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Determination of Extracellular Molecules Produced by Vibrio Harveyi Using MS/MS

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DETERMINATION OF EXTRACELLULAR MOLECULES PRODUCED BY *VIBRIO HARVEYI* USING MS/MS

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by

Jose G. Roble

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To: Dean Michael R. Heithaus
College of Arts and Sciences

This thesis, written by Jose G. Roble, and entitled Determination of Extracellular Molecules Produced by *Vibrio harveyi* Using MS/MS, having been approved in respect to style and intellectual content, is referred to you for judgment.

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DEDICATION

I dedicate this thesis to my parents. Their unconditional support, advice, understanding and love have made it possible for me to pursue my dreams.

Dedico esta tesis a mis padres. Su apollo incondicional, asesoramiento, comprensión y amor han hecho posible que persiga mis sueños

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ABSTRACT OF THE THESIS

DETERMINATION OF EXTRACELLULAR MOLECULES PRODUCED BY *VIBRIO HARVEYI* USING MS/MS

by

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Professor John Makemson, Major Professor

Quorum sensing (QS) is a process that allows bacteria to sense the population density of cells around them by communicating with each other via autoinducer molecules. This cross-communication is crucial in the regulation of bacterial processes such as bioluminescence, virulence, and biofilm formation.

Previous research by Milburn and Makemson on *Vibrio harveyi* suggested that in addition of the known biosynthesis of three well-characterized autoinducers, dozens of unknown molecules are also produced and released to the environment by *V. harveyi*. This study was performed using electrospray tandem mass spectrometry with the purpose of detection and characterization of the extracellular molecules produced by *V. harveyi*, and assessment of their relationship to QS. A total of 11 molecules were characterized, from which three could be related to QS. These findings provide a glimpse of the nature of novel secondary metabolites produced by *V. harveyi* and provide the groundwork for further research.

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ABREVIATIONS AND ACRONYMS

ACN	Acetonitrile
AHL	Acyl homoserine lactone
AI	Autoinducer
AI-2	Autoinducer 2
ASW	Artificial seawater
CAI-1	*Vibrio Cholerae* autoinducer
DPD	4,5-dihydroxy-2,3-pentanedione
Ea-C_8-CAI-1	*Vibrio harveyi* derivative of CAI-1
ESI-MS	Electrospray ionization mass spectrometry
ESI-MS-MS	Electrospray ionization tandem mass spectrometry
HAI-1	*Vibrio harveyi* AHL
HCD	High cell density
HLS	Homoserine lactone
HPLC	High performance liquid chromatography
IR	Infrared spectrometry
LCD	Low cell density
LLE	Liquid-Liquid extraction
m/z	mass-to-charge ions
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PQS	*Pseudomonas* quinolone signal
QS	Quorum sensing

SPE	Solid-Phase extraction
TIC	Total Ion Chromatogram

A. INTRODUCTION

Bacteria are able to communicate with each other through a chemical language. Instead of using words they use molecules that allow them to talk with members of their species, genera, and even other domains. Nealson *et al*. first described the process in 1970, which they termed autoinduction, and now it is known as quorum sensing (QS; Fuqua *et al.* 2001). The process can be described as a cell-to-cell chemical communication that can detect and measure the cell density of the surrounding population. Quorum sensing regulates expression of suites of genes and ultimately physiological processes using small and easily diffusible molecules called autoinducers (AIs), which can be species specific or global AI (Eberhard *et al.* 1981; Ng and Bassler, 2009).

Quorum sensing regulates a broad range of bacterial processes, including the regulation of virulence factors, biofilm formation, bioluminescence, growth, antibiotic resistance, metabolism, expression of survival genes, and others (Shadaba and Opal, 2008). Understanding the molecular basis of QS activation and inhibition is important in the medical field, where the search for molecules that could block quorum sensing is an active field of research. By blocking the QS-mediated virulence in pathogens such as *Pseudomonas aeruginosa*, one could prevent the production of disease causing virulence factors. Quorum sensing inhibitors would target only the release of virulence factors, and prescribing them alongside with antibiotics could be more powerful than treatments with antibiotics alone.

Until recently the methods used for detecting AIs in cell media have included cell line bioreporters and thin layer chromatography (TLC). The previous techniques can

reveal the presence of AI molecules but they have a low level of sensitivity compared to other techniques in the analysis of molecules. With the development of analytical tools such as high performance liquid chromatography and high definition mass spectrometry (HPLC/MS), it is possible to produce a comprehensive identification, structural and quantitative analysis of all the molecules and its derivatives found in cell culture supernatants.

a. Ecology and Distribution of *Vibrio harveyi*

Vibrio harveyi is a Gram-negative, marine, bioluminescent, facultative anaerobe bacterium that propels itself with the use of polar and peritrichous flagella. In the planktonic state it can be found at the surface of warm coastal waters (Ruby *et al*., 1980), and also inside the gut of fish and invertebrates in a commensal relationship (Makemson and Hermosa, 1999; Owens and Busico-Salcedo, 2006).

b. Bacterial Luciferase

Bioluminescence occurs when light is produced and emitted by an organism through a chemical reaction. Bioluminescence can be seen in many organisms such as animals, fungi and microbes. Their bioluminescence is achieved by the action of the inducible enzyme luciferase. The bacterial luciferase reaction consumes oxygen and reducing power.

Bacterial luciferase catalyzes the following reaction in which one of the byproducts is photon energy:

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + h\nu \text{ (light)}$$

In the reaction, $FMNH_2$ and an aliphatic aldehyde (RCHO) in the presence of oxygen are converted to FMN, an aliphatic acid, water and light (Ziegler and Baldwin, 1981). At the genetic level in bacteria, all the genes coding for the enzyme are found in the *lux* operon (Hastings, 1983), or *luxCDABEG*, with *luxR* acting as its master regulator (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984). In *V. harveyi*, the luciferase gene is composed of LuxAB, LuxCD and LuxE code for enzymes that recycle the aliphatic acid back to the aliphatic aldehyde (Boylan *et al.*, 1989), and LuxG and other proteins reduce FMN using NADH (Zenno and Saigo, 1994). The *lux* operon is regulated by a QS regulatory system in which a homoserine lactone (HSL), a boronated furanone, and an aliphatic alcohol/ketone act as autoinducers to control the expression of *luxR*.

It was suggested that the Lux system is used in bacteria to remove excess oxygen to counteract oxidative stress (Visick *et al*., 2000; Timmins *et al*., 2001; Bose *et al*., 2007), or to remove excess reductant such as NADH (Burgois *et al*., 2001). However, animals (fish and invertebrates) with light organs populated by different species of luminous bacteria are able to harness the light for hunting and escape tactics (Hastings *et al.*, 1987).

Luciferase can be thought as a mixed-function oxidase (Hastings and Balny, 1975). One of the unusual features of the luciferase reaction is that it is very slow, so slow that at room temperature, one cycle of the reaction takes place in a few seconds. Consequently, it has long-lived intermediates (Hastings and Gibson, 1963). The luciferase system is also affected by catabolite repression (glucose inhibits luciferase synthesis), which can be reversed with cAMP (Nealson *et al*., 1972). Another compound

that affects the synthesis of luciferase is arginine, dramatically increasing the light emission in minimal media (Coffey, 1967; Makemson and Hastings, 1979).

Luciferase is composed of two protein subunits α and β. The enzyme shows specificity towards aldehydes that contain 8-16 carbons (Hastings *et al*., 1963; Watanabe and Wakamura, 1972). Specifically, *V. harveyi*'s luciferase has a molecular weight of 79,000 (α = 42,000 and β = 37,000; Tu 1978). Subunit α contains the active site and all the catalytic properties of the enzyme (Rogers and McElroy, 1955; Meighen, 1978). Although subunit β is not involved in the reaction, the reaction cannot happen if it is absent, as it provides conformational stability for the enzyme (Cline, 1973). Both subunits can be recombined from different species to form chimeras with varying properties compared to the parent luciferase α subunit (Hosseinkhani *et al*., 2005).

The decay rate of the light reaction is species dependent, ranging from slow, intermediate, and fast, with fast rates being 5-10 times faster than slow rates (Ruby *et al*., 1978). The fast class of luciferase is found in *Photobacterium leiognathi*, *Photobacterium phosphoreum*, *Aliivibrio fischeri*, and *Vibrio logei*, with decay rates of more than 0.6 s^{-1} (Nealson, 1978; Nealson and Hastings, 1979). The intermediate class is found in *Shewanella woodyi*, with a decay rate of 0.2 - 0.3 s^{-1} (Makemson *et al*., 1997). Lastly, the slow luciferase is found to contain a rate of 0.02 - 0.1 s^{-1}, and it is found in *V. harveyi* and *Vibrio splendidus* species.

c. Major Classes of Autoinducers

Gram-positive bacteria use modified oligopeptides as autoinducer molecules, with unique amino acid sequences and level of modification, making them species specific. Gram-negative bacteria use different classes of AI molecules. The first class of AI molecules is acyl homoserine lactones (AHL), which are highly common in proteobacteria. These molecules are composed of a homoserine lactone ring with acyl groups of different lengths attached to them, providing an amphipathic nature for membrane diffusion (Fuqua *et al*., 2001). Acyl homoserine lactones are used for intra-species communication. Another class of HSL molecules contains a p-coumaric group instead of an acyl group (**Figure 1**; Schaefer *et al*., 2008). A second autoinducer (AI-2), first discovered in *V. harveyi*, is derived from the molecule 4,5-dihydroxy-2,3-pentanedione (DPD). It is synthesized by *luxS* gene product found in hundreds of bacterial genomes, Gram-positive and Gram-negative, thus used for interspecies communication (Schauder *et al.*, 2001). One peculiarity of the chemical structure of AI-2 is that it can incorporate the element boron in its structure (Chen *et al*., 2002). A third autoinducer only found in *V. harveyi* and *Vibrio cholerae* is (S)-3-hydroxytridecan-4-one (CAI-1), which plays an important role in the QS system of *V. cholerae* (Higgins *et al*., 2007).

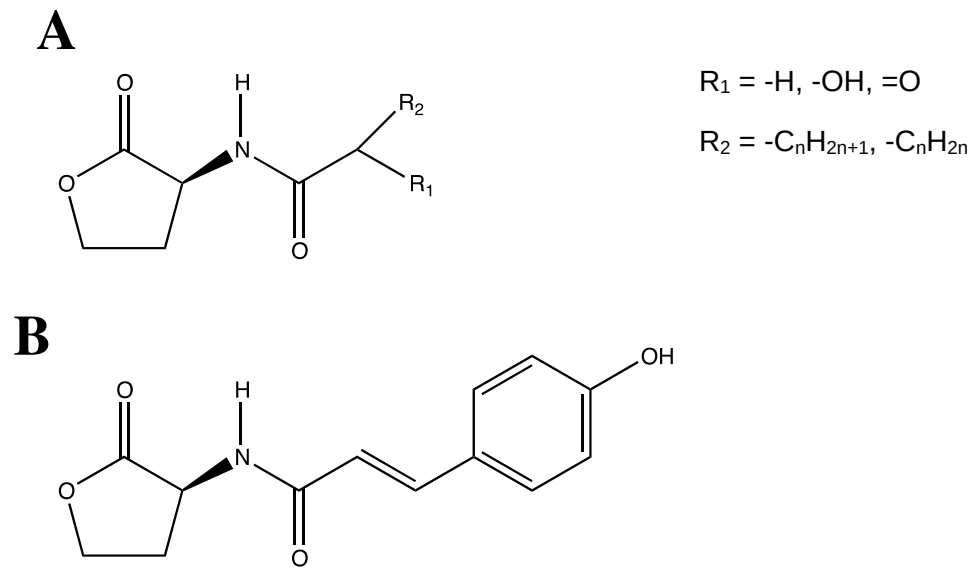


Figure 1 – Two classes of HLS. **A)** Acyl homoserine lactone **B)** N-(p-Coumaroyl)-L-homoserine lactone

d. Quorum Sensing Systems

i. Quorum Sensing in Gram-Positive Bacteria

In Gram-positive bacteria, the quorum sensing mechanism is regulated by AIs that are usually small oligopeptides (Ji *et al.*, 1995). Precursor peptides are modified inside the cell and are secreted as cell density increases. A membrane-bound histidine kinase protein detects the oligopeptides, starting a phosphorylation cascade that in turn activates a response regulator, which activate the targeted genes (Ng and Bassler, 2009). The phosphorylation cascade also promotes the transcription of the genes that code for the precursor peptide, creating a positive feedback mechanism. It should also be noted that the genes for the precursor peptide, the histidine kinase, and the response regulator usually form an operon, thus they get transcribed together (Ji *et al.*, 1995). The QS system is species-specific with many different variations of the oligopeptides, histidine

kinases, and response regulator, and with each species having a unique amino acid sequence of the precursor peptide (Pestova *et al.*, 1996).

ii. Quorum Sensing in *Aliivibrio fischeri*

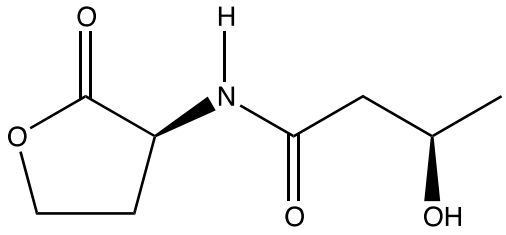
In *A. fischeri*, the QS mechanism is regulated by two proteins LuxI and LuxR, which activate bioluminescence (Engebrecht and Silverman, 1984). LuxI is responsible for the production of N-3-(oxo-hexanoyl)-homoserine lactone (C_6-oxo-3-HSL; Schaefer *et al*., 1996). As more AI is produced, their concentration starts to increase outside the cell, diffusing back into the cell and binding more frequently to LuxR, forming a stable complex. This complex will then activate the *luxICDABE* operon that codes for luciferase (Stevens *et al*., 1994). Without the AHL, LuxR is very unstable and gets degraded, and only with a high concentration of the autoinducer can the stable AHL-LuxR complex be formed. The system contains a positive feedback mechanism where the AHL-LuxR complex will promote the synthesis of the LuxI protein (Engebrecht and Silverman, 1984).

iii. Quorum Sensing in *Vibrio harveyi*

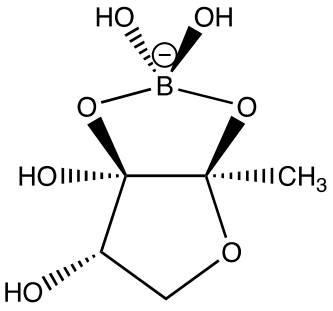
In *V. harveyi*, QS regulates bioluminescence and other processes such as a type III secretion membrane protein and a metalloprotease (Henke and Bassler, 2004; Callahan and Dunlap, 2000). *Vibrio harveyi* relies on three membrane bound histidine kinases to mediate QS. LuxM synthesizes the HAI-1 autoinducer, N-(β-hydroxybutyryl) homoserine lactone which is detected by the histidine kinase HAI-1 receptor LuxN (Bassler *et al.*, 1993). AI-2 is produced by LuxS and detected by LuxPQ, the histidine kinase receptor (Bassler *et al*., 1994[a]). Lastly, (Z)-3-aminoundec-2-en-4-one (Ea-C8-

CAI-1), a derivative of *V. cholerae* CAI-1 autoinducer but with a shorter 8-carbon tail, is produced CqsA synthase and detected by the CqsA histidine kinase receptor (**Figure 2**; Henke and Bassler 2004; Ng *et al.*, 2011).

