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Autoinducers extracted from microbial mats reveal a surprising diversity of *N*-acylhomoserine lactones (AHLs) and abundance changes that may relate to diel pH

Alan W. Decho,[1]* Pieter T. Visscher,[2] John Ferry,[3] Tomohiro Kawaguchi,[1] Lijian He,[3] Kristen M. Przekop,[2] R. Sean Norman[1] and R. Pamela Reid[4]

[1]*Department of Environmental Health Sciences, Arnold School of Public Health, and* [3]*Department of Chemistry and Biochemistry, College of Arts and Sciences, University of South Carolina, Columbia, SC 29208, USA.*

[2]*Department of Marine Sciences, University of Connecticut, 1084 Shennecossett Rd., Groton, CT 06340-6097, USA.*

[4]*Division of Marine Geology and Geophysics, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, USA.*

Summary

Microbial mats are highly structured and diverse communities, and one of the earliest-known life assemblages. Mat bacteria interact within an environment marked by strong geochemical gradients and fluctuations. We examined natural mat systems for the presence of autoinducers involved in quorum sensing, a form of cell–cell communication. Our results revealed that a diverse array of *N*-acylhomoserine lactones (AHLs) including C_4- to C_{14}-AHLs, were identified from mat extracts using mass spectrometry (MS), with further confirmation by MS/MS-collision-induced dissociation (CID), and additions of external standards. Microelectrode measurements showed that mats exhibited diel pH fluctuations, ranging from alkaline (pH 9.4) during daytime (net photosynthesis) to acidic (pH 6.8) during darkness (net respiration/fermentation). Under laboratory conditions, AHLs having shorter acyl-chains were degraded within the time frame that daily alkaline pH (> 8.2) conditions exist in mats. Intensive sampling of mats after full day- or night-time incubations revealed that accumulations of extractable shorter-chain AHLs (e.g. C_8- and C_{10}-AHLs) were significantly ($P < 0.001$) diminished during daytime. Our study offers evidence that stabilities of AHLs under natural conditions may be influenced by the proximal extracellular environment. We further propose that the ancient periodicity of photosynthesis/respiration in mats may potentially drive a mechanism for diel differences in activities of certain autoinducers, and hence bacterial activities mediated through quorum sensing.

Received 19 May, 2008; accepted 7 August, 2008. *For correspondence. E-mail awdecho@gwm.sc.edu; Tel. (+1) 803 777 6584; Fax (+1) 803 777 3391.

Introduction

Quorum sensing (QS) is a form of microbial chemical communication that relies on the production and release of molecular signals into the extracellular environment, and their subsequent concentration-dependent detection by proximal cells resulting in gene modulation (Fuqua *et al*., 1996; Fuqua and Greenberg, 2002). Bacteria are now known to employ a range of small molecules as signals (Camilli and Bassler, 2006).

The *N*-acylhomoserine lactones (AHLs) are a highly conserved class of QS signals used by a wide range of Gram-negative proteobacteria (Fuqua *et al*., 2001). They consist of a five-member homoserine lactone with an amide-linked acylated side-chain (Fuqua *et al*., 2001; Miller and Bassler, 2001; Waters and Bassler, 2005). When bacteria are present in high local abundances AHLs may accumulate to high concentrations (e.g. nM to μM) externally, and upon reaching a threshold concentration (nM) within cells form a complex with a regulator protein that induces changes in gene expression (Hanzelka and Greenberg, 1995). Laboratory culture studies have shown over 70 different species of bacteria that produce AHLs (Waters and Bassler, 2005), and the presence of complete QS circuits in 68 different bacterial genomes (Case *et al*., 2008) that have been sequenced thus far. To date, AHLs have not been found in *Archaea*.

Quorum sensing is thought to occur within bacterial communities under a range of natural conditions (Horswill *et al*., 2007). While a great deal has been learned from laboratory studies concerning the molecular mechanisms

of QS, comparatively little has been understood regarding AHLs and their functions in natural systems (McLean *et al*., 1997; Bachofen and Schenk, 1998; Miller *et al*., 2005; Horswill *et al*., 2007). During their movement through the extracellular environment [via diffusion or lipid vesicle carriers (Mashburn and Whiteley, 2005)] to neighbouring cells the physical/chemical integrity of signals may be compromised by rapidly changing geochemical/photochemical variables. An important step in assessing the relevance of bacterial signalling under natural conditions will be in understanding the persistence and modifications of signals within the '*in situ*' extracellular environments of microbiota.

Microbial mats are complex assemblages of spatially organized bacteria from different functional groups (Des Marais, 1990), and represent one of the earliest life assemblages extending 3.4–3.5 Gy bp (Shen *et al*., 2001; Tice and Lowe, 2004; Allwood *et al*., 2006; Canfield, 2006). Mats display the highest metabolic rates documented, and have had a substantial impact on the evolution of the Earth (Canfield *et al*., 2000; Jørgensen, 2000). These laminated, organo-sedimentary biofilms typically exhibit tightly coupled cycles of photosynthetic production and heterotrophic consumption (Des Marais, 1990) and sharp geochemical gradients over small (μm to mm) spatial scales. The high diversities, abundances and activities of cells within the different functional groups of mat communities (Ley *et al*., 2006; Feazel *et al*., 2008) present an ideal platform for initiating studies of QS in natural systems.

Earlier studies showed that the structural integrity of AHLs was susceptible to alkaline conditions (Voelkert and Grant, 1970; Yates *et al*., 2002). Photosynthetically dominated mat environments are characterized by sharp geochemical (e.g. pH, O_2) gradients, which fluctuate between acidic and alkaline conditions over a diel (i.e. 24 h) cycle (Des Marais, 1990; Visscher and van Gemerden, 1991). Hence, the strong fluctuations in pH that typically occur in mat environments may represent a substantial constraint to AHL-mediated QS.

