figure A-12). The adsorption of short to medium-chain AHLs (C4-C8) to the sludge was found to be experimentally insignificant. These results thus strongly support that the rapid signal degradation observed was mainly attributed to the microbial activity than and the alkaline pH or physical adsorption.

Figure 5-1. Degradation of AHLs based on the function of acyl chain length in the floccular sludge environment. The degradation value is expressed as the signal half-life. A mixture of unsubstituted (A) and oxo-substituted (B) AHLs was added to the live (open circles) or the heat inactivated (solid circles) sludge sample to a final concentration of 5 μ M for each AHL species. The sludge mixture was incubated at room temperature with constant shaking at 200 rpm. Residual AHLs were extracted and quantified using LC-MS/MS at different time points. The signal half-life for each AHL was estimated based on the zero-order or first-order kinetics. All results are shown as Mean $\pm$ SEM, with three independent replicates.

5.3.2 AHL inactivation is mediated by intracellular enzymatic activities

To confirm the source of the QQ activity in the floccular sludge environment, exogenous AHL (3OC6-HSL) was independently added to sludge biomass and sludge supernatant for 18 h. Residual 3OC6-HSL was assessed using the *E. coli* JBA357 biosensor (Andersen *et al.*, 2001). There was no obvious degradation observed for signals added to the sludge free supernatant, while in contrast, complete inactivation of exogenous 3OC6-HSL was observed in the presence of floccular biomass (Figure 5-2). Specifically, synthetic 3OC6-HSL was degraded by intracellular enzymatic activities derived from the sludge biomass as demonstrated by progressive degradation of AHL signal incubated with the crude protein extracts from the floccular sludge community (Figure 5-3). The signal half-life for 3OC6-HSL incubated with 1 mg crude protein extract was approximately 7 fold lower than that when incubated with the heat inactivated control, suggesting that the sludge community produced enzymes that can catalyse the degradation of AHLs. When the sludge inactivated signals were subsequently acidified, functional AHLs were recovered that activated the biosensor although the amount of signal recovered decreased with time of incubation (Table 5-3). This indicates that lactonases may account for some of the QQ activity observed (Yates *et al.*, 2002).

Figure 5-2. AHL inactivation is mediated by the sludge biomass activities. Inactivation of synthetic 3OC6-HSL by sludge biomass (A and B) and sludge supernatant (C and D) were compared. Different concentrations of synthetic 3OC6-HSL were added to the sludge biomass or the sludge supernatant (solid bars). Corresponding negative controls, i.e. heat inactivated sludge biomass and heat inactivated sludge supernatant (open bars), were included. The samples were incubated for 18 h and the residual 3OC6-HSL was measured using the *E. coli* JBA357 96 well AHL detection bioassay. The x-axis represents the concentration of 3OC6-HSL added to each of the samples at time 0 h. All results are shown as the Mean $\pm$ SEM, with three independent replicates. RFU represents relative fluorescence unit.

Figure 5-3. AHL inactivation is mediated by intracellular enzymatic activities. Inactivation of 2 μ M of exogenous 3OC6-HSL by 1 mg of total protein extracts from the sludge biomass (open circles) were assessed over a period of 120 min at room temperature. Negative controls, where 3OC6-HSL were added to the heat inactivated protein extracts, were included (solid circles). Residual 3OC6-HSL in each treatment was quantified using the *E. coli* JBA357 96 well bioassay at 0, 30, 60 and 120 min post incubation. The signal half-life for each AHL was estimated based on the zero-order or first-order kinetics. All results are shown as Mean $\pm$ SEM, with three independent replicates.

Table 5-3. Recovery of AHLs from quorum quenching experiments as an indication of lactonase activities in the floccular sludge community.

AHL ^a	% of signal recovered by acidification ^b (Mean $\pm$ SD) ^c		
	Quorum Quenching Reaction Time (h)		
	1	4	12
3OC6-HSL	100 $\pm$ 0	100 $\pm$ 0	62.5 $\pm$ 17.7
3OC8-HSL	100 $\pm$ 0	80 $\pm$ 0	60 $\pm$ 0
3OC10-HSL	83.3 $\pm$ 23.6	0 $\pm$ 0	0 $\pm$ 0
3OC12-HSL	75 $\pm$ 35.6	0 $\pm$ 0	0 $\pm$ 0

^a Five micromolar of synthetic AHLs were added to the sludge samples.

^b % of signal recovered is expressed as the proportion of signal recovered from the total signal inactivated [(HCl-H₂O)/(Total-H₂O)]*100%.

^c Data represent the mean of two independent replicate experiments.

5.3.3 The majority of community isolates are capable of quenching AHLs

To further assess the generality of QS and QQ activities in the sludge community, we have examined the ability of a diverse range of isolates cultured from the floccular sludge system, to both synthesize and degrade AHLs. A total of 330 isolates including 307 strains of bacteria and 23 strains of fungi, which correspond to 50 bacterial and 4 fungal genera, respectively, were investigated (Table A-4). The bacterial isolates

represented 3.5% of the community members identified by meta-rRNA sequencing (Table A-5). AHL production was primarily determined based on activation of different biosensors, including *E. coli* JBA357 (Andersen *et al.*, 2001), *A. tumefaciens* A136 (Fuqua *et al.*, 1995) and *C. violaceum* CV026 (McLean *et al.*, 1997). The AHL profile of each putative signal producer was subsequently determined using LC-MS/MS. AHL degradation was assayed using AHLs with short (3OC6-HSL), medium (3OC8-HSL) and long (3OC12-HSL) acyl chains. While only about 10% of the isolates were found to produce AHLs, 58.1% of the isolates were able to quench at least one of the AHLs examined (Figure 5-4). A relatively small proportion of the isolates, 4.8%, both produced and quenched AHLs. The remaining 36.7% of the isolates neither produced nor quenched AHLs. All of the AHL quenchers were capable of inactivating the long-chain AHL, 3OC12-HSL. While 77% of the quenchers could only degrade the long-chain AHLs, approximately 23% could degrade long-chain AHLs as well as short and/or medium-chain AHLs.

Figure 5-4. Distribution of microbial isolates according to their capacity to produce and/or quench AHLs. A total of 330 isolates, including 307 and 23 strains of bacterial and fungal origins, respectively, were cultured from the floccular sludge community during the bioreactor operational period from week 40 to 44. All isolates were screened for AHL production as detected by different bioassays and LC-MS/MS, as well as for their capability to quench 5 μ M of AHLs with short (3OC6-HSL), medium (3OC8-HSL) and long (3OC12-HSL) acyl chains within a 2-h incubation time. Residual AHLs were quantified using the *A. tumefaciens* A136 agar based spot bioassay.

5.3.4 AHL producers and quenchers are of diverse origins

The evolutionary relationship among the bacterial isolates, based on their 16S rRNA sequences, indicated that there was no specific clade associated with QQ activity while the QS organisms were all classified as alpha, beta, or gamma Proteobacteria (Figure 5-5). The classification of QS isolates as exclusively Proteobacteria was consistent with previous work which had indicated that approximately 10% of isolates were QS and were all Proteobacteria (Taylor *et al.*, 2004; d'Angelo-Picard *et al.*, 2005; Morgan-Sagastume *et al.*, 2005). The QS isolates identified in this study represented genera that have previously been reported to be AHL producers, such as *Acidovorax*, *Pantoea*, *Rhizobium*, *Rhodobacter*, *Sphingomonas* and *Stenotrophomonas*, as well as novel genera that have never been reported to synthesize AHLs, including *Frateuria*, *Lysobacter* and *Shinella* (Figure 5-5 and Table 5-4). The LC-MS/MS analysis further revealed that the isolates produced AHLs of various acyl chain lengths ranging from 4 to 14 carbons, with or without modifications at the C3 position (Table 5-4). Every signal producing isolate synthesized more than one type of AHL and multiple strains could produce the same AHLs. In contrast, the AHL quenchers were classified into four different bacterial phyla, including the Proteobacteria, Actinobacteria, Bacterioidetes and Firmicutes, as well as the Fungi (Figure 5-5 and Table A-4). While some of the QQ isolates represented genera that are commonly reported to degrade AHLs, such as *Ochrobacterium*, *Variovorax*, *Bacillus* and *Rhodococcus*, most of the QQ isolates identified here have not been previously shown to do so. These novel QQ organisms were distributed amongst genera of *Novosphingobium*, *Acidovorax*, *Rheinheimera*, *Tsukamurella*, *Flavobacterium*, *Candida* and others. The number of genera involved in quenching of 3OC12-HSL, 3OC8-HSL and 3OC6-HSL, were found to be 37, 12 and 8, respectively (Table A-4). Interestingly, most of the signal producers had close relatives, same genus or species that were capable of degrading the AHLs (Figure 5-5). In some cases, the same AHL producing strain may even be capable of degrading its own signals, e.g. *Rhizobium borbori* N065. In addition, there was considerable variation even at the species level. For example, 10 strains of *Stenotrophomonas* sp. with identical 16S rRNA sequences were able to produce AHLs while the remaining 6 strains did not. Likewise, not every strain of the same species was able to quench AHLs, as was the case for *Stenotrophomonas maltophilia*. The dedicated QS and QQ characteristics of the cultivable community suggest that the community communication

is highly complicated with the engagement of species of multiple origins *in situ* and it is likely that these QS and QQ activities are important for maintaining the community functions measured here.

Figure 5-5 (next page). Evolutionary relationship of 100 representative bacterial isolates (taxa) based on the 16S rRNA gene sequences. Each representative was selected for their uniqueness in terms of AHL production and/or quenching properties. The evolutionary relationship was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary relationship of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree was drawn to scale, with branch lengths reflecting evolutionary distances. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1,031 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. The out groups, retrieved from the GenBank database, are underlined. The isolated strains are classified as producer (▲), quencher (■), producer and quencher (◆), and neither producer nor quencher (○). The genera in which AHL production (@) or AHL degradation (*) activities have been reported in the literature are indicated. The closest relative with at least 97% sequence similarity for each strain, except for those strains highlighted in bold, is shown.

Table 5-4. LC-MS/MS profiling of AHLs produced by the representative isolates cultured in LB5 and R2A media.

Strain	Closest Match	C4-HSL	C6-HSL	3OC6-HSL	C7-HSL	C8-HSL	3OC8-HSL	C10-HSL	3OC10-HSL	C12-HSL	3OC12-HSL	3OHC12-HSL	C14-HSL	3OC14-HSL
Alpha-proteobacteria														
N065	<i>Rhizobium borbori</i>													
R055	<i>Rhizobium borbori</i>													
R023	<i>Rhodobacter maris</i>													
R074	<i>Rhodobacter maris</i>													
R076N	<i>Rhodobacter maris</i>													
S009a	<i>Rhodobacter maris</i>													
S011a	<i>Rhodobacter maris</i>													
N016	<i>Shinella fusca</i>													
R054a	<i>Sphingomonas</i> sp.													
R060	<i>Sphingomonas</i> sp.													
Beta-proteobacteria														
R081	<i>Acidovorax facilis</i>													
Gamma-proteobacteria														
N027a	<i>Frateriia</i> sp.													
R011a	<i>Frateriia</i> sp.													
R037	<i>Lysobacter brunescens</i>													
R053	<i>Lysobacter brunescens</i>													
R092	<i>Lysobacter brunescens</i>													
R067d	<i>Pantoea stewartii</i>													
N025	<i>Stenotrophomonas</i> sp.													
N064	<i>Stenotrophomonas</i> sp.													
P026NR	<i>Stenotrophomonas</i> sp.													
P026NS	<i>Stenotrophomonas</i> sp.													
P088	<i>Stenotrophomonas</i> sp.													

