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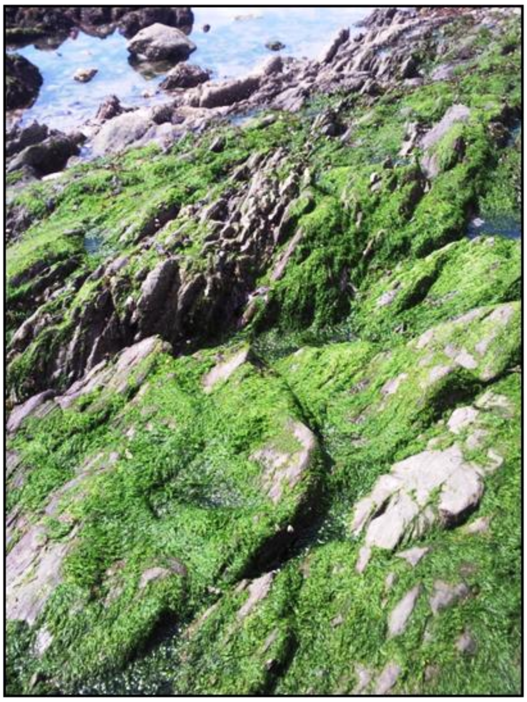
The Signal Based Relationship between the Green Seaweed *Ulva* and its Indigenous Bacterial Community



Matthew S. Twigg M.Sc. B.Sc. (hon.)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

December 2012

For Mum and Dad

"They say the sea is cold but the sea contains the hottest blood of all"

D. H. Lawrence

Declaration

Unless otherwise acknowledged the work presented in this thesis is my own. No part has been submitted for another degree in the University of Nottingham or any other institute of learning

Matthew S. Twigg M.Sc. B.Sc. (hon.)

December 2012

Abstract

This project has focused on the relationship between the green seaweed *Ulva*, commonly found in the intertidal zone of the UK coastline and its cognate bacterial community. It has previously been reported that motile *Ulva* zoospores are attracted to *N*-Acylhomoserine lactones (AHLs), signalling molecules utilised by Gram-negative bacteria in a density dependent form of cellular communication termed quorum sensing (QS) and produced by several biofilm dwelling species of marine bacteria. The species represented in the bacterial community associated with *Ulva* spp. were identified by generating a 16S rDNA phylogenetic clone library from bacterial DNA isolated from the surface of the seaweed. These data revealed that the majority of the population belonged to the *Proteobacteria* or *Bacteroidetes* phyla. In order to investigate whether QS signalling affected the rate of zoospore germination in addition to zoospore attraction, *Ulva* zoospores were settled and allowed to grow on synthetic AHLs, biofilms derived from AHL-producing model organisms and strains relevant to the *Ulva* epiphytic population which were shown to produce AHLs. Results from these experiments revealed that AHLs affected zoospore germination and the early growth of the *Ulva* germling as zoospores germinated and grown in the absence of AHLs were significantly longer than those germinated in the presence of AHLs. We therefore hypothesise that reduced germling growth in the presence of AHLs allows *Ulva* to obtain a healthy epiphytic bacterial community that is vital for the seaweed's later development. Further understanding of *Ulva* growth biology could have potential applications in preventing marine biofouling by this genus of seaweed.

This study progressed to characterise AHL production in a number of strains of *Shewanella* and *Bacteroidetes* bacteria, which, for differing reasons were deemed relevant to *Ulva* biology. Although data presented by this thesis showed AHL production in these bacterial groups, AHL synthase and response regulator sequences could not be identified in the published genome sequences from either *Shewanella* or the *Bacteroidetes*. This study also identified an AHL inactivating acylase enzyme in an environmental *Shewanella* isolate. This acylase, AacS, was shown to degrade a variety of synthetic AHLs and the AHLs produced by *Yersinia pseudotuberculosis*. This study has therefore increased the range of marine bacteria known to be producing AHLs, however the lack of AHL synthase and response regulator genes in the genomes of these bacteria leads to the conclusion that many marine bacteria possess novel, yet to be characterised AHL-mediated QS systems.

Finally, this study screened a number of extracts from marine microalgae for compounds that act as agonists or antagonists to AHL-mediated QS. Although no AHL mimics were identified data presented by this thesis showed extracts to affect the luminescence produced in *lux*-based AHL bio-reporters in the presence of exogenously added signal, affect a number of QS regulated phenotypes in marine pathogens and effect QS regulated genes in the human pathogen *Pseudomonas aeruginosa*. As such, we hypothesise that these microalgae have the ability to produce quorum-quenching compound(s). Further characterisation of quorum-quenching compound(s) produced by microalgae may be beneficial in the bio-control of pathogenic bacteria in aquaculture and may act as candidates for novel antibiotics.

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I would also like to take the opportunity to thank the countless numbers of teachers and lectures I have had at Berrycombe School, Bodmin Community College, King's College London and The University of Nottingham. Without these institutions and the people who work/ worked at them I would have never got to a point in my education where I have the ability, knowledge and importantly, the confidence to write this thesis. I would like to give special mention to four people from my education history who are of particular note. Jenny Carthew, for teaching a young dyslexic kid how to read and write; Alan Swan, for triggering my interest and love of science; Martin Dixon, for teaching me that biology was indeed the best of all those sciences and finally, Dr. Ann Wood at King's College for getting me through my first degree and for teaching me most of what I know about microbiology!

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AHL Production and Inactivation in Marine Bacteria Indigenous to the Ulva *Epiphytic Community*

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Acronyms and Abbreviations

Adv.	Average
AHL	*N*-acylhomoserine lactone
AQ	2-Alkyl-4-quinolones
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
d.H_2O	Distilled Water
CCAP	Culture Collection of Algae and Protozoa
DCM	Dichloromethane
DMSP	Dimethylsulphoniopropionate
DMS	Dimethylsulphide
DNA	Deoxyribonucleic Acid
d.NTP	Deoxyribonucleotide Triphosphate
DOTUR	Distance-Based Operational Taxonomic Unit and Richness
EDTA	Ethylenediaminetetraacetic Acid
FITC	Fluorescein Isothiocyanate
HF	High Fidelity
HPLC	High Performance Liquid Chromatography
HSL	Homoserine Lactone
LCMS	Liquid Chromatography Mass Spectroscopy
m/v	Mass to Volume
NCIMB	National Collection of Industrial, food and Marine Bacteria
OD	Optical Density

o/n	Over Night
ORF	Open Reading Frame
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PHQ	2-*n*-pentyl-4-quinolinol
rDNA	Ribosomal Deoxyribonucleic Acid
RLU	Relative Light Units
RNA	Ribonucleic Acid
RPM	Rotations Per Minute
SDS	Sodium Dodecyl Sulphate
SE	Standard Error
SEM	Scanning Electron Micrograph
TAE	Tris-acetate-EDTA
TLC	Thin Layer Chromatography
UV	Ultra Violet
wt	Wild Type
w/v	Weight to Volume
v/v	Volume to Volume
5’	5 Prime (DNA Orientation)
3’	3 Prime (DNA Orientation)

List of Bacterial Signal Molecules

C4-HSL	*N*-butanoyl-L-homoserine lactone
3-oxo-C4-HSL	*N*-(3-oxobutanoyl)-L-homoserine lactone
3-OH-C4-HSL	*N*-(3-hydroxybutanoyl)-L-homoserine
C6-HSL	*N*-hexanoyl-L-homoserine lactone
3-oxo-C6-HSL	*N*-(3-oxhexananoyl)-L-homoserine lactone
3-OH-C6-HSL	*N*-(3-hydroxyhexanoyl)-L-homoserine lactone
C8-HSL	*N*-octanoyl-L-homoserine lactone
3-oxo-C8-HSL	*N*-(3-oxooctanoyl)-L-homoserine lactone
3-OH-C8-HSL	*N*-(3-hydroxyoctanoyl)-L-homoserine lactone
C10-HSL	*N*-decanoyl-L-homoserine lactone
3-oxo-C10-HSL	*N*-(3-oxodecanoyl)-L-homoserine lactone
3-OH-C10-HSL	*N*-(3-hydroxydecanoyl)-L-homoserine lactone
C12-HSL	*N*-dodecanoyl-L-homoserine lactone
3-oxo-C12-HSL	*N*-(3-oxododecanoyl)-L-homoserine lactone
3-OH-C12-HSL	*N*-(3-hydroxydodecanoyl)-L-homoserine lactone
C14-HSL	*N*-Tetradecanoyl-L-homoserine lactone
PQS	(*Pseudomonas* Quinolone Signal) 2-heptyl-3-hydroxy-4-quinolone
HHQ	2-heptyl-4-quinolone

Units of Measurement

Mass

kg	Kilograms
g	Grams
mg	Milligrams
µg	Micrograms
ng	Nanograms

Length

m	Metre
mm	Millimetre
µm	Micrometre
nm	Nanometre

Substance Concentration

M	Molar
mM	Millimolar
µM	Micromolar
nM	Nanomolar

Amount of Substance

mol	Moles
mmol	Millimoles
μmol	Micromoles

Volume

L	Litre
ml	Millilitre
μl	Microlitre

Time

h	Hours
min	Minutes
s	Seconds

Temperature

^{o}C	Degrees Celsius

Molecular Biology

bp	Base pair
kb	Kilobase pair
Da	Dalton
kDa	Kilo Dalton

Electrical Potential

kV	Kilovolts
V	Volts

Sound Frequency

MHz	Megahertz

Chapter 1

General Introduction

1.1 Bacterial Quorum Sensing

1.1.1 The Discovery and General Overview of Bacterial Quorum Sensing

Since the foundation of the science of microbiology by Antoni Van Leeuwenhoek in 1664, the classical view of the bacterial world has been that of an autonomous, single celled organism. This individual bacterial cell reproduces via binary fission and strives for its survival within a particular environmental niche. The black and white picture presented by this view, is however completely incorrect. In order to survive, bacteria have developed means to recognise, co-operate or outcompete other prokaryotic and eukaryotic organisms within their local environment. One such method is facilitated by the production and secretion of chemical signalling molecules. These signalling molecules play a vital role in the microbial phenomenon known as ‘quorum sensing’ (QS) (reviewed by Fuqua *et al.*, 1994).

The term ‘quorum sensing’ was first adopted in an review paper by Fuqua *et al.* in 1994 and is defined as being a population size-dependent form of cell-to-cell communication facilitated via the production and transduction of small signalling molecules (reveiws by Williams *et al.*, 2007; Fuqua *et al.*, 1994). Although the term quorum sensing was first adopted in 1994 the process of population-dependent bacterial communication was initially recognised through the study of bioluminescence in the marine bacterium *Vibrio fischeri* during the 1970’s (Fuqua *et al.*, 1994; Nealson *et al.*, 1970). *V. fischeri* has the ability to emit bioluminescence, (Figure 1.1), due to the action of luciferase that oxidises reduced flavin and a long chain fatty aldehyde that is in turn provided by a fatty acid reductase (Hastings *et al.*, 1985; Belas *et al.*, 1982). The enzymatic components required for bacterial bioluminescence are transcribed from the *lux* operon. This operon consists of 7 genes; *luxA* and *luxB* which, when translated, provide the two subunits of luciferase; the gene products of *luxC*, *luxD* and *luxE* provide the components of the fatty acid reductase and the gene products of *luxG* and *luxH* provide the reduced flavin substrate (Meighen, 1991). Nealson *et al.* (1970) observed that in *V. fischeri* lag phase and early log phase cultures, luciferase expression was repressed, however during mid log phase luciferase was produced and bioluminescence occured (Nealson *et al.*, 1970). Nealson *et al.* (1970) also observed that once luciferase production was initiated the rise in bioluminescence increased at a greater rate than bacterial growth (Nealson *et al.*, 1970). The initiation of luciferase transcription and subsequent rapid rise in bioluminescence was attributed to conditioning of the culture medium by some, then unknown, factor (Nealson *et al.*, 1970). Further study into *V. fischeri* confirmed that culture conditioning initiated luciferase transcription, as the addition of cell free supernatant from late log phase cultures into fresh cultures resulted in the early induction of bioluminescence (Hastings and Nealson, 1977).

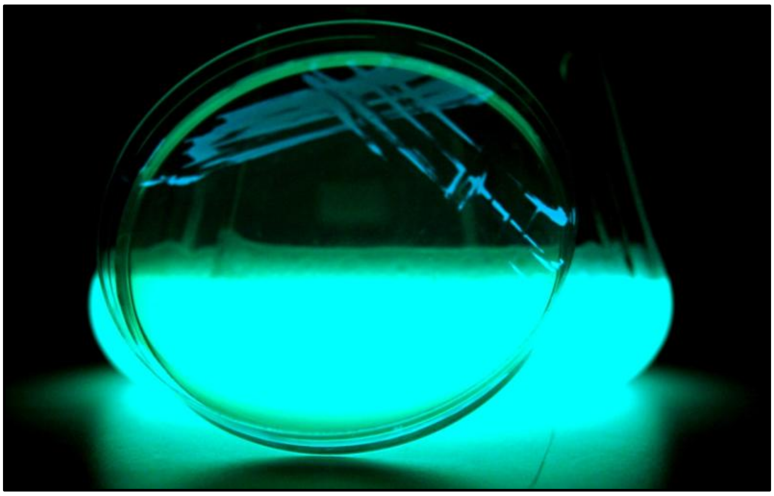


Figure 1.1. Bioluminescent stationary phase culture of *V. fischeri*. *V. fischeri* cultures are bioluminescent due to the actions of the enzyme luciferase, which is positively regulated via quorum sensing.

The molecule responsible for luciferase induction and therefore bioluminescence in *V. fischeri* was identified as *N*-(3-oxohexanoyl) homoserine lactone (Eberhard *et al.*, 1981). This molecule is one of a larger family of small diffusible signal molecules known as *N*-acyl homoserine lactones (AHLs). Its discovery lead to the general paradigm of bacterial QS; that a bacterial cell or population of bacterial cells is able to produce and respond to the presence of molecules, more widely referred to as signalling molecules, in the extracellular environment, to bring about a population-wide change in behaviour (reveiwed by Fuqua *et al.*, 1994). At low bacterial densities the concentration of signal molecules is low but as the bacterial density increases so does the concentration of the signal to a point where the concentration of signalling molecules passes a threshold and is detected by signal molecule response regulators. At this point the bacterial population is termed to be 'quorate', the signal is then transduced and target gene expression is triggered (Figure 1.2) (review by Bassler, 2002). In addition to regulating gene expression in the bacterial population, QS signal molecules may in some cases positively auto-regulate their own production leading to a further increased concentration of signal molecules when the bacterial population is quorate. This auto-regulation of cognate signal molecule production and the expression of other genes has been termed autoinduction, leading to QS signal molecules often being referred to as autoinducers (review by Miller and Bassler, 2001).

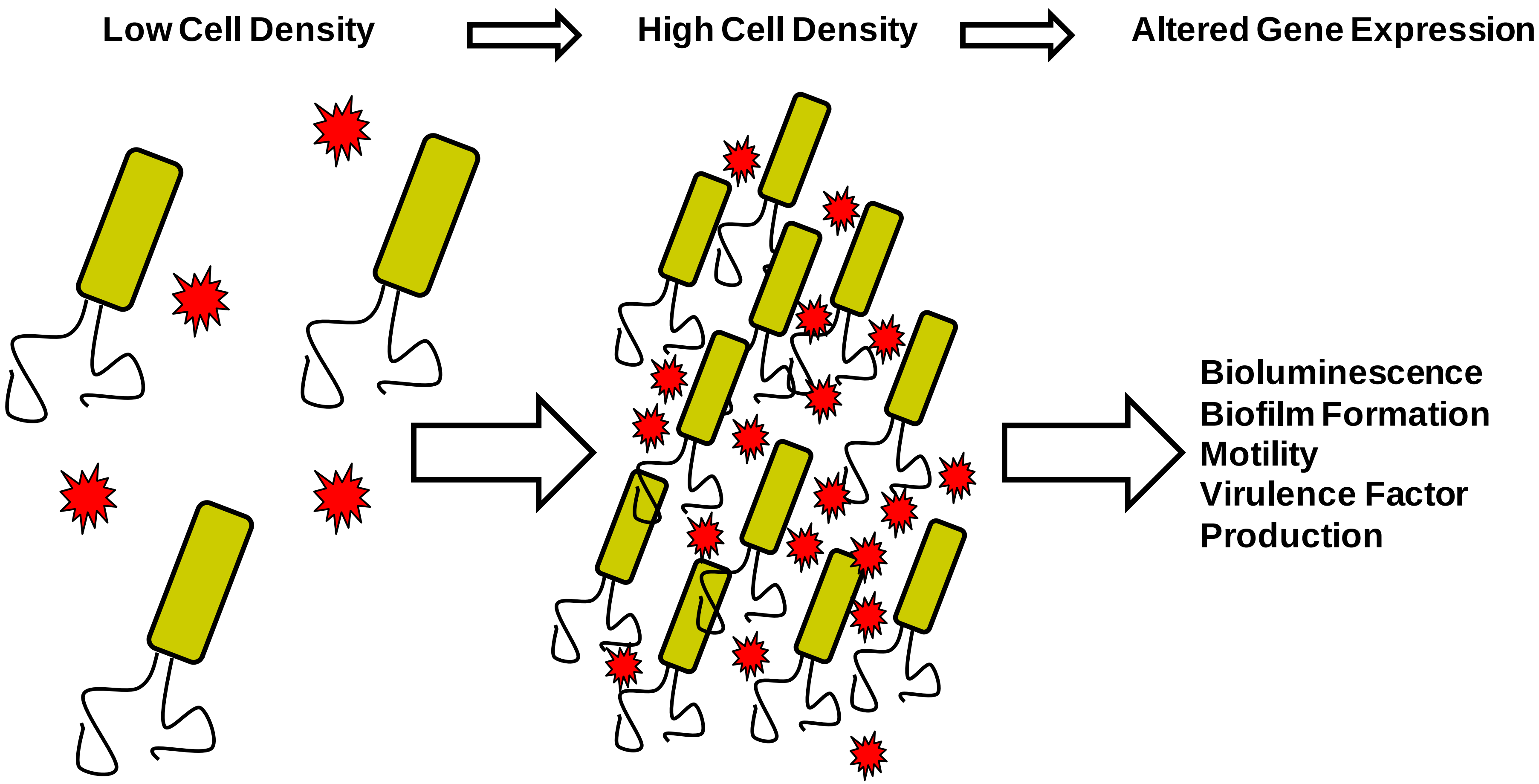


Figure 1.2. Illustration of bacterial quorum sensing. Bacteria can produce and export signalling molecules (red stars) to the extracellular environment. At low cell densities signal molecule concentration is low, as the cell density increases signal molecule concentration will also increase. At a specific threshold concentration signal molecules are detected by the bacteria effecting a change in the populations gene expression.

1.1.2 AHL-mediated Quorum Sensing

AHLs are by far the best characterised class of QS signal molecules. The chemical composition of an AHL autoinducer is that of a five-membered homoserine lactone ring *N*-acylated with a fatty acid side chain at α-position 1 (Eberhard *et al.*, 1981). AHL acyl side chains range from 4 to 18 carbons in length, can either be saturated or un-saturated and may contain hydroxyl or oxy substitutions on the third carbon (Figure 1.3) (Marketon *et al.*, 2002; Schaefer *et al.*, 2000). The number of carbons in the acyl side chain is usually even however side chains with an odd number of carbons have been reported in different species including *Rhizobium leguminosarum* and *Yersinia pseudotuberculosis* (Ortori *et al.*, 2007; Horng *et al.*, 2002). In order to be biologically active, AHLs have to possess an acyl side chain of at least 4 carbons in length (Yates *et al.*, 2002). This minimum side chain length requirement is due to the effect of pH on the homoserine lactone ring. Acyl side chains composed of 4 or more carbon atoms stabilise the structure of the AHL, maintaining biological activity (Chhabra *et al.*, 2003; Yates *et al.*, 2002).

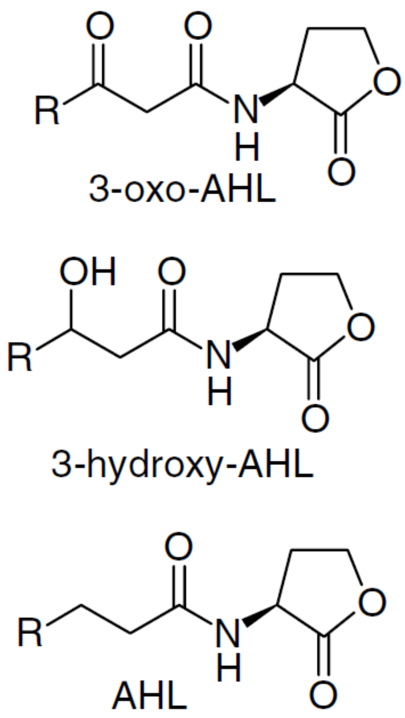


Figure 1.3. General chemical structures of AHL signalling molecules. AHLs are composed of a homoserine lactone ring with an acyl fatty acid side chain ranging from 4 to 18 carbons in length. The acyl fatty acid side chains can be saturated or unsaturated and possess hydroxyl or oxy substitutions on the third carbon (Figure sourced from Williams *et al.*, 2007).

AHLs are synthesised by signal synthase proteins that were first studied in the marine bacterium *V. fischeri*. The signal synthase protein in *V. fischeri* is LuxI, a protein with a relative mass of 25 kDa, produced as a result of the expression of the gene *luxI* (Devine *et al.*, 1989; Engebrecht and Silverman, 1984). LuxI acts as an enzyme catalysing the formation of an amide bond between S-adenosylmethionine and 3-oxo-hexanoyl-CoA in order to produce 3-oxo-hexanoyl-HSL, the cognate AHL of *V. fischeri* (Eberhard *et al.*, 1991). Other LuxI type AHL synthase enzymes carry out the same function but recognise different acylated-acyl carrier protein conjugates which provide the differing fatty acid biosynthetic precursors required to produce the wide range of AHLs used by bacteria (Moré *et al.*, 1996).

AHLs move to the extracellular environment either by free diffusion across the plasma membrane, or in the case of some long chain AHLs which have increased hydrophobicity, export to the extracellular environment is achieved via an efflux pump (Aendekerk *et al.*, 2002; Kaplan and Greenberg, 1985). This is the case for *Pseudomonas aeruginosa*, which employs the MexAB-OprM pump for the export of 3-oxo-C12-HSL (Aendekerk *et al.*, 2002).

When AHL concentration in the extracellular environment is sufficient to reach a threshold, LuxR, a twin domain 27 kDa protein binds the AHL at the amino terminus and the resulting complex binds DNA via a classic helix-turn-helix motif in the C-terminus (Fuqua *et al.*, 1994; Slock *et al.*, 1990; Engebrecht and Silverman, 1984). The *luxR* gene is found at the same locus as *luxI* within the *V. fischeri lux* operon (Devine *et al.*, 1989). The AHL/LuxR complex has been shown to bind to palindromic DNA sequences, 18-20 bp in length upstream from the genes they regulate, termed *lux* boxes (Figure 1.4) (Gray *et al.*, 1994).

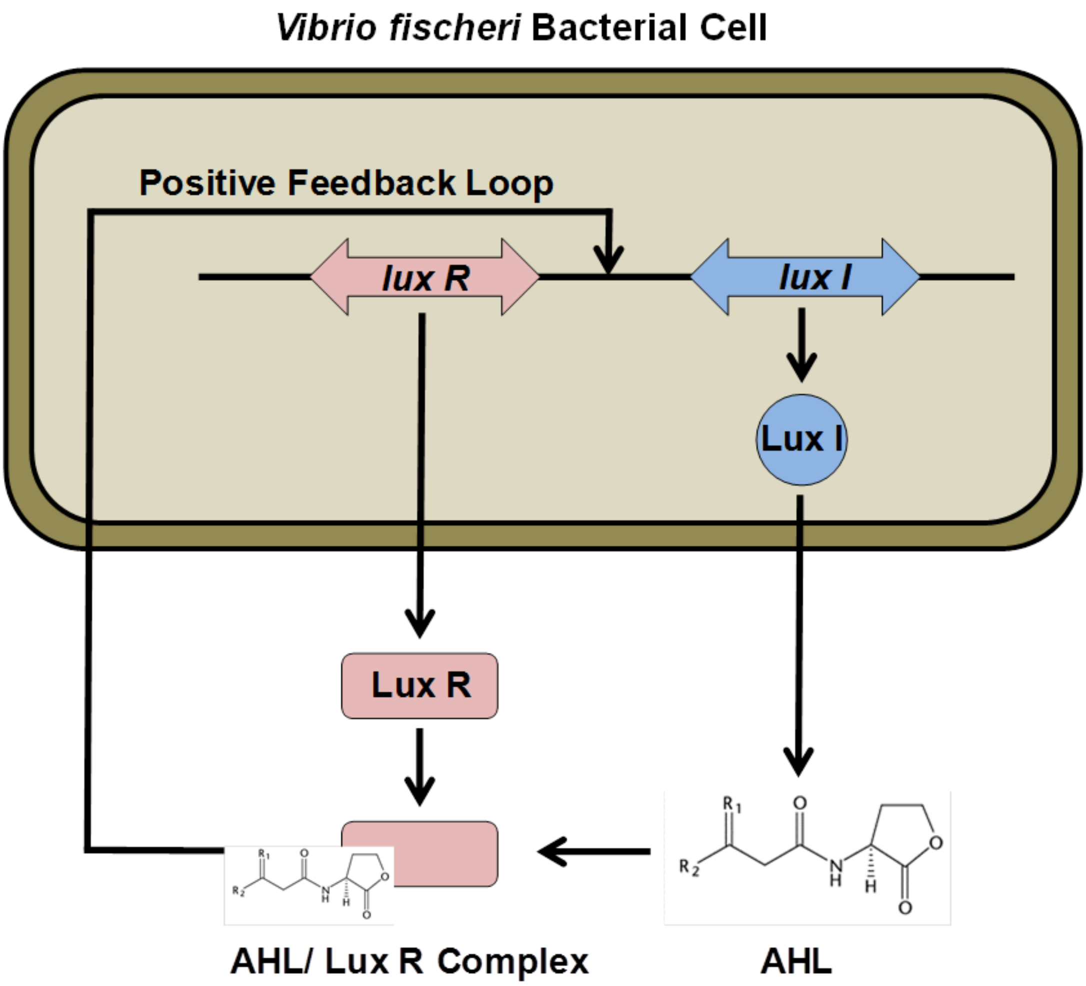


Figure 1.4. Schematic of the AHL-mediated QS system present in *V. fischeri*. LuxI produces AHL signalling molecules which are exported to the extracellular environment. LuxR acts as the AHL response regulator. AHL/LuxR complexes bind palendromic DNA sequences upstream of both *luxI* and genes regulated by AHL-mediated QS, altering gene expression and causing a positive feedback loop which increases AHL production. Many Gram-negative bacteria possess AHL-mediated QS systems.

Since the initial identification of *N*-(3-oxohexanoyl)-L-homoserine lactone as the autoinducer molecule in *V. fischeri* many other bacterial species have been reported to possess LuxR/I homologues capable of producing and transducing AHL molecules. AHL-mediated QS has also been proven to regulate a wide viariety of different phenotypes aside from bioluminescence. These include virulence, biofilm formation, siderophore production and motility (reveiwed by Williams *et al.*, 2007). A number of AHL-mediated QS systems belonging to different bacteria are listed alongside the phenotype(s) they regulate in Appendix 1. Although it is clear that AHLs are produced by a great number of bacterial species, all current research suggests that AHL-mediated quorum sensing is however restricted to Gram-negative bacteria only (reveiwed by Williams *et al.*, 2007).

In addition to the LuxR/I systems described above and shown in Appendix 1, DNA sequencing of bacterial chromosomes has revealed the presence of a number of *luxR* genes unconnected with *luxI*-type AHL synthase genes (Patankar and Gonzalez, 2009). The resultant proteins expressed by these genes have been termed LuxR orphans which in contrast to the 'classical' LuxR homologue do not transduce AHLs produced by a cognate synthase but are capable of interacting with AHLs to regulate gene expression (Patankar and Gonzalez, 2009). LuxR orphans have been identified in bacterial species possessing a functional LuxR/I system and include; QscR in *P. aeruginosa*, ExpR and NesR in *Sinorhizobium meliloti* and VirR and ExpR2 in *Erwinia sp*. (Barnard and Salmond, 2007; Pellock *et al.*, 2002; Chugani *et al.*, 2001). Additionally there are a number of LuxR orphans in bacteria where there is no functional LuxI and therefore no cognate AHLs produced, these include; SdiA in *Escherichia coli* and *Salmonella enterica* and XccR in *Xanthomonas campestris* (Zhang *et al.*, 2007; Ahmer, 2004; Ahmer *et al.*, 1998). It is possible that LuxR orphans may enable bacteria to sense the presence of rival species in their proximal environment or gain an advantage by using exogenously produced AHLs to regulate their own gene expression without the metabolic burden of producing cognate AHLs. This hypothesis is supported by the observations of non AHL-producing bacteria such as *E. coli* possessing orphan LuxR homologues and of the *P. aeruginosa* LuxR orphan, QscR's, ability to detect 3-oxo-C10-HSL (Lee *et al.,* 2006; Ahmer, 2004). Another hypothesis is that orphan LuxR homologues exist to form heterodimers with LuxR proteins linked to *luxI* orthologes. Transcriptional regulation as a result of LuxR heterodimerisation has been observed in *Agrobacterium tumefaciens* (Chai *et al.*, 2001).

AHL synthesis and transduction is not only limited to LuxI and LuxR type proteins, a further family of proteins has also been shown to facilitate AHL-mediated QS. Similar to the discovery of LuxI and LuxR, these proteins were also discovered during the study of bioluminescence in a marine bacterium, *Vibrio harveyi*. *V. harveyi* possesses an AHL synthase protein termed LuxM which catalyses the synthesis of its cognate AHL, 3-OH-C4-HSL (Bassler *et al.*, 1993; Cao and Meighen, 1989). Structural analysis of LuxM showed it to have no homology to LuxI; however the two synthase genes catalyse AHL production using the same substrates (Hanzelka *et al.*, 1999; Bassler *et al.*, 1993). 3-OH-C4-HSL synthesised by LuxM is not transduced by a LuxR homologue, AHL detection and transduction is however carried out by a membrane bound two component sensor kinase protein termed LuxN (Freeman *et al.*, 2000). At low cell densities and, therefore, low AHL concentration LuxN acts as a kinase and under such conditions phosphate is transferred to a cytoplasmic phosphotransferase protein LuxU and then onto the σ^{54} dependent activator LuxO. Phosphorylation of LuxO results in the repression of bioluminescence in *V. harveyi* (Freeman *et al.*, 2000; Freeman and Bassler, 1999a; Freeman and Bassler, 1999b). At high cell densities and therefore high AHL concentration, AHLs interact with LuxN switching its function from a kinase to a phosphatase, resulting in the loss of phosphate from the LuxU/O phosphor-relay cascade and, therefore, the loss of repression of bioluminescence (reveiwed by Cámara *et al.*, 2002). LuxM/N-type QS systems homologous to the *V. harveyi* system have been identified in other marine *Vibrio* spp. including; *V. fischeri* (AinS/R) and *V. anguillarium* (VanM/N), where they occur along side LuxI/R-type QS systems (Milton *et al.*, 2001; Hanzelka *et al.*, 1999; Gilson *et al.*, 1995).

1.1.3 Alkyl Quinolone Signalling

A second method of signal induced gene expression regulation employed by Gram-negative bacteria is the use of alkyl quinolone (AQ) signal molecules. AQ signalling was first discovered within the opportunistic pathogen *P. aeruginosa*. *P. aeruginosa* possesses two AHL-mediated QS systems, LasR/I and RhlR/I (Latifi *et al.*, 1995). These systems operate in a hierarchical manner, with the LasR/I system acting as a transcriptional regulator of the RhlR/I system (Latifi *et al.*, 1996). *P. aeruginosa* also possesses another signal system unrelated to homoserine lactone signalling molecules, but instead relies on the 4-quinolone group of small molecules commonly associated with antibacterial functions (Deziel *et al.*, 2005; Pesci *et al.*, 1999). The specific quinolone identified as the signalling molecule in *P. aeruginosa* was 2-heptyl-3-hydroxy-4-quinolone; this molecule was given the generic name of *Pseudomonas* Quinolone Signal (PQS) (Figure 1.5) (Pesci *et al.*, 1999). PQS is initially produced from chorismate that is converted to anthanylate by an anthralinate synthase enzyme encoded by *phnA* and *phnB* (Essar *et al.*, 1990). Anthralinate is then further converted to the direct precursor of PQS, 2-heptyl-4-quinolone (HHQ) by PqsA, B, C and D which are transcribed from the *pqs* operon (Gallagher *et al.*, 2002). The final stage in PQS biosynthesis is the conversion of HHQ to PQS by PqsH (Deziel *et al.*, 2004; Gallagher *et al.*, 2002). HHQ is also believed to be a QS signalling molecule as it is exported to the extracellular environment where it can be taken up by other bacterial cells (Figure 1.5) (Deziel *et al.*, 2004). The biosynthesis of PQS appears to be connected with a multidrug efflux pump, MexGHI-OpmD. This pump prevents the build up of anthranilate, which is toxic to the cell (Aendekerk *et al.*, 2002).

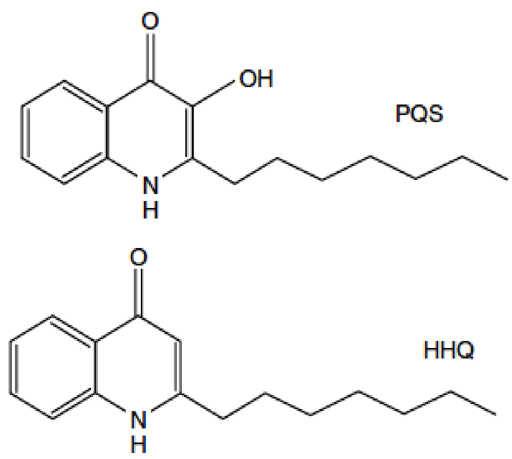


Figure 1.5. Chemical structures of the alkyl quinolones produced and used as signalling molecules by *P. aeruginosa.* 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-quinolone (HHQ) (Figure sourced from Fletcher *et al.*, 2007).

PQS is connected to both of the AHL-mediated QS systems in *P. aeruginosa* as *pqsH* is transcriptionally regulated by the Las system, as is *pqsR* (also known as *msvR*), the master regulator of the *pqs* operon (Deziel *et al.*, 2005; Wade *et al.*, 2005). The association of PQS with the Rhl system was established through the elastase gene, *lasB*. PQS acts as a regulator of *lasB*, but only in the presence of a functioning Rhl system. It was therefore shown that PQS regulated the Rhl system in regards to the transcriptional activation of *lasB* (Pesci *et al.*, 1999). As the Las system regulates PQS, which, in turn, regulates the Rhl system, it can be stated that PQS provides a regulatory bridge between the two AHL-mediated QS systems in *P. aeruginosa* (McKnight *et al.*, 2000). Other studies have demonstrated that PQS is mainly, but not exclusively, synthesised during the stationary phase of the growth cycle and, therefore, PQS has no direct connection with sensing cell density (Diggle *et al.*, 2003). Its function in up-regulating Rhl is therefore hypothesised to be in order to up-regulate Rhl-controlled genes, which would reduce stress on the bacterial cell during stationary phase (Diggle *et al.*, 2003; McKnight *et al.*, 2000). Although no clear molecular mechanisms of how PQS achieves regulation have been defined it has been shown that the protein PqsE acts as an effector of PQS-dependent virulence factors as *pqsE* mutants have a parental PQS profile but also have attenuated virulence (Diggle *et al.*, 2003; Gallagher *et al.*, 2002). It is clear that PQS is a prominent factor in *P. aeruginosa* virulence as mutants which lack the ability to produce PQS have been shown to have reduced virulence in a *Caenorhabditis elegans* nematode model, due to an impaired ability to mediate poisoning via the production of cyanide (Gallagher and Manoil, 2001). PQS is also known to be involved in iron sequestration and as such can trigger an iron starvation response (Bredenbruch *et al.*, 2006).

Although quinolone based signalling has mainly been studied in *P. aeruginosa*, other bacterial species such as *Burkholderia pseudomallei*, *Pseudomonas fluorescens* and the marine bacteria *Pseudomonas bromoutilis* and *Alteromonas* sp. have been shown to produce quinolones, similar in structure to PQS (Appendix 1) (Fletcher *et al.,* 2007; Diggle *et al.,* 2006; Long *et al.,* 2003). *Alteromonas* sp. strain SWAT5 was isolated from marine snow and has been shown to produce alkyl quinolones, specifically 2-*n*-pentyl-4-quinolinol, (PHQ). These quinolones appear primarily to be involved in antibiosis, inhibiting the growth of phytoplankton and algae. However, due to PHQs structural similarities to PQS it could play a role in QS related signalling (Long *et al.,* 2003).

1.1.4 Quorum Sensing in Gram-Positive Bacteria

In Gram-positive bacteria QS is facilitated by a number of different autoinducing peptides. These peptides are produced through the cleavage of oligopeptides and interact with two-component receptor kinases on the cell surface which in turn initiate phospho-transfer cascades, ultimately leading to response regulators which alter gene expression (reviewed by Reading and Sperandio, 2006). The agr autoinducer produced by *Staphylococcus aureus* is referred to as an autoinducing peptide (AIP) and is produced due to the actions of gene products transcribed from the *agr* operon. In *S. aureus,* AIP-mediated QS regulates toxin and protease secretion (reviewed by Novick, 2003). Other peptide mediated QS systems have been found in Gram-positive bacteria a number of which are shown in Appendix 1.

1.1.5 AI-2 and LuxS, a Universal Signalling Language?

AHL-mediated signalling is unique to Gram-negative bacteria. Gram-positive bacteria utilise small peptides as autoinducers to facilitate QS (Dunny and Leonard, 1997). There is, however, a QS system that may transcend this barrier. This system uses a small, diffusible furanosyl borate diester referred to as autoinducer-2 (AI-2) (Figure 1.6) (reviewed by Xavier and Bassler, 2003). As with LuxM/N-type AHL-mediated QS systems, AI-2-mediated QS was discovered through the study of a marine bacterium, *V. harveyi* (reviewed by Xavier and Bassler, 2003).

AI-2 is produced as a result of the actions of an iron containing S-ribosylhomocysteinase enzyme LuxS (Zhu *et al.*, 2003; Schauder *et al.*, 2001). AI-2 is produced during the activated methyl cycle in which *S*-adenosylmethionine (SAM) is converted to S-ribosylhomocysteine (SRH). LuxS acts upon SRH to produce the AI-2 precursor molecule 4,5-dihydroxy-2,3-pentanedione, which in turn spontaneously converts to AI-2 (Miller *et al.*, 2004). Within *V. harveyi* AI-2 regulates bioluminescence alongside the LuxM/N AHL system discussed earlier (Schauder *et al.*, 2001). After reaching threshold concentration AI-2 binds its cognate receptor, a periplasmic protein termed LuxP which in turn interacts with a hybrid sensor kinase protein LuxQ, the signal is then relayed through the LuxU, LuxO phospho-relay system in the same manner as the LuxM/N system (Schauder *et al.*, 2001).

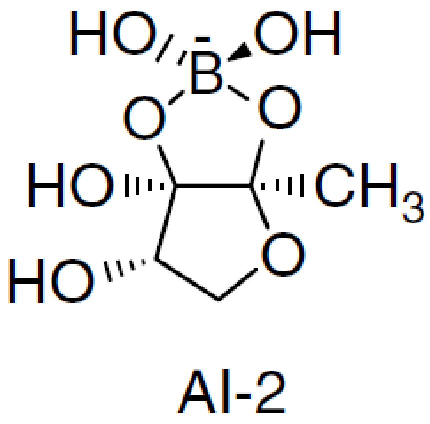


Figure 1.6. Chemical structure of AI-2 produced by *Vibrio harveyi* (Figure sourced from Williams *et al.*, 2007).

In addition to the regulation of bioluminescence in *V. harveyi*, AI-2 mediated QS has been speculated to regulate gene expression in *Vibrio cholera*, *E. coli* and *Salmonella typhurium* (reviews by Xavier and Bassler, 2005; Miller *et al.*, 2004; Cámara *et al.*, 2002). The *luxS* gene is found in over 60 species of bacteria, from a multitude of different taxonomic groups including many Gram-positive bacteria and in many cases mutation of the *luxS* gene abolished AI-2 production. This has lead to the proposition that AI-2 is a universal bacterial signalling molecule (reviewed by Xavier and Bassler, 2003). In the last few years many studies detailing the effect of *luxS* deletion on an array of bacteria have been published (Stroeher *et al.*, 2003; Lyon *et al.*, 2001). However, it is difficult to interpret the resultant phenotypic changes as a defect in QS, or due to a defect in the activated methyl cycle of the cell, as most studies do not clearly separate the two. Sun *et al.* (2004) investigated the phylogenetic distribution of all genes involved in the synthesis of AI-2, as well as the corresponding *lux* genes making up the signal cascade necessary for the detection of AI-2 by analysing 138 complete genomes sequences (Sun *et al.*, 2004). The signal transduction cascade for AI-2 was found to be restricted to *Vibrio* species. Indeed virulence factor production in the pathogenic marine bacterium *Vibrio vulnificus* is regulated by *luxS* (Sun *et al.*, 2004; Kim *et al.*, 2003). McDougald *et al.* (2003) demonstrated that AI-2 could induce starvation adaptation and stress response in the marine *Vibrio*, *V. vulnificus* and *V. angustum* S14. Signals produced from a range of other *Vibrio* species were also able to induce this response (McDougald *et al.*, 2003). Thus, at least for *Vibrio* species AI-2 does function as a quorum sensing molecule, and interspecies AI-2 signalling between *Vibrio* species has been demonstrated. The role of LuxS and AI-2 with regards to bacterial signalling and QS therefore remains a hotly contested subject area.

1.2 Signal-Mediated Interactions

1.2.1 Inter and Intra-species Signalling

Outside laboratory conditions it is rare to find bacteria growing in single species cultures; for example in the marine environment a litre of surface seawater, on average contains 10^9 bacterial cells from an estimated 1 million separate species (Curtis *et al.*, 2002). In many environments, including the marine environment, bacteria are often found in multi-species biofilms as opposed to planktonic culture (Munn, 2004). A biofilm can be defined as an organised aggregate of bacterial cells adhered to a surface, often encased in a self produced extracellular polysaccharide matrix (Costerton *et al.*, 1995). Bacteria growing in multi-species biofilms are able to detect and exploit signals produced by both members of their own species and members of different species within the biofilm. Phenotypic response to exogenously produced signal was initially observed in *Chromobacterium violaceium*, which will produce a purple pigment, violacein, when exposed to C6-HSL produced by other bacteria (McClean *et al.*, 1997). Another example is the ability of *E. caratova* to responded to exogenously produced AHLs using the AHL receptor ExpR2 (Sjöblom *et al.*, 2006). Intra-species signalling often mediates co-operative behaviour as is seen in the QS regulation of antimicrobial secondary metabolite production within a biofilm that disrupts the growth of competitive bacteria (Barnard *et al.*, 2007). Examples of such behaviour include; the production of antimicrobial peptides by lactic acid bacteria such as *Carnobacterium piscicola* and *Lactococcus lactis*; production of phenazines by *Pseudomonas chlororaphis*; production of carbapenems by *E. caratova*; production of pyocyanin by *P. aeruginosa* and production of streptomycin by *Streptomyces griseus* (Barnard *et al.*, 2007; Horinouchi, 2002; Quadri, 2002; Pessi and Haas, 2000; Pierson *et al.*, 1994).

In contrast to intra-species signalling mediated co-operative behaviour, examples of competitive behaviour due to intra-species signalling have also been observed in biofilm communities. Naturally occurring *P. aeruginosa* QS mutants often gain advantages within *Pseudomonas* biofilms as they have the ability to either take advantage of signal without the imposed metabolic cost of producing signal, or fail to produce signal-mediated responce but take advantage of ‘public goods’ produced by other bacteria in response to signaling (reviewed by Diggle *et al.*, 2007). Similar intra-species competitive behaviour has been observed in *S. aureus*. *S. aureus* strains can be grouped according to the type of autoinducing peptides they produce; signal peptides produced by one group have been found to be inhibitory to strains from another group (Ji *et al.*, 1997).

Signalling can also be utilised in a competitive or co-operative manner at the inter-species level. The use of QS to gain advantage over a different species has been observed in *P. aeruginosa* (An *et al.*, 2006). When co-cultured with *A. tumefaciens*, AHL-producing *P. aeruginosa* effectively takes over the biofilm, this behaviour was not seen when co-culturing *P. aeruginosa* QS mutants with *A. tumefaciens*. It was therefore assumed that signalling was conferring a growth advantage to *P. aeruginosa* (An *et al.*, 2006). This effect was seen to be reversed when co-culturing *P. aeruginosa* with indole producing *E. coli* strains as indole inhibits *P. aeruginosa* QS facilitating the survival of *E .coli* (Chu *et al.*, 2012). In a mixed species biofilm, *Burkholderia cenocepacia* has the ability to detect and respond to the signals produced by *P. aeruginosa*. The detection of this signal was found to be unidirectional conferring *B. cenocepacia* with the ability to sense the presence of *P. aeruginosa* in a process termed 'QS eavesdropping' and is a clear example of inter-species competitive interaction (Riedel *et al.*, 2001).

Examples of inter-species co-operation are less prominent in the literature than examples of competitive behaviour, however signal mediated inter species co-operation has been observed. Duan *et al.* (2003) showed an increase in lung damage caused by infection with *P. aeruginosa* co-cultured with avirulent oropharyngeal commensal strains in a rat lung model in comparison to infection with pure cultures of *P. aeruginosa* or oropharyngeal bacteria (Duan *et al.*, 2003). Gene analysis showed that co-culturing up regulated many genes linked to

Pseudomonas virulence including many genes regulated by AI-2. As *P. aeruginosa* does not produce AI-2 this up regulation was attributed to AI-2 production by the oropharyngeal bacteria during the infection (Duan *et al.,* 2003). In a *Drosophila* model, oropharyngeal bacteria, normally avirulent in the host, were shown to cause killing when co-cultured with *P. aeruginosa* (Sibley *et al.*, 2008). This increase in virulence was again attributed to the up-regulation of virulence genes due to inter-species signalling (Sibley *et al.*, 2008). A co-operative relationship modulated by QS signal molecules was also seen between *Pseudomonas savastanoi*, a pathogen of the olive tree (*Olea europaea*) and strains that form part of the plants epiphytic commensal population. AHLs produced by the epiphytic bacteria were able to restore virulence in QS mutants of *P. savastanoi* in an infection model. Additionally, disease caused by *P. savastanoi* was aggravated by the presence of the AHL-producing epiphytes (Hosni *et al.*, 2011). In vivo the opportunistic pathogens *P. aeruginosa* and *Burkholderia cepacia* are often found together in mixed species biofilms (Riedel *et al*., 2001). In mixed species consortia formed in vitro from these two species *B. cepacia* demonstrates swarming motility, a phenotype not seen in pure culture (Venturi *et al.*, 2010). In the same experiment *B. cepacia* was also shown to facilitate the entry of *P. aeruginosa* into an environment *P. aeruginosa* cannot tolerate individually (Venturi *et al.*, 2010). Computer modelling attributed this co-operative behaviour to QS cross talk between the two species (Venturi *et al.*, 2010).

1.2.2 Inter-Kingdom Signalling

In the majority of environments bacteria are in contact not only with other prokaryotic cells but also eukaryotic cells. These can be cells of a host that they are infecting, as part of a symbiotic relationship or cells co-inhabiting a particular environmental niche. As members of all biological kingdoms appear to possess the ability to carry out some form of signalling, a great deal of research has been focused on understanding if signalling mediates parasitic, symbiotic or commensal inter-kingdom interactions.

Examples of bacterial signalling mediating inter-kingdom co-operative behaviour have been observed in environments such as the human gut micro flora and the marine environment. Cell free supernatant from the probiotic strain *Bacillus subtilis* have been shown to stimulate the heat shock protein Hsp27 in CaCO2 colonic epithelial cells, protecting the epithelium from oxidative damage (Fujiya *et al.*, 2007). The stimulation of hsp27 is thought to be mediated by QS signal peptides produced by the bacteria and additionally has the effect of providing the bacteria with a suitable environmental niche optimal for growth (Fujiya *et al.*, 2007). Also in the gut the commensal organisms *Bacteroides fragilis* has been shown to produce a signal polysaccharide that has protective benefits to the host, suppressing harmful pro inflammatory cytokine responses to *Helicobacter* infection (Mazmanian *et al.*, 2008). Although not an example of a non-bacterial organism responding to bacterial signalling molecules an example of inter-kingdom symbiotic behaviour involving bacterial QS can be found in the marine environment. This is the association between *Vibrios* and squid. As previously stated, a number of *Vibrio* species have the ability to bioluminesce when growing in an increased population density. Squid species such as the bobtailed squid (*Euprymna scalopies*) have developed specific light organs that harbour populations of these bioluminescent bacteria. The bioluminescence provided by the bacteria gives the squid advantages with regards to either hunting for prey or avoiding predation. The bacteria are in turn provided with an ample source of nutrients from the animal enabling them to maintain a high population density, required for the facilitation of QS (Boettcher and Ruby, 1995).

Bacterial signalling molecules have also been shown to act as cues mediating responses from species outside the bacterial kingdom; such behaviour has been seen in the human body and in varying environmental niches. AHL signalling molecules such as 3-oxo-C12-HSL have been shown to effect human cancer cell lines through the regulation of Thymidylate synthase production (Dolnick *et al.*, 2005). Additionally, AHLs produced by *P. aeruginosa* have been shown to mediate an inflammatory response in the Cystic Fibrosis lung, where there are specific receptors in host epithelial tissue that recognise these AHLs (Jahoor *et al.*, 2008). The Alfalfa plant (*Medicago sativa*) also has the ability to detect AHL signalling molecules produced by bacterial plant pathogens and up-regulate genes protecting against infection (Mathesius *et al.*, 2003). The fresh water microalgae *Chlamydomonas* has been shown to produce riboflavin which may act as an AHL mimic (Teplitski *et al.*, 2004). As such riboflavin has been shown to activate QS regulated genes in *P. aeruginosa* through activation of the LasR receptor (Teplitski *et al.*, 2004). One of the more interesting examples of a eukaryotic organisms using AHL signalling molecules as cues to mediate behaviour has been observed in the green seaweed *Ulva*. AHLs have been shown to attract the plants motile zoospore during *Ulva* reproduction and, as the focus of this thesis, will be reviewed in section 1.4 (reviewed by Joint *et al.*, 2007).

1.3 Quorum Sensing in the Marine Environment

1.3.1 Abundance of AHL-producing Bacteria within the Marine Environment

The marine environment is the largest ecosystem on the planet. Oceans and seas cover 71% of the Earth's surface, which amounts to a total area of 3.6×10^{8} km^{2} and an estimated total volume of 1.4×10^{21} litres (Munn, 2004). The marine environment is sub-divided into multiple zones depending on water column depth. Bacteria have been proven to be present in all of these zones but are most prevalent at the surface interface between water and atmosphere, the neuston (Munn, 2004). A study by Whitman *et al.* (1998) estimated that in the top 200 m of the worlds ocean there are 3.6×10^{28} prokaryotic cells (Whitman *et al.*, 1998). In spite of these vast numbers of prokaryotic life the marine environment can be considered a stressful environment as regards to microbial life; the overall nutrient concentration in seawater is relatively low, as is temperature, however salinity is high. As such, marine bacteria are highly adapted to the conditions found in the specific marine environmental niches they occupy (Munn, 2004). Owing to low nutrient conditions, physical processes within the oceans, viral lysis and the sheer size of the marine environment, bacterial populations do not generally increase over a population density of 10^{5} to 10^{6} cells ml^{-1} (Munn, 2004). As QS is a population density-dependent process, the majority of QS regulated gene expression occurs at bacterial population with densities above approximately 10^{8} cells ml^{-1} (Fuqua *et al.*, 1996). Specialised niches do exist, however, which would promote the growth of dense microbial populations: the colonisation of host animal light organs by the bioluminescent bacteria *V. fischeri*, and the role QS plays within this symbiosis is a well studied example (Lee and Ruby, 1994). AHL-producing bacteria are also found in abundance in the oceans and in a variety of different environments. These include; the aggregated organic and inorganic particles, commonly referred to as marine snow; algae; invertebrates such as corals and sponges and in biofouling communities (Tait *et al.*, 2010; Tait *et al.*, 2009; Mohamed *et al.*, 2008; Wagner-Döbler *et al.*, 2005; Taylor *et al.*, 2004; Gram *et al.*, 2002). Populations of between 10^8 and 10^9 cells ml^{-1} have been found within marine snow and given that AHL-producing bacteria can be isolated from theses marine snow aggregates, this is a good indication that QS occurs within aggregated communities (Gram *et al.*, 2002; Ploug, 2000). In addition to a significant number of marine *Vibrio* species shown to be producing QS signal molecules other signal molecule producing marine species include strains of *Shewanella* spp., *Sulfitobacter* spp., *Flavobacterium* spp., *Glaciecola* spp., *Tenacibaculum* spp., *Roseobacter* spp., *Flammevirga* spp., *Pseudoalteromonas* spp. and *Thalassomonas* spp. (Romero *et al.*, 2010; Tait *et al.*, 2009; Huang *et al.*, 2008; Milton, 2006). Interestingly, although QS signal molecule production has been shown to be abundant in marine bacteria, homologues of the genes currently implicated in both the synthesis and transduction of these signal molecules are absent in the genomes of many marine species, other than *Vibrio* spp. (Tait *et al.*, 2009). This has lead to the prediction that there may be a number of yet to be discovered novel AHL-mediated QS systems in marine bacteria.

1.3.2 Constraints on AHL-mediated QS within the Marine Environment

The main constraints of AHL-mediated QS in the marine environment are AHL hydrolysis by seawater and inactivation of AHLs by bacteria possessing AHL inactivating enzymes. AHL signal molecules are known to undergo hydrolysis in an increased pH and/or temperature and acyl side chain length manner (Yates *et al.*, 2002). AHLs with 3-oxo groups and short acyl side chains are particularly susceptible to hydrolysis but all AHLs will hydrolyse given suitable conditions. Seawater is typically pH 8.0-8.2 and therefore AHLs are not stable. In fact, AHL hydrolysis in seawater has been followed using HPLC; as expected AHL inactivation took place over a short time period, with AHLs hydrolysed by the alkaline pH of sea water. AHLs with shorter acyl side chains were inactivated faster than AHLs with longer side chains with AHL inactivation taking place according to first-order degridation kinetics (Figure 1. 7)(Hmelo and Van Mooy, 2009). Degridation rate coefficients for C6-HSL, 3-oxo-C6-HSL and 3-oxo-C8-HSL in natural seawater were 0.043 h^{-1} (± 0.003), 0.116 h^{-1} (± 0.005) and 0.148 h^{-1} (± 0.002) (Hmelo and Van Mooy, 2009). Temperatures above 22^{o}C also caused a marked increase in the hydrolysis of C6-HSL, 3-OH-C6-HSL and 3-oxo-C10-HSL in seawater (Hmelo and Van Mooy, 2009; Tait *et al.*, 2005). Interestingly, natural seawater inactivated AHLs up to 57% faster than artificially prepared seawater. As protease K treatment of natural seawater reduced this rate of AHL inactivation to levels seen in artificially prepared seawater, it was proposed that natural sea water contains AHL-inactivating enzymes which increase the rate of AHL turnover beyond the level accounted for by hydrolysis due to high pH (Hmelo and Van Mooy, 2009).

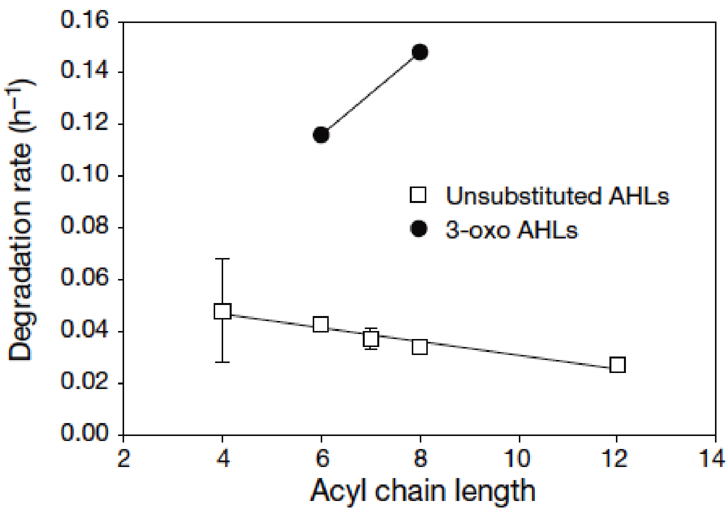


Figure 1.7. Degradation rate coefficients (± SE) of various AHLs in natural seawater. AHLs with shorter acyl side chains are inactivated at an increased rate in comparrsion to long chain AHLs. AHLs with 3-oxo substitutions are inactivated at an significantly increased rate to non-substituted AHLs (Figure sourced from Hmelo and Van Mooy, 2009).

Many marine bacteria have been shown to produce AHLs with long acyl side chains. This could be an artefact of rapid hydrolysis of short chain AHLs in the marine environment (Gram *et al.*, 2002). Diffusion of AHLs from marine biofilms into the seawater is also effected by acyl side chain length with long chain AHLs possessing a slower rate of diffusion in comparison with AHLs with short acyl side chains (Tait *et al.*, 2005). Rapid AHL inactivation due to the pH of seawater most likely constrains QS in the marine environment to either the close proximal environment to the cell producing the signal or to areas that are protected from seawater pH such as marine biofilms. In fact the majority of marine bacteria shown to be engaging in AHL-mediated QS have been isolated from either biofilm communities or aggregated particulates such as marine snow (Tait *et al.*, 2009; Gram *et al.*, 2002).

The evidence that AHL inactivation in non-sterile natural seawater takes place at an increased rate in comparison to sterile artificially produced seawater due to the actions of AHL inactivating enzymes poses the question of how widespread enzymatic AHL inactivation may be in the marine environment (Hmelo and Van Mooy, 2009). There are two broad classes of AHL inactivating enzymes; AHL lactonases and AHL acylases (Dong and Zhang, 2005). Lactonase enzymes inactivate AHLs by disrupting the homoserine lactone ring by hydrolysis of the lactone bond producing an *N*-acyl homoserine analogue (Figure 1.8) (Dong and Zhang, 2005). The first AHL lactonase to be identified, AiiA, was isolated from a *Bacillus* sp. strain found in soil samples (Dong *et al.*, 2000). Since this discovery AHL lactonases have been identified in *P. aeruginosa*, *Microbacterium testaceum*, *Ochrobactrum* sp., and *Rhodococcus* sp. (Mei *et al.*, 2010; Wang *et al.*, 2010; Schipper *et al.*, 2009; Uroz *et al.*, 2008). Acylase enzymes however, inactivate AHLs by cleaving the acyl side chain from the homoserine lactone ring by hydrolysing the amide linkage producing a fatty acid and homoserine lactone (Figure 1.8) (Dong and Zhang, 2005). AHL acylases have been found to be produced by *P. aeruginosa*, *Streptomyces* sp. and *Comamonas* sp. (Uroz *et al.*, 2007; Park *et al.*, 2005; Huang *et al.*, 2003). AHL inactivating enzymes have now been found to be produced by a wide range of marine bacteria including isolates from the *Alphaproteobacteria* (*Hyphomonas* sp.), *Gammaproteobacteria* (*Shewanella* sp. and *Alteromonas* sp.), *Firmicutes* (*Oceanobacillus* sp.), *Actinobacteria* (*Rhodococcus erythropolis*) and *Bacteriodetes* (*Tenacibaculum discolour*) phyla (Romero *et al.*, 2011; Tait *et al.*, 2009; Morohoshi *et al.*, 2008).

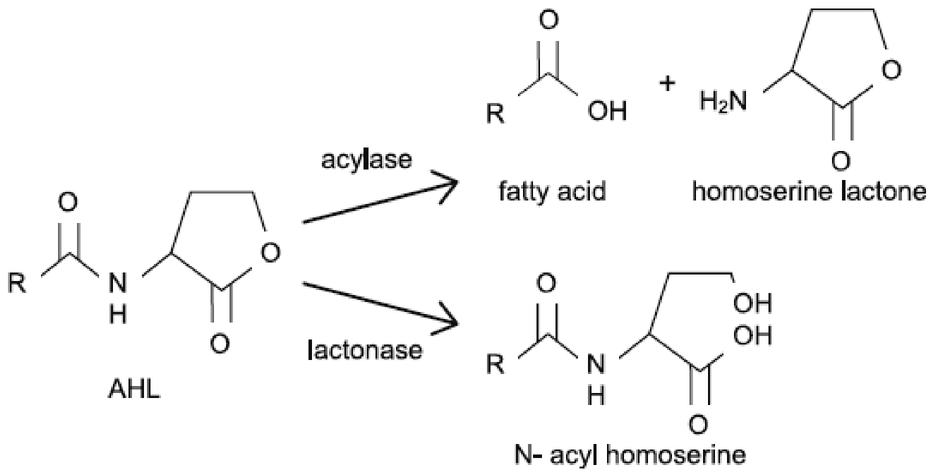


Figure 1.8. Enzymic mechanisms of AHL inactivation. AHL inactivating enzymes either act by hydrolysis of the homoserine lactone ring or by cleaving the acyl side chain from the homoserine lactone ring (Figure sourced from Defoirdt *et al.*, 2004).

1.3.3 Milky Seas Phenomenon

Despite the low bacterial densities found within the water column, and the instability of AHLs in seawater, AHL-producing bacteria have been isolated from the open ocean (Bruhn *et al.*, 2007). The function of AHL-mediated QS within these bacteria is currently unknown. However, QS within the marine environment has been shown to produce spectacular effects on a large scale. A phenomenon termed ‘milky seas’ has been recorded in shipping logs and fictional literature for over a hundred years (Nealson and Hastings, 2006). Milky seas are large patches of sustained luminescence on the surface of the ocean. This sustained luminescence differs from that caused by dinoflagellate blooms which ‘sparkle’ only when agitated or disturbed. The largest recorded instance of milky sea activity spanned an area of approximately 14,300 Km^2, (roughly the size of the US state Connecticut), and was detected using U.S. defence satellite sensor systems (Miller *et al.*, 2005). Water samples were taken from areas of milky seas and shown to contain *V. harveyi* bacteria in association with the microalga *Phaeocystis* (Lapota *et al.*, 1988). Based on knowledge of *V. harveyi* and its interactions with microalgae a hypothesis for the milky sea phenomenon was put forward. The milky seas are thought to be attributed to the breakdown of algal blooms which causes a temporary, but substantial, increase in nutrient availability on the surface of the ocean. *V. harveyi* associated with the algae take advantage of the increased nutrient concentration, growing to a population size where signal molecule concentration reaches near sufficient amounts to activate luciferase gene expression, causing luminescence (Nealson and Hastings, 2006). The actions of grazing marine organisms preying on these bacterial and algal blooms does however reduce the bacterial densities to levels below that required for the sustained bioluminescence seen during milky sea events. This has lead to the question of whether microalgae to which the *V. harveyi* bacteria are associated with produce molecules that mimic AHL signalling molecules and therefore activate bioluminescence. Molecules that have this ability to mimic AHLs have been shown to be produced by the freshwater microalgal species *Chlamydomonas reinhardtii* where they can effect bacterial QS (Teplitski *et al.*, 2004).

1.4 *Ulva*

1.4.1 The Genus Ulva

Species of seaweed belonging to the the genus *Ulva* are part of the plant phyla *Chlorophyta* (Green seaweed) first described by Carl Linnaeus in 1753 (Linné and Salvius, 1753). Species of *Ulva* are colloquially referred to as sea cabbage, gutleaf or grass kelp due to their spread leaf or tubular leaf morphology (Figure 1.9) (Burrow, 2001). According to the current World Register of Marine Species, there are 130 separate species of *Ulva* globally, 11 of which are indigenous to the UK coast line where they are found colonising the rocky shore environment within the intertidal zone (Guiry, 2012; Burrow, 2001). In older publications many species of *Ulva* are often classified as *Enteromorpha* spp., however these two *Chlorophyta* genera were amalgamated in 2003 (Hayden, 2003).

Figure 1.9. Examples of different species of *Ulva* endemic to the UK coastline. From top left; *Ulva compressa*, *Ulva intestinalis*, *Ulva linza* and *Ulva lactuca*.

Species of *Ulva* are economically important as they significantly contribute to marine biofouling; the colonisation of manmade marine surfaces such as sea walls and ships' hulls by algae and marine animals (Munn, 2004; Callow, 1986). *Ulva* growth on ships' hulls has the effect of causing hydrodynamic drag in the water which has the effect of increasing fuel usage by up to 10% (Munn, 2004; Callow and Callow, 2002). The US navy estimates this loss of fuel efficiency due to biofouling to cost over $1 billion per year (Schultz *et al.*, 2000). In addition to increasing hydrodynamic drag on ships hulls *Ulva* fouling can block waste effluent and cooling pipes (Figure 1.10) (Munn, 2004; Callow and Callow, 2002).

Figure 1.10. An example of *Ulva* spp. biofouling. *Ulva* spp. colonisation causing a blockage of an effluent pipe (figure sourced from Callow and Callow, 2002).

1.4.2 **Ulva** *Zoospore Settlement*

Ulva spp. reproduce either sexually by the production of biflagellate gametes or asexually through the production of quadriflagellate zoospores (Callow *et al.*, 1997; Miyake, 1931). *Ulva* zoospores are naked, pear shaped, up to 7 µm in length and 5 µm in diameter (Figure 1.11) (Callow *et al.*, 1997).

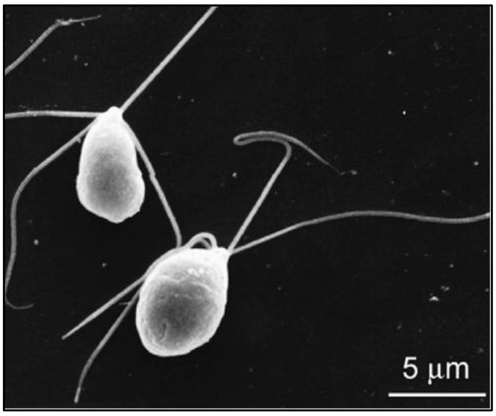


Figure 1.11. SEM of *Ulva* zoospores. *Ulva* zoospores are pear shaped and possess 4 flagella which confer motility (figure sourced from Callow and Callow, 2006).

In the UK *Ulva* spore release tends to take place during spring and summer, approximately 1-2 h after the high tide, potentially using photoperiod and temperature as sporulation cues (reveiwed by Maggs and Callow, 2001). During sporulation zoospore release rate has been estimated at 5.3 X 10^5 spores per plant per day from the fertile tip of the *Ulva* thallus, the released zoospores will then rapidly settle on available substrata, germinate and grow into an adult plant (Callow *et al.*, 2002; Maggs and Callow, 2001). The driving signal dictating that *Ulva* zoospores will settle on the surface of the rocks at the bottom of intertidal zone rock pools is light. *Ulva* zoospores are negatively phototactic and will therefore swim away from a light source (Callow and Callow, 2000). In the natural environment, this negative phototaxis allows the zoospores to swim away from the surface of the seawater to the substrata at the bottom of rock pools (Callow and Callow, 2000). *Ulva* zoospore settlement to surface substrata then involves a transition from a free-swimming state to a non-motile adhered state (Callow *et al.*, 1997). Adherence is a two stage process; primary adhesion to a surface is temporary, involving the zoospore testing the surface for optimal conditions (Callow *et al.*, 1997). Upon selection of an optimum surface the zoospore secretes an *N*-linked, polydisperse glycoprotein from Golgi bodies in the apical end of the spore which cross links with cell wall matrix components forming a physical connection between the spore, adhesive and substratum (Callow and Callow, 2000). This process is referred to as secondary adherence (Callow and Callow, 2000). The strength of the adhesive holding the zoospore onto the surface has shown to increase with time. After 4 hours contact a pressure of up to 250 kPa is required to quantitatively dislodge the settled zoospores. Put in practical terms, to dislodge zoospores attached to the non-treated hull of a ship, the ship would need to travel at a speed of 42 knots in order to generate sufficient flow conditions. Such speeds are beyond the range of most vessels (Finlay *et al.*, 2002). Observations of secondary zoospore adherence have shown that physical properties of a surface will influence where *Ulva* zoospores form permanent attachments, these properties include surface topology and surface chemistry (Callow *et al.*, 2002; Callow *et al.*, 2000). *Ulva* zoospores have been shown to preferably settle on rougher surfaces. Synthetic surfaces with micro valleys and pillars encourage increased zoospore settlement in comparison to a flat surface. Settlement was most pronounced where the diameters of the surface topography were identical to the dimensions of the zoospore. The reason for preferential selection of a rough surface is due to settlement on such surfaces being more energetically favourable (Callow *et al.*, 2002). The wettability of a surface will also affect *Ulva* zoospore settlement (Callow *et al.*, 2000). Surfaces with an increased advancing water contact angle (θ_{AW}) have a decreased wettability and are therefore hydrophobic (Zisman, 1964). Using surfaces of varied wettability formed from polymers with differing percentages of monomers containing either hydrophilic hydroxyl groups or hydrophobic methyl groups Callow *et al.* 2000 showed that as a surface becomes less wettable the number of zoospores preferentially settling on the surface increases (Figure 1.12) (Callow *et al.*, 2000).

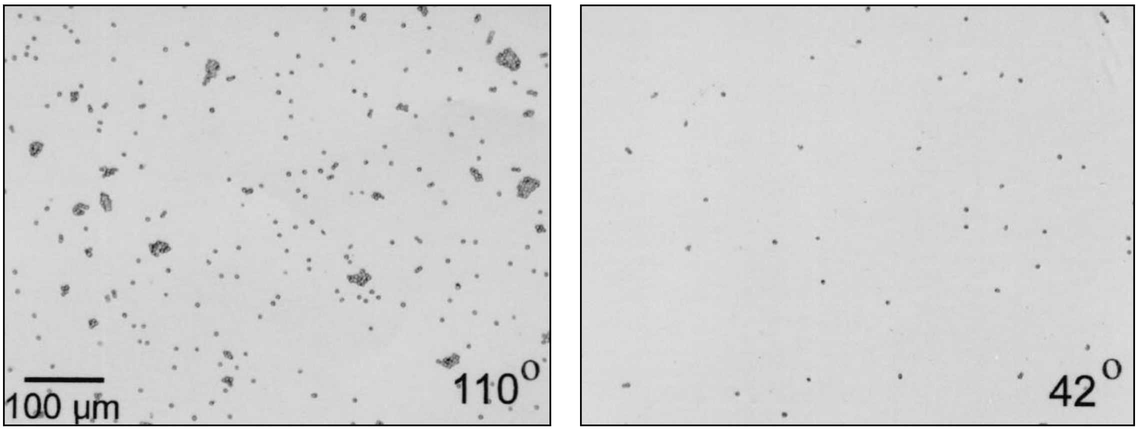


Figure 1.12. Effect of wettability on *Ulva* zoospore settlement. Increased zoospore settlement was observed on hydrophobic surfaces with high θ_{AW} (110°) in comparison to hydrophilic surfaces with low θ_{AW} (42°) (figure sourced from Callow *et al.*, 2000).

Aside from the physical properties of a surface another key factor in primary *Ulva* zoospore settlement is the presence of bacteria which grow as biofilms on marine surfaces. Studies showed that *Ulva* zoospores were preferentially attracted to substrata covered with mixed species bacterial biofilms (Dillon *et al.*, 1989). Additionally Joint *et al.* (2000) showed that zoospore settlement increases as bacterial density within the biofilm is increased, with zoospore preferentially settling on microcolony structures within the biofilm (Joint *et al.*, 2000). Patel *et al.,* (2003) isolated bacteria from the surface of rocks colonised by *Ulva* spp. and showed that the bacteria present fell into three main groups; *Gammaproteobacteria*, *Alphaproteobacteria* and the *Bacteroidetes* (previously called the *Cytophaga-Flavobacteria-Bacteroid* group) (Patel *et al.*, 2003). After producing single species biofilms of each strain, zoospore settlement assays showed zoospores predominantly settled on biofilms composed of bacteria composed of *Vibrio* spp. and *Shewanella* spp., part of the *Gammaproteobacteria* phylum (Patel *et al.*, 2003). However, as this study was a culture based study it is not a completely accurate sample of the bacterial population growing on and around *Ulva* spp.. Tait *et al.,* (2009) further assessed the bacterial population associated with *Ulva* spp. by producing a clone library of 16S rDNA from scrapings from of rocks colonised by *Ulva* spp.. This showed that the population was dominated by the *Rhodobacteraceae*, a family of *Alphaproteobacteria* and the *Flavobacteriaceae*, a family of *Bacteroidetes* (Tait *et al.*, 2009).

The possibility that the zoospores were responding to AHL signals produced by the biofilm was tested using the fish pathogen *Vibrio anguillarum* as a model (Joint *et al.*, 2002). *V. anguillarum* possesses a LuxR/I homologue termed VanR/I and a LuxM/N homologue termed VanM/N (Milton *et al.*, 2001; Milton *et al.*, 1997). *V.anguillarum* strains mutated to be defective in AHL production, (*V.anguillarum* NB10 Δ*vanM*, Δ*vanI* and Δ*vanMI*), were used in zoospore settlement assays. Increased zoospore settlement was evident on biofilms of the AHL-producing *V. anguillarium* wt and Δ*vanI* biofilms but not on biofilms of the Δ*vanM* (Joint *et al.*, 2002). The VanM/N system exerts hierarchical control over the VanR/I system, therefore the Δ*vanI* mutant only lacks the ability to produce 3-oxo-C10-HSL. Whereas, mutation of the VanM synthase, (Δ*vanM*), and mutation of both synthase genes, (Δ*vanMI*), also prevents 3-OH-C6-HSL production, completely rendering *V. anguillarum* AHL deficient (Milton, 2006; Milton *et al.*, 2001; Milton *et al.*, 1997). The evidence that zoospores do not settle on biofilms composed of the Δ*vanM* mutant but do settle on the Δ*vanI* mutant suggests that the zoospores are responding to the presence of AHLs regulated by the VanM/N system. Zoospore settlement was also disrupted in biofilms composed of the *V. anguillarum* Δ*vanMI* double AHL synthase mutant (Joint *et al.*, 2002). As mutations in *vanI/M* may have impacted on multiple phenotypes within *V. anguillarium* which may have affected zoospore settlement, *E. coli* biofilms expressing recombinant *vanI* and *vanM* were subjected to zoospore settlement assays. Spores preferentially settled on the recombinant strains over wt *E. coli* biofilms, which do not produce AHLs (Joint *et al.*, 2002). Additionally, synthetic AHLs were tested for their ability to enhance zoospore settlement. AHLs with acyl side chains of C6 to C14 were shown to increase zoospore settlement (Joint *et al.*, 2002).

Tait *et al* (2005) repeated the zoospore attraction assays using the model organism *V. anguillarum.* In this study a wt strain (NB10), *vanM* mutant strain (NB10 Δ*vanM*) and a strain which expressed the AHL lactonase AiiA (NB10 *aiiA*) were tested for their ability to attract zoospores. Zoospores were shown selectively to settle on the wt but not on the QS mutant or the lactonase expressing strains (Tait *et al.*, 2005). Zoospores were also shown to be attracted to biofilms composed of AHL-producing *Aeromonas hydrophila* and *E. coli* expressing a range of recombinant AHL synthases. The link between signal molecule production and zoospore attraction was further investigated by analysis of zoospore response to specific AHLs. Zoospores were shown to be attracted to all biofilms that produced AHLs; however, increased settlement was seen in biofilms producing longer chain AHLs. Biofilms producing AHLs with hydroxyl or oxy groups did not cause significant differences in zoospore attraction in comparison to biofilms producing un-substituted AHLs (Tait *et al.*, 2005). Increased settlement of zoospores on biofilms with longer chain AHLs is not surprising as longer chain AHLs are less likely to be hydrolysed by the alkaline pH of seawater, which would prevent zoospore attraction (Hmelo and Van Mooy, 2009; Tait *et al.*, 2005). Continuous production of AHLs in a natural biofilm would also be a promoting factor for zoospore attraction (Tait *et al.*, 2005). Tait *et al*, (2005) also showed that the zoospores are attracted to specific parts of the biofilm**,** namely the bacterial microcolonies (Figure 1.13). Using Gfp AHL biosensors, these microcolonies were shown to be areas of high AHL production, further confirming the hypothesis that *Ulva* zoospore were attracted to AHLs (Tait *et al.*, 2005).

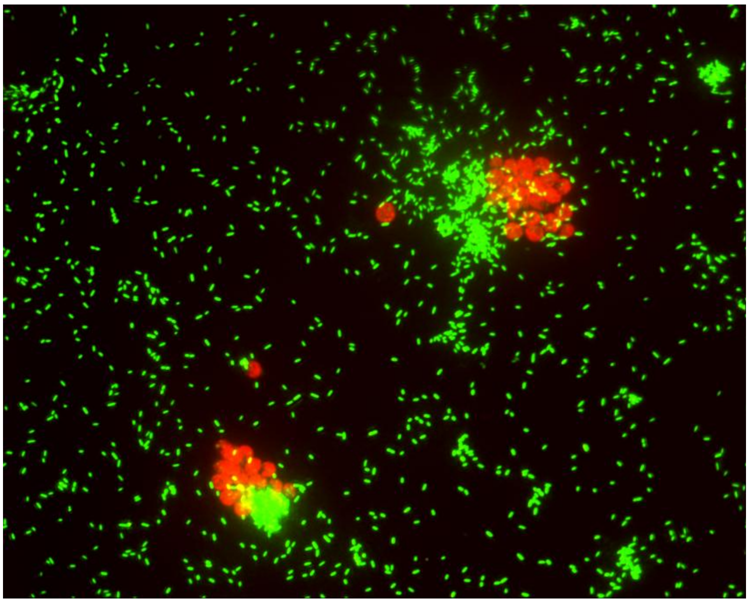


Figure 1.13. AHL production positively effects *Ulva* zoospore settlement. Ulva zoospores (red) were seen to preferentially settle on bacterial microcolonies within the biofilm, (dense green assemblages), where AHL concentration is at its greatest (figure sourced from Tait *et al.*, 2005).

The initial work carried out by Joint *et al.*, (2002) and Tait *et al.*, (2005) connecting *Ulva* zoospore settlement with QS was carried out using a model marine organism *V. anguillarium* (Tait *et al.*, 2005; Joint *et al.*, 2002). Tait *et al.*, (2009) also showed that AHLs are produced by bacteria growing on and around *Ulva*. Specifically AHL production was identified in bacterial species not previously known to be producers of signal molecules; these include species of *Sulfitobacter* sp., *Glaciecola* sp., *Marinobacterium* sp. and *Shewanella* sp. AHLs extracted from these bacteria and identified by mass spectroscopy were shown to possess acyl side chains of varying lengths containing both oxy and hydroxyl substitutions (Tait *et al.*, 2009). *Shewanella* isolates were shown to produce AHLs during the late exponential stage of growth, around 12 h post inoculation. In stationary phase, (>18 h post inoculation), long chain AHLs could no longer be detected indicating the expression of an AHL degrading enzyme by this species.

Turnover of AHLs was found to influence *Ulva* zoospore attraction to a *Shewanella* biofilm. Zoospores were attracted to biofilms of medium density where AHL concentration is highest and not to high density biofilms where signal molecule degradation had taken place causing reduced concentration of AHL (Tait *et al.*, 2009).

The process of how zoospores become associated with bacterial microcolonies producing AHLs was initially thought to be a chemotactic response, but this appears not to be the case. Wheeler *et al.* (2006) used *V. anguillarium* biofilms and synthetic AHLs and showed that signal molecules affect the swimming speeds of the zoospores. Zoospores exposed to wt *V. anguillarum* NB10 biofilms were shown to have reduced swimming speeds compared to those exposed to a biofilm composed of *V. anguillarum* NB10 Δ*vanM* and this result was replicated when zoospores were exposed to synthetic AHLs (Figure 1.14) (Wheeler *et al.*, 2006). The presence of AHLs on a surface clearly caused a reduction is zoospore swim speed which had the effect of keeping the zoospores in close proximity to the AHL source, in turn promoting zoospore settlement. Chemokinesis was most profound when zoospores are exposed to longer chain AHLs, with 3-oxo-C12 HSL causing the greatest reduction in zoospores swim speed (Wheeler *et al.*, 2006). Wheeler *et al.* (2006) proposed a model for zoospore settlement which states negative phototaxis is the driving factor to force zoospores down through the water column and that AHLs act as short range locator cues, modulating zoospore motility for favourable surface selection (Figure 1.14) (Wheeler *et al.*, 2006).

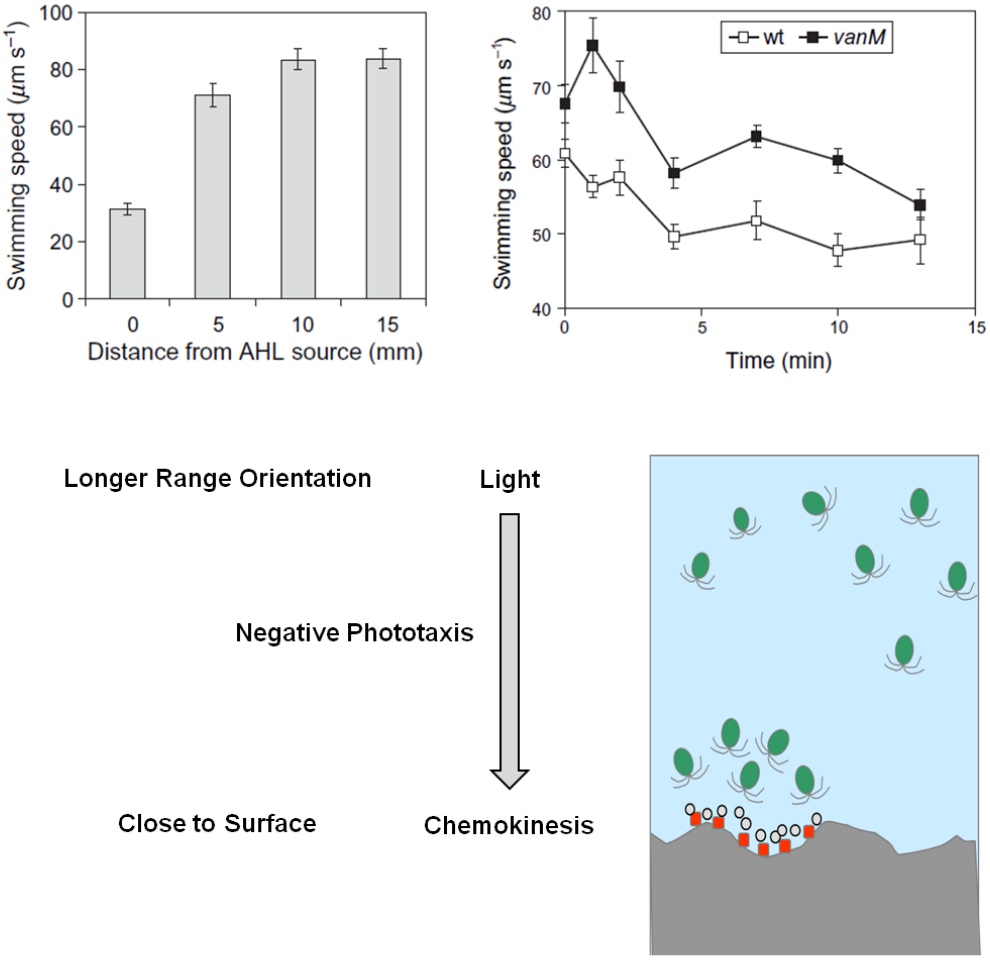


Figure 1.14. AHLs affect *Ulva* zoospore swim speed. *Ulva* zoospore swim speed was seen to increase as the distance from a synthetic AHL source increased (Top left) (sourced from Wheeler *et al.*, 2006). Zoospores exposed to AHL producing *V. anguillarium* wt biofilms had reduced swim speed in comparison to zoospore exposed to AHL deficient *V. anguillarium* Δ*vanM* biofilms (Top right) (sourced from Wheeler *et al.*, 2006). Diagram demonstrating the major cues which act on *Ulva* zoospore determining settlement (Bottom) (Provided by Dr. Karen Tait).