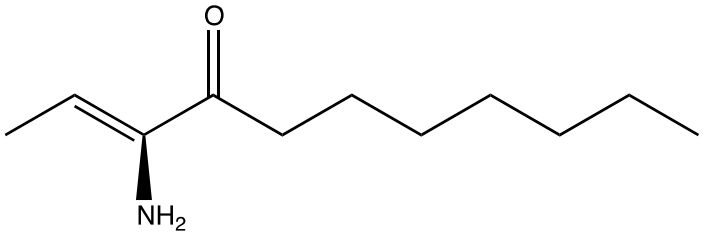
Depending on the extracellular AI levels, LuxN, LuxPQ, and CqsA receptors are bi-functional and can either act as kinases or phosphatases (Ng *et al*., 2011). At a low cell density (LCD), there are low AI levels, and the three membrane-bound receptors behave as kinases transferring their phosphate group to LuxU, a phosphate transfer protein. The LuxU protein then transfers the phosphate to LuxO, which when phosphorylated acts as a transcriptional activator and promotes the transcription of five small regulatory RNAs (sRNAs) called *Qrr* 1-5. Together with the RNA chaperone Hfq, the five sRNAs inhibit transcription of *luxR* (**Figure 3A**; Bassler *et al*., 1994[b]; Waters and Bassler, 2006). In *V. harveyi*, LuxR is the master QS regulator, affecting the expression of up to 30 different genes, including activation of the bioluminescence genes: *luxCDABE* (Pompeani *et al*., 2008). As long as there is a low concentration of LuxR present inside the cell, synthesis of luciferase is repressed and the bacteria only produce a small amount of light. At high cell density (HCD), the membrane receptors act as phosphatases after being bound by the AIs and dephosphorylate LuxU, in turn removing the phosphate from LuxO and therefore inactivating it, and transcription of the five *Qrr* sRNAs is terminated (Freeman and Bassler, 1999; Lilley and Bassler, 2000). Without the repression from the sRNAs, *luxR* mRNA is fully transcribed and translated to LuxR, which promotes the transcription of the *luxCDABE* operon, required for the synthesis of luciferase, as well as the repression of metalloprotease synthesis and a type III secretion system (**Figure 3B**; Miyamoto *et al*., 1994; Henke and Bassler, 2004; Callahan and Dunlap, 2000).



HAI-1



AI-2



Ea-C8-CAI-1

Figure 2 – Autoinducers synthesized by *Vibrio harveyi*

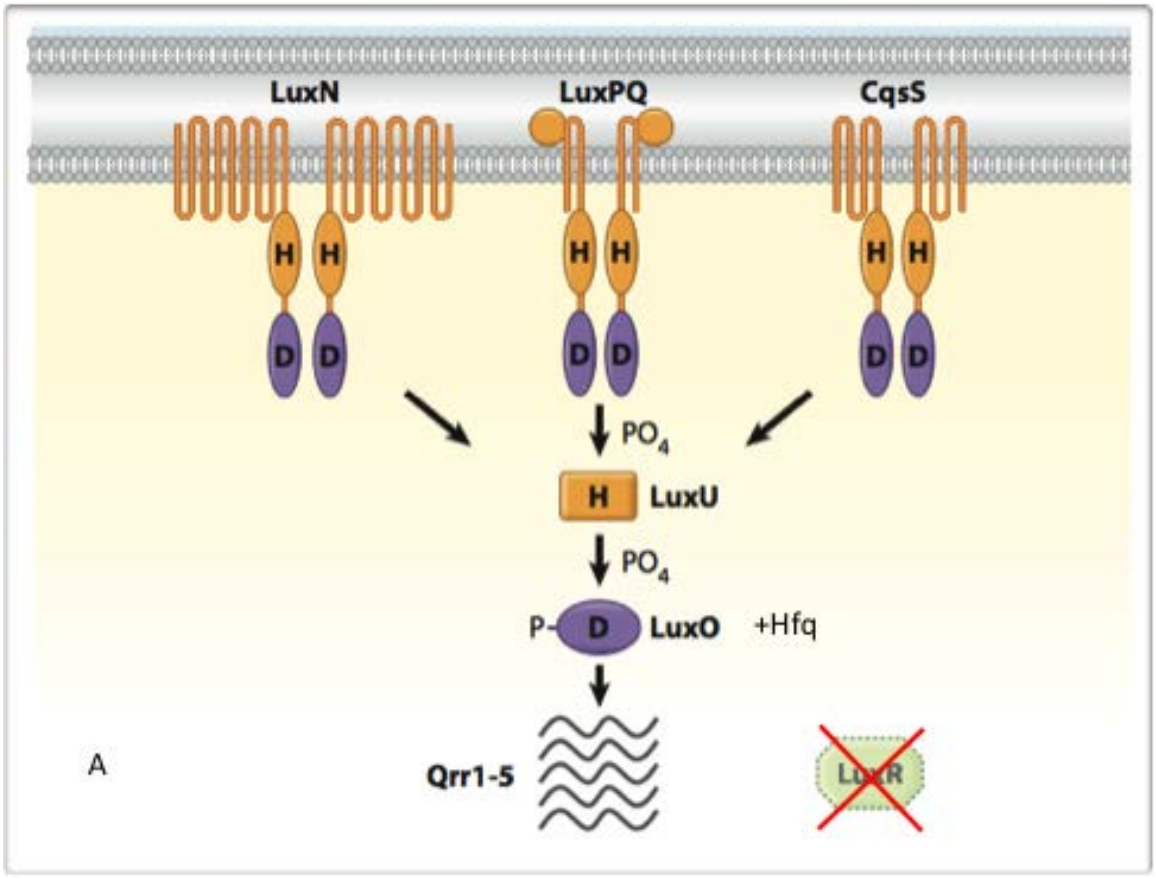


Figure 3A – Quorum Sensing at LCD. Integration of the three autoinducers through the phosphorylation signal cascade (LuxU, LuxO) producing five small RNAs, which repress LuxR at low cell density (Adapted from Ng and Bassler, 2009)

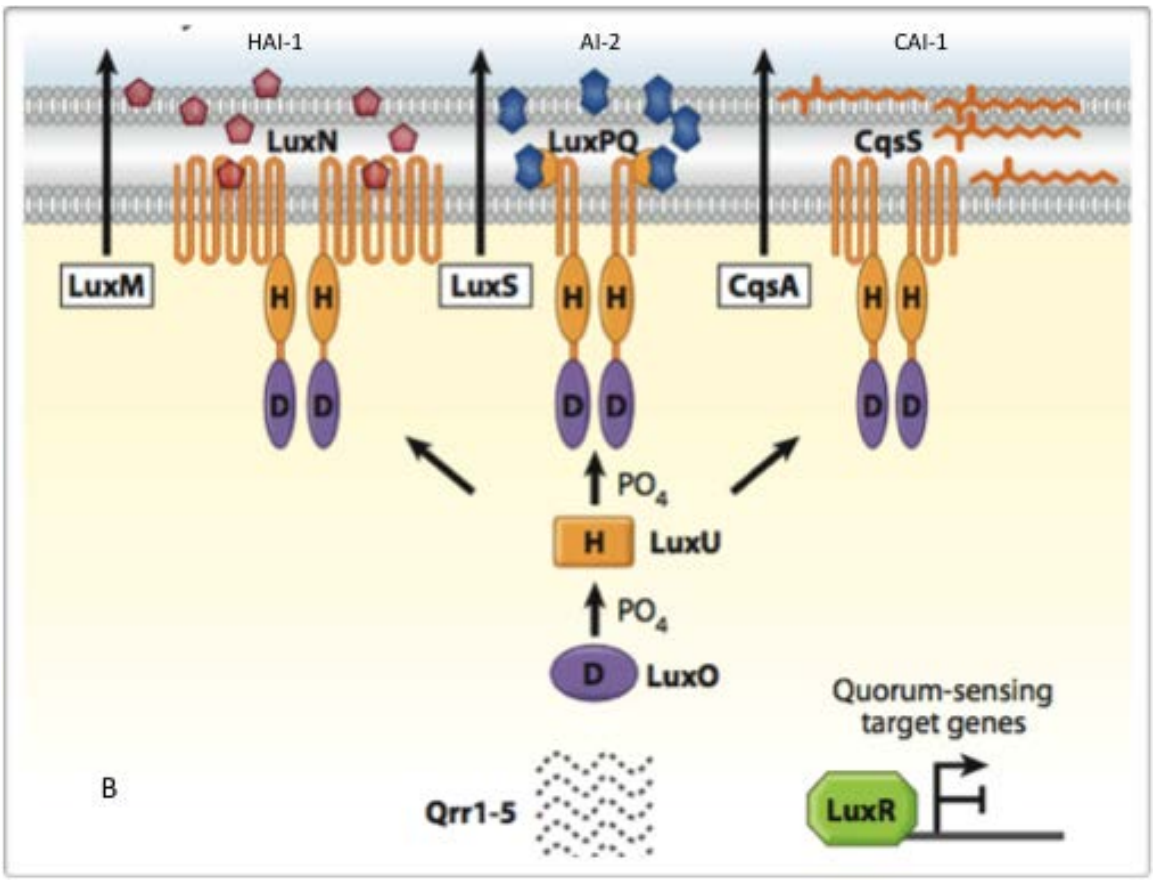


Figure 3B – Quorum Sensing at HCD. At high cell density phosphorylation is reversed, thus relieving inhibition of luxR (Adapted from Ng and Bassler, 2009)

iv. Quorum Sensing in *Vibrio cholerae*

Vibrio cholerae is a Gram-negative human pathogen and the causative agent of the life threatening disease cholera. Once ingested, the pathogen releases the cholera toxin, which causes the symptoms of the disease (Kaper *et al.*, 1995). The organism exhibits a QS mechanism similar to *V. harveyi* with a few variations. First, it does not produce HAI-1, and therefore lacks the LuxN receptor (Ng and Bassler, 2009). Also, instead of producing five sRNAs it only produces four (Lenz *et al*., 2004). The third and most important difference is that even though these two organisms possess a strikingly similar QS system, they use them to regulate different cellular processes. *Vibrio cholerae*

uses its system to repress biofilm formation and the production of virulence factors, and promotes the synthesis of proteases (Jobling and Holmes, 1997; Kovacikova and Skorupski, 2002; Hammer and Bassler, 2003). When *V. cholerae* is ingested it promotes the formation of biofilms to survive the acidic environment of the stomach. Once the AI levels increase, QS genes repress the biofilm genes, and then the bacterium moves to the intestines. Once the bacterium first colonizes the intestines the AI concentration is low, where the expression and release of the cholera toxin is promoted. Once more the AI concentration starts to increase, QS genes repress the virulence factor expression and synthesis of proteases is promoted, which aid in the escape of the organism from the host (Zhu and Mekalanos, 2003).

Since *V. harveyi* and *V. cholerae* produce the similar autoinducer CAI-1, and although it promotes completely different processes in the two organisms, their QS systems are strikingly similar, something that makes them ideal models for study. *V. harveyi* can be used a safe model to study the QS system since is harmless to humans, cost effective, and easy to grow in the laboratory. Therefore, by studying QS with non-harmful models we can gain knowledge that can be later used to treat diseases such as cholera.

v. Quorum Sensing in *Pseudomonas aeruginosa*

Another interesting system is the one found in the Gram-negative, opportunistic pathogen *P. aeruginosa*. The organism generally infects immunocompromised patients with cystic fibrosis, AIDS, severe burns, and cancer, usually resulting in the death of the patient (Mendelson *et al.,* 1994; Storey *et al.,* 1998). It is also most alarming that it is the number one bacterium found in nosocomial infections, such as pneumonia and post-

surgery wound infections (Brewer *et al*., 1996). The organism displays two QS circuits, one of them being the same as *A. fischeri*, containing homologs of LuxI and LuxR, named LasI and LasR. There is also another circuit that involves the proteins RhlI and RhlR, which work together with the LasI/LasR circuit to regulate the activation of virulence factors that causes pathogenicity (Brint and Ohman, 1995; Passador *et al*., 1993). Since the mechanism is similar to *A. fischeri* it will not be covered in detail, but it differs slightly by the discovery of a new autoinducer termed *Pseudomonas* quinolone signal (PQS) that does not code for an AHL, but instead, codes for the molecule 2-heptyl-3-hydroxy-4-quinolone (Pesci *et al*., 1999). It is suggested that this autoinducer serves as some sort of link that connects the LasI/R and RhlI/R pathways (Miller and Bassler, 2001).

