

RESEARCH ARTICLE

Diverse profiles of *N*-acyl-homoserine lactone molecules found in cnidarians

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Abstract

Many marine habitats, such as the surface and tissues of marine invertebrates, including corals, harbour diverse populations of microorganisms, which are thought to play a role in the health of their hosts and influence mutualistic and competitive interactions. Investigating the presence and stability of quorum sensing (QS) in these ecosystems may shed light on the roles and control of these bacterial communities. Samples of 13 cnidarian species were screened for the presence and diversity of *N*-acyl-homoserine lactones (AHLs; a prevalent type of QS molecule) using thin-layer chromatography and an *Agrobacterium tumefaciens* NTL4 biosensor. Ten of 13 were found to harbour species-specific, conserved AHL profiles. AHLs were confirmed in *Anemonia viridis* using liquid chromatography tandem mass spectrometry. To assess temporal role and stability, AHLs were investigated in *A. viridis* from intertidal pools over 16 h. Patterns of AHLs showed conserved profiles except for two mid-chain length AHLs, which increased significantly over the day, peaking at 20:00, but had no correlation with pool chemistry. Denaturing gel electrophoresis of RT-PCR-amplified bacterial 16S rRNA showed the presence of an active bacterial community that changed in composition alongside AHL profiles and contained a number of bands that affiliate with known AHL-producing bacteria. Investigations into the quorum sensing-controlled, species-specific roles of these bacterial communities and how these regulatory circuits are influenced by the coral host and members of the bacterial community are imperative to expand our knowledge of these interactions with respect to the maintenance of coral health.

Introduction

There is a growing appreciation for the fundamental dependence that all biomes have upon their microbial constituents, which holds true for many marine ecosystems (Azam & Worden, 2004). Bacteria are ubiquitous in the marine environment, colonising eukaryotes and exhibiting a range of interactions from beneficial mutualism to pathogenic relationships, which often affect their eukaryotic host. While a wealth of knowledge exists for a few well-studied model symbioses, such as the interaction between *Vibrio fischeri* and *Euprymna scolopes* (e.g. Ruby & Lee, 1998), little is known about the interactions that occur within most marine bacterial communities or the structure and maintenance of the relationship between these communities and eukaryotic hosts, such as corals

(Knowlton & Rohwer, 2003; Weis *et al.*, 2008; Kvennefors *et al.*, 2012).

Corals harbour diverse microbial communities (Rohwer *et al.*, 2002) which carry out functions such as nitrogen fixation, nitrogen cycling and sulphur cycling (Lesser, 2004; Raina *et al.*, 2009). They may also protect the coral from potential pathogens by producing antimicrobial compounds (Nissimov *et al.*, 2009; Shnit-Orland & Kushmaro, 2009) and exhibiting antibiofilm activity (Thenmozhi *et al.*, 2009). Findings that particular bacterial communities are often specific to specific species of coral (e.g. Ritchie & Smith, 1997; Littman *et al.*, 2009) and that particular bacterial groups are found across many species of coral (e.g. Bourne & Munn, 2005; reviewed by Mouchka *et al.*, 2010) strengthen the concept that specific bacterial–coral associations may play fundamental roles in

coral health, particularly as the alteration of these communities often coincides with a change in health status of the coral host (Bourne *et al.*, 2008). Interactions within these bacterial communities and between bacteria and the coral host may select for certain bacterial symbionts that provide nutritional and protective benefits (Ritchie, 2006). Therefore, elucidating the mechanisms behind these interactions, such as bacterial signal exchange, may provide a greater insight into the driving forces in coral reef ecology (Golberg *et al.*, 2011) and the possibility for the biological control of some coral diseases (Teplitski & Ritchie, 2009).

Quorum sensing (QS), or cell-to-cell communication is a well-studied form of bacterial chemical communication that relies on the production and release of extracellular signal molecules and their subsequent concentration-dependent detection and allows bacteria to alter behaviour on a population-wide scale (Williams, 2007). Phenotypic activities such as surface attachment, biofilm formation, virulence and antibiotic production are known to be under quorum sensing control (Williams *et al.*, 2000; Dunne, 2002; Miller *et al.*, 2002; Zhu & Mekalanos, 2003; Diggle *et al.*, 2007; Duerkop *et al.*, 2009). Many of these phenotypes may be important in maintaining coral-associated bacterial communities and protecting the coral host. However, while much laboratory-based information exists on quorum sensing, comparatively little is known about how quorum sensing operates in the complex setting of the natural environment (Horswill *et al.*, 2007) and how it affects microbial diversity and function (Valle *et al.*, 2004).

N-acyl-homoserine lactone (AHL)-based communication systems in Gram-negative bacteria represent one of the most intensively studied classes of QS systems. They are used by a wide range of *Proteobacteria* (Fuqua & Greenberg, 2002; Williams, 2007) and have more recently been found in *Cyanobacteria* (Sharif *et al.*, 2008) and the *Bacteroidetes* (Huang *et al.*, 2008; Romero *et al.*, 2010; Twigg *et al.*, 2013). These bacterial groups are found in abundance in coral-associated bacterial communities (Rohwer *et al.*, 2002). AHLs have also been suggested to play an important role in cross-kingdom interactions between bacteria and eukaryotes in the marine environment, for example, in mediating the settlement of larvae of the polychaete *Hydroides elegans*, the bryozoan *Bugula neritina* (Dobretsov *et al.*, 2007) and the barnacle *Balanus improvisus* (Tait & Havenhand, 2013) and zoospores of the macroalga *Ulva lactuca* (Joint *et al.*, 2002). In addition, AHLs have been found to promote the release of carpospores from the red alga *Acrochaetium* sp. (Weinberger *et al.*, 2007). Marine organisms also produce compounds known to interfere with AHL signalling (reviewed by Dobretsov *et al.*, 2009), such as the halogenated

furanones produced by the red alga *Delisea pulchra* (Givskov *et al.*, 1996; Rasmussen *et al.*, 2000) and manoa-lide monoacetate found in the marine sponge *Luffariella variabilis* (Skindersoe *et al.*, 2008). Such studies suggest that the presence and control of AHL signalling may play an important role in the ecology of higher organisms within the marine environment.

However, AHLs are highly susceptible to rapid, base-catalysed abiotic degradation, losing their activity. They are unstable and short-lived in aqueous media with pH levels in the range of seawater (Yates *et al.*, 2002; Tait *et al.*, 2005). Recent evidence suggests that abiotic degradation of AHLs alone may occur more slowly than predicted in natural seawater; however, enzymatic degradation of AHLs has been suggested to play an important role in the high AHL degradation seen in seawater (Hmelo & Van Mooy, 2009). Bacteria capable of this type of degradation also appear to be unusually prevalent within marine systems (Romero *et al.*, 2011). The limited density of bacteria encountered in the open ocean and low chemical stability of AHLs in seawater have led to the suggestion that AHL-mediated QS activity may be concentrated in specific microhabitats (Hmelo & Van Mooy, 2009), such as marine biofilms (Huang *et al.*, 2007; Tait *et al.*, 2009), marine snow (Gram *et al.*, 2002), the surfaces of algae, and associated with invertebrates such as sponges and corals. Although AHL-producing bacteria have been isolated from corals (e.g. Golberg *et al.*, 2011), there is little evidence to date to demonstrate that QS is active *in situ* in bacteria associated with higher marine organisms.