Marine stromatolites are a type of laminated, lithifying microbial mat (Grotzinger and Knoll, 1999). The mats are composed of five major functional groups of bacteria: cyanobacteria, sulfate reducers [sulfate-reducing bacteria (SRB)], fermenters, aerobic heterotrophs, sulfur oxidizers (Reid *et al*., 2000), and the *Archaea* (R.S. Norman, unpublished). Together, the bacterial functional groups form three distinct microbial mat communities, termed Types 1, 2 and 3 (Reid *et al*., 2000). Repeated cycling of the three communities results in newly lithified layers (i.e. laminae), and ultimately, the upward growth of the stromatolite structure (Reid *et al*., 2000). Precipitated laminae are a defining feature of fossil stromatolites, and are currently thought to occur through closely regulated interactions of autotrophic production by cyanobacteria and heterotrophy, notably the SRB (Reid *et al*., 2000; Visscher *et al*., 2000). The Type-2 stromatolite mats (used in our study) contain an EPS-rich heterotrophic biofilm on their surface that is underlain by a dense layer of cyanobacteria, and exhibit precipitation of a microcrystalline layer of calcium carbonate (i.e. laminae).

Herein, the mat environments of marine stromatolites (Fig. 1) were examined for the presence and activities of AHLs in QS, and to assess how certain geochemical changes such as pH may influence their stability and accumulation. We specifically focused on the lithifying Type-2 mats for extractions of autoinducers because of their relatively high cell abundances and activities.

Results and discussion

Interactions among mat bacteria are likely complex, but poorly understood and occur within an environment marked by strong environmental fluctuations. Quorum sensing is a fundamental interaction among bacteria (Fuqua *et al*., 1996). Our results revealed that a diverse range of AHL autoinducers were extractable from natural microbial mats. The AHLs included C_4-, C_6-, *oxo*-C_6-, C_7-, C_8-, *oxo*-C_8-, C_{10}-, C_{12}- and C_{14}-AHLs, and were detectable from mat extracts using mass spectrometry (MS). This represents the first (to our knowledge) extractions and specific identifications of AHLs from microbial mats. Diel cycling within the mat environment appeared to significantly reduce the relative abundances of certain AHLs, through alkaline pH lactonolysis. This result was supported by concurrent geochemical measurements of pH in natural mats over a diel cycle, and by laboratory experiments of pH-mediated AHL degradation.

Characterizations of AHLs

Extractions using dichloromethane (DCM), and subsequent purification by LC/MS (i.e. liquid chromatography/mass spectrometry) (Fig. 2) revealed putative AHLs having retention times and a mass/charge (*m/z*) corresponding to AHL standards (Morin *et al*., 2003). In-source collision-induced dissociation (CID) was used for further spectral interpretation. Selectivity was based on the MS/MS fragmentation of the molecular $[M+H]^+$ ions, and on their relative intensities. Upon CID, the protonated precursor form of each AHL gave rise to the characteristic product ion *m/z* 102, a protonated homoserine lactone (Morin *et al*., 2003). Subsequent MS/MS fragmentation patterns of individual peaks corresponded to C_4-, C_6-, *oxo*-C_6-, C_7-, C_8-, *oxo*-C_8-, C_{10}-, C_{12}- and C_{14}-AHLs (Table 1). Each AHL that was identified showed the characteristic fragment ions including *m/z* 102, specific for the lactone (Morin *et al*., 2003; Fekete *et al*., 2007). Auto-