1.5 Project Overview and General Experimental Aims

This project has set about expanding our knowledge of bacterial/algal interactions in the marine environment. The interactions investigated by this project involve bacterial QS signal molecules and utilise both the macroalga *Ulva* and a number of species of microalgae to highlight the cross kingdom biological implications of bacterial signalling in the marine environment. As such, the primary experimental aim of this thesis was to further address the relationship between the green seaweed *Ulva* and its indigenous epiphytic bacterial community. This was be achieved by isolating and characterising key microbial groups colonising the surface of *Ulva* spp. and investigating the extent of AHL-mediated signalling and QS within these groups. The importance of AHL signalling molecules to the germination process of *Ulva* zoospores was also be investigated, as well as examining whether microalgae produced compounds that either stimulated or inhibited AHL-mediated bacterial signalling.

This thesis contains the following results chapters:

- **Chapter 3** characterises the composition of *Ulva*'s epiphytic bacterial community, the signalling molecules produced by these bacteria and experimentally investigates the hypothesis that AHLs effect *Ulva* zoospore germination.
- **Chapter 4** is an investigation of AHL signalling, QS and AHL degradation in two groups of bacteria found to be prominent in the *Ulva* epiphytic community, namely the Bacteroidetes and *Shewanella*.
- **Chapter 5** investigates whether microalgae produces compounds which could interefere with QS-mediated responses in bacteria.

Chapter 2

Materials and Methods

2.1 Culture Media and Growth Conditions

2.1.1 Culture Media

The media used to culture bacteria and microalgae is listed below. Solid media plates were routinely made with 1.5% w/v agar (*Oxoid* No.1), semi solid LB agar were made using 0.3% w/v agar (*Oxoid* No.1). Media was sterilised by autoclaving at 121^{o}C for 15 min. Seawater for marine media was obtained from the Western English Channel and filtered through a 0.2 µm nitrocellulose filter prior to use.

- Actinomycete Isolation Media consisted of 22 g premixed Actinomycete Isolation Media (*Difco*) in 1 L d.H_2O.
- F/2 medium consisted of 1 ml 75 g L^{-1} $NaNO_3$ solution, 1 ml 5 g L^{-1} NaH_2PO_4 H_2O solution in 1 L 0.2 µm filtered seawater, autoclave. Post autoclave 1 ml F/2 trace metal solution (Appendix 2) and 0.5 ml F/2 vitamin solution (Appendix 1) was added (Guillard and Ryther, 1963).
- Luria Bertani medium (LB) consisted of 10 g tryptone (*Oxoid*), 5 g yeast extract (*Oxoid*) and 10 g NaCl in 1 L of d.H_2O (Sambrook and Russell, 2001).
- Luria-Bertani Lennox (Ylb) broth 10 g tryptone (*Oxoid*), 5 g yeast extract (*Oxoid*) and 5 g NaCl in 1 L of d.H_2O (Lennox, 1955)
- Marine medium (MB) consisted of either 47.4 g premixed marine broth (*Difco*) in 1 L of d.H_2O or 5 g peptone (*Difco*) and 1 g yeast extract (*Oxoid*) in 1 L 0.2 µm filtered seawater with no further additive (Zobell, 1941).

- R2A medium consisted of 18.2 g premixed R2A media (*Difco*) in 1 L d.H_2O.
- Seawater medium, made with 0.2 µm filtered seawater and 1.5% agar (*Oxoid* No1) medium was not supplemented with further vitamins or nutrients.
- Terrific Broth (TB) consisted of 12 g tryptone, 24 g yeast extract 4 ml 100% glycerol and 100 ml TB phosphate buffer (0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) in 1 L d.H_2O (Sambrook and Russell, 2001).
- Tryptone Soy Broth (TSB) consisted of 30 g premixed TSB media (*Oxoid*) in 1 L d.H_2O.
- SOC Media consisted of 20 g tryptone, 5 g yeast extract and 0.5 g NaCl in 950 ml d.H_2O. After desolving these components, 10 ml of 250 mM KCl and 5 ml $MgCl_2$ were added and the volume adjusted to 980 ml with d.H_2O prior to autoclaving. Post autoclaving 20 ml 1 M sterile glucose solution was added to the media (Sambrook and Russell, 2001).

2.1.2 Culture Media Supplements

Media was supplemented when needed with the following antibiotics and chemicals at the working concentrations shown. Antibiotic and chemical stocks were prepared in accordance with (Sambrook and Russell, 2001), filter sterilised where appropriate and stored as per manufacturer's instructions: ampicillin (Amp) at 50-100 µg ml^{-1} , carbenacillin (Cb) at 100 µg ml^{-1}, chloramphenicol (Cm) at 30 µg ml^{-1}, gentamycin (Gm) 10 µg ml^{-1}, tetracycline (Tet) 10-20 µg ml^{-1}, isopropyl-

1-thio-β-D-galactopyranoside (IPTG) at 64 μg ml^{-1} and 5-bromo-4-chloro-3 indolyl β-D-galactoside (X-Gal) at 64 μg ml^{-1} (*Sigma-Aldrich*).

2.1.3 Bacterial Growth Conditions

Unless otherwise stated all marine bacteria, *C. violaceum* and *Yersinia pseudotuberculosis* strains were routinely cultured at 30^{o}C. *E. coli* and *P. aeruginosa* strains were cultured at 37^{o}C. All Bacterial cultures grown in liquid broth were agitated during growth at 200 rpm to allow adequate mixing of the culture and aeration during growth. Unless otherwise stated all bacterial liquid cultures were grown in 10 ml volumes of culture media. Bacterial growth was monitored by measuring the absorbance of a culture at an optical density (OD) of 600 nm using a 67 Series Spectrophotometer (*Jenway*).

2.2 Bacteria Strains and Plasmids

2.2.1 Bacterial Strains

During the course of this study, a number of bacterial strains were isolated from the marine environment or obtained from the culture collections at PML and the NCIMB. These strains are described in the results chapters of this thesis. Non-marine and marine strains used in various assays as either vectors, bio-reporters or model organisms are listed in Table

Table 2.1. Bacterial stains used in this study for cloning, maintaining vectors and as bio-reports

Strain	Description	Reference/ Source
Aeromonas hydrophila		
AH1-N	Wild type *Aeromonas hydrophila* strain	(Swift *et al.*, 1999)
E. coli		
DH5α	*supE*44 *ΔlacU*169 (Φ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(Sambrook and Russell, 2001)
JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gypA*96 *relA*1 *thi* D (*lac-proAB*)	(Yanisch-Perron *et al.*, 1985)
BL21	*hsd*S gal (λ*dts* 857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene *1*)	(Sambrook and Russell, 2001)
C. violaceum		
CV026	*cviI*::mini-Tn*5* derivative of ATCC 31532, Km^R, AHL^-	(McClean *et al.*, 1997)
P. aeruginosa		
PAO1	Wild-type, Nottingham strain	(Stover *et al.*, 2000)
PAO1 *rhlI::lux*	*Pseudomonas rhlI*::*lux* fusion bio-reporter	Dr. James Lazenby, Uni of Nottingham
PAO1 *lasI::lux*	*Pseudomonas lasI*::*lux* fusion bio-reporter	Dr. James Lazenby, Uni of Nottingham
PAO1 *rhlA::lux*	*Pseudomonas rhl::lux* fusion bio-reporter	Dr. James Lazenby, Uni of Nottingham
PAO1 *lasB::lux*	*Pseudomonas lasB*::*lux* fusion bio-reporter	Dr. James Lazenby, Uni of Nottingham
Vibrio Strains		
V. anguillarium NB10	Wild type, serotype 01, clinical isolate from the Gulf of Bothnia	(Norqvist *et al.*, 1989)
V. anguillarium NB10 Δ*vanI vanM*	Double AHL synthase mutant	(Tait *et al.*, 2009)
V. coralliilyticus LMG20984T	Wild type *Vibrio* species isolated from diseased *Pocillopora damicornis*	(Tait *et al.*, 2010)
V. tubiashii NCIMB 1337	Wild type *Vibrio* species isolated from *Crassostrea virginica*	(Tubiash *et al.*, 1970)
Y. pseudotubercolosis		
YPIII	*Y. pseudotuberculosis* wild type strain	(Rosqvist *et al.*, 1988)

2.2.2 Plasmids

Table 2.2. Plasmids used in this study. All plasmids were stored in d.H_2O at -20^{o}C.

Plasmid	Description	Reference/ Source
pBBRIMCS-5	Broad host range vector containing *lacZ*, Cm^r and a multiple cloning site.	(Kovach *et al.*, 1994)
pCOLD	Protein expression vector	(Hayashi and Kojima, 2008)
pCOLD*aacS*	pCOLD expression vector harbouring *aacS* ORF as an *Nde* I/*Eco* RI fragment in frame with *cspA* prmotor and his tag	This study
pET3a	Cloning vector used as wt control in *E. coli Ulva* zoospore germination assay	(Tait *et al.*, 2005)
pET*VanI*2	Plasmid expressing *V. angullarium* AHL synthase VanI	(Tait *et al.*, 2005)
pGEM T Easy	Cloning vector with an f1 origin of replication containing *lacZ*, Amp^r and a multiple cloning site.	*Promega*
pGEM::*aiiA*	pGEM vector containing Cm^r and the AHL lactonase *aiiA* from *Erwinia carotovora*.	S. Atkinson, Uni of Nottingham
pME6000	Shuttle vector with a pBBR1 origin of replication containing Tet^r and *lacZ.*	(Maurhofer *et al.*, 1998)
pMH655	Plasmid containing Gm^r and the Quorum Sensing Inhibitor System (QSIS).	(Rasmussen *et al.*, 2005)
pMT01	pBBRIMCS1-5 vector containing *aiiA* from pSU18::*aiiA*.	This study
pMW47.1	Plasmid expressing *P. aeruginosa* AHL synthase RhlI	(Latifi *et al.*, 1995)
pSB536	*N*-AHL bio-reporter composed of a pUC18 derived plasmid containing Amp^r and a fusion of *AyhR* and *lux* promotor from *Vibrio fisheri* to the *lux* operon from *Photorhabdus luminescens.*	(Swift *et al.*, 1997)
pSB401	*N*-AHL bio-reporter composed of a pACYC184 derived plasmid containing Tet^r and a fusion of *luxR* and *lux* promotor from *Vibrio fisheri* to the *lux* operon from *Photorhabdus luminescens.*	(Winson *et al.*, 1998)
pSB1142	*N*-AHL bio-reporter composed of a pACYC184 derived plasmid containing Tet^r and a fusion of *lasR* and *lasI* promotor from *Pseudomonas aeruginosa* to the *lux* operon from *Photorhabdus luminescens.*	(Winson *et al.*, 1998)
pT7T3	Cloning vector used as wt control in *E. coli Ulva* zoospore germination assay	(Tait *et al.*, 2005)
pT7T3*luxI*	Plasmid expressing *Vibrio fisheri* AHL synthase *LuxI*	(Tait *et al.*, 2005)

2.3 Isolation of Marine Bacteria from *Ulva* spp.

Marine bacteria were isolated from rocks colonised by *Ulva* spp., the *Ulva* holdfast-rock interface and from the thallus of wild *Ulva* spp. Bacterial isolates were all obtained from either Wembury beach, Devon, UK (50°19'00'' N 4°05'03'' W), or Polzeath beach, Cornwall, UK (50°34'39'' N 4°55'03'' W).

Isolation of strains from the rocks colonised by *Ulva* spp. and from the *Ulva* holdfast/rock interface was carried out in accordance with the method described by Tait *et al.* 2009 taking scrapings from the rocks and/or *Ulva* holdfast, plating onto seawater agar (described in Section 2.1.1), and incubating for 15 days at 15°C. Resultant single colonies were then isolated onto MB agar (Tait *et al.* 2009).

Isolation of strains from the *Ulva* thallus was carried out by adapting the methodology described by Patel *et al.* (2003). Intialy *Ulva* thallus tissue was vortexted in 30 ml strerile Phosphate Buffer Solution (PBS) for approximately 3 min. The resultant PBS supernatant was decanted, serially diluted in sterile PBS to a range of dilutions factoring between 10^{-3} and 10^{-7} and plated onto either seawater agar, MB agar, Actinomycete Isolation Agar or R2A agar, (described in Section 2.1.1). The resultant isolation plates were cultured for 72 h at 30°C, before single colonies were indervidually isolated on to MB agar (Patel *et al.* 2003).

2.4 Preparation and Manipulation of DNA

2.4.1 Preparation of Chromosomal DNA

Chromosomal DNA from bacterial cell pellets obtained by centrifugation of stationary phase cultures or from cells collected from the surface of mature *Ulva* thallus material was extracted either using the DNeasy Blood and Tissue Kit (*Qiagen*) or Wizard Genomic DNA Purification Kit (*Promega*). Chromosomal DNA extracts using both kits were performed as per manufacturer's instructions. Final elution of DNA was in sterile molecular grade d.H_2O (*Sigma-Aldrich*) at 50^oC.

2.4.2 Plasmid Extraction

Plasmid DNA was extracted from stationary phase cultures using a Qiaquick Mini Prep Kit (*Qiagen*) used as per manufacturer's instructions. Final elution of DNA was in sterile molecular grade d.H_2O (*Sigma-Aldrich*) at 50^oC.

2.4.3 Cleaning of PCR Product DNA

DNA from PCR products was cleaned using either a Qiagen PCR Purification Kit (*Qiagen*) or a Wizard DNA Clean-Up System (*Promega*) used as per manufacturer's instructions. Final elution of DNA was carried out using sterile molecular grade d.H_2O (*Sigma-Aldrich*) at 50^oC.

2.4.4 Restriction Digest

Restriction digests of plasmid DNA was carried out on ice in a total volume of 20 µl using 0.2-1 µg of DNA, 1 µl of restriction endonuclease, restriction buffer at 1 X concentration and the remaining volume made up with sterile molecular grade d.H_2O. Restriction digests were incubated at 37^{o}C for 2-3 h, unless otherwise stated. Restriction endonucleases were purchased with appropriate restriction buffer from *Promega* or *New England Biolabs* and were stored in accordance with the manufactures instructions at -20^{o}C.

2.4.5 DNA Analysis by Agarose Gel Electrophoresis

DNA samples were analysed by electrophoresis using agarose gels composed of 0.8-1.5% ultra pure agarose (*Invitrogen*), in 1 X TAE buffer, (80 mM Tris-acetate, 19 mM EDTA at pH 7.6-7.8). To visualise DNA fragments ethidium bromide was added to the melted agarose gels at a final concentration of 10 µgml^{-1}. Prior to loading onto the gel 6 X loading buffer (*Promega*) was added to DNA samples. To judge DNA fragment sizes appropriate DNA ladder in 6 X loading buffer (*Promega*) was added to gels alongside samples. Gels were run in horizontal gel apparatus (*Fisher Scientific*, *Bio-Rad*) in 1 X TAE buffer at 90-120 V depending on the volume of the gel. DNA samples were visualised on the gels using a UV transluminator (*UVP*).

2.4.6 DNA Purification from Agarose Gels

DNA was purified from agarose gels by cutting the required fragments from the gel. Fragments were then extracted and cleaned using a Qiagen Gel Extraction Kit (*Qiagen*), in accordance with manufacturer's instructions. Final elution of DNA was carried out in sterile molecular grade d.H_2O (*Sigma-Aldrich*) at 50^{o}C.

2.4.7 DNA Quantification

Quantification of DNA was carried out by micro spectrophotometry measuring absorbance at 260 nm using a NanoDrop 2000 (*Thermo Scientific*).

2.5 PCR

2.5.1 PCR Primers

PCR primers used in this study are listed in table 2.3. PCR primers used in this study were routinely designed 'by eye' looking at DNA sequence data. The degenerate primers used to amplify putative *Shewanella* AHL acylase genes were designed from the analysis of genomic DNA sequence located up-stream and down-stream of the putative AHL acylase ORFs (Sbal687_3701 and Sput200_0855 respectively) annotated in the genomes of *Shewanella baltica* OS678 (CP002383) and *Shewanella putrafaciens*. 200 (CP002457). The primer MTaacF was designed from conserved sequence in both genomes located 10 bp up-stream from the putative AHL acylase ORF start codon, includes the start codon plus the starting 6 bp of the putative AHL acylase ORF. The primer MTaacR was designed from conserved sequence in both genomes located 104 bp down-stream from the putative AHL acylase ORF stop codon. All primers were checked for secondary structures and primer dimmer formation using the primer analysis software provided on the *Eurofins* oligonucleotide orders webpage. Oligonucleotide primers were synthesised either by *Sigma-Aldrich* or *Eurofins* and stored at -20^{o}C in molecular grade water at a concentration of 100 µM. Primers were diluted to a working concentration of 10 µM prior to being added to PCR reaction mixes.

Table 2.3. List of PCR primers used in this study. Primer sequences are listed using the IUPAC 1-letter code abbreviations.

Primer	Sequence (5'-3')	Anneal Temp.	Additional Information	Reference
96bfm	GAGTTTGATYHTGGCTCAG	53	Used to type marine bacteria. Amplified 16S rDNA corresponding to positions 9-1512 of the *E. coli* 16S rDNA sequence	(Muhling *et al.*, 2008)
1152uR	ACGGHTACCTTGTTACGACTT	53	Used to type marine bacteria. Amplified 16S rDNA corresponding to positions 9-1512 of the *E. coli* 16S rDNA sequence	(Muhling *et al.*, 2008)
341F	CCTACGGGAGGCAGCAG	53	Used to amplify 16s rDNA to produce clone library. Amplified 16S rDNA corresponding to positions 341-926 of the *E. coli* 16S rDNA sequence	(Muyzer *et al.*, 1993)
907R	CCGTCAATTCMTTTGAGTTT	53	Used to amplify 16s rDNA to produce clone library. Amplified 16S rDNA corresponding to positions 341-926 of the *E. coli* 16S rDNA sequence	(Muyzer *et al.*, 1993)
MT*aac*F	GATAATAATAATGAAATTC	43	Used to amplify *Shewanella* AHL acylase genes (see Section 2.5.1 text).	This study
MT*aac*R	TGTTAAWTTTTWACAAKTRYRT	43	Used to amplify *Shewanella* AHL acylase genes (see Section 2.5.1 text).	This study
MRI*aac*F	CTCCACCTACCGAACCTGAA	53	Upstream primer designed to amplify *aac* gene in *Shewanella onediensis* MR1	(Tait *et al.*, 2009)
MRI*aac*R	TGCAGCATCAACTCAGTGGT	53	Downstream primer designed to amplify *aac* gene in *Shewanella onediensis* MR1	(Tait *et al.*, 2009)
B1*aac*F	CATGGTGCTAGGTAATC	53	B1*aac* clone Sequencing Primer	This study
B1*aac*R	GCTGTCCAGCCTCTG	53	B1*aac* clone Sequencing Primer	This study
A1*aac*F	GCCGCGTATCGTTGG	53	A1*aac* clone Sequencing Primer	This study
A1*aac*R	CAGTGATAGCTGTCCAGC	53	A1*aac* clone Sequencing Primer	This study
*Nde*IB1*aac*-F	TTTTTTTTT**CATATG**AAATTCAA CAAACTCGCGATCGCTATGGG	53	Forward primer used to amplify B1*aac* ORF which engineers *Nde* I site (highlighted in bold)	This study
B1*aacEco*RI-R	TTTTTTTTT**CTGCAG**TTATGGTT TTTGTAGTGTCAGCTCAGTCGTCG	53	Reverse primer used to amplify B1*aac* ORF which engineers *Eco* RI site (highlighted in bold)	This study
M13F	GTAAAACGACGGCCAGT		General DNA sequencing primer	(Sambrook and Russell, 2001)
M13R	GGAAACAGCTATGACCATG		General DNA sequencing primer	(Sambrook and Russell, 2001)

2.5.2 General PCR Protocol Using Non-Proofreading DNA Polymerase

Amplification of DNA using a non-proofreading DNA polymerase was carried out using GoTaq DNA Polymerase (*Promega*). Reactions were made as per manufactures instructions to a total volume of 50 µl and contained a final concentration of 1 X GoTaq Flexi Buffer (*Promega*), 1 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM forward and reverse primers, approximately 250 ng chromosomal DNA or 1 µl of boiled bacterial colony suspended in d.H_2O and 1 unit of GoTaq DNA polymerase. Amplification was carried out in a LabCycler thermocycler (*SensoQuest*) using an initial de-naturation step of 96^{o}C for 2 min followed by 35 cycles of 95^{o}C for 1 min, primer anneal temperature for 30 s and 72^{o}C for 1 min per 1 kb of template sequence. Finally 1 step of 72^{o}C for 10 min was used to remove the polymerase from the DNA.

2.5.3 General PCR Protocol Using Proofreading Enzyme DNA Polymerase

When required a proofreading DNA polymerase was used to carry out PCR, the enzyme used was Phusion High-Fidelity DNA Polymerase (*NEB*). Reactions were made to a total volume of 50 µl as per manufacturer's instructions and contained a final concentration of 1 X Phusion HF Buffer (*NEB*), 10 mM dNTPs, 0.5 µM forward and reverse primers, approximately 250 ng chromosomal DNA or 1 µl of boiled bacterial colony suspended in d.H_2O, 3% DMSO and 1 unit Phusion High-Fidelity DNA Polymerase. Amplification was carried out in a LabCycler thermocycler (*SensoQuest*) using an initial de-naturation step of 98^{o}C for 30 s followed by 30-35 cycles of 98^{o}C for 10 s, primer annealing temperature for 30 s and 72^{o}C for 30 s per 1 kb of template sequence. Finally 1 step of 72^{o}C for 5 min was used to remove the polymerase from the DNA.

2.6 Cloning

2.6.1 DNA Ligation

Purified DNA was ligated into vectors in a 1:3, 1:5 or 1:10 ratio of vector to insert depending on the concentration of each. 1-2 µl T4 DNA Ligase (*Promega*) and 1 X DNA ligase buffer (*Promega*) was used for all ligation reactions. Total volume of ligation was between 10-20 µl DNA ligations were carried out by initially cooling the reaction to 1^{o}C followed by overnight incubation at 18^{o}C.

2.6.2 Preparation of Electro-competent Bacteria

E. coli were made electro-competent by growing seed cultures to a mid log phase growth point (OD_{600} of between 0.6 and 0.8). Cells were then washed 3 times in sterile ice-cold 10% glycerol at 1 X, 0.5 X and 0.1 X volume respectively at 4^{o}C. After washing cells were re-suspended in 50 µl of sterile ice-cold 10% glycerol and stored at -80^{o}C in 20 µl aliquots (Sambrook and Russell, 2001). Electro-competent *Shewanella* cells were prepared by washing mid log phase culture 3 times 1 X, 0.5 X and 0.033 X volume of sterile ice-cold 1 M D-Sorbitol followed by re-suspending in 20 µl sterile ice-cold 1 M D-Sorbitol (Myers and Myers, 1997).

2.6.3 Transformation of Bacteria via Electroporation

Prior to electroporation DNA was dialysed using 0.025 µm millipore filters (*Millipore Corporation*). Electroporation was carried out in 2 mm electroporation cuvettes using 20 µl of competent cells and 1-10 µl of DNA. An electroporation pulse of 2.5 kV was delivered using the BioRad Gene Pulsar connected to a BioRad pulse controller (*BioRad Laboratories*). The competent cells were recovered in 1 ml of either LB or SOC media and incubated for 1 h at 37°C before being plated onto LB media supplemented with appropriate antibiotics, X-Gal and IPTG (Sambrook and Russell, 2001). Electroporation of *Shewanella* was carried out in accordance with Myers and Mayers 1997 with using the same equipment as detailed above, recovery of electroporated cells was carried out at 30^{o}C using media appropriate for the strain (Myers and Myers, 1997).

2.6.4 Chemical Transformation of **E. coli**

Chemically competent *E. coli* JM109 cells were purchased from *Promega*, stored and used in accordance with the manufactures instructions. 1-10 µl of DNA was added on ice to 40 µl aliquots of chemically competent *E. coli* JM109 cells. DNA and Cells were incubated on ice for 20 min, heat shocked at 42°C for 3 seconds and returned to ice for a further 2 min. Transformations were recovered in 1 ml of either LB or SOC media and incubated for 1 h at 37°C before being plated onto LB media supplemented with appropriate antibiotics, X-Gal and IPTG (Sambrook and Russell, 2001).

2.6.5. 16S rDNA Clone Library Construction

Epiphytic bacteria were obtained from the surfaces of *Ulva* thallus material collected from Wembury beach, UK (50^{o}19’00’’N 4^{o}05’03’’W) by prolonged vortexing in sterile phosphate buffered solution. Bacteria were pelleted by centrifugation and chromosomal DNA was extracted as described in Section 2.4.1. Using the extracted chromosomal DNA as a template,16S rDNA corresponding to nucleotides 341-926 of the *E. coli* 16S rDNA sequence was amplified via PCR (see Section 2.5.2) using primers 341F and 907R (see Table 2.3). The amplified 16S rDNA was cloned into the pGEM T easy vector and transformed into *E. coli* DH5a via electroporation (see Sections 2.6.1 and 2.6.3). A total of 96 clones were selected for sequencing using the M13F and M13R universal sequencing primers (see Section 2.7.1).

2.6.6. **Shewanella** *Genomic Libary Constuction and QSIS Screening*

Shewanella strain P3 was grown to stationary phase and chromosomal DNA was extracted as described in section 2.4.1. P3 chromosomal DNA was digested separately using *Eco* RI, *Hind* III and *Bam* HI (Promega) for 12h at 37^{o}C as described in section 2.4.4. The digested chromosomal DNA was ligated into shuttle vector pME6000 digested with the corresponding restriction enzyme (see Section 2.6.1). Ligations were transformed into competent *E. coli* DH5α pMH655 *via* electroporation (see Section 2.6.3). Subsequent transformant colonies were screened for AHL degrading genes by picking individual colonies onto LB media containing 15% sucrose, 20 μM synthetic 3-oxo-C12-HSL, ampicillin 50 μg ml^{-1} and tetracycline 10 μg ml^{-1} and then onto LB media containing ampicillin 50 μg ml^{-1} and tetracycline 10 μg ml^{-1} (adapted from method used by Rasmussen *et al.* 2005). Colonies that showed the same level of growth on media containing AHLs and sucrose as on media where AHLs and sucrose were omitted were selected for further study and therefore assayed for AHL degrading activity using the microtitre plate method detailed in section 2.9.5.

2.7 DNA Sequencing and Sequence Analysis Techniques

2.7.1 DNA Sequencing

Sequencing reactions were carried out at the University of Nottingham DNA Sequencing Facility, Queens Medical Centre, Nottingham. The non-radioactive Taq Dye Primer Cycle sequencing kit was used according to the manufacturers recommendations (Applied Biosystems), and the resulting sequencing reactions were analysed by 8% polyacrylamide gel electrophoresis using an Applied Biosystems 373A automated sequencer.

2.7.2 DNA Sequence Analysis

Initial DNA sequence analysis was carried out using DNA Baser (*Heracle BioSoft*) and Lasergene version 7.0 software package (*DNAStar*). Comparisons of DNA and protein sequences were performed using the Basic Local Alignment Search Tool (BLAST) algorithms available at the NCBI's web page (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted out using the method previously used by Tait *et al.* 2009. Alignment of clone library 16S rDNA sequences and the subsequent formation of phylogenetic trees was carried out using the Molecular Evolutionary Genetics Analysis (MEGA) tool (Tamura *et al.*, 2011). The 16S rDNA alignment was carried out using a ClustalW alignment algorithm and the subsequent phylogenetic tree was produced from a neighbour-joining and bootstrap analysis performed with 1000 replications at a sequence similarity cut off of 97% (Tait *et al.* 2009). The species richness and the comparison of the *Ulva* thallus 16S rDNA clone library to the rocky shore and seawater bacterial populations was carried out using the Distance-Based Operational Taxonomic Unit and Richness (DOTUR) program (Schloss and Handelsmen 2005).

2.7.3 Protein Sequence Analysis

Consensus sequences of LuxI-type AHL synthase, LuxR-type AHL response regulator, LuxM-type AHL synthase, LuxN type AHL response regulator and AHL inactivating enzymes were produced from alignments of published protein sequences imported from the NCBI protein database (See Appendices 4-8). Protein alignments were carried out on the MegAlign program, part of the Lasergene version 7.0 software package (*DNAStar*) using a ClustalW alignment algorithm. The resultant majority sequence was exported from MegAlign to the SeqBuilder, program part of the Lasergene version 7.0 software package (*DNAStar*) and used as consensus sequences in BLAST search of both *Shewanella* and *Bacteroidetes* genomes using the NCBI Genomes BLAST program.

Resultant hits from the BLAST searches were analysed for conserved structural/functional motifs using the Pfam sequence search facility provided by the Sanger Institute (http://pfam.sanger.ac.uk/) (Punta *et al.* 2012).

2.8 Protein Expression and Analysis

2.8.1 Protein Expression

Putative *Shewanella* AHL degrading enzymes were expressed using the pCOLD expression vector (Hayashi and Kojima, 2008). The ORF of *aacS* was amplified from a pGEM*aacS* template with primers NdeIB1*aac*-F and B1*aac*EcoRI-R using a proof reading DNA polymerase (*NEB*). The resultant PCR product was cleaned and digested with *Nde* I and *Eco* RI using the appropriate buffer for double digest, (*Promega*). The *Nde* I/ *Eco* RI *aacS* fragment was cloned into the pCOLD expression vector ensuring that it was in frame with the His tag and *cspA* promoter present on the pCOLD vector. The resultant pCOLD*aacS* construct was transformed into *E. coli* BL21 and grown to stationary phase in LB cb_{100} at 37^{o}C. BL21 pCOLD*aacS* and BL21 pCOLD cultures were used separately to seed 50 ml TB cb_{100} to an OD_{600} of 0.05 which were grown to an OD_{600} of 0.4 at 37^{o}C, left to stand at 15^{o}C for 30 min before continuing to culture, with shaking, at 15^{o}C for 24 h. Successful protein expression was indicated by a twofold reduction in the growth of BL21 pCOLD*aacS*, as measured by OD_{600}, in comparison with BL21 pCOLD (Hayashi and Kojima, 2008).

2.8.2 Cell Lysis and Protein Extraction

Cell lysis was achieved by sonication. Cultures were centrifuged at 10,000 rpm for 3 min to pellet the cells. Cells were re-suspended in sterile PBS and sonicated on ice at a frequency of 10-12 MHz for 10 min in repeated cycles 10 s sonication followed by 20 s on ice (Sambrook and Russell, 2001).

Unlysed cells were removed by centrifugation at 5,000 rpm for 5 min at 4^oC. 500 µl of the resultant supernatant was removed and centrifuged at 13,000 rpm for 30 min at 4^oC to pellet inclusion bodies and membranes. The soluble fraction was transferred to a fresh microtube and the insoluble fraction re-suspended in 1 ml sterile PBS both were re-centrifuged at 13,000 rpm for 30 min at 4^oC. Protein fractions were prepared for analysis using sample buffer composed of 60 µl β mercaptoethanol to 1ml bromophenol blue. An equal volume sample buffer was added to the soluble fraction and the insoluble fraction re-suspended in 1 ml sample buffer, both were boiled for 5 min prior to analysis via SDS-PAGE (Sambrook and Russell, 2001).

2.8.3 SDS-Polyacrylamide Gel Electrophoresis

Proteins were analysed using a 12% polyacrylamide gel. Gels were made individually using glass casting plates and casting frames (*Bio-Rad*). Resolving gels were made from 4 ml 30% (w/v) acrylamide/bis (37.5:1); 2.5 ml 1.5 M Tris-HCl at pH 8.8; 100 µl 10% (w/v) SDS and 3.35 ml d.H_2O, set with; 100 µl 10% (w/v) ammonium persulfate and 15 µl TEMED. Stacking gels were made from 830 µl 30% (w/v) acrylamide/bis (37.5:1); 620 µl 1.5 M Tris-HCl at pH 6.8; 50 µl 10% (w/v) SDS and 2.5 ml d.H_2O, set with; 50 µl 10% (w/v) ammonium persulfate and 5 µl TEMED. Samples were loaded onto the gel along with appropriate protein size markers and run in 1 X SDS running buffer composed of 14 g Glycine, 3 g Tris and 1 g SDS in 1 L d.H_2O. All SDS PAGE was carried out in a Mini-Protean Tetra Electrophoresis System (*Bio-Rad*) and gels were run for 80 min at 150 v (Sambrook and Russell, 2001). Post electrophoresis gels were washed 3 X in d.H_2O and stained for 1 h with Simply Blue SafeStain (*Invitrogen*). Gels were de-stained on a shaking platform o/n in 100 ml H_2O.

2.9 Extraction and Analysis of AHLs

2.9.1 Production of Synthetic AHLs

Synthetic AHLs were produced by Alex Thurman at the School of Molecular Medical Science, University of Nottingham. AHL stocks were suspended in 1 ml HPLC grade methanol and stored at -20^{o}C. Synthetic AHLs produced for this study and their abbreviations are listed at the front of this thesis.

2.9.2 Extraction of AHLs from Culture Supernatant

AHLs were extracted from culture supernatants using a method adapted from (McClean *et al.*, 1997). Bacteria were grown to stationary phase in 20 ml cultures and centrifuged to pellet cells. Culture supernatants were decanted and acidified to pH 2.0 using 1 M HCl. AHLs were extracted from the acidified culture supernatant using 0.5 vol dichloromethane. The solvent layer was removed and dried either under nitrogen gas or via rotary evaporation or using a vacuum RC1022 centrifuge (*Thermo Scientific*). Extractions were then reconstituted in 2 ml acetonitrile, re-dried and stored as dried extracts at -20^{o}C.

2.9.3 AHL Detection Using Bio-reporters

Detection of AHLs using bio-reporters was carried out using either a Petri dish based assay or a microtitre plate based assay using cell free supernatant extract prepared as described above. AHL bio-reporters used were the *lux*-based *E. coli* pSB536, pSB401 and pSB1142 and the pigment-based CV026 (Winson *et al.*, 1998; McClean *et al.*, 1997; Swift *et al.*, 1997). The Petri dish based assay was conducted using the method as described in McClean *et al.* 1997. Luminescence as a result of bio-reporter activation was recorded using a luminographer (*Hamamatsu*); activation of CV026 produced a purple pigment (McClean *et al.*, 1997). Microtitre plate based AHL detection assays were carried in accordance with the method described by Tait *et al.* (2009) using the *lux*-based bio reporters *E. coli* pSB536, pSB401 and pSB1142 (Winson *et al.*, 1998; Swift *et al.*, 1997). A black clear bottomed 96 well microtitre plate (*Greiner Bio One*) was used for all assays with 50-100 µl of cell free supernatant extract dried to each well assayed. Bio-reporter activation was either measured at a set point after 3 h incubation at 37^{o}C with luminescence being measured at 450 nm using a *Berthold* MITHRAS microtitre plate reader, or tracked over a 12 h incubation period with bioluminescence and OD being measured every 30 min using a Infinite 200 PRO series microtitre plate reader (*Tecan*). Intensity of bio-luminescence was calculated in Relative Light Units (RLU) (Tait *et al.*, 2009).

2.9.4 AHL Detection via LCMS

AHL detection in bacterial extracts via Liquid Chromatogarphy Mass Spectroscopy Liquid Chromatography was conducted by Mary Bruce at the Department for Molecular Medical Sciences at The University of Nottingham. Liquid Chromatography was carried out using the Agilent 1200 series HPLC, comprising degasser, binary pump, column heater and autosampler using the methodology as previously described by Yates *et al.* 2002 (Yates *et al.*, 2002). Mass Spectroscopy was carried out using the Bruker HCT Plus ion trap in multiple reaction mode (MRM) and Hystar software, ions were introduced using positive ion electrospray from the Agilent HPLC system. Using the Smartfrag option on the software, the trap was set to isolate from full scan and then fragment ions at m/z 172.1 and 190.1. The ion charge control was used to prevent charge overload in the trap. The instrument was optimised using the smart parameter setting for m/z 172.The monitored mass range was 50 - 250 m/z. Data analysis was carried out using the Bruker Data Analysis version 3.3 package. Extracted ion chromatograms (EIC) of m/z 102.1 and 172.3 were produced from the positive ion MSMS of m/z 172.1 and 190.1 respectively. Retention times and peak spectra were matched to the 1 µM standard (x6) injected at the beginning of each method set. Injections of sample solvent were also monitored to assess carryover (Ortori *et al.*, 2007).

2.9.5 AHL Inactivation Assay

AHL inactivating activity was measured using the *lux*-based bio-reporters *E. coli* pSB536, pSB401 and pSB1142 (Winson *et al.*, 1998; Swift *et al.*, 1997). Strains being assayed were grown overnight to stationary phase along with a non AHL-inactivating control. Cultures were then diluted to an OD_{600} of 0.01 in media seeded with appropriate AHL at a concentration of 1.24 µM and grown to stationary phase. Cultures were extracted in either DCM or acidified ethyl acetate as described in section 2.8.2 and residual AHL concentration measured using an appropriate bio-reporter using the microtitre plate assay as described in section 2.8.3.

2.10 Microalgae Assays

2.10.1 Microalgal Strains and Growth Conditions

In order to investigate microalgal/bacterial interactions three separate axenic cultures of microalgae were acquired from the Culture Collection of Algae and

Protozoa (CCAP, Oban, UK). These cultures were; *Nannochloropsis oculata* (Eugstigmatophyte) (CCAP 849/1); *Tetraselmis suecica* (Prasinophyte) (CCAP 66/8) and *Isocrysis galbana* (Haptophyte/ Prymnesiophyceae (CCAP 927/1). All three species were routinely cultured from a starting inoculate of 10 ml of stationary phase culture in 1 L F/2 media, (Guillard and Ryther, 1963). In order to maintain an axenic state all microalgal cultures were supplemented with an antibiotic cocktail of ampicillin (200 µg ml^{-1}), streptomycin (100 µg ml^{-1}) and kanamycin (50 µg ml^{-1}). In addition, all three cultures were viewed under a microscope at 100 X magnification, confirming that all three cultures lacked any bacterial contamination. During growth microalgal culture were incubated at 18^{o}C in the close proximity to a fluorescent white light source with a 16 h light, 8 h dark cycle. Microalgal growth was monitored by measuring the absorbance of a culture at an Optical Density (OD) of 600 nm using a 67 Series Spectrophotometer (*Jenway*).

2.10.2 Extraction of Microalgal Cultures

QS mimics and QS inhibitory compound(s) were obtained by solvent extraction of microalgal cultures. Solvent extraction was carried out 2 X using 0.5 vol dichloromethane in accordance with the method previously detailed in Section 2.9.2 for the extraction of AHLs from the cell free supernatants of bacterial cultures. For experiments using the *lux*-based *E.coli* AHL bio-reporters, separate extracts from individual cultures were used. Experiments using the *P. aeruginosa* transcriptional fusion bio-reporters and marine bacteria protease assays used pooled extracts from 3 X 1 L microalgal cultures. This allowed a greater number of experiments to be performed and overcame any inconsistency in the resultant quorum-quenching activity between extracts from separate cultures.

2.10.3 AHL Mimic Assay

The presence AHL mimic compounds in the extracts from microalgal cultures was assayed for using the microtitre plate assay, based on the method described by Tait *et al.* (2009) for the detection of AHLs in marine bacterial culture extracts and is outlined previously (Section 2.9.3) (Tait *et al.*, 2009). A volume 30 µl of microalgal culture extracts was used in all assays, co-cultured with 300 µl of the *lux*-based AHL bio-reporters *E. coli* pSB536, pSB401 and pSB1142 (Winson *et al.*, 1998; Swift *et al.*, 1997).

2.10.4 Quorum-quenching Activity in Microalgae

Quorum-quenching activity by microalgal species was measured using the *lux*-based AHL bio-reporters *E. coli* pSB536, pSB401 and pSB1142 (Winson *et al.*, 1998; Swift *et al.*, 1997) and *lux*-based *P. aeruginosa* transcriptional fusion reporters PAO1 *lasI*::*lux*, PAO1 *RhlI*::*lux*, PAO1 *LasB*::*lux* and PAO1 *RhlA*::*lux* (un-published). All microalgal quorum-quenching assays were carried out using a 96 well microtitre plates based technique adapted from the AHL detection assay, (Tait *et al.*, 2009), described in section 2.9.3. When using the *E. coli* AHL bio-reporters 1.24 µM C4-HSL, C6-HSL and 3-oxo-C12-HSL were dried to wells of black clear-bottomed 96 well microtitre plate (*Greiner Bio One*). In addition to the AHLs a volume of solvent extract from the microalgal species representative of 20 ml original culture or an equal volume of solvent extract from F/2 medium and HPLC grade solvent was also dried to the 96 well microtitre plate. When using the *P. aeruginosa* transcriptional fusion bio-reporters the synthetic AHLs were negated from the assay as these reporters have the ability to produce both C4-HSL and 3-oxo-C12-HSL, the volume of microalgal extract, F/2 medium extract and solvent control was however the same (a volume representing 20 ml original culture). In all assays 300 µl of appropriate bio-reporter was added to each well. Each experimental condition was assayed in triplicate. Assay plates were incubated at 37^{o}C in an Infinite Pro200 microtitre plate reader (*Tecan*) over a 12 h period. Absorbance at OD_{600} and bio-reporter luminescence at 100 nm in each well was measured every 30 min. Bio-reporter activity over the 12 h period was recorded as a function of luminescence (RLU)/OD_{600}, these measurements were used to calculate total bio-reporter luminescence over the 12 h period from each extract assayed.

2.10.5 Fractionation

In order to initiate the process of identifying compound(s) responsible for observed quorum-quenching activity by microalgae, extracts from stationary phase microalgal cultures were fractionated. Fractionation was performed by initially re-suspending the concentrated pooled extract from 3 X 1 L cultures of each microalgal species in 1 ml methanol. The re-suspended extract was then diluted 10^{-1} in a volume of 10 ml methanol. Subsequently the diluted extracts were applied to Oasis HLB solid phase extraction cartridges (*Waters*) by running the extracts through the cartridge. The extract was allowed to elute through the cartridges by gravity and the run-through collected. A methanol:d.H_2O dilution series was prepared, (10% methanol – 100% methanol, in 10% increments, 10 ml volumes). Each methanol fraction was applied to the HLB solid phase extraction cartridges, in order, stating with the 100% methanol fraction. Each of the 10 resultant fractions eluted from the cartridges were concentrated via rotary evaporation, re-suspended in 1 ml 100% methanol and assayed with the *P.*

aeruginosa transcriptional fusion bio-reporters using the method described previously in Section 2.10.3.

2.10.6 Protease Assay

The fractionated extracts from stationary phase microalgae cultures were assayed for effect on protease production in the marine pathogens *Vibrio anguillarium, Vibrio coralliilyticus, Vibrio tubiashii* and *Aeromonas hydrophila* (See table 2.1). 10 ml o/n cultures of each bacterium were diluted 1:50 and 150 µl of diluted culture were added to 15 µl of each solvent fraction that had been previously air-dried to remove all traces of methanol within microtubes. The subsequent cultures were grown for 16 h, centrifuged and the supernatant used in protease activity assays, carried out, black clear bottomed 96 well microtitre plate (*Greiner Bio One*) using the Protease Fluorescent Detection Kit (*Sigma*), as per the manufactures instructions. Controls included media blank and solvent negative control. Protease activity was calculated as the quantity (ng) of trypsin released by the degradation of casein labelled with fluorescein isothiocyanate (FITC). Fluorescence was measured as per as per the manufactures instructions (Protease Fluorescent Detection Kit - *Sigma*) using a Infinite Pro200 microtitre plate reader (*Tecan*) at 485 nm excitation and 535 nm emission.

2.11 *Ulva* Zoospore Germination Assay

2.11.1 Biofilm Growth

All biofilms were grown on 25 mm X 25 mm glass cover slides within biofilm incubation chambers. Cover slides were placed vertically in holders within the biofilm incubation chambers, submerged in 300 ml filtered seawater and sterilised. Bacteria were grown to stationary phase in appropriate culture media, re-suspended in sterile filtered seawater and adjusted to an OD_{600} of 1.0 prior to inoculation of sterile biofilm incubation chambers. Biofilm incubation chambers were inoculated separately with 0.5, 1, 2 and 4 ml volumes of bacteria. Marine biofilms were grown for 48-72 h at room temperature (≈ 22°C). Biofilms of *E. coli* strains were grown in 70% filtered sea water for 24 h in order to reduce osmotic stress on the bacteria by salinity (Tait *et al.*, 2005).

2.11.2 Biofilm Density Measurement

Biofilm density was determined as per the method described by Joint *et al.* 2002 with microscope image analysis at 40 X magnification, using a Reichert Jung Polyvar microscope and an Optronics Magna Fire SP camera. Image ProPlus Version 5 imaging software was used to measure the area of bacteria within the field of view. The density of bacteria per unit area of slide (L) was determined by $L = x/A$, where x is the total area covered by bacteria and A is the area of the total field of view (Joint *et al.*, 2002).

2.11.3 **Ulva** ***Zoospore Release***

Ulva zoospore release was carried out in accordance with methodology of Callow *et al.* 1997. Mature *Ulva* spp. thallus material was collected from the rocky shore environment at Wembury beach, Devon, UK (50°19’00’’N 4°05’03’’W), at 1-2 h post high water during the period of a spring tide. The *Ulva* thallus material was dried between paper towels over night at a constant temperature of 15°C. After drying the thallus apical tips were cut into sterile filtered sea water on ice and agitated to stimulate

Ulva zoospore release. After which the thallus material was discarded and the zoospore suspension was exposed to an overhead light source for approximately 15 min in order to select for healthy, active zoospores by exploiting their negative photo-tactic phenotype. Excess sea water and non active zoospores were discarded and the zoospore suspension containing the active zoospore was diluted to an OD_{600} of 0.5 using sterile filtered sea water (Callow *et al.*, 1997).

2.11.4 **Ulva** ***Zoospore Germination Assay***

15 ml of the final *Ulva* zoospore suspension was used to inoculate biofilms or sterile glass slides within 90 mm Petri dishes. *Ulva* zoospores were settled onto the glass slides or biofilms in the dark for 1 h (Tait *et al.*, 2005). Post settlement zoospore slides were transferred from the 90 mm Petri dish to sterile 60 mm Petri dishes and submerged in 10 ml sterile filtered seawater, 70% sterile filtered sea water was used with zoospore assays using *E. coli* biofilms. For assays using synthetic AHLs, the appropriate AHL was added to the separate dishes at final concentrations of 0.5, 5, 10, 20, 30, 40 and 50 µM. Zoospore slides were incubated at 18^{o}C in proximity to a light source with a 16 h light, 8 h dark cycle for 24 and 48 h. At the 24 and 48 h time points zoospore slides were removed to sterile 60 mm Petri dishes, fixed with 2% (v/v) glutaraldehyde and stained with dilute carbol fuschin.

Ulva zoospore slides were viewed at 10 X magnification using a Reichert Jung Polyvar microscope with attached Optronics Magna Fire SP camera. The lengths of 300 *Ulva* zoospores and germlings were measured from randomly selected images of each experimental condition using Image ProPlus Version 5 imaging software. Measurements were exported to MS Excel and average zoospore/germling length was calculated. In all *Ulva* germination experiments a successfully germinated *Ulva* zoospore was defined as having a length equal to or greater than 15 μm.

2.11.5 Statistical Analysis

Statistical analysis of *Ulva* zoospore germination assays was carried out by performing ANOVA and PERMANOVA tests using statistical analysis software packages Minitab 16 (*Minitab*) and PRIMER 6 (*PRIMER-E*).

Chapter 3

The Effect of Bacterial Signal Molecules on *Ulva* Zoospore Germination

3.1 Introduction

3.1.1 Effects of Marine Bacteria on Ulva *Zoospore Settlement*

Ulva spp reproduce either via the production of gametes or the production of motile quadriflagellate zoospores (reveiwed by Maggs and Callow, 2001). The *Ulva* zoospore body is pear shaped, approximately 5-8 µm in length and 5 µm in diameter at the widest point (Callow *et al.*, 2002). *Ulva* spp sporulation takes place during the summer period and in most instances approximately 1-2 h after the high tide. During sporulation *Ulva* zoospores are released from the apical tips of the *Ulva* thallus, zoospores then proceed to adhere to surfaces in their proximal environment (Callow *et al.*, 2000). Adherence of zoospores to a surface is a two stage process. Initial adhesion to a surface is temporary and involves the zoospore testing the surface for optimal conditions followed by the secretion of glycoprotein from Golgi bodies in the apical end of the zoospore. Upon identifying an optimum surface, secondary adherence is permanent (Finlay *et al.*, 2002; Callow *et al.*, 2000). Following permanent adhesion *Ulva* zoospores germinate and mature into an adult plant (Callow *et al.*, 2000). *Ulva* zoospores utilise their flagella in order to travel from the site of sporulation on the *Ulva* thallus to the surface they eventually adhere too. Flagella driven motility in *Ulva* zoospores is primarily governed by negative phototaxis, ensuring that the zoospores are oriented and move towards a solid substrate such as the rock surface which provides optimal substratum for adherence and germination (Callow and Callow, 2000). Factors acting as important cues for the selection of an optimal surface for zoospore adherence include the topology of the surface, wettability, the surface chemistry and the presence of a bacterial biofilm (Callow *et al.*, 2002; Callow and Callow, 2000; Dillon *et al.*, 1989).

Further investigation of the relationship between *Ulva* zoospore settlement and bacterial biofilms revealed that *Ulva* zoospores preferentially settle on bacteria producing AHL signal molecules (Tait *et al.*, 2009; Tait *et al.*, 2005; Joint *et al.*, 2002). In investigating the method by which AHLs cause preferential settlement of *Ulva* zoospores Wheeler *et al.* (2006) demonstrated that in the presence of AHLs zoospore motility is significantly reduced, causing zoospores to cluster and eventually settle at areas of increased AHL concentration (Wheeler *et al.*, 2006). In addition to AHLs having an effect on the settlement of *Ulva* zoospores, AHLs have also been shown to affect sporulation in other species of algae. High concentrations of C4-HSL were shown to up-regulate sporulation in *Acrochaetium* spp. This effect was inhibited by a number of halogenated furanone compounds, AHL structural homologues produced by algae such as *Delisea pulchra* (Weinberger *et al.*, 2007; Manefield *et al.*, 1999).

3.1.2 Bacterial Population Associated with the Rocky Shore Environment

Ulva spp. grow on the rocks present in the intertidal zone of the UK coastline which is an environment heavily colonised by marine bacteria which form biofilms on the surfaces of these rocks (Munn, 2004). A 16S rDNA phylogenetic analysis of the intertidal rocky shore environment has revealed the presence of a diverse microbial population (Tait *et al.* 2009). The rocky shore population was found to be dominated by *Alphaproteobacteria* and *Flavobacteria*; however other bacterial groups such as the *Sphingobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Bacilli*, *Cyanobacteria* and *Verrucomicrobiae* were also shown to be present (Figure 3.1). It was possible to isolate bacteria from a number of these groups, (*Flavobacteria*, *Deltaproterobacteria*, *Gammaproteobacteria* and *Bacilli*) and grow them in laboratory culture, however culture based isolations did not reflect the population as defined by molecular methods (Tait *et al.*, 2009).

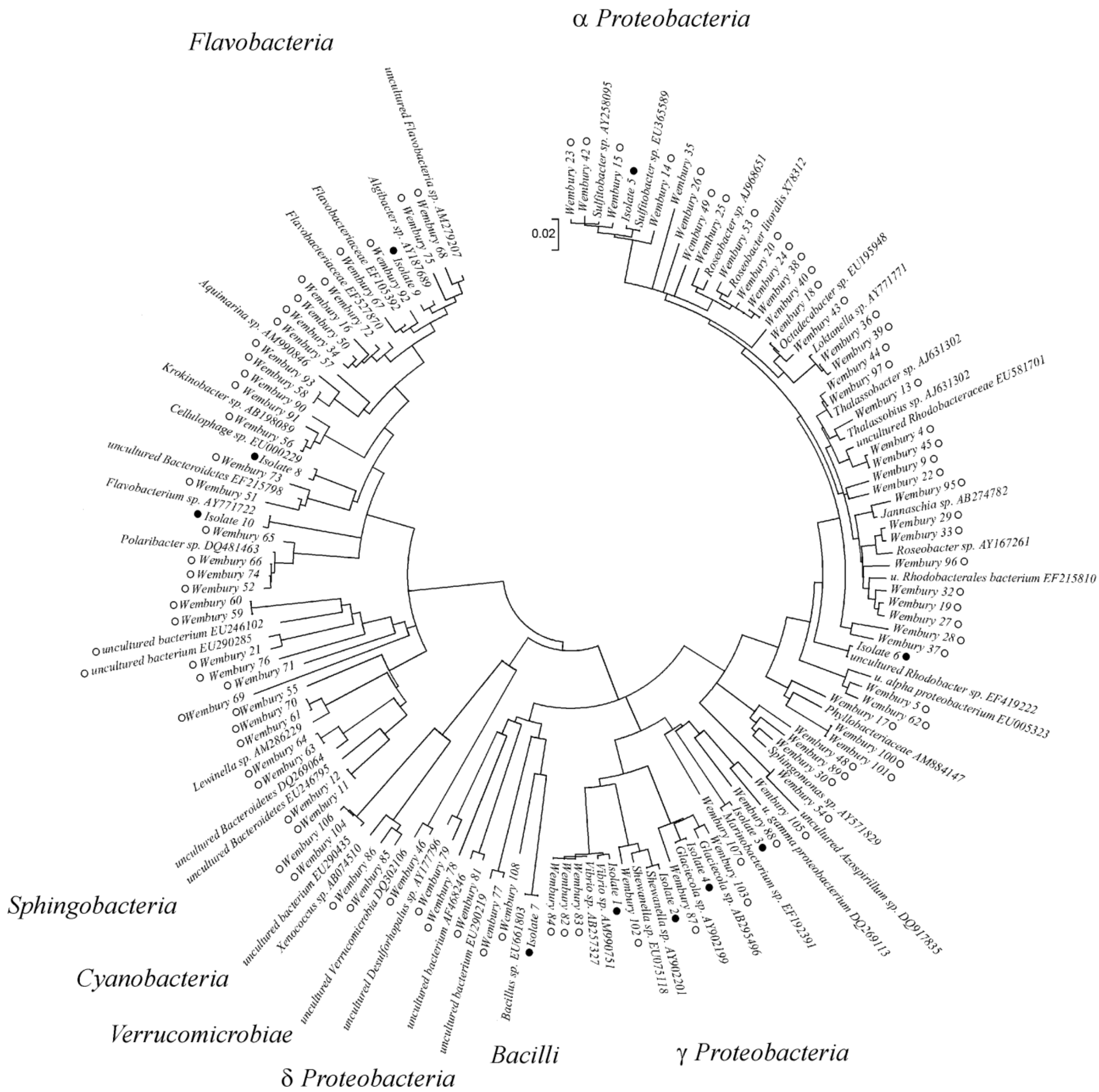


Figure 3.1. Phylogenetic tree showing the bacterial population present on biofilms in the rocky shore environment where *Ulva* spp. are found growing. The phylogenetic tree was produced from an alignment of 16S sequences from clones (○) and isolates (●) taken from rocky shore biofilms. The reference strain sequences were obtained from Genbank (Figure sourced from Tait *et al.*, 2009).

3.1.3 Bacterial Effects on Ulva *spp. Growth and Morphology*

In addition to their effect on *Ulva* zoospore settlement, bacteria appear to have a profound impact on the germination of the *Ulva* zoospore and growth of the *Ulva* plantlet. In 1980, Provasoli and Pintner showed that when grown axenically, *Ulva lactuca* assumed a different morphology to when grown in the presence of marine bacteria. The morphology observed in axenic culture is an atypical 'pin cushion' growth as opposed to wild type foliaceous growth (Provasoli and Pintner, 1980). This phenomenon of aberrant morphological differences in *U. luctuca* cultures when grown axenically has additionally been observed in *Ulva linza*, *Ulva pertusa* and *Ulva compressa* (Marshall *et al.*, 2006; Nakanishi *et al.*, 1996). Adding back bacterial strains to axenic *U. linza* cultures has been proven to restore wild type morphology (Figure 3.2).

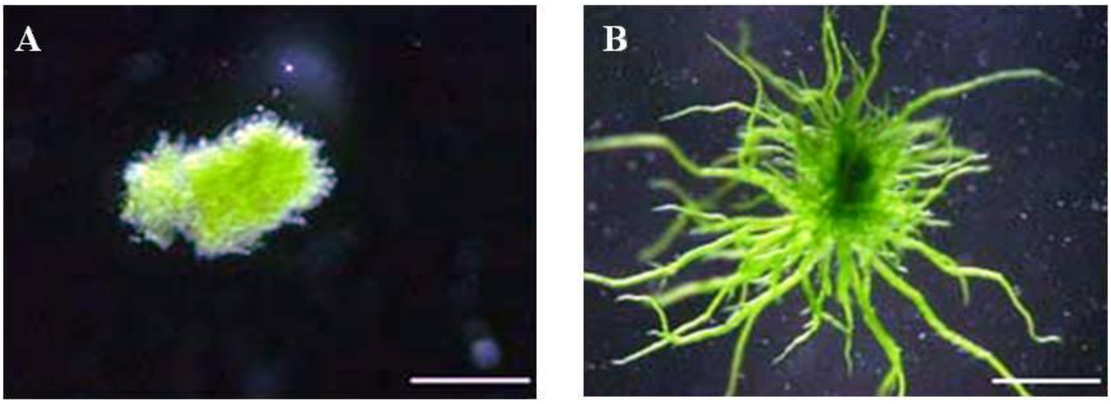


Figure 3.2. Images comparing the aberrant morphology associated with axenic *Ulva* culture and the wild type morphology. (A) 28 day old axenic culture of *U. linza*, (**B)** 28 day old initially axenic culture of *U. linza* inoculated with marine bacterial strain UC19 (*Cytophaga* sp.). The scale bars represent 1 mm (Figure sourced from Marshall *et al.*, 2006).

Of 1555 bacterial strains isolated from *U. pertusa*, 676 strains (41%) showed morphogenesis activity (Nakanishi *et al.*, 1996). In *U. linza*, a number of different strains isolated from the algae not only induced morphological change in axenic cultures but also increased the relative growth rate of the plant (Marshall *et al.*, 2006). From 38 unique isolates, 5 affected both morphology and growth of *U. linza* when added to axenic cultures. Phenotypic analysis of marine bacterial strains that affected either morphology or growth of *U. linza* showed that they grouped to the *Proteobacteria*, *Bacteroidetes* and Gram positive cocci, and that the bacterial effect on *U. linza* growth was independent of bacterial phylogeny (Marshall *et al.*, 2006). The effect of bacteria on the morphology of green alga is not only restricted to the genus *Ulva*. *Monostroma oxyspermum* cannot differentiate without the presence of a bacterial population and approximately 1% of 1159 bacterial strains isolated from green alga were shown to induce morphological changes in *M. oxyspermum* (Matsuo *et al.*, 2003; Tatewaki *et al.*, 1983).

As yet there is no scientifically proven mechanism established as to why bacteria affect the morphology of *Ulva* spp. and other green algae; however there are reports of marine bacteria producing plant growth regulators and vitamins which may affect the morphological differentiation of algae (Croft *et al.*, 2006; Maruyama *et al.*, 1986). A primary example of a plant hormone produced by marine epiphytic bacteria that influences growth morphology is thallusin, shown to effect *Ulva intestinalis*, *U. pertusa* and *M. oxyspermum* (Matsuo *et al.*, 2005). Additionally other algal growth hormones such as cytokinin-type hormones, auxin-type hormones and indole-3-acetic acid have been shown to be produced by marine bacteria (Bradley, 1991; Maruyama *et al.*, 1986). Other hypotheses include bacteria being responsible for supplying nitrogen to the algae based upon isolates from green alga possessing the nitrogenase gene *nifH* and/or bacteria being responsible for the turnover of plant hormones affecting growth and morphology (Ashen and Goff, 2000; Chisholm *et al.*, 1996).

3.1.4 Experimental Aims

Previous work has focused on the bacterial community present in the environment where *Ulva* spp. are found growing, and the relationship between *Ulva* spp. zoospore settlement and bacterial signalling molecules. This study attempted to define the bacterial population present on the surface of the mature *Ulva* thallus via phylogenetic analysis of a 16S rDNA clone library and isolate strains of marine bacteria, representative of this population from the *Ulva* thallus. Marine bacteria representative of the *Ulva* spp. cognate population were then screened for the production of both AHL and AHQ type signal molecules in order to assess if these bacteria were actively engaged in signalling. Based upon previous work which showed that AHLs affect *Ulva* spp. zoospore settlement and that marine bacteria have a profound effect on *Ulva* spp. growth morphology we hypothesised that bacterial signal molecules may not only affect *Ulva* zoospore settlement but also *Ulva* zoospore germination and the early growth of the *Ulva* germling. This hypothesis was tested by exposing germinating *Ulva* zoospores to bacterial biofilms composed of strains indigenous to the *Ulva* spp. bacterial population, biofilms of transgenic *E. coli* which expressed various AHL synthase genes, and exogenously adding synthetic AHLs to zoospores settled on sterile glass slides prior to germination.

3.2 Results

3.2.1 16S rDNA Clone Library of the **Ulva** *spp. Bacterial Population*

To determine the bacterial population associated with the surface of *Ulva*, a clone library of 16S rDNA was constructed and 76 clones sequenced, aligned and compared with the Genbank database using the BLAST program. A phylogenetic tree resulting from the analysis of this alignment shows the species richness present on the *Ulva* thallus to be dominated by the phyla *Proteobacteria* (65.79%) and *Bacteroidetes* (34.21%) (Figure 3.3). At class level, the proteobacterial population was dominated by *Alphaproteobacteria*, which had a relative abundance of 39.47%, with many clones clustering to the *Rhodobacteraceae* family (34.42% of total library). The remaining proteobacterial clones all clustered with known *Gammaproteobacteria* (22.36%) and *Epsilonproteobacteria* (3.94%), with gammaproteobacterial sequences clustering to the *Alteromonadaceae*, *Vibrionaceae*, *Oceanospirillales* and *Chromatiales*. The *Bacteroidetes* population was dominated by the *Flavobacteria* and *Sphingobacteria* classes, which had relative abundances of 14.47% and 19.74% of the total clone library respectively.

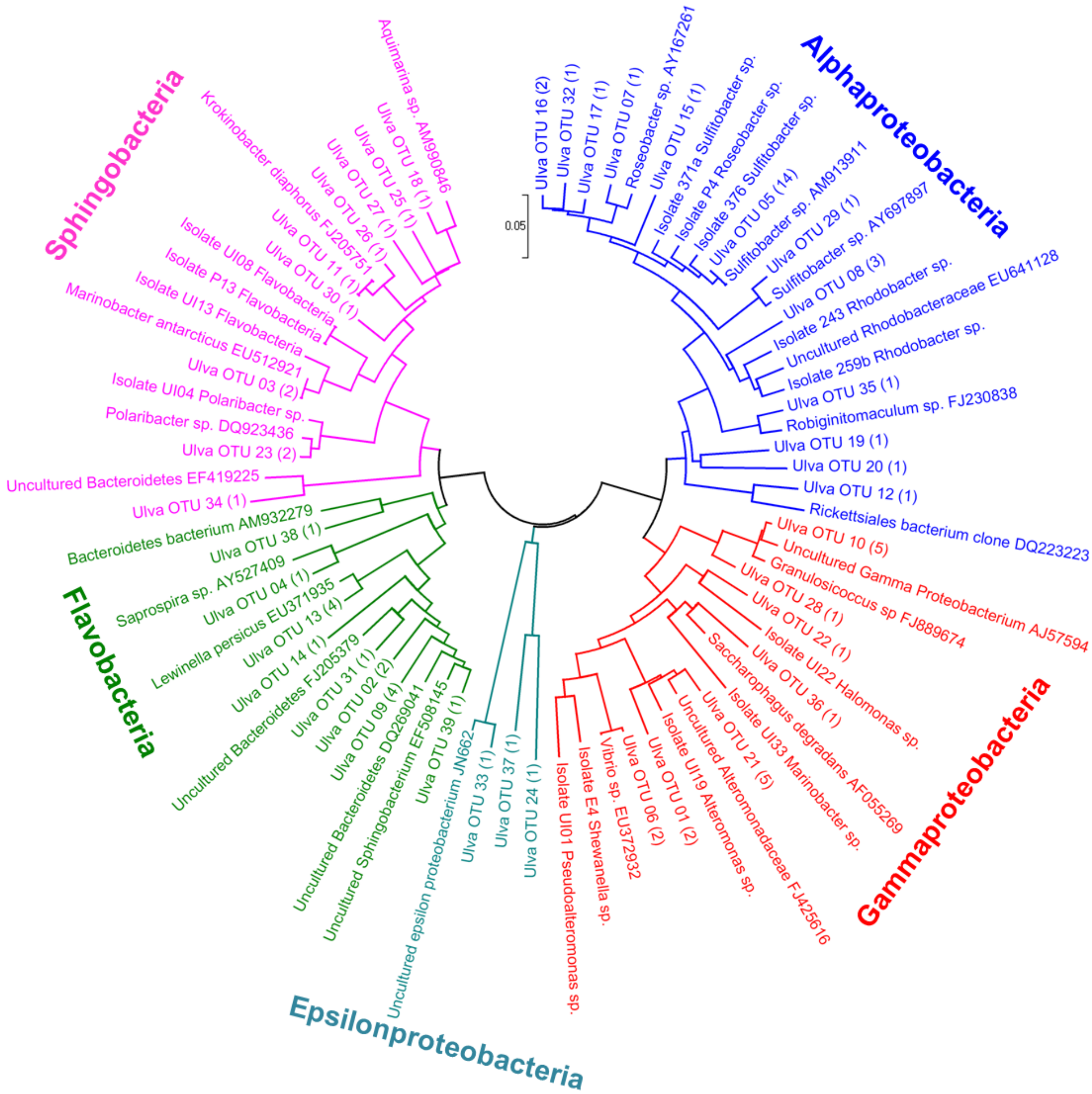
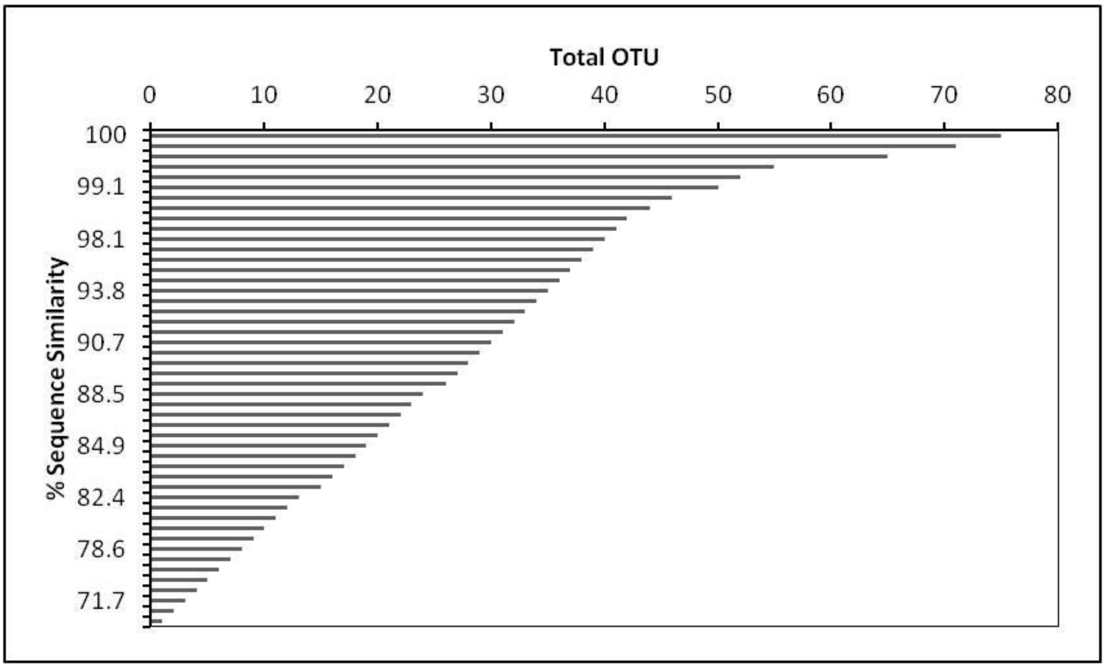


Figure 3.3. Phylogenetic tree of the *Ulva* spp. thallus bacterial population. The tree resulted from a sequence alignment of 16S rDNA from bacterial clones and isolates obtained from the *Ulva* thallus at 97% sequence similarity. The reference strains were taken from Genbank. In brackets is the number of clones in each Operational Taxonomic Unit (OTU). The tree topology is based on neighbour-joining and bootstrap analysis was performed with 1000 replications.

The Distance-Based Operational Taxonomic Unit and Richness (DOTUR) program (Schloss and Handelsman, 2005), showed that reducing the percentage sequence similarity when analysing the 16S rDNA clone library reduced the total number of OTUs present in the clone library (Figure 3.4).



% Sequence Simliarity	Total OTU
100%	75
99%	50
97%	39
95%	36
90%	27

Figure 3.4. DOTUR analysis of *Ulva* spp. thallus 16S clone library. Graph displaying the number of OTUs in the 16S rDNA *Ulva* spp. thallus clone library against the percentage sequence similarly between clones. A reduction in sequence similarity reduced the total OTU in the clone library. The table below the graph displays OTU at the standard cut-off points of 99%, 97%, 95% and 90% sequence similarity.

The top five most abundant OTUs in the *Ulva* spp. thallus clone library were identified by comparison to the Genbank genomic sequence database via BLAST analysis. The phylogeny and Genbank accession number of hits identified as having the strongest homology to each OTU are listed in Table 3.1.

Table 3.1. Identity of most abundant OTUs in *Ulva* spp. thallus clone library. Table displaying BLAST hits in the Genbank database with the greatest homology to the top 5 most abundant OTUs in the *Ulva* spp. thallus 16S clone library.

OTU Number	Number of clones in each OTU	Phylogeny	Genbank Accession Number
Ulva OTU 5	14	*Sulfitobacter* sp	AM913911
Ulva OTU 10	5	Uncultured *Alteromonadaceae*	FJ425616
Ulva OTU 21	5	Uncultured *Gammaproteobacteria*	EU005276
Ulva OTU 9	4	Uncultured *Sphingobacteria*	FN433448
Ulva OTU 13	4	*Lewinella agarilytica*	AM286229

3.2.2 Comparison of **Ulva** *Thallus Population to Rocky Shore and Sea Water Column Population*

The 16S rDNA clone library of the *Ulva* thallus bacterial population was compared to two other similar phylogenetic analyses. One, a 16S rDNA clone library detailing the bacterial population of the rocky shore habitat at Wembury Bay colonised by *Ulva* spp. carried out in June 2007 (Tait *et al.*, 2009). The other was a 16S rRNA tagged 454 pyrosequenceing data set, also carried out in June 2007, detailing the bacterial population present in the seawater column at the L4 sample site in the Western English Channel, 50^{o}15'N, 04^{o}13'W (Gilbert *et al.*, 2010; Gilbert *et al.*, 2009). The percentage relative abundances of bacterial phyla were compared between the three populations, and it was found that in each population the *Proteobacteria* and *Bacteroidetes* phyla dominanted, however the percentage relative abundances of these phyla differed between each population. The *Ulva* thallus and rocky shore populations showed a greater percentage abundance of *Bacteroidetes* clones than the water column population. However, the abundance of *Cyanobacteria* in the water column was much greater than in the *Ulva* thallus population, where no *Cyanobacteria* clones were seen and in the rocky shore population, where *Cyanobacteria* relative abundance was only 2.19%. In addition to these larger differences in phylum abundance, the *Ulva* thallus population lacked the low abundances of other bacterial phyla such as *Fusobacteria, Fermicutes* and *Verrucomicrobia* which were present in both the rocky shore population and the water column population (Figure 3.5).

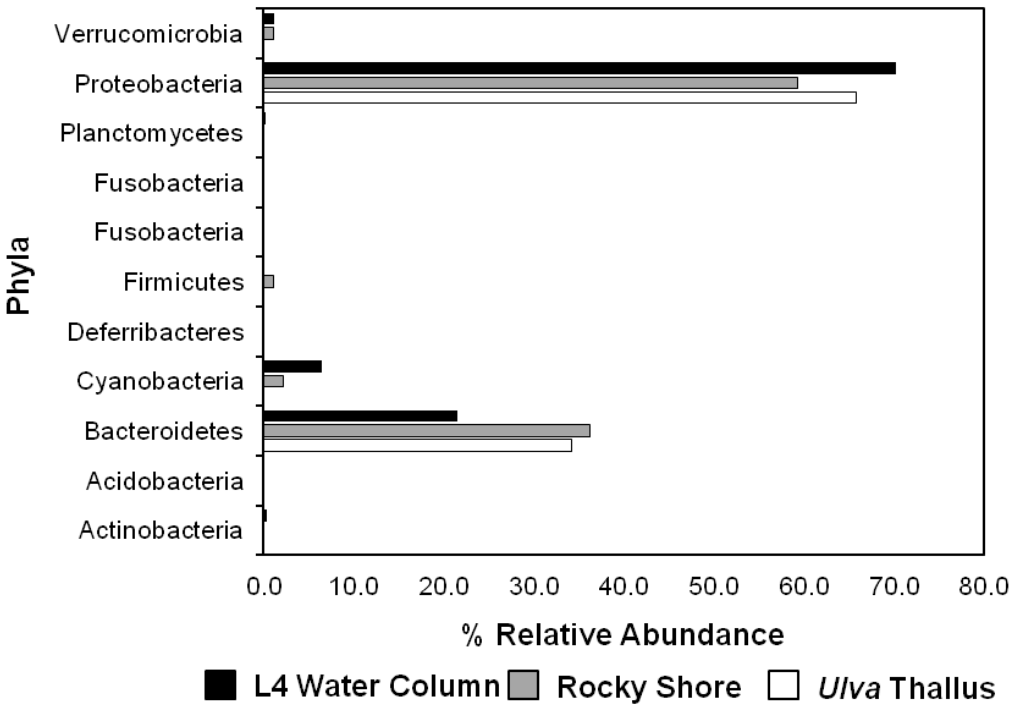
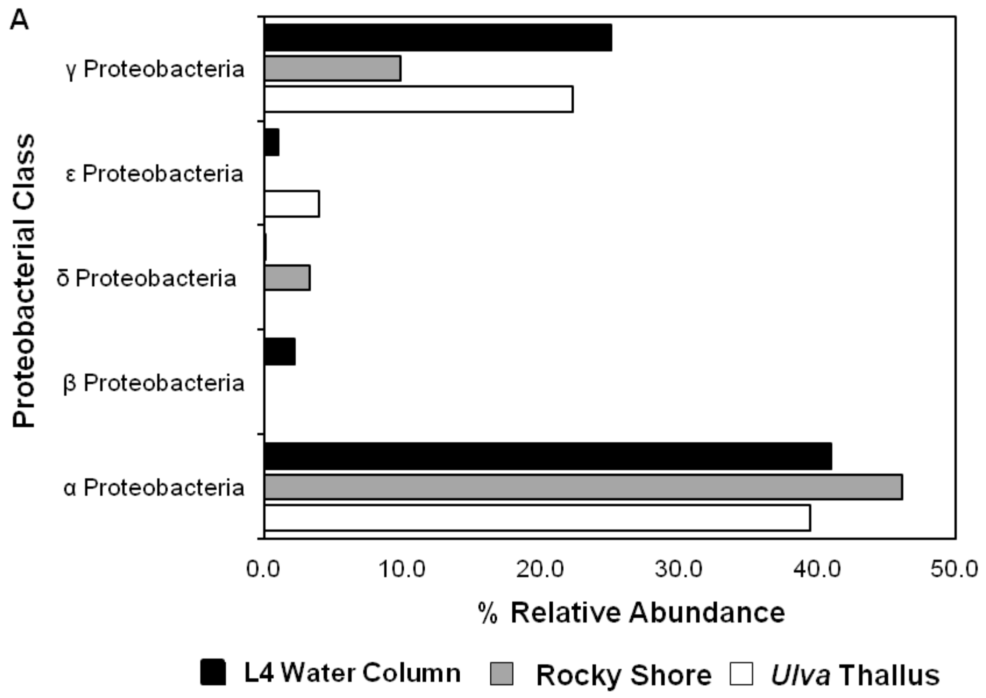


Figure 3.5. Relative abundance of bacterial phyla in three separate marine environmental niches. Comparison of percentage relative abundance of bacterial phyla present in the clone libraries representing the *Ulva* thallus, rocky shore (Tait *et al.*, 2009) and L4 seawater water column (Gilbert *et al.*, 2009) bacterial populations.

The two dominating phyla, *Proteobacteria* and *Bacteroidetes,* in the three populations were further dissected to compare percentage relative abundances at class level. As in the *Ulva* thallus population the *Alphaproteobacteria* and

Gammaproteobacteria were the dominant two classes of *Proteobacteria* present in the rocky shore (46.15% and 9.89% respectively) and water column populations (41.03% and 25.09% respectively), with the *Alphaproteobacteria* being the most dominant class of *Proteobacteria* in all three populations. However, the rocky shore clone library contained a greater fraction of *Alphaproteobacteria*. The rocky shore and water column also contained *Betaproteobacteria* and *Deltaproteobacteria*; these were not seen in the *Ulva* thallus population (Figure 3.6A). The two classes present in the *Bacteroidetes* phyla in all three populations were the *Flavobacteria* and the *Sphingobacteria*; however the percentage relative abundances of each class differed dramatically between each population (Figure 3.6B). The ratio of *Sphingobacteria*: *Flavobacteria* differed markedly in each data set: *Ulva* thallus 37:63; rocky shore 79:21 and water column 6:94.



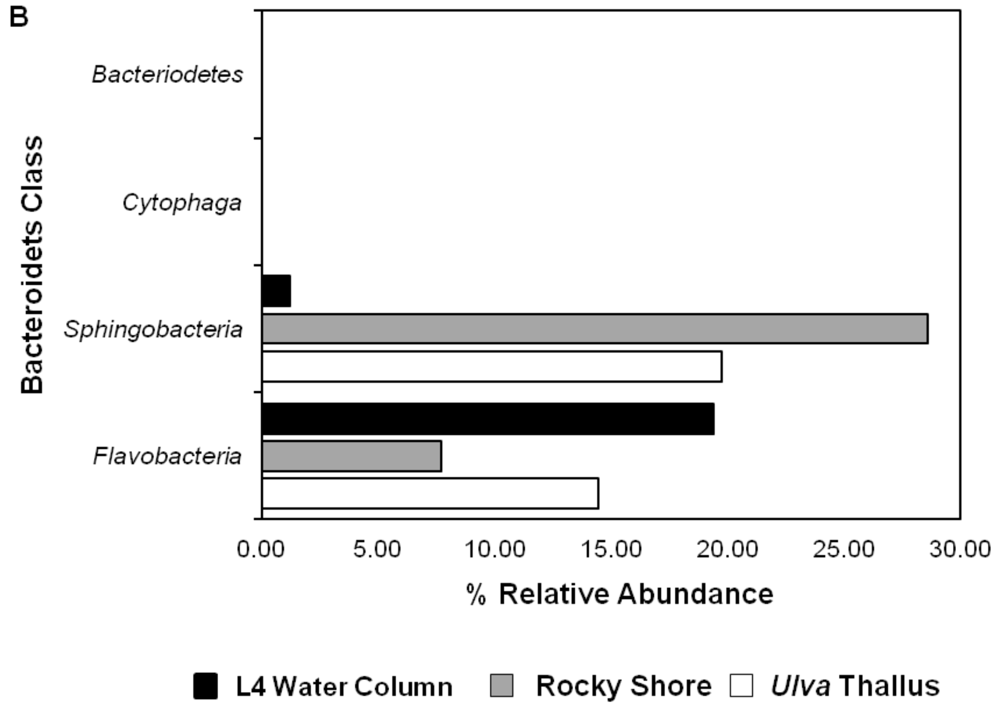


Figure 3.6. Relative abundance of bacterial classes in three separate marine environmental niches. Comparison of percentage relative abundance of proteobacterial classes (**A**) and *Bacteroidetes* classes (**B**) present in the clone libraries representing the *Ulva* thallus, rocky shore (Tait *et al.*, 2009) and L4 seawater water column (Gilbert *et al.*, 2009) bacterial populations.

3.2.3 Isolation and Typing of Marine Bacteria

In order to gain a further insight into the relationship *Ulva* has with the bacterial community that colonises the algae, strains were selected that represented the *Ulva* thallus epiphytic population as identified by the 16S clone library, (see Section 3.2.1). Bacterial strains were isolated from the thallus of wild *Ulva* spp. colonising rocks at Wembury beach, Devon in September 2008 and from the holdfast of wild *Ulva* spp. colonising rocks at New Polzeath Beach, Cornwall in September 2008. A total number of 46 isolates were obtained. Some of these isolates possessed the same colony morphologies and were therefore grouped accordingly. From the 40 isolates with distinct colony morphology, representative strains were selected and characterised by phylogenetic typing of their 16S rDNA gene. The majority of the strains isolated directly from the thallus surface and holdfast of *Ulva* spp. belonged to the *Gammaproteobacteria*, however strains of *Alphaproteobacteria*, *Flavobacteria* and Gram-positive *Actinobacteria* were also identified (Table 3.2). Bacteria from the PML culture collection were also added to this collection, these strains were isolated from the rocks colonised by *Ulva* spp. at Wembury beach in January 2001. Strains obtained from the PML culture collection were also characterised by phylogenetic typing of their 16S rDNA to verify phylogeny and how they related to the *Ulva* thallus clone library.

Table 3.2. Phylogenetic identification of marine bacterial strains based on their 16S rDNA. Table listing the identity and phylogeny of strains taken from the PML culture collection (PML CC) and those isolated from *Ulva* thallus and holdfast tissue.