To better understand the role of QS in marine microhabitats, this study investigated the presence and diversity of AHLs in 13 species belonging to four orders of the Phylum *Cnidaria*, Class *Anthozoa*. The temporal role and stability of AHLs *in situ* was studied in the snakelocks anemone, *Anemonia viridis*, in intertidal pools over a 16-h period. Patterns of AHL molecules were recorded alongside denaturing gradient gel electrophoresis of the bacterial 16S rRNA to determine bacterial community composition and compared with the changing pool chemistry recorded over the section of the diurnal cycle.

Materials and methods

Cnidarian sampling

Replicate samples were collected from a number of cnidarian species from a number of sources (Table 1). Sections were removed from colonies aseptically and treated as described in Table 1. To compare the AHL profile in different tissues of *A. viridis*, tentacles were collected alongside whole individuals. For the temporal study of

Table 1. Summary of cnidarian species tested for the presence of putative AHLs with TLC

	Species	Sample wet weight (g)	Method	AHL signal detected	AHLs detected	Chain length	Replicated
<i>Gorgonacea</i> (seafans)	<i>Eunicella verrucosa</i>	2.0*	I (A1)	Yes	2	M, L	No replicates
	Gorgonian sp.	2.4–6.0*	D (A2)	No	–	–	Yes
<i>Alcyonacea</i> (soft corals)	<i>Discosoma</i> sp. (1)	0.1–0.79	D (A2)	Yes	2	M, L	Yes
	<i>Discosoma</i> sp. (2)	3.2	D (A2)	Yes	2	L	No replicates
	<i>Sinularia</i> sp.	8.0	D (A2)	Yes	2	M, L	No replicates
	Mushroom coral	4.5	D (A2)	No	–	–	No replicates
<i>Scleractinia</i> (hard corals)	<i>Balanophyllia regia</i>	5.1*	F (NE1)	Yes	1	L	Replicates combined
	<i>Pocillopora</i> sp.	0.085–0.73	I (NE2)	No	–	–	Yes
	<i>Favia</i> sp.	0.016–0.088	I (NE2)	Yes	1	M	Yes
	<i>Acropora eurystoma</i>	0.21–0.29	I (NE2)	Yes	2	M	No
	<i>Stylophora pistillata</i>	Standardised by protein content	I, F (NE2)	Yes	2	M, L	Yes
<i>Actinaria</i> (sea anemones)	<i>Anemonia viridis</i>	0.6–17.0	I, F (A2, NE1)	Yes	1–6	S, M, L	Yes
	<i>Aptasia</i> sp.	1.6	D (A2)	Yes	4	S, M, L	No replicates

Table summarises sample weight, methods used, including when tissue homogenisation was carried out after collection [on frozen tissue (F), immediate analysis (< 2 min; I) or delayed analysis (< 30 min; D)] and whether the samples were from the aquarium (A) or the natural environment (NE), detection of signal, number of putative AHLs detected (based on spot number), estimated chain length of putative AHLs (short (S), C2–C6; medium (M), C6–C10; long (L), C10–14; estimated by their position relative to standards) and whether the AHL profile was repeatable. For *Sinularia* sp. and *Aptasia* sp., where TLC plates were smeared, only distinguishable AHLs have been counted. Aquarium samples were collected from Plymouth Marine Laboratory, UK (A1), the National Marine Aquarium, Plymouth, UK (A2). Samples from the natural environment were collected from South-West England (NE1) and the Red Sea, Eilat, Israel (NE2). * denotes fragments that contained high percentages of skeletal material.

the stability of AHLs within *A. viridis*, five rock pools of varying size (pool 1: 4 × 3 m; 2: 4 × 1 m; 3: 0.3 × 0.3 m; 4: 3 × 0.5 m; 5: 1 × 4 m), substratum (pool 1–2, sediment; pool 3–5, rock) and position (1, 2: low shore; 3, 4, 5: mid shore) on the shore were tagged with small floating buoys at Mount Batten, Southwest England (50°21'25.78"N 4° 7'39.28"W), on 10 September 2011. One *A. viridis* (brown variety) was collected from each pool at low tide (08:00), and a further individual was collected every 3 h until 23:00, to include a tidal cycle and periods of light and dark, with sunlight from 06:45 (dawn) to 19:45 (dusk). Samples taken for the last two time points were taken after dusk. All samples were washed three times in 0.2 µm filtered, sterile phosphate-buffered saline (PBS; pH 6.5), immediately snap frozen in liquid nitrogen and stored at –80 °C [shown to not affect AHL detection with *Stylophora pistillata* samples (Fig. 1c)]. Salinity, temperature, pH (YSI 63), dissolved oxygen (YSI 85) and access of the pool to the main body of water were recorded at each time point.

***Anemonia viridis* RNA extraction and reverse transcription**

On ice, two cuttings of tentacles (0.03 g each) were taken from each sample using sterile, RNase-treated equipment, and RNA was extracted using the RNeasy extraction kit (Qiagen). Samples were first placed in 1 mL RTL buffer

(from kit) with 10 µL 2-mercaptoethanol and homogenised using a needle homogeniser (1 min). Homogenate was then drawn into a sterile syringe and expelled five times before centrifuging at 18 000 g for 3 min. The manufacturer's protocol for total extraction of RNA from animal tissues was then followed. Samples were eluted in 60 µL RNase-free water and stored at –80 °C. RNA was quantified by spectrophotometry (Nanodrop 1000, Thermo Scientific) and diluted to 39 ng µL^{–1} in RNase-free water. cDNA was synthesised using 0.1 µL of diluted RNA using the QuantiTect Reverse Transcription kit (Qiagen) with the provided blend of oligo-dT and random primers.

***Anemonia viridis* PCR amplification and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA**

A nested PCR approach (Mühling *et al.*, 2008) was used to amplify 16S rRNA for DGGE firstly using 0.2 µM primers 9bfm and 1512uR (5' GAGTTTGATYHTGGCT-CAG-3' and 5' ACGGHTACCTTGTTACGACTT-3', respectively), 2 mM MgCl₂, 0.2 mM of each dNTP, 1× PCR buffer (Promega, Southampton, UK), 0.25U of Go Taq Flexi (Promega) and 0.4 µg cDNA in a 25 µL reaction using the following conditions: 1 cycle at 96 °C for 4 min; 35 cycles at 96 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min and one final extension cycle at 72 °C

