Chemical Challenges to Bacterial AHL Signaling in the Environment

Alan W. Decho,*,† Rebecca L. Frey,‡ and John L. Ferry‡

Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, South Carolina 29208, United States, and Department of Chemistry and Biochemistry, College of Arts and Sciences, University of South Carolina, Columbia, South Carolina 29208, United States

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1. Introduction

Since the seminal observation that bacterial cells produce intracellular molecules that trigger bioluminescence,[1] a great deal has been learned regarding the process now termed quorum sensing (QS).[2] QS is a form of density-dependent cell-to-cell chemical communication where bacteria produce, release, and respond to extracellular signaling molecules.[2] QS is now recognized to be widespread, occurring not only between bacteria[3] but also in the interactions between microorganisms and eukaryotic (e.g. mammalian and algae) cells.[4−6] This review addresses emerging information regarding quorum sensing and the effect of natural attenuation on the chemical stability of extracellular signaling cues. It focuses on a major class of cues, the *N*-acylhomoserine lactones (AHLs). We examine the literature describing how the dynamic chemistry of natural environments presents limitations and offers unanticipated avenues for cell-to-cell chemical signaling.

Alan W. Decho holds degrees of B.A. (1979) (Eastern Connecticut State University), M.S. (1982) (Ohio University), and Ph.D. (1987) (Louisiana State University). He then conducted research as a Fulbright Hays Postdoctoral Fellow at the CSIRO Marine Laboratories in Queensland, Australia, followed by a National Research Council (NRC) Fellowship at the USGS Laboratories in Menlo Park, CA, and the State University of New York at Stony Brook (1989−1993). In 1994, he moved to his position at the University of South Carolina (USC), where he is currently Professor of Environmental Health Sciences in the Arnold School of Public Health. His current research focuses on microbial biofilm interactions such as chemical communication with the extracellular matrix, and their roles in environmental- and health-related processes.

1.1. A Primer on Terminology

Chemical signaling is a multidisciplinary topic that combines the relevant terminologies of microbiology, chemical ecology, and chemistry. For the sake of clarity, we begin this review with a primer of some terms whose use may be context-dependent for some readers. A *signal*, by definition, is a molecule or medium that has evolved to communicate qualitative and quantitative information to another organism

* To whom correspondence should be addressed. Phone: 803 777-6584. Fax: 803-777-3391. E-mail: awdecho@mailbox.sc.edu.
† Department of Environmental Health Sciences, Arnold School of Public Health.
‡ Department of Chemistry and Biochemistry, College of Arts and Sciences.

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Rebecca L. Frey received her bachelor's degree (2006) from King's College, Wilkes-Barre, PA, with a triple major in chemistry, neuroscience, and psychology. She recently received her Ph.D. in analytical chemistry under the guidance of Professor John L. Ferry at the University of South Carolina. Her research interests include fate and transport of organic chemicals in the environment, photochemically driven oxidation, combinatorial chemistry, trace organic analysis, applications of analytical chromatography and techniques, and modeling of emerging contaminants in the environment.

John L. Ferry obtained his B.S. (1990) from the University of Illinois—Urbana and his M.S. (1993) and Ph.D. (1996) in Environmental Sciences and Engineering from the University of North Carolina at Chapel Hill under the direction of Prof. William H. Glaze. He held a postdoctoral position (1996−98) at the University of Texas—Austin with Prof. Marye Anne Fox, and he is currently an Associate Professor in the Department of Chemistry and Biochemistry at the University of South Carolina. His main research interest is studying the natural mechanisms by which organic molecules are degraded in the environment, particularly focusing on applying combinatorial techniques to map the chemistry of complex environmental systems.

about their immediate environment.[7] We use the broader term "*cue*" throughout to include specific signal molecules but also other molecules (e.g. metabolic products) that may simply be utilized in communication, although they may have other origins or purposes.[8] *Environmental fate* is a term often used in environmental chemistry to describe the manifold of processes, some thermodynamically controlled and some kinetically controlled, which encompass the possible ways organic molecules may degrade in the environment. It usually implies some degree of specificity, in contrast to *natural attenuation*, a more general term used to communicate that the concentration of organic molecules falls with time in environmental matrices. Other terms in this review address the functionality of cues such as "*agonists*," which promote a response; or "*antagonists*," which reduce a response. Finally, because this review addresses natural systems, we use the term "*ecotype*" to encompass groups of highly similar natural bacteria contained within the same species which exhibit small genetic differences that may affect their ecological interactions such as communication.

1.2. The Natural Microbial Microbiome

The Earth's biomass of microbes accounts for approximately half of the total biomass of living organisms and is estimated to contain $3.5- 5.5 \times 10^{17}$ g C or $4- 6 \times 10^{30}$ cells.[9] Natural microbial communities are complex and often biologically diverse when compared with any of their counterparts that can be assembled in the laboratory. The advent of molecular-based technologies has enabled the characterization of these communities based on their specific 16S rDNA sequences[10−12] at the resolution of genera, individual species, and even ecotypes (i.e., strains) of bacteria.[13] In the natural environment, microbial populations can self-organize into communities. Some natural microbial communities may contain thousands of different species having different metabolic pathways (e.g. photosynthetic/chemosynthetic, aerobic/anaerobic organisms).[14−18] In highly structured communities, chemical, biological, and even physical effects of natural conditions add to the complexity and heterogeneity of conditions encountered by bacteria. The appearance of a community is defined by the presence of microbially associated heterogeneities that resolve the community from the greater environment. These heterogeneities can manifest as gradients in local chemical concentrations or solution properties so sharp they attain the appearance of physical membranes. In highly structured communities, these boundaries may fluctuate temporally as well as spatially, as is the case in microbial mat biofilms fluctuating across the diel cycle.[19] However, relatively little is known about how microspatial heterogeneities and temporal fluctuations in the intensity and position of chemical gradients influence the natural attenuation of molecules that are involved in communication within complex microbial communities. Such small-scale communities are a cornerstone of ecological systems but additionally represent reservoirs for pathogens that may survive in a diversity of environments, from marine waters to drinking water distribution systems[20] and pose threats to human health.[21,22] Biofilm formation and its continued persistence is now realized to be closely related to the regulation of microbial communication. Understanding the relationship between environmental conditions and chemical communication is a next essential step in defining the larger implications of cell−cell interactions.

1.3. Extracellular Environments: General Considerations

Characterization of the aquatic environment can be transformed from geographic constraints (e.g. seawater, groundwater) to the molecular by treating the hydrosphere as a complex solution in which dissolved materials vary over wide ranges (geographically and temporally). Parameterizing the aquatic environment as a range of dissolved constituents/descriptors in this fashion allows the construction of a multidimensional parameter space that house various forms of QS-based processes. Parameters may consist of single molecules or ion descriptors (e.g. pH, the concentration of dissolved O_2), aggregated descriptors of several distinct molecular species (e.g. salinity, total phosphorus), or physical descriptors (e.g. temperature, viscosity). QS occurs widely across parameter space in conditions corresponding to

solutions as varying as those found on ice surfaces, hydrothermal vent fields, beaches, water distribution systems, etc. As AHLs traverse the extracellular environment while diffusing between/among cells, they will encounter different concentrations of oxidants and ions, and a range of pH conditions, all of which can potentially attenuate the AHL cues by effecting their degradation. The interaction of local environmental parameters with the QS process can be predicted, but the predictions are constrained by the limits of what is known about the processes that degrade organic molecules in the environment. The interaction between environmental conditions and QS can be thought of as either "inhibitory" to information transfer (through processes that degrade cues past the point of recognition) or "supportive" (through processes that preserve cues or transform them into more active forms).