The QS induced virulence factors produced by *P. aeruginosa* include an elastase encoded by *lasB*, ExotoxinA encoded by *toxA*, a phosphatase encoded by *aprA*, and a metalloprotease encoded by *lasA*. (Jones *et al*., 1993; de Kievit and Iglewski, 2000). Another characteristic of *P. aeruginosa* is the ability to respond to stressful conditions such as starvation and drugs by forming biofilms and selecting for mutants. One study found that QS mutants lacking the ability to produce elastase selected for mutations that would restore their ability to produce the exotoxin when exposed to starvation (Van Delden *et al*., 1998). The ability to mutate under disadvantageous environments proves problematic when trying to use drugs against the organism.

Besides regulating the release of several virulence factors, the QS mechanism in *P. aeruginosa* also plays a role in biofilm formation. Specifically it was shown that colonies that had a mutant *lasI* gene did not produce a mature biofilm, with the process

stopping at early stage in development (Davies *et al.*, 1998). If exogenous LasI autoinducer were added to the mutants, they would successfully form biofilms, directly linking LasI with biofilm formation. This discovery has potential therapeutic applications in treating cystic fibrosis patients infected with *P. aeruginosa* because they are usually found in biofilms inside the lungs (Singh *et al*., 2000).

Considering how pathogens rely on QS to mediate the release of toxins and for biofilm formation, research on the QS systems behind this process is of interest for the potential medical applications in treating the diseases caused by these pathogens. Insights might result in valuable approaches for the development of medicaments, especially QS blockers.

vi. luxS Role in Quorum Sensing Regulated Virulence

Quorum sensing is responsible for regulating the release of virulence factors in several pathogens either Gram-positive or Gram-negative. Specially, it seems the *luxS* gene is responsible for the regulation of virulence factors in several bacteria species. This makes sense if we take under consideration that *luxS* can be found in hundreds of bacterial genomes (Coulthurst *et al*., 2004).

One study focused on several Gram-negative *Serratia marcescens* strains, a pathogen that infects a wide range of hosts, including humans (Coulthurst *et al*., 2004). The pathogen is a problem in hospitals where it causes a number of nocosomial infections, which in most cases *S. marcescens* display antibiotic resistance (Hejazi and Falkiner, 1997). In the study, it was found that two *S. marcescens* strains where the *luxS* activity was knocked down could not produce AI-2, resulting in a moderate decrease in the production of carbapenem antibiotic, prodigiosin (a red pigment), and the exotoxin

hemolysin. It was also shown that the virulence of one of these strains was reduced when it infected the nematode *Caenorhabditis elegans* (Coulthurst *et al*., 2004).

Another example of the virulence regulation by *luxS* is found in *V. vulnificus*. This Gram-negative bacterium is also an opportunistic pathogen that causes septicemia and severe wound infections. The pathogen enters the body through the ingestion of contaminated raw seafood, or when coming in contact with open wounds. Healthy individuals are mostly safe, but infection can prove deadly to patients with compromised immunity, or individuals with liver damage, i.e. caused by heavy alcohol consumption (Tacket *et al*., 1984; Park *et al*., 1991). It has been shown that *luxS* directly regulates the production of haemolysin and metalloproteases. Mutants of *luxSw* (homolog of *luxS*) displayed a decreased production of proteases involved with pathogenicity, as well as a decrease in hemolysin production. These mutants were allowed to infect HeLa cells, and cytotoxicity levels were decreased considerably, as well as decreased mortality in mice infected with the same mutants (Kim *et al*., 2003).

Yet another example of the QS regulation of virulence factors can be found in the anaerobic Gram-positive bacterium *Clostridium perfringens.* This organism is the causative agent of the disease gas gangrene, where a great part of tissue dies (necrosis) and a foul gas is produced as a byproduct of the metabolism of the bacteria (Hatheway, 1990). *Clostridium perfrnigens* produces an array of toxins, including the alpha, theta, and kappa families, coded by the genes *plc*, *pfoA*, and *colA* (Rood, 1998). This organism is also associated with food poisoning caused by a sporulation-associated enterotoxin, encoded by the *cpe* gene (Rood, 1998). Ohtani *et al*. (2002) found a direct relationship

between the *luxS* homolog in this species and the expression and production of the toxins, suggesting that *luxS* promotes transcription of the toxin genes.

These three examples merely provide an insight into how *luxS*, a gene found in hundreds of bacterial genomes, is responsible for the regulation and activation of virulence factors in several bacterial species. Therefore, it should be a target of study to create QS blockers to specifically neutralize *luxS,* for the potential treatment of these and several other diseases.

vii. Quorum Sensing Regulated Biofilm Formation

Another area of rigorous study is the role that QS might have in the regulation of biofilm formation. This is extremely important because of the properties that bacteria acquire when they form these structures. Once in a biofilm, the organisms become resistant to most drugs and antibiotics, the production of virulence factors is increased, and there has even been evidence of horizontal gene transfer between species when in a biofilm (Antonova and Hammer, 2011).

One example where biofilm formation is regulated by QS is in *Escherichia coli*, where AI-2 serves as a transcriptional regulator by controlling motility (Gonzales-Barrios *et al*., 2006). It has been shown that *luxS* is responsible for the regulation of 404 genes in *E. coli*, including the genes responsible for the production of the Shiga toxins (Stx), and the genes responsible for motility (flagellar formation) and chemotaxis (Gonzales-Barrios *et al*., 2006). All these processes are responsible for the pathogenicity of the organism (Sperandio *et al.*, 2001). The gene *luxS* is also responsible for early biofilm formation in strain D39 of *Streptococcus pneumoniae*, the causative agent of pneumonia (Vidal *et al.*, 2011). In *P. aeruginosa*, the membrane protein PA2663 increases biofilm formation,

which in itself promotes virulence and enhances QS (Attila *et al*., 2008). Furthermore, it has been shown that *V. cholerae* while in a biofilm, increases the process of horizontal gene transfer with other bacterial species (Antonova and Hammer, 2011). Usually, horizontal gene transfer in pathogens proves to be very dangerous, since the DNA uptake by the organism could contain genes such as virulence factors, thus increasing pathogenicity and antibiotic resistance of the recipient bacteria (Antonova and Hammer, 2011).

viii. Molecules that Attenuate Quorum Sensing

Advances have been made that find QS inhibitors in several bacterial species; here a few will be mentioned. Traditional Chinese medicinal herbs have been known for their antimicrobial properties, and have been used to treat infections for a long time, thus it was presumed by current researchers that these plants might contain natural QS inhibitors (Zhu *et al*., 1998). Then it was discovered that in the medicinal plant rhubarb there was a compound that displayed QS inhibitory properties. The compound name is emodin, and in *P. aeruginosa* it increases the proteolysis of the QS protein LasR, inhibiting biofilm production. A mixture of emodin and ampicillin successfully killed *P. aeruginosa* more efficiently than the antibiotic did alone (Ding *et al*., 2011). Furthermore, natural compounds found in garlic also displayed QS inhibitory properties in *P. aeruginosa*, specifically organosulfur compounds that are thought to act as antagonists of the *lasI/R* and *rhlI/R* systems (Cady *et al.*, 2012). Interestingly, the *V. cholerae* autoinducer CAI-1 effectively inhibits biofilm formation in *P. aeruginosa* and in high concentrations it also inhibited growth. It is believed that CAI-1 disrupts the membrane interactions with the QS receptors (Ganin *et al*., 2012). Since we know that the

production of QS molecules is increased with biofilm formation, being able to use these compounds in a drug therapy can help fight *P. aeruginosa* infections (Perez *et al*., 2012).

Moreover, a class of molecules called halogenated furanones is also a group of effective QS inhibitors. These compounds are produced by the red alga *Delisea pulchra* and effectively interfered with the expression of the LuxR protein in *E. coli* containing the *lux* operon, without affecting the growth of the bacteria (Manefield *et al*., 1999; Zhang *et al.*, 2011). Honey is another substance that has been shown to slow biofilm formation, with low concentrations attenuating as much as 98% of biofilm formation in *E. coli* (Lee *et al.*, 2011). This discovery makes honey a great tool for combating infections at home, since most households have it.

e. Analytical Techniques to Detect Extracellular Molecules

There are several methods utilized to characterize autoinducers and other extracellular molecules. First it is necessary to extract the molecules from bacterial culture supernatants prepared by centrifuging bacterial cultures. One challenge that arises from extraction is that liquid cultures contain bacterial extracellular molecules but also media components. Therefore, one solution is to minimize the amount of nutrients added to the media in order to reduce any potential interference from media components. The most common used methods of extraction of AI molecules are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). In LLE an organic solvent such as dichloromethane, hexane, ethyl acetate, or ethyl esters are used for the extraction, then the solvent is dried leaving only the extracted molecules, which are usually re-dissolved in methanol or in acetonitrile-water (McClean *et al.*, 1997; Chambers *et al*., 2005; Pearson *et al*., 1994; Pearson *et al*., 1995). In SPE, the sample is first extracted with an

organic solvent such as methanol or ethyl acetate. The sample is dried and re-dissolved in hexane-ethyl acetate, and then it is run through a column with a solid phase consisting of silica, and basic, neutral or acid aluminum. The column is first washed with a buffer and then several fractions are eluted in sequence, and samples to be further analyzed are re-dissolved (Wang *et al*., 2011).

i) AHL Bioreporters

One way to detect the presence of extracellular molecules is the use of cell line bioreporters. There are three major types of bioreporters, which utilize a phenotypic response to detect AHLs, such as violacin production, β-galactosidase activity, and bioluminescence. *Chromobacterium violaceum* (strain CV026) is induced in the presence of AHLs of side chain lengths of C4-C12 to produce the purple pigment violaceum, providing a visual determination for AHLs (Cataldi *et al*., 2013). Another type of bioreporter is the plasmid pSB401 that contains the *luxCDABE* operon, which is usually harbored by *E. coli* (Winson *et al*., 1998). The bioreporter responds mostly to C_6-3-oxo-HSL, but also responds to C_6-HLS, C_8-3-oxo-HSL, and C_8-HSL. When those HSLs are present, bioluminescence is induced. Lastly, *Agrobacterium tumefaciens* bioreporters contain a plasmid with a *lacZ* gene fused to *traG*, which needs the transcription factor TraR that is activated in the presence of AHLs. Beta-galactosidase activity is then promoted, which can be easily detected (Cataldi *et al*., 2013). Acyl homoserine lactone bioassays are restricted to the detection range of the reporter strain used, and for a complete coverage of all AHL, multiple strains are required, as well as the corresponding set of standards for each strain (Cha *et al.*, 1998).

ii) Mass Spectrometry

High performance liquid chromatography (HPLC) separation is used before mass spectrometry. For separation with HPLC, the sample first extracted from LLE or SPE is re-dissolved in methanol or acetonitrile (ACN), run through a C_8 or C_{18} reverse-phase column, and eluted with a 70% ACN isocratic solution in water or water-methanol (Lewenza and Sokol, 2001; Morin *et al*., 2003). The separation of molecules using HPLC is cost effective and easy to perform, and with high levels of selectivity

Techniques such as biosensors cannot determine the structure and concentration of HSLs with precision. Another set of analytical methods is employed to this end, including spectroscopy techniques such as electrospray mass spectrometry (ESI-MS) and electrospray tandem-mass spectrometry (ESI-MS-MS), nuclear magnetic resonance (NMR), and infrared spectrometry (IR; Zhu *et al.*, 1998). Mass spectrometry detects the mass to charge ratio of ionized molecules, and using this information the mass of a compound can be determined. One advantage of ESI-MS is that it requires only small concentrations of the sample, usually detecting picomolar concentrations (Makemson *et al.*, 2006). Usually mass spectrometry is coupled with reverse-phase HPLC, as described above, for the determination of AHL, although mass spectrometry can also be coupled with gas chromatography (GC; Teplitski *et al.*, 2003; Cataldi *et al.*, 2004).

Another available technique used for the characterization of extracellular molecules is high-resolution mass spectrometry (HRMS). The HRMS technique coupled with high-resolution separation techniques provides a fast and reliable method for the characterization of extracellular molecules in bacterial culture supernatants. Two MS techniques are specially popular and reliable in the determination of extracellular

molecules in Gram-negative bacteria; these are Fourier-transform ion cyclotron MS (FTICR) and quadrupole time-of-flight (qTOF) MS. The utilization of ultra-high performance liquid chromatography with HRMS achieves a high degree of mass accuracy, and the molecular formulas of extracted molecules can be determined with accuracy and reliability (Guan *et al.*, 1996). Specifically, the combination of high performance liquid chromatography with of TOF instruments provides great sensitivity and accuracy in the measurements, as well as versatility, since it can be utilized in simple mass as well as tandem mass analyses (Morris *et al.*, 1996; Chernushevich *et al.*, 2001).

The robust technique FTICR-MS can be used to characterize molecules with known or unknown structure in media extracts. If FTICR is coupled with nano-electrospray ionization and HPLC, there is a notable reduction of noise, sample size, as well as a higher degree of sensitivity. The technique is especially useful in the identification of molecules that have low abundance in samples with other high abundant species (Cataldi *et al.*, 2013). More generally, the utilization of electrospray ionization (ESI) is helpful in the determination of chemical structure of molecules found in media extracts. Coupling the ESI ion source to either FTICR-MS or qTOF-MS and ultra HPLC is a powerful tool in the determination of chemical structure of unknown molecules.

In one study, the utilization of LC-MS-MS allowed for the identification of AHLs in the marine bacterium *V. vulnificus* in a complex media without any need to purify the sample (Morin *et al.*, 2003). Three criteria were utilized in the characterization of the unknown AHLs: MS-MS fragmentation patterns, the peak intensity for each ion, and retention time in the separation analysis.