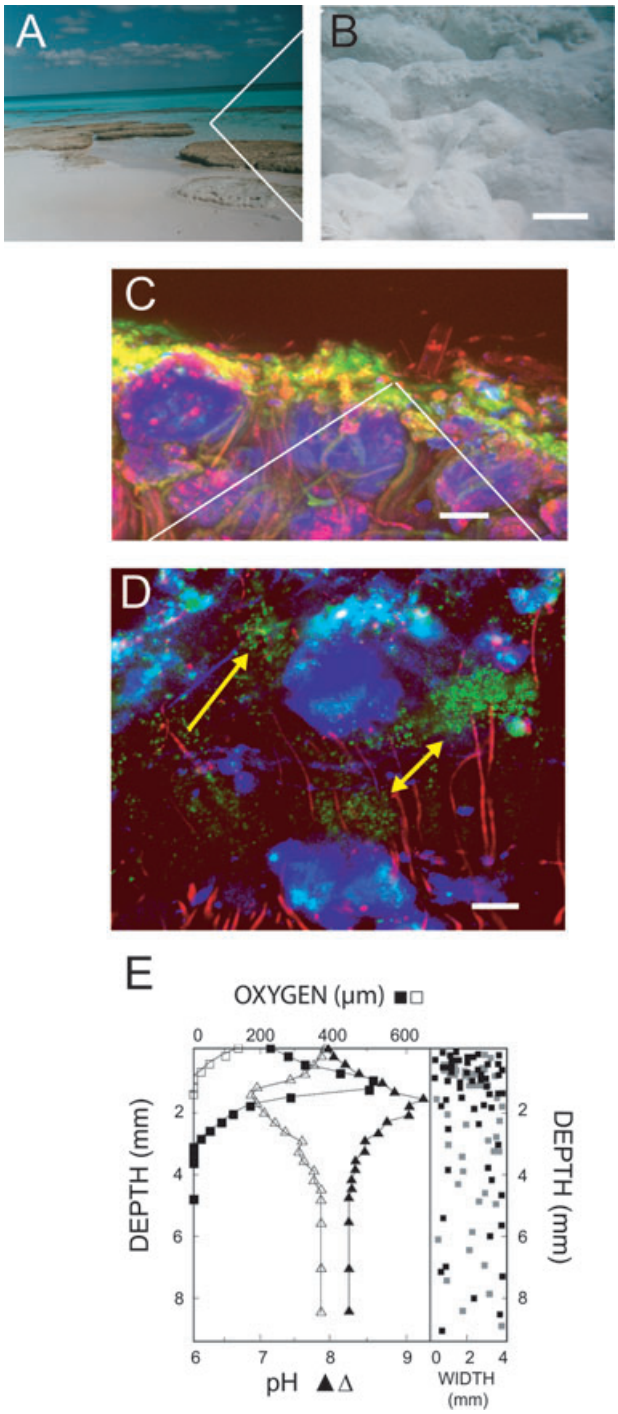

Fig. 1. Vertical zonation and activities of microorganisms within surface layers of marine stromatolite mats.
A. Stromatolite mats occur in a shallow, subtidal, open-water marine environment at the study site (Highborne Cay, Bahamas).
B. Mats form layered structures having repeating laminae of $CaCO_3$ precipitation. Activities of *N*-acylhomoserine lactone (AHL) autoinducers, involved in quorum sensing, were localized within the surface mat layer of stromatolites, where cyanobacteria and heterotrophic bacteria occur in close association (scale bar = 10 cm).
C. Low-magnification image of surface mat cross-section after fluorescence *in situ* hybridization (FISH) coupled to imaging by confocal scanning laser microscopy (CSLM). Sulfate-reducing microbial (SRM) community (dsrAB oligoprobe = green fluorescence), cyanobacteria (red filaments) and carbonate ooids (sediment grains in blue) abundantly occurred in this surface layer (scale bar = 50 µm).
D. Higher-magnification imaging reveals clusters (yellow arrow) of individual SRM cells (green) in proximity to carbonate precipitates (blue) within surface biofilm (scale bar = 10 µm).
E. During daylight hours, intense photosynthetic activity within surface mats produces alkaline pH conditions (day = ▲; night = △), as determined by microelectrode probing. Despite high O_2 concentrations (day = ■; night = □), SRM communities remain active during daylight, as revealed by silver-foil microautoradiography (panel on right; Visscher *et al.*, 2000). It is proposed that alkaline pH conditions generated by daytime photosynthesis result in selective hydrolysis of shorter-chain AHLs. This potentially limits the accumulation of certain AHLs to periods of darkness. Note that the scales of depth profiles of pH, O_2 and sulfide and sulfate reduction rates (expressed as pixels) are the same.

Table 1. *N*-acylhomoserine lactone (AHL) autoinducers detectable using mass spectrometry (MS) in sample extracts from Highborne Cay stromatolite mats.

	Parent ion	Fragmentation ions		
AHL	$[M+H]^+$ (*m/z*)	Lactone moiety (*m/z*)	Acyl-chain moiety (*m/z*)	Relative occurrence in samples
C_4-	172	102	71[a]	*
C_6-	200	102	99	**
3-*oxo*-C_6-	214.1	102	113	*
C_7-	213	102	112	**
C_8-	228.1	102	127	***
3-*oxo*-C_8-	242	102	141	*
C_{10}-	256	102	155	***
C_{12}-	284	102	183	**
C_{14}-	312	102	211	*

a. Exception C_4-AHL does not produce $[M=H-101]^+$. Instead $[M+H-18]^+$=154 was used.
Using collision-induced dissociation (CID), the $[M+H]^+$ ion, derived from the parent AHL molecule, decomposes into two 'fragmentation ion' products corresponding to the lactone moiety (*m/z* 102) and the acyl-chain moiety $[M+H-101]^+$. The relative occurrence of AHLs in samples ($n = 60$) are represented by: *, less common (occurrence in less than 30% of samples); **, common (approximately 30–70% of samples); ***, very common (70–100%). *m/z* = mass/charge ratio.

induction activities of putative signals in extracts were confirmed using thin-layer chromatography (TLC) bioassays (Shaw *et al.*, 1997) employing the sensitive bacterial reporter strain *Agrobacterium tumefaciens* NTL4 (pCF218) (pCF372) expression system for screening of AHL molecules (Fuqua and Winans, 1996).

Initial preliminary sampling revealed that detectable (i.e. using bioassays) accumulations of signals, which occurred within natural mat extracts, were localized to uppermost (< 1 mm depth) surface of Type-2 mats (Reid *et al.*, 2000). Previous studies showed that the surface biofilms of Type-2 mats contained high microbial cell