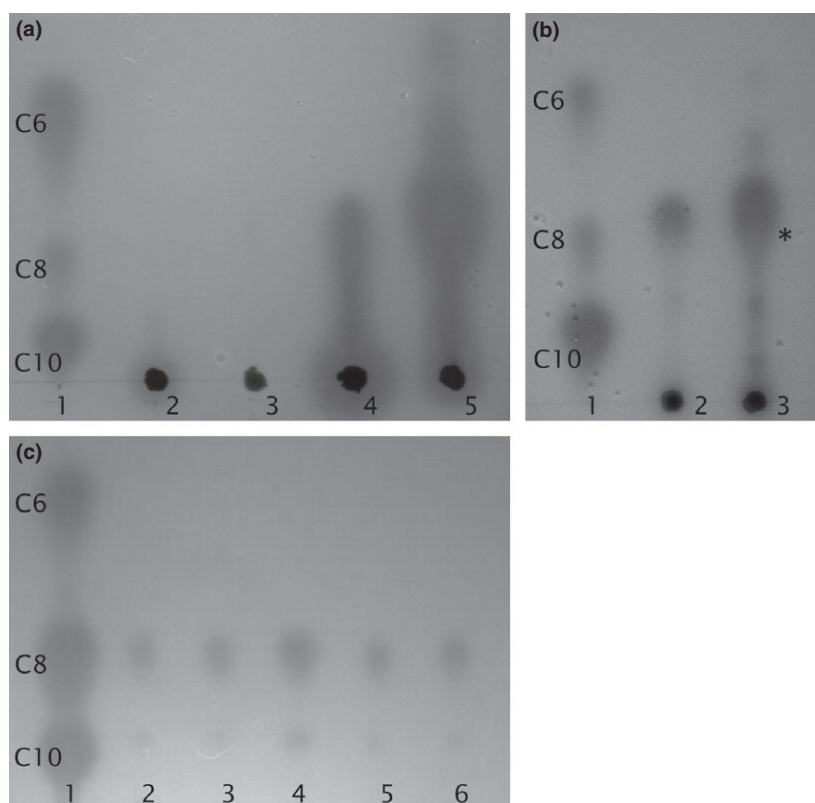


Fig. 1. Reverse phase Si thin-layer chromatography profiling of AHLs extracted from cnidarians. Five microlitre of *N*-acyl-homoserine lactone synthetic standards were used as markers: 5 μ M *N*-(3-oxohexanoyl)-L-homoserine lactone (C6), 0.2 μ M *N*-(3-oxooctanoyl)-L-homoserine lactone (C8) and 0.5 mM of *N*-(3-oxodecanoyl)-L-homoserine lactone (C10) (Lane 1). Images show profiles from *Discosoma* sp. (a2), Mushroom coral (a3), *Sinularia* sp. (a4), *Aptasia* sp. (a5); *Anemonia viridis* (green variety) tentacles (0.6 g; b2) and whole individual (17 g; b3); and five fragments of *Stylophora pistillata* (natural environment samples taken from three colonies on different days; frozen, C2-4 (colony 1, 2 and 3, respectively) and analysed within 2 h (maintained in aquaria), C5-6 (colony 1 and 2, respectively). Star represents AHL with tail shape.

for 5 min. Each PCR was conducted in triplicate. PCR products were then diluted 1 : 10 with dH₂O and re-amplified using primers 341f (5'-CCTACGGGAGG-CAGCAG-3') with a 40-bp GC clamp and 907r (5'-CCGTCAATTCMTTTGAGTTT-3'; Mühling *et al.*, 2008). The reaction mixture contained 0.5 μ M of each primer, 0.15 mM of each dNTP, 1.5 mM MgCl₂, 1 $\times$ PCR buffer, 0.15U of Go *Taq* Flexi and 1 μ L cDNA in a total volume of 60 μ L. Temperature cycling for PCR amplification was 1 cycle at 94 $^{\circ}$ C for 5 min; 32 cycles at 94 $^{\circ}$ C for 1 min, 65 $^{\circ}$ C for 1 min (decreasing by 0.5 $^{\circ}$ C every cycle with the last 10 cycles at 55 $^{\circ}$ C) and 72 $^{\circ}$ C for 1 min, and then one final extension at 72 $^{\circ}$ C for 7 min. PCR products were pooled, quantified by spectrophotometry (Nanodrop) and diluted to 20 ng μ L⁻¹ in nuclease-free water. DGGE was performed using the INGENYphorUTM System (Ingeny, Netherlands) with 15 μ L PCR products and an 8% polyacrylamide gel with urea and formamide as denaturants (20–60% gradient), at 60 $^{\circ}$ C with a constant voltage of 60 V for 18 h. Subsequently, gels were stained with ethidium bromide (50 μ g mL⁻¹) for 1 h and then washed with dH₂O for 30 min. Samples were run twice to check for reproducible sample patterns. Prominent DGGE bands were excised, re-amplified in a PCR using primers 341f and 907r (as above, but without the GC clamp) and directly sequenced on an ABI 3730 XL

automated sequencer (LGC Genomics, Germany). Only one strand of the DNA fragment was sequenced, proving sufficient for taxonomic identification. The 16S rRNA sequences were compared with GenBank sequences (NCBI database) using the BLAST algorithm and aligned using CLUSTALW in MEGA 4 (Molecular Evolutionary Genetics Analysis; Tamura *et al.*, 2007), with the closest match to sequences from BLAST searches. Sequences were submitted to GenBank under accession numbers KC862081–KC862091.

Sample homogenisation and *A. viridis* protein analysis

Cnidarian samples were either homogenised immediately after collection, after a maximum of 30 min or snap frozen and homogenised once removed from the freezer (Table 1). If homogenisation was delayed (30 min), cnidarian samples were kept on ice to reduce bacterial activity (including that of AHL-degrading enzymes). All cnidarian samples were washed three times in 0.2 μ m filtered, sterile phosphate-buffered saline (PBS; pH 6.5), placed in 2 mL sterile PBS (pH 6.5) and homogenised until no visible tissue remained. For the temporal study, samples were removed from the freezer, defrosted on ice, homogenised (1 min) and 100 μ L of *A. viridis* samples

was placed in a microcentrifuge tube and kept at 4 °C in the dark (24 h) for protein analysis. Total protein was measured using Bradford quick start dye reagent using the manufacturer's protocol (Bio-Rad). Protein values were taken in triplicate, with a blank of sterile PBS. Bovine serum albumin (BSA) was used to create a standard curve, achieving an R^2 of 0.98 before using this to determine protein content of samples.

Dichloromethane extraction of AHLs

Dichloromethane (10 mL) was added to all cnidarian samples immediately after homogenisation to extract AHLs. The mixtures were shaken vigorously for 1 min, centrifuged at 4 000 g for 5 min and the solvent layer removed. This process was repeated three times, the solvent layers combined, evaporated to dryness and the dried extract re-suspended in 50 µL acetonitrile.

Visualisation of AHLs from cnidarian tissue

The extracts were applied to RP18 F₂₅₄ reverse phase Si thin-layer chromatography (TLC) plates (20 cm × 20 cm; VWR International). For *A. viridis* samples, the amount of sample applied was normalised to 2.8 mg total protein. A mobile phase of 60% (v/v) methanol was used to separate AHL molecules by acyl chain length (number of carbons) and carbon 3 substitution as seen in Shaw *et al.* (1997). TLC plates were overlaid with the sensitive biosensor *Agrobacterium tumefaciens* NTL4 (pCF218)(pCF372) (Fuqua & Winans, 1996), following the methodology of Mohamed *et al.* (2008). Following incubation at 30 °C overnight, the plates were examined for the presence of blue spots, indicative of AHL production. *N*-acyl-homoserine lactone synthetic standards were used as markers to allow estimation of putative AHL chain lengths by comparing their migration distance to that of the standards [5 µL of stock containing 5 µM *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), 0.2 µM *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL) and 0.5 mM *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C10-HSL)]. Area-density analysis of spots on IMAGEPRO PLUS software determined the amount and detectable presence of various putative AHLs.