2. Chemical Communication and AHL Cues

2.1. Overview

QS is a process where cells produce molecular cues that are released outside the cell, traverse the extracellular environment mostly by diffusion, and are perceived by other cells in proximity, or by the same (i.e. self) cells.[23] Generally speaking, when the ambient (and intracellular) concentration of a cue reaches a "threshold," a concentration-dependent response often results in changes in gene expression.[24] The metabolic changes associated with QS allow a functional coordination of cells (i.e. group behaviors) for activities that are generally favorable to bacteria when in high abundances (e.g. light production, swarming, virulence, extracellular enzyme production, biofilm formation).[25−29] QS has been classically considered to be a cell-density dependent phenomenon where a group of similar bacteria is able to monitor its population and alter its gene expression accordingly.

Several different classes of signal cues are now recognized to be involved in bacterial QS. These include: *N*-acyl-L-homoserine lactones (AHLs), autoinducing oligopeptides (AIP), cyclic dipeptides, or 2,5-diketopiperazines (DKPs), CA-1; furanosyl diesters (also called autoinducer-2, AI-2), γ-butyrolactones (GBL), *Pseudomonas* quinolone signals (PQS), and diffusible signaling factors (DSF) (reviewed in refs 26, 30−36 There are likely to be many as yet uncharacterized cues and perhaps novel cue classes.

2.1.1. Background on the AHL System

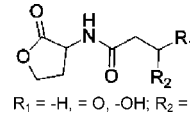
Bacteria are grouped into two major categories based on a simple differential staining response, called the Gram stain, which is indicative of fundamental structural differences in bacteria. Gram-negative bacteria have an inner (plasma) cell membrane and outer membrane, while the Gram-positive bacteria have an inner (plasma) membrane only. With regard to AHL-based communication, the Gram negatives and Gram positives also exhibit major differences. This becomes potentially important in natural environments because both groups often exist in spatial proximity to each other and certain cues that are produced by one group can have antagonist (even lethal) effects on the other group.

AHL-based communication systems have now been found associated with over 70 different genera of Gram negative proteobacteria which are a major grouping of bacterial species.[37] In its simplest form, the system consists of a cue (i.e., AHL), enzymes to produce the AHLs, an AHL-binding protein, and a specific DNA site (*lux* box), which is directly upstream from the promoter region for set of genes that is up- (or down-) regulated by the AHL−protein complex. In this system, two major types of intracellular proteins are involved: a cue synthase enzyme(s) that produces the AHL and a receptor protein(s) that recognizes and binds the cue upon entering the cell.[23] One of the best characterized examples of this is the luxI/luxR families of proteins. Enzymes from the luxI synthase family produce AHLs, which are then released into the surrounding environment. As local concentrations of AHLs increase and upon reaching a threshold (e.g. typically nanomolar concentrations), they will complex with luxR proteins within cells. Subsequent binding of the AHL/luxR complex to the *lux* box region of DNA results in subsequent activation and upregulation (or in some cases down-regulation) of QS-regulated genes.[38] While all AHLs are composed of a conserved homoserine lactone, the incorporated fatty acid side chain can vary in length (4−18 carbon atoms), the level of saturation, and side chain (*oxo*- or *hydroxyl*-) substitutions.[39] Seemingly subtle differences in substitutions occurring at predetermined sites on the molecule confer specificity to the AHL cues and affect its resulting functions to cells.[40] For example, a C6-AHL can represent a completely different cue than a 3-*oxo*-C6-AHL.

2.2. Molecular Properties of AHLs

The structure of AHLs determines not only their signaling function but also their modes of interaction with environmental factors during cell-to-cell transit. AHLs consist of a five-membered ring containing varied amide-linked side chains and identified by a range of 4−18 carbons in length (Figure 1). Several hundred structural variants of the basic AHL molecule have been discovered, synthesized, and characterized (a partial list can be found in Supporting Information Table S1−S3) The acyl side chain can be saturated or unsaturated and may contain a substituted *hydroxyl*- or *oxo*- functional group located at the C3 position.[41,42] QS depends on signaling molecules which are stable on the time scale of diffusion through the extracellular matrix, meaning quorum-sensing regulated gene expression may depend on such factors as the rate of cues degradation as a function of environmental conditions, calling distance, and diffusivity of the cue.[43] A survey of the AHL structures available in the peer reviewed literature (Supporting Information Table S1) reveals several interesting trends that typically correlate with various specific environmental fates.

Certain AHLs that have been recently isolated from marine bacteria exhibit methyl branching and double bonds associated with their acyl chains.[44] The presence of a methyl group, however, has profound effects on the activation of the AHL receptor protein, requiring approximately a 10× higher concentration of AHL than those of the specific AHL.[44] The structural diversity of AHL-related compounds result in large differences in genetic responses and activity.[40,45]



R_1 = -H, = O, -OH; R_2 = $-C_nH_{2n+1}$, $-C_nH_{2n}$

Figure 1. The molecular structure of *N*-acylhomoserine lactones.

2.2.1. Physical Properties

Molecular Weight. AHLs range in size from 171.9 Da for a C4- to 367.57 Da for an unsubstituted C18-AHL. The AHLs in Supporting Information Tables S1−S4 (253 total structures) fall between the molecular weights of 171.90 and 638.80 g/mol (Figure 2). These mass ranges were selected to exclude AHL−protein adducts from the survey; although these molecules are intellectually interesting, they are not considered further here. Molecular weights for neutral organics correlate to several physical properties and in this case are particularly illustrative for their effects on partitioning between aqueous and organic phases (Figure 3) and aqueous solubility (Figure 4) (both figures based on structural subunit contribution calculations).

Solubility, Diffusivity, and Dispersion. These properties are critical for parametrizing limits on the threshold concentration of an AHL required to elicit a response and the mechanism of their movement between cells. Low-molecular-weight AHLs tend to be relatively water soluble, and it is unlikely communal responses based on their presence would ever be limited by their solubility. However, as molecular weights increase significantly above 520 g/mol, the saturation concentration of the AHL is expected to drop below 1 μM (Figure 3). The micromolar cutoff, although an arbitrary limit, defines the upper mass ranges of the AHL family reviewed in the Supporting Information (Table S1). The implication of this observation is that, at high molecular weights, the solubility of AHLs may be so low that communication can only happen over a short range. This is based on the assumption that the attenuation of the (AHL) based on three-dimensional diffusion alone (i.e., distance from the cell of origin), starting from a low concentration as required by high molecular weight, may result in concentrations too low to trigger a response based on the expected distances between an AHL-producing cell and an AHL-perceiving cell (i.e. "calling distance" hypothesis[46]). Experimentally, this has been verified. Gantner and colleagues examined the movement of AHLs between cells using an engineered reporter strain of *Pseudomonas putida* that becomes fluorescent when quorum sensing is occurring. They determined that on plant surfaces most signaling (via

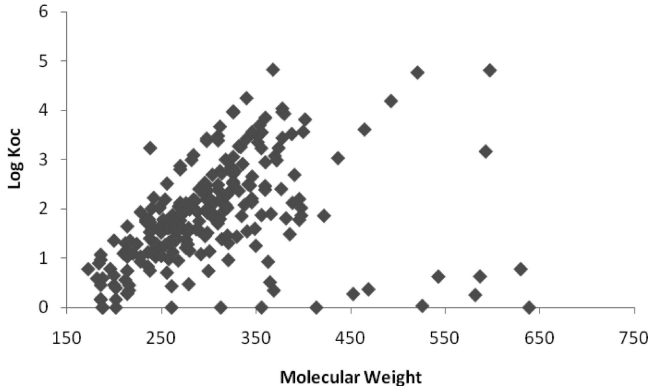


Figure 2. Log of the estimated K_{oc} (organic carbon adsorption coefficient) based on structural subunit contributions vs the molecular weight of the AHL. High-molecular-weight AHLs are generically predicted to adsorb to organic surfaces rather than staying in the aqueous phase. The figure is suggestive of limitations on signal cue transmission based on the organic content of the immediate environment. All data in graph refer to AHL structures that have been published in the peer-reviewed literature with molecular masses of 650 Da and below. The references for each datum are listed in the Supporting Information tables.