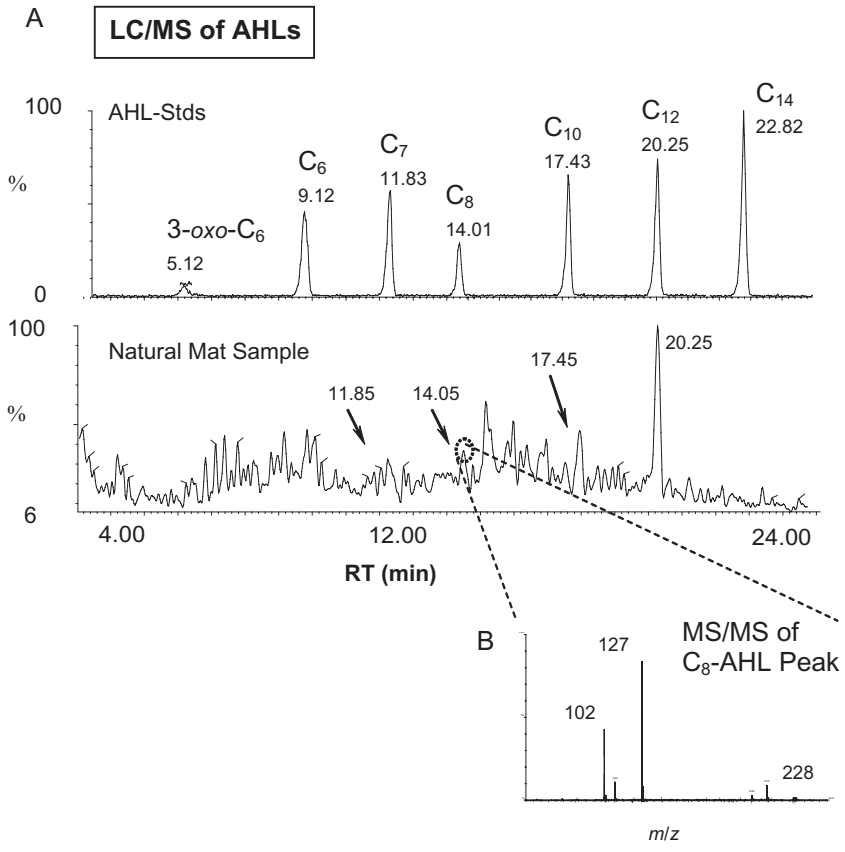

Fig. 2. A. Mass spectra (LC/MS) of an AHL sample extract (bottom) from a Highborne Cay stromatolite mat and AHL standards (top; C_4- to C_{14}-AHLs). Spectrum (shown here) represents a typical daytime natural mat sample, and contains numerous peaks using LC/MS, including peaks whose retention times (RT) corresponded to C_7-, C_8-, C_{10}- and C_{12}-AHL (stds).
B. MS/MS was used to fragment isolated peaks (e.g. C_8-AHL) from natural mat samples. Fragmentation spectra shows parent molecular ion $[M+H]^+$ ($m/z = 228$) of a putative C_8-AHL, and two fragmentation ion products corresponding to the lactone ($m/z = 102$) and acyl-chain ($m/z = 127$) moieties, which matches the fragmentation pattern of the C_8-AHL std.

abundances and activities, and suggested that SRB were a numerically dominant and metabolically active component (Visscher *et al*., 2000; Decho *et al*., 2005). Examinations of intact surface mats were conducted using fluorescence *in situ* hybridization (FISH) (Amann *et al*., 1999) coupled to imaging by confocal scanning laser microscopy (CSLM). Imaging using the oligoprobe dsrAB, which targets the sulfite reductase complex (Wagner *et al*., 1998; Zverlov *et al*., 2005), indicated that assemblages of sulfate-reducing microorganisms (SRM) were abundant and tightly clustered within the surface 130 μm of mats (Fig. 1C and D). This presented a potential microspatial landscape conducive to bacterial QS.

In order to assess the activities of SRM in this microspatial region, and their relative activities during light versus dark conditions, incubations were conducted using intact mat cross-sections and ^{35}S-sulfate-coated silver foil (Visscher *et al*., 2000). Results showed that sulfate-reducing activities peaked at the surface of the mat (Fig. 1E), just above the zone of maximum oxygen concentrations and pH (i.e. maximum photosynthetic production). The results also demonstrated that sulfate reduction occurred during daytime, that rates were higher during daytime than nighttime, and that activities were closely coupled to photosynthesis, which is typical for microbial mats (Canfield and Des Marais, 1991; Fründ and Cohen, 1992; Visscher *et al*., 1992; Jørgensen, 1994).

Microelectrode measurements of diel pH changes

It is well established that photosynthetic mats are fueled by autotrophic production (Des Marais, 1990). Temporal patterns of production and consumption result in strong diel fluctuations in O_2 and pH (Fründ and Cohen, 1992; Visscher *et al*., 2000; Megonigal *et al*., 2003) due to the net predominance of oxygenic photosynthesis during daylight and net heterotrophic respiration and fermentation during darkness. Microspatial associations of cyanobacteria with heterotrophic bacteria drive a strong diel coupling of autotrophic production and heterotrophic consumption (Revsbech *et al*., 1983; Decho *et al*., 2005).

Geochemical depth profiles of natural mats were conducted using microelectrodes (Visscher *et al*., 2000; 2002), and indicated strong diel shifts in pH, ranging from 6.8 to 9.4 (Fig. 1E). Profiles peaked close to the depth where the maximum oxygen concentrations were found (during daylight). Prior work has shown that this coincides with the zone of maximum photosynthesis (Canfield and Des Marais, 1991; Fründ and Cohen, 1992; Visscher *et al*., 2002). Previously, microelectrode profiles revealed

Table 2. The pH-mediated hydrolysis over time of *N*-acylhomoserine homoserine lactones (AHLs) having different acyl-chain lengths (C_6–C_{14}).[a]

	AHL[a]						
pH	C_6	C_7	C_8	C_{10}	C_{12}	C_{14}	*n*
6.18	79 ± 0.01	150 ± 0.01	89 ± 0.01	53 ± 0.01	64 ± 0.01	75 ± 0.001	3
7.2	34 ± 0.01	34 ± 0.01	28 ± 0.01	23 ± 0.01	27 ± 0.01	23 ± 0.01	24
8.2	4.2 ± 0.02	4.2 ± 0.03	5.7 ± 0.02	8.7 ± 0.02	22 ± 0.01	36 ± 0.01	24
8.7	2.4 ± 0.02	2.3 ± 0.02	3.1 ± 0.02	4.6 ± 0.02	10.9 ± 0.01	36 ± 0.01	24
9.55	0.52 ± 0.09	0.50 ± 0.06	0.73 ± 0.18	1.15 ± 0.04	1.10 ± 0.09	11.4 ± 0.11	3

a. AHLs having different acyl-chain lengths, designated as C_6–C_{14}, were used to determine the effect of pH on AHL hydrolysis. Values expressed as half-life ($t_{1/2}$) in hours, represent mean ± SE (*n* = sample size).

that alkaline conditions (i.e. > pH 8.2) typically prevailed in these surface mats for approximately 8–10 h per day (Visscher *et al*., 2002).

Laboratory studies showed that alkaline pH conditions resulted in hydrolysis of the lactone moiety of an AHL (Voelkert and Grant, 1970). It was assumed here that hydrolysis of an AHL lactone deactivated its signalling capabilities (Dong *et al*., 2000; Yates *et al*., 2002). Agar-plate bioassays, conducted using alkaline-treated AHLs and the reporter strain *A. tumefaciens* (as previously described), confirmed this deactivation (data not shown) (Kawaguchi *et al*., 2008). Given the magnitude of the pH range of the mats, it was likely that the structural integrity, and hence activities, of the AHLs may be influenced on a daily basis by pH changes within photosynthetic microbial mats. If AHLs were being constantly produced by cells, but rapidly degraded by alkaline pH conditions, they may not reach locally high enough (*c.* nM) concentrations to achieve autoinduction in neighbouring cells. Therefore, the question arose: Do signals produced within a microbial mat persist long enough to accumulate to sufficient extracellular concentrations to stimulate autoinduction?