LC-MS/MS identification of acyl-homoserine lactones from cnidarian tissue

AHLs were identified by liquid chromatography in tandem with mass spectrometry detection. The HPLC system used was a Shimadzu SIL-HTc autosampler with two Shimadzu LC-10ADvp pumps. Chromatographic separation was achieved with a Phenomenex Gemini C18

reversed phase column (3.0 µm, 100 × 3.0 mm) using a constant mobile phase flow rate of 450 µL min⁻¹. Mobile phases consisted of aqueous 0.1% (v/v) formic acid (A) and 0.1% (v/v) formic acid in MeOH (B). The binary gradient began initially at 10% B and ran isocratically for the first 1 min before increasing linearly to 99% B over 9 min. After a further 5 min at this composition, the gradient was returned to 10% B over the next 1 min and allowed to re-equilibrate for 4 min. The MS system used was a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer. MS detection was operated in a multiple reaction monitoring (MRM) mode, screening the LC eluent for all unsubstituted AHLs, 3-oxo-AHLs and 3-OH-AHLs with acyl chain lengths of 4, 6, 8, 10, 12 and 14 carbon atoms long (Ortori *et al.*, 2011).

Statistics

GelCompar (Applied Maths) was used to identify DGGE bands within the bacterial profiles and construct a binary matrix based on the presence and absence of aligned bands. In Primer-E6 (Clarke & Gorley, 2006), a Bray-Curtis similarity matrix of DGGE bands (presence/absence data) and a Euclidian distance similarity matrix (square root transformed) of AHL profiles were performed. Ways of reducing the dimensions of the environmental variables were investigated using primary components analysis (PCA); however, Eigen values were low, and 3 axes were required to explain 70% of the variability. PERMANOVA analysis of AHL profiles with time point and pool as fixed terms was therefore used to establish differences between pools and sampling times. Covariates (normalised environmental factors) were added to the model one by one to assess their importance in determining AHL profiles. The lack of replication was dealt with by PERMANOVA by excluding the highest order interaction term (pool × time). The violation of sphericity, which will affect the distribution of *F* values in PERMANOVA, was accounted for using a strict critical level of 0.01 for *P*-values to determine significant results. Hierarchical clustering (group average) was used to visualise similarities in AHL profiles and bacterial communities between samples. SIMPROF tests tested for structure in the subset of data corresponding to each branch of a dendrogram (1000 permutations, significance level 5%). To assess variability (as a measure of disturbance) of anemone bacterial communities and AHL profiles between pools and sampling times, multivariate dispersion (MvDISP) was performed. RELATE was used to investigate similarity between the AHL profile and bacterial community matrices. MINITAB 6.0 was used to carry out a two-way ANOVA to establish differences in the presence of prominent DGGE bands between pools and sampling times.

Results

Presence of putative AHLs in cnidarian samples

TLC was used to separate putative AHLs, detected using the sensitive *A. tumefaciens* reporter; this confirmed the presence of putative AHLs in 10/13 (77%) of the cnidarian species tested. A diverse range of putative AHLs of various chain lengths were detected in both natural and aquarium cnidarians; representative results are shown in Fig. 1. Autoinduction activities were detected in fragments as small as 0.016 g, suggesting high concentrations of these molecules in the tissues. Presence and diversity and putative chain length of AHLs are summarised in Table 1.

Repeatability and species specificity of AHL profiles

The majority of areas with biosensor induction occurred with similar migration distances to the mid- and long-chain synthetic AHL standards 3-oxo-C8-HSL and 3-oxo-C10-HSL on TLC plates suggesting the presence of mid- and long-chain AHLs in cnidarian extracts (Table 1). In contrast, putative short-chain AHLs were only detected in the *Actinaria*, a result that is unrelated to fragment weight (Table 1). TLC analysis also revealed spots with tail shapes, for example in *Anemonia viridis* (Fig. 1b3, represented by *), which could indicate the presence of a 3-oxo- substitution on the AHL compound (Shaw *et al.*, 1997).

TLC plates suggested different degrees of diversity of putative AHLs between extracts, varying from 1 to 6 different spots depending on the origin of the extract (Table 1). Most abundant levels of AHLs and greatest diversity were detected in the *Alcyonacea* and the *Actinaria*. In the *Actinaria*, analysis of whole individual (Fig. 1b2: 4.45–17.1 g) vs. tentacles (Fig. 1b3: 0.6–0.97 g) showed AHLs to be concentrated in the tentacles when related to weight ($n = 3$). All putative AHL profiles from fragments of the same cnidarian species showed good reproducibility when extracted from individuals on different days (*A. viridis*, $n = 3$ and *S. pistillata*, $n = 3$) and after different methods of analysis (snap-frozen and fresh samples of *S. pistillata*).

Temporal and spatial stability of AHLs in *A. viridis*

To further investigate spatial and temporal stability of AHLs within a cnidarian host: bacterial consortium, *A. viridis* was chosen due to the level of AHLs detected in previous analysis and its availability locally. Presence of putative AHLs was assessed in whole *A. viridis* from five

rock pools of varying size, substratum and position. Samples were taken to include a tidal cycle and periods of light and dark, with samples for the last two time points taken after dusk. Due to position, pools 3, 4 and 5 (mid shore) were subjected to longer periods without connection to the main body of water at low- and mid tide compared with pools 1 and 2 (low shore).

Profiles of detectable AHLs remained relatively stable in anemones from different pools; representative results are shown in Fig. 2. Four distinct spots were identified on TLC plates, suggesting the production of a variety of AHL molecules by bacteria associated with *A. viridis*. Slight variability in the position of biosensor induction areas in some samples is likely to be either the result of other compounds in the sample affecting migration or the presence of a modified form of AHL. For the purpose of this analysis, the former was assumed. All four induction areas were found in line with, or close to, the mid- and long-chain synthetic AHL standards 3-oxo-C8-HSL and 3-oxo-C10-HSL. However, not all areas of induction were found in each sample (Fig. 2).

Even though detectable AHL profiles remained relatively stable in all samples, some variation was observed over the monitoring period. This was quantified in a crude analysis of induction area of the biosensor using area–density analysis with IMAGE PROPLUS software, where values corresponded to AHL detected from 2.8 mg *A. viridis* protein (Fig. 3). Induction areas were classified due to their position on TLC plates and labelled spots 1–4 (Fig. 3).

Spot 1, located above AHL standard 3-oxo-C8-HSL on the TLC plates, was found in all samples tested. With the

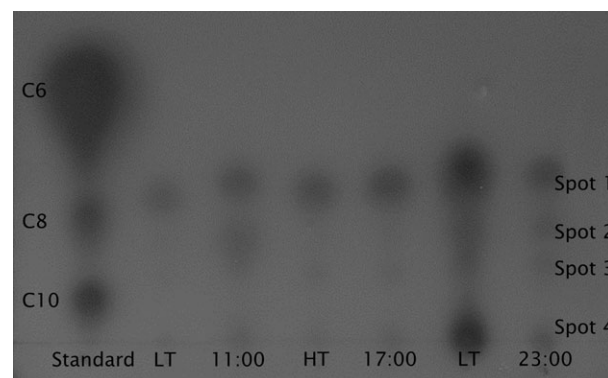


Fig. 2. Reverse phase Si thin-layer chromatography profiling of AHLs extracted from *Anemonia viridis*. Standard lane shows 5 μ L of AHL synthetic standard made up of 5 μ M *N*-(3-oxohexanoyl)-L-homoserine lactone (C6), 0.2 μ M *N*-(3-oxooctanoyl)-L-homoserine lactone (C8) and 0.5 mM of *N*-(3-oxodecanoyl)-L-homoserine lactone (C10); and dichloromethane extract corresponding to 2.8 mg total protein from *A. viridis* in pool 4 at 08:00 (LT), 11:00, 14:00 (HT); 17:00, 20:00 (LT) and 23:00.