The HPLC-MS-MS method has also been used to identify the presence of AHLs in several members of the Vibrionaceae family, with interesting results. According to this study, species such as *A. fischeri* produce up to eight different AHLs something that has never been reported before (Purohit *et al.*, 2013).

B. MATERIALS AND METHODS

a. Bacterial Cultures and Liquid-Liquid Extraction

The MAV strain of *V. harveyi* was a gift to Dr. Makemson from a colleague. The MAV strain was isolated Woods Hole in the 1960s, and corresponds to ATCC number 14126 from the American Type Culture Collection (www.atcc.org). The cultures were maintained on agar slants in screw-capped tubes during the length of this project. HAI-1 was purchased from Sigma-Aldrich as a control for the MS detection of HAI-1.

The minimal media used contained 0.2% ammonium chloride as the nitrogen source, 0.05% glycerol phosphate as phosphorous source, growth factors, 0.3% glycerol as the carbon and energy source, and 75% Artificial Seawater (ASW). The ASW composition consisted of 0.5M NaCl, 25mM $MgSO_4$, 25mM $MgCl_2$, and 10mM KCl (**Table 1**). The pH of the media was adjusted to 7.5 using 0.1 M NaOH. The media was utilized specifically to enhance the signal of the mass spectrometric analysis; since it only contains one organic molecule and the rest are inorganic salts there would be minimum interference from the media on the mass spectrometry analysis. The *V. harveyi* cultures were inoculated and grown at 25 °C with aeration using a rotary shaker at 200 rpm. Growth was measured as optical density using a Spectronic 20 spectrophotometer (Milton Roy Company, Ivyland, PA) at 660 nm, and luminescence measured using a calibrated photomultiplier photometer.

The starter culture was grown overnight using glycerol marine media to hasten the growth. The next day, the cells were centrifuged at 6000 rpm and resuspended in fresh minimal media. The new culture was grown for a period of 24 hours, and 2.5 ml aliquots removed every four hours to measure OD_{660nm}, luminescence, and to extract secondary

products. Aliquots were centrifuged at 6000 rpm for 10 minutes. The supernatant was filtered through 0.2 μm pore filters to remove bacterial cells. The cell free culture supernatant was then extracted 3 times with acidified ethyl acetate (99.5% ethyl acetate and 0.5% acetic acid) using a 1:1 volume ratio. The top layer containing the organic phase with the extracellular molecules was collected and the aqueous phase containing the inorganic salts from the media was discarded. The ethyl acetate extract was dried with a flow of N_2 gas, and the dried extracellular media components remaining were re-dissolved in ACN-water (20% v/v), ready for injection into the mass spectrometer. A media extract containing no cultures was extracted using the same procedure described above serving as a media control for the MS analysis.

Table 1 - Media for Growth of *Vibrio harveyi*

Nutrient	**Glycerol Marine Media**	**Minimal Media**
Carbon Source	Glycerol 0.3%	Glycerol 0.3%
Nitrogen Source	Peptone 0.5%	NH_4Cl 0.2%
Phosphorous	Glycerol phosphate 0.2%	Glycerol phosphate 0.2%
Iron	Yeast Extract 0.1%	Ferric Ammonium Sulfate, 1 μM
Growth Factors	Yeast Extract 0.1%	0.0001% Yeast Extract.
Buffer	None	$HEPES_1$

$HEPES_1$ = pH buffer added at 10 mM
ASW_2 = Artificial Sea Water (0.5M NaCl, 25mM $MgSO_4$, 25mM $MgCl_2$, 10mM KCl)

b. HPLC-MS/MS and Data Analysis

The cell free extracts were injected into a 1200 Infinity Series HPLC (Agilent Technologies, CA), where the molecules in the sample are pressure pumped with a solvent (mobile phase) through a solid material (stationary phase). The molecules then interact with and adsorb onto the solid material and elute from the column at different rates. For this specific experiment, the separation column used was a reverse phase ZORBAX Eclipse Plus C_{18} Rapid Resolution High Definition (RRHD; Agilent Technologies, CA), with octadecyl carbon chain (C_{18})-bonded silica as stationary phase and acetonitrile/water as the mobile phase. The column dimensions were 2.1 x 100 mm, and 1.8 μm particle size. The column allowed for the separation of nonpolar molecules from pH 2-9. The polar characteristics of the mobile phase cause hydrophobic molecules to adsorb strongly to the stationary phase. Polar compounds will have less affinity to the bonded silica and will elute first from the column.

The HPLC was equipped with a 6530 Accurate Mass Q-TOF LC/MS (Agilent Technologies, CA), with an Agilent Jetstream (AJS) ESI ion source. The acquisition method for the HPLC was set to the following parameters: the draw and ejection speed were set to 200.0 μL/min the, sample volume injected was 5 μl, the column temperature was set at 30°C, and the flow rate was 0.400 mL/min. The low-pressure limit was set to 0 bar and the high-pressure limit to 900 bar. Solvent A contained water, 5 mM ammonium formate and 0.1% formic acid at pH 4, and solvent B was ACN. At minute 1 the solvent composition was 95% solvent A and 5% solvent B, and at minute 10 the solvent gradient had changed to 5% solvent A and 95% solvent B.

The MS was run in two modes: full scan ESI-MS and targeted ESI-MS/MS both in positive mode. The full scan detected all ions ranging from m/z 100-1000 at a scan rate of 10 spectra/sec. The targeted ESI-MS/MS detected ions ranging from m/z 50-1700 with an ESI-MS scan rate of 10 spectra/sec and ESI-MS/MS scan rate of 3 spectra/sec. The optimized parameters for all MS runs were the following: the drying gas temperature was 300°C, with a gas flow of 7 L/min, and the nebulizer gas pressure was 30 psig. The sheath gas temperature and flow were 375°C and 11 L/min respectively. The capillary voltage was 4000V, nozzle voltage was 500V, fragmentor voltage 135V, skimmer voltage at 65V, and octopole RF peak of 750V.

The data were analyzed with Mass-Hunter Workstation software (Agilent Technologies, CA). The program allows for the control of the MS instruments and specific tools for the quantitative and qualitative analysis of the MS data acquired. For the full scan MS, the raw data were integrated and the peak spectra extracted for each ion. The targeted MS/MS data were analyzed using the function ‘Find compounds by targeted MS/MS’ which provided extracted chromatograms for each ion as well as the peak spectra. From the resulting data, the compound formulas were generated.

C. RESULTS

a. *Vibrio harveyi* Growth Curve

A standard growth and luminescence curve were performed on *V. harveyi* MAV. Two things can be determined from the growth curve: growth rate and the generation time (the time it takes for a population to double in size). By measuring growth and luminescence, it can be determined when autoinduction takes place, which is the point when the population density surpasses a threshold that activates QS genes. Because luminescence in *V. harveyi* is induced by QS, bioluminescence increases at a rate faster than growth (**Figure 4**) when this event takes place. The growth rate of *V. harveyi* is calculated by using a first order rate equation. In minimal media active growth occurred from 4 hrs to 20 hrs, at a rate of 0.138 hrs^{-1}. The generation time was 5.03 hrs. Quorum sensing mediated induction of bioluminescence started at 13 hours, when the OD was 0.100.

Bioluminescence was measured using a calibrated photometer. One light unit (LU) measured by the photometer can be converted to quanta/sec, the total amount of photons being emitted by the cell culture per unit time. The conversion factors for the calibrated photometer were different depending on the voltage used (1000V: 1 LU = $6.02x10^6$ quanta/sec, 500V: 1 LU = $1.08x10^9$ quanta/sec)

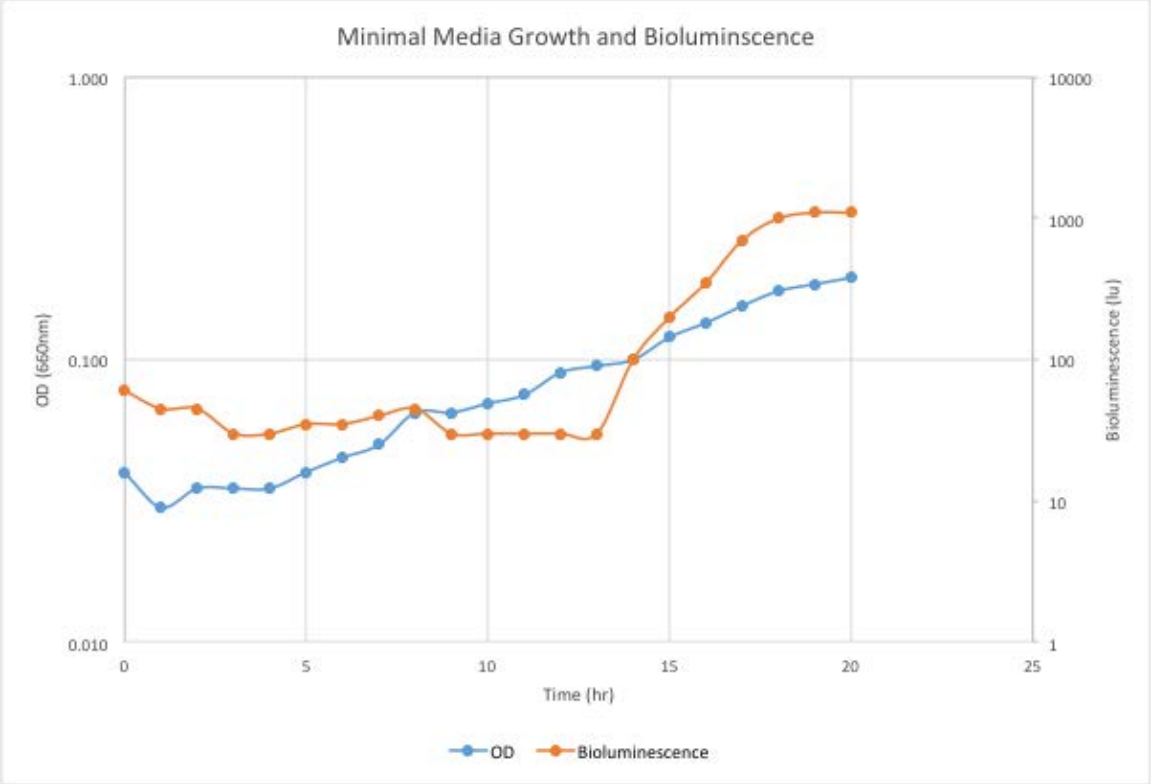


Figure 4 – Growth and Luminescence curve of *V. harveyi* in Minimal Glycerol Marine Broth. Autoinduction of the *lux* operon can be seen at 13 hours by a sudden increase in bioluminescence. Growth rate was 0.138 hr^{-1} and generation time was 5.03 hrs

b. Tandem Mass Spectrometry Analysis

The cell free extracts were first run through full scan ESI-MS to determine the ions present in the media at different times. Each experiment consisted of nine samples, including a media control, a HAI-1 control, and seven media extracts (0hr, 4hrs, 8hrs, 12hrs, 16hrs, 20hrs, 24hrs). The media control was sterile minimal media with no extracellular molecules and served to detect any peaks already present in the media and not produced by *V. harveyi*. The HAI-1 control contained HAI-1 dissolved and served as a control for the detection of HAI-1 from the extracted media. Several of these experiments were performed in order to build an ion profile where focus was given to ions that would show in several experiments. Ions that were present in the media extract

control or difficult to replicate were not further analyzed because the primary aim of these experiments was to identification of extracellular molecules that could be replicated in subsequent experiments. After an ion profile was built, it was further analyzed by re-running the samples using a Targeted ESI-MS/MS method, where the masses previously detected were the only ones being selected for scanning. **Table 2** (page 32) summarizes the finding of these experiments, showing the ions further analyzed in Targeted ESI-MS/MS mode. All molecules detected were single charged. Parameters such as retention time, product ions, base peak, and peak area provide information that can help identify of the extracellular molecules. The list of molecules is ordered on the basis of increasing retention time. Ions with short retention times are more polar than the ions with long retention times, therefore the molecules with a high RT are hydrophobic and the molecules with low RT are hydrophilic in nature. Sometimes the molecules are ionized with adducts of different masses. These adducts are made during the electrospray ion formation and are not from the medium extracted sample. The most common adducts are ammonium ($[M+17]^+$), water ($[M+18]^+$), sodium ($[M+23]^+$), potassium ($[M+39]^+$), and acetonitrile ($[M+41]^+$). In some cases the mass-to charge ions (m/z) will have adducts represented by the difference in mass between the m/z and the actual mass of the molecule. The proposed formulas were determined using the Mass-Hunter molecular formula algorithm, where only C, O, H, N, S, elements were selected when generating the formulas. The score **(Table 2)** depicts the confidence of the molecular formula selected, with 100 being the highest score and with scores of 70 less suggesting that the proposed formula are less probable.

The analysis of the peak area for each ion shows the intensity of the extracellular molecules at a specific growth curve time points, which in turn is related to the concentration of the extracellular molecule in the sample (**Figure 5A**). The peak corresponding to HAI-1 (m/z 188) was detected in all samples with increasing intensity over time, and the highest peak area was recorded at 24 hours, showing the gradual accumulation of HAI-1. **Figure 5B** shows that m/z 227.1734 had the highest intensity of all the ions, showing a peak area 8 times higher than the rest of the ions. Also, the data show that the intensity of m/z 227.1734 was constant during the first 16 hours of growth; afterwards there was almost a doubling in intensity for the remainder of the growth curve, indicating QS is affecting the expression of the extracellular molecule. On the other hand, m/z 267.1718 could not be detected at the 0-hour sample, but then showed a 5x increase in peak area from 8-16 hours, and a decrease in intensity afterwards **(Figure 5A)**. The decrease in concentration after QS activation could mean that the bacterial cells might have imported the molecule, or perhaps that it ceased to be produced and began to naturally break down in the media. Another molecule (m/z 354.2825) decreased from 0-12 hours to a point where the signal could not be detected by the mass spectrometer, then started to increase in intensity to match the same peak area that it showed at the beginning of the experiment, which could mean that it only starts being synthesized after a specific population density has been achieved.