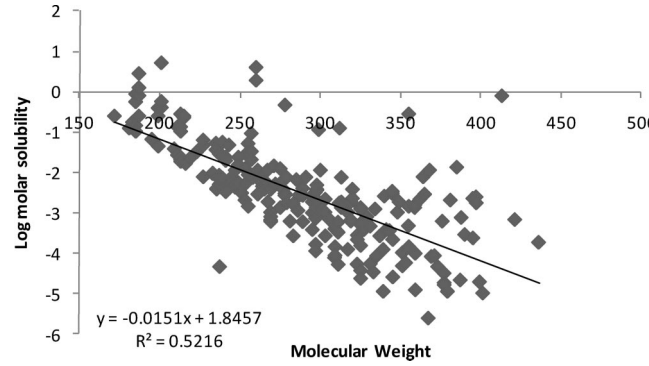


Figure 3. Log of the estimated molar solubility of AHLs based on the structural subunit contribution method. AHLs are generally polar, and water solubility is unlikely to be a limiting factor for most chemical cues reported. All points refer to AHL structures that have been published in the peer-reviewed literature with molecular masses of 650 Da and below. References are listed in Supporting Information Table S1.

AHLs) occurred over relatively short distances (<10 um), however, some AHLs were able to travel a relatively long distance (up to 78 μm) and still induce quorum sensing (i.e., fluorescence). Hence, they have referred to these distances as the cell−cell "calling distances."

"Calling Distances" of AHLs. Chemical signaling under natural conditions can operate efficiently over relatively short distances (e.g. 10 s micrometers). However, owing to diffusion constraints, binding, and chemical modifications of cues, can quorum sensing operate over much longer distances? If so, over what distances can one bacterium potentially detect the presence of another? Biological theory predicts that diffusion over relatively long distances (e.g. 100 s micrometers) will be inefficient for directed transport of molecules, especially in three dimensions. In the more complex and heterogeneous geo- and physicochemical milieu of natural environments, the calling distance for AHLs will be further complicated by the diffusion-slowing properties of gel-like EPS matrices, the alteration/degradation of AHLs versus distance, and the physicochemical properties of the AHL itself. It is relevant that many isolates of bacteria secrete several types of cues, each of which can exhibit different diffusivities and different susceptibilities to chemical modification by the local environment. This leads to the possibility that certain types of cues may be better suited over relatively short distances while other types of cues are better suited to longer calling distances.

Working from this premise and broadly assuming only one dimension, rates of diffusion (D_{aq}) for AHL-like molecules should be relatively rapid in water, even at a mass of 600 Da. A one micrometer travel distance should be less than 30 s away (i.e. cell−cell) (Figure 4). Measured half-life hydrolysis rates of small AHLs (e.g. C6), even under relatively high pH (e.g. pH 9.5), are near 30 min[47] and would be slower than diffusion times between cells spaced just a few micrometers apart. Therefore, AHL travel time between closely spaced cells should be relatively fast (e.g. seconds) assuming one dimension. These diffusivities, however, represent near-maximal rates because they are calculated for 25 °C, and realizing that diffusion in nature occurs in three-dimensions (i.e. not one-dimension), with hydrolyses times being pH-dependent.

Speculatively, these properties also have bearing on the mode of transport of AHLs between cells. As molecular weight increases, the fugacity of AHLs in the liquid phase

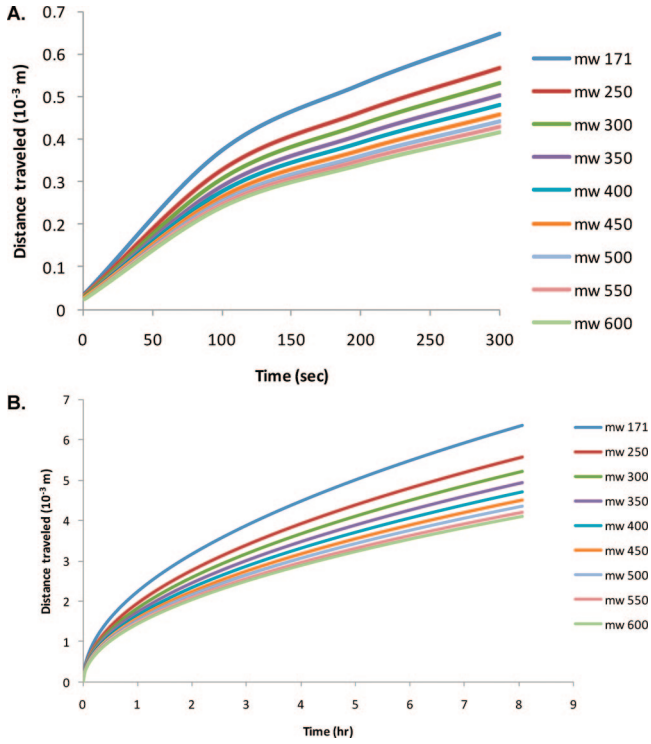


Figure 4. The distance AHLs travel through solution over a fixed time is modeled based on their molecular weight (MW). As AHLs get larger, their dispersion times (via diffusion) and ability to evoke a communal response are predictably slowed. Calculated one-dimensional travel[72] for AHLs ranging from unsubstituted C4 to 600 Da are shown over periods of (A) 5 min and (B) over 8 h (at 25 °C).

increases and they become more prone to associate with organic phases (Figure 2). One implication of this is that their transport may be more appropriately described as resembling chromatographic movement, where there is partitioning between a mobile phase (water) and a stationary phase (e.g., sediment particles or a biofilm comprised of cells and extracellular polymers). An estimated log K_{oc} of 3 or higher suggests a minimum of 99.9% AHL would be expected to partition into the organic phase. In this case, the strictly diffusive movement of large (mw > 350 g/mol) may become insignificant, as static diffusion would be severely limited by the case of a molecule that favored the stationary phase. Utilization of a high-molecular-weight AHL in a biofilm may require the presence of a moving aqueous phase within the biofilm community to ensure sufficiently short response times.

Short acyl-chain AHLs freely diffuse in/out of cells.[24] Longer chain AHLs use active-efflux mechanisms such as multidrug efflux pumps to transport AHLs in/out of cells.[48] The movement of AHLs once outside the cell is not well understood. Small molecule movement is largely mediated by passive diffusion in their water medium, and diffusivity is influenced by spherical molecular diameter (i.e., hydrodynamic radius) and surface reactivity with other molecules according to Fick's Law.[49] Small cue molecules (e.g., a C4-AHL), therefore, typically diffuse more rapidly than larger molecules (e.g., a C14-AHL), all else being similar. Larger cue molecules are further restricted in relative mobility by other factors such as solubility and their capacity to form weak interactions (e.g. London forces) with other molecules. The shorter-chained AHLs (e.g., C4- to C8-AHL) are highly water soluble, whereas AHLs having extended acyl chain lengths (e.g. C10- to C18-AHL) are progressively less soluble (Figure 2). Therefore, it can be predicted that relatively long-chain AHLs should not easily move between cells in a diffusion-mediated manner.[50] It also should be noted that chemical alterations, such the addition of an *oxo*-group at the C-3 position, or lysis of the lactone, will make an AHL slightly more hydrophilic and therefore more soluble in water; the diffusivities, however, should remain the same, all else being equal. If one assumes that an AHL must exist or be packaged in a relatively water-soluble form in order to disperse, then increased solubility would result in a higher concentration of the cue being able to disperse, as opposed to being hydrophobically bound to a surface (e.g. sediment particle).