Strain	Identification	Phylogeny (Phylum, Class)	Genbank Accession Number	% Sequence Identity	*Ref*
I259b	*Rhodobacter* sp.	*Proteobacteria, Alphaproteobacteria*	N/A	N/A	PML CC
I243	*Rhodobacter* sp.	*Proteobacteria, Alphaproteobacteria*	N/A	N/A	PML CC
I371a	*Sulfitobacter* sp. KMUT3	*Proteobacteria, Alphaproteobacteria*	AB583769	98%	PML CC
I376	*Sulfitobacter* sp. KMUT 3	*Proteobacteria, Alphaproteobacteria*	AB583769	99%	PML CC
P4	*Roseobacter denitrificans* NBRC15277	*Proteobacteria, Alphaproteobacteria*	DQ915623	99%	PML CC
E4	*Shewanella* sp. SCSA3	*Proteobacteria, Gammaproteobacteria*	AM884345	97%	PML CC
483	*Sulfitobacter* sp. KMUT3	*Proteobacteria, Alphaproteobacteria*	AB583769	98%	PML CC
P13	*Favobacterium* sp. BSw21403	*Bacteroidetes, Flavobacteria*	FJ748511	98%	PML CC
UI01	*Pseudoalteromonas* sp. DJLY29	*Proteobacteria, Gammaproteobacteria*	EU169492	99%	This Study
UI08	*Cellulophaga* sp. BSw21403	*Bacteroidetes, Flavobacteria*	FJ748511	98%	This Study
UI09	*Winogradskyella eximia* KMM	*Bacteroidetes, Flavobacteria*	AY521225	98%	This Study
UI18	*Rhodobacteraceae* bacterium ROS8	*Proteobacteria, Alphaproteobacteria*	AY841782	99%	This Study
UI13	*Cellulophaga* sp. RE2-13	*Bacteroidetes, Flavobacteria*	AF539758	98%	This Study
UI36	*Aeromicrobium* sp DR8	*Actinobacteria, Actinobacteria*	FJ464983	98%	This Study
UI20	*Pseudomonas* sp. LD12	*Proteobacteria, Gammaproteobacteria*	AM913883	99%	This Study
UI11	*Pseudoalteromonas* sp. 8	*Proteobacteria, Gammaproteobacteria*	DQ642811	99%	This Study
UI19	*Alteromonas* sp. MA112	*Proteobacteria, Gammaproteobacteria*	AB491743	99%	This Study
UI33	*Marinobacter* sp. SCSWD16	*Proteobacteria, Gammaproteobacteria*	FJ461454	99%	This Study
UI39	*Marinobacter* sp. H96B3	*Proteobacteria, Gammaproteobacteria*	FJ746575	99%	This Study
UI22	*Halomonas* sp. NAH1	*Proteobacteria, Gammaproteobacteria*	EU239362	99%	This Study
UI12	*Marinobacter flavimaris* CJHH25	*Proteobacteria, Gammaproteobacteria*	EU169559	99%	This Study
UI04	*Polaribacter* sp. CS05	*Proteobacteria, Gammaproteobacteria*	EU477168	99%	This Study
UI32	*Marinobacter* sp. QJWW107	*Proteobacteria, Gammaproteobacteria*	FJ384492	99%	This Study
RUBI01	*Kocuria rhizophila* ES_145con	*Actinobacteria, Actinobacteria*	EU934094	99%	This Study
RUBI02	*Vibrio rumoiensis* LAR3	*Proteobacteria, Gammaproteobacteria*	DQ530292	99%	This Study
RUBI03	*Paracoccus* sp. jx9	*Proteobacteria, Alphaproteobacteria*	FJ539115	100%	This Study
RUBI04	*Kocuria* sp. BBN2C-02d	*Actinobacteria, Actinobacteria*	FJ357623	99%	This Study
RUBI05	*Vibrio rumoiensis* LAR03	*Proteobacteria, Gammaproteobacteria*	DQ530292	98%	This Study
RUBI06	*Micrococcus* sp. LZXC21	*Actinobacteria, Actinobacteria*	DQ659067	99%	This Study

3.2.4 QS Signal Molecule Characterisation using AHL Bio-reporters

The Gram-negative strains isolated from the *Ulva* thallus and the *Ulva* holdfast rock interface in this study were assayed for AHL signal molecule production using AHL bio-reporter strains. The *lux*-based *E. coli* JM109 bio-reporters harbouring pSB536 for the detection of C4-HSL, pSB401 for the detection of C6-HSL and C8-HSL and pSB1142 for the detection of C10-HSL and C12-HSL preferentially were used (Winson *et al.*, 1998; Swift *et al.*, 1997). The initial method employed to assay signal molecule production involved streaking the test strain against the bio-reporter on agar plates composed of two separate culture media, one capable of supporting the bio-reporter and the second capable of supporting the test strain. This method proved to be inconclusive for all strains assayed with the exception of those isolated from the holdfast rock interface. Of the six strains isolated from this interface one, RUBI03 (*Paracoccus* sp.) showed activation of the JM109 pSB401 bio-reporter, indicating the production of either C6-HSL or C8-HSL.

As the T-streak method proved inconclusive for strains isolated from the *Ulva* thallus a 96 well plate assay was adopted to screen acidified cell free supernatant extracts obtained from these strains for AHL production using the *E. coli* AHL bio-reporters listed previously. Table 3.3 lists the strains assayed using the 96 plate well technique and shows which bio-reporters were activated by each strain. In the majority of strains where activation of bio-reporters was seen, pSB536 was found to be the most prevalently activated reporter. As JM019 pSB536 is activated by the short chain C4-HSL, activation of this bio-reporter by cell free supernatant extracts indicates production of this signal molecule. Strain UI19 (*Alteromonas* sp.), RUBI03

(*Paracoccus* sp.), 483 (*Sulfitobacter* sp.) and E4 (*Shewanella* sp.) showed activation of the pSB401 bio-reporter indicating the production of either C6-HSL or C8-HSL.

Table 3.3. Table listing marine bacterial strains assayed for AHL production via 96 well plate method. Strains activating the bio-reporter suggesting AHL production are marked with a **+**, Strains marked with **-** denotes no activation of the bio-reporter.

Strain	**Identity**	**Bio-reporter Activation**		
		pSB536	**pSB401**	**pSB1075**
UI01	*Pseudoalteromonas* sp.	-	-	-
UI08	*Cellulophaga* sp.	+	-	-
UI09	*Winogradskyella eximia*	-	-	-
UI18	*Rhodobacteraceae* bacterium	-	-	-
UI13	*Cellulophaga* sp.	+	-	-
UI20	*Pseudomonas* sp.	+	-	-
UI11	*Pseudoalteromonas* sp.	-	-	-
UI19	*Alteromonas* sp	+	+	-
UI39	*Marinobacter* sp.	-	-	-
UI33	*Marinobacter* sp.	+	-	-
UI22	*Halomonas* sp.	+	-	-
UI12	*Marinobacter flavimaris*	-	-	-
UI04	*Polaribacter* sp.	+	-	-
UI32	*Marinobacter* sp.	-	-	-
RUBI02	*Vibrio rumoiensis*	-	-	-
RUBI03	*Paracoccus* sp.	+	+	-
RUBI06	*Vibrio rumoiensis*	-	-	-
E4	*Shewanella* sp.	+	-	+
483	*Sulfitobacter* sp.	-	+	-

3.2.5 QS Signal Molecule Characterisation using LCMS

The screening of marine bacteria directly isolated from *Ulva* for signal molecule production using bio-reporters showed that a number of strains were producing AHLs. This method of screening however did not provide data relating to specific AHL production for each strain. In order to gain more insight into the AHLs produced by bacteria that colonise *Ulva*, Liquid Chromatography coupled with Mass Spectroscopy (LCMS) was utilised. Acidified cell free supernatant extracts of strains isolated from *Ulva* and strains selected from the PML culture collection that were present in the *Ulva* clone library were analysed for the presence of individual AHLs using non quantitative LCMS. This detects un-substituted, 3-oxy-substituted and 3-hydroxyl-substituted homoserine lactones with fatty acid side chains ranging from 4 to 14 carbons in length. Extracts from acidified sterile marine broth and methanol were used as negative controls (Table 3.4). The results of the LCMS screen confirmed the AHL profiles which had previously been seen using bio-reporter based assays: bacteria associated with *Ulva* produce a wide range of AHL signal molecules. Particularly prevalent were C4-HSL and AHLs with fatty acid side chains 8 carbons in length, both un-substituted and substituted with either 3-oxy or 3-hydroxyl groups. Examples of compound mass spectra showing 3-oxo-C8-HSL in extracts of I376 (*Sulfitobacter* sp.) and C4-HSL in UI20 (*Pseudomonas* sp.) are displayed in Figure 3.7.

Table 3.4. Table showing AHLs produced by strains representative of *Ulva*'s epiphytic bacterial community as identified by non-quantitative LCMS analysis. Boxes in the table shaded grey denotes production of that AHL by the strain.

<table>
<tr><th>Strain</th><th>Identity</th><th colspan="6">AHL</th></tr>
<tr><td></td><td></td><th colspan="6">Un-substituted Series (X-HSL)</th></tr>
<tr><td></td><td></td><th>C4</th><th>C6</th><th>C8</th><th>C10</th><th>C12</th><th>C14</th></tr>
<tr><td>RUBI03</td><td>Parracoccus sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td style="background:grey"></td></tr>
<tr><td>UI20</td><td>Pseudomonas sp.</td><td style="background:grey"></td><td></td><td style="background:grey"></td><td></td><td></td><td></td></tr>
<tr><td>UI19</td><td>Alteromonas sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI33</td><td>Marinobacter sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>I371a</td><td>Sulphitobacter sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>243</td><td>Roseobacter sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>I376</td><td>Sulfitobacter sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>P13</td><td>Flavobacterium sp.</td><td style="background:grey"></td><td style="background:grey"></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI08</td><td>Cellulophaga sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI13</td><td>Cellulophaga sp.</td><td style="background:grey"></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>438</td><td>Roseobacter sp.</td><td style="background:grey"></td><td style="background:grey"></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><th colspan="6">3-oxo Group Series (3-oxo-X-HSL)</th></tr>
<tr><td></td><td></td><th>C4</th><th>C6</th><th>C8</th><th>C10</th><th>C12</th><th>C14</th></tr>
<tr><td>RUBI03</td><td>Parracoccus sp.</td><td></td><td></td><td style="background:grey"></td><td></td><td></td><td></td></tr>
<tr><td>UI20</td><td>Pseudomonas sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI19</td><td>Alteromonas sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI33</td><td>Marinobacter sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>I371a</td><td>Sulphitobacter sp.</td><td></td><td></td><td style="background:grey"></td><td></td><td style="background:grey"></td><td></td></tr>
<tr><td>243</td><td>Roseobacter sp.</td><td></td><td></td><td style="background:grey"></td><td></td><td></td><td></td></tr>
<tr><td>I376</td><td>Sulfitobacter sp.</td><td></td><td></td><td style="background:grey"></td><td></td><td></td><td></td></tr>
<tr><td>P13</td><td>Flavobacterium sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI08</td><td>Cellulophaga sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI13</td><td>Cellulophaga sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>438</td><td>Roseobacter sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><th colspan="6">3-hydroxyl Group Series (3-OH-X-HSL)</th></tr>
<tr><td></td><td></td><th>C4</th><th>C6</th><th>C8</th><th>C10</th><th>C12</th><th>C14</th></tr>
<tr><td>RUBI03</td><td>Parracoccus sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI20</td><td>Pseudomonas sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI19</td><td>Alteromonas sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI33</td><td>Marinobacter sp.</td><td></td><td></td><td style="background:grey"></td><td></td><td></td><td></td></tr>
<tr><td>I371a</td><td>Sulphitobacter sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>243</td><td>Roseobacter sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>I376</td><td>Sulfitobacter sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>P13</td><td>Flavobacterium sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI08</td><td>Cellulophaga sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>UI13</td><td>Cellulophaga sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>438</td><td>Roseobacter sp.</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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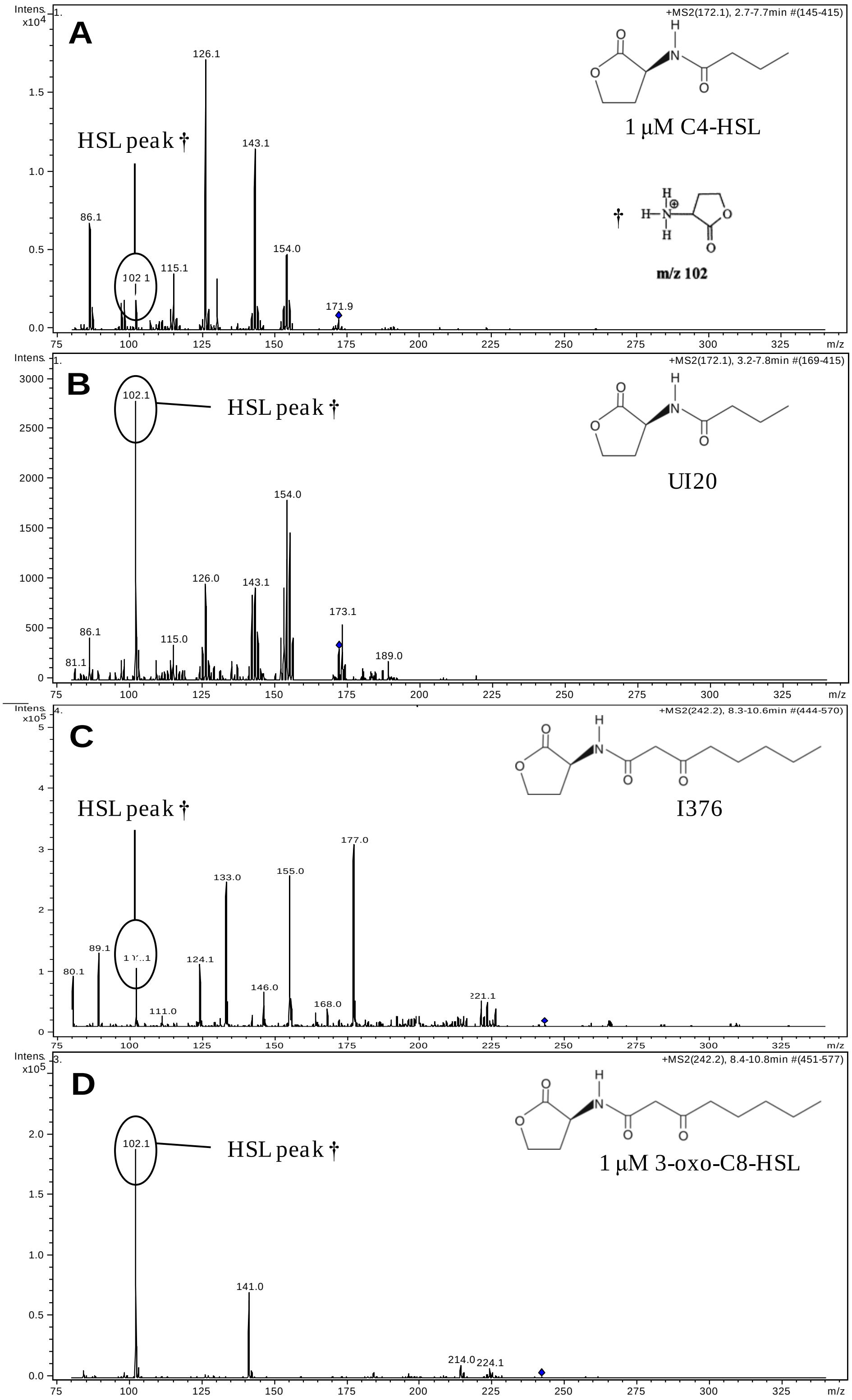


Figure 3.7. Screening for AHL production in marine bacterial isolates via LCMS. Examples of product ion spectra from the breakdown of nominal precursor ion of m/z 172 (C4-HSL), 1 µM C4-HSL standard (A), UI20 (*Pseudomonas* sp.) (B) and of nominal precursor ion m/z 172 (C4-HSL), I376 (*Sulfitobacter* sp.) and 1 µM 3-oxo-C8-HSL standard (D). The peak representing in daughter ion m/z 102.1 (homoserine lactone) highlighted in all spectra.

In addition to assaying for AHL production the same strains were assayed for the production of AQ type signal molecules using non-quantitative LCMS. The AQs assayed were the PQS type quinolones with alkyl chains of 7, 9 and 11 carbons; HHQ type quinolones with alkyl chains of 7, 9 and 11 carbons and N-oxide AHQs with alkyl chains of 7 and 9 carbons. LCMS analysis failed to show any strong evidence of AQ production in the marine bacterial stains assayed (data not shown).

3.2.6 **Ulva** *spp. Zoospore Germination Response when Exposed to* **V. anguillarium** *Biofilms*

V. anguillarium produces 3-oxo-C10-HSL, C6-HSL and 3-hydroxy-C6-HSL directed by the AHL synthase homologues *vanI* and *vanM*, (Milton *et al.*, 2001; Milton *et al.*, 1997), and was utilised in the initial experiments exploring the relationship between AHL-producing bacteria and *Ulva* zoospore settlement (Tait *et al.*, 2005; Joint *et al.*, 2002). *V. anguillarium* is, therefore, a viable model organism to investigate the effect of AHL-producing biofilms on *Ulva* zoospore germination. As the amount of AHLs produced depends on biofilm density, the effect on *Ulva* germination on biofilms of varying density was compared. Repeated observations after 72 h incubation, using length equal to or greater than 15 µm to define a successfully germinated zoospore, *Ulva* spp. germlings grown on AHL expressing *V. anguillarium* wt biofilms had reduced average lengths when compared to *Ulva* spp. germlings grown on biofilms composed of a *V. anguillarum* Δ*vanI/M* mutant (Figure 3.8). As the zoospores were incubated on the *V. anguillarium* biofilms for 72 h prior to being fixed and stained for image analysis, 100% of the settled *Ulva* spp. zoospores had germinated and in many cases it was difficult to select and measure individual *Ulva* germlings as the biofilm slides were crowded with germinated zoospores. As a result of this observation it was decided to reduce the zoospore incubation time to 48 h and add a sample point at 24 h for future experiments, in order to better assay the period of zoospore germination.

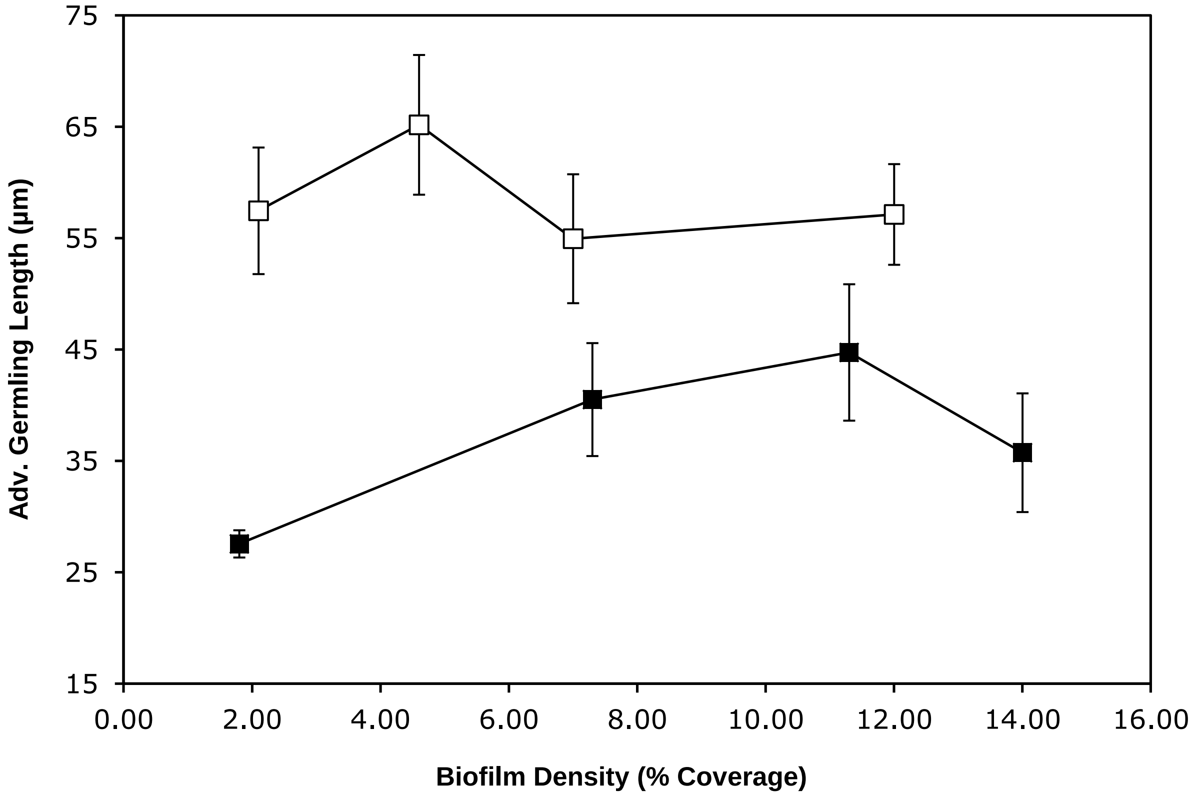


Figure 3.8. Effect of *V. anguillarum* biofilms on *Ulva* zoospore germination. Average length of *Ulva* spp. germlings grown on biofilms composed of *V. anguillarum* wt (■) and *V. anguillarium* Δ*vanI/M* (□) of varying bacterial density. Error bars represent 95% confidence intervals.

3.2.7 Ulva *Zoospore Germination Response when Exposed to Biofilms Composed of Marine Bacteria Indigenous to* Ulva's *Bacterial Population*

Although the germling growth on biofilms of *V. anguillarium* wt was reduced when compared to growth on biofilms of the *V. anguillarium* Δ*vanI/M* mutant, this strain is not known to be associated with the mature algae. Similar experiments were therefore conducted with bacteria isolated from the *Ulva* thallus. The 16S clone library of bacteria associated with *Ulva* spp. showed both *Sulfitobacter* spp. (an alphaproteobacterial species) and Uncultured *Alteromonadales* to be highly abundant within the population (Section 3.2.1). On the basis of these findings two strains; 483 and E4 were selected for repeated experiments investigating zoospore response to *Ulva*'s indigenous bacteria. Strain 483 is a strain of *Sulfitobacter* and E4 a strain of *Shewanella*, a member of the *Alteromonadale* order. Strain 483 produces C8-HSL and E4 produces C4-HSL and C8-HSL as shown by activations of AHL bio-reporters pSB536 and pSB401, (Table 3.3). Both 483 and E4 were transformed with the AHL lactonase *aiiA* from *Bacillus sp.* 240B1, cloned into the broad host vector pBBRIMCS-1 (pMT01). Successful transformation with the pMT01 plasmid rendered cognate AHLs biologically inert as *aiiA* hydrolyses the homoserine lactone ring. This was confirmed as both 483 pMT01 and E4 pMT01 failed to activate *lux*-based AHL bio-reporters pSB536 and pSB401 (data not shown). Biofilms of strains 483 and E4 transformed with empty pBBRIMCS-1 vector which produced AHLs and 483 and E4 transformed with pMT01 were grown from a range of starting inoculums on glass cover slides. *Ulva* zoospores were settled onto the biofilms, incubated as described previously and sampled at 24 h and 48 h.

The varying volumes of inoculate used to grow biofilms of *Shewanella* sp. E4 and *Sulfitobacter* sp. 483 produced biofilms with bacterial densities ranging from approximately 35 to 75% coverage, with no major difference in growth observed between strains carrying the pBBRIMCS-1 or pMT01 plasmid.

Repeated observations showed that at the 24 h sample point there was little observable difference in average germling length when zoospores were exposed to the AHL-producing *Shewanella* sp. E4 pBBRIMCS-1 biofilms in comparison to biofilms composed of *Shewanella* sp. E4 pMT01 which did not produce AHLs (Figure 3.9). However, at higher biofilm densities average germling length was increased on *Shewanella* sp. E4 pMT01 in comparison to *Shewanella* sp. E4 pBBRIMCS-1 (Figure 3.9). In contrast, the percentage of zoospores that had germinated at the 24 h sample point was increased on biofilms composed of *Shewanella* sp. E4 pMT01 (Table 3.5). At the 48 h sample point, average germling length was greatly increased on biofilms composed of *Shewanella* sp. E4 pMT01 in comparison to *Shewanella* sp. E4 pBBRIMCS-1 biofilms (Figure 3.9). As at 24 h, percentage germination of zoospores was increased on *Shewanella* sp. E4 pMT01 biofilms in comparison to *Shewanella* sp. E4 pBBRIMCS-1 biofilms at the 48 h sample point (Table 3.5).

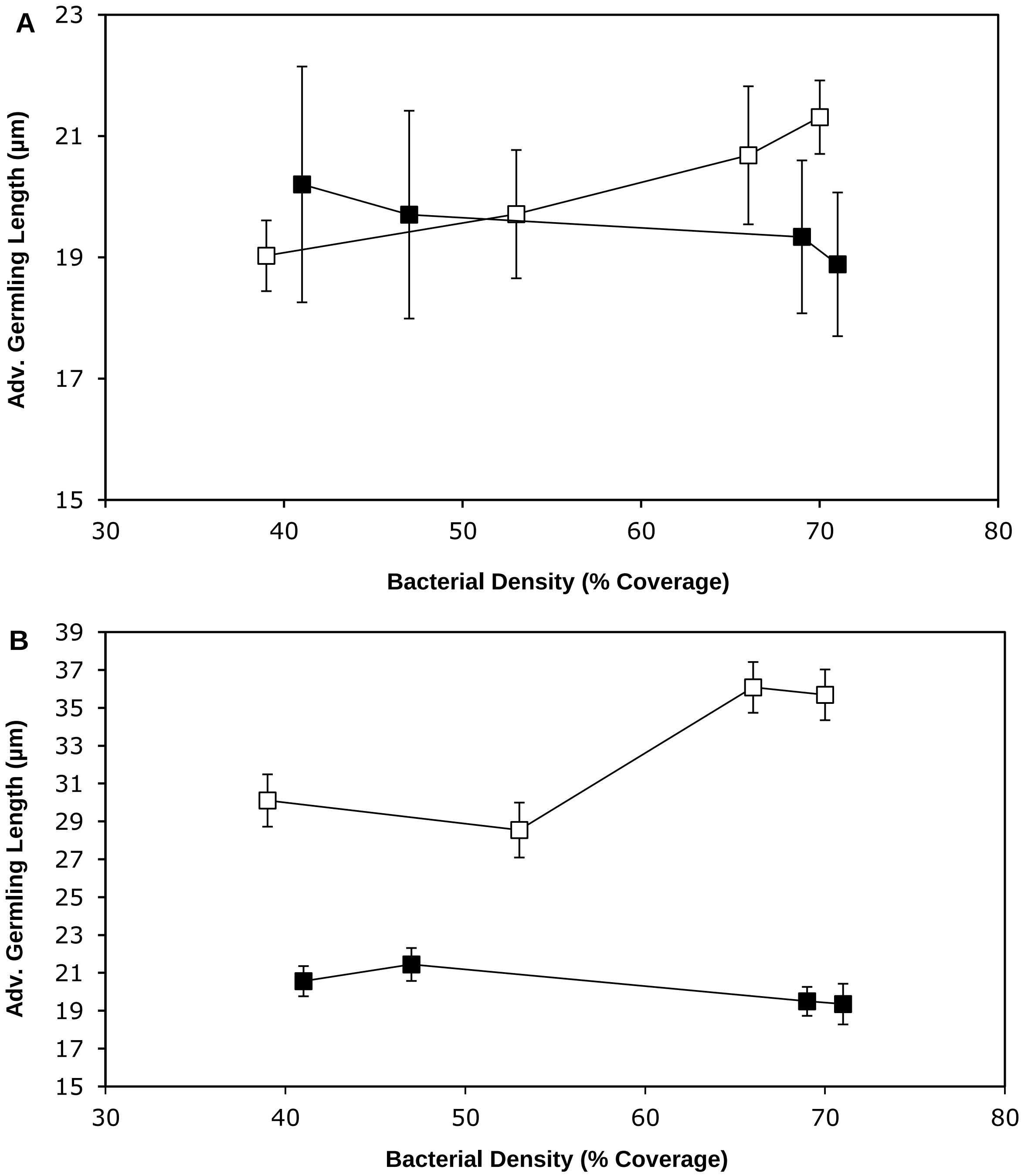


Figure 3.9. Effect of *Shewanella* biofilms on *Ulva* zoospore germling growth. Average germling length of zoospores exposed to biofilms composed of AHL-producing *Shewanella* sp. E4 pBBRIMCS-1 (■) and non AHL-producing *Shewanella* sp. E4 pMT01 (□) at 24 h incubation (**A**) and 48 h incubation (**B**). Error bars represent 95% confidence intervals.

Table 3.5. ***Ulva*** **zoospore percentage germination in the presence of** ***Shewanella*** **biofilms.** Table comparing percentage of *Ulva* zoospores that germinated when exposed to biofilms of increasing inoculate volume composed of non AHL-producing *Shewanella* sp. E4 pMT01 and AHL-producing *Shewanella* sp. E4 pBBRIMCS (E4 wt) at both 24 h and 48 h incubation.

		Percentage *Ulva* Zoospore Germination (24 h)		**Percentage *Ulva* Zoospore Germination (48 h)**	
	Strain	**E4 pMT01**	**E4 wt**	**E4 pMT01**	**E4 wt**
Inoculum Volume (ml)	**0.5**	48	10	76	56
	1	27	9	77	65
	2	44	13	87	51
	4	73	18	87	47

Repeated observations also showed that at both 24 h and 48 h sample points average germling length was increased on biofilms composed of non AHL-producing *Sulfitobacter* sp. 483 pMT01 in comparison to AHL-producing *Sulfitobacter* sp. 483 pBBRIMCS-1 biofilms (Figure 3.10). This pattern was also observed with regards to percentage germination of *Ulva* zoospores with germination being greater on *Sulfitobacter* sp. 483 pMT01 biofilms in comparison to *Sulfitobacter* sp. 483 pBBRIMCS-1 biofilms at both 24 h and 48 h sample points (Table 3.6).

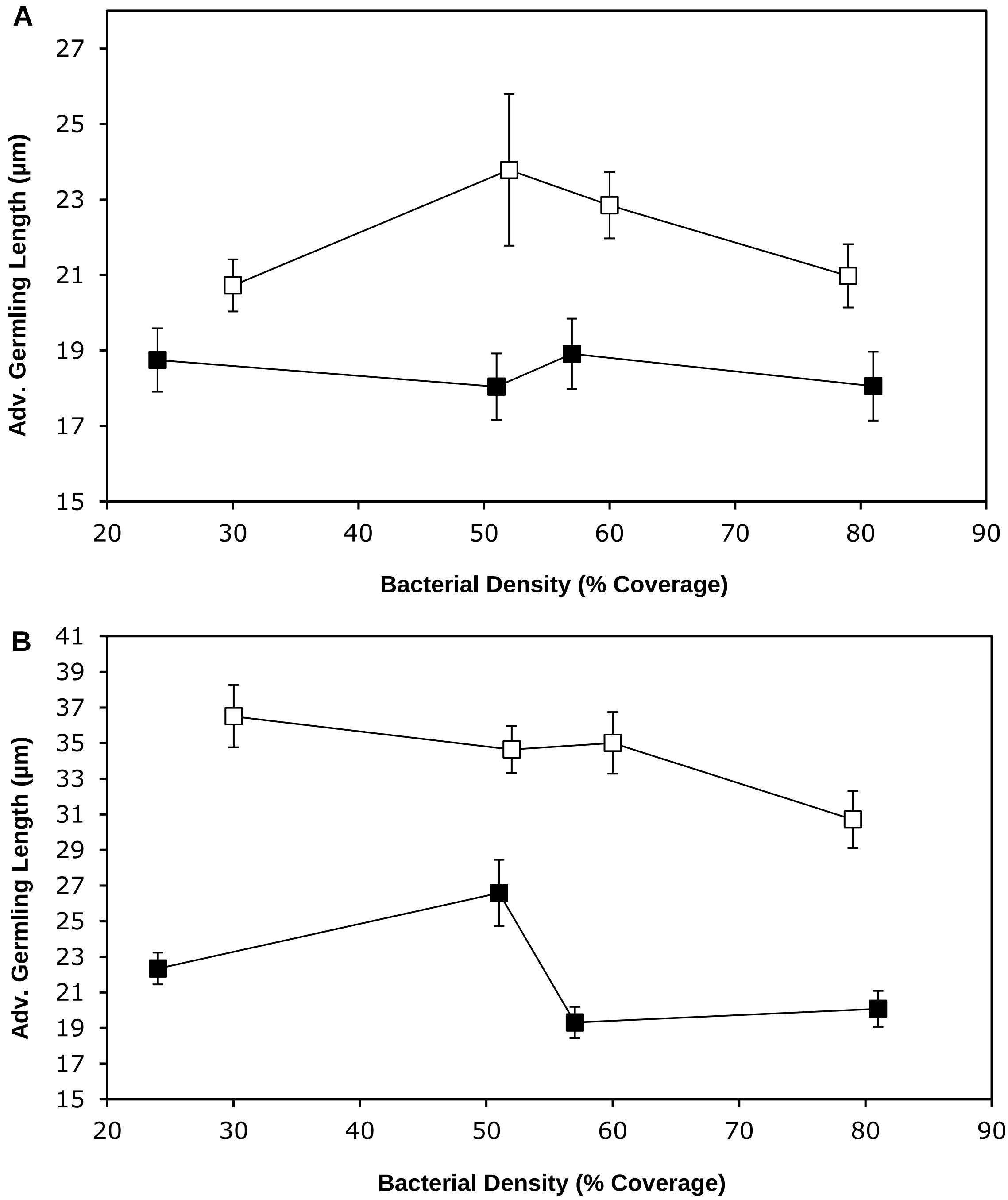


Figure 3.10. Effect of *Sulfitobacter* biofilms on *Ulva* zoospore germling growth. Average germling length of zoospores exposed to biofilms composed of AHL-producing *Sulfitobacter* sp. 483 pBBRIMCS-1 (■) and non AHL-producing *Sulfitobacter* sp. 483 pM101 (□) at 24 h incubation (**A**) and 48 h incubation (**B**). Error bars represent 95% confidence intervals.

Table 3.6. ***Ulva*** **zoospore percentage germination in the presence of** ***Sulfitobacter*** **biofilms.** Table comparing percentage of *Ulva* zoospores that germinated when exposed to biofilms of increasing inoculate volume composed of non AHL-producing *Sulfitobacter* sp. 483 pMT01 and AHL-producing *Sulfitobacter* sp. 483 pBBRIMCS (483 wt) at both 24 h and 48 h incubation.

		Percentage *Ulva* Zoospore Germination (24 h)		**Percentage *Ulva* Zoospore Germination (48 h)**	
	Strain	**483 pMT01**	**483 wt**	**483 pMT01**	**483 wt**
Inoculum Volume (ml)	**0.5**	57	21	80	75
	1	35	18	85	60
	2	64	17	80	60
	4	56	14	76	61

3.2.8 Ulva *Zoospore Germination Response when Exposed to Transgenic* E. coli *Biofilms*

The introduction of AiiA into *Shewanella* sp. E4 and *Sulfitobacter* sp. 483 may have altered phenotypes other than AHL production that may have affected zoospore germination. In order to overcome this problem and to expand the observations of the relationship between AHLs and *Ulva* zoospore germination seen with exposure to biofilms of indigenous marine bacteria, *Ulva* zoospore germination was assayed using biofilms composed of transgenic *E. coli* strains which expressed various recombinant AHL synthase genes. These AHL synthases were *rhlI* from *P. aeruginosa* (directing the production of C4-HSL), *luxI* from *V. fischeri* (directing the production of 3-oxo-C6-HSL) and *vanI* from *V. anguillarum* (directing the production of 3-oxo-C10-HSL) (Hanzelka *et al.*, 1997; Milton *et al.*, 1997; Parsek *et al.*, 1997). For each transgenic *E. coli* strain, zoospore germination was compared to *E. coli* possessing vector plasmids without recombinant AHL synthase homologues. These strains provided a constant supply of AHLs, facilitating a more stable assay for monitoring the response of *Ulva* zoospores to AHLs. Owing to the detrimental effect of the osmotic pressure of seawater on the growth and survival of *E. coli* biofilms, zoospore slides were incubated for a reduced time of 24 h in 70% sterile filtered seawater prior to being fixed and stained. At 24 h incubation, there was a small yet significant decrease in average germling length when *Ulva* zoospores were settled and grown on biofilms composed of *E. coli* expressing *rhlI* and *vanI*, (one-way ANOVA $P < 0.01$). Although the response to *E. coli* expressing *luxI* was not found to be significant, (one-way ANOVA $P > 0.01$), a modest decrease in the presence of this biofilm was also apparent (Figure 3.11). Marked reductions in the percentage of germinated *Ulva* zoospores were however observed on biofilms of all AHL expressing transgenic *E. coli* strains at 24 h, 33%, 19% and 22% respectively in the *rhlI*, *luxI* and *vanI* expressing stains in comparison to 78% 73% and 60% on the biofilms of the respective AHL deficient vector control stains (Table 3.7).

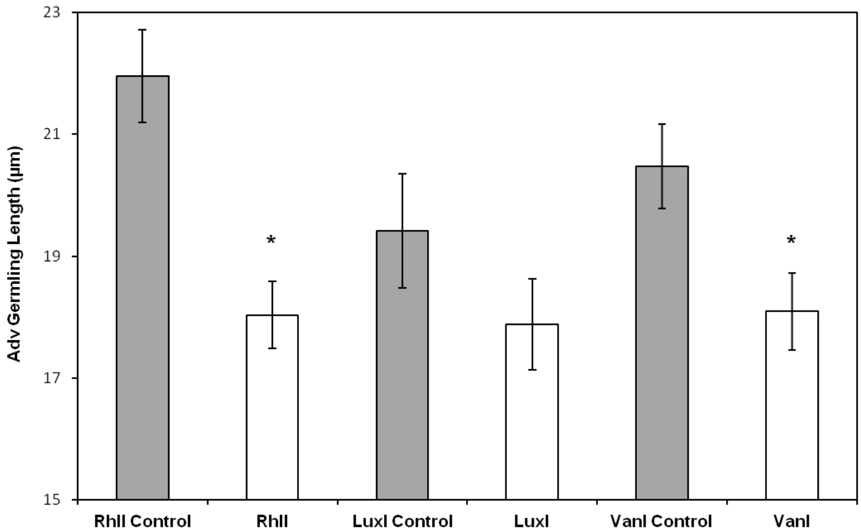


Figure 3.11. Effect of transgenic *E. coli* biofilms on *Ulva* germling growth. Average *Ulva* germling length when exposed to transgenic *E. coli* biofilms expressing recombinant AHL synthase homologues in comparison to biofilms composed of non AHL-producing *E. coli* controls at 24 h incubation. Error bars represent 95% confidence intervals, * marks values significantly different to controls (one-way ANOVA $P < 0.01$).

Table 3.7 *Ulva* zoospore percentage germination in the presence of transgenic *E. coli* biofilms. Table comparing percentage of *Ulva* zoospores that germinated when exposed to biofilms composed of AHL synthase expressing *E. coli* and wt *E. coli* controls at 24 incubation.

	AHL Expressing	**Control**
***rhlI* (C4-HSL)**	33	78
***luxI* (3-oxo-C6-HSL)**	19	73
***vanI* (3-oxo-C10-HSL)**	22	60

3.2.9 Ulva *Zoospore Germination in the Presence of Synthetic AHLs*

Previously, increased *Ulva* zoospore settlement has been seen on agarose films containing synthetic AHLs with fatty acid side chains ranging from 6 to 14 carbons in length (Tait *et al.*, 2005). In addition, many of the bacteria associated with *Ulva* have been shown to produce a wide range of signalling molecules. Having identified AHL-producing biofilms to inhibit the early growth of *Ulva* germlings, this study investigated the relationship between *Ulva* zoospore germination and synthetic AHL signalling molecules. *Ulva* zoospores were first settled onto sterile glass surfaces, exposed to set concentrations of exogenous C4-HSL, C6-HSL, C8-HSL, C10-HSL and C12-HSL and incubated for a period of 24 h and 48 h under the condition described in section 3.2.6.

Under all experimental conditions both the average length of *Ulva* germlings and percentage of germination of *Ulva* zoospores was greater after 48 h incubation compared to 24 h incubation. Two-way PERMANOVA analysis to determine the effect of AHL concentration and incubation time on germling length revealed a significant effect of AHL concentration for all AHLs assayed ($P < 0.01$). However, germling length in different AHL concentrations was not the same after 24 h and 48 h of incubation ($P > 0.01$). For clarity, only the results for the 48 h time point are displayed here, however the results from the 24 h time point are discussed within the main text and can be found in Appendix 3.

At the 24 h sample point there was a significant decrease in average germling length at 0.5 µM C4-HSL in comparison to when no exogenous C4-HSL was added (one-way ANOVA $P < 0.01$). However, no significant difference in average germling length was observed at any other C4-HSL concentration in comparison to when no exogenous AHL was added (one-way ANOVA $P > 0.01$). At the 48 h sample point there was a significant reduction in average germling length at a C4-HSL concentration of 5 µM in comparison to when no exogenous C4-HSL is presen, (one-way ANOVA $P < 0.01$) (Figure 3.12). However, there was no significant difference in average germling length at 0.5 μM and 50 μM C4-HSL in comparison to when no exogenous AHL was added (one-way ANOVA $P > 0.01$). (Figure 3.12).

At the 24 h sample point there was a significant decrease in average germling length in the presence of C6-HSL at 5 μM in comparison to when no exogenous C6-HSL was added (one-way ANOVA $P < 0.01$). No significant differences in germling length observed at any other C6-HSL concentration in comparrsion to when no exogenous C6-HSL was present (one-way ANOVA $P > 0.01$). At the 48 h sample point there was a significant reduction in average germling length in the presence of 50 μM C6-HSL compared to when no exogenous C6-HSL was present (one-way ANOVA $P < 0.05$) (Figure 3.12). No significant difference in average germling length was seen at 0.5 μM and 5 μM C6-HSL in comparrsion to when no exogenous C6-HSL was present (one-way ANOVA $P > 0.01$) (Figure 3.12).

At the 24 h sample point there was a significant increase in average germling length in the presence of 50 μM C8-HSL in comparison to when no exogenous C8-HSL was present (one-way ANOVA $P < 0.01$). However, no significant differences in germling length were observed at any other C8-HSL concentration in comparrsion to when no exogenous C8-HSL was present (one-way ANOVA $P > 0.01$). At the 48 h sample point there was also a significant increase in average zoospore length in the presence of 50 μM C8-HSL (one-way ANOVA $P < 0.01$) (Figure 3.12). No significant difference in average germling length was seen at 0.5 μM and 5 μM C8-HSL in comparrsion to when no exogenous C8-HSL was present (one-way ANOVA $P > 0.01$) (Figure 3.12).

At the 24 h sample point there was no significant difference in average germling length in the presence of C10-HSL at any concentration compared to when no exogenous C10-HSL was present (one-way ANOVA $P > 0.01$). At the 48 h sample point there was a significant decrease in average germling length at 50 µM C10-HSL compared to when no exogenous C10-HSL was present, however there was an increase in germling length at 5 µM (one-way ANOVA $P < 0.01$) (Figure 3.12). No significant difference in average germling length was seen at 0.5 µM C10-HSL compared to when no exogenous C10-HSL was present (one-way ANOVA $P > 0.01$) (Figure 3.12).

Repeated observations at the 24 h sample point showed average germling length was significantly increased at 0.5 µM C12-HSL and significantly decreased at 50 µM C12-HSL in comparison to where no exogenous C12-HSL was present (one-way ANOVA $P < 0.01$). At the 48 h sample point there was a significant decrease in average germling length at 50 µM C12-HSL in comparison to where no exogenous C12-HSL was present (one-way ANOVA $P < 0.01$) (Figure 3.12).

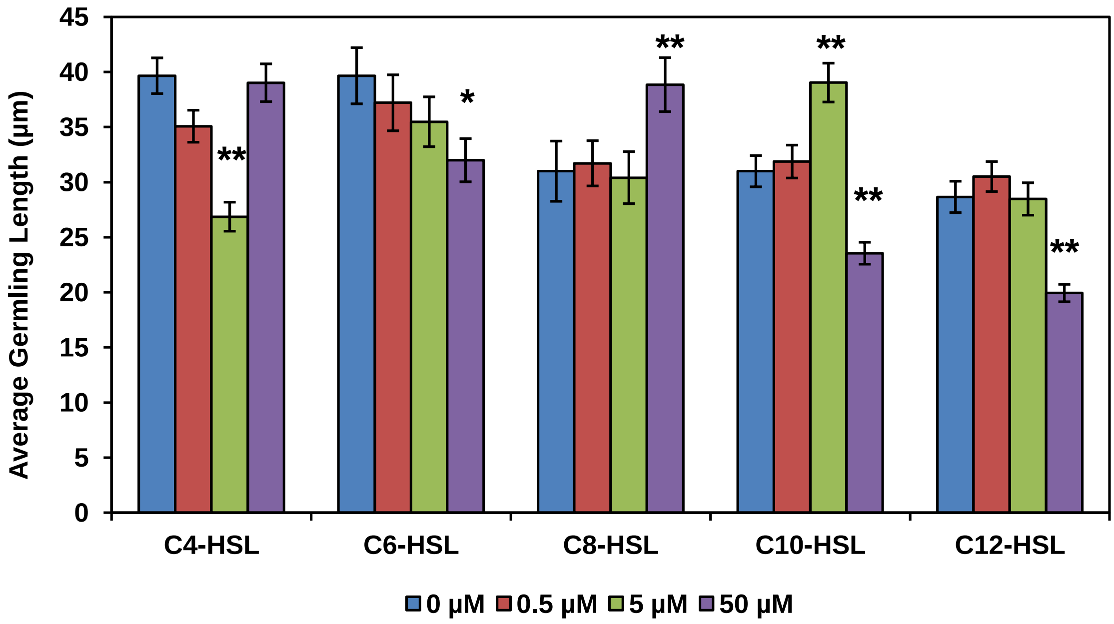


Figure 3.12. Effect of exogenously added synthetic AHLs on *Ulva* germling growth. Average *Ulva* germling length when exposed to set concentrations of various AHLs at 48 h incubation. Error bars represent 95% confidence intervals and asterisks show those values that differed significantly from that of 0 µM AHL (one-way ANOVA * = $P < 0.05$, ** = $p < 0.01$).

As with *Ulva* zoospore germination assays using bacterial biofilms the percentage germination of zoospore was recorded at the 24 h and 48 h sample points in the presence of all synthetic AHLs assayed. At the 24 h sample point, percentage zoospore germination was reduced at 0.5 µM C4-HSL and 50 µM C4-HSL in comparison to when no C4-HSL was present (18%, 20% and 34% respectively). However it was increased at 5 µM C4-HSL (45%) in comparison to when no C4-HSL was present (34%) (Table 3.8). At the 48 h sample point, percentage germination of *Ulva* zoospores was reduced when exposed to C4-HSL at all concentrations assayed with the greatest reduction in percentage germination at 5 µM C4-HSL in comparison to when no C4-HSL was present (61% and 86% respectively) (Table 3.8).

At the 24 h sample point percentage zoospore germination was reduced in the presence of C6-HSL compared to when no exogenous C6-HSL was present, particularly at 0.5 μM and 50 μM C6-HSL where percentage germination was 11% and 14% respectively compared to 35% at 0 μM C6-HSL (Table 3.8). As with the 24 h sample the percentage zoospore germination at the 48 h sample point was also reduced when exogenous C6-HSL was present, with the greatest reduction seen at 5 μM C6-HSL in comparison to when no C6-HSL was present (78% and 86% respectively) (Table 3.8).

At both the 24 h and 48 h sample points the percentage zoospore germination was increased when exogenous C8-HSL was present in comparison to when no exogenous C8-HSL was present (Table 3.8). The exception to this trend was where zoospore germination was recoreded in the presence of 5 μM C8-HSL at the 48 h sample point where percentage germination was slightly reduced comparison to when no C8-HSL was present (78% and 80% respectively) (Table 3.8).

At the 24 h sample point there was an increase in percentage zoospore germination at 0.5 μM and 5 μM C10-HSL compared to when no exogenous C10-HSL was present (28%, 23% and 21% respectively). However, at 50 μM C10-HSL percentage germination was reduced compared to when no exogenous C10-HSL was present (17% and 21% respectively) (Table 3.8). At the 48 h sample point percentage germination was increased at 0.5 μM and 5 μM C10-HSL with a substantial fall in germination at 50 μM C10-HSL of 64% compared to 80% when no exogenous C10-HSL was present (Table 3.8).

At the 24 h sample point percentage zoospore germination was notably reduced at 50 µM C12-HSL; 17% germination at 50 µM C12-HSL compared to 64% germination at 0 µM C12-HSL (Table 3.8). At 48 h, increased percentage zoospore germination was seen at 0.5 µM C12-HSL, germination was then substantially reduced below the level seen when no exogenous C12-HSL was present. At C12-HSL concentrations of 5 µM and 50 µM, 70% and 46% respectively compared to 80% when no C12-HSL was present (Table 3.8).

Table 3.8. *Ulva* zoospore percentage germination in the presence of exogenously added synthetic AHLs. Table showing the percentage *Ulva* zoospore germination in the presence of set concentrations of various AHLs at both 24 h and 48 h incubation.

	Percentage *Ulva* Zoospore Germination (24 h)				**Percentage *Ulva* Zoospore Germination (48 h)**			
AHL Conc. (µM)	**0**	**0.5**	**5**	**50**	**0**	**0.5**	**5**	**50**
C4-HSL	34	18	45	20	86	82	61	84
C6-HSL	35	11	30	14	86	85	78	80
C8-HSL	21	38	35	38	80	81	78	88
C10-HSL	21	28	23	17	80	80	89	64
C12-HSL	64	78	72	17	69	83	79	46

As C12-HSL was the most stable synthetic AHL in seawater due its increased acyl side chain, experiments assayed for effect on *Ulva* zoospore germination were repeated using C12-HSL concentrations ranging between 5 µM and 50 µM. With the notable exception of at 20 µM, no significant difference in average germling length was observed at any concentration of C12-HSL in comparison to when no exogenous C12-HSL was present at the 24 h sample point (one-way ANOVA $P > 0.001$) (data not shown). However, at the 48 h sample point average germling length was significantly reduced when C12-HSL was present at all concentrations assayed compared to when no exogenous C12-HSL was present (one-way ANOVA $P < 0.001$) (Figure 3.13).

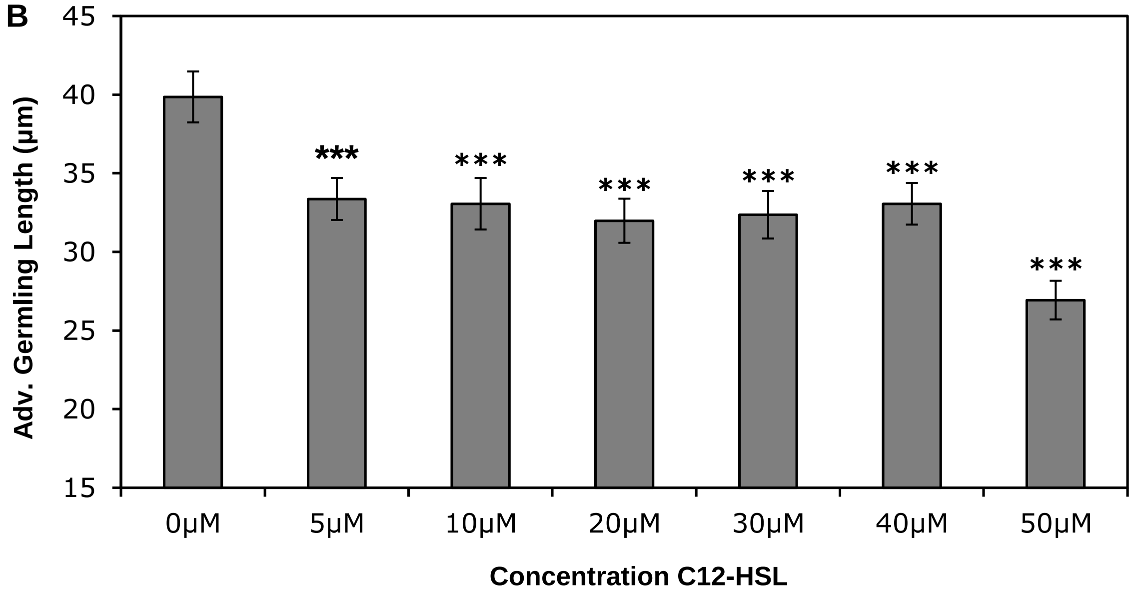


Figure 3.13. Effect of exogenously added synthetic C12-HSL on *Ulva* germling growth. Average *Ulva* germling length when exposed to a range of C12-HSL concentrations at 48 h incubation. Error bars represent 95% confidence intervals and asterisks show those values that differed significantly from that of 0 µM AHL (one-way ANOVA =$P < 0.001$).

The percentage of germinated *Ulva* zoospores was also recorded for each concentration of C12-HSL at both the 24 h and 48 h sample points. At the 24 h sample point the percentage of germinated zoospores was reduced at all C12-HSL concentrations, with the exception of 40 µM (39%), compared to when no exogenous C12-HSL was present (35%) (Table 3.9). At the 48 h sample point percentage germination was reduced in the presence of C12-HSL at all concentrations in comparison to when no exogenous AHL was present; particularly at 50 µM C12-HSL where zoospore germination was at 65% in comparison to 86% when no exogenous C12-HSL was present (Table 3.9).

Table 3.9. *Ulva* zoospore percentage germination in the presence of exogenously added synthetic C12-HSL. Table showing the percentage *Ulva* zoospore germination in the presence of set concentrations of C12-HSL at both 24 h and 48 h incubation.

C12-HSL Conc. (μM)	Percentage *Ulva* Zoospore Germination (24 h)	Percentage *Ulva* Zoospore Germination (48 h)
0	35	86
5	29	78
10	27	71
20	23	77
30	25	78
40	39	80
50	27	65

3.3. Discussion

3.3.1 *The Bacterial Population Associated with* Ulva

This study has identified the epiphytic bacterial community residing on the surface of the macroalgae *Ulva* that was found growing in the intertidal rocky shore environment of southwest region of the UK coastline. The population present on the surface of the *Ulva* thallus is composed of bacteria from the phyla *Proteobacteria* and *Bacteroidetes*. The dominant classes of bacteria are *Alphaproteobacteria, Gammaproteobacteria*, *Flavobacteria* and *Sphingobacteria*. The bacterial population present on the *Ulva* thallus was found to be highly similar to the population present within biofilms found on the rocks colonised by *Ulva* (Tait *et al.*, 2009). The same dominant groups were represented in both populations; however, the relative abundance of each class differed between the populations seen in the two environments.

The epiphytic population present on *Ulva*, identified by this study was also remarkable similar to the epiphytic bacterial population present on the surface of *Ulva australis*, an antipodeanian member of the *Ulvacean* family (Burke *et al.*, 2011). Using a large number of 16S rRNA clones Burke *et al.* (2011) showed the *U. australis* population to be composed of *Proteobacteria* and *Bacteroidetes* at the phylum level, dominated by *Alphaproteobacteria*, *Gammaproteobacteria* and *Flavobacteria*. At the species level this study showed a significantly large amount of variability between clone libraries from different individual *U. astralis* samples collected in the same area (Burke *et al.*, 2011). This dominance of *Proteobacteria* and *Bacteroidetes* observed by both this study and the study into the epiphytic population of *U. australis* is replicated in the epiphytic communities present on other algal species such as *Laminaria* *hyperborean*, *Laminaria saccharinam*, (Bengtsson *et al.*, 2010, Staufenberger *et al.*, 2008) and endophytic communities of *Bryopsis* sp. (Hollants *et al.*, 2011)

The *Ulva* thallus population identified by this study was also similar to the bacterial population observed in the water column of the Western English Channel; however as with the rocky shore population (Tait *et al.*, 2009), the water column population shows greater bacterial diversity and an increased range of bacterial phyla (Gilbert *et al.*, 2009). Variability between the *Ulva* epiphytic population and the surrounding seawater bacterial population was also observed in *U. australis* (Burke *et al.*, 2011). The similarities between the *Ulva* thallus population and the rocky shore and water column populations suggests *Ulva* acquires its bacterial population from the rocks on which it colonises and the seawater to which it is exposed during the tidal cycle. However, the difference in three populations supports a hypothesis that some bacteria are better adapted for epiphytic growth on a biotic surface. The main difference between the *Ulva* thallus population and the rocky shore was that the relative abundance of the *Sphingobacteria* class of *Bacteroidetes* is much higher in the rocky shore population. The sphingobacterial population in both the *Ulva* thallus and rocky shore populations is in turn much greater than that found in the water column population. The difference between Sphingobacterial abundance in the *Ulva* thallus, the rocky shore and the water column is expected as costal environments tend to possess a higher abundance of *Bacteroidetes* in comparison with the open ocean (Alonso *et al.*, 2007). Additionally, *Sphingobacteria* are also commonly found in areas of reduced salinity such as the intertidal zone (Alonso *et al.*, 2007). Another factor that would explain the difference between the *Ulva* thallus population and that of the seawater is the increased nutrient availability present on the *Ulva* thallus; algal species exude a number of nutrients such as organic carbon compounds that bolster microbial growth compared to the open ocean environment which has limited nutrient availability (Pregnall, 1983). A surprising result was the difference in abundance of *Sphingobacteria* seen in the *Ulva* thallus population compared to that of the rocky shore population. This difference may be due to *Sphingobacteria* being better adapted to local environmental conditions present on the rocks, or more likely, due to unaccounted differences in environmental conditions present at the location and time of sampling such as local wheather activity and/or nutrient levels in the seawater. One such condition, that could affect *Bacteroidetes* populations, is the occurrence of a phytoplankton bloom as it has been reported that *Bacteroidetes* populations are increased at the time of such blooms (Pinhassi *et al.*, 2004).

Microbial communities are highly influenced by the seasonal changes that take place in the marine environment. This has been comprehensively demonstrated by phylogenetic, meta-genomic and metabolomic studies conducted over broad time periods at the L4 sampling site in the Western English Channel which show significant changes in bacterial populations depending on the time of sampling (Gilbert *et al.*, 2010; Gilbert *et al.*, 2009). The bacterial community on the surface of *Ulva* has also been shown to have a high degree of temporal variability (Tujula *et al.*, 2010). Density Gradient Gel Electrophoresis (DGGE) studies analysing the *U. australis* epiphytic population over one year identified up to 40% seasonal variability, this study also puts forward the case for *Ulva* spp. possessing a core bacterial population which remains unchanging throughout the year. This hypothesis was however discredited by 16S rRNA phylogenetic analysis of the *U. australis* community (Burke *et al.*, 2011; Tujula *et al.*, 2010). In addition, *Ulva* itself undergoes drastic growth changes throughout the year with biomass and production being at the lowest during the winter period (Maggs and Callow, 2001). Therefore, the bacterial population on *Ulva* spp. will alter in response to these conditions. Seasonal differences in the bacterial community in response to the changing growth cycle of algae has been demonstrated in the brown algal species *Laminaria hyperborean* and *Laminaria saccharina* (Bengtsson *et al.*, 2010; Staufenberger *et al.*, 2008). The bacterial population identified in this study can only be attributed to the time of year when sampling was carried out. In order to gain a more comprehensive understanding of the *Ulva* bacterial population, sampling and phylogenetic analysis must be conducted throughout the course of *Ulva*'s annual growth cycle.

3.3.2 Signal Molecule Production by Marine Bacteria Representative of the **Ulva** *Bacterial Population*

Through various detection methods this study has shown that many of the bacteria isolated from the *Ulva* thallus and/or representing the *Ulva* bacterial population are actively producing a range of AHL signal molecules, (see section 3.2.4 and 3.2.5). AHL production by bacterial species isolated from the marine environment and specifically from *Ulva* has been reported previously (Tait *et al.*, 2009; Mohamed *et al.*, 2008; Gram *et al.*, 2002). Initially AHL detection assays were conducted via both T streak and plate based methods using bio-reporters to detect the presence of AHLs (Winson *et al.*, 1998; McClean *et al.*, 1997). In general the T streak method proved inconclusive; potentially due to abiotic disruption of the homoserine lactone ring via hydrolysis under the increased pH of marine broth, abolishing the biological activity of the AHL (Hmelo and Van Mooy, 2009; Yates *et al.*, 2002). The original T streak method did however produce one clear result. Isolate RUBI03, identified as a strain belonging to the genus *Paracoccus* was shown to induce bioluminescence in the bio-reporter *E. coli* pSB401 suggesting that its cognate AHLs possess fatty acid side chains 6 to 8 carbons in length, later confirmed via LCMS analysis. These data are intriguing given that Steindler and Venturi (2007) showed that *Paracoccus* spp. produced long chain AHLs (Steindler and Venturi, 2007). Assaying dichloromethane extractions from acidified culture supernatants identified AHL production by a wider range of bacteria isolated from *Ulva*. Performing signal molecule analysis via LCMS to characterise AHLs being produced by different strains isolated from *Ulva* and strains representative of the *Ulva* population proved far more beneficial than both T streak and plate based detection assays as it unequivocally revealed which AHLs were being produced by each strain assayed. LCMS analysis showed a wide range of signal molecules being produced by the marine bacteria isolated from the *Ulva* thallus. It was interesting to note that the AHLs being produced by the strains isolated from *Ulva* were, in general, found to be producing short to mid range sized fatty acid side chains and not the longer side chains which are more stable in sea water and predominantly show increased *Ulva* zoospore settlement (Tait *et al.*, 2005). The predominant AHLs identified to be present in rocky shore biofilms were C8-HSL and C10-HSL: these molecules, along with the longer chain C12-HSL and C14-HSL were also seen to be produced by bacteria isolated from the rocky shore (Tait *et al.*, 2009). Although both bacteria isolated from the rocky shore and bacteria isolated from the *Ulva* thallus surface show a wide range of AHL production, the *Ulva* thallus isolates tend to produce AHLs with shorter fatty acid side chains. The production of AHLs with short to medium length fatty acid side chains differs from other studies that have investigated AHL production in bacterial strains, isolated from a variety of marine environments, which show a predominant bias to long chain AHL production (Wagner-Döbler *et al.*, 2005; Taylor *et al.*, 2004; Gram *et al.*, 2002).

3.3.3 *Effect of AHLs on* Ulva *Zoospore Germination*

A relationship between *Ulva* and bacterial signalling molecules has been established with regards to *Ulva* zoospore settlement, with *Ulva* zoospores being shown to preferentially settle on bacterial biofilms which produce AHLs and on agarose slides permeated with synthetic AHLs (Tait *et al.*, 2005; Joint *et al.*, 2002). Preferential settlement of zoospores was also shown to be abolished either by the hydrolysis of AHLs due to the alkaline pH of sea water, the actions of AHL-inactivating enzymes such as the lactonase *aiiA* and by AHL inactivation from other bacteria such as *Shewanella* spp. (Tait *et al.*, 2009; Tait *et al.*, 2005). The method by which *Ulva* zoospores sense AHLs remains uncertain although it has been shown that in response to an AHL source *Ulva* zoospore motility is reduced, therefore allowing an accumulation of zoospores at the AHL source promoting increased settlement (Wheeler *et al.*, 2006).

In addition to affecting *Ulva* zoospore settlement bacteria have also been shown to affect the growth of *Ulva* spp. and other marine algae (Matsuo *et al.*, 2003). Axenic cultures of the green alga *M. oxyspermun* and *U. linza* lack the ability to differentiate naturally. Adding cognate bacterial isolates back to axenic *U. linza* cultures restores wild type morphology; additionally five isolates were shown to increase the growth rate of the plant (Marshall *et al.*, 2006; Matsuo *et al.*, 2003). Based on the reported effects bacteria have on the growth and morphology of *Ulva* and on the attraction effects AHLs have on *Ulva* zoospore settlement the prediction that synthetic AHLs, and bacteria activity producing AHLs, would increase *Ulva* zoospore germination was a reasonable assumption. However, this study has shown that *Ulva* zoospore germination and the early growth of the *Ulva* germling is reduced when AHLs are present. The effect of AHLs on *Ulva* zoospore germination was initially observed when exposing germinating *Ulva* zoospores to varying concentrations of exogenously added synthetic AHLs. High concentrations of C6-HSL, C10-HSL and C12-HSL significantly reduced both the percentage zoospore germination and the length of *Ulva* zoospore germlings in comparison to when no exogenous AHL was added. Increased consistency in observations of the effect of AHLs on both percentage zoospore germination and average *Ulva* germling length, was observed when exposing zoospores to high concentrations of C12-HSL. In addition, exposing zoospores to concentrations ranging between 5 μM and 50 μM of C12-HSL also affected percentage zoospore germination and germling length, with germling growth and percentage germination both in general reduced in the presence of C12-HSL in comparison to when no exogenous C12-HSL was present. The increased consistency seen when assaying zoospore germination in response to long chain AHLs can be explained by the fact that AHLs are unstable in seawater and therefore have a short time period of being biologically active. The longer the AHLs fatty acid side chain the more resistant the AHL is to being deactivated by the increased pH of seawater (Hmelo and Van Mooy, 2009). Interestingly, the presence of high concentrations of C8-HSL appeared to have the opposite effect and increase *Ulva* germling length. This contradictory result may suggest that *Ulva* zoospores exhibit a differential response to various AHLs, further study using C8-HSL and the 3-oxo and 3-hydroxyl AHL derivatives would be required to confirm this speculation. It is also of interest to note that shorter chain AHLs, shown by this study to be produced by *Ulva*'s indigenous bacterial population, had a reduced effect on *Ulva* zoospore germling length when added exogenously, however the longer chain AHLs which are generally reported to be produced by many marine bacteria had a clear effect, reducing *Ulva* zoospore germling length. This difference in the effect on *Ulva* germination between long chain and short chain AHLs may however be due to the high rate of turnover of C4-HSL attributed to relatively high pH of seawater during the experiment as observed in previous studies (Tait *et al.*, 2005). To address this, experiments using RhlI-expressing *E. coli* showed a significant decrease in *Ulva* germling length. In this experiment the biofilm would have been constantly producing C4-HSL which would replace the hydrolysed signal in contrast with the experiment using exogenously added C4-HSL where replacement of hydrolysed signal did not occur.

Exposing *Ulva* zoospores to biofilms composed of bacteria which were producing AHLs had the effect of reducing both the percentage of zoospores which germinated and the average length of *Ulva* germlings, suggesting a reduced growth rate when compared to bacterial biofilms lacking the ability to produce AHLs. Reduced *Ulva* germling growth and percentage germination in the presence of AHL-producing biofilms was observed with *V. anguillarium*, *E. coli* producing AHLs from recombinant AHL synthases and bacteria indigenous to *Ulva*'s population. As previously discussed, biofilms composed of signalling bacteria are producing a continuous source of AHLs therefore maintaining an *in situ* AHL concentration gradient increasing the reliability of experimental observations of zoospore germination over a 48 h period. Inhibition of algal growth and spore germination by bacteria has been previously reported (Dobretsov *et al.*, 2006). A primary example of marine bacteria which effects spore germination is the gammaproteobacterial species *Pseudoalteromonas tunicate*, which produces an extracellular component that prevented spore germination in both *U.*

lactuca and the red alga *Polysiphonia* spp. (Egan *et al.*, 2001). *P. tunicate* has been identified as coloniser of *Ulva* and this study also isolated *Pseudoalteromonas* from the surface of *Ulva* (Rao *et al.*, 2006). Additionally a number of bacteria isolated from the marine environment, including a *Vibrio* isolate have been shown to disrupt *Ulva* zoospore germination (Bernbom *et al.*, 2011).

Experimental evidence showing AHLs have a reducing effect on *Ulva* zoospore germination and the early growth of the *Ulva* germling presents a misnomer; why do AHLs promote zoospore settlement but reduce zoospore germination and growth? This study and studies by Tait *et al.* have demonstrated that *Ulva* zoospores and germlings are exposed to a variety of AHL-producing bacteria in the natural environment (Tait *et al.*, 2009; Tait *et al.*, 2005). Therefore, this study speculates that the observation of reduced zoospore germination and germling growth when *Ulva* is exposed to AHLs and AHL-producing bacteria reflects what takes place in the natural environment as the plant is always going to be exposed to AHL-producing bacteria. The observation that *Ulva* zoospore germination and growth is greater in the absence of AHLs is most likely an artefact of the experiments conducted by this study as *Ulva* is unlikely to experience such conditions in the intertidal rocky shore environment. However, in the natural environment fluctuations in the local concentrations of AHLs due to the production of AHL inactivating enzymes by marine bacteria may also have an effect on the germination of the *Ulva* zoospore and early germling growth. Previous studies have shown *Ulva* requires a healthy epiphytic bacterial community to form wild type growth morphology (Marshall *et al.*, 2006; Matsuo *et al.*, 2003). This study therefore hypothesises that *Ulva* uses the AHL signalling by marine bacteria as a cue to reduce early growth in order to become sufficiently colonised by bacteria present in rocky shore biofilms. The observation by this study that *Ulva* appears to obtain its epiphytic bacterial community from the biofilms present on the rocky shore lends further credence to this theory. Although this study has clearly shown that AHLs affect not only *Ulva* zoospore settlement but also *Ulva* germination, further research is needed to prove the hypothesis that reduced *Ulva* growth due AHL cues promotes successful colonisation of *Ulva*. The positive effects with regards to the AHL-producing bacteria of increased *Ulva* zoospore settlement and reduced zoospore growth are unknown, therefore the relationship between *Ulva* and bacteria cannot currently be considered as mutualistic. The negative effect of AHLs on *Ulva* germination could potentially be exploited by industry as an antifouling treatment, as *Ulva* related biofouling presents a significant burden to the marine economy.

3.3.4 Summary Conclusions and Future Directions

This study has identified the epiphytic bacterial community and species richness present on the thallus of a mature *Ulva* plant and used these data to select strains for further research. AHL profiling of these selected strains showed bacteria associated with *Ulva* produce a range of AHL signalling molecules. Additionally, the AHL-mediated interaction between *Ulva* and bacteria has been shown not just to effect *Ulva* zoospore settlement but also *Ulva* germination and early growth of the *Ulva* germling. In conclusion, it is likely from the phylogenetic study of the *Ulva* bacterial community that horizontal transmission of bacteria takes place between the rocky shore biofilms and the *Ulva* plants colonising those rocks. The finding that AHLs reduce zoospore germination and growth is significant as it adds further complexity to the relationship *Ulva* has with the bacteria present in the marine environment. Further experimentation is required to investigate why AHLs have such a negative effect on zoospore germination and growth if we are to truly understand this relationship.

Research in this area should progress in the following directions:

- Identification of the mechanism by which AHLs effect *Ulva* zoospore settlement, germination and germling growth. Previous studies have implicated AHL mediation of calcium efflux to control settlement (Joint *et al.,* 2007). This work could act as a starting point to identify AHL-mediated alterations in *Ulva* zoospores or *Ulva* germlings.
- Carrying out comparative protein/RNA expression profiles of *Ulva* zoospores/germlings exposed to and deprived of AHLs, may also identify where AHL signalling molecules are acting, mediating the behavioural alterations observed by this study and previous work by Tait *et al.*
- Another area of research would be to identify the levels of AQ production in the environment colonised by *Ulva*. Initial work by this study failed to identify AQ production in marine bacteria associated with *Ulva*, however a targeted LCMS approach may reveal AQ production omitted by this study.
- If AQ signalling molecules are present in the environment colonised by *Ulva,* investigation of their effects on *Ulva* zoospore settlement, germination and germling growth could be conducted. However, preliminary data not presented in this thesis exposing *Ulva* zoospores to AQ producing and non-producing *P. aeruginosa* biofilms did not show any significant differences in germination or germling growth. This work would need repeating to rule out any effect of AQs on the *Ulva* growth cycle.

Chapter 4

AHL Production and Inactivation in Marine Bacteria Indigenous to the *Ulva* Epiphytic Community

4.1 Introduction

The green seaweed *Ulva* possesses an indigenous bacterial population growing in biofilms on the surface of the thallus and the holdfast. This community appears to originate from the rocks colonised by *Ulva* spp. and the seawater it is exposed to during the tidal cycle. Bacteria from the *Altermonadaceae* family, to which *Shewanella* belongs and from the phylum *Bacteroidetes*, are found in high abundance within this community. These groups of bacteria are also abundant in the biofilms found on rocks in the intertidal zone and in the general marine environment.

4.1.1 The Genus **Shewanella**

Shewanella spp. were first isolated from spoiled butter in 1931 and identified as *Achromobacter putrefaciens*, (Derby and Hammer, 1931). However, after much reclassification the genus *Shewanella* was created in 1985, owing to phylogenetic work from 5S rRNA sequence data. The genus was named after the microbiologist who initially isolated the bacterium, Dr James Shewan (MacDonell and Colwell, 1985). *Shewanella* bacteria are Gram-negative, rod shaped and lack the ability to form spores. *Shewanella* cells are in general 2-3 μm in length, 0.4-0.7 μm in diameter and possess a single polar flagellum conferring swimming motility (Venkateswaran *et al.*, 1999). On agar, *Shewanella* colonies appear smooth and circular with regular edges. Many *Shewanella* species appear to have a pinkish colouration when grown on agar or in liquid broth. Two species of *Shewanella* have be shown to be bioluminescent; *Shewanella hanedai* and an isolate from the Alboran Sea which was designated *Shewanella woodyi* (Makemson *et al.*, 1997). These species were shown to possess *lux* genes homologous to the *V. fischeri lux* operon, potentially acquired via horizontal transfer (Urbanczyk *et al.*, 2008; Makemson *et al.*, 1997).

To date there are currently 56 typed species of *Shewanella* (NCBI Taxonomy). The phylogenetic relationship between 39 different species based on 16S rDNA sequencing is shown in Figure 4.1. *Shewanella* bacteria have been isolated from a variety of different environments and are widely distributed in both marine and fresh water (reviewed by Hau and Gralnick, 2007). *Shewanella* spp. can grow at a range of different temperatures, however most *Shewanella* strains display optimal growth at 16-30^{o}C, as a result they have a wide ranging environmental distribution (reviewed by Hau and Gralnick, 2007). A number of species such as *Shewanella frigidimarina* and *Shewanella livingstonensis* have been proven to be either psychrotolerant or psychrophillic, growing at temperatures below 5^{o}C (Bozal *et al.*, 2002; Brown and Bowman, 2001). In addition to growth at low temperature, *Shewanella* spp. such as *Shewanella benthica*, *Shewanella violacea* and *Shewanella piezotolerans* have been proven to be barophillic, growing at high pressures (Wang *et al.*, 2008; Kato and Nogi, 2001).

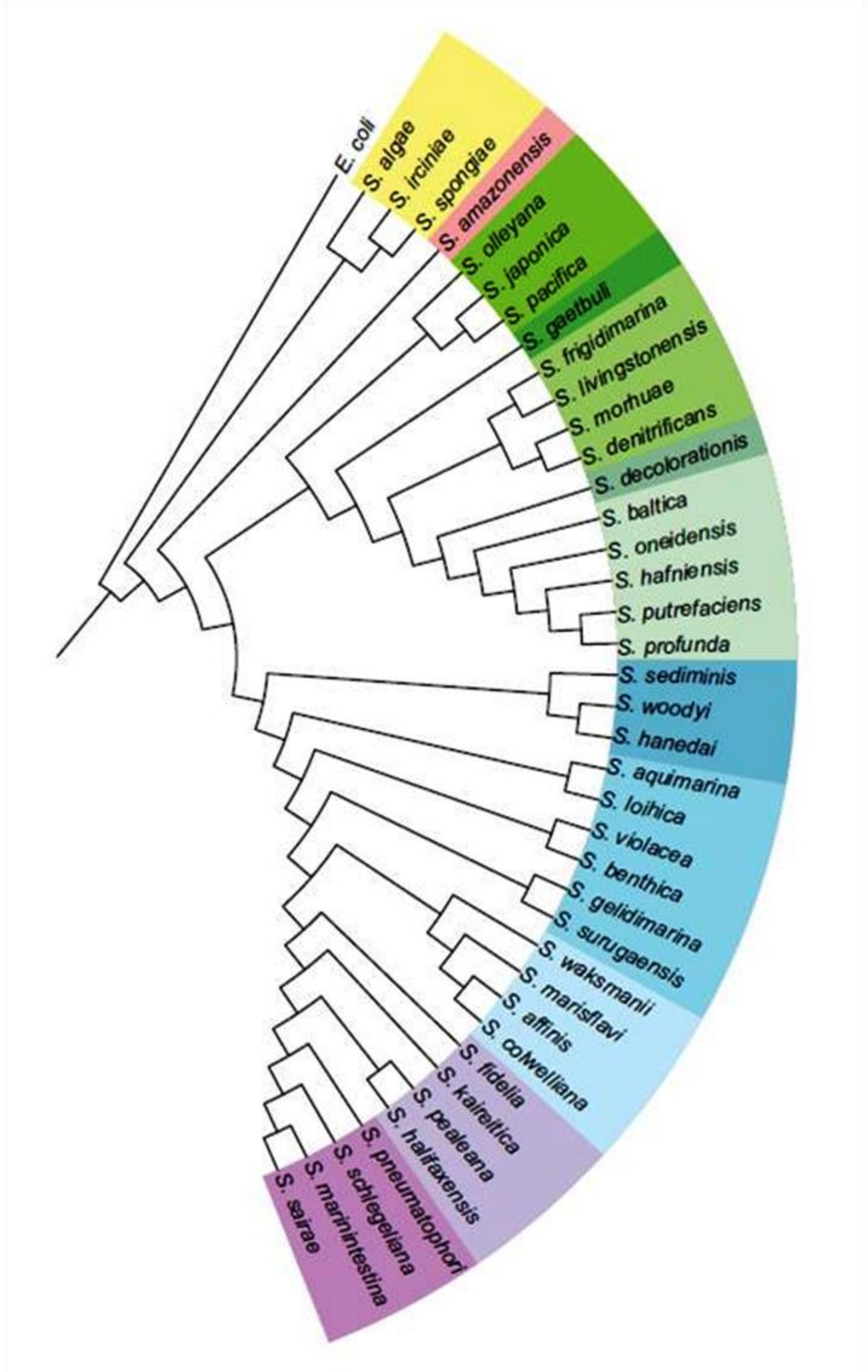


Figure 4.1. Phylogenetic analysis of 16S rDNA from 39 type-strain species of *Shewanella*. *Shewanella abyssi* was not considered in this analysis as obtaining 16S rDNA from this species is experimentally difficult. The different colour blocks represent *Shewanella* species isolated from similar environments (figure sourced from Hau and Gralnick, 2007).

Shewanella spp. are facultative anaerobes and it is their anaerobic metabolism which makes this genus of bacteria of interest to science: *Shewanella* spp. show a greater diversity in their ability to utilise compounds and elements as electron acceptors than any other group of bacteria (reviewed by Nealson and Scott, 2006). *Shewanella* spp. do not however have the ability to utilise complex organic carbon sources during metabolism and show a preference for lactate, pyruvate and simple amino acids (reviewed by Venkateswaran *et al.*, 1999; Scott and Nealson, 1994). When growing on lactate in an anaerobic environment, *Shewanella* spp. release CO_2 and acetate which can be utilised by other anaerobic bacteria such as methanogens. In turn, *Shewanella* spp. can themselves utilise many metabolic products produced as a result of fermentation by other anaerobic bacteria (Kato and Nogi, 2001).

Shewanella spp. have been shown to reduce more than 20 different electron acceptors and as such sequenced *Shewanella* genomes have been found to contain a large variety of cytochrome genes in comparison with other bacteria with similar genomes sizes (reviewed by Nealson and Scott, 2006). *Shewanella* spp. can use Oxygen, Nitrate, Sulphate and elemental sulphur as electron acceptors along with elemental metal oxides including Fe (III), Mn (IV), U (VI), Cr (VI), Tc (VII) and arsenate (reviewed by Nealson and Scott, 2006). Many enzymes involved in the reduction of metal oxides are found on the outer membrane of *Shewanella* bacteria (Beliaev *et al.*, 2001). Metal reduction by bacteria accounts for the majority of the carbon cycling in a number of different environmental niches, including the marine environment (Lovley and Phillips, 1988). In the central Baltic Sea the majority of the organic carbon cycling at the oxic/anoxic interface is due to the metabolic actions of *Shewanella baltica* (Ziemke *et al.*, 1997). *Shewanella* spp. are unique amongst aerobic bacteria as they have the ability to use elemental sulphur as an electron acceptor producing hydrogen sulphide, as such they can grow at environmental interfaces with sulphate-reducing bacteria such as those found in the Black Sea (Moser and Nealson, 1996).

There have been a number of proposals to exploit the metabolic diversity of *Shewanella* bacteria for the purposes of bioremediation, (the biotic decontamination of environments contaminated with organic and inorganic pollutants). Using *Shewanella* spp. to decontaminate land and ground water contaminated with uranium, technetium, cobalt and chromium have been experimented within a laboratory setting (Hau and Gralnick, 2007). In addition to using *Shewanella* spp. for the bioremediation of environments contaminated with inorganic metal pollutants, it has been proposed to use *Shewanella* spp., in concert with other bacteria, to decontaminate land contaminated with halogenated solvents, as *Shewanella* spp. possess the ability to utilise halogenated organic compounds as electron acceptors (Becker *et al.,* 2005; Picardal *et al.*, 1995).

A number of species of *Shewanella* have been shown to either be pathogenic or cause the spoilage of food. Human disease caused by *Shewanella* spp. are highly rare opportunistic infections, occurring mainly in geographic regions having warm climates and usually caused by exposure to contaminated seawater (Holt *et al.*, 2005). There have only been two species of *Shewanella* shown to be associated with human infection; *Shewanella algae* and *Shewanella putrifaciens* (Khashe and Janda, 1998). These species have been associated with acute ear infection, skin and soft tissue infection following trauma, acute eye infection and more seriously bacteraemia (Pagani *et al.*, 2003; Butt *et al.*, 1997; Holt *et al.*, 1997; Domínguez *et al.*, 1996). In addition to causing human infection *Shewanella* bacteria are often implicated in food spoilage.

Shewanella spp. are the dominant organisms found on stored fish products and account for the rotting fish smell due to the production of trimethylamine and hydrogen sulphide (Gram and Huss, 1996). Many of the species that cause food spoilage (i.e. *S. putrefaciens*) are able to utilise a wider number of carbon sources in comparison to other *Shewanella* species (Bowman *et al*., 2000).

4.1.2 AHL Production in **Shewanella**

AHL production was first characterised in a *Shewanella* spp. by Tait *et al*., 2009, in which it was demonstrated that a rock-pool isolate *Shewanella* sp. B21 produced 3-oxo-C4-HSL, 3-oxo-C10-HSL and 3-oxo-C12-HSL. AHL production was shown to take place during the late exponential growth phase; an unusual result, as AHL production is normally seen in stationary phase bacterial cultures. *Shewanella* biofilms were also shown to produce AHLs with long chain AHLs being produced during early biofilm growth and short chain AHL production taking place during the later stages of biofilm growth (Figure 4.2) (Tait *et al*., 2009). AHL production has also been observed in a *Shewanella* strain (MIB015) isolated from the intestinal tracts of fish (Morohoshi *et al*., 2008). AHL inhibition by *Shewanella* spp. during stationary phase has also been observed. Cell free supernatant extracts from cultures over 18 h old failed to activate AHL bio-reporters and disrupted AHL signalling by the Alphaproteobacterial strain *Sulfitobacter* sp. BR1 (Tait *et al*., 2009). The turnover of AHL signalling molecules by *Shewanella* spp. was additionally shown to detrimentally affect *Ulva* zoospore settlement (Tait *et al*., 2009).