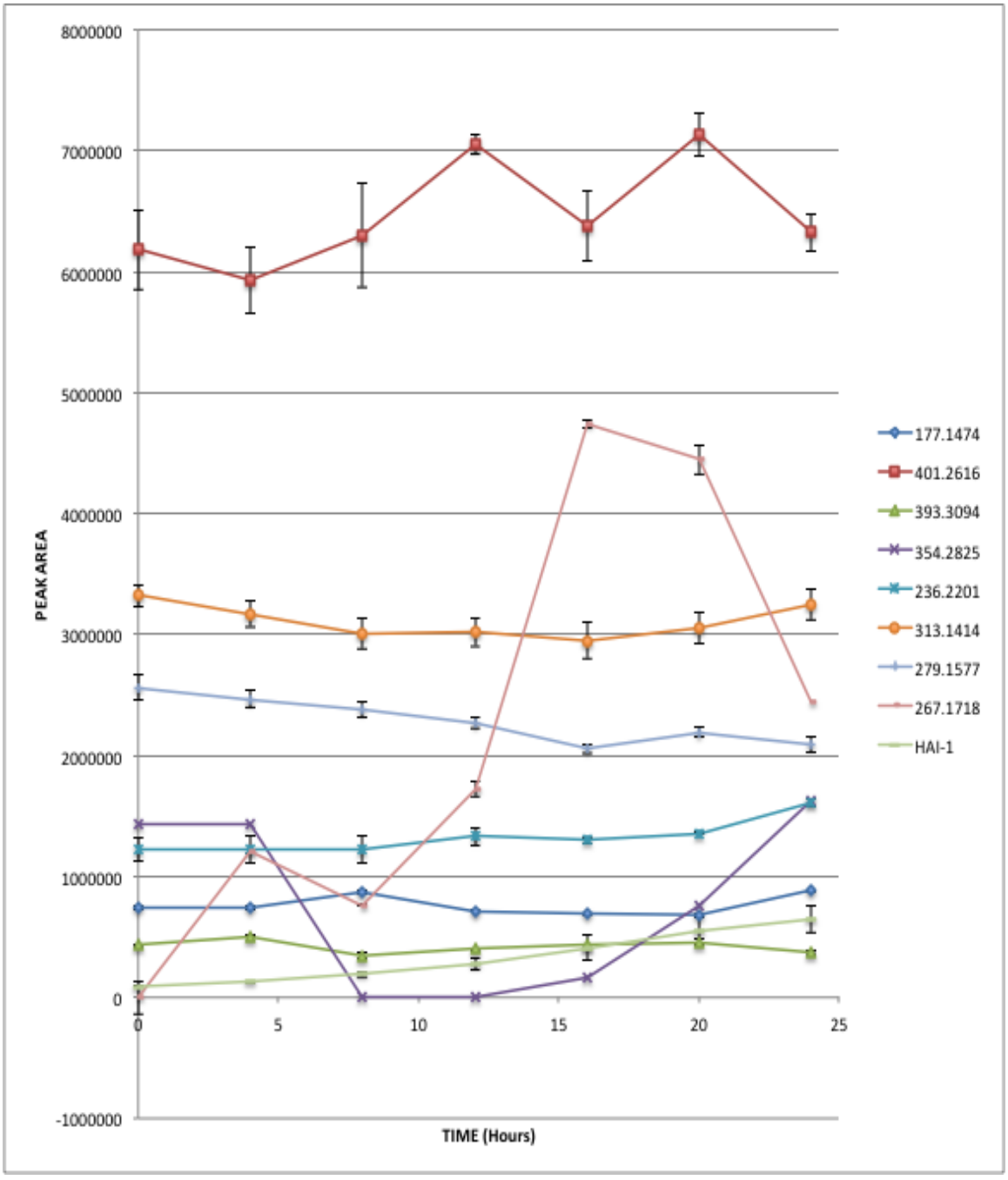


Figure 5A – Peak area over time for extracellular molecules. The peak area was calculated from the corresponding TIC+ spectra.

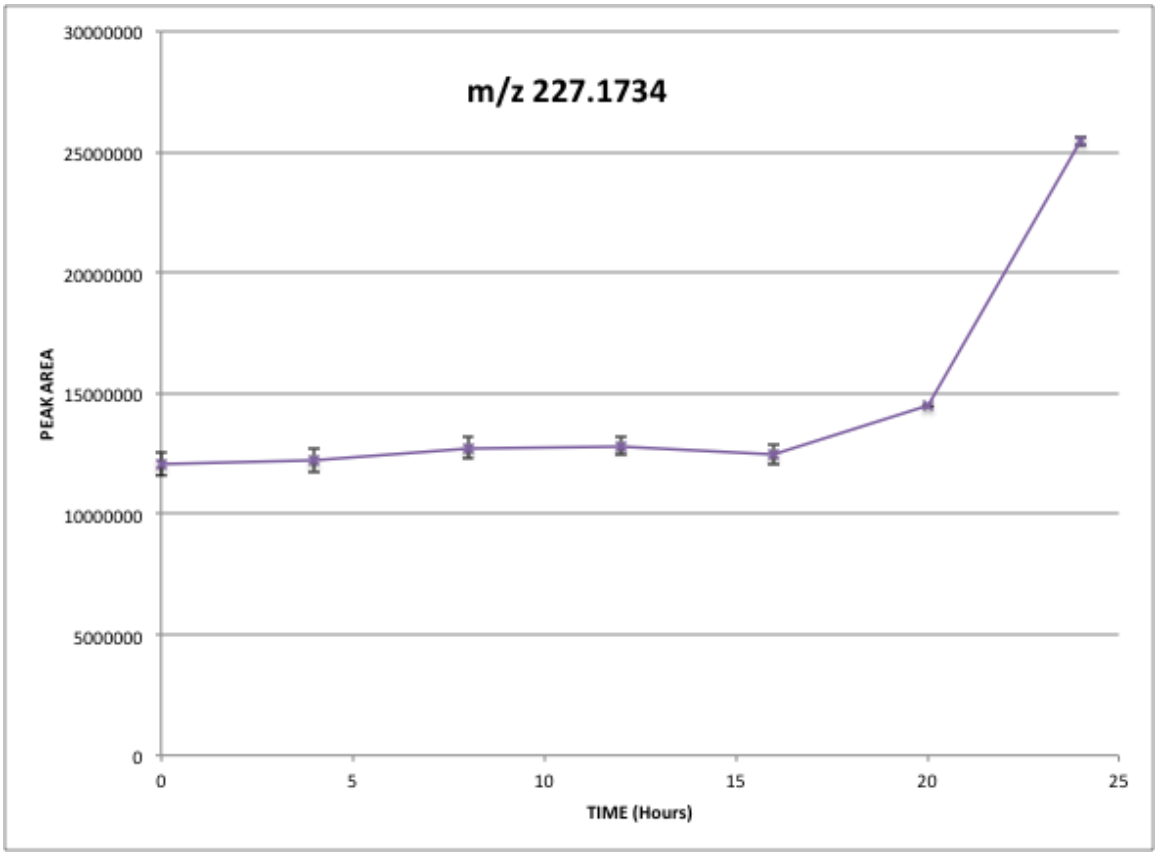


Figure 5B – Peak area over time for m/z 227.1734

The extraction of the product ion chromatogram shows a graphical representation of the distribution of the product ions from the second stage of mass analysis. **Figure 6** shows the total ion chromatogram (TIC+) scans and the +ESI product ion chromatogram for HAI-1 and selected molecules. The secondary ion analysis can help piece together the fragment ions to produce a molecular formula, along with the utilization of other analytical methods such as NMR and IR spectrometry.

The TIC+ scan shows the peak intensity over the retention time, and from here the peak area is calculated. The +ESI chromatogram shows the product ions for each m/z peak. Detection of HAI-1 **(Figure 6A)** at m/z 188.0906 was achieved in all samples, with

the presence of a fragment ion of mass 102.0546, a signature peak indicative of the presence of an AHL, which represents the unacylated lactone ring (Frommberger *et al*., 2004). **Figure 6B** shows a chromatogram for the HAI-1 control displaying the same signature peak, and thus confirming the presence of HAI-1 in the extracted media. **Figure 6C** shows the chromatogram for m/z 227.1740 that has a base peak of mass 100.1111 and an ion fragmentation pattern of 3 more daughter ions. Of special interest is the peak m/z 401.2628, which contains one fragment with mass 210.1472 which could represent the sodium adduct of HAI-1 ([HAI-1-Na^+]$^+$; **Figure 6D**). It is unknown what makes up the rest of the molecular mass of the ion. Furthermore, m/z 295.2001 contains one fragment ion with mass 171.1359 which could represent C_4-HLS (**Figure 6E**). In both cases m/z 102 was not detected, which could not be an HSL. Lastly, Figure 6F shows the chromatogram for m/z 267.1730, a molecule that it is thought to be mostly hydrophobic (high retention time). The TIC for the rest of the extracellular molecules can be found in the appendix.

Table 2 – List of Extractable Extracellular Molecules Produced by *Vibrio harveyi* Analyzed Using HPLC-ESI-MS-MS.

Compound	m/z	Mass	Base Peak	Product Ions	RT (min)	Peak Area	Proposed Formula	Score	Samples found (hours)
1	227.1734	204.1842	100.1111	83.0852, 100.1111, 117.1387, 209.1635	3.203	12889041	C10 H24 N2 O2	97.13	0, 4, 8, 12, 16, 20, 24
2	453.3411[a]	430.3519	453.3417	227.1734	4.163	43702935	C22 H46 N4 O4	97.93	0, 12, 16, 20, 24
3	177.1474	176.1397	59.0495	59.0495, 69.0693, 89.0605, 107.0697, 133.1003, 159.0405, 179.1415	5.218	761265	C7 H18 N3 O2	75.68	0, 4, 8, 12, 16, 20, 24
4	401.2616	378.2725	210.1473[b]	83.0849, 100.1117, 182.1529, 210.1473, 242.1737, 259.2007, 337.2103, 369.2367	5.475	6712122	C18 H38 N2 O6	97.87	0, 4, 8, 12, 16, 20, 24
5	393.3094	392.3021	375.2986	266.2464, 375.2986	7.04	421559	C21 H38 N5 O2	98.37	0, 4, 8, 12, 16, 20, 24
6	295.2001	271.2358	171.1363[c]	57.0698, 73.0281, 89.0585, 107.0878, 171.1363	7.333	875310	C11 H27 N8	81.16	4, 12, 16, 20, 24
7	354.2825	353.2753	103.0752	59.0465, 87.0440, 103.0752, 119.0701, 163.1324	8.695	1463647	C13 H35 N7 O4	94.13	0, 4, 16, 20, 24
8	236.2201	235.2121	89.0597	118.1222, 149.0231, 177.0547, 199.1685, 219.1938, 255.1940[d]	8.883	1270646	C8 H25 N7 O	92.41	0, 4, 8, 12, 16, 20, 24
9	267.1718	244.1825	98.9842	80.9741, 98.9842, 109.0991	9.202	1126405	C17 H24 O	84.2	4, 8, 12, 16, 20, 24
10	313.1414	312.1345	91.0539	91.0539, 149.0227, 261.2223, 279.2308	9.995	3177220	C17 H18 N3 O3	96.52	0, 4, 8, 12, 16, 20, 24
11	279.1577	278.1507	149.0221	121.0248, 149.0221	10.177	2644343	C14 H20 N3 O3	97.23	0, 4, 8, 12, 16, 20, 24

[a] m/z 453.3411 is the dimer adduct of m/z 227.1734

[b] Base peak 210.1473 might correspond to [HAI-Na^+] adduct

[c] Base peak 171.1363 might correspond the AI C4-HLS

[d] Product ion 255.2940 might correspond to [(Ea-C8-CAI)-$C_2H_3N^+$] adduct

Figure 6 – Chromatograms and TIC Spectra of Selected Extracellular Molecules. All chromatograms except the HAI-1 control correspond to 16-hour samples

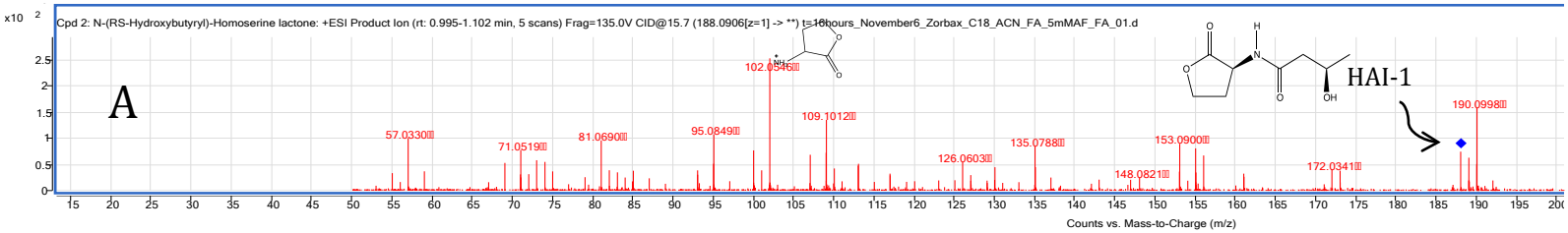


Figure 6A – ESI-MS/MS Product Ion chromatogram for experimental HAI-1. Fragment peak at 102.0546 represent the lactone ring.