Laboratory studies of pH-mediated AHL hydrolyses

The acyl side-chain of known AHLs ranges in length from 4 to 18 carbons, and confers specificity to the AHL signal (Camilli and Bassler, 2006). Therefore, it was important to determine if AHLs having different acyl-chain lengths were equally susceptible to pH-mediated hydrolysis, especially over the relevant diel time frames (e.g. 8–10 h) that alkaline conditions occur in natural mats.

GC/MS analyses were used to track, with high temporal resolution (i.e. <0.5 h), the degradation (i.e. via lactonolysis) of AHLs under different pH conditions. Degradation half-lives ($t_{1/2}$) ranged from approximately 30 min to > 100 h (Table 2). Differential degradation of AHLs, at a given pH, occurred as a function of acyl-chain length (Table 2). This result was consistent with previous work (Voelkert and Grant, 1970). Relatively rapid hydrolyses occurred in AHLs having acyl-chain lengths less than C_{10}. Degradation of C_6- to C_{10}-AHLs occurred within the same time frame (i.e. < 10 h) during which natural mat communities experienced alkaline conditions (Fig. 3), as evidenced by our microelectrode data (Fig. 1E). AHLs having longer acyl-chains (C_{12} to C_{14}) were significantly less susceptible to hydrolysis of the lactone. The C_4- could not be analysed using our GC-based approach.

Day versus night changes in mat AHLs

Given the well-established diel cycle of alkaline/acidic pH conditions that occurs in photosynthetic mats (Des Marais, 1990), the shifts should differentially affect the persistence and accumulation of AHLs, depending on their acyl-chain length. Our laboratory-based results predicted that in natural mats, *in situ* concentrations of shorter-chain AHLs should be higher after a period of full darkness (i.e. approximately 10–12 h), and comparatively lower after a period of full daylight (i.e. approximately

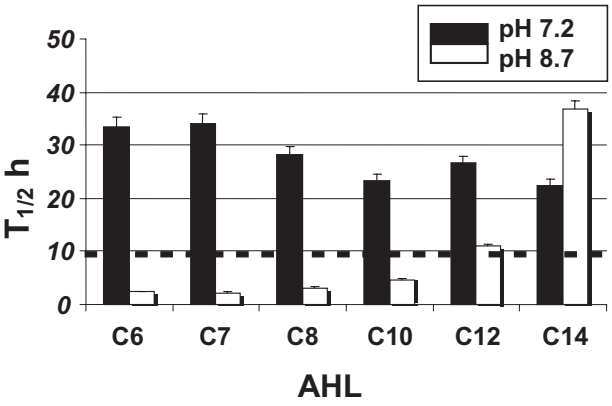

Fig. 3. Graph illustrating the relative degradation of AHLs having different acyl-chain lengths (C_6–C_{14}) when exposed to slightly acidic (pH 7.2) and alkaline (pH 8.7) conditions. Degradation is expressed as half-life ($t_{1/2}$) in hour. Bars contained within the box (i.e. hatched line) indicate that significant degradation of AHLs may occur within the approximate 10 h daily time period in which alkaline conditions (i.e. > pH 8.2) occur in stromatolites mats. Each value represents the mean ± SE. [Correction added on 10 November 2008, after first online publication: the x-axis labels for Figure 3 were corrected.]

10–12 h). Assuming that bacteria produced AHLs at a near constant rate over a 24 h cycle, changes in day versus night concentrations should largely reflect degradation.

We examined changes in the relative abundances of extractable AHLs from natural mats after day versus night exposures. A separate and more intensive sampling approach (i.e. five replicates, with three pooled samples per replicate) allowed us to quantify the two most abundant AHLs (i.e. C_8- and C_{10}-AHLs) present in extracts. Results of two-way analysis of variance (ANOVA) showed a significant main effects term, indicating that significant ($P = 0.0481$) decreases occurred in mean concentrations of C_8-AHL (*c.* 45% decrease) and C_{10}-AHL (*c.* 31% decrease) in samples extracted immediately following daylight incubations (Fig. 4). Calculations of C_8- and C_{10}-AHL concentrations were based on an extraction efficiency of *c.* 48%, as determined using external standards (i.e. C_8 and C_{10}).

It follows that on a daily basis, higher abundances of these two AHLs will typically occur at night, then decrease during daylight conditions. While it is conceivable that differential AHL synthesis may occur as a function of energy levels or light-mediated phenomena, we were not able to experimentally distinguish such effects in our study. We expect that other geochemical and photochemical (e.g. UV, oxidants) factors additionally may have contributed to the observed results. Finally, AHLs may be enzymatically degraded by bacteria after uptake and hydrolysis using intracellular lactonases (Leadbetter and Greenberg, 2000; Lee *et al*., 2002; Lin *et al*., 2003; Riaz *et al*., 2008; Uroz *et al*., 2008) or amidohydrolases (Dong *et al*., 2000; 2001). We did not measure lactonase activities in our study, therefore we cannot discount the potential contribution of enzymatic degradation of AHLs by microbiota.