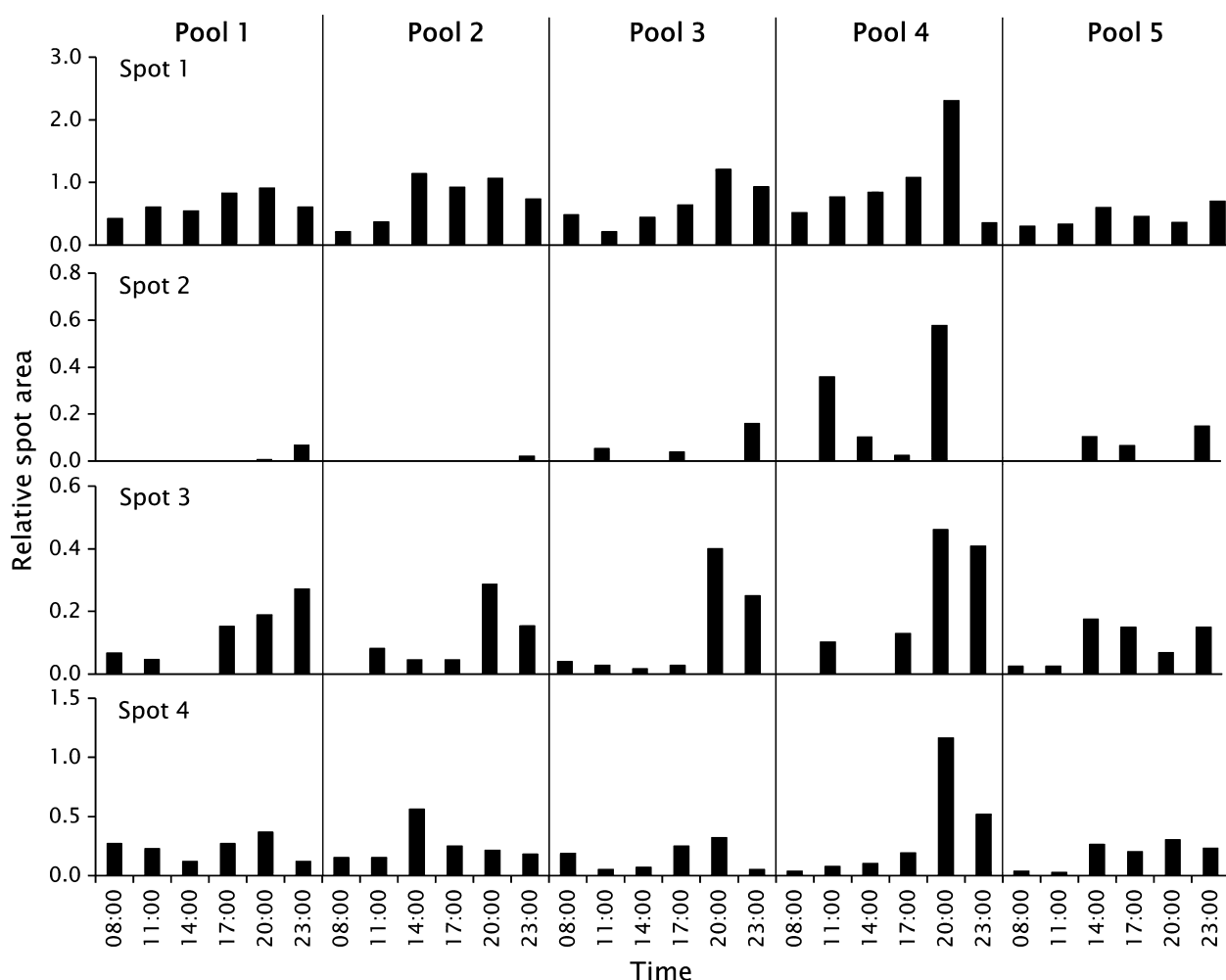


Fig. 3. Analysis of spots from *Anemonia viridis* extracts from reverse phase Si thin-layer chromatography plates based on area and density analysis using IMAGEPRO PLUS. Values correspond to relative area that *Agrobacterium tumefaciens* bioreporter was induced by 2.8 mg *A. viridis* protein.

exception of pool 5, spot 1 increased in concentration throughout the day, to peak at 20:00 and then decreased in concentration. Although not present in all samples and less abundant, spot 3 increased in concentration at 20:00 for pools 2, 3 and 4, and at 17:00 for pool 1 and 14:00 for pool 5. Spots 2 and 4 showed no obvious trends. Spot 2 was more abundant in pools 3, 4 and 5. Spot 4 was also found in all samples. Samples from pool 5 showed similar patterns to all the other pools; however, spots were smaller at 20:00.

Hierarchical clustering (based on a Euclidean distance resemblance measure) and SIMPROF analysis shows statistically significant evidence of genuine clusters based on AHL profiles, separating samples into two clusters, one containing samples taken at 08:00 and 11:00 (except pool 4, 11:00) and one containing samples taken at 20:00 and 23:00 (except for pool 5, 20:00). This suggests differences in

AHL profiles as a result of time of day (Supporting Information, Fig. S1A). Samples from 14:00 and 17:00 were found in both clusters. Samples at 14:00 (high tide) were the most variable (1.39) in a multivariate dispersion (MvDISP) analysis. A further MvDISP analysis looking at the variability of AHLs within pools showed pool 1 to have the least variable AHLs over the experimental time period (0.67); with pool 4 having the most varied AHLs (1.46).

PERMANOVA analysis of AHL profiles with time point and pool as fixed terms showed significant differences between time points (Pseudo- $F = 2.98$, $P < 0.005$), but not between pools (Type 1 model). Covariates (normalised environmental factors) were added to the model one by one to assess their importance in determining AHL profiles. This included dissolved oxygen, which was highest at 11:00 for all pools with an average of 12.0 mg L^{-1} and dropped significantly by 23:00 with an average of 0.79 mg L^{-1} , and

temperature, which was also highest at 11:00 with an average of 20.2 °C and dropped to an average of 15.1 °C by 23:00. However, none of the environmental factors recorded had significant interactions with the model.