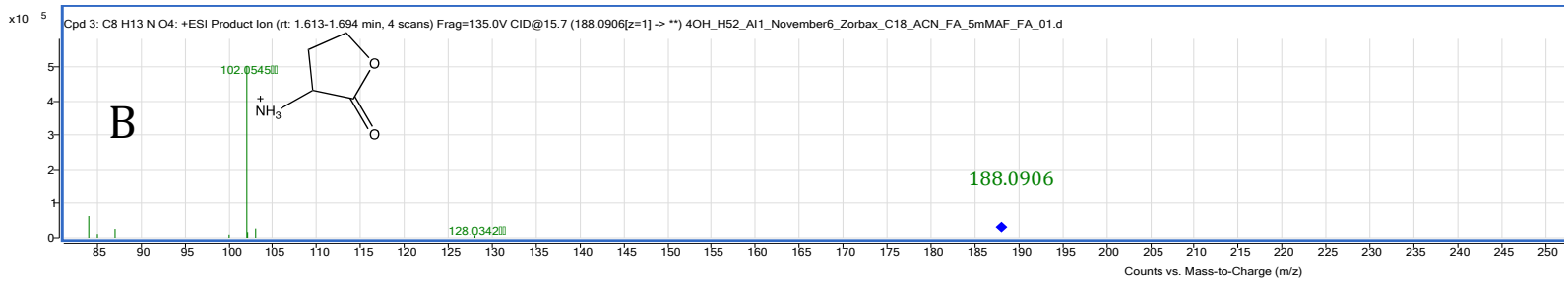


Figure 6B – ESI-MS/MS Product Ion chromatogram of HAI-1 control

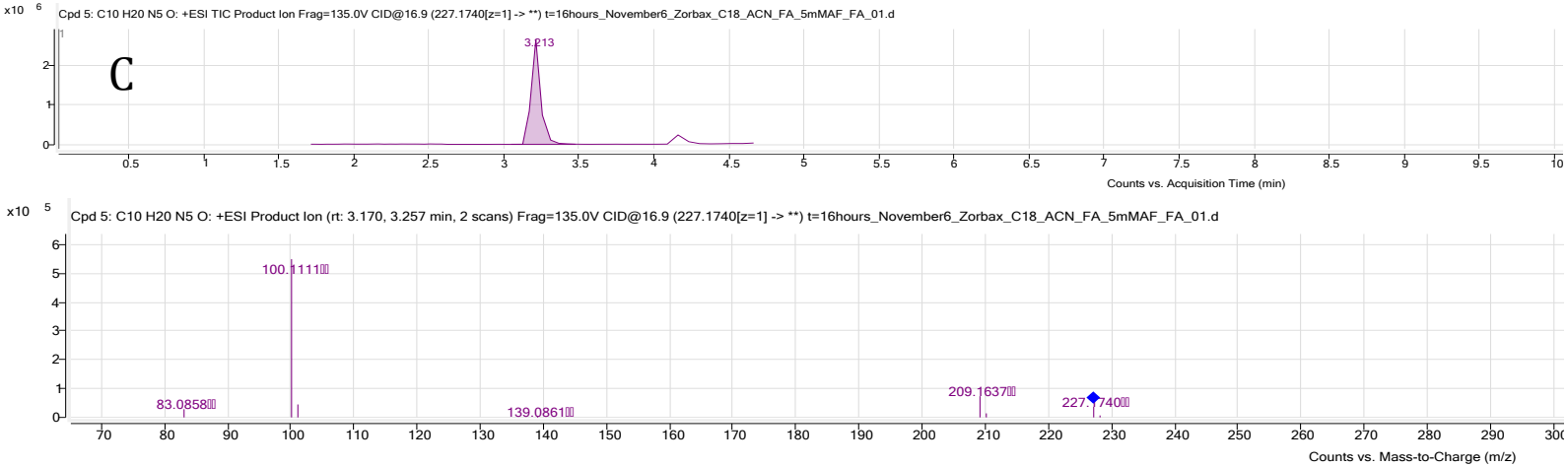


Figure 6C – ESI-MS/MS Product Ion chromatogram for m/s 227.1740. This peak had the highest intensity of all the peaks studied

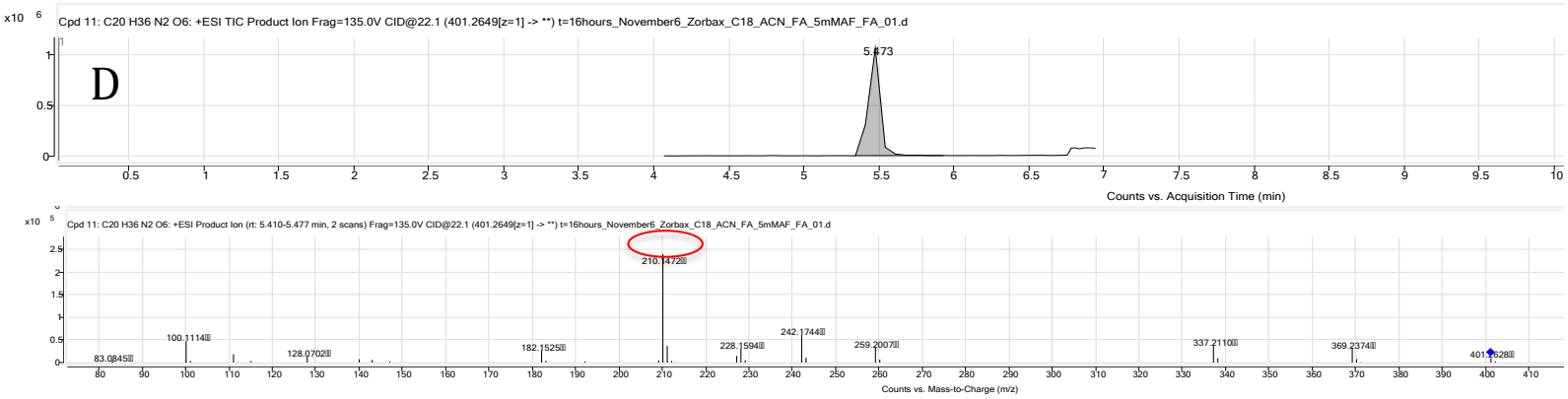


Figure 6D – ESI-MS/MS Product Ion chromatogram for m/z 401.2628. Base ion of mass 210.1472 circled in red might represent [HAI-1-Na^+] adduct.

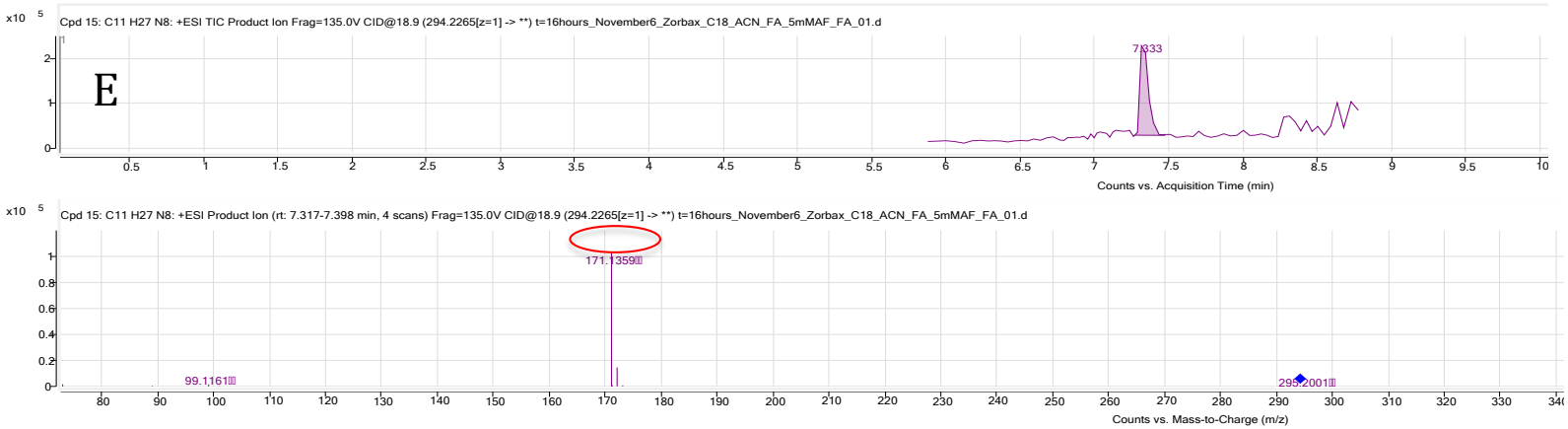


Figure 6E – ESI-MS/MS Product Ion chromatogram for m/z 295.2001. Base peak of mass 171.1359 might represent C_4-HLS

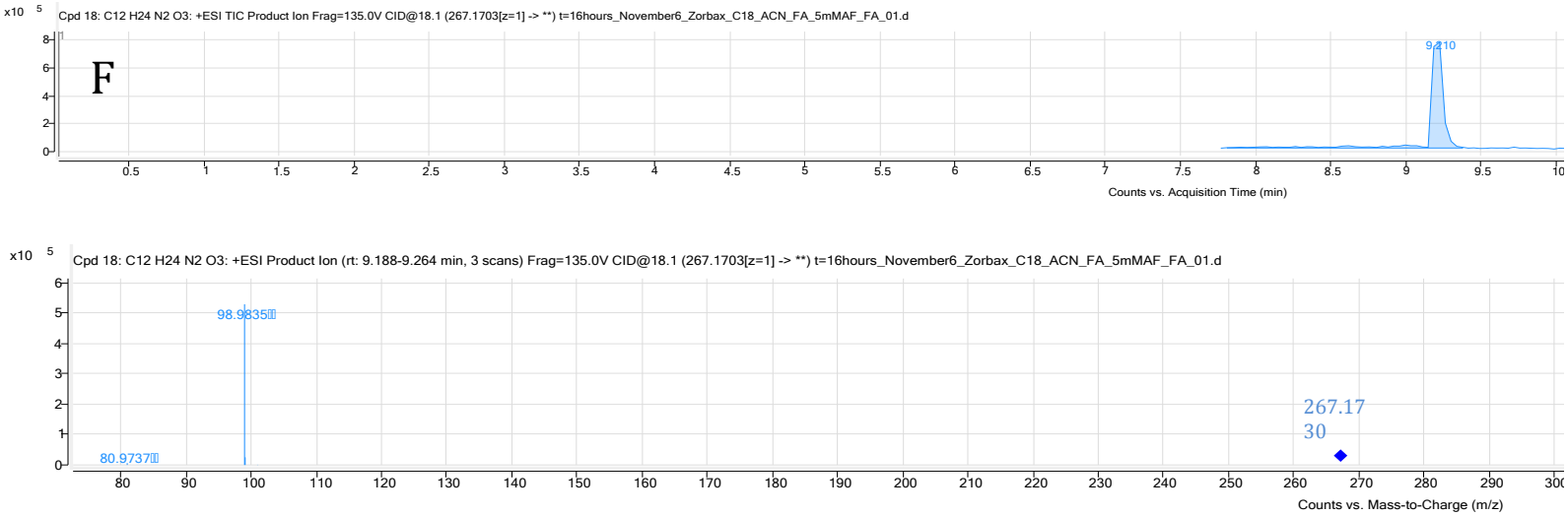
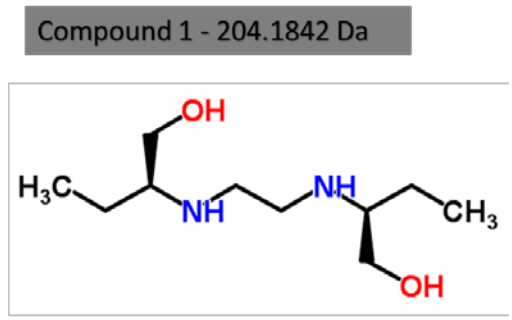


Figure 6F – ESI-MS/MS Product Ion chromatogram for m/z 267.1730

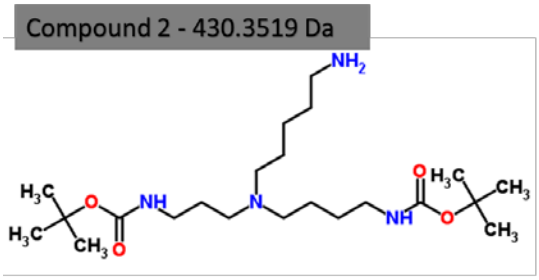
c. Generation of Chemical Structures

The chemical structure generation was done using the ChemSpider Database (**Figure 7**; Royal Society of Chemistry, 2015). The parameters used to generate the chemical structures were the molecular formula and molecular mass generated from the MS data analysis. Each generated structure contains the ChemSpider database ID where more properties of these compounds can be found. Anyone of the structures generated might correspond to the actual definitive structure of the secondary metabolites but at this point it is not known which. In some cases such as Compound 1, 9, 10, and 11 there were hundreds of structures generated and they were not added, only the structures corresponding to molecules of known function for Compound 1 and 9 are shown. From Compound 1, one of the structures generated corresponds to Ethambutol, an antibacterial agent utilized to treat tuberculosis (Thomas *et al.,* 1961). Two of the possible structures for Compound 9 are Phantolide and Falcarinol. Phantolide is a musk fragrance used in creams and other products, and Falcarinol is a natural occurring substance found mainly in carrots (Crosby and Aharonson, 1967; Dong *et al*., 2014). Compound 6, 7, and 8 did not generate any results.

Figure 7 - Generated Chemical Structures for the Extracellular Metabolites of *V. harveyi* using Chemspider Database. Each structure contains the database ID.