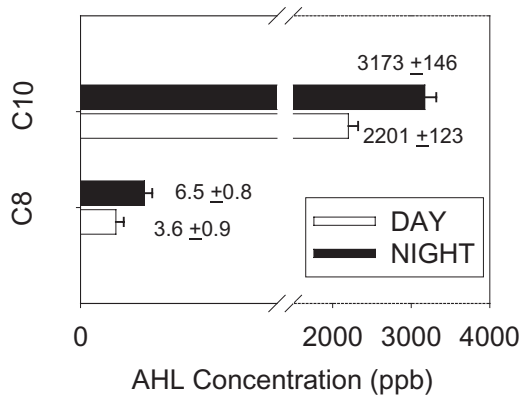

Fig. 4. Concentrations of two most abundant AHLs, extracted during day versus night conditions from natural microbial mats. Values are expressed in p.p.b. as mean ± SE ($n = 5$). Each *n* measurement is the result of three-pooled sample cores (8.0 mm diameter, approximately 1 mm depth), triple-extracted in dichloromethane/water, then quantified against standards using LC/MS and Q-TOF MS/MS (mass spectrometry). Values were standardized according to sediment dry weight. C_8- and C_{10}-AHLs refer to: *N*-octanoyl-L-homoserine lactone and *N*-decanoyl-L-homoserine lactone respectively.

Accurate and precise measurements of QS signals are complicated by the inherent microspatial (i.e. micrometer-scale) variability in microbial processes that occurs within a microbial mat. This presents an obstacle to estimating the relevant (to QS) concentrations of AHLs experienced by cells under natural conditions. For example, the coring devices used for mat sampling (in AHL extractions) were relatively large (e.g. 8 mm diameter), while many clusters of microbial cells (Fig. 1D) that were observed using CSLM were comparatively small (e.g. 10–60 μm diameter). Cell distributions typically exhibited much microspatial heterogeneity (i.e. clustering) within the mats (A.W. Decho, in preparation). AHL molecules will likely accumulate to higher concentrations within small 'clusters' (e.g. tens of micrometres) of bacteria, when compared with areas having lower cell densities. Therefore, a given sample effectively represents the 'mean AHL concentration' estimated over several millimetres of heterogeneously distributed cells. Additionally, we expect that AHLs will be localized by the diffusion-slowing properties of the EPS matrix of mat biofilms (Decho, 1990). The protective effect against hydrolysis of AHLs by bacterial EPS in mat biofilms is not known and is currently under investigation.

It was noteworthy that the C_7-AHL was relatively common, though not abundant, in mat extracts, occurring in approximately 60% of samples (Table 1). The C_7-AHL has been relatively uncommon in most known laboratory strains of bacteria that produce AHLs (Horng *et al*., 2002; Cataldi *et al*., 2007; Poonguzhali *et al*., 2007). It is not known if this signal may represent a partial degradation product of a longer-chain AHL, especially since fatty acid chains are typically synthesized having even numbers (e.g. C_6, C_8, C_{10}, etc.) (Parsek *et al*., 1999). Also, C_4-AHLs were not detectable in most samples (< 10%). This was likely due to our use of DCM as a solvent (Pearson *et al*., 1999). Multiple AHL signals were detectable in extracts from natural mats. However, we expect that many bacterial functional groups may have contributed to the detectable AHL pool.

Interestingly, the SRM resided in the uppermost mat surface where highly oxic (and alkaline pH) conditions exist during daylight. This was evidenced by FISH/CSLM using the dsrAB oligoprobe, which targets the dissimilatory (bi)sulfite reductase gene complex. This includes many lineages of SRB, and Archaea (Wagner *et al*., 1998; Loy *et al*., 2004; Zverlov *et al*., 2005; Leloup *et al*., 2007; Lücker *et al*., 2007). We suspect that the SRM, which were abundant in our surface mats, may have contributed to our detectable AHL pool. However, AHLs have not yet

been isolated from the deltaproteobacteria or other SRM groups. Production of AHLs by SRB is currently under examination (Decho/Norman/Visscher). Our results showed that the SRM were also most active during the day, although they are physiologically challenged by the presence of O_2 (Fournier *et al*., 2003). It has been proposed that SRM, especially the SRB, may benefit from close, proximal associations with other bacteria such as sulfide oxidizers, or may utilize specific metabolic alternatives to cope with high O_2 levels (Cyprionka, 2000; Fournier *et al*., 2003; Baumgartner *et al*., 2006).

Our results present the likely possibility that bacterial communities utilize QS within the surface microbial mats of stromatolites. The diversity and range of long- and short-acyl-chain AHL signals that are found in stromatolite mats may have evolved to operate with a periodicity determined, in part, by diel geochemical cycles within their environment. Since alkaline pH is destructive to shorter-chain AHLs, bacteria may utilize diel pH periodicity to alternate their day/night autoinduction of specific sets of genes. It is proposed herein that bacteria may differentially exploit AHLs as autoinducers based on their relative proclivity to decay in response to alkaline conditions. The differential susceptibility of signals to lactonolysis may result in a biogeochemically mediated mechanism to permit a short temporal window for signalling. Alkaline hydrolyses during daylight conditions would limit accumulation (and autoinduction activities) of short acyl-chain AHLs to night-time, when more acidic pH conditions and a net predominance of respiratory pathways prevailed in mats. In contrast, intercellular signalling using longer-chain AHLs may potentially occur over the entire diel cycle (i.e. even during the high-pH conditions of daylight). Such a mechanism would allow shorter-chain AHLs to autoinduce genes only during periods of darkness, while other, longer-chain AHLs may autoinduce throughout the diel cycle. Thus, bacteria within mats could utilize signalling pathways that are closely in tune with the diel cycling of autotrophic production and heterotrophic respiration.

Quorum sensing influences a wide range of microbial processes including biofilm formation, exopolysaccharide secretion and virulence (Davies *et al*., 1998; Fuqua *et al*., 2001; Taga and Bassler, 2003; Bassler and Losick, 2006). The functions of genes that are regulated by AHL-mediated QS in this system are not yet known, and are currently under investigation. Given the recent realization of the very high diversities of microbial mats (Ley *et al*., 2006) it is likely that different bacterial species, within or even between functional groups, may communicate with each other using the same AHL signal(s). Recent studies have found that AHL-dependent QS systems are able to adapt rapidly in order to respond to the presence of new bacteria and their AHLs (Collins *et al*., 2005). This provides potential for facilitating the development of (and adaptation of existing) consortia. Also, some bacteria have been shown to possess parallel or hierarchical QS systems which allow cells to integrate multiple signals, and thus enabling greater flexibility in their regulation of genes (Henke and Bassler, 2004). While our study has focused on AHLs, it is likely that other forms of signal molecules, such as AI-2, AI-3, peptides (Camilli and Bassler, 2006), and even metabolites (Monds and O'Toole, 2008) may be utilized within mat communities.