Detected by LC-MSMS
 Undetectable by LC-MSMS

5.4 DISCUSSION

The study of interspecies interactions is crucial for developing a systematic understanding of the physiology of high density, matrix encased microbial communities, which are now widely appreciated as the dominant mode of growth for bacteria in most natural and engineered ecosystems (Straight and Kolter, 2009; Elias and Banin, 2012). Communication via diffusible signalling molecules has been proposed to be one of the important interacting mechanisms among species of diverse origins (Elias and Banin, 2012), and there is growing evidence that complex communities in natural environments are highly communicative with the constant accretion of a plethora of signals within the microbial consortia (Decho *et al.*, 2009; Chong *et al.*, 2012; Van Mooy *et al.*, 2012). While studies on simple dual-species systems have revealed many exciting phenotypic responses as consequences of signalling mediated interactions (Elias and Banin, 2012), the global effect of signal accumulation and hence species interactions in complex communities is largely unknown, similarly there is a dearth of information on how regulation of communication systems is mediated. By building on the technological advances in meta-sequencing and analytical chemistry as well as the application of an improved culture dependent microbiological approach, it has been possible to begin unravelling the complexity of community communication by deciphering the details of AHL mediated signalling system in a floccular sludge community.

Using an activated floccular sludge community as a model ecosystem, this study has shown that community level communication involves the concerted activities of QS and QQ (Figure 5-1 and Section 3.3.3: Figure 3-5) that are mediated by members of different taxa, including both bacteria and fungi (Figure 5-5 and Table A-4). Whereas the proportion of QS populations, specifically the AHL producers (10%) in this cultivable sludge community is consistent with those in other environments (d'Angelo-Picard *et al.*, 2005; Cirou *et al.*, 2007), the frequency of AHL quenchers (QQ populations) was considerably higher (60%) than that reported by other studies, approximately 5-15% of cultivable isolates (d'Angelo-Picard *et al.*, 2005; Cirou *et al.*, 2007). The discovery here, that a significant fraction of the community has QQ activity, is a consequence of testing for QQ activity using a range of AHLs as targets, including the long-chain AHLs, which have not typically been used to detect QQ activity. It was

particularly interesting to note that while all of the QQ isolates in this study could degrade the long-chain AHLs, only a small proportion of those QQ organisms (23 %) were able to degrade the short and medium-chain AHLs (Figure 5-4) and this is consistent with the AHL degradation kinetics *in situ* (Figures 5-1 and A-12). The sludge community degraded AHLs with long acyl chains at the fastest rate (3OC12-HSL $t_{1/2}$ 0.66 ± 0.06 h), followed by the degradation of medium (3OC8-HSL $t_{1/2}$ 1.39 ± 0.16 h) and short-chain (3OC6-HSL $t_{1/2}$ 2.57 ± 0.29 h) AHLs (Figure 5-1). Thus, the QQ activities of the spectrum of isolates collected were more generally reflected in the types of signals that were degraded by the whole community *in situ*. This observation also corresponded with the profile of AHLs that were identified in the sludge community, where only AHLs with short or medium acyl chains were detected, while signals with long acyl chains ≥ 10 carbons were not detected (Section 3.3.3: Figure 3-5).

Although it would be tempting to speculate that the lack of long-chain AHLs in the sludge was due to the absence of producer strains, this was clearly not the case as almost every QS isolate produced at least one long-chain AHL (Table 5-4), suggesting that the sludge community has the capacity to synthesize the long-chain signals *in situ*. Alternatively, it is possible that the hydrophobic, long-chain AHLs were not detected due to partitioning into the sludge biomass as has been observed for microbial mats (Decho *et al.*, 2009). While a small proportion (10-30%) of exogenously added long-chain AHLs (C10-C12) were observed to adsorb to the sludge biomass, it is highly unlikely that adsorption alone could account for progressive reduction of the remaining 70-90% of long-chain AHLs (Figure A-12). Instead, the data presented here demonstrate that the rapid signal decay was mainly attributed to microbial activity and that community QQ activity was responsible for the selective removal of long-chain signals. In support of this, metagenomic analysis of the sludge community revealed the presence of multiple putative QQ genes, including 109 AHL acylase and 46 AHL lactonase homologs (data not shown). Acylases have been shown to preferentially degrade the long-chain AHLs, in contrast to AHL lactonases that show no such specificity for AHLs at all acyl chain lengths (Huang *et al.*, 2003; Wang *et al.*, 2004; Park *et al.*, 2005; Chen *et al.*, 2009). The high proportion of AHL acylases, along with the presence of isolates that produce long-chain AHLs strongly supports the conclusion

that the long-chain AHLs are preferentially removed from the community as a consequence of specific biological activity *in situ* and that the differential QQ activity of individual community members collectively contributes to the modulation of overall community signalling events.

Based on the preferential loss of long-chain AHLs and the strong positive correlation of the remaining AHLs with community metabolism, the results presented here suggest that QQ activity favouring the accumulation of short and medium-chain AHLs plays a role in the regulation of nitrogen metabolism via the nitrite shortcut pathway (Section 3.3.3: Figure 3-6). Indeed, there is clear precedence for the role in AHL mediated signalling in multiple aspects of nitrogen metabolism (Batchelor *et al.*, 1997; Yoon *et al.*, 2002; Toyofuku *et al.*, 2007; De Clippeleir *et al.*, 2011). For example, AHL based signalling has been shown to be involved in negative regulation of nitrite accumulation (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007). In *P. aeruginosa*, increased levels of the short-chain AHL, C4-HSL downregulates the expression of denitrification enzymes via RhlR, reducing the build-up of toxic nitrite and nitric oxide (NO) in the medium, and thus increasing its survival (Yoon *et al.*, 2002; Toyofuku *et al.*, 2007). The activation of QS, via the *rhlI* and *rhlR* system, is in turn controlled by the nitrogen responsive sigma factor, RpoN (Thompson *et al.*, 2003). This highly regulated QS sensing system may also be relevant and beneficial at the community level since both nitrite and NO, the by-products of denitrification, are toxic to most microorganisms when they accumulate to high concentrations (Fang, 1997). Selective accumulation of short and medium-chain AHLs, as a consequence of preferential signal degradation, may therefore protect the whole community from intoxication by high levels of nitrite and NO by down regulating their production in the community. However, the specific role of AHLs with different acyl chain lengths in the regulation of nitrite metabolism remains to be addressed.

AHL based signalling has also been demonstrated to play a role in the nitrification process by promoting oxidation of ammonium to nitrite (Batchelor *et al.*, 1997; De Clippeleir *et al.*, 2011). For example, exogenous addition of short-chain AHL, 3OC6-HSL was found to accelerate the recovery of *Nitrosomonas europaea* from starvation and also its capability to oxidize ammonium to nitrite (Batchelor *et al.*, 1997).

Importantly, many ammonium oxidizing organisms (AOBs), including *N. europaea*, *Nitrospira multiformis* and *Nitrospira hamburgensis* that are commonly involved in nitrite production in water and wastewater treatment facilities (Arp *et al.*, 2007), have been documented to either produce AHLs (Burton *et al.*, 2005) or to encode AHL syntheses in their genomes (Chain *et al.*, 2003; Norton *et al.*, 2008). In our system, there were at least two species from *Nitrospira* and one species from *Nitrosomonas* present among the top 30 most abundant community members (Table A-5). A metagenomic analysis of the sludge community further revealed the presence of a pair of putative AHL synthase and receptor genes, with more than 76% protein sequences matched to that of the *N. hamburgensis* QS system (data not shown). These findings thus suggest a potential link between the AOBs, AHL QS signalling and nitrite metabolism at the community level and that preferential QQ activity of the community may favour the regulation of nitrification process based on the short and medium-chain AHL signalling system.

Although QQ may serve to modulate community signalling as discussed, the QQ activity is most likely not an altruistic behaviour on the part of the quenchers, but potentially provides the quenchers with a competitive advantage. AHLs can be utilised by bacteria as a source of carbon and nitrogen, the latter of which is often limiting in many habitats (Leadbetter and Greenberg, 2001; Huang *et al.*, 2003). Given that most of the QQ activity is intracellular and hence the breakdown products would be available to the AHL degrading cell, the acquisition of nutrients may be one of the evolutionary drivers leading to the widespread nature of QQ activities. Alternatively, AHLs and their derivatives have been shown to have some antimicrobial activities. For example, long-chain AHLs, e.g. 3OC12-HSL, and the derivatives, i.e. tetramic acids, have antimicrobial activity, particularly against Gram positive bacteria (Kaufmann *et al.*, 2005; Romero *et al.*, 2011). The long-chain AHL produced by *Rhizobium leguminosarum* was originally classified as a bacteriocin based on its antimicrobial activity (Gray *et al.*, 1996; Schripsema *et al.*, 1996). Furthermore, *P. aeruginosa* outcompetes *Staphylococcus aureus* when the two are grown in co-culture and this effect is the result of 3OC12-HSL mediated growth inhibition of *S. aureus* (Qazi *et al.*, 2006). To increase their survival, the competitors are therefore expected to evolve strategies to counter against QS regulated behaviour and QQ could be one such

mechanism. In line with this argument, QQ potential has been shown to increase survival of *Bacillus* species in the rhizosphere, where the QQ mutant was always outgrown by the wildtype strain (Park *et al.*, 2008). Therefore, inactivation of AHLs by QQ enzymes may represent a defensive strategy to protect bacteria from the inhibitory effects of AHLs (Dong *et al.*, 2001; Yang *et al.*, 2005).

The co-occurrence of QS and QQ activities identified here is likely to be common in many other habitats. The organisms that are known to be AHL producers or quenchers, including Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria phyla, have recently expanded to include Cyanobacteria (Romero *et al.*, 2008; Sharif *et al.*, 2008), Acidobacteria (Bijtenhoorn *et al.*, 2011; Kalia *et al.*, 2011), Bacteroidetes (Huang *et al.*, 2008; Romero *et al.*, 2010; Rashid *et al.*, 2011) and Archaea (Kalia *et al.*, 2011; Zhang *et al.*, 2012), suggesting that AHL mediated community communication as characterized by both QS and QQ activities is ubiquitous and prevalent in natural environments. Further, many of these species co-exist or even co-dominate the same niches, such as in the rumen (Hughes *et al.*, 2010; Koenig *et al.*, 2011), microbial mats (Decho *et al.*, 2009) and soil/rhizosphere (d'Angelo-Picard *et al.*, 2005; Cirou *et al.*, 2007). This is also likely to be true for used water treatment facilities, which have an extremely high microbial diversity, including many QS and QQ organisms identified in this study and in the literature. Indeed, as indicated above, preliminary data suggest that multiple QS and QQ genes are present in the activated sludge community from a full scale plant. These data not only strongly support the co-occurrence of QS and QQ activities in the bioreactor community but also that community behaviour is in some part controlled by the sum activity of the QS and QQ populations present along with the specificity of the QQ activity for particular AHL signals.

In conclusion, it has been demonstrated here that microbially mediated QQ is prevalent in the floccular sludge community, consistent with the observation that a large proportion of community members (> 70%; identified by culture-independent meta-rRNA sequencing) was negatively associated with the community AHL production (Figure 3-7). The overall AHL profile of the community was observed to be in part controlled through the QQ specificity for long-chain AHLs, leading to the selective accumulation of short to medium-chain AHLs. The observation that QS and QQ

activities were associated with phylogenetically diverse bacterial species that are ubiquitous in many different natural habitats, engineered ecosystems or even clinical settings, strongly suggests that AHL mediated community communication could be a general feature of complex communities, and that the concerted QS and QQ activities may play a significant role in the selection of stable and functional communities.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUDING REMARKS

6.1 GENERAL DISCUSSION

QS signalling via small diffusible chemical molecules has been proposed as one of the important mechanisms that mediates interactions among diverse microbial species in nature (Elias and Banin, 2012). Support for this first emerged from detection of signalling molecules *in situ* in a wide range of natural habitats (Steidle *et al.*, 2001; Gantner *et al.*, 2006; Decho *et al.*, 2009) and was further supported by reports on the regulation of interspecies phenotypic changes via QS signalling in synthetic dual-species model systems (Riedel *et al.*, 2001; Dulla and Lindow, 2009; Elias and Banin, 2012). Conceivably, different QS signalling systems, in particular the AHL based QS, have been exploited in nature, however, the global effect and the role of QS signalling in these habitats remain largely unknown. The findings described in this thesis not only support the hypothesis that AHL signalling mediates interspecies interactions in complex environments, but also suggest that AHL based QS signalling is closely linked to the regulation of community metabolism (Chapter 3) and community assembly (Chapter 4). Importantly, this work provides insights into the QS biology at the community level with the identification of several key features unique to the community QS, such as the competing activity of quorum sensing and quorum quenching populations, highlighting the limitations of our understanding of QS biology as derived from conventional single species, planktonic population studies.