13433 (+)-S,S-Ethambutol



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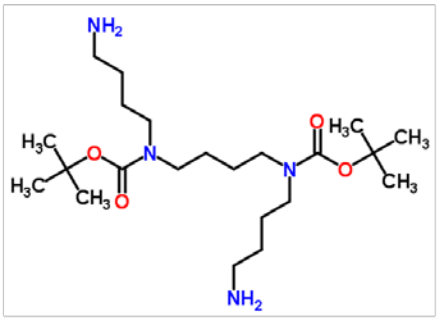

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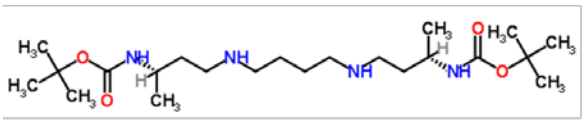

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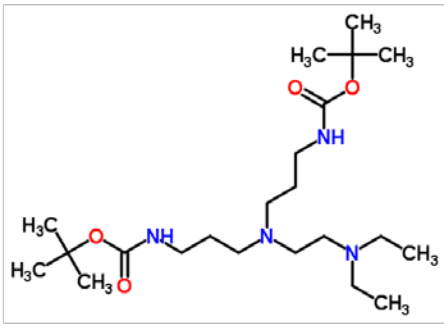

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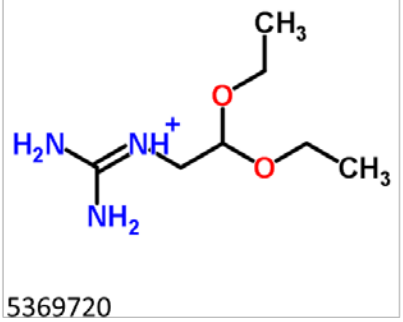
Compound 3 - 176.1397 Da



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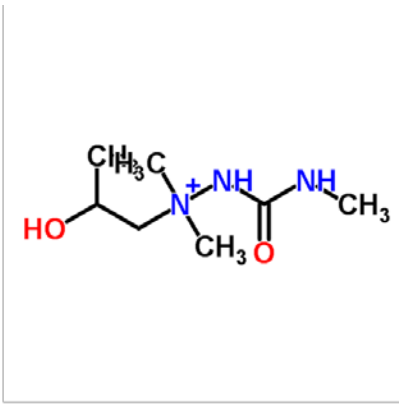
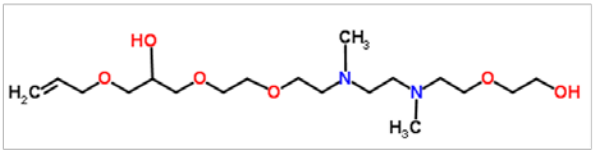


8256424

Compound 4 – 378.2725 Da



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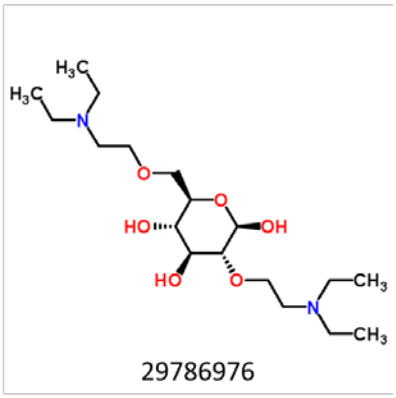

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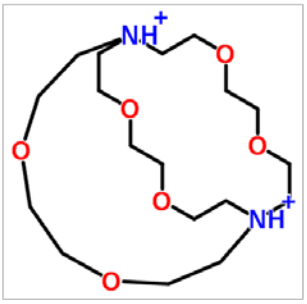

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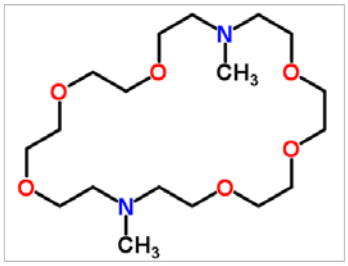
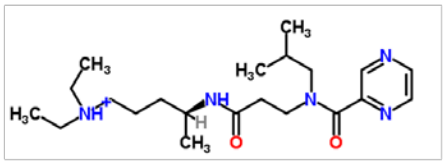


3619863

Compound 5 – 392.3021 Da



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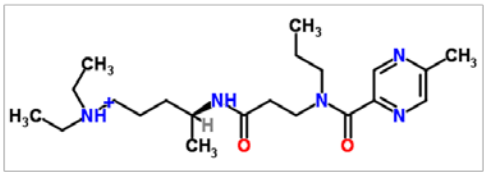
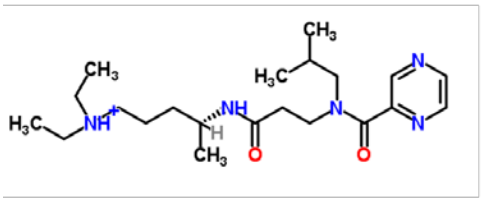


5625094



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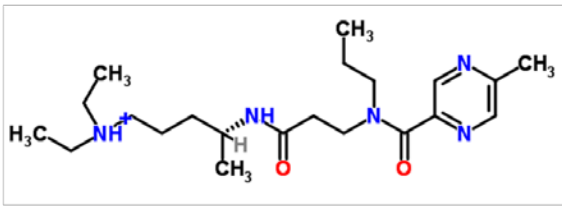
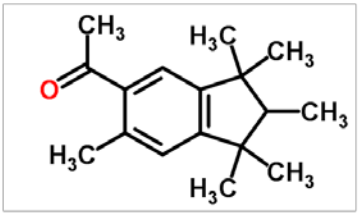
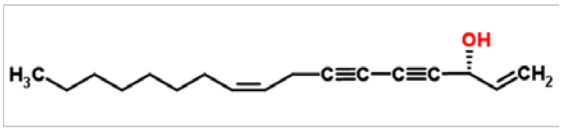


5625100

Compound 9 – 244.1825 Da



42929 Phantolide



4444589 (R)-(-)-Falcarinol

D. DISCUSSION

Analysis of the growth curve of *V. harveyi* was important in order to find a relationship between the extracellular molecules detected and their role in quorum sensing. From **Figure 4** it appears that the activation of QS genes takes place at about 13 hours, therefore the samples at 16hrs, 20hrs, and 24hrs, reflect this change in gene expression, and thus molecules displaying a change in intensity during these times are possibly be related to QS. From the 11 molecules characterized in this experiment, 8 of them maintained constant intensity suggesting that they are either produced at increasingly lower rates or they are a constant contaminant of the medium or reagent. Because these molecules are not present in the medium blank, they are most likely produced by the bacteria. Therefore, we can conclude that it is improbable for these molecules to be related to quorum sensing. The remaining 3 molecules exhibited a change in intensity corresponding with QS activated gene expression. Mass-to-charge 227.1740 exhibited an intensity curve similar to the *V. harveyi* growth curve (**Figure 4** and **5B)**, maintaining a constant concentration before the activation of QS and almost doubling its concentration during QS activation. This change in concentration shows that m/z 227.1740 is a QS regulated extracellular metabolite. The concentration of this molecule in the extracted samples was the highest, indicating that it is synthesized at a higher rate inside the cell or it is more stable than the rest of the molecules detected. On the other hand, the concentration of m/z 267.1703 increased from 0-16 hours and then dropped considerably after QS activation, suggesting that the molecule might be converted to something else or degraded.

Lastly, m/z 354.2825 was not detectable from 4-16 hours, suggesting a suppression of this metabolite once bacteria were inoculated into fresh media at 0-hours (change in population density). It is only after the population density increased and QS is activated that the molecule was detected again. Thus, the data show that these three extracellular molecules are affected by the activation of QS in *V. harveyi*.

Of the three known AIs produced by *V. harveyi*, only HAI-1 was detected in the extracted media. There was a gradual increase in the intensity of HAI-1 throughout the growth curve, indicating an accumulation in the media, although there is a sharper increase during the 20-24 hour interval. The concentration of HAI-1 was low compared to other molecules detected, which agree with findings by Anetzberger *et al.* (2012), which showed that the concentrations of autoinducers vary depending on the growth phase. The study showed that HAI-1 and CAI-1 activities becomes more prominent later during stationary phase, and that AI-2 concentration accumulates early during growth, peaks during mid-exponential growth phase and eventually declines during stationary phase. The findings agree with the low intensity of HAI-1 detected in this study for growth in minimal media.

The analytical method used did not detect the presence of AI-2 and Ea-C_8-CAI-1, which could be the result of the structure of the molecules. In the case of AI-2, it has been determined that it contains a low ionization potential, rendering it extremely difficult to detect using mass spectrometry. Furthermore, the low stability of the precursor DPD at high concentration restricts the concentration of the molecule (Campagna *et al*., 2009). Similarly, the mass spectrometry analysis done on CAI-1 involves the production of derivatives that would produce adducts of known mass. Spirig *et al*. (2008) utilizes an

oximation reaction between CAI-1 and O-(2,3,4,5,6-pentafluorobenzyl) hydroxyl- amine hydrochloride (O-PFB) to produce known adduct products. Similarly, Ea-C_8-CAI-1 modified with trimethylsilylated methoxyamine has been potentially detected using GC-TOF-MS (Anetzberger *et al*., 2012). Thus, the most probable explanation for the lack of detection is that Ea-C8-CAI-1 fragments in a manner that it went undetected without the use of stabilizing derivatives.

Some of the extracted extracellular molecules were of interest since they might represent modified metabolites of AIs. The m/z 401.2616 contained a base peak of mass 210.1472 that could represent the sodium adduct of HAI-1. Furthermore, the base ion with mass 171.1359 corresponding to m/z 294.2258 could represent C_4-HLS. There is no evidence that *V. harveyi* is able to synthesize C_4-HSL; therefore its detection in the sample cannot be explained, but it could be caused by a natural reaction of HAI-1 in the media. In both cases a peak at m/z 102 was not detected, a signature peak found in samples containing HSLs, depicting the lactone ring without the acyl group. Lastly, a product ion of mass 255.2940 present in the m/z 236.2201 might correspond to the acetonitrile adduct of Ea-C_8-CAI, although the $[M+H]^+$ ion corresponding to the AI was not detected. Although it is not possible to assert with certainty that these metabolites are modified AIs, the idea that *V. harveyi* and possibly other species are able to further modify the AI molecules is interesting, and it would further expand our knowledge on the ways bacterial cells are able to utilize QS to communicate with each other. Also, it is possible this is the first time Ea-C_8-CAI is detected without the use of stabilizing derivatives.

It should be taken into consideration the possibility of reactions taking place during the ionization process, such as dimerization and trimerization. Makemson *et al.* (2006) reported that when using acetonitrile in the extraction and electrospray ionization process, a number of dimers and trimmers could be found. Nevertheless, there was no evidence of dimerization taking place, probably because a different acquisition method was used in this study.

In this project, an attempt was made to determine the chemical structure of the unknown extracellular metabolites. There are a few methods that currently are available to help identify completely unknown metabolites; here molecular networking in metablomics will be briefly mentioned. Metablomics is the analysis of small molecules usually with a molecular weight of less than 1000 Da (Feighn, 2001). In the case of MS/MS, fragmentation data can be compared with mass spectral libraries found online. One such tool is called Multistage Elemental Formula (MEF), developed by Rojas-Chertó *et al.* (2001) to assess the elemental composition of MS data, and with the MEF fragmentation created is easier to find the chemical structure. Nonetheless, there are some challenges with the determination of the chemical structure of extracellular molecules. Crude media extract samples are very complex and dynamic with dozens of unknown molecules. Also, the ESI ionization process generates a series of a chemical adduct species derived from one parent metabolite that could lead to false positives in identification (Brown *et al.*, 2009). It was not possible to utilize the MEF tool for the *Vibrio harveyi* extracellular samples, instead, the ChemSpider database was utilized to generate a list of possible chemical structures for the compounds extracted (see **Figure 7**).

E. CONCLUSIONS

One of the aims of this work was to characterize the extracellular molecules produced by *V. harveyi* when grown in minimal media using electrospray tandem mass spectrometry. There has been no previous investigation on the nature of any extracellular molecules except for the autoinducers this microbe synthesizes. The second aim of this work was to determine whether the molecules found were related to the quorum sensing mechanism of *V. harveyi*. This study has characterized a total of 11 molecules being actively produced and secreted by *V. harveyi*, with molecular masses ranging from 200 to 400 Daltons. These molecules are small enough to diffuse out of the cell, a characteristic of autoinducers.

There are three molecules (m/z 227.1740, m/z 267.1703, m/z 354.2825) whose concentrations were affected during the activation of quorum sensing. This is an indication that changes in gene expression after the activation of quorum sensing were affecting the rate at which these molecules were being synthesized or degraded.