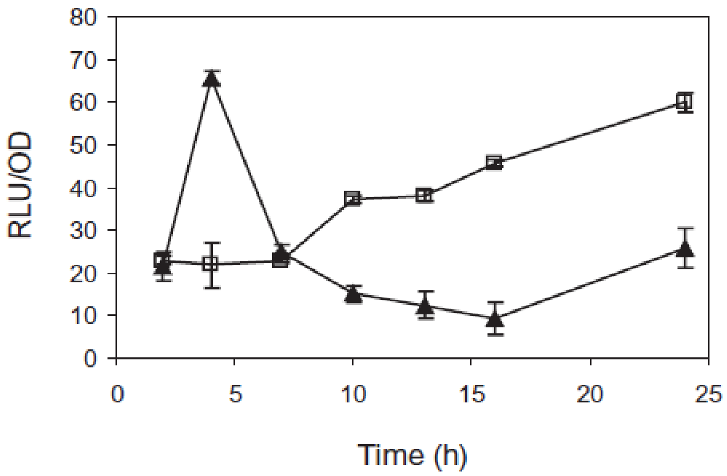


Figure 4.2. AHL production in *Shewanella* spp. biofilms. Luminescence as a function of growth (RLU/OD) was observed in the *lux*-based short chain AHL bio-reporter *E. coli* pSB536 upon exposure to extract from mature *Shewanella* spp. biofilms (□). In contrast, extract from early growth stage *Shewanella* spp. biofilms induced luminescence in the *lux*-based long chain AHL *E. coli* bio-reporter pSB1075 (▲) (figure sourced from Tait *et al.*, 2009).

4.1.3 The Phylum **Bacteroidetes**

Bacteria belonging to the phylum *Bacteroidetes* were first described by Winogradsky as non-spore forming Gram-negative mainly rod shaped bacteria with gliding motility (Kirchman, 2002). After a considerable amount of re-classification the current taxonomic position of the *Bacteroidetes* phylum was created via the consolidation of the *Cytophaga-Flavobacteria*/ *Flexibacter-Bacteroidetes* (CFB) division in 2002 (Garrity *et al.*, 2002; Paster *et al.*, 1985). Following the consolidation of these divisions the newly created *Bacteroidetes* phylum was taxonomically divided into four separate classes of bacteria; *Bacteroidia*, *Flavobacteria*, *Sphingobacteria* and *Cytophagia* (Krieg *et al.*, 2010). The foremost characterised class of *Bacteroidetes* are the *Bacteroidia* which consist mainly of strict anaerobic bacteria. Members of this class are highly dominant in bacterial populations colonising the human gastrointestinal tract

(Eckburg *et al.*, 2005). The *Bacterodia* species *Bacteroides fragilis* is known to be an opportunistic human pathogen causing anaerobic bacteraemia and acute diarrheal disease (Lassmann *et al.*, 2007; Myers *et al.*, 1984). Other *Bacteroidia* species such as *Prevotella intermeddia* are commonly found colonising the oral cavity and are associated with oral infections such as dental cares and periodontal abscesses (Tanaka *et al.*, 2008).

Of more interest to environmental microbiology are the *Bacteroidetes* classes *Flavobacteria*, *Cytophagia* and *Sphingobacteria*. These classes are broad groups of heterotrophic bacteria found colonising fresh water, salt water, soil and sediment environments (Martinez-Alonso *et al.*, 2010; Kormas *et al.*, 2006; Lydell *et al.*, 2004; Eilers *et al.*, 2001). *Flavobacteria* and *Sphingobacteria* have also been shown to be associated with marine phytoplankton and marine animals such as sponges (Grossart *et al.*, 2005; Webster *et al.*, 2001). Several Fluorescence *in situ* Hybridisation (FISH) studies of various open ocean water environments and costal bacterial plankton populations have shown that *Bacteroidetes* bacteria are the most abundant group of bacteria present (Kirchman, 2002; Cottrell and Kirchman, 2000). In freshwater environments FISH studies have also shown that *Bacteroidetes* bacteria are highly abundant in biofilm populations (Manz *et al.*, 1999). In addition to studies using FISH to characterise marine bacterial populations, phylogenetic clone libraries of marine environmental niches have also shown high abundances of *Bacteroidetes* species (Tait *et al.*, 2009; Alonso *et al.*, 2007). *Bacteroidetes* populations do however tend to be underrepresented in 16S rDNA clone libraries in comparison with FISH based studies, this may be due to the limited copy of the 16S rDNA gene in the genomes of *Bacteroidetes* species (Alonso *et al.*, 2007).

It is not just the sheer abundance of the *Bacteroidetes* in the marine environment that makes this phylum of bacteria of interest to science. The *Bacteroidetes* are metabolically specialised to degrade complex organic matter such as polysaccharides and proteins; an example being cellulose that is insoluble in seawater (Thomas *et al.*, 2011; Edwards *et al.*, 2010). Fibres of cellulose immersed in the Irish Sea were colonised by biofilms composed of *Bacteroidetes* species that within one month were able to breakdown the cellulose, liberating a source of carbon that could be utilised by other marine organisms higher up the trophic levels (Edwards *et al.*, 2010; Kirchman, 2002). In the ocean, dissolved organic matter (DOM) accumulates into flocculent particles colloquially referred to as marine snow (Alldredge and Silver, 1988). Bacteria readily colonise marine snow particles and can reach densities of between 10^6-10^8 cells per aggregate, a figure much greater than that of planktonic bacteria in the marine environment (Munn, 2004). *Bacteroidetes* have been shown to be highly abundant in these marine snow communities (DeLong *et al.*, 1993), possibly metabolising the DOM in marine snow and hence significantly contributing to the cycling of organic carbon in the marine environment (Kirchman, 2002).

4.1.4 AHL Production in Non Proteobacterial Gram-negative Bacteria

Since the initial discovery of bacterial QS in the marine bacterium *V. fischeri* the predominant focus of QS research has been centred in the *Proteobacteria* phylum, with a bigger emphasis on bacterial species that act as human pathogens (Williams *et al.*, 2007; Hastings and Nealson, 1977). Aside from these pathogens, the number of marine species reported to be engaging in AHL-mediated QS, since the initial discovery in *V. fischeri*, has increased and includes bacteria such as *V. harveyi, V. anguillarium,* *Aeromonas hydrophyla* and *Roseobacter* sp., (Milton, 2006; Gram *et al.*, 2002; Swift *et al.*, 1999; Hastings and Nealson, 1977). However, in spite of this, little research has been carried out into AHL-mediated QS in other Gram-negative phyla outside the *Proteobacteria* (reviewed by Williams, 2007). In recent years, the range of bacteria producing AHLs has been extended beyond the *Proteobacteria* phylum with AHL-producing bacteria being identified in the *Cyanobacteria* and *Bacteroidetes* phyla (Romero *et al.*, 2010; Sharif *et al.*, 2008). The initial study to show AHL production by species grouping to the *Bacteroidetes* phylum was a study in which an strain of *Flavobacteria* isolated from the North Sea activated the *gfp*-based short chain AHL bio-reporter *E. coli* pJBA132. However the specific AHL activating this bio-reporter was not identified (Wagner-Döbler *et al.*, 2005). In a similar study, Huang *et al.*, (2009) showed AHL production in marine biofilms over a number of days using both *C. violacium* and *Agrobacterium tumefaciens* AHL bio-reporters. In the same paper the biofilms which activated both reporters were shown to contain bacteria whose 16S rDNA clustered to the *Bacteroidetes* phylum (Huang *et al.*, 2009). The first AHL identified being produced by *Bacteroidetes* bacteria was C4-HSL which was being synthesised by a number of strains of *Tenacibaculum maritimun* (Romero *et al.*, 2010). Studies have also showed that *T. maritimum* had the ability to quench the activity of C10-HSL (Romero *et al.*, 2011; Romero *et al.*, 2010).

4.1.5 The Relevance of **Shewanella** *and the* **Bacteroidetes** *within the* **Ulva** *Epiphytic Population*

The reason for selecting the *Bacteroidetes* phylum for further studies in this thesis was primarily due to this phyla's abundance within the *Ulva* spp. epiphytic population as seen in the 16S rDNA clone library. In particular, 36.49% of clones in the library grouped to the *Bacteroidetes* phylum (see Section 3.2.1 and Figure 3.3). Additionally a *Bacteroidetes* strain (*Cytophaga* sp. MBIC 04683) was shown to produce the plant growth effector thallusin, implicated to affect to the growth morphology of *Ulva* spp. (Matsuo *et al.* 2005).

The *Altermonadaceae*, the family to which *Shewanella* belongs to are also abundant within the *Ulva* spp. epiphytic population with 9.21 % of clones from this library grouping to the *Altermonadaceae*, this figure represents 41.18 % of the clones grouping to the *Gammaproteobacteria* phylum within the *Ulva* spp. 16S clone library (see Section 3.2.1 and Figure 3.3). These two groups of bacteria were also found in abundance within the biofilms present on the rocks where *Ulva* spp. can be found colonising (Tait *et al.*, 2009). The *Shewanella* are of particular interest as they can be isolated from both rocky shore biofilms and from the *Ulva* thallus (Tait *et al.*, 2009). *Shewanella* isolates from both these niches have been shown to produce AHLs and interestingly turn over their cognate signal and inhibit the signalling of other AHL-producing bacteria found in the rocky shore biofilms, affecting *Ulva* zoospore settlement (Tait *et al.*, 2009). Additionally AHL synthase and response regulator genes have not, as yet, been identified in the genus *Shewanella*. All these above factors make *Shewanella* bacteria of interest for further study and relevant to this thesis.

4.1.6 Experimental Aims

Bacterial belonging to the *Bacteroidetes* phylum and the genus *Shewanella* have been shown to be abundant within the epiphytic bacterial population of *Ulva* and interact with the seeweed affecting the *Ulva* growth process. The aims of this study were to further investigate the extent of AHL production and inhibition by members of these two groups of bacteria. The presence of AHL synthases, AHL response regulators and AHL inhibiting enzymes within both *Shewanella* spp. and the *Bacteroidetes* phylum was investigated using *in silico* analysis of genomic sequences. Owing to previous research that showed AHL inhibition by *Shewanlla* spp. to directly effect *Ulva* zoospore settlement, AHL inhibition by members of the *Shewanella* genus was therefore investigated using a number of molecular biological techniques.

4.2 Results

4.2.1 AHL Production in **Bacteroidetes** *and* **Shewanella**

4.2.1.1 AHL Production in **Shewanella**

AHL production has already been reported in *Shewanella* isolates from the rocks colonised by *Ulva* spp. and from fish intestines (Tait *et al.*, 2009; Morohoshi *et al.*, 2008). This study utilised a number of *Shewanella* strains provided by Dr K Tait isolated from *Ulva* spp. and decaying fish. These strains were identified by 16S rDNA typing and screened for AHL production using the *lux*-based bio-reporters *E. coli* pSB536, pSB401 and pSB1142. AHL bio-reporter assays showed that these strains were producing both short and long chain AHLs as cell free supernatant extracts activated both the bio-reporters pSB536 and pSB1142 (Table 4.1).

Table 4.1 ***Shewanella*** **strains screened for AHL production.** A number of *Shewanella* strains isolated from decaying fish were identified by 16S rDNA typing and screened for AHL production using AHL bio-reporters.

					Bio-reporter Activation *		
Strain	**Phylogenetic Identity**	**Accession Number**	**% Identity**	**Source**	**pSB536**	**pSB401**	**pSB1142**
A1	*Shewanella baltica* gene for 16S rRNA, partial sequence, strain: X1410	AB205580	99	Fish	+	-	+
A2	*Shewanella sp.* CsQ2 16S Ribosomal RNA Gene	EU075118	98	Fish	+	-	+
A4	*Shewanella baltica* strain KB30 16S Ribosomal RNA Gene	JF327458	98	Fish	+	-	+
A7	*Shewanella sp.* MPU12 gene for 16S ribosomal RNA, partial sequence	AB334772	98	Fish	+	-	+
A8	*Shewanella sp.* MPU12 gene for 16S ribosomal RNA, partial sequence	AB334772	97	Fish	+	-	+
B1	*Shewanella putrefaciens* SS6 16S ribosomal RNA gene, partial sequence	JX032786	97	Fish	+	+	+
B8	*Shewanella baltica* 16S rDNA X1410	AB902206	98	Fish	-	-	+
P3 †	*Shewanella sp.* 16S ribosomal RNA P3	AY902206	100	Rock	+	-	+

***** *Strains marked with a + were shown to induce bioluminessence in the bio-reporter, strains marked with a – failed to induce bioluminessence in the bio-reporter.*

† *Data provided by Dr. Karen Tait*

4.2.1.2 AHL Production in the **Bacteroidetes**

The 16S rDNA phylogenetic typing showed that a number of strains isolated from *Ulva* spp. were *Bacteroidetes* bacteria. Strains UI08 and UI13, isolated from the *Ulva* thallus were identified as being *Cellulophaga* sp. members of the *Flavobacteria* class of *Bacteroidetes*. Phenotypically both UI08 and UI13 possessed similar colony morphology of bright yellow-pigmented rough circular colonies. After several days incubation on the bench at ≈ 22^{o}C both strains spread across plates and UI08 showed agarlytic activity. Acidified cell free supernatant extracts from strains UI08 and UI13 showed activation of the *E. coli* pSB536 AHL bio-reporter suggesting the presence of short chain AHLs (see Section 3.2.4 and Table 3.3).

In order to positively identify the specific AHLs being produced by these *Bacteroidetes* strains, solvent extracts from cell free stationary phase cultures of both UI08 and UI13 were analysed by LCMS. In addition to these two *Bacteroidetes* strains isolated from the *Ulva* thallus the stationary phase cell free supernatant extract from *Bacteroidetes* strain P13, isolated from biofilms found on rocks colonised by *Ulva* sp. were also assayed for short chain AHL production using LCMS. Extraction ion chromatograms and their corresponding compound mass spectra from the LCMS analysis showed all three strains produce C4-HSL (Figure 4.3). LCMS analysis also showed the presence of C4-HSL with the lactone ring opened, indicating that a proportion of C4-HSL was being inactivated by either the alkaline pH of marine broth media or by the actions of an AHL lactonase.

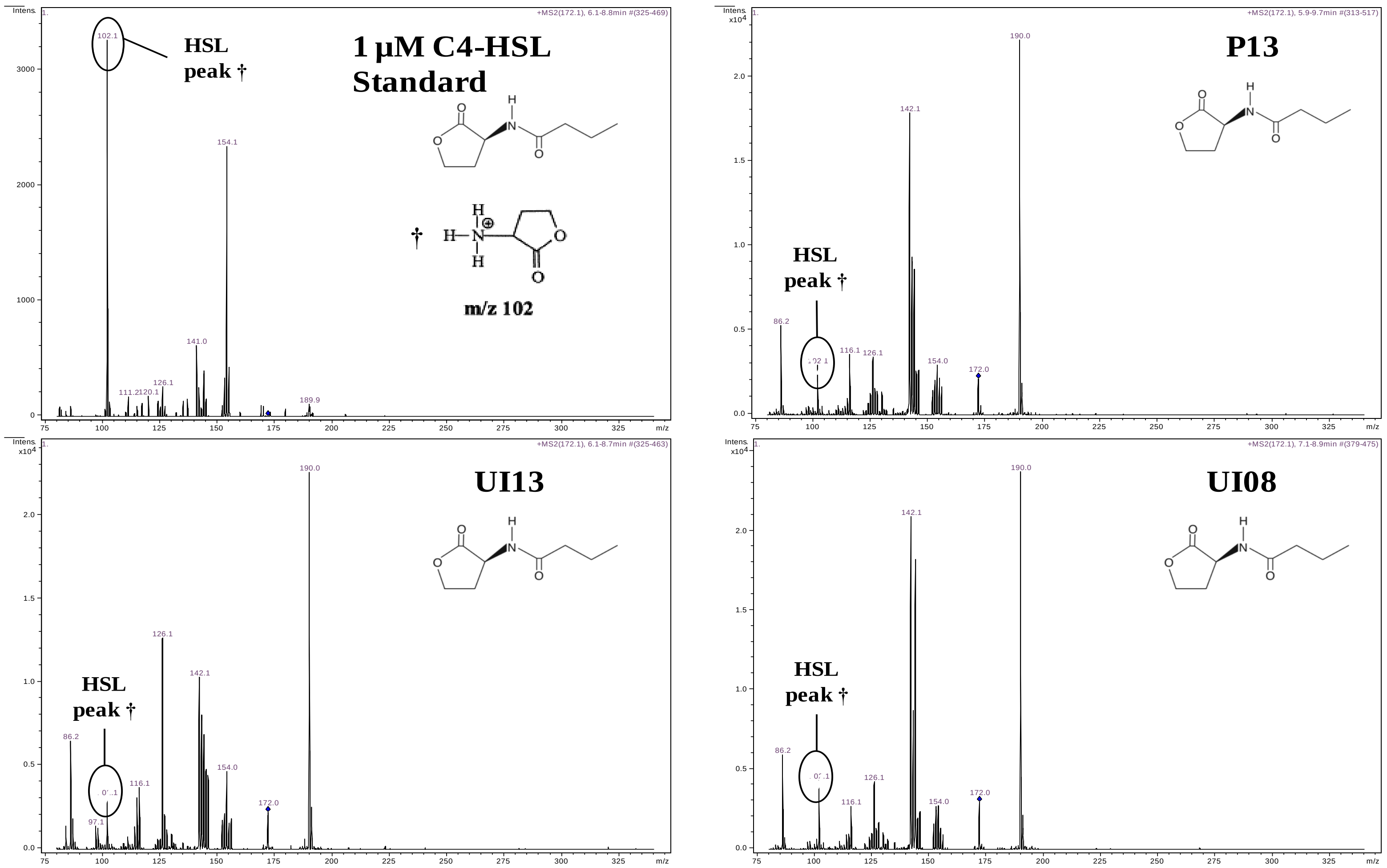


Figure 4.3. LCMS analysis of *Bacteroidetes* strains isolated from *Ulva* spp. and rocky shore biofilms. Compound mass spectra from the breakdown of precursor ion m/z 172 (C4-HSL) of C4-HSL standard P13, UI13 and UI08. Daughter ion of m/z 102.1 (HSL) labelled in all spectra.

The Bacteroidetes strains isolated from *Ulva* spp. which were shown to produce C4-HSL all grouped within the *Flavobacteria* class. As the *Bacteroidetes* population present on *Ulva* spp. also contains bacteria from the *Sphingobacteria* class, it was decided to look for AHL production in strains of *Bacteroidetes* from a wider range of phylogenetic backgrounds. A number of *Bacteroidetes* strains from a range of phylogenetic backgrounds were selected from the PML culture collection and the National Collection of Industrial, food and Marine Bacteria (NCIMB) to be screened for AHL production, (Table 4.2).

Table 4.2. List of *Bacteroidetes* strains from varying phylogenetic backgrounds obtained from the PML and NCIMB culture collections.

Strain	Phylogenetic Identity	Phylogeny	Accession Number	% Identity	Source
1363	*Saprospira grandis* 16S rRNA partial sequence	*Sphingobacteria*	AB071781	99	Rock pool
476	*Flexibacter aggregans* gene for 16S rDNA IFO15975	*Cytophagia*	AB078039	95	Seawater
14225	*Algoriphagus chordae* Strain LMG 21970 16S ribosomal RNA	*Cytophagia*	NR025603	98	Algae
397	*Cytophaga marinoflava* 16S ribosomal RNA gene	*Cytophagia*	AF203475	98	Seawater
14312	*Lewinella marina* MKG38 16S ribosomal RNA partial sequence	*Sphingobacteria*	NR041594	99	Marine Sediment
699	*Balneola alkaliphila* CM41_14b 16S ribosomal RNA	*Sphingobacteria*	NR044367	92	Seawater

All strains listed in table 4.2 were screened for AHL production using LCMS with the exception of strain 699. Strain 699 was discounted from this screening as it displayed exceedingly slow growth, only reaching an OD_{600} of 0.3 in 14 days. In all

Bacteroidetes strains screened only C4-HSL was detected in the supernatant extracts, (Figure 4.4).

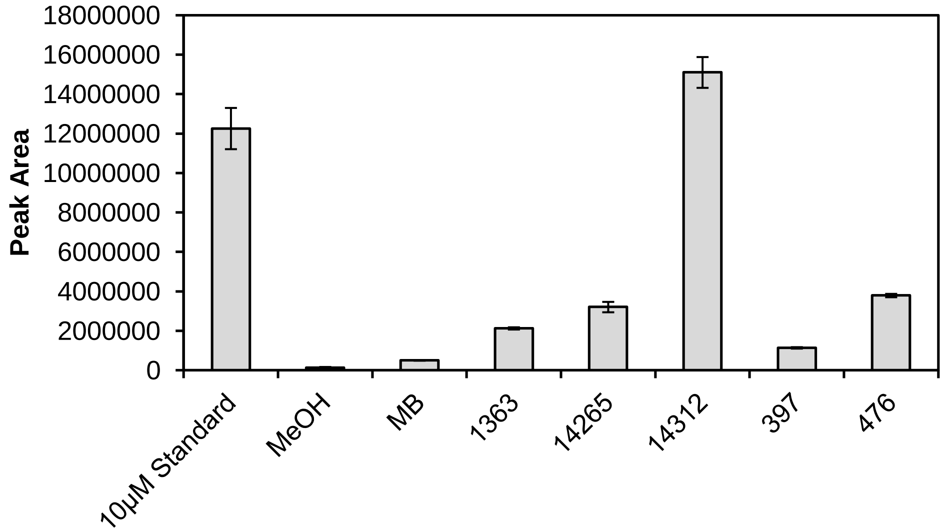


Figure 4.4. Screening of *Bacteroidetes* strains of varying phylogeny for AHL production via LCMS. Graph showing the areas of the C4-HSL extraction ion chromatogram peaks from the LCMS analysis of *Bacteroidetes* strains of differing phylogeny. As controls synthetic C4-HSL, extract from marine broth (MB) and Methanol were used. Error bar represent standard deviation from the mean peak area.

4.2.2 **In silico** ***search for*** **Shewanella** ***spp. AHL Synthase and AHL Response Regulator Sequences***

Annotated and semi-annotated *Shewanella* genome sequences were screened for AHL synthase and AHL response regulator protein sequences. To accomplish this, AHL synthase and AHL response regulator consensus sequences were compiled from the majority consensus of ClustalW alignments of 24 published LuxI and 31 published LuxR homologues (Appendices 4 and 5). Consensus sequence were aditionally compiled from the majority consensus of ClustalW alignments of LuxM homologues from *V. fischeri*, *V. anguillarium* and *V. harveyi* (Milton *et al.*, 2001, Hanzelka *et al.*, 1999, Bassler *et al.*, 1993), and 10 published LuxN protein sequences (Appendices 6 and 7). This enabled the screening for the LuxM/N-type two component AHL synthese and response regulator systems. All 4 consensus sequences, (Figure 4.5), were used to screen protein sequences from 19 separate *Shewanella* genomes using the Genomic BLASTp program (*NCBI*).

LuxI Consensus Sequence

```
MLXIFDXXXVSYDXLSEEKXXXKELFRLRKKXFKDRLGWDVNCXNGMXXXEFDXXQYDNXNTRYLLGIXD
XXXGQXVGCVRLIETTXXXPNMLTGTFFXLLXDDGALPEXXGXYIXESSRFFXXXDKARARLLXGNXXXY
PLSLVXLFLSMINYARANGYTGIVTVVSRAMERILKRSGWPIERIGQXXGHXXEKGEXIYLXLHLPIDXX
XQERLARRINQPLQGPSSXLLTWPLSLP
```

LuxR Consensus Sequence

```
XXXXXXXXXXXXMXILFFDNESINEXIKNYXDRKLKXYGFDKYAYGXXNKXXXXKXPPDXFIISNYPXEW
VERYTKNNYQXIDPVVLTAXRRFSPFXWDXXXNIXIFSXLKXSKIFNLAREYGIVNXGYTFVLHDANNXN
LAMLSLASDDSDXNXIDXRIEXXKXKLQMLLILXHEKMLXLYRLXXXXXNXXNXKMNPKAILSPRENEIL
YWASMGKTYQEIAIILGISEXTVKFHIGNVVKKLGXXVLNAKXAIRLXVELGLIKXXXXXXXXXXXPXXX
XXXXXX
```

LuxM Consensus Sequence

```
MLSLLSXXXXXXXPVQDSCPTLVASALIQNWSVRDTWLSFTYAPQXXNYCFPSYGYSEFTRLQLFTPSSL
SKCYXXEFDNEFKXQLSDTQAVCEVFTLRLTVXXXXYFLYLAQKELMSVLHQAGYKXXXXXXXIIEQPFM
LNFYRAIDAKAYFHSFTGYCDLNDDGKQTYRGFWNFEMMVKAFSNIDFRGYKRXRASRKRGSLERDEHV
```

LuxN Consensus Sequence

```
XXMLDLGLEAIXYPKAITLLATVAVVLXWLXYYCYRLKQKNEXVIFGTHHAPYIAYSXCIIAWIXSNAYF
HTDLLPELGASAAIFMAKLANLASFLAFAFAYYFSCQLAAEQRKGKVHLWQKGIFVTLTVYSLXINLXPG
LTVEHVDIVGPSQFVIEFGPHTXYFFIGLXSFVILTLXNLXAMRANSSKLTLAKTNYMIAGILVFMLSTA
VIHXGITYFLGDFSLTWLPPALSISEMLFVGYALLTSRFYSVKYLAYLTLSXLLVCAIYVIPLGAIFIPL
TEDNQWLIAIPICALIGITWHLLYKRVSRYASFLIYGNKXTPVXQILALEEEFKXSIDDAMRXLGSLLNI
PNDKLQLVNSNYNETFYEDYLSSNRSVLVXDELSEELDYKXXSAKRSXKALYXKMSSNNTALVMPLFGEG
KSVTHLLISSHKSNNQLFSNEEISALQTLLTRVQSTIEADRKXRQSRALANSIAHEMRNPLAQVQLHFEA
LKQHIDSXAPXSQXXXXXXXXXXIKQXIENGQAAIQRGRQLIDIILREVSDSSPEHEPXTMTSIHKAVDQ
AVSHYGFENEKIIERIRLPQQNDFVAKLNETLFNFVIFNLIRNAIYYFDSYPDSQIEIXTXTGXYENKLI
FRDTGPGIDEAILHKIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGRIECXSKLGEFTEFHLYFPVVPNAP
KAETLRXXXXXXXXXXXXXXXXXXXXXXXXTPYFNSWKQNXSTTENXXXXXXXXXXXXXXXXXXXXXXXX
KTXXXVKPXRQXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXAPTVLIVDDKEVQRTLVQMYLNRLGVNSLQANNGENAVELFKSXKVDLILMDVQMPVMNGFDA
SQIIKXXSPQTPIIALSGESGERELXMISKLMDGRLEKPTXLNALQQVLDXWLXKXXASNXXXXXXXXXX
XXX
```

Figure 4.5. AHL synthase and AHL response regulator consensus sequences. The consensuses were generated from the majority consensus of ClustalW sequence alignments of multiple LuxI, LuxR, LuxM and LuxN homologues, X represents regions where no majority consensus was present in the alignment (see Appendices 4-7).

BLASTp screening using the LuxI-type AHL synthase consensus sequence yielded no hits to any sequences within the 19 *Shewanella* genomes searched. BLAST screening using the LuxR type AHL response regulator consensus sequence yielded 164 hits to sequences within *Shewanella* genomes. Most of these hits possessed very low similarity scores (< 25%) to the consensus sequence and any similarity was due to the presence of helix-turn-hexix DNA binding domains. The top hit (percentage Identity 34%), corresponded to SVI_2459, which was annotated as a LuxR family response regulator in *Shewanella violacea* DSS12. This sequence had regions of similarity to the LuxR consensus sequence in both the C-terminal and N-terminal regions. Structural analysis of this sequence using the Pfam database (Punta *et al.*, 2012), showed both an AHL-binding domain and a LuxR type DNA-binding domain to be present, characteristic of the LuxR family of proteins (Kolibachuk and Greenberg, 1993). Analysis of the upstream and downstream ORFs of SVI_2459 using Artemis genome viewer showed no sequences with any similarity to the LuxI consensus.

BLASTp screening using the LuxM-type AHL synthase consensus sequence produced three hits in the *Shewanella* genomes. These hits possessed very low sequence identity and amino acid coverage values, (28% and 27% respectively), so are therefore unlikely to be true LuxM homologues (data not shown). BLAST screening using the LuxN type AHL response regulator consensus sequence yielded a number of hits within the *Shewanella* genomes. These hits possessed sequence identity values of up to 46% and amino acid coverage values of up to 52%. However, the genomes of these top 5 BLAST hits lacked any sequences resembling LuxU and LuxO which are key elements in the phospho relay cascade integral to the function of the two component AHL response regulator system (Freeman and Bassler, 1999a; Freeman and Bassler, 1999b). It is therefore most likely that these hits are not true LuxN homologues.

4.2.3 **In silico** ***Search for*** **Shewanella** ***spp. AHL Inactivating Enzyme Sequences***

In order to screen for potential AHL-inactivating enzymes, consensus sequences were constructed from protein sequences of published AHL lactonases and AHL acylases, (Appendix 8), using the same approach as with the construction of the LuxI/R consensus sequences (see Section 4.2.2). The resultant consensus sequences (Figure 4.6) were used to screen 19 separate *Shewanella* genomes. No potential AHL lactonase sequences were identified within these *Shewanella* genomes. Using the AHL acylase consensus sequence 26 hits were identified within the *Shewanella* genomes including *S. putrefaciens, S. baltica* and *Shewanella amazonensis*. With the exception of one, all the identified sequences possessed between 29-40% identity with the consensus and 90-94% amino acid sequence coverage. The top 5 hits are displayed in table 4.3.

AHL Lactonase Consensus Sequence

```
MXXXXXKLXXLQSGXXRCDXXHISMNXGXXXXGKXYXXXXXXXXXEIPVPAXLIXHXDGFTLIDTGLXXEG
AXDPSGRWXXGSXXXXQXXPXXXXSXEQGCVXEQLKXXXGLXPXDIXYVVLSHLHLDHAGAIGRFPNAXH
IVQRXEYEYAFAXXXWFXXGAXXXXYIRKDFXXXXXXLPXLNWQFXNGIEDDRYXVXPGVXXTLLXXFTP
GHXPGHQSXLVRLPKXGPFLLTIDAAYTLDXXXXXXALAGXLXXTIDXXQSVQKLRQXAERYXATVIFGH
DPEQWARFXKXAPXFYX
```

AHL Acylase Consensus Sequence

```
MXXNXXXRXXXXAXLAXLXGXXXLXGSTXXTXGXXXXPSXXXXXGLSAXIRRTXYGXPHIRAKDXASLGX
GXGYAXAQDNXCLLADGFXTXNGERSRYFGPEXXXDFSLXXXXXXXXXNLXSDIFXKGLXDXEXLEAXWX
XXXPXXXXXXXRELLRGXAAGXNRYLADXXXXXXXAAXXCRGXPWVRPITXXDVXRXGXXLAVLGGAGXF
LDGIVAAQPPGAXPXXXXXVAXXXXPEXAAFXVXXQRXLXARXAXXXXXXXXXXDXGSNAXAFGXXXTAN
GRGMLLGNPHFPWTGXXRFXQSHLTIPGKLDVMGASLXGXPVXVXIGFNKDXAWTHTVSTGXHFTLYXLX
LDPGDPTXYLVDGXPXPMTKRTVXIXVKGXDGGLERVXXTXYXTIYGPXVXXPXGXLPXPWTXXXAYALR
DANXXNTRXVDXWLXINQARDVAXVRQAFKXLQGXPWVNTIAADRAGNAXFADXSVVPYLXXXLXXRCAT
PXLXXAXFXXXGLXILDGSXXRCAWSXDXXXXXXGITPPARMPVLERXDXVQNSNDSAWLTNPAXPLTGF
SPVXGSXAXPISLRTRXXLXXXXSRLAGKXXXXXXXXXXXXLXAMVFXNRXFAXELVLGDLLAXCXAXPG
XXAVXXXXXXXXVRXACAALXQWDRKXXXDSRXGHLXFREFXXXAXXXXQAXXWKXXFDPADPVXTPRGL
XTDXPXVXXXXXXALADAXAEXXXAGXALDAPLGDVQFVXRXXXXXXXXGXRXXIPGGXEXEGVXNKXQT
VXXGDXXXXXXXXXXXXXXXXXXXXXXXXXXXYLEVXXGXSYIQAVTFDDEGXPVARGXLTYSXSSXP
RSPHFXDQTRLYSGXKXWXTLPFSEADIAADPXXLXVLRLXEXX
```

Figure 4.6. AHL inactivating enzyme consensus sequences. The consensuses were generated from the majority consensus of ClustalW sequence alignments of multiple AHL lactonase and AHL acylase homologues, X represents regions where no majority consensus was present in the alignment (Appendix 8).

Table 4.3 BLAST hits resulting from screening *Shewanella* genomes for AHL inactivating acylases. Table listing NCBI accession number, sequence annotation, percentage identity, and percentage amino acid coverage values of the top 5 AHL acylase BLAST hits from the screening of *Shewanella* genomes.

AHL Acylase			
Accession Number	**Sequence Annotation**	**% Identity**	**% Coverage**
YP_964683	Peptidase S45, penicillin amidase, *Shewanella sp.* W3-18-1	39	93
YP_006008798	Acyl-homoserine-lactone acylase, *S. putrefaciens* 200	39	94
YP_001182388	Peptidase S45, penicillin amidase, S. putrefaciens 200	39	94
NP_716547	*aac* gene product, *S. oneidensis* MR1	40	93
YP_001367743	Peptidase S45, penicillin amidase, *S. baltica* OS185	40	93

4.2.4 **In silico** *Search for* **Bacteroidetes** *AHL Synthases and AHL Response Regulator Sequences.*

Having identified AHL production in a number of *Bacteroidetes* strains, annotated and semi-annotated *Bacteroidetes* genome sequences were screened for AHL synthase and AHL response regulator protein sequences using the LuxI, LuxR, LuxM and LuxN consensus sequences compiled for the *in silico* analysis of *Shewanella* genomes (Figures 4.5). These consensus sequences were used to screen protein sequences from 180 separate *Bacteroidetes* genomes using the Genomic BLAST program (*NCBI*).

BLAST screening using the LuxI-type AHL synthase consensus sequence yielded a total of 5 hits all of which had a low % similarity and % coverage score (< 38% and < 36% respectively). Similarity to the consensus was only apparent to the C-terminal ends of the identified hits. Aligning the BLAST hits to published LuxI homologues showed that no sequence similarity was present in the reported active site region located between residues 25 and 70 (Hanzelka *et al.*, 1997). It is therefore unlikely that these hits are true LuxI homologues.

BLAST screening using the LuxR-type AHL response regulator consensus sequence yielded over 100 hits all of which had a slightly higher but still low % similarity and % coverage score in comparison to the LuxI hits (< 50% and 24% respectively). Again, these hits only possessed regions of similarity to the C-terminal end of the consensus. Structural analysis using the Pfam database (Punta *et al.*, 2012) of the top 5 BLAST hits showed LuxR-type DNA-binding domains to be present within the protein sequences however all 5 hits lacked AHL-binding domains, characteristic of the LuxR family of proteins (Kolibachuk and Greenberg, 1993). Additionally, there were no *luxI*-type predicted gene sequences near these BLAST hits suggesting the absence of any LuxI/R system within the genomes searched.

BLAST screening using the LuxM-type AHL synthase consensus produced one hit in the *Bacteroidetes* genomes. This hit possessed very low sequence identity and amino acid sequence coverage (36% and 21% respectively). It is therefore unlikely that these hits are true LuxM homologues. BLAST screening using the LuxN-type AHL response regulator consensus sequence produced over 135 hits in *Bacteroidetes* genomes with many having high levels of similarity over the entirety of the sequences identified (48% and 49% respectively). Structural analysis using the Pfam data base, (Punta *et al.*, 2012), of the BLAST hits showed them to be transmembrane proteins containing phosphokinase domains. The top 5 BLAST hits were added to an alignment of published LuxN homologues compiled by Swem *et al.* 2008. The BLAST hits lacked many of the conserved residues that are thought to be required for the activity of LuxN (Swem *et al.*, 2008). As with the *Shewanella* LuxN BLAST hits the genomes of these *Bacteroidetes* top 5 BLAST hits also lacked any sequences resembling LuxU and LuxO and therefore again it is most likely that these hits are not true LuxN homologues.

4.2.5 **In Silico** *Search for* **Bacteroidetes** *AHL-Inactivating Enzymes*

AHL inactivation by *Bacteroidetes* bacteria has been reported (Romero *et al.*, 2011, Romero *et al.*, 2010). Therefore, in addition to carrying out *in silico* screens for putative AHL synthase and response regulator sequences, a screen for AHL-inactivating enzymes was conducted. The AHL lactonase and AHL acylase consensus sequences used to screen *Shewanella* genomes (Figure 4.6) were used to screen 180 separate *Bacteroidetes* genomes. The AHL lactonase consensus sequence produced 53 BLAST hits in *Bacteroidetes* genomes. The top 5 hits are displayed in Table 4.4 and have a percentage identity score with a 14% - 59% range and a percentage amino acid coverage score with a 46% to 24% range.

Table 4.4. BLAST hits resulting from screening *Bacteroidetes* genomes for AHL inactivating lactonases. Table lists NCBI accession number, sequence annotation, % Identity and % amino acid coverage values of the top 5 AHL lactonase BLAST hits from the screening of *Bacteroidetes* genomes.

AHL Lactonase Hits				
Hit Number	**Accession Number**	**Sequence Annotation**	**% Identity**	**% Coverage**
BLAST Hit 1	ZP_01051078.1	Metallo-beta-lactamase superfamily protein [*Dokdonia donghaensis* MED134]	59	25
BLAST Hit 2	YP_004430745.1	AttM/AiiB family protein [*Krokinobacter* sp. 4H-3-7-5]	63	27
BLAST Hit3	YP_003715653.1	AttM/AiiB family protein [*Croceibacter atlanticus* HTCC2559]	60	24
BLAST Hit 4	ZP_01692265.1	Zn-dependent hydrolase GumP [*Microscilla marina* ATCC23134]	47	25
BLAST Hit 5	ZP_03967553.1	possible metal-dependent hydrolase [*Sphingobacterium spiritivorum* ATCC 33300]	14	46

The top 5 BLAST hits were added to a protein sequence alignment containing the sequences of 3 published AHL lactonases; AiiA from *Bacillus* sp. 240B1. (Dong *et al.*, 2000), AiiB from *Agrobacterium tumefaciens* and AttM from *A. tumefaciens* (Carlier *et al.*, 2003). The sequence alignment (Figure 4.7) showed that all five hits contained high levels of sequence similarity and that all 5 hits contained the conserved motif HXHXDH~H~D~H, a structural motif encompassing the Zn-binding domain previously shown to be integral for the function of an AHL lactonase (Liu *et al.*, 2007; Dong *et al.*, 2000).

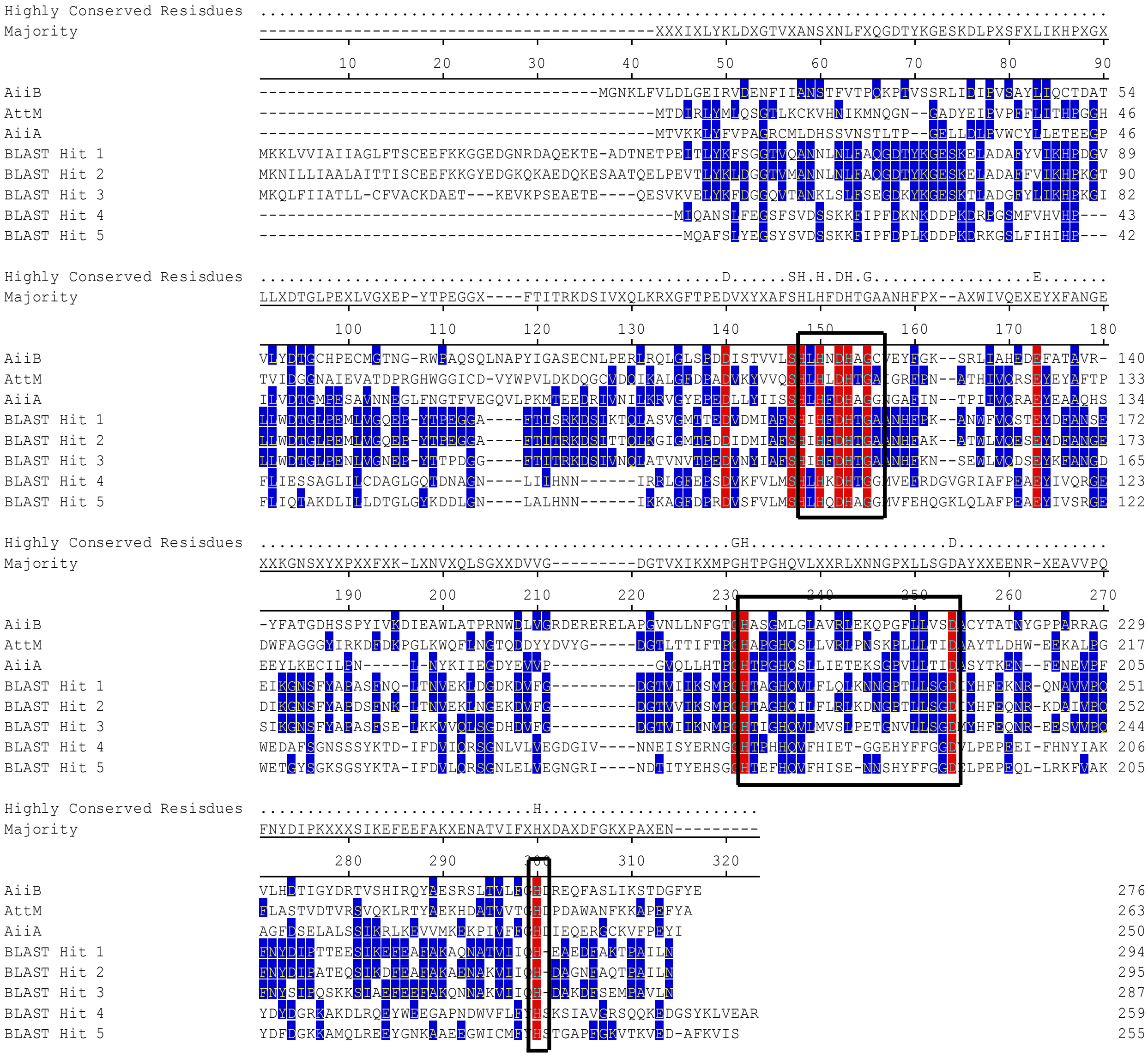


Figure 4.7. Protein sequence alignment of AHL lactonase BLAST hits with the AHL lactonases; AiiB, AttM and AiiA. BLAST hits 1-5 (see Table 4.4) identified by screening *Bacteroides* genomes using the AHL lactonase consensus sequence were aligned against published AHL lactonase sequences. Areas of sequence similarity are highlighted in blue. Residues highlighted in red are where sequence similarity is conserved throughout the alignment. The residues enclosed by black boxes indicate conserved residues that form the Zn-binding domain present in AHL lactonases.

Screening *Bacteroidetes* genomes using the AHL acylase consensus sequence produced 59 BLAST hits. The top 5 hits are displayed in Table 4.5 and have a percentage identity score with a 29%-47% range and a percentage coverage score with a 24% to 29% range.

Table 4.5. BLAST hits resulting from screening *Bacteroidetes* genomes for AHL inactivating acylases. Table listing NCBI accession number, sequence annotation, % identity and % amino acid coverage values of the top 5 AHL acylase BLAST hits from the screening of *Bacteroidetes* genomes.

AHL Acylase Hits				
Hit Number	**Accession Number**	**Sequence Annotation**	**% Identity**	**% Coverage**
BLAST Hit 1	YP_003194092.1	Penicillin amidase superfamily protein [*Robiginitalea biformata* HTCC2501]	30	24
BLAST Hit 2	ZP_01692595.1	Glutaryl 7-aminocephalosporanic acid acylase [*Microscilla marina* ATCC 23134]	29	24
BLAST Hit3	ZP_03701247.1	Peptidase S45 penicillin amidase [*Flavobacteria* bacterium MS024-3C]	47	29
BLAST Hit 4	YP_445269.1	Penicillin amidase superfamily protein [*Salinibacter ruber* DSM 13855]	43	29
BLAST Hit 5	YP_003571196.1	Penicillin amidase [*Salinibacter ruber* M8]	43	29

The BLAST hits from the AHL acylase screen were aligned with the AHL acylase sequences of AiiD from *Ralstonia* sp. XJ12B (Lin *et al.*, 2003), PvdQ from *P. aeruginosa* PAO1 (Huang *et al.*, 2003) and AhlM from *Streptomyces* sp. M664 (Park *et al.*, 2005). The alignment (Figure 4.8) shows areas of sequence similarity between the BLAST hits and the known AHL acylase sequences. Additionally there is a highly conserved glycine-serine paring present in all the BLAST hits that is present in AHL acylases and which has been previously shown to be integral to the post translational modification of the acylase propeptide (Lin *et al.*, 2003).

Figure 4.8. Protein sequence alignment of AHL Acylase BLAST hits and known AHL acylases; AiiD, PvdQ and AhlM. BLAST hits 1-5 (see Table 4.5) identified by screening *Bacteroides* genomes using the AHL acylase consensus sequence were aligned against published AHL acylase sequences. Areas of sequence identity are highlighted in blue. Residues highlighted in red are regions of sequence identity conserved throughout the alignment. The residues enclosed in black boxes indicate conserved glycine-serine pairing common in acylase sequences.

4.2.6 AHL Inactivation by Shewanella *sp.*

Shewanella spp. have been previously shown to produce AHLs in mid log phase and then to inactivate them during stationary growth phase (Tait *et al.*, 2009). To identify the gene responsible for AHL inactivation, a genomic library of *Shewanella* sp. P3 chromosomal DNA, a strain shown previously to have good AHL inactivating activity (Tait, un-published data), was constructed via restriction digest, transformed into an *E. coli* host and screened using the Quorum Sensing Inhibitor Selector (QSIS) system present on plasmid pMH655. The plasmid pMH655 constitutively expresses the *lasR* AHL response regulator gene and also possesses the levansucrase gene *sacB*, which is under the control of the LasR regulated promoter, P_{lasB} (Rasmussen *et al.*, 2005). The *Shewanella* sp. P3 library was screened by growing transformant colonies on media containing sucrose, and seeded with exogenous 3-oxo-C12-HSL. Bacteria possessing the QSIS system and no AHL inhibitor sequence would not survive, as the exogenous 3-oxo-C12-HSL will be transduced by LasR, activating P_{lasB} allowing the expression of *sacB* resulting in cell death due to the presence of the sucrose in the media. If an AHL inactivating enzyme sequences was present, exogenously added AHLs will be inactivated and the *sacB* gene will not be expressed, resulting in cell survival in the presence of sucrose. After screening approximately 6000 transformant clones, 6 clones appeared to have increased growth after an 18h period and as such were selected as potentially having AHL inactivating activity.

The six clones putatively possessing a gene encoding an AHL inactivating enzyme were grown in the presence of synthetic 3-oxo-C12-AHL. This AHL was selected as a previous study showed that *Shewanella* spp inactivate 3-oxo-C12-HSL during stationary phase (Tait *et al.*, 2009). The *lux*-based AHL bio-reporter *E. coli* pSB1142 was used to detect any inactivation of the signal molecule in the culture supernatants of these clones using a microtitre plate reader over a 24h period. Inactivation of AHLs would present itself as a loss or absence of luminosity by the bio-reporter over time. The six clones were compared to a positive control, an *E. coli* strain carrying the QSIS system and expressing the AHL inactivating lactonase gene *aiiA*, (*E. coli* JM109 pSU18*aiiA* pMH655). Of the six clones assayed which had been incubated with 3-oxo-C12-HSL, supernatants from clones 1 and 4 were shown not to cause any activation of the bio-reporter. The AHL inactivating positive control strain showed an initial activation of the bio-reporter followed by a fall in luminescence indicating AHL inactivation. Clones 2, 3, 5 and 6 showed activation of the bio-reporter, as did the negative control *E. coli* DH5α pMH655 which had been seeded with 3-oxo-C12-HSL (Figure 4.9).

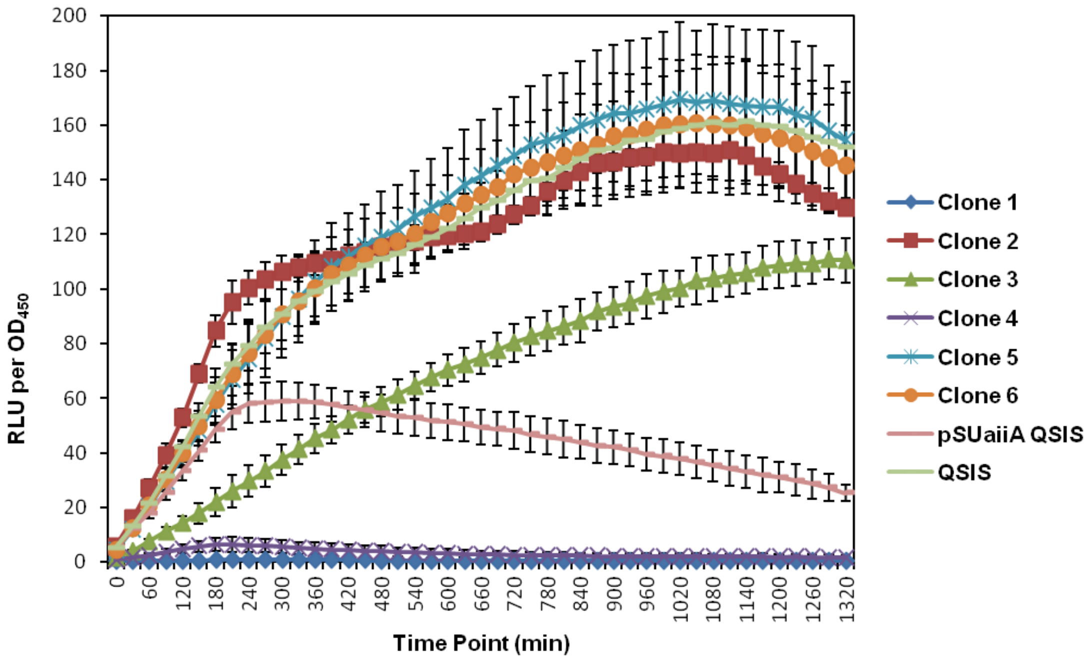


Figure 4.9. AHL degradation assay of *Shewanella* sp. P3 genomic library clones. Clones possessing suspected AHL inactivating gene sequences as identified through QSIS screening were assayed for AHL inactivation in the presence of 3-oxo-C12-HSL over a 24 h period at 37°C.

Clones 1 and 4 which appeared to show AHL inactivation were subjected to miniprep plasmid extraction in order to detect insert DNA within the pME6000 shuttle vector. Although the clones appeared to show AHL inhibiting activity using the bio-reporter neither of the clones possessed insert DNA so were therefore false positive results.

4.2.7 Putative AHL Acylase AacS

4.2.7.1 Screening **Shewanella** *Strains for the* **aac** *Gene*

Screening a *Shewanella* genomic library for AHL inactivating enzymes produced a number of false positive results and was therefore abandoned. Previous work by Tait *et al.* (2009) showed that AHL inactivation in *Shewanella* takes place via the actions of an acylase enzyme which cleaves the acyl side chain from the homoserine lactone ring rendering the AHL biologically inactive (Tait *et al.*, 2009). A putative AHL acylase has been identified in a *Shewanella* strain isolated from a fish intestinal tract in Japan (*Shewanella* sp. MIB015). This acylase showed high levels of similarity to the Aculeacin A acylase gene *aac* in *S. oneidensis* strain MR1 (Morohoshi *et al.*, 2008). Using degenerate primers designed from sequence data of the upstream and downstream regions of putative AHL acylases found within genomic sequences of *S. baltica*, *S. putrefaciens* and *S. oneidensis* the isolates listed in Table 4.6 were screened by PCR for potential AHL inactivating enzymes with similarity to *aac*. The majority of strains screened produced PCR fragments of approximately 2.5 Kb; the expected size of the *Shewanella aac* gene. These fragments were cloned into the vector pGEM T easy, transformed into *E. coli* DH5α and subsequently sequenced using the universal primers M13F and M13R. The resultant sequences were compared to the

NCBI nucleotide database using a BLAST search and found to be homologous to either *aac* from *S. oneidensis* MR1, or to putative AHL acylase genes identified during genomic sequencing of various *Shewanella* species (Table 4.6).

Table 4.6. *Shewanella* strains screened for the presence of the *aac* gene. Table listing the *Shewanella* strains screened via PCR for the *aac* gene, their phylogenetic identity as identified by 16S rDNA typing, where they were sourced from and whether they possessed *aac* homologues.

Strain	Phylogenetic Identity	Accession Number	% Identity	Source	*aac* Gene †
A1	*Shewanella baltica* gene for 16S rRNA, partial sequence, strain: X1410	AB205580	99	Fish	+
A2	*Shewanella sp.* CsQ2 16S Ribosomal RNA Gene	EU075118	98	Fish	+
A4	*Shewanella baltica* strain KB30 16S Ribosomal RNA Gene	JF327458	98	Fish	-
A7	*Shewanella sp.* MPU12 gene for 16S ribosomal RNA, partial sequence	AB334772	98	Fish	+
A8	*Shewanella sp.* MPU12 gene for 16S ribosomal RNA, partial sequence	AB334772	97	Fish	-
B1	*Shewanella putrefaciens* SS6 16S ribosomal RNA gene, partial sequence	JX032786	97	Fish	+
B8	*Shewanella baltica* 16S rDNA X1410	AB902206	98	Fish	-
E1	*Shewanella sp.*		99	Fish	+
NCIMB 2157	*Shewanella hanaidai*	N/A	N/A	NCIMB	+
NCIMB 13526	*Shewanella woodyi*	N/A	N/A	NCIMB	+
MR1	*Shewanella onediensis*	N/A	N/A	PML	+

† *Strains possessing an* aac *homologues are marked with* +, *strains not found to possess an* aac *homologue are marked with* -

4.2.7.2 AHL Inactivation by the **Shewanella putrafaciens** ***B1*** **aac** ***Clone***

The putative AHL inactivating gene clone pGEMB1*aac* from strain B1 (identified as *S. putrefaciens*), harboured by *E. coli* DH5α was selected for further study. The DNA sequence and translated protein sequence of this putative AHL acylase from *S. putrefaciens* B1 (named *aacS* and AacS respectively) can be seen in Figure 4.10.

***aacS* DNA Sequence 2,550bp**

ATGAAATTCAACAAACTCGTGATTGCTATGGGAATGGCCTGCGGTGTAATACTGACCGGCTGTAACGATA
GCGAAGATAGCACTACGCCTACCGAACCTGAAACTCAACTGCAAGCTTTTGCCCCCAATGGTTTACTCAA
AGCCAGTATTCGCCGTACTACCTTTGGCGTGCCGCATATCCAAGCAGACAACTTAGAAAGTTTAGGTTTT
GGTAGTGGTTATGCGCAGGCACAGGACAACTTATGTGTATTAGCCGATGGTTTTATCAAGGCGAACTCAC
AGCGTTCTATGTACTTTGGTCCCCATGCGTCGATTGACTTCACTACGGGTCAACCTACGGCGGAAGATAA
CGGTAACCTTATCTCAGATTTTGCCTATAAAGCGTTAAAGATCAGAGCGCAAGCCGAAGAAAAATGGCCG
AAATTTAGTGAAAACTCTCGGGCACTTATCCAAGGTTTTACCTCTGGTTATAACCAATATCTTGCTGATG
TCGAAGCGGGCACACAAACGGCAGAACCTTTCTGTGGCGGTCAGCCTTGGGTGAAACCCATAGTGCCAGA
GGATGTGGTGACTTATTTGTTTTCTATCGCCTTATTACCGGGCGCAGCTAACTTTCTCGATCTGATTTTT
TACGCTAACCCAGGAGATGCACAGGAATACATGCCGCGTATCGTTGGGCCCGCGCCTAGCCAAGACCAAA
CTGCGTTTGTGGCCGATATGCAGTCTAAGTTGATTGCCCGCGCGGCTCGTATCACGACACCAGAAACCAA
TCCCCGCGATTTAGgTTCAAATGGTTGGGGTTTAGGGAAGGATAAAACCGAAAaTGGTAAgGGCATGGTG
CTAGGTAATCCGCATTTCCCGCATACGGGTAACCTGCGTTTTTGGCAATCCCATATTACGATCCCAGGGC
ATTTAGATATGATGGGCGGCTCGTTAGTGGGTATGCCTGGACCGATTAATATTGGTTTCaATAAAGATCT
CGCTTGGACTCATACCTTCTCTACCGCTGAGCATTTTGTGATGTATAACCTTGAGTTAGTCTCGGGTGAT
CGGATGCAGTATTTGTTTGATGGTAAACCTATGCCGATCACCAAAGAGACAGTATCGGTTCTAGTGAATG
CAGGTCCTGCGGGCATGCTGGTCGCCGAGAAAGATATTTATACTACGGCCAAAGGCCCTATGGTCGAAGC
TCCTCCTGCTTTAGCGCCTTTTGGTTGGGATGATGGCAGTGCCTTTATGATCCAAGATGCCAATATGGGG
ACTATGGACCCTGTTGACCATTGGTTAGCCATGAACATGGCGACGAACAAAGAAGAGTTCCAACAGGCAT
TTAAGGACTACGATGGTGTCATCTTCAATAACACTATGTACGCCGATAAAGAGGGCAATGCTTTTTATAT
CGACGACTCAACTGTGCCAGGATTATCTGAATCGGCAGTTGTGTTGTTAAAAACCTCACCGGATATTAAA
GCGGCTAAGGCAAAAGCCGGATTTACGATTTTACCCGGTAACACTTCGTTGTTTAGCTTCGATGGTCCTA
CGCCCTATGAGCGTGCGCCCAAGCTTGAGCGCAGCGACTTTGTGCAAAACTCCAATGACTCCTTTTGGTC
GACTAACTTGAATGAACCGCTGACTTACTACTCGCCTATTTTACGGCGCAGAAGGCTGGACAGCTATCAC
TGCGGACACGGATGGGCTATGCCGCGGGGGGCTGATGGCAAGTTCAGCTGGAAGAACTCGAAGCGGGCTG
TGCTGTCGAATCGCAGTTATCTCGCCGAGTTAGTGCTGCCTGACTTGATTGCCCAGTGTGATGCCCAAGG
CAGTACACCTGTGGTGGTGTCGGCAAGTTTATCTAAGGATGTCTCTTTGGCTTGTGCGGCATTAAAGGCG
TGGAACGGTAAGCAAGATAACGACAGTAAAGGTGGTGCTTTACTACGTGAATTTGCCCATCTATTCAGCC
AAAAGACCATGTTGACCCAAGGATTCGATCCCGCTAATGCGGCAACGACACCTAAAACCTTGACTACGGA
TGGCAGTGCCTTAAAGGCCTTGGCGCACAGTGCGCTTAACCTTGAGGCCGCAGGCTTTGCATTAGATGCG
CCATTGGGGGATGTGCAATTTGTGGAGAAATCGCTGCCAGATGGAACGCCAAGCGGGTCGCGTTTACCTT
GGCCGGGTAGTCATAATGCTGAAGGTGGATTCAACGTGTTTTCAACCAGTTTGTCGGGTGATGACACTTT
GATCCCGCAGCACAAATACGCCGCTGTGATGGATGTGGTCACAGGCAAGGCGATGAGTTCTGGCTTAACG
GCGAAAGGATACCAAGTGCGTTACGGTTCGAGTTGGATGATGGCGGTGAACTTTACCGACGAGGGGCCTG
TGGCGCGGGGGATTTTAACTTACTCTGAGTCGAGTAATATCTTAACGCCTGCGTTTGCTGATCAAAGTAT
CTTGTACTCGAGTGCTAAAAGCTTCCGTCCTTTGTTGTTCAAAGAGGCTGACATAGCACCTGCTGTAGTG
TCGACGACTGAGCTGACACTACAAAAACCA

AacS Protein Sequence 850 amino acid residues, predicted size 91.82 KDa

MKFNKLVIAMGMACGVILTGCNDSEDSTTPTEPETQLQAFAPNGLLKASIRRTTFGVPHIQADNLESLGF
GSGYAQAQDNLCVLADGFIKANSQRSMYFGPHASIDFTTGQPTAEDNGNLISDFAYKALKIRAQAEEKWP
KFSENSRALIQGFTSGYNQYLADVEAGTQTAEPFCGGQPWVKPIVPEDVVTYLFSIALLPGAANFLDLIF
YANPGDAQEYMPRIVGPAPSQDQTAFVADMQSKLIARAARITTPETNPRDLGSNGWGLGKDKTENGKGMV
LGNPHFPHTGNLRFWQSHITIPGHLDMMGGSLVGMPGPINIGFNKDLAWTHTFSTAEHFVMYNLELVSGD
RMQYLFDGKPMPITKETVSVLVNAGPAGMLVAEKDIYTTAKGPMVEAPPALAPFGWDDGSAFMIQDANMG
TMDPVDHWLAMNMATNKEEFQQAFKDYDGVIFNNTMYADKEGNAFYIDDSTVPGLSESAVVLLKTSPDIK
AAKAKAGFTILPGNTSLFSFDGPTPYERAPKLERSDFVQNSNDSFWSTNLNEPLTYYSPILRRRRLDSYH
CGHGWAMPRGADGKFSWKNSKRAVLSNRSYLAELVLPDLIAQCDAQGSTPVVVSASLSKDVSLACAALKA
WNGKQDNDSKGGALLREFAHLFSQKTMLTQGFDPANAATTPKTLTTDGSALKALAHSALNLEAAGFALDA
PLGDVQFVEKSLPDGTPSGSRLPWPGSHNAEGGFNVFSTSLSGDDTLIPQHKYAAVMDVVTGKAMSSGLT
AKGYQVRYGSSWMMAVNFTDEGPVARGILTYSESSNILTPAFADQSILYSSAKSFRPLLFKEADIAPAVV
STTELTLQKP

Figure 4.10. *aacS* DNA sequence and translated protein sequence.

In order to assay the ability of the *aacS* clone from *S. putrefaciens* B1 to inactivate AHLs, the *E. coli* DH5α pGEM*aacS* strain was grown to stationary phase in media seeded with 1.25 µM C4-HSL, C6-HSL and C12-HSL. An AHL concentration of 1.25 µM was found via reapeated AHL inactivation assays exposing a range of exogenous AHL concentrations to the AHL lactonase AiiA to be sufficient to activate the bio-reporters but low enough for AHL inactivation to be detected (data not shown). AHLs were extracted from the resultant culture supernatant using acidified ethyl acetate and detected using appropriate *E. coli* bio-reporters in a 96 well plate assay (Figure 4.11). After 4 h incubation *E. coli* DH5α pGEM*aacS* failed to activate the AHL bio-reporters pSB536, pSB401 and pSB1142 indicating that the AHLs in seeded media had been degraded. Extracts from culture supernatants from *E. coli* DH5α pGEM*aacS*, which had been acidified to pH 2.0 prior to extraction, also failed to activate the AHL bio-reporters. Based on previous work characterising enzymic methods of AHL inactivation this result indicated that the AHLs were inactivated through the actions of an acylase as acidification would counter act AHL inactivation via a lactonase enzyme (Yates *et al.*, 2002) (Figure 4.11).

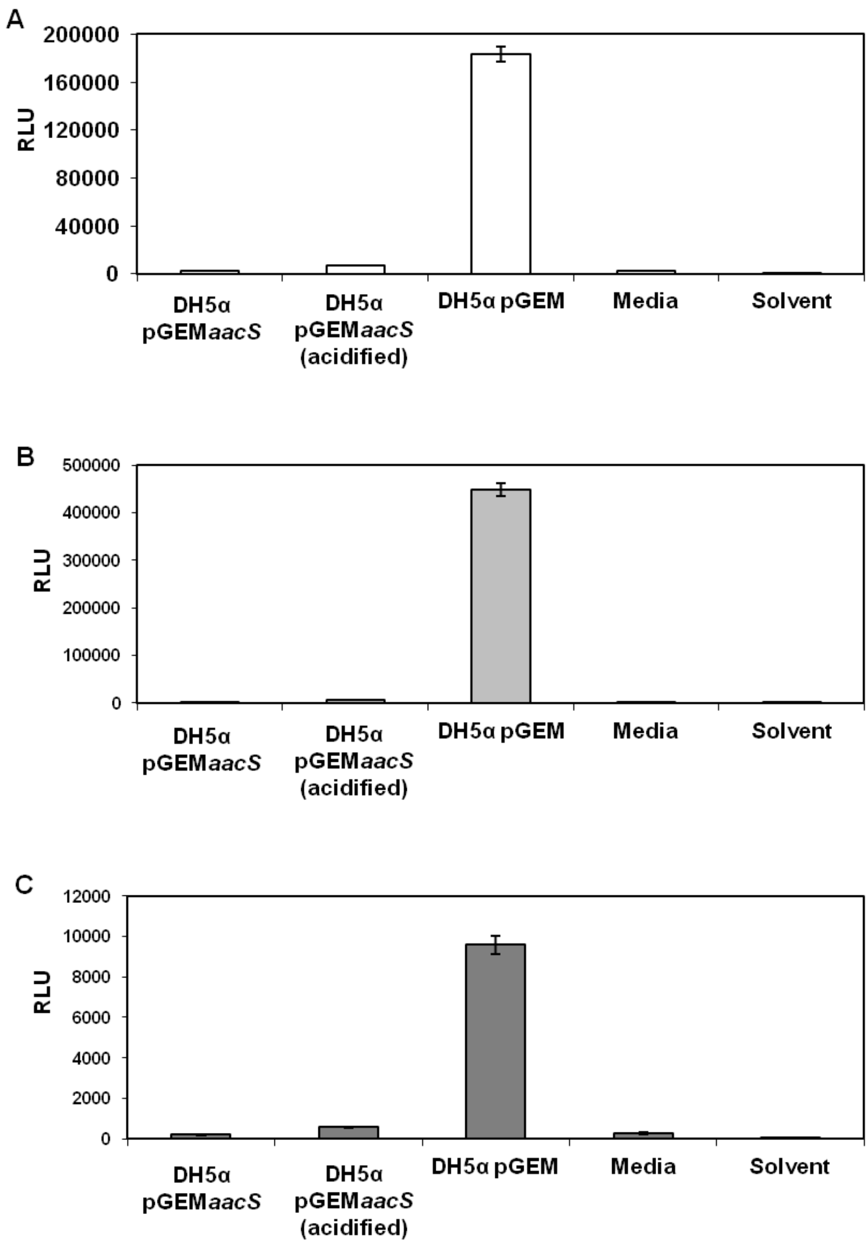


Figure 4.11. Initial AHL Inactivation Assays. Luminescence (RLU) resulting from the activation of AHL bio-reporters (**A**) *E. coli* pSB536, (**B**) *E. coli* pSB401 and (**C**) *E. coli* pSB1142 in the presence of cell free supernatant extracts from *E.coli* DH5α pGEM*aacS*, *E.coli* DH5α pGEM*aacS* (acidified) and *E.coli* DH5α pGEM cultured in media seeded with 1.25 µM C4-HSL, C6-HSL and 3-oxo-C12-HSL respectively. Extracted LB media and solvent are plotted as negative controls. Error bars represent standard deviation from the mean.

4.2.7.3 AacS Protein Expression

The putative AHL acylase from *S. putrefaciens* B1, AacS, was expressed in order to purify the resultant protein for further studies. Expression was carried out using the pCOLD expression system (Hayashi and Kojima, 2008). The pCOLD vector regulates expression using the *E. coli* cold shock protein (*cspA*) promoter, only allowing expression at 15^{o}C. This vector also adds an N-terminal his-tag onto the expressed protein allowing easy purification. The *aacS* ORF was amplified via PCR and cloned into the pCOLD vector creating the expression construct pCOLD*aacS*. This expression construct was transformed into *E. coli* BL21 and cultured for 24 h at 15^{o}C. The successful expression of the acylase gene from *E. coli* pCOLD*aacS* resulted in an approximately two-fold decrease in growth, as measured by OD_{600}, in comparison to *E. coli* pCOLD cultured under the same conditions.

The recombinant AacS protein from *E. coli* BL21 pCOLD*aacS* was analysed on a 12% polyacrylamide gel (Figure 4.12). The expressed AacS protein was present in the insoluble fraction of the protein extraction. The size of this expressed protein was between 50 KDa and 60 KDa, much smaller than the predicted 91.82 KDa, indicating that there may have been post-translational modification. This assumption of post-translational modification in given further credence by the findings of Lin *et al.* who state that AHL acylases possess conserved glycine-serine parings which are integral to post-translational modification (Lin *et al.* 2003).

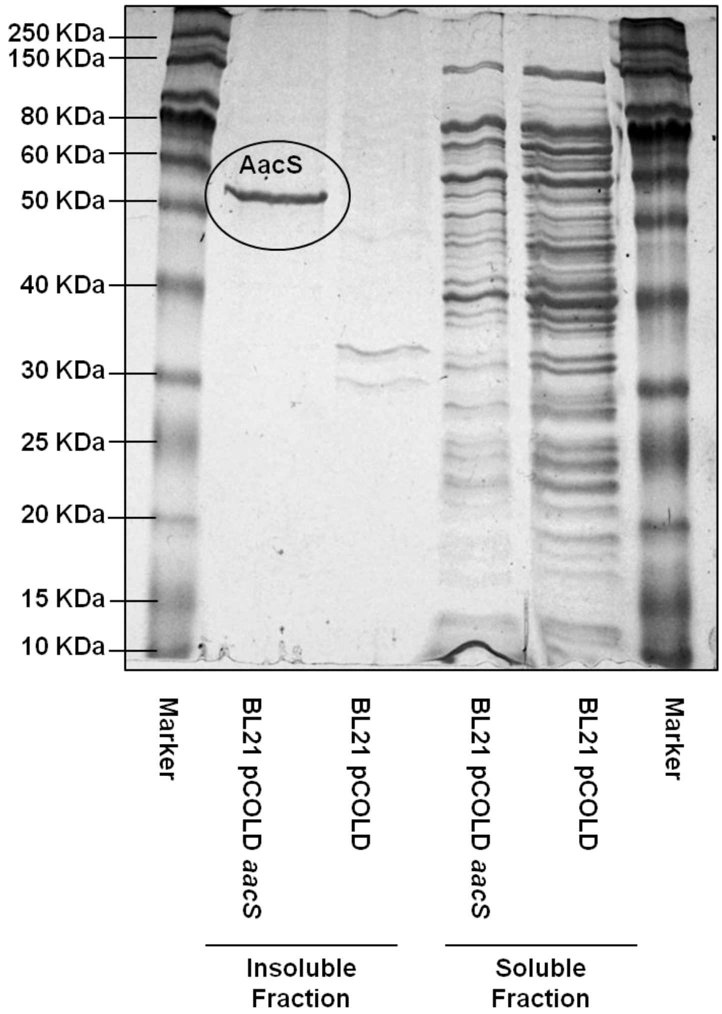


Figure 4.12. Expression of the *S. putrefaciens* **B1 recombinant AHL inactivating enzyme AacS from *E. coli* BL21 pCOLD*aacS*.** Polyacrylamide gel showing soluble and insoluble proteins extracted from both *E. coli* BL21 pCOLD*aacS* and *E. coli* BL21 pCOLD. The expressed AacS protein is marked in the *E. coli* BL21 pCOLD*aacS* insoluble fraction. The protein size marker used was the ColourPlus Pre-stained Protein Ladder, Broard Range 10-230 KDa (*NEB*).

4.2.7.4 AacS AHL Inactivation Specificity Assays

The specificity of AacS was tested by assaying its ability to inactivate a range of different AHLs. *E. coli* DH5α pGEM*aacS* and *E. coli* DH5α pGEM were grown separately to stationary phase in LB media supplemented with different AHLs at a concentration of 1.25 μM. Cell free supernatants from these cultures were extracted using DCM and assayed for AHL inactivation as described in section 2.9.3. Bioluminescence and absorbance was measured over a 12 h period every 30 min and the total amount of bioluminescence as a function of absorbance was plotted against time (Figure 4.13). Extracts from *E. coli* DH5α pGEM grown in media seeded with AHL activated the appropriate bio-reporters as expected. In contrast a complete absence of bio-reporter activation was observed with extracts from *E. coli* DH5α pGEM*aacS* grown in media seeded with C4-HSL, C10-HSL, 3-oxo-C10-HSL, 3-OH-C10-HSL, C12-HSL, 3-OH-C12-HSL and C14-HSL (Figure 4.13 A and C). With the exception of 3-OH-C8-HSL, a reduction in bio-reporter activation was observed with extracts from *E. coli* DH5α pGEM*aacS* grown in media seeded with the remaining AHLs in comparison to extract from *E. coli* DH5α pGEM (Figure 4.13 B). Extracts from the LB culture media and solvent had no effect on the AHL bio-reporters (data not shown).

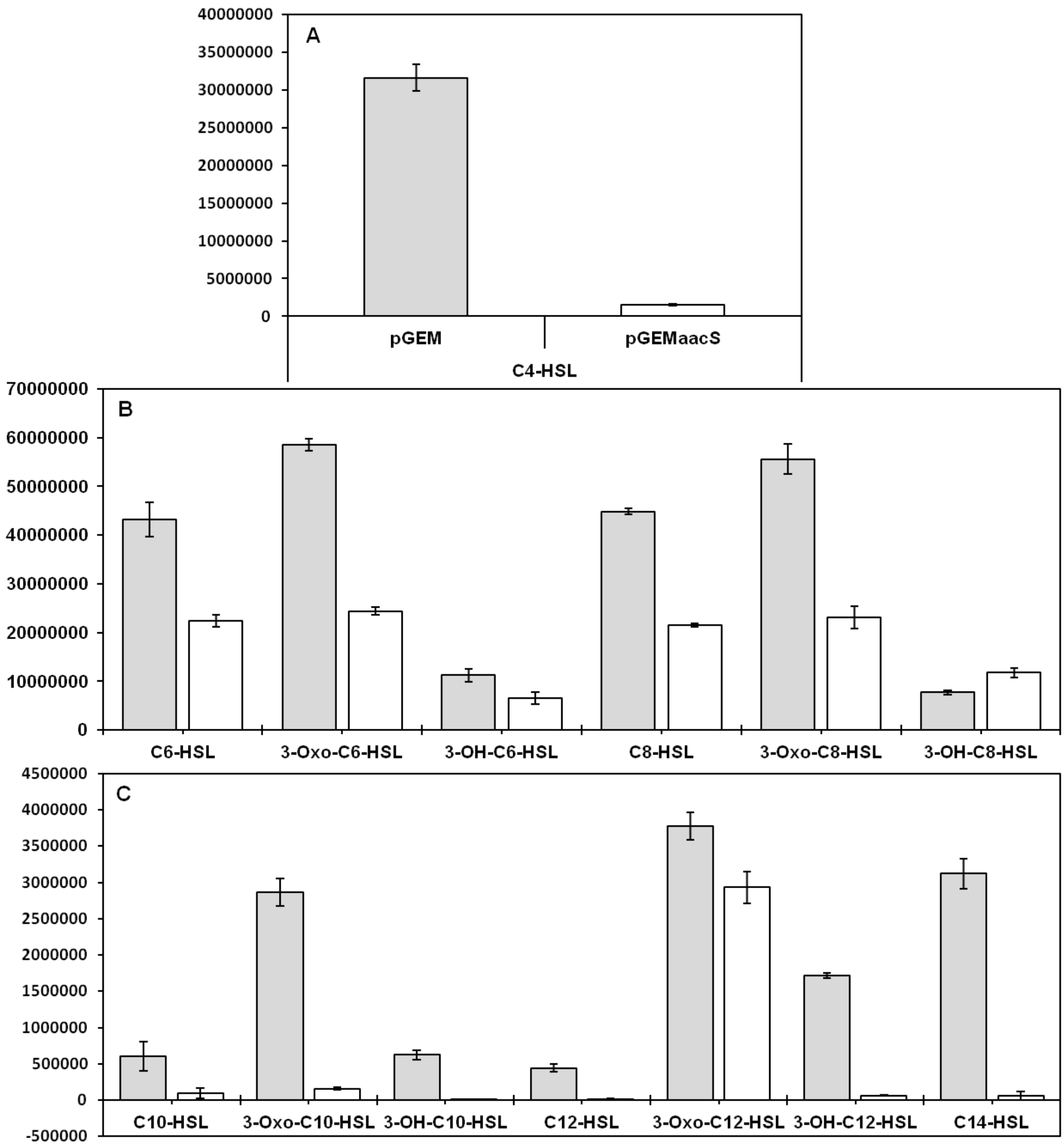


Figure 4.13. AacS AHL Inactivation Specificity Assay. AHL bio-reporter *E. coli* pSB536 (**A**), pSB401 (**B**), and pSB1142 (**C**) activation in the presence of cell free supernatant extracts of DH5α pGEM*aacS* (white bars), DH5α pGEM (grey bars) grown in LB seeded with C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-OH-C6-HSL, C8-HSL, 3-oxo-C8-HSL, 3-OH-C8-HSL, C10-HSL, 3-oxo-C10-HSL, 3-OH-C10-HSL, C12-HSL, 3-oxo-C12-HSL, 3-OH-C12-HSL and C14-HSL. Error bars represent standard deviation from the mean.

In addition to assaying the specificity of AacS to inactivate synthetic AHLs exogenously added to culture media, AacS was also assayed for its ability to inactivate the AHLs produced by the human pathogen *Yersinia pseudotuberculosis*. *Y. pseudotuberculosis* has the ability to produce 24 separate AHL's. Of these 24 only eight were found to be present at levels above 17% of the total AHL output and were therefore produced at abundancies of biological significance (Ortori *et al.*, 2007). These eight are 3-oxo-C6-HSL, 3-oxo-C7-HSL, C6-HSL, 3-OH-C8-HSL, 3-oxo-C8-HSL, C8-HSL, 3-oxo-C10-HSL and C7-HSL (Ortori *et al.*, 2007). The pGEM*aacS* vector was transformed into *Y. pseudotuberculosis* YPIII and the resultant strain YPIII pGEM*aacS* subsequently grown to stationary phase. AHLs from cell free supernatants of YPIII pGEM*aacS*, the parental YPIII (without plasmid) and, to act as a positive control for AHL inactivation in *Yersinia*, YPIII harbouring the AHL lactonase *aiiA* on the pGEM vector were extracted using acidified ethyl acetate. These extracts were assayed for their ability to activate the AHL bio-reporters *E. coli* pSB401 and pSB1142. Extract from YPIII pGEM*aacS* cultures activated the bio-reporter *E. coli* pSB401 to a comparable level as extract from YPIII wt cultures (Figure 4.14). This result indicates that AacS failed to inactivate the shorter chain AHLs produced by *Y. pseudotuberculosis*. Extract from YPIII pGEM*aacS* cultures did not activate the bio-reporter pSB1142, showing comparable levels of bio-reporter activation to extract from the AHL-inactivating YPIII pGEM*aiiA* cultures (Figure 4.14). This indicated that AacS preferentially inactivates the longer chain AHLs produced by *Y. pseudotuberculosis*; a result consistent with AHL inactivation assays using synthetic AHLs.

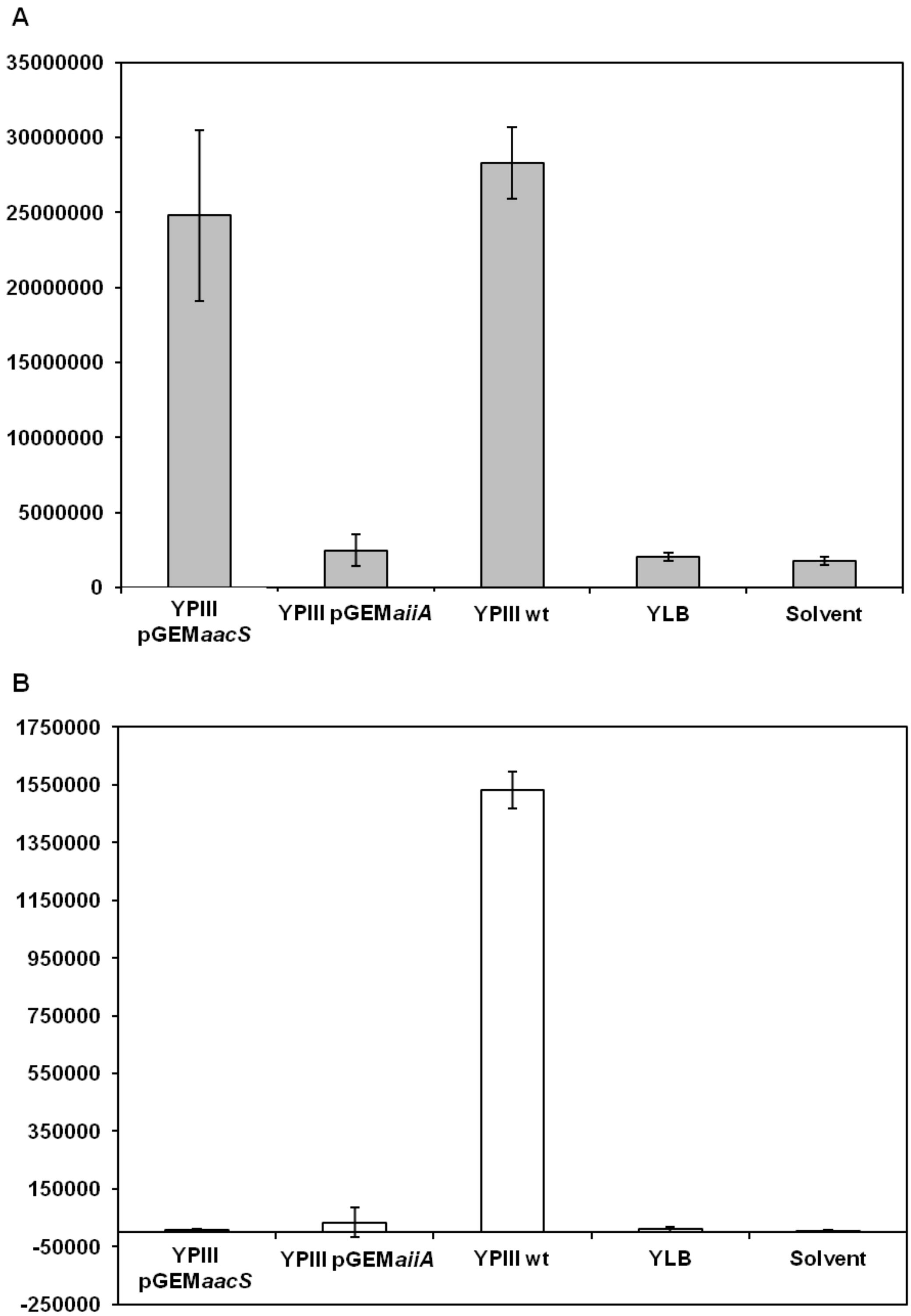


Figure 4.14. AacS AHL inactivation assay within a *Y. pseudotuberculosis* host. *E. coli* pSB401 (**A**) and pSB1142 (**B**) total luminescence as a function of growth in the presence of cell free supernatant extracts of YPIII pGEM*aacS*, YPIII wt and YPIII pGEM*aiiA*. Solvent and media controls are also plotted. Error bars represent standard deviation from the mean.