At the population level, QS signalling operates as a function of cell density and signal concentration (Lazdunski *et al.*, 2004). Activation of QS occurs only at specific signal threshold concentrations that accumulate in the extracellular environment and this is classically linked to high cell densities or biomass (Williams *et al.*, 2007; West *et al.*, 2012). Similarly, in both floccular (Chapter 3) and granular (Chapter 4) sludge bioreactor systems, AHL signals were found to accumulate in the bulk liquid medium of the bioreactors. However, in contrast to the nanomolar signal concentrations in population studies that are typically reported for QS induction, the extracellular signal concentrations in the sludge communities were present at sub-nanomolar levels, consistent with the findings of endogenous picomolar signal concentrations in the soil

environment (Wang and Leadbetter, 2005). The finding that signals were present in two separate bioreactor systems and that in both cases, the signal concentration could activate the bioreporter strain *in situ* strongly suggest that these low levels of signals are indeed biologically relevant. Although the function of AHL signalling in soil communities remains to be established (Wang *et al.*, 2004; d'Angelo-Picard *et al.*, 2005), strong, positive associations between AHL mediated QS and sludge community nitrite metabolism as well as the development of microbial granules were clearly demonstrated in this thesis (Chapters 3 and 4), further supporting the relevance of low signal concentrations in the sludge environment. Based on these observations it may be postulated that low AHL concentrations are more typical of complex communities and that these low concentrations are sufficient to control the QS phenotypes. Thus, there is a difference in the minimum inducing signal concentration as compared to laboratory based studies of bacterial populations. The lower induction threshold for environmental communities may reflect the challenges in signal accumulation. For example, in complex environments, signals would be constantly diluted, degraded, or sequestered due to interaction with the abiotic components of the environment, e.g. soil, as well as changes in the community composition, competition with signal degrading organisms and changes in metabolic activity of signal producers. Bacteria equipped with QS systems of high sensitivity will be able to detect and to respond swiftly to minor, yet significant, changes in the immediate milieu either for cooperation or competition between different bacterial species for survival. Indeed, such supersensitive QS systems have recently been reported in two members of the genus *Bradyrhizobium*, which respond to low picomolar levels of signal concentrations, i.e. 10 pM (Ahlgren *et al.*, 2011; Lindemann *et al.*, 2011). While it remains to be seen if such high sensitive QS systems are common in nature, other mechanisms may exist to explain how the extracellular AHL signals might induce QS in a complex environment at signal levels that are orders of magnitude lower than that of model QS systems in the laboratory. Some potential avenues to explore include changes in global regulators that influence QS activation as a consequence of community metabolism or perhaps via synergistic induction where a secondary signal is required to sensitise the bacteria for QS induction as exemplified by co-modulation of gene expression by both LasIR and RhlIR QS systems in *P. aeruginosa* (Schuster *et al.*, 2003).

Many of the community members of the floccular or granular sludge that were shown to be QS associated in this thesis work (Chapters 3 and 4), have either been previously demonstrated to synthesize AHLs or were isolated and shown to be the AHL producers (Chapter 5). Thus, these organisms are likely to contribute to AHL production in the sludge bioreactors. On the other hand, several community members that negatively associate with QS signalling were found to degrade AHLs when they were isolated, suggesting that these AHL quenchers are responsible for the signal turnover in the sludge environment. Accretion of extracellular AHL signals for the sludge communities appears to depend on the dynamic abundance of these specific AHL producers and quenchers instead of relying exclusively on the total sludge biomass as would be expected for a microbial population QS signalling. Given the high diversity of the sludge communities, the contribution of multiple bacterial species to the observed signal accumulation in the bioreactors may not be entirely surprising. Indeed, many different bacterial isolates from a single environment, such as the soil and the leaf surfaces, have exhibited the capability to contribute to signal accumulation in their habitats (d'Angelo-Picard *et al.*, 2005; Dulla and Lindow, 2009). As such, these findings may strongly support the concept of interspecies interactions or communication in complex environments from both the perspectives of signal pooling (i.e. signal generation by different AHL producers) and signal sharing (i.e. uptake of AHLs accumulated in the environments by different AHL responders). The later is especially important in order to promote system wide communication between the individual sludge particles, i.e. flocs or granules that can often vary in distance due to convection generated by the frequent gas flow supplied to the bioreactors. This would presumably enable a truly global coordination of community behaviour and hence achieve communal cooperation, such as development of microbial granules and modulation of toxic metabolite levels. However, it may also be argued that the functional calling distance may make communication challenging at such scale. While it has been determined that the maximum calling distance between a signal producer and a signal responder can be long as 78 μm (Gantner *et al.*, 2006), interfloccular or intergranular communication, i.e. interparticle signalling, may scale up to millimetres to centimetres in the bioreactor context. Alternatively, it has been suggested that interspecies signalling within individual flocs or granules, which often appear as islands or constellations of communities, i.e. intraparticle signalling, may be more

important than interactions between flocs or granules for coordination of community behaviour. Higher signal concentrations would be expected to exist around the origin of signal production despite the possibility of neighbouring populations being AHL quenchers. Indeed, our data suggest that AHLs could accumulate in the flocs to more than 20 nM while signals accumulated in the granules were predicted to be as high as 300 nM, consistent with the observation in microbial mats where high concentrations of AHLs (e.g. 12 μ M of C10-HSL) were found to accumulate within the mats (Decho *et al.*, 2009). It is thus highly likely that QS induction would be active in the flocs and granules examined here.

Nevertheless, given the recent discoveries that bacterial species can respond to low picomolar levels of signals (Ahlgren *et al.*, 2011; Lindemann *et al.*, 2011) and that low extracellular signal concentrations were strongly and positively associated with the community nitrogen metabolism (Chapter 3) as well as granulation (Chapter 4), it remains a possibility that the low extracellular signal concentrations may also be involved in driving the QS regulated community behaviour. It is possible that different levels of signal concentrations are essential to modulate diverse community activities, as has been demonstrated in the case of *P. aeruginosa* where the transcription of different QS regulated genes were activated or repressed according to a signal gradient (Schuster *et al.*, 2003). Further experiments involving restricted enzymatic degradation of AHLs in the bioreactors, i.e. AHLs in the bulk liquid versus AHLs within the sludge biomass, would thus be essential to distinguish between the role of the intraparticle and interparticle signalling in this highly complex ecosystem.

It was observed that isolates from the sludge communities had the capacity to synthesize a wide spectrum of AHLs, covering all of the synthetic AHLs available (Chapter 5). This potentially could lead to a situation where all signals are present in the community at inducing concentrations. However, despite this capacity, only short to medium and not the long-chain AHLs were found *in situ* in the bulk liquid (Chapters 3 and 4). This suggests either a preferential role of the short to medium-chain AHLs or that the phenotypes associated with the long-chain AHLs were selected against in these communities. It would be particularly useful to determine the specific phenotypes regulated by the different AHLs, e.g. 3OC6-HSL or 3OC8-HSL versus 3OC12-HSL, in

the isolates and to further verify if those QS phenotypes or gene circuits are activated or repressed in the sludge community where the long-chain AHLs were below the detection limit. While the long-chain AHLs were not detected in the bulk liquid medium and hence may not participate in the interfloccular or intergranular communication, they may nonetheless accumulate in the sludge biomass due to their hydrophobic nature to mediate interspecies interactions in the flocs or granules. Indeed, examples of long-chain AHLs accumulating in the biofilm structures have been demonstrated in *P. aeruginosa* and microbial mats (Charlton *et al.*, 2000; Decho *et al.*, 2009), although it remains to be determined if such long-chain AHLs are also present in the sludge biomass. If they do, it may further suggest functional partitioning of QS signalling based on specific AHL structures in complex communities where the short to medium-chain AHLs are chiefly engaged in interparticle communication while the long-chain signals are more likely to be involved in the intraparticle signalling within the flocs or granules. In agreement with this hypothesis, Singh *et al.* (2000) proposed that short-chain C4-HSL, instead of the long-chain 3OC12-HSL, as the key signal mediating QS processes in the biofilm context.

Although selective QS signalling in the sludge communities can be in part attributed to the hydrophobic nature of individual AHLs and hence differential mass transfer for each type of signals, the differential QS signalling behaviour may also be driven by specific microbial community QQ activity, where the long-chain AHLs were found to be preferentially degraded relative to the short and medium-chain AHLs (Chapter 5). In essence, these microbially mediated QQ activities may effectively behave as a regulatory system to modulate the accumulation of particular types of AHLs at the community level, and therefore contribute to the specific mode of AHL based QS signalling for coordination of community behaviour. Given that the sludge community composition established here is commonly seen in many other natural habitats, it would be tempting to hypothesize that such AHL based QS and QQ regulation of community communication may be widespread across different environments and play an important role to drive the structure and function of the respective local communities.

6.2 CONCLUDING REMARKS

AHL based QS signalling biology was first described as a population phenomenon and was proposed as an important mechanism to increase the fitness of a bacterial population through coordinated regulation of phenotypes. In this thesis, it has been shown that QS mediated cooperative behaviour is also relevant in complex microbial communities. Further, this QS driven cooperation may be important to facilitate control of metabolism, such as the levels of nitrification/denitrification metabolites (Chapter 3) as well as aggregation into dense particles through the control of EPS production (Chapter 4). These cooperative behaviours are therefore not only important for the survival of individual species, but the overall success of the whole community, consistent with the concept of QS regulation in populations. From the operational perspective, however, QS signalling at the community level may behave differently from that of at the population level. In addition, the accumulation of signals in the system is a function of specific populations of signal producers and signal quenchers, rather than being directly related to the total biomass as observed for single-species systems. While the specific role of microbially mediated QQ activities in sludge communities remains to be determined, the current results strongly suggest they play an important role in regulating the QS behaviour of the community. For example, the long-chain AHLs were more sensitive to QQ activity and any phenotypes controlled by those AHLs would be hence less likely to be induced in the system used here.

More importantly, the current findings are also of practical significance in terms of water and wastewater treatment processes. For example, supplementation of AHLs to a floccular sludge was able to induce EPS production, which is essential to granulation. This suggests that amendment of granulation systems with AHLs might be a strategy to either enhance granule formation or to maintain the mature granules. It would be particularly interesting to examine the long-term effect of AHLs to the granulation process by signal add-back to further confirm our observations. Also, it would be equally important to determine how QS signalling can be integrated with other environmental factors, such as short settling times and intensive hydrodynamic shear force, to rapidly and reproducibly generate dense microbial granules. In addition, it is critical to understand the role of microbially mediated QQ in relation to the accumulation of AHLs over time and if these QQ processes can be exploited to

enhance granulation. In summary, the findings in this thesis provide novel insights into the complexity of AHL mediated QS signalling at the community level, and also an experimental framework for future research aimed at the manipulation of microbial communities based on signalling processes.

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APPENDIX

Figure A-1 (next page).

Figure A-1 (continued from previous page). Cycle study analysis of the floccular sludge community in selected weeks of operation (Weeks 1-82). The concentrations of ammonium (solid circle), nitrite (open triangle), nitrate (solid triangle) and orthophosphate (open circle) in the biomass-free supernatant were measured according to the APHA standard engineering method (Eaton *et al.*, 2005). The dotted lines separate different phases of reactor operation during a cycle, i.e. anaerobic, aerobic and anoxic.