Bacteria produce and release lipid vesicles that can be used to package enzymes, DNA, and other molecules.[51,52] The vesicles are derived from the outer cell membrane of Gram negative -bacteria and are "blebbed" off into the extracellular matrix of the biofilm environment.[53] Larger, more hydrophobic, cue molecules called *Pseudomonas aeruginosa* quinolone signals (PQS) have been shown to be packaged within these lipid vesicles and vesicles released from bacteria for dispersion.[54] Experiments have shown that other molecules, which possess a more hydrophilic periphery, can be used to chelate AHL cues.[55] If this occurs under natural conditions, such packaging could serve to protect an AHL from degradation or modification during transit from one cell to another. However, unless the cue is able to be released from the shuttle molecule after travel, the cue may not be able to enter the cell and will remain inactive.

2.2.2. Reactivity of AHLs

Environments just outside of the protective confines of the cell are characterized by geochemical, photochemical, and physical parameters that can potentially modify or compromise the structural stability of an AHL.[36,56] The ability of AHLs to function as cues will depend on the types and magnitude of these geochemical challenges and the transit times (e.g. diffusivities) for a cue to move from cell−cell, a process that will determine the lengths of exposure to geochemical modifiers.

Abiotic Hydrolysis. Alkaline conditions occur with regularity in many natural systems, especially within biofilm microenvironments. Most photosynthetic biofilm systems, such as microbial mats, exhibit dramatic shifts in pH (e.g. pH 6.5 − 9.5) over a 24 h period (diel cycle).[57] Rapid photosynthesis during the sunlit hours results in depletion of aqueous CO_2, in some cases driving pH >9.5 during daylight hours due to the relative dominance of phototrophic processes, while more acidic conditions (e.g., pH <6.5) occur during night hours due to bacterial respiration.[58] Such mat environments are highly productive centers of C cycling in the present day and were important in shaping the biogeochemistry of the early Earth.[59,60] Under alkaline conditions (e.g. > pH 7.1), AHLs are susceptible to hydrolysis by attack of H_2O or HO^- at the carbonyl of the lactone ring with conversion to the corresponding signaling-inactive γ-hydroxy carboxylate (Figure 5).[47,61−63]

The rate of hydrolysis is proportional to the molecular weight of the AHL, with higher molecular weight AHLs hydrolyzing more slowly than lower ones ($t_{1/2}$ on the order of 10^2 min for C4 and 10^3 min for C14).[64] This pattern of reactivity is consistent with the general hydrolysis of carboxylic esters in aqueous systems and has been variously

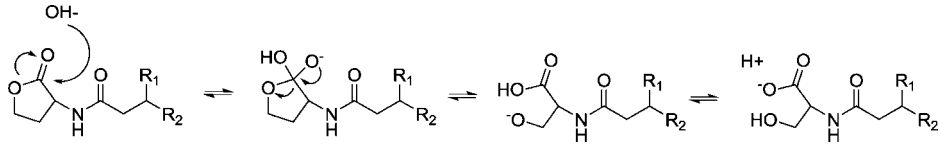


Figure 5. Base-catalyzed hydrolysis of acyl AHLs. R_1 = H, O, OH; R_2 = *n*-alkyl chain, C2−C14.

attributed to factors such as the stability of the transition state, solubility of the starting materials, and increasing steric hindrance with larger esters.[64−67] Lactone hydrolysis (whether caused by abiotic or biotic factors) is reversible under some environmental conditions, particularly at acidic pHs.[66] An implication of this is that in photosynthetic biofilms or eutrophic surface waters, active signaling may be a pulsed phenomenon, reflective of cycling between open and closed forms of the AHL during photosynthetically driven pH variation. Given the relative instability of AHLs to alkaline pH conditions, it is interesting AHLs were found to be present in extracts from bacteria or archaea growing, in part, under alkaline conditions. The haloalkaliphilic archeaon, *Natronococcus occultus*, was able to produce AHL-positive compounds (using an AHL biosensor) during growth at pH 10.[68] Cyanobacterial isolates were recently shown to produce AHLs.[69] The cyanobacterium *Gloeothece* PCC6909 was reported to produce an *N*-octanoyl-homoserine lactone, which resulted in changes in the expression of 43 proteins.[69] Although AHL-based activities have been found associated with phototrophic biofilms,[70,71] this was the first report of AHL production by a cyanobacterial isolate. These observations are intriguing because photosynthesis raises the pH, which should result in AHL hydrolysis. The effects of other common nucleophiles (e.g., reduced sulfur compounds, halides, dissolved organic matter, etc.) on AHL lifetimes have not yet been reported, although these species are known to catalyze the hydrolysis of other esters.[67,65,72,73] In microbial mats, sulfide, for example, is known to react rapidly with low-molecular-weight carbon compounds,[74] especially during nighttime conditions. Therefore, the acyl side chains of AHLs (especially those containing *oxo*- and *hydroxyl*- functional groups) could especially reactive with sulfides during this time.

Biotic Hydrolyses: Enzymatic Degradation of AHLs. The degradation of AHLs can occur by both biological and chemical mechanisms. Biological mechanisms include hydrolyses by enzymes, usually produced by other bacteria. Bacteria, such as Gram positives, which are not known to produce AHLs, often produce two major groups of enzymes that can hydrolyze AHLs: lactonases and acylases, which hydrolyze the lactone and acyl chains, respectively. The AHL-lactonase hydrolyzes the ester bond of the lactone (of an AHL), which results in an acyl homoserine having reduced biological activity.[75,76] Amino acylases cleaves the peptide (amide) bond of an AHL to yield a fatty acid and a homoserine lactone, which results in the loss of any detectable biological activity.[77,78] The fatty acid product is used by bacteria as a carbon source, and the nitrogen from the amide bond is available as ammonium via the action of lactonases. This provides a mechanism for Gram positive bacteria to coexist in the presence of AHL-producing bacteria. Similarly, most known Gram negative, AHL-producing bacteria will possess enogenous capabilities to degrade their own and other AHLs.[79] These AHL acylases can also regulate the relative proportions of two AHLs that are synthesized by a cell to provide more tightly controlled production of AHLs.[80,80] Currently, all known forms of acylases and lactonases are intracellular enzymes and hence require cellular uptake of the AHL in order for hydrolysis to occur.[75−78,81,82]

Oxidation. Sharp microscale (e.g., micrometer) redox gradients have been shown to occur in biofilms in water pipes, condensers, photosynthetic microbial mats, and marine sediments (e.g., conversion from aerobic to sulfate-reducing communities).[83] Associated film surfaces are likely to experience exposure to oxidants generated naturally in situ or applied for technological purposes, either of which could lead to the inadvertent oxidation of AHLs. The interaction of natural organic matter and photoactive metals with sunlight leads to the generation of a suite of reactive species, including carbon centered radicals, and the reactive oxygen species (ROS, including HO, $^1O_2$, HOO/O_2^-, H_2O_2, and various organoperoxides).[84−93] The redox cycling of some transition metals, particularly Fe, can also lead to ROS formation in the absence of sunlight. The advanced oxidation processes are technological approaches for water remediation based on oxidation of dissolved organics by technologically generated HO (i.e., through radiolysis of water, reduction of ozone by hydrogen peroxide, etc.).[94] Nonradical oxidants that can oxidize AHLs include the hypohalites, generated biotically or applied as technologies (i.e., disinfectants). Certain marine algae produce haloperoxidases, which generate oxidized halogens (e.g. HOCl) and react specifically with C3-*oxo*-AHLs to negate chemical signaling activities.[95] These changes may be particularly important to biofilms growing in oxidizing environments such as the surfaces of sunlit metal oxide particles at beaches; drinking water distribution systems with disinfectants or food surfaces during electron beam treatment, etc.[88] The reactivity of AHLs with oxidants, including hypohalites and hydroxyl radicals, has been examined.[65,96] Both of these chemical species are applied during water purification as reagents for the control of pathogens and dissolved trace organics, applications that can potentially result in the attenuation of AHLs and subsequent inhibition of QS.[97−101] Oxidation of AHLs by hypochlorite and hypobromite is dependent on the structure of the acyl side chain. These initial studies show that acylated groups, which contain the *oxo* forms, are readily oxidized.[101,102] Inclusion of an *oxo*-group in the three position led to rapid oxidation and halogenations with a product profile consistent with oxidation of the enol form of the 3-*oxo*-AHL, whereas AHLs without additional functional groups on the acyl chain were unreactive with added hypohalites.[95,103] In contrast, all AHLs tested were reactive with hydroxyl radical. Oxidation of AHLs with photogenerated HO was been shown to lead to the unspecific formation of corresponding *oxo* analogues, suggesting partial oxidation of biofilms with HO may lead to increases in signaling activity (Figure 6).[101,102] Most ROS oxidations are nearly activationless, and their rates would not be expected to be sensitive to temperature over the biologically relevant range.