4.3. Discussion

4.3.1 AHL Production in **Shewanella** *and* **Bacteroidetes** *Strains Abundant in the* **Ulva** *Epiphytic Bacterial Population.*

This chapter reports AHL signal molecule production and inactivation in bacteria belonging to the genus *Shewanella* and the bacterial phylum *Bacteroidetes*. Both either can be isolated from the *Ulva* thallus surface or from other marine environmental niches colonised by *Ulva* spp. In an attempt to identify AHL synthases, response regulators and AHL inactivating protein sequences, this study screened both *Shewanella* and *Bacteroidetes* genomes via *in silico* analysis. Owing to the greater amount of previous work conducted on AHL signalling and inactivation in *Shewanella* spp. and the effects of both these phenotypes on the biology of both *Ulva* spp. and the bacteria present in rocky shore biofilm populations, this study also went on to investigate AHL inactivation within a number of different *Shewanella* stains.

4.3.1.1 AHL Production by the **Bacteroidetes**

The majority of research into AHL-mediated QS has focused on the proteobacterial phylum, specifically in species which are involved in human pathogenesis (reveiwed by Williams *et al.*, 2007). However, in the last few years an increasing number of marine bacterial strains belonging to *Cyanobacteria* and *Bacteroidetes* phyla have been reported to produce AHL signalling molecules (Huang *et al.*, 2009; Sharif *et al.*, 2008). Initial studies reporting AHL signalling in biofilms containing *Bacteroidetes* bacteria and strains of the *Bacteroidetes* genus *Flavobacterium* presented conflicting results and were limited in their detection methods; relying on the activation of AHL bio-reporters to prove the existence of AHL signalling mechanisms (Huang *et al.*, 2009; Wagner-Döbler *et al.*, 2005). This study and others has found that the sole use of bio-reporters to confirm AHL signalling is unreliable when analysing marine strains. This is due to the conditions of the marine culture media such as the increased pH affecting bio-reporter activation due to the hydrolysis of AHLs (Romero *et al.*, 2010; Tait *et al.*, 2005). True confirmation of AHL signal molecule production requires chemical analysis of cell free supernatant extracts using high performance liquid or gas chromatography coupled with mass spectroscopy (Ortori *et al.*, 2007). Presently, AHL production by *Bacteroidetes* has only been proved unequivocally using LCMS in *T. maritimum,* which has been confirmed to be a producer of C4-HSL (Romero *et al.*, 2010). Using LCMS as a detection method for AHL production, this study has extended the range of *Bacteroidetes* species proven to be producing AHLs to include *Cellulophaga* strains isolated from the *Ulva* thallus and other *Bacteroidetes* strains from varying taxonomic backgrounds (Table 3.3 and Figure 4.4). Having shown AHL production in the *Sphingobacteria* strain *Lewinella marina* and the *Cytophaga* strains *Cytophaga marinoflava*, *Algoriphagus chordate*, *Flexibacter aggregans* and *Saprospira grandis* this is additionally the first study to demonstrate that *Bacteroidetes* strains outside of the *Flavobacteria* class produce AHLs. This study has shown that the *Bacteroidetes* make up a substantial percentage of the epiphytic bacterial community of the green seaweed *Ulva* (Figure 3.5). Previous work has also shown the *Bacteroidetes* to be prevalent in biofilm communities on the rocks where *Ulva* spp. are found colonising (Tait *et al.*, 2009). *Ulva* zoospore settlement, *Ulva* zoospore germination and the early growth of the *Ulva* germling is affected by the production of AHLs by the bacteria in the plants proximal environment, (Tait *et al.*, 2005; Joint *et al.*, 2002). Owing to the prevalence of *Bacteroidetes* in this proximal environment and the evidence of AHL production by a wide range of *Bacteroidetes* strains from varying phylogenetic backgrounds, it is therefore likely that the *Bacteroidetes* play a significant role in *Ulva* spp. growth and development.

Interestingly, the only AHL being found to be produced by *Bacteroidetes* strains assayed in this study was the short chain C4-HSL. C4-HSL production appears to be ubiquitous within the *Bacteroidetes* phylum as *T. maritimum* was also shown to be only producing C4-HSL, (Romero *et al.*, 2010). The sole production of C4-HSL is at odds with other marine bacterial strains which tend to produce AHLs with longer acyl side chains (Tait *et al.*, 2009). AHLs with acyl short side chain, such as C4-HSL, have been shown to be rapidly hydrolysed by the alkaline pH of seawater rendering them biologically inactive (Hmelo and Van Mooy, 2009; Yates *et al.*, 2002). As such the production of C4-HSL by *Bacteroidetes* may be to facilitate communication between cells within close proximity to each other potentially limiting the possibility of 'eavesdropping' by competitive bacterial species. The production of a single type of signal molecule makes the *Bacteroidetes* differ from proteobacterial species such as marine *Vibrio* spp., which in general produce two specific types of AHL, and human pathogens such as *Y. pseudotuberculosis* which has been shown to produce a range of different AHLs (Atkinson *et al.*, 2006; Milton, 2006).

4.3.1.2 Speculative Role of AHL Signalling in the **Bacteroidetes** *and* **Shewanella sp.**

The primary purpose of bacterial AHL production is to regulate gene expression and therefore various phenotypes in a cell density-dependent manner (Williams *et al.*, 2007). Many proteobacterial phenotypes have been connected to AHL-mediated QS and in the cyanobacterial phyla AHLs have been shown to regulate alkaline phosphatases, affecting phosphorus acquisition in *Trichodesmium* consortia (Van Mooy *et al.*, 2012; Williams *et al.*, 2007). Currently no phenotype within *Shewanella* spp. or the *Bacteroidetes* phylum has been shown to be regulated by AHLs; however a number of potential candidate phenotypes can be proposed. Flavobacterial species such as *Flavobacterium johnsoniae* and *Flavobacterium psychrophilum* exhibit rapid gliding motility linked to a novel protein secretion mechanism (Sato *et al.*, 2010; Jarrell and McBride, 2008). *Shewanella* is also a highly motile bacterial species with swimming motility being conferred by the presence of a single polar flagella (Paulick *et al.*, 2009). Motility is a phenotype widely regulated by AHL-mediated quorum sensing in a number of proteobacterial species including *Y. pseudotuberculosis*, *P. aeruginosa* and *Serratia liquefaciens* (Atkinson *et al.*, 1999; Glessner *et al.*, 1999; Lindum *et al.*, 1998). Swimming motility, the type of motility exhibited by *Shewanella* has been conclusively shown to be regulated by QS in *Y. pseudotuberculosis*, where the AHL-mediated QS system YpsR/I controls the expression of FlhDC, the flagella master regulator (Atkinson *et al.*, 2008). Similar regulatory mechanisms with regard to swimming motility could account for AHL production in *Shewanella* spp. In addition motility similar to the type reported to be exhibited by *Flavobacteria* has also been reported to be regulated by AHL-mediated quorum sensing systems in *Serratia marcescens* (Morohoshi *et al.*, 2007). Virulence factor production may also be a candidate as to why these two groups of bacteria are producing AHLs. A number of *Bacteroidetes* strains such as *F. psychrophilum* are implicated in fish disease and have been shown to produce virulence factors such as secreted proteases and cytolytic toxins (Duchaud *et al.*, 2007; Nematollahi *et al.*, 2003). The regulation of similar virulence factors by

AHL-mediated quorum sensing systems in a range of bacterial pathogens has been widely reported (Williams, 2007). If *Bacteroidetes* virulence is regulated by AHL-mediated QS it could be of importance not just in marine ecosystems but also in the field of human disease due to the prevalence of *Bacteroidetes* bacteria in intestinal and dental infections, (Tanaka *et al.*, 2008; Lassmann *et al.*, 2007). As little is known about *Shewanella* pathogenesis this study is unable to connect AHL production to virulence factor expression within this group of bacteria. Another *Bacteroidetes* phenotype that could potentially be regulated by AHL-mediated quorum sensing is pigment production. In general *Bacteroidetes* species are reported to be highly pigmented, (Kirchman, 2002), a characteristic also observed in the strains assayed for AHL production by this study. Pigment production has been reported to be connected to AHL-mediated quorum sensing in a number of bacteria including violacein production in *C. violaceum* and pyocyanin production in *P. aeruginosa*, of which both pigments additionally act as antimicrobials (Dietrich *et al.*, 2006; McClean *et al.*, 1997). Secondary metabolite and exoenzyme production and export are regulated by AHL-mediated QS in a number of different bacteria (reviewed by Williams *et al.*, 2007). *Shewanella* species such as *S. putrefaciens* produce and export siderophores, which are utilised in iron accession (Gram, 1994). AHL-mediated QS has been implicated in the regulation of siderophore production in *Burkholderia pseudomallei* and *P. aeruginosa* (Song *et al.*, 2005; Stintzi *et al.*, 1998). *Bacteroidetes* bacteria have also been shown to produce exoenzymes that facilitate the degradation of complex organic matter such as chitin and cellulose (Teeling *et al.*, 2012; Cottrell and Kirchman, 2000). As the production of exoproteases and other exoenzymes has been reported to be regulated by AHL-mediated QS in marine bacteria such as *Aeromonas hydrophyla* and *Aeromonas salmonicida*, potential QS regulation of this phenotype in the *Bacteroidetes* is not unfounded (Swift *et al.*, 1999). AHL production in *Shewanella* spp. may also be used in the regulation of organism's novel metabolic processes such as the use of nitrate and nitrites. Nitrogen metabolism has been shown to be regulated by QS in *P. aeruginosa* (Juhas *et al.*, 2004). It is however important to stress that currently these candidate phenotypes are only speculations as to why both *Shewanella* and *Bacteroidetes* strains are producing AHLs; further phenotypic analysis of these bacteria would be required to link any of these behaviours to AHL production.

4.3.2 AHL Synthase, AHL Response Regulator and AHL Inactivating Enzymes in **Shewanella** *and the* **Bacteroidetes**

Although this study and previous work have established AHL production in both *Shewanella* spp. and the *Bacteroidetes* phylum, as yet no AHL synthase or response regulator has been identified in these two groups of bacteria (Romero *et al.*, 2010; Huang *et al.*, 2009; Tait *et al.*, 2009). One putative AHL synthase sequence has been identified in the flanking DNA upstream of the *lux* operon in *S. hanedai*, however AHL production by the product of this gene has not been investigated and the sequence of this gene does not appear in other *Shewanella* genomes (Kasai *et al.*, 2007). This study screened 19 separate *Shewanella* and 180 separate *Bacteroidetes* genomic sequences in an attempt to identify putative AHL synthases and response regulators but failed to identify any sequences with homology to LuxI in either *Shewanella* or the *Bacteriodetes*. Screening for AHL response regulator homologues produced a number of results, however in both the *Shewanella* and the *Bacteroidetes* genomes the resultant hits did not possess AHL binding domains so therefore cannot be classed as AHL transducing proteins. Only one identified LuxR hit, found in genome of *S. violacea,* was shown to possess both an AHL binding domain and a LuxR type DNA binding domain. However, this *S. violacea* genome lacked any ORF with homology to *luxI*. This putative *S. violacea* LuxR sequence therefore maybe an orphan LuxR homologue, which has the ability to detect and transduce exogenously produced AHL signals, similar to those reported in *P. aeruginosa*, *E. caratova* and *E. coli* (Barnard and Salmond, 2007; Chugani *et al.*, 2001). Further investigation, *in vivo*, using *S. violacea* would need to be conducted to confirm this theory. Similar false positive results were seen when screening *Shewanella* and *Bacteroidetes* genomes for two component AHL response regulators similar to the LuxN system in *V. harveyi* (Bassler *et al.*, 1993). *Bacteroidetes* genomes that possessed hits to the LuxN consensus lacked the constitutive LuxM type AHL synthases. A speculative theory that these hits may be 'orphan' LuxN homologues is not plausible as these genomes also did not contain LuxO and LuxU homologues, the components essential for the function of LuxN as an AHL receptor/response regulator (Freeman and Bassler, 1999a; Freeman and Bassler, 1999b). It is therefore credible to assume that both *Shewanella* and the *Bacteroidetes* possess novel and yet to be identified AHL synthase/response regulator systems.

In general, AHLs are biologically inactivated by two classes of enzyme; AHL lactonases which catalyse the hydrolysis of the homoserine lactone ring and AHL acylases which facilitate the cleavage of the fatty acid side chain from the homoserine lactone ring (Dong *et al.*, 2000; Leadbetter and Greenberg, 2000). AHL inactivation has been identified in a number of marine bacteria including two species of *Bacteroidetes*; *T. maritimum* and *T. discolor* (Romero *et al.*, 2011; Romero *et al.*, 2010). This study screened *Bacteroidetes* genomic sequences for putative AHL lactonase and AHL acylase sequences. Genomic screening revealed a number of hits to both AHL lactonase and AHL acylase consensus sequences. Further bioinformatic analysis of these sequences revealed conserved amino acid residues to be present that are integral for AHL lactonase and acylase activity (Lin *et al.*, 2003; Dong *et al.*, 2002). The presence of these integral conserved residues within the identified hits supports the idea that these sequences are putative novel AHL inactivating enzymes. In order to confirm these hits as true AHL lactonase or acylase enzymes, AHL inactivation assays would need to be carried out. The putative AHL inactivating enzyme sequences identified in *Bacteroidetes* genomes by this study were however, not found in the genomes of strains known to be either producing or degrading AHLs. As this study failed to identify any AHL lactonase or AHL acylase sequences within the genomes of *Bacteroidetes* strains currently reported to be either actively producing or inactivating AHLs, it is credible to assume that *Bacteroidetes* bacteria possess novel AHL inactivating enzymes yet to be alluded.

4.3.3 AHL Inactivation by **Shewanella** *Aculeacin A Acylase.*

AHL inactivation has additionally been reported in *Shewanella* spp., where long chain AHLs are rapidly inactivated during the stationary growth phase (Tait *et al.*, 2009). The study by Tait *et al.* also identified that AHL inactivation by *Shewanella* spp. takes place through the actions of an acylase enzyme. This method of AHL inactivation was first identified using AHL bio-reporters and then confirmed in HPLC experiments which showed a reduction in 3-oxo-C12-HSL coupled to a rise in homoserine lactone levels after incubation with *Shewanella* sp. BL21 (Tait *et al.*, 2009). There has also been one study to identify an AHL acylase in a *Shewanella* strain isolated from fish intestinal tracts. Morohoshi *et al.* (2008) reported that the aculeacin A acylase gene (*aac*) cloned from *Shewanella* sp. MIB015 possessed the ability to inactivate AHLs and shared 26.8% identity with the AHL acylase AiiD from *Ralstonia* sp. XJ12B (Morohoshi *et al.*, 2008; Lin *et al.*, 2003). *In silico* analysis carried out in this study revealed a number of putative AHL acylase sequences in genomes from various different *Shewanella* species including *S. ondeiensis*, *S. putrefaciens*, *S. baltica* and *S. amonzoniensis*. Many of these sequences also possessed high levels of identity to Aac from *Shewanella* sp. MIB015. Owing to these high levels of sequence identity, this study screened a number environmental isolates for homologues of the *aac* gene using PCR primers derived from the sequences identified during *in silico* screening. PCR screening showed that the majority of the strains assayed did possess *aac* homologues and, as such, it can be inferred that the *aac* gene is highly conserved within the *Shewanella* genus. The *aac* gene from a *Shewanella* strain, B1, (identified as most closely related to *S. putrefaciens*), *aacS*, was selected for further study. AacS was shown to possess 33% identity with the AHL acylase PvdQ found in multiple *Pseudomonas* species (Huang *et al.*, 2003). Purification of AacS with the intention to carry out AHL inactivation assays was attempted. However, expression of AacS resulted in the protein being present within the insoluble fraction of the protein extraction and, due to time constraints, it was not possible to carry out the purification of this protein. Interestingly, the purified AacS protein expressed in this study was smaller than the size predicted by analysis of the AacS sequence. This may be due to post-translational modification of the polypeptide. Previous studies have indicated that AHL acylase proteins are modified and that a serine-glycine paring within the acylase is integral to this modification (Lin *et al.*, 2003). To confirm that the AacS protein expressed using the pCOLD system by this study is an active AHL acylase, it may be possible to run the protein through a non-denaturing gel and overlay the gel with a bio-reporter that has the ability to detect AHL inactivation.

The AHL inactivating activity of AacS was however assayed using the recombinant clones, harbouring *aacS* within the pGEM vector. AacS was shown to inactivate a wide variety of AHLs with the exception of 3-OH-C8-HSL. Inactivation was found to be most pronounced in AHLs with long acyl side chains. In addition to the inactivation of synthetic long chain AHLs, AacS also inactivated the long chain AHLs produced by *Y. pseudotuberculosis*, however had little to no effect on the other AHLs this bacterium produces. Inactivation of long chain AHLs is consistent with previous results that showed long chain AHL inactivation in *Shewanella* biofilms and with the activity of AHL acylases PvdQ from *P. aeruginosa* and AiiD from *Ralstonia* sp. XJ12B (Tait *et al.*, 2009; Huang *et al.*, 2003; Lin *et al.*, 2003). AHL inactivation by Aac homologues does not however appear to be consistent in all *Shewanella* strains as previous work demonstrated that the Aac from *S. oneidensis* MR1 did not show AHL inactivating activity, as such it is likely that *Shewanella* spp. may possess another AHL acylase (Tait *et al.*, 2009).

In general, bacteria inactivate AHLs in order to gain a competitive advantage over other bacteria growing within a particular environmental niche (Dong *et al.*, 2004). For example *Bacillus thuringiensis* produces an AHL lactonase that has the ability to inhibit AHL signalling in *E. carotovora*. The inactivation of this signal results in the 'disarming' of *E. carotovora* as it inhibits antibiotic and virulence factor expression preventing the spread of *E. cartovora* and therefore conferring *B. thuringiensis* with an advantage (Dong *et al.*, 2004). AHL acylase production by *Shewanella* spp. has been shown to inactivate the AHL signals produced by bacteria found growing in the same environmental niche (Tait *et al.*, 2009). It is therefore possible that *Shewanella* produces AHL inactivating enzymes in order to gain a competitive advantage over these bacteria. This still however poses the question of why does *Shewanella* inactivate its own AHL signal molecules? As *Shewanella* appears to inactivate its cognate signal during stationary phase it may be that inactivation of signal is to prevent autoinduction of AHLs which are no longer required during this growth stage, therefore preventing the metabolic burden associated with AHL production. An example of a bacterium using an AHL inactivating enzyme to turn over its cognate AHL signal molecules for this purpose is not unprecedented. *Agrobacterium tumefaciens* inactivates cognate 3-oxo-C8-HSL production using an AHL lactonase, AttM (Carlier *et al.*, 2003). As with *Shewanella* AHL inactivation, AHL inactivation by AttM also takes place during stationary phase and in this case is triggered by a stress alarmone (p)ppGpp (Zhang *et al.*, 2004). This inactivation of cognate AHLs terminates energy consuming processes regulated by AHL-mediated QS and allows the *A. tumefaciens* to timely adapt to starvation stress (Zhang *et al.*, 2004). Another explanation of *Shewanella* cognate AHL inactivation may also be that *Shewanella* inactivates its long chain AHLs at stationary phase in order to switch to the regulation of gene expression by short chain AHLs, as it is these short chain AHLs that are found in mature *Shewanella* biofilms (Tait *et al.*, 2009). As with potential roles of AHL production in *Shewanella* presented in section 4.3.1.2, the purposes for AHL inactivation by *Shewanella* are only speculations and require proving via experimental investigation.

4.3.4 Summary Conclusions and Future Directions

In summary, this study has further extended the range of *Bacteroidetes* bacteria known to be producing AHLs. This study has also further investigated AHL inactivation by *Shewanella* bacteria and shown that the AHL acylase Aac is highly conserved in *Shewanella* spp. and is responsible for long chain AHL inactivation. In spite of this, our knowledge of AHL signalling and QS in *Shewanella* and the *Bacteroidetes* phylum is still in its infancy as no AHL synthase and response regulator systems have yet been identified within these two groups of bacteria. Further research into AHL production and QS in both *Shewanella* and the *Bacteroidetes* would be of benefit to our understanding of marine ecosystems due to the abundance of these groups of bacteria within the marine environment. In addition to furthering our understanding of the marine environment researching AHL production and QS in the *Bacteroidetes* may provide benefit to medical science, as these bacteria are responsible for a large number of common infections.

Research in this area should progress in the following directions:

- Carry out mutation of AacS in *S. putrafaciens* B1. Construction of an AacS knock out mutant has been initiated by this study, however is not discussed in this thesis. An AacS mutation construct has been constructed via PCR amplification of an 80 bp region in the centre of *aacS* which was ligated into the suicide vector pKNOCK, (Alexeyev, 1999), in order to perform homologous recombination, an approach previously shown to successfully construct knock out mutants in *Shewanella* spp. (Bodor *et al.,* 2011). A potential *S. putrafaciens* B1 Δ*aacS* strain has been constructed but not tested.

- Assay *Shewanella* sp. Δ*aacS* mutant for AHL production at all stages of growth using the method outlined in section 2.9.2 and 2.9.3 to confirm AacS as the AHL inactivating enzyme in *Shewanella* and to identify if AacS is the only AHL inactivating enzyme present.
- Use *Shewanella* sp. Δ*aacS* mutant to identify *Shewanella* spp. AHL synthase and response regulators using a transposon library or genomic library approach as previously used in enteric bacteria (Hao *et al.*, 2010; Swift *et al.*, 1993).
- Screen an AHL-producing *Bacteroidetes* strain for AHL synthase and response regulators using a transposon library or genomic library approach as previously used in enteric bacteria (Hao *et al.*, 2010; Swift *et al.*, 1993).

Chapter 5

Quorum-quenching Activity in Microalgae

5.1. Introduction

The inhibition of bacterial QS has been proven to affect an array of phenotypes such as virulence factor expression (Dong *et al.*, 2000). The targeting and subsequent inhibition of key components in QS systems is often referred to as quorum-quenching (reveiwed by Zhang, 2003). Quorum-quenching has been shown to be achieved either by the production of AHL inhibiting enzymes such as AHL lactonases and AHL acylases discussed in Section 1.3.2 or chemically by AHL inhibiting compounds (reveiws by Dong and Zhang, 2005, Zhang, 2003). Such compounds have been identified to be produced by a number of different organisms including other bacteria, terrestrial plants and marine eukaryotes (reveiwed by Zhang, 2003). The production of quorum-quenching compounds by marine microalgae and its potential effects on both human and marine pathogens is the focus of the data presented in this chapter.

5.1.1. Algal Compounds have the Ability to Inhibit Bacterial Quorum Sensing

Bacterial/algal associations have been studied for a long period of time and as such interactions between these two kingdoms have been well documented. A number of these interactions are discussed previously (Sections 1.2.2, 3.1.1, 3.1.3 and 3.3.3). Recent studies however have focused on bacterial/algal interactions involving bacterial signalling. Algae have been shown to interfere with bacterial QS in order to inhibit colonisation, which may lead to infection (Teplitski *et al.*, 2004, Hentzer *et al.*, 2002). Algae accomplish this interference using two methodologies; either by producing molecules that disrupt bacterial QS by quorum-quenching activity or by producing molecules that mimic bacterial QS signals (Teplitski *et al.*, 2004, Manefield *et al.*, 2002). Algal quorum-quenching behaviour was first observed in a species of macroalgae, the red seaweed *Delisea pulchra* which was shown to produce halogenated furanones which are chemically similar to AHLs (Manefield *et al.*, 1999). The furanone compounds were proven to affect QS regulation of the motility master operon *flhDC* within *Serratia liquefaciens*, reducing swarming motility (Rasmussen *et al.*, 2000). Halogenated furanone disruption of QS was further confirmed by observations that furanones produced by *D. pulchra* reduced *V. fischeri* bioluminescence by 50 to 100 fold, a clear example of quorum-quenching activity (Rasmussen *et al.*, 2000). Halogenated furanone compounds are produced by *D. pulchra* within vesicles in the algas thallus and are then exported out onto the surface of the thallus and are in greatest concentration at the apical tips of the plant (Dworjanyn *et al.*, 1999). Manefield *et al.* (2002) proposed a biochemical mechanism by which furanone compounds produced by *D. pulchra* disrupt QS regulated gene expression. Manefield suggests that furanones interact with the signal response regulator LuxR forming a LuxR-Furanone complex. This complex causes a conformational change within LuxR leading to its increased susceptibility to proteolytic degradation within the bacterial cytosol, thus abolishing or severely disrupting QS gene regulation (Manefield *et al.*, 2002). Halogenated furanones have also been shown to effect virulence factor expression in *P. aeruginosa* and reduce the expression of genes thought to be regulated by AI-2 in both *E. coli* and *Salmonella enterica* (Janssens *et al.*, 2008, Ren *et al.*, 2004, Hentzer *et al.*, 2002). It is not only *D. pulchra* which has the ability to disrupt QS, the bryozoan *Flustra foliacea* has also been shown to produce brominated alkaloid compounds. The brominated alkaloid compounds have been proven to reduce the growth of bacteria isolated from *F. foliacea*. Using a *P. aeruginosa* model the brominated alkaloids were shown to block AHL-dependent QS and have a phenotypic effect on the bacteria by reducing extracellular protease production (Peters *et al.*, 2003). Oxidised halogen compounds produced by many marine macroalgae have been shown to affect bacterial biofilms (Wever *et al.*, 1991). Borchardt *et al.* (2001) showed that such compounds also react with 3-oxo substituted AHLs forming halogenated homoserine lactone and fatty acids (Borchardt *et al.*, 2001). In the same study oxidised bromide compounds produced by the brown alga *Laminaria digitata* were shown to react and therefore biologically inactivate AHLs produced by *P. aeruginosa* biofilms (Borchardt *et al.*, 2001). QS inhibitory compounds effecting C8-HSL and the *A. tumefaciens* TraR AHL response regulator have also been shown to be produced by the red algae *Ahnfeltiopsis flabelliformis* (Liu *et al.*, 2008). A broad ranging study was undertaken by Skindersoe *et al.* (2008) investigating QS inhibition using extracts from 284 marine organisms in conjunction with the Quorum Sensing Inhibitor Selector (QSIS) systems described in section 4.2.7 (Skindersoe *et al.*, 2008, Rasmussen *et al.*, 2005). This study showed that 23% of the 284 extracts tested showed some QS inhibitory activity including 5 out of 35 algal extracts (Skindersoe *et al.*, 2008). The study also went on to characterise QS inhibition by three C_{25} sesterterperne metabolites produced by the marine sponge *Luffariella variabilis* (Skindersoe *et al.*, 2008).

Less research has been carried out on the effect of molecules produced by microalgal species, in spite of studies showing complex bacterial communities to be present on these algae (Fukami *et al.*, 1997). One example of such behaviour has been demonstrated in the freshwater microalgae *Chlamydomonas reinhardtii*, which has been shown to produce a number of molecules that stimulated the AHL response regulators LasR (*P. aeruginosa*) and CepR (*Pseudomonas putida*). These compounds did not show any effect on other AHL response regulators including LuxR (*V. fischeri*), AhyR (*A. tumefaciens*) and CivR (*C. violaceum*), indicating that the mimic activity was receptor specific (Teplitski *et al.*, 2004). Owing to their abundance in both the marine and freshwater environments and the increasing economical importance of microalgae, further research into their interactions with bacteria is warranted.

5.1.2. Microalgae, Ecology and Biotechnology

5.1.2.1 Microalgal Ecology

Microalgae are eukaryotic, unicellular algal species (reveiwed by Satyanarayana *et al.*, 2011). In the marine environment microalgae are found existing either individually, in chains, or in groups and are therefore classed as part of the marine phytoplankton (reveiws Satyanarayana *et al.*, 2011; Munn, 2004). Microalgal metabolism is driven by photosynthesis; as such these organisms are limited to the photic zone of the marine water column (Munn, 2004). The photosynthetic activity of marine microalgae, (including cyanobacterial *species*), accounts for the majority of carbon fixation in the marine environment and approximately 50% of global carbon fixation (Munn, 2004). When living, microalgae can be prey for zooplankton and other higher marine organisms and in death microalgal organisms provide carbon sources for heterotrophic marine microbes in the form of dissolved organic matter (Munn, 2004). These key roles in carbon fixation and in the biological marine carbon cycling makes microalgae a significant group of organisms in the study of marine ecosystems and ocean processes. In addition to being found in the marine environment microalgae can additionally be found in fresh water environments (Harris, 2001).

5.1.2.2 Usage of Microalgae in Aquaculture and Industry

Microalgae or the metabolic products of microalgae are already used in a number of biotechnological applications including use in the cosmetics industry, food industry, aquaculture and use as biofuels. In the cosmetics industry frozen microalgal bio-matter is utilised in the production of anti wrinkle creams and the high proportions of carbohydrates, proteins, omega-3 and omega-6 oils produced by microalgae are utilised in the food industry (reveiwed by Guil-Guerrero *et al.*, 2004). Microalgae can be used in the production of biofuels, where lipids produced by microalgae are utilised as replacements for fossil fuels in the production of diesel or utilised in the production of bio-ethanol; a substitute for petroleum in the production of motor fuel (John *et al.*, 2011, Li *et al.*, 2008). The main economic purpose of growing microalgal species is however for usage in aquaculture (reveiwed by Borowitzka, 1997). Aquaculture can be defined as the farming of aquatic organisms such as fish, molluscs, crustaceans and aquatic plants. Statistics provided by the 2012 United Nations Food and Agriculture Organisation (UNFAO) report into world fisheries and aquaculture show inland and marine aquaculture production to have risen from 47.3 million tonnes in 2006 to 63.6 million tonnes in 2011. Total aquacultural algal production has also seen a distinct rise from 3.8 million tonnes in 1990 to 19 million tonnes in 2010 providing an economic value of $5.7 billion in 2010 to the global economy (UNFAO, 2012). The majority of this production is related to the farming of macroalgal species, which are farmed for hydrocolloids and in both animal and human food production (Abbott, 1996, Bixler, 1996). However the production of marine microalgae in aquaculture has also seen a rise in the last 20 years with a total of 3.1 million tonnes produced in 2010, (UNFAO, 2012). Aquacultural production of microalgae has multiple uses including; food sources or feed additives in the commercial rearing of aquatic animals for direct human consumption; food sources for commercial fish species at the larval stage; food sources for rearing rotifers which are intern used has a food in aquacultural fisheries and for the quality enhancement of aquacultural produce by providing pigments such as astaxanthin (Chien and Shiau, 2005; Borowitzka, 1997). Microalgal cultures can be reared from two sources either from natural populations or from unialgal cultures (Austin and Day, 1990; New, 1990). A great deal of research has been focused on the usage of optimal strains of microalgae in order to gain the highest levels of aquacultural production at reduced economic cost to the producer. This has lead to the discoveries of microalgal strains with specific biological properties that are of benefit to aquaculture such as high lipid containing strains of *Tetraselmis* spp. which have been shown to enhance the growth of oyster larvae (Wikfors *et al.*, 1996).

5.1.3. Experimental Aims

This study investigated three microalgal species indigenous to the marine environment: *Nannochloropsis oculata*, *Tetraselmis suecica* and *Isochrysis galbana*. These species were selected for study due their importance in aquaculture in the rearing of rotifers, crustaceans and molluscs respectively (Borowitzka, 1997). By further understanding the biology of these commercially significant species, particularly focusing on how they may interact with bacterial populations, it may be possible to exploit such interactions for commercial benefit. Based on research discussed previously, this study investigated the possibility that these three microalgal species interact with bacteria by modulating bacterial QS (Teplitski *et al.*, 2004, Manefield *et al.*, 2002). As such, extracts from axenic cultures of all three species were assayed for their ability to produce QS mimic molecules or actively quench bacterial AHL-mediated signalling.

5.2. Results

5.2.1. Activation of AHL Bio-reporters by Microalgal Extracts

Axenic *N. oculata*, *T. suecica* and *I. galbana* cultures were subjected to solvent extraction as described in Section 2.10.1. The resultant extracts were concentrated via rotary evaporation and assayed for their ability to produce bioluminescence in the *lux*-based AHL bio-reporters *E. coli* DH5α pSB536, JM109 pSB401 and JM109 pSB1142. If the extracts contained compounds that interacted with and activated the various AHL response regulator genes contained in these reporters, bioluminescence would occur due to the transcription of a *lux* cassette also contained in these reporters (Winson *et al.*, 1998, Swift *et al.*, 1997). The bio-reporters produced approximately the same levels of bioluminescence after co-incubation with extracts from all three microalgal species as co-incubation with extracted F/2 media and solvent negative controls (Figure 5.1). A minor increase in the bioluminessence produced in pSB536 was observed after exposure to the microalgal extracts in comparrsion to the solvent and media controls (Figure 5.1). This rise is however thought to be due to pSB536 possesing a 'leaky' promoter and not due actual activation of the bio-reporter.The AHL bio-reporters did however produce bioluminescence when exposed to appropriate synthetic AHLs: C4-HSL, C6-HSL and 3-oxo-C12-HSL, respectivly (Figure 5.1).

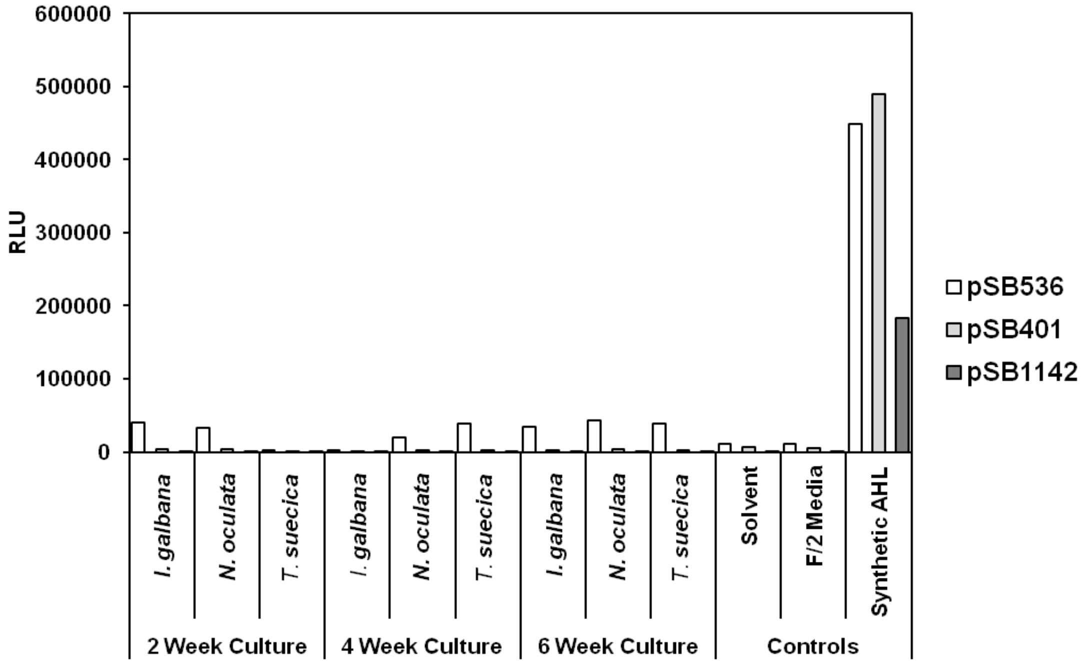


Figure 5.1. Activation of AHL bio-reporters by microalgal extracts. AHL bio-reporters exposed to extracts from *N. oculata*, *T. suecica* and *I. galbana* cultures grown for 2, 4 and 6 weeks produced levels of bioluminessence comparative to both solvent and F/2 media controls, indicating a lack of any compound within the extract that could activate these reporter stains. Synthetic AHL controls (C4-HSL, C6-HSL and 3-oxo-C12-HSL) activated all three bio-reporters, indicating that the reporters were acting as expected.

5.2.2. Quorum-quenching Activity by Marine Microalgal Cultures

N. oculata, *T. suecica* and *I. galbana* extracts were also assayed for the ability to disrupt bioluminescence in the *E.coli lux*-based AHL bio-reporters in the presence of exogenously added AHLs. This was achieved by co-incubating *E.coli* pSB536, pSB401 and pSB1142 with C4-HSL, C6-HSL and 3-oxo-C12-HSL respectively in the presence of extracts from the three microalgal cultures grown for 2, 4 and 6 weeks.

In the presence of synthetic AHLs, the F/2 media control extracts had no effect on bioluminescence (Figures 5.2-5.4), whereas a number of the microalgal extracts repeatedly had an effect on the levels of bioluminescence produced by the AHL bio-reporters in the presence of synthetic AHLs. Significantly reduced levels of bioluminescence were observed using the bio-reporter pSB536 in the presence extracts from 2 wk old cultures of *I. galbana* and extracts from 4 wk old cultures of *T. suecica* when compared to the F/2 media control (2-Tailed T Test $P < 0.01$) (Figure 5.2). When exposing the bio-reporter pSB401 to extracts from 2 and 4 wk old cultures of *T. suecica* significantly reduced bioluminescence was observed in comparison to F/2 media extracts (2-Tailed T Test $P < 0.01$) (Figure 5.3). Allthough not found to be significant, reductions in pSB401 bioluminessence after exposure to extracts from 6 wk old cultures of *I. galbana* and extracts from 2 and 6 wk old cultures of *N. oculata* in comparison to F/2 media extracts was also apparent (Figure 5.3). Finally, significantly reduced levels of bioluminescence were observed in the bio-reporter pSB1142 after exposure to extracts from 2, 4 and 6 wk old cultures of *I. galbana*; extracts from 2 and 6 wk old cultures of *N. oculata* and extracts from 2, 4 wk old cultures of *T. suecica*, in comparison with F/2 media extracts (2-Tailed T Test $P < 0.01$) (Figure 5.4). Extracts from both F/2 media and the microalgal cultures did not affect the growth of any of the AHL bio-reporters used in this assay and the solvent used to reconstitute these extracts did not affect the AHL bio-reporters (Figure 5.2-5.4).

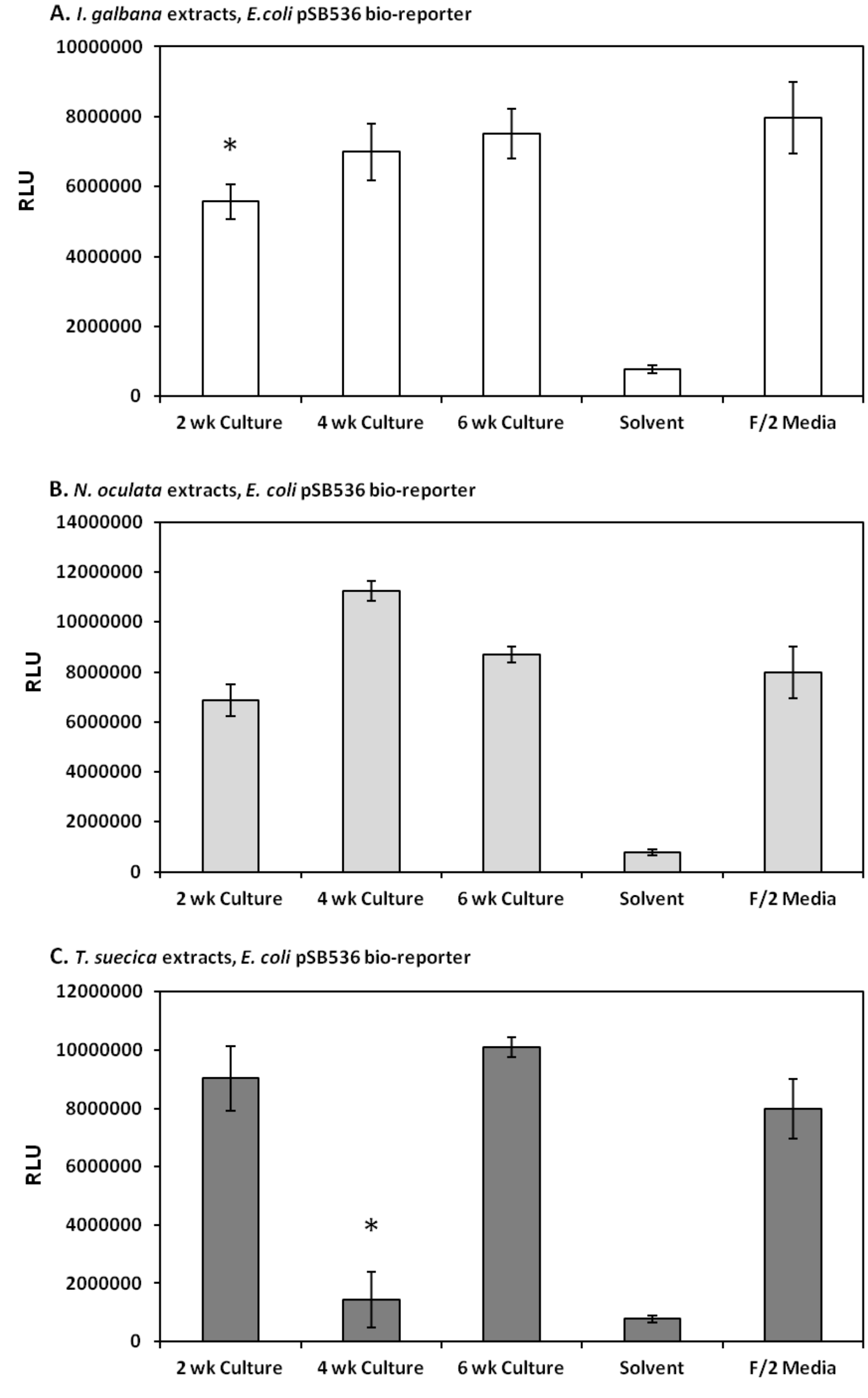


Figure 5.2. Microalgae quorum-quenching assay using AHL bio-reporter pSB536. Total luminescence produced from the bio-reporter JM109 pSB536 cultured with microalgal extracts and extracted F/2 media in the presence of C4-HSL. Significantly reduced luminescence was observed after exposure to extracts from *I. galbana* and *T. suecica* cultures grown for 2 and 4 weeks respectively. A solvent control was shown to have no effect on the bio-reporter. Error bars represent standard deviation from the mean. Asterix represent significantly significant difference from the control (2-Tailed T Test $P < 0.01$).

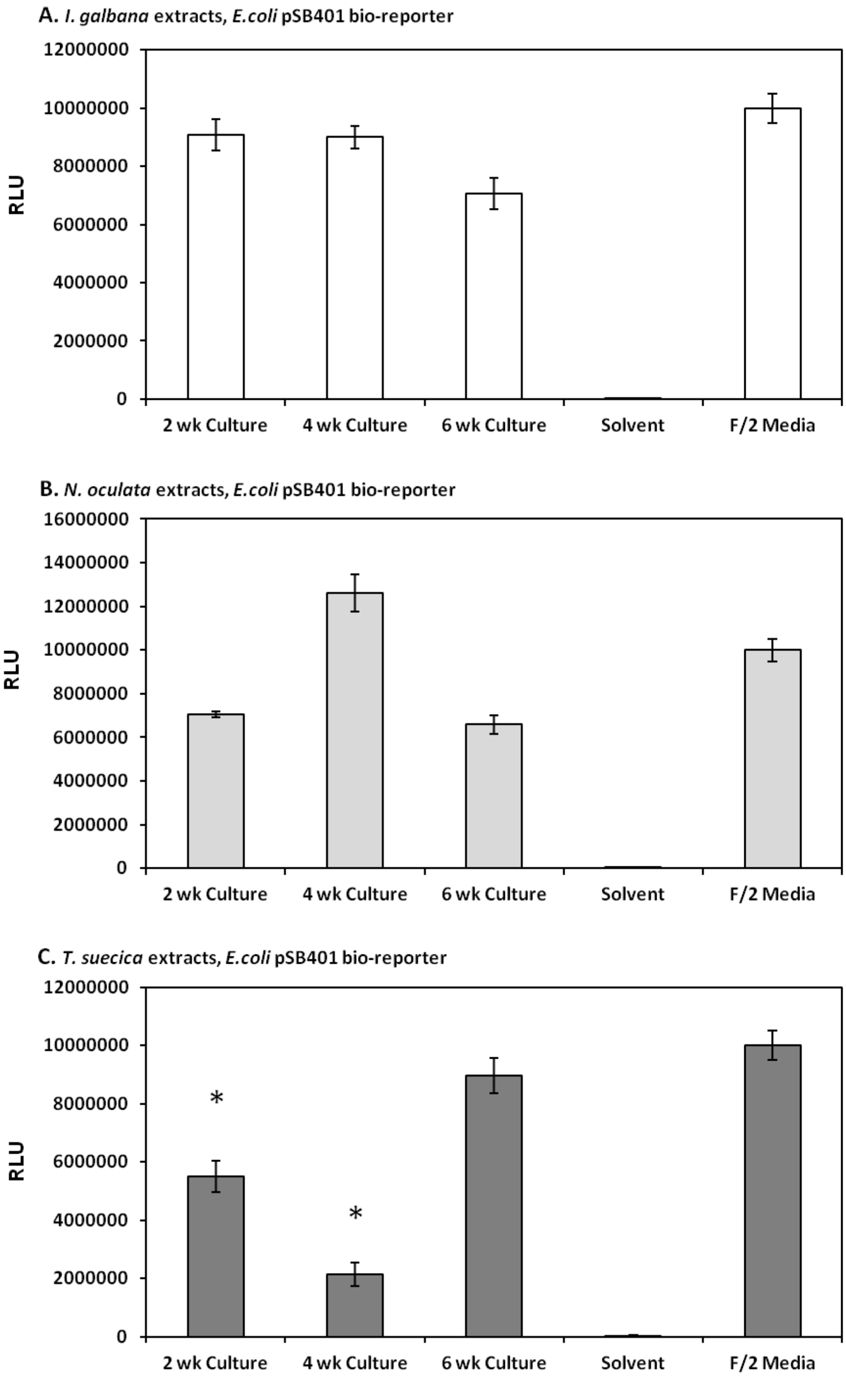


Figure 5.3. Microalgae quorum-quenching assay using AHL bio-reporter pSB401. Total luminescence produced from the bio-reporter JM109 pSB401 cultured with microalgal extracts and extracted F/2 media in the presence of C6-HSL. Significantly reduced luminescence was seen after exposure to extracts from *T. suecica* cultures grown for 2 and 4 weeks. A solvent control was shown to have no effect on the bio-reporter. Error bars represent standard deviation from the mean. Asterix represent significantly significant difference from the control (2-Tailed T Test $P < 0.01$).

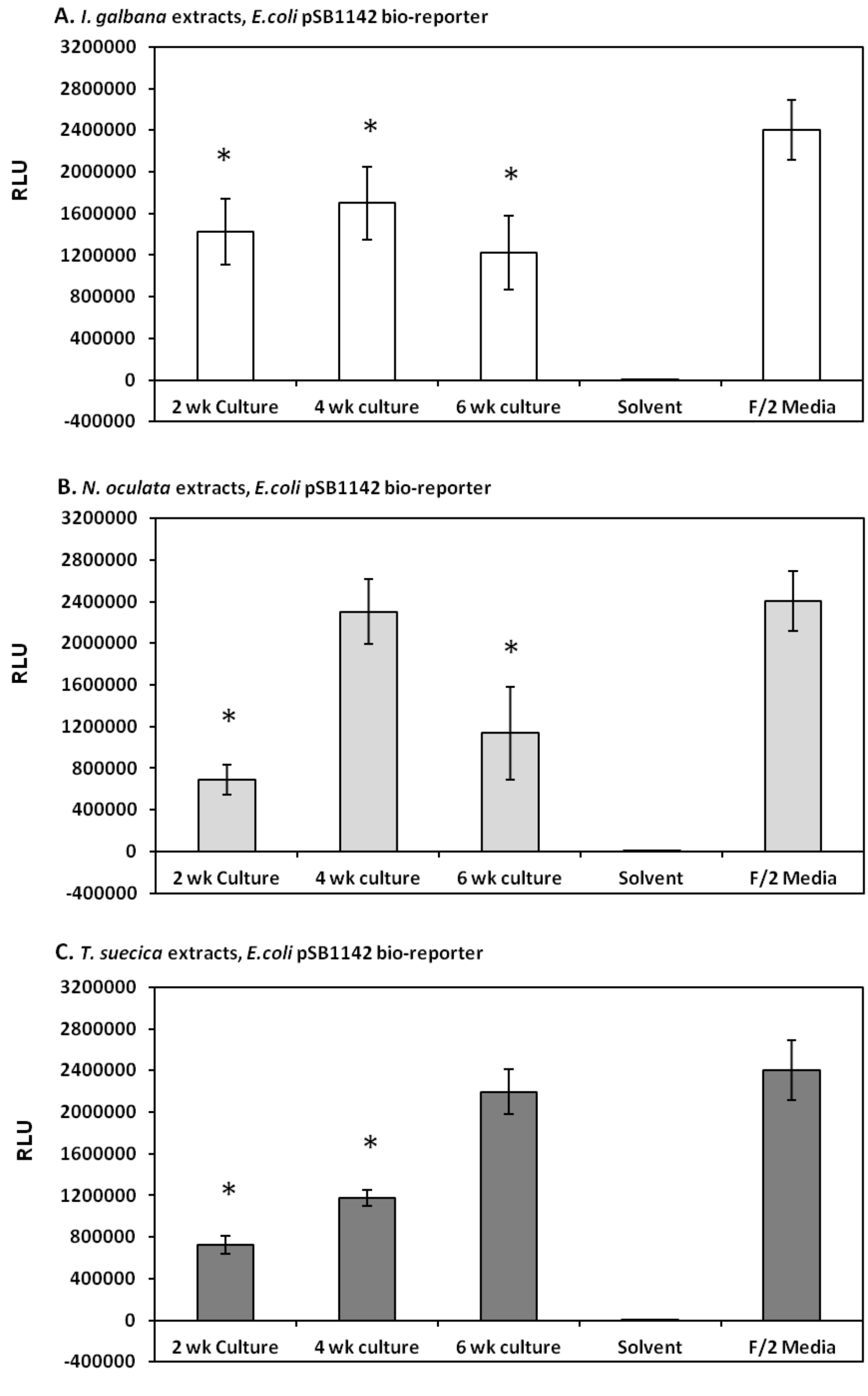


Figure 5.4. Microalgae quorum-quenching assay using AHL bio-reporter pSB1142. Total luminescence produced from the bio-reporter JM109 pSB1142 cultured with microalgal extracts and extracted F/2 media in the presence of 3-oxo-C12-HSL. Significantly reduced luminescence was seen after exposure to extracts from *I. galbana* cultures grown for 2, 4 and 6 weeks, extracts from *N. oculata* cultures grown for 2 and 6 weeks and extracts from *T. suecica* cultures grown for 2 and 4 weeks. A solvent control was shown to have no effect on the bio-reporter. Error bars represent standard deviation from the mean. Asterix represent significantly significant difference from the control (2-Tailed T Test $P < 0.01$).

5.2.3. Microalgal Quorum-quenching Activity Effects **P. aeruginosa** *Virulence*

As extracts from microalgal cultures affected the activation of AHL bio-reporters in the presence of their cognate signal molecules. Extracts micro algal cultures were also assayed for their ability to affect the expression of *P. aeruginosa* genes regulated by QS. *P. aeruginosa* PAO1 *lux*-based transcriptional fusions to *rhlI*, *lasI*, *rhlA* and *lasB* (Unpublished, provided by Dr. James Lazenby) were cultured in the presence of pooled extracts from a number of stationary phase axenic cultures of *N. oculata*, *T. suecica* and *I. galbana*. Pooled extracts were used inorder to scale up the number of experiments which could be carried out and to overcome incosistanceies observed in previous assays using extracts from separate stationary phase microalgal cultures.

Several of the extracts from microalgal cultures did produce lower amounts of bioluminescence in the *P. aeruginosa* transcriptional bio-reporters in comparison to extract from F/2 culture media (Figure 5.5). Significantly reduced levels of bioluminescence were seen in the *rhlI*, *lasI* and *rhlA* bio-reporters upon exposure to extracts from the three microalgal cultures (2-Tailed T Test $P < 0.01$) (Figures 5.5). Significantly reduced of bioluminescence were also seen in the *lasB* bio-reporter when exposed to extracts from *I. galbana* and *N. oculata* cultures (2-Tailed T Test $P < 0.01$) (Figure 5.5).

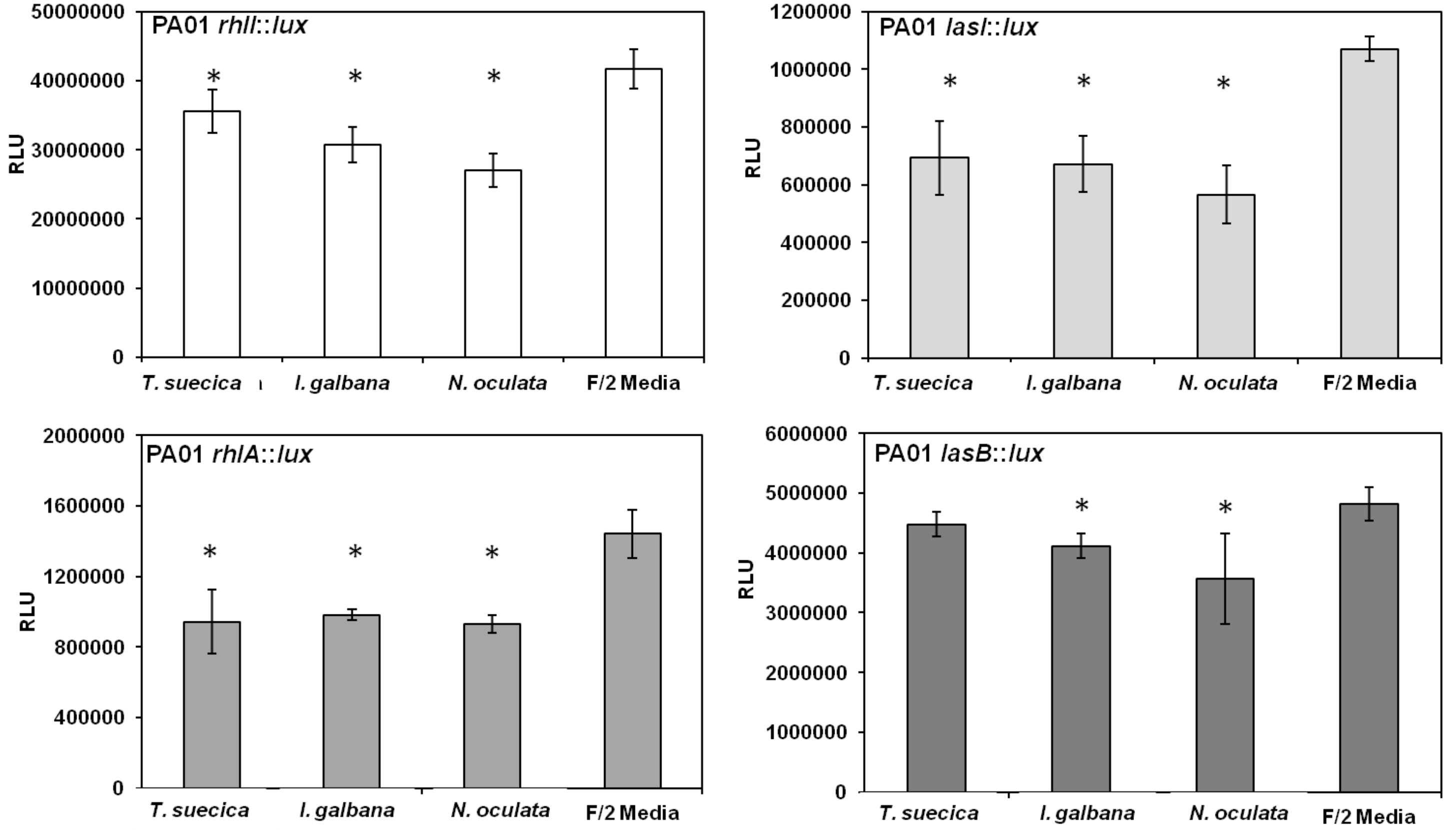


Figure 5.5. Microalgal quorum-quenching assay using *P. aeruginosa* transcriptional bio-reporters. Total bioluminescence obtained from bio-reporters *P. aeruginosa* PAO1 *rhlI::lux*, PAO1 *lasI::lux*, PAO1 *rhlA::lux* and PAO1 *lasB::lux* co-incubated with pooled extracts from stationary phase microalgal cultures and F/2 media over a 12 h period. Asterix mark extracts, which caused significant reductions in reporter bioluminescence in comparison to the F/2 media control (2-Tailed T Test $P < 0.01$). Error bars represent standard deviation from the mean.

The microalgal extracts were checked to ensure that they did not have any effect on the activity of the *lux* genes. This was achieved by repeating the assay using a *P. aeruginosa* PAO1 strain harbouring the mini CTX::*lux* construct constituently expressing the lux gene under the Km constitutive promoter (Becher and Schweizer, 2000). After carrying out 2-tailed Tests no significant reduction in bioluminescence was observed when the strain was cultured with extracts from the three microalgal cultures in comparison to culturing the strain with an extract from F/2 media (2-Tailed T Test $P > 0.01$) (Figure 5.6).

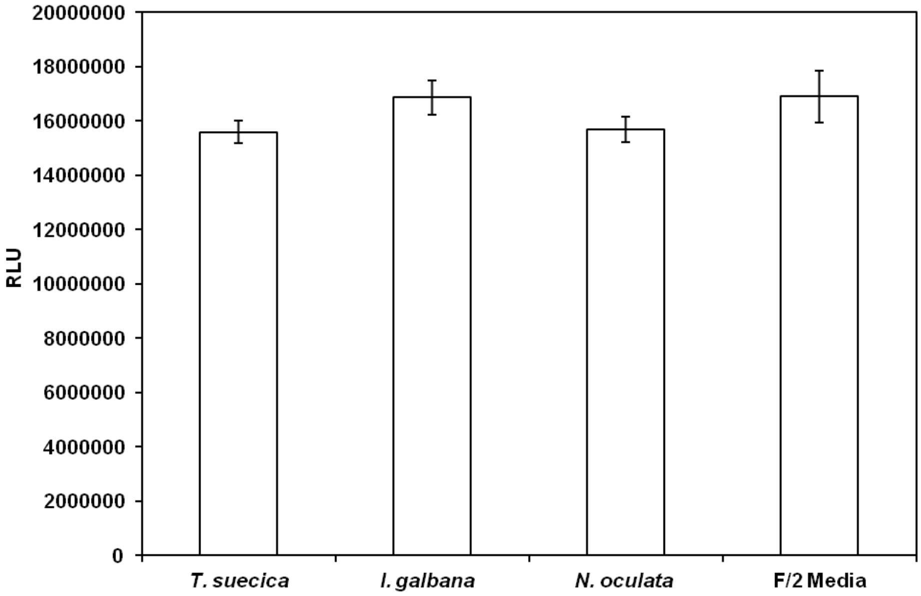


Figure 5.6. PAO1 mini CTX::*lux* bioluminescence upon exposure to microalgal extracts. Extracts from the three micro algal species were assayed with PAO1 harbouring the mini CTX::*lux* construct expressing under the constitutive P_{km} promoter to ascertain if they affected the bioluminescence process itself. Comparing bioluminescence after exposure to each microalgal extract to bioluminescence after exposure to F/2 media showed no significant differences (2-Tailed T Test $P > 0.01$). Error bars represent standard deviation from the mean.

5.2.4. Fractionation of Extracts Microalgal Cultures

In an attempt to identify the compounds produced by the microalgae which were showing activity against the bio-reporter and transcriptional fusions, extracts from stationary phase cultures of the three microalgal species were fractionated using solid phase extraction columns as described in section 2.10.5.

5.2.4.1* P. aeruginosa *Virulence Assay

The re-constituted fractions were assayed for their ability to affect the *P. aeruginosa lux*-based transcriptional fusions described in section 5.2.4. Small but significant reductions in bioluminessence were observed in the PAO1 *lasI*::*lux* and PAO1 *lasB*::*lux* reporters when exposed to the 60% Methanol *I. galbana* fraction (2-Tailed T Test $P < 0.05$) (Figure 5.7). The 70% Methanol *N. oculata* fraction also showed small but significantly reduced bioluminessence in the PAO1 *rhlI*::*lux* in comparrsion to the solvent control but not the F/2 media control (2-Tailed T Test $P < 0.05$). However, the 70% Methanol *N. oculata* fraction did significantly reduce bioluminessence in the PAO1 *rhlA*::*lux* reporter in comparrsion to both the solvent and F/2 media controls (2-Tailed T Test $P < 0.05$) (Figure 5.8). Other fractions had no observable effect on the *P. aeruginosa* bio-reporters (Data not shown).

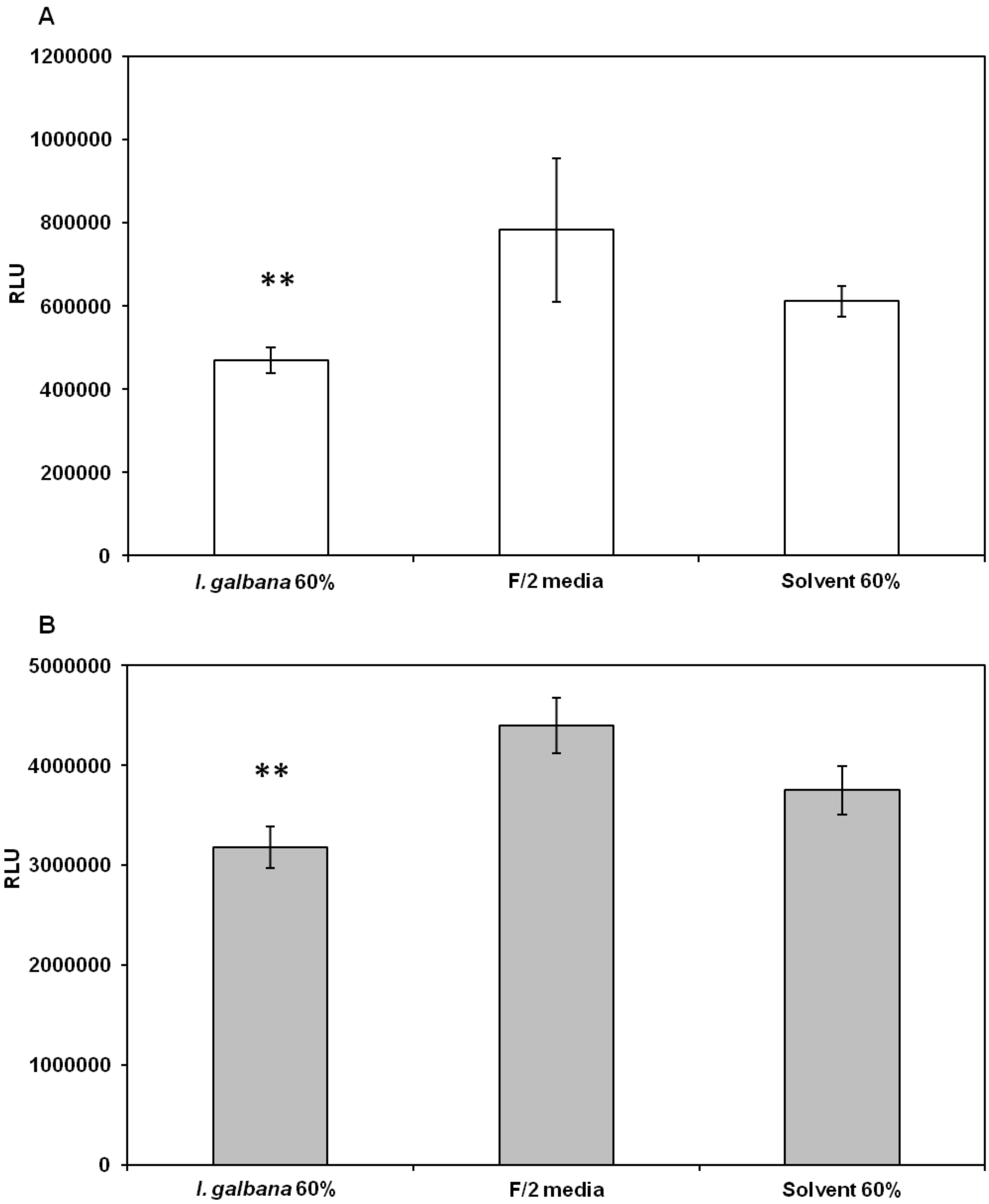
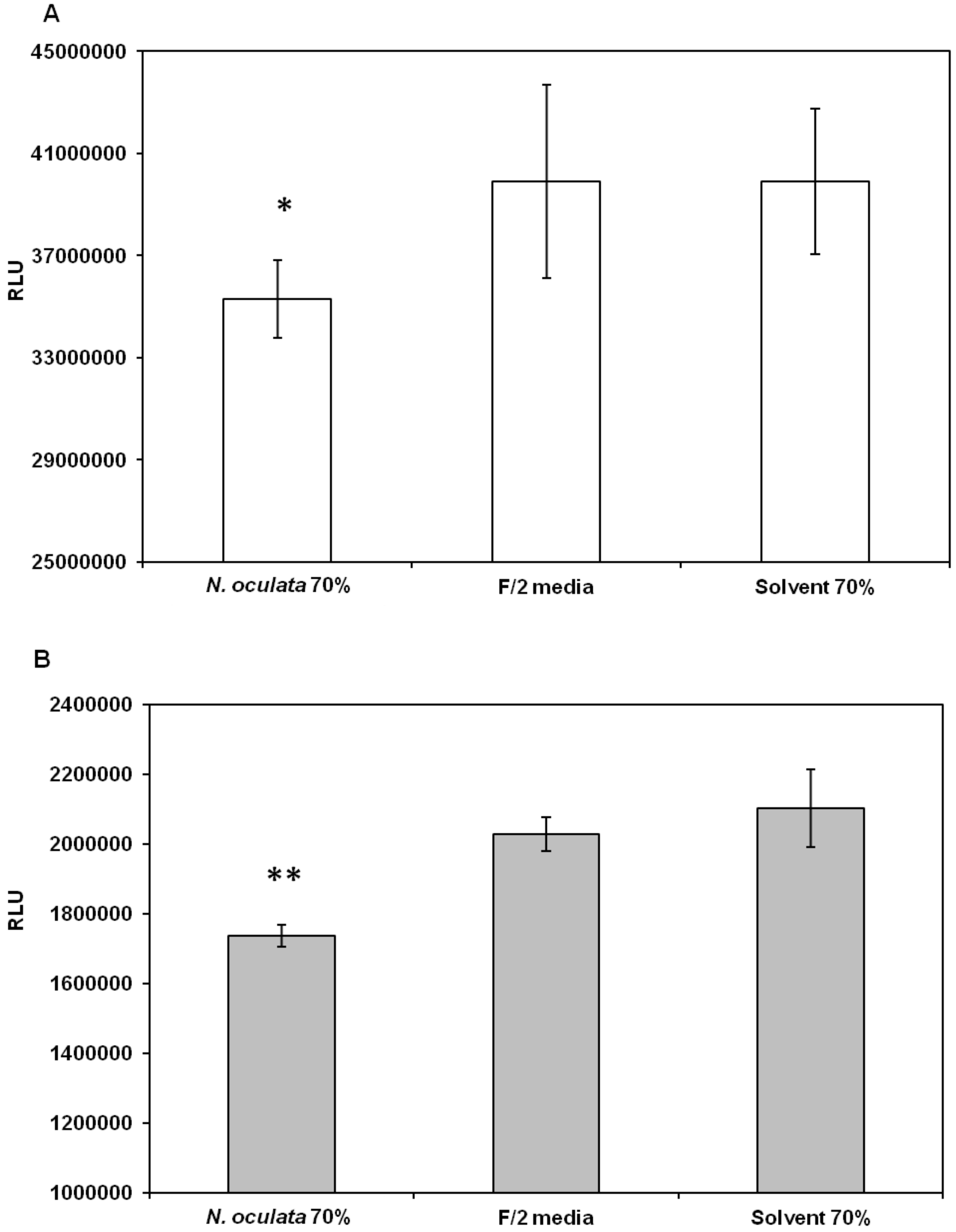


Figure 5.7. *P. aeruginosa* transcriptional bio-reporter assay using fractionated *I. galbana* culture. Total bio-reporter luminescence produced by (**A**) PAO1 *lasI*::*lux* and (**B**) PAO1 *lasB*::*lux* after 12 h exposure to 60% *I. galbana* fraction. ** Marks significant reductions in bioluminescence is seen in both reporters exposed to the 60% methanol *I. galbana* fraction in comparison to the F/2 media and solvent controls (2-Tailed T Test $P < 0.05$). Error bars represent standard deviation from the mean.



5.8. *P. aeruginosa* transcriptional bio-reporter assay using fractionated *N. oculata* culture. Total bio-reporter luminescence produced by (**A**) PAO1 *rhlI*::*lux* and (**B**) PAO1 *rhlA*::*lux* after 12 h exposure to 70% *N. oculata* fraction. * Marks significant reduction in bioluminescence was seen the *rhlI* reporter in comparison to the solvent control (2-Tailed T Test $P < 0.05$). ** Marks significant reduction in bioluminescence seen in the *rhlA* reporter in comparison to both F/2 media and solvent controls (2-Tailed T Test $P < 0.05$). Error bars represent standard deviation from the mean.

5.2.4.2 Marine Bacteria Protease Assays

The fractionated extracts from the three microalgal cultures were also used to investigate any effects on protease production in 4 species of marine bacteria, *V. anguillarium*, *Vibrio tubiashii*, *Vibrio corrallilyticus* and *Aeromonas hydrophila*. These species are commonly found to cause infection in fish species reared for aquacultural purposes via the AHL regulated production of protease enzymes that act as virulence factors; (Tait *et al.*, 2010, Croxatto *et al.*, 2002, Swift *et al.*, 1999). As such, inhibition of AHL signalling may affect protease production. The four bacterial strains were co-cultured with the fractionated extracts from *T. suecica*, *I. galbana* and *N. oculata* and resulting protease production in each bacterium was compared between the fractions. Protease activity was determined using the methodology described in section 2.10.5. Initial assays comparing the different fractions from each microalgal culture indicated reductions in protease activity when cultures of *A. hydrophila* were exposed to the 60% fraction from *I. glabana* and when cultures of *V. tubiashii* were exposed to the 60% fraction from *I. glabana* and the 70% fractions from both *T. suecica* and *N. oculata* extracts (Appendix 9). As such, these fractions were used to repeat the assay in all four bacterial strains comparing protease activity to cultures exposed to a solvent control.

The 60% fraction of *I. galbana* extract and the 70% fraction of *N. oculata* extract caused significant reductions in *A. hydrophila* protease activity in comparison to the solvent control (2-Tailed T Test $P < 0.05$) (Figure 5.9). However, only the 70% fraction of *N. oculata* extract significantly affected protease activity in *V. anguillarium* and in *V. tubiashii* (2-Tailed T Test $P < 0.05$) (Figure 5.9). No significant reductions in protease activity were observed in *V. corrallilyticus* upon exposure to any of the solvent fractions assayed in comparison to the solvent control (2-Tailed T Test $P > 0.05$).

Culture OD_{600} measurments showed that in comparison to the solvent control the fractions from all three microalgal culture extracts did not affect bacterial growth (data not shown).

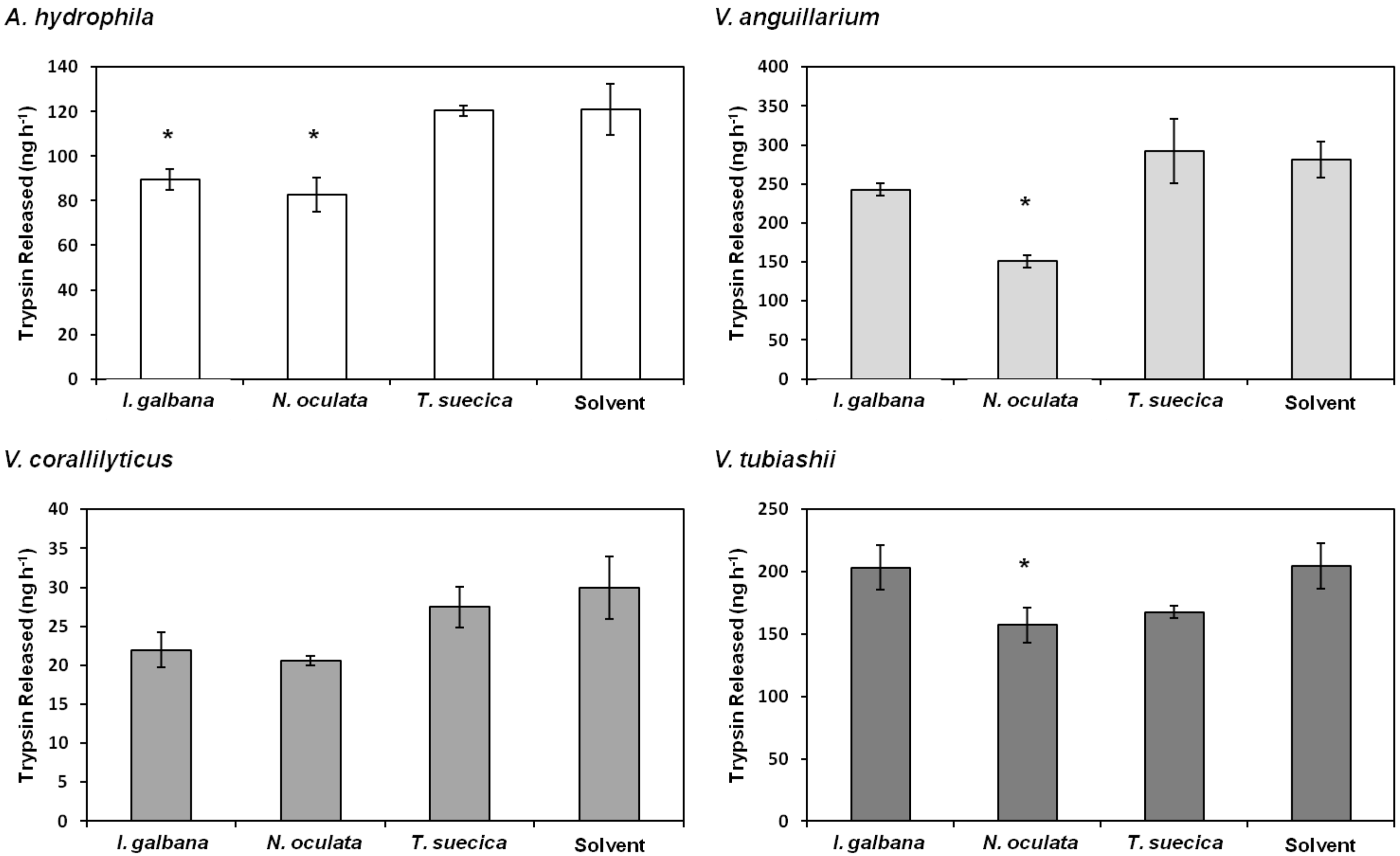


Figure 5.9. Protease assay of fractionated extracts from microalgal cultures. Protease activity in marine bacteria as a result of co-incubation with the 60% fraction from *I. glabana* extract and from the 70% fractions from *N. oculata* and *T. suecica* extracts in pathogenic marine bacteria. The 60 % fraction of *I. glabana* extract significantly reduced protease activity in *A. hydrophila*. The 70% fraction *N. oculata* extract significantly reduced protease activity in *A. hydrophila, V. anguillarium* and *V. tubiashii*. The Error bars represent standard deviation from the mean. Asterix represent significantly significant difference from the control (2-Tailed T Test $P < 0.05$).

5.3. Discussion

5.3.1 Quorum-quenching Activity if Marine Microalgae

Previous studies investigating algal/bacterial interactions in the marine environment have shown that large scale surface water bioluminescence, commonly referred to as milky sea events, result from AHL-mediated QS by *V. fischeri* bacteria associated with algal plankton blooms (Nealson and Hastings, 2006). As the bacterial densities measured during milky sea events was below that previously reported to initiate AHL-mediated regulation of bioluminescence in *V. fischeri*, it has been theorised that the bioluminescence apparent during such events may be stimulated by QS mimic compounds produced by microalgae (Nealson and Hastings, 2006, Fuqua *et al.*, 1994). This hypothesis was given credence due to the evidence that secondary metabolite production by freshwater microalgal species *C. reinhardtii* mimicked AHL bacterial signalling molecules (Teplitski *et al.*, 2004). As such, this study investigated potential AHL mimic production and quorum-quenching activity in three microalgal species found in the marine environment. The species were *I. glabana*, *N. oculata* and *T. suecica*, species which are commonly grown for usage in aquaculture, acting as feed stocks for bivalve molluscs, crustaceans and rotifers, which are in turn used to feed reared fish species (reviewed by Borowitzka, 1997). In further investigating if these algae can modulate bacterial behaviour via the stimulation or inhibition of QS, it may be possible to exploit such behaviour for aquacultural or medical benefit.

This study failed to identify any secondary metabolite being produced by these three microalgal species that had the ability to activate AHL response regulators AhyR (*A. hydrophyla*), LuxR (*V. fischeri*) and LasR (*P. aeruginosa*), as extracts from these microalgae did not produce bioluminescence in reporters pSB536, pSB401 and pSB1142, respectively (Winson *et al.*, 1998; Swift *et al.*, 1997). This does not entirely rule out the possibility that these species produce compound(s) which mimic AHLs, as such compounds may affect AHL response regulators other than the three present in theses bio-reporters. It may also be the case that any mimic compound(s) interact with these AHL response regulators inhibiting AHL-mediated QS and there for would obviously not produce bioluminescence in these reporters.

This study went onto investigate if these species were producing compound(s) that inhibited the activation of these AHL response regulators in the presence of exogenous AHLs. When culturing the *lux*-based AHL bio-reporter pSB536 with extract from *I. glabana* and *T. suecica*; pSB401 with extract from *T. suecica* and pSB1142 with extracts from all three cultures in the presence of their cognate AHLs bioluminescence was significantly decreased in comparison to culturing with extracted sterile F/2 microalgal media. The microalgae used in this study were obtained as axenic cultures, grown in conditions that would prevent bacterial contamination and checked for bacterial contamination via microscopy throughout the study supporting the notion that inhibition of AHL bio-reporter luminescence is due to the microalgal species and not epiphytic bacteria. In addition, extracts from all three microalgal cultures were shown to have no effect on the transcription of the genes in the *lux* operon that encode the bioluminescence producing enzyme luciferase. As such, it is likely that compounds(s) produced by these microalgae are inhibiting AHL-mediated QS in the bio-reporters accounting for the reductions in bioluminescence observed in the presence of exogenous AHLs. Natrah *et al.* (2011) published results that support this study's finding that axenic strains of marine microalgae produce secondary metabolite(s) that inhibit the activation of AHL bio-reporters in the presence of their cognate signal molecules (Natrah *et al.*, 2011). The study published by Natrah *et al.* (2011) only shows inhibition of AHL bio-reporters by extracts from microalgal cultures and does not identify any compound(s) that may be causing such inhibitory activity. The study does however included the freshwater microalgae *Chlorella vulgaris* and *Chlorella saccharophila* to the list of species known to be producing potential QS inhibitors (Natrah *et al.*, 2011). With the exception of bioluminescence in the fish pathogen *V. harveyi*, in which they show a reduction in luminescence due to the presence of microalgal extracts, using a *V. harveyi* AHL bio-reporter; the Natrah *et al.* (2011) study does not investigate if this quorum-quenching activity effects any genes regulated by AHL-mediated QS (Natrah *et al.*, 2011).

This current study did investigate if extracts from microalgal species effected the expression of genes known to be, in part regulated by AHL-mediated QS using the opportunistic human pathogen *P. aeruginosa*. *P. aeruginosa* possesses two AHL-mediated QS systems, the LasR/I system which directs the production and transduction of 3-oxo-C12-HSL and the RhlR/I system which directs the production and transduction of C4-HSL (Latifi *et al.*, 1995). Further research has also shown that these two QS systems function in a hierarchical fashion, with the LasR/I systems regulating the RhlR/I system (reviewed by Latifi *et al.*, 1996). These two AHL-mediated QS systems, alongside other regulatory systems are responsible for the regulation of a large amount of genes in *P. aeruginosa*, including many responsible for the production of extracellular virulence factors, biofilm formation, siderophore production and auto-regulation of their own AHL synthase genes (reviewed by Whiteley *et al.*, 1999). Extracts from all microalgal cultures were shown to significantly affect the luminescence produced by *lux*-based transcriptional fusions reporting the transcription of both *lasI* and *rhlI*, with reduced luminescence observed in the presence of extracts from all three microalgal cultures. Additionally a number of the extracts effected *lux*-based transcriptional fusions reporting the transcription of two *P. aeruginosa* genes, which are in part regulated by AHL-mediated QS, *lasB* and *rhlA*. Both these gene provide important roles in *Pseudomonas* pathogenicity (Reviewed by Whitelely *et al*., 1999). All three extracts produced significant reduction in biolumminesnce in the *rhlA* bio-reporter and extracts from *I. galbana* and *N. oculata* significant reduced biolumminesnce in the *lasB* bio-reporter. The *lasB* gene encodes a metalloprotease, elastase, which acts as a major virulence factor in *P. aeruginosa* infections (Fukushima *et al*., 1997, Gambello and Iglewski, 1991). The *rhlA* gene belongs to a gene cluster along with *rhlB*. These two genes encode rhamnosyltransferase, which catalyses the production of rhamnolipids; glycolipids produced by *P. aeruginosa* and used as a surfactant promoting motility (Soberón-Chávez *et al*., 2005, Lang and Wullbrandt, 1999). *P. aeruginosa rhlI* mutants lack the ability to produce rhamnolipids and are therefore *rhlI* is implicated in the regulation of the *rhlAB* gene cluster (Ochsner and Reiser, 1995). As the microalgal extracts were shown to reduce bioluminescence in these transcriptional bio-reporters and in the *lux*-based *E. coli* AHL bio-reporters it is possible that these extracts contain compound(s) which may inhibit AHL-mediated QS in *P. aeruginosa.*

In addition to studying potential quorum-quenching activity by marine microalgae on QS regulated *P. aeruginosa* genes, this study also initiated the identification of potential quorum-quenching compound(s) by fractionating the extracts obtained from the three microalgal cultures. The separate solvent fractions were tested for their ability to inhibit bioluminescence in *lux*-based transcriptional bio-reporters of genes regulated by QS in *P. aeruginosa* and also to inhibit protease activity in 4 marine bacteria shown to be pathogenic to fish species reared in aquaculture, *A. hydrophila*, *V. anguillarium*, *V. coralliyticus* and *V. tubiashii*. Results showing inhibition of bioluminescence in *P. aeruginosa* virulence factor transcriptional fusion bio-reporters by the fractionated extracts were not as clear as when the whole extract was assayed using the same reporters. The lack of clear bio-reporter inhibition using the fractionated extracts may indicate that the compound(s) causing inhibition when assaying the whole extract did not bind to the solid phase extraction columns used by this study in the fractionation process. In spite of this, small by significant reductions in the bioluminescent resulting from the activation of the *lasI* and *lasB* bio-reporters were observed after exposure to the *I. galbana* 60% methanol fraction and in the bioluminescent resulting from the activation of the *rhlI* and *rhlA* bio-reporters when exposed to the *N. oculata* 70% fraction.