Figure A-2. Time-course detection of AHLs, released *in situ* by the floccular sludge community, with *E. coli* JBA357 biosensor. The live sludge sample (solid bars), or the heat inactivated sludge sample (open bars) were co-incubated with the biosensor at room temperature with a constant shaking at 200 rpm over a period of 8 h. Perception of AHLs by the biosensor induces the expression of green fluorescent proteins (GFP), which can be detected using a microplate reader at 488 nm and reported as relative fluorescent units (RFU). The heat inactivated sludge sample serves as a background fluorescent control for the sludge biomass (auto-fluorescence) as well as a negative control since all the existing AHLs (if any) in the sludge sample had been heat-denatured and hence, the denatured signal molecules which will not be detected by the biosensor.

Figure A-3 (previous page). Total ion chromatogram of a mixture of 13 standard AHLs added to the blank sludge supernatant sample matrix (A) and an extract from the sludge supernatant sample (B), as analysed using LC-MS. Isolated peaks eluted at different times from the sample, e.g. peak eluted at 3.72 min, were subjected to fragmentation to yield an MS/MS spectrum (C). The example given here (C) shows that the 3.72 min peak contains a parent ion $[M+H]^+$ at m/z 182, which is composed of two key product ions corresponding to the lactone ring at (m/z 102) and the acyl side chain (m/z 99), a fragmentation pattern matching with that of the synthetic C6-HSL (C, insert).

Figure A-4. TLC profiling of AHLs released by the floccular sludge community into the supernatant over a period of 82 weeks. Samples extracted from 50 ml of sludge supernatants (treated effluents), which were collected on different bioreactor operational weeks as indicated by an initial 'W' followed by the number of the week, were chromatographed on a C18 reverse phase thin-layer plate, and developed with methanol:water (60:40, v/v). The spots were visualized using the *A. tumefaciens* A136 biosensor. Standard AHLs corresponding to unsubstituted AHLs (S1), i.e. C7-HSL at 100 pmol and C8-HSL at 100 pmol, as well as oxo-substituted AHLs (S2), i.e. 3OC6-HSL at 2 pmol, 3OC8-HSL at 0.5 pmol, 3OC10-HSL at 10 pmol and 3OC12-HSL at 200 pmol, were included for comparison. The letter 'a' indicates the presence of 3OC8-HSL while letter 'b' represents the presence of 3OC6-HSL in the respective samples.

Figure A-5. LC-MS/MS profiling of AHLs released by the floccular sludge community into the supernatant over a period of 82 weeks. Samples were extracted and analysed from 50 mL of sludge supernatants (treated effluents), which were collected on different bioreactor operational weeks, were analysed using MRM approach. The identity and quantity of individual AHLs in each sample, such as C4-HSL (A), C6-HSL (B), 3OC6-HSL (C), C8-HSL (D), and 3OC8-HSL (E), were assessed in comparison with 13 standard AHLs. The total amount of different AHLs present is represented in (F). All results are shown as Mean $\pm$ SEM, three independent extractions per sample point.

Figure A-6. Successional changes in microbial diversity over a period of 82 weeks as assessed by the Simpson (circles) and the Shannon-Weaver (squares) diversity indexes. The dotted line (week 16th) separates the low dissolved oxygen (DO) supplied period, i.e. 1.0–1.5 mg L⁻¹, and the high DO supplied period, i.e. 2.0–3.0 mg L⁻¹.

Figure A-7. Long-term system performance of the granular sludge community over a period of 22 weeks. Total nitrogen removal including the removal of NH_4^+ -N, NO_2^- -N and NO_3^- -N (A), and total phosphorus removal (B), were determined as per cycle study. The dotted lines separate the different developmental phases of microbial granulation process, i.e. Phase I to Phase V.

Figure A-8. LC-MS/MS profiling of AHLs released by the sludge community during granulation. Samples extracted from 50 ml of sludge supernatants (treated effluents), which were collected on different bioreactor operational weeks, were analysed using MRM approach. The identity and quantity of individual AHL present in each sample, such as C4-HSL (A), C6-HSL (B), 3OC6-HSL (C), C7-HSL (D), C8-HSL (E) and 3OC8-HSL (F), were assessed in comparison with the MRM profiles of 13 standard AHLs. All results are shown as Mean $\pm$ SEM, three independent extractions per sample point. The dotted lines separate the different developmental phases of granulation, i.e. Phase I to Phase V, while n.d. represents 'not determined'.

Figure A-9. Successional changes in microbial diversity during granulation as assessed by the Simpson (circles) and the Shannon-Weaver (squares) diversity indexes.

Figure A-10. The correlation between AHLs accumulated in the bulk liquid of the bioreactor and the abundance of specific community members: (A) the *Xanthomonadaceae* bacterium (Tag 6) and (B) the *Comamonadaceae* bacterium (Tag 17). Expression of 3OC8-HSL (grey bars) correlates positively with the abundance of Tag 6 (upper panel, circles) and negatively with Tag 17 (bottom panel, circles). n.d. represents 'not determined'. (C) LC-MS/MS profile of AHLs released by the overnight cultures of *Lysobacter brunescens* R037 (open bars) and *Stenotrophomonas* sp. P088 (solid bars) isolated from the bioreactor. The 16S rRNA sequences of the R037 and P088 match 100% to *Xanthomonadaceae* bacteria, Tag 6 and Tag 14, respectively. The identity and quantity of individual AHLs present in each sample were compared with the multiple reaction monitoring profiles of 13 standard AHLs. All results are shown as the Mean $\pm$ SEM, three independent extractions per sample point. (D) Inactivation of AHL by *Diaphorobacter nitroreducens* R042 isolated from the bioreactor. Five micromolar 3OC12-HSL was added to the overnight culture of R042 for 2 h and the remaining 3OC12-HSL was assessed by the *A. tumefaciens* A136 spot bioassay. *E. coli* JM109 was included as the negative control. The 16S rRNA sequence of the R042 matches 100% with *Comamonadaceae* bacterium (Tag 17).

Figure A-11. The pH (triangles) and the dissolved oxygen (DO; circles) profiles of an operational cycle of the bioreactor. Each cycle consists of two operating phases. The dotted lines separate the different stages of the bioreactor within a 6 h operational cycle: Ana, anaerobic; OX, aerobic; Ano, anoxic.

Figure A-12. The kinetics of AHL inactivation mediated by the floccular sludge community. A mixture of unsubstituted (A) and oxo-substituted (B) AHLs was added into the live (open symbols) or the heat inactivated (solid symbols) sludge sample to a final concentration of 5 μ M for each AHL species. The mixture was incubated at room temperature with constant shaking at 200 rpm. Residual AHLs were extracted and quantified using LC-MS/MS at different time points, and normalized to the respective negative controls (heat inactivated controls) at 0 h. The linear and non-linear regression curves were modelled from three independent sets of experiments. All results are shown as Mean $\pm$ SEM.

Figure A-13. The kinetics of AHL inactivation at different pH. A mixture of unsubstituted (A) and oxo-substituted (B) AHLs was added into the fresh SWW media at pH 6.7 (circles), pH 7.2 (squares) and pH 8.3 (triangles) to a final concentration of 5 μ M for each AHL species. The mixture was incubated at room temperature with constant shaking at 200 rpm. Residual AHLs were extracted and quantified using LC-MS/MS at different time points. The signal half-life for each AHL was estimated based on the zero-order or first-order kinetics.

Table A-1. Multi MRMs and optimized MS/MS parameters for AHL detection and quantification.

AHL	Screening time (min)	Retention time (min)	Transitions*	Q1 (V)	CE (V)	Q3 (V)
C4-HSL	2.40-2.80	2.60	172.2 → 102.1	-20.0	-10.0	-20.0
			172.2 → 71.1	-20.0	-15.0	-14.0
3OC6-HSL	2.57-2.97	2.77	214.2 → 102.1	-17.0	-10.0	-21.0
			214.2 → 71.1	-17.0	-20.0	-14.0
C6-HSL	3.52-3.92	3.72	200.2 → 99.1	-15.0	-13.0	-21.0
			200.2 → 102.1	-23.0	-11.0	-22.0
3OC8-HSL	3.78-4.18	3.98	242.2 → 102.1	-13.0	-10.0	-21.0
			242.2 → 71.3	-13.0	-25.0	-14.0
C7-HSL	4.21-4.61	4.41	214.2 → 113.1	-11.0	-11.0	-22.0
			214.2 → 102.1	-16.0	-9.0	-20.0
C8-HSL	4.85-5.25	5.05	228.2 → 127.1	-17.0	-10.0	-28.0
			228.2 → 102.1	-17.0	-10.0	-11.0
3OC10-HSL	5.05-5.45	5.25	270.3 → 102.1	-21.0	-15.0	-21.0
			270.3 → 169.1	-21.0	-15.0	-13.0
C10-HSL	5.88-6.28	6.08	256.3 → 102.1	-10.0	-10.0	-21.0
			256.3 → 155.1	-20.0	-13.0	-12.0
3OC12-HSL	6.02-6.42	6.22	298.3 → 102.1	-12.0	-15.0	-21.0
			298.3 → 71.2	-12.0	-25.0	-15.0
3OHC12-HSL	6.05-6.45	6.25	300.4 → 102.1	-16.0	-15.0	-20.0
			300.4 → 74.1	-16.0	-30.0	-15.0
C12-HSL	6.64-7.04	6.84	284.3 → 102.1	-15.0	-10.0	-12.0
			284.3 → 183.3	-14.0	-13.0	-23.0
3OC14-HSL	6.75-7.15	6.95	326.4 → 102.1	-13.0	-15.0	-21.0
			326.4 → 225.2	-13.0	-15.0	-17.0
C14-HSL	7.24-7.64	7.44	312.4 → 102.1	-12.0	-15.0	-21.0
			312.4 → 57.2	-12.0	-35.0	-21.0

*Two most prominent transition ions generated from each precursor ion based on the optimized MS/MS parameters were chosen. The first MRM transition was used for quantification while the second MRM transition was used for identity confirmation.

Table A-2. Meta-rRNA studies: Statistics of microbiota data (Chapter 3).

Week	V6 Sequences	Unique V6 tag	Simpson (1/D)	Shannon (H')	Shannon-Weaver (e^{H'})
1	8331	191	5.1	2.8	16.9
9	7958	227	10.0	3.2	24.7
13	7455	169	7.9	2.8	17.1
16	12794	215	6.1	2.7	14.8
30	8183	154	3.1	2.1	8.4
34	10232	147	4.8	2.3	9.5
40	9243	143	3.4	2.1	8.3
44	11030	156	5.1	2.2	9.2
48	10205	160	6.3	2.5	12.3
51	6981	132	5.4	2.2	9.4
59	4801	111	5.8	2.4	11.4
61	9713	159	5.8	2.4	11.4
65	10869	180	5.6	2.5	12.3
73	12269	171	3.8	2.3	10.2
77	12829	198	4.8	2.4	11.1
82	13270	184	3.9	2.1	7.9

Table A-3. Meta-rRNA studies: Statistics of microbiota data (Chapter 4).