Identification of AHLs from *A. viridis* by LC-MS/MS

Subsequent analysis of *A. viridis* DCM extracts with LC-MS/MS revealed the identity of a number of AHLs. Results confirm the presence of five AHLs, namely *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-OH-C6-HSL), *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-OH-C8-HSL) and *N*-(3-hydroxydecanoyl)-L-homoserine lactone (3-OH-C10-HSL). Figure 4 shows the MS spectra acquired from chromatographic runs of DCM extracts and synthetic standards, showing strong evidence for the presence of these AHLs in the extracts, with chromatographic peaks that display matching retention times. This confirms patterns seen in TLC plates. The spots did not tail like their 3-oxo derivatives (the standards), suggesting these are 3-OH or 3-unsubstituted AHLs (Shaw *et al.*, 1997). While 3-hydroxy-substituted compounds migrate with the same mobility at their 3-oxo analogs, 3-unsubstituted derivatives have been shown to migrate with characteristic retention times, below that of their 3-oxo analogs (Shaw *et al.*, 1997). This suggests that spot 1 is *N*-hexanoyl-L-homoserine lactone (C6-HSL), spot 2 is *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-OH-C8-HSL), spot 3 is *N*-octanoyl-L-homoserine lactone (C8-HSL) and spot 4 is *N*-(3-hydroxydecanoyl)-L-homoserine lactone (3-OH-C10-HSL), although this spot runs slightly below the standard suggesting the presence of an altered form. While *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-OH-C6-HSL) is not detected in the TLC analysis, LC/MS analysis shows this AHL to be present in very low quantities in comparison with the other detected AHLs. TLC analysis also relies on the ability of the biosensor to pick up AHLs, whereas LC/MS is not limited by this bias.

Comparing putative AHL profiles to the active bacterial community in *A. viridis*

Cluster analysis (based on a Bray–Curtis resemblance measure) and SIMPROF of PCR-DGGE profiles of 16S rRNA isolated from *A. viridis* RNA extracts revealed differences between samples (Fig. S1B). Samples clustered into three statistically significant groups, with samples from 23:00 (pool 2–5) clustering separately to other samples, as seen in the AHL profile analysis (Fig. S1A). Similarly, samples from 08:00 and 11:00 clustered

together except for samples from pool 1 (08:00) and 2 (08:00 and 11:00), which correspond to pools with sediment substratum and access to main body of water at mid tide. 20:00 samples were found in the third cluster (except pool 2) and joined by an assortment of samples from other times and pools. Multivariate dispersion analysis (MvDISP) of pools showed samples from pools 1 and 2 were the most dispersed throughout the experimental time period. In an MvDISP analysis of sampling times, samples at high tide (14:00) were shown to have the most dispersed bacterial community (1.257), which matches dispersion seen in AHL profiles at high tide. Samples from 20:00 and 23:00 are almost as discrete (1.103 and 1.174 respectively). A RELATE analysis of the Euclidian distance AHL matrix and the Bray–Curtis bacterial community matrix revealed significant but low similarity ($\rho = 0.16$, $P < 0.05$).

A number of DGGE bands were present in over 10 *A. viridis* samples, including bands that closely affiliated with an uncultured bacteria originally isolated from the coral *Montastraea faveolata* (19/30 samples; GU118683), an uncultured gammaproteobacterium (13/30 samples; GQ347793) and an uncultured *Cyanobacteria* (17/30 samples; FJ53106) (Table 2) as well as a further five unidentified bands. While some bands were found in a varying number of anemones at all time points, such as the band affiliating with an uncultured gammaproteobacterium (GQ347793), some patterns in the activity of *A. viridis* bacteria were found with time (Table 2). Most notably, four bands found at all other time points were not present in samples taken at 23:00, including a band affiliated with an uncultured bacteria originally isolated from *M. faveolata* (GU118683) and a band affiliated with a *Saprospira* sp. (AB088635). However, no significant differences were found in the presence of abundant bands (found in > 10 samples) between pools or sampling times (two-way ANOVA; pool $F = 0.12$, time $F = 2.59$, $P > 0.05$).

Discussion

Presence of putative AHLs in a variety of cnidarians

This study has established the presence of putative AHL molecules in the majority of cnidarian species tested. The lack of detectable AHL molecules in the remaining three species may be due to the restricted amount of soft tissue available and/ or the detection limits of the biosensor, as opposed to an absence of signal molecules in these species. LC-MS/MS data confirmed the presence of a number of AHLs in *A. viridis* extracts: C6-HSL, C8-HSL, 3-OH-C6-HSL, 3-OH-C8-HSL and 3-OH-C10-HSL, which matched patterns seen on TLC plates. A range of AHLs

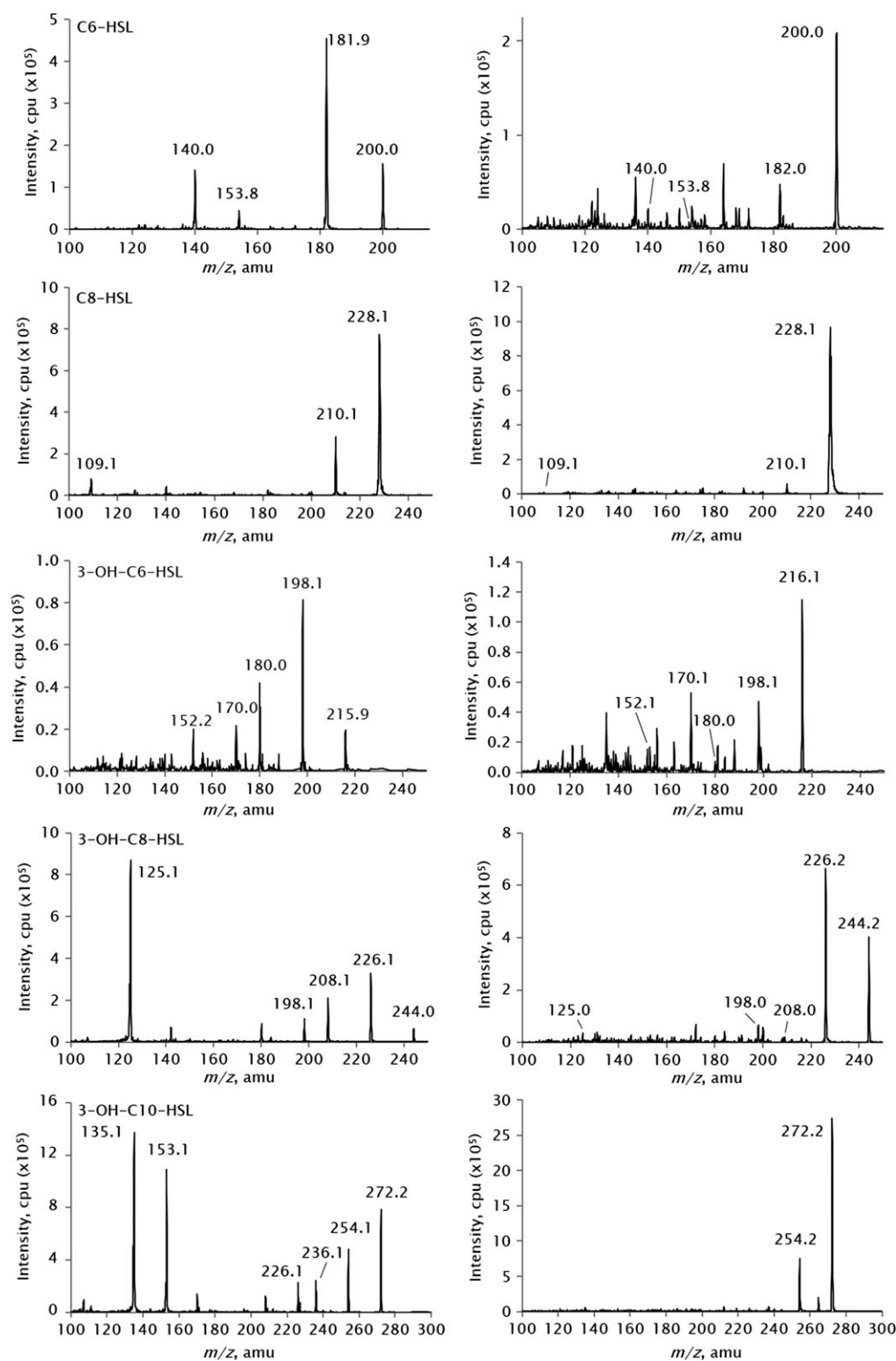


Fig. 4. Spectra acquired from chromatographic runs of synthetic standards (left) and *Anemonia viridis* DCM extract (right) showing strong evidence for the presence of C6-HSL, C8-HSL, 3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C10-HSL (as labelled) in the extracts, with the main diagnostic fragment peaks present in both.