The fractionated microalgal extracts were also show to decrease protease activity in all four marine bacterial pathogens assayed. Previous studies have shown that all four of these species produce AHLs and have also shown them to possess AHL-mediated QS systems (Santos Ede *et al.*, 2011, Temperton *et al.*, 2011, Tait *et al.*, 2010, Milton *et al.*, 2001, Swift *et al.*, 1997). Previous studies have also shown that in *A. hydrophila* and in various *Vibrio* species, the production of protease virulence factors is regulated by both AHL and AI-2 mediated QS systems (Milton, 2006, Swift *et al.*, 1999). As such, the inhibition of protease activity in marine pathogens observed by this study, has a result of exposure to extracts from microalgal cultures, may indicate quorum-quenching activity by compound(s) produced by these marine microalgae. Interestingly, the fractions from microalgal extracts effecting the transcription of AHL regulated genes in *P. aeruginosa* were, in the most part, consistent with those that affected protease activity in the marine pathogens. As such, it is therefore likely that any compound(s) inhibiting AHL-mediated QS are present in these fractions.

The evidence that different solvent fractions from the two species affected different *P. aeruginosa* bio-reporters and protease activity in marine pathogens may indicate the production of a range of secondary metabolites that elute into different fractions, which in combination have the ability to effect AHL regulated gene expression by potential quorum-quenching abilities. Previous studies have shown eukaryotic species including *C. reinhardtii*, *D. pulchra* and *Medicago truncatula* to produce up to 30 separately identifiable compounds that inhibit bacterial QS (Teplitski *et al.*, 2004, Gao *et al.*, 2003, Manefield *et al.*, 2002). Further fractionation combined with chemical analysis techniques such as LCMS or nuclear mass spectroscopy would be required to positively identify the compound(s).

The biological aim of producing compound(s), which inhibit AHL regulated QS may by to disrupt bacterial signalling in order to prevent infection. This is clearly demonstrated to be the case for the production of halogenated furanones by the marine macroalgae *D. pulchra* as these compounds were shown to prevent swarming motility in *S. liquefaciens* reducing the bacteria's ability to colonise. Previous studies have shown marine microalgae have the ability to modulate the bacterial population to which they are associated with (Sapp *et al.*, 2007, Salvesen *et al.*, 2000). Inhibition of bacterial QS by producing quorum-quenching compounds may be one method by which microalgae can control the bacterial population to their advantage. As microalgal cultures are heavily used in aquaculture it may be possible to utilise these antimicrobial properties to prevent disease in reared fish populations. Inhibition of the fish pathogen *V. anguillarium* by *Roseobacter* spp. due to co-culturing with *N. oculata* has been recently reported (Sharifah and Eguchi, 2011). It is of note that both *V. anguillarium* and *Roserbacter* clade bacteria have been reported to regulate gene expression using AHL-mediated QS. It maybe that quorum-quenching activity by *N. oculata* is promoting *Roseobacter* growth at the expense of *V. anguillarium*. Results presented by this study showing inhibition of protease production in marine pathogens noted to effect aquacultural stocks by three microalgal species currently used as feed sources in aquaculture further expands these findings, lending credence to the potential of exploiting microalgal quorum-quenching to control infection in aquaculture. In addition, this study has shown that compound(s) produced by marine microalgae may affect virulence gene expression in human pathogens. This makes microalgae and the quorum-quenching compounds they produce attractive candidates for novel antimicrobials as they can reduce the virulence of a pathogen without inducing a selective pressure, causing antimicrobial resistance (Müh *et al.*, 2006, Zhang, 2003).

5.3.2. Summary Conclusions and Future Directions

This study has identified quorum-quenching activity in three marine microalgal species. This activity has been shown to effect AHL bio-reporters bioluminescence in the presence of various exogenous AHLs, the expression of a number of *P. aeruginosa* virulence factors previously shown to be in part regulated by AHL-mediated QS and protease expressing in a number of marine bacterial pathogens. Although advanced by this study, research into QS inhibition by marine microalgae is clearly still in its infancy. As yet, no compound(s) have been identified as the cause of the quorum-quenching activity seen in this study. The identification of these compounds is clearly the next step in this research.

Research in this area should progress in the following directions:

- Continue fractionation of extracts from stationary phase microalgal cultures to purify and therefore identify quorum-quenching compound(s) produced by these species. This can be conducted by continuing with the method laid out in this thesis (Section 2.10.5) or by the use of reverse-phase C18 analytical HPLC (see Teplitski *et al.*, 2004), assaying each fraction for quorum-quenching activity using AHL bio-reporters (see Section 2.10.4).
- Once quorum-quenching compound(s) have been sufficiently purified, initiate compound(s) identification using chemical analysis techniques such as LCMS, or for more precise identification, nuclear magnetic resonance spectroscopy.

Chapter 6

General Discussion

6.1 General Discussion

The first aim of this study was to examine the relationship the green seaweed *Ulva* has with its epiphytic bacterial community, concentrating on the impacts of AHL signal molecule production by these bacteria. As such, this thesis has characterised *Ulva*'s epiphytic bacterial population in terms of both taxonomic composition and the AHL signal molecules being produced by members of this population. As previous work has shown that AHL signal molecule production affects the settlement of *Ulva* zoospores and that bacteria affect the growth morphology of *Ulva* spp. This thesis presented data examining the effects AHLs, and bacteria producing AHLs have on *Ulva* zoospore germination and the early growth of the *Ulva* germling. This thesis then progressed to investigate AHL production and degradation in two groups of bacteria that can be readily isolated from biofilms present on both the surface of the *Ulva* thallus and on the rocks colonised by *Ulva* spp., *Shewanella* and the *Bacteroidetes*. Finally, this thesis diverged into a study of quorum-quenching behaviour in three species of marine microalgae prolifically used in aquaculture.

Marine algae have been shown to possess diverse populations of epiphytic bacteria; these bacteria in general exist in aggregated biofilm communities and as such will behave in a different manner to planktonic bacteria in the water column. These epiphytic bacterial populations have been shown to interact with their algal host organisms in a number of different ways, many of which have been discussed in this thesis (Tait *et al.,* 2009; Rao *et al.*, 2006; Miller *et al.*, 2005; Matsuo *et al.,* 2003,). In some cases, these interactions may be symbiotic. Examples of potential symbiotic interactions include the utilisation of bacterially produced cyanocobalamin (Vitamin

B_{12}) by algae, many of which are cyanocobalamin auxotrophs (Croft *et al.*, 2006). In return the bacteria benefit from carbon sources produced by algal metabolism which provide growth substrates (Croft *et al.*, 2006; Croft *et al.*, 2005). However, the true symbiotic nature of this interaction has been questioned with suggestions that algae merely scavenge vitamins (Droop, 2007). These interactions have also been proven to be dynamic and can be either beneficial or detrimental to the host. The prominent microalgal species *Emiliania huxleyi* and bacteria from the *Roseobacter* clade show both a mutualistic and pathogenic interaction depending on the senescence of the algal host and is mediated by small molecule production by both host and symbiont (Seyedsayamdost *et al.*, 2011). In healthy *E. huxeyli* cultures the algae provide a substrata and nutrients for bacterial growth and the bacteria produce antibiotics preventing algal infection. As the algae matures it releases a signal (pCA) which prompts *Roseobacter* species to produce algaecides killing the host and allowing the bacteria to access the subsequent nutrient released proliferate to a healthy host (Seyedsayamdost *et al.*, 2011).

6.2 *Ulva* Zoospore Settlement, Germination and Germling Growth

Small molecule mediated algal/bacterial interactions have also been proven to take place utilising the signalling molecules that many species, including those from the marine environment, produce for bacterial QS. A prime example is the interaction between bacteria and *Ulva* spp., where AHL signalling molecules attract *Ulva* zoospores to microcolonies within bacterial biofilms via the mediation of zoospore swim speed (Wheeler *et al.*, 2006; Tait *et al.*, 2005; Joint *et al.*, 2002). A key finding presented in this thesis has shown that the interaction between *Ulva* spp. and their indigenous signal molecule producing bacterial population does not just affect zoospore settlement but also the germination process and the early growth of the *Ulva* zoospore. In the presence of AHLs *Ulva* zoospore germination and the growth of the resultant *Ulva* germlings were both shown to be retarded. This reduction in the rate of early growth may allow the *Ulva* germling to obtain an indigenous epiphytic bacterial population which is of benefit as bacteria have been shown to modulate the healthy differentiation of *Ulva* spp. into mature adult plants. This hypothesis is supported by data presented in sections 3.2.1 and 3.2.2 that show *Ulva* possess an epiphytic bacterial population similar to the previously characterised population found in rocky shore biofilms (Tait *et al.*, 2009).

The interactions between *Ulva* spp. and bacteria is often cited as an example of 'cross kingdom communication' owing to AHL bacterial signalling molecules, which are normally involved in population density dependent communication, mediating these interactions (Joint *et al.*, 2007). However, the data presented in this study and the previous studies into AHL-mediated *Ulva*/bacterial interactions only reveals the algal response to AHL signal molecules. As yet, no data has been presented showing *Ulva* spp. to produce any form of signal molecule which mediates a phenotypic change in bacterial populations. As true communication requires both communicating partners to understand and participate in the communicative process, labelling this interaction as an example of cross kingdom communication may be a somewhat broad ranging statement. Additionally, very little is understood of the mechanism behind reduced swimming speed of *Ulva* zoospores by AHLs and this study has not eluded any mechanism for how AHLs modulate zoospore germination and germling growth. As such, it is most likely that *Ulva* spp. utilise the AHLs being produced by members of its indigenous population as cues for various growth processes, be this zoospore settlement, zoospore germination or the early growth of *Ulva* germlings. Since the interaction between AHL-producing bacteria and *Ulva* has been established, the mechanisms which drive this interaction should be investigated. Joint *et al.* (2007) propose that an influx of calcium into the cytosol caused by the presence of AHLs is responsible for the modulation of *Ulva* zoospore swim speed, which in turn leads to increased zoospore settlement in the presence of AHL-producing biofilms (Joint *et al.*, 2007; Wheeler *et al.*, 2006). This hypothesis was supported by manganese quenching experiments in which the fluorescence of a calcium indicator dye was reduced as a result of manganese entering the cell, a direct result of calcium influx channels opening when zoospores were exposed to high concentrations of 3-oxo-C12-HSL (Joint *et al.*, 2007; Taylor *et al.*, 1996). Calcium has also been shown to affect flagella swim speed in other marine eukaryotes such as *Chlamydomonas* spp. (Kamiya and Witman, 1984). Comparative genomic and proteomic analysis of *Ulva* to both bacterial and micro-eukaryotic species sequence information may identify potential receptors which have the ability to detect AHLs. Such analysis may also identify subsequent signalling pathways regulating these previously reported alterations in calcium influx which appear to modulate swim speed. However, to accomplish this, it will be necessary to first sequence the genomes of key *Ulva* species. Additionally, comparisons of both RNA and protein expression profiles between *Ulva* zoospores or germlings exposed to AHLs with those not exposed to AHLs may also provide information on the transcription of proteins involved in modulating this interaction and answer the question of whether AHLs produced by bacteria elicit a change in *Ulva*'s gene expression.

There is currently no direct evidence that epiphytic bacteria benefit from being associated with *Ulva* spp. However, it is possible to hypothesise that epiphytes may obtain nutrients because of the association with *Ulva* spp. A number of studies have shown that bacteria belonging to the *Rhodobacteraceae* have the ability to degrade dimethylsulphoniopropionate (DMSP) to dimethylsulphide (DMS) (Wagner-Döbler and Biebl, 2006). DMSP is a sulphonium compound produced by a range of marine algae including *Ulva*, this compound is released into the water and its degradation can provide a source of sulphur for marine bacteria (Wagner-Döbler and Biebl, 2006; Yoch, 2002; Van Alstyne *et al.*, 2001). Species belonging to the *Rhodobacteraceae* have been shown to be able to degrade DMSP via two biochemical pathways and are also closely associated with marine algae, including, as this study has shown, *Ulva* (Miller and Belas, 2004). It is therefore possible that bacteria benefit by being provided with a source of sulphur from the DMSP being produced by *Ulva* spp. Similar relationships have been observed between *Rhodobacteraceae* bacteria and the microalgal species *Pfiesteria* spp. and DMSP has also been shown to act as a chemo-attractant to these bacteria (Miller and Belas, 2004). As this is only a hypothesis as to how bacteria benefit from association with *Ulva* spp., the *Ulva*/bacterial interaction cannot yet be truly considered as symbiotic. It would therefore be interesting to observe if the bacterial population associated with *Ulva* spp. changes during the growth of the plant in response to the different nutrients available as a result of the different stages in plant growth. Currently the only studies looking at *Ulva* bacterial populations either have taken snap shots from one point in the *Ulva* life cycle as this study did, or have tracked seasonal change in the *Ulva* bacteria/population (Burke *et al.*, 2011; Tujula *et al.*, 2010).

In the presence of a variety of synthetic AHLs *Ulva* zoospore germination and germling growth was assessed. There was no clear pattern of specificity seen with regards to which AHLs affected germination or germling growth, although assays were more consistent using AHL with long acyl side chains. This increased consistency was attributed to long chain AHLs being more stable in the alkaline pH of seawater (Hmelo and Van Mooy, 2009; Tait *et al.*, 2009). As bacterial biofilms producing AHLs with short and mid-length acyl side chains showed significant effects on *Ulva* zoospore germination and germling length in this study, and to *Ulva* zoospore settlement in previous studies (Tait *et al.*, 2005), it is possible that *Ulva* does not respond to a specific AHL as a cue during zoospore settlement, germination and early growth. This would be consistent with data presented by this study and previous studies suggesting that the bacteria associated with *Ulva* spp. and the surfaces colonised by *Ulva* spp. produce a wide variety of different AHL signalling molecules (Tait *et al.*, 2009). As other signalling molecules such as alkyl quinolones have been shown to be present in the marine environment being produced by marine bacteria such as *Alteromonas* spp., (Long *et al.*, 2003), it would be interesting to investigate if these molecules affect *Ulva* zoospore settlement and germination. As such, similar assays to those used by this study could be carried out using synthetic alkyl quinolone molecules and model organisms such as *P. aeruginosa*, which produce these quinolone molecules.

This study has increased our understanding of the complex relationship that exists between the algae and bacteria and in to the future the commercial benefits of these observations may be far reaching. *Ulva* is a prominent species involved in marine macrofouling which incurs massive costs to the marine industry (Callow and Callow, 2002). Currently antifouling methods involve treating submerged surfaces with chemical paints, which can be detrimental to the marine environment (Thomas *et al.*, 2001; Tolosa *et al.*, 1996). A greater understanding of *Ulva* growth and development in conjunction with associated bacterial species may lead to new antifouling treatments that are more effective with reduced environmental implications. This study and previous work suggest that AHL signalling is beneficial to the growth processes of *Ulva* spp. therefore one such avenue of research for producing novel antifouling treatments may be to develop treatments that inhibit AHL signalling and therefore reduce *Ulva* settlement and affect the wild type growth process of the *Ulva* germling. Alkyl quinolone molecules have additionally been shown to be algaecidal, inhibiting the growth of phytoplankton at concentrations above 10 µM (Long *et al.*, 2003). As such, investigating the effects quinolone producing bacteria have on *Ulva* growth may be another avenue for novel antifouling treatments.

6.3 AHL-mediated Quorum Sensing in *Shewanella* and the *Bacteroidetes*

Section 4.2.2 described AHL signal molecule production in two groups of marine bacteria, *Shewanella* and the *Bacteroidetes*. These two bacterial groups were deemed of relevance to this study for separate reasons. *Shewanella* spp. can be readily isolated from *Ulva* spp. and has previously been shown to modulate *Ulva* zoospore settlement via the production and subsequent inactivation of AHLs. The *Bacteroidetes* are of relevance as this study has shown that they are highly abundant in the *Ulva* epiphytic community and previous work has shown similar abundance in rocky shore biofilms colonised by *Ulva* spp. (Tait *et al.*, 2009). This study has shown AHL production by a number of different strains of both *Shewanella* and *Bacteroidetes* bacteria. Concerning the *Bacteroidetes*, this is of particular interest as AHL production in this phylum is currently a novel topic for research and expands our understanding of AHL signal molecule production to bacteria outside of the proteobacterial phylum. As previous work has only proven AHL production in one strain of *Bacteroidetes*, the discovery that a number of *Bacteroidetes* strains from differing taxonomic backgrounds produce AHLs, specifically C4-HSL, points towards conserved signal molecule production by this bacterial phylum. The study of *Bacteroidetes* AHL production also underpinned the importance of using the latest techniques to identify AHL production. In this study the use of LCMS to characterise AHL production in both the *Bacteroidetes* and in other marine isolates produced an accurate AHL profile which would have been virtually impossible using traditional bio-reporters because of the alkaline nature of marine media hydrolysing AHLs (Yates *et al.*, 2002).

This study has increased the range of *Shewanella* bacteria known to be producing AHLs to include strains of *S. putrafaciens* and *S. baltica*. As it is clear that both the *Bacteroidetes* and *Shewanella* spp. are actively producing AHLs this study attempted to identify AHL synthase and response regulators in both groups. *In silico* analysis failed to identify any sequences homologous to LuxI/LuxR or LuxM/LuxN. This study therefore concludes that both these groups of bacteria possess novel, yet to be identified, AHL synthases and response regulators. Given the abundance of these two groups and other bacteria in the marine environment and the expanding list of marine bacteria known to be producing AHLs, it is possible that there is a wide variety of novel systems used by marine bacteria to produce and detect AHLs. Further genomic sequencing of the ocean's bacterial populations and subsequent bioinformatic analysis currently being carried out by studies such as Global Ocean Sampling Expedition may reveal such novel systems (Venter *et al.*, 2004). Alternatively, the identity of the AHL synthase(s) may be revealed using a transposon library constructed in *Bacteroidetes* strains which are actively producing AHLs. AHL bio-reporters which detect C4-HSL production would then be used to identify mutants which were unable to produce AHLs, this technique does however present drawbacks owing to the high levels of background light associated with C4-HSL bio-reporters which could lead to a number of false positive results. A *Bacteroidetes* genomic library may also be used to identify novel AHL synthases using bio-reporters which respond to C4-HSL. These strategies have been used for the detection of AHL-mediated QS systems in a number of enteric bacteria and in environmental metagenomic libraries (Hao *et al.*, 2010; Swift *et al.*, 1993). In *Shewanella*, the screening of genomic libraries being expressed in a number of different AHL bio-reporters to identify AHL synthases and transducers has been exhaustively attempted and lead a number of false positive results (Tait *et al.*, 2009). The turnover of *Shewanella* AHLs by the expression of AHL acylases complicates this process and as such mutating the cognate *Shewanella* AHL inactivating enzyme would be required before attempting this screening procedure. As this study has identified a conserved AHL acylase in a number of *Shewanella* strains, its mutation would be the next logical step to determining if *Shewanella* spp. express more than one AHL inactivating enzyme. If this proved successful, then putative AHL synthase and response regulator screening genes could be screened in a transposon library generated from the newly constructed mutant.

As well as investigating AHL production in *Shewanella* spp. this study also concentrated on the phenomenon of AHL inactivation by *Shewanella*. Enzymic AHL inactivation by bacteria is generally carried out either by AHL lactonase enzymes or

AHL acylase enzymes, both of which inactivate AHLs via hydrolysis but at different points in the AHL molecule (Lin *et al.*, 2003; Dong *et al.*, 2000). AHL inactivation in *Shewanella* spp. has been observed in previous studies and one study has identified an acylase enzyme in an environmental *Shewanella* isolate with the ability to cleave the homoserine lactone ring from the acyl side chain of AHLs known to be produced by *Shewanella* (Tait *et al.*, 2009; Morohoshi *et al.*, 2008). Via *in silico* screening this study found putative AHL acylase sequences to be present in a number of different *Shewanella* genomes. Using this genomic information, this study confirmed the presence of sequences with strong identities to AHL acylase genes in a number of *Shewanella* strains isolated from the marine environment. The identified acylase, named AacS, cloned from one such strain inactivated synthetic AHLs and the long chain AHLs produced the human pathogen *Y. pseudotuberculosis*. Inactivation of long chain AHLs was consistent with previous studies investigating AHL turn over by *Shewanella* spp. (Tait *et al.*, 2009). AHL inactivation by *Shewanella* is not a unique phenotype in the marine environment as AHL inactivating activity in other marine bacteria such as *Vibrio* spp. and in the *Bacteroidetes* has been reported (Romero *et al.*, 2011; Romero *et al.*, 2010; Tait *et al.*, 2010). AHL inactivation would therefore affect algal bacterial interactions mediated by QS signal molecules. Tait *et al.* (2009) have already shown that the enzymatic inactivation of AHLs in poly-microbial biofilms abolishes the attraction of *Ulva* zoospores during settlement (Tait *et al.*, 2009). Data presented in this thesis showed both *Ulva* zoospore germination to be repressed by AHLs. This study also revealed poly-microbial communities composed of *Vibrio* spp., *Bacteroidetes* and *Shewanella* spp. to be present on *Ulva* spp. It is therefore conceivable that enzymic inactivation of AHLs will have stimulatory effects on germination as oppsed to the negative effect reported by Tait *et al.* (2009) on zoospore settlement (Tait *et al.*, 2009).

6.4 Quorum-quenching by Marine Microalgae

Finally, this study investigated the effects marine microalgae have on bacterial QS. The microalgae selected for this study are species commonly utilised in aquaculture (Muller-Feuga, 2000; Borowitzka, 1997). Many bacteria shown to be pathogenic to fish populations reared in aquaculture have been shown to possess AHL-mediated QS systems, such bacteria include *A. hydrophyla*, *A. salmonicida*, *V. anguillarium*, *V. tubiash*, *V. vulnificans* and *Yersinia ruckeri* (Temperton *et al.*, 2011; Fernández *et al.*, 2007; Milton, 2006; Swift *et al.*, 1997). Infection in aquacultural stocks is routinely prevented by supplementing feed with antibiotics; this has the potential to increase the prevalence of antibiotic resistant bacteria which is currently a significant cause for concern for both the UK Health Protection Agency and the World Health Organisation (Cabello, 2006). As such, alternative treatments that do not exert a selective pressure on pathogenic bacteria are currently being investigated.

One such alternative is to inhibit QS in these pathogens as QS often regulates virulence factor expression (Fernández *et al.*, 2007; Swift *et al.*, 1999). Inhibition of QS can result from the production of signal molecule inactivating enzymes, such as AHL lactonases and acylases, (Section 1.3.2), or by the production of compounds which have the ability to quench QS (Lin *et al.*, 2003; Dong *et al.*, 2000; Manefield *et al.*, 1999). Compounds that inhibit QS by quenching the effects of AHL type signalling molecules have been shown to be produced by a number of different marine organisms, including the freshwater microalgae *C. reinhardtii* (Skindersoe *et al.*, 2008; Teplitski *et al.*, 2004). This study has identified that the marine microalgal species *N. oculata*, *I. galbana* and *T. suecia* also produce and excrete a compound(s) that appears to have the ability to inhibit AHL-mediated QS. This was shown using AHL bio-reporters (Section 5.2.2). In assays using marine bacterial fish pathogens and in the opportunistic human pathogen *P. aeruginosa*, the expression of a number of genes previously linked with AHL-mediated QS were also shown to be inhibited by microalgal extracts. As such this is the first study to demonstrate a phenotypic effect of putative microalgal quorum-quenching apart from in an AHL bio-reporter. This study did not identify specifically what compound(s) were causing this apparent quorum-quenching activity; however, purification and characterisation of this compound(s) may reveal novel antibiotic compounds capable of attenuating virulence. Such compounds may be of benefit to the bio-control of marine pathogens that can affect aquacultural productivity and potentially human health and in the control of human pathogens in a broader medical setting.

Exploiting microalgal quorum-quenching compound production to attenuate virulence in marine fish pathogens detrimental to aquaculture benefits productivity without resorting to supplementing feed with antibiotics routinely used to treat human infections. This could both reduce the likelihood of spreading antibiotic resistance and reduce aquacultural costs as we are already using these microalgal species as food stocks. Additionally, exploiting quorum-quenching compound production by microalgal feed stocks in the bio-control of marine pathogens that effect human health would also be beneficial to aquaculture. The main route of transmission of the pathogenic marine *Vibrio* species such as *Vibrio parahaemolyticus* is through the ingestion of raw oysters (Daniels *et al.*, 2000). *V. parahaemolyticus* has been shown to regulate its virulence factors via QS and the *V. harveyi* like AHL synthase LuxM has been shown to be present in the *V. parahaemolyticus* genomes (Henke and Bassler, 2004; Makino *et al.*, 2003). By inhibiting QS, virulence may be reduced to levels sufficient to prevent disease in oysters and subsequently prevent human disease. This is compounded by data presented in section 5.2.4.2 that showed extracts from these microalgae effected QS mediated protease production in a number of marine pathogens both detrimental to aquacultural production and similar to marine pathogens that effect human health such as *V. parahaemolyticus* and *V. vulnificus*.

Sections 5.2.3 and 5.2.4.1 revealed that putative quorum-quenching compounds produced by the marine microalgae affected the expression of genes associated with virulence in the opportunistic human pathogen *P. aeruginosa*. This is of importance as the development of novel antimicrobial compounds, specifically those that do not induce a selective pressure on bacterial populations is a prominent area of current medical research. As such, it would be of interest to investigate how the production of quorum-quenching compounds in microalgae is controlled. Understanding how these compounds are produced, it may be possible to culture microalgae under conditions that select for quorum-quenching compound production. The initial step in further understanding quorum-quenching in microalgae would be to advance the work in this study by identifying these compounds(s). This can be achieved by continuing the fractionation of microalgal extracts initiated by this study and screening the resultant fractions with AHL bio-reporters. This study used solid phase extraction cartridges to carry out fractionation, however a technique such as reverse-phase HPLC could also be used and has proven to be successful in previous studies in identifying quorum-quenching compound(s) (Teplitski *et al.*, 2004). Identification of the quorum-quenching compound(s) would be best carried out using chemical analysis technique such as mass spectroscopy as has been used previously in the identification of similar acting compounds produced by freshwater microalgal species which inhibit bacterial QS, (Teplitski *et al.*, 2004), or full characterisation using techniques such as nuclear magnetic resonance spectroscopy. These compounds could then be easily purified and investigated for use in medicine to tackle both acute and systemic bacterial infections, where virulence is highly regulated by QS, such as in *P. aeruginosa*.

From an ecological perspective, it would also be interesting to investigate if microalgal quorum-quenching activity could affect macroalgal/bacterial interactions such as those observed in *Ulva* zoospore settlement, *Ulva* zoospore germination and the early growth of *Ulva* spp. germlings in a similar way to enzymic AHL inactivation by bacteria such as *Shewanella*. If so, it would add a deeper layer of complexity to these bacterial/algal interactions taking place in the marine environment. Additionally if such microalgal quorum-quenching compounds do affect these interactions, they may also have potential usages in the production of novel antifouling treatments. Overall, this thesis has furthered our understanding of how bacterial signalling molecules and their inactivation either by enzymes or by quorum-quenching compounds facilitate bacterial/algal interactions in the marine environment. It is also likely that as interactions are taking place between epiphytic bacteria and macroalgae like *Ulva*, similar bacterial/algal interactions may be facilitated by the production and turnover of QS signalling molecules in other marine species. As such, our current understanding of the importance of QS signalling in the marine environment and its potential biological, environmental and biotechnological implications is limited and therefore merits further research.

References

ABBOTT, I. A. 1996. Ethnobotany of seaweeds: clues to uses of seaweeds. *Hydrobiologia*, 326-327**,** 15-20.

AENDEKERK, S., GHYSELS, B., CORNELIS, P. & BAYSSE, C. 2002. Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium. *Microbiology,* 148**,** 2371-2381.

AHMER, B. M. 2004. Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. *Molecular Microbiolology,* 52**,** 933-945.

AHMER, B. M., VAN REEUWIJK, J., TIMMERS, C. D., VALENTINE, P. J. & HEFFRON, F. 1998. *Salmonella typhimurium* encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. *Journal of Bacteriology,* 180**,** 1185-1193.

ALEXEYEV, M. F. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria. *BioTechniques,* 26**,** 824-828.

ALLDREDGE, A. L. & SILVER, M. W. 1988. Characteristics, dynamics and significance of marine snow. *Progress In Oceanography,* 20**,** 41-82.

ALONSO, C., WARNECKE, F., AMANN, R. & PERNTHALER, J. 2007. High local and global diversity of Flavobacteria in marine plankton. *Environmental Microbiology,* 9**,** 1253-1266.

AN, D., DANHORN, T., FUQUA, C. & PARSEK, M. R. 2006. Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 3828-3833.

ASHEN, J. B. & GOFF, L. J. 2000. Molecular and ecological evidence for species specificity and coevolution in a group of marine algal-bacterial symbioses. *Applied and Environmental Microbiology,* 66**,** 3024-3030.

ATKINSON, S., CHANG, C. Y., PATRICK, H. L., BUCKLEY, C. M. F., WANG, Y., SOCKETT, R. E., CÁMARA, M. & WILLIAMS, P. 2008. Functional interplay between the *Yersinia pseudotuberculosis* YpsRI and YtbRI quorum sensing systems modulates swimming motility by controlling expression of *flhDC* and *fliA*. *Molecular Microbiology,* 69**,** 137-151.

ATKINSON, S., GOLDSTONE, R. J., JOSHUA, G. W. P., CHANG, C.-Y., PATRICK, H. L., CÁMARA, M., WREN, B. W. & WILLIAMS, P. 2011. Biofilm Development on *Caenorhabditis elegans* by *Yersinia* Is Facilitated by Quorum Sensing-Dependent Repression of Type III Secretion. *PLoS Pathogens,* 7**,** e1001250.

ATKINSON, S., SOCKETT, R. E., CAMARA, M. & WILLIAMS, P. 2006. Quorum sensing and the lifestyle of *Yersinia*. *Current Issues in Molecular Biology,* 8**,** 1-10.

ATKINSON, S., THROUP, J. P., STEWART, G. S. & WILLIAMS, P. 1999. A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Molecular Microbiology,* 33**,** 1267-1277.

AUSTIN, B. & DAY, J. G. 1990. Inhibition of prawn pathogenic *Vibrio spp.* by a commercial spray-dried preparation of *Tetraselmis suecica*. *Aquaculture,* 90**,** 389-392.

BAINTON, N. J., BYCROFT, B. W., CHHABRA, S. R., STEAD, P., GLEDHILL, L., HILL, P. J., REES, C. E., WINSON, M. K., SALMOND, G. P., STEWART, G. S. & ET AL. 1992. A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. *Gene,* 116**,** 87-91.

BARNARD, A. M., BOWDEN, S. D., BURR, T., COULTHURST, S. J., MONSON, R. E. & SALMOND, G. P. 2007. Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria. *Philosophical Transactions of the Royal Society London B Biological Sciences,* 362**,** 1165-1183.

BARNARD, A. M. & SALMOND, G. P. 2007. Quorum sensing in *Erwinia* species. *Analytical and Bioanalytical Chemistry,* 387**,** 415-423.

BASSLER, B. L. 2002. Small talk. Cell-to-cell communication in bacteria. *Cell,* 109**,** 421-424.

BASSLER, B. L., WRIGHT, M., SHOWALTER, R. E. & SILVERMAN, M. R. 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Molecular Microbiology,* 9**,** 773-786.

BECHER, A. & SCHWEIZER, H. P. 2000. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *Biotechniques,* 29**,** 948-952.

BECK VON BODMAN, S. & FARRAND, S. K. 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *Journal of Bacteriology,* 177**,** 5000-5008.

BECKER, J. G., BERARDESCO, G., RITTMANN, B. E. & STAHL, D. A. 2005. The Role of Syntrophic Associations in Sustaining Anaerobic Mineralization of Chlorinated Organic Compounds. *Environmental Health Perspectives,* 113**,** 310-316.

BELAS, R., MILEHAM, A., COHN, D., HILMAN, M., SIMON, M. & SILVERMAN, M. 1982. Bacterial bioluminescence: isolation and expression of the luciferase genes from *Vibrio harveyi*. *Science,* 218**,** 791-793.

BELIAEV, A. S., SAFFARINI, D. A., MCLAUGHLIN, J. L. & HUNNICUTT, D. 2001. MtrC, an outer membrane decahaem c cytochrome required for metal reduction in *Shewanella putrefaciens* MR-1. *Molecular Microbiology,* 39**,** 722-730.

BENGTSSON, M. M., SJOTUN, K. & OVREAS, L. 2010. Seasonal dynamics of bacterial biofilms on the kelp *Laminaria hyperborea*. *Aquatic Microbial Ecology,* 60**,** 71-83.

BERNBOM, N., NG, Y. Y., KJELLEBERG, S., HARDER, T. & GRAM, L. 2011. Marine bacteria from Danish coastal waters show antifouling activity against the marine fouling bacterium *Pseudoalteromonas sp*. strain S91 and zoospores of the green alga *Ulva australis* independent of bacteriocidal activity. *Applied and Environmental Microbiology,* 77**,** 8557-8567.

BIXLER, H. J. 1996. Recent developments in manufacturing and marketing carrageenan. *Hydrobiologia,* 326-327**,** 35-57.

BOBROV, A. G., BEARDEN, S. W., FETHERSTON, J. D., KHWEEK, A. A., PARRISH, K. D. & PERRY, R. D. 2007. Functional quorum sensing systems affect biofilm formation and protein expression in *Yersinia pestis*. *Advances in Experimental Medicine and Biology,* 603**,** 178-191.

BODOR, A. M., JANSCH, L., WISSING, J. & WAGNER-DOBLER, I. 2011. The *luxS* mutation causes loosely-bound biofilms in *Shewanella oneidensis*. *BMC Research Notes,* 4**,** 180.

BOETTCHER, K. J. & RUBY, E. G. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. *Journal of Bacteriology,* 177**,** 1053-1058.

BORCHARDT, S. A., ALLAIN, E. J., MICHELS, J. J., STEARNS, G. W., KELLY, R. F. & MCCOY, W. F. 2001. Reaction of Acylated Homoserine Lactone Bacterial Signaling Molecules with Oxidized Halogen Antimicrobials. *Applied and Environmental Microbiology,* 67**,** 3174-3179.

BOROWITZKA, M. 1997. Microalgae for aquaculture: Opportunities and constraints. *Journal of Applied Phycology,* 9**,** 393-401.

BOWMAN, J. P., REA, S. M., MCCAMMON, S. A. & MCMEEKIN, T. A. 2000. Diversity and community structure within anoxic sediment from marine salinity meromictic lakes and a coastal meromictic marine basin, Vestfold Hills, Eastern Antarctica. *Environmental Microbiology,* 2**,** 227-237.

BOZAL, N., MONTES, M. J., TUDELA, E., JIMENEZ, F. & GUINEA, J. 2002. *Shewanella frigidimarina* and *Shewanella livingstonensis* sp. nov. isolated from Antarctic coastal areas. *International Journal of Systematic and Evolutionary Microbiology,* 52**,** 195-205.

BRADLEY, P. M. 1991. Plant hormones do have a role in controlling growth and development of algae. *Journal of Phycology,* 27**,** 317-321.

BREDENBRUCH, F., GEFFERS, R., NIMTZ, M., BUER, J. & HÄUSSLER, S. 2006. The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environmental Microbiology,* 8**,** 1318-1329.

BROWN, M. V. & BOWMAN, J. P. 2001. A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). *FEMS Microbiology Ecology,* 35**,** 267-275.

BRUHN, J. B., GRAM, L. & BELAS, R. 2007. Production of Antibacterial Compounds and Biofilm Formation by Roseobacter Species Are Influenced by Culture Conditions. *Applied and Environmental Microbiology,* 73**,** 442-450.

BURKE, C., THOMAS, T., LEWIS, M., STEINBERG, P. & KJELLEBERG, S. 2011. Composition, uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*. *ISME Journal,* 5**,** 590-600.

BURROW, E. M. 2001. *Seaweeds of the British Isles, Volume 2: Chlorophyta,* London, Natural History Museum.

BUTT, A. A., FIGUEROA, J. & MARTIN, D. H. 1997. Ocular infection caused by three unusual marine organisms. *Clinical Infectious Diseases,* 24**,** 740.

CABELLO, F. C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology,* 8**,** 1137-1144.

CALLOW, J. & CALLOW, M. 2006. The *Ulva* Spore Adhesive System. *In:* SMITH, A. M. & CALLOW, J. A. (eds.) *Biological Adhesisves.* Berlin: Springer.

CALLOW, J. A., STANLEY, M. S., WETHERBEE, R. & CALLOW, M. E. 2000. Cellular and molecular approaches to understanding primary adhesion in *Enteromorpha*: an overview. *Biofouling,* 16**,** 141-150.

CALLOW, M. E. 1986. Fouling Algae from 'In-service' Ships. *Botanica Marina,* 29**,** 351-357.

CALLOW, M. E. & CALLOW, J. A. 2000. Substratum location and zoospore behaviour in the fouling alga *Enteromorpha*. *Biofouling,* 15**,** 49-56.

CALLOW, M. E. & CALLOW, J. A. 2002. Marine biofouling a sticky problem. *Biologist,* 49**,** 1-5.

CALLOW, M. E., CALLOW, J. A., ISTA, L. K., COLEMAN, S. E., NOLASCO, A. C. & LOPEZ, G. P. 2000. Use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores. *Applied and Environmental Microbiology,* 66**,** 3249-3254.

CALLOW, M. E., CALLOW, J. A., PICKETT-HEAPS, J. D. & WETHERBEE, R. 1997. Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules: Quantitative settlement studies and video microscopy. *Journal of Phycology,* 33**,** 938-947.

CALLOW, M. E., JENNINGS, A. R., BRENNAN, A. B., SEEGERT, C. E., GIBSON, A., WILSON, L., FEINBERG, A., BANEY, R. & CALLOW, J. A. 2002. Microtopographic Cues for Settlement of Zoospores of the Green Fouling Alga *Enteromorpha*. *Biofouling,* 18**,** 229-236.

CAMARA, M. HARDMAN, A. WILLIAMS, P. & MILTON, D. 2002. Quorum Sencing in *Vibrio cholerae*. *Nature Genetics*, 32, 217-218.

CAO, J. G. & MEIGHEN, E. A. 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *Journal of Biological Chemistry,* 264**,** 21670-21676.

CARLIER, A., UROZ, S., SMADJA, B., FRAY, R., LATOUR, X., DESSAUX, Y. & FAURE, D. 2003. The Ti plasmid of *Agrobacterium tumefaciens* harbors an *attM*-paralogous gene, *aiiB*, also encoding *N*-acyl homoserine lactonase activity. *Applied and Environmental Microbiology,* 69**,** 4989-4993.

CHAI, Y., ZHU, J. & WINANS, S. C. 2001. TrlR, a defective TraR-like protein of *Agrobacterium tumefaciens*, blocks TraR function *in vitro* by forming inactive TrlR:TraR dimers. *Molecular Microbiology,* 40**,** 414-421.

CHHABRA, S. R., HARTY, C., HOOI, D. S., DAYKIN, M., WILLIAMS, P., TELFORD, G., PRITCHARD, D. I. & BYCROFT, B. W. 2003. Synthetic analogues of the bacterial signal (quorum sensing) molecule *N*-(3-oxododecanoyl)-L-homoserine lactone as immune modulators. *Journal of Medical Chemistry,* 46**,** 97-104.

CHIEN, Y.-H. & SHIAU, W.-C. 2005. The effects of dietary supplementation of algae and synthetic astaxanthin on body astaxanthin, survival, growth, and low dissolved oxygen stress resistance of kuruma prawn, *Marsupenaeus japonicus* Bate. *Journal of Experimental Marine Biology and Ecology,* 318**,** 201-211.

CHISHOLM, J. R. M., DAUGA, C., AGERON, E., GRIMONT, P. A. D. & JAUBERT, J. M. 1996. Roots in Mixotrophic Algae. *Nature,* 381**,** 382.

CHU, W., ZERE, T. R., WEBER, M. M., WOOD, T. K., WHITELEY, M., HIDALGO-ROMANO, B., VALENZUELA, E., JR. & MCLEAN, R. J. 2012. Indole production promotes *Escherichia coli* mixed-culture growth with *Pseudomonas aeruginosa* by inhibiting quorum signaling. *Applied and Environmental Microbiology,* 78**,** 411-419.

CHUGANI, S. A., WHITELEY, M., LEE, K. M., D'ARGENIO, D., MANOIL, C. & GREENBERG, E. P. 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences U S A,* 98**,** 2752-2757.

COSTERTON, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. & LAPPIN-SCOTT, H. M. 1995. Microbial Biofilms. *Annual Review of Microbiology,* 49**,** 711-745.

COTTRELL, M. T. & KIRCHMAN, D. L. 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Applied and Environmental Microbiology,* 66**,** 5116-5122.

CROFT, M. T., LAWRENCE, A. D., RAUX-DEERY, E., WARREN, M. J. & SMITH, A. G. 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature,* 438**,** 90-93.

CROFT, M. T., WARREN, M. J. & SMITH, A. G. 2006. Algae need their vitamins. *Eukaryotic Cell,* 5**,** 1175-1183.

CROXATTO, A., CHALKER, V. J., LAURITZ, J., JASS, J., HARDMAN, A., WILLIAMS, P., CÁMARA, M. & MILTON, D. L. 2002. VanT, a Homologue of *Vibrio harveyi* LuxR, Regulates Serine, Metalloprotease, Pigment, and Biofilm Production in *Vibrio anguillarum*. *Journal of Bacteriology,* 184**,** 1617-1629.

CURTIS, T. P., SLOAN, W. T. & SCANNELL, J. W. 2002. Estimating prokaryotic diversity and its limits. *Proceedings of the National Academy of Sciences,* 99**,** 10494-10499.

DANIELS, N. A., MACKINNON, L., BISHOP, R., ALTEKRUSE, S., RAY, B., HAMMOND, R. M., THOMPSON, S., WILSON, S., BEAN, N. H., GRIFFIN, P. M. & SLUTSKER, L. 2000. *Vibrio parahaemolyticus* Infections in the United States, 1973–1998. *Journal of Infectious Diseases,* 181**,** 1661-1666.

DE KIEVIT, T. R., GILLIS, R., MARX, S., BROWN, C. & IGLEWSKI, B. H. 2001. Quorum-Sensing Genes in *Pseudomonas aeruginosa* Biofilms: Their Role and Expression Patterns. *Applied and Environmental Microbiology,* 67**,** 1865-1873.

DECHO, A., W. 2000. Microbial biofilms in intertidal systems: an overview. *Continental Shelf Research,* 20**,** 1257-1273.

DEFOIRDT, T., BOON, N., BOSSIER, P. & VERSTRAETE, W. 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. *Aquaculture,* 240**,** 69-88.

DEFOIRDT, T., BOSSIER, P., SORGELOOS, P. & VERSTRAETE, W. 2005. The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. *Environmental Microbiology,* 7**,** 1239-1247.

DELONG, E. F., FRANKS, D. G. & ALLDREDGE, A. L. 1993. Phylogenetic Diversity of Aggregate-Attached vs. Free-Living Marine Bacterial Assemblages. *Limnology and Oceanography,* 38**,** 11.

DENG, M.-D. & COLEMAN, J. R. 1999. Ethanol Synthesis by Genetic Engineering in Cyanobacteria. *Applied and Environmental Microbiology,* 65**,** 523-528.

DERBY, H. A. & HAMMER, B. W. 1931. *Bacteriology of butter: Bacteriological studies on surface taint butter*, Agricultural Experiment Station, Iowa State College of Agriculture and Mechanic Arts.

DEVINE, J. H., SHADEL, G. S. & BALDWIN, T. O. 1989. Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC7744. *Proceedings of the National Academy of Sciences U S A,* 86**,** 5688-5692.

DEZIEL, E., GOPALAN, S., TAMPAKAKI, A. P., LEPINE, F., PADFIELD, K. E., SAUCIER, M., XIAO, G. & RAHME, L. G. 2005. The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of *N*-acyl-L-homoserine lactones. *Molecular Microbiology,* 55**,** 998-1014.

DEZIEL, E., LEPINE, F., MILOT, S., HE, J., MINDRINOS, M. N., TOMPKINS, R. G. & RAHME, L. G. 2004. Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proceedings of the National Academy of Sciences U S A,* 101**,** 1339-1344.

DIETRICH, L. E., PRICE-WHELAN, A., PETERSEN, A., WHITELEY, M. & NEWMAN, D. K. 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Molecular Microbiology,* 61**,** 1308-1321.

DIGGLE, S. P., GRIFFIN, A. S., CAMPBELL, G. S. & WEST, S. A. 2007. Cooperation and conflict in quorum-sensing bacterial populations. *Nature,* 450**,** 411-414.

DIGGLE, S. P., LUMJIAKTASE, P., DIPILATO, F., WINZER, K., KUNAKORN, M., BARRETT, D. A., CHHABRA, S. R., CAMARA, M. & WILLIAMS, P. 2006. Functional genetic analysis reveals a 2-Alkyl-4-quinolone signalling system in the human pathogen *Burkholderia pseudomallei* and related bacteria. *Chemistry & Biology,* 13, 701-710.

DIGGLE, S. P., WINZER, K., CHHABRA, S. R., WORRALL, K. E., CAMARA, M. & WILLIAMS, P. 2003. The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Molecular Microbiology,* 50, 29-43.

DILLON, P. S., MAKI, J. S. & MITCHELL, R. 1989. Adhesion of *Enteromorpha* swarmers to microbial films. *Microbial Ecology,* 17**,** 39-47.

DOBRETSOV, S., DAHMS, H. U. & QIAN, P. Y. 2006. Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling,* 22**,** 43-54.

DOLNICK, R., WU, Q., ANGELINO, N. J., STEPHANIE, L. V., CHOW, K.-C., SUFRIN, J. R. & DOLNICK, B. J. 2005. Enhancement of 5-Fluorouracil Sensitivity by an rTS Signaling Mimic in H630 Colon Cancer Cells. *Cancer Research,* 65**,** 5917-5924.

DOMÍNGUEZ, H., VOGEL, B. F., GRAM, L., HOFFMANN, S. & SCHAEBEL, S. 1996. *Shewanella alga* Bacteremia in Two Patients with Lower Leg Ulcers. *Clinical Infectious Diseases,* 22**,** 1036-1039.

DONG, Y. H. & ZHANG, L. H. 2005. Quorum sensing and quorum-quenching enzymes. *Journal of Microbiology,* 43 Spec No**,** 101-109.

DONG, Y.-H., ZHANG, X.-F., XU, J.-L. & ZHANG, L.-H. 2004. Insecticidal *Bacillus thuringiensis* Silences *Erwinia carotovora* Virulence by a New Form of Microbial Antagonism, Signal Interference. *Applied and Environmental Microbiology,* 70**,** 954-960.

DONG, Y. H., GUSTI, A. R., ZHANG, Q., XU, J. L. & ZHANG, L. H. 2002. Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. *Applied and Environmental Microbiology,* 68**,** 1754-1759.

DONG, Y. H., XU, J. L., LI, X. Z. & ZHANG, L. H. 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proceedings of the National Academy of Sciences U S A,* 97**,** 3526-3531.

DROOP, M. R. 2007. Vitamins, phytoplankton and bacteria: symbiosis or scavenging? *Journal of Plankton Research,* 29**,** 107-113.

DUAN, K., DAMMEL, C., STEIN, J., RABIN, H. & SURETTE, M. G. 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Molecular Microbiology,* 50**,** 1477-1491.

DUBERN, J.-F., LUGTENBERG, B. J. J. & BLOEMBERG, G. V. 2006. The *ppuI-rsaL-ppuR* Quorum-Sensing System Regulates Biofilm Formation of *Pseudomonas putida* PCL1445 by Controlling Biosynthesis of the Cyclic Lipopeptides Putisolvins I and II. *Journal of Bacteriology*, 188**,** 2898-2906.

DUCHAUD, E., BOUSSAHA, M., LOUX, V., BERNARDET, J. F., MICHEL, C., KEROUAULT, B., MONDOT, S., NICOLAS, P., BOSSY, R., CARON, C., BESSIERES, P., GIBRAT, J. F., CLAVEROL, S., DUMETZ, F., LE HENAFF, M. & BENMANSOUR, A. 2007. Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. *Nature Biotechnology,* 25**,** 763-769.

DUNNY, G. M. & LEONARD, B. A. 1997. Cell-cell communication in Gram-positive bacteria. *Annual Reviews in Microbiology,* 51**,** 527-564.

DWORJANYN, S. A., DE NYS, R. & STEINBERG, P. D. 1999. Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*. *Marine Biology,* 133**,** 727-736.

EBERHARD, A., BURLINGAME, A. L., EBERHARD, C., KENYON, G. L., NEALSON, K. H. & OPPENHEIMER, N. J. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry,* 20**,** 2444-2449.

EBERHARD, A., LONGIN, T., WIDRIG, C. A. & STRANICK, S. J. 1991. Synthesis of the *lux* gene autoinducer in *Vibrio fischeri* is positively autoregulated. *Archives of Microbiology,* 155**,** 294-297.

EBERL, L., WINSON, M. K., STERNBERG, C., STEWART, G. S., CHRISTIANSEN, G., CHHABRA, S. R., BYCROFT, B., WILLIAMS, P., MOLIN, S. & GIVSKOV, M. 1996a. Involvement of *N*-acyl-L-hormoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Molecular Microbiology,* 20**,** 127-136.

ECKBURG, P. B., BIK, E. M., BERNSTEIN, C. N., PURDOM, E., DETHLEFSEN, L., SARGENT, M., GILL, S. R., NELSON, K. E. & RELMAN, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-1638.

EDWARDS, J. L., SMITH, D. L., CONNOLLY, J., MCDONALD, J. E., COX, M. J., JOINT, I., EDWARDS, C. & MCCARTHY, A. J. 2010. Identification of Carbohydrate Metabolism Genes in the Metagenome of a Marine Biofilm Community Shown to Be Dominated by Gammaproteobacteria and Bacteroidetes. *Genes,* 1**,** 371-384.

EGAN, S., JAMES, S., HOLMSTRÖM, C. & KJELLEBERG, S. 2001. Inhibition of algal spore germination by the marine bacterium *Pseudoalteromonas tunicata*. *FEMS Microbiology Ecology,* 35**,** 67-73.

EILERS, H., PERNTHALER, J., PEPLIES, J., GLOCKNER, F. O., GERDTS, G. & AMANN, R. 2001. Isolation of novel pelagic bacteria from the German bight and their seasonal contributions to surface picoplankton. *Applied and Environmental Microbiology,* 67**,** 5134-5142.

EL-SAYED, A. K., HOTHERSALL, J. & THOMAS, C. M. 2001. Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586. *Microbiology,* 147**,** 2127-2139.

ENGEBRECHT, J. & SILVERMAN, M. 1984. Identification of genes and gene products necessary for bacterial bioluminescence. *Proceedings of the National Academy of Sciences U S A,* 81**,** 4154-4158.

ESSAR, D. W., EBERLY, L., HAN, C. Y. & CRAWFORD, I. P. 1990. DNA sequences and characterization of four early genes of the tryptophan pathway in *Pseudomonas aeruginosa*. *Journal of Bacteriology,* 172**,** 853-866.

FERNÁNDEZ, L., MÉNDEZ, J. & GUIJARRO, J. A. 2007. Molecular virulence mechanisms of the fish pathogen *Yersinia ruckeri*. *Veterinary Microbiology,* 125**,** 1-10.

FINLAY, J. A., CALLOW, M. E., SCHULTZ, M. P., SWAIN, G. W. & CALLOW, J. A. 2002. Adhesion Strength of Settled Spores of the Green Alga *Enteromorpha*. *Biofouling,* 18**,** 251-256.

FLETCHER, M. P., DIGGLE, S. P., CRUSZ, S. A., CHHABRA, S. R., CAMARA, M. & WILLIAMS, P. 2007. A dual biosensor for 2-alkyl-4-quinolone quorum-sensing signal molecules. *Environmental Microbiology,* 9**,** 2683-2693.

FREEMAN, J. A. & BASSLER, B. L. 1999a. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. *Molecular Microbiology,* 31**,** 665-677.

FREEMAN, J. A. & BASSLER, B. L. 1999b. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. *Journal of Bacteriology,* 181**,** 899-906.

FREEMAN, J. A., LILLEY, B. N. & BASSLER, B. L. 2000. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. *Molecular Microbiology,* 35**,** 139-149.

FUJIYA, M., MUSCH, M. W., NAKAGAWA, Y., HU, S., ALVERDY, J., KOHGO, Y., SCHNEEWIND, O., JABRI, B. & CHANG, E. B. 2007. The *Bacillus subtilis* Quorum-Sensing Molecule CSF Contributes to Intestinal Homeostasis via OCTN2, a Host Cell Membrane Transporter. *Cell host & microbe,* 1**,** 299-308.

FUKAMI, K., NISHIJIMA, T. & ISHIDA, Y. 1997. Stimulative and inhibitory effects of bacteria on the growth of microalgae. *Hydrobiologia,* 358**,** 185-191.

FUKUSHIMA, J., ISHIWATA, T., YOU, Z., ISHII, T., SHIGEMATSU, T., KURATA, M., CHIKUMARU-FUJITA, S., BYCROFT, B. W., STEWART, G. S. A. B., KAWAMOTO, S., MORIHARA, K., WILLIAMS, P. & OKUDA, K. 1997. Dissection of the promoter/operator region and evaluation of *N*-acylhomoserine lactone mediated transcriptional regulation of elastase expression in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters,* 146**,** 311-318.

FUQUA, C., WINANS, S. C. & GREENBERG, E. P. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annual Reviews in Microbiology,* 50**,** 727-751.

FUQUA, W. C. & WINANS, S. C. 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumour metabolite. *Journal of Bacteriology,* 176**,** 2796-2806.

FUQUA, W. C., WINANS, S. C. & GREENBERG, E. P. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology,* 176**,** 269-275.

GALLAGHER, L. A. & MANOIL, C. 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *Journal of Bacteriology,* 183**,** 6207-6214.

GALLAGHER, L. A., MCKNIGHT, S. L., KUZNETSOVA, M. S., PESCI, E. C. & MANOIL, C. 2002. Functions required for extracellular quinolone signalling by *Pseudomonas aeruginosa*. *Journal of Bacteriology,* 184**,** 6472-6480.

GAMBELLO, M. J. & IGLEWSKI, B. H. 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *Journal of Bacteriology,* 173**,** 3000-3009.

GAO, M., TEPLITSKI, M., ROBINSON, J. B. & BAUER, W. D. 2003. Production of Substances by *Medicago truncatula* that Affect Bacterial Quorum Sensing. *Molecular Plant-Microbe Interactions,* 16**,** 827-834.

GARRITY, G. M., BELL, J. A. & LILBURN, T. G. 2002. *Taxonomic outline of the prokaryotes. Bergey's manual of systematic bacteriology, Second Edition*, Springer-Verlag, New York.

GILBERT, J. A., FIELD, D., SWIFT, P., NEWBOLD, L., OLIVER, A., SMYTH, T., SOMERFIELD, P. J., HUSE, S. & JOINT, I. 2009. The seasonal structure of microbial communities in the Western English Channel. *Environmental Microbiology,* 11**,** 3132-3139.

GILBERT, J. A., FIELD, D., SWIFT, P., THOMAS, S., CUMMINGS, D., TEMPERTON, B., WEYNBERG, K., HUSE, S., HUGHES, M., JOINT, I., SOMERFIELD, P. J. & MUHLING, M. 2010. The taxonomic and functional diversity of microbes at a temperate coastal site: a 'multi-omic' study of seasonal and diel temporal variation. *PLoS One,* 5**,** e15545.

GILSON, L., KUO, A. & DUNLAP, P. V. 1995. AinS and a new family of autoinducer synthesis proteins. *Journal of Bacteriology,* 177**,** 6946-6951.

GLESSNER, A., SMITH, R. S., IGLEWSKI, B. H. & ROBINSON, J. B. 1999. Roles of *Pseudomonas aeruginosa las* and *rhl* Quorum-Sensing Systems in Control of Twitching Motility. *Journal of Bacteriology,* 181**,** 1623-1629.

GONZALEZ, A., BELLENBERG, S., MAMANI, S., RUIZ, L., ECHEVERRIA, A., SOULERE, L., DOUTHEAU, A., DEMERGASSO, C., SAND, W., QUENEAU, Y., VERA, M. & GUILIANI, N. 2012. AHL signaling molecules with a large acyl chain enhance biofilm formation on sulfur and metal sulfides by the bioleaching bacterium *Acidithiobacillus ferrooxidans*. *Applied Microbiology and Biotechnology*. DOI 10.1007/s00253-012-4229-3.

GRAM, L. 1994. Siderophore-Mediated Iron Sequestering by *Shewanella putrefaciens*. *Applied and Environmental Microbiology,* 60**,** 2132-2136.

GRAM, L., GROSSART, H.-P., SCHLINGLOFF, A. & KIØRBOE, T. 2002. Possible Quorum Sensing in Marine Snow Bacteria: Production of Acylated Homoserine Lactones by Roseobacter Strains Isolated from Marine Snow. *Applied and Environmental Microbiology,* 68**,** 4111-4116.

GRAM, L. & HUSS, H. H. 1996. Microbiological spoilage of fish and fish products. *International Journal of Food Microbiology,* 33**,** 121-137.

GRAY, K. M., PASSADOR, L., IGLEWSKI, B. H. & GREENBERG, E. P. 1994. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. *Journal of Bacteriology,* 176**,** 3076-3080.

GROSSART, H. P., LEVOLD, F., ALLGAIER, M., SIMON, M. & BRINKHOFF, T. 2005. Marine diatom species harbour distinct bacterial communities. *Environmental Microbiology,* 7**,** 860-873.

GUIL-GUERRERO, J. L., NAVARRO-JUÁREZ, R., LÓPEZ-MARTÍNEZ, J. C., CAMPRA-MADRID, P. & REBOLLOSO-FUENTES, M. 2004. Functional properties of the biomass of three microalgal species. *Journal of Food Engineering,* 65**,** 511-517.

GUILLARD, R. R. L. & RYTHER, J. H. 1963. Studies of marine planktonic diatoms. *I. Cyclotella* nana Hustedt and *Detonula confervacea* (Cleve). *Canadian Journal of Microbiology,* 8**,** 229-239.

GUIRY, M. D. 2012. *Ulva Linnaeus, 1753. In: Guiry, M.D. & Guiry, G.M. (2012). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. Accessed through: World Register of Marine* [Online]. Available: http://www.marinespecies.org/aphia.php?p=taxdetails&id=144296 [Accessed 29/07/2012 2012].

HAMMER, B. K. & BASSLER, B. L. 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Molecular Microbiology,* 50**,** 101-104.

HANZELKA, B. L., PARSEK, M. R., VAL, D. L., DUNLAP, P. V., CRONAN, J. E., JR. & GREENBERG, E. P. 1999. Acylhomoserine lactone synthase activity of the *Vibrio fischeri* AinS protein. *Journal of Bacteriology,* 181**,** 5766-5770.

HANZELKA, B. L., STEVENS, A. M., PARSEK, M. R., CRONE, T. J. & GREENBERG, E. P. 1997. Mutational analysis of the *Vibrio fischeri* LuxI polypeptide: critical regions of an autoinducer synthase. *Journal of Bacteriology,* 179**,** 4882-4887.

HAO, G., ZHANG, H., ZHENG, D. & BURR, T. J. 2005. *luxR* Homolog *avhR* in *Agrobacterium vitis* Affects the Development of a Grape-Specific Necrosis and a Tobacco Hypersensitive Response. *Journal of Bacteriology,* 187**,** 185-192.

HAO, Y., WINANS, S. C., GLICK, B. R. & CHARLES, T. C. 2010. Identification and characterization of new LuxR/LuxI-type quorum sensing systems from metagenomic libraries. *Environmental Microbiology,* 12**,** 105-117.

HARRIS, E. H. 2001. *Chlamydomonas* as a model organism. *Annual Review of Plant Physiology and Plant Molecular Biology,* 52**,** 363-406.

HASTINGS, J. W. & NEALSON, K. H. 1977. Bacterial bioluminescence. *Annual Reviews in Microbiology,* 31**,** 549-595.

HASTINGS, J. W., POTRIKUS, C. J., GUPTA, S. C., KURFURST, M. & MAKEMSON, J. C. 1985. Biochemistry and physiology of bioluminescent bacteria. *Advances in Microbial Physiology,* 26**,** 235-291.

HAU, H. H. & GRALNICK, J. A. 2007. Ecology and biotechnology of the genus *Shewanella*. *Annual Reviews in Microbiology,* 61**,** 237-258.

HAYASHI, K. & KOJIMA, C. 2008. pCold-GST vector: a novel cold-shock vector containing GST tag for soluble protein production. *Protein Expression and Purification,* 62**,** 120-127.

HAYDEN, H. S. 2003. Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European Journal of Phycology,* 38**,** 277-294.

HENKE, J. M. & BASSLER, B. L. 2004. Quorum Sensing Regulates Type III Secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *Journal of Bacteriology,* 186**,** 3794-3805.

HENTZER, M., RIEDEL, K., RASMUSSEN, T. B., HEYDORN, A., ANDERSEN, J. B., PARSEK, M. R., RICE, S. A., EBERL, L., MOLIN, S., HØIBY, N., KJELLEBERG, S. & GIVSKOV, M. 2002. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology,* 148**,** 87-102.

HMELO, L. & VAN MOOY, B. A. S. 2009. Kinetic constraints on acylated homoserine lactone-based quorum sensing in marine environments. *Aquatic Microbial Ecology,* 54**,** 127-133.

HOLLANTS, J., LEROUX, O., LELIAERT, F., DECLEYRE, H., DE CLERCK, O. & WILLEMS, A. 2011. Who is in there? Exploration of endophytic bacteria within the siphonous green seaweed *Bryopsis* (Bryopsidales, Chlorophyta). *PLoS One,* 6**,** e26458.

HOLT, H. M., GAHRN-HANSEN, B. & BRUUN, B. 2005. *Shewanella algae* and *Shewanella putrefaciens*: clinical and microbiological characteristics. *Clinical Microbiology and Infection,* 11**,** 347-352.

HOLT, H. M., SØGAARD, P. & GAHRN-HANSEN, B. 1997. Ear infections with *Shewanella alga*: a bacteriologic, clinical and epidemiologic study of 67 cases. *Clinical Microbiology and Infection,* 3**,** 329-333.

HORINOUCHI, S. 2002. A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in *Streptomyces griseus*. *Frontiers in bioscience,* 7**,** d2045-57.

HORNG, Y. T., DENG, S. C., DAYKIN, M., SOO, P. C., WEI, J. R., LUH, K. T., HO, S. W., SWIFT, S., LAI, H. C. & WILLIAMS, P. 2002. The LuxR family protein SpnR functions as a negative regulator of *N*-acylhomoserine lactone-dependent quorum sensing in *Serratia marcescens*. *Molecular Microbiology,* 45**,** 1655-1671.

HOSNI, T., MORETTI, C., DEVESCOVI, G., SUAREZ-MORENO, Z. R., FATMI, M. B., GUARNACCIA, C., PONGOR, S., ONOFRI, A., BUONAURIO, R. & VENTURI, V. 2011. Sharing of quorum-sensing signals and role of interspecies communities in a bacterial plant disease. *ISME Journal,* 5**,** 1857-1870.

HUANG, J. J., HAN, J. I., ZHANG, L. H. & LEADBETTER, J. R. 2003. Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. *Applied and Environmental Microbiology,* 69**,** 5941-5949.

HUANG, Y.-L., KI, J.-S., LEE, O. O. & QIAN, P.-Y. 2009. Evidence for the dynamics of Acyl homoserine lactone and AHL-producing bacteria during subtidal biofilm formation. *ISME Journal,* 3**,** 296-304.

HUANG, Y. L., KI, J. S., CASE, R. J. & QIAN, P. 2008. Diversity and acyl-homoserine lactone production among subtidal biofilm-forming bacteria. *Aquatic Microbial Ecology,* 52**,** 185-193.

HUBER, B., RIEDEL, K., HENTZER, M., HEYDORN, A., GOTSCHLICH, A., GIVSKOV, M., MOLIN, S. & EBERL, L. 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology,* 147**,** 2517-2528.

JAHOOR, A., PATEL, R., BRYAN, A., DO, C., KRIER, J., WATTERS, C., WAHLI, W., LI, G., WILLIAMS, S. C. & RUMBAUGH, K. P. 2008. Peroxisome proliferator-activated receptors mediate host cell proinflammatory responses to *Pseudomonas aeruginosa* autoinducer. *Journal of Bacteriology,* 190**,** 4408-4415.

JANSSENS, J. C. A., STEENACKERS, H., ROBIJNS, S., GELLENS, E., LEVIN, J., ZHAO, H., HERMANS, K., DE COSTER, D., VERHOEVEN, T. L., MARCHAL, K., VANDERLEYDEN, J., DE VOS, D. E. & DE KEERSMAECKER, S. C. J. 2008. Brominated Furanones Inhibit Biofilm Formation by *Salmonella enterica* Serovar Typhimurium. *Applied and Environmental Microbiology,* 74**,** 6639-6648.

JARRELL, K. F. & MCBRIDE, M. J. 2008. The surprisingly diverse ways that prokaryotes move. *Nature Reviews Microbiology,* 6**,** 466-476.

JI, G., BEAVIS, R. & NOVICK, R. P. 1997. Bacterial interference caused by autoinducing peptide variants. *Science,* 276**,** 2027-2030.

JI, G., BEAVIS, R. C. & NOVICK, R. P. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proceedings of the National Academy of Sciences U S A,* 92**,** 12055-12059.

JOHN, R. P., ANISHA, G. S., NAMPOOTHIRI, K. M. & PANDEY, A. 2011. Micro and macroalgal biomass: A renewable source for bioethanol. *Bioresource Technology,* 102**,** 186-193.

JOINT, I., CALLOW, M. E., CALLOW, J. A. & CLARKE, K. R. 2000. The attachment of *Enteromorpha* zoospores to a bacterial biofilm assemblage. *Biofouling,* 16**,** 151-158.

JOINT, I., TAIT, K., CALLOW, M. E., CALLOW, J. A., MILTON, D., WILLIAMS, P. & CAMARA, M. 2002. Cell-to-cell communication across the prokaryote-eukaryote boundary. *Science,* 298**,** 1207-1207.

JOINT, I., TAIT, K. & WHEELER, G. 2007. Cross-kingdom signalling: exploitation of bacterial quorum sensing molecules by the green seaweed *Ulva*. *Philosophical Transactions of the Royal Society London B Biological Sciences,* 362**,** 1223-1233.

JUHAS, M., WIEHLMANN, L., HUBER, B., JORDAN, D., LAUBER, J., SALUNKHE, P., LIMPERT, A. S., VON GÖTZ, F., STEINMETZ, I., EBERL, L. & TÜMMLER, B. 2004. Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology,* 150**,** 831-841.

KAMIYA, R. & WITMAN, G. B. 1984. Submicromolar levels of calcium control the balance of beating between the two flagella in demembranated models of *Chlamydomonas*. *The Journal of Cell Biology,* 98**,** 97-107.

KAPLAN, H. B. & GREENBERG, E. P. 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *Journal of Bacteriology,* 163**,** 1210-1214.

KASAI, S., OKADA, K., HOSHINO, A., IIDA, T. & HONDA, T. 2007. Lateral Transfer of the *lux* Gene Cluster. *Journal of Biochemistry,* 141**,** 231-237.

KATO, C. & NOGI, Y. 2001. Correlation between phylogenetic structure and function: examples from deep-sea *Shewanella*. *FEMS Microbiology Ecology,* 35**,** 223-230.

KHASHE, S. & JANDA, J. M. 1998. Biochemical and Pathogenic Properties of *Shewanella alga* and *Shewanella putrefaciens*. *Journal of Clinical Microbiology,* 36**,** 783-787.

KIM, S. Y., LEE, S. E., KIM, Y. R., KIM, C. M., RYU, P. Y., CHOY, H. E., CHUNG, S. S. & RHEE, J. H. 2003. Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. *Molecular Microbiology,* 48**,** 1647-1664.

KIRCHMAN, D. L. 2002. The ecology of Cytophaga-Flavobacteria in aquatic environments. *FEMS Microbiology Ecology,* 39**,** 91-100.

KOLIBACHUK, D. & GREENBERG, E. P. 1993. The *Vibrio fischeri* luminescence gene activator LuxR is a membrane-associated protein. *Journal of Bacteriology,* 175**,** 7307-7312.

KORMAS, K. A., TIVEY, M. K., VON DAMM, K. & TESKE, A. 2006. Bacterial and archaeal phylotypes associated with distinct mineralogical layers of a white smoker spire from a deep-sea hydrothermal vent site (9 degrees N, East Pacific Rise). *Environmental Microbiology,* 8**,** 909-920.

KOVACH, M. E., ELZER, P. H., HILL, D. S., ROBERTSON, G. T., FARRIS, M. A., ROOP, R. M., 2ND & PETERSON, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene,* 166**,** 175-176.

KOVACH, M. E., PHILLIPS, R. W., ELZER, P. H., ROOP, R. M., 2ND & PETERSON, K. M. 1994. pBBR1MCS: a broad-host-range cloning vector. *Biotechniques,* 16**,** 800-802.

KRIEG, N. R., LUDWIG, W., WHITMAN, W. B., HEDLUND, B. P., PASTER, B. J., STALEY, J. T., WARD, N., BROWN, D. & PARTE, A. 2010. *Bergey's Manual of Systematic Bacteriology,* New York, Springer-Verlag.

LANDINI, P., JUBELIN, G. & DOREL-FLAMAN, C. 2006. The Molecular Genetics of Bioadhesion and Biofilm Formation Biological Adhesives. *In:* SMITH, A. M. & CALLOW, J. A. (eds.). Springer Berlin Heidelberg.

LANG, S. & WULLBRANDT, D. 1999. Rhamnose lipids--biosynthesis, microbial production and application potential. *Applied Microbiology and Biotechnology,* 51**,** 22-32.

LAPOTA, D., GALT, C., LOSEE, J. R., HUDDELL, H. D., ORZECH, J. K. & NEALSON, K. H. 1988. Observations and measurements of planktonic bioluminescence in and around a milky sea. *Journal of Experimental Marine Biology and Ecology,* 119**,** 55-81.

LASSMANN, B., GUSTAFSON, D. R., WOOD, C. M. & ROSENBLATT, J. E. 2007. Re-emergence of anaerobic bacteremia. *Clinical Infectious Diseases,* 44**,** 895-900.

LATIFI, A., FOGLINO, M., TANAKA, K., WILLIAMS, P. & LAZDUNSKI, A. 1996. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Molecular Microbiology,* 21**,** 1137-1146.

LATIFI, A., WINSON, M. K., FOGLINO, M., BYCROFT, B. W., STEWART, G. S., LAZDUNSKI, A. & WILLIAMS, P. 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Molecular Microbiology,* 17**,** 333-343.

LEADBETTER, J. R. & GREENBERG, E. P. 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *Journal of Bacteriology,* 182**,** 6921-6926.

LEE, J.-H., LEQUETTE, Y. & GREENBERG, E. P. 2006. Activity of purified QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Molecular Microbiology,* 59**,** 602-609.

LEE, K.-H. & RUBY, E. G. 1994. Effect of the Squid Host on the Abundance and Distribution of Symbiotic *Vibrio fischeri* in Nature. *Applied and Environmental Microbiology,* 60**,** 1565-1571.

LENNOX, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology,* 1**,** 190-206.

LEWENZA, S., CONWAY, B., GREENBERG, E. P. & SOKOL, P. A. 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. *Journal of Bacteriology,* 181**,** 748-756.

LI, Q., DU, W. & LIU, D. 2008. Perspectives of microbial oils for biodiesel production. *Applied Microbiology and Biotechnology,* 80**,** 749-756.

LI, Y. H., TANG, N., ASPIRAS, M. B., LAU, P. C., LEE, J. H., ELLEN, R. P. & CVITKOVITCH, D. G. 2002. A quorum-sensing signalling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *Journal of Bacteriology,* 184**,** 2699-2708.

LIN, Y. H., XU, J. L., HU, J., WANG, L. H., ONG, S. L., LEADBETTER, J. R. & ZHANG, L. H. 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Molecular Microbiology,* 47**,** 849-860.

LINDUM, P. W., ANTHONI, U., CHRISTOPHERSEN, C., EBERL, L., MOLIN, S. & GIVSKOV, M. 1998. *N*-acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. *Journal of Bacteriology,* 180**,** 6384-6388.

LINNÉ, C. V. & SALVIUS, L. 1753. *Caroli Linnaei ... Species plantarum :exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas,* Holmiae :, Impensis Laurentii Salvii.

LIU, D., THOMAS, P. W., MOMB, J., HOANG, Q. Q., PETSKO, G. A., RINGE, D. & FAST, W. 2007. Structure and specificity of a quorum-quenching lactonase (AiiB) from *Agrobacterium tumefaciens*. *Biochemistry,* 46**,** 11789-11799.

LIU, H., KOH, K., KIM, J., SEO, Y. & PARK, S. 2008. The effects of betonicine, floridoside, and isethionic acid from the red alga *Ahnfeltiopsis flabelliformis* on quorum-sensing activity. *Biotechnology and Bioprocess Engineering,* 13**,** 458-463.

LONG, R. A., QURESHI, A., FAULKNER, D. J. & AZAM, F. 2003. 2-n-Pentyl-4-quinolinol produced by a marine *Alteromonas sp.* and its potential ecological and biogeochemical roles. *Applied and Environmental Microbiology,* 69**,** 568-576.

LOVLEY, D. R. & PHILLIPS, E. J. P. 1988. Novel Mode of Microbial Energy Metabolism: Organic Carbon Oxidation Coupled to Dissimilatory Reduction of Iron or Manganese. *Applied and Environmental Microbiology,* 54**,** 1472-1480.

LYDELL, C., DOWELL, L., SIKAROODI, M., GILLEVET, P. & EMERSON, D. 2004. A population survey of members of the phylum Bacteroidetes isolated from salt marsh sediments along the east coast of the United States. *Microbial Ecology,* 48**,** 263-273.

LYON, W. R., MADDEN, J. C., LEVIN, J. C., STEIN, J. L. & CAPARON, M. G. 2001. Mutation of *luxS* affects growth and virulence factor expression in *Streptococcus pyogenes*. *Molecular Microbiology,* 42**,** 145-157.

MACDONELL, M. T. & COLWELL, R. R. 1985. Phylogeny of the Vibrionaceae, and Recommendation for Two New Genera, *Listonella* and *Shewanella*. *Systematic and Applied Microbiology,* 6**,** 171-182.

MAGGS, C. A. & CALLOW, M. E. 2001. *Algal Spores*, John Wiley & Sons, Ltd.

MAKEMSON, J. C., FULAYFIL, N. R., LANDRY, W., VAN ERT, L. M., WIMPEE, C. F., WIDDER, E. A. & CASE, J. F. 1997. *Shewanella woodyi* sp. nov., an Exclusively Respiratory Luminous Bacterium Isolated from the Alboran Sea. *International Journal of Systematic Bacteriology,* 47**,** 1034-1039.

MAKINO, K., OSHIMA, K., KUROKAWA, K., YOKOYAMA, K., UDA, T., TAGOMORI, K., IIJIMA, Y., NAJIMA, M., NAKANO, M., YAMASHITA, A., KUBOTA, Y., KIMURA, S., YASUNAGA, T., HONDA, T., SHINAGAWA, H., HATTORI, M. & IIDA, T. 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V cholerae*. *The Lancet,* 361**,** 743-749.

MANEFIELD, M., DE NYS, R., KUMAR, N., READ, R., GIVSKOV, M., STEINBERG, P. & KJELLEBERG, S. 1999. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology,* 145**,** 283-291.

MANEFIELD, M., RASMUSSEN, T. B., HENZTER, M., ANDERSEN, J. B., STEINBERG, P., KJELLEBERG, S. & GIVSKOV, M. 2002. Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology,* 148**,** 1119-1127.

MANZ, W., WENDT-POTTHOFF, K., NEU, T. R., SZEWZYK, U. & LAWRENCE, J. R. 1999. Phylogenetic Composition, Spatial Structure, and Dynamics of Lotic Bacterial Biofilms Investigated by Fluorescent in Situ Hybridization and Confocal Laser Scanning Microscopy. *Microbial Ecology,* 37**,** 225-237.

MARKETON, M. M., GLENN, S. A., EBERHARD, A. & GONZÁLEZ, J. E. 2003. Quorum Sensing Controls Exopolysaccharide Production in *Sinorhizobium meliloti*. *Journal of Bacteriology,* 185**,** 325-331.

MARKETON, M. M. & GONZALEZ, J. E. 2002. Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. *Journal of Bacteriology,* 184**,** 3466-3475.

MARKETON, M. M., GRONQUIST, M. R., EBERHARD, A. & GONZÁLEZ, J. E. 2002. Characterization of the *Sinorhizobium meliloti sinR/sinI* Locus and the Production of Novel *N*-acyl Homoserine Lactones. *Journal of Bacteriology,* 184**,** 5686-5695.

MARSHALL, K., JOINT, I., CALLOW, M. E. & CALLOW, J. A. 2006. Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. *Microbial Ecology,* 52**,** 302-310.

MARTINEZ-ALONSO, M., ESCOLANO, J., MONTESINOS, E. & GAJU, N. 2010. Diversity of the bacterial community in the surface soil of a pear orchard based on 16S rRNA gene analysis. *International Microbiology,* 13**,** 123-134.

MARUYAMA, A., MAEDA, M. & SIMIDU, U. 1986. Occurrence of plant hormone (cytokinin)-producing bacteria in the sea. *Journal of Applied Microbiology,* 61**,** 569-574.

MATHESIUS, U., MULDERS, S., GAO, M., TEPLITSKI, M., CAETANO-ANOLLÉS, G., ROLFE, B. G. & BAUER, W. D. 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proceedings of the National Academy of Sciences USA,* 100**,** 1444-1449.

MATSUO, Y., IMAGAWA, H., NISHIZAWA, M. & SHIZURI, Y. 2005. Isolation of an algal morphogenesis inducer from a marine bacterium. *Science,* 307**,** 1598.

MATSUO, Y., SUZUKI, M., KASAI, H., SHIZURI, Y. & HARAYAMA, S. 2003. Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga *Monostroma oxyspermum*. *Environmental Microbiology,* 5**,** 25-35.

MAURHOFER, M., REIMMANN, C., SCHMIDLI-SACHERER, P., HEEB, S., HAAS, D. & DEFAGO, G. 1998. Salicylic Acid Biosynthetic Genes Expressed in *Pseudomonas fluorescens* Strain P3 Improve the Induction of Systemic Resistance in Tobacco Against Tobacco Necrosis Virus. *Phytopathology,* 88**,** 678-684.

MAZMANIAN, S. K., ROUND, J. L. & KASPER, D. L. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature,* 453**,** 620-625.

MCCLEAN, K. H., WINSON, M. K., FISH, L., TAYLOR, A., CHHABRA, S. R., CAMARA, M., DAYKIN, M., LAMB, J. H., SWIFT, S., BYCROFT, B. W., STEWART, G. S. & WILLIAMS, P. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology,* 143**,** 3703-3711.

MCDOUGALD, D., SRINIVASAN, S., RICE, S. A. & KJELLEBERG, S. 2003. Signal-mediated cross-talk regulates stress adaptation in *Vibrio* species. *Microbiology,* 149**,** 1923-1933.

MCKNIGHT, S. L., IGLEWSKI, B. H. & PESCI, E. C. 2000. The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. *Journal of Bacteriology,* 182**,** 2702-2708.

MEI, G.-Y., YAN, X.-X., TURAK, A., LUO, Z.-Q. & ZHANG, L.-Q. 2010. AidH, an Alpha/Beta-Hydrolase Fold Family Member from an *Ochrobactrum sp.* Strain, Is a Novel *N*-acylhomoserine Lactonase. *Applied and Environmental Microbiology,* 76**,** 4933-4942.

MEIGHEN, E. A. 1991. Molecular biology of bacterial bioluminescence. *Microbiology Reviews*, 55**,** 123-142.

MILLER, M. B. & BASSLER, B. L. 2001. Quorum sensing in bacteria. *Annual Reviews in Microbiology,* 55**,** 165-199.

MILLER, T. R. & BELAS, R. 2004. Dimethylsulfoniopropionate Metabolism by Pfiesteria-Associated Roseobacter spp. *Applied and Environmental Microbiology,* 70**,** 3383-3391.

MILLER, S. D., HADDOCK, S. H. D., ELVIDGE, C. D. & LEE, T. F. 2005. Detection of a bioluminescent milky sea from space. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 14181-14184.

MILLER, S. T., XAVIER, K. B., CAMPAGNA, S. R., TAGA, M. E., SEMMELHACK, M. F., BASSLER, B. L. & HUGHSON, F. M. 2004. *Salmonella typhimurium* Recognizes a Chemically Distinct Form of the Bacterial Quorum-Sensing Signal AI-2. *Molecular Cell,* 15**,** 677-687.

MILTON, D. L. 2006. Quorum sensing in *Vibrio*s: Complexity for diversification. *International Journal of Medical Microbiology,* 296**,** 61-71.

MILTON, D. L., CHALKER, V. J., KIRKE, D., HARDMAN, A., CAMARA, M. & WILLIAMS, P. 2001. The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl)homoserine lactone and *N*-hexanoylhomoserine lactone. *Journal of Bacteriology,* 183**,** 3537-3547.

MILTON, D. L., HARDMAN, A., CAMARA, M., CHHABRA, S. R., BYCROFT, B. W., STEWART, G. S. & WILLIAMS, P. 1997. Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI/vanR* locus and identification of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone. *Journal of Bacteriology,* 179**,** 3004-3012.

MIYAKE, K. 1931. On the Conjugation of the Gametes and the Development of the Zoospores in Ulvaceae. *Journal of the College of Agriculture,* 11**,** 341-357.

MOHAMED, N. M., CICIRELLI, E. M., KAN, J., CHEN, F., FUQUA, C. & HILL, R. T. 2008. Diversity and quorum-sensing signal production of Proteobacteria associated with marine sponges. *Environmental Microbiology,* 10**,** 75-86.

MOROHOSHI, T., NAKAZAWA, S., EBATA, A., KATO, N. & IKEDA, T. 2008. Identification and characterization of *N*-acylhomoserine lactone-acylase from the fish intestinal *Shewanella* sp. strain MIB015. *Bioscience, Biotechnology and Biochemistry,* 72**,** 1887-1893.

MOROHOSHI, T., SHIONO, T., TAKIDOUCHI, K., KATO, M., KATO, N., KATO, J. & IKEDA, T. 2007. Inhibition of Quorum Sensing in *Serratia marcescens* AS-1 by Synthetic Analogs of *N*-acylhomoserine Lactone. *Applied and Environmental Microbiology,* 73**,** 6339-6344.