It has been suggested that autoinducers (e.g. AHL) may additionally function as diffusion probes (Redfield, 2002) and chemical cues (Keller and Surette, 2006). A recent study by Gantner and colleagues showed that AHLs associated with bacteria on plant roots operated most efficiently over short distances (e.g. 4–12 μm), termed 'calling distances' (Gantner *et al*., 2006). However, cell-to-cell communication also occurred over much longer distances, extending up to 78 μm, and far outside the boundaries of quorums (clusters of cells). It was suggested that such long-distance sensing would allow bacteria to mount a response to the presence of a few neighbouring competitors or collaborators (Gantner *et al*., 2006). Within the dense and diverse communities of bacteria in mats, calling distances may provide important spatial information for bacteria to constructing and maintaining assemblages and consortia. Also, the structural integrity of a signal from a source bacterium may be chemically modified by distance, time or the environment. It is prudent to speculate that the biogeochemical hydrolyses of shorter-chain AHL, relative to longer-chain AHLs, or other potential modifications could provide a further mechanism for spatial and even diel sensing activities.

Finally, the important role of microbial mats in the Earth's evolution (Des Marais, 1997) makes these systems an intriguing platform from which to study complex signalling interactions. Our study suggests that chemical communication may have evolved relatively early in the history of life. This is evidenced by the early appearance of microbial mats in the fossil record (Des Marais, 2000; Shen *et al*., 2001; Canfield, 2006).

Experimental procedures

Sampling location

All sampling of mats was conducted in a subtidal (i.e. approximately 10–20 cm depth at low-water) marine environment at Highborne Cay in the Exuma island chain of the Bahamas (24°42′N, 76°51′W) (Fig. 1) that has been previously described (Reid *et al*., 2000). All samples were collected from Type-2 stromatolite mats, which posses a developing surface precipitate layer of $CaCO_3$ (Reid *et al*., 2000). Initial laboratory sample processing was conducted aboard the R.V. Walton Smith (University of Miami). For further details see http://www.stromatolites.info.

Confocal microscopy and FISH

Samples of freshly collected intact stromatolites were trimmed with a sterile razor into blocks (approximately $2 \times 2 \times 2$ cm), and immediately fixed in 6% (w/v) paraformaldehyde in filtered (0.2 μm) seawater (SW). Samples were returned to the field laboratory (i.e. R.V. Walton Smith) and sliced into vertical approximately 3-mm-thick vertical cross-sections, gently placed between adjacent glass-slides, then returned (in 6% paraformaldehyde in SW) to the Decho laboratory for later analyses. Mat cross-sections were prepared for FISH (Amann *et al*., 1999), and observed using CSLM (Decho and Kawaguchi, 1999). Oligoprobes were custom-synthesized (GeneDetect (Auckland, New Zealand) using 16S rRNA oligonucleotide ProbeBase sequence of probe dsrAB (GD1001-CS with GreenStar*™ FITC fluorescent labelling) designed to target the dissimilatory (bi)sulfite reductase genes (*dsrAB*) of many recognized lineages of SRB (Wagner *et al*., 1998; Minz *et al*., 1999; Zverlov *et al*., 2005; Lücker *et al*., 2007) and likely other groups of SRM (Loy *et al*., 2004; Leloup *et al*., 2007). Appropriate nonsense controls were used for each. DAPI (4′6′-diamidino-2-phenylindole) and propidium iodide (Molecular Probes, Eugene, OR) were used for general bacteria staining.

Extraction of AHLs

Samples were collected from mats by cutting approximately 4×4 cm ($\times$ 2 cm depth) sections of mats using a sterile razor, then quickly returned to the laboratory. Under a dissecting microscope, the uppermost surfaces (approximately 1–2 mm) of the mats were removed, and immediately placed in DCM (20 ml). Extraction of AHLs was conducted using standard protocols (Shaw *et al*., 1997; Schaefer *et al*., 2000; Zhu *et al*., 2003), except that a 1:1 DCM : water solvent system was used instead of ethyl acetate. DCM was dried under a gentle flow of filtered (0.2 μm) N_2 gas, and stored at −20°C. All samples were triple-extracted, and all resulting AHL values were standardized according to sediment dry weight.

Characterization using MS

All standards and mat extracts were analysed at the USC Mass Spectrometry Center (Department Chemistry and Biochemistry). Identification of AHLs was determined using a Waters Micromass Quattro LC triple quadrupole mass spectrometer (LC/MS) system. All samples were chromatographed by HPLC (ES Industries AquaSep C18 column; mobile phase flow rate of 0.2 μl min^{-1}), and prepared with 20% acetonitrile for MS analysis. Effluent was ionized by electrospray ionization (ESI) and detected in the positive ion mode (Morin *et al*., 2003). Limits of detection (LOD) and quantification (LOQ) for analytes were calculated with a signal-to-noise ratio of 3.3 and 10 respectively. Mass spectrometry was conducted in multiple reaction monitoring (MRM) mode using two characteristic transitions for each AHL. Identifications of putative AHLs were made using three complementary approaches: (i) comparisons were made using ion product peaks of AHL standards and putative AHL peaks derived from extracts, (ii) analyses of in-source CID ion products were used to assist in AHL identification – a cone voltage of 40 V was employed to perform CID, and (iii) additions of external standards (i.e. AHLs) to existing samples. A full scan, ranging from *m/z* 100 to 350, was used to acquire MS spectra, and precursor ion scan mode was employed to obtain MS/MS data.