Week	V6 Sequences	Unique V6 tag	Simpson (1/D)	Shannon (H')	Shannon-Weaver (e^{H'})
0	2188	88	4.2	2.2	8.9
2	4321	130	5.4	2.8	16.5
4	1518	87	4.0	2.5	12.3
6	4993	111	2.9	2.1	7.8
7	2996	103	5.1	2.4	11.1
8	5140	121	5.3	2.3	10.2
10	4267	97	4.4	2.1	8.5
11	3891	86	3.8	1.9	6.7
12	4301	90	4.2	2.1	8.3
14	4709	108	4.7	2.3	9.6
15	6037	150	7.1	2.8	16.3
17	5117	146	8.1	2.9	17.7
19	3901	139	8.1	2.9	17.7
20	3633	132	5.7	2.6	13.8
21	5055	133	3.6	2.2	9.1
22	5544	149	3.4	2.3	10.1

Table A-4. Quorum sensing and quorum quenching profiles of individual isolates.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Alpha-proteobacteria								
A009	KC252645	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
A010	KC252646	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
A011	KC252647	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
A012	KC252648	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
A026N	KC252662	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
N027b	KC252701	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
R007	KC252836	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
R008	KC252837	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
R017N	KC252848	<i>Agrobacterium tumefaciens</i> strain 137	FR828338.1	100	--	--	--	--
R093N	KC252927	<i>Bosea</i> sp. Dv3	FJ774000.1	99	--	--	+	+
R096	KC252930	<i>Bosea</i> sp. Dv3	FJ774000.1	99	--	+	+	+
N008	KC252681	<i>Bosea thiooxidans</i> strain Q8	FJ581444.1	99	--	+	+++	+++
N014	KC252686	<i>Bosea thiooxidans</i> strain Q8	FJ581444.1	99	--	--	--	+++
N026	KC252699	<i>Bosea thiooxidans</i> strain Q8	FJ581444.1	99	--	+	+	+++
N001	KC252673	<i>Bosea vestrisii</i> strain 34635	NR_028799.1	99	--	+	+	+++
N031	KC252705	<i>Bosea vestrisii</i> strain 34635	NR_028799.1	99	--	+	+	+++
R045	KC252874	<i>Bosea vestrisii</i> strain 34635	NR_028799.1	99	--	+	+	+++
R071N	KC252905	<i>Bosea vestrisii</i> strain 34635	NR_028799.1	99	--	+	+	+
R078N	KC252912	<i>Bosea vestrisii</i> strain 34635	NR_028799.1	99	--	+++	+++	+
N018	KC252690	<i>Bosea vestrisii</i> strain 34635	NR_028799.1	99	--	--	--	--
N061	KC252734	<i>Brevundimonas diminuta</i> strain 3P04AD	EU977701.1	100	--	+	+++	+++
P025	KC252762	<i>Brevundimonas diminuta</i> strain 3P04AD	EU977701.1	100	--	+++	+++	+++
P051	KC252792	<i>Brevundimonas diminuta</i> strain 3P04AD	EU977701.1	100	--	+++	+++	+++
P053	KC252794	<i>Brevundimonas diminuta</i> strain 3P04AD	EU977701.1	100	--	+++	+++	+++
R057	KC252887	<i>Brevundimonas diminuta</i> strain 3P04AD	EU977701.1	100	--	+++	+++	+++
R043	KC252872	<i>Brevundimonas olei</i> strain MJ15	GQ250440.2	98	--	--	--	+

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Alpha-proteobacteria								
R095	KC252929	<i>Mesorhizobium ciceri</i> strain NBRC 100389	AB681164.1	98	--	--	--	+++
N002N	KC252674	<i>Novosphingobium</i> sp. FW-6	JQ349046.1	98	--	+	+++	+++
N058	KC252730	<i>Ochrobactrum anthropi</i> strain W-7	EU187487.1	100	--	+++	+++	+++
R005a	KC252833	<i>Ochrobactrum anthropi</i> strain W-7	EU187487.1	100	--	+++	+++	+++
R006	KC252835	<i>Ochrobactrum anthropi</i> strain W-7	EU187487.1	100	--	+++	+++	+++
R058	KC252888	<i>Ochrobactrum anthropi</i> strain W-7	EU187487.1	100	--	+++	+++	+++
N065	KC252738	<i>Rhizobium borbori</i> strain DN365	GU356639.1	99	+++	+++	+++	+++
R055	KC252885	<i>Rhizobium borbori</i> strain DN365	GU356639.1	98	+++	--	--	--
R087	KC252921	<i>Rhodobacter gluconicum</i>	AB077986.1	99	--	--	--	--
R023	KC252851	<i>Rhodobacter maris</i> strain JA276	NR_042629.1	97	+++	--	--	--
R074	KC252908	<i>Rhodobacter maris</i> strain JA276	NR_042629.1	97	+++	--	--	--
R076N	KC252910	<i>Rhodobacter maris</i> strain JA276	NR_042629.1	97	+++	--	--	--
S009a	KC252938	<i>Rhodobacter maris</i> strain JA276	NR_042629.1	97	+++	--	--	--
S011a	KC252941	<i>Rhodobacter maris</i> strain JA276	NR_042629.1	97	+++	--	--	--
N020	KC252692	<i>Rhodobacter</i> sp. XJ-1	GU184187.1	99	--	--	--	--
R077N	KC252911	<i>Rhodobacter</i> sp. XJ-1	GU184187.1	100	--	--	--	+++
R049	KC252878	<i>Roseomonas terrae</i> strain DS-48	NR_044188.1	99	--	+++	+++	+++
R089	KC252923	<i>Sphingobium yanoikuyae</i> strain R5-725	JQ659783.1	99	--	--	--	--
R054a	KC252883	<i>Sphingomonas</i> sp. PA225	AM900788.1	96	+++	--	--	--
R060	KC252890	<i>Sphingomonas</i> sp. PA225	AM900788.1	96	+++	--	--	+
N016	KC252688	<i>Shinella fusca</i> strain DC-196T	FM177879.1	100	+++	--	--	--
Beta-proteobacteria								
P070N	KC252810	<i>Achromobacter spanius</i> strain G191	JN629044.1	99	--	--	--	--
A013	KC252649	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A014	KC252650	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A015	KC252651	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Beta-proteobacteria								
A016N	KC252652	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A017	KC252653	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A023	KC252659	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A029	KC252664	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A030	KC252665	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A031	KC252666	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A032	KC252667	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A033	KC252668	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A034	KC252669	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A035	KC252670	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
A036	KC252671	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
P029	KC252767	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
P072	KC252812	<i>Achromobacter xylosoxidans</i> strain NCPP-44	AB547225.1	100	--	--	--	--
N035b	KC252710	<i>Acidovorax delafieldii</i> strain PCWCS4	GQ284437.1	100	--	--	--	+++
R081	KC252915	<i>Acidovorax facilis</i> strain 228	EU730927.1	100	+++	--	--	--
P062	KC252803	<i>Alcaligenes</i> sp. LSBA1977	FJ908708.1	99	--	--	--	--
R042*	KC252871	<i>Diaphorobacter nitroreducens</i> strain NA10B	NR_024782.1	96	--	--	--	+++
P027	KC252765	<i>Delftia tsuruhatensis</i> strain BN-HKY6	HQ731453.1	100	--	--	+++	+++
P035a	KC252770	<i>Delftia tsuruhatensis</i> strain BN-HKY6	HQ731453.1	100	--	--	+++	+++
P039	KC252776	<i>Delftia tsuruhatensis</i> strain BN-HKY6	HQ731453.1	100	--	--	+++	+++
R025N	KC252853	<i>Hydrogenophaga flava</i> strain NBRC 102514	AB681848.1	99	--	--	--	--
R066	KC252896	<i>Paludibacterium yongneupense</i> strain 5YN8-15	NR_042552.1	97	--	--	--	--
R052	KC252881	<i>Thauera selenatis</i> strain AX39	NR_026474.1	99	--	--	--	--
R086	KC252920	<i>Thauera selenatis</i> strain AX39	NR_026474.1	99	--	--	--	--
S004b	KC252935	<i>Variovorax paradoxus</i> strain E4C	AF209469.1	100	--	--	+	+++

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Gamma-proteobacteria								
N024	KC252697	<i>Acinetobacter junii</i> strain tu13	FJ544395.1	99	--	--	--	+++
N029N	KC252703	<i>Acinetobacter junii</i> strain tu13	FJ544395.1	99	--	--	--	+++
N030N	KC252704	<i>Acinetobacter junii</i> strain tu13	FJ544395.1	99	--	--	--	+++
S003	KC252934	<i>Acinetobacter junii</i> strain tu13	FJ544395.1	99	--	--	--	+++
S010	KC252940	<i>Acinetobacter junii</i> strain tu13	FJ544395.1	99	--	--	--	+++
R088	KC252922	<i>Acinetobacter schindleri</i> strain W1-2	FJ373024.1	99	--	--	--	--
R027N	KC252855	<i>Acinetobacter</i> sp. 8A18N1	HQ246229.1	100	--	--	--	+++
R028	KC252856	<i>Acinetobacter</i> sp. 8A18N1	HQ246229.1	100	--	--	--	+++
S005	KC252936	<i>Acinetobacter</i> sp. 8A18N1	HQ246229.1	100	--	--	--	+++
P054	KC252795	<i>Aeromonas caviae</i> strain JXZ-3	JF496552.1	100	--	--	--	--
P055	KC252796	<i>Aeromonas caviae</i> strain JXZ-3	JF496552.1	100	--	--	--	--
P086	KC252824	<i>Aeromonas caviae</i> strain JXZ-3	JF496552.1	100	--	--	--	--
P087	KC252825	<i>Aeromonas caviae</i> strain JXZ-3	JF496552.1	100	--	--	--	--
P005	KC252743	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P006	KC252744	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P009	KC252747	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P010N	KC252748	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P013N	KC252751	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P014	KC252752	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P016N	KC252754	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P019	KC252756	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P020	KC252757	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P023	KC252760	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P024	KC252761	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P028N	KC252766	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P035b	KC252771	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Gamma-proteobacteria								
P036	KC252772	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P037	KC252773	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P038b	KC252775	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P041a	KC252778	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P042bN	KC252782	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P043N	KC252783	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P046a	KC252786	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P061	KC252802	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P065	KC252806	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P073	KC252813	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
P074	KC252814	<i>Citrobacter freundii</i> strain MRB070408-2	GU126683.1	99	--	--	--	--
N063	KC252736	<i>Enterobacter ludwigii</i> strain BIHB 336	FJ859683.1	99	--	--	--	--
N027a	KC252700	<i>Frateuria</i> sp. Ni-H2-1	EU170476.1	100	+++	--	--	+
R011a	KC252840	<i>Frateuria</i> sp. Ni-H2-1	EU170476.1	100	+++	--	--	+
R037	KC252866	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R044	KC252873	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R053	KC252882	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R068a	KC252901	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R069	KC252903	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R072	KC252906	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R082	KC252916	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R092	KC252926	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R094	KC252928	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
R097	KC252931	<i>Lysobacter brunescens</i> strain F2	GQ859167.1	100	+++	--	--	+++
P058	KC252799	<i>Klebsiella pneumoniae</i> strain SDM45	GU997596.1	99	--	--	--	--
R067c	KC252899	<i>Pantoea ananatis</i> strain C-2-3	GU324769.1	99	--	--	--	--

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Gamma-proteobacteria								
R067d	KC252900	<i>Pantoea stewartii</i> subsp. <i>stewartii</i> strain GSPB 2626	AF373198.1	99	+++	--	--	--
P001	KC252739	<i>Pseudomonas otitidis</i> strain TNAU 45	GQ339108.1	99	--	--	--	+++
P038a	KC252774	<i>Pseudomonas otitidis</i> strain TNAU 45	GQ339108.1	99	--	--	--	+++
P040	KC252777	<i>Pseudomonas otitidis</i> strain TNAU 45	GQ339108.1	99	--	--	--	+++
P046b	KC252787	<i>Pseudomonas otitidis</i> strain TNAU 45	GQ339108.1	99	--	--	--	+++
P085	KC252823	<i>Pseudomonas otitidis</i> strain TNAU 45	GQ339108.1	99	--	--	--	+++
P090	KC252828	<i>Pseudomonas otitidis</i> strain TNAU 45	GQ339108.1	99	--	--	--	+++
P080	KC252818	<i>Pseudomonas veronii</i> strain ZW	GU357489.1	99	--	--	--	+
P060	KC252801	<i>Pseudoxanthomonas japonensis</i> strain NBRC 101033	AB681337.1	100	--	--	--	+++
P079N	KC252817	<i>Pseudoxanthomonas japonensis</i> strain NBRC 101033	AB681337.1	100	--	--	--	+++
N012	KC252684	<i>Pseudoxanthomonas</i> sp. 11_4K	EF540482.1	99	--	--	--	+++
R083	KC252917	<i>Rheinheimera chironomi</i> strain EIF90	HM480359.1	99	--	--	--	+++
A005	KC252640	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
A006	KC252641	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
A007	KC252642	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
A008a	KC252643	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
A019	KC252655	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
A021N	KC252657	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
A022	KC252658	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
A027	KC252663	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
N005	KC252677	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
P003N	KC252741	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P007	KC252745	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Gamma-proteobacteria								
P008	KC252746	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P011	KC252749	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P012	KC252750	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P015	KC252753	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P017N	KC252755	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
P021	KC252758	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
P022	KC252759	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
P056	KC252797	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P059	KC252800	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
P064N	KC252805	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P067	KC252808	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P069	KC252809	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P071	KC252811	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	--
P075	KC252815	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
P076	KC252816	<i>Stenotrophomonas maltophilia</i> strain CCF0025	GU391033.1	100	--	--	--	+
N017	KC252689	<i>Stenotrophomonas maltophilia</i> strain SPd	FJ405363.1	99	--	--	--	+
N022b	KC252696	<i>Stenotrophomonas maltophilia</i> strain SPd	FJ405363.1	99	--	--	--	--
N036	KC252712	<i>Stenotrophomonas maltophilia</i> strain YSP48	JF894170.1	99	--	--	--	+
R015N	KC252845	<i>Stenotrophomonas maltophilia</i> strain YSP48	JF894170.1	99	--	--	--	+
R040N	KC252869	<i>Stenotrophomonas maltophilia</i> strain YSP48	JF894170.1	99	--	--	--	+
N006NR	KC252678	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	--	--	--	--
N006NS	KC252679	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	--	--	--	--
N025	KC252698	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
N054	KC252726	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	--	--	--	--
N059a	KC252731	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
N060	KC252733	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	--	--	--	--