MORÉ, M. I., FINGER, L. D., STRYKER, J. L., FUQUA, C., EBERHARD, A. & WINANS, S. C. 1996. Enzymatic Synthesis of a Quorum-Sensing Autoinducer Through Use of Defined Substrates. *Science,* 272**,** 1655-1658.

MOSER, D. P. & NEALSON, K. H. 1996. Growth of the facultative anaerobe *Shewanella putrefaciens* by elemental sulfur reduction. *Applied and Environmental Microbiology,* 62**,** 2100-2105.

MUHLING, M., WOOLVEN-ALLEN, J., MURRELL, J. C. & JOINT, I. 2008. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *ISME Journal,* 2**,** 379-392.

MULLER-FEUGA, A. 2000. The role of microalgae in aquaculture: situation and trends. *Journal of Applied Phycology,* 12**,** 527-534.

MUNN, C. B. 2004. *Marine Microbiology: ecology and fundementals,* London, BIOS Scientific Publishers.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology,* 59**,** 695-700.

MYERS, C. R. & MYERS, J. M. 1997. Replication of plasmids with the p15A origin in *Shewanella putrefaciens* MR-1. *Letters in Applied Microbiology,* 24**,** 221-225.

MYERS, L. L., FIREHAMMER, B. D., SHOOP, D. S. & BORDER, M. M. 1984. *Bacteroides fragilis*: a possible cause of acute diarrheal disease in newborn lambs. *Infection and Immunity,* 44**,** 241-244.

MÜH, U., SCHUSTER, M., HEIM, R., SINGH, A., OLSON, E. R. & GREENBERG, E. P. 2006. Novel *Pseudomonas aeruginosa* Quorum-Sensing Inhibitors Identified in an Ultra-High-Throughput Screen. *Antimicrobial Agents and Chemotherapy,* 50**,** 3674-3679.

NAKANISHI, K., NISHIJIMA, M., NISHIMURA, M., KUWANO, K. & SAGA, N. 1996. Bacteria that induce morphogenesis in *Ulva pertusa* (Chlorophyta) grown under axenic conditions. *Journal of Phycology,* 32**,** 479-482.

NATRAH, F. M. I., KENMEGNE, M. M., WIYOTO, W., SORGELOOS, P., BOSSIER, P. & DEFOIRDT, T. 2011. Effects of micro-algae commonly used in aquaculture on acyl-homoserine lactone quorum sensing. *Aquaculture,* 317**,** 53-57.

NEALSON, K. & SCOTT, J. 2006. Ecophysiology of the Genus *Shewanella* The Prokaryotes. *In:* DWORKIN, M., FALKOW, S., ROSENBERG, E., SCHLEIFER, K.-H. & STACKEBRANDT, E. (eds.). Springer New York.

NEALSON, K. H. & HASTINGS, J. W. 2006. Quorum Sensing on a Global Scale: Massive Numbers of Bioluminescent Bacteria Make Milky Seas. *Applied and Environmental Microbiology,* 72**,** 2295-2297.

NEALSON, K. H., PLATT, T. & HASTINGS, J. W. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology,* 104**,** 313-322.

NEMATOLLAHI, A., DECOSTERE, A., PASMANS, F. & HAESEBROUCK, F. 2003. *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of Fish Diseases,* 26**,** 563-574.

NEW, M. B. 1990. Freshwater prawn culture: a review. *Aquaculture,* 88**,** 99-143.

NORQVIST, A., HAGSTROM, A. & WOLF-WATZ, H. 1989. Protection of rainbow trout against vibriosis and furunculosis by the use of attenuated strains of *Vibrio anguillarum*. *Applied and Environmental Microbiology,* 55**,** 1400-1405.

NOVICK, R. P. 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology,* 48**,** 1429-1449.

OCHSNER, U. A. & REISER, J. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences USA,* 92**,** 6424-6428.

ORTORI, C. A., ATKINSON, S., CHHABRA, S. R., CAMARA, M., WILLIAMS, P. & BARRETT, D. A. 2007. Comprehensive profiling of *N*-acylhomoserine lactones produced by *Yersinia pseudotuberculosis* using liquid chromatography coupled to hybrid quadrupole-linear ion trap mass spectrometry. *Analytical and Bioanalytical Chemistry,* 387**,** 497-511.

PAGANI, L., LANG, A., VEDOVELLI, C., MOLING, O., RIMENTI, G., PRISTERÀ, R. & MIAN, P. 2003. Soft Tissue Infection and Bacteremia Caused by *Shewanella putrefaciens*. *Journal of Clinical Microbiology,* 41**,** 2240-2241.

PARK, S. Y., KANG, H. O., JANG, H. S., LEE, J. K., KOO, B. T. & YUM, D. Y. 2005. Identification of extracellular *N*-acylhomoserine lactone acylase from a *Streptomyces sp.* and its application to quorum-quenching. *Applied and Environmental Microbiology,* 71**,** 2632-2641.

PASTER, B. J., LUDWIG, W., WEISBURG, W. G., STACKEBRANDT, E., HESPELL, R. B., HAHN, C. M., REICHENBACH, H., STETTER, K. O. & WOESE, C. R. 1985. A Phylogenetic Grouping of the Bacteroides, Cytophagas, and Certain Flavobacteria. *Systematic and Applied Microbiology,* 6**,** 34-42.

PATANKAR, A. V. & GONZALEZ, J. E. 2009. Orphan LuxR regulators of quorum sensing. *FEMS Microbiology Reviews,* 33**,** 739-756.

PATEL, P., CALLOW, M. E., JOINT, I. & CALLOW, J. A. 2003. Specificity in the settlement - modifying response of bacterial biofilms towards zoospores of the marine alga *Enteromorpha*. *Environmental Microbiology,* 5**,** 338-349.

PAULICK, A., KOERDT, A., LASSAK, J., HUNTLEY, S., WILMS, I., NARBERHAUS, F. & THORMANN, K. M. 2009. Two different stator systems drive a single polar flagellum in *Shewanella oneidensis* MR-1. *Molecular Microbiology,* 71**,** 836-850.

PELLOCK, B. J., TEPLITSKI, M., BOINAY, R. P., BAUER, W. D. & WALKER, G. C. 2002. A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *Journal of Bacteriology,* 184**,** 5067-5076.

PESCI, E. C., MILBANK, J. B., PEARSON, J. P., MCKNIGHT, S., KENDE, A. S., GREENBERG, E. P. & IGLEWSKI, B. H. 1999. Quinolone signalling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences U S A,* 96**,** 11229-11234.

PESSI, G. & HAAS, D. 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *Journal of Bacteriology,* 182**,** 6940-6949.

PETERS, L., KÖNIG, G. M., WRIGHT, A. D., PUKALL, R., STACKEBRANDT, E., EBERL, L. & RIEDEL, K. 2003. Secondary Metabolites of *Flustra foliacea* and Their Influence on Bacteria. *Applied and Environmental Microbiology,* 69**,** 3469-3475.

PICARDAL, F., ARNOLD, R. G. & HUEY, B. B. 1995. Effects of electron donor and acceptor conditions on reductive dehalogenation of tetrachloromethane by *Shewanella putrefaciens* 200. *Applied and Environmental Microbiology,* 61**,** 8-12.

PIERSON, L. S., 3RD, KEPPENNE, V. D. & WOOD, D. W. 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *Journal of Bacteriology,* 176**,** 3966-3974.

PINHASSI, J., SALA, M. M., HAVSKUM, H., PETERS, F., GUADAYOL, O., MALITS, A. & MARRASE, C. 2004. Changes in bacterioplankton composition under different phytoplankton regimens. *Applied and Environmental Microbiology,* 70**,** 6753-6766.

PIPER, K. R., BECK VON BODMAN, S. & FARRAND, S. K. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature,* 362**,** 448-450.

PLOUG, H. 2000. Bacterial growth and grazing on diatom aggregates: Respiratory carbon turnover as a function of aggregate size and sinking velocity. *Limnology and Oceanography,* 45**,** 1467-1475.

PREGNALL, A. M. 1983. Release of dissolved organic carbon from the estuarine intertidal macroalga *Enteromorpha prolifera*. *Marine Biology,* 73**,** 37-42.

PROVASOLI, L. & PINTNER, I. J. 1980. Bacteria induced polymophism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). *Journal of Phycology,* 16**,** 196-201.

PUNTA, M., COGGILL, P. C., EBERHARDT, R. Y., MISTRY, J., TATE, J., BOURSNELL, C., PANG, N., FORSLUND, K., CERIC, G., CLEMENTS, J., HEGER, A., HOLM, L., SONNHAMMER, E. L., EDDY, S. R., BATEMAN, A. & FINN, R. D. 2012. The Pfam protein families database. *Nucleic Acids Research,* 40**,** D290-301.

PUSKAS, A., GREENBERG, E. P., KAPLAN, S. & SCHAEFER, A. L. 1997. A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. *Journal of Bacteriology,* 179**,** 7530-7537.

QUADRI, L. E. N. 2002. Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria. *Antonie van Leeuwenhoek,* 82**,** 133-145.

QUINONES, B., DULLA, G. & LINDOW, S. E. 2005. Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Molecular Plant-Microbe Interactions,* 18**,** 682-693.

RAO, D., WEBB, J. S. & KJELLEBERG, S. 2006. Microbial colonization and competition on the marine alga *Ulva australis*. *Applied and Environmental Microbiology,* 72**,** 5547-5555.

RASMUSSEN, T. B., BJARNSHOLT, T., SKINDERSOE, M. E., HENTZER, M., KRISTOFFERSEN, P., KOTE, M., NIELSEN, J., EBERL, L. & GIVSKOV, M. 2005. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *Journal of Bacteriology,* 187, 1799-1814.

RASMUSSEN, T. B., MANEFIELD, M., ANDERSEN, J. B., EBERL, L., ANTHONI, U., CHRISTOPHERSEN, C., STEINBERG, P., KJELLEBERG, S. & GIVSKOV, M. 2000. How *Delisea pulchra* furanones affect quorum sensing and swarming motility in *Serratia liquefaciens* MG1. *Microbiology,* 146**,** 3237-3244.

READING, N. C. & SPERANDIO, V. 2006. Quorum sensing: the many languages of bacteria. *FEMS Microbiology Letters,* 254**,** 1-11.

REN, D., BEDZYK, L. A., YE, R. W., THOMAS, S. M. & WOOD, T. K. 2004. Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signalling system of *Escherichia coli*. *Biotechnology and Bioengineering,* 88**,** 630-642.

RIEDEL, K., HENTZER, M., GEISENBERGER, O., HUBER, B., STEIDLE, A., WU, H., HØIBY, N., GIVSKOV, M., MOLIN, S. & EBERL, L. 2001. *N*-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology,* 147**,** 3249-3262.

RIVAS, M., SEEGER, M., HOLMES, D. S. & JEDLICKI, E. 2005. A Lux-like quorum sensing system in the extreme acidophile *Acidithiobacillus ferrooxidans*. *Biological Research* , 38**,** 283-297.

RODELAS, B., LITHGOW, J. K., WISNIEWSKI-DYE, F., HARDMAN, A., WILKINSON, A., ECONOMOU, A., WILLIAMS, P. & DOWNIE, J. A. 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. viciae. *Journal of Bacteriology,* 181**,** 3816-3823.

ROMERO, M., AVENDANO-HERRERA, R., MAGARINOS, B., CAMARA, M. & OTERO, A. 2010. Acylhomoserine lactone production and degradation by the fish pathogen *Tenacibaculum maritimum*, a member of the Cytophaga-Flavobacterium-Bacteroides (CFB) group. *FEMS Microbiology Letters,* 304**,** 131-139.

ROMERO, M., MARTIN-CUADRADO, A. B., ROCA-RIVADA, A., CABELLO, A. M. & OTERO, A. 2011. Quorum-quenching in cultivable bacteria from dense marine coastal microbial communities. *FEMS Microbiology Ecology,* 75**,** 205-217.

ROSQVIST, R., SKURNIK, M. & WOLF-WATZ, H. 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. *Nature,* 334**,** 522-525.

SALVESEN, I., REITAN, K. I., SKJERMO, J. & ØIE, G. 2000. Microbial environments in marine larviculture:Impacts of algal growth rates on the bacterial load in six microalgae. *Aquaculture International,* 8**,** 275-287.

SAMBROOK, J. & RUSSELL, D. W. 2001. *Molecular Cloning A Laboratory Manual,* New York, Cold Spring Harbor Laboratory Press.

SANTOS EDE, O., ALVES, N., JR., DIAS, G. M., MAZOTTO, A. M., VERMELHO, A., VORA, G. J., WILSON, B., BELTRAN, V. H., BOURNE, D. G., LE ROUX, F. & THOMPSON, F. L. 2011. Genomic and proteomic analyses of the coral pathogen *Vibrio coralliilyticus* reveal a diverse virulence repertoire. *ISME Journal,* 5**,** 1471-1483.

SAPP, M., SCHWADERER, A., WILTSHIRE, K., HOPPE, H.-G., GERDTS, G. & WICHELS, A. 2007. Species-Specific Bacterial Communities in the Phycosphere of Microalgae? *Microbial Ecology,* 53**,** 683-699.

SATO, K., NAITO, M., YUKITAKE, H., HIRAKAWA, H., SHOJI, M., MCBRIDE, M. J., RHODES, R. G. & NAKAYAMA, K. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proceedings of the National Academy of Sciences,* 107**,** 276-281.

SATYANARAYANA, K. G., MARIANO, A. B. & VARGAS, J. V. C. 2011. A review on microalgae, a versatile source for sustainable energy and materials. *International Journal of Energy Research,* 35**,** 291-311.

SCHAEFER, A. L., HANZELKA, B. L., PARSEK, M. R. & GREENBERG, E. P. 2000. Detection, purification, and structural elucidation of the acylhomoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Methods in Enzymology.* Academic Press.

SCHAUDER, S., SHOKAT, K., SURETTE, M. G. & BASSLER, B. L. 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Molecular Microbiology,* 41**,** 463-476.

SCHIPPER, C., HORNUNG, C., BIJTENHOORN, P., QUITSCHAU, M., GROND, S. & STREIT, W. R. 2009. Metagenome-Derived Clones Encoding Two Novel Lactonase Family Proteins Involved in Biofilm Inhibition in *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology,* 75**,** 224-233.

SCHLOSS, P. D. & HANDELSMAN, J. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology,* 71**,** 1501-1506.

SCHULTZ, M. P., FINLAY, J. A., CALLOW, M. E. & CALLOW, J. A. 2000. A turbulent channel flow apparatus for the determination of the adhesion strength of microfouling organisms. *Biofouling,* 15**,** 243-251.

SCOTT, J. H. & NEALSON, K. H. 1994. A biochemical study of the intermediary carbon metabolism of *Shewanella putrefaciens*. *Journal of Bacteriology,* 176**,** 3408-3411.

SEYEDSAYAMDOST, M. R., CARR, G., KOLTER, R. & CLARDY, J. 2011. Roseobacticides: Small Molecule Modulators of an Algal-Bacterial Symbiosis. *Journal of the American Chemical Society,* 133**,** 18343-18349.

SHARIF, D. I., GALLON, J., SMITH, C. J. & DUDLEY, E. 2008. Quorum sensing in Cyanobacteria: *N*-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium *Gloeothece* PCC6909. *ISME Journal,* 2**,** 1171-1182.

SHARIFAH, E. N. & EGUCHI, M. 2011. The Phytoplankton *Nannochloropsis oculata* Enhances the Ability of *Roseobacter* Clade Bacteria to Inhibit the Growth of Fish Pathogen *Vibrio anguillarum*. *PLoS ONE,* 6**,** e26756.

SHAW, P. D., PING, G., DALY, S. L., CHA, C., CRONAN, J. E., JR., RINEHART, K. L. & FARRAND, S. K. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proceedings of the National Academy of Sciences USA,* 94**,** 6036-6041.

SIBLEY, C. D., DUAN, K., FISCHER, C., PARKINS, M. D., STOREY, D. G., RABIN, H. R. & SURETTE, M. G. 2008. Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathogens,* 4**,** e1000184.

SJÖBLOM, S., BRADER, G., KOCH, G. & PALVA, E. T. 2006. Cooperation of two distinct ExpR regulators controls quorum sensing specificity and virulence in the plant pathogen *Erwinia* carotovora. *Molecular Microbiology,* 60**,** 1474-1489.

SKINDERSOE, M., ETTINGER-EPSTEIN, P., RASMUSSEN, T., BJARNSHOLT, T., DE NYS, R. & GIVSKOV, M. 2008. Quorum Sensing Antagonism from Marine Organisms. *Marine Biotechnology,* 10**,** 56-63.

SLOCK, J., VANRIET, D., KOLIBACHUK, D. & GREENBERG, E. P. 1990. Critical regions of the *Vibrio fischeri luxR* protein defined by mutational analysis. *Journal of Bacteriology,* 172**,** 3974-3979.

SOBERÓN-CHÁVEZ, G., AGUIRRE-RAMÍREZ, M. & SÁNCHEZ, R. 2005. The *Pseudomonas aeruginosa* RhlA enzyme is involved in rhamnolipid and polyhydroxyalkanoate production. *Journal of Industrial Microbiology & Biotechnology,* 32**,** 675-677.

SOKOL, P. A., SAJJAN, U., VISSER, M. B., GINGUES, S., FORSTNER, J. & KOOI, C. 2003. The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections. *Microbiology,* 149**,** 3649-3658.

SONG, Y., XIE, C., ONG, Y. M., GAN, Y. H. & CHUA, K. L. 2005. The BpsIR quorum-sensing system of *Burkholderia pseudomallei*. *Journal of Bacteriology,* 187**,** 785-790.

SPOLAORE, P., JOANNIS-CASSAN, C., DURAN, E. & ISAMBERT, A. 2006. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering,* 101**,** 87-96.

STAUFENBERGER, T., THIEL, V., WIESE, J. & IMHOFF, J. F. 2008. Phylogenetic analysis of bacteria associated with *Laminaria saccharina*. *FEMS Microbiology Ecology,* 64**,** 65-77.

STEINDLER, L. & VENTURI, V. 2007. Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiology Letters,* 266**,** 1-9.

STINTZI, A., EVANS, K., MEYER, J.-M. & POOLE, K. 1998. Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: *lasR/lasI* mutants exhibit reduced pyoverdine biosynthesis. *FEMS Microbiology Letters,* 166**,** 341-345.

STOVER, C. K., PHAM, X. Q., ERWIN, A. L., MIZOGUCHI, S. D., WARRENER, P., HICKEY, M. J., *et al.* 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature,* 406**,** 959-964.

STROEHER, U. H., PATON, A. W., OGUNNIYI, A. D. & PATON, J. C. 2003. Mutation of *luxS* of *Streptococcus pneumoniae* Affects Virulence in a Mouse Model. *Infection and Immunity,* 71**,** 3206-3212.

SUN, J., DANIEL, R., WAGNER-DOBLER, I. & ZENG, A.-P. 2004. Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. *BMC Evolutionary Biology,* 4**,** 36.

SWEM, L. R., SWEM, D. L., WINGREEN, N. S. & BASSLER, B. L. 2008. Deducing receptor signalling parameters from *in vivo* analysis: LuxN/AI-1 quorum sensing in *Vibrio harveyi*. *Cell,* 134**,** 461-473.

SWIFT, S., KARLYSHEV, A. V., FISH, L., DURANT, E. L., WINSON, M. K., CHHABRA, S. R., WILLIAMS, P., MACINTYRE, S. & STEWART, G. S. 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. *Journal of Bacteriology,* 179**,** 5271-5281.

SWIFT, S., LYNCH, M. J., FISH, L., KIRKE, D. F., TOMÁS, J. M., STEWART, G. S. A. B. & WILLIAMS, P. 1999. Quorum Sensing-Dependent Regulation and Blockade of Exoprotease Production in *Aeromonas hydrophila*. *Infection and Immunity,* 67**,** 5192-5199.

SWIFT, S., WINSON, M. K., CHAN, P. F., BAINTON, N. J., BIRDSALL, M., REEVES, P. J., REES, C. E., CHHABRA, S. R., HILL, P. J., THROUP, J. P. & ET AL. 1993. A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Molecular Microbiology,* 10**,** 511-520.

TAIT, K., HUTCHISON, Z., THOMPSON, F. L. & MUNN, C. B. 2010. Quorum sensing signal production and inhibition by coral-associated *Vibrios*. *Environmental Microbiology Reports,* 2**,** 145-150.

TAIT, K., JOINT, I., DAYKIN, M., MILTON, D. L., WILLIAMS, P. & CAMARA, M. 2005. Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga *Ulva* to bacterial biofilms. *Environmental Microbiology,* 7**,** 229-240.

TAIT, K., WILLIAMSON, H., ATKINSON, S., WILLIAMS, P., CAMARA, M. & JOINT, I. 2009. Turnover of quorum sensing signal molecules modulates cross-kingdom signalling. *Environmental Microbiology,* 11**,** 1792-1802.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution,* 28**,** 2731-2739.

TANAKA, S., YOSHIDA, M., MURAKAMI, Y., OGIWARA, T., SHOJI, M., KOBAYASHI, S., WATANABE, S., MACHINO, M. & FUJISAWA, S. 2008. The relationship of *Prevotella intermedia*, *Prevotella nigrescens* and *Prevotella melaninogenica* in the supragingival plaque of children, caries and oral malodor. *Journal of Clinical Paediatric Dentistry,* 32**,** 195-200.

TATEWAKI, M., PROVASOLI, L. & PINTNER, I. J. 1983. Morphogenesis of *Monostroma oxyspermum* (Kütz.) Doty (Chlorophyceae) in axenic culture, especially in bialgal culture. *Journal of Phycology,* 19**,** 409-416.

TAYLOR, A. R., MANISON, N. F. H., FERNANDEZ, C., WOOD, J. & BROWNLEE, C. 1996. Spatial Organization of Calcium Signaling Involved in Cell Volume Control in the Fucus Rhizoid. *The Plant Cell Online,* 8**,** 2015-2031.

TAYLOR, M. W., SCHUPP, P. J., BAILLIE, H. J., CHARLTON, T. S., DE NYS, R., KJELLEBERG, S. & STEINBERG, P. D. 2004. Evidence for acyl homoserine lactone signal production in bacteria associated with marine sponges. *Applied and Environmental Microbiology,* 70**,** 4387-4389.

TEELING, H., FUCHS, B. M., BECHER, D., KLOCKOW, C., GARDEBRECHT, A., BENNKE, C. M., KASSABGY, M., HUANG, S., MANN, A. J., WALDMANN, J., WEBER, M., KLINDWORTH, A., OTTO, A., LANGE, J., BERNHARDT, J., REINSCH, C., HECKER, M., PEPLIES, J., BOCKELMANN, F. D., CALLIES, U., GERDTS, G., WICHELS, A., WILTSHIRE, K. H., GLÖCKNER, F. O., SCHWEDER, T. & AMANN, R. 2012. Substrate-Controlled Succession of Marine Bacterioplankton Populations Induced by a Phytoplankton Bloom. *Science,* 336**,** 608-611.

TEMPERTON, B., THOMAS, S., TAIT, K., PARRY, H., EMERY, M., ALLEN, M., QUINN, J., MACGRATH, J. & GILBERT, J. 2011. Permanent draft genome sequence of *Vibrio tubiashii* strain NCIMB 1337 (ATCC19106). *Standards in Genomic Sciences,* 4**,** 183-90.

TEPLITSKI, M., CHEN, H., RAJAMANI, S., GAO, M., MERIGHI, M., SAYRE, R. T., ROBINSON, J. B., ROLFE, B. G. & BAUER, W. D. 2004. *Chlamydomonas reinhardtii* Secretes Compounds That Mimic Bacterial Signals and Interfere with Quorum Sensing Regulation in Bacteria. *Plant Physiology,* 134**,** 137-146.

THOMAS, F., HEHEMANN, J. H., REBUFFET, E., CZJZEK, M. & MICHEL, G. 2011. Environmental and gut bacteroidetes: the food connection. *Frontiers in Microbiology,* 2**,** 93.

THOMAS, K. V., FILEMAN, T. W., READMAN, J. W. & WALDOCK, M. J. 2001. Antifouling Paint Booster Biocides in the UK Coastal Environment and Potential Risks of Biological Effects. *Marine Pollution Bulletin,* 42**,** 677-688.

THROUP, J. P., CAMARA, M., BRIGGS, G. S., WINSON, M. K., CHHABRA, S. R., BYCROFT, B. W., WILLIAMS, P. & STEWART, G. S. 1995. Characterisation of the *yenI/yenR* locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acylhomoserine lactone signal molecules. *Molecular Microbiology,* 17**,** 345-56.

TOLOSA, I., READMAN, J. W., BLAEVOET, A., GHILINI, S., BARTOCCI, J. & HORVAT, M. 1996. Contamination of Mediterranean (Côte d'Azur) coastal waters by organotins and irgarol 1051 used in antifouling paints. *Marine Pollution Bulletin,* 32**,** 335-341.

TUBIASH, H. S., COLWELL, R. R. & SAKAZAKI, R. 1970. Marine *Vibrio*s Associated with Bacillary Necrosis, a Disease of Larval and Juvenile Bivalve Mollusks. *Journal of Bacteriology,* 103**,** 271-272.

TUJULA, N. A., CROCETTI, G. R., BURKE, C., THOMAS, T., HOLMSTROM, C. & KJELLEBERG, S. 2010. Variability and abundance of the epiphytic bacterial community associated with a green marine Ulvacean alga. *ISME Journal,* 4**,** 301-311.

ULRICH, R. L., DESHAZER, D., BRUEGGEMANN, E. E., HINES, H. B., OYSTON, P. C. & JEDDELOH, J. A. 2004. Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *Journal of Medical Microbiology,* 53**,** 1053-1064.

UNFAO 2012. The State of World Fisheries and Aquaculture - 2012 (SOFIA). Rome: Fisheries and Aquaculture Dept, UN Food and Agriculture Organisation.

URBANCZYK, H., AST, J. C., KAEDING, A. J., OLIVER, J. D. & DUNLAP, P. V. 2008. Phylogenetic Analysis of the Incidence of *lux* Gene Horizontal Transfer in Vibrionaceae. *Journal of Bacteriology,* 190**,** 3494-3504.

UROZ, S., OGER, P., CHHABRA, S., CÁMARA, M., WILLIAMS, P. & DESSAUX, Y. 2007. *N* -acyl homoserine lactones are degraded via an amidolytic activity in *Comamonas sp*. strain D1. *Archives of Microbiology,* 187**,** 249-256.

UROZ, S., OGER, P. M., CHAPELLE, E., ADELINE, M. T., FAURE, D. & DESSAUX, Y. 2008. A *Rhodococcus* qsdA-encoded enzyme defines a novel class of large-spectrum quorum-quenching lactonases. *Applied and Environmental Microbiology,* 74**,** 1357-1366.

VAN HOUDT, R., GIVSKOV, M. & MICHIELS, C. W. 2007. Quorum sensing in *Serratia*. *FEMS Microbiology Reviews,* 31**,** 407-424.

VAN MOOY, B. A., HMELO, L. R., SOFEN, L. E., CAMPAGNA, S. R., MAY, A. L., DYHRMAN, S. T., HEITHOFF, A., WEBB, E. A., MOMPER, L. & MINCER, T. J. 2012. Quorum sensing control of phosphorus acquisition in Trichodesmium consortia. *ISME Journal,* 6**,** 422-429.

VENKATESWARAN, K., MOSER, D. P., DOLLHOPF, M. E., LIES, D. P., SAFFARINI, D. A., MACGREGOR, B. J., RINGELBERG, D. B., WHITE, D. C., NISHIJIMA, M., SANO, H., BURGHARDT, J., STACKEBRANDT, E. & NEALSON, K. H. 1999. Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *International Journal of Systematic Bacteriology,* 49**,** 705-724.

VENTER, J. C., REMINGTON, K., HEIDELBERG, J. F., HALPERN, A. L., RUSCH, D., EISEN, J. A., WU, D., PAULSEN, I., NELSON, K. E., NELSON, W., FOUTS, D. E., LEVY, S., KNAP, A. H., LOMAS, M. W., NEALSON, K., WHITE, O., PETERSON, J., HOFFMAN, J., PARSONS, R., BADEN-TILLSON, H., PFANNKOCH, C., ROGERS, Y.-H. & SMITH, H. O. 2004. Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science,* 304**,** 66-74.

VENTURI, V., BERTANI, I., KERÉNYI, Á., NETOTEA, S. & PONGOR, S. 2010. Co-Swarming and Local Collapse: Quorum Sensing Conveys Resilience to Bacterial Communities by Localizing Cheater Mutants in *Pseudomonas aeruginosa*. *PLoS ONE,* 5**,** e9998.

WADE, D. S., CALFEE, M. W., ROCHA, E. R., LING, E. A., ENGSTROM, E., COLEMAN, J. P. & PESCI, E. C. 2005. Regulation of Pseudomonas Quinolone Signal Synthesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 187**,** 4372-4380.

WAGNER-DÖBLER, I. & BIEBL, H. 2006. Environmental Biology of the Marine Roseobacter Lineage. *Annual Review of Microbiology,* 60**,** 255-280.

WAGNER-DÖBLER, I., THIEL, V., EBERL, L., ALLGAIER, M., BODOR, A., MEYER, S., EBNER, S., HENNIG, A., PUKALL, R. & SCHULZ, S. 2005. Discovery of Complex Mixtures of Novel Long-Chain Quorum Sensing Signals in Free-Living and Host-Associated Marine Alphaproteobacteria. *ChemBioChem,* 6**,** 2195-2206.

WANG, F., WANG, J., JIAN, H., ZHANG, B., LI, S., WANG, F., ZENG, X., GAO, L., BARTLETT, D. H., YU, J., HU, S. & XIAO, X. 2008. Environmental Adaptation: Genomic Analysis of the Piezotolerant and Psychrotolerant Deep-Sea Iron Reducing Bacterium *Shewanella piezotolerans* WP3. *PLoS One,* 3**,** e1937.

WANG, W.-Z., MOROHOSHI, T., IKENOYA, M., SOMEYA, N. & IKEDA, T. 2010. AiiM, a Novel Class of *N*-acylhomoserine Lactonase from the Leaf-Associated Bacterium *Microbacterium testaceum*. *Applied and Environmental Microbiology,* 76**,** 2524-2530.

WANG, Y. H., ZHANG, L. Q., LI, J. Y., WANG, J. H. & WANG, H. M. 2008. The quorum-sensing system AvsR-AvsI regulates both long-chain and short-chain acyl-homoserine lactones in *Agrobacterium vitis* E26. *Antonie Van Leeuwenhoek,* 93**,** 267-273.

WATERS, C. M., LU, W., RABINOWITZ, J. D. & BASSLER, B. L. 2008. Quorum Sensing Controls Biofilm Formation in *Vibrio cholerae* through Modulation of Cyclic Di-GMP Levels and Repression of *vpsT. Journal of Bacteriology,* 190**,** 2527-2536.

WEBSTER, N. S., WILSON, K. J., BLACKALL, L. L. & HILL, R. T. 2001. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Applied and Environmental Microbiology,* 67**,** 434-444.

WEINBERGER, F., BELTRAN, J., CORREA, J. A., LION, U., POHNERT, G., KUMAR, N., STEINBERG, P., KLOAREG, B. & POTIN, P. 2007. Spore release in *Acrochaetium sp.* (Rhodophyta) is bacterially controlled. *Journal of Phycology,* 43**,** 235-241.

WEVER, R., TROMP, M. G. M., KRENN, B. E., MARJANI, A. & VAN TOL, M. 1991. Brominating activity of the seaweed *Ascophyllum nodosum*: impact on the biosphere. *Environmental Science & Technology,* 25**,** 446-449.

WHEELER, G. L., TAIT, K., TAYLOR, A., BROWNLEE, C. & JOINT, I. 2006. Acyl-homoserine lactones modulate the settlement rate of zoospores of the marine alga *Ulva intestinalis* via a novel chemokinetic mechanism. *Plant Cell and Environment,* 29**,** 608-618.

WHITELEY, M., LEE, K. M. & GREENBERG, E. P. 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences,* 96**,** 13904-13909.

WHITMAN, W. B., COLEMAN, D. C. & WIEBE, W. J. 1998. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences,* 95**,** 6578-6583.

WIKFORS, G. H., PATTERSON, G. W., GHOSH, P., LEWIN, R. A., SMITH, B. C. & ALIX, J. H. 1996. Growth of post-set oysters, *Crassostrea virginica*, on high-lipid strains of algal flagellates *Tetraselmis spp. Aquaculture,* 143**,** 411-419.

WILLIAMS, P. 2007. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology,* 153**,** 3923-3938.

WILLIAMS, P., WINZER, K., CHAN, W. C. & CAMARA, M. 2007. Look who's talking: communication and quorum sensing in the bacterial world. *Philosophical Transactions of the Royal Society London B Biological Sciences,* 362**,** 1119-1134.

WINSON, M. K., SWIFT, S., FISH, L., THROUP, J. P., JØRGENSEN, F., CHHABRA, S. R., BYCROFT, B. W., WILLIAMS, P. & STEWART, G. S. A. B. 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiology Letters,* 163**,** 185-192.

WISNIEWSKI-DYE, F. & DOWNIE, J. A. 2002. Quorum-sensing in *Rhizobium*. *Antonie Van Leeuwenhoek,* 81**,** 397-407.

XAVIER, K. B. & BASSLER, B. L. 2003. LuxS quorum sensing: more than just a numbers game. *Current Opinion in Microbiology,* 6**,** 191-197.

XAVIER, K. B. & BASSLER, B. L. 2005. Regulation of Uptake and Processing of the Quorum-Sensing Autoinducer AI-2 in *Escherichia coli*. *Journal of Bacteriology,* 187**,** 238-248.

VAN ALSTYNE, K. L., WOLFE, G. V., FREIDENBURG, T. L., NEILL, A. & HICKEN, C. 2001. Activated defense systems in marine macroalgae: evidence for an ecological role for DMSP cleavage. *Marine Ecology Progress Series,* 213**,** 53-65.

YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene,* 33**,** 103-119.

YATES, E. A., PHILIPP, B., BUCKLEY, C., ATKINSON, S., CHHABRA, S. R., SOCKETT, R. E., GOLDNER, M., DESSAUX, Y., CAMARA, M., SMITH, H. & WILLIAMS, P. 2002. *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infection and Immunity,* 70**,** 5635-5646.

YOCH, D. C. 2002. Dimethylsulfoniopropionate: Its Sources, Role in the Marine Food Web, and Biological Degradation to Dimethylsulfide. *Applied and Environmental Microbiology,* 68**,** 5804-5815.

ZHANG, H.-B., WANG, C. & ZHANG, L.-H. 2004. The quormone degradation system of *Agrobacterium tumefaciens* is regulated by starvation signal and stress alarmone (p)ppGpp. *Molecular Microbiology,* 52**,** 1389-1401.

ZHANG, L., JIA, Y., WANG, L. & FANG, R. 2007. A proline iminopeptidase gene upregulated in planta by a LuxR homologue is essential for pathogenicity of *Xanthomonas campestris* pv. campestris. *Molecular Microbiology,* 65**,** 121-136.

ZHANG, L.-H. 2003. Quorum-quenching and proactive host defense. *Trends in Plant Science,* 8**,** 238-244.

ZHU, J., DIZIN, E., HU, X., WAVREILLE, A.-S., PARK, J. & PEI, D. 2003. S-Ribosylhomocysteinase (LuxS) Is a Mononuclear Iron Protein. *Biochemistry,* 42**,** 4717-4726.

ZIEMKE, F., BRETTAR, I. & HÖFLE, M. G. 1997. Stability and diversity of the genetic structure of a *Shewanella putrefaciens* population in the water column of the central Baltic. *Aquatic Microbial Ecology,* 13**,** 63-74.

ZISMAN, W. A. 1964. Relation of the Equilibrium Contact Angle to Liquid and Solid Constitution. *In:* FOWKES, F. M. (ed.) *Contact Angle, Wettability, and Adhesion.* American Chemical Society.

ZOBELL, C. E. 1941. Studies on marine bacteria.I. The cultural requirements of heterotrophic aerobes. *Journal of Marine Research***,** 173-188.

Appendices

Appendix 1: Bacterial QS Systems

Table listing a number of bacteria known to regulate gene expression via QS, the type of signal molecule they produce, the synthase and response regulator genes and the phenotypes regulated.

Bacteria	Signal Molecule	Synthase/ response regulator	Phenotypes Regulated	References
Acidithiobacillus ferrooxidans	AHLs	AfeR/I	Biofilm formation	(Rivas *et al.*, 2005)
			Nutrient acquisition	(Gonzalez *et al.*, 2012)
Aeromonas hydrophila	AHLs	AhyR/I	Biofilm formation	(Swift *et al.*, 1997)
			Exoproteases	(Swift *et al.*, 1999)
			Virulence	(Lynch *et al.*, 2002)
Aeromonas salmonicida	AHLs	AsaR/I	Exoprotease	(Swift *et al.*, 1997)
				(Swift *et al.*, 1999)
Agrobacterium tumefaciens	AHLs	TraR/I	Plasmid Conjugation	(Fuqua and Winans, 1994)
				(Piper *et al.*, 1993)
Agrobacterium vistis	AHLs	AvsR/I	Virulence	(Wang *et al.*, 2008)
				(Hao *et al.*, 2005)
Berkholderia cenocepacia	AHLs	CepR/I	Exoenzymes	(Lewenza *et al.*, 1999)
	AQs		Biofilm formation	(Sokol *et al.*, 2003)
			Moltility	(Huber *et al.*, 2001)
			Siderophore production	

Burkholderia pseudomallei	AHLs	BpsR/I	Virulence	(Song *et al.*, 2005) (Ulrich *et al.*, 2004)
Chromobacterium violaceum	AHLs	CviR/I	Virulence Pigmentation	(McClean *et al.*, 1997)
Enterobacter agglomerans	AHLs	EagR/I	?	(Swift *et al.*, 1993)
Enterococcus faecalis	Autoinducing Peptides	Cyl operon	Cytolysin production	(Reading and Sperandio, 2006)
Erwinia carotova	AHLs	CarR/I	Carbapenem production Virulence	(Bainton *et al.*, 1992) (Barnard and Salmond, 2007)
Obesumbacterium proteus	AHLs	OprR/I	?	(Swift *et al.*, 1999)
Pantoea stewartii	AHLs	EsaR/I	Exopolysaccharide production	(Beck von Bodman and Farrand, 1995)
Pseudomonas aeruginosa	AHLs AQs	LasR/I, RhlR/I PQS operon	Exoenzyme Biofilm formation Motility Virulence	(Latifi *et al.*, 1996) (Glessner *et al.*, 1999) (Latifi *et al.*, 1995) (De Kievit *et al.*, 2001)
Pseudomonas fluorescens	AHLs	PhzR/I	Mupirocin production	(Shaw *et al.*, 1997) (El-Sayed *et al.*, 2001)
Pseudomonas putida	AHLs	PpuR/I	Biofilm formation	(Dubern *et al.*, 2006)
Pseudomonas syringae	AHLs	PsyR/I	Exopolysaccharide production Motility Virulence	(Swift *et al.*, 1999) (Quinones *et al.*, 2005)
Rhizobium leguminosarum	AHLs	RhiR/I	Root nodulation Plasmid transfer	(Rodelas *et al.*, 1999) (Wisniewski-Dye and Downie, 2002)
Rhodobacter sphaeroides	AHLs	CerR/I	Cellular aggregation	(Puskas *et al.*, 1997)

Serratia liquifacines	AHLs	SwrR/I	Motility	(Eberl *et al.*, 1996)
			Biofilm formation	(Horng *et al.*, 2002)
			Biosurfactant production	(Van Houdt *et al.*, 2007b)
Serratia marcescens	AHLs	SpnR/I	Motility	(Horng *et al.*, 2002)
			Biofilm formation	(Van Houdt *et al.*, 2007a)
			Biosurfactant production	(Eberl *et al.*, 1996)
Sinorhizobium meliloti	AHLs	SinR/I	Root nodulation	(Marketon and Gonzalez, 2002)
			Symbiosis	(Marketon *et al.*, 2003)
Staphylococcus aureus	Autoinducing	Arg operon	Virulence	(Novick, 2003)
	Peptides		Biofilm formation	(Ji *et al.*, 1995)
Streptococcus mutans	Autoinducing	Com operon	Biofilm formation	(Reading and Sperandio, 2006)
	Peptides			(Li *et al.*, 2002)
Vibrio anguillarum	AHLs	VanR/I, VanM/N	Virulence	(Milton *et al.*, 1997)
				(Defoirdt *et al.*, 2005)
Vibrio cholera	CAI-1	CqsA	Biofilm formation	(Hammer and Bassler, 2003)
	AI-2	LuxS		(Waters *et al.*, 2008)
Vibrio fisheri	AHLs	LuxR/I, AinS	Bioluminescence	(Engebrecht and Silverman, 1984)
				(Hastings and Nealson, 1977)
Vibrio harvyi	AHLs	LuxM/N	Bioluminescence	(Bassler *et al.*, 1993)
	AI-2	LuxS		
Yersinia enterocolylica	AHLs	YenR/I	Motility	(Throup *et al.*, 1995)
				(Atkinson *et al.*, 2008)
Yersinia pestis	AHLs	YpeR/I	Biofilm formation	(Throup *et al.*, 1995)
				(Bobrov *et al.*, 2007)

Yersinia pseudotuberculosis	AHLs	YpsR/I, YtbR/I	Biofilm Formation	(Atkinson *et al.*, 1999)
			Motility	(Atkinson *et al.*, 2011)

Appendix 2: F/2 Media Supplements

F/2 Trance Metal Solution

	In 1 l d.H_2O (Autoclave)
Na_2EDTA	4.36 g
$FeCl_3$ $6H_2O$ (Ferric Chloride)	3.15 g
Primary trace metals	1ml of each stock solution

Primary Trace Metal Solutions

	In 10 ml d.H_2O (filter sterilise)
$CuSO_4$ $5H_2O$	0.100 g
$ZnSO_4$ $7H_2O$	0.220 g
$CoCl_2$ $6H_2O$	0.100 g
$MnCl_2$ $4H_2O$	0.180 g
$NaMoO_4$ $2H_2O$	0.063 g

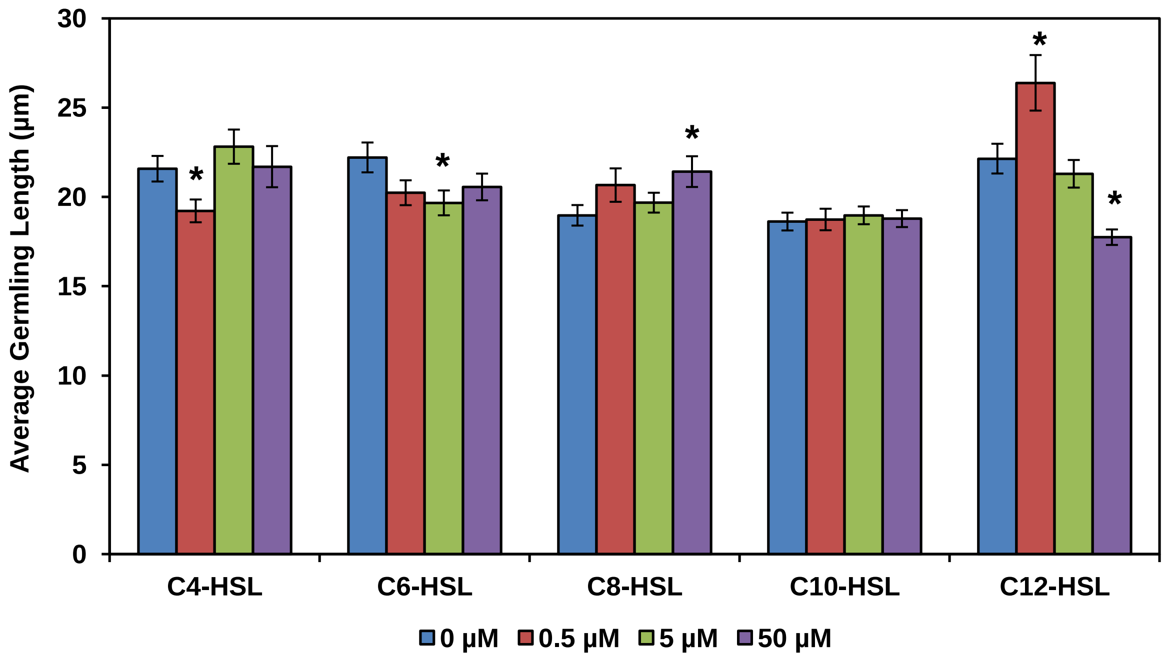
F/2 Vitamin Stock solution

		In 100 ml d.H_2O (Filter sterilise)
Biotin	0.10 $mgml^{-1}$ solution	1.00 ml
Vitamin B_{12}	1.00 $mgml^{-1}$ solution	0.10 ml
Thiamine HCl		0.02 g

Appendix 3: *Ulva* Zoospore Germination in the Presence of Synthetic AHLs

Graph displaying the average length of *Ulva* germlings exposed to various concentrations of synthetic AHLs after 24 h incubation. Error bars represent 95% confidence limits.

* Represents where average zoospore length is significantly different from that where no exogenous AHL was added (0 µM) (one-way ANOVA $p < 0.05$).



Appendix 4: Construction of LuxI-Type AHL Synthase Consensus Sequences

LuxI-Type AHL Synthase Consensus Sequence

LuxI Consensus Sequence

MLXIFDXXXVSYDXLSEEKXXXKELFRLRKKXFKDRLGWDVNCXNGMXXXEFDXXQYDNXNTRYLLGIXD
XXXGQXVGCVRLIETTXXXPNMLTGTFFXLLXDDGALPEXXGXYIXESSRFFXXXDKARARLLXGNXXXY
PLSLVXLFLSMINYARANGYTGIVTVVSRAMERILKRSGWPIERIGQXXGHXXEKGEXIYLXLHLPIDXX
XQERLARRINQPLQGPSSXLLTWPLSLP

Protein Sequences of Published LuxI-Type AHL Synthases

AhyI (*Aeromonas hydrophyla*)

MLVFKGKLKEHPRWEVENELYRFRNRVFSDRLGWDVESHRGLEQDSFDTPDTHWVLIEDEEGLCGCIRLL
SCAQDYMLPSIFPTALAGEAPPRSNDVWELTRLAIDAERAPRLGNGISELTCVIFREVYAFAKAQGIREL
VAVVSLPVERIFRRLGLDIERLGHRQAVDLGAVRGVGIRFHLDERFARAVGQPLQGAYDEARELVTE

AsaI (*Aeromonas salmonicida*)

MLVFKGKLKEHPRWEVENELYRFRNRVFSDRLGWDVESHRGLEQDSFDTPDTHWVLIEDEEGLCGCIRLL
SCAQDYMLPSIFPTALAGEAPPRSSDVWELTRLAIDANRAPRMGNGVSELTCVIFREVYAFARAKGIREL
VAVVSLPVERIFRRLGLPIERLGHRQAVDLGAVRGVGIRFHLDERFARAVGHPMQGEYADARELVTE

CarI (*Erwinia carotovora)*

MLEIFDVNHTLLSETKSGELFTLRKETFKDRLNWAVQCTDGMEFDFTQYDNNNTTYLFGIKDNTVICSLR
FIETKYPNMITGTFFPYFKEINIPEGNYLESSRFFVFTDKSRAKDILGNEYPISSMLFLSMINYSKDKGY
DGIYTIVSHPMLTILKRSGWGIRVVEQFTGLSEKEERVYLVFLPVDDENQEALARRINRSGTFMSNELKQ
WPLRVPAAIAQA

CerI (*Rhodobacter sphaeroides)*

MIFIIDSLNLREHADIVKDMFRLRKRVFADRLGWDVQISQGMERDFTRFDDLDPAHVVSVDDEGRVVGCM
RLLQTTGPHMLSDVFSSILDGEPPLRSATLWEATRFFTCVDTDRLVSGRARNSIAYVTSEVMIGAFEFAM
SAGVTDAVAVIDPVMDRVLKRSGNAPQFTGYVGTPKPMGKVTALAALMDCSEERVKRIRDFAGIYHDVTQ
PQTVIA

EagI (*Pantoea agglomerans*)

MLEIFDVSYNDLTERRSEDLYKLRKITFKDRLDWAVNCSNDMEFDEFDNSGTRYMLGIYDNQLVCSVRFI
DLRLPNMITHTFQHLFGDVKLPEGDYIESSRFFVDKNRAKALLGSRYPISYVLFLSMINYARHHGHTGIY
TIVSRAMLTIAKRSGWEIEVIKEGFVSENEPIYLLRLPIDCHNQHLLAKRIRDQSESNIAALCQWPMSLT
VTPEQV

EchI (*Erwinia chrysanthemi)*

MLEIFDVSFSLMSNNKLDEVFTLRKDTFKDRLDWAVNCINGMEFDEYDNEHTTYLLGVKEGKVICSVRFI
EIKYPNMITGTFYSYFDNLKIPEGNYIESSRFFVDRDRVRNLIGTRNPACVTLFLAMINYARKYHYDGIL
TIVSHPMLTLLKRSGWRISIIQQGLSEKQERIYLLHLPTDDDSRHALIERITQMTQAESEQLKTLPLLVP
LA

EsaI (*Pantoea stewartii)*

MLELFDVSYEELQTTRSEELYKLRKKTFSDRLGWEVICSQGMESDEFDGPGTRYILGICEGQLVCSVRFT
SLDRPNMITHTFQHCFSDVTLPAYGTESSRFFVDKARARALLGEHYPISQVLFLAMVNWAQNNAYGNIYT
IVSRAMLKILTRSGWQIKVIKEAFLTEKERIYLLTLPAGQDDKQQLGGDVVSRTGCPPVAVTTWPLTLPV

ExpI (*Pectobacterium carotovorum)*

MLEIFDVSYTLLSEKKSEELFTLRKETFKDRLNWAVKCINGMEFDQYDDDNATYLFGVEGDQVICSSRLI
ETKYPNMITGTFFPYFEKIDIPEGKYIESSRFFVDKARSKTILGNSYPVSTMFFLATVNYSKSKGYDGVY
TIVSHPMLTILKRSGWKISIVEQGMSEKHERVYLLFLPVDNESQDVLVRRINHNQEFVESKLREWPLSFE
PMTEPVG

LasI (*Pseudomonas aeruginosa*)

MIVQIGRREEFDKKLLGEMHKLRAQVFKERKGWDVSVIDEMEIDGYDALSPYYMLIQEDTPEAQVFGCWR
ILDTTGPYMLKNTFPELLHGKEAPCSPHIWELSRFAINSGQKGSLGFSDCTLEAMRALARYSLQNDIQTL
VTVTTVGVEKMMIRAGLDVSRFGPHLKIGIERAVALRIELNAKTQIALYGGVLVEQRLAVS

LuxI (*Vibrio fischeri*)

MTIMIKKSDFLAIPSEEYKGILSLRYQVFKQRLEWDLVVENNLESDEYDNSNAEYIYACDDTENVSGCWR
LXPTTGDYMLKSVFPELLGQQSAPKDPNIVELSRFAVGKNSSKINNSASEITMKLFEAIYKHAVSQGITE
YVTVTSTAIERFLKRIKVPCHRIGDKEIHVLGDTKSVVLSMPINEQFKKAVLN

PhzI (*Pseudomonas chlororaphis)*

MHMEEHTLNQMSDELKLMLGRFRHEQFVEKLGWRLPAHPSQAGCEFTWDQYDTEHARYLLAFNEDRAIVG
CARLIPTTFPNLLEGVFGHTCAGAPPKHPAIWEMTRFTFTTREPQLAMPLFWRSLKTASLAGADAIVGIV
NSTMERYYKINGVHYERLGPVTVHQNEFTKILAIKLSAHREHHRSAVAPSAFMSDTLLRETA

PsyI (*Pseudomonas syringae)*

MSSGFEFQLASYTTMPVTLLETLYSMRKKIFSDRLEWKVRVSHAFEFDEYDNAATTYLVGSWNGVPLAGL
RLINTCDPYMLEGPFRSFFDCPAPKNAAMAESSRFFVDTARARSLGILHAPLTEMLLFSMHNHAALSGLQ
SIITVVSKAMARIVRKSGWEHHVLSTGEASPGETVLLLEMPVTADNHQRLLGNIALRQPVTDDLLRWPIA
LGVSGSAPQACMHSAA

RhlI (*Pseudomonas aeruginosa)*

MIELLSESLEGLSAAMIAELGRYRHQVFIEKLGWDVVSTSRVRDQEFDQFDHPQTRYIVAMGRQGICGCA
RLLPTTDAYLLKEVFAYLCSETPPSDPSVWELSRYAASAADDPQLAMKIFWSSLQCAWYLGASSVVAVTT
TAMERYFVRNGVILQRLGPPQKVKGETLVAISFPAYQERGLEMLLRYHPEWLQGVPLSMAV

SolI (*Rhodobacter sphaeroides)*

MRTFVHGGGRLPEGIDAALAHYRHQVFVGRLGWQLPMADGTFERDFTQYDRDDTVYVVARDEGGTICGCA
RLLPTTRPYLLKDVFASLLMHGMPPPESPEVWELSRFTFAARSGAPCPRSGRADWAVRPMLASVVQCAAQ
RGARRLIGATFVSMVRLFRRIGVRAHRFTAGPVRCIGGRPVVACWIDIDASTCAALGIPSASAAPGPVLQ

SwrI (*Serratia liquefaciens*)

MIELFDVDYNLLPDNRSKELFSLRKKTFKDRLDWLVNCENNMEFDEYDNRHATYIFGTYQNHVICSLRFI
ETKYPNMISDGVFDTYFNDIKLPDGNYVEASRLFIDKARIQALQLHQAPISAMLFLSMINYARNCGYEGI
YAIISHPMRIIFQRSGWHISVVKTGCSEKNKNIYLIYMPIDDANRNRLLARINQHATKMG

TraI (*Agrobacterium tumefaciens*)

MRILTVSPDQYERYRSFLKQMHRLRATVFGGRLEWDVSIIAGEERDQYDNFKPSYLLAITDSGRVAGCVR
LLPACGPTMLEQTFSQLLEMGSLAAHSGMVESSRFCVDTSLVSRRDASQLHLATLTLFAGIIEWSMASGY
TEIVTATDLRFERILKRAGWPMRRLGEPTAIGNTIAIAGRLPADRASFEQVCPPGYYSIPRIDVAAIRSA
A

VanI (*Vibrio anguillarum*)

MTISIYSHTFQSVPQADYVSLLKLRYKVFSQRLQWELKTNRGMETDEYDVPEAHYLYAKEEQGHLVGCWR
ILPTTSRYMLKDTFSELLGVQQAPKAKEIYELSRFAVDKDHSAQLGGVSNVTLQMFQSLYHHAQQYHINA
YVTVTSASVEKLIKRMGIPCERLGDKKVHLLGSTRSVALHIPMNEAYRASVNA

YenI (*Yersinia enterocolytica)*

MLKLFNVNFNNMPERKLDEIFSLRKITFKDRLDWKVTCIDGKESDQYDDENTNYILGTIDDTIVCSVRFI
DMKYPTMITGPFAPYFSDVSLPIDGFIESSRFFVEKALARDMVGNNSSLSTILFLAMVNYARDRGHKGIL
TVVSRGMFILLKRSGWNITVLNQGESEKNEVIYLLHLGIDNDSQQQLINKILRVHQVEPKTLETWPIIVP
GIIK

YepI (*Yersinia pestis)*

MLEIFDVRXDEXTDIRSEDLYKLRKKTFKDRLNWEVNCSNGMEFDEYDNSDTRYLLGIYQGQLICSVRFI
ELHLPNMITHTXNALFDXXALPKRGYIESSRFFVDKTRAKLLFGNHYPISYLFFLSIINYSRHNGYTGIY
TIVSRAMLTILKRSGWQVEVIKXAHITEKERIYLLHLPIDRDNQARLXLQVNQRLQDPCSVLSTWPISLP
VMPESA

YpeI (*Yersinia pestis)*

MLKVFNVNFDRMSENKLDEIFTLRKITFKDRLDWKVTCIDGKESDQYDDENTNYLLGTIDDTLVCSVRFV
EMQYPTMITGPFAPYFRDLDLPIDGFIESSRFFVEKALARDKLGNNGSLSAILFLSMVNYARNRGYKGIL
TVVSRGMYTILKRSGWGITVINQGESEKNEVIYLLHLSIDSNSQQQLIRKIQRVHNIDTHTLASWPLVVP
SMTK

YpsI (*Yersinia pseudotuberculosis)*

MLKVFNVNFDRMSENKLDEIFTLRKITFKDRLDWKVTCIDGKESDQYDDENTNYLLGTIDDTLVCSVRFV
EMQYPTMITGPFAPYFRDLDLPIDGFIESSRFFVEKALARDKLGNNGSLSAILFLSMVNYARNCGYKGIL
TVVSRGMYTILKRSGWGITVINQGESEKNEVIYLLHLSIDSNSQQQLIRKIQRVHNIDTHTLESWPLVVP
SMTK

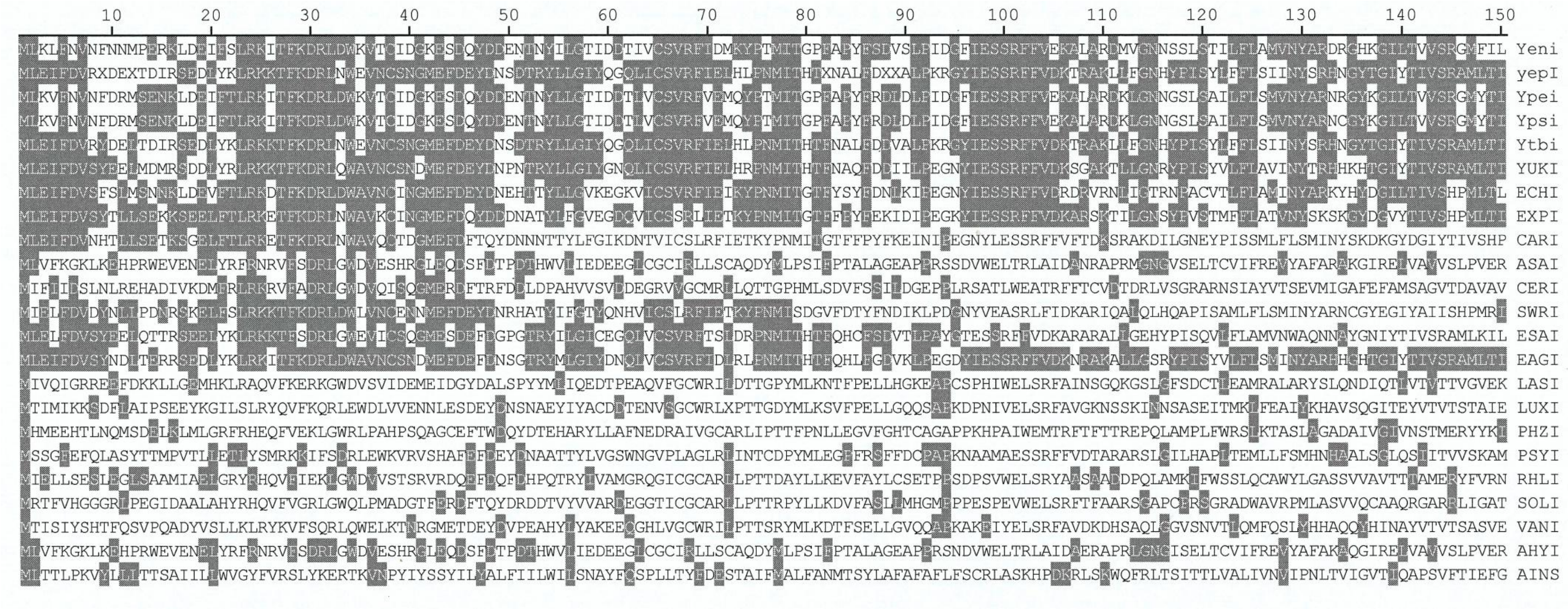
YtbI (*Yersinia pseudotuberculosis)*

MLEIFDVRYDELTDIRSEDLYKLRKKTFKDRLNWEVNCSNGMEFDEYDNSDTRYLLGIYQGQLICSVRFI
ELHLPNMITHTFNALFDDVALPKRGYIESSRFFVDKTRAKLLFGNHYPISYLFFLSIINYSRHNGYTGIY
TIVSRAMLTILKRSGWQVEVIKEAHITEKERIYLLHLPIDRDNQARLLLQVNQRLQDPCSVLSTWPISLP
VMPESA

YukI (*Yersinia ruckeri)*

MLEIFDVSYEELMDMRSDDLYRLRKKTFKDRLQWAVNCSNDMEFDEYDNPNTRYLLGIYGNQLICSVRFI
ELHRPNMITHTFNAQFDDIILPEGNYIESSRFFVDKSGAKTLLGNRYPISYVLFLAVINYTRHHKHTGIY
TIVSRAMLTILKRSGWQFDVIKEAFVSEKERIYLLRLPVDKHNQALLASQVNQVLQGSDSALLAWPISLP
VIPELV

Lux I-Type AHL Synthase Sequence Alignment



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              160       170       180       190       200       210       220
LKRSGWNITVLNQGESEKNEVIYLLHLGIDNDSQQQLINKILRVHQVEPKTLETWPIIVPGIIK.                Yeni
LKRSGWQVEVIKXAHITEKERIYLLHLPIDRDNQARLXLQVNQRLQDPCSVLSTWPISLPVMPESA.              yepI
LKRSGWGITVINQGESEKNEVIYLLHLSIDSNSQQQLIRKIQRVHNIDTHTLASWPLVVPSMTK.                Ypei
LKRSGWGITVINQGESEKNEVIYLLHLSIDSNSQQQLIRKIQRVHNIDTHTLESWPLVVPSMTK.                Ypsi
LKRSGWQVEVIKEAHITEKERIYLLHLPIDRDNQARLLLQVNQRLQDPCSVLSTWPISLPVMPESA.              Ytbi
LKRSGWQFDVIKEAFVSEKERIYLLRLPVDKHNQALLASQVNQVLQGSDSALLAWPISLPVIPELV.              YUKI
LKRSGWRISIIQQGLSEKQERIYLLHLPTDDDSRHALIERITQMTQAESEQLKTLPLLVPLA                   ECHI
LKRSGWKISIVEQGMSEKHERVYLLFLPVDNESQDVLVRRINHNQEFVESKLREWPLSFEPMTEPVG              EXPI
MLTILKRSGWGIRVVEQFTGLSEKEERVYLVFLPVDDENQEALARRINRSGTFMSNELKQWPLRVPAAIAQA.        CARI
IFRRLGLPIERLGHRQAVDLGAVRGVGIRFHLDERFARAVGHPMQGEYADARELVTE.                       ASAI
IDPVMDRVLKRSGNAPQFTGYVGTPKPMGKVTALAAIMDCSEERVKRIRDFAGIYHDVTQPQTVIA.              CERI
IFQRSGWHISVVKTGCSEKNKNIYLIYMPIDDANRNRLLARINQHATKMG                               SWRI
TRSGWQIKVIKEAFLTEKERIYLLTLPAGQDDKQQLGGDVVSRTGCPPVAVTTWPLTLPV                     ESAI
AKRSGWEIEVIKEGFVSENEPIYLLRLPIDCHNQHLLAKRIRDQSESNIAALCQWPMSLTVTPEQV               EAGI
MMIRAGLDVSRFGPHLKIGIERAVALRIELNAKTQIALYGGVLVEQRLAVS                              LASI
RFLKRIKVPCHRIGDKEIHVLGDTKSVVLSMPINEQFKKAVLN.                                     LUXI
NGVHYERLGPVTVHQNEFTKILAIKLSAHREHHRSAVAPSAFMSDTLLRETA.                            PHZI
ARIVRKSGWEHHVLSTGEASPGETVLLLEMPVTADNHQRLLGNIALRQPVTDDLLRWPIALGVSGSAPQACMHSAA     PSYI
GVILQRLGPPQKVKGETLVAISFPAYQERGLEMLLRYHPEWLQGVPLSMAV                              RHLI
FVSMVRLFRRIGVRAHRFTAGPVRCIGGRPVVACWIDIDASTCAALGIPSASAAPGPVLQ.                    SOLI
KLIKRMGIPCERLGDKKVHLLGSTRSVALHIPMNEAYRASVNA                                      VANI
IFRRLGLDIERLGHRQAVDLGAVRGVGIRFHLDERFARAVGQPLQGAYDEARELVTE.                       AHYI
PFAPLFFLNAFLFVILTSINFFKL                                                         AINS
```

Appendix 5: Construction of LuxR-Type AHL Response Regulator Consensus Sequences

LuxR-Type AHL Response Regulator Consensus Sequence

LuxR Consensus Sequence
XXXXXXXXXXXXMXILFFDNESINEXIKNYXDRKLKXYGFDKYAYGXXNKXXXXXKXPPDXFIISNYPXEW
VERYTKNNYQXIDPVVLTAXRRFSPFXWDXXXNIXIFSXLKXSKIFNLAREYGIVNXGYTFVLHDANNXN
LAMLSLASDDSDXNXIDXRIEXXKXKLQMLLILXHEKMLXLYRLXXXXXNXXNXKMNPKAILSPRENEIL
YWASMGKTYQEIAIILGISEXTVKFHIGNVVKKLGXXVLNAKXAIRLXVELGLIKXXXXXXXXXXXPXXX
XXXXXX

Protein Sequences of Published LuxR-Type AHL Response Regulators

AcuR (*Aeromonas veronii*)

MKQEQLFEYLEHFTSVTDGDRLAGLIGRFTVDMGYDYYGFTLIIPMSMQRPKVVLFNQCPISWVQTYTDN
NMLACDPVIQLARKQTLPIYWNRLDERARFLQEGSMDVMGLAAEFGLRNGISFPLHGAAGENGILSFITS
ERASSDLLLESSPILSWMANYIFEAAIRVVRLRDSDQQAALTDRETECLFWASEGKTSGEIACILGITER
TVNYHLNQVTRKTGSMNRYQAIAKGISSGILLPNLEQVVVTNFPKLMQ

AhyR (*Aeromonas hydrophyla*)

MKQDQLLEYLEHFTSVTDGDRLAELIGRFTLGMGYDYYRFALIIPMSMQRPKVVLFNQCPDSWVQAYTAN
HMLACDPIIQLARKQTLPIYWNRLDERARFLQEGSLDVMGLAAEFGLRNGISFPLHGAAGENGILSFITA
ERASSDLLLESSPILSWMSNYIFEAAIRIVRVSLREDDPQEALTDRETECLFWASEGKTSGEIACILGIT
ERTVNYHLNQVTRKTGSMNRYQAIAKGVSSGILLPNLEQVVVTNFPKLMQ

AsaR (*Aeromonas salmonicida*)

MKQDQLLEYLEHFTSVTDGDRLAELIGRFTLGMGYDYYRFALIIPMSMQRPKVVLFNQCPDSWVQAYTAN
HMLACDPIIQLARKQTLPIYWNRLDERARFLQEGSLDVMGLAAEFGLRNGISFPLHGAAGENGILSFITA
ERASSDLLLESSPILSWMSNYIFEAAIRIVRVSLREDDPQEALTDRETECLFWASEGKTSGEIACILGIT
ERTVNYHLNQVTRKTGSMNRYQAIAKGVSSGILLPNLEQVVVTNFPKLMQ

BsmR (Burkholderia pseudomallei)

MRAAMGNWAEDLLAGLDSARSEEEAFRSVETAAAALDFEYCAYGLRVPWPLSRPRIETRSNFPEQWKRRY
VEAGFLDVDPILAHGRRSQQPVVLAETLFASAHQMWVEAQSFGLRFGWAQSSFDAYGGMGMLALVRSCEP
VTAAELDAKEYRMRWLVRTAHAALGRMMLPKLMADPERGLTEREVEVLKWAADGKTSGEISKILAISVDT
VNFHVKNAILKLRTANKTAAVVRAAMLGLLS

BviR (*Burkholderia cepacia*)

MQAWREKYLNGFATAKSEADVFLEFSADVRALGFDHCSFGLRIPLPISKPQFMLQSNYPQTWVERYVSQN
YFAVDPTVRHGLSRMSPLIWRADSQTQCVQFWEEADQHGLRHGWCMPSVSRTGAIGLITMVRSGEPIEER

ELAEKGYQMSWLANTANYAMSMHLLQRLVPEYTVELTVREREALQWSAAGKTYAEIGKIMHVDDRTVKFH
LVNAMRKLNAANKTEAAVKATMLGLLF

CarR (*Erwinia carotovora*)

MDHEIHSFIKRKLKGVGDVWFSYFMMSKNSTSQPYIISNYPEAWMKEYIKKEMFLSDPIIVASLARITPF
SWDDNDIVTLRAKNQDVFISSVQHDISSGYTFVLHDHDNNVATLSIANHLEDANFEKCMKNHENDLQMLL
VNVHEKVMAYQRAINDQDNPPDNSRNALLSPRETEVLFLVSSGRTYKEVSRILGISEVTVKFHINNSVRK
LDVINSRHAITKALELNLFHSPCEPVVMKHMDAR

CerR (*Rhodobacter sphaeroides*)

MDIIDLSTVATDDASFLDYIDQLCQKLGFDYASYATTSPMTGAVQFTGYANYPDSWKMHYMRRNLHRVDP
TIHKSALSIAPVDWSRFERDERFRAVFFAAEDFGITFTPQGLTVPVRGPYGDRGLLSVTRNCARPEWEKH
KRAVIGELQVAAVHLHDAVMRSDVISRFTALRQPRLSTREIEVLQWAAAGKSQTDIGDILGISHRTVEVH
LRSAREKLGTLSTVQAVGFTRAIGLGLVYPR

CinR (*Rhizobium leguminosarum*)

MQENEHSTTYPDAFSAMKNAATVAAALDEFQSHYPIDFVTFHLARTIVDNVDAPFVRTTYPDSWVSRYLL
NDYINVDPIIREGFSRQLPFDWREIDITETAQEFMVDAELHGIGTNGVSVPIVDKSRRSLLSINSQKSDE
EWTLIERQFLPEWLELGFLLHRKAVFELHGENDPVPALGSREIECLHWASRGKDSKDIGKILGLSEHTTR
GYLKSARYKLGCPTLSAAIAHAVHLNLITPHVGTPS

EchR (*Erwinia chrysanthemi*)

MSISFSNFDFINSTIQNYLNRKLKSYGDLKYAYLIMNKKKPTDVVIISNYPSEWVEIYRSNNYQHIDPVI
LTAINKISPFSWDDDLVISSKLKFSRIFNLSKEYDIVNGYTFVLHDPGNNLATLSFMFEENRSGELEEIV
QNNKEKLQMLLISAHEKLTSLYREMSKNKNNSKSQEPNIFSQRENEILYWASMGKTYQEIALILGITTST
VKFHIGNVVKKLGVLNAKHAIRLGVEMNLIKPVEPVKARS

EsaR (*Pantoea stewartii*)

MFSFFLENQTITDTLQTYIQRKLSPLGSPDYAYTVVSKKNPSNVLIISSYPDEWIRLYRANNFQLTDPVI
LTAFKRTSPFAWDENITLMSDLRFTKIFSLSKQYNIVNGFTYVLHDHMNNLALLSVIIKGNDQTALEQRL
AAEQGTMQMLLIDFNEQMYRLAGTEGERAPALNQSADKTIFSSRENEVLYWASMGKTYAEIAAITGISVS
TVKFHIKNVVVKLGVSNARQAIRLGVELDLIRPAASAAR

ExpR (*Pectobacterium carotovorum*)

MSQLFYNNETISRIIKSQFDMALSHYGDIKYAYMVLNKKKPTEILIISNHHDEWREIYQANNYQHIDPVV
IAALNKITPFPWDEDLLVSTQLKMSKIFNLSREHNITNGYTFVLHDHSNNLVMLSIMIDESNVSNIDDVI
ESNKDKLQMTLMTIHAETISLYREMIRNKEDERSNDKDIFSQRENEILYWASMGKTYQEIALILDIKTGT
VKFHIGNVVKKLGVLNAKHAIRLGIELQLIRPVQS

LasR (*Pseudomonas aeriginosa*)

MALVDGFLELERSSGLEWSAILQKMASDLGFSKILFGLLPKDSQDYENAFIVGNYPAAWREHYDRAGYAR
VDPTVSHCTQSVLPIFWEPSIYQTRKQHEFFEEASAAGLVYGLTMPLHGARGELGALSLSVEAENRAEAN
RFMESVLPTLWMLKDYALQSGAGLAFEHPVSKPVVLTSREKEVLQWCAIGKTSWEISVICNCSEANVNFH
MGNIRRKFGVTSRRVAAIMAVNLGLITL

LuxR (*Vibrio fischeri*)

MKNINADDTYRIINKIKACRSNNDINQCLSDMTKMVHCEYYLLAIIYPHSMVKSDISILDNYPKKWRQYY
DDANLIKYDPIVDYSNSNHSPINWNIFENNAVNKKSPNVIKEAKSSGLITGFSFPIHTANNGFGMLSFAH
SEKDNYIDSLFLHACMNIPLIVPSLVDNYRKINIANNKSNNDLTKREKECLAWACEGKSSWDISKILGCS
KRTVTFHLTNAQMKLNTTNRCQSISKAILTGAIDCPYFKS

MalR (*Burkholderia pseudomallei*)

MHDFLQFWLNEFSRSENPQHVISVLTRAAATLGYEYAAYGMRRPFPISNPPILMVSNYPARWQERYIEAR
FANIDGAVKAALGSDRPVTWSAPANASKSAFWAEALSFGIAHGWSSASRGADGAIGVLTLSRTQDPIDTA
EKFRNESIVHWLANVAHASMAPFLPAADEFDPDLTRRETDVLKWTADGKTAYEIALILSISESTVNFHVK
NIVSKLGSTNKIQAVAKAALMGML

OccR (*Agrobacterium tumefaciens*)

MNLRQVEAFRAVMLTGQMTAAAELMLVTQPAISRLIKDFEQATKLQLFERRGNHIIPTQEAKTLWKEVDR
AFVGLNHIGNLAADIGRQAAGTLRIAAMPALANGLLPRFLAQFIRDRPNLQVSLMGLPSSMVMEAVASGR
ADIGYADGPQERQGFLIETRSLPAVVAVPMGHRLAGLDRVTPQDLAGERIIKQETGTLFAMRVEVAIGGI
QRRPSIEVSLSHTALSLVREGAGIAIIDPAAAIEFTDRIVLRPFSIFIDAGFLEVRSAIGAPSTIVDRFT
TEFWRFHDDLMKQNGLME

PhzR (*Pseudomonas chlororaphis*)

MELGQQLGWDAYFYSIFARTMDMQEFTAVALRALRELRFDFFRYGFTMCSVTPFMRPRTYMYGNYPEDWV
QRYQAANYAVIDPTVKHSKVSSSPILASNELFRGCPFTDLWSEANDSNLRHGLAQPSFNTQGRVGVLSLA
RKDNPISLQEFEALKVVTKAFAAAVHEFTKISELESDVRVFNTDVEFSGRECDVLRWTADGKTSEEIGVI
MGVCTDTVNYHHRNIQRKFTIGASNRVQASRYAVAMGYI

ProR (*Serratia proteamaculans*)

MDTHLQPLMDALLTSQPDRKVFLSQLAPCAQALGFEYFSYTVFSCYPASRPKMLIEGNFPECYLEDYRKL
RVYLQDPVIEQAHHSTLQFYWDEHFYQDKPELWWRMAQFGIREGWSQSVKDCYGRLGILTFAGKSIPVQS
PQASARNETFFLWLAQMVHKTLREALISVNDEAIKDVLTLREKDILRWCSEGKTSEETALLMGLSERTVN
FHIGNSIKKLSVANKTAATAKAVYLQLI

PsyR (*Pseudomonas syringae*)

MEVRTVKAQLDCPPLKINGAPAPLRQLIEDFENDLHHIGDFTYAYFSTPKTRNVKPVILSNYPDSWLKSY
VASNYHLIDPIIKHAWHSITPFFWREAECCSGRRTDDFLKRSAKYQLSSGATFTLHDASWLFAALSLCNA
RQQNDFDQRIREKAADIQMSLIRFHDRLIKTRAPHELFPQPAQCKLSTRETGVLKWVAMGKSYSEIAEIF
SISERTVKFHMSNVSSKLKVRTAKQAVYKAINMGMV

RaiR (*Rhizobium etli*)

MSPSHAEQFSFFLLSGPDLRIADIAGSGNDAGRSRPHLCDIAYGSPCDLAGATDSNPLLMLTYPPEWVKQ
YRDRDYFSIDPVVRLGRRGFLPVEWSASGWDSGRAYGFFKEAMAFGVGRQGVTLPVRGPQGERSLFTVTS
NHPDAYWRQFRMDSMRDLQFLAHHLHDRAMVLSGMRKVADLPRLSRRELQCLEMTANGLLAKQICARLSI
SVSAVQLYLASARRKLTVATTSEQLLGPRRSN

RhiR (*Rhizobium leguminosarum*)

MKEESSAVSNLVFDFLSESASAKSKDDVLLLFGKISQYFGFSYFAISGIPSPIERIDSYFVLGNWSVGWF
DRYRENNYVHADPIVHLSKTCDHAFVWSEALRDQKLDRQSRRVMDEAREFKLIDGFSVPLHTAAGFQSIV
SFGAEKVELSTCDRSALYLMAAYAHSLLRAQIGNDASRKIQALPMITTREREIIHWCAAGKTAIEIATIL
GRSHRTIQNVILNIQRKLNVVNTPQMIAESFRLRIIR

RhlR (*Pseudomonas aeriginosa*)

MRNDGGFLLWWDGLRSEMQPIHDSQGVFAVLEKEVRRLGFDYYAYGVRHTIPFTRPKTEVHGTYPKAWLE
RYQMQNYGAVDPAILNGLRSSEMVVWSDSLFDQSRMLWNEARDWGLCVGATLPIRAPNNLLSVLSVARDQ
QNISSFEREEIRLRLRCMIELLTQKLTDLEHPMLMSNPVCLSHREREILQWTADGKSSGEIAIILSISES
TVNFHHKNIQKKFDAPNKTLAAAYAAALGLI

SmaR (*Erwinia billingiae*)

MSNSFFNNTSINISIKNYLEKNLKVFNNIKYAYAIMNKKNPNDFAIISNRMEWFDFYTKNNLQFIDPVLI
TASCCFTPFLWDENIMISSGLKMPKIFNMAKNYDVINGYTFVLHDHNHNLVVLSIIMDKSCDDDIEKIIV
DKKNDLQMLLLTTHEKLITLYQEINDTHQFNKKNQKEILSKRENEILYWASMGKSYQEIALILGIKLTTV
KYHVGNAVKKLGVTNAKHAIRLGVELKLIRPILPDAE

SolR (*Rhodobacter sphaeroides*)

MEPDFQDAYHAFRTAEDEHQLFREIAAIARQLGFDYCCYGARMPLFTPVSKPAVAIFDTYPAGWMQHYQA
SGFLDIDPTVRAGASSSDLIVWPVSIRDDAARLWSDFTARDAGLNIGVARSSWTAHGAFGLLTLARHADP
LTAAELGQLSIATHWLANLAHTLMSPFFTLVPQLVPESNAVLTTREREVLCWTGEGKTAYEIGQILRISE
RTVNFHVNNVLLKLAATNFTKVQAVVKAIATGLI

VanR (*Vibrio angullarium*)

MYKILRLIQENQQITSHDDLENVLNGLNNLIGHEFFLFGLSFQPTLKTSETLVTDNYPNSWRQQYDESGF
MHIDPIVKYSITNFLPIRWDDAKRVNNDGRVIFEEARCNGLKAGFSIPIHGLRGEFGMISFATSDTKSYD
LNQQSIHTSQLIVPLLAHNIGNITRYHKDAKPRAVLTAREVQCLAWAAEGKSAWEIATIINTSERTVKFH
FSNACKKLGATNRYQAITKAILGGYINPYL

VsmR (*Pseudomonas aeruginosa*)

MRNDGGFLLWWHGLRCEMQPIHDSQGVFAVLEKEVRRLGFDYYAYGVRHTIPFTRPKTEVHGTYPKAWLE
RYQMQNYGAVDPAILNGLRSSEMVVWSDRLFDQSRMLWNEARDWGLCVGATLPIRAPNNLLSVLSVARDQ
QNISSFEREEIRLRLRCMIELLTQKLTDLEHPMLMSNPVCLSHREREILQWTADGKSSGEIAIILSISES
TVNFHHKNIQKKFDAPNKTLAAAYAAALGLI

YenR (*Yersinia enterocolytica*)

MIIDYFDNESINEDIKNYIQRRIKTYGDLCYSYLVMNKKTPLHPTIISNYPLDWVKKYKKNSYHLIDPVI
LTAKDKVAPFAWDDNSVINKKSTDSAVFKLAREYNIVNGYTFVLHDNSNNMATLNISNGSDDSISFDERI
EINKEKIQMLLIITHEKMLGLYQSNSDKNENRNTQIERDIFSPRENEILYWASVGKTYAEISIILGIKRS
TVKFHIGNVVRKLGVLNAKHAIRLGIELKLIKPI

YpeR (*Yersinia pestis*)

MIINFFDNESINEDIKNYIQRRIKAYGNIRYSYLLMNKKVPLHPAIISNYPLDWVKKYKKNSYHLIDPVI
LTAKGKVAPFAWDDNSVINIKSTDSAVFNLAREYNIVNGYTFVLHDNNNNMATLNVSSGDDDSIFFDESI
EVNKEKIQMLLIFIHDKMLGLYNKSHHENNTLNKKENKREIFSPRENEILYWASVGKTYSEIAIILGIKK
STVKFHIGNIVRKLGVLNAKHAIRLGIELQLIKPI

YpsR (*Yersinia pseudotuberculosis*)

MIINFFDNESINEDIKNYIQRRIKAYGNIRYSYLLMNKKVPLHPAIISNYPLDWVKKYKKNSYHLIDPVI
LTAKGKVAPFAWDDNSVINIKSTDSAVFNLAREYNIVNGYTFVLHDNNNNMATLNVSSGDDDSIFFDESI
EVNKEKIQMLLIFIHDKMLGLYNKSHHENNTLNKKENKREIFSPRENEILYWASVGKTYSEIAIILGIKK
STVKFHIGNIVRKLGVLNAKHAIRLGIELQLIKPI

YspR (*Yersinia pestis*)

MHSVFNRSNEVIETLRDYIDRKLTIYDSPKYTYMVINKKNPGDIFIVTSYPNEWAELYTNNNYQNIDPVV
LIAFRRFSPFSWDENITVLSELKLSKIFTLSKKYNIVNGFTFVLHDTMNNLAMLSLIMDDSALNGVESRV
LNDRDRLQMLLIETHEKMLTLSQRNMNIQERQGKGMPGKAILSPRENEVLYWASMGKTYQEIAIITNITP
RTVKYHIGNVVKKLGVINAKQAIGLGVELEIIKPILA

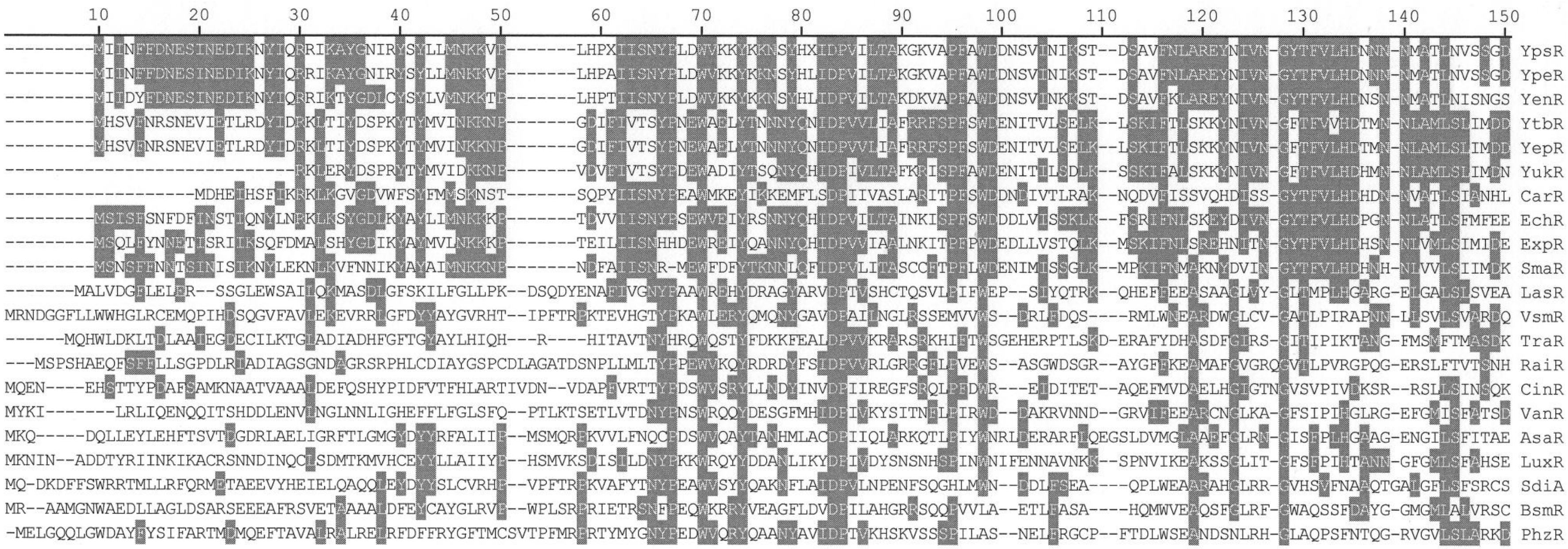
YtbR (*Yersinia pseudotuberculosis*)

MHSVFNRSNEVIETLRDYIDRKLTIYDSPKYTYMVINKKNPGDIFIVTSYPNEWAELYTNNNYQNIDPVV
LIAFRRFSPFSWDENITVLSELKLSKIFTLSKKYNIVNGFTFVLHDTMNNLAMLSLIMDDSALNGVESRV
LNDRDRLQMLLIETHEKMLTLSQRNMNIQERQGKGMPGKAILSPRENEVLYWASMGKTYQEIAIITNITP
RTVKYHIGNVVKKLGVINAKQAIGLGVELEIIKPILA

YukR (*Yersinia ruckeri*)

RKLERYDSPRYTYMVIDKKNPVDVFIVTSYPDEWADIYTSQNYQHIDPIVLTAFKRISPFAWDENITILS
DLKSSKIFALSKKYNIVNGFTFVLHDHMNNLAMLSLIMDNNADKGLNSRIESDKDRLQMNLIKIHEKMLM
LEQNKLGVSNGKNTDTSGKGILSPRENEVLHWASMGKTYPEIALIAGITTRTVKHHMGNVVKKLGVINAR
QAIRLGVELELIKPVLV

Lux R-Type AHL Response Regulator Sequence Alignment