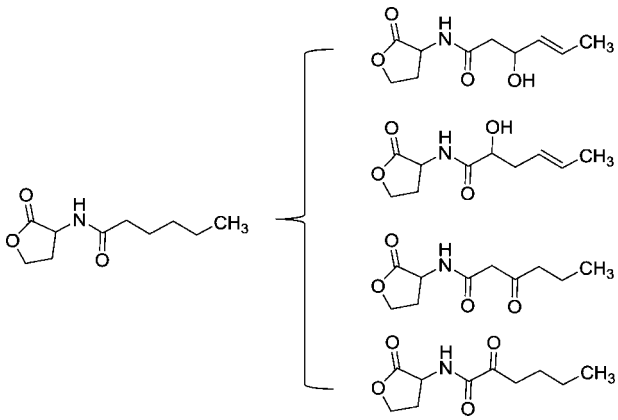


Figure 6. Oxidation of AHLs with photogenerated HO was shown to lead to the unspecific formation of AHL analogues. Adapted with permission from ref 101. Copyright 2009 John Wiley & Sons.

2.3. Agonistic and Antagonistic Analogues of AHLs in Nature

Chemical structures of AHLs can be modified in numerous ways to form analogues that still retain some degree of induction activity, which can be positive or negative. A wide range of AHL analogues have been synthesized in the laboratory and tested for agonistic (i.e. positive) and antagonistic (i.e. negative) activities.[27,44,95,104−111] They will not be discussed further here.

2.3.1. Furanones

Under natural conditions, some organisms are known to produce compounds that can inhibit bacterial quorum sensing, specifically those based on AHL-signaling. Some of the best examples of an environmentally derived analogue are the halogenated (i.e. bromonated) furanones produced by the seaweed *Delisea pulchra* that produce structural analogues for AHLs and result in QS inhibition.[105,112] Binding of the furanone to the (AHL) receptor induces a conformational change in luxR, which results in a noncompatible complex for subsequent gene expression.

2.3.2. Tetramic Acids

It is also notable that cues such as AHLs can be converted to forms exhibiting antibacterial activities. The partial degradation products of AHLs, specifically homoserine lactones and homoserines, can be toxic to other bacteria.[113] Alterations of 3-*oxo*-AHLs, produced by Gram negative bacteria, resulted in conversions to tetramic acid products (e.g. 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione), which then exhibited antibacterial effects against Gram positive cells due to transmembrane changes in pH and resulting cell lysis.[114] Interestingly, Fe concentrations are limiting in many natural environments and the tetramic acids additionally exhibit Fe-binding properties comparable to siderophores. Hence, AHLs can be environmentally modified to products having additional roles as antimicrobials and Fe chelators. A range of naturally occurring tetramic acid products have now been found.[115] It is now realized that chemical cues, previously thought to function exclusively as signals, likely have multiple functions to cells. The multifunctional nature of cues (e.g. PQS in Fe sequestration) has been recently been addressed and adds to an emerging view of QS cues.[116] Under natural conditions, a given cue can have several functions depending on the organism perceiving the cue and concentration. Many antibiotics exhibit signaling properties at low concentrations.[117,118]

Analytical tools provide insight into the complex array of compounds encountered by bacteria in natural systems.[41,64,103,119−121] HPLC is an effective method in the fractionation and preparation of AHLs for structural analysis. Once a single HPLC peak has been detected, it is important to assign a structure to the QS cue molecule on the basis of its spectroscopic properties. Elucidation by LC-MS approaches has presented remarkable insight into the identity of both natural and synthetic autoinducers.[41] Further confirmation of the structure of AHLs often follows using proton or carbon-13 NMR and infrared spectroscopy, which provides identifications of functional groups in an organic molecule. However, microbial cells must distinguish between molecules having useful information content (e.g., cues) for communication or sensing and background organic compounds (e.g. chemical noise and nutrients). The molecular mechanisms that may accomplish this, however, are not well understood.

3. Adaptability of the AHL System

3.1. Ability of Bacteria to Perceive New Cues

3.1.1. Variability within the AHL System

Within the realm of natural communities, the ability of AHL-based communication to exhibit flexibility and to adapt is likely an important characteristic for bacteria. In this way, AHL cues that are synthesized by one type of bacterium and can be perceived by another type of bacterium, and an association can be built. Flexibility in the types of AHLs that are synthesized has been observed in specific bacterial cultures and is rooted in the apparent flexibility of their AHL-synthases. Bacterial monocultures often have the enzymatic capability to produce several different types of AHLs. Some bacteria, for example, possess several *luxR*/*luxI* systems. Each synthase is able to make a set of different AHLs.[122] Because the majority of AHL synthases that have been characterized can accept several acyl-ACPs, this enables a given bacterium the potential to produce a range of AHLs using de novo syntheses. Up to 24 different AHLs have been observed to be synthesized by a single bacterium.[123] Under a given set of growth conditions, however, only a few (e.g. 1−3) AHLs are typically synthesized by a bacterium. Thus, the inherent enzymatic machinery appears to have the potential to allow changes in the types of AHLs that are produced by a given bacterium. This adaptability in the cues produced, under the right conditions, can facilitate the development of efficient metabolic associations between different bacteria that have recently come into spatial proximity within a community.

3.1.2. Variability in AHL-Receptor Proteins and the Adaptation to Exogenous AHLs

LuxR proteins, which bind AHLs,[38] typically demonstrate a high level of specificity for their cognate AHL. Such specificity regulates perception of AHLs and establishes an efficient signaling system for a single (or closely related group of) species to regulate gene expression within a mixed-species environment. However, in contrast to AHLs, whose structures are highly conserved, the amino acid sequences of different luxR homologues can exhibit much variability across bacterial genera, species, or even strains and often

have relatively low (15−25%) shared identity in amino acid sequences.[124] The variability in luxR homologues, in part, may reflect the ability of bacteria to adapt to new cues (from other bacteria). A wide range of different luxR homologues have now been characterized in bacteria.[125] Small differences in the acyl chain length and/or substitution of an AHL affects the binding efficiency to a luxR receptor protein, and the subsequent ability of the luxR−AHL complex to activate transcription.[126] Hence, the binding of a given luxR receptor protein with a noncognate AHL does not necessarily result in changes in gene expression. There is, however, the possibility for the AHL system, specifically their luxR homologues, to adapt to a new AHL over time. Leadbetter and colleagues[127] showed that when laboratory cultures of a bacterium, which normally utilize a 3-*oxo*-C6-AHL, were exposed over many generations to exogenous C8-AHLs, they developed increased sensitivity and gene activation for the latter AHL. Analyses indicated that only small changes (i.e., only three or fewer residue changes via mutation) in luxR proteins were needed to allow the bacterium *Vibrio fischeri* to adapt to the C8-AHL)[118] and later a C4-AHL.[128] An adaptive feature of such plasticity is that, in natural environments, bacteria are often in proximity to other bacteria (that utilize other AHL cues). Studies by Winans and colleagues[129,130] revealed that substitutions (or deletions) of an amino acid at specific locations on traR (i.e., a luxR-type transcription factor) were key to directing changes in the binding of the AHL to traR and in the subsequent binding of the traR/AHL complex to the promoter site. The ability of a bacterium to adapt rapidly to a new AHL provides a mechanism by which one bacteria may adapt to and form communicative associations with other bacteria and develop coordinated sets of activities. Hence it has been suggested that there may be a distinct evolutionary advantage to maintaining such plasticity (and adaptability) in the AHL system.[127]