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Gamma-proteobacteria								
N064	KC252737	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
P002	KC252740	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
P026NR	KC252763	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
P026NS	KC252764	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	+++
P042aR	KC252780	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	--	--	--	--
P042aS	KC252781	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	--	--	--	--
P045	KC252785	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
P063	KC252804	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
P088	KC252826	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	--
R063	KC252893	<i>Stenotrophomonas</i> sp. M2T2B9	GQ246699.1	100	+++	--	--	+
R038	KC252867	<i>Thermomonas brevis</i> strain R-13291	NR_025578.1	97	--	--	--	--
R039N	KC252868	<i>Thermomonas brevis</i> strain R-13291	NR_025578.1	97	--	--	--	--
R051*	KC252880	<i>Dokdonella</i> sp. CC-YHH031	GQ281768.1	94	--	--	--	+++
Actinobacteria								
N038	KC252714	<i>Agromyces mediolanus</i> strain CNF186	D45054.1	99	--	--	--	--
N021b	KC252694	<i>Brevibacterium aureum</i> strain Enb17	AY299093.1	99	--	--	--	+++
N035c	KC252711	<i>Brevibacterium aureum</i> strain Enb17	AY299093.1	99	--	--	--	+++
N028	KC252702	<i>Microbacterium flavum</i> strain YM18-098	NR_041562.1	100	--	+	--	+++
R024N	KC252852	<i>Microbacterium flavum</i> strain YM18-098	NR_041562.1	100	--	+	+	+++
N056	KC252728	<i>Microbacterium hydrocarbonoxydans</i> strain HR98	JF700471.1	98	--	--	--	+
R075N	KC252909	<i>Microbacterium hydrocarbonoxydans</i> strain HR98	JF700471.1	98	--	--	--	+
N019N	KC252691	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
N021a	KC252693	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	--
N022a	KC252695	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Actinobacteria								
N039	KC252715	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
N050	KC252724	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
N050	KC252724	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
N055	KC252727	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100		--	--	+
R009	KC252838	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R010	KC252839	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R012N	KC252842	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R013	KC252843	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R014N	KC252844	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R026	KC252854	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R067a	KC252897	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R070N	KC252904	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
R079	KC252913	<i>Microbacterium laevaniformans</i> strain 1ck28	JQ229794.1	100	--	--	--	+
N037	KC252713	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
N049	KC252723	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
P032	KC252769	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
P048a	KC252789	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
P052	KC252793	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+++	--	+++
P081	KC252819	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
P082	KC252820	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
P083	KC252821	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
R073	KC252907	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
R084	KC252918	<i>Microbacterium oxydans</i> strain OL-4	HQ202812.1	100	--	+	--	+++
N041	KC252716	<i>Pimelobacter simplex</i> strain S151	AY509240.1	99	--	--	--	+++
N042	KC252717	<i>Pimelobacter simplex</i> strain S151	AY509240.1	99	--	--	--	+++
N044	KC252718	<i>Pimelobacter simplex</i> strain S151	AY509240.1	99	--	--	--	+++

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Actinobacteria								
N053	KC252725	<i>Pimelobacter simplex</i> strain S151	AY509240.1	99	--	--	--	+++
N057	KC252729	<i>Pimelobacter simplex</i> strain S151	AY509240.1	99	--	--	--	+++
N062	KC252735	<i>Pimelobacter simplex</i> strain S151	AY509240.1	99	--	--	--	+++
P047	KC252788	<i>Rhodococcus erythropolis</i> strain ZJB-0910	GU726138.1	100	--	+++	+++	+++
R011b	KC252841	<i>Rhodococcus erythropolis</i> strain ZJB-0910	GU726138.1	100	--	+++	+++	+++
N003	KC252675	<i>Tsukamurella tyrosinosolvens</i> strain IFM 10623	AB478956.1	100	--	--	+++	+++
N034N	KC252708	<i>Tsukamurella tyrosinosolvens</i> strain IFM 10623	AB478956.1	100	--	--	+++	+++
R019	KC252849	<i>Tsukamurella tyrosinosolvens</i> strain IFM 10623	AB478956.1	100	--	--	+++	+++
R020	KC252850	<i>Tsukamurella tyrosinosolvens</i> strain IFM 10623	AB478956.1	100	--	--	+++	+++
Bacterioidetes								
A001	KC252636	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
A002	KC252637	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+
A003	KC252638	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
A004	KC252639	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
A018	KC252654	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
A020	KC252656	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
A024	KC252660	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
A025	KC252661	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
A037	KC252672	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
N004	KC252676	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
N032	KC252706	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
N033	KC252707	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Bacterioidetes								
N035a	KC252709	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+
N045	KC252719	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
N046	KC252720	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
N047	KC252721	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
N048	KC252722	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R001	KC252829	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R002	KC252830	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R003	KC252831	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R004	KC252832	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R005b	KC252834	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R059	KC252889	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R061	KC252891	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R062	KC252892	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R064	KC252894	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R065	KC252895	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R067b	KC252898	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R068b	KC252902	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R085	KC252919	<i>Chryseobacterium indologenes</i> strain McR-1	JF894157.1	99	--	--	--	+++
R048	KC252877	<i>Cloacibacterium normanense</i> strain tu29	FJ544401.1	100	--	--	--	+++
R091	KC252925	<i>Cloacibacterium normanense</i> strain CCUG 46293	NR_042187.1	98	--	--	--	--
N010*	KC252683	<i>Dyadobacter fermentans</i> strain DSM 18053	CP001619.1	87	--	--	--	+++
R050*	KC252879	<i>Dyadobacter fermentans</i> strain DSM 18053	CP001619.1	87	--	--	--	+++
R098*	KC252932	<i>Dyadobacter fermentans</i> strain DSM 18053	CP001619.1	87	--	--	--	+++
N015b	KC252687	<i>Elizabethkingia anophelis</i> strain 5.33	EF426430.1	99	--	--	--	--
R029a	KC252857	<i>Elizabethkingia anophelis</i> strain 5.33	EF426430.1	99	--	--	--	--

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	30C6-HSL	30C8-HSL	30C12-HSL
Bacterioidetes								
R029b	KC252858	<i>Elizabethkingia anophelis</i> strain 5.33	EF426430.1	99	--	--	--	--
R036N	KC252865	<i>Elizabethkingia anophelis</i> strain 5.33	EF426430.1	99	--	--	--	--
N009	KC252682	<i>Flavobacterium banpakuense</i> strain 15F3	GQ281770.1	97	--	--	--	+++
P041b	KC252779	<i>Flavobacterium banpakuense</i> strain 15F3	GQ281770.1	97	--	--	--	+++
R030N	KC252859	<i>Flavobacterium</i> sp. NL124	AB636296.1	99	--	--	--	--
R041	KC252870	<i>Flavobacterium</i> sp. NL124	AB636296.1	99	--	--	--	--
R046	KC252875	<i>Flavobacterium</i> sp. NL124	AB636296.1	99	--	--	--	+++
N007N	KC252680	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+++
N013N	KC252685	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+++
R031	KC252860	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+++
R032N	KC252861	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	--
R033	KC252862	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+++
R034N	KC252863	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	--
R035N	KC252864	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	--
R047N	KC252876	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+++
R056	KC252886	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+
R080N	KC252914	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+
R090N	KC252924	<i>Pedobacter composti</i> strain TR6-06	NR_041506.1	99	--	--	--	+++
R016a	KC252846	<i>Sphingobacterium mizutaii</i> strain LMG 8340	JF708889.1	100	--	--	--	+++
R016b	KC252847	<i>Sphingobacterium mizutaii</i> strain LMG 8340	JF708889.1	100	--	--	--	+++
P031	KC252768	<i>Sphingobacterium mizutaii</i> strain NBRC 14946	JF708889.1	98	--	--	--	+++
P004	KC252742	<i>Sphingobacterium multivorum</i> strain IAM14316	NR_040953.1	99	--	--	--	--
P044	KC252784	<i>Sphingobacterium multivorum</i> strain IAM14316	NR_040953.1	99	--	--	--	+
P049	KC252791	<i>Sphingobacterium multivorum</i> strain IAM14316	NR_040953.1	99	--	--	--	+
P057	KC252798	<i>Sphingobacterium multivorum</i> strain IAM14316	NR_040953.1	99	--	--	--	--
P066N	KC252807	<i>Sphingobacterium multivorum</i> strain IAM14316	NR_040953.1	99	--	--	--	--

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	30C6-HSL	30C8-HSL	30C12-HSL
Bacterioidetes								
P084	KC252822	<i>Sphingobacterium multivorum</i> strain IAM14316	NR_040953.1	99	--	--	--	+
P089	KC252827	<i>Sphingobacterium multivorum</i> strain DW-1	EU240954.1	99	--	--	--	--
Firmicutes								
N059b	KC252732	<i>Bacillus firmus</i> strain N12-3	HM030743.1	99	--	--	--	+
A008b	KC252644	<i>Bacillus megaterium</i> strain As-30	JF895489.1	100	--	--	--	+
R054bN	KC252884	<i>Bacillus niabensis</i> strain m10	JF411233.1	99	--	--	--	--
P048b	KC252790	<i>Staphylococcus condimentii</i> strain F-2	NR_029345.1	100	--	--	--	--
S001N	KC252933	<i>Staphylococcus haemolyticus</i> strain HNMCTR1	JF775574.1	100	--	--	--	--
S006N	KC252937	<i>Staphylococcus haemolyticus</i> strain HNMCTR1	JF775574.1	100	--	--	--	--
S009b	KC252939	<i>Staphylococcus haemolyticus</i> strain HNMCTR1	JF775574.1	100	--	--	--	--
S011b	KC252942	<i>Staphylococcus haemolyticus</i> strain HNMCTR1	JF775574.1	100	--	--	--	--
Fungi								
A028	KC252943	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	--
N043N	KC252944	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	--
N052	KC252945	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	+++
P030	KC252946	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	--
P068	KC252947	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	--
P077	KC252948	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	--
P078	KC252949	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	--
P091N	KC252950	<i>Candida sojae</i> strain CBS 7871	JQ647916.1	100	--	--	--	--
P018	KC252951	<i>Candida tropicalis</i> isolate ypy06	HQ412611.1	100	--	--	--	+
P033	KC252952	<i>Candida tropicalis</i> isolate ypy06	HQ412611.1	100	--	--	--	+
P034N	KC252953	<i>Candida tropicalis</i> isolate ypy06	HQ412611.1	100	--	--	--	--
P050	KC252954	<i>Candida tropicalis</i> isolate ypy06	HQ412611.1	100	--	--	--	--
N011	KC252955	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++
N023	KC252956	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++

Table A-4. Continued.