```
          160       170       180       190       200       210       220       230       240       250       260       270       280
131 DDSIFFDESIEVXKEKIQMLLILFHDKMLGLYNKSHHENNTLNKKENKREIFSPRENEILYWASVGKTYSEIAIILGIKKSTVKFHIGNIVRKLG--VLNAKHAIRLGIELQLIK----PI                    YpsR
131 DDSIFFDESIEVNKEKIQMLLIFIHDKMLGLYNKSHHENNTLNKKENKREIFSPGENEILYWASVGKTYSEIAIILGIKKSTVKFHIGNIVRKLG--VLNAKHAIRLGIELQLIK----PI.                   YpeR
131 DDSISFDERIEINKEKIQMLLIITHEKMLGLYQSNSDKNENRN-TQIERDIFSPRENEILYWASVGKTYAEISIILGIKRSTVKFHIGNVVRKLG--VLNAKHAIRLGIEIKLIK----PI                    YenR
131 SALNGVESRVLNDRDRLQMLLIETHEKMLTLSQRNMNIQERQGKGMPGKAILSPRENEVLYWASMGKTYQEIAIITNITPRTVKYHIGNVVKKLG--VINAKQAIGLGVELEIIK----PILA.                 YtbR
131 SALNGVESRVLNDRDRLQMLLIETHEKMLTLSQRNMNIQERQGKGMPGKAILSPRENEVLYWASMGKTYQEIAIITNITPRTVKYHIGNVVKKLG--VINAKQAIGLGVELEIIK----PILA                  YepR
111 NADKGLNSRIESDKDRLQMNLIKIHEKMIMLEQNKLGVSNGKNTDTSGKGILSPRENEVLHWASMGKTYPEIALIAGITTRTVKHHMGNVVKKLG--VINARQAIRLGVELELIK----PVLV                  YukR
121 EDAN-FEKCMKNHENDLQMLLVNVHEKVMAYQRAIN--DQDNPPDNSRNALLSPRETEVLFLVSSGRTYKEVSRILGISEVTVKFHINNSVRKLD--VINSRHAITKALELNLFHSPCEPVVMKHMDAR            CarR
131 NRSGELEEIVQNNKEKLQMLLISAHEKLTSLYREMS--KNKNNSKSQEPNIFSQRENEILYWASMGKTYQEIALILGITTSTVKFHIGNVVKKLG--VLNAKHAIRLGVEMNLIK----PVEPVKARS             EchR
131 SNVSNIDDVIESNKDKLQMTLMTIHAETISLYREMI--RNKEDERSNDKDIFSQRENEILYWASMGKTYQEIALILDIKTGTVKFHIGNVVKKLG--VLNAKHAIRLGIELQLIR----PVQS                  ExpR
130 SCDDDIEKIIVDKKNDLQMLLLTTHEKLITLYQEIN--DTHQFNKKNQKEILSKRENEILYWASMGKSYQEIALILGIKLTTVKYHVGNAVKKLG--VTNAKHAIRLGVELKLIR----PILPDAE               SmaR
134 ENRAEANRFMESVLPTLWMLKDYALQSGAGLAFEHP--------VS-KPVVLTSREKEVLQWCAIGKTSWEISVICNCSEANVNFHMGNIRRKFG--VTSRRVAAIMAVNLGLITL                         LasR
141 QNISSFEREEIRLR---LRCMIELLTQKLTDLEH-----P--MLMS-NPVCLSHREREILQWTADGKSSGEIAIILSISESTVNFHHKNIQKKFD--APNKTLAAAYAAALGLI                           VsmR
135 PVID-LDREIDAVAAAATIGQIHARISFLRTTP-----------TAEDAACVDPKEATYLRWIAVGKTMEEIADVEGVKYNSVRVKLRERMKRFD--VRSKAHLTALAIRRKLI                           TraR
143 PDAYWRQFRMDSMR-DLQFLAHHLHDRAMVLSG---------MRKVADLPRLSRRELQCLEMTANGLLAKQICARLSISVSAVQLYLASARRKIT--VATTSEQLLGPRRSN                             RaiR
138 SDEE-WTLIERQFLPEWLELGFLLHRKAVFELHG----------ENDPVPALGSREIECLHWASRGKDSKDIGKILGLSEHITRGYLKSARYKLG--CPTLSAAIAHAVHINLIT----PHVGTPS               CinR
136 TKSY--DLNQQSIHT--SQLIVPLLAHNIGNITR-----Y--HKDAKPRAVLTAREVQCLAWAAEGKSAWEIATIINTSERTVKFHFSNACKKLG--ATNRYQAITKAILGGYIN----PYL                   VanR
142 RASSDLLLESSPILSWMSNYIFEAAIRIVRVSLR---------EDD-PQEALTDRETECLFWASEGKTSGEIACILGITERTVNYHLNQVTRKTG--SMNRYQAIAKGVSSGILLPNLEQVVVTNFPKLMQ.        AsaR
143 KDNYIDSLFLHACMN--IPLIVPSLVDNYRKIN---------IANNKSNNDLTKREKECLAWACEGKSSWDISKILGCSKRTVTFHLTNAQMKLN--TTNRCQSISKAILTGAIDC---PYFKS                 LuxR
141 RREIPILSDELQLK---MQLLVRESLMALMRLND-----E--IVMT-PEMNFSKREKEIIRWTAEGKTSAEIAMILSISENTVNFHQKNMQKKIN--APNKTQVACYAAATGLI                           SdiA
139 EPVTAAELDAKEYR---MRWLVRTAHAALGRMML-----P--KLMADPERGLTEREVEVLKWAADGKTSGEISKILAISVDTVNFHVKNAILKLR--TANKTAAVVRAAMLGLLS                          BsmR
144 NPISLQEFEALKVVTKAFAAAVHEFTKISELESD-----V--RVFN-TDVEFSGRECDVLRWTADGKTSEEIGVIMGVCTDTVNYHHRNIQRKFTIGASNRVQASRYAVAMGYI.                          PhzR
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Appendix 6: Construction of LuxM-Type AHL Synthase Consensus Sequences

LuxM-Type AHL Synthase Consensus Sequence

```
LuxM Consensus Sequence
MLSLLSXXXXXXXPVQDSCPTLVASALIQNWSVRDTWLSFTYAPQXXNYCFPSYGYSEFTRLQLFTPSSLSKCYX
XEFDNEFKXQLSDTQAVCEVFTLRLTVXXXXYFLYLAQKELMSVLHQAGYKXXXXXXXIIEQPFMLNFYRAIDAK
AYFHSFTGYCDLNDDGKQTYRGFWNFEMMVKAFSNIDFRGYKRXRASRKRGSLERDEHV
```

Protein Sequences of Published LuxM-Type AHL Synthases

LuxM (*V. harveyi*)

MLSLLSLSQVGKHFIVLKHPVQDSCPTLVASALIQNWSVRDTWLSFTYAPQFSNEQWNYCFPSYGYSEFTRLQLF
TPSSLSKCYSLPEFDNEFKLQLSDTQAVCEVFTLRLTVSGNAQQKLYFLYLAQKELMSVLHQAGYKIGFTIIEQP
FMLNFYRAIDAKAYFHSFTGYCDLNDDGKQTYRGFWNFEMMVKAFSNIDFRGYKRAVRASRKRGSLERDEHV

VanM (*V. anguillarium*)

MKIISSLGSRLTNSLPIEKKQRALVEFVINTYQPQQRADLFRALTEHRKNQLLNLFPEHHNKSFSILFELMDYRE
LIRRYPNTFGEEIAHLEQAVSECYSHWLDFWCECEIAAIKTLFPIEADAPHRIELPLKDCAYRGFLIDQIEDSEL
WVTTPSHPQKMPIKDAITLSNLELFIKGEKWYEMLPLLSLSQKGKHFVLLKHPNNEASPTLVASMLVQDWSVNQT
WLSYAQQFSNEQWQFCLPDHSYDVLMELQLFKPALSKCDSLPEFDHQFRRQLTGTQAVCEVLRLTVSGNAQQKLY
FLYLAQKKLIQILNQMGYKIGFTIIEQPFILNFYQTIEPKSYFRSGYNDLNNNGKQSYRGFWMIERMDKVFSDTN
FRDYRHAVSQRRKYDLTKRKEKEYA

AinS (*V.fischeri*)

MLTTLPKVYLLLTTSAIILLWVGYFVRSLYKERTKVNPYIYSSYILYALFIILWILSNAYFQSPLLTYFDESTAI
FMALFANMTSYLAFAFAFLFSCRLASKHPDKRLSKWQFRLTSITTLVALIVNVIPNLTVIGVTIQAPSVFTIEFG
PFAPLFFLNAFLFVILTSINFFKL

LuxM-Type AHL Synthase Sequence Alignment

```
                                 10        20        30        40        50        60        70        80        90       100       110       120       130
V anguillarium.pro MKIISSLGSRLTNSLPIEKKQRALVEFVINTYQPQQRADLFRALTEHRKNQLLNLFPEHHNKSFSILFELMDYRELIRRYPNTFGEEIAHLEQAVSECYSHWLDFWCECEIAAIKTLFPIEADAPHRIEL 130
V fischeri.pro     ---------------------------------------------------------------------------------------------------------------------------------- 0
V harveyi.pro      ---------------------------------------------------------------------------------------------------------------------------------- 0

                                140       150       160       170       180       190       200       210       220       230       240       250       260
V anguillarium.pro PLKDCAYRGFLIDQIEDSELWVTTPSHPQKMPIKDAITLSNLELFIKGEKWYEMLPLLSLSQKGKHFVLLKHPNNEASPTLVASMLVQDWSVNQTWLS--YAQQFSNEQWQFCLPDHSYDVLMELQLFKP 258
V fischeri.pro     ----------------------------------------------------------MLTTLPKVYLLLTTS---AIILLWVGYFVRSLYKERTKVN------------PYIYSSYILYALFIILWILS 57
V harveyi.pro      -----------------------------------------------------MLSLLSLSQVGKHFIVLKHPVQDSCPTLVASALIQNWSVRDTWLSFTYAPQFSNEQWNYCFPSYGYSEFTRLQLFTP 77

                                270       280       290       300       310       320       330       340       350       360       370       380       390
V anguillarium.pro -ALSKCDSLPEFDHQFRRQLTGTQAVCEV--LRLTVSGNAQQKLYFLYLAQKKLIQILNQMGYKIGFTIIEQPFILNFYQTIEPKSYFR--SGYNDLNNNGKQSYRGFWMIERMDKVFSDTNFRDYRHAV 383
V fischeri.pro     NAYFQSPLLTYFDESTAIFMALFANMTSY----LAF---AFAFLFSCRLASKHPDKRLSKWQFRLTSITTLVALIVNVIPNLTVIGVTIQAPSVFTIEFG--PFAPLFFLNAFLFVILTSINFFKL     174
V harveyi.pro      SSLSKCYSLPEFDNEFKLQLSDTQAVCEVFTLRLTVSGNAQQKLYFLYLAQKELMSVLHQAGYKIGFTIIEQPFMLNFYRAIDAKAYFHSFTGYCDLNDDGKQTYRGFWNFEMMVKAFSNIDFRGYKRAV 207

                                400
V anguillarium.pro SQRRKYDLTKRKEKEYA                                                                                                                  400
V fischeri.pro                                                                                                                                        174
V harveyi.pro      RASRKRGSLERDEHV                                                                                                                    222
```

Appendix 7: Construction of LuxN-Type AHL Response Regulator Consensus Sequences

LuxN-Type AHL Response Regulator Consensus Sequence

XXMLDLGLEAIXYPKAITLLATVAVVLXWLXYYCYRLKQKNEXVIFGTHHAPYIAYSXCIIAWIXSNAYF
HTDLLPELGASAAIFMAKLANLASFLAFAFAYYFSCQLAAEQRKGKVHLWQKGIFVTLTVYSLXINLXPG
LTVEHVDIVGPSQFVIEFGPHTXYFFIGLXSFVILTLXNLXAMRANSSKLTLAKTNYMIAGILVFMLSTA
VIHXGITYFLGDFSLTWLPPALSISEMLFVGYALLTSRFYSVKYLAYLTLSXLLVCAIYVIPLGAIFIPL
TEDNQWLIAIPICALIGITWHLLYKRVSRYASFLIYGNKXTPVXQILALEEEFKXSIDDAMRXLGSLLNI
PNDKLQLVNSNYNETFYEDYLSSNRSVLVXDELSEELDYKXXSAKRSXKALYXKMSSNNTALVMPLFGEG
KSVTHLLISSHKSNNQLFSNEEISALQTLLTRVQSTIEADRKXRQSRALANSIAHEMRNPLAQVQLHFEA
LKQHIDSXAPXSQXXXXXXXXXXIKQXIENGQAAIQRGRQLIDIILREVSDSSPEHEPXTMTSIHKAVDQ
AVSHYGFENEKIIERIRLPQQNDFVAKLNETLFNFVIFNLIRNAIYYFDSYPDSQIEIXTXTGXYENKLI
FRDTGPGIDEAILHKIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGRIECXSKLGEFTEFHLYFPVVPNAP
KAETLRXXXXXXXXXXXXXXXXXXXXXXXXTPYFNSWKQNXSTTENXXXXXXXXXXXXXXXXXXXXXXXX
KTXXXVKPXRQXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXAPTVLIVDDKEVQRTLVQMYLNRLGVNSLQANNGENAVELFKSXKVDLILMDVQMPVMNGFDA
SQIIKXXSPQTPIIALSGESGERELXMISKLMDGRLEKPTXLNALQQVLDXWLXKXXASNXXXXXXXXXX
XXX

Protein Sequences of Published LuxN-Type AHL Response Regulators

LuxN *(P. phosphorem***)**

MPDLPLLLFSEPRGALLFFAAGIILAWLGYFSFTLFTSRPGANRNVYYPYLAYSVSIFLWILSNAYFQSP
LLTYYSESTAVTMALFANLVSFCAFISAYSFSCRLISTQPDSNLSLYQKLFISIISLYALIINSSPGLTV
KHVDIVAPGDFVIIFGPQTSWFFLCLMSAVFLTFHNFLIYKKAGSPLIQKKSQYMILGVIIFMLSTLIVH
LIIPFMLDDFSLTWVPPALAIFETLLIGYALLFNRFYSPRYIISQFISHLVNVTLYLSPYLLIIAIGYED
NPLLIGLWIALIGLGWKSSLIQIKRGTNRLLYGKNGSPSENIQRVIGHFQYSTEYGLGKLNELLNTRSGQ
ILNINTHSDLAALKIYFEGKHSVLVKDELEFQIQYETHTELSNISWLKKNMDANNSALVLPIVSKNGDIS
HLFMVSKKDRDGLFSSEEIDALQVLFEQANQYIRSEEQVRKSQVLAGSIAHEIRNPLSKIQYHFERIDAD
LFDVNNNSAHPFLSEQMKGLYKELTESKKAVQLGTRFIDIIIDEIKGNSINSQTFSSHSAGRLTEQALSE
YGFVGNTYQARIIANTQNDFQFWGNETLFSFVMFNLVKNALHYFSQYPQSTLSIHLERGESENCIIVTDT
GPGIADNVIPHIFDEFYTLGKSDGSGLGLAYCRRVINAFGGNIHCQSKYGSYTRFTLTFPIINEERIPNN
LFNELKEALTGKQVLVIGHKENTTLISSLLSGFNIIVSTVDNGKSAAKYIGNNNVDFAFYDLSLSPTQFE
ALKKIRSGDFGANAQKIPLIALSNENTRSTRFDTNVFQGEFRISDSLPLFAQSLKLLIDSGSLKPLGHLI
GKRVLVVDDMQINRMLVQSYLAQEGITVLQAHNGSVALCIAEQERPDLILMDIHMPEMDGLEVSRILRQR
GYNIPIIALSGECCNEVTKEISQYMNAYLMKPITRQQLIQKLQYWIPESEADKVISKQDIHIVHSI

LuxN *(P. profundem)*

MHDFIQSTLANMVAIFLVAIALVVVIWATYFARILAKHLPGSSRQVYFPYTLYSVFISAWILSNAYFQSD
LLVYFGADTAIIMALLANIFSGLAFAYAFLFSCRLVSERTSFQLKTWQWILFSLTCIIILVTNCVPGLNV
KSVDIEGIGSFVIHFGPTIGVFFGNLLLLLILTLGNFILSSRSQLKLKQIKANYMIFGMMAFIISTFFAH
FLIPIFLNDFSKAWLPPALSIIEVIIVGYALLHHRFYSIRYIGLITLSFVINAAIYIIPIASVGFVGTQD
STLLLVIWTLITGICWYKSLAIIRRSVNRLLYKEKGDPVENICNLIGEFSYSTDQAVIKLNQVLNAKSGR
IQKVSGNTENNIFVSYFHGNRSVLIKEEIEYQLKHEKPEGTKELSNVTREMVNMGVSLVLPITNERNEVT
QLYMVSKEKENVLFSSEEIMGLQLLFDKANCFIVTEDKIRKSQVLVGTIAHEIRNPLTKIKYHFERIDAD
MFGIENTSLSPFASKEMKKIYQELSEGQKAVQLGSRFIDAILDELRGESIGTTLFDNYSVAKLTHQALND
FCFNSEEHKLRINIDTQSDFFFHGSDTLYSFVLFNLIKNAVYYFDTYPNSQIRIYFQKERNYNKVHVVDT
GPGISPDHQKHILEEFYTNGKVQGNGLGLSYCKRVIESFGGTITCQSELGEYTEFILSFPSIDEKIHSEM
SKEKIKSYLTGMSGLVLGSVEVGNWLSSEFKSLGVELCTAPDVKTGLHHLSQQAVDFIIMDHMLLNREMG
SIKMLRAGTHGHQAQTTPMFLYGYTENSEHLNSIELSPFFQGQIDGINDHQAFLHSLESLIDNDLFAKLG
SLIGKTVLVVDDMQVNRMLVQAYLASEGITVVQASSGDEAIEKVKKEPFNLVLMDIQMPGMSGIEATHQI
RHLFDAIPIVALSGEYNEEITRAISETMNDHLVKPINKQQLLQTLTKWMT

LuxN *(V. algiolyticus)*

MLDVHLHGLFYPKAMALYATVLIVFAWLLYYCYRLKQKSESILGSHHAPYIAYSSCIIVWISSNAYFHTD
LLPELGSVGGIFMAKLANLASFFAFAFAFYFSCQLTADVKKTAVKVWQKVVFVALATYSLYINLVPNLTV
ENVTISGPSQFVIEFGPHTSYFFISLLAFVVLTLLNLIAMRANSSKLTLAKSNYMIAGILVFMLSTAVIH
LGMTYFLGDFSLTWLPPALSISEMLFVGYALLTSRFYSAKYLTYLTVSALLVCAIFVLPLGAIFIPISED
NQWLVAVPLCALIGITWHLLFKRVSRYASYFIYGKRHTPVQQILGLEEEFKRSIDDAMRQLASLLNIPNN
KLQLVTSNYTETFYEEYLPSSKSVLVLDELSEEIDYASSSKGSMRKLYERMRSSNTALVMPLFGRGKSVT
HLLISSHKIDNKLFSNEEISALQTLLVRIQSTIEADRKVRQSRALANSIAHEMRNPLAQVQLQFEALKQH
IESNASLDTLKREIDKGEAAIQRGRQLIDIILREVSDSSPEHEPLALTSIHKAIDQAVSRYGFENDQIIE
RINLPQAHDFVAKLNETLFNFVIFNLIRNAIYYFDSYPDSQIEIRTQTGAYENILIFRDSGPGIDSSILH
KIFDEFFSYQKSGGSGLGLGYCQRVMRSFGGRIECQSELNEFTEFYLYFPVVPNAPKPETLRAPDFDSWK
ATASHSENHSAQHVQVCTDAPTVLIVDDKEVQRTLVQMYLKRLGVNSLQANNGASAVELFHSHKIDLVLM
DVQMPVMNGFDASQRIKQITSSVPIIALSGESGARELELISKLMDDRLEKPTTLNALQVVIQRWLQNENF
APSNTF

Van N (*V. anguillarium)*

MLNLNLDPILYPKAITLIAAVAMVLVWLTYYCYRLKQKNEVIFGTHHASYIAYSSCIIAWIGSNAYFHTD
WLVELGVNRAIFMAEIANISASLAFVFAYYFSCQLSAEQRKGKVHLWQRLIFITIAAYSVLINLQSNLTV
KHVDIVGPSEFVIEFGPHTPYFFNAMLCSVILTLFNLVVMRTNSSKLALAKTNYIIAGILVYMLSTLVIH
IGITFFFQDFSLSWLPPALSISEMMFVGYALITSRFYSVKYLAYLCLNTALVCGVLFIPLGAIFIPLTDS
NQWLIAIPLCALIGITWNPLYKRLSRYASLLIYGNQQTPVEQILALEDDFKRSIDDAMRRLGQLLYIADD
KLQFVNSNYNETVYERYLSSKQTALVFDELFEKLDNKTAAKNSIKALYDKMSSNNTALVMPLFGHSKLVT
HLLISPHKINNQMFSNEEIAALQTLLTRIQSIIEADRRVCQSRALANSIAHEMRNPLAQVQLHFEILKQH
IDSQAPAQQIKQDIENGQAAIQRGRQLIDIILREVSDSSPEHEPITMTSIHKAVDQAVSQYGFENEKVIE
RIHLPQQDDFVAKLNETLFNFVIFNLIRNAIYYFDSYPNSQIEITTQIGTYENILIFRDTGPGIDDAISY
KIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGRVECKSKLGEFTEFHLYFPMVPNAPQADSLRTPDFKSWQ
QPKPNTEQRTVDNIQPIDKPFLINNKAPTVLIVDDKEVQRSLVQMYLNQLGVNNLQANNGENAVEIFKAN
SIDLILMDIQMPVMNGFEASQIIKAHSPQVPIIALSGESGERELEMISKLMDGRLEKPTSLNALQQVISH
WLNKDIVPNAHTAKSGTVI

LuxN *(V. angustum)*

MADLYQAVTTNVIAIFLIAISAVIAVWTGYFARFLHSKPSLSHDKRIYFPYIIYTSFISLWILSNAYFQS
SLLIERSDIVAVNIALAANIFSGLAFIFAYLFSCRITSKKDNFSLTFTQKFLLYTSIIITLLTNIIPRIN
ITSIDIKAIGVFYINFGELSFIFFGMLIIILLSTIINLLILHKNNTCINRVKAKYMITGIIAFISSTFLI
HFIAAVIFHDFTAAWLPPALSVIEVFLIGYALFNSRFYSLKYIIFITSSTFINIIFYTAPVILLELYHIK
ETPFFLVLWTLITGFFWHRTLRLVRLFANKIIYHKKGNPVENITKIISEFKISTDLGISKLNTVIHSNNG
IIVQVSNKNQLLRDYFKTGRNILLKQDLDVLLNDNVLADNHLHLVSEQLHKMGVTLVVPILDESKKITHF
YIASKEMSNVLFSCEEIMGLQRLFERANRFIDTEEKVRKSQVLAGSIAHEIRNPLSKIKYHFEKIDSDFL
SVHKESINSLATLEIEKIHQELTEGKKALQLGTKFSDVILDELRGSSISTSFFQHYSAASLTSQALNDFS
LYSEEHKKRIHLEATNNFYFYGSDTLFSFVLFNLLKNAVYYFDTFPESHISIQFEKGLKHNKIHVRDTGP
GITEEQLENLFDEFYSFGKVSGNGLGLAYCKKVMESFSGSISCHSILGEFTEFTLTFPAINIQSNGELTN
PRIKQHLSGQSCLILSASSLSKKLTESFNGLNMNIECSNDPSIGFTRIKDCPFNFIVIDHRLYITHYDQI
SMLREGKYGYLAQITPIFIFNSTSINLNNDRINVPKYTQGYIDTLNGALAFECSLEAIINDTKFAPLGSL
NDKTVLVVDDMHANRLLVKAYLSKEGINVIQAASGYEAIEQVKKNNIDLIFMDIHMPGMNGIETAKQLKE
LDSTKPIIAISGEYGEKIVSDIHKVMDDYIVKPIEKSTLVSLTSKWLIINKVKD

LuxN *(V. fischeri)*

MLTTLSKVYLLLTTSAIILLWVGYFVRSLYKERTKVNPYIYSSYIFYALFIILWILSNAYFQSPLLTYFD
ESAAIFMALFANMTSYLAFAFAFLFSCRLASKHPDKRLSKWQFGLTSITTFAALIVNVIPNLTVIGVTIQ
APSVFTIEFGPFAPLFFLNAFLFVILTSINFFKLRKSNIKLNKEKSIYLMVGIFIYMISTIASQIIIPVI
WADFSYTWVPPALSVTEALLIGYTLLYHRLYSFKYLLFWSLSYSINLILYLIPIIIIYDLTTPSDLLYIC
IIEIIFTGLFWDKTLKKTKKIASIIIYKDKQTPVEKIYKIAEEFKYSSSNAIIKLASILNTPKEELLLIG
KNTNYNIFIPHLNQSHSALVKDELDYQIHYSPKTANAELHQVQEKMSESKTALILPIFGENKLISHFLIS
ANKHDNTTFSNEEISAIQWVLTKVQGYIESERKVRQSQALANSIAHEMRNPLSQLQYHFEKIKHHYQKNT
EHEKQEQLIKNELNQGCLAIQKGAQLIDIILSEAKNTAISDDLFHHHSISLLTQQIIDEYVFDSEEMKQK
ITLDLEDDFIVNINDTLYGFILFNLLRNATYYFDEYNSSISIRLVKGFATNKLIFRDTGPGIDSHILPNI
FDDFFTHNKEGGSGLGLSYCLRVMHAFEGNIACYSTKGEFTEFVLSFPHIEGDINALNSHKSNTPPLINK
KDNSLKTVLIVDDKKVQRMLIHTFINKDNLTLLQAENGEEAVEIATNNKLDLIFMDSRMPVMNGIDAAKK
IKIIYPNLPIIALTGESSHEEISAITQVMDGYLTKPVSKAQLQQVVDKWL

LuxN *(V. harveyi)*

MFDFSLEAIVYAKAISLLATVAVVMMWLFYYCYRLKQKNEVIFGTHHAAYIAYSVCIIAWISSNAYFHTD
LLPELGASAGMFMAKFANLASFFAFAFAYYFSCQLAAEQRKGKVHRWQQGIFVSLTVYSLFINLRPGLTV
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LGMTYFMGDFSLTWLPPALSISEMLFVGYALLTSRFYSVKYIAYLALSVLLVCAIFVLPLGAIFIPLTES
NQWLIAIPICALIGITWQLLYKKTSRYASFLIYGDKKTPVQQILSLEEDFKLSIDDAMRRLGKLLQIPND
KLRLVTSNYNETFYEEYLSSNRSVLVFDELSEELEYKVSAKRSMKALYDKMSSNNTALVMPLFGQGKSVT
HLLISPHKSNNQMFSNEEISAVQTLLTRVQSTIEADRRIRQSRALANSIAHEMRNPLAQVQLQFEALKQH
IENHAPVEQITLDIENGQAAIQRGRQLIDIILREVSDSSPEHEPIAMTSIHKAVDQAVSHYGFENEKIIE
RIRLPQHTDFVAKLNETLFNFVIFNLIRNAIYYFDSYPDSQIEISTKTGPYENTLIFRDTGPGIDETISH
KIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGRIECKSKLGTFTEFHLYFPVVPNAPKADTLRTPYFNDWK
QNKRSNEHKVAPNVQINNQSPTVLIVDDKEVQRALVQMYLNQLGVNSLQANNGENAVEVFKANHVDLILM
DVQMPVMNGFDASQRIKELSPQTPIVALSGESGERELDMINKLMDGRLEKPTTLNALRHVLGNWLNKNTA
SSACEAERE

LuxN *(V. paraheamolyticus)*

MLDIGLSGLLYPKAITLFATVAVVLVWLLYYCYRLKQKNEVILGSYHAPYIAYSTCIIIWISSNAYFHTD
LLPLLGSEGGIFMAKLANLASFFAFAFAFYFSCQLAAEQKKGKVKLWQQGIFVALTVYSLVINLRPNLTV
ENVLIDGPSQFVIEFGPHTSYFFMGLVTFVVMTLTNLISMRANSSKLSIAKNNYMIAGILVFMLSTAVIH
LGMTYFLGDFSLTWLPPALSISEMLFVGYALLTSRFYSAKYLAYLTISVLFVCTIFVLPLGAVFIPMSED
NQWLISIPICALIGITWHLVYKRVSRVASFFIYGNRQTPVQQILALEEEFKRSIDDAVHQLSTLLNIPND
KLQLVTSNYTETFYEDYLHSNDSVLVLDELSERLDEKPSSKGSIKALYERMRSSNTALVMPLFGREKSVS
HLLISSHKSDNKLFSNEEISALQTLLIRVQNTIESDRKIRQSRALANSIAHEMRNPLAQVQLQFEALKQH
IDSNASDDKIRSDIEKGQAAIQRGRQLIDIILREVSDTSAVHEPLSLTSIHKAVDLAVSRYGFENEHIIE
RVKLPTQNDFVAKINETLFNFVIFNLIRNAIYYFDSYPDSQIEIRTLVGPYENTLVFRDTGPGIDDSILH
KIFDDFFSFQKSGGSGLGLGYCQRVMRSFGGRIECKSVTNEFTEFYLHFPVVPNAPKVETLRTPNFYNWN
QKVKTKPSPEPVVQINKDAPTVLIVDDKEVQRTLVQMYLNRLGVNSLQANNGANAVELFQSHQVDLVLMD
VQMPVMNGFDASEKIKQCSPTTPIIALSGESGEKELEMIAKLMDGRLEKPTTLNALRDVLVRWLHFDKIS
VTNSYQIANE

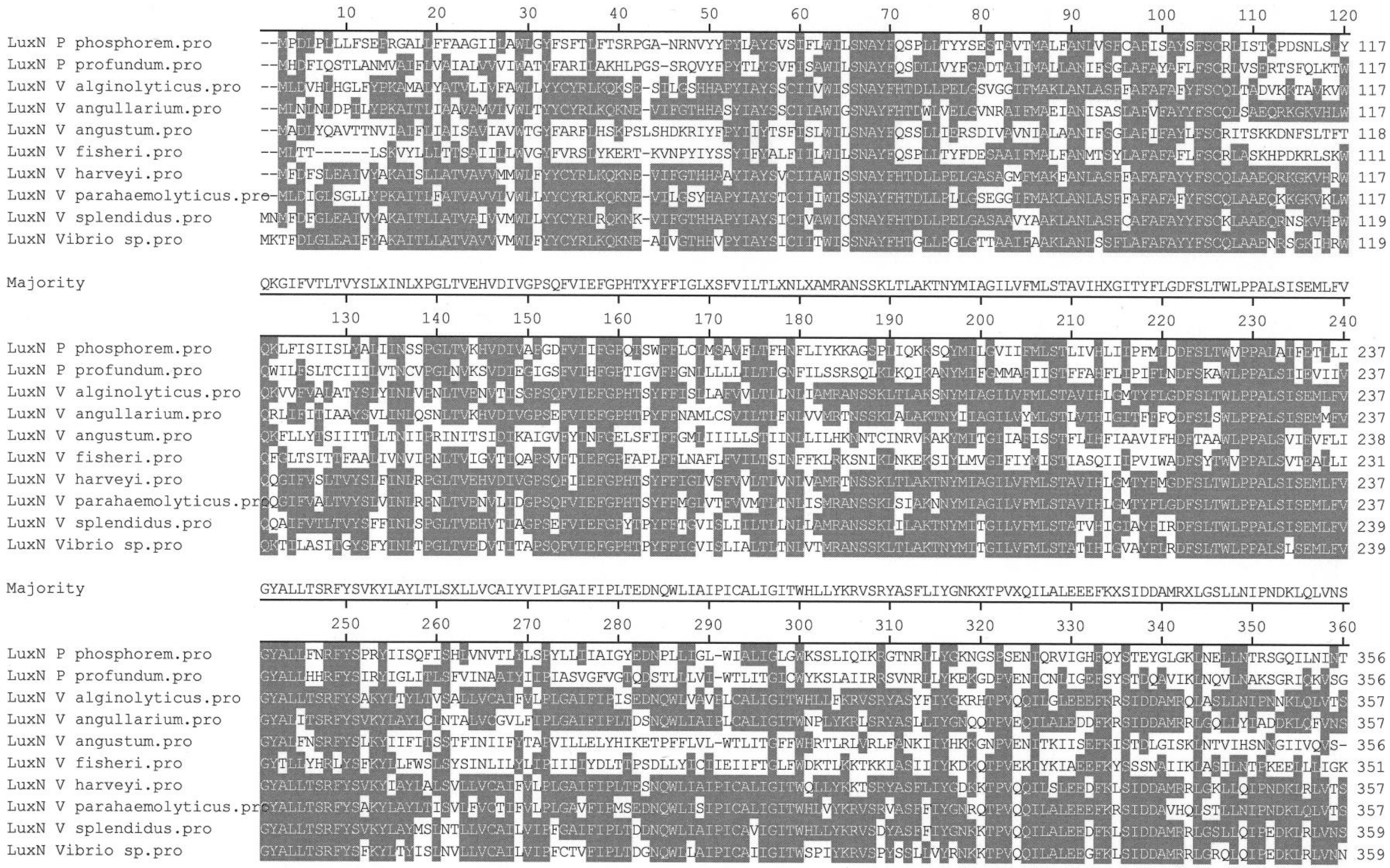
LuxN *(V. splendidtus)*

MNMFDFGLEAIVYAKAITLLATVAIVVMWLLYYCYRLRQKNKVIFGTHHAPYIAYSICIVAWICSNAYFH
TDLLPELGASAAVYAAKLANLASFCAFAFAYYFSCKLAAEQRNSKVHPWQQAIFVTLTVYSFFINLSPGL
TVEHVTIAGPSEFVIEFGPYTPYFFTGVISLIILTLLNLLAMRANSSKLILAKTNYMITGILVFMLSTAT
VHIGIAYFIRDFSLTWLPPALSISEMLFVGYALLTSRFYSVKYLAYMSLNTLLVCAILVIPFGAIFIPLT
DDNQWLIAIPICAVIGITWHLLYKRVSDYASFFIYGNKKTPVQQILALEEDFKLSIDDAMRRLGSLLQIP
EDKLRLVNSNYNETFYEDYLSTNKSVLVFDELSQELDYTAPAKRSIKALYDKMSSNDTALVMPLFGQGKS
VTHLLVSSHKSNDQMFSNEEISALQTLLTRVQSTIEADRRIRQSRALANSIAHEMRNPLAQVQLQFELLK
QHIDNQAPAKQILLDIENGQAAIQRGRQLIDIILREVSDSSPEHGPITMTSIHKAVDQAVSHYGFENEKI
IERIRLPPHADFVAKLNETLFNFVIFNLIRNAIYYFDSYPDSQIEISTKTGAYENVLTFRDTGPGIDEAI
VHKIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGKVECHSKLGEFTEFHLYFPVVPNAPKADALRTPYFND
WKSNQAATENKTNVDAKPDNQAATQNSEPTSTLTPGNHLAPTVLIVDDKEVQRTLVQMYLSRLGVNSLQA
KNGENAVELFKTHKVDLILMDVQMPIMNGFDASQIIKARSPQTPIIALSGESGQHELDMISKLMDGRLEK
PTSLKALQHVLDNWLEKGWASNTSKETESEE

LuxN *(Vibrio sp.)*

MKTFDLGLEAIFYAKAITLLATVAVVVMWLFYYCYRLKQKNEAIVGTHHVPYIAYSICIITWISSNAYFH
TGLLPGLGTTAAIFAAKLANLSSFLAFAFAYYFSCQLAAENRSGKIHRWQKTILASITGYSFYINLTPGL
TVEDVTITAPSQFVIEFGPHTPYFFIGVISLIALTLTNLVTMRANSSKLTLAKTNYMITGILVFMLSTAT
IHIGVAYFLRDFSLTWLPPALSLSEMLFVGYALLTSRFYSFKYLTYISLNVLLVCAILVIPFCTVFIPLT
DGNQWLLAIPICAIIGITWSPIYKRVSPYSSLLVYRNKKTPVQQILALEEGFKLSIDDAMRRLGRQLQIP
EDKLRLVNNNYNETFYEDYLSSKESVLVFDELSEELDDTALAKRSLKALYDKMSSNNTALVMPLFGHKKS
VTHLLVSSHKSNNRMFSNEEISALQTLLTRVQSTIEADRRIRQSRALANSIAHEMRNPLAQVQLHFEVLK
QHIDNQAPTQQILTDIENGQAAIQRGRQLIDIILREVSDSSPEHGPITMTSIHKAVDQAVSHYGFENEKI
IERIRLPQHADFVAKLNETLFNFVIFNLIRNAIYYFDSYPDSQIEISTKTGSYENVLTFRDTGPGIDEAI
VHKVFDDFFSFQKSGGSGLGLGYCQRVMRSFGGRVECHSKLGEFTEFHLYFPIVPNAPKAETLRTPYFNG
WKHNQSTEDKAEADVKPESQTPSGDIEPEPASTLTESKQTERTQAENQPASSHLAPTVLIVDDKEVQRTL
VQMYLSRLGVNSLQAKNGENAVELFRSHKVDLILMDVQMPIMNGFDASQIIKARSPQTPIIALSGESGQR
ELDMIRKLMDGRLEKPTSLNALQHLLDNWLEKGWAPNASKETENE

LuxN-Type AHL Response Regulator Sequence Alignment



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                               370       380       390       400       410       420       430       440       450       460       470       480
LuxN P phosphorem.pro          HSDLAALKIYFEGKHSVLVKDELEFQIQYETHTELSNISWLKKNMDANNSALVLEIVSKNGDISHLFMVSKKDRDGLFSSEEIDALQVLFEQANQYIRSEEQVRKSQVLAGSIAHEIRNP 476
LuxN P profundum.pro           NTENNIFVSYFHGNRSVLIKEEIEYQLKHEKPEGTKELSNVTREMVNMGVSLVLPITNERNEVTQLYMVSKEKENVLFSSEEIMGLQLLFDKANCFIVTEDKIRKSQVLVGTIAHEIRNP 476
LuxN V alginolyticus.pro       NYTETFYEEYLPSSKSVLVLDELSEEIDYA-SSSKGSMRKLYERMRSSNTALVMPLFGRGKSVTHLLISSHKIDNKLFSNEEISALQTLLVRIQSTIEADRKVRQSRALANSIAHEMRNP 476
LuxN V angullarium.pro         NYNETVYERYLSSKQTALVFDELFEKLDNK-TAAKNSIKALYDKMSSNNTALVMPLFGHSKLVTHLLISPHKINNQMFSNEEIAALQTLLTRIQSIIEADRRVCQSRALANSIAHEMRNP 476
LuxN V angustum.pro            -NKNQLLRDYFKTGRNILLKQDLDVLLN-DNVLADNHLHLVSEQLHKMGVTLVVEILDESKKITHFYIASKEMSNVLFSCEEIMGLQRLFERANRFIDTEEKVRKSQVLAGSIAHEIRNP 474
LuxN V fisheri.pro             NTNYNIFIPHLNQSHSALVKDELDYQIHYSPKTANAELHQVQEKMSESKTALILPIFGENKLISHFLISANKHDNTTFSNEEISAICWVLTKVQGYIESERKVRQSQALANSIAHEMRNP 471
LuxN V harveyi.pro             NYNETFYEEYLSSNRSVLVFDELSEELEYK-VSAKRSMKALYDKMSSNNTALVMPLFGQGKSVTHLLISPHKSNNQMFSNEEISAVQTLLTRVQSTIEADRRIRQSRALANSIAHEMRNP 476
LuxN V parahaemolyticus.pro     YTETFYEDYLHSNDSVLVLDELSERLDEK-PSSKGSIKALYERMRSSNTALVMPLFGREKSVSHLLISSHKSDNKLFSNEEISALQTLLIRVQNTIESDRKIRQSRALANSIAHEMRNP 476
LuxN V splendidus.pro          NYNETFYEDYLSTNKSVLVFDELSQELDYT-APAKRSIKALYDKMSSNDTALVMPLFGQGKSVTHLIVSSHKSNDQMFSNEEISALQTLLTRVQSTIEADRRIRQSRALANSIAHEMRNP 478
LuxN Vibrio sp.pro             NYNETFYEDYLSSKESVLVFDELSEELDDT-ALAKRSLKALYDKMSSNNTALVMPLFGHKKSVTHLIVSSHKSNNRMFSNEEISALQTLLTRVQSTIEADRRIRQSRALANSIAHEMRNP 478

Majority                       LAQVQLHFEALKQHIDSXAPXSQ----------IKQXIENGQAAIQRGRQLIDIILREVSDSSPEHEPXTMTSIHKAVDQAVSHYGFENEKIIERIRLPQQNDFVAKLNETLFNFVIFNL
                               490       500       510       520       530       540       550       560       570       580       590       600
LuxN P phosphorem.pro          LSKICYHFERIDADLFDVNNNSAHPFLSEQMKGLYKELTESKKAVQLGTRFIDIIIDEIKGNSINSQTFSSHSAGRLTEQALSEYGFVGNTYQARIIANTQNDFQFWGNETLESFVMFNL 596
LuxN P profundum.pro           LTKIKYHFERIDADMFGIENTSLSPFASKEMKKIYQELSEGQKAVQLGSRFIDAIIDELRGESIGTTLFDNYSVAKLTHQALNDFCENSEEHKLRINIDTQSDFFHGSDTLYSFVLFNL 596
LuxN V alginolyticus.pro       LAQVQLQFEALKQHIESNASLDT----------LKREIDKGEAAIQRGRQLIDIILREVSDSSPEHEPLALTSIHKAIDQAVSRYGFENDQIIERINLPQAHDFVAKLNETLFNFVIFNL 586
LuxN V angullarium.pro         LAQVQLHFEILKQHIDSQAPAQQ----------IKQDIENGQAAIQRGRQLIDIILREVSDSSPEHEPITMTSIHKAVDQAVSQYGFENEKVIERIHLPQQDDFVAKLNETLFNFVIFNL 586
LuxN V angustum.pro            LSKIKYHFEKIDSDFLSVHKESINSLATLEIEKIHQELTEGKKALQLGTKFSDVILDELRGSSISTSFFQHYSAASLTSQALNDFSLYSEEHKKRIHLEATNNFYFYGSDTLESFVLFNL 594
LuxN V fisheri.pro             LSQLQYHFEKIKHHYQKNTEHEKQ------EQLIKNELNQGCLAIQKGAQLIDIILSEAKNTAISDDLFHHHSISLLTQCIIDEYVFDSEEMKQKITLDLEDDFIVNINDTLYGEILFNL 585
LuxN V harveyi.pro             LAQVQLQFEALKQHIENHAPVEQ----------ITLDIENGQAAIQRGRQLIDIILREVSDSSPEHEPIAMTSIHKAVDQAVSHYGFENEKIIERIRLPQHTDFVAKLNETLFNFVIFNL 586
LuxN V parahaemolyticus.pro     AQVQLQFEALKQHIDSNASDDK----------IRSDIEKGQAAIQRGRQLIDIILREVSDTSAVHEELSLTSIHKAVDLAVSRYGFENEHIIERVKLPTQNDFVAKINETLFNFVIFNL 586
LuxN V splendidus.pro          LAQVQLQFELLKQHIDNQAPAKQ----------ILLDIENGQAAIQRGRQLIDIILREVSDSSPEHGPITMTSIHKAVDQAVSHYGFENEKIIERIRLPPHADFVAKLNETLFNFVIFNL 588
LuxN Vibrio sp.pro             LAQVQLHFEVLKQHIDNQAPTQQ----------ILTDIENGQAAIQRGRQLIDIILREVSDSSPEHGPITMTSIHKAVDQAVSHYGFENEKIIERIRLPQHADFVAKLNETLFNFVIFNL 588

Majority                       IRNAIYYFDSYPDSQIEIXTXTGXYENKLIFRDTGPGIDEAILHKIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGRIECXSKLGEFTEFHLYFPVVPNAPKAETLR-------------
                               610       620       630       640       650       660       670       680       690       700       710       720
LuxN P phosphorem.pro          VKNALHYESQYEQSTLSIHLERGESENCIIVTDTGPGIADNVIPHIFDEEYTLGKSDGSGLGLAYCRRVINAFGGNIHCQSKYGSYTRETLTFEIINEERIPNNLFNELKEALTGKQVLV 716
LuxN P profundum.pro           IKNAVYYFDTYPNSQIRIYFQKERNYNKVHVVDTGPGISPDHQKHILEEFYTNGKVQGNGLGLSYCKRVIESFGGTITCQSELGEYTEEILSFPSIDEKIHSEMSKEKIKSYLTGMSGLV 716
LuxN V alginolyticus.pro       IRNAIYYFDSYPDSQIEIRTQTGAYENILIFRDSGPGIDSSILHKIFDEFFSYQKSGGSGLGLGYCQRVMRSFGGRIECQSELNEFTEEYLYFPVVPNAPKPETLR------------- 692
LuxN V angullarium.pro         IRNAIYYFDSYPNSQIEITTQIGTYENILIFRDTGPGIDDAISYKIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGRVECKSKLGEFTEFHLYFPMVPNAPQADSLR------------- 692
LuxN V angustum.pro            LKNAVYYFDTFPESHISIQFEKGLKHNKIHVRDTGPGITEEQIENLFDEEYSFGKVSGNGLGLAYCKKVMESFSGSISCHSILGEFTEETLTFEAINIQSNGELTNPRIKQHLSGQSCLI 714
LuxN V fisheri.pro             LRNATYYFDEY-NSSISIRLVKCFATNKLIFRDTGPGIDSHILPNIFDDFFTHNKEGGSGLGLSYCLRVMHAFEGNIACYSTKGEFTEEVLSFPHIEGDIN--A--------------- 686
LuxN V harveyi.pro             IRNAIYYFDSYPDSQIEISTKTGPYENTLIFRDTGPGIDETISHKIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGRIECKSKLGTFTEFHLYFPVVPNAPKADTLR------------- 692
LuxN V parahaemolyticus.pro     RNAIYYFDSYPDSQIEIRTLVGPYENTIVFRDTGPGIDDSILHKIFDDFFSFQKSGGSGLGLGYCQRVMRSFGGRIECKSVTNEFTEEYLHFPVVPNAPKVETLR------------- 692
LuxN V splendidus.pro          IRNAIYYFDSYPDSQIEISTKTGAYENVLTFRDTGPGIDEAIVHKIFDDFFSYQKSGGSGLGLGYCQRVMRSFGGKVECHSKLGEFTEFHLYFPVVPNAPKADALR------------- 694
LuxN Vibrio sp.pro             IRNAIYYFDSYPDSQIEISTKTGSYENVLTFRDTGPGIDEAIVHKVFDDFFSFQKSGGSGLGLGYCQRVMRSFGGRVECHSKLGEFTEFHLYFPIVPNAPKAETLR------------- 694
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                              730       740       750       760       770       780       790       800       810       820       830       840
                              |---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
LuxN P phosphorem.pro         IGHKENTTLISSLLSGFNIIVSTVDNGKSAAKYIGNNNVDFAFYDLSLSPTQFEALKKIRSGDFGANAQKIPLIALSNE--NTRSTRFDTN-VFQGEFRISDSLPLFAQSLKLLIDSGSL 833
LuxN P profundum.pro          LGSVEVGNWLSSEEKSLGVELCIAPDVKTGLHHLSQQAVDFIIMDHMLLNREMGSIKMLRAGTHGHQAQTTPMFLYGYTENSEHLNSIELSPFFQGQIDGINDHQAFLHSLESLIDNDLF 836
LuxN V alginolyticus.pro      ----------AEDEDSWKATASHSEN-------------------------HSAQHVQVCTD---------------------------------------------------------- 719
LuxN V angullarium.pro        ----------TEDEKSWQQPKPNTEQ-------------------------RIVDNIQEIDK---------PFLINN------------------------------------------- 725
LuxN V angustum.pro           LSASSLSKKLTESFNGLNMNIECSNDPSIGFTRIKDCPFNFIVIDHRLYITHYDQISMLREGKYGYLAQITPIFIFNSTSINLNNDRINVPKYTQGYIDTLNGALAFECSLEAIINDTKF 834
LuxN V fisheri.pro            -------------LNSHKSNTPPLIN-------------------------KKDNSLK-------------------------------------------------------------- 706
LuxN V harveyi.pro            ----------TPYFNDWKQNKRSNEH-------------------------KVAPNVQINNQ---------S------------------------------------------------ 720
LuxN V parahaemolyticus.pro   ----------TPNFYNWNQKVKTKP--------------------------SPEPVVQINKD---------------------------------------------------------- 718
LuxN V splendidus.pro         ----------TPYFNDWKSNQAATEN-------------------------KTNVDAKEDNQAATQ--NSEPTSTLTPG----------------------------------------- 736
LuxN Vibrio sp.pro            ----------TPYFNGWKHNQS-TED-------------------------KAEADVKEESQTPSGDIEPEPASTLTESK-------------------------------QTERTQAEN 747

Majority                      -------APTVLIVDDKEVQRTLVQMYLNRLGVNSLQANNGENAVELFKSXKVDLILMDVQMPVMNGFDASQIIKXXSPQTPIIALSGESGERELXMISKLMDGRLEKPTXLNALQQVLD
                              |---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
                                       850       860       870       880       890       900       910       920       930       940       950       960
LuxN P phosphorem.pro         KPLGHLIGKRVLVVDDMQINRMLVQSYLAQEGITVLQAHNGSVALCIAEQERPDLILMDIHMPEMDCLEVSRILRQRGYNIPIIALSGECCNEVTKEISQYMNAYLMKPITRQQLIQKLQ 953
LuxN P profundum.pro          AKLGSLIGKTVLVVDDMQVNRMLVQAYLASEGITVVQASSGDEAIEKVKKEPFNLVLMDIQMPGMSGIEATHQIRHLFDAIPIVALSGEYNEEITRAISETMNDHLVKPINKQQLLQTLT 956
LuxN V alginolyticus.pro      -------APTVLIVDDKEVQRTLVQMYLKRLGVNSLQANNGASAVELFHSHKIDLVLMDVQMPVMNGFDASQRIKQITSSVPIIALSGESCARELELISKLMDDRLEKPTTLNALQVVIQ 832
LuxN V angullarium.pro        ------KAPTVLIVDDKEVQRSLVQMYLNQLGVNNLQANNGENAVEIFKANSIDLILMDIQMPVMNGFEASQIIKAHSPQVPIIALSGESGERELEMISKLMDGRLEKPTSLNALQQVIS 839
LuxN V angustum.pro           APLGSLNDKTVLVVDDMHANRLLVKAYLSKEGINVIQAASGYEAIEQVKKNNIDLIFMDIHMPGMNGIETAKQLKELDSTKPIIAISGEYGEKIVSDIHKVMDDYIVKPIEKSTLVSLTS 954
LuxN V fisheri.pro            ---------TVLIVDDKKVQRMLIHTFINKDNLTLLQAENGEEAVEIATNNKLDLIFMDSRMPVMNGIDAAKKIKIIYPNLPIIALTGESSHEEISAITQVMDGYLTKPVSKAQLQQVVD 817
LuxN V harveyi.pro            --------PTVLIVDDKEVQRALVQMYLNQLGVNSLQANNGENAVEVFKANHVDLILMDVQMPVMNGFDASQRIKELSPQTPIVALSGESGERELDMINKLMDGRLEKPTTLNALRHVLG 832
LuxN V parahaemolyticus.pro   -------APTVLIVDDKEVQRTLVQMYLNRLGVNSLQANNGANAVELFQSHQVDLVLMDVQMPVMNGFDASEKIKQCSPTTPIIALSGESGEKELEMIAKLMDGRLEKPTTLNALRDVLV 831
LuxN V splendidus.pro         ----NHLAPTVLIVDDKEVQRTLVQMYLSRLGVNSLQAKNGENAVELFKTHKVDLILMDVQMPIMNGFDASQIIKARSPQTPIIALSGESGQHELDMISKLMDGRLEKPTSLKALQHVLD 852
LuxN Vibrio sp.pro            QPASSHLAPTVLIVDDKEVQRTLVQMYLSRLGVNSLQAKNGENAVELRSHKVDLILMDVQMPIMNGFDASQIIKARSPQTPIIALSGESGQRELDMIRKLMDGRLEKPTSLNALQHLLD 867

Majority                      XWLXKXXASN---X---------
                              |---------|---------|
                                       970       980
LuxN P phosphorem.pro         YNIPESEADKVISKQDIHIVHSI                                                                                                  976
LuxN P profundum.pro          KKMT                                                                                                                     960
LuxN V alginolyticus.pro      RWLQNENFAPSNTF                                                                                                           846
LuxN V angullarium.pro        HWLNKDIVPNAHTAKSGTVI                                                                                                     859
LuxN V angustum.pro           KWLIINKVKD                                                                                                               964
LuxN V fisheri.pro            KWL                                                                                                                      820
LuxN V harveyi.pro            NWLNKNTAS-SACEAERE                                                                                                       849
LuxN V parahaemolyticus.pro   RWLHFDKISVTNSYQIANE                                                                                                      850
LuxN V splendidus.pro         NWLEKGWASNTSKETESEE                                                                                                      871
LuxN Vibrio sp.pro            NWLEKGWAPNASKETENE                                                                                                       885
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Appendix 8: Protein Sequences of Published AHL Degrading Enzymes

AHL Lactonase Sequences

AhlD (*Arthrobacter sp.* IBN110)

MEKDQLKVRVLETGVMEADMAWLLLKPGRIIADRNNKERQREWGEIPTHAVLIEHPEGRILWDTGVPRDW
SSRWQESGMDNYFPVKTESSSESGFLDSSLAQVGLEPADIDLLILSHLHLDHAGNARLFDNGKTKIVANR
KELEGVQEIMGSHLGGHLKADFEGLKIDAIEGDTEIVPGVSVIDTPGHTWGTMSLQVDLPDDGTKIFTSD
AVYLRDSFGPPAIGAAVVWNNLLWLESVEKLRRIQERTNAEMIFGHESEQTSQIRWAHQGHYQ

AhlK (*Klebsiella pneumoniae* 342)

MMPEIKLFMFQSGTQHCRYQHIRMNQGVGEHYEIPVPWFLLTHPDGFTLIDGGLAVEGLKDPSGYWGSTV
EQFKPVMSEEQGCVEQLKRIGIAPEDIRYVVLSHLHSDHTGAIGRFPHATHVVQRQEYEYAFAPDWFTSG
AYCRRDFDRPQLNWLFLNGLSDDHYDLYGDGTLQCIFTPGHSPGHQSFLIRLPGGTNFTLAIDAAYTLDH
YHEKALPGLMTSATDVAQSVRKLRQLTERYHAVFIPGHDPEEWKKNRLAPACYY

AiiA (*Bacillus sp.* 240B1)

MTVKKLYFLPAGRCMLDHSSVNSTLTPGKLLNLPVWCYLLETTEGPILIDTGMPESAVDNEDLFKGTFVE
GQILPKMKPDDRIVNILKRVGYAPEDLLCVISSHLHFDHAGGNGSFSHAPIIVQRTEHDAALHRAEYLKE
CILPDLNYQMIEGDYEVMPGVQLLYTPGHSPGHQSILVKTEKSGSVLLTIDASYTQENFEQGVPFAGFDS
EMASQSINRLKEIVLDEKPIVFFGHDMEQEKRCKTFPEFL

AiiB (*Agrobacterium tumefaciens* C58)

MGNKLFVLDLGEIRVDENFIIANSTFVTPQKPTVSSRLIDIPVSAYLIQCTDATVLYDTGCHPECMGTNG
RWPAQSQLNAPYIGASECNLPERLRQLGLSPDDISTVVLSHLHNDHAGCVEYFGKSRLIAHEDEFATAVR
YFATGDHSSPYIVKDIEAWLATPRNWDLVGRDERERELAPGVNLLNFGTGHASGMLGLAVRLEKQPGFLL
VSDACYTATNYGPPARRAGVLHDTIGYDRTVSHIRQYAESRSLTVLFGHDREQFASLIKSTDGFYE

AiiM (*Microbacterium testaceum* LB037)

MILAHDVSGSGPLLVLLHGITEDRRSWDPVDFTDGFTVVRVDLRGHGASAAEEPYDIPTLATDVHDTLAQ
LAENDVIPGELPVIVGHSMGGIVATAYGALFPARAIVNVDQPLQLAGMQGQVQQAEGMLRGADFPLFIHG
MFAQMAGGLDAEELARVNGIRSPRQDVVLGMWRPLLEDSPEELAALVSGLTRIPEDVPYLVITGLDAGPE
YAAWLQREIPQAVQEVWQPPTHYPHLVDPARFVERVEAFVR

AttM (*Agrobacterium tumefaciens* C58)

MLQSGTLKCKVHNIKMNQGNGADYEIPVPFFLITHPAGHTVIDGGNAIEVATDPRGHWGGICDVYWPVLD
KDQGCVDQIKALGFDPADVKYVVQSHLHLDHTGAIGRFPNATHIVQRSEYEYAFTPDWFAGGGYIRKDFD
KPGLKWQFLNGAQDDYYDVYGDGTLTTIFTPGHAPGHQSFLVRLPNSKPLLLTIDAAYTLDHWEEKALPG
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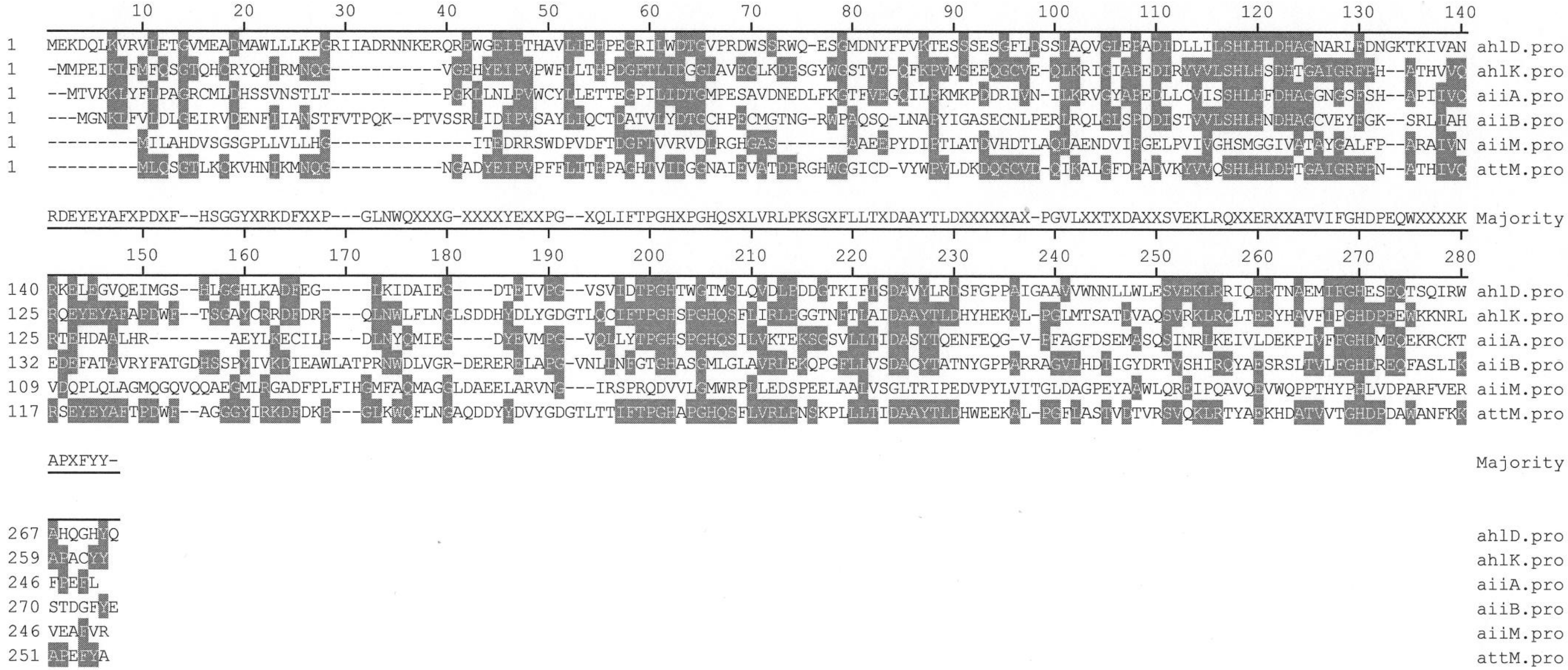
AHL Lactonase Sequence Alignment



AHL Acylase Sequences

Aac (*Shewanella sp.* MIB015)

MKFNKLMIAMGMACGVMLTGCNDSEDSTLPTEPETQLQTFAPNGLLKANIRRTTYGVPHIQADNLESLGF
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QFSERSRALIQGFTAGYNQYLADVEVGKQTAEPFCGGQPWVKPIVPEDVVTYLFSIALLPGAANFLDLIF
YANPGDGQEYMPRIVGPAPSQEQTAFVSDMQSKLLARAARITTPETNPRDLGSNGWGLGKDKTENGRGMV
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RLQYLFDGQPMPINKETVSILVNAGPAGMLVAEKDIYTTAKGPMVEAPPSLAPFGWDDGSAFMIQDANMA
NMDPVDHWLAMNLATNKDEFQQAFKDYDGVIFNNTMFADKEGNAFYIDDSTVPGLSESAVVVLKTSPDIK
AAKQRAGFTILPGNTSLFSFSGPTPYERAPKLERSDFVQNSNDSFWSTNLNEPLTYFSPMYGPEAGQLSL
RTRMGLTLMQDAAGSDGKFNLEELEAAVLSNRSYLAELVLQDLIAQCEAQGSTPVVVSASLSKDLTSACA
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SGMTAKGYQVRYGSSWMMAVSFTDEGPVARGILTYSESSNILSPSFTDQSNLYSSSKSFRPLLFKEADIA
PAVISTTELTLQKAQ

AhlM (*Streptomyces sp.* M664)

MRLRNRLRLLGVAGLALFTVSASLPPATASGAGQERHPSGGGLSAVIRYTEYGIPHIVAKDFAQLGFGTG
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AADVTRTQWWTRYGPVVTSMGAALPLPWTATTAYALNDPNATNLRMADTGLGFSKARSTKDVERSLRRNQ
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GIFGPGRMPVLKNQPYVENSNDSAWLTNADRPLTGYERVFGTIATPRSMRTRGAIEDVASMADKGRLRVA
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WRKTSAVPAAELWKTPFDPADPVRTPRGLNTAAPGVGRALADAVAELRAAGIALDAPLGKHQFVVRNGKR
LPIGGGTESLGIWNKTEPVWNAAGGGYTEVSSGSSYIQAVGWDDSRCPVARTLLTYSQSENPRSRHFSDQ
TRLYAGERWVTSRFCEKDIARSPDLRVVRVHERR

AiiD (*Ralstonia sp.* XJ12B)

MMQGFALRGTLAMAALAALAGCASSTDGRWGSLSDTGLSAEIRRTGFGIPHIRANDYASLGYGMAYAYAQ
DNLCLLADQVVTVNGERSKTFGPEGTVTVSFKPIPNLQSDAFFKGIFDEDGLRAGYAQMSPEARELLRGY
IAGFNRYLKDTPPANFPAACRNAAWVRPLTLGDMMRMGEEKAIQASAGAMLAGIVAAQPPGRTPVAEREI
PPQAVDTVALDRELQLRDMPIGSNGWAFGADATANRRGVLLGNPHFPWTTTNRFYQVHLTVPGKLDVMGA
SIAAFPVVSIGFNKDVAWTHTVSTGRRFTLFELKLAEGDPTTYLVDGTPHKMTTRTVAFDVKLPDGRLER
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TIATDRNGRALFADVSTTPDVPAAELQRCAPSPLAGKLFKDAGLVLLDGSRGTCNWQVDPASPVPGLVAP
ARMPVLERDDYVANSNDSSWLTNPAQKLTGFSPVMGSVDVPQRLRTRIGLIEIGRRLAGTDGLPGNRIDL
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AQVHAVEFDPADPVHTPRGLRMNDATVRTAVFKALKEAVGAVRKAGFALDAPLGTVQAAHAPDGSIALHG
GEEYEGVLNKLQTLPIGPKGLPVYFGTSYIQTVTFDDQGPVADAILTYGESTDHASPHAFDQMRAYSGKH
WNRLPFSEAAIAADPALKVMRLSQ

AiiO (*Ochrobactrum sp.* A44)

MTINYHELETSHGRIAVRESEGEGASLLMIHGNSSSGAIFAPQLEGEIGKKWRVIAPDLPGHGKSSDAID
PDRSYSMEGYADAMTEVMQKLGIADAVVFGWSLGGHIGIEMIARYPAMRGLMITGTPPVAREEVGQGFKS
GPDMALAGQEIFSERDVESYARSTCGEPFETSLLDIVARTDGRARRIMFEKFGNGTGGNQRDIVAEAKLP
IAVVNGRDEPFVELDFVSKVKFGNLWDGKTHVIDNSGHAPFREAPAEFDAYLARFIGDCTK

PvdQ (*Pseudomonas aeruginosa* PA01)

MGMRTVLTGLAGMLLGSMMPVQADMPRPTGLAADIRWTAYGVPHIRAKDERGLGYGIGYAYARDNACLLA
EEIVTARGERARYFGSEGKSSAELDNLPSDIFYAWLNQPEALQAFWQAQTPAVRQLLEGYAAGFNRFLRE
ADGKTTSCLGQPWLRAIATDDLLRLTRRLLVEGGVGQFADALVAAAPPGAEKVALSGEQAFQVAEQRRQR
FRLERGSNAIAVGSERSADGKGMLLANPHFPWNGAMRFYQMHLTIPGRLDVMGASLPGLPVVNIGFSRHL
AWTHTVDTSSHFTLYRLALDPKDPRRYLVDGRSLPLEEKSVAIEVRGADGKLSRVEHKVYQSIYGPLVVW
PGKLDWNRSEAYALRDANLENTRVLQQWYSINQASDVADLRRRVEALQGIPWVNTLAADEQGNALYMNQS
VVPYLKPELIPACAIPQLVAEGLPALQGQDSRCAWSRDPAAAQAGITPAAQLPVLLRRDFVQNSNDSAWL
TNPASPLQGFSPLVSQEKPIGPRARYALSRLQGKQPLEAKTLEEMVTANHVFSADQVLPDLLRLCRDNQG
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RQALADAAAEVEKSGIPDGARWGDLQVSTRGQERIAIPGGDGHFGVYNAIQSVRKGDHLEVVGGTSYIQL
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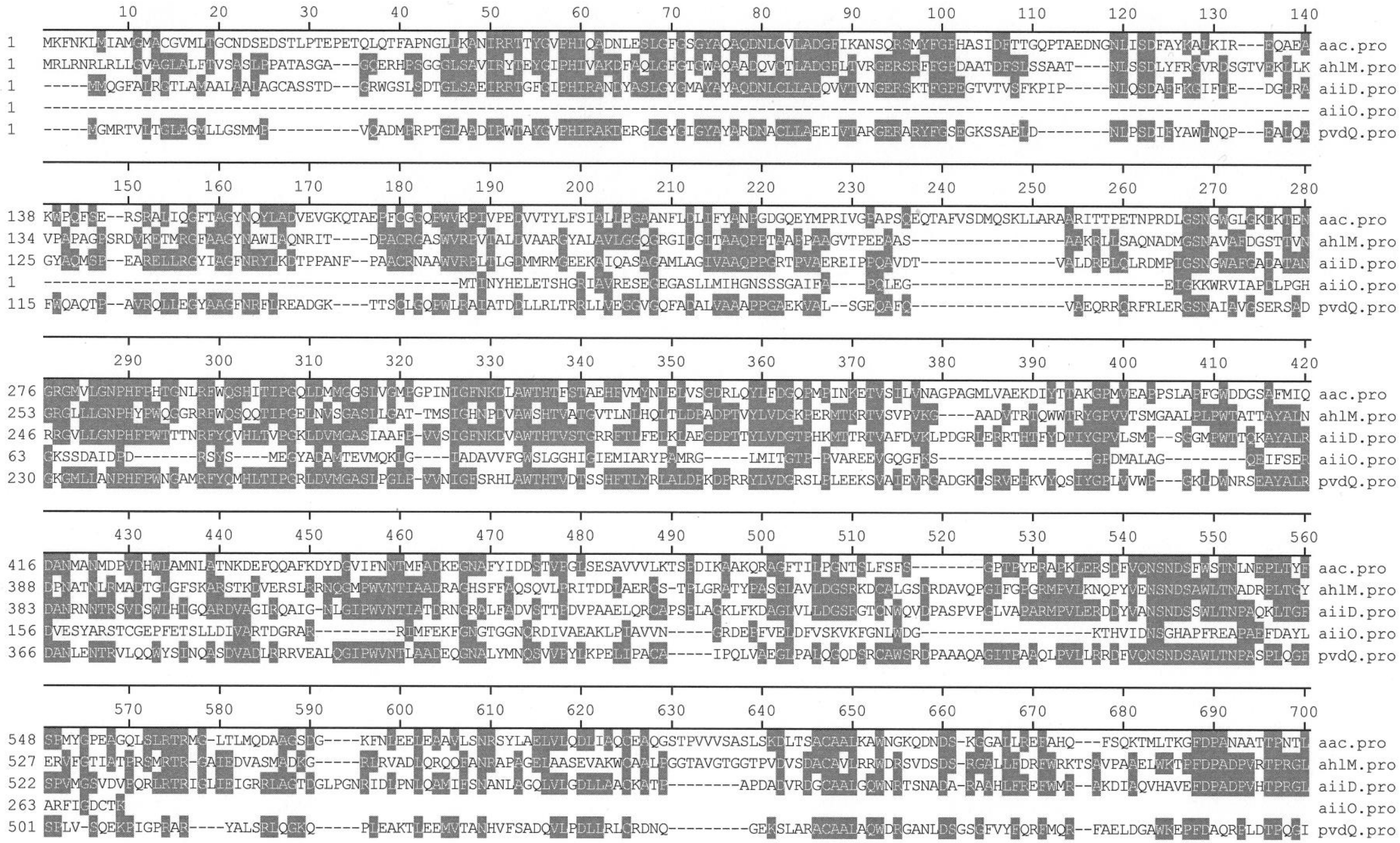
AHL Acylase Sequence Alignment



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660 N----TAAPGVGRALADAVAELRAAGIALDAPLGKHQFVVR-------NGKRLPIGGGTESLGIWNKTEPVWNAAGGGY-------------------------TEVSSGSSYIQAVGWDDSRCPVARTLLTYSQSENPR ahlM.pro
651 RMNDATVRIAVFKALKEAVGAVRKAGFALDAPLGTVQAAHA-------PDGSIALHGGEEYEGVLNKLQTLPIGPKG-------------------------LPVYFGTSYIQTVTFDD-QGPVADAILTYGESTDHA aiiD.pro
271                                                                                                                                              aiiO.pro
620 ALDRPQVATQVRQALADAAAEVEKSGIPDGARWGDLQVSTR-------GQERIAIPGGDGHFGVYNAIQSVRKGD---H-------------------------LEVVGGTSYIQLVTFPE-EGPKARGLLAFSQSSDPR pvdQ.pro

          850       860       870       880
813 SPSFTDQSNLYSSSKSFRPLLFKEADIAPAVISTTELTLQKAQ     aac.pro
764 SRHESDQTRLYAG-ERWVTSRFCEKDIARSEDLRVVRVHERR      ahlM.pro
756 SPHAFDQMRAYSG-KHWNRLPFSEAAIAADPALKVMRLSQ        aiiD.pro
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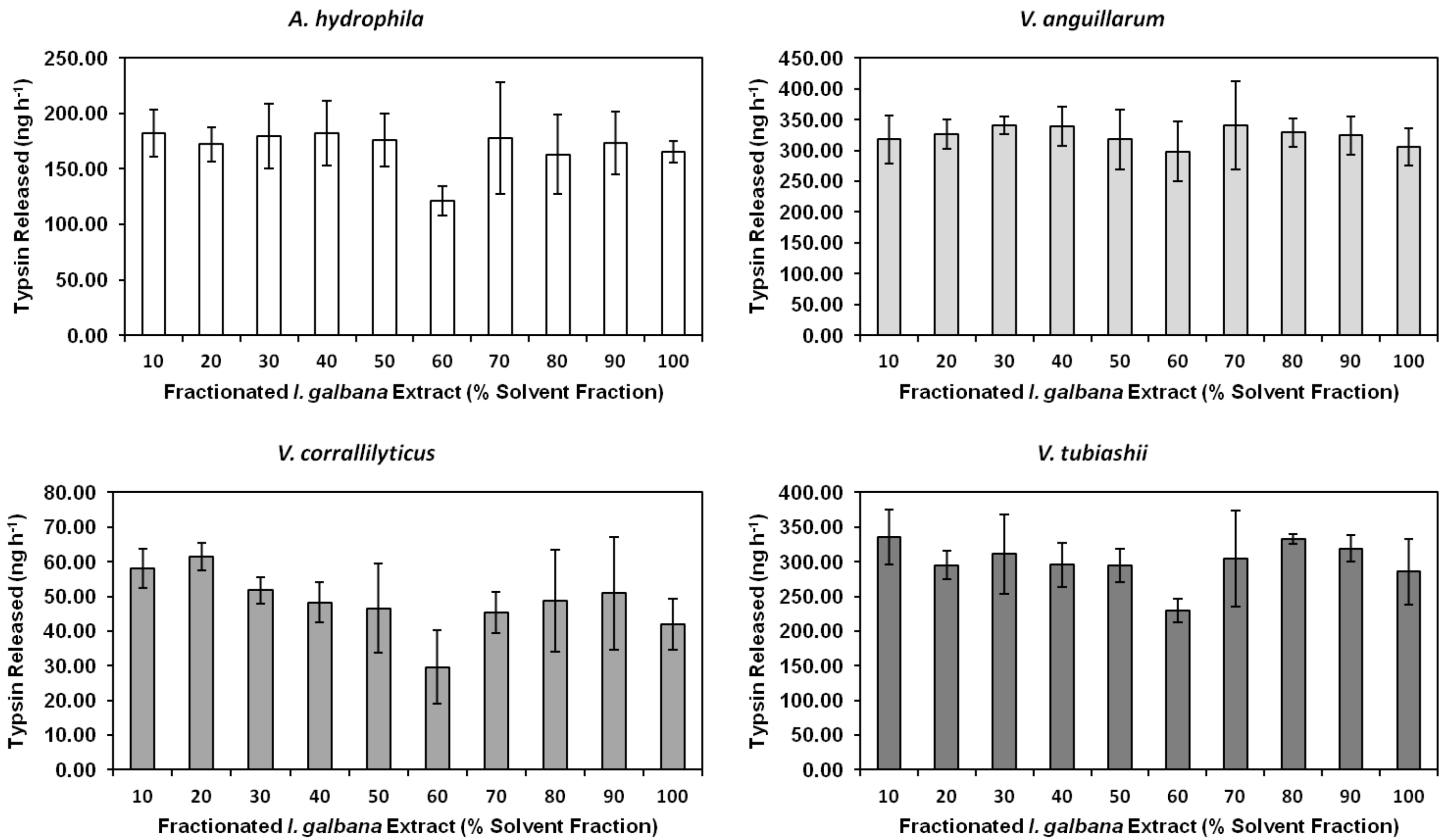
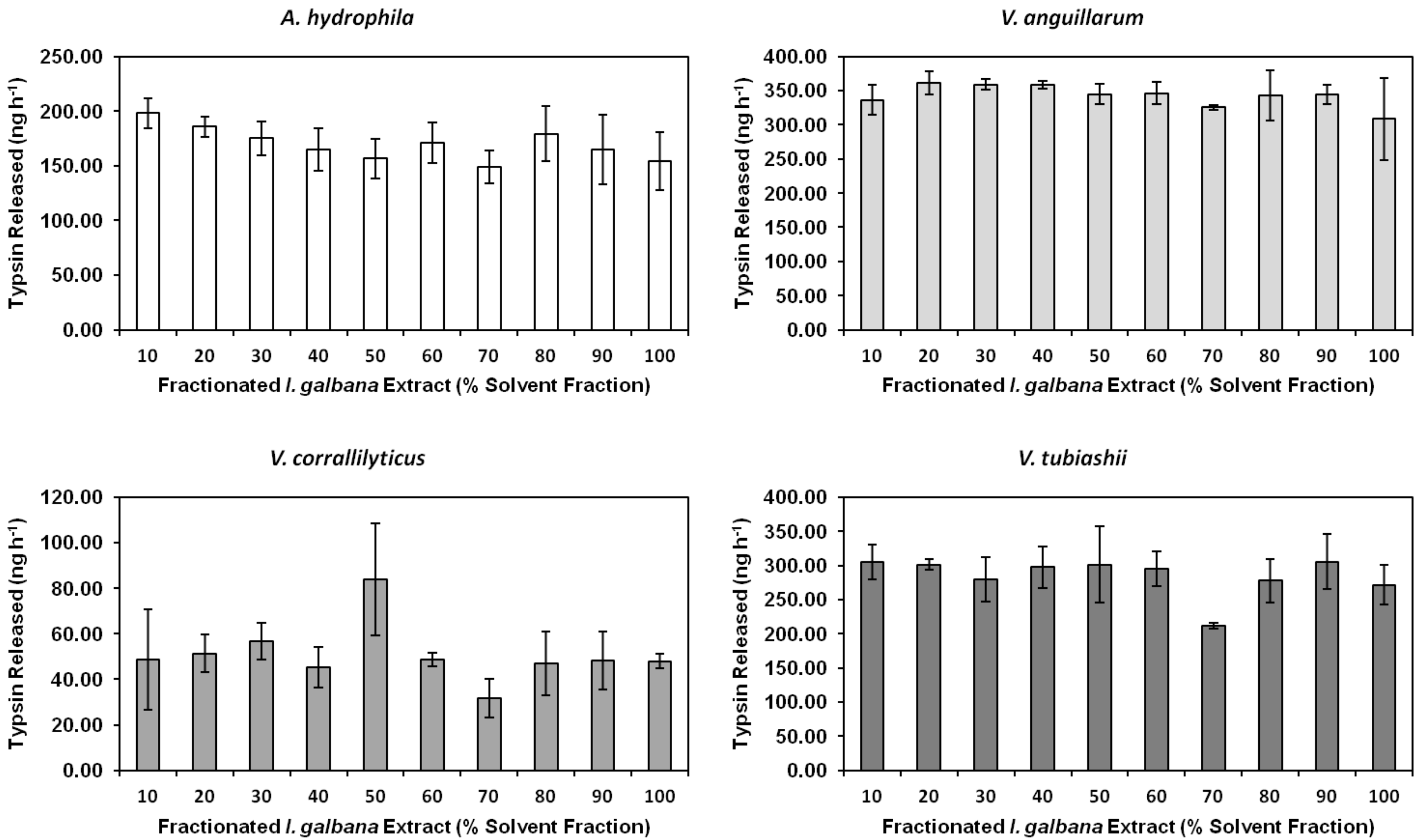
Appendix 9: Initial Microalgal Protease Assays

Protease activity of in four marine bacterial pathogens exposed to fractionated extracts from stationary phase *I. glabana* culture. Error bars represent standard deviation from the mean.



Protease activity of in four marine bacterial pathogens exposed to fractionated extracts from stationary phase *N. oculata* culture. Error bars represent standard deviation from the mean.



Protease activity of in four marine bacterial pathogens exposed to fractionated extracts from stationary phase *T. suecica* culture. Error bars represent standard deviation from the mean.