AHL standards. A total of nine different AHLs were analysed and used the following AHL standards (Sigma Chem., St Louis, MO): C_4 = *N*-butanoyl-L-homoserine lactone; C_6 = *N*-hexanoyl-L-homoserine lactone; 3-*oxo*-C_6 = *N*-(3-*oxo*-hexanoyl)-L-homoserine lactone; C_7 = *N*-heptanoyl-L-homoserine lactone; C_8 = *N*-octanoyl-L-homoserine lactone; 3-*oxo*-C_8 = *N*-(3-*oxo*-octanoyl)-L-homoserine lactone; C_{10} = *N*-decanoyl-L-homoserine lactone; C_{12} = *N*-dodecanoyl-L-homoserine lactone; and C_{14} = *N*-tetradecanoyl-L-homoserine lactone.

External standard additions. To further confirm precursor ion identity (i.e. putative AHL peaks determined by MS) and peak locations, mat extract samples were re-analysed by MS after additions of external standards (C_4- to C_{14}-AHLs). To determine AHL extraction efficiencies, adjacent samples of mats homogenates were then spiked with AHL standards (1 mg each, of C_8- and C_{10}-acyl homoserine lactones), extracted and analysed by MS.

Activities of AHLs. Activities of AHLs were determined using TLC bioassays (Shaw *et al*., 1997; Zhu *et al*., 2003) using the reporter bacterium, *A. tumefaciens* NTL4 (pCF218) (pCF372) for screening of AHL molecules (Fuqua and Winans, 1996).

Intensive day versus night AHL sampling

A separate and more intensive sampling approach was used to quantitatively examine the *in situ* concentrations of extractable AHLs that accumulated in mats during daylight conditions and night-time conditions. Samples were at two times: (i) 'Night'; mat samples collected just prior to sunrise (06:00 h) after a diel period of full ambient darkness (approximately 10 h), and (ii) 'Day'; mat samples collected (17:00 h) after exposure to full ambient sun (approximately 10 h). Samples were collected using a Harris corer (8 mm diameter, Ted Pella). Samples were collected to a depth of approximately 1–2 mm, and immediately placed in DCM. A total of five replicates were collected per sample time. Each replicate consisted of three-pooled (8 mm core) subsamples, which were randomly collected from a mat surface. All samples were triple-extracted (as described above). The two most abundant AHLs (i.e. C_8- and C_{10}-AHLs) present in extracts were identified by MS. All resulting AHL concentration values were standardized according to sediment dry weight. External standards were used to determine extraction efficiencies. Standardized concentration values were analysed using a two-way factorial analysis of variance (ANOVA) (SAS, 2004). Results were analysed for significance of the main effects [i.e. time (day versus night) and AHLs], and interaction terms.

Determination of pH, O_2 and sulfate reduction activity

Mapping of sulfate reduction rates within stromatolite mats was conducted using silver foil coated with $Na_2{}^{35}SO_4$, following

determination of geochemical depth profiles (Visscher *et al*., 2000). The two-dimensional distribution of sulfate reducing activity was measured with a strip of silver foil (20 by 50 mm, 50 μm thick) coated with $Na^{35}SO_4$ (0.25 mCi foil^{-1}; GE Healthcare-Amersham Biosciences). Following determination of geochemical depth profiles, a stromatolite mat sample was cut near the location where the microelectrodes were deployed. The surface of the mat was marked on the Ag foil and the sample was incubated in the dark under a *c*. 20% oxygen atmosphere for 6 h, after which the $^{35}SO_4^{2-}$ was washed off. The two-dimensional distribution of $Ag^{35}S$ was digitized within 5 days using a Bio-Rad Molecular Imager System GS-525. Pixel intensity was displayed as black (high activity) or grey (low activity) (Fig. 1E, right). Depth profiles of O_2 and pH were obtained using both glass (UniSense) and needle (Diamond General) microelectrodes in combination with a motor-driven micromanipulator (National Aperture). Polarographic measurements for O_2 were carried out with a picoammeter (UniSense PA 2000), and pH measurements using a high-impedance mV (Microscale Measurements) system.

Previously, profiles of O_2, S^{2-} and pH were measured in Type-2 mats during an entire diel cycle (Visscher *et al*., 2002). Here, we performed measurements during peak photosynthetic activity and at the end of the dark period to verify the values measured earlier. Depth profiles were determined in triplicate with a vertical resolution of *c.* 100 μm during the end of the dark period and the middle of the afternoon under ambient light and temperature conditions in samples submersed in *c.* 5 cm of water (Visscher *et al*., 2000).

Laboratory experiments of pH-mediated AHL hydrolysis

Laboratory AHL hydrolysis experiments were carried out in 40 ml EPA vials with Teflon-lined septa. Solution pH was adjusted using 0.02 N HCl or NaOH at 26 ± 1°C. Samples were extracted using methylene chloride, and concentrated with nitrogen gas to approximately 1 ml. Gas chromatography (i.e. Varian 3800 GC) with coupled MS/MS (i.e. a Varian Saturn 2000 MS/MS system) was employed for direct AHLs separation and determination, modified from Cataldi and colleagues (2007), using phenathrene-d10 as an internal standard. A fused silica capillary column (i.e. J&W Scientific 30 m DB-5MS, 0.25 mm internal diameter and 0.25 μm film thickness) was used with He carrier gas (constant flow = 1.3 ml min^{-1}). The operation conditions were as follows: He carrier gas was set at a constant flow rate of 1.3 ml min^{-1}. The GC injection port and transfer lines were operated isothermally at 220°C and 280°C respectively. The oven temperature programme was held at the initial temperature at 90°C for 2 min, then increased to 280°C at 15°C min^{-1}, then held at that temperature for 3 min. The ion trap and the manifold temperature were set at 220°C and 80°C respectively. The ion trap was operated in selective ion mode. Degradation half-lives ($t_{1/2}$) were calculated as the time for loss of half of activity of each AHL.

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