These findings provide the groundwork for more research on these extracellular molecules. A number of phenotypic and analytical studies can be performed to further characterize the molecular structure of these compounds and their role in the metabolism, quorum sensing, and other cellular processes of *V. harveyi.*

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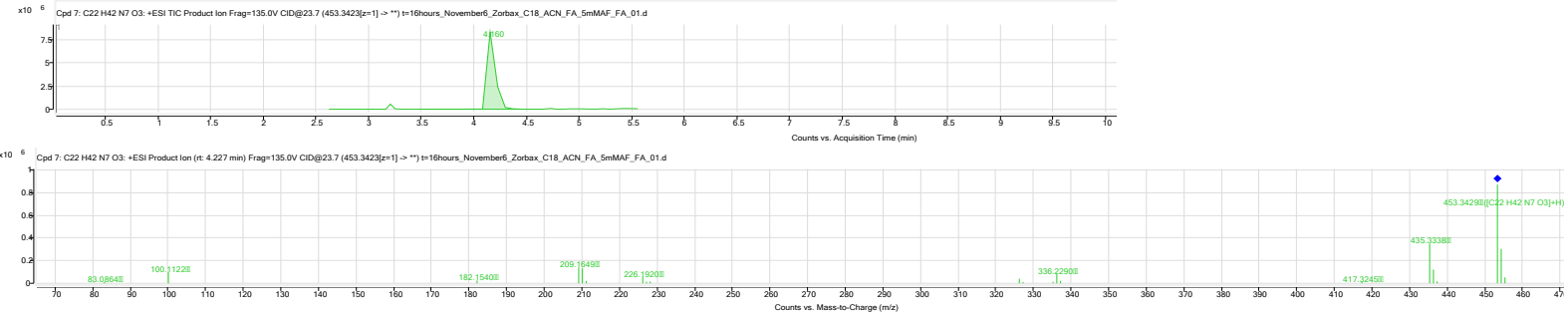
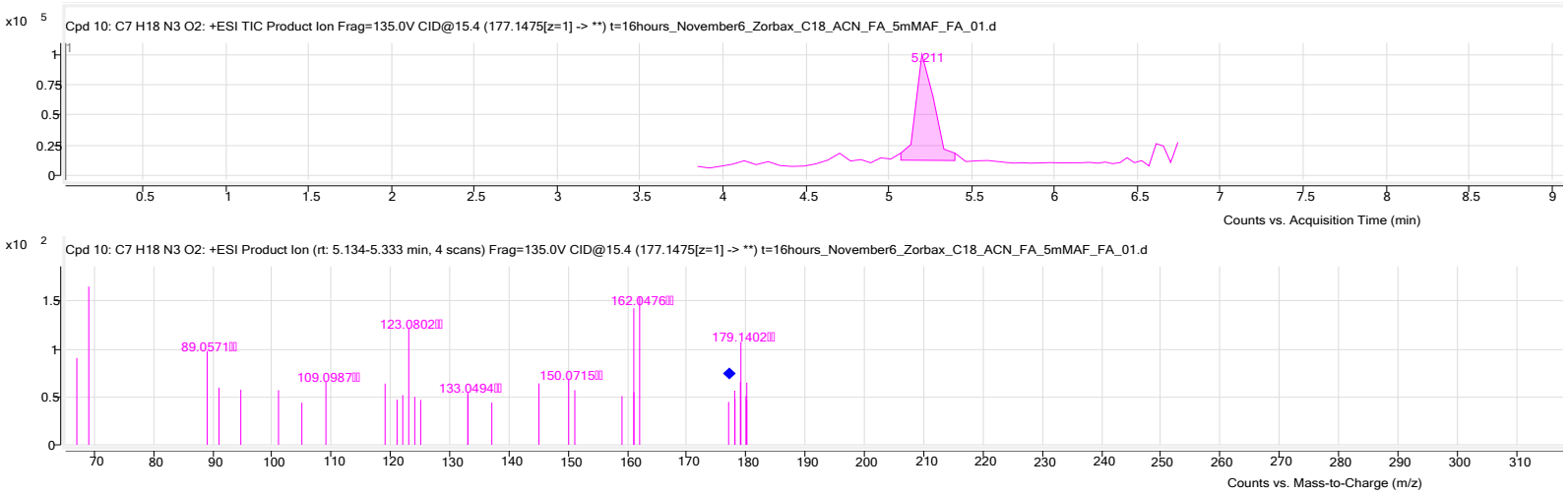
G. APPENDICES

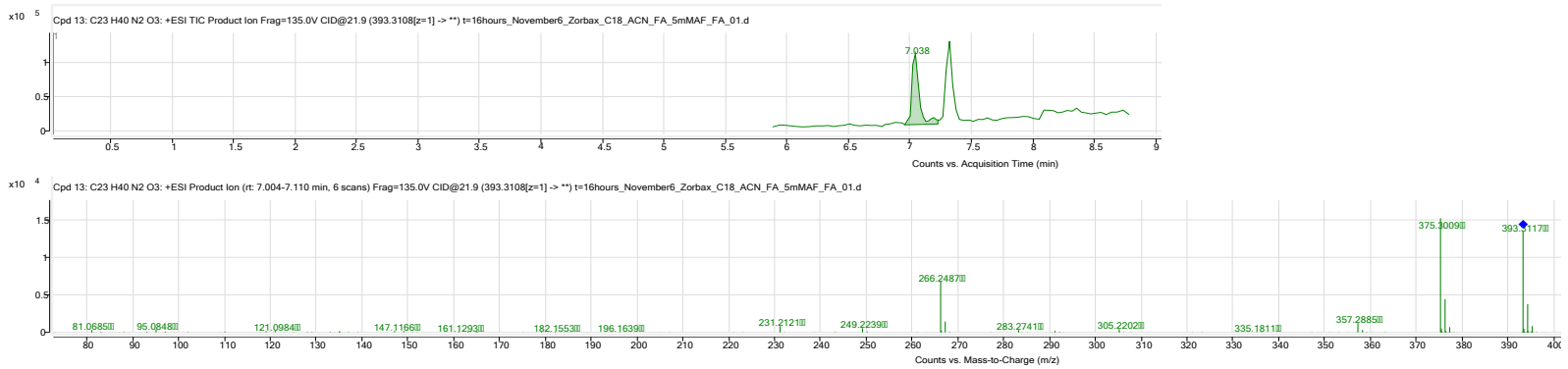
a. Product Ion Chromatograms for the Rest of Extracted Extracellular Molecules Produced by *V. harveyi*



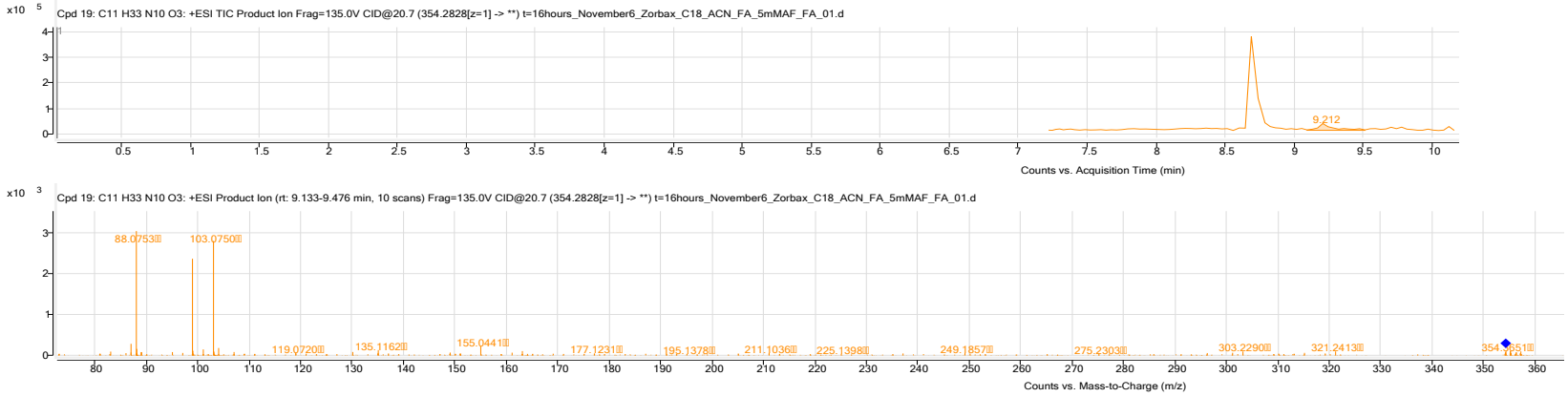
1. ESI-MS/MS Product Ion Chromatogram for m/z 453.2429



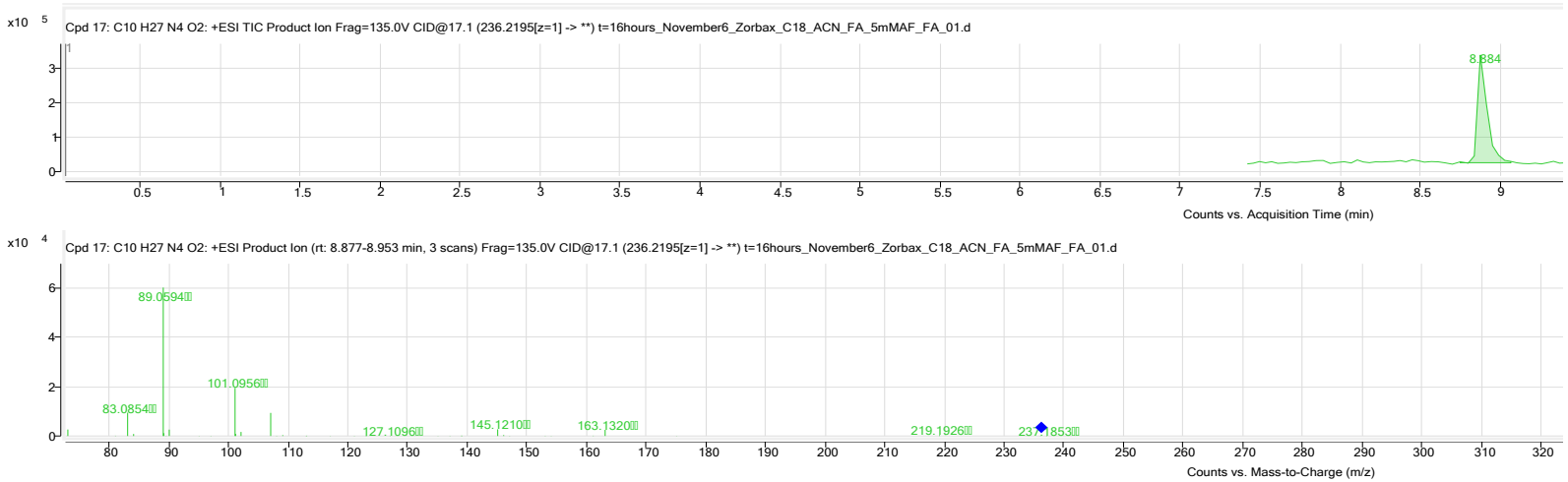
2. ESI-MS/MS Product Ion Chromatogram for m/z 177.1474



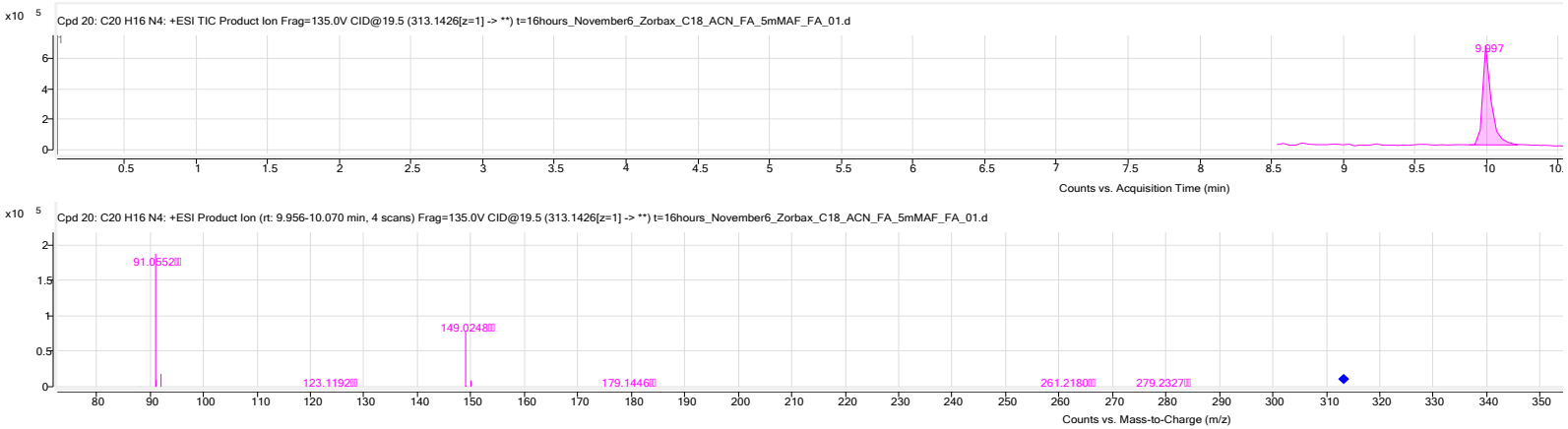
3. ESI-MS/MS Product Ion Chromatogram for m/z 393.3094



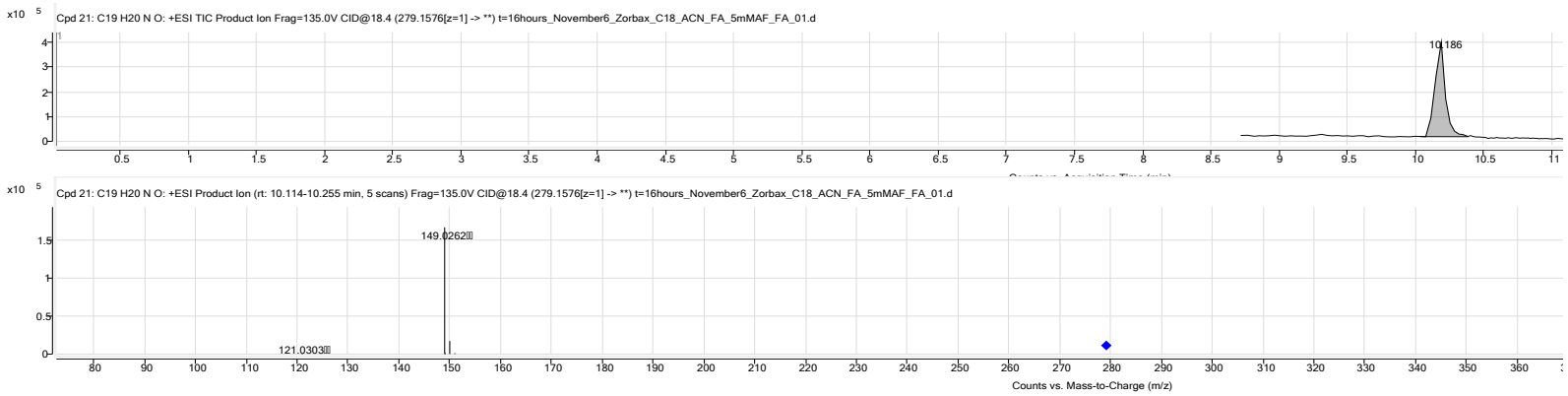
4. ESI-MS/MS Product Ion Chromatogram for m/z 354.2825



5. ESI-MS/MS Product Ion Chromatogram for m/z 236.2201



6. ESI-MS/MS Product Ion Chromatogram for m/z 313.1414



7. ESI-MS/MS Product Ion Chromatogram for m/z 279.1577