Some bacteria (e.g. *Escherichia coli* and *Salmonella* sp.) do not synthesize their own AHLs, as evidenced by a lack of genes encoding AHL synthase enzymes (i.e. luxI homologues). Yet these same bacteria sometimes possess AHL receptor proteins (i.e., appropriate luxR homologues) that can perceive the AHL and effect the up-regulation of genes.[131] Such have been referred to as incomplete QS circuits.[132] Thus these bacteria can respond to AHLs released by other types of bacteria. Indeed, some bacteria are known to have more luxR homologues (i.e. AHL receptor proteins) than the number of recognized AHL synthases in the genome.[132,133] It has been proposed that extra types of luxR homologues may serve to detect (i.e., eavesdrop on) other types of bacteria.[134] The extra luxR homologues were initially termed "'*orphan proteins*,"[133] although their functions are now known[132] but may contribute to the expansion of regulatory networks or integrations of environmental controls to quorum sensing systems. Under natural conditions, the presence of multiple QS systems (and luxR orphans), and/or the ability to adapt to new AHLs could provide bacteria with the potential to interact with other, outsider bacteria. While researchers are starting to examine these possibilities, it is clear that QS regulated gene expression is much more complex than originally thought.[50]

3.2. Cues Generated from Environmental Substrates

Expression of AHL systems in some bacteria can be significantly affected by nutritional conditions[135] and hence the availability of external substrates. However, is it possible for cues to be constructed, in part, from molecules that are present in the natural environment? The synthesis of AHLs occurs through pathways involving the substrate SAM and an appropriate fatty acid, both of which are synthesized within cells.[136,137] If molecules from the natural environment can be used as specific substrates for the synthesis of cues, then there is the potential for production of environment-specific cues.

In support of this idea, it was recently determined that molecules from the outside environment can be used as substrates for the production of homoserine lactones by cells. A new class of homoserine lactone cues, called *p*-coumaroyl-homoserine lactones, were characterized from an anoxygenic phototrophic bacterium *Rhodopseudomonas palustris*.[34] Uniquely, the origin of *p*-coumaroyl-homoserine lactones is a substrate molecule, *p*-coumaric acid, which is sequestered from the outside environment (rather than from cellular-based fatty acid pools). This study was the very first to show utilization by cells of an environmentally derived substrate for the production of cue molecules. Given the plethora of different organic molecules that are present in extracellular environments, this can represent a means by which microbial cells can utilize alternative substrates (i.e., rather than intracellular fatty acids) to generate cues that reflect, and possibly develop, substrate-specific responses to their local environment.

3.3. Odd-Number Chain AHLs

Another interesting, but as yet poorly understood, aspect of AHLs that integrates with natural environments relates to the occurrence of odd-numbered acyl chains. There are now numerous reports of C7-AHLs that have been extracted from both laboratory cultures[44,119,138−142] and recently from natural environments.[47] As previously mentioned, AHLs have fatty acid based acyl chains. The mechanics of fatty acid synthesis typically involve the stepwise additions of two carbon units donated through acyl carrier proteins (ACP) units to nascent acyl chains. This results in the production of even-numbered carbon chains (e.g. 4, 6, 8 carbons).[143] AHLs are then synthesized by the amide-coupling of *S*-adenosylmethionine (SAM) and the acyl moiety of the cognate ACP.[144] Because the acyl chain of an AHL, in part, is a result of fatty acid synthesis[144,145] it might be expected that AHLs will primarily consist of even number chains (e.g. C4 to C18, etc). However, bacteria are known to produce odd-numbered fatty acids, but as a general rule, having relatively long chains (e.g., >C15) and in low abundances (i.e., 0.1% of total fatty acids).[146] Therefore, it is not known if the detected odd-numbered AHLs result from synthesis (e.g., use of propionyl-CoA and malonyl-CoA as acyl chain starter and extender units)[139] or are the result of postsecretion modifications. Speculatively, it is possible that some of these are the product of abiotic oxidation of the acyl side chain. Successive one-electron oxidation of alkanes and carboxylic acids is known to lead to the mineralization of organic carbon, a process that effectively converts methylenes and methyl groups to carbon dioxide.[147,148]

4. Natural Systems

4.1. General Considerations

Rather than living as homogeneous liquid cultures of cells as bacteria do when grown in the laboratory, bacteria in nature are often living within the enclosed confines of biofilms where they coexist as groups of cells surrounded with a matrix of extracellular polymeric secretions (EPS). The biofilm provides a three-dimensional architecture that can more efficiently localize chemical cues than open water.[149] As AHLs diffuse between/among cells they traverse the extracellular environment and encounter a range of concentrations of oxidants and ions, as well as changes in pH, all of which can be potentially destabilizing to AHL molecules. If signaling can be analogous to verbal language, then natural environments are filled with a cacophony of molecules that must be distinguished from and sorted. This assumes that the secreted AHL remains in its intact molecular form. However, the extracellular environment can modify AHLs and thus alter their activities through photochemical, physicochemical, and geochemical processes. This presents a likely possibility that the natural environment itself can be used as a selective filter to destroy or modify certain types of AHLs (and other cues) in a predictable manner, and in doing so, provide important sensing information (and/or feedback) to cells.

4.1.1. AHLs in Open Oceans

AHLs have now been extracted and characterized from many environmental isolates of bacteria that have been grown under laboratory conditions. To date, however, quorum sensing-like activities have been demonstrated, albeit indirectly, in only a few types of natural environments. In the open ocean environment, one of the most intriguing examples of natural quorum sensing was detected by satellite imaging where expansive areas approximating 15400 km^2 of the northwestern Indian Ocean exhibited bioluminescent surface waters over three consecutive nights.[150,151] This "milky ocean" phenomenon was thought to result from massive blooms of the luminescent bacterium *Vibrio harveyi* (i.e. having a total bacterial population approximating 4×10^{22} cells). Laboratory studies have shown that bacterial cells of *V. harveyi* produce light in the presence of low nm concentrations of a 3-hydroxybutanoyl-AHLs and a furanone borate diester, called AI-2.[152,153] Many anecdotal references to the milky ocean phenomenon have been noted over the centuries in ship captain's logs. Light production by symbiotic bacteria within the light organs of marine animals such as squid is also well documented.[154] Here, bioluminescence is a result of the luxR/luxI system and was first observed in the laboratory culture flasks of the *Vibrio fischeri*,[155] a bioluminescent commensal bacterium that inhabits the light organs of certain marine squid.[156]

4.1.2. Microbial Mats and Other Natural Biofilm Environments

AHLs[47] or evidence of their activities,[71,157] have been found in microbial mats and phytoplankton aggregates.[70] Isolates of bacteria from marine (snow) aggregates,[158] open waters,[159−161] and sponges showed AHL activities as determined by extractions and biosensors. These constitute the only reports to our knowledge of AHL-based quorum sensing evidence in natural environments at present. The putative occurrence of bacterial QS has been observed directly or indirectly in a number of natural environments. These have included detection using agar plate reporter assays,[71,157] extractions of AHLs from marine sediments (i.e., microbial mats),[47] and the presence of QS genes in arctic soils.[162]

Microbial mats typically contain very high diversities (e.g., thousands of different species) of bacteria. Therefore, mats have represented interesting sites to investigate chemical cues such as AHLs and microbial interactions. A bacterium living in a mat environment will be subjected to diel changes in geochemical conditions and may experience conditions that may alter signaling stability differently during day vs night. Microbial mats consist of horizontal layers of bacteria that are spatially organized into "functional groups" or "clades,"[58] which are groups of bacteria having similar biological (i.e. metabolic) activities. The activities of these microorganisms result in sharp gradients (e.g., pH, oxygen, sulfide) in the extracellular medium over microspatial (i.e. micrometer) scales.[74,163−165] Further, the aqueous environment in microbial mats is also influenced by photochemical and geochemical reactions that occur, often interactively, with biological reactions and products.[93,166,167] Here, different functional groups of bacteria may exist in microspatial (e.g. micrometers) proximity to each other.[74]