Table 2. Most common affiliations in the NCBI database for bands successfully excised, re-amplified and sequenced from 16S rRNA PCR-DGGE of RNA extracts of *Anemonia viridis*

Most common affiliation (NCBI)	Accession number	Sequence similarity (%)	Presence at sampling times						Frequency in samples
			8:00	11:00	14:00	17:00	20:00	23:00	
Uncultured Cyanobacterium	FJ53106	99	3	2	2	0	3	1	11
Uncultured Cyanobacterium	FJ53106	99	1	1	1	1	1	1	6
<i>Saprospira</i> sp.	AB088635	94	3	2	1	3	1	–	10
Uncultured Mollicute	EF137402	87	2	2	2	1	1	–	8
Uncultured Gammaproteobacterium	GQ347793	97	1	3	1	5	2	1	13
Uncultured Sphingobacterium	EF664090	86	1	3	1	1	3	1	10
Uncultured coral bacterium	GU118683	96	3	2	3	2	1	–	11
Uncultured coral bacterium	GU118683	96	2	–	2	5	4	1	14
<i>Flexibacter littoralis</i>	AB681019	99	–	–	1	3	–	2	6
<i>Geobacillus</i> sp.	KC551260	99	1	4	1	1	2	–	9
<i>Geobacillus</i> sp.	KC551260	99	1	1	1	3	1	2	9
<i>Geobacillus thermoleovorans</i>	NR07491	99	1	1	1	3	3	1	10
Total			19	21	17	28	22	10	

with varying chain length were detected in other species with profiles that were species specific and reproducible over replicates. For *A. viridis* and *S. pistillata*, the AHL profile detected was also reproducible between fragments collected from different locations and times, suggesting a stable role for QS in cnidarian-associated bacterial communities.

Although only a crude image analysis of high-quality TLC plates was possible in this study, it is estimated that the assays detected a range of AHLs in the low μM range: 3–7 μM AHLs from *Actinaria*, 5–11 μM for *Alcyonacea* tested and 0.07–0.2 μM for *Scleractinia* tested. Previous studies have found addition of 2 μM AHLs to induce changes in bacterial community and function in activated sludge (Valle *et al.*, 2004) and 1–100 nM to stimulate defences in *Medicago truncatula*, modulating primary and secondary metabolism (Mathesius *et al.*, 2003). This suggests that AHLs in cnidarians are present in concentrations that are able to induce phenotypic change in bacterial populations and possibly in the cnidarian host.

Extracts showed a differing degree of diversity of AHLs detected throughout the samples, which do not seem to relate to amount of wet tissue used and may instead relate to bacterial diversity and/or the number of phenotypes being controlled within the coral holobiont. The presence of different AHL molecules could be the result of a single bacterial species or functional group, but is more likely to be an accumulation of an array of interactions within and across functional groups allowing for rapid responses to a range of environmental changes experienced within these ecosystems. Most abundant levels and greatest diversity of AHLs were detected in the *Alcyonacea* and the *Actinaria*, which may relate to the number of bacteria found within these tissues or the

importance of AHL quorum sensing (QS) in these species.

Short-chain AHLs were only detected in the *Actinaria*, while the majority of the spots seen on the TLC plates ran at similar rates to the mid- and long-chain synthetic AHL standards 3-oxo-C8-HSL and 3-oxo-C10-HSL, suggesting a dominance of longer chain AHLs associated with the cnidarian species tested. Short-chain AHLs are known to degrade more rapidly in seawater (Yates *et al.*, 2002; Hmelo & Van Mooy, 2009) and thus longer chain AHLs may be better optimised to function in a seawater medium. This is consistent with numerous reports that AHL-producing marine bacteria often produce long-chain rather than short-chain AHLs (Schaefer *et al.*, 2002; Wagner-Döbler *et al.*, 2005). However, it may be possible that these short-chain AHLs are effective in the environment over shorter distances (Hmelo & Van Mooy, 2009) or are buffered from seawater chemistry by their invertebrate host, only to be broken down more rapidly once the animals are removed from their natural environment, thus preventing detection.

These data suggest an important role for AHL molecules within cnidarian-associated bacterial communities and complement growing evidence that bacterial communities and their association with higher organisms may involve QS and QS regulation (Rice *et al.*, 2005; Huang *et al.*, 2007; Hunt *et al.*, 2012). While this is the first study to determine the diversity of AHLs *in situ*, bacteria isolated from the elkhorn coral *Acropora palmata* (4%; Alagely *et al.*, 2011), various other corals (30%; Golberg *et al.*, 2011) and *Vibrio* spp. from healthy and diseased corals (Tait *et al.*, 2010), have been found to be capable of affecting (stimulating or inhibiting) QS reporters. The production of stable AHLs may inhibit subcommunities

of bacteria (Golberg *et al.*, 2011), may promote specific bacterial colonisation of invertebrate surfaces (Taylor *et al.*, 2004; Mohamed *et al.*, 2008) or may be responsible for defence against invasive microorganisms by inducing genes for the production of antimicrobials. The production of antimicrobials active against planktonic bacteria, coral pathogens and biomedically important microorganisms has been shown by bacteria isolated from a number of corals (Ritchie, 2006; Nissimov *et al.*, 2009; Shnit-Orland & Kushmaro, 2009), as well as from other invertebrates such as sponges (Thakur *et al.*, 2004) and ascidians (Karthikeyan *et al.*, 2009). QS-positive *Aptasia pallida* bacterial isolates have also been found to be capable of inhibiting both swarming and biofilm production in the coral pathogen *Serratia marcescens* PDL100 (while not affecting growth) and stopping the progression of disease by *S. marcescens* in *A. pallida* (Alagely *et al.*, 2011). Although the mode of action of these isolates has yet to be determined, in combination with the results presented here, these studies indicate the importance of establishing the role of QS in the maintenance of coral health and during disease, as is suggested by Teplitski & Ritchie (2009). Furthermore, data showing that: (1) Caribbean gorgonians can antagonise and stimulate QS activity (Hunt *et al.*, 2012); (2) that soft corals (order Alcyonacea) exhibit the highest levels of QS inhibitory activity compared with other marine species tested (Skindersoe *et al.*, 2008); and (3) that high levels of QS molecules occur in these ecosystems (as shown in this study) suggest that complex coral–bacterial relationships rely, at least in part, on QS and QS inhibition for sustaining a balanced community.