Strain Information		Closest Match			QS	QQ		
Strain ID	NCBI Accession No.	NCBI ID	NCBI Accession No.	% of Matching	Bio_MS	3OC6-HSL	3OC8-HSL	3OC12-HSL
Fungi								
N040	KC252957	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++
N051	KC252958	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++
R021	KC252959	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++
R022	KC252960	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++
S002	KC252961	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++
S008	KC252962	<i>Cryptococcus curvatus</i> strain ATCC 10567	EU266558.1	100	--	--	--	+++
S004a	KC252963	<i>Meyerozyma guilliermondii</i> strain W56260-07	HQ693808.1	100	--	--	--	+
S007	KC252964	<i>Meyerozyma guilliermondii</i> strain W56260-07	HQ693808.1	100	--	--	--	+
R018	KC252965	<i>Trichosporon montevidense</i> isolate CLOA70	GU299461.1	99	--	--	+	+++

QS – Quorum sensing assessed by both bioassays and LC-MS/MS: -- Negative; +++ Positive

QQ – Quorum quenching: -- Negative; + Weak positive; ++ Medium positive; +++ Strong positive

QS Bio_MS – QS was assessed by both bioassays (*E. coli* JBA357, *A. tumefaciens* A136 and *C. violaceum* CV026) and LC-MS/MS.

* Classification shown in Figure 5-5: R042 – Comamonadaceae bacterium; R051 – Xanthomonadaceae bacterium; N010, R050, R098 – Cytophagaceae bacterium.

All the experiments were independently repeated for three times.

APPENDIX

Table A-5. The top 200 most abundant floccular sludge community member and the corresponding isolates.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
1	GTGTTTCAGGTTCTCTTGCGAGCACTCCCAAATC	Candidatus <i>Accumulibacter</i> (Genus)	
2	GTCTCGGGGCTCCTTTTCGGCACCCCCATCTCT	NA	
3	GTCTCGGGGCTCCTTGCGGCACCTCCCATCTCT	NA	
4	GTGTTTCAGGTTCTCTTGCGAGCACTCCCGAATC	Candidatus <i>Accumulibacter</i> (Genus)	
5	GTGTTCTGGCTCCCGAAGGCACCCTCGCCTCTC	<i>Thauera</i> (Genus)	<i>Thauera</i> sp.- R052; R086
6	GTCTCGGGGCTCCTTGCGGCACCGTCCATCTCT	Gammaproteobacteria (Class)	
7	GTCTCGGGGTTCTTGCGGCACCTGCCATCTCT	NA	
8	GTGTCCAGGCTCCCTTTTCGGGCACCAAGCCATC	Rhodocyclaceae (Family)	
9	GTCTTGGGGCTCCTTTTCGGCACCATCCATCTCT	NA	
10	GTGTGGCGTCCAGCCGAAGTGAAGTCCCAGGTC	NA	
11	TGCTTTGTGTCCCGTAGGAAAATACCGTTTCCG	NA	
12	GTGATCGCTGCCCGAAGGCTCGTTCCGCTTTCG	NA	
13	GAGCACGCTGGTATTGCTACCTCGTCAGGCTTT	<i>Nitrospira</i> (Genus)	
14	GTCACCGATCCAATAAATGAAGGAGTCCTTTT	Alphaproteobacteria (Class)	
15	GTCTCGGGGCTCCTTGCGGCACCTGTCCATCTCT	Gammaproteobacteria (Class)	
16	GTGTTTCAGGCTCCCTTGCGGGCACTCCCGAATC	NA	
17	GTGTCCAGGCTCTCTTTTCGAGCACCAAGCCATC	Rhodocyclaceae (Family)	
18	GTGCACGTTCCGGTCTTGCGACTGTGGCCCCGC	NA	
19	GTGTTCTGGCTCCCGAAGGCACCCTCGGCTCTC	<i>Thauera</i> (Genus)	
20	GTGTTCAAATTCTCTTTTCGAGCACGATTCCATC	NA	
21	GAGCACGCTGGTATTGCTACCTCGTTAGGCTTT	<i>Nitrospira</i> (Genus)	
22	GTCTCGGGGCTCCTTGCGGCACCCCCATCTCT	NA	
23	GTGTTTAGATTTCCTTTTCGGACACAATTTTCATC	<i>Nitrosomonas</i> (Genus)	
24	TACAGACTGTGTATTGCTACAAAGCCCTCTTTC	NA	
25	ATATAGCGGCCCCGAAGGCGCAAACATCTCTGCT	NA	
26	GTCACCGGTCCAGCCGAAGTGAAGGAAACGATC	Phyllobacteriaceae (Family)	
27	TACAAACTGTGTATTGCTACAAAGCCCTCTTTC	NA	
28	GTCTCTCGGCTCCCTCGTGAGAGGGCACCTCCG	NA	
29	CGTAATATGTCCGAAGAAATATAGCTTTCACCA	Sphingobacteriales (Order)	

APPENDIX

Table A-5. Continued.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
30	GTGTCTCGGTTCTCTTTTCGAGCACCTCCGCATC	NA	
31	GTGCATGTTCCACCCTTGCGGGTGTGGCTCTGC	NA	
32	GTGTGGTAGCCACCCGAAGTGAAGGACCAAGAT	NA	
33	GTGTTCAAATTCCCTTTTCGGGCACGATTTTATC	NA	
34	GTGTCCAGATTCCCTTTTCGGGCACCCCCACCTC	NA	
35	GTGCTAGCTCCCCGAAGGGTCGTTCCGTTTTCA	Caldilineaceae (Family)	
36	GTGCAAAGGCTCCCCGAAGGGCACCCCAACTTT	NA	
37	GTACAAGCTCCCCGAAGGGTCACTCACCTTTTCG	NA	
38	GTGCATGTTCCGGTCTTGCGACTGTGGCTCTAC	NA	
39	GTGCAAGCTCCCTTGCGGGTCGTTACCTTTTCG	Deltaproteobacteria (Class)	
40	GTGTCCCGGTTCCCTTTTCGGGCACCAAACCATC	NA	
41	AGTTTCGTGCTCCGAAGAGAATCCTGCTTTCAC	NA	
42	GTGTGACGTCCAGCCGAAGTGAAGACCGGTCT	<i>Amaricoccus</i> (Genus)	
43	GTGTTACGGTTCTCTTTTCGAGCACCAAGCCATC	Comamonadaceae (Family)	<i>Diaphorobacter nitroreducens</i> - R042
44	GTGTTACAGGTTCTCTTGCGAGCACTCCCAAATA	NA	
45	GTGCAAAGCGGGTATTGCTACCCTATCCGCCTT	NA	
46	ATACAGGCAACCGACTTAACGGTTCATTCGACT	Chlorobi (Phylum)	
47	TGATATCTGTCCTTGCGGACTAATCCATTTCTG	NA	
48	GTGATAGCTTCCCGAAGGATCGTTCCACTTTTCG	NA	
49	AGTTTCGTGTCCCGAAGGAAAGACCCCTTTTCAG	NA	
50	GTGCAGGTCACACCCGAAGGTAATCAGCCAGCT	NA	
51	GTGTTCCGGCTCCCTTTTCGGGCACTCCCAAATC	NA	
52	GTGTCTTAATTCCCTTTTCGGGCACTTTTACATT	NA	
53	AACTACAGGTTCTCCGAAGAGCACCCCGACCTT	Myxococcales (Order)	
54	ATATAGCGGCCCCGAAGGCGACCCTGTCTCCAGG	Deltaproteobacteria (Class)	
55	GTGCAAAGCGGGTATTGCTACCCTATCCGGCTT	<i>Prostheobacter</i> (Genus)	
56	GTGTAACAGTTCACGGTTTCCCGTGAAACCCGA	NA	
57	GTGCTAGCTCCCCGAAGGGTCGTTCCGCTTTTCG	NA	
58	GTCTCGGGGCTCCTTTTCGGCACCACTCATCTCT	NA	

Table A-5. Continued.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
59	GTG TTCAGGTTCTCTTGCGAGATCGGAAGAGCG	NA	
60	GTG TTCAGGTTCTCTTGCGAGATCGGAAGAGCA	NA	
61	TGTTTCGTGTCCCGAAAGACTATACCGTTTCCG	NA	
62	TGTTTCGTGTCCCGAAGGAAAGTCCCCTTTCAG	Bacteroidetes (Phylum)	
63	GTGTTCCAGTTCCCTTTTCGGGCACACCCAAATC	<i>Dechloromonas</i> (Genus)	
64	GTGCATGTTCCACCCGAAGGCGTGGCTCGGCTT	Planctomycetaceae (Family)	
65	GTGATGGTCCACCCGAAGTGACGACAAGCTTTC	NA	
66	GTAATGGCTCCCCGAAGGGTCGTTCCGCTTTCG	NA	
67	TGTAGAGAGTCCGAAGAAAGAAGTGTTTCCACC	NA	
68	GTCTCTGGATTCCCTTGCGGGCACGCCGACTTT	NA	
69	GTGTGGCGTCCGGCCGAACCGACCCCCAGGTCT	NA	
70	TACTGGCAGTGTATTGCTACAAAAAGAGCTTTC	NA	
71	GTG TTCAGGTTCTCTTGCGAGAGATCGGAAGAG	NA	
72	TGTAGAGAGTCCGAAGAAATAAGTGTTTCCACC	NA	
73	CGCACGCCGTCCGAAGAAGAGGATGTTTCCACC	NA	
74	GTCTCGGGGCCCCCTTTCGGCACCCCCCATCTCT	NA	
75	TGTTTCGTGTCCCGAAGGAAAGTCTCCTTTCAG	NA	
76	GTCACCGGTCCAGCCGAAGTGAAGGAATCCATC	<i>Mesorhizobium</i> (Genus)	
77	GCACAGGATTCTCTTGCGAGTTGGCCGACGTT	NA	
78	TGATACCGGTCCCGAAGGAAAATCAGCTTTCAC	NA	
79	TGCTTTGTGTTCTATTGCTAGACCTACAGGCTT	NA	
80	AGTTTCGCGTCATTTCTGAAAGACTCTTTTCGG	Bacteroidetes (Phylum)	
81	GTGTTCAAATTCTTTTCGGGCACGATTTTATC	NA	
82	GTGTGGCGTCCGGCCGAACCGACTCCCCGCTCT	Alphaproteobacteria (Class)	
83	GTG TTCAGGAGATCGGAAGAGCACACGTCTGAA	NA	
84	GTG TTCAGGTTCTCTTGCGAGCACAGATCGGAA	NA	
85	TGAGAACCGTCCGAAGAAAGGTCTGTTTCCAGA	NA	
86	TACTATCCGTATATTGCTATAAAGACACCTTTC	Bacteroidetes (Phylum)	
87	TGTTTCGTGTCATTGCTGACTGTAACATCTCTG	NA	