In terms of AHL stability, significant environmental extremes can be encountered while in mat environments. AHLs are produced by bacteria and possibly archaea in mats in hypersaline (i.e. high salt) ponds, where microorganisms encounter an extreme range of ion concentrations and even desiccation. Therefore, it is important to understand the stability of, and structural changes in AHLs that can occur under these environmental conditions. In hypersaline ponds, for example, the microbial communities, consisting of bacteria, archaea, and diatoms, spend intermittent periods at varying ionic conditions that can range from less than 10 g l^{-1} to over 350 g l^{-1} (salinity). Bacteria and diatoms are typically more active at lower ionic conditions, while archaea tend to be the most active group at higher ionic conditions in these systems. It is of interest to understand how such ranges in ionic concentrations may affect the structure, functions, and activities of AHLs. Llamas and colleagues[168] isolated C4-, C6-, C8-, and C12-AHLs from several isolates of a halophilic bacterium *Halomonas* sp. grown at 75 g l^{-1} salinity. Low osmolarity conditions may indirectly influence quorum sensing. Aldehyde content (i.e. a substrate for luciferase) in cultures of *V. fischeri* affected luminescence output by cells. Reducing the aldehyde content reduced luminescence output. While this effect was most pronounced at low osmolarity, it was independent of the *lux* promoter (genes).[156] In these systems, changes in ionic concentration can occur relatively rapidly (e.g. within days to weeks). Increases in salinity result from progressive evaporation and typically occur over weeks to months. Decreases in salinity, however, can occur quite rapidly due to rain events or rapid additions of ocean seawater (often adjacent to ponds) during storm events.[169]

In some mats, an alkaline pH (>9) exists for almost half of the day (i.e. 8−10 h)[57] and can potentially contribute to hydrolyses of AHLs.[47] The stability of an AHL against alkaline hydrolysis has been shown to vary depending on the acyl chain length.[47,62,63,170] AHLs having longer acyl chains (>12 carbons) tend to be more resistant to hydrolysis than their shorter chain counterparts. If cues are degraded

too rapidly by environmental conditions, they may not be produced rapidly enough by cells in order to affect autoinduction in neighboring cells. This would imply that the activity of shorter-chain AHLs could be restricted to times of low or no photosynthesis. During daylight conditions and its associated alkaline pH, only long-chain AHLs would remain largely intact. However, both longer-chain and shorter-chain AHLs might remain active during the slightly acidic conditions at night. A recent study confirmed that AHLs were relatively stable in seawater and that the relative stability increases with the chain length.[63]

The supersaturated O_2 concentrations during daylight facilitates the generation of oxidants through reactions involving carbonates, Fe(II), and certain types of organic matter and photochemically mediated Fenton reactions.[86] As a result, concentrations of oxidants (such as H_2O_2 or $OH^{\bullet}$) might reach high levels within mats. Sulfides, especially abundant during nighttime anoxia are highly reactive.[74] While the periodic geochemical changes in microbial mats are well documented, the effects of these changes on signaling molecules and their efficacy on QS require further study.

4.2. Interactions with Organic Matter

4.2.1. Protective and Confining Nature of the Biofilm EPS Matrix

Microbial cells growing under natural conditions abundantly release extracellular polymeric secretions (EPS), especially within biofilms.[149] The EPS are composed of both large and small molecules which surround microbial cells and impart important properties to attached- and aggregate-associated microbial communities.[171−173] This forms an often-protective three-dimensional gel-like matrix surrounding cells.[174] One result of the secreted matrix is to localize extracellular processes, such as extracellular enzyme production, sequestration of nutrients, pH, and ionic conditions.[175] Biofilm formation and the secretion of EPS has now been shown to be regulated by quorum sensing in various bacteria.[28,176] The in situ fabric of EPS has not yet been examined at high magnifications using cryo-electron tomography but will likely be important in regulating the movement of chemical cues within a biofilm. Thus far, examinations of the hydrated state of some EPS gels using atomic force microscopy (AFM) have shown that the matrix consists of an interlocking network of polymeric fibrils having solvent (water) cavities among the fibrils,[177] presumably through which diffusion of small molecules occurs through the gel.

The mobility of cues such as AHLs can be influenced strongly by the presence of an EPS matrix depending on its density, frequency of linkages, and composition. Biofilm EPS exists in different compositions and physical states and densities ranging from very loose soluble polymers to dense, cohesive gel states.[178] Differences in EPS density, composition, and properties can occur over microspatial scales and have been collectively referred to as EPS "microdomains."[178,179] The concept of EPS microdomains becomes important to the diffusive movement of AHLs and other types of signal cues. In turn, bacteria are capable of manipulating the gel−solution state of EPS through the actions of extracellular enzymes.[180] The extent to which this occurs, however, is not known.

A polymeric gel, owing to its inherent properties, can slow or even confine the diffusive movement of molecules such as an AHL. While in a dense gel state, frequent linkages are

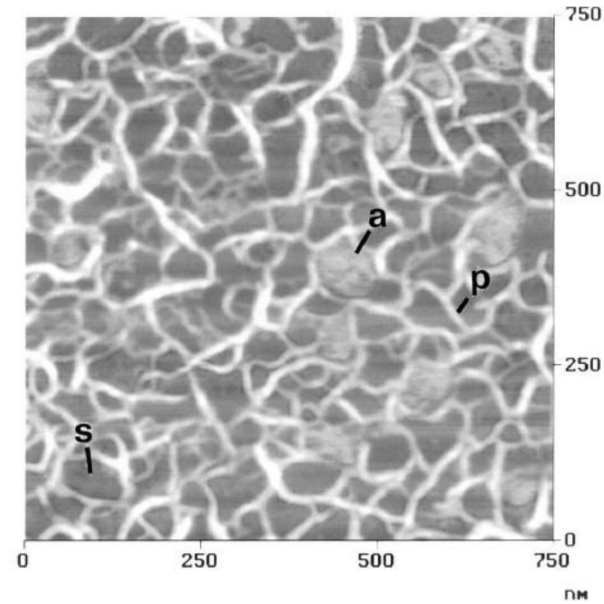


Figure 7. Atomic-force micrograph (AFM) showing the physical network structure of the EPS matrix through which AHLs will diffuse while traveling from cell to cell (p = EPS polymers, s = solvent cavities, a = artifact). Adapted with permission from ref 177. Copyright 1999 Elsevier.

formed between adjacent EPS molecules. The frequency of linkages and spacing between adjacent molecules will influence the ability of molecules, such as AHLs, to diffuse through the matrix (Figure 7). A second key issue of the EPS matrix influencing the diffusive movement of AHLs is the concentration of charged ions or groups within the matrix (i.e., charge density of the gel) relative to concentrations just outside the matrix. This is called the *Donnan potential* difference.[181] When Donnan potentials are elevated due to accumulations of charged ions within a gel (e.g. EPS), the diffusive movement of even small molecules may be reduced compared to that of water. Therefore, as an AHL diffuses through an EPS matrix, the AHL potentially can act as a nonreacting solute or sorb reversibly or irreversibly, and/or react stoichiometrically or catalytically with EPS or other biofilm components. This has been referred to as the reaction-diffusion mechanism.[182] Biofilm diffusion coefficients (compared with those of water) for various types of organic molecules have been measured or calculated and typically ranging from as high as 0.72 to 0.047 (water = 1.0) and have been shown to relate to the molecule's size, reactivity, and densities of the biofilm.[182−183,189] New approaches such as pulsed-field gradient nuclear magnetic resonance (NMR) are providing important information on the microspatial variability of diffusion within biofilms.[189] Many important structural features occur in the EPS matrix, which must be observed in situ and are lost upon their extraction.[149] It is now established that extracellular DNA (i.e., eDNA) is found in many EPS.[149] It is thought to contribute to the structural stability of the biofilm.[190] DNA contains many charged moieties and can contribute to Donnan potential differences. These features are relevant to the movement, accumulation, and chemical modifications of AHLs. Modeling studies showed that both diffusion and convection processes occur within the biofilm matrix.[191]