Temporal stability of putative AHLs in *A. viridis* and their relationship to environmental conditions and bacterial communities

Investigations into the temporal stability of AHLs produced *in situ* in *A. viridis* revealed remarkable similarity between AHL profiles over the 16-h observational period. This suggests the presence of a specific bacterial community and a particular, stable role for these AHLs that do not directly relate to changes in the environmental conditions seen during the diurnal cycle in these rock pools. However, the increased variability in detected AHLs and bacterial communities in anemones at high tide suggests (1) some level of environmental control beyond the variables measured in this study that changes with tide, (2) a disruption to the bacterial community and/or function that may be the result of an influx of new bacterial species, brought in with high tide, attempting to colonise the anemone tissue and (3) a correlation between bacterial activity and intensity of AHLs. The increase in certain

AHLs over the day, with abundance peaking at 20:00, also suggests that AHL abundance is at least partly controlled by environmental factors that are correlated with time of day. As previous studies have shown that temperature can affect AHL production both positively (Hasegawa *et al.*, 2005; Latour *et al.*, 2007) and negatively (Tait *et al.*, 2010) and considering the fluctuation in temperature over the day (15.1–20.2 °C), we expected to see a temperature effect. However, the model indicates that this is not the case and suggests that such changes in AHL production may only occur at higher temperature thresholds as seen in coral-associated *Vibrio* spp. (Tait *et al.*, 2010).

The stability and pattern of putative AHL abundance, despite fluctuations in rock pool pH (7.0–8.5) is also surprising, considering the findings of a study on microbial mats by Decho *et al.* (2009), which found daily shifts in AHL profiles. Significantly more medium-chain (C6 to C10) AHLs were found after mats were subjected to night time conditions, consisting of a dark, more anoxic and acidic environment, in comparison with mats kept in day time conditions (Decho *et al.*, 2009) due to alkaline lactonolysis at higher pHs (Yates *et al.*, 2002) found during the day. In contrast, long-chain AHLs (C12 and C14) showed a less significant change. Decho *et al.* (2009) suggested that this may allow bacteria to utilise diel pH periodicity to alternate their day/night induction of specific sets of genes. In the present study, an increase in two medium-chain AHLs was observed throughout the day, peaking at 20:00 (just after dusk) and thus not following the hypothesised pH-dependent pattern observed in the microbial mats studied by Decho *et al.* (2009). This suggests that either *A. viridis* controls internal pH, allowing medium-chain AHL molecules to remain active after periods of high external pH and/or that the change in conditions, that is the lack of light, anoxic environment or the decrease in pH, may result in changing bacterial function or metabolic activity that is associated with bacterial communities throughout the day.

This study was designed to limit the possibility of AHL degradation by bacterial enzymes known to be produced by marine bacteria (Romero *et al.*, 2011) or potentially by *A. viridis* tissue (as seen with gorgonian tissue extracts; Hunt *et al.*, 2012) during tissue processing. This was ensured by immediately snap freezing samples on collection and carrying out tissue homogenisation and dichloromethane extractions in prompt succession, immediately after thawing tissue on ice. However, there is a small possibility that changes in the production of AHL-degrading substances by bacteria or *A. viridis* during processing could have contributed to the fluctuations in AHL abundance observed, acting on AHLs during homogenisation (1 min). All these factors warrant further investigation.

DGGE profiles of 16S rRNA from *A. viridis* showed the presence of a number of bands identified as belonging to groups of known quorum-sensing bacteria. Two bands, relating most closely to an uncultured bacterium originally isolated from the coral *Montastraea faveolata* and found in 19 of 30 of the samples, have a secondary affiliation with a *Reichenbachiella* sp. (93% similarity), a member of the *Bacteroidetes*. This group, although not as well studied, has been found to produce a number of AHL molecules (Huang *et al.*, 2008; Romero *et al.*, 2010; Twigg *et al.*, 2013) including C14-HSL, C8-HSL, 3-oxo-C4-HSL and 3-oxo-C6-HSL from four isolates in subtidal biofilms (Huang *et al.*, 2008). Another band was identified as an uncultured member of the *Cyanobacteria* and found in 17 of 30 anemones. *Cyanobacteria* have also recently been found to use QS in a study of the epilithic colonial cyanobacterium *Gloeotheca* PCC6909, which produces C8-HSL (Sharif *et al.*, 2008). Finally, a band identified as an uncultured member of the *Gammaproteobacteria*, found in 13 of 30 samples, is part of the most intensively studied and largest class of known AHL producers. In a recent study of cultivated coral-associated bacteria, AHL producers were found to frequently cluster in the *Proteobacteria*, and of these 90% were affiliated with the *Gammaproteobacteria* (Golberg *et al.*, 2011). Two clades of bacteria consistently detected in *Porites astreoides*, namely a *Roseobacter* clade and a *Marinobacter* sp. (Sharp *et al.*, 2011), also belong to groups of known QS bacteria. Ainsworth & Hoegh-Guldberg (2009) have shown bacterial aggregates in both *Acropora aspera* and *Stylophora pistillata* from the Great Barrier Reef to be made up of only one morphotype, identified as members of the *Gammaproteobacteria*, and they suggested that this group may prove to be a universal symbiont group associated with corals, further indicating the potential role for QS in these systems.

In this study, no one band in DGGE community profiles based on 16S rRNA amplification from RNA extracts was present in every sample, leading to the conclusion that the AHL profiles obtained from *A. viridis* samples are the result of a consortium of bacteria which contribute to the overall AHL pool. Results from Golberg *et al.* (2011) suggest the same, with 30% of coral bacterial isolates found to produce AHL molecules; thus, AHL-QS appears to be widespread in coral bacteria. However, this study has also shown AHLs associated with *A. viridis* to be concentrated in the tentacles. A recent investigation using light and scanning electron microscopy of the anemone *Metridium senile* showed bacterial aggregates living in the tentacles. Sequence analysis of these aggregates revealed three subgroups of *Proteobacteria* including the gammaproteobacterium *Endozoicomonas elysicola* (98%) (Schuett *et al.*, 2007). Together, this suggests the possibility of a conserved

group of bacteria found in the tentacles, which may be expressing a different set of genes, based on different quorum sensing circuits and which carry out different functions to those found in other compartments of the anemone. As this genus is found in a number of marine invertebrates (Martínez-García *et al.*, 2007; Schuett *et al.*, 2007), including a number of corals (Kurahashi & Yokota, 2006; Yang *et al.*, 2010), and is a member of a prominent group of quorum sensing bacteria, they are a prime target for further investigation into their quorum sensing pathways and the functions that these systems control.

In conclusion, this study has shown for the first time that AHL signals can be detected *in situ* in the coral holobiont. The presence of spatially conserved AHL signal profiles in cnidarian extracts suggests that AHL signalling is at least one of the mechanisms by which coral-associated bacteria may maintain their communities and that AHL-producing bacteria may have an important role in the coral holobiont. Further, the finding that the species-specific nature of these AHL profiles links to the species-specific nature of coral-associated bacteria (Ritchie & Smith, 1997; Rohwer *et al.*, 2001, 2002; Littman *et al.*, 2009) calls for further investigation into the quorum sensing-controlled, species-specific roles of these bacterial communities.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Cluster analysis (group average) and SIMPROF test of AHL abundance (A) and PCR-DGGE of 16S rRNA (B) in *A. viridis* samples.