APPENDIX

Table A-5. Continued.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
88	GTGTCCAGATTCCCTTTCGGGCACTCCCCAATC	Betaproteobacteria (Class)	
89	GTGTTCCGATTCCCGAAGGCACTCCCGCATCTC	Gammaproteobacteria (Class)	<i>Frateuria</i> sp. - N027a; R011a
89	GTGTTCCGATTCCCGAAGGCACTCCCGCATCTC	Gammaproteobacteria (Class)	<i>Dokdonella</i> sp. - R051
90	TCTCACTCGCTCCCACGAGGGGGCACCCAGACAT	NA	
91	CGATTGCCGTTCCCGAAGGAAACCCCGCTATTT	Acidobacteriaceae (Family)	
92	TGCTCTTTGTTCTTGCGAAAAAATCCATCTCTG	NA	
93	GTGCAGAGGCCCGAAGGGAATTACTATCTCTA	NA	
94	GTCTCGGGGCTCCTTTCGGCACCCCCCATCCCT	NA	
95	TGCTCCATGTCCTTGCGGAAGAGTCCATCTCTG	Bacteroidetes (Phylum)	
96	GTGCAGGTCACACCCGAAGGTAATCATCCGACT	Planctomycetales (Order)	
97	GAGCACGCTGGTATTGCTACCTCGTTAAGCTTT	NA	
98	GTGAGATCGGAAGAGCACACGTCTGAACTCCAG	NA	
99	GTGTTCAAGTTCTCTTGCGAGCACTCCCGAATA	NA	
100	GTGTTACGGCTCTCTTTCGAGCACTCCTGCGTC	<i>Ottowia</i> (Genus)	
101	GTGCACGAGTCCCGAAGGACGCTACCATCTCTG	NA	
102	GTGTTCAAGAGATCGGAAGAGCGTCGTGTAGGG	NA	
103	GTGCAAAGTCATAGATTACTCTATGAAAGATA	NA	
104	GTGTTCAAGTTCTCAGATCGGAAGAGCACACGT	NA	
105	GTATAGGCTCCCCGAAGGGTCGTTCCGCTTTCA	NA	
106	GTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAG	NA	
107	GTGCAACCGGTTATTGCTAACCTAGCTTACTT	NA	
108	GTGTTCAAGTTCTCTTGCGAGCACTCCAGATC	Betaproteobacteria (Class)	
109	GTGTTGCAGCTCCCTTTCGGGCACTTTCTCATC	NA	
110	GTCTCGGGGCTCCTTTCGGCAGATCGGAAGAGC	NA	
111	GTCTCACGGTTCCCGAAGGCACCAATCCATCTC	Xanthomonadaceae (Family)	<i>Pseudoxanthomonas</i> sp. - P060; P079N
111	GTCTCACGGTTCCCGAAGGCACCAATCCATCTC	Xanthomonadaceae (Family)	<i>Lysobacter</i> sp. - R069; N012; R092; R097; R037; R082; R053; R044; R068a; R094; R072
112	GTCTCACGATTCCCGAAGGCACCCCCGCATCTC	NA	

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Table A-5. Continued.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
113	GTCTCGGGGCCCCCTTGCGGCACCTCCCATCTCT	NA	
114	GTGTTTCAGAGATCGGAAGAGCACACGTCTGAAC	NA	
115	GTCTTTGGGCTCCTTGCGGCACCCCCATATCTC	NA	
116	GTGTCCAGGCTCCCTTGCGGGCACCCCTCTCGTT	NA	
117	GTCACCGGTCCAGCCGAAGTGAAGGTTACCATC	<i>Mesorhizobium</i> (Genus)	
118	GTGTCCAGGTTCCCTTGCGGGCACCCCCAGATC	NA	
119	CTGCAAAAGCTCCCCGAAAGGCACCACCATATT	NA	
120	GAGCACGCTGGTATTGCTACCTCGTAAGGCTTT	NA	
121	GCGCAGGACCCTTACGGACCCCATATCTCTATG	NA	
122	GTCTCGGGGCTCCTTTTCGGCGCCCCCCCATCTCT	NA	
123	GTGTATCTGCCTATTGCTAGGGGTCCAAACTTT	NA	
124	GTCACGGCTCCTTGCGGTTCGGTCCCCTTTCGGT	Chloroflexi (Phylum)	
125	TGTTTCGTGTAGTATTGCTACCAAAAAACCCTT	NA	
126	GTGCAAACCTCTCCTTGCGGAGTCGTCACCCTTT	NA	
127	GTGCGGCAGCCTCGAAGGCCTCCGATCTTTTCGA	Verrucomicrobia (Phylum)	
128	GTCTCGGGGCTCCTTTTCGGCCCCCCCCCATCTCT	NA	
129	GTGTTTCAGAGATCGGAAGAGCGTCGTGTAGGGA	NA	
130	CGCATCGTGCCCCGAAGGGAGATCGGCTTTCAC	NA	
131	GTGATAGTCCACCCGAAGTGACGACAAGCTTTC	<i>Verrucomicrobium</i> (Genus)	
132	GTGTCCAGGCTCTCTTTCGAGCACGAATCCATC	<i>Zoogloea</i> (Genus)	
133	GTAGACCAGCCCCGAAGGGAAGGATACTTTCAT	NA	
134	GTGTGGCATCCAGCCGAAGTGAAGGACCATCT	<i>Methylophilus</i> (Genus)	
135	GTGTGGTATCCAGCCGAAGTGAAGGACCATCT	<i>Rhodobacter</i> (Genus)	
136	GTCACCGGTCCAGCCGAAGTGAAGGATTCCATC	<i>Mesorhizobium</i> (Genus)	
137	GTGTGGCGTCCAGCCGAAGTGAAGGAGCAGGTC	NA	
138	GTCTCGGGGCTCCTTTTCGGCACCCCCCATCTCC	NA	
139	GTGTTGTATCCAGCCGAAGTGAAGGAACCATCT	Rhodobacteraceae (Family)	<i>Rhodobacter</i> sp. - R087
140	GTGTTTCAGGTTCTCAGATCGGAAGAGCGTCGTG	NA	
141	TGAAAGACGTCCGAAGAAATATCTGTTTCCAAA	NA	<i>Flavobacterium</i> sp. - R041; R046; R030N

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Table A-5. Continued.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
142	GTGTTCCCGCCAGCCGAAGGATTCCATC	Rhizobiales (Order)	
143	GTTTAAACTCCCTTGCGGGTCGATCCAATTTCT	NA	
144	GTGTGCAGGTCACCGAAGTGAAGAGATCCGTCT	<i>Devosia</i> (Genus)	
145	GTCTCAGTGTTCCCGAAGGCACCAATCCATCTC	Gammaproteobacteria (Class)	<i>Thermomonas</i> sp. - R038; R039N
146	GTCACCGCGCCTCCGAAGAGGACCATCGATCTC	NA	
147	GTGCTGGTTCCGGTCTTGCGACTGTGGCTCTAC	NA	
148	GTGCACGTTCCATTCTTGCGATTGTCATCGATG	NA	
149	GTGTTACGGTTCCCGAAGGCACAATCTCATCTC	<i>Conchiformibius</i> (Genus)	
150	GTCTCGGGGCTCCTTGCGGCCCTCCCATCTCT	NA	
151	GTGTTCAAGTTCTCTTGCGAGCACTCCCAAGAT	NA	
152	GTGCATGTTCCACCCGAAGGCGTGGCTCTGCTT	Planctomycetales (Order)	
153	GTGTTCAAGTTCTCTTGCGAGCAGATCGGAAGA	NA	
154	GTGTCCAGGCTCCCTTTTCGGAGATCGGAAGAGC	NA	
155	GTGTGGCGTCCAGCCGAAGTGAAGTCCTAGGTC	NA	
156	GGTCTAGCACCCCTCGAAGGACGTCCCATTCTG	NA	
157	GTGTCCAGGCTCCCTTTTCGGGCACCAAGAGATC	NA	
158	TGTTTCGTGTCCCGAAGGAAAGTTCCCTTTCAG	NA	
159	GTTTTCAAGTTCCCTTACGGGCACTCCCGCATT	NA	
160	GTCTCGGGGCTCCTTTTCGAGATCGGAAGAGCGT	NA	
161	GTGCAAAGTGGGTATTGCTACCCTATCCGCCTT	NA	
162	GTGCACGCTCCCCGAAGGGTCGTTCCGCTTTCG	Anaerolineaceae (Family)	
163	GTGCTTGTTCCACTCTTGCGAGCGTGGCTCCTG	NA	
164	GCGTTCAGGTTCTCTTGCGAGCACTCCCAAATC	NA	
165	GTGCACGTTCCACCCGAAGGCGTGGCTCTCCTT	<i>Planctomyces</i> (Genus)	
166	TGCTACCTCTCCATTGCTGGAACGCCATCTTTC	NA	
167	TGTTTCGTGTCCCGAAGGAAAAACGGCTTTCAC	Sphingobacteria (Class)	
168	GTGTTGCAGTTCTCTTTCGAGCACTTCTCATC	NA	
169	GTGCCCAGATTCCCTTGCGGCACTCCACATCTC	<i>Azonexus</i> (Genus)	
170	GTCTCGGGGCTCCTTTTCAGATCGGAAGAGACA	NA	

Table A-5. Continued.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
171	GTCTCGGGGCTCCTTGCGGCACCTCCCAGATCG	NA	
172	GTCTCTCGATTCTTCTTGCGAAGGCACCCAC	NA	
173	GTGCAAGCTGGTATTGCTACCTCGTCACCCTTT	NA	
174	GTCACCGGTCCAGCCGAAGTGAAGGGATCCATC	Phyllobacteriaceae (Family)	
175	GTCTCGGGGCTCCTTTTCGAGATCGGAAGAGCAC	NA	
176	GTGCACGTTCCAGCCGAAGCTGTGGCCCCGCTT	Planctomycetaceae (Family)	
177	GTGTGGGATCCGGCCGAACCGACAACAATCATC	NA	
178	GTGTCCAGGCTCCCTTTTCGGGCACCAAGCAGAT	NA	
179	GTGTTTCAGGTTCTCTTGCGAGCCCTCCCAAATC	NA	
180	GTGTTCCAGCTCCCTTTTCGGGCACTCCCAAATC	Rhodocyclaceae (Family)	
181	TGCTCCTTGTTCTTGCGAAAAAATCCATCTCTG	Cytophagales (Order)	
182	GTGTCCTGTCCGGCCGAACCGACAGACCCGGTC	<i>Defluviicoccus</i> (Genus)	
183	GTGTTTCAGGCTCCCTTGCGGGCACTCCCAAATC	NA	
184	GTGCACGTTGTACCCGAAGGCCTGGCTCCTGTT	Planctomycetales (Order)	
185	GTGTTTCAGGTTCTCTTGCGAGATCGGAAGAGCAC	NA	
186	GTCCCGGGGCTCCTTTTCGGCACCCCCATCTCT	NA	
187	GTCTCGGGGCTCCTTGCGGCACCTCCCCTCTCT	NA	
188	GTCTCGGGGCTCCTTGCGGCGCCTCCCATCTCT	NA	
189	TGGAATCAGCCCCAAAGGGCGATCACCTTTCAG	NA	
190	GTGTTCTCGCCAGCCGAAGTGAAGAAAGTCATC	NA	
191	GTGTTCAAGATCGGAAGAGCGTCGTGTAGGGAA	NA	
192	GTGTCCACGTCCCCGAAGGGAAGAAATCCATCT	NA	
193	GTCTCGGGGCTCCTTTTCAGATCGGAAGAGCGTC	NA	
194	GTCTCGGGGCTCCTCGCGGCACCGCCCATCTCT	NA	
195	GTGCTTGTTCCACCCAGAGGGTGTGGCTCCTCT	NA	
196	GTATACCGACCTTGCGGGGAGAACATTTCTGAA	<i>Frigoribacterium</i> (Genus)	
197	GTGTTTCAGGTTCTCTTAGATCGGAAGAGCGTCG	NA	
198	GTCTCGGGGCTCCTTTTCGGCACCCCCATCTCTG	NA	
199	GTGTCCAGGCTCTCTTTTCGAGCACCAAACCATC	<i>Thiomonas</i> (Genus)	

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Table A-5. Continued.

Abundance ranking	V6 tag	Identity	Isolates matching to V6 tag
200	GTCTCGGGGCTCCTTTTCGGCACCCCAGATCGGA	NA	
>200	GTCTCAATGTTCCCGAAGGCACCAATCTATCTC	<i>Pseudomonas</i> (Genus)	<i>Pseudomonas veronii</i> - P080;
>200	GTGTGCGATCCAGCCGAAGGAACCATCT	<i>Rhodobacter</i> (Genus)	<i>Rhodobacter</i> sp. - R077N;N020;
>200	GTGTTCCGGCCAGCCGAAGAAAGGCATC	<i>Bosea</i> (Genus)	<i>Bosea thiooxidans</i> - N014;N026;N008; <i>Bosea</i> sp. - R093N;R096;

NA – Not applicable. Unclassified based on the taxonomy classification pipeline stated in Materials and Methods (Chapter 3: Section 3.2.11).