There is the potential for chemical signaling to occur over distances exceeding those which can be predicted for efficient three-dimensional diffusion. Eukaryotic cells are known to transport large molecules very rapidly using the process of

facilitated diffusion. Here, diffusion, which normally occurs in three dimensions, is reduced to either one or two dimensions. This is thought to occur by the presence of filament tracks using an energy dependent mechanism, which shuttles molecules in a particular direction and often at faster rates (i.e. 100−1000×) than those predicted by passive diffusion.[192] An interesting and recent study by Nielsen and colleagues showed that when the conditions in the water overlying sediments were changed, the metabolic processes of microbial communities deep in the sediment also changed. The changes, however, occurred more rapidly than could be accounted for by diffusion. It was postulated that some form of communication may have been occurring between the surface and subsurface microbes, perhaps via EPS "nanowires."[193] The exact mechanism remains to be verified. Currently, however, a great deal is not understood regarding the EPS matrix and its roles in chemical communication under natural conditions.

Within and outside of biofilms, AHLs can also be used as extracellular "sensors" that provide cells with information regarding their proximal environment such as local diffusion conditions.[194] This is a broader process that has been termed "efficiency sensing."[195] Gauging the relative diffusivity of molecules released by cells into the outside environment is important in assessing whether a cell should expend energy to conduct extracellular processes (e.g. production and release of extracellular enzymes, virulence factors, construction of biofilms). If a cue diffuses away rapidly, it may not be cost effective for a cell to produce and release extracellular enzymes. Keller and Surrette[35] calculated that energy expenditures involved in synthesizing cue-like molecules were comparatively less than those needed for producing enzymes. Within the confines of a biofilm, cellular expenditures of energy (e.g. for production and release of extracellular enzymes) should result in greater benefit when compared with cells in the open water. A final point to be noted is that the relative physiological status of natural bacterial cells can vary from a high-activity state to a relatively inactive state, the latter which have been termed as persister cells or viable by nonculturable states. It is not known how active a signal-receiving bacterium must be in order to receive and respond to signal cues.

4.2.2. Microspatial Patterning of Bacteria and Relationship to AHLs

A final important aspect of the EPS matrix and its role in QS is that the matrix provides a three-dimensional scaffold where microbial cells can move and orient themselves relative to one another.[149] Recent in situ imaging studies of natural biofilms using confocal microscopy have shown that microbial cells often exist in highly heterogeneous spatial arrangements.[47,196] In short, bacteria exist in dense clusters of cells or aggregations, with less dense cells existing in between clusters (Figure 8). The relative spacing of cells within a cluster is important in facilitating the rapid diffusion of cues between cells.[197,198] The microspatial arrangement of microbial cells within natural environments affects the ability of individual (and the collective group of) cells to gather nutrients and get rid of wastes and their abilities to exert positive or negative interactions. Recent studies have shown that under experimental conditions, bacteria migrate to cavities and other spaces to form clusters, a process that offers certain advantages.[199] Further, under experimental conditions, even a single cell can initiate self-induced quorum sensing.[200]

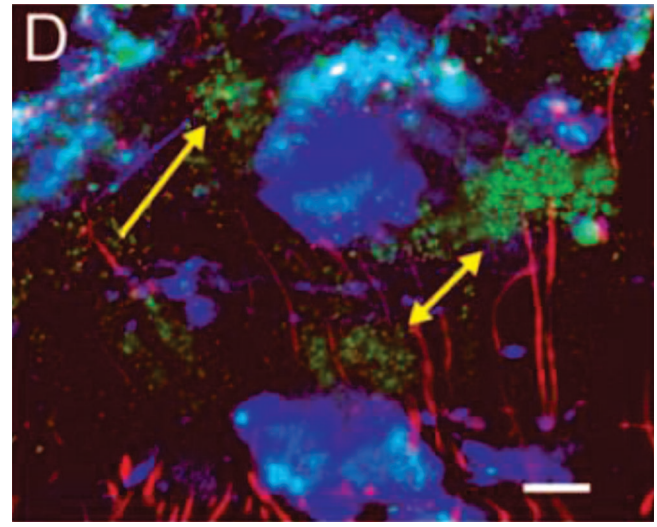


Figure 8. Confocal micrograph showing the microspatial heterogeneity that exists within a microbial mat. Dense clusters (yellow arrows) of individual bacterial cells (green dots) are observed and are likely places for chemical commnication. Cells are sulfate-reducing bacteria and were stained with a fluorescent 16S oligoprobe *dsrAB* (red = cyanobacteria; blue = $CaCO_3$ precipitates; scale bar = 10 μm) Adapted with permission from ref 47. Copyright 2009 John Wiley & Sons.

Modeling studies have shown that signaling among bacteria within a complex community is not necessarily required for the formation of stable, cooperative, and coordinated associations and even consortia.[201] However, aspects of community behavior may be enhanced by the ability to coordinate activities.[202] Recent sociobiology modeling studies of self-organization in biofilms predicted that certain levels of organization can arise without active coordination (e.g., signaling) and that biofilms should not develop entirely as cooperative units.[201,203] Cooperative associations are not always beneficial because cheaters (i.e., those who do not cooperate but gain from cooperation) will undermine the broader stability.[204−206] However, cooperative behaviors, cheating, and other social interactions tend to be enhanced in environments that exhibit relatively frequent disturbances[207,208] such as natural biofilms. It is suggested here that cooperation will occur transiently at small spatial scales (e.g. within clusters) but not among all cells in a biofilm. Hence, QS should be thought of as a network of signaling clusters occurring in small localized patches, and coinciding with the many clusters of cells that are observed within mats.[47]

5. Summary and Future Directions

There is an emerging realization that the process of QS is not a communication-only activity but rather represents a subset of much broader activities that are conducted by bacteria. A developing consensus is that AHLs and other classes of molecules that have been traditionally considered as signals additionally serve other functions to microbial cells such as diffusion and efficiency "sensors." Chemical alterations within the environment that occur postrelease of AHLs can limit the efficiency of their use in signaling but also contribute to their own sensing functions. However, given

that cues can travel relatively long distances, they could be utilized to sense collaborators, competitors, or their environment.[48]

The presence of AHLs has been directly observed in a limited number of natural systems. Given our present understanding of the physical/chemical stability and diffusivity of AHLs, it appears that QS in natural biofilms will be confined to relatively small-sized (e.g. 10s micrometers) patches containing clusters of cells and raises the additional possibility that chemical cues are altered by natural environments. Alterations of cues such as AHLs provide a mechanism for these molecules to be used as environmental sensors, especially if the alterations occur in a chemically predictable manner that reflects the proximal environment. If the chemical alterations are perceivable by receiver cells, then important environmental information is gained when compared to the exchange of intact cues. Little is known, however, about the range of functions of QS within natural systems. Understanding this will require more pointed determinations of how subtle environmental changes affect gene regulation. Also, if the environment alteration of cues occurs in a predictable manner, the modified cues could bind to different receptors and provide a unique flexibility for the regulation of gene expression in response to environmental changes. Finally, other important directions for future work include examinations of how the EPS matrix influences the dispersion and protection of AHLs and in the identification of "naturally produced" AHL analogues. Natural environments provide much inherent variability over microspatial (e.g., micrometer) scales and provide opportunities to investigate other roles for QS-related cues within microbial communities.

6. Acknowledgments

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7. Supporting Information Available

Tables showing a list of structures and important physical−chemical properties of acylated homoserine lactone (AHL) cues and their analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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